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Original Research

Profile of Ciliate Protozoan and its Effects on Metabolites in Rumen under Herbal Drug Treated Feeding

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Abstract

Ruminants possess a specific ecosystem consisting of bacteria, protozoa, and fungi where a unique activity of feed fermentation takes place in it. The end products of fermentation will be VFAs and gases. The present study was designed to see the effects of self-compounded herbal drugs which were given to two groups of calves along with one control group. The herbal mixture was composed of some herbs like chirayita, kutki, cordimom, guruchi, kalmegh, bhumiamalki, neemcchal, anis seed or ajwain, black pepper piplamul, ghee-kunwar, azmoda, yeast along with ammonium chloride and certain other minerals. Nine Claves were divided into three groups with three calves in each group. The period of each trial was of three months including one month of pre-experimental feeding. The experimental trials were conducted in a switch-over design. Results indicated positive effects of the compounded herbal drug on PH, VEAs, and microbial population (Protozoan). The high level of the drug (D3=80gm) improved the rumen ecosystem (PH, VFAs, and protozoan number) compared to the control group (D1=without drug) and a low dose of the drug (D2=40gm). This herbal drug had no adverse effect on the animal body.

Keywords: Herbal drug, Rumen ecosystem, Fermentation

Introduction

Ruminant animal having a unique ability to not only harvest feed and roughages but also degrade complex fibre sources and it converted into readily digested essential nutrients in their complex and advanced digestive tract. The digestive tract of ruminants having an anaerobic condition and inhabitants' various types of microbes performs their activities such as breakdown complex feed and fermentation. Total protozoan which constitutes a significant fraction of ruminal biomass appears to be influenced by the dietary regime, an equilibrium is maintained in the rumen between microbial population and chemical concentration (VFAs) when fermented complex fibre, starches, and sugars produce VFAs, CO_2, and gases. Lipids are partially breakdown into glycerol and fatty acids while protein is partially degraded to NH_3, UFA, and gases. The large protozoa biomass contributes about 40-80% microbial biomass (Harrison and McAllan1980) and their ability to attack the major components of feeds (Coleman1985) indicates that they may serve an important role in ruminal fermentation. The rumen volume, chemical status, pH, and the whole environment will be changed following defaunation. Ruminal Indigestion occasionally becomes problematic defaunation takes place resulting in bloat, acidosis, and other toxics.

Changing in rumen fermentation is possible to increase the nutrient utilization that subsequently improves the efficiency of production by form animals (Wang and Wang 2016). Manipulation or changing the rumen microbial ecosystem by using some chemical compounds as feed additives have been practiced for a long time during the 1970's decades. But it has side or adverse effects and creates some health problems for animals and human beings also (Marashi Sarai, 2007). Therefore, public acceptability is reduced to using these compounds in Animal nutrition, and using of these compounds also ban by European Union in January 2006 (Anadon 2006). In view of restrictions and limitations of the usage of antibiotics recently various researchers (Gautam *et al* 2013, Mazher Hasmi 2014; Faniyl *et al*., 1916; Kolte *et al.,* 2009; Patra *et al*., 2019) reported on the change in rumen content which has brought by using the herbal mixture. Now using of herbs or herbal preparations is preferred over chemical additives and it is becoming a new trend in livestock production (Makkar *et al*. 2007, Tomkins *et al*. 2015, Yildiz *et al.*, 2015; Knapp *et al*., 2014, Khattab *et al.,* 2017). Herbs are a potential source of therapeutics and nutritive aids and have a significant role in health systems all over the world for both humans and animals (Prajakta Kuralkar *et al*., 2021). Although from ancient times and now still in India mostly herbal preparation are used to prevent or cure various diseases and tumors. Many attempts were carried out to use herbs as feed additives to improve the efficiency of feed utilization and productive performance of farm animals such as sheep, goats, buffaloes, and cows (Shohata *et al*., 2007; Swereb, 2012). An additional benefit of using these herbs in animals' rations was the preventive solution to avoid the side effects of chemicals (El-Kholany *et al*., 2015).

Herbs can replace antibiotics for raising calves (Wenk, 2002). Some herbs are used as a mixture and provided to animals to increase feed intake due to their flavoring and medicinal value and other unidentified factors in chemical forms also play an important role in enhancing animal performance. Besides these herbs are less expensive and safe than chemical additives. This is an example of the sustained production of animals. An herbal mixture of 32 herbs has been added to compose an herbal product batisa, which is used to improve the appetite of animals and helps in curing different types of indigestion by increasing the protozoan mass, bacterial biomass, and absorption of nutrients. The present study was designed to determine the effect of the self-compounded herbal drug on microbial profile and ruminal metabolites.

Materials and Methods

The present study has been conducted at Dairy Farm, Banaras Hindu University, Varanasi, U.P. India. The study was designed to see the effect of self-compounded herbal drugs on the various types of ciliated protozoa vis-a-vis the metabolites present in the rumen. The herbal drugs comprise several herbs like chirayata, Kutki, Cardamom, Guruchi, Piplamul, Azmoda, Gheekwar, Kalmegh, Bhumiamalki, Neemchhal and yeast along with ammonium chloride, and some other minerals. As these herbs have properties, like stomachic, carminative, antioxidant, anti-inflammatory laxative, cardiotonic, purgative, and emmenagogue, etc. and improve digestion as well, they also reduce the number of protozoa which are responsible for methane production. The drugs for the present experiment were compounded in the following ratios-

Composition of Self-Compounded Herbal Drug

Kutki (10%), Chirayita (10%), Guruchi (10%), Kalmegh (10%), Bhumiamlki (10%), Piplamul (10%), Gheekwar (10%) , Neemchhal (10%), Ginger (4%), Cardimom (3%), Black pepper (3%), Azmoda (2%), Saunf (2%), Ajwain

(1%), Black salt (2%) , Ammonium Chloride (1%), yeast 1%) and minerals (1%). These ratios of herbal powder were for one Kg.

In the study nine healthy cross-bred calves were selected nearly 3 to 6 months of age and randomly divided in to three groups D_1, D_2 and D_3. To minimize the experimental errors three trials were conducted in switchover design in three different seasons i. e. summer, winter, and Rainy. Each trial was conducted for up to 3 months including one month of the pre-experimental trial period. During each trial of the self-compounded drug, the calves were supplemented feeding ration into three levels of herbal drugs (D_1=without herbal drug, D_2= 40gm. Herbal drug and D_3=80gm). Experimental animals were kept on normal feeds consisting of wheat bhusa and concentrate. Data were recorded at fortnightly intervals i.e., 0, 15, 30, 45, and 60 days.

Collection and Processing of Rumen Liquor

Rumen liquor was collected from non-fistulated animals with help of a stomach tube at 0 hours (immediately before feeding) by using an aspiration bottle and collection was done once a day for the last 3 days of each feeding trial. On the day of the collection of rumen, liquor water was offered to the animal at 8 a.m. Immediately after collection, about 100 ml. of a representative sample of liquor was brought to the laboratory in a flask closed with a rubber stopper to maintain an anaerobic condition during transportation. Further, each sample of liquor was strained with two layers of muslin cloths to remove the course debris. About 50 ml. of strained rumen liquor (SRL) was poured into the plastic bottle containing a few drops of 10% mercuric chloride and was mixed thoroughly for the protozoal count. For protozoal counts 50 ml. SRL was preserved by diluting with 50 ml. formalin (10% formaldehyde) (Promasnaia, M. (1966), Franzolin and Dehority, 1996b) in another bottle of 50 ml. SRL was preserved with 1-2 drops of 25% H_2So_4 for TVFA estimation.

Identification and Counting of Protozoa

The rumen protozoa were identified under temporary preparation using acidified Methylene blue (0.5 gm. Methylene blue and 2 ml. acetic acid with 100 ml. distilled water) as nuclear stain and Lugol's iodine solution (2 gm. Iodine, 2gm, Potassium chloride and adding 300 ml. distilled water) for skettal plates (Dehority, 1993) Sketches by Ogimoto and Imai in (1981) and Williams and Coleman (1992) for meet the basis of identification. The pH of rumen fluid was determined by a digital pH meter or automatic pH meter immediately after straining. Collected liquor was strained with the help of muslin cloth. Data were statistically tested as per the method recommended by Snedecor and Cochran (1994).

Results and Discussion

During the present study following attributes were taken into consideration to see the effect of herbal drugs.

Table 1: Rumen pH, TVFA, and Microbial population in Rumen of cross breed Calves

Parameter	**Treatment group**		
	D1 (Control)	**D2**	**D3**
pH*	8.34	7.13	6.21
TVFA**(meq/l)	25.8	36.5	59.1
1-Acetic Acid	12.49 (48.64%)	19.67(53.48%)	43.37 (74.27%)
2-Propionic Acid	6.89 (26.76%)	8.64 (24.02%)	7.20 (12.42%)
3- Butyric Acid	4.41 (16.19%)	5.73 (15.67%)	6.22 (10.46%)
4- Other VFAs	2.22 (07.47%)	2.33 (6.59%)	2.17 (02.74%)
Microbial population ** (Protozoan)	3.305×10^5	5.23×10^5	6.915×10^5
a- Holotricha*	0.838×10^5 (25.4%)	1.246×10^5 (20.71%)	1.63×10^5 (23.07%)
1- Isotricha*	0.441×10^5	0.4546×10^5	0.97×10^5
2- Dasytricha*	0.398×10^5	0.598×10^5	0.76×10^5
b- Entodeniomorphids*	2.46×10^5 (74.59%)	3.837×10^5 (79.21%)	5.767×10^5(76.92%)
1- Entodenium**	2.30×10^5 (69.73%)	3.267×10^5(64.28%)	5.226×10^5(69.7%)
2- Diplodenium	0.267×10^5	0.83×10^5	0.89×10^5
3- Epidinium	0.155×10^5	0.23×10^5	0.34×10^5

*Note: * Significant (P < 0.05), ** Significant (P<0.01)*

Rumen pH: - The mean value of ruminal pH was recorded as 8.34, 7.13 and 6.31 in D_1, D_2 & D_3 respectively (Table-1). The positive effect of the herbal drug (D_2 and D_3) on rumen pH was found significant ($P< 0.05$) Observations indicated that higher pH was recorded in the control group (D_1) as the animals were fed only normal feed-course material (Bhusa and straw). Coarse material creates an alkalinity condition and produces a lesser amount of VFA in the rumen. Reduction in ruminal pH might be due to the production of organic acids (VFAs) at a high rate in the rumen in fermentation activities of the increased number of protozoa in treated groups (D_2 & D_3). (Kolte *et al*. 2009). Herbs have properties to improve microbial biomass in the rumen.

Rumen pH was observed changing at different days of the interval as maximum pH 8.00, 8.10, 8.15, and 8.34 at 15, 30, 45, and 60 days respectively in the control group and minimum 6.9, 6.75, 6.53, and 6.31 at corresponding days in D3 level (Table-2). Results are showing significant differences amongst different days of interval in various treatments (D_1, D_2, and D_3). Progressive changes were observed in the pH of the rumen contents at different days of intervals. Changing was observed greater in the D_3 level followed by D_2, and D_1 levels. Reduction in ruminal pH was associated with herbal drug incorporation and it continued to fall up to 60 days in D_3, and D_2 levels but pH was recorded as 8.0, 8.10, 8.15, and 8.34 at different days of intervals in the control D_1 level.

Table 2: Rumen pH at different days of Interval

Treatment	Days of Intervals			
	15	**30**	**45**	**50**
D_1	8.00	8.10	8.15	8.34
D_2	7.97	7.90	7.50	7.13
D_3	6.90	6.75	6.53	6.31
CD at 5%	NS	S	S	S

Total Protozoa Population: The data presented in Table No. 1 showed an average number of total protozoa as 3.30×10^5, 5.23×10^5, and 6.915×10^5 / ml. in D_1, D_2 & D_3 respectively. Observations indicate that the effect of the drug on the protozoa population was found significant ($P< 0.05$). It seems that by nature herbs use in the experiment have properties to improve microbial population in the rumen. The compounded herbal drug act to maintain a balance between ruminal bacteria and pathogens and gradual improvement in protozoal concentration in both treated groups D_2 and D_3 respectively. It seems that the ciliate population is increased by the utilization of bacterial protein amino acid contains in the rumen which has improved by supplementation of herbal drugs. Results show that increased microbial activity in the rumen through improving roughage digestion and utilization of bacterial protein amino acids content by protozoa. Coleman (1972) has indicated that the amino acids of bacterial origin are incorporated into protozoal protein without any interconversion of one amino acid to the other and are almost part of the bacterial protein utilized for protozoal protein synthesis.

Holotrichs- The data pertaining in Table-1 showed that the number of holotrichs was recorded as 0.838×10^5, 1.246×10^5, and 1.63×10^5 per ml. in D_1, D_2, and D_3 respectively. Among holotrichs the genera Isotricha and Dasytricha numbers were recorded as positive in both treated groups D_2 and D_3 than in control D_1 (Table No.1). The findings of the study show a significant effect ($P<0.05$) of the herbal drug on holotrich population.

Isotricha – The average number of Isotricha was observed more (0.4546×10^5 & 0.97×10^5/ml.) in D_2 & D_3 compared to D_1 (0.441×10^5). Results indicated a significant ($P<0.05$) improvement in Isotricha. The concentration of Isotricha has reported an increase in the treated group (D_2 & D_3) in comparison to the control D_1 group which might be due to better predation capability. Isotricha is larger cell size protozoa represent only 5% of the total ciliate population and 35% of the total protozoal volume or 40% of the protozoal nitrogen (Abe *et al*., 1981; Holler and Harmeyer 1964). Both the species of Isotricha chemotactically 80% attach to soluble carbohydrates within 2-5 hrs. of feeding with entering feed in the rumen (Oripin and Letcher, 1978).

Dasytricha – The findings of the study (Table-1) have shown that the dasytricha number was recorded as 0.398×10^5, 0.598×10^5, and 0.97×10^5/ml in D_1, D_2, and D_3 respectively. Dasytricha number was found more in both treated groups D_2, and D_3 compared with D_1. The result indicated a significant ($P< 0.05$) effect of the herbal drug on Dasytricha population. All the strains of Holotrichs protozoa have a chemotactic affinity to the soluble sugar and Dasytricha shows similar activity to attachment with soluble sugar with lucerne stem fragments (Bouchop, 1989). The concentration of *D. ruminantium* improvement might be due to the utilization of soluble sugar and predation of bacterial biomass by both species of Isotricha. (Coleman, 1975a).

In the nutrition of Holotrich protozoa is played an important role and utilize both ruminal (Gutierrez, 1958) and non-rumen strains of bacteria (Wallece and Coleman, 1967). Bacterial biomass may be increased in the rumen by improving roughage (diet) digestion. Herbal additives may have secondary metabolites such as tannins, saponins, and essential oil which act as a natural rumen modifier (Knapp *et al*., 2014). Herbal drugs have such properties to change the rumen microbial population by improving ruminal fermentation efficiency. (Khiaosa-ard & Zebeli, 2013).

Entodiniomorphids: The data pertaining in Table-1 indicates that the number of entrodiniomorphids is 2.46 x 10^5/ml, 3.837 x 10^5/ ml. and 5.76 x 10^5/ml. at three different levels of herbal drugs D_1, D_2, and D_3. These findings showed a significant ($P< 0.05$) effect of the herbal drug on the entodiniomorphids numbers. The number of various species of Entodioniomorphids is presented in Table No. 1. The data were shown that different species Entodinium, Diplodinium, Epidinium, amongst Entodiniomorphida and they were found as predominant genera in reports of the study.

Entodinium– The average number of Entodinium was found as 2.30 x 10^5, 3.267 x 10^5, and 5.26 x 10^5 in D. D_2 and D_3 groups respectively. The findings indicated that the effect of the compounded herbal drug on Entodinium population was significant ($P < 0.01$). The experiment findings showed that Entodinium numbers were recorded as positive in both treated groups (D_2 & D_3) than control (D_1). This might be due to (ruminatohric) effect of herbal drugs and a sufficient amount of roughage (Cellulosic material in feeds) given to the animal. Entodinium is inhabited in the largest percentage of total protozoa present in the rumen of almost domesticated and wild animals. This genus has protozoan smallest in size and different spp. more than 100 have been reported (Williams and Coleman 1992).

Diplodinium: - The concentration of Diplodinium recorded in D_1, D_2, and D_3 were found as 0.287 x 10^5, 0.83 x 10^5 and 0.89 x 10^5 /ml respectively. The findings of the study indicated that the effect of the herbal drug on Diplodinium population was non-Significant ($P<0.05$) but the effect of the D_3 level was observed better than D_2 level (Table No. 1).

Epidinium: - The average population of Epidinium counts was recorded which varied from 0.155 x 10^5/ ml, 0.230 x 10^5/ml, and 0.34 x 10^5/ml in D_1, D_2, and D_3 groups respectively. Results indicated that the effect of the herbal drug on Epidinium number was non-significant($P<0.05$) (Table No.1). Epidinium species are an important component of B type population in the rumen of a cow. Sadhana *et.al.* (1997) has been reported that the protozoal number varied from 1.5 x 10^4/ml. at a wheat straw – concentrate feeds to 1.9 x 10^4/ml. (at wheat straw GNC) in cattle. Irrespective of preformed proteins the most predominant genera in cattle were Isotricha and Desytricha of Holotrichs followed by Entodiniomorphs and may be possible to their being proliferated 10 times faster than Holotrichs. In the present study, data indicated that Entodinium was highly, and consistently frequent genera of Entodiniomorphs- Diploinium and Epidinium were recorded as having increasing trend in number in both treated groups D_2, and D_3 but were found non-significance.

The concentration and composition of Protozoan in rumen are influenced by many factors. It seems that feeds containing concentrate between 40–60% will be supported to maximum protozoal numbers with almost species of the protozoan. Ruminal pH goes down below 6 at high concentrate feeding due to a protozoal number decrease and Entodinium species are mostly present in the rumen (Dehority and Orpin, 1988).

Bryant and Small (1960) and Eadie (1962 a) reported that pH was of primary importance in the establishment and maintenance of the ruminal protozoal population. High concentrations (above 60%) generally markedly reduced the ruminal pH. Gradual fall in Ph is generally associated with a reduction in the protozoan population. A number of studies supported the findings that defaunation or markedly reduction in protozoan numbers is the result of low ruminal pH (Srinivas and Krishnamoorty, 2016).

In the present study, the effect of pH on different species of protozoa was observed to be significant ($p<0.01$). The Data pertaining to Table-3 and figure no. 2 indicated that inverse relationship between a large number of protozoans and the pH of the rumen. The maximum number of protozoan holotricha (0.97, 0.76 x 10^5 Isotricha and Dasitricha respectively) and Entodiniomorphs (5.22 x 10^5 - Entodinium , 0.89 x 10^5 Deplodinium and 0.34 x 10^5 Epidinium) respectively at minimum pH 6.31 in D_3 level. In the presence of a large number of protozoa, ruminal pH goes down linearly (multiple regression coefficients ($r =1$)) from 8.132 to 6.31 at D_1 to D_3 level.

Protozoa regulate ruminal pH to check the fall from normal to the acidic condition- Acidosis. The multiple regression equations were calculated by using pH as the dependent variable and different protozoa as independent variables and the equation is presented below: -

Table 3: Effect of Different Protozoal spp. on Rumen pH

Treatments	Rumen pH	Protozoal population (x 10^5)				
		Isotricha	**Dasytricha**	**Entodinium**	**Diplodianium**	**Epidinium**
D_1	8.13	0.45	0.39	2.30	0.83	0.15
D_2	7.13	0.55	0.59	3.26	0.83	0.23
D_3	6.31	0.97	0.76	5.22	0.89	0.34

*Multiple regression coefficient R = 1***

$Y= 9.7_2 – 0.276\ x_1 – 0.1989x_2 – 1.523\ x_3 – 0.350x_4 – 0.1007x_5$

The regression equations between pH and various protozoan species were calculated separately and are given as –

$Y= 0.27\ x_1 + 2.6415R^2 = 0.8373$*(between pH (y) and Isotricha* x_1*)*

$Y= -\ 0.198\ x_2 + 2.057\ R^2 = 1$ *(Regression equation between pH (y) and Desitricha* x_2*)*

$Y= 1.5255\ x_3 + 1.4527\ R^2 = 0.9442$ *(Regression equation between pH (y) and Entodinium* x_3*)*

$Y = 0.3503\ x_4 + 3.1808\ R^2 = 0.863$*(Regression equation between pH (y) and diplodinium* x_4*)*

$Y = 0.1007\ x_5 + 0.9656\ R^2 = 0.2727$ *(Regression equation between pH (y) and Epidinium* x_5*)*

These equations indicate that there was a linear inverse relationship between rumen pH and different protozoal species. The equations are indicated that Dasytricha and Entodinium played a major role in the reduction of ruminal pH. In equations no. 2 and 3, R^2 values were recorded as one and 0.9442 respectively. In these equations seems that the contribution of different protozoan species has shown how much pH was reduced by individual protozoal species.

The presence of holotrichs creates a defense mechanism against acidosis and maintains ruminal pH at an optimum level (Oxford, 1955; Machie *et al.,* 1978) although Isotricha represent only 5% of total ciliate number but 35% in volume. Purser and Moir (1959) found a linear regression of mean daily protozoan population density and minimum daily pH. Protozoa is played an important role in the stabilization of the environmental pH during ruminal fermentation and may be significant to bacterial growth and enzymatic activities which are inhibited at lower pH (Stewart, 1977).

A number study supported the relationship between a protozoan number and ruminal pH. When the pH falls below 5.5 the protozoans' cells start disintegrating and lysis completely takes place. The lower pH associated with the absence of protozoa (Bryant and Small, 1960; Abe & Kumeno,1973; Lyle *et al*. 1981) and the production of ruminal fermentation VFAs level is directly affected by ruminal pH.

Warner (1962) reported a minimum division time of 5.5hr. for Entodinia in sheep fed hey. Nakamura and Kanegasaki (1969) found that it took 6 days for the protozoa to increase 2-4 x 10^5 to a 7 – 12 x 10^5 /g of rumen liquor when sheep were changed from hey to a diet containing 29% concentrate. The findings of the present investigation indicated that the increased protozoa population in treated groups D_2 & D_3. might be due to in nature many herbs improve microbial biomass by actions in the rumen which improve fibre digestion.

Variations in Ciliate Population

Observations of the study related to the effect of the herbal drug on different species of protozoa at various (15, 30, 45, and 60) days of intervals were recorded in three groups of treatment D_1, D_2, and D_3 (Table No.4). The findings indicated that Isotricha number increase significantly ($P < 0.05$) but maximum number recorded at 15-, 30-, 45- and 60-days interval D_3, in compression to control group D_1. Findings indicated that Isotricha population is significantly different at different days of intervals in various treatment groups.

Similarly, Dasytricha spp. was reported as an increase at different days of intervals 15, 30, 45, and 60 days in the D_3 level while a minimum number of Desytricha was recorded in the D_1 level at different days of interval (15, 30, 45 and 60 days) (Table No.4) Results showed significant differences in Desytricha number among D_1, D_2 and D_3 level at 15, 30, 45 and 60 days of interval. Bhatia *et al*. (1998) have reported that the relative occurrence of protozoal

genera of the holotrichs Isotricha and Desytricha in cattle (5.7%, 4.3%) and buffalo (3.6, 2.7%) were the most predominant than other.

Table 4: Changes in Protozoal number at different days of interval (x105/ ml.)

Species	Treatment											
	D_1				D_2				D_3			
	Days of interval				Days of interval				Days of interval			
	15	30	45	60	15	30	45	60	15	30	45	60
Holotricha	0.26	0.34	0.56	0.84	0.46	0.74	0.94	1.24	0.76	1.26	1.36	1.63
Isotricha	0.15	0.24	0.43	0.44	0.21	0.33	0.48	0.54	0.40	0.60	0.79	0.97
Dasitricha	0.10	0.16	0.21	0.40	0.29	0.33	0.42	0.60	0.36	0.50	0.57	0.76
Entodinimorphids	1.90	2.08	2.15	2.46	2.55	2.60	3.07	3.83	4.005	4.50	5.03	5.76
Entodinium	1.243	1.725	1.948	2.3	1.25	1.67	2.67	3.26	2.765	2.50	3.613	5.226
Diplodinium	0.005	0.01	0.05	0.02	0.02	0.04	0.09	0.2	0.05	0.09	0.07	0.12
Epidinium	0.04	0.05	0.08	0.3	0.03	0.06	0.1	0.5	0.02	0.02	0.15	0.48
Total	3.005	3.16	3.3	3.30	3.65	4.1	4.69	5.23	4.02	4.72	5.65	6.91

The findings recorded at 15, 30, 45, and 60 days of interval in the treatment D_1, D_2, and D_3 indicated that the number of Entodinium was increased significantly ($P < 0.05$) at different days of interval in the D_3 level. The number of Entodinium was reported to increase due to the larger number of bacteria present in the rumen with the addition of Yeast (Dawson *et al*.1990) which was incorporated with the herbal drug. *Entodinium caudatum* engulfs any bacterium (Coleman and Hall, 1969).

Diplodinium population was reported to increase at 15, 30, 45, and 60 days of interval in D_3 level compared with D_1 and D_2. The maximum number of Diplodinium was found as 0.89×10^5 in the D_3 level compared to 0.267×10^5 and 0.83×10^5 in D_1, and D_2 levels respectively. The effect of the compounded herbal drug on Diplodinium was found to be not significantly different ($P < 0.05$) among 3 levels of drug D_1, D_2, and D_3. But the effect of the D_3 level was found better than the D_2 level. The number of Epidinium was observed to increase but it was found not significant among the three levels of treatments.

Several studies reported that plants have anti-inflammatory activities and strong +antioxidant. It has been found that many herbs contain active compounds such as lemongrass, turmeric, quelangal, rosemary clove, and Cinnamomum, etc. modify rumen fermentation positively affect VFAs protein-carbohydrate degradation, and reduces ruminal hydrogenation (Khattab *et al*. 2017)[15]. Changing in the number of Holotrichs and Entodiniomorphids both are affected positively by herbal mixture.

Metabolic Products of Carbohydrate Fermentation

T.V.F.A.:- The present study data pertaining in Table No.1 indicated that the effect of the herbal drug on Acidic Metabolites – (acetic acid, propionic, butyric, and other VFA) was found significant ($P<0.01$). The maximum TVFA production (59.1 meq/lt.) was recorded at the D_3 level compared to D_1 (25.8 meq/ lt.) and D_2 (36.5 meq/lt.).

Acetic Acid: - The average Acetic acid production was recorded as 12.49, 19.67, and 43.39 meq/lt. in D_1, D_2 and D_3 respectively. Results show a significant difference ($P< 0.05$). The Acetic Acid level was found high in D_3 (43.37 meq /lt.) than in D_1 (12.49 meq /lt.) and D_2 (19.67 meq/lt.) (Table No.1)

Propionic Acid: In Table No. 1 data showed the effect of the herbal drug on the propionic acid level. It was recorded as 6.89, 8.64, and 7.2 meq/lt. in D_1, D_2 and D_3 level respectively. These findings showed no significant difference among the three levels of herbal drugs D_1, D_2, and D_3.

Butyric Acid – The butyric acid level was measured as 4.41, 5.73, and 6.22 meq/lt in D_1, D_2, D_3 groups respectively. The herbal drug effect on Butyric Acid was found non-significant ($P< 0.05$) (Table No.1).

Other VFA – The data presented in Table No. 1 indicated to the effect of the herbal drug on other VFA no significant difference amongst the three levels of drug D_1, D_2, and D_3. The maximum level of other VFA (2.33 meq/lt.) was recorded in D_2 followed by D_1 (2.2 meq/lt.) and D_3 (2.17 meq/lt.)

The main end products of carbohydrate fermentation in the rumen are VFAs, H_2 and Co_2 gases, and storage polysaccharides. The proportions of the metabolite's formation are affected by the concentration and type of carbohydrate available in the rumen (Prins and Van Hoven 1977). In addition, environmental pH, temperature, metabolic interaction with methanogens, low O_2 levels, and antibiotics can affect the proportion of the metabolites produced. Gutierrez (1955)[54] reported that the holotrichs contributed some 10% of the total acids in the rumen. The rates of metabolite formation are not constant and influenced by various factors. After carbohydrate fermentation by holotrichs the end Products formed as lactic, acetic, butyric acid, H_2, CO_2, storage polysaccharide, and also formic acid.

In the soluble and easily digestible degradable portion of corbohydrate fermentation, the major role is played by two genera of Holotrichs- Isotricha and Dasytricha. In organic Acids/ VFAs production at a high rate of fermentation, the principal end product is lactic acid while in low formation rates the main end products are butyric and acetic acid.

Variations in VFAs Production

The data pertaining to Table No. 5 shows the number of TVFAs at different days of intervals 15, 30, 45, and 60 days. Maximum TVFAs are produced in the D_3 level at 15, 30, 45, and 60 days compared with D_1 & D_2 levels. The minimum amount of TVFAs was recorded in D_1 as 20.2, 21.5, 22.8 25.8 meq/lt. at 15, 30, 45, and 60 days respectively compared to D_2 at 22.3, 26.5, 30.8, and 36.4 meq/lt. at different days of interval respectively. Results show a significant ($P< 0.05$) improvement at 45 (46.8 meq/lt.) and 60 days (59.1 meq/lt.). Observations indicated that the effect of the compounded herbal drug on TVFAs at 15, 30, 45, and 60 days was found to be better but at 45 and 60 days was found significantly (in D_3) more positive than D_1, and D_2 levels. TVFA is comprised of Acetic, Butyric, propionic, and other VFA. These volatile fatty acids are considered a major source of energy in ruminants.

Table 5: Changes in VFA Production on different days of Interval

VFA (Meq/lt.)	Treatment											
	D_1 (days interval)				D_2 (days interval)				D_3 (days interval)			
	15	30	45	60	15	30	45	60	15	30	45	60
Acetic Acid	10.11	9.11	10.11	12.49	12.44	10.56	10.56	19.87	21.22	26.56	32.33	43.37
Propionic Acid	4.10	4.64	5.31	6.85	0.08	5.30	6.62	8.67	5.40	6.46	7.25	7.33
Butyric Acid	2.68	3.01	3.39	4.38	2.72	3.57	4.37	5.73	3.60	4.46	5.19	6.33
Other VFA	1.01	1.18	1.41	1.93	1.07	1.43	1.84	2.42	1.20	1.15	1.31	1.61
TVFA	20.2	21.5	22.8	25.8	22.3	26.5	30.8	36.4	30.0	38.5	46.8	59.1

The acetic acid level in the treatments D_1, D_2, and D_3 at different days of intervals 15, 30, 45, and 60 days was recorded as maximum averages of 21.22, 26.56, 32.33, and 45.37 meq/lt respectively in D_3 compared with D_1 (10.11, 9.11, 10.11 and 12.49 meq/lt.) and D_2 (12.44, 15.56, 18.56, 19.67) respectively. Observations indicated that acetic acid significantly ($P<0.05$) increased in D_3 level at 45 & 60 days compared with 15, and 30 days of intervals.

The propionic acid concentration observed at different days of interval shows that amount of propionic acid increase at a non-significantly rate ($P<0.05$). The effect of the compounded herbal drug on the butyric acid level was found positive in the D_3 level at different days of intervals 15, 30, 45, and 60 days compared with the D_1, and D_2 groups. Results show improvement in butyric acid levels at different days of the interval but it was found non-significant.

Herbal drug effect on other VFAs was observed positive at different days of the interval but when data was subjected to the test of significance it showed non-significant ($P< 0.05$).

In goats, normal TVFA concentration ranges between 6-12 meq/lt. of rumen liquor (Chakravarti, 2006). After treatment by D_2 and D_3 level results were found positive than control D_1 indicating the stomachic effect of the herbal drug on TVFA levels. Similar reports of various studies supported the finding of the study (Pal *et al*., (1994) Desai (1998). It might be due to the regeneration of VFA-producing microbes present in the rumen. It seems that the stimulatory effect of herbal supplementation on the multiplication of microorganisms and therefore the level of TVFA increased TVFA level through the incorporation of herbal drugs reported by Sardar *et al*. (1997), Manjunatha *et al*. (1998) and Pankaj *et al*. (2008). The net TVFA production per Kg. D.M. may be 6.2 mol/kg. of DM for a 60% concentrate and 40% roughage diet (Suttan *et.al.*, 2003; Siciliane-Jones and Murphy,1989).

The molar proportion of the VFAs was variable and changed often inconstant manner during the fermentation process. Shifts in VFAs proportions may be important in two respects. Where the presence or absence of protozoa is associated with large changes in the ratio of acetate plus butyrate to propionate, there can be significant changes in methane formation and synthesis of milk fat. In defaunation, propionate production may be increased in double proportions while the production of methane goes down halved and the grass energy increased by 5%. Methanogenic bacteria attached with protozoa and thus protozoa may be responsible for methane production differences in the molar proportions of acetate, propionate, and butyrate produced during fermentation affects the amount of hydrogen available for methane production (Wolin M.J., 1960) as does the site of digestion (fermentation vs gastric) and the extent of digestion. It seems that the proportions of VFAs are also influenced by the diet.

The molar proportions of propionic acid increase largely on defaunation at the expense of acetic and butyric acid. Most protozoa produce acetic and butyric Acid on fermentation of sugar but no propionic acid is produced. Propionic acid has an inhibitory effect on holotrich protozoa and large entadiniomorphids (Kobayashi T. and H. Itabashi, 1986).

Generally, the molar proportion of acetic is less affected than either propionate or butyrate but has a tendency to be slightly lower in the rumen of defaunated animals (Conrad *et al*., 1964; Males & Pursar, 1970). The incorporation of single protozoan species (Polyplastran multivasiculattum or Entodinium) to meroxans lambs resulted in an increase in the ruminal VFA concentration and changes in a molar proportion of the principal acetate, propionate, and Butyrate.

Rumen pH, Protozoa Population, and VFA's Concentration

The data presented in Table No. 1 indicated that rumen pH was lower in two D_2 (7.13) and D_3 (6.31) groups in comparison to control D_1(8.34). Similarly, the protozoa population was recorded as 3.305, 5.23 & 6.91 x 10^5 / ml. and TVFA was 25.8, 36.5, and 59.1 meq/lt. in D_1, D_2 and D_3 groups respectively. Observations show that the minimum pH was at the D_3 level simultaneously with the maximum protozoal number and TVFA level. Results indicated the positive impact of an enhanced number of protozoan and increased TVFA concentration on reduction in the ruminal pH. It seems that the production of TVFA is associated with a reduction in ruminal pH because organic acid production creates an acidic condition in the rumen and therefore fall in ruminal pH **(Fig.5).** Observations show that a linearly inverse relationship between protozoan population and rumen pH. (Table 3 and Fig.-2). It indicated that in the presence of a large number of protozoa, ruminal pH goes down linearly (multiple regression coefficients (r=1).

$$y = 9.72 - 0.276\, x_1 - 0.198\, x_2 - 0.1523 x_3 - 0.350\, x_4 - 0.1007\, x_5$$

The regression equations indicate the contribution of different protozoan spp. and how much pH was reduced by individual protozoan spp. A number of studies supported the relationship between the protozoan population and rumen pH (44, 39, 42, 45). Protozoa regulate to check the following ruminal pH. Holotrichs create a defense mechanism against acidosis and maintain ruminal pH. A fall in pH is associated with the absence of protozoa (defaunation) when falling below 5.5 pH protozoa start disintegrating and complete lysis (39, 45, 46) takes place. In defaunated and faunated animals' ruminal pH varies from below normal (6.2 to 6.9) and normal (6.5 to 7.1) respectively according to the diet which is taken by animals. Herbal additives act as natural rumen modifiers (14) and have properties to change the rumen microbial population by improving ruminal fermentation efficiency (9, 35).

VFA proportions are variable and it change may be in either the presence or absence of protozoan, associated with large changes in the ratio of acetate plus butyrate to propionate. Shift proportions of VFAs are affected by protozoa. Jouany *et al*. (1981) indicated that the proportions of VFAs are often changed by the presence or absence of protozoans in an inconstant manner. The main end product of carbohydrate fermentation is VFAs. The proportions of the metabolism formation are affected by the concentration and type of carbohydrate (Prins and Van Hoven 1977). Production of TVFA is drastically affected by the number of protozoa and their activities.

Holotrichs protozoa act on and break down the soluble and degradable portion of the diet is taken and fermented by holotrichs and converted into organic acids Lactic, butyric, and acetic acid as major products with H_2, Co_2 Formic acid, and propionic acid. (Gutierrez, 1955; Williams and Harfoot, 1976; Prins and Von Hoven, 1977).

Entodiniomorphid protozoans have been responsible to break down the starch in the diet (Hungate, 1966). Starch fermentation by Entodiniomorphid at a slow rate produces mainly acetic and butyric acid. Protozoa can increase in number in response to the added starch and sugar in the diet.

The effect of protozoal population on VFA production was recorded in Tables- 6a to 6d it showed linearly positive significance ($P<0.05$). Acetic acid was recorded as 12.49, 19.69, and 43.67 meq/liter in D_1, D_2, and D_3 groups, respectively. Similarly, the protozoan population (Isotricha, Desytricha, Entodinia, Deplodinia, and Epidinia) correspondingly increased in all groups (Table 6a).

The data presented in Tables 6a to 6d and Fig. 6 showed a significant effect of different protozoan species on various VFAs. This is indicated by the positive effect of a large number of different protozoan species to enhance propionic, butyric, and other VFA levels. VFA increased linearly (multiple regression coefficient r=1) with increasing protozoan population.

Table 6(a): Effect of different protozoans on the acetic acid level in the rumen. Multiple regression coefficient r = 1**

Treatments	**Acetic acid (meq/l)**	**Protozoal Population (x 10^5/ml.)**				
		Isotricha	**Dasytricha**	**Entodinium**	**Diplodinium**	**Epidinium**
D_1	12.49	0.45	0.39	2.30	0.26	0.15
D_2	19.69	0.55	0.59	3.26	0.83	0.23
D_3	43.67	0.97	0.76	5.22	0.89	0.34

***Significant (p<0.01)*

Table 6(b): Effect of different protozoans on the propionic acid level in the rumen. Multiple regression coefficient r = 1**

Treatments	Propionic acid (meq/l)	Protozoal Population (x 10^5/ml.)				
		Isotricha	Dasytricha	Entodinium	Diplodinium	Epidinium
D_1	6.86	0.45	0.39	2.30	0.26	0.15
D_2	8.64	0.55	0.59	3.26	5.83	0.23
D_3	7.20	0.97	0.76	5.22	0.89	0.34

***Significant (p<0.01)*

Table 6(c): Effect of different protozoans on the butyric acid level in the rumen. Multiple regression coefficient r = 1**

Treatments	**Butyric acid (meq/l)**	**Protozoal Population (x 10^5/ml.)**				
		Isotricha	**Dasytricha**	**Entodinium**	**Diplodinium**	**Epidinium**
D_1	4.41	0.45	0.39	2.03	0.26	0.15
D_2	5.73	0.55	0.59	3.26	5.83	0.23
D_3	6.22	0.97	0.76	5.22	0.89	0.34

***Significant (p<0.01)*

Table 6(d): Effect of different protozoans on other VFA acid levels in the rumen. Multiple regression coefficient r = 1**

Treatments	**Other VFA (meq/l)**	**Protozoal Population (x 10^5/ml.)**				
		Isotricha	**Dasytricha**	**Entodinium**	**Diplodinium**	**Epidinium**
D_1	2.20	0.45	0.39	2.03	0.26	0.15
D_2	2.33	0.55	0.59	3.26	5.83	0.23
D_3	1.91	0.97	0.76	5.22	0.89	0.34

***Significant (p<0.01)*

The multiple regression equations were calculated and presented below: -

$Y= 28.51 + 0.0168\, x_1 + 0.0114\, x_2 + 0.087x_3 - 0.0421\, x_4 + 0.10056x_5$
(Acetic acid as dependent variable and different protozoan species as independent variables)
$Y= 29.71 - 0.0573\, x_1 + 0.6459\, x_2 + 0.087x_3 + 0.2105x_4 + 0.0071x_5$
(Proponic acid as dependent variable and different proteozoan species as independent variables)
$Y= 18.09 + 0.2099x_1 + 0.1886\, x_2 + 1.252x_3 - 0.3528x_4 + 0.084x_5$
(Butyric acid as dependent variable and different protozoan species as independent variables)

$Y= 9.39 - 1.138x_1 + 0.53 x_2 + 5.30x_3 - 0.4782x_4 + 0.324x_5$
(Other VFA as the dependent variable and different protozoan species as independent variables)

A soluble and degradable portion of the diet is taken and fermented by holotrichs while starch breaks down by the entodiniomorphids. Molar proportions of acetate are less affected than either propionate or butyrate but have a tendency to be slightly lower in the rumen of defaunated animals. Most protozoans produce acetic and butyric acid on fermentation of sugar but no propionic acid. Propionic acid has an inhibitory effect on holotrichs and large entodiniomorphids protozoans (Kobayashi and Etabashi, 1986). The findings of the present study are supported by various workers of present studies.

Conclusion

The present study revealed a positive response of herbal drugs on rumen pH, TVFA, individual VFA (Acetic, Propionic, Butyric, and other VFA), and protozoal population. Herbs can modify the metabolism and metabolites of ruminants. Thus, we concluded that by supplementation of herbal additives in the diet of ruminants, the ruminal ecosystem can be improved.

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Conflict of Interests

There is no conflict of interest.

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