

Changes in *in vitro* rumen fermentation parameters of crossbred cattle (*Bos taurus*) and buffalo (*Bubalus bubalis*) in response to diet at different time intervals

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ABSTRACT

Assessment of the diet adaptation period is critical for feeding experiments in ruminants to have significant changes in the concerned parameters. An *in vitro* trial was conducted to investigate the impact of the sampling at different time periods (day 0, 11, 21, and 60) to determine the adaptation period of feed treatment over the ruminal fermentation parameters in crossbred Karan-Fries cattle (*Bos taurus*) and Murrah (*Bubalus bubalis*) heifers. Two types of total mixed rations (TMRs), viz. high forage diet (HFD) having roughage and concentrate ratio (R:C) as 70:30 and high concentrate diet (HCD) having R:C as 40:60 were formulated, and fed to cattle and heifers. Rumen liquors were collected from all the animals on 0, 11, 21, and 60 days. HFD and HCD diets were incubated with strained rumen liquor collected from cattle and buffalo adopting Menke's *in vitro* gas procedure. The results showed that most of the *in vitro* rumen fermentation parameters, viz. total volatile fatty acids (TVFAs) (mM/ml), acetate (%), propionate (%), butyrate (%), acetate propionate ratio (A:P), partitioning factor (PF) and microbial biomass production (MBP) (mg), *in vitro* dry matter digestibility (IVDMD) (%) and *in vitro* organic matter digestibility (IVOMD) (%) were significantly different at day 0 and 11 compared to day 21. Afterward, up to day 60, there were no effects reported except increased IVDMD and IVOMD for buffalo compared to day 21. As a result of our findings, it can be concluded that there are significant differences in rumen fermentation parameters at 11 and 21 days and that the time period for cattle and buffalo should not be reduced to 11 days from 21 days in order to achieve proper feed adaptation and stabilize the rumen fermentation process. Further research is needed to investigate the impacts of the adaption period at various time intervals.

Keywords: Adaptation period, Crossbred cattle, *In vitro* rumen fermentation, Menke's total gas estimation, Murrah buffalo

In nutritional studies of ruminants, the diet adaptation period, given for stabilizing the fermentation processes is very critical (Brown *et al.* 2006). The residual effects of the previously fed feed, vast storage capacity of the rumen, and sensitive microbial ecosystem are the prime causes of temporal dynamics of the rumen (Qui *et al.* 2021). The time-lapse essential for the transition from high-forage to high-grain ration and vice-versa helps to establish a stable microbial community and results in significant metabolic changes in the rumen (Fernando *et al.* 2010). This particular period adapts the animals in the form of structural changes in the rumen like ruminal papillae and functional changes in terms of the composition of ruminal microbiota (Estevam *et al.* 2020). In the absence of a proper adaptation period, the animals may develop ruminal acidosis, ruminitis, and other health complications, which further hamper the animal production.

Previously, a longer than 16-30 days preliminary

period was recommended in steers with a fluctuating dietary composition (Nicholson *et al.* 1965). Generally, 21 day's time period has been considered as standard for the stabilization of the fermentation processes for basal nutrition experiments of ruminants (Vasconcelos and Galyean 2007). However, some reports claimed 11 days (Sirohi *et al.* 2013, Saleem *et al.* 2013) and 14 days (Estevam *et al.* 2020) for the adaptation from high roughage to a high concentrate diet. Moreover, a shorter adaptation period is appreciated from the economic point of view, especially in beef cattle (Pinto and Millen 2018); however, a less than 14 days adaptation period caused compromised animal performance viz. inferior carcass traits (Brown *et al.* 2006). An attempt to shorten the adaptation period from 14 days using virginiamycin as a sole feed additive has not been recommended in Nellore cattle (Rigueiro *et al.* 2021). Likewise, Nellore and crossbreed Angus-Nellore cattle did not get benefited from the shortening adaptation period to 9 days from 14 days after consuming a high concentrate (86%) fattening ration (Watanabe *et al.* 2022).

In vitro procedures involving Menke's gas production and digestibility estimation apparently reflect the animal's

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internal environment; and are routinely used to evaluate the nutritional experiments due to their convenience, adaptability, efficiency, it's ease of adoption, repeatability, minimized use of animals, and lesser budget needed than for *in vivo* evaluation of feeds (Dixit *et al.* 2016). Therefore, the present investigation was carried out to evaluate the *in vitro* fermentation characteristics at different time periods of feeding trials in crossbred cattle and buffalo.

MATERIALS AND METHODS

Rumen liquor was collected from 12 crossbred cattle and Murrah heifers each, maintained on high forage diet (HFD) having roughage and concentrate ratio (R:C) as 70:30 and high concentrate diet (HCD) having R:C as 40:60 at day 0, 11, 21 and 60 after initiation of feeding trial. The proximate composition of total mixed rations (TMRs) as per the standard procedures is given in Table 1 (AOAC 2005). The procedure followed is depicted in Fig. 1.

Table 1. Chemical composition of TMRs used for *in vitro* trial

Attribute (%)	*Treatment	
	HFD	HCD
OM	91.68±0.89	92.92±0.72
CP	9.28±0.24	12.34±0.53
EE	2.74±0.02	3.77±0.02
TA	8.31±0.36	7.08±0.25
NDF	54.42±1.49	43.63±1.58
ADF	30.23±0.12	26.86±0.25

*HFD-Sugargraze (70%) + Concentrate mix. (30%); HCD-Sugargraze (40%) + Concentrate mix. (60%); OM, organic matter; CP, crude protein; EE, ether extract; TA, total ash; NDF, neutral detergent fibre; ADF, acid detergent fibre.

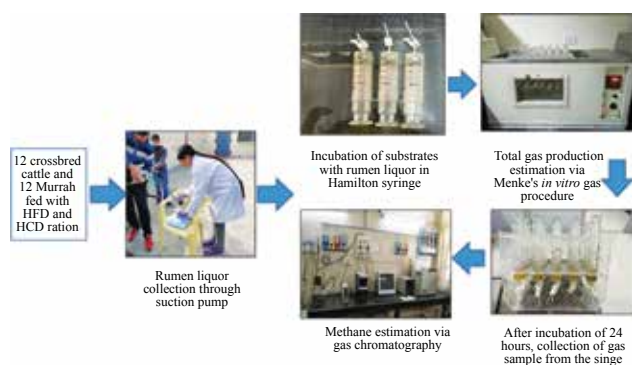


Fig. 1. Procedure followed.

The rumen liquor was collected from a rubber pipe inserted through the mouth and fitted with a vacuum pump; then filtered through a four-layered muslin cloth. Further, centrifuged at 3000 rpm for 10 min and individual volatile fatty acids (IVFA) were estimated by taking 1 ml of the supernatant treated with 25% metaphosphoric (4 ml) prepared in 1N H₂SO₄ and kept for 3-4 h at ambient temperature (Erwin *et al.* 1961). For *in vitro* gas production, each TMR (200±5 mg) was incubated in 100 ml calibrated glass syringes filled with 30 ml of buffered rumen inoculums and placed in the water

bath adjusted at 39±0.5°C for 24 h (Menke *et al.* 1979, Menke and Steingass 1988). Gas produced (mL/200 mg substrate) by fermentation during 24 h was measured from the movement of piston upside by visual assessment of the calibrated scale on the syringe and net gas produced by the substrate was calculated from corresponding blank values. After 24 h incubation, a suitable (200 µl) aliquot of gas was withdrawn from the tip of the incubation syringe using air tight Hamilton syringe and analyzed for methane content with the help of Gas chromatograph (Nucon 5700, India) fitted with stainless steel column packed with Porapak-N and Flame Ionization Detector (FID).

$$\text{Methane (\%)} = \frac{\text{Area covered by the sample}}{\text{Area covered by the standard of methane}} \times 50$$

For digestibility estimation, after incubation of 24 h, the syringe contents were transferred to centrifuge tubes. The tubes were centrifuged at 3,000 rpm for 10 min and the pellets were used for estimation of IVDMD and IVOMD. The pellets were then transferred to 500 ml spout-less beakers by dissolving with 100 ml of neutral detergent solution (NDS). Beakers were kept on the heater and refluxing was done at 100°C for 1 h from the start of boiling. The contents in the beakers were filtered under a vacuum through pre-weighed crucibles and washed with hot water. Crucibles containing residue were oven-dried till constant weight (65°C, 48 h), then weighed and the dry residues were ashed at 550°C. IVDMD and IVOMD were calculated from the disappearances of dry matter (DM) and organic matter (OM) with the help of the following equations:

$$\text{IVDMD (\%)} = \frac{(\text{DM taken for incubation} - \text{NDF residue})}{(\text{DM taken for incubation})} \times 100$$

$$\text{IVOMD (\%)} = \frac{(\text{OM taken for incubation} - \text{residue OM})}{(\text{OM taken for incubation})} \times 100$$

Partitioning factor (PF) and MBP were calculated as per the standard equations using truly degraded organic matter (TDOM) (Blümmel *et al.* 1997, Blümmel *et al.* 2005).

$$\text{PF} = \frac{\text{TDOM (mg)}}{\text{Net gas production}}$$

where, TDOM was calculated by multiplying IVOMD (%) by OM content (mg) of the substrate.

$$\text{MBP (mg)} = \text{TDOM (mg)} - (\text{Net gas volume (mL/g)} \times \text{Stoichiometric factor})$$

where, the stoichiometric factor considered for concentrates and mixed diets was 2.34 and 2.20, respectively.

Statistical analysis was done using completely randomized design, one-way classification (Snedecor and Cochran 1994). Significant differences among different treatments were identified using Duncan's Multiple Range Test and a P<0.05 was considered to be statistically significant. All the statistical analysis was done using SPSS version 16.

RESULTS AND DISCUSSION

The *in vitro* rumen fermentation characteristics at 0, 11, 21, and 60 days for crossbred cattle and Murrah buffalo consuming HFD and HCD rations are presented in Table 2 and Table 3, respectively. Our findings indicated that acetate, net gas, IVDMD, and IVOMD of crossbred cattle fed were higher on HFD ration on day 21 compared to days 0 and 11, and the values remained statistically similar to the value reported on day 60 (Table 2). Propionate and acetate: propionate ratio (A:P) of crossbred cattle on HFD; however were higher even at day 11 compared to day 0. Additionally, the values for TVFA, acetate, A:P, methane, IVDMD and IVOMD of crossbred cattle on HCD were also found to be significantly ($P<0.05$) different at day 21 as compared to day 0 and 11 (Table 2). Some of the parameters viz. propionate, butyrate, net gas, PF and MBP of crossbred cattle on HCD achieved statistically different concentration on day 11 as compared to day 0. One of the probable explanations might be the most pronounced increase in copy numbers of fibrolytic bacteria at day 11 in high roughage consuming animals (Sirohi *et al.* 2013). In crossbred cattle, there was no effect of sampling days on TVFA, methane, and MBP on high fibrous ration (R:C=70:30).

For Murrah heifer rumen liquor samples, significantly ($P<0.05$) higher values of net gas, PF and methane on the day 21 reflected a more or less similar trend as observed in cattle (Table 3). TVFA, IVDMD and IVOMD, both on HFD and HCD, achieved their statistically ($P<0.05$) different concentrations earlier, i.e. at day 11 as compared

to day 0. The levels of acetate, butyrate, methane, PF, and MBP remained statistically comparable in relation to the adaptation days for HCD diet in Murrah heifers. However, high roughage diet could not produce significant changes in concentration of propionate, butyrate, A:P and MBP in Murrah heifers.

A stable rumen fermentation process is associated with host's physiology and health. The microbial ecosystem present in rumen remains in temporal dynamics as diet changes in composition and reflects in the pattern of rumen fermentation characteristics (Qiu *et al.* 2019). The levels of individual VFAs were found highest at 14-day post-high concentrate diets in Holstein cows (Mamuad *et al.* 2017). In consonance with the present findings, Karan-Fries cattle had higher concentrations of IVFAs on day 11 as compared to day 0, then further decreased up to day 21, although non-significantly (Sirohi *et al.* 2013). There are definite effects of the source of rumen liquor and roughage concentrate ratio over the fermentation parameters; however, we have restricted our study to the effect of adaptation time only. Although the specific bacterial communities might be changed quantitatively in response to the feeding, but the stabilization of rumen ecosystem requires a definite time lag (Li *et al.* 2009). Following a shift from high forage diet to high concentrate in beef steers, rumen microbiome stabilized in approximately 9 weeks of adaptation time (Clemmons *et al.* 2019). The time duration of 3 to 28 days has been previously reported to witness considerable changes in the microbial communities (Tajima *et al.* 2000), when switched from high forage to high concentrate ration.

Table 2. *In vitro* fermentation characteristics at days 0, 11, 21, and 60 in crossbred cattle heifers

Particular	Diet	0 Day	11 Day	21 Day	60 Day
TVFA (mM/L)	HFD	82.46±1.53	82.30±1.07	85.13±1.72	83.13±1.09
	HCD	84.29 ^a ±0.67	86.96 ^a ±0.75	92.62 ^b ±1.45	90.29 ^b ±2.01
Acetate (%)	HFD	65.03 ^a ±0.15	66.09 ^a ±0.35	69.02 ^b ±0.16	69.13 ^b ±0.24
	HCD	64.28 ^a ±0.44	64.12 ^a ±0.33	61.82 ^b ±0.16	62.57 ^b ±0.30
Propionate (%)	HFD	25.63 ^a ±0.27	23.32 ^b ±0.46	20.20 ^b ±0.32	19.29 ^b ±0.73
	HCD	22.82 ^a ±0.54	24.76 ^b ±0.64	28.79 ^c ±0.18	29.39 ^c ±0.34
Butyrate (%)	HFD	9.34 ^a ±0.12	10.59 ^{ab} ±0.61	10.78 ^{ab} ±0.31	11.58 ^b ±0.46
	HCD	9.23 ^a ±0.46	11.63 ^b ±0.70	9.39 ^a ±0.26	8.04 ^a ±0.04
A:P	HFD	2.54 ^a ±0.03	2.93 ^b ±0.06	3.02 ^b ±0.05	2.90 ^b ±0.10
	HCD	2.83 ^a ±0.09	2.60 ^a ±0.07	2.15 ^b ±0.01	2.13 ^b ±0.03
Net gas (ml/200 mg)	HFD	29.17 ^a ±0.61	30.17 ^a ±0.31	34.33 ^b ±0.84	35.17 ^b ±0.88
	HCD	28.17 ^a ±0.80	35.08 ^b ±0.51	40.33 ^c ±0.80	39.17 ^c ±0.67
Methane (% total gas)	HFD	31.75±1.84	32.92±1.79	36.64±0.67	36.26±0.81
	HCD	32.21 ^a ±0.75	32.75 ^a ±0.61	27.48 ^b ±1.16	27.01 ^b ±0.66
PF	HFD	4.07 ^a ±0.10	3.77 ^{ab} ±0.04	3.68 ^{ab} ±0.13	3.60 ^b ±0.10
	HCD	4.26 ^a ±0.14	3.43 ^b ±0.06	3.22 ^b ±0.10	3.22 ^b ±0.07
MBP (mg)	HFD	50.33±2.06	44.45±0.87	45.59±3.27	43.97±2.61
	HCD	53.82 ^a ±3.22	38.08 ^b ±1.80	35.19 ^b ±3.32	38.62 ^b ±2.13
IVDMD (%)	HFD	55.01 ^a ±0.80	56.03 ^a ±0.32	62.67 ^b ±0.70	62.31 ^b ±0.60
	HCD	56.33 ^a ±0.40	58.58 ^a ±0.39	65.97 ^b ±0.50	67.80 ^b ±2.81
IVOMD (%)	HFD	63.78 ^a ±0.89	63.80 ^a ±0.21	67.59 ^b ±1.13	68.50 ^b ±0.54
	HCD	64.63 ^a ±1.53	65.34 ^a ±0.91	69.68 ^b ±0.73	70.80 ^b ±0.41

Means bearing different superscripts in the same row differ significantly ($P<0.05$).

Table 3. *In vitro* fermentation characteristics at days 0, 11, 21, and 60 in buffalo heifers

Particular	Diet	Day 0	Day 11	Day 21	Day 60
TVFA (mM/L)	HFD	87.72 ^a ±0.99	102.89 ^b ±0.84	101.89 ^b ±0.76	104.22 ^b ±1.70
	HCD	88.14 ^a ±0.58	103.31 ^b ±0.54	102.31 ^b ±0.73	108.14 ^b ±1.19
Acetate (%)	HFD	62.36 ^a ±0.39	65.96 ^b ±0.74	65.22 ^b ±0.73	64.64 ^{ab} ±0.33
	HCD	63.83±0.63	63.82±0.64	61.84±0.51	62.12±0.12
Propionate (%)	HFD	23.43±0.81	23.70±0.46	23.76±0.74	22.76±0.87
	HCD	23.32 ^a ±0.14	24.82 ^{ab} ±0.66	28.05 ^b ±0.79	27.62 ^{ab} ±1.01
Butyrate (%)	HFD	9.21±0.36	9.55±1.61	11.19±0.46	12.97±1.00
	HCD	13.34±0.60	11.81±0.76	10.72±0.86	10.77±1.12
A:P	HFD	2.68±0.09	2.79±0.05	2.76±0.12	2.86±0.11
	HCD	2.74 ^a ±0.03	2.58 ^a ±0.08	2.21 ^b ±0.07	2.27 ^b ±0.09
Net gas (ml/200 mg)	HFD	28.25 ^a ±0.31	29.67 ^a ±0.38	33.83 ^b ±0.38	35.17 ^b ±1.09
	HCD	28.67 ^a ±1.87	36.50 ^b ±0.47	36.33 ^b ±1.67	37.17 ^b ±1.82
Methane (% total gas)	HFD	32.89 ^a ±1.92	33.04 ^a ±0.48	36.90 ^b ±1.76	39.78 ^b ±1.94
	HCD	32.85±1.31	30.34±0.03	29.48±1.49	28.02±0.70
PF	HFD	4.21 ^a ±0.06	4.11 ^a ±0.10	3.77 ^b ±0.06	3.70 ^b ±0.11
	HCD	4.08±0.28	3.40±0.03	3.57±0.15	3.69±0.18
MBP (mg)	HFD	52.82±1.29	52.24±2.35	48.33±1.45	47.18±2.46
	HCD	47.34±5.06	38.81±0.82	43.38±4.26	48.53±4.04
IVDMD (%)	HFD	54.60 ^a ±0.36	61.13 ^b ±0.35	65.66 ^c ±0.43	64.89 ^c ±0.98
	HCD	54.59 ^a ±1.10	60.85 ^b ±0.44	67.25 ^b ±0.73	68.38 ^c ±1.61
IVOMD (%)	HFD	63.82 ^a ±0.59	65.99 ^b ±0.88	68.84 ^{bc} ±0.47	70.42 ^c ±1.49
	HCD	61.55 ^a ±0.95	67.51 ^b ±0.28	68.91 ^b ±0.70	73.50 ^c ±0.80

Means bearing different superscripts in the same row differ significantly (P<0.05).

Similarly, Sirohi *et al.* (2013) found significantly increased copy numbers of fibrolytic bacteria, viz. *Ruminococcus albus*, *R. flavefaciens*, *Butyrivibrio fibrisolvens* and *Fibrobacter succinogens* on day 11 in rumen of cattle fed on high fibre diet and the total bacterial and total anaerobic fungi population also increased by nearly 50% on day 11 in high fiber diet than day 0 (Sirohi *et al.* 2013). An adaptation period of 11 days was further suggested; where it was found sufficient to allow wash-in of the experimental diets before the metabolite's measurements (Saleem *et al.* 2013). In case of long-term fattening steers, a three-month period had not been found sufficient to stabilize the fermentation process (Qiu *et al.* 2021). Swamp buffalo can be adapted to a rice straw-based diet with urea supplementation within 2 weeks of feeding (Wanapat *et al.* 2016). Nellore cattle had been found to adapt to a corn-based ration in 14 days without affecting the performance (Estevam *et al.* 2020). On the other hand, dairy cows fed a hay diet, transitioned to a 65% grain diet for 3 weeks, had a significantly altered density and diversity of microbiota in the rumen (Hook *et al.* 2011). A unique composition of rumen microflora, for example, genus *Oscillospira* in buffalo heifers in the companion *in vivo* trial may also decide the adaptation efficiency of the animals (Dixit *et al.* 2022). Nellore bulls adapted to high concentrate diets for 21 days had been observed with a larger rumen wall absorptive surface area as compared to the animals adapted for 14 days; however, after a longer feeding period up to 84 days, there was non-significant difference (Parra *et al.* 2019). A similar study on yaks revealed that the adaptation period of 16 days to a high nutrition diet

had a stabilized gut micro-ecology in relation to relative abundances of *Erysipelotrichia*, *Gammaproteobacteria*, *Saccharimonadia*, and *Coriobacteriia* (Zhang *et al.* 2020).

As a result of our findings, we can conclude that rumen fermentation parameters differ significantly between 11 and 21 days, as compared to 0 day; and that the time period for cattle and buffalo should not be reduced to 11 days from 21 days in order to achieve proper feed adaptation and stabilize the rumen fermentation process. More research is needed to better understand the effects of the adaptation period at different time intervals.

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