CHAPTER - 3
MATERIALS AND METHODS
Materials and Methods

The present study was carried out in Plant Animal Relationship division at Indian Grassland and Fodder Research Institute, Jhansi (U.P). The materials used, techniques employed and procedures followed to execute the technical programme of study are presented in this chapter. The contents of the chapter are broadly distributed in to following parts.

3.1 Collection and preparation of feed and fodder samples
3.2 Preparation of roughage protein dietary regimens
3.3 Mineral supplementation (Dietary treatments)
3.4 Proximate constituents estimation
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   3.7.4 Processing of feed, residue and faeces samples
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3.8 Rumen metabolites estimation
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3.1 Collection and preparation of feed and fodder samples

Feed samples of protein sources namely groundnut cake (GNC), cotton seed cake (CSC), mustard seedcake (MSC) and linseed cake (LSC) were collected from feed store of Plant Animal Relationship Division while roughage wheat straw, mixed grass were collected from experimental farm of Indian Grassland and Fodder Research Institute, Jhansi.

Initially the samples of roughages and protein sources were dried in hot air oven at 65-70 °C for 4 days till a constant weight is attained. The dried samples were ground through 2 mm sieve using electrically operated Willey mill. The ground samples were then stored in Tarson make sample bottles after proper labelling for further chemical and biochemical estimations.

3.2 Preparation of roughage protein dietary regimes

Roughage protein dietary combinations were prepared using wheat straw and grass as roughage and groundnut cake, cotton seed cake, mustard seed cake and linseed cake as protein sources. The following dietary regimes were formulated:

Wheat straw-groundnut cake

Wheat straw-cotton seed cake

Wheat straw-mustard cake

Wheat straw-linseed cake

Grass- groundnut cake

Grass- cotton seed cake

Grass- mustard cake

Grass- linseed cake

The crude protein contents of the above dietary regimes were 12%.
To achieve 12 % crude protein in the roughage-protein dietary regimen following amount/quantity of roughage and protein was mixed in the composite sample (roughage-protein combination).

<table>
<thead>
<tr>
<th>S. No</th>
<th>Roughage protein combination</th>
<th>Roughage</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wheat straw-groundnut cake</td>
<td>81.40</td>
<td>18.50</td>
</tr>
<tr>
<td>2</td>
<td>Wheat straw-cotton seed cake</td>
<td>71.31</td>
<td>28.60</td>
</tr>
<tr>
<td>3</td>
<td>Wheat straw-mustard cake</td>
<td>76.50</td>
<td>23.50</td>
</tr>
<tr>
<td>4</td>
<td>Wheat straw-linseed cake</td>
<td>82.60</td>
<td>17.39</td>
</tr>
<tr>
<td>5</td>
<td>Grass-groundnut cake</td>
<td>80.50</td>
<td>19.50</td>
</tr>
<tr>
<td>6</td>
<td>Grass-cotton seed cake</td>
<td>70.10</td>
<td>29.80</td>
</tr>
<tr>
<td>7</td>
<td>Grass-mustard cake</td>
<td>75.40</td>
<td>24.50</td>
</tr>
<tr>
<td>8</td>
<td>Grass-linseed cake</td>
<td>81.70</td>
<td>18.20</td>
</tr>
</tbody>
</table>

3.3 Mineral supplementation (Dietary treatments)

Different roughage protein dietary regimens prepared for the study (mentioned in 3.2) were supplemented with mineral mixture. The mineral mixture used in the study was of Alembic made and its composition is mentioned in Table 3.1. The level of mineral mixture supplementation varied from 0-2.5%.

Each dietary regimen was supplemented with different levels of mineral mixtures and for each diet/substrate treatments were as follows:

- $T_0$: Control (0 %)
- $T_1$: 1.0 %
- $T_2$: 1.5 %
- $T_3$: 2 %
- $T_4$: 2.5 %
Table 3.1 Composition of mineral mixture

Flavoured mineral feed enriched with amino acids. Each 100gm provides

- Calcium          -30gm
- Phosphorus       -12gm
- Manganese        - 0.12gm
- Iodine           - 0.1gm
- Iron             - 0.6gm
- Copper           - 0.1gm
- Cobalt           - 0.02gm
- Zinc             - 0.215gm
- Lysine           - 0.45gm
- DL-Methionine    - 0.2gm
- Fluorine not exceeding - 0.03gm

3.4 Proximate constituents estimation

The quantitative chemical analysis of various constituents in feed and faeces is the first step and essential prerequisite to estimate the nutritive value of feeds. The method of analysis followed in the present investigation for the estimation of dry matter and proximate principles were essentially those recommended by A.O.A.C. (1980). Methods employed were as follows.

Each ground sample of feed (grass and protein sources) and their dietary regimens were spread in a thin layer over glazed paper before weighing so that the sample attained the room temperature and humidity. Suitable quantities were weighed for estimation of different constituents using single pan balance (Metteler AE 2000).
Dry Matter

The moisture content of the sample was determined so the analytical results could be presented on the dry matter or moisture free basis. A suitable quantity of the sample was weighed in aluminium moisture cup. The sample was dried at 100± 5°C in a hot air oven for about 24 hrs the samples was brought to room temperature in a dessicator and weighed. the loss in weight of the material on drying was the moisture content. Percent dry matter was calculated using the formula given below.

\[
\text{Dry matter (\%)} = \frac{\text{Weight of dried sample}}{\text{Weight of fresh sample}} \times 100
\]

Crude Protein

A representative feed sample of 2g was accurately weighed in already weighed aluminium scoop and transferred in to a kjeldahl flask. About 25 ml of concentrated sulphuric acid and digestion mixture (sodium / potassium sulphate and copper sulphate in the ratio of 10:1) were added in to the flask. the contents were digested first at low temperature then at high one till frothing subsides. the digestion was continued till a blue greenish solution was left in the flask. Then flask was cooled and all the content were quantitatively transferred in to a 250 ml capacity round bottom volumetric flask. Kjeldahl flask washed with distilled water to make 250 ml final volume in the round bottom flask. About 20 ml boric acid solution was taken in conical flask and put it under the condenser with tip beneath surface of after it 10 ml of digested material from volumetric flask was put in distillation tube. to this 10-15 ml of 40% NaOH was added the contents were allowed to distil till a volume of 40-50 ml was obtained in conical flask. The conical flask with distilled contents was removed and titrated with standard sulphuric acid (N/10 H₂SO₄)
The percentage of nitrogen in sample was calculated by using the factor 1ml of N/10 H₂SO₄ equal to 0.0014 gm of nitrogen and percentage crude protein was calculated by multiplying the nitrogen percentage by 6.25.

\[
\text{Total-N} (\%) = \frac{\text{Volume of N/10H}_2\text{SO}_4 \times 0.014 \times \text{aliquot factor}}{\text{Weight of sample taken on DM basis}} \times 100
\]

\(\%\) Crude protein = \(\%\) Nitrogen \(\times\) 6.25

**Total Ash**

Weigh exactly about five gm samples in to a tarred silica basin and desmoke on heater. Transfer the basin in to a muffle furnace and ash at a temperature of 550-600\(^0\) c for three hour. Cool in a desiccators and weigh the silica basin.

\[
\text{Total ash} (\%) = \frac{(\text{Wt. of silica basin + ash}) - \text{wt. of the empty silica basin}}{\text{Weight of sample taken}} \times 100
\]

**3.4 Cell wall determination**

The different fibre fractions (cell wall constituents) in the biological samples were estimated following the method of Goering and Von soest (1970). The procedures followed are mentioned below.

**Neutral detergent Fibre (NDF)**

NDF or the cell wall constituents which include hemicellulose, cellulose lignin and silica were determined in feeds residue and faecal samples the following reagent were used for their estimation -

**Neutral detergent solution**

**Reagents:**

Distilled water - 1 litre

Sodium lauryl sulphate - 30 g
Di sodium ethylene di amino -18.61g
Tetra acetate (EDTA) dehydrate Sodium borate decahydrate -6.81g
Di sodium hydrogen phosphate (anhydrate) -4.5 g
2-Ethoxy ethanol - 10 ml

Both EDTA and sodium borate decahydrate were put in a large beaker
containing some of the distilled water. The beaker was shaken and heated until
they were dissolved. The sodium lauryl sulphate and 2- ethoxy ethonal were
added to it while disodium hydrogen phosphate put in separate beaker containing
some distilled water and heated until it was dissolved then both the solutions
were mixed properly and volume was made to 1 litre.

3-Decahydronephelene

4- Acetone

Procedure - one gm air-dried sample (ground to pass through 20- 30 mesh
screen) was taken in 500 ml spout less beaker. About 100ml of neutral detergent
solution along with 2 ml of decahydronephelene were poured in to these beakers.
The beakers put on soxlet apparatus and the samples were refluxed for 60
minutes, taking from on set of boiling. The solution was filtered in weighed
crucible and the samples were washed repeatedly with hot water. The washing
was also done twice with acetone. Crucibles were then dried in hot air oven at
100 +/- 50c for overnight and weighed. The NDF was calculated as follows-

\[
NDF(\%) = \frac{(\text{Weight of crucible + residue of sample}) - (\text{weight of crucible})}{\text{Weight of sample taken}} \times 100
\]
Acid Detergent Fiber

Reagents

1- 20% Acid detergent solution was prepared by dissolving 20 g of acetylene trimethyl ammonium bromide (CTAB) in 1N H₂SO₄ and volume was made to 1 litre.

2- Acetone

Procedure - Weigh 2.0 g powered sample in to a 500 ml spout less beaker. To these beakers 100ml of acid detergent solution and 2ml of decaline were added reflux the sample for one hour. Filter through a tarred printered / sinetral glass crucible wash the beaker by hot distilled water till all the sample is transferred to the crucible thoroughly wash the content of the crucible till there is no detergent solution. Final 2-3 washing was given with acetone. Dry crucible is an oven at a temperature of 100+- 5°C for over night. Put the crucible in a dessicator and weigh . The ADF was then calculated as below.

\[
NDF (\%) = \frac{(\text{Weight of crucible} + \text{residue of sample}) - (\text{weight of crucible})}{\text{Weight of sample taken}} \times 100
\]

Acid detergent lignin (ADL)

Reagent

72% sulphuric acid (w/w)

For ADL estimation sample left after ADF estimation were treated with 72% H₂SO₄ for 3-4 hours. Treated crucibles were washed with cold water for 3-4 times till no acid left. During washing lump was broken with glass rod. Washed crucibles were then dried in hot air oven at 70 °C for two successive days and kept in muffle furnace at 600 OC for 3 hours for ashing.

ADL of feed forage sample was calculated by following formula
\[
\text{% ADL} = \frac{\text{Weight of crucible + Lignin) - \text{weight of crucible + Ash}}}{\text{Weight of sample}} \times 100
\]

**Cellulose**

To determine cellulose credits after 72 % \(\text{H}_2\text{SO}_4\) treatment were ashed at \(500^\circ\text{C}\) for 3 hours and cellulose was estimated as the difference between this weight (ash weight) and original weight of crucible (lignin weight).

\[
\text{Cellulose\%} = \frac{\text{Wt. of lignin crucible - weight of crucible + Ash}}{\text{Weight of sample taken for ADF estimation}} \times 100
\]

**Hemi-cellulose**

The hemi-cellulose contents of roughages (wheat straw, grass), protein sources (groundnut cake, cotton seed cake, mustard cake and linseed cake), residue and faeces were estimate by difference using following formula.

\[
\text{Hemi-cellulose} = \text{% NDF} - \text{ADF}\%
\]

**3.5 In vitro dry matter digestibility**

In vitro DM digestibility of roughage-protein dietary combinations was estimated adopting technique of Tilley and Terry (1963) with rumen inoculums of sheep. In the present study only the first stage of technique was used and samples were incubated for 24 and 48 hours.

**Reagents**

1) Phosphate carbonate buffer,

<table>
<thead>
<tr>
<th>Composition</th>
<th>g/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{NaHCO}_3)</td>
<td>9.80</td>
</tr>
<tr>
<td>(\text{NaHPO}_4.2\text{H}_2\text{O})</td>
<td>7.00</td>
</tr>
<tr>
<td>(\text{KCl})</td>
<td>0.57</td>
</tr>
<tr>
<td>(\text{NaCl})</td>
<td>0.47</td>
</tr>
<tr>
<td>(\text{MgSO}_4.7\text{H}_2\text{O})</td>
<td>0.12</td>
</tr>
<tr>
<td>(\text{CaCl}_2)</td>
<td>0.04</td>
</tr>
</tbody>
</table>
To prepare the buffer first five chemicals were dissolved in 500 ml water in volumetric flask of one litter. The contents were stirred well till dissolved and made to volume up to mark. Just before use CaCl$_2$ kept at 39 °C was added and then CO$_2$ was passed through the buffer solution until pH was between 6.8-7.1.

**Method**

About 0.5 g of oven-dried sample was taken in 50ml conical flask fitted with gas release valve. To these sample 50 ml (40 ml of CO$_2$ saturated phosphate carbonate buffer and 10ml strained rumen liquor) buffer and rumen inoculum’s mixture was added. The rumen liquor was collected from sheep using stomach tube and filtered through double layer of muslin cloth before use. CO$_2$ was passed over the surface of contents for few second and then stopper was fitted on the flasks immediately. One flask having no sample was used as blank. After it the same was incubated at 39 °C for a period of 24 hour. These flasks were swirl occasionally during the incubation. After 48 hrs of incubation, flasks were removed from the incubator and contents were filtered through pre-weighed sintered crucible and residue was washed with water. The filtrate of triplicate samples was pooled and used for ammonia estimation. Crucibles were dried in hot air oven (65± 5 °C) and weighed when constant weight is obtained. The loss in DM of sample was calculated as *In vitro* dry matter digestibility using below following formula.

DM disappearance = Weight of sample - (weight of residue sample - weight of residue blank)

\[
    \text{DM disappearance} \\
    \text{DM digestibility\%} = \frac{\text{DM disappearance}}{\text{Weight of sample}} \times 100
\]
**Source of inoculum for in vitro enumeration of anaerobic microbes**

Different roughage and protein dietary combinations subjected to in vitro dry matter digestibility were also subjected to first stage of Tilley and Terry technique where roughage-protein combinations supplemented with varying levels of mineral mixture were incubated for 48 hours. After it the incubated samples were filtered and the filtrate was used as source of inoculum to estimate the rumen microbes (total viable, amylolytic, cellulolytic and proteolytic bacteria along with ruminal fungi, protozoa counts, their generic distribution) and cellulase enzyme activity.

3.7 *In vivo* experiments

3.7.1 Selection of animal

For the in vivo studies 20 adult sheep of Muzafarnagari breed were selected from the livestock farm of Plant Animal Relationship Division. These animals were distributed randomly into 4 dietary groups with 4 animals in each. The description of the animals is given in Table 3.2
Table 3.2: Description of animals

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Weight of animals (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_0$</td>
</tr>
<tr>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>Mean Body weight</td>
<td>29</td>
</tr>
</tbody>
</table>
3.7.2 Feeding of animals

The animals were tied with iron chains individually and were offered required amount of grass and groundnut cake supplemented/fortified with different levels of mineral mixture (T₀- 0, T₁-1.0, T₂-1.5, T₃-2.0 and T₄-2.5%). Animals were first offered groundnut cake supplemented with mineral mixture followed by offering of mixed grass daily between 9.30 to 10.00 a.m. The animals were having free access to drinking water. The animals were maintained on these dietary regimens for 70 days.

3.7.3 Digestibility trial

After about 70 days of feeding a digestion trial of 5 days duration was conducted. The animals were put in metabolic cages and samples of feed offered, residue left and faeces of each animal were collected daily. The representative samples of faeces, feed and residue were taken for subsequent biochemical analysis.

3.7.4 Processing of feed, residue and faeces samples

Sample of dry mixed grass, groundnut cake residue were dried and then grinded by the electrically operated willey will using 2mm sieve for further analysis. For dry matter and faecal-N estimation 100 and 10gm of sample was pooled during the collection period. For faecal -N sample was preserved in glass bottle with 40% H₂SO₄ solution. The faecal sample kept for DM estimation was further grinded with willey will to estimate the cell wall polysaccharide and ash contents.

3.7.5 Collection and processing of rumen liquor samples

After the end of digestion trial rumen liquor samples were collected from individual animal to estimate the TVFA, pH, rumen metabolites and rumen microbes. Rumen liquor sample were taken before feeding of animal by putting a pressure tube into rumen through mouth. The SRL was collected in thermos
having temperature around 38°C. The SRL samples collected were filtered through double layer of musclin cloth. The pH of SRL was estimated just after collection of rumen liquor.

Rumen liquor collected from individual animal was further processed for rumen metabolites and microbial parameters. For rumen metabolites samples were preserved with HgCl2 while for microbial studies sample were preserved with 10% formal saline solution.

3.6 Rumen metabolites estimation

Total-nitrogen

Total nitrogen in the rumen liquor was estimated by micro Kjeldhal method. In a digestion tube 5ml of SRL was digested with 7.5 ml of concentrated sulphuric acid in presence of digestion mixture (sodium sulphate and copper sulphate in 10:1 ratio). Digested sample was transferred to 100ml volumetric flask and volume was made to 100ml with distilled water .A suitable aliquot was used for nitrogen estimated by micro- kjeldahl method.

Ammonia Nitrogen By Conway Diffusion Method

In this method SRL is treated with saturated solution of potassium carbonate (K₂CO₃) in the outer chamber of Conway disc and ammonia liberated is trapped in 2% boric acid indicator in the inner chamber of disc keep it for three hrs. Now titrate it against N /100 H₂SO₄ for quantative estimation of ammonia - N from the factor (1ml of 0.01N H₂SO₄ =0.00014 gm nitrogen).

TCA Soluble and TCA Perceptible nitrogen

Precipitate 5ml of SRL with 5ml of 20%TCA and keep over night in refrigerated condition. Next day centrifuge it at 3000 rpm for 15 minutes .5ml of supernatant with TCA soluble N was used for nitrogen estimation by micro
kjeldahl method. TCA precipitated -N was calculated by deducting TCA soluble N from total nitrogen of SRL.

**Total Volatile Fatty Acid (TVFA)**

TVFA in the rumen liquor were estimated by the method of Barnett and Reid (1956). One ml of SRL (preserved in HgCl₂) was distilled in the presence of 1ml of potassium oxalate buffer (5% oxalic acid and 10 % of potassium oxalate in the ration of 1: 1) in markhams distillation apparatus about 80-100 ml of distillate was collected in a conical flask placed in ice bath which was immediately titrated against N/50 NaOH Solution using phenolphthaline indicator amount of TVFA was calculated (1ml N/50 NaOH= 0.02 Millimole TVFA) and expressed per 1000 ml of SRL.

**Rumen liquor pH**

Rumen liquor collected from the sheep through stomach tube and was filtered immediately through double layer of muslin cloth. The ph of filtered rumen liquor was determine using electronic pH meter (Systronic -10)

**3.7 Anaerobic microbes**

**3.7.1 Bacteria**

**Techniques**

**Total viable bacteria counts**

For determination of viable bacteria counts anaerobic dilution fluid was prepared adopting the technique given by of Brayant & Burkey (1953). Dilution fluid was prepared using the below mentioned ingredients -

**Composition Of Dilution Fluid**

- Mineral solution I - 15.00 ml
- Mineral solution II - 15.00 ml
- Resazurin (0.1%) - 0.1 ml
Cysteine HCL - 0.05 gm
Sodium bicarbonate - 0.4 gm
Distilled water - 100 ml (volume)

The above-mentioned ingredients except cysteine-HCL and sodium bicarbonate were mixed, boiled and cooled under CO₂ atmosphere. After addition of cysteine HCL and sodium bicarbonate the resazurin gets completely reduced. After it, 9ml of this dilution was dispensed into test tube continuously gassed with CO₂. Test tubes were properly stoppered and sealed with aluminium caps with crimpier before autoclaving at 15lb pressure for a period of 20 minutes.

For total viable bacteria counts media was prepared as reported by Kurihara et al (1968) with the following ingredients -

Bacto-casitone - 1.0 gm
Bacto - yeast extract - 0.25 gm
Mineral solution 1 - 15.00 ml
Mineral solution II - 15.00 ml
Clarified rumen liquor - 20.00 ml
Resazurin (0.1%) - 0.1 ml
Glucose - 0.2 gm
Starch (added as solution in 15 ml water) -0.5 gm
Sodium bi carbonate - 0.4 gm
Cystein -HCL -0.05 gm
Distilled water - 100 ml (volume)

All above mentioned ingredients except cysteine HCL and sodium bicarbonate were boiled under CO₂ till the resazurin turns pink. Media was cooled and added with cysteine- HCL and sodium bicarbonate under CO₂ to get final pH of 6.8 in the media from this media. 9 ml was dispended in to CO₂
gassed test tubes were then properly stoppered and sealed before autoclaving till their use.

For culturing total bacteria (MPN) freshly drawn rumen liquor along with digesta from sheep was strained through two layers of muslin cloth from strained rumen liquor, one ml was taken with the help of sterilized inoculation syringe (1-2ml capacity) and then inoculated in to tubes containing 9ml of sterilized dilution fluid successive dilutions were prepared up to 10-10 from this dilution in the same manner. This dilution fluid was used to inoculate a set of three tubes in duplicate containing culture media with one ml of each dilution ($10^8$, $10^9$, $10^{10}$). Three dilutions were inoculated using one set for each dilution. The inoculated tubes were incubated at $38 \pm 1^\circ C$, tubes showing microbial growth were identified and scored after 5 to 10 days of inoculation for total viable count. Most probable numbers of these bacteria were computed using Mc Crady's (1918) table on the basis of positive tubes.

**Amylolytic Bacteria**

Estimation of starch hydrolysing bacteria was done by adding the 0.1 ml iodine -potassium iodide solution (1: 2) in the tubes. Tubes showing brown colour on addition were considered as positive and those become black were treated as negative numbers were computed on the basis of positive tubes using Mc Crady table.

**Cellulolytic Bacteria**

For culturing cellulolytic bacteria from rumen of sheep, what man No.1 filter paper strip was used as a source of cellulose in the media. The media was prepared as described by Mann (1968) with the following ingredients.
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-casitone</td>
<td>0.25 gm</td>
</tr>
<tr>
<td>Bacto yeast extract</td>
<td>0.06 gm</td>
</tr>
<tr>
<td>Clarified rumen liquor</td>
<td>20 ml</td>
</tr>
<tr>
<td>Mineral solution 1</td>
<td>15 ml</td>
</tr>
<tr>
<td>Mineral solution 11</td>
<td>15 ml</td>
</tr>
<tr>
<td>Resazurin (0.1%)</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.025 gm</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.05 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml (volume)</td>
</tr>
<tr>
<td>Cellulose added</td>
<td>whatman No.1 filter paper strip</td>
</tr>
</tbody>
</table>

All the ingredients except the cysteine -HCL and sodium bicarbonate were mixed in the media flask and boiled under CO₂. After resazurin has turned pink, the flask was cooled to 450c under CO₂. Cysteine- HCL and sodium bicarbonate were added and pH of media adjusted to 6.5 from culture flask 9ml of media was dispensed in to test tube gassed with CO₂. These tubes were autoclaved and then stored at room temp. Till use dilution of rumen inoculum up to 10 -10 was made with the help of sterilized syringe as mentioned earlier. A set of three media tubes for each dilution (10-8, 10-9 10-10) was inoculated with sterilized syringe (1-2 ml capacity) the inoculated tubes were incubated at 38 +/10c and observations were recorded after 1st, 2nd, 3rd, 7th, 14th and 21st days of incubation. cellulolytic bacteria growths shows pitting followed by disintegration of filter paper strip . Most probable number of cellulolytic bacteria was computed based on positive tubes using McCrady's table (1918).

**Proteolytic Bacteria**

For enumerating the proteolytic bacteria composition of media used described by Abou - Akkada and Blackburn (1963) was followed.
The ingredients of the media are as follows.

- **Mineral solution 1**  - 15 ml
- **Mineral solution 11**  - 15 ml
- **Cysteine -HCL**  - 0.05 gm
- **Clarified rumen liquor**  - 10 ml
- **Tryptose**  - 0.3 gm
- **Agar**  - 2.5 gm
- **Resazurin**  - 0.1 ml
- **Sodium bicarbonate**  - 0.5 gm
- **Distilled water**  - to make 100 ml of volume
- **Glucose**  - 0.1 gm

Method of inoculation and dilution preparation is same as described for total viable bacteria except the dilution were made up to \(1 \times 10^9\) and tubes inoculated were of \(10^8, 10^9, 10^{10}\) dilution. The inoculated tubes were incubated for 9-12 days. Opacity as indicator of proteolysis was recorded by adding one ml of cultured sample and one ml of 1N HCL. Most probable number of proteolytic bacteria was computed using MC crady table based on the positive tubes on the positive tubes (proteolysis).

**Estimation of Fungal Population**

For the period under report the method of Theodorou and Trinci (1988) for culturing of anaerobic ruminal fungi was followed to estimate the fungal population in the rumen of sheep. This procedure relies on most probable no. (MPN) tables to provide a viable cell count. The composition of fungal media used is given below.
Mineral solution 1 - 15 ml
Mineral solution 11 - 15 ml
Clarified rumen liquor -15 ml
Bacto-casitone (difco) - 1.0 gm
Yeast extract (difco) - 0.25 gm
Sodium bicarbonate - 0.6 gm
Resazurin (0.1%) - 0.1 ml
Cysteine - HCl - 0.1 gm

Above ingredients were pooled and 100 ml volume was made with distilled water, boiled and cooled while CO₂ gas passed through it for at least 10 minutes before adding the cysteine - HCl. Gassing was continued till the colour of media changes from pink to straw. Dispensing of 45 ml media in to each gassed bottle was sealed with aluminium crimps and then autoclaved at 15lb /sq inch or (115°C) for 20 min.

Mineral solution - K₂HPO₄ - 3.0 gm/ L
Mineral solution 11 - KH₂PO₄ -3.0 gm/ L
(NH₄)₂SO₄ - 6.0 gm/ L
NaCl 6.0 gm/L
MgSO₄ - 0.6 gm/L
CaCl₂ - 0.6 gm/ L

Before inoculation 0.5 ml of antibiotic was added in each autoclaved media bottle (chloramphenicol to give to 25ug /ml in the media ie stock 2.5mg /ml) and warm to 390c before use.

Method of Inoculation

Approximately 10 gm of unfiltered sample of rumen digesta collected from animals (sheep) was weighed and transferred to a plastic bag containing 45 ml of fungal media being gassed out continuously with CO₂. Contents of bags
were mixed thoroughly from bag; 5ml contents were taken in a sterile pipette and add to the 2nd bottle being gassed with CO₂. Bottle contents were mixed thoroughly and add 10 ml of mixed bottle contents to each of three culture tubes (gassed) containing 0.1 to 0.2 gm pre-sterilized dried wheat straw. Here make sure to flame gassing hooks and tube caps between each bottle. Mark these tubes as 10² dilutions. Take another 5ml from 10² and add to the third bottle and mark them as 10³ flame gassing hooks between bottles and don’t touch the pipette to the medium. Repeat above-mentioned steps till the final dilution (usually up to 10⁶) is attained. Incubate inoculated tubes at 39 °C over night then examine the tubes for signs of fungi (eg. presence of zoospores) and observe positive tubes. Keep these tubes at 39 °C for 9 days. Observe the tubes every day and record when each becomes positives and how many replicates of each dilution are positive. The MPN of fungus was computed using MC Crady (1918) table based on the positive tubes.

3.7.2 Protozoa and their generic distribution

Standard method described by moir (1951) was followed for counting the total protozoal population from the formalized strained rumen liquor. For the generic identification of protozoa, 1-2 ml of strained rumen liquor samples were added few drops of methyl green saline solution (10ml to 30% formaldehyde, methyl green 0.06 gm sodium chloride 0.8 gm and distilled water 90 ml).

A drop of strained liquor was put on slide and covered with coverglass. Observation (x450) was recorded as per the morphological features complied by kudo (1960) Hungate (1966) and ogimote and Imai (1981) to identify the ciliates.

3.8 Cellulase enzyme

Cellulase enzyme activity in the strained rumen liquor was estimated as per the method of Mandel’s and Weber (1969) in which C1 or exo. B 1-4 glucanase (filter paper degrading) activity was determined by using what’s man
no.1 filter paper as substrate. What's man no.1 filter strip weighing 50 mg was suspended in 1ml of 0.05m sodium citrate buffer of pH 4.8 at 60 °C for 10 minutes and an appropriate amount of diluted culture filtrate was then added. The mixture was incubated at 60 °C for 30 minutes. The total sugar concentrate in the reaction mixture was determined by method of Nelson (1944). Enzyme activity in international unites is expressed as u moles of glucose released min⁻¹ ml⁻¹ of culture filtrate.

Citrate buffer - 0.5M pH 4.8
A- 0.1M solution of citric acid (21.01gm) in 1000 ml.
B- 0.1M solution of citric acid (29.41gm) in 1000ml.
Mix 23ml of A+ 27 ml of B diluted to 100 ml 0.05M.

Reagent For Sugar Estimation -

Reagent A
Sodium carbonates -25 gm
  Sodium potassium tartarate - 25 gm
Sodium bicarbonate -20gm
Sodium sulphate -20gm
  Make the volume 1000 ml with distilled water.

Copper reagent B
CuSO₄ 6H₂O - 15gm
H₂O - 100 ml H₂SO₄ -2-3 drops (acidification)

Reagent C -
  Mix 25 ml of reagent A + 1ml of reagent B.
**Arsenomolybdate reagent**

Solution - a

Ammonium molybdate 25 gm
H₂O - 450ml
H₂SO₄ - 21ml

Solution - b

Sodium arsenate - 3gm
H₂O - 25ml

Mix a+b solution and incubate at 370 c for 24 hr before use

To inactive enzyme activity 2 ml of copper reagent C was added from this mixture one ml was added in to tubes, which were then kept for 30 minutes in boiling water bath. After cooling 1ml of the arsenomolybdate reagent was added. Samples were mixed thoroughly by inverting and diluted to 25 ml. A stable blue colour developed was read at 520 nm. Glucose at concentration between 25- 250. μg was used as standard. To this, 1 ml of diluted culture filtrate was added and the

Mixture was incubated at 60 °C for 30 min. The total reducing sugar were estimated as mentioned earlier, enzyme activity is expressed in international units as μ moles of glucose released min -1 ml-1 of the culture filtrate.

3.9 Statistical Analysis

Data recorded on the In vitro experiments were statistically analysed as per methods of Sedecor and Cochran (1968). Results of in vivo studies were subjected to statistical analysis as per SPSS package (version 10.0).