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Effects of Replacing Alfalfa Hay with Amaranthus caudatus Hay on Digestibility, Methane Emissions, and Microbial Protein Efficiency in Ruminant Diets

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ABSTRACT: The purpose of the study was to evaluate the effects on fermentation parameters of replacing alfalfa hay in ruminant diets with amarant (Amaranthus caudatus) hay. Diets containing amaranth hay at 0%, 10%, 20%, and 30% were created using in vitro gas generation, preserving isocaloric and nitrogenic levels. There were significant changes in true digestible dry matter (TDDM), true digestibility (TD), gas and methane output per digested dry matter (DM), and microbial protein, but not in methane (CH_4) production. Substituting alfalfa with amaranth hay resulted in relatively higher microbial protein synthesis compared to gas and methane production, supporting the potential for up to 30% replacement. This change improved microbial protein and decreased methane output. To assess its effect on feed intake and overall animal production, more in vivo studies are required.

Keyword: *Amaranthus caudatus, In vitro digestibility, methane, microbial protein, partitioning factor*.

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INTRODUCTION

The nutrition of ruminant animals, such as cattle and sheep, plays a crucial role in their overall health and productivity (Gallo et al., 2015). These diets consist of a combination of grains, protein feeds, by-products, hay, and forages (Metzler-Zebeli, 2012). It is essential to evaluate the suitability of different feed sources to ensure optimal nutrition and health for ruminant animals. The nutritional value of a feed must be considered as a combination of numerous parameters that determine the nutrient intake of ruminant animals (Nogoy et al., 2020; Shadi et al., 2020). Assessing the in vitro digestibility of animal feeds provides valuable information on their potential utilization by ruminants and their ability to support maintenance and growth requirements (Gallo et al., 2015). Additionally, evaluating methane production is important due to the contribution of methane emissions from ruminant animals to greenhouse gas emissions and climate change (IPCC, 2001). Understanding rumen fermentation dynamics underscores the need for feed strategies that lower methane emissions and contribute to reducing the greenhouse effect (Palangi & Lackner, 2022; Curzaynz-Leyva et al., 2018).

Amaranth (*Amaranthus spp*.) comprises approximately 60-70 species, with around 40 species native to the Americas. It is cultivated in both temperate and tropical climates and is used both as a grain and a vegetable. Amaranth is highly nutritious, containing various vitamins and minerals, and is also used as animal feed (Alegbejo, 2013; Ampapon et al., 2022).

By substituting Amaranthus caudatus hay for alfalfa in ruminant diets, the study aims to ascertain how fermentation parameters like gas and methane production, true digestibility, partitioning factor, microbial protein, and the efficiency of microbial protein synthesis are affected.

MATERIALS AND METHODS

Experimental design

To determine the effect of amaranth hay on methane production and digestibility in diets, the *in vitro* gas production technique was used to simulate rumen fermentation (Menke et al., 1979). The feed ingredients used in the diets were obtained from a specialized feed mill. These ingredients were ground to pass through a 1 mm sieve and stored in airtight bags until use. Amaranth hay was substituted for alfalfa hay in the diets at levels of 0%, 10%, 20%, and 30%.

Feedstuffs Collection and Chemical Analysis

Sampling was conducted in June, with amaranth hay harvested from Balıkesir province. The dry matter (DM, method 930.15), crude protein (CP, method 954.01), total ash (method 942.05), and ether extract (EE, method 920.39) contents of the amaranth hay and other feed ingredients were determined using standard methods as specified by AOAC (1990). The CP content of amaranth hay was calculated based on its total nitrogen content (N x 6.25). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of the diets were determined according to the method described by Van Soest et al. (1991). The metabolizable energy (ME) and organic matter digestibility (OMD) of the amaranth hay and other feed ingredients were calculated according to Menke and Steingass (1979).

$$ME\ (MJ/kg\ dm) = 1.68 + 0.1418GP + 0.073CP + 0.217EE - 0.028CA \quad (1)$$

$$OMD\ (\%) = 14.88 + 0.8893GP + 0.448CP + 0.651CA \quad (2)$$

GP: 24-hour incubation results in gas production in the samples (200 mg/DM)
CP: Crude protein (%)
EE: Ether extract (%)
CA: Crude ash (%)

Table 1.

Ingredients	DM (%)	CA (%)	EE (%)	CP (%)	GP(ml)	ME(MJ/kgDM)	OMD (%)
Alfalfa	94.41	9.41	1.53	18.64	47.70	9.87	72.77
Amaranth	93.21	14.14	3.80	12.80	36.33	8.13	62.16
Soybean meal	91.12	7.33	2.41	52.86	55.74	13.76	82.97
Wheat straw	92.44	4.80	1.37	3.87	31.83	6.64	72.67
Oat grain	90.78	3.03	2.12	11.25	61.80	11.64	76.85

DM: Dry Matter. CA: Crude Ash. EE: Ether extract. CP: Crude Protein. GP: Gas production 200 mg DM, ME: Metabolic Energy. OMD: Organic Matter Digestibility.

TMR Preparation

The metabolizable energy (ME) levels and crude protein (CP) contents of the rations were formulated based on the nutrient requirements for 45 kg body weight sheep, as outlined by the NRC (2007) (Table 1). The rations were developed using the data solver add-in feature in Microsoft Excel (2016). Diets were prepared as isocaloric and isonitrogenous in order to see that the by-products released as a result of fermentation of the diets were due to partial replacement applied to the diet.

Rumen Fluid Collection

Rumen fluid for the experiment was sourced from a local slaughterhouse, following the methodology described by Palangi et al. (2024). The rumen fluid was collected from three healthy Awassi sheep, each weighing an average of 55 ± 5 kg and aged around 13 ± 2 months. The collection was carried out within 10 minutes after slaughter, filtered through cheesecloth, and kept at 38°C in anaerobic conditions. It was transported to the animal nutrition laboratory within 30 minutes of collection. The incubation medium was prepared with buffer solutions and rumen fluid, as outlined by Menke et al. (1979). The rumen fluid was combined with buffer solutions (reduced and mineral solutions) at a ratio of 1:2 (v/v).

Gas Production, Methane Measure and *In Vitro* Digestibility

Pre-calibrated 100 ml glass syringes (Model Fortuna, Häberle Labortechnik, Lonsee-Ettlenschieβ, Germany) were used to measure total gas volume and methane production during ruminal fermentation, according to the procedure established by Menke et al. (1979). Each syringe contained 500 mg of dry matter (DM) sample, and a mixture of buffer solution and rumen fluid in a 1:2 ratio (totaling 40 ml) was added. The syringes were incubated in a water bath at 39°C for 24 hours. After incubation, the total gas volume was recorded. Methane volume was measured using an infrared methane analyzer (Sensor Europe GmbH, Erkrath, Germany), following the method described by Goel et al. (2008). All treatments were conducted with four replicates to ensure the reliability and reproducibility of the results.

The methane volume (CH4, ml) was calculated using the formula:

Table 2.

Substitution rate (%)				
	0	**10**	**20**	**30**
Alfalfa hay	500	400	300	200
Amaranthus caudatus	-	100	200	300
Oil	40	40	40	40
Soybean meal	316.37	347.92	379.47	411.01
Oat grain	44.08	32.21	20.34	8.47
Wheat straw	73.53	53.86	34.18	14.50
Salt	10	10	10	10
Calcium carbonate	15	15	15	15
Min-Vit Mix	1	1	1	1
Total (g)	1000	1000	1000	1000
ME (kcal/kg DM)	2400	2400	2400	2400
CP (%)	18	18	18	18
EE (%)	5.70	5.95	6.21	6.46
CA (%)	7.49	8.03	8.62	9.21
NDF (%)	68.94	67.07	61.18	47.53
ADF (%)	23.28	21.14	17.85	13.22

ME: Metabolisable Energy, CP: Crude protein calculated. EE: Ether extract calculated. CA: Crude ash calculated. NDF: Neutral detergent fiber. ADF: Acid detergent fiber.

$$CH4\ (ml)\ =\ Total\ Volume\ Gas\ (ml)\ *\ CH4\ (\%) \quad (3)$$

Values for true digestible dry matter (TDDM), true digestibility (TD), partitioning factor (PF), microbial protein production (MP), and efficiency of microbial protein production (EMP) were determined according to the formulas provided by Blümmel et al. (1997a) and Vercoe et al. (2010).

$$TDDM\ =\ Dry\ Matter\ Incubated\ (mg)\ -\ Remaining\ Dry\ Matter\ (mg) \quad (4)$$
$$TD\ (/\%)\ =\ (TDDM/Dry\ Matter\ Incubated)\ *\ 100 \quad (5)$$
$$PF\ =\ (TDDM/Gas\ Production\ Incubated) \quad (6)$$
$$MP\ =\ (TDDM\ -\ (2.2\ *\ Gas\ Production\ Incubated)) \quad (7)$$
$$EMP\ =\ (((TDDM\ -\ (2.2*\ Gas\ Production\ Incubated))\ /TDDM)\ *\ 100 \quad (8)$$

Statistical Analysis

The statistical analysis of the data obtained in this study was conducted using one-way ANOVA in the SPSS 20.0 software package (SPSS, 2011). Differences between the means were determined using Duncan's multiple comparison test (Duncan, 1955).

RESULTS

Table 3 presents the effects of partially replacing alfalfa hay with amaranth hay on fermentation parameters and microbial protein values. There were no statistically significant differences in in vitro gas production (ml/incubated substrate) or methane emissions (CH4 ml/incubated substrate) between the diets (P>0.05). Both total gas production and the resulting greenhouse gas emissions are influenced by several factors, including the nutrition of ruminants, feed digestibility, and the type of feed material used. This study is novel in its exploration of increasing levels of amaranth hay in ruminant diets, specifically considering total gas and methane production based on a substrate solely derived from chemically characterized factory feed (as detailed in Table 1). While the partial substitution of alfalfa with amaranth hay did not significantly affect gas or methane production (ml/incubated substrate or %), it did lead to significant changes in TDDM, TD, gas production per digested DM, methane per digested DM, PF, MP, and EMP of the diets (P<0.001).

TDDM (mg) and TD (%) values ranged from 304.79 mg to 358.75 mg and 65.20% to 77.22%, respectively. Replacing alfalfa hay with amaranth hay significantly increased both TDDM and TD (P<0.05). However, this substitution also led to a reduction in gas and methane production per unit of digested DM. Gas production ranged from 253.45 ml to 301.61 ml per digested DM, while methane production ranged from 35.60 ml to 44.12 ml per

Table 3.

	Substitutions rate (%)					P values		
	%0	%10	%20	%30	SEM	L	Q	C
GP (ml)/ Incubated	91.91	92.25	92.58	90.91	1.08	.457	.227	.574
CH_4 (ml)/ Incubated	13.44	12.98	12.78	12.77	0.46	.171	.515	.963
CH_4 (%)	14.63	14.07	13.79	14.05	0.49	.233	.277	.875
TDDM (mg)	304.79^{a}	336.53^{b}	343.90^{b}	358.75^{c}	6.08	.000	.085	.137
TD (%)	65.20^{a}	72.40^{b}	74.06^{b}	77.22^{c}	1.26	.000	.061	.118
Gas (ml)/g digested DM	301.61^{c}	274.23^{b}	269.42^{b}	253.45^{a}	6.27	.000	.234	.128
CH_4(ml)/g digested DM	44.12^{b}	38.57^{a}	37.22^{a}	35.60^{a}	1.58	.000	.117	.397
PF	3.31^{a}	3.64^{b}	3.71^{b}	3.94^{c}	0.87	.000	.439	.158
MP (mg)	102.57^{a}	133.58^{b}	140.22^{b}	158.73^{c}	6.95	.000	.240	.138
EMP (%)	33.64^{a}	39.66^{b}	40.72^{b}	44.23^{c}	1.38	.000	.234	.128

^{abc}There is no difference between averages with the same symbol and in the same row. SEM: Standart Error Mean (P<0.05). GP: Gas Production 500 mg DM. CH_4 (ml) and CH4 (%): Methane production amount of rations 500 mg DM. TD: True digestibility. TDDM: True digestible dry matter 500 mg DM. PF: Partitioning factor. MP: Microbial protein. EMP: Efficiency of microbial protein.

digested DM. Additionally, the partial replacement of alfalfa hay with amaranth hay resulted in an increase in microbial protein (MP) within the diets.

DISCUSSION

Total Volume Gas and *In vitro* Digestibility

In vitro gas production methods are commonly used to assess the energy content of feedstuffs (Hariadi and Santoso, 2009; Ayasan et al., 2021; Besharati et al., 2021). The volume of gas generated *in vitro* is strongly linked to the degradable nutrients and the net energy released during the fermentation of organic matter in the incubated substrates (Zicarelli et al., 2011). In this study, gas production is expressed both as gas per incubated substrate and gas per digested dry matter (DM). Although replacing alfalfa hay with amaranth hay did not significantly alter gas production per incubated substrate, it significantly affected gas production per digested DM. Thus, relying on gas production per incubated substrate alone when comparing diets may lead to inaccurate conclusions, making gas production per digested DM a more useful and accurate metric (Selçuk et al., 2024)

The incubated substrate can either be utilized for gas production or microbial protein synthesis. Gas production is often viewed as an inefficient outcome of fermentation. Interestingly, despite the lack of a significant difference in gas production per incubated substrate, microbial protein synthesis increased when amaranth hay replaced alfalfa hay. This increase in microbial protein is difficult to explain solely based on gas production per incubated substrate. However, when gas production is presented in relation to digested or fermented dry matter (DM), the reason for the rise in microbial protein becomes clearer. Thus, expressing gas production as gas per digested DM during fermentation is considered more accurate and meaningful compared to gas production per incubated substrate (Navarro-Villa et al., 2011; Yanez-Ruiz et al., 2016). Enhancing the fixation of substrates by rumen microbes to improve intestinal protein supply while simultaneously reducing methane emissions is a widely recognized strategy in ruminant nutrition (Putri et al., 2024; Kim et al., 2024). Therefore, selecting forages with high in vitro true degradability but low gas production per unit of degraded substrate appears to be a sound recommendation (Olatunji & Garba, 2013; Sakita et al., 2020).

As indicated in Table 3, the partial replacement of alfalfa hay with Amaranthus hay in the diets led to a statistically significant increase in PF value ($P<0.01$). Theoretically, an increase in PF value reflects higher voluntary feed intake and improved microbial efficiency, indicating that a greater amount of degraded substrate is being incorporated into microbial biomass (Blümmel et al., 1997b; Araújo et al., 2012; Sembiring & Baba, 2022).

Table 3 also shows that replacing alfalfa hay with amaranth hay increased the true digestibility of dry matter (TDDM) in the diets. This improvement in TDDM is likely linked to the neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents listed in Table 1. As the inclusion of amaranth hay increased, the NDF and ADF levels decreased. However, this did not result in higher gas production. Instead, the microorganisms were provided with a more digestible substrate that generated less gas and, in turn, enhancing microbial protein synthesis. The data suggest that incorporating amaranth hay into the diets shifted the fermentation process from gas production toward microbial protein synthesis. Adjusting the fermentation process in this way is beneficial for improving the efficiency of nutrient utilization in the rumen. Typically, increased gas production during fermentation is associated with lower feed utilization efficiency. The more gas that is produced, the less efficient the feed utilization becomes. Microbial efficiency is defined as the proportion of substrate energy that is incorporated into microbial cells, and it is inversely related to the production of short-chain fatty acids (SCFA). Microbial efficiency determines the availability of microbial protein, which allows the animal to make use of non-protein nitrogen via rumen fermentation. The net metabolizable protein available to the animal consists of truly digestible microbial protein and the undegraded feed protein (Thirumalesh & Krishnamoorthy, 2013). As shown in Table 3, with increased amaranth hay inclusion, the metabolic pathways shift from gas production to microbial protein synthesis.

The way methane production is presented, much like gas production, is essential for accurate interpretation. As shown in Table 3, methane production expressed per incubated substrate or as a percentage reveals no significant difference between the diets. However, when methane production is calculated per digested substrate, a significant reduction is observed. Replacing alfalfa hay with amaranth hay clearly results in a notable decrease in methane emissions. This reduction is likely linked to the carbohydrate profile present in amaranth hay. However,

a more detailed analysis of the carbohydrate composition of amaranth hay would provide valuable insights and help clarify the observed decrease in methane production. The lack of this specific analysis is a limitation of the current study in explaining the reduction in methane emissions.

CONCLUSION

In conclusion, the fermentation pattern was changed by replacing alfalfa hay with amaranth hay, which increased microbial protein synthesis while decreasing gas and methane generation. Because amaranth hay is highly digested and has anti-methanogenic qualities, it can replace up to 30% of alfalfa hay in ruminant diets, lowering methane emissions and boosting the generation of microbial protein. To assess the effect of this substitution on feed intake and overall animal performance, more in vivo research is necessary.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

All the authors of this manuscript have contributed significantly towards the execution of this work.

DATA AVAILABILITY

All relevant data are within the manuscript, and the datasets generated and/or analysed during this study are available from the corresponding author upon reasonable request.

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