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***In vitro* evaluation of rumen-protected b-complex vitamins using a protected fat-protected casein carrier system**

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Abstract

This study evaluated a protected fat-protected casein carrier system for protection of B-complex vitamins under *in vitro* gas production. Treatments included substrate alone (CG), substrate with protected fat alone (T₁), substrate with protected fat and B-complex vitamins (T₂), substrate with protected fat and protected casein (T₃), and substrate with protected fat, protected casein and B-complex vitamins (T₄). Gas production, fermentation kinetics, dry matter digestibility, and total volatile fatty acids were measured. Protected fat alone and protected fat with B-complex vitamins did not affect gas production, whereas T₃ reduced gas production during active fermentation ($p < 0.05$), with the lowest values observed in T₄ ($p < 0.05$). Despite lower gas production, dry matter digestibility and total volatile fatty acid concentration were unchanged among treatments. These results suggest that the protected fat-protected casein matrix reduced ruminal degradation of the carrier system without impairing fermentation.

Keywords: B-complex vitamins, protected casein, protected fat, *In vitro* rumen, fermentation, Gas production kinetics

1. Introduction

B-complex vitamins are essential for energy metabolism, protein synthesis, nucleic acid formation, and many enzyme-mediated processes that support growth, reproduction, immunity, and overall health in ruminants (Duplessis *et al.*, 2014; Sacadura *et al.*, 2008) ^[1, 2]. Although rumen microbes can synthesize these vitamins, the amount produced is often not enough to satisfy the needs of fast-growing, high-yielding, or stressed animals. Moreover, a substantial portion of supplemented B-complex vitamins may be degraded in the rumen before reaching the small intestine, which limits their absorption and biological usefulness (Santschi *et al.*, 2005; Schwab *et al.*, 2006) ^[3, 4]. For this reason, rumen-protected vitamin supplementation has attracted interest as a means of improving vitamin supply and supporting animal performance.

To reduce ruminal degradation while allowing release in the lower gut, several protection methods such as encapsulation and coating with lipid- or protein-based materials have been developed. An ideal carrier should remain stable in the rumen, avoid disturbing normal fermentation, and release the protected nutrient after the rumen. Protected fat is commonly used because it is largely resistant to microbial fermentation (Sergio *et al.*, 2012), while protein-based materials can strengthen the protective matrix; among them, casein is particularly attractive because of its good film-forming and encapsulating properties (Rodriguez *et al.*, 1975) ^[5]. *In vitro* rumen fermentation offers a practical way to test such carrier systems before animal trials, and gas production techniques are widely used to assess fermentation behavior and microbial degradation (Fekri *et al.*, 2025) ^[7]. Therefore, the present study was designed to evaluate a protected fat-protected casein carrier system for B-complex vitamins under *in vitro* rumen conditions by measuring gas production, fermentation kinetics, dry matter digestibility, and total volatile fatty acid production.

2. Materials and methods

2.1 Protection of B complex vitamins

B-complex vitamins were protected using two carrier materials, namely rice bran oil and casein. Rumen-protected rice bran oil (PF) was prepared by converting the free fatty acids into calcium salts. The calcium soap of rice bran oil was synthesized in the laboratory following the double decomposition method (Kumar *et al.*, 2017)^[8]. Rumen-protected casein (PP) was produced by treating the protein with 40% (v/v) formaldehyde at a level equivalent to 1% of its protein content (Aasiwal *et al.*, 2015)^[9]. The protected carrier materials were incorporated into the substrate at a rate of 1 mg per 200 mg, corresponding to a daily supplementation level of 25 g/d of the protected ingredient per animal. Feed-grade B-complex vitamins were procured from M/s Varsha Multi Tech, Bangalore, India, and the vitamin supplementation levels were determined based on the recommendations reported by Ashwin and Srinivas (2019)^[10]. Half of the total B-complex vitamin requirement (50%) was encapsulated using the respective carrier materials and subsequently evaluated using the *in vitro* gas production (IVGP) technique.

2.2 Substrate for *in vitro* study

A total mixed ration (TMR) with a roughage-to-concentrate ratio of 70:30 was used as the substrate for the *in vitro* gas production (IVGP) study. The roughage component comprised 75% ragi straw (RS) as the dry fodder and 25% green fodder, which consisted of para grass, guinea grass, hybrid napier, and maize in a ratio of 3:1:1:1, respectively. The ragi straw and concentrate supplement (CS) were dried in a hot air oven at 90 ± 5 °C until a constant weight was achieved (AOAC 2005; Method No. 934.01). In contrast, the green fodders (para grass, guinea grass, hybrid napier, and maize) were shade-dried at ambient temperature (approximately 32 °C) until constant weight. All dried feed ingredients were then ground to pass through a 1-mm sieve, mixed and stored in airtight plastic containers.

2.3 *In vitro* experimental groups

The *in vitro* experiment was conducted using a completely randomized design comprising five treatment groups, each with 15 replicates. The treatment groups included: CG (substrate alone), T₁ (substrate supplemented with PF), T₂ (substrate supplemented with PF and B-complex vitamins), T₃ (substrate supplemented with PF and PP), and T₄ (substrate supplemented with PF, PP, and B-complex vitamins). The effectiveness of B-complex vitamin protection against ruminal degradation was assessed by comparing *in vitro* gas production, fermentation characteristics, and gas production kinetic parameters across the different treatment groups.

2.4 *In vitro* gas production

Rumen fluid was collected before the morning feeding (08:00 h) from rumen-cannulated cattle maintained on a diet similar to the experimental ration, ensuring a minimum 6 h interval after the last water intake. The rumen liquor was transported to the laboratory in a pre-warmed thermos flask and filtered through a double layer of muslin cloth to obtain strained rumen liquor (SRL). For the *in vitro* gas production (IVGP) assay, 200 mg of substrate was incubated with 10 mL of SRL and 20 mL of buffer medium (Srinivas and Krishnamoorthy, 2005)^[11] under continuous CO₂ flushing to

maintain anaerobic conditions (Menke and Steingass, 1988)^[12]. Gas production was recorded periodically over 72 h incubation after blank correction. At the end of incubation, the filtrate was analyzed for total volatile fatty acids (TVFA) using the Markham distillation method (Barnett and Reid, 1956)^[13], and *in vitro* dry matter digestibility was determined according to Lokesh *et al.* (2017)^[14]. Data were analyzed by one-way ANOVA using SPSS version 19.

3. Results and Discussion

3.1. Effect of protected fat, protected casein and B-complex vitamins on *in vitro* gas production

In vitro gas production profile of the substrate supplemented with protected fat (PF), protected casein (PP) and protected B-complex vitamins is presented in Table 1. Gas production during the initial incubation period (1-2 h) did not differ among treatments. However, significant differences were observed during the active fermentation phase (4-60 h). At 4 h, treatments containing protected casein (T₃ and T₄) produced significantly lower gas than the control and PF-only treatment ($p < 0.05$). At 12 h, T₂ recorded the highest gas production, while T₃ and T₄ remained comparable. From 18 to 60 h, T₄ consistently exhibited the lowest gas production, with significant reductions at 36, 48 and 60 h ($p < 0.05$), whereas no significant differences were observed at 72 h.

The comparable gas production between the control, PF (T₁) and PF supplemented with B-complex vitamins (T₂) indicates that protected fat alone had minimal influence on ruminal fermentation. In contrast, the inclusion of protected casein (T₃) and its combination with protected B-complex vitamins (T₄) reduced gas production during the later stages of incubation, suggesting reduced ruminal fermentability of the protected carrier matrix.

3.2. *In vitro* fermentation kinetics and digestibility of substrates supplemented with protected fat, protected casein and B-complex vitamins

The inclusion of protected fat (PF) alone (T₁) or in combination with B-complex vitamins (T₂) did not significantly alter total gas production compared with the control, indicating that PF exerted minimal influence on ruminal fermentation (Table 2). The addition of Ca salts of fatty acids was more or less inert at ruminal pH and did not affect fiber digestion (Chalupa W, 1975; Jenkins and Palmquist, 1984)^[15, 16]. Sirohi *et al.*, (2001)^[17] also reported that the addition of 7.5% of Ca salts of FA supplementation did not affect digestibilities of OM and DM besides no effect on the rumen fermentation. Treatments containing protected casein (PP) (T₃ and T₄) exhibited lower gas production during the active fermentation phase (4-60 h), with T₄ producing the lowest cumulative gas (31.76 ml) and asymptotic gas yield (31.20 ml) ($p < 0.05$). Furthermore, T₄ showed a shorter lag time and half-life with higher fermentation rate constants than the other treatments, whereas *in vitro* dry matter digestibility and total volatile fatty acid concentration remained unaffected ($p > 0.05$). Rodriguez *et al.* (1975)^[5] found that formaldehyde treatment of casein at 0.25 to 3% reduced the solubility of protein by 10% at pH 6.8 and 30% at pH 2.5. Decrease in gas production while using protein sources (casein) might be due to the production of ammonia during fermentation which neutralizes and lowers gas production (Fekri *et al.*, 2025)^[7].

Protected fat alone acted as a rumen-inert ingredient, the incorporation of protected casein limited microbial access to the carrier matrix, thereby decreasing its fermentability. The further reduction in gas production in T₄ suggests that the protected fat-protected casein matrix effectively entrapped the B-complex vitamins and reduced their exposure to ruminal microbial degradation. Since dry matter digestibility

and total volatile fatty acid concentration were maintained across treatments, the lower gas production is more likely to reflect reduced degradation of the protected nutrient complex rather than impaired ruminal fermentation, indicating that the combination of protected fat and protected casein provided an effective carrier system for improving the ruminal protection of B-complex vitamins.

Table 1: *In vitro* gas production (ml/200 mg DM) of substrate supplemented with protected fat, protected casein and protected B-complex vitamins at different incubation intervals

Treatment	Incubation intervals (hr)												
	0	0.5	1	2	4	8	12	18	24	36	48	60	72
Substrate (CG)	-	-	0.20	0.78	2.74 ^b	4.11	4.90 ^a	6.35 ^c	4.89 ^a	6.26 ^b	2.55 ^b	2.35 ^b	0.98
CG+PF (T ₁)	-	-	0.20	0.58	2.03 ^a	4.91	5.59 ^{ab}	5.48 ^b	4.81 ^a	6.26 ^b	2.50 ^b	2.41 ^b	1.44
T ₁ +Vit.B (T ₂)	-	-	0.10	0.86	2.49 ^{ab}	4.32	6.72 ^c	5.76 ^{bc}	5.61 ^b	5.86 ^b	2.02 ^b	1.92 ^{ab}	1.35
CG+PF+PP (T ₃)	-	-	0.10	0.93	1.76 ^a	4.55	6.45 ^{bc}	4.56 ^a	5.49 ^b	5.68 ^{ab}	1.78 ^{ab}	1.69 ^{ab}	1.12
T ₃ +Vit.B (T ₄)	-	-	0.00	0.75	1.88 ^a	4.77	6.32 ^{bc}	4.58 ^a	4.80 ^a	4.68 ^a	1.12 ^a	1.12 ^a	0.75
SEM	-	-	0.11	0.13	0.24	0.26	0.29	0.29	0.12	0.39	0.26	0.30	0.21
P Value	-	-	0.69	0.43	0.03*	0.20	0.001**	0.001**	0.001**	0.04*	0.001**	0.03*	0.14

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

Table 2: *In vitro* fermentation kinetics and digestibility of substrate with protected fat, protected casein and 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)	36.12 ^b	0.35 ^b	17.58 ^b	3.95 ^a	8.11 ^a	34.97 ^b	4.92 ^a	69.58	15.91
CG+PF (T ₁)	36.26 ^b	0.33 ^b	17.62 ^b	3.94 ^a	8.42 ^a	35.15 ^b	4.95 ^a	69.85	15.46
T ₁ +Vit.B (T ₂)	36.27 ^b	0.31 ^{ab}	16.18 ^{ab}	4.40 ^{ab}	9.20 ^{ab}	35.40 ^b	5.57 ^{ab}	70.28	14.96
CG+PF+PP (T ₃)	34.06 ^{ab}	0.30 ^{ab}	15.79 ^{ab}	4.56 ^{ab}	9.46 ^{ab}	33.23 ^{ab}	5.79 ^{ab}	69.19	15.01
T ₃ +Vit.B (T ₄)	31.76 ^a	0.28 ^a	14.22 ^a	5.09 ^b	10.32 ^b	31.20 ^a	6.50 ^b	68.34	15.25
SEM	1.24	0.02	0.75	0.27	0.47	1.18	0.37	0.68	0.32
P Value	0.05*	0.03*	0.02*	0.03*	0.02*	0.08*	0.03*	0.62	0.48

Values bearing different alpha * $p < 0.05$ and ** $p < 0.01$

4. Conclusion

The incorporation of protected casein along with protected fat reduced ruminal fermentation of the carrier matrix, as reflected by lower gas production, while maintaining dry matter digestibility and TVFA concentration. These findings suggest that the protection strategy effectively reduced ruminal degradation of the fat, casein-based vitamin carrier without adversely affecting overall fermentation. However, further *in vivo* studies are warranted to validate the protective efficiency of the carrier system and its practical application in ruminant feeding.

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