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Effect of *Acacia melanoxylon* R. Br. Inclusion on the Chemical Composition, Fermentation Dynamics, and In Vitro Digestibility of *Medicago sativa* L. Silage

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Abstract

This study evaluated the effect of *Acacia melanoxylon* inclusion in *Medicago sativa* silage on chemical composition, fermentation quality, in vitro digestibility, gas production, and energy value. Due to its high moisture content, *M. sativa* presents challenges for ensiling. *A. melanoxylon*, a woody legume with high dry matter (DM) content, was tested as a structural additive. Five treatments were prepared—control (100% *M. sativa*) and mixtures with 6, 12, 24, and 48% *A. melanoxylon* (fresh basis)—and ensiled for 45 days under vacuum. Silages were analyzed for DM, crude protein, fiber fractions, pH, ammonia nitrogen, in vitro digestibility, gas production kinetics, and estimated energy values (*ME* and *NEL*). Increasing *Acacia* raised DM (17.75 ± 0.04 → 28.45 ± 0.11%) and reduced pH (5.86 ± 0.01 → 4.53 ± 0.01) and NH_3-N/Total N (11.38 ± 0.10% → 8.05 ± 0.10%), indicating improved fermentation quality. Conversely, crude protein, digestibility (IVDMD 62.61 ± 0.05% → 48.02 ± 0.16%), and cumulative gas at 96 h decreased, as did energy values (*ME* 5.91 → 4.45 MJ/kg DM; *NEL* 3.13 → 2.02 MJ/kg DM) at higher inclusion levels; gas-kinetic parameters reflected the same trend (lower b and c). Overall, *A. melanoxylon* acts as a structural co-ensiling option that increases DM and supports fermentation quality while clearly delineating nutritional and fermentability trade-offs; low-to-moderate inclusion (6–12%) appears advisable to balance process benefits against acceptable nutritional penalties.

Keywords: *Acacia melanoxylon*; *Medicago sativa*; silage quality; dry matter; in vitro gas production; digestibility; forage legumes; fermentation profile; ruminant nutrition

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1. Introduction

The conservation of forages through ensiling is a fundamental strategy to ensure a continuous and reliable supply of feed for ruminants, particularly in production systems where seasonal variability limits pasture availability [1]. While grasses are most commonly used for silage due to their high content of water-soluble carbohydrates and favorable fermentation profile, legumes such as *Medicago sativa* L. (alfalfa) are also widely employed because of their high crude protein content, mineral richness, and functional bioactive compounds [2,3].

However, *M. sativa* presents specific challenges for silage making. Its low concentration of soluble sugars, high buffering capacity, and elevated protein content can hinder

acidification, promoting proteolytic fermentation and the formation of undesirable nitrogenous compounds such as ammonia and biogenic amines [4,5]. Furthermore, when ensiled with a dry matter (DM) content below 30%, effluent losses are common, compromising both silage quality and environmental safety [6]. To mitigate these issues, strategies such as field wilting, co-ensiling with energy-rich crops, or the addition of structural or functional additives have been explored [7,8]. Evidence from mixed-forage systems also indicates that blending alfalfa with other roughages can improve fermentation quality and in vitro digestibility, and inclusion of alfalfa enhanced the nutritive value of straw–grass mixed silages in Tibet [9].

Among these strategies, the inclusion of high-DM woody legumes has emerged as a promising approach to improve the physical structure of the silage mass, enhance fermentation dynamics, and, in some cases, contribute to methane mitigation due to the presence of condensed tannins [10,11]. *Acacia melanoxylon* R. Br., a leguminous tree from the Fabaceae family, is widespread in the Azores, where it behaves as an invasive species. This evergreen tree is characterized by its high biomass yield, persistent canopy, and dense foliage. It also exhibits high nitrogen-fixing ability and strong regeneration capacity, including fire-induced germination [12,13]. From a zootechnical perspective, *A. melanoxylon* has been considered a potential alternative protein source and is particularly rich in condensed tannins [14]. Although its direct inclusion in ruminant diets is limited by antinutritional factors and low digestibility, its combination with highly digestible forages such as M. sativa could provide a local, pragmatic solution to increase pre-ensiling DM and improve packing/anaerobiosis while managing trade-offs in digestibility and fermentability [15,16].

Beyond classical nutritional endpoints, silage evaluation increasingly uses in vitro gas production because total gas and kinetic parameters reflect the extent and rate of rumen microbial fermentation under standardized conditions; gas arises mainly from CO_2 and CH_4 during carbohydrate fermentation, so higher volumes generally indicate more extensive degradation (albeit not methane partitioning), and gas at specific time points (e.g., 24 h) is commonly used together with crude protein and ether extract to estimate metabolizable and net lactation energy via the Menke–Steingass equations [17]. In parallel, ensiling performance must also account for process attributes achieving adequate dry matter (DM) to curb effluent, promoting rapid acidification, minimizing proteolysis (NH_3–N), and ensuring aerobic stability, because structural additives that raise DM improve packing and anaerobiosis may increase fiber and lignin, with predictable impacts on digestibility and in vitro fermentability [18]. In our study region, *M. sativa* is widely conserved as hay/haylage/silage, while unmanaged stands of the invasive tree *Acacia melanoxylon* generate abundant high-DM biomass; together these factors motivate testing *A. melanoxylon* as a structural co-ensiling complement to raise pre-ensiling DM and support fermentation quality, while explicitly quantifying the associated trade-offs in digestibility, energy value, and gas production [9–11].

Although this work is grounded in an island agroecosystem, its scope extends beyond a single locality. The core question, whether a locally abundant, high-DM woody legume can serve as a structural co-ensiling resource for alfalfa applies to many humid temperate regions where alfalfa is harvested at low DM and woody or tannin-rich biomass is accessible (including areas invaded by *Acacia* spp.). The mechanisms involved (DM management, packing/anaerobiosis, proteolysis control, and the digestibility–fermentability trade-off linked to fiber/lignin and tannins) are generalizable, while quantitative outcomes depend on chemotype, inclusion rate, maturity, and management. Hence, the results are transferable in principle and cautiously extrapolatable to similar contexts, acknowledging that in vitro assays are proxies and do not replace in vivo trials [9–11,15].

In many humid temperate regions, improving silage quality is constrained by low pre-ensiling DM and limited access to water-soluble carbohydrates. Where invasive woody biomass is abundant, co-ensiling can provide a locally available structural resource to raise DM and support fermentation. Among potential species, *A. melanoxylon is* particularly suitable because it is widespread as an invasive tree, generates substantial residues through routine control, and combines high DM with a condensed-tannin profile. These features create a pragmatic opportunity for valorization while addressing ensiling constraints.

We tested whether incremental inclusion of *A. melanoxylon* in *M. sativa* silage functions as a structural co-ensiling strategy that increases dry matter and improves fermentation quality (pH, NH_3-N), while quantifying the nutritional trade-offs and the effects on fermentability captured by in vitro gas production kinetics. We hypothesized that low-to-moderate inclusion levels (6–12%) would improve ensiling conditions relative to alfalfa-only silage, with limited penalties to digestibility, whereas higher inclusion levels would increasingly constrain fermentability and energy value [17].

2. Materials and Methods

2.1. Study Area and Conditions

The study site lies in the municipality of Angra do Heroísmo (Terceira, Azores; ~300 m a.s.l.), on volcanic soils classified as Andosols derived from pyroclastic parent materials (vitric Hapluand), which are well suited to agriculture due to their favorable physical properties. The local climate is temperate oceanic with a dry-summer subtype (Köppen Csb). At this elevation, monthly temperatures ranged from 11.6 to 20.4 °C, total annual precipitation was 1280.3 mm, average monthly wind speed was 0.32–1.61 m s^{-1}, relative humidity remained high (86.1–95.0%), and incoming solar radiation was low (51.9–171.6 W m^{-2}); rainfall typically peaks in January–February and is lowest in July [19].

2.2. Sample Collection and Preparation

In December 2024, *Medicago sativa* L. was harvested on Terceira Island (Azores, Portugal) from a commercial field in the municipality of Angra do Heroísmo at the vegetative stage, prior to flowering. On the same day, leaves of *Acacia melanoxylon* were collected from unmanaged, wild-growing stands within the municipality of Angra do Heroísmo, selecting healthy, fully expanded foliage without inflorescences and avoiding lignified twigs and petioles.

M. sativa was air-dried naturally for 48 h and then chopped into fragments of 2–3 cm. *A. melanoxylon* leaves were subjected to the same wilting and chopping procedure.

2.3. Silage Preparation

Five treatments were prepared, corresponding to different inclusion levels of *A. melanoxylon* in the *M. sativa*:

TC—Control (100% *M. sativa*)

T1—94% *M. sativa* + 6% *A. melanoxylon*

T2—88% *M. sativa* + 12% *A. melanoxylon*

T3—76% *M. sativa* + 24% *A. melanoxylon*

T4—52% *M. sativa* + 48% *A. melanoxylon*

Each treatment was prepared in triplicate (three independent mini-silos; totaling 15 silage units). Approximately 2.5 kg of each mixture was packed into airtight plastic bags, vacuum-sealed to ensure anaerobic conditions, and stored in a dark room at ambient temperature (18–25 °C) for 45 days. All laboratory determinations were run in duplicate (technical replicates) and averaged per mini-silo, which served as the experimental unit for subsequent analyses.

2.4. Chemical Analysis

At the end of the fermentation period, silages were opened and sampled. A portion of each sample was frozen for later analysis of pH and ammonia nitrogen (NH_3-N/Total N). The remaining sample was oven-dried at 65 °C in a forced-air oven until constant weight and ground to 1 mm in a Retsch mill (Haan, Germany).

Chemical composition was assessed using standard Weende methods [20], including, Dry Matter (DM), method 930.15, Ash, method 942.05, Ether Extract (*EE*), method 920.39, Crude Protein (*CP*), method 954.01.

Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL) were determined according to Goering and Van Soest [21].

pH was measured using a potentiometric method with a glass electrode [22], and ammonia nitrogen in silage water extracts was determined by acidifying 10 mL of filtrate with 10 mL 0.2 N HCl (1 h, room temperature) and steam-distilling on a Kjeltec 2300 [23].

2.5. In Vitro Digestibility, Gas Production, and Energy Estimation

The in vitro digestibility of dry matter (IVDMD) and organic matter (IVOMD) was determined using the method of Tilley and Terry [24], modified by Alexander and McGowan [25].

Gas production was assessed according to Menke and Steingass [17]. For each sample, 200 mg of dry matter were incubated in 100 mL calibrated glass syringes with 30 mL of buffered rumen fluid at 39 °C, in an incubator equipped with a motor-driven rotor to provide continuous agitation. Gas volume was recorded at several time points over a 96-h incubation period. The gas production kinetics were modeled using the equation developed by Ørskov and McDonald [26,27].

The metabolizable energy (*ME*) content of the silages was estimated using the Equation (1) proposed by Menke and Steingass [17], based on gas production, crude protein, and ether extract:

$$ME\left(\frac{\text{MJ}}{\text{KgDM}}\right) = 2.20 + 0.136 \times GP + 0.057 \times CP + 0.0029 \times EE^2 \quad (1)$$

Net energy for lactation (*NEL*) was subsequently estimated using the following Equation (2) [28]:

$$NEL\left(\frac{\text{MJ}}{\text{KgDM}}\right) = 0.101 \times GP + 0.051 \times CP + 0.11 \times EE \quad (2)$$

where *GP* is the gas production (mL/200 mg DM after 24 h), *CP* is crude protein (g/kg DM), and *EE* is ether extract (g/kg DM).

2.6. Rumen Fluid Collection

Rumen fluid was collected from the slaughterhouse on Terceira Island immediately after the slaughter of healthy dairy cows, following the procedure described by [28]. The fluid was filtered through four layers of cheesecloth and kept under anaerobic conditions until use.

2.7. Statistical Analysis

The study used a completely randomized design (CRD) with five inclusion levels of A. melanoxylon (0, 6, 12, 24, 48% on a fresh basis) and three independent mini-silos per level. Mixtures were randomly allocated to mini-silos; the experimental unit was the individual mini-silo. Data were tested for normality (Shapiro–Wilk) and homogeneity of variances (Levene). One-way ANOVA was applied to compare treatment effects on

chemical composition, digestibility, energy value, and gas-kinetic parameters (b, c), with Tukey's HSD for post hoc comparisons. In addition, polynomial trend tests (linear and quadratic) using the actual inclusion percentages (0, 6, 12, 24, 48%) were conducted to evaluate dose–response patterns across the ordered gradient. For time-course gas data, repeated-measures ANOVA was applied to cumulative gas (within-subject factor: time; between-subject factor: inclusion level). Statistical significance was declared at $p < 0.05$. Analyses were performed using IBM SPSS Statistics, version 27.

3. Results

3.1. Pre-Ensiling Chemical and Digestibility Profiles of M. sativa and A. melanoxylon

The results presented in Figure 1 correspond to the chemical composition and in vitro digestibility of *M. sativa* and *A. melanoxylon* before ensiling. Significant differences ($p < 0.05$) were observed between species for most parameters. *M. sativa* exhibited higher crude protein (25.98 ± 0.16%), lower fiber fractions (NDF: 50.09 ± 0.11%; ADF: 34.02 ± 0.20%) and greater digestibility values (IVDMD: 70.61 ± 0.14%; IVOMD: 62.76 ± 0.10%) compared to *A. melanoxylon*. In contrast, *A. melanoxylon* presented higher dry matter (43.73 ± 0.07%), NDF (68.90 ± 0.08%), and ADF (50.27 ± 0.15%) contents. No significant differences were detected for acid detergent lignin (ADL) and ether extract (*EE*). Crude ash was significantly higher in *M. sativa* (9.75 ± 0.13%) than in *A. melanoxylon* (5.44 ± 0.02%).

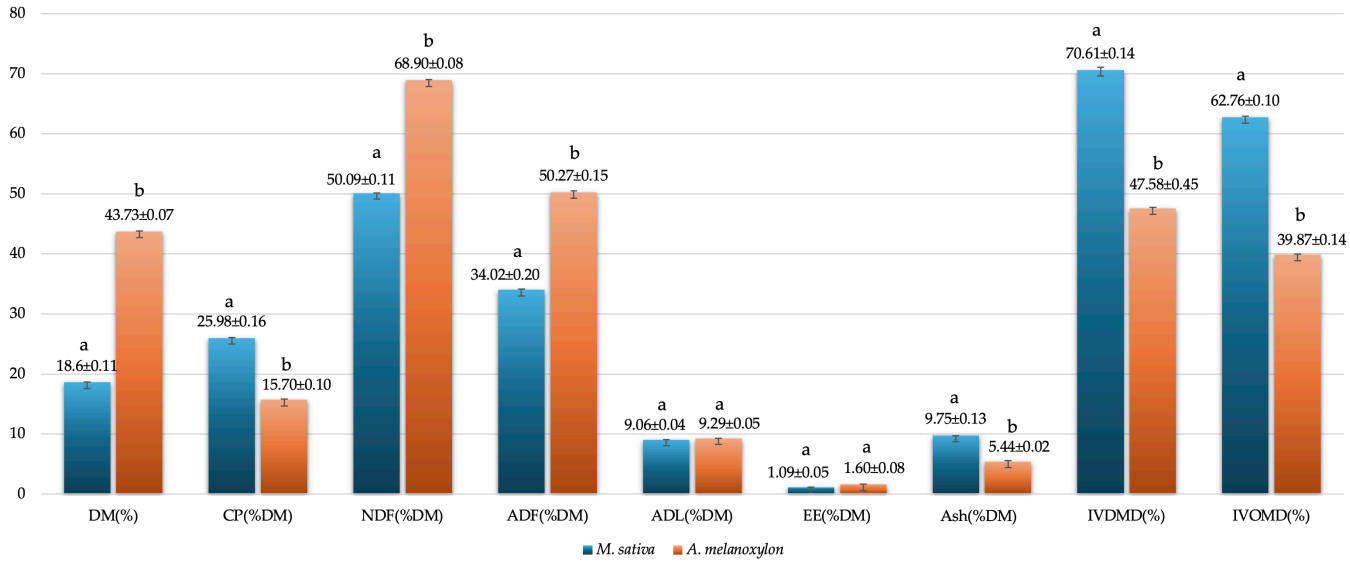


Figure 1. Chemical composition and in vitro digestibility of *M. sativa* and *A. melanoxylon*. Bars represent the mean ± standard error (n = 3). *CP*: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; *EE*: ether extract; IVDMD: in vitro dry matter digestibility; IVOMD: in vitro organic matter digestibility. Different letters above bars indicate significant differences between species for each parameter ($p < 0.05$).

3.1.1. Pre-Ensiling Gas Production Profiles of *M. sativa* and *A. melanoxylon*

The in vitro gas production kinetics and cumulative gas production of *M. sativa* and *A. melanoxylon* prior to ensiling are shown in Table 1. Significant differences ($p < 0.05$) were observed for most kinetic parameters and gas volumes between the two species. M. sativa showed a markedly higher potential gas production (parameter b) and fermentation rate (c), as well as a longer lag phase (Lag t), compared to A. melanoxylon. Cumulative gas production was consistently higher in *M. sativa* across all time points from 8 h onwards ($p < 0.001$). No significant difference was observed at 4 h ($p = 0.063$), and the soluble fraction (a) was also not significantly different ($p = 0.091$).

Table 1. In vitro gas production kinetics and cumulative gas production (mL/0.2 g DM) of *M. sativa* and *A.melanoxylon* prior to ensiling.

	A. melanoxylon	***M. sativa***	***p* Value**
Kinetics of Reaction			
a (mL/0.2 g DM)	2.46 ± 0.1242	−1.6244 ± 0.2035	0.091
b (mL/0.2 g DM)	12.36 ± 0.1595	39.9572 ± 0.2374	<0.001
c (mL/h)	0.0250 ± 0.0100	0.0364 ± 0.0006	<0.001
Lag t (h)	0.00 ± 0.00	1.00 ± 0.0156	<0.001
Gas production (mL/0.2DM)			
4 h	3.33 ± 0.24	3.98 ± 0.15	0.063
8 h	3.91 ± 0.03	8.38 ± 0.14	<0.001
12 h	4.64 ± 0.11	12.54 ± 0.21	<0.001
24 h	6.35 ± 0.26	21.47 ± 0.25	<0.001
48 h	8.92 ± 0.12	31.26 ± 0.25	<0.001
72 h	10.95 ± 0.3	35.59 ± 0.17	<0.001
96 h	12.15 ± 0.19	37.29 ± 0.15	<0.001

Values are expressed as mean ± SEM (n = 3). "a" represents the gas produced from the immediately soluble fraction (mL/0.2 g DM), "b" is the gas production from the insoluble but fermentable fraction (mL/0.2 g DM), "c" is the rate constant of gas production (mL/h), and "Lag t" is the lag time before gas production begins (h). Differences between means were considered statistically significant at $p < 0.05$.

In addition to the kinetic parameters, Figure 2 shows the cumulative gas production over 96 h of in vitro incubation for *M. sativa* and *A. melanoxylon*. A significantly higher gas production was observed for *M. sativa* at all time points from 8 h onwards ($p < 0.001$), reaching a maximum of 37.29 mL/0.2 g DM at 96 h. In contrast, *A. melanoxylon* exhibited a limited and gradual fermentation pattern, with a final value of only 12.15 mL/0.2 g DM. These results confirm the slower fermentative behavior of *A. melanoxylon* as already indicated by the kinetic parameters (Table 1), particularly the lower potential gas volume (b), the absence of a lag phase, and the reduced fermentation rate (c). This profile suggests a lower digestibility of *A. melanoxylon*, possibly related to its higher fiber and lignin contents.

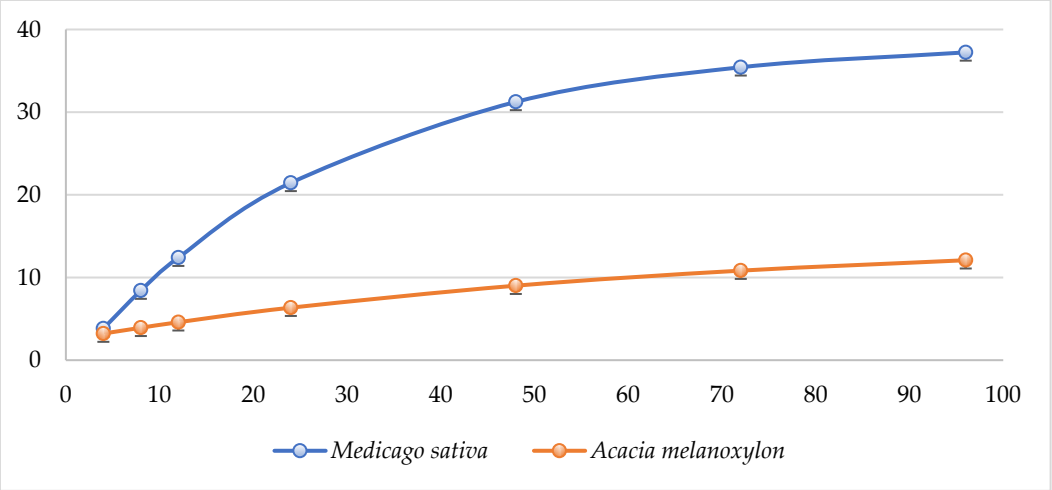


Figure 2. Cumulative in vitro gas production (mL/0.2 g DM) of *M. sativa* and *A. melanoxylon* over 96 h of incubation.

3.1.2. Effect of Ensiling on *M. sativa* Composition and Digestibility

A comparison between fresh *M. sativa* and the ensiled control (TC) is presented in Figure 3. Ensiling led to a slight but significant decrease in crude protein (from 25.98 ± 0.10% to 22.85 ± 0.19%, $p < 0.05$), and in vitro digestibility (IVDMD decreased

from 70.61 ± 0.46% to 62.61 ± 0.05%; IVOMD from 62.76 ± 0.14% to 54.14 ± 0.04%, $p < 0.05$). Fiber contents, particularly NDF and ADF, increased slightly after ensiling (NDF: 50.09 ± 0.08% to 53.94 ± 0.20%; ADF: 34.02 ± 0.19% to 35.29 ± 0.20%). These changes are consistent with typical fermentation losses and nutrient transformations during the ensiling process.

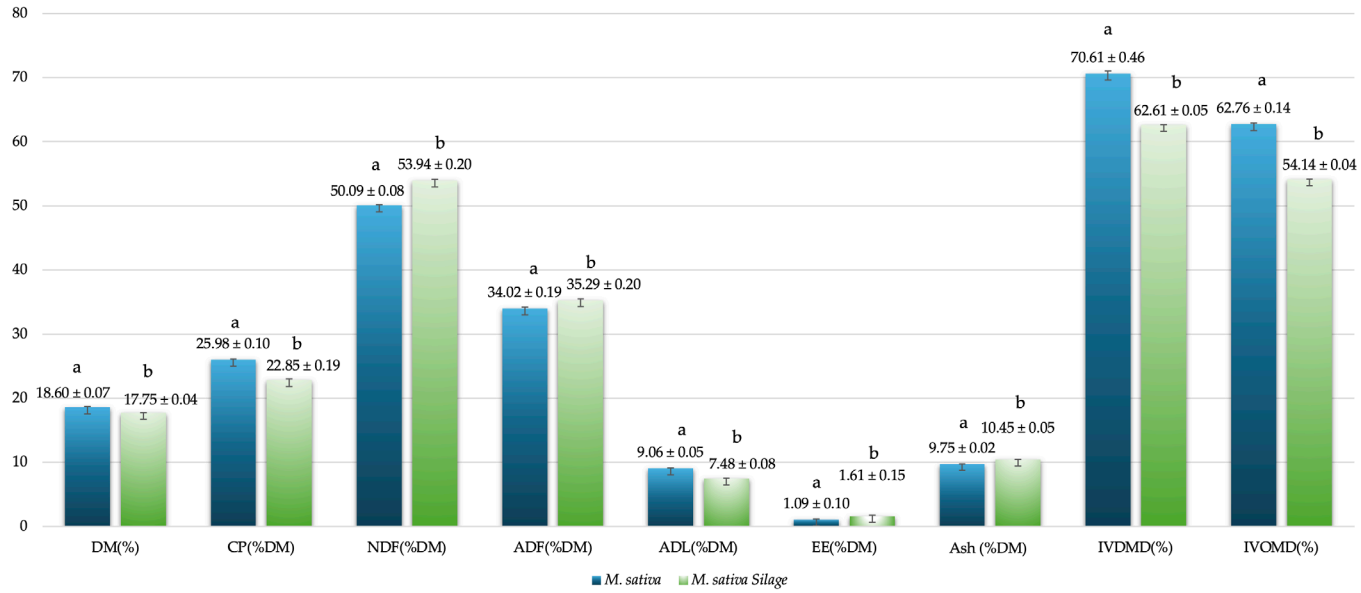


Figure 3. Chemical composition and digestibility parameters of *M. sativa* before and after the ensiling process (*M. sativa* Silage). Values are expressed as mean ± standard error (n = 3). Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$).

3.2. Chemical Analysis of M. sativa Silages with Different Inclusion Levels of A. melanoxylon

The chemical composition and in vitro digestibility of the experimental silages are presented in Table 2 and Figure 4. Significant differences ($p < 0.05$) were observed among treatments for most parameters. Dry matter (DM) content increased progressively with the inclusion level of *A. melanoxylon*, ranging from 17.75 ± 0.04% in the control (TC) to 28.45 ± 0.11% in T4. Conversely, crude protein (*CP*) content decreased with higher *A. melanoxylon* inclusion, with T4 presenting the lowest value (19.85 ± 0.09%).

Table 2. Chemical composition and in vitro digestibility of *M. sativa* silages with increasing levels of *A. melanoxylon* inclusion.

Parameters		Treatment				
		TC	**T1**	**T2**	**T3**	**T4**
DM (%)	Mean ± SEM CV (%)	17.75 ± 0.04 0.2	17.84 ± 0.09 0.48	19.65 ± 0.31 1.6	22.53 ± 0.06 0.28	28.45 ± 0.11 0.38
CP (%DM)	Mean ± SEM CV (%)	22.85 ± 0.19 0.82	23.45 ± 0.15 0.62	21.32 ± 0.15 0.7	21.31 ± 0.05 0.22	19.85 ± 0.09 0.45
NDF (%DM)	Mean ± SEM CV (%)	53.94 ± 0.20 0.37	53.09 ± 0.05 0.1	57.95 ± 0.13 0.22	58.36 ± 0.13 0.22	63.12 ± 0.08 0.13
ADF (%DM)	Mean ± SEM CV (%)	35.29 ± 0.20 0.57	42.08 ± 0.11 0.25	48.25 ± 0.15 0.31	48.70 ± 0.17 0.36	53.01 ± 0.17 0.31
ADL (%DM)	Mean ± SEM CV (%)	7.48 ± 0.08 1.01	9.74 ± 0.05 0.54	9.91 ± 0.05 0.53	9.93 ± 0.03 0.27	9.98 ± 0.03 0.27

Table 2. *Cont.*

Parameters		Treatment				
		TC	**T1**	**T2**	**T3**	**T4**
EE (%DM)	Mean ± SEM	1.61 ± 0.15	2.10 ± 0.05	1.43 ± 0.06	2.25 ± 0.07	1.62 ± 0.12
	CV (%)	9.41	2.52	4.25	3.03	7.71
ASH (%DM)	Mean ± SEM	10.45 ± 0.05	11.56 ± 0.23	10.28 ± 0.19	9.35 ± 0.09	7.53 ± 0.07
	CV (%)	0.44	2	1.83	0.98	0.87
IVDMD (%)	Mean ± SEM	62.61 ± 0.05	62.50 ± 0.06	56.79 ± 0.20	54.36 ± 0.07	48.02 ± 0.16
	CV (%)	0.08	0.1	0.36	0.13	0.33
IVOMD (%)	Mean ± SEM	54.14 ± 0.04	53.79 ± 0.14	47.99 ± 0.12	45.07 ± 0.12	40.61 ± 0.22
	CV (%)	0.06	0.26	0.25	0.28	0.54

Data are presented as mean ± standard error of the mean (SEM), based on three replicates per treatment. DM: Dry Matter; *CP*: Crude Protein; NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber; ADL: Acid Detergent Lignin; *EE*: Ether Extract; IVDMD: In Vitro Dry Matter Digestibility; IVOMD: In Vitro Organic Matter Digestibility; CV: Coefficient of Variation. Values are mean ± SEM (n = 3). Treatment pairwise differences (Tukey's HSD, $p < 0.05$) are shown in Figure 4 for each variable.

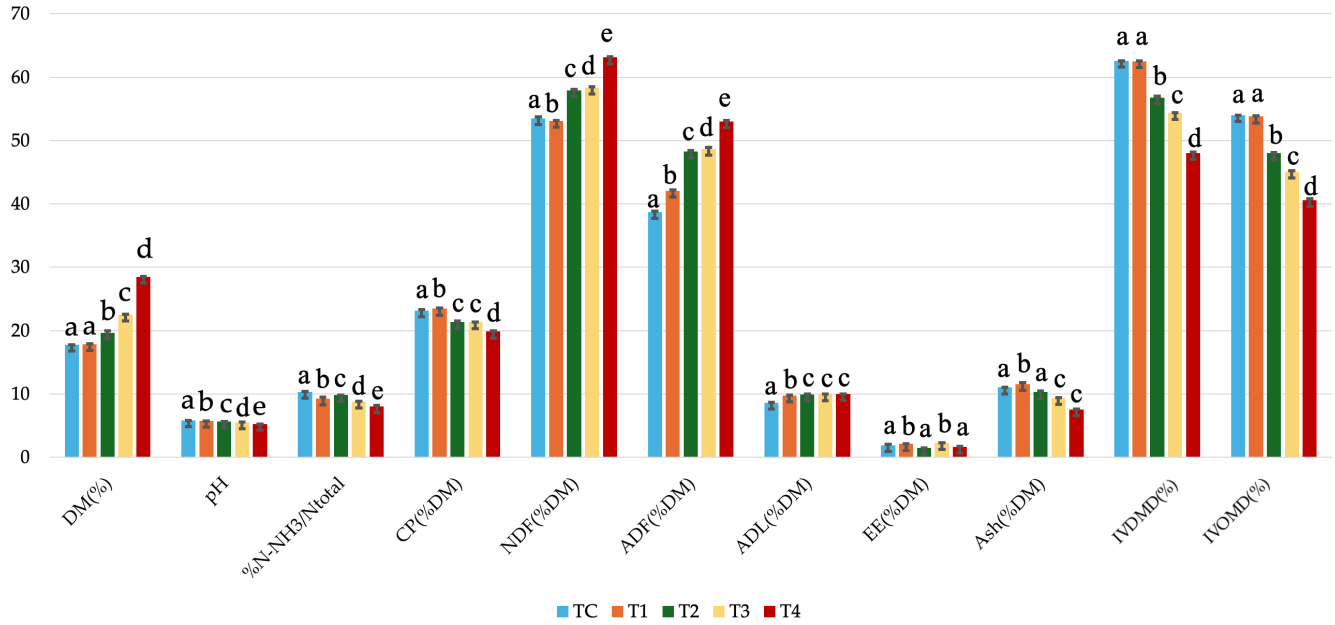


Figure 4. Chemical composition and in vitro digestibility of *M. sativa* silages with increasing inclusion levels of *A. melanoxylon*. Different letters above bars indicate significant differences between treatments ($p < 0.05$).

Fiber fractions also increased with the inclusion of *A. melanoxylon*. Neutral detergent fiber (NDF) rose from 53.94 ± 0.20% in TC to 63.12 ± 0.08% in T4, while acid detergent fiber (ADF) and acid detergent lignin (ADL) followed the same trend, reaching 53.01 ± 0.17% and 9.98 ± 0.03% in T4, respectively. These increases were accompanied by a decline in both in vitro dry matter digestibility (IVDMD) and in vitro organic matter digestibility (IVOMD), which decreased significantly ($p < 0.001$) in the highest inclusion treatments, particularly T4 (48.02 ± 0.16% and 40.61 ± 0.22%, respectively).

3.2.1. Fermentation Profile of Silages

The fermentation characteristics of the silages, including pH and ammonia nitrogen (NH_3-N/Total N), are shown in Table 3. A clear and significant decrease ($p < 0.001$) in pH was observed with increasing *A. melanoxylon* inclusion, from 5.86 ± 0.01 in TC to

4.53 ± 0.01 in T4. Similarly, the NH_3-N/Total N ratio followed a decreasing trend, ranging from 11.38 ± 0.10% in the control silage to 8.05 ± 0.10% in T4.

Table 3. pH and ammonia nitrogen (NH_3-N % of total N) content of *M. sativa* silages with increasing inclusion levels of *A. melanoxylon*.

Parameters		Treatment				
		TC	T1	T2	T3	T4
pH	Mean ± SEM	5.86 ± 0.01	5.72 ± 0.01	4.85 ± 0.01	4.76 ± 0.01	4.53 ± 0.01
	CV (%)	0.11	0.17	0.18	0.18	0.19
N-NH_3/N (%)	Mean ± SEM	11.38 ± 0.10	9.25 ± 0.20	9.82 ± 0.01	8.76 ± 0.08	8.05 ± 0.10
	CV (%)	0.84	2.19	0.10	0.89	1.22

Data are presented as mean ± standard error of the mean (SEM), based on three replicates per treatment. Treatment pairwise differences (Tukey's HSD, $p < 0.05$) are shown in Figure 4 for each variable.

These results suggest that A. melanoxylon inclusion improved the fermentation quality of the silages, promoting lower pH values and reducing proteolysis, as indicated by the lower ammonia nitrogen content. Despite the high fiber and lignin contents observed in the previous section, the ensiling process appears to have been effective across treatments.

3.2.2. Estimated Energy Content of Silages

The energy values of the silages, expressed as net energy for lactation (*NEL*) and metabolizable energy (*ME*), are presented in Figure 5. Both parameters followed a similar decreasing trend as the inclusion level of *A. melanoxylon* increased. The control treatment (TC) exhibited the highest energy values (3.13 ± 0.016 MJ/kg DM for *NEL* and 5.91 ± 0.010 MJ/kg DM for *ME*), while the lowest values were recorded in T4 (2.02 ± 0.012 MJ/kg DM for *NEL* and 4.45 ± 0.005 MJ/kg DM for *ME*). These results suggest that increasing proportions of *A. melanoxylon* in *M. sativa* silage consistently reduce its energetic value.

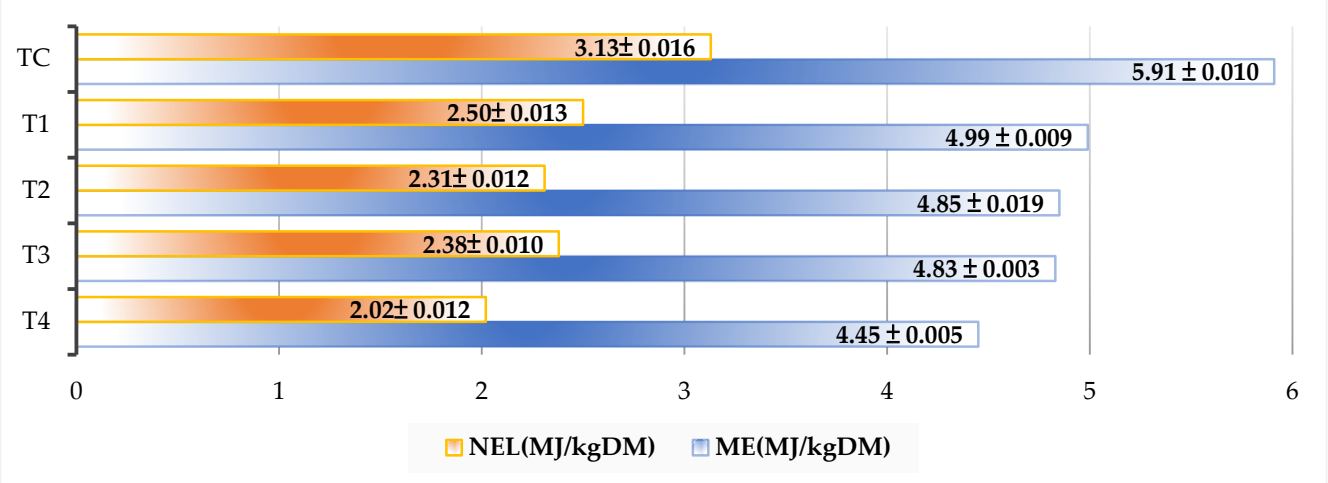


Figure 5. Net energy for lactation (*NEL*, MJ/kg DM) and metabolizable energy (*ME*, MJ/kg DM) of *M. sativa* silage (TC) and silages with increasing inclusion levels of *A. melanoxylon* (T1–T4). Bars represent mean ± standard error of the mean (SEM) from three replicates (n = 3).

3.2.3. Gas Production Kinetics and Fermentability of Silages

Gas production kinetics and cumulative gas production of *M. sativa* silages with increasing levels of *A. melanoxylon* inclusion are presented in Table 4. Significant differences were observed among treatments for the kinetic parameters *b* (volume of gas produced from the insoluble but fermentable fraction), *c* (rate of gas production), and

lag time, with $p < 0.001$. However, parameter *a* (gas produced from the soluble fraction) did not differ significantly between treatments ($p = 0.06$), indicating a comparable initial fermentation behavior.

Table 4. Gas Production Kinetics and Cumulative Gas Volume of *M. sativa* Silages with Increasing Inclusion Levels of *A. melanoxylon*.

	Treatment					*p* Value
	TC	**T1**	**T2**	**T3**	**T4**	
Kinetics of Reaction						
a (mL/0.2 g DM)	−1.95 ± 0.15	−0.87 ± 0.08	−0.15 ± 0.05	−1.25 ± 0.1	−0.03 ± 0.12	0.06
b (mL/0.2 g DM)	32.72 ± 0.48	28.43 ± 0.52	22.97 ± 0.23	25.08 ± 0.59	24.72 ± 0.22	<0.001
c (mL/h)	0.04 ± 0.002	0.02 ± 0.002	0.01 ± 0.001	0.03 ± 0.002	0.02 ± 0.001	<0.001
Lag t (h)	1.67 ± 0.06	1.31 ± 0.05	0.4 ± 0.08	1.86 ± 0.11	0.12 ± 0.11	<0.001
Gas Production (mL/0.2 g DM)						
4 h	2.75 ± 0.13	1.48 ± 0.12	1.44 ± 0.28	1.28 ± 0.4	2.18 ± 0.4	0.10
8 h	6.65 ± 0.17	3.47 ± 0.23	3.12 ± 0.24	3.47 ± 0.32	4.17 ± 0.14	<0.001
12 h	10.14 ± 0.31	5.36 ± 0.2	4.52 ± 0.1	5.59 ± 0.13	5.98 ± 0.16	<0.001
24 h	17.76 ± 0.26	10.35 ± 0.19	8.24 ± 0.14	10.64 ± 0.08	10.6 ± 0.26	<0.001
48 h	25.51 ± 0.05	17.2 ± 0.07	13.51 ± 0.1	16.91 ± 0.38	16.58 ± 0.18	<0.001
72 h	28.59 ± 0.27	21.26 ± 0.04	17.05 ± 0.25	20.32 ± 0.09	20.2 ± 0.17	<0.001
96 h	29.74 ± 0.26	23.9 ± 0.26	19.07 ± 0.21	21.95 ± 0.19	22.04 ± 0.09	<0.001

Values are presented as mean ± standard error of the mean. Kinetic parameters: a—gas production from the immediately soluble fraction; b—gas production from the insoluble fraction; c—gas production rate constant; Lag t—lag time before gas production begins. Differences were considered significant at $p < 0.05$.

For cumulative gas production, no significant differences were observed at 4 h of incubation ($p = 0.10$), but from 8 h onwards, treatments differed significantly ($p < 0.001$). The control treatment (TC) consistently exhibited higher gas volumes across all time points compared to silages with *A. melanoxylon* inclusion. Treatments T2 and T4 produced the lowest cumulative gas volumes at later incubation times, suggesting a reduction in fermentative activity with increasing levels of *A. melanoxylon*.

A general decreasing trend in gas production and fermentation rate was observed as the inclusion level of *A. melanoxylon* increased.

Figure 6 illustrates the cumulative gas production profiles over 96 h of in vitro fermentation for silages containing increasing inclusion levels of *A. melanoxylon*. The control treatment (TC), composed solely of *M. sativa*, showed the highest gas production throughout the incubation period, reaching 29.74 ± 0.26 mL/0.2 g DM at 96 h. In contrast, the inclusion of *A. melanoxylon* resulted in lower gas volumes, with T2 displaying the most pronounced reduction in fermentability. Treatments T1 and T3 exhibited similar gas production kinetics, while T4 produced intermediate values. Overall, the data indicate a negative dose-dependent effect of *A. melanoxylon* inclusion on gas production, supporting the results presented in Table 4.

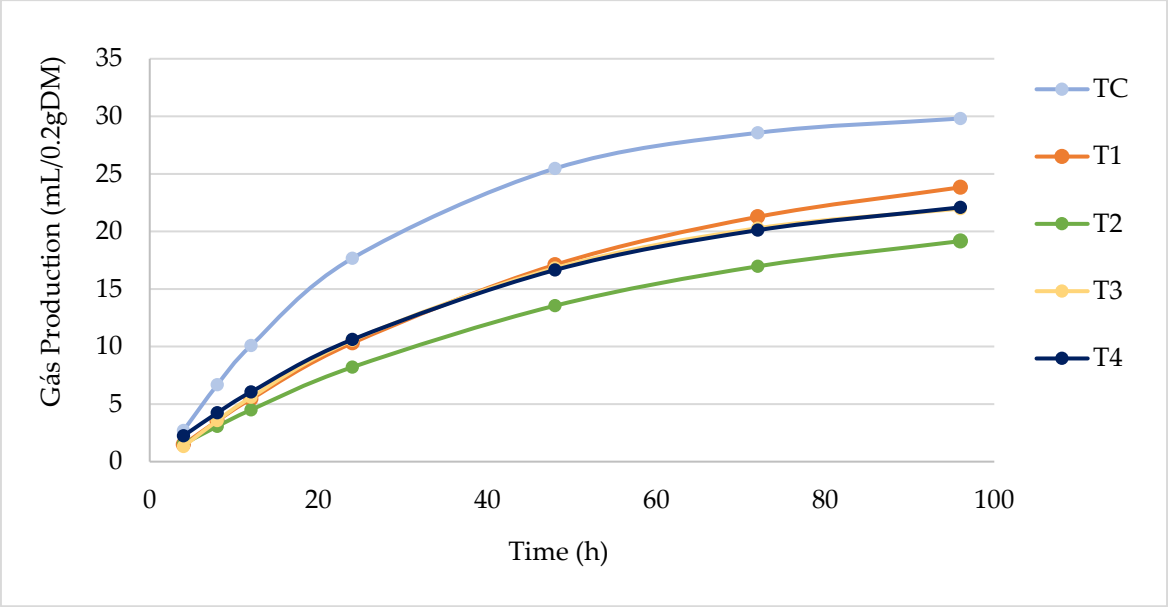


Figure 6. Cumulative gas production profiles during 96 h of in vitro fermentation of silages with varying *A. melanoxylon* inclusion levels.

4. Discussion

The present study aimed to evaluate the effect of including *A. melanoxylon* in *M. sativa* silage on chemical composition, fermentation quality, digestibility, energy value, and in vitro fermentability. Due to its high moisture content, *M. sativa* is prone to excessive effluent loss and undesirable fermentation when ensiled alone, particularly when harvested in the vegetative stage [29]. In this context, biomass sources with higher dry matter (DM) content, such as *A. melanoxylon*, may serve as effective structural complements to improve the physical and fermentative characteristics of the silage.

4.1. Impact of Ensiling on Fresh M. sativa Quality

A comparison between fresh *M. sativa* and the ensiled control (TC) confirmed typical alterations associated with the ensiling process. Slight but significant reductions were observed in crude protein content (from 25.98 ± 0.10% to 22.85 ± 0.19%, $p < 0.05$), as well as in digestibility parameters (*IVDMD* decreased from 70.61 ± 0.46% to 62.61 ± 0.05%; IVOMD from 62.76 ± 0.14% to 54.14 ± 0.04%, $p < 0.001$). Fiber contents, including NDF and ADF, increased slightly after ensiling (NDF: 50.09 ± 0.08% to 53.94 ± 0.20%; ADF: 34.02 ± 0.19% to 35.29 ± 0.20%), consistent with literature on proteolysis and fiber concentration due to fermentation losses [18,30]. These results highlight the importance of assessing any additional changes introduced by the inclusion of *A. melanoxylon* on top of the baseline modifications caused by ensiling itself.

Mechanistically, these shifts reflect the low water-soluble carbohydrate (WSC) content and high buffering capacity of *M. sativa*, which slow acidification and favour proteolysis/deamination; the conversion of true protein into non-protein nitrogen, including ammonia nitrogen (NH_3-N), helps explain the observed *CP* decrease. In parallel, the removal/fermentation of soluble components and effluent losses lead to an apparent concentration of cell-wall fractions rather than de novo fibre formation, accounting for the modest increases in NDF and ADF and for the penalties in IVDMD/IVOMD typically reported in alfalfa silages made at lower packing DM [6,18,31].

From a process viewpoint, these baseline responses in *M. sativa*—delayed pH decline, greater proteolysis and reduced digestibility—underscore the central role of pre-ensiling

DM management for achieving rapid acidification and preserving protein. They also provide a clear rationale for testing high-DM structural complements such as *A. melanoxylon*: by elevating DM they are expected to improve packing and anaerobiosis and to support lactic fermentation. At the same time, increasing the proportion of structural biomass inevitably raises fiber and lignin and dilutes readily fermentable components, which can depress digestibility, energy value, and in vitro gas production; therefore, the practical challenge is to identify low-to-moderate inclusion ranges that secure process benefits (higher DM, improved packing/anaerobiosis) while keeping nutritional penalties within acceptable bounds [7–11,17,18]

4.2. Chemical Composition and Gas Production Kinetics Prior to Ensiling

Prior to ensiling, *M. sativa* and *A. melanoxylon* showed distinct nutritional and fermentative profiles. As expected, *M. sativa* exhibited higher crude protein (CP), lower fiber fractions (NDF, ADF, and ADL), and superior in vitro digestibility (IVDMD and IVOMD), consistent with its classification as a high-quality forage legume [32,33]. In contrast, *A. melanoxylon* presented markedly higher DM and lignin contents, and correspondingly lower digestibility, limiting its value as a standalone forage source.

Fermentation kinetics further emphasized these differences. *M. sativa* showed significantly higher cumulative gas production and faster fermentation kinetics (higher b and c values), while *A. melanoxylon* produced minimal gas, exhibited no measurable lag phase, and had a lower fermentation rate. This profile is characteristic of woody species with high concentrations of structural carbohydrates and lignin [34,35]. The absence of a lag phase and the reduced fermentation rate are consistent with limited microbial access to lignified tissue [6].

Altogether, these results support the rationale for using *A. melanoxylon* only as a complementary biomass in ensiling mixtures. Its high DM content may aid in achieving optimal moisture conditions for ensiling, but its nutritional limitations suggest it should be used at restricted inclusion levels.

4.3. Effect of A. melanoxylon Inclusion on Silage Composition and Digestibility

Post-ensiling analyses revealed that increasing levels of *A. melanoxylon* inclusion resulted in a progressive rise in DM content, confirming its potential to improve silage structure and reduce effluent losses [36]. However, this benefit came at the cost of nutritional quality. *CP* content declined, while fiber fractions and ADL increased significantly, particularly in T3 and T4. These changes were reflected in reduced IVDMD and IVOMD values, indicating impaired microbial degradability of the silage substrate [37,38].

Interestingly, while ether extract (*EE*) content showed no clear trend, ash content decreased, likely due to the dilution of *M. sativa*'s higher mineral fraction. Despite these shifts, fermentation indicators such as pH and ammonia nitrogen ($N\text{-}NH_3/N$) showed favorable trends. The reduction in pH across treatments suggests that *A. melanoxylon* inclusion did not compromise lactic acid production; on the contrary, the drier biomass may have improved compaction and anaerobiosis [36,39]. The observed decrease in $N\text{-}NH_3/N$ ratios indicates reduced proteolysis and better protein preservation during ensiling [40,41].

4.4. Energy Value and In Vitro Fermentation After Ensiling

The metabolizable energy (*ME*) and net energy for lactation (*NEL*) values decreased progressively with *A. melanoxylon* inclusion, consistent with the reductions observed in digestibility and the increase in fiber fractions. The TC showed the highest energy values (5.91 MJ/kg DM *ME*; 3.13 MJ/kg DM *NEL*), while T4 exhibited the lowest (4.45 and 2.02 MJ/kg DM, respectively). This trend corroborates literature indicating that lignified or

phenolic-rich forages reduce energy availability due to limited fermentation and microbial access [42,43].

Cumulative gas production also declined significantly with higher *A. melanoxylon* inclusion. Treatments T2–T4 showed consistently lower gas volumes, particularly beyond 24 h of incubation, suggesting reduced rumen fermentation potential. The b and c parameters further confirmed reduced fermentability, while lag time was variable among treatments. This variability may be explained by differential microbial responses to secondary compounds, such as tannins, present in *A. melanoxylon* [44].

These results are consistent with findings from studies involving other woody legumes or phenolic-rich forages, where partial inclusion may aid in DM management but excessive levels compromise degradability and energy release [45].

5. Conclusions

This study evaluated *A. melanoxylon* not only as a nutritional diluent but primarily as a structural co-ensiling strategy in *M. sativa* silage, showing that improvements in dry matter and fermentation quality are accompanied by predictable reductions in digestibility, energy value, and in vitro gas production as inclusion increases. Low to moderate inclusion (6–12%) offered the best trade-off supporting ensiling conditions (higher DM, lower pH and NH_3-N) while keeping nutritional penalties within acceptable bounds, whereas higher inclusion markedly constrained fermentability and energy. These findings support the judicious, partial use of *A. melanoxylon* to enhance *M. sativa* ensilability and warrant in vivo validation.

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