DOI: http://dx.doi.org/10.1590/1678-992X-2018-0247

ISSN 1678-992X

SCIENTIA AGRICOLA

Research Article

Methane mitigation and ruminal fermentation changes in cows fed cottonseed and vitamin E

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Edited by: Antonio Faciola

Received September 25, 2018
Accepted May 03, 2019

ABSTRACT: Inherently, ruminant production of methane (CH_4), a greenhouse gas (GHG), causes animal energy losses. Cottonseed is a lipid source and is used sometimes to enhance energy density in cattle diets. It also can mitigate enteric CH_4. Lipids release peroxides in the rumen, and antioxidants have the ability to neutralize them. Thus, a lipid and antioxidant source can benefit rumen fermentation. The aim of this study was to evaluate rumen fermentation parameters from cows fed cottonseed and vitamin E. Six cannulated cows were arranged in a replicate 3 × 3 latin square. Treatments were: 1) Control, 2) CS (30 % corn replaced by cottonseed) and 3) CSVitE (30 % corn replaced by cottonseed, plus 500 IU VitE). Results were compared by orthogonal contrast. When compared to the control diet, cottonseed inclusion reduced enteric CH_4 emissions by 42 %. Production of acetate, butyrate and the acetate to propionate ratio were respectively 34 %, 47 % and 36 % lower with the cottonseed treatments. Energy lost in the rumen as CH_4 and energy release as butyrate were reduced by 26 % and 32 % respectively. Propionate and intestinal energy release were, respectively, 43 % and 35 % higher with cottonseed treatments. Furthermore, as a nutritional strategy to mitigate enteric CH_4, cottonseed has positive effects on short chain fatty acid (SCFA) production and gastrointestinal energy release. Vitamin E did not result in improvements in ruminal fermentation. Further studies evaluating levels of vitamin E in association with different amounts and sources of lipids are required.
Keywords: SCFA, enteric methane, oilseed, antioxidant

Introduction

Enteric methane emissions from ruminants are problematic with respect to the energy utilization efficiency of feed, and they also have an environmental impact. According to Buddle et al. (2011), 5 % to 9 % of gross energy consumed by animals is lost as CH_4. This represents 14 % of anthropogenic GHGs and 25 % of the CH_4 emissions from human activities (Gerber et al., 2013). Methane mitigation feeding strategies are therefore a priority for improving animal productivity and environmental sustainability (Beauchemin et al., 2011), although resources relative to this subject and the relationship between methane mitigation and animal performance could be more thoroughly reported in the relevant literature.

Incorporating oilseeds into cattle diets has been shown to reduce enteric CH_4 emissions, as reported by Beauchemin et al. (2008) and Martin et al. (2010). Although the influence of lipids in methane emissions, digestibility and rumen fermentation vary between studies, this may be associated with the type and concentration of fatty acids in diet (Grainger and Beauchenin, 2011).

Ruminal microorganisms are predominantly anaerobes and have a less developed antioxidant capacity (Brioukhanov and Netrusov, 2004). Dietary lipids, if not biohydrogenated in terms of potential oxidation, can be significant contributors to the load of peroxides in the rumen. According to Andrews et al. (2006), it is possible that raising peroxide concentrations would be enough to create an oxidative stress condition in ruminal microorganisms that could compromise their activity for optimal growth.

A common way to attempt to reduce or prevent lipid peroxidation is through the use of antioxidants. In recent years, supplemental vitamin E in the diet of ruminants has been studied for its potential role in preventing lipid peroxidation (Bloomberg et al., 2011). The reasoning being that supplementing vitamin E to relieve oxidation effects could be beneficial to rumen microorganisms by improving fermentation production and changing the gastrointestinal availability of energy for the cows. Feeding an antioxidant, such as α-tocopherol, alleviates the negative effect of a high level of unsaturated fat supplementation on microbial growth and SCFA utilization by the rumen microflora (Hino et al., 1993).

In this study, we began by hypothesizing *that* oilseed not only has beneficial effects on rumen fermentation, but also produces a number of undesirable effects in terms of peroxidation products, *and that* vitamin E could decrease the negative effects of fatty acids on ruminal fermentation. The overall aim of this research was therefore to investigate the effects of cottonseed and vitamin E inclusion on methane and SCFA production, as well as to evaluate the energy release in the gastrointestinal tract of cows.

Materials and Methods

Study location and ethical care

The study was conducted at Pirassununga, São Paulo, Brazil (Latitude: –21.996; Longitude: –47.4268

21°59′46″ S, 47°25′36″ W; Altitude: 625 meters). The experiment was approved by and complied with the guidelines set out by the Ethics Committee by the Use of Animals of the University of São Paulo, under application number nº 009/2013, in respect of animal experimentation and care of animals used for scientific purposes.

Animals, housing and feeding

Six Holstein dairy cows were sorted into individual pens that had free access to water and a sand bed. They were neither pregnant nor lactating; they had ruminal cannula, and the average of their body weights was 876 kg (± 16.1). Cows were fed ad libitum twice daily (08h00 and 16h00) with the same amount in each meal. The feed was weighed daily and offered to each animal after the feed residue from the previous day had been removed. The vitamin E amount offered was 500 IU per animal per day. To administer this, a powder containing 50 % alpha tocopheryl acetate was top-dressed on the diet and immediately mixed with a pitchfork at the bunker. This inclusion rate for Vitamin E was determined according to Scrist et al., (1998), who stated that the addition of Vitamin E at a rate of 500 mg kg^{-1} of DM improves both feed efficiency and economic justifications.

Experimental design and treatments

A replicated 3 × 3 Latin squared design with 3 periods was used. Three dietary treatments were defined: 1) C – Control diet, 2) CS - Control diet with 30 % cottonseed replacing ground corn grain, and 3) CSVitE – the CS diet supplemented with vitamin E. The dietary levels for fatty acids and cottonseed levels were determined according to Andrae et al. (2001). Diets had a forage-concentration rate of 16:84, in which sugar bagasse was the main fiber source. Soybean meal and urea were the primary protein sources; for energy content, ground corn grain was replaced by cottonseed. Nutritional demands were estimated by the NRC system (2001) with the aim of daily mean weight gain of 1.2 kg d^{-1}. The ingredients and chemical compositions of the experimental diets are presented in Table 1.

Table 1 – Ingredients and chemical composition of dietary treatments.

	Dietary treatments		
	Control	CS	CSVitE
Ingredient			
Sugarcane bagasse, g kg^{-1} of DM	134	134	134
Cottonseed, g kg^{-1} of DM	-	304	304
Ground corn grain, g kg^{-1} of DM	572	281	281
Citrus pulp, g kg^{-1} of DM	183	183	183
Soybean meal, g kg^{-1} of DM	81.7	81.7	81.7
Minerals, g kg^{-1} of DM	60.0	60.0	60.0
Limestone, g kg^{-1} of DM	40.0	40.0	40.0
Urea, g kg^{-1} of DM	13.7	2.70	2.70
Vitamin E, mg kg^{-1} of DM	-	-	500
Chemical composition			
DM, g kg^{-1}	891	910	910
CP, g kg^{-1} of DM	158	160	160
RDP[1], g kg^{-1} of CP	302	366	366
RUP[2], g kg^{-1} of CP	69.8	63.4	63.4
EE, g kg^{-1} of DM	26.1	76.9	76.9
NDF, g kg^{-1} of DM	234	357	357
ADF, g kg^{-1} of DM	171	265	265
Lignin, g kg^{-1} of DM	55.3	136	136
Ca, g kg^{-1} of DM	15.7	18.2	18.2
P, g kg^{-1} of DM	12.7	14.7	14.7
Hemicellulose[3], g kg^{-1} of DM	63.0	92.0	92.0
Cellulose[4], g kg^{-1} of DM	115	136	136
OM[5], g kg^{-1} of DM	829	845	845
NFC[6], g kg^{-1} of DM	525	328	328
Gross energy, MJ kg^{-1} of DM	17.3	17.8	17.8
[7]Vitamin E, mg kg^{-1} of DM	14.0	7.00	507

DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; Ca = calcium; P = phosphorus; NFC = Non-fibrous carbohydrate; [1]RDP = rumen degradable protein estimated according to NRC (2001); [2]RUP = rumen undegradable protein estimated according to NRC (2001); [3]Hemicellulose = NDF – ADF; [4] Cellulose = ADF – Lignin; [5]OM = DM – mineral; [6]NFC = 100 – (CP + NDF + EE + ash); [7]Vitamin E: estimated according to NRC (2001).

Feed intake

Feed intake was measured from the 11th to the 15th days of each period by weighing feeds offered to and refused by the cows. Refusal was recorded once daily and the feeding rate was adjusted to yield orts on the basis of at least 10 % of the amount supplied (on an as-fed basis). Dry matter intake was determined by multiplying feed intake by respective dietary dry matter.

Ruminal emptying

The last 2 days of each period - before morning feeding and 3 h after morning feeding respectively - ruminal contents were manually removed according to Chilibroste et al. (2000). Using a strainer, ruminal contents were separated into solid and liquid phases, and were then weighed and sampled. Immediately thereafter, ruminal contents were returned into the rumen. The solid and liquid samples were dried at 60 °C (forced-air oven) for 72 h in order to determine the dry matter content of each sample. Ruminal solid mass was calculated by the sum of solid and liquid content adjusted by its respective dry matter content.

Nutrient digestibility and fecal output

Dry matter digestibility and fecal output were determined using chromium oxide as an external marker as according to Bateman (1970). From the 8th until the 17th, 15 grams per head per day of indigestible marker was placed twice daily (08h00 and 16h00 before feeding) via rumen fistula accordingly. Feces were manually collected twice a day from the rectum from the 13th until the 17th at 08h00 and 16h00 after feeding. A composite of 200 g samples was then analyzed for chromium oxide concentration according to Conceição et al. (2007).

Determination of Methane and SCFA production

SCFA, CH_4 and N-NH_3 productions were determined by the ex situ ruminal fermentation technique (Rodrigues et al., 2012; Perna Junior et al., 2017). The principle of this technique consists of leaving the ruminal content samples in bottles (as a micro-rumen), which are then incubated in a water bath at 39 °C. This simulates the prevailing conditions of the rumen (presence of microorganisms, anaerobic environment, a temperature of 39 °C, natural saliva, and physiological rumen pH).

Ruminal content samples were collected on the 18th day of each period through the ruminal cannula at 0, 3, 6, 9 and 12 h after the morning meal. On this day, animals were fed once in the morning. The evening meal was offered only after the collection of the 12 h sample. Approximately 300 mL of rumen fluid (using a motorized vacuum pump) and 300 g of solid content (with hands) were collected at each sampling time from three different parts of the rumen (dorsal sac in the front, middle and back). The two fractions were mixed in the proportion of 66 % liquid phase and 33 % solid phase and homogenized before preparation for analysis of SCFA, CH_4 and N-NH3 using the ex situ ruminal fermentation technique (Perna Junior et al., 2017).

For each sample, four bottles were prepared; two were used for incubation (T_{30}) and two were used as blanks (T_0). The mixed rumen contents (30 mL) were pressed through a funnel into a 50 mL capacity bottle, which was then capped with rubber corks and sealed with an aluminum seal. The bottles were then flushed with CO_2 through needles for input and output to ensure an anaerobic environment. After 30 min of incubation, fermentations were stopped by autoclaving at 121 °C (250 °F) and 100 kPa (15 psi) above atmospheric pressure for 15 min. The measurements of total gas volume produced in incubated (T_{30}) and not incubated (T_0) bottles were taken using a pressure transducer connected to a syringe with a needle. The gas volume was obtained from the sum of the volume obtained at the transducer plus the head space. CH_4 concentration was determined by gas chromatography, injecting 0.5 mL of gas from each bottle, according to Kaminski et al. (2003) in a controlled temperature environment (25 °C).

The volume of liquid in the incubated (T_{30}) and not incubated (T_0) bottles was calculated as the difference between the weight of the bottle sample after drying in an oven with forced air circulation at 105 °C for 24 h and the weight of the bottles before heating in the oven. The solid content of the bottles was obtained by measuring the weight difference between the bottle containing the sample after drying in the oven and the weight of the empty bottle (before filling with ruminal content sample). For SCFA analyses (of acetate, propionate and butyrate), a fraction of ruminal fluid from each bottle was centrifuged at 2,000 × g for 20 min, and 2.0 mL of the supernatant was added to 0.4 mL of formic acid and frozen at minus 20 °C for further analyses, according to Erwin et al. (1961). SCFA were measured by gas chromatography using a glass column, 1.22 m in length and 0.63 cm in diameter, packed with 80/120 Carbopack B-DA/4 %.

The quantification of CH_4 production was obtained by multiplying the total volume of gas (mL) by the CH_4 concentration in the gas phase, the mmol mL^{-1} obtained in the incubated bottle (T_{30}). This result was subtracted from the value that was produced in the bottle not incubated (T_0). The individual quantification of SCFA was obtained by multiplying the liquid volume (mL) by the concentration of each SCFA (mmol mL^{-1}) obtained in the incubated bottle (T_{30}), and this value was also subtracted from that obtained from the bottle not incubated (T_0). CH_4 and SCFA production was identified according to Perna Junior et al., 2017.

To determine energy from the fermentative products (CH_4 and SCFA), each product was multiplied by their respective combustion heat, so the relative energy loss (REL) was the ratio between the energy in the methane produced and the energy sum in all the quantified fermentation products (CH_4 and SCFA), expressed as a percentage. In making this examination, values and data from the literature were used that assumes: acetic, propionic, butyric, CH_4 and CO_2 had 3.49, 4.98, 5.96, 13.16 and 0.0 kcals per gram, or conversely, 209.40, 368.52, 524.48, 210.56 and 0.0 kcals per mol, respectively. The relative energy loss was calculated according to Perna Junior et al., 2017.

Determination of nitrogen-ammonia concentration and balance

For N-NH_3 concentration determination, a 2.0 mL centrifuged sample of each bottle (after microbial inactivation) was mixed with 1 mL of 1 N of H_2SO_4 solution. The tubes were then immediately frozen until the colorimetric analyses were carried out, according to the method described by Kulasek (1972). The balance was obtained by subtracting the N-NH_3 concentration after 30 min of incubation (T_{30}) from the baseline (T_0). With this procedure it is possible to evaluate whether on balance of ammonia production in the rumen is positive or negative. In this paper, this information was expressed in terms of changes in concentration (mg dL^{-1}) per hour.

Notably in this study, the *ex situ* methodology was capable of evaluating N-NH_3 concentrations from the rumen for microorganisms, and through its incubation process, it is possible to determine the N-NH_3 balance.

Gastrointestinal energy released

Gross energy intake (MJ d^{-1}) was calculated by multiplication of DMI (kg) and diet gross energy (MJ kg^{-1}). To calculate the energy release when expressed as MJ d^{-1}, the acetate, butyrate, propionate and methane productions (g kg^{-1} d^{-1}) were multiplied by their respective combustion heat (MJ g^{-1}), and then multiplied by their ruminal solid mass (kg). The energy release in the rumen, when expressed in terms of % gross energy intake (GEI) or % digestive energy (DE), was obtained by dividing ac-

etate, propionate, butyrate and methane releases (MJ d^{-1}) by gross energy intakes (MJ d^{-1}) or digestive energy (MJ d^{-1}) and then multiplying the result by 100.

Methane release in the cecum and colon (C&C) was considered to be 5 % of total methane release. Enteric methane is produced mainly in the rumen (95 %) and, to a smaller extent (5 %), in the large intestine (Dini et al., 2012).

Energy release in the intestine (MJ d^{-1}) was calculated from the gross energy intake (MJ d^{-1}) subtracted from the respective acetate, propionate, butyrate and methane releases in the rumen (MJ d^{-1}), plus the feces' gross energy (MJ d^{-1}) and methane releases in the cecum and colon (MJ d^{-1}). The following equation 1 refers to:

$$ERI = GEI - (C2 + C3 + C4 + \text{feces GE} + C\&C) \quad (1)$$

where: *ERI* = energy release in the intestine (MJ d^{-1}), *GEI* = gross energy intake (MJ d^{-1}), C2 = acetic (MJ d^{-1}), C3 = propionic (MJ d^{-1}), C4 = butyric (MJ d^{-1}), feces GE = energy release in the feces (MJ d^{-1}), and C&C methane = methane release in cecum and colon (MJ d^{-1}).

The energy release in the intestine, expressed in terms of % GE or % DE, was obtained by dividing energy release in the intestine (MJ d^{-1}) by GEI (MJ d^{-1}) or DE (MJ d^{-1}) and then, multiplying the result by 100. Energy release in the feces, expressed in terms of % GEI, was obtained by dividing feces' energy content (MJ d^{-1}) by gross energy intake (MJ d^{-1}) and then multiplying the result by 100.

Laboratory analysis

Pooled feed ingredients, as well feces samples, were collected and stored at −20 °C. Samples were dried at 60 °C for 48 h and milled through a 1mm screen using a Wiley mill. The DM content was processed at 100 °C for 4 h followed by cold weighing (method 930.15, AOAC, 1995). Nitrogen content was determined by the micro Kjeldahl method (AOAC, 1995) using a nitrogen distiller, which was then multiplied by 6.25 to determine CP. EE was determined using light petroleum ether in the Soxhlet apparatus (method 920.39, AOAC, 1995). GE was determined by combustion using an adiabatic calorimeter bomb according to AOAC (1995). NDF, ADF and lignin were determined using the sequential method with heat stable α-amylase (method 973.18, AOAC, 1995) using a fiber digester.

Statistical analysis

The data were analyzed using the MIXED procedure of SAS (Statistical Analysis System, version 9.0). Cows in each period were considered to be experimental units. Before the actual analysis, the data were first analyzed for the presence of disparate information ("outliers") and the normality of residuals (Shapiro-Wilk). An individual observation was considered outlier when standard deviations in relation to the mean were greater than +3 or less than −3.

For the ruminal solid mass, gross energy intake, and energy release data, the model used included both the fixed effect of treatments and the random effects of square, period, and animals within the square. These variables were analyzed using the following model:

$$Y_{ijkl} = \mu + T_i + P_j + S_k + A_l(S_k) + e_{ijkl}$$

where: Y_{ijkl} = the dependent response variable, μ = the overall mean, T_i = the treatment effect, P_j = the period effect, S_k = the square effect, $A_l(S_k)$ = animals within square effect, and e_{ijkl} = the residual error term.

For the methane SCFA and ammonia variables, data were analyzed using mixed models (PROC MIXED). From 15 different covariance structures tested, the selected model was chosen based on the lower value of the Corrected Akaike Information Criterion (AICC), (Wang and Goonewardene, 2004). In this model, the treatment, time and interaction treatment*time effects were considered fixed. The effects of period, square, and the animal within the square were considered random. These variables were analyzed using the following model:

$$Y_{ijklm} = \mu + T_i + P_j + S_k + A_l(S_k) + T_I + (T_i \times TI)_{ij} + e_{ijklm}$$

where: Y_{ijklm} = the dependent response variable, μ = the overall mean, T_i = the treatment effect, P_j = the period effect, S_k = the square effect, $A_l(S_k)$ = cows within square effect, TI = the time effect, $(T_i \times TI)_{ij}$ = the interaction treatment and time, and e_{ijklm} = the residual error term.

Contrast statements were used to evaluate differences between means, such as "C1" -Control vs. CS and CSVitE and "C2" - CS vs. CSVitE. Statistical significance was declared at $p \leq 0.05$.

Results

Due to the replacement of ground corn grain for cottonseed, the CS and CSVitE had higher values for rumen degradable protein (RDP), neutral digestive fiber (NDF), acid detergent fiber (ADF) and lignin, ether extract (EE), Hemicellulose and Cellulose. CS and CSVitE had lower values for rumen ungradable protein (RUP) and non-fibrous carbohydrate (NFC), when compared to the Control (Table 1).

Ruminal parameters

No effect was observed for the $N\text{-}NH_3$ concentration in the 0 and 30 min time frames. For the remaining hourly periods, animals fed vitamin E had a lower $N\text{-}NH_3$ balance (0.85 vs. 1.99 mg dL^{-1}) compared to those animals fed no vitamin E (Table 2).

The inclusion of cottonseed in the diet, with or without vitamin E, decreased acetate ($p < 0.0028$), butyrate ($p < 0.0001$), and methane ($p < 0.0001$) production compared to the Control. Propionate production was similar among treatments. Furthermore, and

Table 2 – Ruminal fermentation of non-lactating cows fed dietary treatments.

	Treatments			SEM	*Probability			
	Control	CS	CSVitE		C1	C2	Time	T × Ti
N-NH_3, mg dL^{-1} h^{-1}								
T_0	22.4	21.9	21.3	0.86	0.590	0.730	0.001	0.001
T_{30}	23.5	23.9	22.2	0.93	0.756	0.349	0.001	0.001
Balance, hours	1.11	1.99	0.85	0.41	0.149	< 0.001	0.150	0.508
Acetate								
T_0, mmol L^{-1}	71.1	67.2	67.1	0.89	0.123	0.967	0.011	0.152
T_{30}, mmol L^{-1}	76.1	71.3	71.0	0.99	0.020	0.913	< 0.001	0.181
Mmol g^{-1} of DM h^{-1}	0.14	0.09	0.09	0.01	0.002	0.841	0.018	0.481
mol kg^{-1} of DM d^{-1}	3.50	2.39	2.23	0.19	0.002	0.725	0.018	0.246
g kg^{-1} of DM d^{-1}	210	143	133	11.8	0.002	0.723	0.018	0.242
EB, MJ kg^{-1} d^{-1}	3.06	2.10	1.95	0.04	0.010	0.709	0.018	0.538
Propionate								
T_0, mmol L^{-1}	15.2	20.7	20.9	0.49	0.001	0.124	< 0.001	0.020
T_{30}, mmol L^{-1}	16.8	22.7	22.9	0.55	0.001	0.921	< 0.001	0.330
Mmol g^{-1} of DM h^{-1}	0.04	0.04	0.04	0.01	0.122	0.993	0.010	0.179
mol kg^{-1} of DM d^{-1}	1.09	1.15	1.10	0.07	0.806	0.784	0.010	0.219
g kg^{-1} of DM d^{-1}	80.9	85.3	82.0	5.51	0.801	0.791	0.010	0.221
EB, MJ kg^{-1} d^{-1}	1.68	1.77	1.70	0.11	0.801	0.791	0.010	0.221
Butyrate								
T_0, mmol L^{-1}	12.7	9.34	9.12	0.29	< 0.001	0.712	0.059	0.075
T_{30}, mmol L^{-1}	14.2	10.3	10.0	0.32	< 0.001	0.725	0.006	0.615
Mmol g^{-1} of DM h^{-1}	0.04	0.02	0.02	0.00	< 0.001	0.504	0.019	0.121
mol kg^{-1} of DM d^{-1}	1.03	0.58	0.50	0.04	< 0.001	0.385	0.019	0.262
g kg^{-1} of DM d^{-1}	90.7	51.3	44.3	4.22	< 0.001	0.388	0.019	0.255
EB, MJ kg^{-1} d^{-1}	2.25	1.28	1.10	0.10	< 0.001	0.388	0.019	0.255
SCFA total								
T_0, mmol L^{-1}	99.0	97.3	97.1	13.3	0.624	0.971	0.004	0.093
T_{30}, mmol L^{-1}	107	104	104	14.6	0.402	0.921	0.001	0.305
Mmol g^{-1} of DM h^{-1}	0.23	0.17	0.16	0.01	0.005	0.805	0.046	0.448
mol kg^{-1} of DM d^{-1}	5.62	4.13	3.84	0.29	0.005	0.657	0.053	0.476
g kg^{-1} of DM d^{-1}	381	280	260	19.6	0.005	0.692	0.049	0.434
EB, MJ kg^{-1} d^{-1}	7.01	5.15	4.76	0.35	0.005	0.641	0.044	0.448
C2:C3 ratio								
Concentration	4.76	3.29	3.34	0.09	< 0.001	0.870	< 0.001	0.324
Production	3.60	2.19	2.41	0.17	0.009	0.613	0.061	0.350
Methane								
T_0, mmol L^{-1}	0.03	0.02	0.02	0.00	< 0.001	0.430	0.627	0.837
T_{30}, mmol L^{-1}	0.15	0.10	0.09	0.00	< 0.001	0.587	< 0.001	0.549
Mmol g^{-1} of DM h^{-1}	0.11	0.06	0.06	0.00	< 0.001	0.323	< 0.001	0.783
mol kg^{-1} of DM d^{-1}	2.73	1.65	1.50	0.08	< 0.001	0.409	< 0.001	0.241
g kg^{-1} of DM d^{-1}	43.7	26.4	24.1	1.42	< 0.001	0.356	< 0.001	0.356
EB, MJ kg^{-1} d^{-1}	2.40	1.45	1.32	0.07	< 0.001	0.358	< 0.001	0.215
REL, %	31.3	29.6	27.3	1.36	0.462	0.598	0.567	0.364

SCFA = short chain fatty acids; C2:C3 ratio = acetate to propionate ratio; RE = relative energy loss; SEM = standard error of the mean; T × TI = interaction treatment time ; NS = $p > 0.10$; *Probability: C1 = contrast 1 (CS and CSVitE vs. control); C2 = contrast 2 (CS vs. CSVitE).

in terms of total SCFA production ($p < 0.0054$), the acetate to propionate ratio ($p < 0.0001$) was reduced by cottonseed supplementation compared to the Control diet. Including cottonseed or vitamin E in diet did not affect the relative energy loss (REL) (Table 2). The interactions between times and treatments for the N-NH_3 ($p < 0.001$) concentration were significant. In T_0 and T_{30} specimens, cottonseed treatments had a *higher* N-NH_3 concentration at 0 h post feeding and a *lower* concentration at 9 h post feeding vs. the Control (Figure 1). No interaction between time and treatment was observed for the acetate, butyrate or methane production; however, the result of the time effect was notable, ($p < 0.05$). The acetate, propionate, butyrate and methane productions were higher at 3 h post feeding and lower at 0 h post feeding (Figure 2).

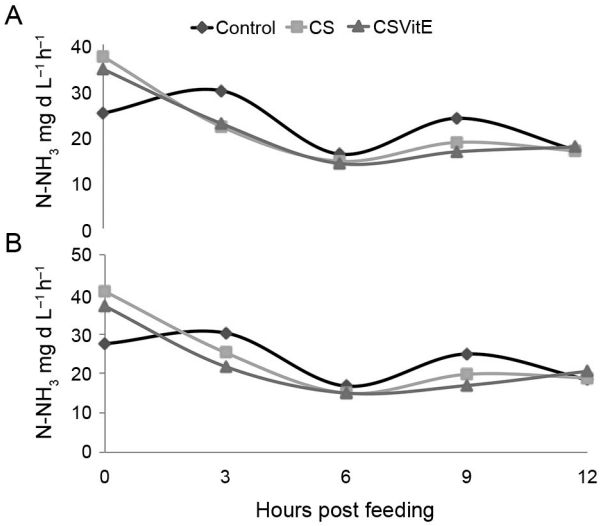

Figure 1 – Ruminal N-NH_3 concentration in the T_0 (A) and T_{30} (B) over 12 h post feeding.

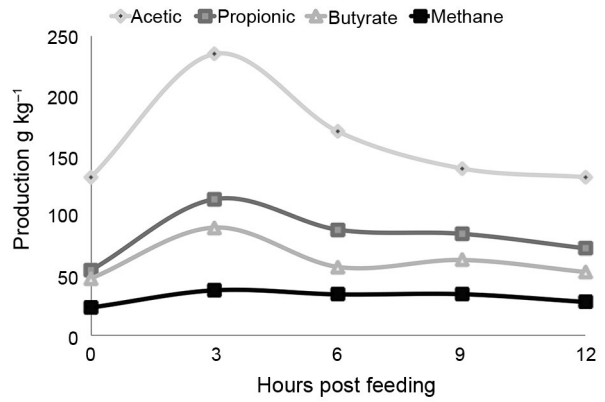

Figure 2 – Average production of acetate, propionate, butyrate and methane over 12 h post feeding.

Table 3 – Estimate of energy release in the gastrointestinal tract of cows fed different diets.

	Treatments			SEM	*Probability	
	Control	CS	CSVitE		C1	C2
Ruminal solid mass, kg	8.29	10.8	11.2	0.45	< 0.001	0.603
Dry matter intake, kg	14.6	15.4	15.4	0.61	0.064	0.952
Dry matter excretion, kg	3.67	4.02	4.06	0.19	0.437	0.912
Gross energy intake, MJ d^{-1}	211	245	245	13.0	0.107	0.979
Digestive energy, MJ d^{-1}	158	169	172	12.5	0.054	0.861
Energy release in the rumen						
Acetate						
MJ d^{-1}	24.6	22.8	20.8	1.17	0.330	0.526
GE, %	11.9	9.88	9.39	0.96	0.295	0.824
DE, %	18.8	15.0	14.4	1.98	0.169	0.842
Propionate						
MJ d^{-1}	13.3	19.2	18.9	1.25	0.034	0.907
GE, %	6.40	8.05	8.09	0.55	0.465	0.977
DE, %	9.82	12.2	12.2	1.06	0.720	0.981
Butyrate						
MJ d^{-1}	19.2	13.9	12.2	1.00	0.003	0.304
GE, %	9.22	5.78	5.07	0.51	< 0.001	0.292
DE, %	14.2	8.60	7.58	0.93	0.002	0.448
Methane						
MJ d^{-1}	20.5	15.7	14.5	1.12	0.001	0.441
GE, %	9.72	5.96	6.59	0.50	< 0.001	0.342
DE, %	15.0	9.87	8.73	0.93	0.003	0.003
Energy release in the intestine						
MJ d^{-1}	63.3	94.1	102	12.1	0.040	0.728
GE, %	27.3	36.1	38.9	3.61	0.023	0.625
DE, %	39.0	51.8	54.7	4.14	0.016	0.646
Energy release in the Feces						
MJ d^{-1}	66.9	75.7	72.8	4.30	0.176	0.702
GE, %	33.3	31.9	31.0	2.33	0.759	0.825

GE = gross energy; DE = digestible energy; SEM = standard error of the mean; NS = $p > 0.10$; *Probability: C1 = contrast 1 (CS and CSVitE vs. control); C2 = contrast 2 (CS vs. CSVitE).

Gastrointestinal energy released

No differences were observed in dry matter intake or dry matter excretion, gross energy intake, or digestive energy. The cottonseed inclusion diet, regardless of the VitE presence, resulted in 32 % higher ruminal solid mass compared to the Control (Table 3).

Comparative results between other treatments vs. the Control were as follows: the propionate and intestine energy releases, when expressed as MJ d^{-1}, were respectively 43 % ($p < 0.0343$) and 57 % ($p < 0.0405$) higher for cows fed cottonseed. Butyrate and methane energy releases, when expressed as MJ d^{-1}, were, respectively, 32 % ($p < 0.0033$) and 26 % ($p < 0.0013$) lower for the cottonseed treatments. The acetate and feces energy releases, when expressed as MJ d^{-1}, were similar among treatments (Table 3).

Specifically, cottonseed treatments were lower vs. the Control in these results: The butyrate energy released expressed as % GEI (5 % vs. 9 %) ($p < 0.002$) and % DE (8 % vs. 14 %) ($p < 0.0021$). The methane energy released expressed as % GEI (6 % vs. 9 %) ($p < 0.005$) and % DE (9 % vs 15 %) ($p < 0.0031$). The intestinal energy released expressed as % GEI (37 % vs. 27 %) ($p < 0.0239$) and % DE (53 % vs. 39 %) ($p < 0.0231$), the latter being the only result that was higher than the Control. The acetate and propionate % GE and % DE energy releases were similar among treatments (Table 3).

Discussion

Ruminal parameters

In the present study, the ruminal ammonia concentration (T_0) was, on average, 21.8 mg dL^{-1}. According to Leng (1990), ruminal N-NH_3 should be greater than 10 mg dL^{-1} for suitable rumen fermentation. Patra et al. (2014), in a meta-analyses study about lipid effects on ruminal parameters, observed that, on average, ruminal N-NH_3 was 12.9 mg dL^{-1}. Here, the minimum was

3.1 mg dL^{-1}, and the maximum 34.5 mg dL^{-1}. These results indicated high variability between the treatments. After (T_{30}) fermentation, the N-NH_3 concentration was, on average, 23.2 mg dL^{-1}. The N-NH_3 balance was positive, on average, 1.31 mg dL^{-1} h^{-1}. Positive balance is an indication that the amount of N-NH_3 in the rumen was sufficient for microorganism growth and microbial protein production.

Although urea concentration was higher in the Control diet, the supplemented diets had a higher NFC concentration (Table 1). The N-NH_3 rapidly released by the urea was in equilibrium with the quick energy available from NFC. Cottonseed treatments also had higher fiber content (Table 1); the energy available was released slowly, as it equilibrated with the N-NH_3 released by protein from cottonseed. As a consequence, the balance of N-NH_3 was similar among treatments. An optimum N-NH_3 level cannot be a fixed value because microorganisms need to utilize the N-NH_3, and consequently, microbial protein production is dependent on carbohydrate fermentation rates (energy availability) (Van Soest, 1994).

Dietary lipid supplementation may influence SCFA production, depending on the composition of the basal diet and the amount of lipid added (Benchaar et al., 2012; Chelikani et al., 2004; Shingfield et al., 2008). In the present study, propionate had similar production levels; however, acetate and butyrate had lower production when cottonseed was included. Propionate is used for glucose production, and it is also the major substrate of hepatic gluconeogenesis. The propionate pathway is more efficient than that of acetate. To convert an mol of glucose in acetate, 251 kcal mol^{-1} is necessary; whereas, only 60 kcal mol^{-1} is needed to convert in propionate (Kozloski, 2002). Additionally, the acetate pathway releases H_2, which causes a simultaneous increase in propionate and a decrease in acetate. This balance contributes to improving the energy efficiency of the animal and decreases free H_2 in the rumen.

Given the results observed in this study, it is possible to conclude that the primary effects of administering cottonseed into the diet are much more dependent on lipids than fiber, as previously illustrated by the decreases in acetic and butyric acids. If the effects of fiber in cottonseed were more prominent than those of lipid effects, we would expect an increase in acetic and butyric acid production, which was not observed here. Conversely, if the effects of cottonseed in rumen fermentation were more dependent on its lower NFC content, a decrease in propionic acid production would be expected. This decrease was not, in fact, observed, thus, it is reasonable to conclude that the carbohydrate type in cottonseed composition is less important than its lipid content.

Acetate and butyrate have cellulose and hemicellulose as major precursors. These fibrous carbohydrates have a slower digestion rate in the rumen than do the non-fiber-carbohydrates. Consequently, fibrous carbohydrate products are released slowly. Cottonseed inclusion increased cellulose and hemicellulose content in the diet, resulting in slower acetate and butyrate production vs. the Control. Sullivan et al. (2005) reported that both the acetate molar proportion and the acetate to propionate ratio decreased linearly as dietary fatty acids (FA) from the whole cottonseed increased.

Moreover, propionate has NFC as its major precursor. This study demonstrated that, although cottonseed diets had lower NFC, they had higher lipids. In the rumen, lipids cannot ferment, rather they are hydrolyzed. The products of lipid hydrolyzation are fatty acids and glycerol, which are quickly fermented by microorganisms and then mostly converted into propionate. Propionate, therefore, had a similar production rate with different precursors.

In the present study, vitamin E had no effect on SCFA. This is in disagreement with in vitro studies. Naziroğlu et al. (2002) supplemented 0.4 mg and 0.8 mg of vitamin E in 100 mL rumen fluid and observed that this inclusion increased acetic and propionate concentrations and decreased butyrate concentration. Hou et al. (2013) reported that supplementing vitamin E at 2 mg per 80 mL in incubation liquid increased in vitro rumen acetate and total SCFA production, as well as decreasing butyrate production. Wei et al. (2015) added 0, 7.5, 15, 30 IU vitamin E kg^{-1} of DM in an in *vitro trial* and observed that supplementing vitamin E not only increased the total SCFA and propionate, but also tended to increase acetate production ($p = 0.084$). This is simply an observation. Our study was an *in vivo* trial and did not reflect these *in vitro* results.

Considering then the level of vitamin E used (as recommended by previous studies), there were no safety concerns about the real vitamin E intake for the cows. The vitamin E was mixed into the concentrate and offered to the animals; thus, the amount of vitamin E cannot have been enough to cancel out or minimize oxidative stress on ruminal microorganism. Further research is certainly necessary to confirm the levels of vitamin E on minimizing oxidative stress in the rumen in order to facilitate positive effects in the ruminal fermentation process. The mechanism(s) by which antioxidant compounds improve the toxic effect of excessive unsaturated fatty acids has not been well documented and may vary according to the antioxidant compound and type of fat (Vázquez-Añón and Jenkins, 2007).

Our experimental results indicate that enteric methane emissions decreased significantly (by approximately 42 %) in animals on the cottonseed diets compared to the basal diet. The reduction in intensity of CH_4 emissions (as g of CH_4 kg^{-1} of DM) with the cottonseed diet was higher in our study than in the studies of both Martin et al. (2008) and Beauchemin et al. (2009b), in which the deceases were only 27 % and 18 %, respectively. This difference may be due to the high lipid content in our cottonseed diet compared with those of the other authors.

Our results suggest that, for each percentage of lipids added in the cows' diet, the result was a reduction of 8 % in the methane emission. This result was higher than that found by Patra et al. (2014). In a meta-analysis study, they concluded that, for each percentage of lipid added, the result was a reduction of 4 % in methane emissions. Again, this difference is likely due to the higher oil level used in the present experiment. According to Beauchemin et al. (2008), there exists a linear relationship between the percentage of lipids added and the reduction in CH_4 emissions.

The inhibitory effect of lipids on enteric methane emissions has been widely reported in studies, although the extent of inhibition appears to be variable (Brask et al., 2013; Grainger and Beauchemin, 2011). Several mechanisms have been recognized for their inhibitory effects of lipids on methane emissions. Lipids inhibit methanogenesis by reducing the metabolic activity and the numbers of ruminal methanogens and protozoa.

The biohydrogenation of unsaturated fatty acids is an alternative hydrogen sink, and it decreases free hydrogen in the rumen (Beauchemin et al., 2009a; Johnson and Johnson, 1995). However, according to Mills et al., 2001, only between 1 and 3 % of H_2 is taken up for biohydrogenation, which consists of H saturation of the double bonds of unsaturated fatty acids.

Lipids are not fermented in the rumen, and, thus, they do not produce a surplus of free hydrogen. Among the SCFA, acetate production releases the highest amount of ruminal free hydrogen; therefore, by decreasing the acetate production, the free hydrogen concentration will be reduced. Consequently, methane production could decline directly, either by reducing the methanogen numbers and/or activity - or indirectly - by production and/or concentration of less hydrogen, when higher cottonseed levels are included in the diets.

The pattern of SCFA production and methane emissions during the day increased rapidly after feeding and then decreased slowly until the next feeding. SCFA and methane production peaked immediately after post-feeding (Figure 2), as had been previously demonstrated by Mao et al. (2010).

Gastrointestinal energy released

Notably, energy release in the rumen is related to SCFA and methane production and their heating powers, as well as to the ruminal solid mass amount. Cottonseed treatments had 32 % higher ruminal solid mass vs. the Control. Ruminal propionate production is an expression of ruminal mass to equate to other similar treatment values. Table 2 demonstrates that the highest ruminal solid mass with cottonseed treatments induced a higher propionate release in the rumen. This was in the order of 42 %, when expressed as MJ d^{-1} vs. the Control. Acetate production from the cottonseed treatments was 34 % lower when expressed as ruminal mass (Table 2) vs. the Control; however, the higher ruminal solid mass for the cottonseed treatments cancels out this lower acetate production (and thereby the acetate released in the rumen) when it is expressed as MJ d^{-1}. This was similar among all the treatments.

In spite of higher ruminal solid mass from the cottonseed treatments, there was an expressive reduction in the butyrate production - in the order of 47 % when expressed as ruminal mass (Table 2). This was enough to further an approximate decrease of 32 % in energy release in the rumen, when expressed as MJ d^{-1} vs. the Control. A similar situation was observed for the methane, in which a lower ruminal methane production, in the order of 42 % when expressed as ruminal mass (Table 2), was enough to further a decrease in ruminal energy release of 26 %, when expressed as MJ d^{-1} for the cottonseed treatments as compared to the Control.

Changes in the energy release site did occur. In the rumen of cows fed cottonseed, less energy was released as methane and butyrate (expressed as MJ d^{-1}) in the percentages of GE or DE; therefore, more energy was released in the intestine due to energy released in the feces. This was similar among all the treatments. The energy release in the intestine (expressed in MJ d^{-1}) was 57 % higher for the cows fed cottonseed compared to cows fed the Control.

Methane production in cattle typically accounts for 5 to 6 % of GEI (Johnson and Ward, 1996); however, values of 2 to 12 % (Johnson and Johnson 1995) have been reported for some diets. Martin et al. (2008), using linseed as a lipid source for lactating cows, maintained a dietary level of 7 % for lipids and observed that CH_4 output represented 6 % of GEI. In the current study, our data are in agreement with these authors; our CH_4 emissions averaged 6 % of GEI in the cottonseed treatments and 10 % of GEI in the Control treatment. Since methane represents a loss of dietary energy, a significant reduction in gas emissions was observed for the cows fed cottonseed, and this indicated that these animals were more efficient in utilizing dietary energy than those in the Control.

Conclusions

Cottonseed inclusion in cattle diets can be considered as a methane mitigation strategy. Changes in the ruminal products, such as lower acetate, butyrate, and methane production are favorable with the use of cottonseed in cattle diets. The inclusion of vitamin

E in vivo studies may not improve ruminal fermentation in cows.

Acknowledgments

The authors thank the University of São Paulo employers, professors, trainees and São Paulo Research Foundation (FAPESP) for the grant number: 2013/11983-5. FAPESP provided funding through scholarship, experimental design, laboratory analysis and English language review.

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