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Dr. R Dhinesh Kumar

Assistant Professor, Livestock
Farm Complex, Veterinary
College and Research Institute,
Namakkal, Tamil Nadu, India

Dr. Bandla Srinivas

Principal Scientist, Dairy
Production Section, Southern
Regional Station, ICAR-
National Dairy Research
Institute, Bengaluru, Tamil
Nadu, India

Dr. G Prabakar

Assistant Professor, Department
of Livestock Production
Management, Veterinary College
and Research Institute,
Udumalpet, Tamil Nadu, India

Dr. S Prakash

Assistant Professor, Department
of Livestock Production
Management, Veterinary College
and Research Institute,
Udumalpet, Tamil Nadu, India

Corresponding Author:

Dr. R Dhinesh Kumar

Assistant Professor, Livestock
Farm Complex, Veterinary
College and Research Institute,
Namakkal, Tamil Nadu, India

Evaluation of formaldehyde-treated protein carriers for rumen protection of b-complex vitamins using the *in vitro* gas production technique

R Dhinesh Kumar, Bandla Srinivas, G Prabakar and S Prakash

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Abstract

B-complex vitamins are essential for normal metabolism in ruminants, but their ruminal synthesis is often insufficient for high-producing animals, as they are extensively degraded in the rumen. This study evaluated fish meal, casein, cottonseed meal, and blood meal as formaldehyde-treated protein carriers for protecting B-complex vitamins using the *in vitro* gas production technique. Treatments comprised substrate alone (CG), substrate with carrier protein (T₁), substrate with carrier and unprotected vitamins (T₂), and substrate with formaldehyde-treated carrier containing protected vitamins (T₃). Fermentation kinetics, gas production, volatile fatty acids, and *in vitro* dry matter digestibility were assessed. Fish meal, casein, and blood meal showed limited protection, as fermentation characteristics remained comparable to unprotected vitamins. In contrast, formaldehyde-treated cottonseed meal reduced fermentation relative to unprotected vitamins while maintaining normal ruminal fermentation, indicating effective protection. Cottonseed meal was the most suitable carrier for rumen protection of B-complex vitamins compared to fish meal, casein and blood meal.

Keywords: B-complex vitamins, rumen protection, cottonseed meal, formaldehyde treatment, *In vitro* gas production

1. Introduction

B-complex vitamins are essential cofactors in energy metabolism, protein synthesis, nucleic acid metabolism, and cellular growth (Duplessis *et al.*, 2014; Sacadura *et al.*, 2008) ^[1, 2]. In ruminants, rumen microorganisms synthesize several B-complex vitamins, endogenous production is often insufficient to meet the requirements of rapidly growing and high-producing animals. In addition, a substantial proportion of dietary B-complex vitamins may be degraded in the rumen before reaching the small intestine, thereby limiting their post-ruminal availability for absorption and utilization by the host animal (Santschi *et al.*, 2005; Schwab *et al.*, 2006) ^[3, 4]. To overcome this limitation, B-complex vitamins must be protected from ruminal degradation and delivered to the lower gastrointestinal tract in a form that permits efficient release and absorption. Protein-based carrier systems treated with formaldehyde provide a simple and economical means of achieving such protection, as formaldehyde can reduce microbial degradation in the rumen while allowing dissociation under acidic conditions in the abomasum and intestine (Ricke *et al.*, 2019) ^[5]. Therefore, the present study was undertaken to compare fish meal, casein, cottonseed meal, and blood meal as carrier proteins for formaldehyde-protected B-complex vitamins using the *in vitro* gas production technique.

2. Materials and Methods

2.1 Protection of B complex vitamins

Protection of B complex vitamins was done by using different protein sources viz. fish meal, casein, cottonseed meal and blood meal as carrier. Rumen protection of protein sources were done by 40% formaldehyde treatment at the rate of 1% of protein content (Aasiwal *et al.*, 2015) ^[6]. These carrier ingredients were added at 1 mg/200 mg of substrate equivalent to supplementing 25g/d of the protected ingredient in the animal diet daily. Feed grade B

complex vitamins were purchased from M/s Varsha Multi tech, Bangalore, India and their requirements in the animals were taken from earlier reported studies by Ashwin and Srinivas (2019) [7]. Fifty percentage of the B complex vitamin was protected through the protein carriers mentioned above and tested using IVGP technique.

2.2 Substrate for *in vitro* study

Total mixed ration (TMR) containing roughage to concentrate ratio of 70:30 was taken as substrate for IVGP studies. Roughage part consisted 3/4th finger millet (*Eleusine coracana*) or Ragi straw (RS) as dry roughage and 1/4th green roughages consisted 3 parts of Para grass (*Brachiaria mutica*) and 1 part each of Guinea grass (*Megathyrsus maximus*), Hybrid Napier (*Pennisetum glaucum* × *Pennisetum purpureum*) and Maize (*Zea mays*). RS and concentrate supplement (CS) were dried overnight in an oven at 90±5 °c to constant weight in a hot air oven (AOAC, 2005; Method No. 934.01). Green grasses such as Para grass, Guinea grass, Napier and Maize were dried at room temperature (32 °c) to constant weight. Dried samples were ground to pass through 1 mm screen. All the constituents were mixed in the above said proportion and stored in the plastic container.

2.3 *In vitro* experimental groups

In vitro studies were conducted using 4 groups as per completely randomized design using 15 replicates each as CG (substrate), T₁ (substrate with one of the carrier bases like blood meal, fishmeal, casein), T₂ (substrate with respective carrier bases and unprotected B complex vitamins) and T₃ (substrate with formaldehyde treated carrier containing protected B complex vitamins). The different treatments were formulated to deduce the effect of natural bypass ability of carrier from anaerobic fermentation in rumen *in vitro* and/or further improvement in protection after chemical treatment by formaldehyde. Comparison of *in vitro* gas production, fermentation characteristics, and gas production kinetics among the treatments was used to identify the most effective carrier for protecting B-complex vitamins from ruminal degradation.

2.4 *In vitro* gas production

Rumen liquor was collected from rumen cannulated cattle where the animal was maintained in the diet similar to the test diet. Rumen liquor was collected before offering feed at 8.00 AM with a minimum of 6 h gap from the last offering of drinking water, collected in thermos flask and brought to the laboratory. Immediately, rumen liquor was strained (SRL) through a double layer muslin cloth. SRL was used as inoculum and taken 10 ml in each syringe along with 20 ml of buffer media (Srinivas and Krishnamoorthy, 2005) [8] along with 200 mg of test feed samples under CO₂ flushing to maintain anaerobic condition (Menke and Steingass, 1988) [9]. Seventy two hours of incubation was made and gas production was measured at frequent intervals after correction for blanks. The filtrate obtained after the complete incubation was used to estimate total volatile fatty acids (TVFA) (Barnett and Reid, 1956) [10] by Markham distillation method and *in vitro* dry matter digestibility was also done (Lokesh et al., 2017) [11]. Analysis of data was done using one-way ANOVA using SPSS version 19.

3. Results and Discussion

3.1 Effect of Fish Meal-Based Protected B-Complex Vitamins on *In vitro* Fermentation Kinetics

The effect of fish meal as a carrier for rumen-protected B-complex vitamins on *in vitro* fermentation kinetics and digestibility is presented in Table 1. Total gas production differed significantly ($p < 0.001$) among the treatments. The control (CG) produced 39.36 ml of gas, whereas the highest gas production (44.13 ml) was observed in T₃. Lag time was significantly ($p < 0.05$) reduced in all fish meal-supplemented treatments compared with the control. The shorter lag phase observed in T₁, T₂, and T₃ suggests faster microbial attachment and colonization of the substrate, possibly due to the additional nitrogen supplied by fish meal, which could stimulate early microbial growth Hoover *et al.*, (1989) [12]. The half-time of fermentation (T_{1/2}) tended to decrease in the supplemented groups ($p < 0.10$), indicating relatively faster fermentation than the control. The gas production (Y_{max}) differed significantly ($p < 0.05$) among treatments. The higher Y_{max} observed in T₃ indicates greater availability of fermentable substrates throughout the incubation period, suggesting that supplementation of B complex vitamins improved ruminal fermentation (Ashwin and Srinivas, 2019) [7] and thus indicated no effect of HCHO treated fish meal on the protection of B vitamins. *In vitro* dry matter digestibility (IVDMD) was significantly ($p < 0.001$) improved by supplementation.

3.2 Effect of Casein-Based Protected B-Complex Vitamins on *In vitro* Fermentation Kinetics

Total gas production in casein supplemented groups showed a marginally significant difference ($p < 0.10$) (Table 2). The increased gas production in the supplemented groups indicates that casein did not impair ruminal fermentation and supported normal microbial activity. Lag time was significantly reduced ($p < 0.01$) in all casein-supplemented treatments compared with the control. The half-time of fermentation (T_{1/2}) was significantly lower ($p < 0.01$) in all supplemented treatments than in the control, suggesting faster fermentation of the substrate. The asymptotic gas production (Y_{max}) also differed significantly ($p < 0.05$). Finlayson and Armstrong (1986) [13] reported that increasing the amount of formaldehyde (HCHO) bound to casein reduced its *in vitro* degradability, although no further decrease was observed when the level exceeded 10.6 g HCHO/kg of treated casein DM whereas in our study degradability was not significantly affected treating casein with HCHO. The increased Y_{max} in T₃ than T₂ indicated that HCHO treatment did not completely protect the B-complex vitamins and their partial availability contributed to increased fermentation and digestion.

3.3 Effect of Cottonseed meal -Based Protected B-Complex Vitamins on *In vitro* Fermentation Kinetics

Total gas production differed significantly ($p < 0.05$) between T₂ and other treatment groups (Table 3). Supplementation with unprotected B-complex vitamins (T₂) significantly increased total gas production to 38.38 mL, whereas the formaldehyde-treated cottonseed meal containing protected B-complex vitamins (T₃) produced 35.91 mL, which was comparable to the control. The higher gas production observed in T₂ indicates enhanced microbial fermentation

following vitamin supplementation, while the reduction in T_3 may be attributed to formaldehyde treatment, which reduced the immediate degradability of the carrier protein. Ashwin and Srinivas (2019) [7] reported that B vitamins were used by most of cellulolytic bacteria and anaerobic fungi for their growth and improving the fermentation *in vitro*. The half-time of fermentation ($T_{1/2}$) tended to differ among treatments ($p < 0.10$), with T_2 and T_3 recording higher values than the control. The asymptotic gas production (Y_{max}) differed significantly ($p < 0.05$). The highest Y_{max} was observed in T_2 (37.30 mL), whereas the control, T_1 , and T_3 recorded comparable values. The increased fermentation potential in T_2 suggests enhanced microbial utilization of fermentable substrates following supplementation with unprotected vitamins. In contrast, the lower Y_{max} in T_3 indicates that formaldehyde treatment reduced the availability of rapidly fermentable nutrients during ruminal incubation.

The observed reduction in fermentation kinetics following formaldehyde treatment is consistent with the protective action of formaldehyde on protein. Formaldehyde forms reversible cross-links between amino groups of proteins, thereby reducing their susceptibility to microbial degradation in the rumen while allowing digestion under the acidic conditions of the abomasum and small intestine (Ricke *et al.*, 2019) [5]. The increased lag time and reduced gas production in T_3 therefore suggest successful protection of the carrier protein rather than inhibition of rumen microbial activity.

3.4 Effect of Blood meal -Based Protected B-Complex Vitamins on *In vitro* Fermentation Kinetics

Total gas production differed significantly ($p < 0.01$) among the treatments. Supplementation with blood meal alone (T_1)

slightly reduced total gas production (36.25 mL) compared with the control (37.25 mL). However, supplementation with unprotected B-complex vitamins (T_2) significantly increased total gas production to 39.97 mL, while the formaldehyde-treated blood meal containing protected B-complex vitamins (T_3) produced 39.56 mL, which was comparable to T_2 . Schor and Gagliostro (2001) [14] found that after 48h of *in situ* incubation of BM and soybean meal (SBM), CP degradability was lower (52%) with BM compared to SBM (98%) besides higher fermentable CP and rate of disappearance. BM is naturally having high RUP content. The addition of B-complex vitamins to BM increased fermentation and even protecting both with HCHO treatment did not curtail the fermentation. BM was found less useful to protect B-complex vitamins than FM, casein, and CSC.

4. Conclusion

The present study demonstrated that the different protein carriers influenced the *in vitro* fermentation characteristics and digestibility of B-complex vitamin preparations to varying degrees following formaldehyde treatment. Among the carrier proteins evaluated, cottonseed meal was identified as the most suitable carrier for protecting B-complex vitamins compared to fish meal, casein and blood meal because it provided effective ruminal protection while maintaining acceptable fermentation characteristics. Further studies involving direct quantification of ruminal degradation, intestinal release, bioavailability, and *in vivo* animal responses are required to validate the efficiency of cottonseed meal in protecting and releasing B-complex vitamins under practical feeding conditions.

Table 1: *In vitro* Gas Production Kinetics and Digestibility of Substrate Supplemented with Fish Meal-Based Protected B-Complex Vitamins

Treatment	Total gas (ml)	Lag Ferm (hr)	$T_{1/2}$ (hr)	Rate constant (%/hr)		Y max (ml)	Ferm Rate (%/hr)	In vitro (%)	
				k1	k2			DMD	TVFA
Substrate (CG)	39.36 ^b	0.32 ^b	17.36 ^b	4.02 ^a	8.78 ^a	38.21 ^b	5.08 ^a	74.23 ^a	17.81
CG+FM (T_1)	35.26 ^a	0.29 ^a	15.43 ^a	4.64 ^b	9.91 ^b	34.57 ^a	5.93 ^b	75.26 ^b	18.30
T_1 +Vit.B (T_2)	42.51 ^{bc}	0.29 ^a	15.91 ^{ab}	4.37 ^{ab}	9.64 ^b	41.63 ^{bc}	5.58 ^{ab}	75.14 ^b	18.50
T_2 +HCHO (T_3)	44.13 ^c	0.28 ^a	15.73 ^a	4.42 ^{ab}	9.80 ^b	43.26 ^c	5.66 ^{ab}	76.80 ^c	17.98
SEM	1.21	0.01	0.51	0.16	0.27	1.20	0.22	0.22	0.28
P Value	0.001***	0.02*	0.06*	0.08*	0.03*	0.001***	0.07*	0.001***	0.12

Values bearing different alpha superscripts in a column differ significantly: * $p < 0.10$, ** $p < 0.05$, *** $p < 0.01$ and **** $p < 0.001$

Table 2: *In vitro* Gas Production Kinetics and Digestibility of Substrate Supplemented with Casein-Based Protected B-Complex Vitamins

Treatment	Total gas (ml)	Lag Ferm (hr)	$T_{1/2}$ (hr)	Rate constant (%/hr)		Y max (ml)	Ferm Rate (%/hr)	In vitro (%)	
				k1	k2			DMD	TVFA
Substrate (CG)	34.20 ^a	0.37 ^b	19.88 ^b	3.50 ^a	7.63 ^a	32.61 ^a	4.36 ^a	74.95	18.10
Sub+casein (T_1)	37.37 ^b	0.33 ^a	18.68 ^a	3.74 ^b	8.46 ^b	36.00 ^b	4.72 ^b	74.53	18.91
T_1 +Vit.B (T_2)	36.24 ^{ab}	0.34 ^a	18.70 ^a	3.72 ^b	8.32 ^b	34.92 ^{ab}	4.69 ^b	76.57	17.03
T_2 +HCHO (T_3)	36.86 ^b	0.35 ^{ab}	18.81 ^a	3.70 ^b	8.09 ^b	35.46 ^b	4.64 ^b	75.44	17.52
SEM	0.87	0.01	0.29	0.06	0.016	0.82	0.08	0.71	0.46
P Value	0.06*	0.01**	0.01**	0.02*	0.01**	0.03*	0.01**	0.46	0.51

Values bearing different alpha superscripts in a column differ significantly: * $p < 0.10$, ** $p < 0.05$ and *** $p < 0.01$

Table 3: *In vitro* Gas Production Kinetics and Digestibility of Substrate Supplemented with Cotton seed Meal-Based Protected B-Complex Vitamins

Treatment	Total gas (ml)	Lag Ferm (hr)	$T_{1/2}$ (hr)	Rate constant (%/hr)		Y max (ml)	Ferm Rate (%/hr)	In vitro (%)	
				k1	k2			DMD	TVFA
Substrate (CG)	34.87 ^a	0.30 ^a	15.51 ^a	4.61	9.51 ^b	34.12 ^a	5.84	71.32	16.03
CG+CSM (T_1)	34.93 ^a	0.30 ^a	16.01 ^{ab}	4.38	9.38 ^{ab}	34.13 ^a	5.56	71.86	15.36
T_1 +Vit.B (T_2)	38.38 ^b	0.32 ^{ab}	17.23 ^b	4.02	8.77 ^{ab}	37.30 ^b	5.08	72.91	15.80
T_2 +HCHO (T_3)	35.91 ^{ab}	0.33 ^b	17.30 ^b	4.02	8.49 ^a	34.87 ^a	5.04	71.77	16.40
SEM	0.89	0.01	0.53	0.20	0.31	0.82	0.27	0.95	0.59
P Value	0.03*	0.05*	0.06*	0.12	0.08*	0.03*	0.12	0.41	0.29

Values bearing different alpha superscripts in a column differ significantly: * $p < 0.10$, ** $p < 0.05$ and *** $p < 0.01$

Table 4: *In vitro* Gas Production Kinetics and Digestibility of Substrate Supplemented with Blood Meal-Based Protected B-Complex Vitamins

Treatment	Total gas (ml)	Lag Ferm (hr)	T _{1/2} (hr)	Rate constant (%)		Y max (ml)	Ferm Rate (%)	In vitro (%)	
				k1	k2			DMD	TVFA
Substrate (CG)	37.25 ^{ab}	0.28	15.03 ^{ab}	4.63 ^{ab}	10.16 ^{ab}	36.64 ^{ab}	5.59	72.69 ^a	16.32
CG+BM (T ₁)	36.25 ^a	0.29	16.00 ^b	4.38 ^a	9.60 ^a	35.48 ^a	5.33	73.01 ^a	16.10
T ₁ +Vit.B (T ₂)	39.97 ^c	0.28	15.26 ^{ab}	4.55 ^{ab}	10.02 ^{ab}	39.27 ^c	5.60	75.23 ^b	17.30
T ₂ +HCHO (T ₃)	39.56 ^{bc}	0.27	14.66 ^a	4.76 ^b	10.33 ^b	38.94 ^{bc}	5.67	74.10 ^{ab}	17.86
SEM	0.87	0.01	0.36	0.10	0.20	0.86	0.25	0.59	0.80
P Value	0.01**	0.12	0.08*	0.09*	0.09*	0.01**	0.49	0.07*	0.29

Values bearing different alpha superscripts in a column differ significantly: * $p < 0.10$, * $p < 0.05$ and ** $p < 0.01$

Conflict of Interest

Not available

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