doi: 10.17221/8402-CJAS

Effects of supplementing vitamin E on *in vitro* rumen gas production, volatile fatty acid production, dry matter disappearance rate, and utilizable crude protein

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ABSTRACT: Two *in vitro* trials were carried out to study the effects of supplementing vitamin E (V_E) on rumen fermentation. In Trial I, four levels of V_E product (purity 50%), i.e. 0, 15, 30, and 60 mg/kg dry matter (DM) of feed (equivalent to 0, 7.5, 15, 30 IU V_E/kg DM) were supplemented to a typical feed mixture, respectively, as experimental treatments. The gas test technique of Menke et al. (1979) was used to measure gas and volatile fatty acid (VFA) production. In Trial II, the *in vitro* incubation technique of Zhao and Lebzien (2000) was used to determine DM disappearance rate and utilizable crude protein (uCP). Four levels of V_E, i.e. 0, 7.5, 15, 30 IU/kg DM were supplemented to the same feed mixture as in Trial I, respectively, as experimental treatments. The results showed that supplementing V_E increased total gas production ($P < 0.01$) and tended to increase methane (CH_4) production ($P = 0.087$). Supplementing V_E also increased total VFA ($P < 0.05$) and propionate ($P < 0.05$), tended to increase acetate production ($P = 0.084$), and significantly increased DM disappearance rate ($P < 0.05$) and uCP ($P < 0.01$). It was concluded that supplementing V_E at 30 IU/kg DM under the conditions of present trials with 11.1 IU/kg DM in the feed mixture improved *in vitro* rumen fermentation of feed mixture. Further research is necessary to confirm the effects of supplementing V_E using *in vivo* trials.

Keywords: vitamin E; rumen fermentation; *in vitro*

INTRODUCTION

Vitamin E (V_E) is one of the fat-soluble vitamins and mainly acts as an antioxidant in metabolism of animals. It was reported that supplementing V_E enhanced the anti-oxidation and palatability characteristics of beef (Bloomberg et al. 2011), elevated α-tocopherol concentration in plasma and suppressed oxidation of lipid in muscle tissue of steers (O'Grady et al. 2001), improved the function of bovine neutrophils and reduced somatic cell counts in milk of dairy cows (Politis et al. 2004), and linearly increased carcass weight and average daily gain of finishing beef cattle (Burken et al. 2012).

The rumen environment is relatively free of oxygen and suitable for the colonization and growth of rumen microbes which are anaerobes. However, a small amount of oxygen, which is harmful to rumen microbes, may go into the rumen with saliva, feeds, and drinking water and diffusion from blood into rumen. Therefore, supplementing V_E to relieve oxidation effects from oxygen could be beneficial to rumen microbes and consequently feed digestion. In *in vitro* rumen fermentation, Hou et al. (2013) reported that supplementing V_E at 2 mg/80 ml incubation liquid increased *in vitro* rumen acetate and total VFA production and decreased butyrate production. Naziroglu et al. (2002) observed that supplementing at 0.8 mg V_E /100 ml incubation liquid increased acetate and propionate production, and Hino et al. (1993) reported that adding β-carotene plus V_E (α-tocopherol) (5 mg/l,

Supported by DSM China Ltd. (Project No. DCL-2013-101).

doi: 10.17221/8402-CJAS

respectively) improved cellulose digestion at the presence of 100 mg/l of safflower oil.

The typical rations for beef cattle in China contain a relatively high proportion of roughages and V_E is usually not added to the rations. It is unclear if supplementing V_E would be beneficial to rumen fermentation. The objectives of the present research were to study the effects of supplementing V_E to a typical feed mixture for beef cattle on *in vitro* rumen gas and volatile fatty acid (VFA) production, dry matter (DM) disappearance rate and utilizable crude protein (uCP) and to investigate the feasibility of supplementing V_E to the rations of beef cattle on rumen fermentation.

MATERIAL AND METHODS

The study was approved by the Animal Care and Use Committee of China Agricultural University.

Animals and feeding. Two 12-month old Simmental male cattle, with an average live weight of 300 ± 4 kg and fitted with permanent rumen fistulas made of polyethylene, were used as the donors of rumen fluid. The ration for the cattle included 6 kg/day wild rye hay and 2 kg/day concentrate mixture (air-dry basis) composed of 58% corn, 20% soybean meal, 18% wheat bran, 2% calcium hydrogen phosphate ($CaHPO_4$), 1% sodium chloride (NaCl), and 1% trace element mixture. The ration was divided into two equal meals which were fed to cattle at 7:00 and 17:00 h, respectively. The cattle had free access to fresh drinking water.

Feed samples and source of V_E. Air-dried feeds including corn, wheat bran, rapeseed meal, cottonseed meal, wild rye hay, and corn silage were milled to pass through a screen of 1 mm in pore size for formulating the feed mixture for incubation (Table 1). The V_E product (50% all-rac-α-tocopheryl acetate, silicate) for the study was supplied by DSM China Ltd. (Shanghai, China).

Preparation of V_E emulsion. 12 mg of V_E product were added to 200 ml of distilled water in a beaker and were mixed well using a magnetic stirrer as the V_E emulsion (concentration: 0.06 mg/ml or 0.06 IU/ml) for the trials.

Experimental design. In Trials I and II, four levels of V_E, i.e. 0, 7.5, 15, and 30 IU/kg DM were added to feed mixture, respectively, as experimental treatments.

Measurement of gas and VFA production. In Trial I, the procedures and preparation of rumen-buffer mixture for *in vitro* rumen fermentation was prepared according to Menke et al. (1979). Glass syringes, with 32 mm in internal diameter and 200 mm in length and with a calibrated volume of 100 ml, were used as incubation vessels. About 200 mg of feed sample was weighed and put inside each syringe. One of four volumes of V_E emulsion (0, 50, 100, 200 µl) was added into each syringe by pipette to obtain four supplementing levels of V_E (0, 7.5, 15, 30 IU/kg DM), respectively, as experimental treatments. Two hours after morning feeding, 250 ml of rumen fluid was taken from the ventral sac of the rumen of each cattle. The rumen fluid was well mixed and filtered through four layers of gauze and kept in a warmed bottle (39°C), then 700 ml buffer mixture and 350 ml rumen fluid were mixed well and kept in a water bath of 39°C and continuously gassed with CO_2. About 30 ml rumen-buffer mixture was sucked into each syringe and the air inside the syringes was expired and the tops of the syringes were sealed. Ten syringes were used as replicates for each treatment. Three syringes containing no feed sample were used as the blanks for each batch of incubation. Then the syringes were kept in an incubator at 39°C for incubation. At the incubation time points of 2, 4, 8, 12, 24, 36, and 48 h, the total gas production was recorded. At the end of incubation, 1 ml gas was sampled using a syringe for analysis. The incubation residue in each syringe was transferred into a centrifugation tube with a volume of 50 ml and centrifuged at 15 000 *g* and 4°C

Table 1. Components and chemical composition of feed mixture

Items	Content
Ingredients (% DM)	
Corn	22.01
Wheat bran	4.96
Rapeseed meal	4.04
Cottonseed meal	4.01
Chinese wild rye	20.01
Corn silage	44.97
Total	100.00
Chemical composition (% DM)[1]	
Crude protein	11.00
Neutral detergent fibre	41.69
Vitamin E (IU/kg DM)[2]	11.1

DM = dry matter

[1,2] analyzed values

doi: 10.17221/8402-CJAS

for 15 min. 1 ml supernatant was taken and mixed with 0.25 ml meta-phosphoric acid (25%, v/v) for VFA analysis.

Measurement of DM disappearance rate and uCP. In Trial II, the procedures and preparation of rumen-buffer mixture for determining DM disappearance rate and uCP were according to the *in vitro* incubation technique of Zhao and Lebzien (2000). Triangular flasks with calibrated volume of 100 ml were used as incubation vessels. About 500 mg of feed mixture (Table 1) was weighed and put into each triangular flask. One of four volumes of V_E emulsion (i.e. 0, 125, 250, 500 µl) was added into each triangular flask by pipette to obtain four levels of V_E (0, 7.5, 15, 30 IU/kg DM), respectively, as experimental treatments. Two hours after morning feeding, 250 ml of rumen fluid was taken from the ventral sac of the rumen of each cattle. The rumen fluid was well mixed and filtered through four layers of gauze, kept in a warmed bottle (39°C), and continuously gassed with CO_2. 50 ml of rumen fluid-buffer mixture was transferred into each flask. Each flask was sealed with a rubber stopper fitted with a glass tube with a rubber balloon at the top to release the gas produced during incubation. Ten flasks were used as replicates for each treatment and three flasks with no feed mixture were used as the blanks for each batch of incubation. The flasks were kept in an incubator for 24 h at 38°C.

At the end of incubation, the incubation residue of each flask was filtered through ashless filter paper and washed twice with distilled water. The volume of the liquid was recorded and 25 ml of liquid was sampled for nitrogen (N) determination. The solid material with the filter paper was collected for DM determination and followed by N determination. The liquid samples were distilled to release excess ammonia and followed by N determination.

Analytical procedures. The dry matter (DM), crude protein (CP), ether extract (EE), and ash of feed mixture were analyzed according to AOAC (1999) and the neutral detergent fibre (NDF) was analyzed according to Van Soest et al. (1991). The V_E content of the feed mixture and the V_E product used in the study was analyzed using high performance liquid chromatography (Agilent 1200 HPLC; Agilent Technologies Inc., Santa Clara, USA) in line with China National Standards GB/T 17812-2008 and GB/T 7293-2006, respectively. The methane (CH_4) and carbon dioxide (CO_2) of gas samples were analyzed using gas chromatography (TP-2060T; Beijing Tianpu Instrument Co. Ltd., Beijing, China). The VFA of liquid samples was analyzed using gas chromatography (TP-2060F; Beijing Tianpu Instrument Co. Ltd.).

Calculations and statistical analysis. The gas and VFA production of feed mixture were calculated as:

$$Y_{sample} = (Y_{incubation\ liquid} - Y_{blank})/DM$$

where:

Y_{sample} = gas (ml/g DM) or VFA production (µmol/g DM)

$Y_{incubation\ liquid}$ = gas (ml/g DM) or VFA production (µmol/g DM) of treatment

Y_{blank} = gas (ml/g DM) or VFA production (µmol/g DM) of blank

DM = DM of feed sample (g)

The total gas production at 2, 4, 8, 12, 24, 36, and 48 h of incubation was fitted to the model of Orskov and McDonald (1979):

$$dp = a + b\ (1 - e^{-ct})$$

where:

dp = *in vitro* gas production at different time points (ml/g DM)

t = incubation time (h)

a = gas production of immediately fermentable fraction (ml/g DM)

b = gas production of slowly fermentable fraction (ml/g DM)

c = gas production rate of fraction b (%/h)

The dry matter (DM) disappearance rate of feed mixture was calculated as:

$$DM_{disappearance\ rate} = (DM_{sample} + DM_{filter\ paper} - DM_{(residues + filter\ paper)} - DM_{blank\ residue})/DM_{sample} \times 100$$

where:

DM_{sample} = DM of feed sample (g)

$DM_{filter\ paper}$ = DM of filter paper (g)

$DM_{(residues + filter\ paper)}$ = DM of solid incubation residue and filter paper (g)

$DM_{blank\ residue}$ = DM of blank (g)

The utilizable crude protein (uCP) (g/kg DM) after incubation was calculated as:

$$uCP = (CP_{liquid} + CP_{solid} - CP_{blank})/DM$$

doi: 10.17221/8402-CJAS

where:
CP_{liquid} = CP in total liquid (g) which was: (CP in 25 ml) × (ml total liquid/25 ml)
CP_{solid} = CP in solid incubation residue (g)
CP_{blank} = CP of blank (g)
DM = DM of feed sample (kg)

Since the ammonia was released during distillation, the CP left in the liquid was considered to be uCP.

The data were subjected to the analytical procedures of GLM of SAS (Statistical Analysis System, Version 9.1, 2003) using the model:

$$Y_i = \mu + V_{Ei} + \varepsilon_i$$

where:
Y_i = observation
μ = general mean
V_{Ei} = effect of vitamin E (i = 1–4)
ε_i = residual error

Orthogonal contrast was performed to evaluate linear and quadratic effects of supplemental V_E. Differences between treatments were considered to be significant when $P < 0.05$, extremely significant when $P < 0.01$, and tended to be significant when $0.05 < P < 0.10$.

RESULTS

In Trial I, the pH of incubation liquid of four treatments at 48 h was 6.92–6.96. The results showed that supplementing V_E at 7.5–30 IU/kg DM increased the total gas production at time points of 24, 36, and 48 h of incubation ($P < 0.001$) (Table 2), the gas production of slowly fermentable fraction (b) ($P < 0.001$), and the sum of immediately and slowly fermentable fractions ($a + b$) ($P < 0.001$), respectively, while did not affect the gas production rate (c) of slowly fermentable fraction of feed mixture (Table 2). The results showed that supplementing V_E at 7.5–30 IU/kg DM tended to increase the 48 h CH_4 production ($P = 0.087$) whereas did not affect the 48 h CO_2 and H_2 production ($P > 0.05$) (Table 3).

The results also showed that supplementing V_E at 15–30 IU/kg DM increased the DM disappearance rate ($P = 0.021$) and uCP of feed mixture ($P = 0.004$) in Trial II (Table 4) and supplementing V_E at 30 IU/kg DM increased the total VFA and propionate production ($P < 0.05$) and tended to increase the acetate ($P = 0.084$) and butyrate ($P = 0.085$) production whereas did not affect the ratio of acetate to propionate in Trial I (Table 4).

Table 2. Effects of supplementing vitamin E (V_E) on *in vitro* rumen gas production (ml/g DM) at different incubation time and gas production parameters

Items	V_E (IU/kg DM)				SEM	*P*-value	*P*-value	
	0	7.5	15.0	30.0			linear	quadratic
Incubation time (h)								
2	24.1	30.0	30.0	28.0	5.48	0.083	0.327	0.049
4	59.2	56.1	61.1	63.0	9.48	0.419	0.200	0.407
8	122.6	120.8	119.6	120.5	9.34	0.904	0.627	0.751
12	168.4	169.6	166.8	169.9	7.97	0.825	0.788	0.855
24	217.8^B	236.8^A	236.2^A	242.6^A	11.52	< 0.001	< 0.001	< 0.001
36	252.3^B	267.3^A	266.8^A	262.6^A	6.63	< 0.001	0.088	< 0.001
48	271.4^B	284.3^A	279.8^A	279.0^A	6.22	0.001	0.246	0.011
Gas production parameters								
a	–6.83	–6.30	–5.41	–6.98	5.03	0.795	0.917	0.633
b	283.9^B	302.4^A	297.8^A	295.7^A	9.80	0.001	0.142	0.005
c	0.072	0.068	0.069	0.073	0.01	0.502	0.553	0.338
a + *b*	277.0^B	296.1^A	292.4^A	288.7^A	8.52	< 0.001	0.123	0.001

a = gas production of immediately fermentable fraction (ml/g DM), *b* = gas production of slowly fermentable fraction (ml/g DM), *c* = gas production rate of fraction *b* (%/h), DM = dry matter, SEM = standard error of the mean, $n = 10$
^{A,B}values within a row with different superscripts are extremely significantly different ($P < 0.01$)

doi: 10.17221/8402-CJAS

Table 3. Effect of supplementing vitamin E (V_E) on 48 h *in vitro* rumen gas production

Items	V_E (IU/kg DM)				SEM	*P*-value	*P*-value	
	0	7.5	15.0	30.0			linear	quadratic
Total gas (ml/g DM)	271.4^{B}	284.3^{A}	279.8^{A}	279.0^{A}	6.2	0.001	0.246	0.011
CH_4 (ml/g DM)	45.7	46.2	47.2	47.4	1.76	0.087	0.018	0.044
CO_2 (ml/g DM)	217.8	222.2	226.3	223.4	7.5	0.104	0.117	0.048
H_2 (µl/g DM)	425	403	408	400	4	0.538	0.254	0.435

DM = dry matter, SEM = standard error of the mean, n = 10

^{A,B}values within a row with different superscripts are extremely significantly different ($P < 0.01$)

DISCUSSION

In i*n vitro* batch culture inoculated with rumen fluid of goats, Hou et al. (2013) reported that supplementing V_E (Sigma, USA) at 0.5, 1, and 2 mg/80 ml incubation liquid (equivalent to 0.19, 0.38, and 0.75IU/30 ml) to 800 mg of feed mixture (70% hay: 30% concentrates) linearly and quadraticly increased the 24 h total VFA and acetate production and the ratio of acetate to propionate and decreased the butyrate production. In *in vitro* rumen fermentation inoculated with rumen fluid from beef steers, Naziroglu et al. (2002) reported that supplementing at 0.8 mg V_E (all-rac-α-tocopherol acetate 50%, silicic acid 50%)/100 ml incubation liquid (equivalent to 0.12 IU/30 ml) increased the 24 h acetate and propionate production and decreased the butyrate production. In *in vitro* incubation inoculated with rumen fluid of goats, Hino et al. (1993) reported that adding β-carotene plus V_E (DL-α-tocopherol) (5 mg/l, equivalent to 0.16 IU/30 ml, respectively) improved the 20 h cellulose digestion at the presence of 100 mg/l of safflower oil.

In Trial I of the present study, supplementing V_E up to 30 IU/kg DM (equivalent to 0.006 IU/30 ml incubation liquid) increased the total VFA and propionate production. The results were in agreement with the results of others mentioned above. The increased VFA production in the present study was consistent with the increased total gas production in Trial I and the DM disappearance rate in Trial II since the DM degradability positively correlated with the total gas production (Doane et al. 1997; Getachew et al. 2004).

In *in vivo* trial, Chikunya et al. (2004) reported that supplementing 500 mg V_E (α-tocopheryl acetate)/kg DM (equivalent to 500 IU/kg DM) to the rations of sheep with 50 g fatty acids/kg DM increased rumen microbial N flow to the proximal duodenum compared to the treatment of 100 mg V_E/kg DM. In Trial II of the present study, supplementing V_E at 15 or 30 IU/kg DM increased the uCP (including undegraded feed CP and microbial

Table 4. Effect of supplementing vitamin E (V_E) on *in vitro* rumen VFA production, DM disappearance rate, and uCP

Items	V_E (IU/kg DM)				SEM	*P*-value	*P*-value	
	0	7.5	15.0	30.0			linear	quadratic
VFA production (mmol/g DM)								
Total VFA	3.60^{b}	3.62^{b}	3.73^{ab}	3.90^{a}	0.212	0.013	0.001	0.005
Acetate	1.23	1.24	1.31	1.31	0.096	0.084	0.023	0.061
Propionate	1.58^{b}	1.63^{ab}	1.63^{ab}	1.75^{a}	0.135	0.037	0.005	0.017
Butyrate	0.59	0.55	0.58	0.61	0.085	0.085	0.432	0.446
Acetate/propionate	0.780	0.767	0.810	0.752	0.088	0.511	0.582	0.555
DM disappearance rate (%)	24.2^{b}	24.8^{ab}	26.1^{a}	26.5^{a}	1.75	0.021	0.004	0.010
uCP (g/kg DM)	204.8^{B}	213.6^{AB}	222.1^{A}	224.0^{A}	12.1	0.004	0.001	0.001

DM = dry matter, VFA = volatile fatty acids, uCP = utilizable crude protein, SEM = standard error of the mean, n = 10

^{a,b}values within a row with different superscripts are significantly different ($P < 0.05$)

^{A,B}values within a row with different superscripts are extremely significantly different ($P < 0.01$)

doi: 10.17221/8402-CJAS

CP) of feed mixture, indicating that supplementing V_E improved the N utilization of the feed mixture in *in vitro* rumen fermentation. The results were in agreement with Chikunya et al. (2004).

Vitamin E is usually considered as a lipophilic antioxidant which is important for protection of cell membranes from oxidation. Although the environment of rumen of adult ruminants is anaerobic and the concentration of oxygen is believed to be very low, the rumen is not absolutely free of oxygen since oxygen dissolved in water, saliva, and blood diffuses into rumen. It was reported that the amount of oxygen diffused into rumen could be as high as 3 mmol/l rumen fluid and maintained at the level for at least 18 h during a day (Stewart and Bryant 1988). Moreover, most species of rumen bacteria are strict anaerobes which are extremely susceptible to oxidative stress under fibrous feeds (Kamra 2005). Therefore, the effects of V_E on rumen fermentation could be mainly attributed to the antioxidation and protection effects for maintaining the integrity of microbial membranes from oxidation (Burton and Traber 1990) and the effects of V_E on improving the growth of rumen protozoa and cellulolytic bacteria (Hino et al. 1993; Naziroglu et al. 2002).

It should be noted that the supplementing level of V_E in the present trials was much lower than that of previous trials. The reason for the experimental design was a consideration of lowering the cost of supplementing V_E in ruminant feeding practice. The results showed that supplementing lower than 7.5 IU V_E/kg DM did not affect DM disappearance rate and uCP of the feed mixture and lower than 15 IU V_E/kg DM did not affect VFA production while supplementing V_E higher than 7.5 IU/kg DM increased total gas production. The results indicated that under the conditions of the present trials, supplementing V_E at 15–30 IU/kg DM would be beneficial to rumen fermentation.

Tagliapietra et al. (2013) found that supplementing vitamin E (all racemic mixture of α-tocopheryl acetate, purity 97.10%) at 8 mg/g feed (equivalent to 7000 IU/kg DM) decreased feed digestion and rumen microbial N when supplied to hay but not when supplied to corn in *in vitro* incubation. The results indicated that both the supplementing level of V_E and the chemical composition of feeds are important to obtain positive effects on rumen fermentation and an interaction may exist between the V_E level and the chemical composition of feed. It is necessary to study the supplementing level of V_E suitable for different feeding regimes of ruminants.

CONCLUSION

Under the conditions of the present trial with 11.1 IU V_E/kg DM in feed mixture, supplementing V_E at 30 IU/kg DM increased *in vitro* rumen total gas and VFA production, DM disappearence rate, and uCP of feed mixture. The results indicated that supplementing V_E to the ration of ruminants may have beneficial effects on rumen fermentation. Further research is needed to confirm the effects of supplementing V_E on nutrient digestion and animal performance with *in vivo* trials.

Acknowledgement. The authors thank DSM China Ltd. for financial support of the trials.

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Received: 2014–09–17
Accepted after corrections: 2015–01–26

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