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Implications of phyto-feed additives supplementation in buffalo calves on rumen fermentation pattern and microbial population

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ABSTRACT

Natural phyto-feed additives have been identified as a potential rumen fermentation modifier by *in vitro* studies and by few short-term *in vivo* trials. However, information on impact on animal performance by their long-term administration is still inadequate. In light of this, the present study was undertaken to examine the rumen fermentation pattern, rumen microbial enzymes and microbial profiles as influenced by long term supplementation of phyto-feed additives to buffalo calves. A six months feeding trial was conducted on 20 male buffaloes (165±4 kg body weight), divided into four groups and fed on diet supplemented with no additive (T0, control), with feed additive FAI @ 1% of dry matter intake (DMI) (T1), with FAII @ 1 ml/kg DMI (T2) and with FAI and FAII switched alternatively after every 15 days (T3). No significant effect was observed on rumen fermentation pattern as well as carboxymethylcellulase, avicelase, xylanase, acetyl esterase, and protease activities in the rumen of buffalo calves. The population density of methanogens, fungi, *Ruminococcus flavefaciens*, and *R. albus* decreased significantly in T3 where FAI and FAII were fed alternately, but *Fibrobacter succinogenes* decreased significantly in T2 where FAII was fed. When compared to the control, the microscopic count of protozoa decreased in all the three supplemented groups. It can be concluded that rumen fermentation, including rumen metabolites and microbial enzymes, were unaffected; however, phyto-feed additives exhibited changes in rumen microbes.

Keywords: Buffalo calves, Microbes, Phyto-feed additives, Protozoa, Rumen

Greenhouse gas (GHG) emissions are a great cause of global warming and climate variations, therefore, are a flaring issue all over the world. Around 16% of the global methane emission is contributed by ruminants (Tseten *et al*. 2022). Within the agricultural sector, 73% of the methane emission comes from livestock, majorly represented by beef (35%) and dairy (30%) cattle, with only 15% from small ruminants and buffalos (Islam and Lee 2019). The CH_4 and N_2O are the most important GHGs from the animal production sector and have very high potential for global warming (GWP), 25 and 298 times more than CO_2, respectively (Møller *et al*. 2022). GHG emissions from livestock activities are expected to rise as demand for the animal products rises (Molho-Ortiz *et al.* 2022). So, the utmost important task is to keep a balance among the animal productivity, consumer demand and environmental protection. Use of plants rich in secondary metabolites (saponins, tannins, essential oils etc) is the most acceptable strategy for reducing methane production in ruminants because they are naturally occurring compounds that are socially acceptable, safe, and easy to feed. Herbs are gaining popularity in the animal industry due to their specific antimicrobial activity, which has demonstrated their ability to modify rumen microbial population to improve rumen fermentation, nitrogen metabolism, animal productivity, and reduce enteric methane emission (Kumar *et al.* 2022). Numbers of plant part have been screened individually or in combinations for their antimethanogenic property using *in vitro* system and some of them showed very promising results (Inamdar *et al*. 2015, Pal *et al*. 2015, Choudhary *et al*. 2022). *In vitro* system is not a true index of *in vivo* system; therefore, feeding trials have to be conducted to validate the potential of a feed additive considering production and health both. There are limited feeding trials using plant parts as feed additive (Patra *et al*. 2011, Yatoo *et al.* 2018), whereas, long-term feeding of these phyto-feed additives are very limited. Therefore, the goal of this study was to observe the effects of long term phyto-feed supplementation on rumen fermentation pattern, microbial and enzyme profiles in buffalo calves.

MATERIALS AND METHODS

The experiment was carried out at the Animal Nutrition Research Shed, Indian Veterinary Research Institute, Bareilly, Uttar Pradesh (India). All experimental protocols were approved and compliant with the guidelines established by Institutional Animal Ethics Committee

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constituted (IAEC) under CPCSEA, New Delhi.

Animals, feed and experimental design: A feeding trial of six months was conducted on 20 male buffalo calves, 12-15 months of age with almost similar live weight (165±4 kg), divided into four equal groups assigned to T0, control; T1 with FAI @1% of DMI; T2 with FAII @ 1ml/kg DMI and T3 with FAI and FAII alternatively for 15 days each, using a completely randomized block design. FAI was a mixture of four plant parts (mixture of garlic, ajwain, harad and soapnut in equal proportion) and FAII was an essential oil (ajwain oil). The animals were dewormed prior to the experiment. Details of the chemical composition of the experimental diet are given in Table 1. Animals were fed as per ICAR (2013) targeting the growth of 500 g/d. The additives were mixed well with the concentrate mixture before offering it to the buffalo calves. The wheat straw was offered after the concentrate mixture was completely consumed by the animals. To meet vitamin A (carotene) requirement, 5 kg chopped green maize fodder per animal was provided once a week. All animals had free access to clean water. Individual feed intake was recorded by measuring feed offered and orts in the morning daily throughout the experiment.

Preparation of phyto feed additives: The plant parts used in this study were garlic bulb (*Allium sativum*), ajwain seed (*Trachyspermum ammi*), harad pulp (*Terminalia chebula*) and soapnut pulp (*Sapindus mukurossi*) in FAI and ajwain oil in FAII. The mixture of herbs (FAI) was prepared by mixing an equal quantity of four herbs. These herbs were procured from local market, sun dried, powdered and mixed to form a uniform mixture.

Rumen fermentation: About 250 mL rumen fluid was collected through the oral cavity using a stomach tube connected to a vacuum pump before the morning feeding in sterilized plastic bottles. After collection, samples were immediately transferred to the lab for further analysis. The pH of rumen liquor, was measured immediately using a pH meter. Subsequently, the rumen liquor was strained through two layers of cheesecloth and the clear rumen liquor was stored at -20°C till further analysis. A subsample for DNA extraction was stored at -80°C till further processing. The rumen liquor was analysed for volatile fatty acids (TVFAs) using Nucon-5765 gas chromatograph (AMIL, New Delhi, India) armed with a double flame ionization detector and glass column (4ft. length and 1/8-inch diameter) packed with chromosorb 101 according to method defined by Cottyn and Boucque (1968) and for ammonia-N concentration (Wheatherburn 1967).

Rumen microbial enzymes activity: The enzymes were extracted from 25 ml rumen liquor mixed with 5 ml each of lysozyme (0.4%) and carbon tetrachloride as per the procedure described by Hristov *et al.* (1999). The activities of avicelase, carboxymethylcellulase (CMCase) and xylanase were estimated using avicel, carboxymethylcellulose and xylan as substrate, respectively (Agarwal *et al*. 2000) and the reducing sugars released after incubation were estimated as per Miller *et al*. (1959). The avicelase, CMCase and xylanase activities (unit) were expressed as nmol glucose (for CMCase and avicelase) and xylose (for the xylanase) produced/ml/min. The protein contents of the enzyme samples were estimated (Lowry *et al*. 1951) and the specific activity was defined as unit per mg protein.

Microscopic count of protozoa: For counting of protozoa, 1 ml rumen liquor was stained with 1 ml methyl green formal saline solution (Kamra *et al*. 1991) and allowed to stand overnight at room temperature. If necessary, further dilution was done with 30% (v/v) glycerol. Counting was done under the microscope in hemocytometer counting chamber.

Enumeration of rumen microbes by real time PCR: The frozen samples of rumen liquor (-80°C) was used to determine microbial populations using real-time qPCR. Extraction of genomic DNA was done from rumen liquor (Yu and Morrison 2004). A 20µl assay mixture containing 10 µl of 2× SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 0.6 µl each of forward and reverse primers, 2 µl DNA and nuclease free

Table 1. Ruminal microbe primers for quantitative PCR assay

Microbe	Primer sequence (5′-3′)	Annealing Temp. (°C)	Size (bp)	Reference
Total bacteria	F-CGG CAACGAGCGCAACCC R-CCATTGTAGCACGTGTGTAGCC	60	130	Denman and McSweeney (2006)
Fibrobacter succinogenes	F-GTTCGGAATTACTGGGCGTAAA R-CGCCTGCCCCTGAACTATC	60	121	Denman and McSweeney (2006)
R. flavefaciens	F-CGAACGGAGATAATTTGAGTTTACTTAGG R-CGGTCTCTGTATGTTATGAGGTATTACC	60	132	Denman and McSweeney (2006)
Fungi	F-GAGGAAGTAAAAGTCGTAACAAGGTTTC R-CAAATTCACAAAGGGTAGGATGATT	60	110	Denman and McSweeney (2006)
Ruminococcus albus	F-CCCTAAAAGCAGTCTTAGTTCG R-CCTCCTTGCGGTTAGAACA	60	175	Koike and Kobayashi (2001)
Protozoa	F-GCTTTCGWTGGTAGTGTATT R-CTTGCCCTCYAATCGTWCT	55	223	Sylvester *et al.* 2004
Methanogen	F-TTCGGTGGATCDCARAGRGC R-GBARGTCGWAWCCGTAGAATCC	60	140	Denman *et al.* 2007

water, was prepared for qPCR amplification. Specific primers were used to enumerate the microbial population of total bacteria, Ruminococcus albus, Fibrobacter succinogenes, Ruminococcus flavefaciens, methanogen, fungi and protozoa (Table 1). The copy number of each microbe was calculated (Ritalathi et al. 2006).

Statistical analysis: Data obtained from this experiment were analysed by two-way analysis of variance (ANOVA) using SPSS 16.0. When a parameter showed significant difference at P<0.05, Duncan's multiple range test was conducted for comparing treatment means.

RESULTS AND DISCUSSION

Rumen fermentation: The mean value of pH, NH_3-N, VFAs and molar proportion of acetate, propionate, butyrate and acetate to propionate ratio did not differ (P>0.05) among all the groups (Table 2). The results indicate that the rumen microbes might be able to tolerate the levels of phyto-feed additives fed to the animals, hence, the functioning of rumen remained normal. This finding probably indicates relatively low contents of anti-methanogenic phytochemical substances or the adaptation of the microbiota to phyto-feed additives (Patra and Yu 2015). Ahmad *et al.* (2021) reported no detrimental effect on rumen fermentation by inclusion of mootral (combination of garlic and citrus powder in 9:1 ratio) at various doses and suggested that the doses studied were not high enough to cause noticeable alterations in the fermentation profile. However, some studies have shown decrease in rumen ammonia nitrogen level by feeding phyto-feed additives. Pawar *et al.* (2021) reported decrease in ammonia nitrogen levels in the rumen liquor of calves by feeding EOs @ 2g/d but TVFAs remained unaffected. A meta-analysis of the data of 23 experiments on feeding of blend of essential oils (Agolin Ruminant(R)) to dairy cows revealed no effect on fermentation parameters including pH, TVFAs and its fractions (Belanche *et al*. 2020).

Table 2. Ingredient and chemical composition of the diet (DM basis) (g/kg dry matter)

Ingredient	Concentrate	Wheat straw	FAI
Physical composition (g/kg feed basis)			
Maize	350	-	-
Soybean meal	240	-	-
Wheat bran	380	-	-
Mineral mixture	20	-	-
Salt	10	-	-
Chemical composition (g/kg dry matter basis)			
Organic matte	925	930	920
Crude protein	203	360	110
Ether extract	30	118	37
NDF	260	800	420
ADF	65	530	220
TA	75	70	76

Rumen microbial enzymes activity: Phyto-feed additives had no effect on the activities of carboxymethylcellulase, avicelase, acetyle esterase, xylanase and protease in any of the treatment group (Table 3). However, increase in CMCase and decrease in protease with no change in avicelase, acetyl esterase, xylanase activities was observed by feeding EOs to buffalo calves (Pawar *et al.* 2021). The difference in the two reports might be due to difference in the dose because same EO was used in the two experiments. No impact of EOs feeding on rumen microbial fibre degrading enzymes was also reported by Kala *et al.* (2017). Agarwal *et al*. (2020) reported significant increase in the activities of xylanase, amylase, α-glucosidase and β-glucosidase, whereas, protease decreased, and CMCase and avicelase did not change by feeding of an herbal mixture of seven plant parts. Regarding herbal feed additives, they are the mixtures of different plant part therefore the response of rumen enzymes varied.

Microbial population: The microbial count of protozoa decreased significantly in all the treated groups as compared to control irrespective of period (Table 4). When, protozoa were assessed by qPCR, the population was numerically down in the treated groups but the difference was non-significant (Table 5). Majewska *et al.* (2021) fed plant additive to sheep and found significant reduction in protozoa count in the rumen fluid. Albores-Moreno *et al.* (2017) reported a reduction of up to 40% in total protozoa counts when ground pods of *Enterolobium cyclocarpum* were included in the ration (30-45% DM). The population density of total bacteria and fungi were not affected by feeding phyto-feed additives but the population of *F. succinogenes, R. albus* and methanogens significantly decreased as compared to other three groups

Table 3. Effect of phyto additives on rumen fermentation parameters in buffalo calves

Parameter	Treatments (T)				Period, Months (P)			SEM	P value		
	T0	T1	T2	T3	1	3	6		T	P	T×P
pH	6.61	6.69	6.62	6.63	6.64	6.63	6.65	0.10	0.078	0.598	0.668
NH_3-N (mg/dl)	10.47	10.96	10.05	10.71	11.53	10.00	10.11	0.33	0.790	0.127	0.927
TVFAs (mM/dl)	9.06	9.16	8.83	9.56	9.38	8.94	9.13	0.20	0.652	0.675	0.964
Acetate (A) %	73.11	71.90	71.80	72.10	72.47	71.63	72.58	0.35	0.550	0.500	0.986
Propionate (P) %	19.04	19.78	19.45	20.08	19.78	19.66	19.33	0.25	0.494	0.740	0.965
Butyrate%	7.86	8.32	8.74	7.82	7.75	8.71	8.09	0.24	0.506	0.279	0.942
A: P ratio	3.85	3.66	3.73	3.60	3.69	3.73	3.78	0.06	0.509	0.689	0.966

T0, control; T1, FAI (blend of garlic harad, ajwain and soapnut in equal proportion @ 1% of DMI); T2, FAII (ajwain oil @ 1ml per kg DMI); T3, FAI and FAII alternatively for every 15 days; T, treatment; P, period; SEM, standard error of mean.

Table 4. Effect of phyto additives on rumen enzyme specific activities (U/mg protein) in buffalo calves

Parameter	Treatments (T)				Period, Months (P)			SEM	P value		
	T0	T1	T2	T3	1	3	6		T	P	T×P
Carboxymethylcellulase	0.39	0.42	0.41	0.43	0.38	0.43	0.42	0.01	0.740	0.204	0.777
Avicelase	0.19	0.19	0.18	0.20	0.19	0.17	0.21	0.01	0.925	0.369	0.952
Xylanase	1.33	1.28	1.29	1.33	1.25	1.30	1.37	0.02	0.793	0.060	0.859
Acetyl esterase	0.37	0.38	0.38	0.41	0.35	0.40	0.41	0.02	0.820	0.267	0.991
Protease	8.87	9.27	8.73	9.31	8.94	8.92	9.27	0.36	0.922	0.909	0.998

T0, control; T1, FAI (blend of garlic harad, ajwain and soapnut in equal proportion @1% of DMI); T2, FAII (ajwain oil @ 1ml per kg DMI); T3, FAI and FAII alternatively for every 15 days; T, treatment; P, period; SEM, standard error of mean.

Table 5. Effect of phyto additives on microbial population (Log10) in rumen liquor of buffalo calves

Parameter	Treatments (T)				Period, Months (P)			SEM	P value		
	T0	T1	T2	T3	1	3	6		T	P	T×P
Protozoa											
Holotrichs	3.95^{X}	3.80^{Z}	3.88^{Y}	3.78^{Z}	3.89^{A}	3.88^{A}	3.79^{B}	0.01	<0.001	0.002	0.002
Entodinimorphs	5.37^{X}	5.30^{Y}	5.31^{Y}	5.32^{Y}	5.28^{B}	5.36^{A}	5.33^{A}	0.07	0.005	0.001	0.526
Total	5.39^{X}	5.31^{Y}	5.32^{Y}	5.33^{Y}	5.30^{B}	5.37^{A}	5.34^{A}	0.07	0.002	0.001	0.459
Protozoa	6.18	5.78	6.33	5.75	6.90^{A}	5.85^{B}	5.28^{B}	0.13	0.326	0.000	0.418
Bacteria											
F. succinogenes	6.69^{XY}	7.07^{X}	6.07^{Y}	4.63^{Z}	5.39^{B}	6.27^{A}	6.69^{A}	0.11	0.000	0.000	0.268
R. flavefaciens	6.71^{X}	6.84^{X}	6.03^{Y}	6.95^{X}	6.39	6.64	6.87	0.11	0.036	0.241	0.171
R. albus	5.80^{X}	6.17^{X}	5.74^{X}	5.04^{Y}	5.51	5.65	5.91	0.10	0.003	0.241	0.023
Methanogens	6.99^{X}	6.98^{X}	6.80^{X}	6.10^{Y}	6.67	6.78	6.71	0.11	0.026	0.915	0.525
Total bacteria	10.56	10.51	10.37	9.96	10.89	10.35	9.82	0.24	0.819	0.222	0.351
Fungi	6.37^{XY}	6.49^{XY}	6.67^{X}	6.08^{Y}	6.02^{B}	6.19^{B}	7.00^{A}	0.08	0.074	0.000	0.000

T0, control; T1, FAI (blend of garlic harad, ajwain and soapnut in equal proportion @1% of DMI); T2, FAII (ajwain oil @1ml per kg DMI); T3, FAI and FAII alternatively for every 15 days; T, treatment; P, period; SEM, standard error of mean. ^{AB}Different superscripts in a column for a parameter differ significantly. ^{XYZ}Different superscripts in a row for a parameter differ significantly. ^{abc}Different superscripts among rows and columns for a parameter differ significantly.

including control in T3 group where FAI and FAII were fed alternatively. The *R. flavefaciens* population was adversely affected in T2 group as compared to control, T1 and T3. This shows that by feeding phyto-feed additive continuously, some rumen microbes may get adapted as the maximum populations barring *R. flavifaciens*, were inhibited in T3 where two phyto-feed additives were fed alternately. Pawar *et al.* (2021) also observed decreased protozoa, methanogens and *F. succinogenes* population by feeding ajwain seed oil to buffalo claves. Similarly, Agarwal *et al.* (2020) reported decreased methanogens and *F. succinogenes* population by feeding herbal mix to buffaloes. The results indicate that methanogens and *F. succinogenes* are the most sensitive microbes to phyto-feed additives and are being affected by most of them. In the rumen, the three bacteria viz., *F. succinogenes, R. flavefaciens* and *R. albus* are considered as the key fibre degrading microbes and are highly explored (Kala *et al*. 2020). But these are the not only fibre degrading microbes (Kala *et al*. 2017) and that is why reduction in the population of these microbes not necessary influence fibre degradation also. When ginger powder along with lime peel powder were fed to sheep, there was reduction in total protozoa, methangens and ammonia nitrogen but the population of *R. albus* and *R. flavifacience* increased along with no change in *F. succinogenes* (Okoruwa and Aidelomon 2020). This rumen microbial changes improved nutrient digestibility reflecting that any change in fibre digestibility is not necessarily associated with the changes in these three key fibre degrading microbial populations. Rumen protozoa population was reduced in majority of experiments on feeding plant additives and since protozoa is one of the major hydrogen suppliers to methanogens, such changes are indicative of reduced enteric methane production.

Methanogens are the prime culprit of methane production in the rumen. Just like protozoa, reduced methanogen population is also an indicative of reduced methane production, but it has been observed that reduced methane reduction is not always associated with reduction in the population of total methanogens or vice versa. Wang *et al.* (2019) demonstrated significant decrease in methanogen population but methane production was not reduced by inclusion of plant feed additives. Similarly, Kumar *et al.* (2019) reported significant decrease in methane production but methanogen population was not affected by dietary supplementation of bromoethanesulphonic acid at variable levels to cattle calves. Compared to control, the population of rumen fungi in all the treated phyto-feed additives fed groups was similar showing no response to the feed additives. The rumen fungi also contribute to the supply of hydrogen to rumen methanogens (Beauchemin *et*

al. 2020) and no change in rumen fungi in the present study indicate that rumen fungi were not playing role neither in increasing or decreasing methane emission in the present study.

It may be asserted from the present study that phyto-feed additives (FAI, a blend of garlic, harad, ajwain, and soap nut @1g per kg DMI and FAII, ajwain oil @1 ml kg DMI) had no effect on rumen fermentation pattern. The alternate feeding of the two phyto-feed additives also did not impart any additional benefit except that the population of protozoa, *R. albus* and methanogens populations in the rumen were suppressed in this group.

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