Middle East Journal of Agriculture Research
Volume: 14 | Issue: 01| Jan. – Mar.| 2025

EISSN: 2706-7955 ISSN: 2077-4605
DOI: 10.36632/mejar/2025.14.1.11
Journal homepage: www.curresweb.com
Pages: 163-171

Assessing the efficacy of graded levels of Azolla (*Azolla pinnata*) on nutritive value and methane production: An *in vitro* rumen fermentation study

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Received: 15 Jan. 2025 **Accepted:** 10 Mar. 2025 **Published:** 30 Mar. 2025

ABSTRACT

This study aimed to investigate the effect of dried Azolla (*Azolla pinnata*) graded levels on nutritive value and methane production using the rumen *in vitro* gas production technique. The experiment consisted of three treatments. The first treatment (T1) was a mix of 30% alfalfa hay and 70% concentrate feed mixture (CFM) as a control. The second (T2) and third treatments (T3) were the same as the control group, but 50% and 100% of soya bean meal (part of CFM) were replaced with dried Azolla for T2 and T3, respectively. The data showed that the inclusion of gradually levels of Azolla in the experimental rations didn't show negative effects on fermentation and nutritive value. Total gas production (GP) after incubation for 24 hours, *in vitro* dry matter degradation (IVDMD), *in vitro* organic matter degradation (IVOMD) and neutral detergent fiber degradation (NDFD) had similar values among the tested rations, but without significant differences. On the other hand, graded levels of Azolla resulted in a significant ($P<0.05$) reduction in metabolizable energy (ME), total digestible nutrients (TDN) and methane production. TDN decreased by 2.63 and 3.50% for T2 and T3 compared to T1, respectively. Methane production was reduced by 31.92 and 47.30% for T2 and T3 compared to T1 respectively. Generally, increasing Azolla levels had no deleterious effects on nutritive value and resulted in a higher reduction in methane production.

Keywords: Azolla, nutritive value, methane production, rumen, *in vitro*, gas production.

1. Introduction

The massive growth of the human population, climate change, wars, and unexpected outbreaks increase food demand that creates severe competition between humans and animals on limited cultivated lands for food or feed production. So, using unconventional feedstuffs in animal feeding practices can provide affordable and sustainable solutions, especially in developing countries (Chisoro *et al*., 2023 and Khidr *et al*., 2024).

Azolla (also called freshwater fern, mosquito fern and duckweed fern) is one of the unconventional feeds which has a promising future to be used for feeding different livestock species and has even been proposed (in cooked form) to be on the human space habitation diet on Mars (Katayama *et al*., 2008 and Rashad, 2021).

Azolla is considered a good source of crude protein because of its successful symbiotic relationship between Azolla and *Anabaena Azolla*e (one of blue-green algae) for nitrogen fixation. Azolla can be applied as a biofertilizer, animal feed, anti-fungal and growth activator in hydroponic sprouting barley, water purifier, biological herbicide against mosquito larvae and atmospheric carbon dioxide (CO_2) sequestration (Ravi *et al*., 2018; Alrefaey *et al*., 2019; Rashad, 2021; El Naggar and El-Mesery, 2022).

Methane production from ruminants has nutritional and environmental concerns. Ruminants lose about 2 to 12% of gross energy as methane. Additionally, methane is one of the greenhouse gases that

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EISSN: 2706-7955 ISSN: 2077-4605 *DOI: 10.36632/mejar/2025.14.1.11*

are responsible for global warming phenomena and its worldwide negative effects (Johnson and Johnson, 1995 and Belanche *et al*., 2025).

Therefore, many feed additives such as ionophores, nitrate and plant secondary metabolites are recommended to be methane mitigators, but incorrect dosage of the ionophores or nitrate can cause animal toxicity and even death. Additionally, using ionophores for long periods may raise antibiotic resistance hazards (Abdelbagi *et al*., 2023 and Ekinci *et al*., 2023). Plant secondary metabolites such as phenolic compounds can decrease methane production through many modes of action like increasing ruminal propionic acid and decreasing ruminal protozoa concentration (Dai *et al*., 2022 and Rabee *et al*., 2024). Azolla has a high content of secondary metabolites like phenolic compounds (Tran *et al*., 2020 and Bouattou *et al*., 2024) that may be responsible for decreasing methane production in rations containing Azolla (Jayasuriya *et al*., 1988).

Studies that investigated both nutritive value and methane production under the effect of Azolla inclusion in ruminant feeding are few. So, this study aimed to investigate the effects of graded levels of dried Azolla (*Azolla pinnata*) on nutritive value and methane production.

2. Materials and Methods

2.1. Azolla cultivation and collection

Azolla was cultivated in South Sinai research experimental station - Desert Research Center (DRC) - Egypt. Azolla cultivation was explained in steps and illustrated in figure (1) as the following:
Azolla cultivation steps:

1- Digging a basin with a width of 2.5 m, a length of 10 m and a depth of approximately 20 cm (with a total volume equal to 5 m^3)
2- Covering the basin using a thick plastic sheet with secured ends and sides
3- Preparing the nutrient solution, which consists of two liters of soaked organic fertilizer + 200 grams of superphosphate as in organic fertilizer /1m^3 of water in the basin
4- Filling the basin with clean and fresh irrigated water (in case of using tap water, store it in an open container for at least one day to chlorine evaporation)
5- Planting Azolla in the basin to cover about 20% of the basin area
6- Harvesting Azolla when the basin is fully covered with Azolla (It takes about 7-10 days)
7- Drying Azolla by spreading it on plastic sheets for air drying under indirect sunlight and then storing it for further applications.

Fig. 1: Azolla cultivation steps

2.2. Experimental design

An *in vitro* experiment was designed to make a comparison between three treatments of total mixed rations (TMR) with two substitution levels of dried Azolla (the chemical composition of Azolla is shown in Table 1) according to El-Hawy (2024) as the following:

T1 Control ration: 30% Alfalfa (*Medicago sativa*) hay and 70% concentrate feed mixture (CFM),
T2: Control ration was partially substituted 50% of soya bean meal with dried Azolla and,
T3: Control ration was partially substituted 100% of soya bean meal with dried Azolla.

The substitution of soya bean meal by Azolla was chosen because soya bean meal as protein source is considered as one of the highest priced imported feed ingredients, and Azolla is considered as an affordable local source of protein that can be produced at farm level.

Table 1: Chemical composition of dried Azolla (% on DM basis).

DM	OM	Ash	CP	EE	NDF	ADF	GE*
92.33	84.46	15.54	18.39	6.67	57.42	31.48	17.47

DM: dry matter, OM: organic matter, CP: crude protein, EE: ether extract, NDF: neutral detergent fiber, ADF: acid detergent fiber, GE (MJ/Kg DM): gross energy. *Calculated value.

2.3. Rumen *in vitro* gas production

In vitro gas production was conducted according to Menke and Steingass (1988) and modified by Ismail *et al.* (2018) to evaluate the effect of different Azolla levels on rumen fermentation characteristics and methane production. For obtaining rumen microorganisms **(**inoculum), rumen fluid was collected from at least three rumens of slaughtered local Egyptian sheep breeds fed on berseem hay (*Trifolium alexandrinum*) ration. The slaughterhouse was near Cairo **-** Egypt. Each treatment was tested in four replicates accompanied by four blank vessels (no substrate). Samples (about 400 mg) of tested rations with different Azolla levels were added separately to the 125 ml incubation vessels. Each vessel was filled with 40 ml of a mixture of 1:3 (v/v) rumen fluids/buffer solution. All vessels were sealed and incubated at 39°C for 24h with shaking then fermentation processes were terminated by putting the glass vessels in cold water. All vessels were filtered in fiber filter bags with 25-micron porosity (ANKOM- USA). The residues in the bags were dried at 70° C in the oven for 48 h to calculate dry matter degradation (IVDMD) then neutral detergent fiber (NDF) was determined in filter bags to calculate NDF degradation (NDFD). Rumen fluid pH was measured using a digital pH-meter. The overall volume of the total gas produced was determined using a glass syringe (100 ml capacity) according to Gao *et al*. (2023). Methane (CH_4) and carbon dioxide (CO_2) gases were measured at 24h of incubation using Gas-Pro detector (Gas Analyzer Crowcon, Model Tetra3, Abingdon, UK) according to Kholif *et al*. (2022). Methane gas (ml) was converted into mass (g) then converted into energy (MJ) according to Ku-vera *et al*. (2013). Quantitative analysis of ammonia-nitrogen (NH_3-N) concentration was carried out as described in the kit's pamphlet of the Biodiagnostic company, Egypt. *In vitro* organic matter degradation (IVOMD), Short chain fatty acids (SCFA) concentrations, metabolizable energy (ME), total digestible nutrients (TDN), and microbial protein (MP) were calculated using equations.

2.4. Chemical analysis

Proximate analysis of feeds including dry matter (DM), total ash, organic matter (OM), crude protein (CP) and ether extract (EE) were determined according to AOAC (2005). Fiber fractions (neutral detergent fiber (NDF) and acid detergent fiber (ADF)) were conducted according to Van Soest and Robertson (1985) using ANKOM Model 220 Fiber Analyser (Macedon, NY, USA). Gross energy (GE) was calculated using equation.

2.5. Calculation

Gross energy (GE):

GE (MJ/KgDM) = 0.0176 OM (g/kg) + 0.0064 CP(g/kg) + 0.0214 EE(g/kg) according to SCA (1990).

Middle East J. Agric. Res., 14(1): 163-171, 2025
EISSN: 2706-7955 ISSN: 2077-4605 *DOI: 10.36632/mejar/2025.14.1.11*

***In vitro* dry matter degradation (IVDMD):**
IVDMD% = [(feed sample weight before incubation) – (feed sample weight after incubation - blank) / (feed sample weight before incubation)] *100.
The values of IVDMD% were calculated according to Tilley and Terry (1963).

***In vitro* organic matter degradation (IVOMD):**
IVOMD % = 24.59 + 0.7984*GP + 0.0496*CP
IVOMD was calculated according to Menke and Steingass (1988) where IVOMD is *in vitro* organic matter degradation, GP is 24h net gas production (ml/200 mg DM) and CP is crude protein percent.

Metabolizable energy (ME):
ME (MJ/kg DM) = 2.20 + 0.136*GP (ml/200 mg DM) + 0.057*CP (DM%)
ME was calculated according to Menke and Steingass (1988) where ME is the metabolizable energy, GP is 24h net gas production (ml/200 mg DM) and CP is crude protein%

Total digestible nutrients (TDN):
TDN% = [ME (MCal/kg DM) + 0.45] / 0.0445309. TDN was calculated according to according to NRC (1989) where TDN is total digestible nutrients.

Short chain fatty acids (SCFA):
SCFA (mM) = 0.0239*GP- 0.0601
Where SCFA is short chain fatty acids. GP is 24h net gas production (ml/200 mg DM) using the equation described by Getachew *et al*. (2000).

Rumen microbial protein (MP):
MP g/ Kg DOM = (19.3 * IVOMD %*6.25)/100.
MP was calculated according to Czerkawski (1986) where MP is rumen microbial protein.

2.6. Statistical analysis

One way analysis of variance (ANOVA) was used to differentiate among means ($P< 0.05$). Duncan's new multiple range test (Duncan, 1955) was used to compare between means. The General Linear Model (GLM) of SAS (1996) was applied.
The following statistical model was adopted:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where: Yij = observation, μ = over all mean, Gi = the effect of the treatments (G = 1, 2, 3; 1 = control; 2 = 50% substitution from soya bean meal by Azolla; 3 =100% substitution from soya bean meal by Azolla) and eij = experimental error, assumed to be randomly distributed (0, $\sigma 2$).

3. Results and discussion

Chemical composition of rations with different Azolla levels is presented in Table (2). Increasing Azolla levels resulted in increasing EE, NDF and ADF content. On the other hand, increasing Azolla levels resulted in decreasing CP and GE content. These differences in chemical composition among the tested rations were related to the chemical composition of Azolla (Table 1). Generally, the variations in the chemical composition of Azolla were dependent on many factors, such as Azolla species and cultivation conditions (El Naggar and El-Mesery, 2022).

3.1. Effect of graded levels of Azolla on *in vitro* fermentation, gas production and nutritive value

The values of *in vitro* fermentation, gas production and nutritive value of different Azolla levels are presented in Table (3). Increasing levels of Azolla had no effects on gas production and the overall mean of treatments was 123.66 ml/0.4g for 24h of incubation. The same trend was noted by Kavya *et al*. (2014) who reported that graded levels of Azolla (0, 3, 6 and 9%) that replaced from mixed ration (Paddy straw and CFM) resulted in non-significant differences in gas production at 24h even with different roughage types such as ragi straw, maize stover and husk and sorghum stover. The values of *in vitro* dry matter degradation (IVDMD), *in vitro* organic matter degradation (IVOMD) and neutral detergent fiber degradation (NDFD) were similar among the treatments (with non-significant differences) and averaged 56.16, 74.72 and 40.38%, respectively. This indicated that increasing Azolla levels didn't negatively affect the values of IVDMD, IVOMD and NDFD%.

Table 2: Chemical composition of different Azolla experimental rations (% on DM basis).

Item	T1	T2	T3
DM	92.50	92.59	93.27
OM	90.81	89.64	88.65
Ash	9.19	10.36	11.35
CP	20.10	15.65	12.22
EE	4.49	4.81	6.29
NDF	48.70	54.25	60.35
ADF	17.72	20.56	24.10
GE*	18.23	17.81	17.73

T1: Control ration, T2: Replacing 50% of soya bean meal from control ration with dried Azolla, T3: Replacing 100% of soya bean meal from control ration with dried Azolla. DM: dry matter, OM: organic matter, CP: crude protein, EE: ether extract, NDF: neutral detergent fiber, ADF: acid detergent fiber, GE (MJ/Kg DM): gross energy. *Calculated value.

Table 3: Effect of graded levels of Azolla on gas production and nutritive value of experimental rations.

Item	T1	T2	T3	±SEM	P- value
GP (ml/0.4g DM)	124.26	122.69	124.04	0.558	0.5074
GP (ml/g IVDMD)	619.60	584.10	593.40	7.859	0.1622
GP (ml/g IVOMD)	454.90^{c}	460.20^{b}	467.80^{a}	1.714	0.001
pH	6.00	5.97	5.98	0.012	0.475
SCFA (mmol)	1.43	1.41	1.42	0.007	0.641
NH_3-N (mg/100 ml)	11.29^{a}	11.11^{a}	10.14^{b}	0.200	0.021
IVDMD%	55.70	56.74	56.04	0.396	0.594
IVOMD%	75.19	74.34	74.63	0.231	0.3431
NDFD%	39.37	40.85	40.92	0.426	0.272
TDN%	73.41^{a}	71.48^{b}	70.84^{b}	0.379	0.002
MP (g/Kg IVOMD)	90.70	89.68	90.02	0.279	0.3449

Means followed by different superscripts within the same row are significantly different ($P \leq 0.05$). T1: Control ration, T2: Replacing 50% of soya bean meal from control ration with dried Azolla, T3: Replacing 100% of soya bean meal from control ration with dried Azolla. SEM: standard error of the mean. GP: gas production, IVDMD: *in vitro* dry matter degradation, IVOMD: *in vitro* dry matter degradation, SCFA: Short chain fatty acids, NH_3-N: ammonia nitrogen, NDFD: neutral detergent fiber degradation, TDN: total digestible nutrients, MP: microbial protein.

Ruminal ammonia concentration decreased significantly ($P<0.05$) by 1.59 and 10.19% for T2 and T3 compared to T1 and this was in parallel with increasing Azolla levels and with decreasing CP content (Table 2). High crude protein content in ration resulted in high ruminal ammonia concentration (Cone and Van Gelder, 1999). Additionally, low ruminal ammonia concentrations in Azolla treatments (T2 and T3) versus control (T1) can be explained by low protein solubility in Azolla as reported by Jayasuriya *et al*. (1988) who described that ruminal ammonia decreased by 54.46% when using barley straw and Azolla instead of the same ration but with wheat bran due to low protein solubility of Azolla (11.4%) compared to wheat bran (28.5%) in the same study. The same results were obtained by Parashuramulu *et al*. (2013) and Indira and Ravi (2014). They found that the soluble protein of Azolla equaled to 18.22 and 16.58%, respectively.

The values of TDN decreased significantly ($P<0.05$) with increasing Azolla levels. Moreover, TDN% in T2 and T3 were lower than T1 by 2.63 and 3.50%, respectively.

3.2. Effect of graded levels of Azolla on energy utilization and methane production

Energy utilization and methane production values are shown in Table (4). Increasing Azolla significantly (P<0.05) decreased digestible energy (DE) and metabolizable energy (ME). It can be noticed that DE decreased by 2.99 and 4.03% and ME decreased by 3.4 and 4.1% for T2 and T3 compared to T1, respectively. Gradually increase of Azolla levels resulted in significantly decreased values of methane production. Conversely, CO_2 production significantly increased in parallel with increasing azolla levels. Methane energy represented a significant (P<0.05) loss of GE (8.93, 6.15 and 4.86%) and of DE (11.32, 7.85 and 6.25%) for T1, T2 and T3, respectively. That agreed with Moss *et al*. (2000) and Leng (2018) who reported that ruminants lose on average 8.5% of their GE and 12.3% of DE as methane. It can be concluded that, increasing Azolla levels decreased the energy loss as methane from both GE (31.13 and 45.80%) and DE (30.65 and 43.99%) in T2 and T3 compared to T1. Methane production values decreased significantly by 31.92 and 47.30% in T2 and T3 compared to control (T1). In the same line, Jayasuriya *et al*. (1988) reported that replacing wheat bran with Azolla in a mixture with barley straw reduced methane production by 81.81%. Moreover, Lester *et al*. (2024) reported that the inclusion of Azolla by 20% of oaten chaff versus 100% of oaten chaff *in vitro* experiment reduced methane production by 3.4%. The variation between *in vitro* studies in methane mitigation with Azolla inclusion may be due to different chemical compositions and levels of Azolla and other tested feedstuffs as well as donor animals (ruminant species) of rumen liquor and *in vitro* experimental conditions.

Table 4: Effect of graded levels of Azolla on energy utilization and methane production of experimental rations.

Item	T1	T2	T3	±SEM	P- value
DE (MJ/Kg DM)	14.38^{a}	13.95^{b}	13.80^{b}	0.086	0.0017
ME (MJ/Kg DM)	11.80^{a}	11.44^{b}	11.32^{b}	0.071	0.0019
ME/GE	0.65	0.64	0.64	0.002	0.224
CH_4 (ml/0.4g DM)	16.51^{a}	11.24^{b}	8.70^{c}	1.007	<.0001
CH_4 (ml/g DM)	41.28^{a}	27.78^{b}	21.85^{c}	2.514	<.0001
CH_4/GP%	13.29^{a}	9.14^{b}	7.03^{c}	0.809	<.0001
CH_4 (ml/g IVDMD g)	82.54^{a}	52.82^{b}	41.77^{c}	5.370	<.0001
CH_4 (ml/g IVOMD g)	60.46^{a}	41.67^{b}	33.06^{c}	3.556	<.0001
CH_4/GE%	8.93^{a}	6.15^{b}	4.86^{c}	0.526	<.0001
CH_4/DE%	11.32^{a}	7.85^{b}	6.25^{c}	0.658	<.0001
CH_4/ME%	13.81^{a}	9.58^{b}	7.63^{c}	0.802	<.0001
CO_2 (ml/0.4g DM)	42.63^{b}	48.91^{a}	47.69^{a}	1.043	0.013
CO_2/CH_4	3.23^{c}	5.42^{b}	6.78^{a}	0.454	<.0001

Means followed by different superscripts within the same row are significantly different (P≤0.05). T1: Control ration, T2: Replacing 50% of soya bean meal from control ration with dried Azolla, T3: Replacing 100% of soya bean meal from control ration with dried Azolla. SEM: standard error of the mean. DE: digestible energy, ME: metabolizable energy, GE: gross energy, CH_4: methane, IVDMD: *in vitro* dry matter degradation, IVOMD: *in vitro* dry matter degradation, CO_2: carbon dioxide.

Decreasing methane production with increasing Azolla levels can be explained by many factors in Azolla such as fat percent, fatty acid profile and secondary metabolites (total phenolic, tannins and flavonoids).

Generally, there is a positive relationship between methane production and total protozoal count. Rumen protozoa provide a host for methane producing bacteria (which live on and within protozoa), protection from oxygen and supplying with metabolic hydrogen that activate methanogenesis hence increase methane production (Newbold *et al*., 1995 and Dai *et al*., 2022).

In the present study, crude fat (ether extract) increased by increasing Azolla levels from 4.49% in control group to 4.81 and 6.29% in T2 and T3, respectively (Table 2). Giger-Reverdin *et al*. (2003) reported that increasing dietary ether extract was negatively correlated with ruminant methane production. Moreover, Chandrababu *et al*. (2024) reported that Azolla (*Azolla pinnata*) had about

36.87% of total fatty acids as unsaturated fatty acids with a higher concentration (13.54%) of Eicosapentaenoic acid (C20:5(3)) that decreased significantly acetate and acetate:propionate ratio (Toral *et al*., 2017). Increasing unsaturated fatty acids creates competition in consuming metabolic hydrogen through ruminal biohydrogenation bacteria and methanogenic bacteria. Additionally, unsaturated fatty acids are toxic to both protozoa and methanogenic bacteria (Hegarty, 1999 and Króliczewska *et al*., 2023). Moreover, Ebeid *et al*. (2020) reported that using gradual levels of *Camelina sativa* oil (from 0 up to 8%) with a total mixed ration (*in vitro* study) resulted in decreased methane production with low concentrations of both protozoa and methanogenic bacteria.

Azolla pinata extract had many phenolic compounds such as Catechin, Syringic acid and Rosmarinic acid (Alrefaey *et al*., 2019). Rosmarinic acid decreased methane production by 15% compared to control. It decreased methyl-coenzyme M reductase that involved in the metabolic pathway of methane production in methanogenic bacteria and the most dominant genus (Methanobrevibacter) of methanogenic bacteria also decreased (Janssen and Kirs, 2008 and Liu *et al*., 2024). Additionally, Catechin decreased methane production through H_2 sinks via cleavage of ring structures of Catechin and decreased total protozoa and methanogens (Oskoueian *et al*., 2013 and Patra *et al*., 2017).

In addition, environmental and nutritional stress stimulate the synthesis of total phenol, flavonoids and condensed tannins in Azolla by 2, 4.7 and 2.7 times compared to non-stressed azolla (Tran *et al*., 2020). These stressful conditions can be provided to produce Azolla with high phenolic compounds content. So, Azolla can not only be used as a feed supplement on a small scale on farms, but it can also be used as an organic feed additive that has potential for reducing methane production without deleterious effects on feed utilization which may support farmers to adopt this ruminant feeding practice for methane mitigation (Grainger and Beauchemin, 2011).

4. Conclusion

It can be concluded that gradually increasing levels of Azolla had no negative effects on nutritive value (IVDMD, IVOMD and TDN). Additionally, increasing Azolla levels resulted in a severe reduction in methane production that reached 47.30% compared to the control ration. Furthermore, future *in vivo* studies are strongly recommended to study Azolla as an affordable and organic methane inhibitor.

5. Acknowledgement

The authors thank all team members of the research program: "Livestock development through feed production and processing" in the Animal and Poultry Production Division, Desert Research Center (DRC), Egypt for their valuable support.

References

Abdelbagi, M., R. Ridwan, A. Fitri, and A. Jayanegara, 2023. Performance, methane emission, nutrient utilization, and the nitrate toxicity of ruminants with dietary nitrate addition: A meta-analysis from *in vivo* trials. Tropical Animal Science Journal, 46(1): 74-84.

Alrefaey, A., A.M. Nofal, A.M. Allam, and E.I. EL-Serag, 2019. A new way to eliminate the root rot fungi in green fodder under the hydroponic conditions. Journal of Environmental Studies and Researches, 9: 99-113.

AOAC. 2005. Official Methods of Analysis. 18th ed. Association of Official Analytical Chemists; Arlington, VA, USA.

Belanche, A., A. Bannink, J. Dijkstra, Z. Durmic, F. Garcia, F.G. Santos, ... and E.M. Ungerfeld, 2025. Feed additives for methane mitigation: A guideline to uncover the mode of action of antimethanogenic feed additives for ruminants. Journal of Dairy Science, 108(1): 375-394.

Bouattou, K., A. Ali-Nehari, and W. Djamai, 2024. Assessment of the biological activities of *Azolla pinnata* growing in the North-West of Algeria. Bionatura Journal, 1, 1-19.

Chandrababu, K.A., U. Parvathy, B. Meenu, and P.K. Binsi, 2024. Unveiling the nutritional treasure trove: A comparative analysis of two Azolla species for potential food applications. Food and Humanity, 3, 100444.

Chisoro, P., I.F. Jaja, and N. Assan, 2023. Incorporation of local novel feed resources in livestock feed for sustainable food security and circular economy in Africa. Frontiers in Sustainability, 4: 1251179.

Cone, J.W., and A.H. van Gelder, 1999. Influence of protein fermentation on gas production profiles. Animal Feed Science and Technology, 76(3-4): 251-264.

Czerkawski, J.W., 1986. An introduction to rumen studies. Pergamon Press, Oxford

Dai, X., K.F. Kalscheur, P. Huhtanen, and A.P. Faciola, 2022. Effects of ruminal protozoa on methane emissions in ruminants-A meta-analysis. Journal of Dairy Science, 105(9): 7482-7491.

Duncan, D.B.,1955. Multiple range and multiple F test. Biometric., 11:1-24.

Ebeid, H.M., F.U. Hassan, M. Li, L. Peng, K. Peng, X. Liang, and C. Yang, 2020. *Camelina sativa* L. oil mitigates enteric in vitro methane production, modulates ruminal fermentation, and ruminal bacterial diversity in buffaloes. Frontiers in Veterinary Science, 7: 550.

Ekinci, İ.B., A. Chłodowska, and M. Olejnik, 2023. Ionophore toxicity in animals: a review of clinical and molecular aspects. International Journal of Molecular Sciences, 24(2): 1696.

El Naggar, S., and H.S. El-Mesery, 2022. *Azolla pinnata* as unconventional feeds for ruminant feeding. Bulletin of the National Research Centre, 46(1): 1–5.

El-Hawy, A.S., 2024. Reproductive and productive efficiency of baladi goats fed on *Azolla pinnata* as an alternative source of ration protein in South Sinai, Egypt. Middle East J Agric. Res., 13(4): 980-990.

Gao, M., A. Cie´slak, H. Huang, M. Gogulski, D. Petric, D. Ruska, A. K. Patra, M. El-Sherbiny and M. Szumacher-Strabel, 2023. Effects of raw and fermented rapeseed cake on ruminal fermentation, methane emission, and milk production in lactating dairy cows. Anim. Feed Sci. Technol. 300, 115644.

Getachew, G., H. Makkar and K. Becker, 2000. Effect of polyethylene glycol on *in vitro* degradability of nitrogen and microbial protein synthesis from tannin-rich browse and herbaceous legumes. Br. J. Nutr., 84 (1): 73-83.

Giger-Reverdin, S., P. Morand-Fehr, and G. Tran, 2003. Literature Survey of the Influence of Dietary Fat Composition on Methane Production in Dairy Cattle. Livest. Prod. Sci., 82, 73–79.

Grainger, C. and K. A. Beauchemin, 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Animal feed science and technology, 166, 308-320.

Hegarty, R.S., 1999. Reducing rumen methane emissions through elimination of rumen protozoa. Australian Journal of Agricultural Research, 50(8): 1321-1328.

Indira, D., and A. Ravi, 2014. Feeding value of Azolla (*Azolla pinnata*) in buffalo calves. International Journal of Food, Agriculture and Veterinary Sciences, 4(2): 23-27.

Ismail, S.A., A.M. Abdel-Fattah, M.A. Emran, H.H. Azzaz, M.S. El-Gamal and A.M. Hashem, 2018. Effect of partial substitution of ration's soybean meal by biologically treated feathers on rumen fermentation characteristics (*in vitro*). Pakistan Journal of Biological Sciences, 21(3): 110-118.

Janssen, P. H., and M. Kirs, 2008. Structure of the archaeal community of the rumen. Appl Environ Microbiol . 2008 Jun;74(12):3619-25. doi: 10.1128/AEM.02812-07.

Jayasuriya, M.C.N., R. Hamilton, S. Uriyapongson, and D.L. Eskew, 1988. Fermentation of straw-based diets containing azolla (Azolla caroliniana Willd) using the Rumen simulation technique (Rusitec). Biological wastes, 24(3): 213-226.

Johnson, K. A. and D. E. Johnson, 1995. Methane emissions from cattle. Journal of Animal Science, 73, 2483–2492.

Katayama, N., M. Yamashita, Y. Kishida, C.C. Liu, I. Watanabe, H. Wada, and S.A.T. Force, 2008. Azolla as a component of the space diet during habitation on Mars. Acta Astronautica, 63(7-10): 1093-1099.

Kavya, K., T.M. Prabhu, R.G. Gloridoss, K.C. Singh, and Y.B. Rajeshwari, 2014. Nutritional evaluation of azolla (*Azolla pinnata*) and its supplementary effect on in vitro digestibility of total mixed ration. Mysore J. Agric. Sci., 48 (3): 413-419.

Khidr, R.E., S. Abo Bakr, B. Farrag, and R. Elawady, 2024. Agricultural residues and their utilization as alternative feed resources in Egypt: A review article. Egyptian Journal of Animal Production, 61(3): 113-127.

Kholif, A.E., G.A. Gouda, and A.K. Patra, 2022 The sustainable mitigation of *in vitro* ruminal biogas emissions by ensiling date palm leaves and rice straw with lactic acid bacteria and *Pleurotus ostreatus* for cleaner livestock production. J. Appl. Microbiol., 132: 2925-2939.

Króliczewska, B., E. Pecka-Kiełb, and J. Bujok, 2023. Strategies used to reduce methane emissions from ruminants: Controversies and issues. Agriculture, 13(3): 602.

Ku-Vera, J.C., J.R. Canul-Solis, A.T. Pineiro,-Vazquez, E.G.Briceno-Poot, J.A. Alayon-Gamboa, A.J. Ayala-Burgos, F.J. Solorio-Sanchez, C.F. Aguilar-Perez, L. Ramirez-Aviles, and O.A. Castelan-Ortega, 2013. Methane emissions from ruminants in the tropics: Implications for global warming and options for mitigation. In: Basile, A. (Ed.), Methane in the Environment: Occurrence, Uses and Production. Nova Science Publishers, Inc., NY, 267-290.

Leng, R.A., 2018. Unravelling methanogenesis in ruminants, horses and kangaroos: the links between gut anatomy, microbial biofilms and host immunity. Animal Production Science, 58(7): 1175-1191.

Lester, R.E., A. Macqueen, E.K. Armstrong, D.T. Dodemaide, G.K. Dwyer, T.S. Mock, ... and L. Webb, 2024. Can freshwater plants and algae act as an effective feed supplement to reduce methane emissions from ruminant livestock?. Science of The Total Environment, 914: 169296.

Liu, Y., X. Li, Q. Diao, T. Ma, and Y. Tu, 2024. In-Silico and in vitro Studies Revealed that Rosmarinic Acid Inhibited Methanogenesis via Regulating Composition and Function of Rumen Microbiota. Journal of Dairy Science.

Menke, K.H. and H. Steingass, 1988. Estimation of the energetic feed value obtained from chemical analyses and gas production using rumen fluid. Anim. Res. Develop., 28: 7-55.

Moss, A.R., J.-P. Jouany, and J. Newbold, 2000. Methane production by ruminants: Its contribution to global warming. Ann. Zootech. 49: 231-253.

Newbold, C.J., B. Lassalas, and J.P. Jouany, 1995. The importance of methanogensis associated with ciliate protozoa in ruminal methane production in vitro. Letts. Appl. Micro. 21: 230–234.

NRC. 1989. Nutrient Requirements of Dairy Cattle. 6th Rev. Ed. National Academy of Sciences, Washington, D.C.

Oskoueian, E., N. Abdullah, and A. Oskoueian, 2013. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. BioMed research international, 1: 349129.

Parashuramulu, S., P.S. Swain, and D. Nagalakshmi, 2013. Protein fractionation and in vitro digestibility of Azolla in ruminants. Journal of Animal and Feed Sciences, 3(3):129–132.

Patra, A., T. Park, M. Kim, and Z. Yu, 2017. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. Journal of animal science and biotechnology, 8: 1-18.

Rabee, A.E., M. Ghandour, A. Sallam, E.A. Elwakeel, R.S. Mohammed, E.A. Sabra, A. Abdel-Wahed, D. Mourad, A. Amin and O.R. Hafez, 2024. Rumen fermentation and microbiota in Shami goats fed on condensed tannins or herbal mixture. BMC Veterinary Research, 20(1): 35.

Rashad, S., 2021. An overview on the aquatic fern Azolla spp. as a sustainable source of nutrients and bioactive compounds with resourceful applications. Egyptian Journal of Aquatic Biology and Fisheries, 25(1): 775-782.

Ravi, R., N.S.H. Zulkrnin, N.N. Rozhan, N.R. Nik Yusoff, M.S. Mat Rasat, M.I. Ahmad, ... and M.F.M. Amin, 2018. Chemical composition and larvicidal activities of *Azolla pinnata* extracts against Aedes (Diptera: Culicidae). PloS one, 13(11): e0206982.

Samanta, G and Y. Tamang, 1995. Feeding value of azolla in goats. Ann Zootech. 44: 62

SAS. 1996. SAS (Statistical Analysis System) Procedure Guide. Version 6.12 edition. SAS institute, INC., Cary, NC, USA.

Standing Committee on Agriculture (SCA). 1990. Ruminants Feeding Standards for Australian Livestock. CSIRO Publications, Australia.

Tilley, J.M.A. and R.A. Terry, 1963. A two-stage technique for the in vitro digestion of forage crops. J. Brit. Grassland Soc., 18:104-111.

Toral, P. G., G. Hervás, D. Carreño, H. Leskinen, A. Belenguer, K.J. Shingfield, and P. Frutos, 2017. In vitro response to EPA, DPA, and DHA: Comparison of effects on ruminal fermentation and biohydrogenation of 18-carbon fatty acids in cows and ewes. Journal of Dairy Science, 100(8): 6187-6198.

Tran, T.L.N., A.F. Miranda, S.W. Abeynayake, and A. Mouradov, 2020. Differential production of phenolics, lipids, carbohydrates and proteins in stressed and unstressed aquatic plants, Azolla filiculoides and *Azolla pinnata*. Biology, 9(10): 342.

Van Soest, P.J., and J.B. Robertson, 1985. Analysis of forages and fibrous foods. Cornell Univ. USA.