

**NUTRITIONAL EVALUATION OF AZOLLA
(*Azolla pinnata*) AND ITS SUPPLEMENTARY EFFECT
ON *IN VITRO* DIGESTIBILITY OF CROP RESIDUES**

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**Affectionately Dedicated
To My Beloved Parents**

Smt. CHINNAMMA

&

Sri. CHANDRASHEKHAR



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Certificate

*This is to certify that the thesis entitled “NUTRITIONAL EVALUATION OF AZOLLA (*Azolla pinnata*) AND ITS SUPPLEMENTARY EFFECT ON *IN VITRO* DIGESTIBILITY OF CROP RESIDUES” submitted by Mr. Ramesh Hebballi., for the degree of Master of Veterinary Science in Animal Nutrition of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, is a record of the research work done by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar titles.*

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LIST OF ABBREVIATIONS

ADDM	- Apparent digestible dry matter
ADF	- Acid detergent fiber
AOAC	- Association of Official Analytical Chemists
ATP	- Adenosine triphosphate
EE	- Ether Extract
EMBS	- Efficiency of microbial biomass synthesis
H ₂	- Hydrogen
MBP	- Microbial biomass production
ME	- Metabolizable energy
N	- Nitrogen
NDF	- Neutral detergent fiber
NDS	- Neutral detergent solution
OM	- Organic matter
PF	- Partitioning factor
RIVIGP	- Rumen <i>in vitro</i> gas production at 24 h
SCFA	- Short chain fatty acids
SF	- Stoichiometric factor
TA	- Total Ash
TDN	- Total digestible nutrients
TDOM	- True digestible organic matter
VFA	- Volatile fatty acids

INTRODUCTION

I. INTRODUCTION

India has the largest cattle population and it has been estimated to constitute about 22 per cent of the total bovine population in the world. But its contribution to national economy is surprisingly low. This is due to the fact that average performance of Indian cattle is far from satisfactory. The low level of production among our cattle is attributed to several factors which need a thorough assessment for bringing about an improvement in their performance. Among various factors chronic malnourishment coupled with poor management is one.

Recent estimates have revealed that there is shortage of 47% concentrate, 38% of green fodder and 40% of dry fodder (Ranjhan, 1990). The pressure on land is so great for cereals and cash crops that hardly any land could be spared for cultivating animal feeds and fodder, and with the increase in human population, it is difficult to expect more feed for the livestock from cultivable area. Hence it is imperative to seek new avenues for augmenting the sources for cattle feed by utilizing various unconventional feed resources efficiently in order to meet the shortage of livestock feed in the country. Further, use of these by-products would be possible to lower cost of animal production and cereals and pulses diverted for human consumption.

A variety of unconventional feed stuffs have been investigated in the past which find use as protein and energy sources for livestock.

Azolla is one such unconventional feed resource and not much work has been carried out to utilize the same as feed ingredient of cattle feed in India.

Azolla belongs to the family Azollaceae and mainly is used as nitrogen (N) source for paddy cropping systems. The paddy field is partially drained and the Azolla is pressed into the soil by feet before re-flooding. It is also used to supply nitrogen, for example in vegetables and it benefits from *Prosopis* as a wind break as well as fertilizing it. Azolla can supply around 25-30 kg of N per hectare (Kannaiyan, 1987). In India Azolla is found floating on the idle water in shallow ditches and in channels. Azolla can fix atmospheric nitrogen with help of blue green algae, *Anabena azollae*, found in cavities of dorsal part of leaves. Azolla contains high amount of nitrogen i.e. 4 to 5 % on dry weight basis. It is also rich in minerals like Ca, P, K and Mg.

In India, Azolla can be easily cultivated and easily available to the farmers. Presently Azolla is used as a good source of manure for rice crops (Singh, 1981). However, the use of Azolla as manure for rice crop may not be an efficient way to utilize its nitrogen and other nutrients because some nitrogen goes waste due to decomposition of Azolla in soil of rice fields. It could be used for feeding to poultry and ruminants directly or as a feed or concentrate replacer it would be highly advantageous proposition, because Azolla does not require intensive

agronomical practices for cultivation as compared to groundnut. In countries of Indo- China, like Vietnam, Azolla as well as other aquatic plants are used in pig, duck and fish production. Therefore an attempt has been made to include Azolla as a feed supplement for *in vitro* studies and to assess its utility as cattle feed.

One of the most important problems in dairy industry is to provide adequate protein and energy for optimum growth and production of dairy animals. Unless this problem is successfully tackled, the desired production can not be achieved. Therefore it is essential to augment the sources of cattle feed by utilizing various unconventional feed resources. Azolla is available in good amount in paddy growing areas and no serious attempt appears to have been made so far in India to study the use of this material as an ingredient in cattle feeds. The present investigation on this aspect therefore is considerable practical and economic importance.

The objectives of the study include:

- I. To study the nutritional evaluation of Azolla.
- II. To evaluate the nutritive value of Azolla by rumen *in vitro* gas production kinetics.
- III. To study the Rumen fermentation characters of crop residues supplemented with Azolla.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Weeds and planktonic algae of both marine and fresh water are considered to be a potential source of food for expanding world's needs (Pirie, 1976), besides which the growth of these plants interferes with both commercial and recreational uses of such waters. Thus the feasibility of harvesting these plants and their possible use as animal feed attracted the attention of naturalists and biologists. Nevertheless, stray and sparse attempts have been made to explore the possibilities of utilization of such aquatic plants by few workers (Patton *et al.*, 1967; Blum and Calet, 1975; Mutzar *et al.*, 1976).

Aquatic plants species, because of their growth habit, appear not to accumulate secondary plant compounds and therefore offer a greater potential than tree leaves as a source of protein for animals (Bacerra *et al.*, 1990). The aquatic plants such as water hyacinth and hydrilla species (Boyed, 1969); Erusian water milfoil (Scheutte and Hoffman, 1921; Bailey, 1965); Duck weed (Culley and Epps, 1973) are approximately equal to alfalfa in nutritional value (Morrison, 1959 and Spencer *et al.*, 1969) including crude protein and crude fiber contents. The lower level of fiber content in some of the aquatic species, depends on location and stage of growth. However, the ash and xanthophylls contents are fairly high, when compared to the conventional forages. Although high ash content is being one of the apparent limitations, in

nutritional value of such aquatic plants, but high xanthophyll contents possibly offset the limitation, and thus makes these aquatic plants, superior to alfalfa meal in imparting color to egg and poultry skin pigmentation (Muztar *et al.*, 1976).

2.1.1 Nutritive value

Muztar *et al.* (1976) tested to evaluate the nutritive value of five different species of dried ground aquatic plants, cladophora, pond weed, milofoil and vallisneria duck weed in layer diets. These species of aquatic plants revealed (Table 2.1) that the waterweeds had extremely variable ash content 18.4 -62.5 per cent, crude protein 5.6- 15.5 per cent but contained relatively less crude fiber and ether extract than dehydrated alfalfa meal. The calcium content varied from 3.0 to 16.7 per cent as compared to 1.9 per cent for dehydrated alfalfa. Lipstein and Hurwitz (1980) reported 574 g of total protein, 22 g fiber, 89 g moisture, 11.7 g phosphorus and 2.0 g calcium per kg in algae meal.

Table 2.1 : Per cent chemical composition of aquatic plants and other plants (dry matter basis)

Plant species	Crude protein	Crude fiber	Ether extract	Ash	Ca.	P	Gross energy
<i>Azolla pinnata</i>	21.4	12.7	2.7	16.2	1.16	1.29	-
Alfalfa	17.7	24.2	3.65	9.9	1.9	0.27	4.16
Cladophora	12.93	14.50	1.22	59.83	4.93	0.28	1.99
Duck weed	15.53	16.64	3.14	18.43	3.03	0.39	4.19
Milofoil	5.61	17.04	0.94	62.53	16.72	0.10	1.82
Pond weed	10.54	20.00	2.90	40.44	11.90	0.19	2.78

2.1.2 Feeding trials

Various workers have conducted feeding trials using different species to determine nutritional value of aquatic plants, Azolla in cat fish and pigs (Liang and Lovell, 1971; Combs and Wallace, 1973), cattle (Hentges, 1970, Hentges, *et. al.*, 1972; Salveson, 1971; Stephens, 1972, and Vetter, 1972) and the duck weed in swine and sheep (Baldwin, 1973 and Culley and Epps, 1973).

Ruminants fed both pelleted and ensiled aquatic plants have failed to show any advantages in their performance than when fed conventional forages. Further, the effect of aquatic plants feeding on growth of rats as well as possible toxic effects in cattle has been reported (Bangall *et al.*, 1973; Buckingham *et al.*, 1978) in *in vitro* digestibility trials which showed that Azolla was readily degraded by rumen micro organisms and indicted its possible usefulness as a protein source for ruminants. Trux *et al.*, (1972) reported that inclusion of duckweed to poultry diets significantly improved the growth rate in comparison to feeding of alfalfa meal. The duckweed is also a good source for pigmentation of egg yolk in birds (Crager *et al.*, 1963).

Mutzar *et al.* (1976) studied the nutritive value of five aquatic plants in egg type male chicks against controls comparing a corn; soybean meal diet containing graded levels of dehydrated alfalfa meal. Alfalfa was considerably lower in ash content as compared to other water

plants. The gross energy of green plants had high negative correlation with ash content ($r = -0.99$).

2.2 Azolla as a source of vegetable protein

Azolla is a delicate, free floating fern from aquatic and semi aquatic habitats, such as still water, ponds, ditches and paddy fields of warm temperature and tropical regions (Konar and Kapoor, 1974). This aquatic plant is of worldwide distribution. There are seven species of Azolla, which are widely distributed in different parts of the world (Svenson, 1944, Lumpkin and Plucknett, 1982) as *Azolla caroliniana* (North America and Caribbean), *Azolla filiculoides* (South America), *Azolla microphylla*, (Tropical and sub tropical America), *Azolla mexicana* (North and South America), *Azolla nilotica* (Upper reaches of Nile to Sudan), *Azolla rubra* (Japan, Korea, Australia and New Zealand and *Azolla pinnata* commonly found in India (Loyal, 1958; Singh, 1981).

The aquatic fern has a high relative growth rate (RGR) and a symbiotic relationship with nitrogen fixing endophytic cyanobacteria called *Anabaena azollae*. This symbiotic relationship gives Azolla a competitive advantage over other floating hydrophytes in environments such as rice fields, which are relatively low in available nitrogen. The symbiosis is of interest to agriculturists, since incorporation and decomposition of Azolla crop in rice field soil can result in increase of available nitrogen to a companion or succeeding rice crop. Azolla can

accumulate more than 10 kg N/ha per day (25 tons/ha of Azolla X 6% dry weight X 4% N X RGR 0.17 ton per day, Lumpkin and Plunkett, 1982). The advantage of Azolla is that, it can produce green matter yield of 200 to 300 tons/ha/year as compared to 30 to 50 tons/ha/year produced by other green manure crops (Stewart, 1977).

Azolla is used as a nitrogenous crop in Vietnam, China, Taiwan and other south east Asian countries (Moore, 1969). Some of the aquatic plants like water hyacinth, duck weed and Azolla are approximately equal to alfalfa meal in crude protein, fiber, amino acid and vitamin content, although ash and xanthophylls content are fairly high in these plants (Muztar *et al.*, 1976). Azolla is capable of assimilating atmospheric nitrogen efficiently, due to presence of an algal symbiont in the leaves (Moore, 1969; Peters, 1975). Azolla has also been used as excellent food for fish, pigs and ducks (Lumpkin and Plucknett, 1982).

2.2.1 Azolla production / multiplication of Azolla

The cultivation of Azolla is a continuous process requiring a certain amount of attention throughout the year (Lumpkin and Plucknett, 1980; Kannaiyan, 1987). Usually a certain area of field within a production unit is set aside for such cultivation. Azolla nurseries are of two types' viz., maintenance and multiplication nurseries.

Azolla in nurseries in small plots (50-100 m²) area are prepared to avoid wind action. A standing water of 3 to 5 cm depth is essential. A pH

of 5.0 to 7.0 is ideal and acidic soils (between pH 4-6) are not suitable unless lime is used to correct pH. Winter temperature in the range of 14°C to 35°C is preferable but the optimum range is 20°C to 30°C. The Azolla inoculum at 0.1 to 0.4 kg / m² is most desirable for production of 8 to 10 tons per hectare green matter in 20 days. Application of super phosphate at 75 kg per hectare is essential for rapid growth of Azolla. Use of carbofuran / furadon at one to two kg per hectare or 0.625 g per m² prevents the rapid spread of insects, parasites and the consequent destruction of Azolla nurseries (Kannaiyan, 1987). At the good growth period the Azolla mat should be separated once in a week.

2.2.2 Proximate principles

The proximate analyses of different species of Azolla are reported by various workers (Table 2.2).

Alalde and Iyayi (2006) reported that Azolla contained 21.4 per cent crude protein, 2.7 per cent ether extract, 16.2 per cent total ash, 1.25 per cent phosphorus and 1.29 per cent calcium. The ADF and NDF values were 26.58 and 39.16 per cent, respectively.

Singh and Subudhi (1978) reported that Azolla contained 24 to 34 per cent crude protein, 3.18 per cent ether extract, 9.1 crude fiber and 10.5 per cent total ash on dry matter basis. The calcium and phosphorus contents were found to be 0.4 to 1.0 per cent and 0.5 to 0.9 per cent

Table 2.2: Per cent chemical composition of different species of azolla (dry matter basis)

Proximate Principle	Species of azolla				
	<i>Azolla microphyla</i> Taklimi. 1990	<i>Azolla filiculoides</i> Buckingham <i>et al.</i>, 1978	<i>Azolla pinnata</i> Castillo <i>et al.</i>, 1982	<i>Azolla pinnata</i> Parnerkar <i>et al.</i>, 1986	<i>Azolla pinnata</i> Alalade and Iyayi, 2006
Dry matter	90	-	89.32		-
Crude Protein	25.33	23 - 42	30.04	15.63	21.4
Ether Extract	3.01	5.05	0.78	2.84	2.7
Crude Fiber	11.60	-	12.38	13.01	12.7
Total ash	23.59	15.54	21.12	-	16.2
Neutral detergent fiber	40.36	39.16	47.85	52.76	36.88
Acid detergent fiber	25.24	26.58	42.16	-	47.08
Calcium	1.70	0.937	1.64	1.64	1.16
Phosphorus	1.05	0.487	1.06	0.56	1.29
Nitrogen free extract	16.91	-	35.68	52.70	47.0

respectively. They also reported that Azolla is rich in vitamin and minerals, xanthophylls and carotinoids.

The freshly harvested Azolla contains 85 to 90 per cent moisture and 0.4 to 6.0 per cent nitrogen (Moore, 1969). The moisture content varied with the stage of maturity of the aquatic plant harvested and the time allowed for the drain /drip post harvesting. However, Thuyet and Taun (1973), reported very low level of crude protein content (13 to 16 per cent) in Azolla.

Biplob Basak *et al.* (2002) reported that Azolla meal contains 25.78 per cent crude protein, 3.47 per cent ether extract, 15.76 total ash and 30.08 Nitrogen free extract. The gross energy was found to be 4243 kcal per kg. The NDF and ADF values were 47.85 and 42.16 per cent respectively.

Parnerkar *et al.* (1986) analyzed the proximate principles in Azolla and reported 15.63 % crude protein, 13.01 % crude fiber, 2.84 % ether extract, 1.64 % calcium, 0.56 % phosphorus on dry matter basis.

2.3 Mechanism of ruminal protein degradation

The protein degradation and assimilation in the ruminant digestive system is illustrated in Fig 2.1. The rumen fermentable pool of proteins includes feed proteins and the endogenous proteins of saliva, sloughed off epithelial cells and the remains of lysed ruminal microbes. All of the

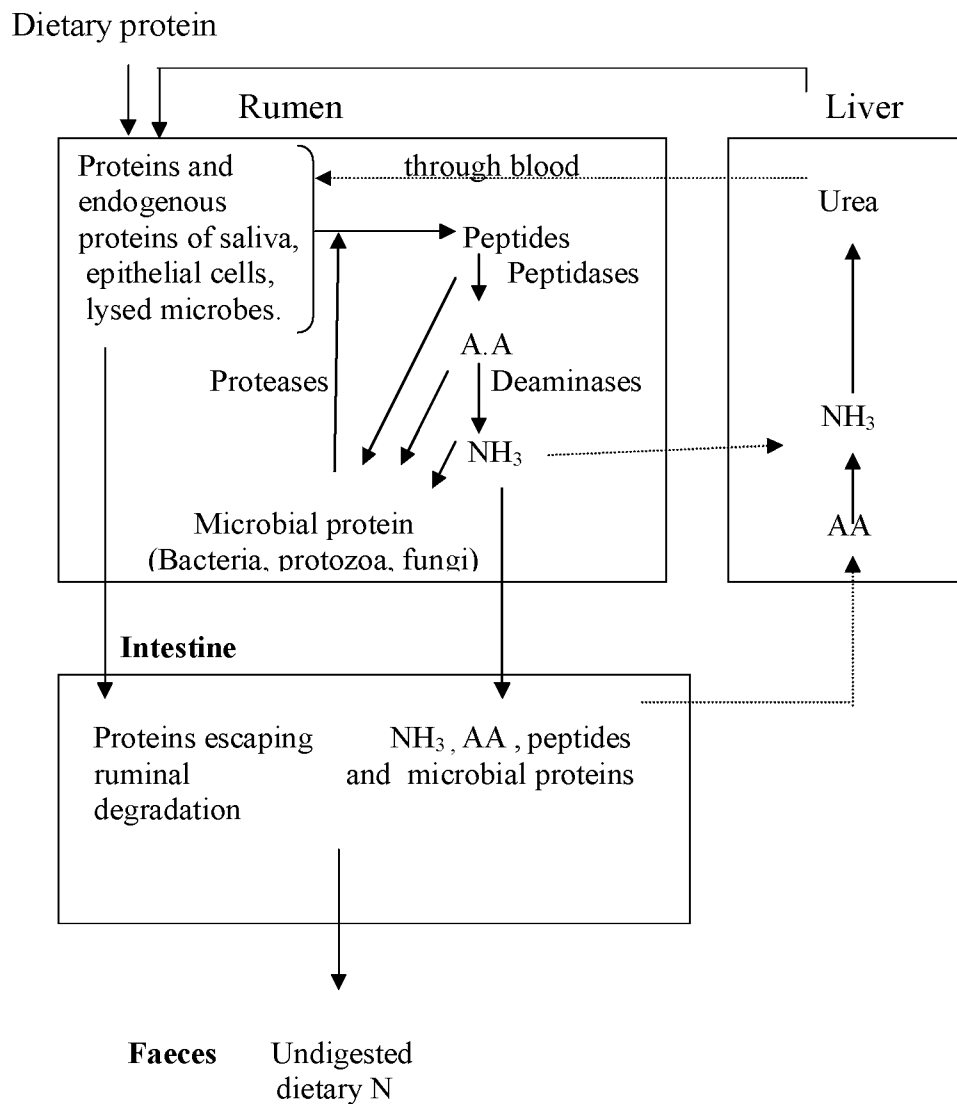
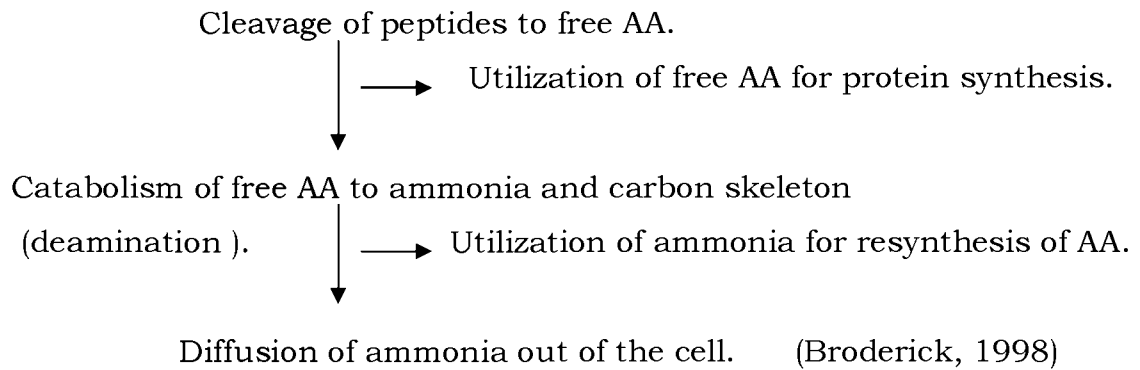


Fig.2.1 Protein degradation and assimilation in the ruminant digestive system

(Wallace, 1996)

enzymatic activity of ruminal protein degradation is of microbial origin. Many strains and species of bacteria, protozoa, and anaerobic fungi participate by elaborating a variety of proteases, peptidases, and deaminases. The liberated peptides, AA and ammonia are nutrients for the growth of ruminal microorganisms. Peptide break down to AA must occur before AA are incorporated into microbial protein, whenever the protein degradation exceeds the AA and ammonia assimilation into microbial proteins, peptides and AA catabolism leads to excessive ruminal ammonia concentrations. Some may escape ruminal degradation to ammonia and become the source of absorbable AA to the host animal (Wallace, 1996).

In the rumen, bacteria are abundant and are the principal microorganisms involved in protein degradation. It was reported that 40% or more of isolated species of rumen bacteria exhibit proteolytic activity (Cotta and Hespel, 1984; Broderick *et al.*, 1991; Wallace, 1996) and nearly 90% of proteolytic activity of rumen bacteria is associated with the cell surfaces (Broderick, 1998). Therefore, the initial step in protein degradation by ruminal bacteria is adsorption of soluble proteins to bacteria (Nugent and Mangan, 1981). Following bacterial uptake of small peptides and free AA, the intracellular events that occur are,



Amino acid catabolism and ammonia production in excess of bacterial needs wastes dietary crude protein and reduces the efficiency of use of rumen degraded protein (RDP). Amino acid deamination by bacteria is carried out by a combination of numerous bacteria with low deaminative activity and a much smaller number of bacteria with high activity (Wallace, 1996). The growth of some bacteria with high deaminating activity can be suppressed by the ionophores such as monensin (Chin and Russell, 1988; Russell *et al.*, 1988), and thereby excessive degradation can be prevented.

Protozoa are also significant participants in ruminal protein degradation. Although they are numerically less than the bacteria (10^{5-6} /ml versus 10^{11-13} /ml), because of their large size, they constitute a significant portion of the total microbial biomass in the rumen (generally less than 10% but some time as high as 50%) (Jouany, 1996; Jouany and Ushida, 1999). Protozoa differ from bacteria in protein metabolism by i) being able to ingest particulate matter such as the bacteria, fungi, and small feed particles. Thus protozoa are more active in degrading

insoluble feed proteins (eg., soybean meal or fish meal) than the soluble feed proteins (eg., Casein) (Hino and Russell, 1987; Jouany, 1996; Jouany and Ushida, 1999). Ingested proteins are degraded within the cell to yield a mixture of peptides and free AA, the AA are incorporated into protozoal protein. ii) having higher proteolytic specific activity than bacteria (Nolan, 1993), iii) not being able to synthesize AA from ammonia, thus being net exporter of ammonia into the rumen (Jouany and Ushida, 1999) iv) being able to release large amount of peptides and AA, as well as peptidases into ruminal fluid.

Much less is known about the involvement of fungi in ruminal protein catabolism. Currently, anaerobic fungi are considered to have negligible effect on ruminal protein digestion, because of their low concentrations in ruminal fluid (10^{3-4} /ml) (Wallace and Monroe, 1986; Jouany and Ushida, 1999).

2.4 Microbial requirements for nitrogen substrates.

Ammonia, amino acids (AA), and peptides are nutrients for the growth of ruminal bacteria. Protozoa cannot use ammonia. Bryant and Robinson (1962) studied the extent of utilization of $\text{NH}_3\text{-N}$ and amino acid carbon by selected species of ruminal bacteria. The results suggested that a significant proportion of the ruminal bacterial population prefer to synthesize much of its cellular constituents from $\text{NH}_3\text{ N}$ and carbon sources other than amino acids, even when the diet

contains large amount of true proteins. Based on pure culture studies, many species of rumen bacteria require NH_3 , and are inefficient in utilizing amino acid carbon. These observations were recently confirmed again by Mould (2005) using gas production as an index of microbial activity.

The mixed ruminal microbial population has essentially no absolute requirement for AA (Virtanen, 1966) as cross feeding among bacteria can meet individual requirements. The ^{15}N studies of Nolan (1975) and Leng and Nolan (1984) indicated that 50% or more of the microbial nitrogen was derived from ammonia and the rest from the peptides and AA. Maeng and Baldwin (1976) reported increased microbial yield and growth on 75% urea + 25% AA-nitrogen compared to 100% urea.

Studies using ^{15}N ammonia or urea infused into the rumen or added as a single dose demonstrated that values for microbial nitrogen derived from ammonia ranged from 18 to 100% (Salter *et al.*, 1979). However, improved microbial growth or efficiency was observed when peptides or AA replaced ammonia or urea as the sole or major source of nitrogen (Cotta and Russell, 1982; Russell and Sniffen, 1984; Griswold *et al.*, 1996). Estimates of the contribution of ammonia versus preformed AA to microbial protein synthesis by the mixed rumen population have been highly variable (Wallace, 1997).

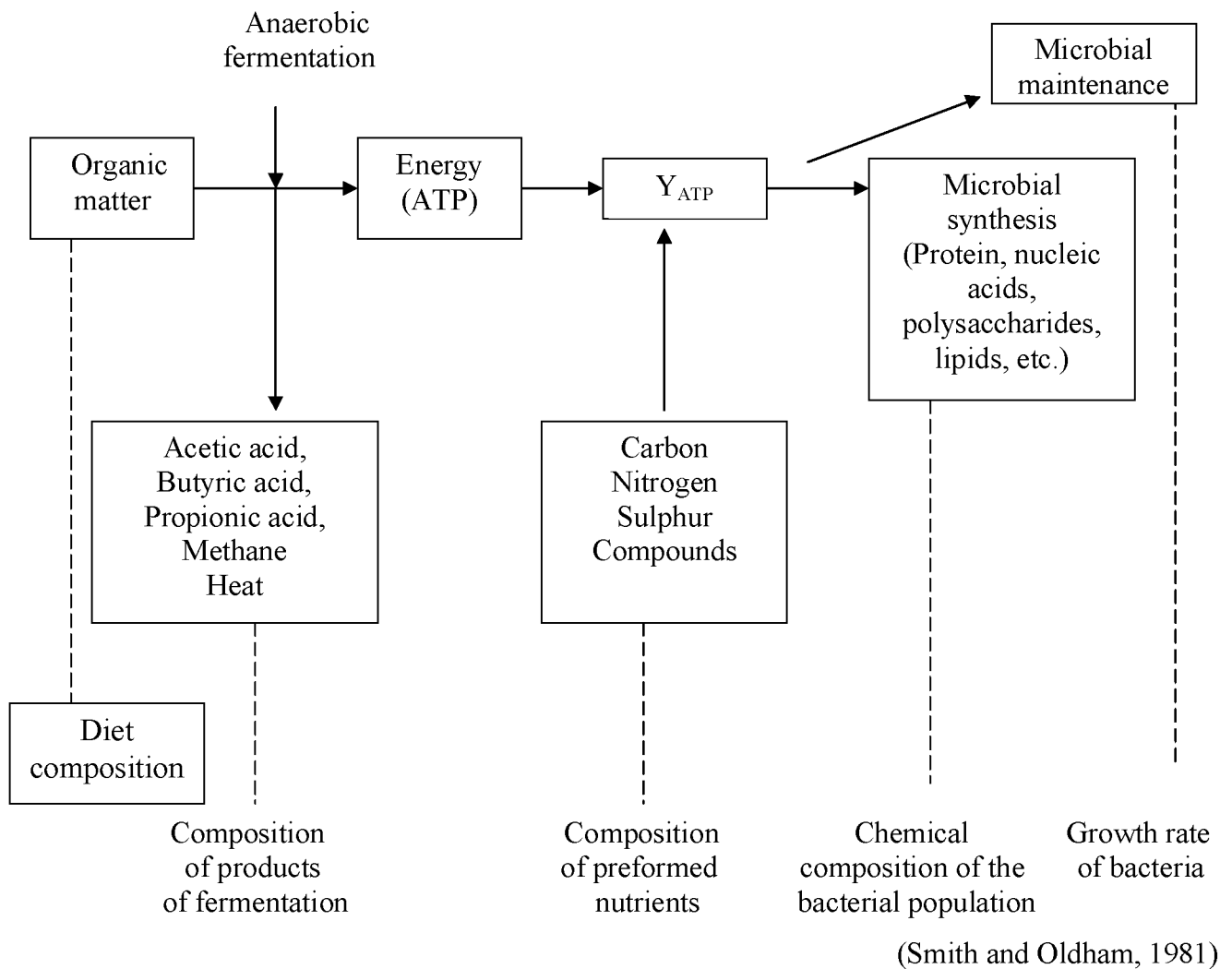
The requirement of peptides, amino acids, and ammonia for growth depends on the source. There are evidences that AA and especially peptides are stimulatory in terms of both growth rate and growth yield for ruminal microorganisms growing on rapidly degraded energy sources (Russell *et al.*, 1983; Chen *et al.*, 1987; Argyle and Baldwin, 1989). However, when energy substrates are fermented slowly, stimulation by peptides and AA does not always occur.

Chikunya *et al.* (1996) demonstrated that when peptides are supplied with rapidly or slowly degraded fibre, microbial growth was enhanced only if the fibre was degraded rapidly. Russell *et al.* (1992) indicated that microorganisms fermenting structural carbohydrates require only ammonia as their N source, while species degrading non structural carbohydrates source will benefit from preformed AA.

2.5 Microbial biomass synthesis

The relationship of a number of factors for microbial biomass synthesis is outlined in Fig. 2.2. Microbial biomass synthesis determines the microbial protein available to the animal and the potential for the use of NPN by rumen fermentation. The net metabolizable protein received by the animal is the sum of these digestible microbial proteins and the UDP (Van Soest, 1994). Therefore, maximizing ruminal microbial protein flow to the duodenum is a desirable nutritional management strategy.

Fig. 2.2 : Relationship of different factors to microbial biomass synthesis



2.5.1 Microbial efficiency

The microbial biomass synthesis in the rumen is dependent on amount of organic matter digested (or ATP yield) in the rumen and the efficiency with which the microbes in the rumen use the organic matter (or ATP) for their growth. The microbial efficiency can be expressed in terms of, $Y_{\text{Glucose...}}$ yield per mole of glucose or as grams of cells per mole of glucose or per 100 g of fermented feed ($Y_{\text{sub...}}$) and (ii) yield of dry cells (or of microbial protein) per mole of ATP ($Y_{\text{ATP...}}$) or per 100 g (or kg) of organic matter either apparently or truly fermented in the rumen (Yokoyama and Johnson, 1979).

The relationship between the amounts of organic matter incorporated into cells versus other products at various efficiencies of microbial growth can be calculated (Leng and Nolan, 1984) as presented in Table 2.3. From the point of measuring microbial protein produced in the rumen, nutritionists often express microbial efficiency as grams of microbial N (or protein) output from the rumen per unit OM apparently or truly fermented. Measurement *in-vivo* indicate that microbial efficiency ranges from 7.6 to 20.3 g microbial crude protein (or 1.2-3.2 g microbial N) per 100 g OM truly fermented in the rumen with a mean value of about 16 g crude protein synthesized per 100 g of OM fermented (Yokohama and Johnson, 1979). For every 100 g of OM truly fermented, around 14.5 to 29.0 g of ruminally available crude protein is needed as shown in Table 2.3 for optimum microbial biomass production.

Table 2.3 Dry matter and nitrogen balance at various efficiencies of microbial growth

True efficiency y_{ATP} (g cells per mole of ATP)	Dry matter destination		Efficiency		Protein needed (g protein per 100g OMF)
	Microbes (%)	VFA, CH₄, CO₂ (%)	True (g cells per 100g OMF)	Apparent (g cells per 100 g OMF)	
8	24.2	75.8	24.2	31.9	14.5
10	30.2	69.8	30.2	43.3	18.1
12	36.3	63.6	36.3	57.0	21.8
14	42.3	57.7	42.3	73.3	25.4
16	48.3	51.7	48.3	93.4	29.0

(Leng and Nolan, 1984)

Average microbial biomass yield for different diets show a marked variation (Table 2.4). Silages and high concentrate diets show lower yield than do forages or mixed diets. Microbial biomass yield from purified diets are highly variable which is probably due to the quality of the diets. Although it is universally agreed that the diets influence microbial biomass synthesis in the rumen, evaluation of diets for microbial biomass synthesis (NRC, 2001; Sniffen *et al.*, 1992) are too complex to be carried as a part of routine feed evaluation. Since rumen in vitro gas production technique (Menke *et al.*, 1979), to determine energy value of feedstuffs measures fermentation gas, its application to measure microbial biomass synthesis was explored (Krishnamoorthy *et al.*, 1991). This study led to the conclusion that the volume of gas is a reflection of total biomass synthesis, but the nature of substrate influenced the efficiency of biomass synthesis (mg biomass / ml gas). Hence, prediction of biomass from gas production requires knowledge about the nature of substrate and its contribution to gas volume. However, Blümmel *et al.*, (1997) proposed a logical argument that in a closed system of rumen fermentation, if the sum of all end products of fermentation equals the true fermented organic matter, a change in mass of one end product should change the mass of the other products as in a chemical reaction. Since the end products of fermented organic matter are waste products (short chain fatty acids (SCFA), gases) and microbial biomass, a reduction in waste products from a known quantity of fermented organic

Table 2.4 Microbial biomass yield (g / kg TDOM). (Van Soest,1982)

	Range	Mean
Purified diets	150-500	337
Mixed diets	100-470	251
Silage diets	110-310	189
Concentrates	130-260	211
forages	160-490	303
ARC (1984)	-	300
Russell et al. (1992)	-	400

matter (FOM), should result in increased biomass. Since gas is one of waste products, with a high positive correlation with SCFA, a reduction in gas volume per unit of FOM would reflect high microbial biomass (Illustrated in Fig 2.2). Therefore, Blümmel *et al.*, (1997b) suggested that the ratio of TDOM to ml of gas produced is a reflection of microbial efficiency and the ratio was termed as Partitioning Factor (PF). If gas production is less per unit of TDOM, the PF would be higher measuring higher microbial efficiency (Illustrated in Fig 2.3). Rymer and Givens (1999) showed that good quality feeds (grasses, silage, wheat, maize, molasses, sugar beet feed and fishmeal), which produce large amount of gas and SCFA, yield small amount of microbial biomass per unit feed truly degraded.

2.5.2 Dietary factors influencing microbial biomass yield

Composition of microbial cells influences the microbial efficiency, because of large variation in cell composition (Table 2.5). Direct storage of lipid and carbohydrates is much more efficient energetically than biosynthesis and storage of protein. Storage rather than synthesis will increase microbial efficiency, when expressed as grams of dry cells per ATP. But, when expressed as grams of protein synthesized per unit of organic matter truly fermented, the microbial efficiency decreases with storage of carbohydrates because ATP is used to polymerize carbohydrates (Yokoyama and Johnson, 1979).

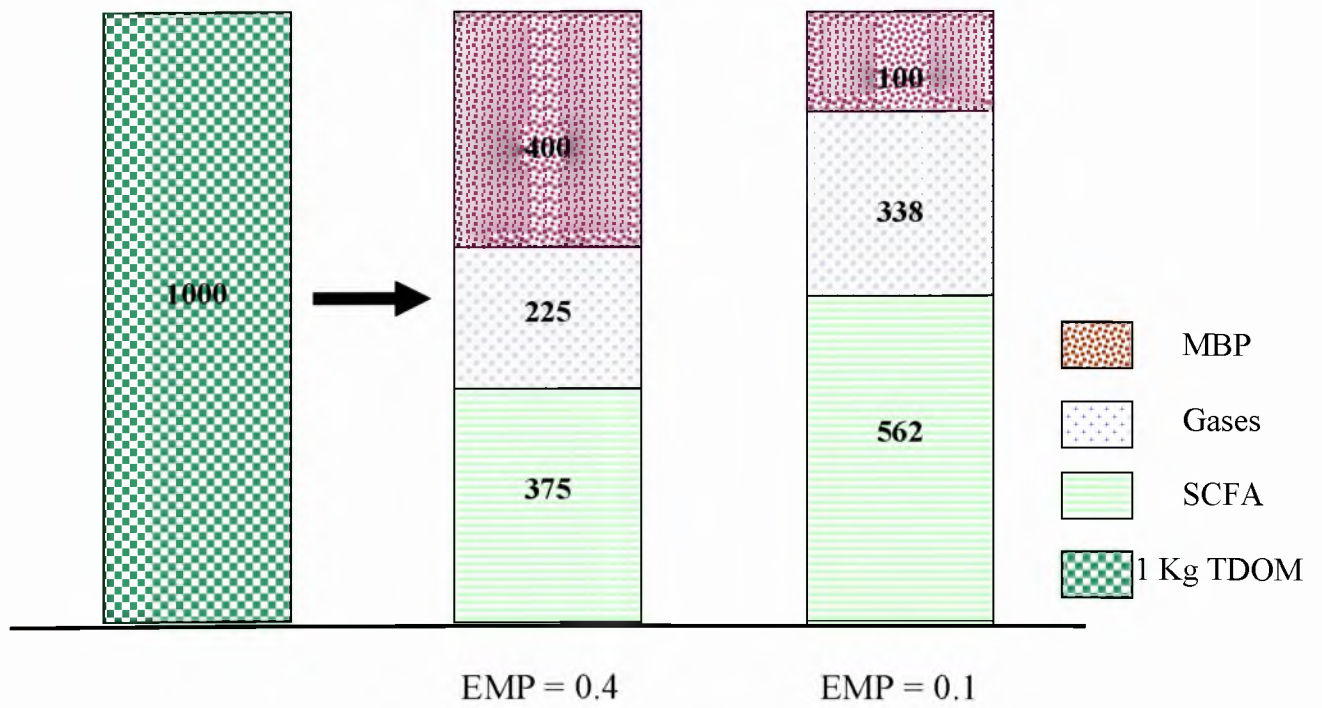


Fig. 2.3 : Partitioning of organic matter truly degraded in the rumen under varying efficiencies of microbial production (EMP)

(Blummel *et al.*, 1997)

Table 2.5 Composition of rumen micro flora

Constituents	Bacteria		Protozoa
	probable	Range	
Total N	10.0	5.00-12.4	3.8-7.9-
True protein	47.5	38-55	-
RNA	24.2	-	-
DNA	3.4	-	-
Lipid	7.0	4-25	-
Polysaccharides	11.5	6-23	-
Peptidoglycan	2.0	-	-
N digestibility	71.0	44-86	76-85

(Van Soest, 1982)

Microbial biomass yield in the rumen largely depends on the availability of carbohydrates and N in the rumen. Several studies indicate that synchronizing for rapid fermentation with fast degradable starch and protein sources stimulates greater synthesis or efficiency of synthesis of microbial biomass. In a study conducted by Sinclair *et al.* (1995), diets from similar sources were synchronized either with rapeseed meal (diet A) or asynchronous with urea (diet B) and the microbial biomass was calculated in sheep. The efficiency of microbial biomass yield was reported to be 11 to 20 per cent greater on feeding diet A compared to diet B. The asynchronous release of ammonia and energy in the rumen results in inefficient utilization of fermentable substrates and reduced microbial biomass yield (NRC, 2001).

MATERIALS AND METHODS

III. MATERIALS AND METHODS

3.1 Location

The experiment was conducted at the department of Livestock Production and Management, Veterinary College, Bangalore, KVAFSU. Bidar.

3.2 Feed Samples

Finger millet straw (FMS) (*Eleusine coracana*) and paddy straw (*Oryza sativa*) commonly used for dairy cattle in Karnataka were selected. The straws were ground to pass through a one mm sieve and preserved in airtight bottles at room temperature while using for chemical analysis and *in vitro* studies. The *Azolla pinnata* was harvested from Azolla tanks (3' X 6') maintained in Veterinary College, Bangalore and Freeze-dried. Freeze dried sample was subjected to ball milling (Retsch® MM 200) and preserved in air tight amber colored bottle in refrigerator while using for *in vitro* studies.

3.3 Chemical Analysis

The samples of Azolla, FMS and Paddy straw were analyzed for the proximate constituents (AOAC, 1984). Fiber fraction was done according to Van Soest *et al.*, (1991). The neutral detergent fiber was determined without amylase and without sodium sulfite.

3.4 Rumen fermentation characteristics

3.4.1 Donor cow and collection of rumen fluid

A crossbred (Holstein-Friesian X *Bos indicus*) lactating dairy cow in late lactation, weighing about 398 kgs, producing 4.5 to 5.0 kg milk per day, fitted with a flexible rumen cannula of large diameter (Bar Diamond Inc., USA) served as the donor of the rumen inoculum. She was fed with a basal diet consisting of finger millet straw at the rate of 6.5 to 7.0 kg/day and supplemented with compound feed mixture (CFM) of 3.0 kg/day in two portions at the time of milking at 06:00 hours and 13:30 hours. The ingredient composition of CFM (% as is) was maize 56.0, wheat bran 20.0, groundnut cake 20.0, sodium sulfate 0.25, mineral mixture 1.5kg, salt 0.5 g, urea 0.5 g and vitamin A, D₃, K, B₂ 0.5 g /50 kg. Drinking water was provided two times a day at 07:30 hours and 14:30 hours. The rumen fluid was collected between 09:00 hours and 09:30 hours.

3.4.2 Kinetics of gas production and metabolizable energy content

Air equilibrated samples of finger millet straw (200 ± 5 mg) and paddy straw were incubated in 100 ml calibrated glass syringes supplemented with Azolla @ 0, 2, 4, 6, 8 percent and Azolla as such in quadruplicates, by the procedure of Menke and Steingass (1988) with 30ml buffered rumen fluid with four blank incubations and the reference standards of roughages and concentrates. The incubations were done in a water bath maintained at 39°C. The readings of displaced syringes

were recorded at different time intervals over 96 hours (Day 1: 10am, 12pm, 2pm, 4 pm, 6 pm, 8pm, 10pm; Day 2: 2am, 6am, 10am, 4pm, 10pm; Day 3: 10am, 10pm; Day 4: 10am, 10pm; and Day 5: 10am). Whenever the syringe readings exceeded 90 ml, the accumulated gas was expelled and the readings were reset to 30.0 ml. The cumulative gas productions at varying intervals of time (2, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36, 48, 60, 72, 84 and 96 h) were calculated. The rate and extent of gas production were determined by non-linear regression using an exponential model, $Y = D (1 - e^{-k(t-l)})$, where, Y is gas volume at time t, D is potential gas production, k is rate at which gas is produced per hour and l is the discrete lag time (Krishnamoorthy *et al.*, 1991). The time of half asymptotic gas production ($t_{1/2}$) was calculated as $t_{1/2} = \ln 2/k$. For determination of ME content, 24 hours net cumulative gas production was corrected for the reference standards. Using chemical composition and net gas production (corrected for blank and reference standard) at 24 hr of incubation, ME was calculated using the following equations.

Roughages

$$ME = 2.2 + 0.1357GP + 0.0057CP + 0.0002859 EE^2$$

And for *Azolla pinnata*

$$ME = 1.06 + 0.1570 GP + 0.0084 CP + 0.022 EE - 0.0081TA$$

Where, ME = Metabolizable energy, MJ/kg DM; GP = gas, ml/200 mg DM; CP, EE and TA are crude protein, ether extract and total ash, respectively in g/kg DM.

3.4.3 Efficiency of rumen microbial biomass synthesis

The feed samples were incubated as explained in section 3.4.2 except that the sample size was 500 ± 10 mg and the inoculum was 40 ml containing double strength buffer (Makkar *et al.*, 1995). The incubations were terminated at $t_{1/2}$ determined in the preceding incubation after recording the gas production. The syringes were kept in ice water for 30 minutes for arresting microbial fermentation. The entire content of syringe was transferred into 50 ml polypropylene centrifuge tubes and centrifuged at 20000 X g for 30 min at 4°C and the syringes were washed thrice with 5-6 ml of distilled water to transfer the contents quantitatively into centrifuge tubes. After complete rinsing of the syringes, centrifugation was once again repeated at 20000 X g for 30 min at 4°C and supernatant was siphoned off and discarded. The residues were dried for 48 hr in forced air oven at 60 °C and weighed. *In vitro* ADDM was calculated as

$t_{1/2} \text{ ADDM} = \text{mg DM of substrate incubated} - (\text{mg DM residue} - \text{mg DM blank})$

The residue in the centrifuged tubes were quantitatively transferred to 500 ml Berzelius beakers and refluxed with neutral

detergent solution for 1 hour followed by filtration on G2 crucibles with repeated washing with boiling distilled water until free of detergent. The recovered residue was dried at 100°C for 24 hr and weighed. In vitro TDDM was calculated as

$$\text{TDDM} = \text{mg of DM substrate incubated} - \text{mg DM of residue after NDS refluxing}$$

Following ashing of the residue at 500°C for 3 h the undigested OM was determined and TDOM was calculated.

$$\text{TDOM} = \text{mg OM of substrate incubated} - \text{mg OM of residue}$$

The microbial biomass production was calculated as

$$(\text{t}_{1/2} \text{ TDOM} - (\text{gas volume at } \text{t}_{1/2} \times \text{stoichiometric factor})).$$
 The stoichiometric factor (SF) of 2.20 for roughages and 2.34 for Azolla were used. (Blummel *et al.*, 1997b).

3.5 Effect of Azolla on gas production and fiber digestion with finger millet straw and paddy straw.

Crop residues like Paddy straw and Ragi straws were selected. The incubations were done in 100ml calibrated syringes as explained in section 3.4.3 with 500 ± 10mg FMS with freeze dried Azolla at different levels. The samples were incubated with 40ml buffered ruminal fluid at 39°C in 6 replicates. The gas production was measured at $\text{t}_{1/2}$ and incubations were terminated to determine ADDM, TDOM, NDF

digestibility and microbial biomass synthesis. The ADDM, TDOM and NDF digestibilities were determined in one set of triplicates as explained in section 3.4.3. The second set of triplicates incubations were subjected to quantitative transfer to centrifuge tubes as explained in section 3.4.3. The residue was washed and freeze dried for six hours followed by 12 h drying in forced air oven at 60°C for purine quantification.

The treatment design is as follows

Treatments	Level of Azolla inclusion
T1 Control	Finger millet Straw
T2	Finger millet Straw with Azolla @ 2%
T3	Finger millet Straw with Azolla @ 4%
T4	Finger millet Straw with Azolla @ 6%
T5	Finger millet Straw with Azolla @ 8%
T6 Control	Paddy Straw
T7	Paddy Straw With Azolla @ 2%
T8	Paddy Straw With Azolla @ 4%
T9	Paddy Straw With Azolla @ 6%
T10	Paddy Straw With Azolla @ 8%
T11	Azolla

3.5.1 Biomass quantification by purine bases.

Purine quantification was done by the spectrophotometer method of Makker and Becker, (1999), after ball milling (Retsch® MM 200) the apparently undigested residue. The Yeast RNA (Sigma) was used as the standard. The absorbance at 260 nm after converting into Yeast RNA

equivalent was taken as an index of microbial biomass. Detailed procedure is in appendix.

3.6 Statistical Analysis

Data was analyzed using standard statistical procedures. The computer programme Graph Pad Prism (200, graph pad Inc, USA) was used to calculate the rate and extent of gas production in the non linear equation. The effect of Azolla at different levels on fermentation characteristics of FMS and paddy straw were analyzed by 2 X 5 factorial experiment using CRD, (Zar J.H., 1999).

RESULTS AND DISCUSSION

IV. RESULTS AND DISCUSSION

4.1 Chemical composition

The chemical composition, rumen *in vitro* gas production and predicted ME content of Azolla, FMS and Paddy straw are presented in Table 4.1. The DM content of Azolla was 4.1 %, FMS 89.25 % and paddy straw was 92.04 %. The CP content was highest (28.52%) in Azolla followed by FMS (4.79%) and paddy straw (3.58%). The Ether extract content was found to be 4.13, 0.55 and 0.75 % in Azolla, paddy straw and FMS respectively. The ash content was 14.43 % in Azolla, 15.6 % in paddy straw and 8.02 % in FMS. The NDF content was 57.71, 73.03 and 67.59 % in Azolla, paddy straw and FMS respectively. Azolla had the highest lignin content of 26.22 per cent on DM basis. The Net Gas production for 24 h observed was 29.3 ml, 28.5 ml and 33.90 ml in Azolla, paddy straw and FMS respectively. In Azolla, Paddy straw and FMS the ME (MJ/ kg DM) was 7.1, 5.5 and 6.38 respectively. Moore (1969) reported that freshly harvested Azolla contains 85 to 90 % moisture and it varied with stage of maturity of the aquatic plant harvested and time allowed for the drain or drip post harvesting. The dry matter content of 10.68 % was reported by Castillo *et al.*, (1982) in *Azolla pinnata*. The crude protein content on DM basis was higher than the reported value of Basak *et al.*, (2002) 25.78%, Alalade and Iyayi (2006) 21.4%. VanHove and Lopez (1982) reported that CP content of Azolla

Table 4.1: Chemical composition, in vitro gas production at 24 hours (IVGP-24h) and predicted metabolizable energy (ME) content of finger millet straw (FMS), paddy straw and *Azolla pinnata*

Varieties	Chemical composition										IVGP-24h(ml/200 mg DM)	ME(MJ/kg DM)
	DM (%)	OM (%)	TA (%)	CP (%)	EE (%)	NDF (%)	NDF-ash (%)	ADF (%)	Hemicellulose (%)	Lignin (%)		
Finger millet straw	89.25	82.4	8.0	4.79	0.75	67.59	9.25	40.64	26.95	4.87	33.90	6.38
Paddy straw	92.04	92.04	15.6	3.58	0.55	73.03	7.41	53.19	19.84	5.61	28.5	5.5
<i>Azolla Pinnata</i>	4.1	85.56	14.43	28.52	4.13	57.71	2.22	46.72	10.99	26.22	29.3	7.1
MEAN	62	87	13	12	2	66	6	47	19	12	31	6

might vary from 13 to 34.5%. Sangianga and VanHove (1989) attributed variations in the nutrient compositions of Azolla meal to differences in responses of Azolla strains to environmental conditions like temperature, light intensity, soil composition which consequently affect their growth, morphology and composition.

The Ether extract and ash corrected NDF of Azolla were higher than values reported by Alalade and Iyayi (2006). The contents of other parameters like total ash, hemicellulose and lignin were more or less same as reported by Alalade and Iyayi (2006).

Alalade and Iyayi (2006) reported the GE value of 2039 k cal/kg for Azolla meal which was around 8.53 MJ GE/ kg DM or 6.99MJ ME/ kg DM (825 of GE is ME). Metabolizable energy is similar to the value (7.59 MJ kg⁻¹) reported by Khatun *et al.* (1999).

The chemical composition and ME content of FMS and paddy straw were found to be similar to the values of Manjunath (2005) and Deepa Ananth (2007), however slight variations may be due to variety of straws.

4.2.1 Kinetics of gas production of azolla.

The kinetics of gas production of Azolla, when incubated alone in the medium showed the following pattern of gas production at different interval of time as shown in Appendix 2.

Table 4.2: The mean fermentation kinetics and microbial biomass synthesis of Azolla

Parameter	Azolla
D (ml/200mg DM)	29.3
k(h ⁻¹)	0.0934
t _{1/2} (h)	7.46
ADDM (mg/gm Dm)	359.5
TDOM (mg/gm DM)	438.88
TDDM (mg/gm DM)	494.7
Net Gas at t _{1/2} (mg/gm DM)	79.76
RNA per gm (mg/gm DM)	16.54
RNA : Gas (ml)	0.21
MBP (mg)	263.40
EMBP	598.31

Table No. 4.3: Gas production kinetics (D, k and $t_{1/2}$) of FMS, Paddy Straw (PS), Azolla levels and their interaction effects

	Straw effect			Azolla effect				Interaction effect			
	D (ml/200mg)	k (h ⁻¹)	$t_{1/2}$ (h)	Azolla levels	D (ml/200mg)	k (h ⁻¹)	$t_{1/2}$ (h)		D (ml/200mg)	k (h ⁻¹)	$t_{1/2}$ (h)
FMS	50.21 ^b ±0.27	0.0462 ^b ±0.0007	15.12 ^a ±0.25	0%	52.87±1.30	0.0365±0.006	20.37±3.3	T1	51.02± 1.0	0.045± 0.002	15.63±0.81
				2%	52.8 ±1.61	0.036±0.006	20.44±3.3	T2	50.52±0.05	0.045± 0.002	15.68±0.87
				4%	52.26±1.61	0.0365±0.006	19.94±3.2	T3	49.98±0.31	0.045±0.001	15.37±0.43
				6%	51.84±1.06	0.038±0.006	19.18±3.1	T4	50.34±0.35	0.047±0.002	14.74±0.52
				8%	51.28±1.47	0.039±0.007	18.94±3.1	T5	49.19± 1.0	0.049±0.0008	14.19±0.22
PS	54.27 ^a ±0.32	0.0282 ^a ±0.0003	24.43 ^b ±0.3					T6	54.72± 0.7	0.028± 0.001	25.11±0.88
								T7	55.08±0.44	0.027±0.001	25.21±0.99
								T8	54.55±0.43	0.028±0.001	24.50±0.57
								T9	53.35±0.7	0.029±0.001	23.62±0.83
								T10	53.37±0.83	0.029±0.008	23.69±0.61
CD	0.75	0.01	0.76								
CV	2.65%	46.2%	7.25%		2.65%	46.2%	7.25%		2.65%	46.2%	7.25%

Note: a, b: Different superscripts in a column differ significantly at $P \leq 0.01$

Where T 1 FMS + Azolla 0%, T 2 is FMS+ Azolla 2%, T 3 FMS+ Azolla 4%, T 4 FMS+ Azolla 6%, T 5 FMS+ Azolla 8%, T 6 PS+ Azolla 0%, T 7 PS+ Azolla 2%, T 8 PS+ Azolla 4%, T 9 PS+ Azolla 6%, T 10 PS+ Azolla 8% levels

The mean fermentation kinetics and microbial biomass synthesis of Azolla are presented in Table 4.2. The potential gas production observed was 29.3 ml, rate of gas degradation was 0.0934 and $t_{1/2}$ was 7.46. The ADDM, TDOM and AUDM were found to be 359.5, 438.88 and 494.7mg respectively at $t_{1/2}$. The RNA /gm, net gas at $t_{1/2}$ and RNA: Gas ratio were found to be 16.54 mg, 79.76 ml and 0.21 ml respectively. The MBP (mg) and EMBS observed were 263.4 and 598.31 respectively.

4.2.2 Kinetics of gas production of straw, Azolla and their interaction

The cumulative gas production at incubation time varying for 2 to 96 hour for the quadruplicate samples of four incubations of two straws supplemented with five levels of Azolla are presented in Appendix 2. The mean values of gas production kinetics like potential gas production (D), rate of degradation (k) and $t_{1/2}$ are presented in Table 4.3. The potential gas production (D) was significantly ($p \leq 0.01$) higher in the paddy straw (54.27 ml) compared to FMS (50.21 ml). The $t_{1/2}$ also showed similar trend which was significantly higher in paddy straw (24.43) than the FMS (15.12). The rate degradation (k) was significantly higher in FMS (0.0462) as compared to paddy straw.

The different levels of Azolla addition on the gas production kinetics like D, k and $t_{1/2}$ was found to be non significant. Similarly,

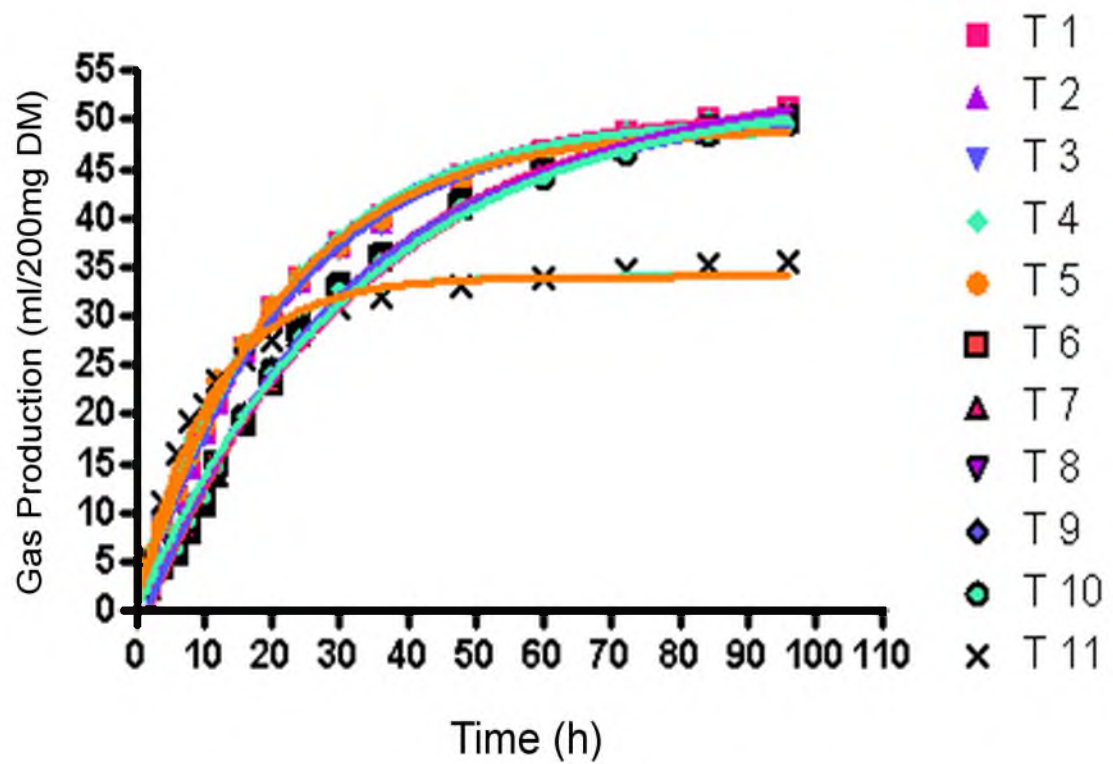


Fig. 4.1 Pattern of *in vitro* gas production on supplementation with azolla to FMS and Paddy straw.

different levels of Azolla addition to straws i.e., interaction effect was also found to be non significant.

The kinetic parameters widely differ depending upon the model used (France *et al.*, 1982). Although different models have been examined to explain gas production patterns, none of these models have been tested for their ability to explain variation in intake and animal performance. Therefore, in this study, the simplest of the models was used (Krishnamoorthy *et al.*, 1991).

The potential gas production (D) represents the total fermented organic matter. Therefore, this is an indirect estimate of energy content of the feedstuffs (Menke *et al.*, 1979). The fermentation kinetics of forages is reported to influence voluntary dry matter intake (Van Soest, 1994). The forages with high fermentation rate are expected to result in higher voluntary dry matter intake and less of residuals or manger waste. Since the residuals in poor quality forages can be as high as 40 to 50% of the offered, selecting fodder varieties with higher rate of fermentation can minimize wastage.

4.2.3 Substrate degradation, gas production & efficiency of microbial biomass production

The different parameters like ADDM, TDOM, NDF Digestibility, RNA /gm, RNA: Gas ratio, net gas @ t $\frac{1}{2}$, EMBS and MBP studied on straws supplemented with five levels of Azolla are presented in

Table 4.4: Substrate degradation (ADDM, TDOM) and NDF digestibility of FMS, Paddy Straw (PS), Azolla levels and their interaction effects

	Straw effect			Azolla effect					Interaction effect		
	ADDM** (gm/0.5gm)	TDOM** (gm/0.5gm)	NDF Digestibility** (gm/0.5gm)	Azolla levels	ADDM* (gm/0.5gm)	TDOM** (gm/0.5gm)	NDF Digestibility (gm/0.5gm)		ADDM** (gm/0.5gm)	TDOM (gm/0.5gm)	NDF Digestibility (gm/0.5gm)
FMS	0.144 ^b ±0.001	0.163 ^b ±0.003	0.288 ^b ±0.004	0%	0.154 ^{ab} ±.004	0.174 ^a ±0.001	0.269±0.007	T1	0.161 ^{ab} ±0.008	0.176± 0.004	0.279±0.005
				2%	0.165 ^{ab} ±0.002	0.163 ^{bc} ±0.001	0.281±0.018	T2	0.168 ^{ab} ±.004	0.161±0.0009	0.307±0.025
				4%	0.133 ^c ±0.002	0.167 ^{abc} ±0.001	0.27±0.009	T3	0.099 ^b ± .025	0.166±0.002	0.283±0.004
				6%	0.145 ^{bc} ±0.01	0.17 ^{ab} ±0.007	0.265±0.013	T4	0.129 ^{ab} ±0.007	0.159± 0.006	0.284±0.006
				8%	0.17 ^a ± 0.01	0.158 ^c ±0.158	0.274±0.012	T5	0.146 ^{ab} ±0.004	0.153± 0.007	0.291±0.007
PS	0.166 ^a ±0.006	0.170 ^a ±0.32	0.255 ^a ±0.001					T6	0.148 ^{ab} ±0.002	0.172± 0.002	0.259±0.002
								T7	0.162 ^{ab} ±0.004	0.165±0.0018	0.256±0.003
								T8	0.167 ^{ab} ±0.007	0.169±0.002	0.257±0.002
								T9	0.161 ^{ab} ±0.02	0.181±0.001	0.247±0.001
								T10	0.194 ^a ±0.005	0.164±0.0011	0.257±0.012
CD	0.01	0.0062	0.01		0.02	0.009			0.086		
CV	0.012	4.95%	5.9%		12.63%	4.95%	5.9%		12.63%	46.2%	5.9%

Note: a, b: Different superscripts in column differ significantly

Where T 1 FMS + Azolla 0%, T 2 is FMS+ Azolla 2%, T 3 FMS+ Azolla 4%, T 4 FMS+ Azolla 6%, T 5 FMS+ Azolla 8%, T 6 PS+ Azolla 0%, T 7 PS+ Azolla 2%, T 8 PS+ Azolla 4%, T 9 PS+ Azolla 6%, T 10 PS+ Azolla 8% levels

Appendix-III. ADDM, TDOM and NDF digestibility of straws, Azolla levels and their interaction are presented in Table 4.4.

ADDM, TDOM were significantly ($P \leq 0.01$ & 0.05) higher in paddy straw, where as the NDF digestibility was significant ($P \leq 0.01$) in FMS. The Azolla levels showed non significant results on NDF digestibility. The 8 % Azolla addition significantly increased TDOM (0.17), followed by 0, 2, & 6 per cent and it was least in 4 % addition (0.133). Where as the TDOM was significantly ($P \leq 0.01$) higher in 0% Azolla inclusion followed by 6%, 4%, 2%, and it was lower in 8% level. The interaction effect of Azolla on TDOM, NDF digestibility was found to be non significant, where as ADDM was found to be significant ($P \leq 0.01$). The ADDM was significantly higher in T 10 (0.194) i.e. paddy straw + 8% Azolla and it was least in T3 (0.99) i.e. FMS + 4% Azolla & other interaction are in between and they are non significant among themselves.

Microbial biomass synthesis indices like RNA/gm, RNA: GAS & net gas at $t_{1/2}$ of straws, Azolla and their interactions are presented in Table 4.5 .The RNA per gm, RNA: GAS ratio were significantly higher in FMS (12.67& 0.122 ml) when compared to paddy straw (10.51& 0.103 ml) respectively.

The net gas at $t_{1/2}$ was significantly higher in paddy straw (118.34ml) than in FMS (104.1ml). Different Azolla levels showed non

Table 4.5: Microbial biomass synthesis indices (RNA per gm, RNA: Gas ratio and net gas at $t_{1/2}$) of FMS, Paddy Straw (PS), Azolla levels and their interactions.

	Straw effect				Azolla effect				Interaction effect		
	RNA** per gm	RNA:GAS**	Net gas at $t_{1/2}$ * (ml/500mg)	Azolla levels	RNA per gm	RNA :GAS**	Net gas at $t_{1/2}$ * (ml/500mg)		RNA per gm	RNA:GAS	Net gas at $t_{1/2}$ (ml/500mg)
FMS	12.67 ^b ±0.37	0.122 ^b ±0.006	104.092 ^a ±12.31	0%	11.11±0.15	0.098 ^b ±0.004	112.51 ^{ab} ±4.7	T1	11.337± 0.7	0.104± 0.01	105.85± 1.7
				2%	11.75±1.15	0.105 ^b ±0.01	118.96 ^a ±4.9	T2	13.39±1.45	0.121±0.017	112.01± 3.5
				4%	11.3±0.5	0.098 ^b ±0.009	116.07 ^{ab} ±5.4	T3	12.017±0.4	0.112±0.002	107.66±1.4
				6%	11.69±1.17	0.110 ^b ±0.01	107.1 ^{bc} ±4.8	T4	13.36±0.7	0.133±0.008	100.207± 1.1
				8%	12.08±0.8	0.151 ^a ±0.007	100.75 ^c ±4.2	T5	13.25±1.18	0.141±0.015	94.733± 4.8
PS	10.51 ^a ±0.16	0.103 ^a ±0.01	118.34 ^b ± 21.68					T6	10.893±0.81	0.092±0.007	119.17±0.33
								T7	10.12±1.06	0.09±0.006	125.913±1.3
								T8	10.59±1.21	0.085±0.009	125.487±2.31
								T9	10.037±0.89	0.088±0.008	113.83±1.436
								T10	10.91±0.25	0.162±0.034	106.773±3.3
CD	1.03	0.013	2.72		0.02	0.024	3.89				
CV	14.16%	22.06%	3.89%		14.16%	22.06%	10.75%		14.16%	22.06%	3.89%

Note: a, b, c: different superscripts in column differ significantly

Where T 1 FMS + Azolla 0%, T 2 is FMS+ Azolla 2%, T 3 FMS+ Azolla 4%, T 4 FMS+ Azolla 6%, T 5 FMS+ Azolla 8%, T 6 PS+ Azolla 0%, T 7 PS+ Azolla 2%, T 8 PS+ Azolla 4%, T 9 PS+ Azolla 6%, T 10 PS+ Azolla 8% levels

significant results with respect to RNA to gas ratio. RNA to gas ratio was significantly higher at 8% Azolla level (0.151) & was least at 0 & 4% level (0.098). The net gas production was significantly higher in 2% Azolla level (118.96 ml) and was least in 8 % Azolla level (100.75 ml). Other levels were found non significant among themselves. The interaction effect of straw & Azolla were found to be non significant with respect to RNA, RNA: GAS & net gas at $t_{1/2}$. Other microbial biomass synthesis indices like EMBS & MBP of straw, Azolla & their interaction are presented in Table 4.6.

The EMBS & MBP were significantly higher in FMS (352.37 & 124.86) when compared to the paddy straw (275.08 & 99.28). There was significantly higher EMBS in 8 % Azolla level (347.3) followed by 6 % (347.33) & 0 % (329.27) & they were non significant among themselves. The EMBS in 4% (290.4) Azolla level was significantly lower than 0%, 6% & 8% level & significantly the lower MBP was seen in 2 % Azolla level (254.84). The MBP was significantly higher in 6% (125.5) inclusive level and 0% (121.97) & both were non significant among themselves. Significantly lower MBP was seen in 8% (118.78) followed by 4% (104.6). The significantly lower MBP was observed in 2% Azolla level (89.66). The interaction effect of straw & Azolla on microbial biomass synthesis indices like EMBS & MBP resulted in non significant results.

Table 4.6: Microbial biomass synthesis indices (EMBS and MBP) of FMS, Paddy Straw (PS), Azolla levels and their interactions.

	Straw effect		Azolla effect			Interaction effect		
	EMBS	MBP	Azolla levels	EMBS	MBP		EMBS	MBP
FMS	352.37 ^a ±12.31	124.86 ^b ±4.9	0%	329.27 ^a ±9.6	121.97 ^a ±13.76	T1	377.59± 6.9	141.44± 5.8
			2%	254.54 ^c ±33.71	89.66 ^c ±12.17	T2	302.23±24.59	106.82±9.06
			4%	290.19 ^b ±39.42	104.6 ^b ±14.55	T3	345.96±1.6	125.26±0.7
			6%	347.33 ^a ±9.41	125.31 ^a ±0.21	T4	360.64±17.13	125.01±10.23
			8%	347.3 ^a ±19.90	118.78 ^{ab} ±4.9	T5	375.45±29.94	125.7±12.59
PS	275.08 ^b ±21.68	99.28 ^a ± 8.5				T6	289.6±9.37	102.5±4.53
						T7	206.86±16.01	72.4±6.2
						T8	234.43±21.51	84.2±8.7
						T9	334.02±9.09	125.61±3.5
						T10	319.16±23.01	111.8±14.5
CD Value	19.71	9.3		31.17	14.84			
C V	9.97%	5.007%		9.97%	5.007%		9.97%	5.007%

Note: a, b, c: Different superscripts in column differ significantly at $P \leq 0.01$

Where EMBS= (MBP/iv TDOM), MBP = TDOM - (ml gas x 2.20)

Addition of Azolla at different levels in two straws studied failed to improve the gas production kinetics like D, k and $t_{1/2}$ and the values were similar to zero per cent Azolla added groups. Similar results were obtained for ADDM, TDOM, NDF digestibility, RNA per gm, net gas at $t_{1/2}$ EMBS and MBP. However an interesting feature noticed in this study was RNA: Gas ratio which increased with 8 per cent level of Azolla inclusion and it was found to be significant. The eight per cent azolla inclusion significantly improved the RNA: Gas ratio, however *in vivo* trials are required to prove the effect of addition of Azolla on microbial biomass synthesis.

SUMMARY AND CONCLUSION

V. SUMMARY AND CONCLUSION

The study was conducted to evaluate inclusion of Azolla on FMS and paddy straw for rumen fermentation characteristics and efficiency of rumen microbial bio mass synthesis. The *in vitro* experiment included chemical evaluation and rumen *in vitro* studies (Hohenheim gas test). The kinetics of rumen fermentation and microbial biomass synthesis were established for Azolla, paddy straw, FMS and also five levels of Azolla viz. 0, 2, 4, 6, and 8 per cent.

The DM, OM, CP, EE, NDF, ADF, Hemicellulose, lignin, IVGP 24 h and ME(MJ/ kg DM) observed in the study were 4.1, 92.04, and 89.25; 85.56, 92.04 and 82.4; 28.52, 3.58 and 4.79; 4.13, 0.55 and 0.75; 57.7, 73.03 and 67.59; 46.72, 53.19 and 40.64; 10.99, 19.84 and 26.95; 26.22, 5.61 and 4.87 per cent; 29.3, 28.5 and 33.9 ml: 7.1, 5.5 and 6.38 MJ/ ME in Azolla, paddy straw and FMS respectively.

The kinetics of gas production for azolla observed was potential gas production (D) 29.3 ml, rate of degradation (k) 0.0934 h^{-1} and $t_{1/2}$ 7.46 h. ADDM 359.5, TDOM 438.88, TDDM 494.7 (mg/ 0.5 gm DM), Net gas at $t_{1/2}$ 79.76 ml, RNA per gm 16.54, RNA: Gas ratio 0.21 ml, MBP 263.4 mg and EMBS 598.31.

In 2 X 5 factorial experiment Azolla was added at rate of 0, 2, 4, 6, and 8 per cent levels in two straws namely FMS and Paddy straw, the

straw effect, Azolla effect and interaction effect were studied. The gas production kinetics like D, k, and $t_{1/2}$ was found to be significant only in straw effect. Azolla effect and interaction effect were found to be non significant. Potential gas production (D) and $t_{1/2}$ were significantly higher in paddy straw, where as k value is higher in FMS.

Substrate degradation like ADDM, TDOM and NDF digestibility were studied in similar fashion as mentioned above. ADDM and TDOM digestibility were found to be significantly higher in paddy straw, where as NDF digestibility was higher in FMS in straw effect. In Azolla effect, 8 per cent Azolla level significantly increased the ADDM, where as zero per cent level significantly increased the TDOM and NDF digestibility was found to be non significant with regard to Azolla effect. In interaction effect TDOM and NDF digestibility were found to be non significant, where as ADDM showed the significance. 8 % Azolla in paddy straw had the significantly higher ADDM and FMS with 4 % Azolla had the significantly lower ADDM.

Microbial bio mass synthesis indices like RNA/gm, RNA: Gas ratio, net gas at $t_{1/2}$, EMBS and MBP were also studied. RNA/gm and RNA: Gas were significantly higher in FMS and Net gas at $t_{1/2}$ was significantly higher in paddy straw. Inclusion of different levels of Azolla was non significant with respect to RNA/gm. RNA: Gas was significantly higher in 8% Azolla levels followed by 6% and it was least in zero per cent Azolla

inclusion level. Net gas at $t_{1/2}$ was significantly higher in 2 % and least in 8% inclusion. The interaction effect for RNA per gm, RNA: Gas and net gas at $t_{1/2}$ were found to be non significant.

EMBS and MBP were significantly higher in FMS in straw effect. The EMBS was significantly higher in 0, 6 and 8% Azolla inclusion levels and interaction effect for EMBS and MBP were found to be non significant .

It is concluded that there could be scope for manipulating rumen fermentation of different roughages by including Azolla as nitrogen source and Azolla can be used as unconventional protein source. It is preliminary study and more of *in vivo* studies are required to depict the fact. In 8 % Azolla inclusion level RNA: Gas ratio was found to be significantly elevated and addition of Azolla can stimulate microbial biomass synthesis. Since dry matter of Azolla is low it is practically impossible to feed such huge quantity of Azolla but regular daily feeding in small quantity along with fibrous crop residues may improve the degradation kinetics of poor quality roughages.

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APPENDICES

APPENDIX-I

Detailed procedure for quantification of purine bases:

Materials required

- 1) 1 and 5ml Pipettes, 15 ml polypropylene tubes, Ball milling machine, centrifuge machine, spectrophotometer and cuvetts
- 2) Reagents required
 - i. 2M HClO_4 (2M per chloric acid).
 - ii. 28.5mM $\text{NH}_4\text{H}_2\text{PO}_4$ and 0.2M $\text{NH}_4\text{H}_2\text{PO}_4$ (ammonium dihydrogen phosphate).
 - iii. 0.4M AgNO_3 (Silver nitrate).
 - iv. 0.5M HCl (Hydrochloric acid).

Procedure

1. The undigested residues of incubations were subjected to ball milling (Retsch® MM 200) for 2 minutes in the 20 1/S frequency.
2. After ball milling, weigh 100 mg of residue into 15ml polypropylene tubes and add 2.5 ml of 2M HClO_4 (2M per chloric acid) and incubate in water bath at 90 to 95°C for 1h.
3. Allow tubes for cooling then add 7.5 ml of 28.5mM $\text{NH}_4\text{H}_2\text{PO}_4$ (ammonium di hydrogen phosphate) and incubate at 90-95°C for 15 minutes.
4. After cooling centrifuge at 5000 rpm for 20 minutes, 0.25ml of supernatant was added to polypropylene tubes containing 4.5 ml 0.2M $\text{NH}_4\text{H}_2\text{PO}_4$ then add 0.25 ml of AgNO_3 (Silver nitrate 0.4M).
5. Mixture is kept over night at 5°C in the dark (Refrigerator).

6. The contents were centrifuged at 6000 rpm for 30 minutes, and the supernatant was discarded.
7. Pellet was washed with 4.5 ml distilled water adjusted to pH 2.0 with H₂SO₄ (Sulfuric acid), and again centrifugation was done.
8. Suspend the pellet in 5ml of 0.5M HCl (Hydrochloric acid), vortex mixed thoroughly and kept in water bath at 90-95°C for 30 minutes after covering the tubes with marble.
9. Centrifuge the contents at 6000 rpm for 30 minutes.
10. Read the absorbance of supernatant at 260 nm against 0.5M HCl in glass cuvetts QG HELLMA (HELLMA®) using spectrophotometer (JENWAY® 6505UV / vis).

RNA equivalents can be calculated using the regression equation:

$$Y \text{ (mg)} = 0.2359 + 14.374 \times \text{OD}$$

Regression equation was obtained by using standard yeast RNA (Sigma®) concentration of 2.5 to 10.0 mg against the O.D at 260 nm.

APPENDIX-II

In vitro net gas production at different intervals of time (ml/200 mg DM) for FMS supplemented with Azolla

Varieties	2h	4h	6h	8h	10h	12h	16h	20h	24h	30h	36h	48h	60h	72h	84h	96h
FMS	5.22	8.96	12.10	16.11	19.39	22.48	27.52	31.40	34.26	38.09	41.33	45.48	48.06	50.14	51.34	52.54
	5.23	8.98	12.59	15.55	18.42	21.80	27.53	31.61	35.08	38.83	41.14	45.30	48.31	49.65	51.37	52.75
	3.26	5.92	8.49	11.29	14.68	17.57	22.85	26.89	29.78	33.27	35.89	40.61	42.03	44.14	45.98	47.12
	5.93	9.56	12.65	16.37	20.23	23.45	29.34	33.38	36.46	39.82	42.49	46.35	48.79	50.49	51.50	52.33
	4.91	8.35	11.45	14.83	18.18	21.33	26.81	30.82	33.90	37.50	40.21	44.43	46.80	48.61	50.05	51.19
FMS+ Azolla 2%	4.65	8.52	11.09	14.82	18.23	21.17	26.33	29.37	33.06	36.19	39.41	43.55	45.86	47.93	49.54	50.60
	4.84	8.74	12.51	15.21	18.65	21.91	27.12	30.65	34.70	38.61	40.79	44.70	47.58	48.79	51.21	52.33
	3.76	6.39	8.51	11.82	15.31	18.03	23.38	27.50	30.63	34.62	37.33	41.19	43.41	45.90	47.58	48.44
	5.98	9.56	13.00	16.67	20.07	23.52	29.18	32.76	35.93	39.51	41.59	45.94	48.07	49.47	50.47	51.15
	4.81	8.30	11.28	14.63	18.07	21.16	26.50	30.07	33.58	37.23	39.78	43.85	46.23	48.02	49.70	50.63
FMS + Azolla 4%	5.25	9.29	12.44	16.07	18.81	22.19	26.84	30.32	33.47	36.35	39.04	43.22	45.40	47.77	48.98	49.76
	4.12	8.29	11.34	14.72	17.31	20.41	25.88	29.67	33.00	36.34	39.21	43.23	46.10	47.58	49.58	50.55
	4.58	7.47	10.42	13.45	17.08	19.80	25.28	29.67	32.39	36.78	39.50	43.48	45.16	47.38	48.78	49.78
	5.15	8.72	12.02	15.28	18.67	22.24	27.48	31.32	34.62	37.92	40.27	44.61	47.14	48.54	49.81	50.62
	4.77	8.44	11.56	14.88	17.97	21.16	26.37	30.24	33.37	36.85	39.50	43.64	45.95	47.82	49.29	50.18
FMS + Azolla 6%	5.67	9.43	12.73	16.35	19.51	22.48	27.41	30.89	34.05	37.21	40.32	44.36	46.82	48.92	50.26	51.19
	4.67	7.99	11.87	14.97	17.69	27.44	25.96	30.31	33.77	37.23	39.96	44.26	47.82	49.29	50.73	51.69
	5.08	8.37	10.76	13.91	17.37	20.21	25.52	29.88	32.99	37.22	39.92	43.61	45.27	47.61	48.87	49.73
	6.07	9.62	13.31	16.96	20.19	23.48	28.69	32.92	35.39	38.81	40.61	44.66	46.77	48.70	49.83	50.50
	5.37	8.85	12.17	15.55	18.69	23.40	26.90	31.00	34.05	37.62	40.20	44.22	46.67	48.63	49.92	50.78
FMS + Azolla 8%	5.79	9.96	12.97	17.70	20.71	23.53	28.58	31.78	34.93	38.22	41.46	45.49	47.81	49.61	50.82	52.02
	4.81	8.13	11.87	15.38	17.83	27.16	25.68	29.75	32.94	36.54	38.99	42.60	45.32	46.80	48.51	49.76
	6.04	9.60	13.08	16.50	20.11	23.09	28.41	32.78	35.49	39.32	41.89	45.32	47.12	48.93	50.46	51.45
	5.54	7.89	11.05	14.43	17.13	20.02	25.38	28.80	31.69	34.98	37.05	41.78	43.77	44.76	45.75	46.70
	5.54	8.90	12.24	16.00	18.95	23.45	27.01	30.78	33.76	37.27	39.85	43.80	46.01	47.53	48.89	49.98

Continued....

***In vitro* net gas production at different intervals of time (ml/200 mg DM) for paddy straw supplemented with Azolla**

Varieties	2h	4h	6h	8h	10h	12h	16h	20h	24h	30h	36h	48h	60h	72h	84h	96h
Paddy straw	2.11	4.13	5.43	8.53	10.65	13.25	18.28	20.45	29.43	31.39	35.21	40.06	43.52	45.94	47.78	48.95
	1.16	3.84	4.38	7.38	9.49	18.52	17.09	21.70	26.00	30.56	33.60	38.97	42.82	44.65	47.12	48.19
	2.76	4.14	5.70	7.62	10.38	12.79	18.58	23.71	27.72	32.84	36.45	41.84	44.56	47.54	49.06	50.44
	3.22	5.28	7.34	9.08	12.17	15.84	22.24	26.85	31.06	35.53	38.39	43.77	46.94	49.94	51.19	52.13
	2.31	4.35	5.71	8.15	10.67	15.10	19.05	23.18	28.55	32.58	35.91	41.16	44.46	47.02	48.79	49.93
Paddy Straw + azolla 2%	2.45	4.39	6.01	8.51	11.24	14.20	19.65	24.51	28.49	32.88	36.67	41.95	45.37	47.87	49.48	51.11
	1.47	4.25	5.35	7.75	9.37	11.91	17.50	22.25	26.41	31.40	34.26	39.53	43.64	45.53	47.93	49.04
	2.32	4.21	5.87	7.89	10.34	13.10	18.53	23.30	27.38	32.29	35.70	40.48	43.41	46.08	47.84	48.93
	2.63	4.64	6.91	9.54	11.90	15.36	21.22	25.99	29.58	34.22	37.41	42.80	46.17	49.10	50.59	51.50
	2.22	4.37	6.04	8.42	10.71	13.64	19.23	24.02	27.96	32.70	36.01	41.19	44.65	47.14	48.96	50.15
Paddy Straw + Azolla 4%	2.48	4.72	6.64	8.88	11.50	14.49	19.72	24.21	28.37	33.09	36.78	41.97	45.29	47.96	49.73	50.67
	2.33	5.83	6.52	8.81	10.85	13.56	19.20	24.00	28.19	32.53	35.56	41.01	44.60	47.06	49.35	50.47
	2.72	4.61	6.27	8.03	10.62	13.12	18.43	23.21	27.03	31.94	35.50	40.67	43.22	45.63	47.78	49.01
	2.37	4.64	6.66	9.29	11.79	15.25	21.12	25.64	29.23	34.14	36.68	42.33	45.31	47.85	49.08	50.39
	2.47	4.95	6.52	8.75	11.19	14.10	19.62	24.26	28.21	32.92	36.13	41.50	44.60	47.13	48.99	50.13
Paddy Straw + Azolla 6%	1.92	4.30	6.07	8.60	10.79	13.78	18.88	23.36	27.66	31.68	34.95	40.00	43.17	45.70	47.61	49.11
	2.76	5.43	7.26	10.54	13.02	15.46	20.98	25.66	29.60	34.23	37.27	42.61	46.22	47.99	50.43	51.69
	2.83	4.44	6.23	8.10	10.67	13.41	18.55	23.30	27.22	32.36	35.50	40.64	43.69	46.08	47.95	49.04
	2.60	4.86	6.99	9.72	12.06	15.49	20.65	25.51	28.68	33.15	36.18	40.74	43.82	46.08	47.55	48.33
	2.53	4.76	6.64	9.24	11.64	14.54	19.76	24.46	28.29	32.85	35.98	41.00	44.22	46.46	48.39	49.54
Paddy Straw + Azolla 8%	2.33	4.85	6.48	9.42	12.31	15.58	20.24	24.30	28.87	33.58	37.26	42.57	45.89	48.40	49.76	51.11
	1.78	4.31	5.99	8.99	11.05	13.49	19.01	23.56	27.49	32.27	35.32	40.94	43.70	46.04	48.20	49.60
	2.97	4.98	6.76	8.64	11.22	14.10	19.38	23.74	27.54	32.42	35.57	40.72	43.25	45.91	47.79	48.75
	2.77	4.65	6.67	9.44	11.94	15.14	20.76	25.28	28.22	33.00	35.55	40.55	43.67	45.82	47.58	48.23
	2.46	4.70	6.48	9.12	11.63	14.58	19.85	24.22	28.03	32.82	35.92	41.20	44.13	46.54	48.33	49.42
Azolla	4.14	9.13	13.54	16.65	18.36	20.70	23.03	24.66	26.36	28.07	29.33	30.41	31.31	32.39	32.48	33.25
	5.49	12.37	17.09	19.83	21.14	22.80	25.14	26.94	28.56	30.04	30.81	32.29	33.46	33.82	34.40	34.67
	6.83	12.13	17.61	21.56	23.94	26.77	28.84	31.04	32.66	34.18	35.13	36.38	36.70	37.69	38.40	38.31
	4.85	10.29	15.45	18.96	20.57	23.05	25.56	27.49	29.42	31.22	32.08	33.42	34.46	35.04	35.22	35.63
	5.33	10.98	15.92	19.25	21.00	23.33	25.64	27.53	29.25	30.88	31.84	33.13	33.98	34.73	35.13	35.47

APPENDIX-III

Microbial biomass synthesis indices measured at different levels of Azolla inclusion at t_{1/2}

Treatments	Different variables per 0.5 g DM								
	iv TDOM (gm)	iv ADDM (gm)	iv AUDM (gm)	RNA-eq	RNA/G (mg)	Gas at t _{1/2} (ml)	RNA:GAS	MBP* (mg)	EMBS
FMS	0.1715	0.1767	0.3956	1.99	11.76	103.01	0.1142	137.63	377.84
	0.1838	0.1544	0.3796	2.14	12.46	108.90	0.1144	152.89	389.55
	0.1716	0.1511	0.3825	1.80	9.79	105.64	0.0849	133.81	365.38
	0.1756	0.1607	0.3859	1.98	11.34	105.85	0.1045	141.44	377.59
FMS+ azolla @2%	0.1621	0.1603	0.3976	2.36	15.47	106.06	0.1459	99.89	341.76
	0.1595	0.174	0.3825	1.87	10.60	118.27	0.0896	90.05	257.11
	0.1621	0.1711	0.3941	2.23	14.10	111.70	0.1263	109.29	307.83
	0.1612	0.1685	0.3914	2.15	11.93	112.01	0.1079	99.67	302.23
FMS+ azolla @4%	0.1623	0.1232	0.3974	1.87	11.20	104.78	0.1069	123.77	349.35
	0.1678	0.125	0.3774	2.10	12.24	108.98	0.1123	125.98	344.46
	0.1677	0.124	0.398	2.03	12.61	109.22	0.1154	126.04	344.07
	0.1659	0.1241	0.3909	2.00	12.02	107.66	0.1086	125.26	345.96
FMS+ azolla @6%	0.1619	0.1222	0.4016	2.24	14.62	100.28	0.1458	132.33	374.92
	0.147	0.1429	0.3889	2.16	13.28	98.25	0.1351	104.80	326.53
	0.1669	0.1213	0.403	1.97	12.20	102.09	0.1195	137.92	380.47
	0.1586	0.1288	0.3978	2.12	12.95	100.21	0.1235	125.02	360.64
FMS+ azolla @8%	0.167	0.1422	0.3958	1.89	11.17	101.06	0.1105	140.62	387.43
	0.1453	0.1542	0.4016	2.33	15.28	97.89	0.1561	100.72	318.65
	0.148	0.1425	0.3914	2.14	13.30	85.25	0.1561	135.96	420.27
	0.1534	0.1463	0.3963	2.12	12.98	94.73	0.1289	125.77	375.45

Continued....

Different variables per 0.5 g DM									
Treatments	iv TDOM (mg)	iv ADDM (gm)	iv AUDM	RNA-eq	RNA/G (mg)	Gas at t 1/2 (ml)	RNA:GAS	MBP (mg)	EMBS
Paddy Straw	0.1701	0.1494	0.391	1.66	10.07	119.24	0.0845	97.29	270.55
	0.1756	0.1432	0.3844	1.54	12.53	118.48	0.1058	111.53	299.66
	0.1709	0.1512	0.3858	1.68	10.08	119.63	0.0843	98.67	272.67
	0.1722	0.1479	0.3871	1.63	10.90	119.12	0.0915	102.50	280.96
PS+Azolla @2%	0.1669	0.1561	0.3658	1.71	9.64	123.68	0.0779	81.36	230.20
	0.1613	0.1695	0.3842	1.94	12.16	128.21	0.0949	60.33	176.19
	0.1663	0.1606	0.3859	1.50	8.56	125.85	0.0968	75.47	214.19
	0.1648	0.1621	0.3786	1.72	10.12	125.91	0.0898	72.39	206.86
PS+Azolla @4%	0.1708	0.1805	0.3693	1.81	10.57	127.50	0.0829	82.45	227.17
	0.1639	0.161	0.4318	1.79	12.71	126.03	0.1009	69.90	201.34
	0.1714	0.1602	0.3734	1.53	8.49	119.93	0.0707	99.98	274.80
	0.1687	0.1672	0.3915	1.71	10.59	124.49	0.0848	84.11	234.43
PS+Azolla @6%	0.1796	0.1828	0.3564	1.96	10.99	115.87	0.0948	120.04	320.16
	0.1808	0.1218	0.3711	1.53	8.24	114.56	0.0719	124.56	330.75
	0.1814	0.1792	0.3545	1.96	10.88	111.06	0.0979	132.24	351.16
	0.1806	0.1613	0.3607	1.81	10.04	113.83	0.0882	125.61	334.02
PS+Azolla @8%	0.179	0.1969	0.3843	1.77	10.63	111.53	0.0953	131.31	348.60
	0.1704	0.1833	0.3751	1.80	10.68	108.90	0.2078	120.74	335.09
	0.1439	0.2015	0.3874	1.84	11.42	100.49	0.1822	83.35	273.80
	0.1644	0.1939	0.3823	1.80	10.91	106.97	0.1618	111.80	319.17
Azolla	0.2001	0.1652	0.3462	3.02	16.03	83.01	0.1931	251.63	579.46
	0.186	0.1721	0.3687	2.96	17.13	77.57	0.2209	232.91	577.15
	0.2205	0.1596	0.3716	2.86	16.45	78.72	0.2090	305.65	638.32
	0.2022	0.1656333	0.3622	2.95	16.54	79.76	0.2077	263.40	598.31

*MBP = TDOM - (ml gas x 2.20)

EMBS = (MBP/ iv TDOM)