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Optimized oral supplementation of vitamins improves feed intake and rumen microbial protein synthesis in Deoni cows

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Abstract: The present study was conducted to optimize oral doses of fat- and water-soluble vitamins in cattle using in vitro gas production model and to ascertain their effects on dry matter intake (DMI), nutrient digestibility, and rumen microbial protein (RMBP) synthesis. Doses optimized using the in vitro model were 3.66, 6.78, 16.3, 41.1, 3.87, 0.323, 4.12, and 0.055 mg/kg DMI, respectively, for thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folic acid, and B_{12} and 500 IU/kg DMI for vitamin K. Thirty-two adult, nonproducing Deoni cows (375 ± 13.4 kg body weight, BW) were randomly divided into four equal groups (n = 8) and fed a basal diet (CON) or diets supplemented with in vitro optimized doses of water-soluble (WS), fat-soluble (FS), or both (WSFS) vitamins. The DMI values in WS and WSFS were higher than that of FS (92.6 and 96.17 vs. 82.85 g/kg $BW^{0.75}$; P = 0.048) but comparable to CON (90.34 g/kg $BW^{0.75}$). The purine derivative creatinine index was significantly higher in the WSFS group compared to CON (270.3 vs. 224.6; P = 0.035). Oral supplementation of optimized doses of vitamins as derived from in vitro trials improved DMI and RMBP production in Deoni cows.

Key words: Cattle, digestibility, in vitro fermentation, purine derivative

1. Introduction

Oral supplementation of vitamins and particularly B vitamins to ruminants is conventionally ignored owing to the general misconception that they are synthesized in the rumen in adequate quantities by rumen microbes. The traditional concept was challenged by Scott and Dehority (1) when they reported that, apart from the ruminant, rumen microbes also have specific requirements for certain B vitamins. Recent studies in this regard indicated that major cellulolytic organisms in the rumen including bacteria like *Ruminococcus* species and *Bacteroides* species and anaerobic fungi like *Neocallimastix* have specific requirements for thiamine, riboflavin, niacin, pyridoxine, biotin, folic acid, and B_{12} (2–4). Rumen microbes play a pivotal role in the digestion of nutrients in the rumen. They utilize low-quality feed ingredients and convert it to high-quality microbial proteins to fulfill the nutritional requirements of the ruminants. In the present scenario, there is an acute shortage of good-quality feeds for feeding of ruminants, especially in the tropics. Measures to improve the efficiency of feed utilization are the need of the hour.

However, the oral dose of vitamins for ruminants is not optimized. The National Research Council (NRC) extrapolated the B vitamin requirements of dairy cattle based on the tissue level requirements of a lactating pig (5). The vitamin requirements suggested by the NRC (5) need to be tested and validated in the rumen or an environment simulating rumen. Hence, the present study aimed to optimize the oral dose of each vitamin for efficient microbial fermentation and to evaluate its impact on feed intake, digestibility, and rumen microbial protein (RMBP) synthesis by taking the NRC recommendations as the reference.

2. Materials and methods

2.1. In vitro gas production model for dose optimization

An in vitro gas production (IVGP) technique (6) was used to optimize the dose of each vitamin. The basal substrate for the in vitro study consisted of ground finger millet straw (*Eleusine coracana*) and concentrate feed in a 70:30 ratio. Ingredient composition of the concentrate feed used in the study is reported in Table 1. Individual feed-grade vitamins were purchased from a commercial source (Varsha Group, Bengaluru, India). The rumen fluid was taken before the morning feeding from a rumen-cannulated, nonlactating, nonpregnant Holstein cow

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Table 1. Ingredient composition of concentrate mixture used in in vitro and in vivo studies.

Ingredients	Inclusion percentage
Maize	25
Molasses	15
Deoiled rice bran	25
Cotton seed cake	12
Soya bean meal	11
Deoiled copra cake	9
Mineral mixture	2
Common salt	1

(about 400 kg BW) fed on 1.5 kg of concentrate feed and ad libitum finger millet straw. It was collected in a prewarmed insulated flask, homogenized using a laboratory blender, and strained through four layers of nylon cloth having a pore size of 100 μm to produce strained rumen liquor. All handling was carried out with continuous flushing by CO_2. The well-mixed and CO_2-flushed rumen fluid was added to the buffered mineral solution (7), which was maintained in a water bath at 39 °C, and mixed. Buffered rumen fluid (30 mL) was dispensed into each syringe containing 200 mg of basal substrate. Ten syringes were used for each treatment and the control (CON). Extrapolated NRC (5) recommendations of vitamins for dairy cows were used as a guideline for deciding doses to be tested. Four doses of each vitamin, X, 2X, 3X, and 5X, were tested, in which X represented the NRC recommendation and 2X, 3X, and 5X represented two, three, and five times the NRC recommendations, respectively. They were compared with CON, in which vitamins were not supplemented. The required dose of each water-soluble vitamin (WS) was reconstituted in 1 mL of distilled water and added to the syringe containing substrate and inoculum. Fat-soluble vitamins (FS) were dissolved in 1 mL of edible oil. The CON syringes were inoculated with either 1 mL of distilled water or edible oil for correction of volume. The syringes were incubated at 39 ± 1 °C in an incubator for 72 h.

In vitro total gas production (TGP) in the syringes was recorded for up to 72 h of incubation. In vitro total volatile fatty acid (TVFA) production in the fermented inoculum was measured using a Markham apparatus with a slight modification of the procedure given by Barnett and Reid (8). Two milliliters of fermented inoculum and 2 mL of oxalate buffer (10% potassium oxalate and 5% oxalic acid in 1:1 ratio) were pipetted into a Markham distillation assembly and distilled. A few drops of phenolphthalein were added to 100 mL of distilled fluid collected in an ice bath. It was titrated against 10 mmol/L NaOH and the development of a pink color was recorded as the endpoint. The TVFA (mmol/100 mL fermented inoculum) was calculated as TVFA = (volume of NaOH used × strength of NaOH × 100) / (volume of fermented inoculum taken). The dose of each vitamin for in vivo study was optimized based on in vitro TGP and TVFA production. Accordingly, two types of vitamin mixtures, a WS mixture and FS mixture, were formulated.

2.2. Animals, experimental design, and diets

The study was carried out at the Deoni Conservation Farm of the Southern Regional Station-National Dairy Research Institute, Bengaluru, India (12°56′37.9752″N, 77°36′33.1992″E) from February to December 2015. Thirty-two adult, nonproducing, multiparous Deoni cows (375 ± 13.4 kg of body weight, BW) were used in the study. A completely randomized design was used with four treatments having eight animals in each treatment. The animals were housed in a head-to-head system and fed according to the Indian Council of Agriculture (ICAR) guidelines (9) with a basal diet consisting of finger millet straw and concentrate supplement in 70:30 ratios. The treatments were as follows: basal diet with no vitamin supplements (CON), or supplemented with the optimized dose of water-soluble vitamins (WS), fat-soluble vitamins (FS), or both (WSFS). All the animals were dewormed with fenbendazole before starting the experiment.

2.3. Experimental protocol and sampling

Experimental feeding was carried out for a period of 90 days. Animals were weighed every 15 days in the morning before offering feed and feeding was adjusted according to BW.

The feed was weighed individually and offered to cows twice daily at 0900 and 1700 hours. Drinking water was provided ad libitum. After 90 days of experimental feeding, a 7-day digestion trial was carried out to evaluate feed intake, digestibility, and RMBP synthesis in the rumen. All 32 animals were used in the digestibility trial. Sampling was done daily at 0900 hours. Whole feces voided by animals daily were collected individually. From each animal, 100 g of feed offered, feed residue, and well-mixed feces were collected and were dried in a hot air oven overnight at 95 to 100 °C on the same day. The samples collected during the digestibility trial from each animal during the 7-day digestibility trial were pooled in separate bags for further analysis. Five hundred milligrams of the well-mixed fecal sample from individual animals was pooled daily in bottles containing 3.5 mL of 10% H_2SO_4 for estimation of the crude protein content of feces. Spot urine samples were collected three times daily at 0800, 1430, and 1800 hours during the 7-day digestibility trial. Collected urine samples in plastic bottles containing 20% H_2SO_4 were transported to the laboratory and stored at –20 °C for evaluation of the purine derivative creatinine

(PDC) index. Spot urine samples for 7 consecutive days were pooled for individual animals and mixed well, and aliquots were taken for estimation of purine derivatives and creatinine.

2.4. Chemical analyses

Finger millet straw, concentrate, and fecal samples were analyzed for dry matter (DM), crude protein (CP), ether extract (EE), total ash (TA), neutral detergent fiber (NDF), and acid detergent fiber (ADF) according to the procedure given by the Association of Official Analytical Chemists (10). The organic matter (OM) was calculated by subtracting total ash from dry matter.

Allantoin, creatinine, and uric acid in the spot urine sample were quantified using high-performance liquid chromatography (HPLC) as described by George et al. (11). Standard solutions of allantoin (Sigma), creatinine (Nice, India), and uric acid (Nice, India), each containing 50 µg/mL (w/v) of respective standards, were separately prepared in HPLC-grade water. HPLC (Model 2489, Waters India Pvt. Ltd.) was run in isocratic conditions using a C-18 reverse-phase column (Waters India Pvt. Ltd., 4.5 × 260 mm, 5 µm) with 10 mmol/L potassium dihydrogen phosphate (pH 4.7) as the mobile phase. Flow rate was fixed at 1 mL/min and the reading was taken at 220 nm. Peaks were standardized for allantoin, uric acid, and creatinine by injecting 20 µL of the respective standard. Urine samples stored at –20 °C during the digestibility trial were thawed, pooled individually, and mixed thoroughly and aliquots of 20 mL were taken. It was filtered through a Millipore filter of 0.2 µm pore size. One milliliter of the filtrate was taken and the volume was made up to 10 mL with HPLC-grade water after adjusting the pH to 7.0 using 10 mmol/L NaOH and 10 mmol/L HCl. The concentration of allantoin, creatinine, and uric acid was calculated by comparing the areas of their peaks with respective standards. Total purine derivative (PD, mmol/L) excreted in the urine was calculated as the sum of allantoin and uric acid in the urine. The PDC index was calculated by dividing total PD in urine by creatinine (mmol/L) in urine and multiplying the result with metabolic body weight ($BW^{0.75}$) (12).

2.5. Statistical analysis

The data are presented as means with standard errors of means (SEMs) for all parameters. Statistical analysis was carried out by one-way analysis of variance using SPSS 20 (13). Post hoc comparisons were performed using Duncan's test. The difference between means was considered statistically significant at 5% level of probability ($\alpha \leq 0.05$).

3. Results

3.1. In vitro dose optimization

The nutrient composition of finger millet straw and concentrate feed is reported in Table 2. The in vitro TGP and TVFA production from substrate fortified with four different doses of each vitamin is reported in Table 3. The in vitro TGP and TVFA were considered as the markers of fermentation and the best dose of each vitamin was decided based on these two attributes. There was a significant increase ($P < 0.01$) in fermentation when substrates were fortified with double (2X) the recommended NRC dose of thiamine, pantothenic acid, pyridoxine, folic acid, and B_{12}. Further increase in the dose did not produce a significant simultaneous improvement in fermentation attributes. For riboflavin, niacin, and biotin, the recommended NRC dose (X) was found to be optimum for optimal microbial fermentation. Although TGP with biotin was highest at 3X, TVFA production remained best at X. Hence, X was chosen as the best dose. Data on the requirement of vitamin C in ruminants were not given by the NRC (5). The doses of vitamin C tested in the present study were 0.5, 1, 1.5, and 2.5 mg/kg DMI. No improvement in fermentation was observed with vitamin C supplementation in the studied range. With FS, vitamin A did not affect in vitro fermentation. Results with vitamin D were inconsistent. Higher TGP with the 3X dose (3600 IU/kg DMI) of vitamin D did not produce a simultaneous increase in TVFA production and thus the result was not conclusive. Vitamin E supplementation negatively impacted fermentation

Table 2. Nutrient composition (g/kg DM) of concentrate feed and finger millet (*Eleusine coracana*) straw.

	Chemical composition*					
	DM	CP	EE	TA	NDF	ADF
Concentrate feed	888	168	34	88	226	92
Finger millet straw	935	54	13	90	713	396

All the observations were averages of 4 measurements. *ADF = Acid detergent fiber corrected for ash and crude protein, CP = crude protein, DM = dry matter, EE = ether extract, NDF = neutral detergent fiber corrected for ash and crude protein, TA = total ash.

Table 3. In vitro total gas production (TGP, mL/g) and total volatile fatty acid production (TVFA, mmol/g) from substrate (concentrate: roughage = 30:70) with or without supplementation of four different doses of each vitamin.

Attributes	Treatment groups*					SEM	P-value
	CON	X	2X	3X	5X		
TGP (mL/g)							
Thiamin	171.9^{a}	191.2^{b}	191.8^{b}	192.7^{b}	179.3^{ab}	5.47	0.008
Riboflavin	149.1^{a}	161.4^{b}	150.8^{a}	159.1^{b}	147.6^{a}	2.14	<0.001
Niacin	155.3^{a}	171.0^{c}	161.8^{b}	160.9^{ab}	160.4^{ab}	2.06	<0.001
Pantothenic acid	197.8^{a}	200.4^{a}	223.8^{b}	226.3^{b}	230.5^{b}	2.47	<0.001
Pyridoxine	168.0^{b}	182.7^{c}	188.2^{c}	169.3^{b}	142.4^{a}	2.15	<0.001
Biotin	163.7^{a}	176.2^{bc}	174.5^{b}	187.6^{d}	182.1^{c}	2.37	<0.001
Folic acid	189.7^{b}	181.0^{a}	208.3^{d}	202.3^{c}	209.4^{d}	1.98	<0.001
B_{12}	181.2^{a}	193.6^{b}	200.2^{b}	196.5^{b}	194.3^{b}	2.67	<0.001
C	194.8^{b}	198.4^{b}	190.5^{ab}	194.9^{b}	185.8^{a}	2.67	0.028
A	237.2^{b}	217.4^{a}	224.0^{a}	226.0^{ab}	225.5^{ab}	3.98	0.029
D	198.0^{c}	177.2^{b}	193.8^{c}	207.4^{d}	168.8^{a}	2.61	<0.001
E	154.8^{c}	143.9^{ab}	149.7^{bc}	136.3^{a}	140.8^{ab}	2.95	<0.001
K	165.4^{a}	186.8^{b}	206.4^{c}	192.7^{b}	196.0^{bc}	3.68	<0.001
TVFA (mmol/g)							
Thiamin	85.00^{a}	86.67^{a}	96.67^{b}	96.67^{b}	101.6^{b}	1.97	<0.001
Riboflavin	75.00^{c}	60.83^{a}	69.17^{b}	73.33^{c}	74.17^{c}	0.98	<0.001
Niacin	70.00^{b}	71.67^{b}	70.00^{b}	62.50^{a}	62.50^{a}	1.17	<0.001
Pantothenic acid	95.00^{b}	90.00^{a}	110.0^{d}	99.17^{c}	86.67^{a}	1.23	<0.001
Pyridoxine	70.00^{a}	69.17^{a}	77.50^{c}	71.67^{ab}	75.00^{b}	1.23	0.004
Biotin	81.67^{a}	100.8^{d}	88.33^{b}	84.17^{a}	93.33^{c}	0.83	<0.001
Folic acid	74.17^{a}	81.67^{b}	84.17^{b}	76.67^{a}	75.00^{a}	0.98	<0.001
B_{12}	88.17^{a}	88.33^{a}	95.83^{b}	95.83^{b}	113.3^{c}	0.83	<0.001
C	136.6^{b}	123.3^{a}	126.6^{a}	126.6^{a}	138.3^{b}	1.05	<0.001
A	99.17	100.0	103.3	102.5	99.17	1.70	0.331
D	96.67	99.17	97.50	94.17	94.17	1.17	0.053
E	93.33^{d}	85.00^{b}	88.33^{c}	76.67^{a}	88.33^{c}	0.98	<0.001
K	91.67^{a}	98.33^{b}	101.6^{bc}	104.1^{c}	101.6^{bc}	1.05	<0.001

*CON = Control (only basal substrate); X, 2X, 3X, and 5X represent basal substrate supplemented with one, two, three, and five times the NRC recommended dose of each vitamin, respectively.

^{abcd} Means with different superscripts in a row differ significantly according to Duncan's test ($P < 0.05$).

parameters. Vitamin K supplementation produced the best result at a 2X dose (500 IU/kg DMI; $P < 0.001$). The best dose of each vitamin along with the NRC recommendation is reported in Table 4.

3.2. In vivo supplementation

The composition of WS and FS mixtures used in the animal trial is reported in Table 5. The feed intake (expressed in kg/day and g/$BW^{0.75}$) and digestibility data are reported in Table 6. The absolute DMI (kg/day) did not vary significantly between groups. The DMI (expressed in g/$BW^{0.75}$) in WS and WSFS was significantly ($P = 0.048$) higher than in FS and comparable to CON. No significant ($P > 0.05$) improvement in nutrient digestibility was observed with oral vitamin supplementation. The metabolic body weight, total PD, creatinine, and PDC index in urine are presented in Table 7. The PDC index was highest in WSFS (332.8; $P < 0.001$), followed by WS (292.1), while it was comparable between FS and CON (232.5 and 247.4, respectively).

Table 4. The optimum oral dose of vitamins for cattle along with respective NRC recommendations (5).

	NRC (2001)	Optimized dose*
Dose (mg/kg DMI)		
Thiamin	1.83	3.66
Riboflavin	6.78	6.78
Niacin	16.3	16.3
Pantothenic acid	20.6	41.1
Pyridoxine	1.94	3.87
Biotin	0.323	0.323
Folic acid	2.06	4.12
B_{12}	0.028	0.055
C	0	0
Dose (IU/kg DMI)		
A	4400	0
D	1200	0
E	20	0
K	0	500

*Doses were optimized from in vitro total gas production (TGP) and total volatile fatty acid (TVFA) data from substrate after fortifying it with test doses of different vitamins.

Table 5. Composition of water-soluble (WS) and fat-soluble (FS) vitamin mixture (g/100 g) used for experimental feeding of cows.

Vitamin	WS	FS
Thiamin	4.802	0
Riboflavin	8.897	0
Niacin	21.39	0
Pantothenic acid	53.93	0
Pyridoxine	5.078	0
Biotin	0.424	0
Folic acid	5.406	0
B_{12}	0.072	0
K	0	100

4. Discussion

The present study was done to investigate the effect of oral supplementation of vitamins on rumen microbes. This was done in two phases: an in vitro study to optimize the dose of each vitamin, followed by an in vivo feeding experiment in nonproducing Deoni cows to validate the in vitro findings. The IVGP model was selected for in vitro studies as it was widely used to evaluate nutritive values of different kinds of feeds. In this model, the effect of treatments in the rumen fermentation is reflected in the amount of gas production in the syringes (14). The TGP and TVFA productions within the syringes were considered as markers to assess the fermentation.

In the present in vitro study, fortification of the basal substrate with double the recommended NRC dose of thiamine significantly improved the TGP and TVFA production. Major cellulolytic bacteria like *Ruminococcus albus* and *Ruminococcus flavefaciens* require thiamine for optimal proliferation (2). Orpin and Greenwood (15) reported that anaerobic fungi in the rumen like *Neocallimastix* have an obligate requirement for thiamine. As the anaerobic fungi in the rumen are involved in the processing of low-quality forages for other microbes, a promoting action of thiamine on rumen anaerobic fungi could be considered as the major possible underlying mechanism.

Literature on riboflavin requirements for rumen microbes is scarce. The majority of the rumen bacteria, except *Ruminococcus albus* strain $B_3$37, did not elicit higher growth responses with riboflavin supplementation (2). In the present study, TGP production with riboflavin was highest with the recommended NRC dose (5) at 6.78 mg/kg DMI. The respective TVFA production data lacked a dose-dependent trend. The present study failed to establish the accurate riboflavin requirement for rumen microbes and, hence, based on the TGP, the NRC recommendation (5) for riboflavin was considered during in vivo vitamin mixture formulation.

MacLeod and Murray (16) reported a stimulatory effect of niacin irrespective of strains on *Ruminococcus* growth in vitro. Higher doses of niacin were hypothesized to produce a significant impact on microbial fermentative activity as it formed an integral component of the coenzymes nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate involved in energy metabolism. However, in the present in vitro study, the optimum dose was observed to be the recommended NRC dose (5) of 16.3 mg/kg DMI. A dose of niacin up to 81.5 mg/kg DMI was tested and found to reduce TGP and TVFA production. The observed optimum dose of niacin in the current study was lower than 400 mg/kg DMI, as concluded by Samanta et al. (17).

Major cellulolytic organisms like *Butyrivibrio fibrisolvens*, *Bacteroides succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* required biotin and pyridoxine in the culture medium for their optimal activity (2). Cruyvagen and Bunge (18) observed higher fermentability and NDF digestibility of roughages in in vitro studies conducted using rumen liquor from cows that were supplemented with 40 mg of biotin for 21 days

Table 6. Intake and digestibility of nutrients in cows supplemented with fat- and/or water-soluble vitamins.

Attributes	Dietary groups*				SEM	P-value
	CON	WS	FS	WSFS		
Intake						
DM, kg	7.94	8.43	6.83	7.72	0.30	0.301
OM, kg	7.22	7.67	6.21	7.02	0.27	0.301
CP, g	665.3	712.5	583.9	638.7	25.17	0.361
EE, g	157.9	168.9	138.3	151.8	5.96	0.352
TA, g	715.5	759.5	614.8	695.9	26.83	0.303
NDF, kg	4.52	4.76	3.82	4.43	0.17	0.260
ADF, kg	2.43	2.56	2.05	2.39	0.09	0.254
Intake (g/kg $BW^{0.75}$)						
DM	90.34^{ab}	92.60^{b}	82.85^{a}	96.17^{b}	2.99	0.048
OM	82.20^{ab}	84.26^{b}	75.39^{a}	87.50^{b}	2.72	0.048
CP	7.57	7.82	7.10	7.95	0.23	0.093
EE	1.80	1.85	1.68	1.89	0.05	0.082
TA	8.14^{ab}	8.34^{b}	7.46^{a}	8.67^{b}	0.27	0.048
NDF	51.22^{ab}	52.50^{ab}	46.72^{a}	55.19^{b}	1.18	0.043
ADF	27.58^{ab}	28.24^{ab}	25.09^{a}	29.76^{b}	0.65	0.042
Digestibility (%)						
DM	60.17	61.53	64.65	60.80	2.97	0.723
OM	62.18	63.65	66.89	62.80	2.93	0.681
CP	57.21	58.72	57.54	58.83	3.64	0.984
EE	70.03	71.37	64.85	70.19	2.86	0.415
TA	39.86	40.12	42.09	40.66	3.68	0.973
NDF	60.61	63.10	67.31	61.52	1.54	0.461
ADF	57.33	57.82	63.06	57.62	1.86	0.701

[ab] Means with different superscripts in a row differ significantly according to Duncan's test ($P < 0.05$).
*CP = Crude protein, DM = dry matter, EE = ether extract, OM = organic matter, TA = total ash, NDF = neutral detergent fiber, ADF = acid detergent fiber, CON = control, WS = supplemented with water-soluble vitamins, FS = supplemented with fat-soluble vitamins, WSFS = supplemented with both water- and fat-soluble vitamins.

(equivalent to 2.67 mg/kg DMI). Lebzien et al. (19) reported that the amount of biotin available in the duodenum of cattle was not dependent on the diet composition or oral biotin supplementation, but on the total RMBP synthesis and fermented OM. In the present study, the effect of different doses of biotin ranging from 0.323 to 1.615 mg/kg DMI was tested. There was a significant increase in TGP and TVFA. Doses above the NRC recommendation (5) did not produce a dose-dependent trend and hence the NRC recommendation was considered optimum. Unlike other vitamins, pyridoxine stimulated the growth of all cellulolytic organisms (16). In the present study, the 2X dose (3.87 mg/kg DMI) stimulated optimal TGP and TVFA production compared to higher doses and therefore it was selected for the in vivo feeding trial.

In the present study, pantothenic acid supplementation promoted microbial fermentation in vitro, with maximum fermentative activity at 41.1 mg/kg DMI. The findings were contrary to an earlier report that suggested its immediate and extensive destruction in the rumen without being available to microbes for their metabolism (20).

Butyrivibrio fibrisolvens had an absolute requirement for folic acid while *Ruminococcus flavifaciens* required only folic acid for its growth (2). Chiquette et al. (21) could not observe any significant effect of folic acid supplementation on rumen fermentation in steers fed either high forage or high concentrate. Vitamin B_{12} stimulated the growth of cellulolytic organisms when it was supplemented to pure cultures of organisms (22). Accordingly, in the present in vitro study, there was a significant increase in TGP by 10%

Table 7. Purine derivative creatinine index in cows supplemented with fat- and/or water-soluble vitamins

Attributes	Dietary groups*				SEM	P-value
	CON	WS	FS	WSFS		
$BW^{0.75}$	88.17	90.76	81.85	80.29	5.17	0.455
Total PD, mmol/L	8.20	8.86	6.98	10.49	0.92	0.109
Creatinine, mmol/L	2.96	2.75	2.45	2.50	0.27	0.553
PDC index	247.4[a]	292.1[b]	232.5[a]	332.8[c]	11.60	<0.001

[ab] Means with different superscripts in a row differ significantly according to Duncan's test ($P < 0.05$).
*$BW^{0.75}$ = Metabolic body weight; PD = purine derivative; PDC = purine derivative creatinine, CON = control, WS = supplemented with water-soluble vitamins, FS = supplemented with fat-soluble vitamins, WSFS = supplemented with both water- and fat-soluble vitamins.

when the diet was fortified with folic acid and B_{12} (4.12 and 0.055 mg/kg DMI, respectively). Supplementation of vitamin C up to 2.5 mg/kg DMI did not improve rumen microbial fermentation, contrary to an earlier observation (23). Bryant and Robinson (2) reported that a mixed culture of normal rumen microbes showed much smaller growth effect upon addition or deletion of vitamins as compared with pure cultures.

The present in vitro study failed to correlate microbial fermentative metabolism with higher oral doses of FS, except for vitamin Hymøller and Jensen (24) infused vitamin D as ergocalciferol and cholecalciferol directly into the rumen of fistulated cows to study its utilization in the rumen. There was no disappearance of vitamin D in the rumen of high-producing dairy cows and hence they concluded that vitamin D was not essential for optimum microbial metabolism. Similarly, in the present study, there was no improvement in fermentation with vitamin D supplementation. Weiss et al. (25) observed that only a minimum quantity of vitamin E was metabolized in the rumen and the major portion of orally supplemented vitamin E was available for absorption in the lower gastrointestinal tract. In an earlier study, there was a reduction in microbial fermentative activity when high fiber diets were fortified with higher doses of vitamin E (23). The present in vitro study also underlined the inhibitory effect of vitamin E on microbial fermentation. Vitamin K_2 is produced in the rumen from the fermentative activity of the rumen microbes. Studies correlating microbial metabolism with vitamin K are scarce. In the present in vitro study, four different doses of vitamin K from 250 to 1250 IU/kg DMI were tested. Optimum fermentative activity was observed at 500 IU/kg DMI.

Schwab et al. (26) reported an increased ruminal synthesis of pyridoxine, folic acid, and B_{12} with a higher proportion of forage and niacin, pyridoxine, and B_{12} with a higher proportion of nonfiber carbohydrates in the ration. They also reported that, across diets, ruminal synthesis of niacin was highest, followed by riboflavin, B_{12}, thiamine, pyridoxine, and folic acid. Diet did not influence ruminal biotin synthesis in their study. As green roughage is considered as the major source of vitamins for ruminants, it was not considered for diet formulation in the present study. Concentrate feed was supplemented in order to fulfill the nutrient requirements of the animals. However, the present study failed to quantify the amount of individual vitamins present in the concentrate feed.

Wolin et al. (27) opined that B-vitamin "cross-feeding" occurs between ruminal microbes; B vitamins produced by one species may be required for the growth of others. Each microbe has its own specific vitamin requirements. As there was no ruminal absorption of B vitamins (26), oral supplementation of critical vitamins could have improved microbial proliferation as evidenced by significant improvement in the PDC index in the WSFS group. Dipu et al. (28) reported that the PDC index was relatively constant and did not vary significantly with the time of collection of urine, and they endorsed the use of the PDC index as a measure of microbial protein supply. They reported that mean PDC index from daily multiple spot urine samples can better correlate with RMBP than that estimated from an inappropriate quantity of urine samples collected over many days. They also opined that the PDC index from spot urine samples could only detect large variations in RMBP supply, particularly when animals are on a low plane of nutrition. In our present study, although we simulated field-level feeding patterns by withholding green forage, nutrient requirements were met by supplementing concentrates. Even in this circumstance, the PDC index from spot urine samples improved significantly with oral vitamin supplementation. The present findings are therefore significant in tropical conditions like those in India, where a majority of animals do not receive good quality forage. The range of PDC index reported in Yerli Kara crossbreed cattle by Cetinkaya et al. (29) varied with the level of feeding and ranged from 32 to 62 when animals

were fed at 40% to 95% levels. Dipu et al. (28) reported a PDC index from 17.41 to 30.47 in buffalos fed from 40% to 95% levels. In the present study, feeding was done to meet the nutrient requirement of animals according to the ICAR (9) and the PDC index estimated ranged from 232 to 332 in different treatment groups, which was five to ten times higher than earlier reported values. This could probably be attributed to the level of feeding, feed composition, and characteristics of the breed used in the study. Dipu et al. (28) reported comparable RMBP when calculated using the whole urine collection method and spot urine samples. Nolan and Kahn (30) reported that the microbial biomass produced in the rumen is the major source of nutrients for the host animals, especially when they are fed with roughages. They reported that low RMBP synthesis in the rumen indicates deficiency of nutrients required for microbial proliferation within the rumen. Higher RMBP observed in the present study with vitamin supplementation may implicate improved nutritional status of the animals. As ruminal B vitamin synthesis improved with high forage diet (26), feeding with a whole roughage-based diet could have given a more conclusive idea. In another observation, B-vitamin concentrations were 10- to 5000-fold higher within the rumen bacteria than in particle-free supernatant (31). Hence, a higher RMBP could improve B-vitamin availability for the host ruminant. In the present study, variation in blood vitamin levels with oral supplementation was not assessed.

In the present study, there was a significant improvement in DMI in the WS and WSFS groups as compared to FS when expressed in terms of metabolic body weight. However, absolute DMI (kg/day) did not vary between the dietary groups. As B-vitamin concentrations within the microbial cells were a thousand-fold higher than rumen supernatant (31), they could be considered as 'vitamin capsules' for the host animal. Studies correlating B vitamin supplementation with DMI are scarce. Among B vitamins, niacin was synthesized in the highest quantity in the rumen when cows were fed with high forage diet (26). Niacin induced flushing (increased blood flow to the skin) that reduced core body temperature and thereby heat stress in animals of tropics (32). Reduction in heat stress is always accompanied by improved feed intake. This could be considered as one of the potential mechanisms behind higher DMI when B vitamins are supplemented in the diet, as the present study was conducted in hot tropical conditions. Earlier reports indicated a higher proliferation of cellulolytic bacteria (2,16,18) and anaerobic fungi (15) and higher rumen fermentability and NDF digestibility (18,20) with various B vitamin supplementation. We could not observe changes in nutrient digestibility in vivo when we supplemented an optimized vitamin mixture orally for Deoni cows.

To our knowledge, no research has been conducted investigating the relationship between dietary supplementation of FS and changes in rumen microbial populations. Chiofalo et al. (33) observed improvement in the shelf-life of milk with dietary vitamin E in ruminants and it was attributed to its antioxidant activity. As the primary aim of the present study was to improve rumen microbial fermentation, and vitamin E was detrimental to fermentative activity, as observed in the in vitro study, it was not used in the in vivo trial. In the present study, although vitamin K improved ruminal fermentation in vitro, it did not influence DMI, RMBP, or nutrient digestibility in vivo.

In conclusion, oral supplementation of optimized doses of B-vitamins improved feed intake and rumen microbial protein synthesis and did not affect nutrient digestibility. Fat-soluble vitamins did not affect microbial fermentation, nutrient intake, or digestibility. The cows used in the present study were nonproducing. As higher vitamin availability (associated with higher microbial proliferation) is directly reflected in the vitamin content and yield of milk, we could not assess the improvement in animal productivity. More research in high/medium-producing cows is needed to support our results. To our knowledge, the present study was the first of its kind that investigated the influence of dietary supplementation of all vitamins simultaneously in an in vivo system.

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