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Influences of Feed Additives on *in vitro* Volatile Fatty Acid Production

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Abstract: This study investigated effects of the feed additives nitrate and fumarate alone or in combination on *in vitro* Volatile Fatty Acid (VFA) production. Rumen fluid was collected from Holstein-Friesian cattle averaging 650 kg in BW (body weight) offered 11.4 kg d^{-1} per animal of concentrate diet containing equal amounts of soybean meal, whole cottonseed and ground corn once a day with free access to grass hay. The feed additives used were nitrate, fumarate and a nitrate-fumarate combination. Concentrations of VFAs were measured using Gas Chromatography. The results of the study revealed that nitrate decreased (p<0.05) individual VFA production compared to the control and all other feed additives. The addition of fumarate had no effect on VFA production. The addition of the nitrate-fumarate combination decreased (p<0.05) butyrate and iso-butyrate production compared to the control and had no effect on all other individual VFAs. The addition of nitrate also increased (p<0.05) the acetate to propionate ratio compared to the control, fumarate or nitrate-fumarate combination. The addition of fumarate and nitrate-fumarate had no effect on the acetate to propionate ratio compared to the control. The current study suggests that nitrate alone may have an adverse effect on microbial fermentation if VFA production is significantly decreased. Therefore, the addition of the nitrate-fumarate combination may reduce the potential negative effects of nitrate on VFA production.

Keywords: *in vitro* Fermentation, Volatile Fatty Acids, Nitrate, Fumarate

Introduction

Nutritional supplements for dairy cows have been shown to influence rumen fermentation. There is also an increasing interest in the use of feed additives that positively impact or alter rumen microbial populations and Volatile Fatty Acid (VFA) profiles. Therefore, it is important to highlight the potential benefits of feed additives that can maintain VFA production and enhance animal efficiency. Dietary manipulation can have profound effects on the profiles and concentrations of VFA in the rumen. Boadi *et al*. (2004) reported that there is a direct relationship between VFA concentration and methane production with acetate, propionate and butyrate noted as the three major VFAs produced in the rumen. Microorganisms in the rumen are responsible for the fermentation of carbohydrates in the diet to VFAs, which the animal uses as energy sources (Boadi *et al*., 2004). The major VFAs produced during fermentation have different functions in the animal (Boadi *et al*., 2004); the non-glucogenic fatty acids (acetate and butyrate) are used for milk fat and long-chain fatty acid synthesis, propionate on the other hand is used for glucose synthesis (Morvay *et al*., 2011). The production of acetate and butyrate leads to free Hydrogen (H_2) to be utilized in the rumen for various processes. Since there is a direct relationship between VFA and CH_4 production it is important to explore how dietary manipulation can shift VFA production and reduce the availability of H_2 for methanogenesis. The addition of organic acids like fumarate have been shown to shift VFA profiles in favor of propionate by enhancing the succinate-propionate pathway (Araújo *et al*., 2011) and prevent the availability of H_2 in the rumen for CH_4 synthesis

(Boadi *et al*., 2004). Several studies (Mao *et al*., 2008; Abdl-Rahman *et al*., 2010; Wood *et al*., 2009; Yu *et al*., 2010) have reported that addition of fumarate stimulated production of propionate in ruminants. It has also been reported (Ungerfeld *et al*., 2007) fumarate can act as an electron acceptor by reducing the availability of Hydrogen (H_2) and increase acetate production in the rumen (Ungerfeld *et al*., 2007).

Electron acceptors like nitrate and other nitro-compounds have been investigated as feed additives *in vitro* (Anderson and Rasmussen, 1998; Bozic *et al*., 2009) and *in vivo* (Hulshof *et al*., 2012; Van Zijderveld *et al*., 2010) for their potential to reduce CH_4 production. Nitrate has been shown to increase the production of acetate while decreasing propionate production (Bozic *et al*., 2009; Zhou *et al*., 2011), however at high concentrations nitrate had no effect on acetate production (Zhou *et al*., 2011). The use of nitrate in the ruminant diet also raises the risk of methemoglobinemia unless the ruminant is allowed to adapt to nitrate by slowly introducing it to the diet (Van Zijderveld *et al*., 2010). Normally, fumarate is converted to succinate and then to propionate, while nitrate is reduced to nitrite that leads to the production of Ammonia (NH_3) in the rumen and both reactions can reduce the availability of H_2 for CH_4 synthesis. Therefore, fumarate consumes H_2 for propionogenesis while nitrate consumes H_2 for its own reduction to NH_3. The risk of nitrate toxicity has led to the investigation of combining feed additives with nitrate to reduce this undesirable effect by increasing the rate of nitrate disappearance *in vivo* (Sar *et al*., 2004) and *in vitro* (Anderson and Rasmussen, 1998; Iwamoto *et al*., 1999). Iwamoto *et al*. (1999) reported that addition of fumarate to nitrate increased the rate of nitrate and nitrite reductions while propionate and acetate production increased. The additive effect of fumarate and nitrate on *in vitro* VFA production is not yet clearly understood.

In vitro rumen fermentation techniques provide knowledge on the fermentation process and how the different feed additives may alter conditions that can affect digestion (Castro-Montoya *et al*., 2012). Although *in vitro* fermentation methods are not substitutes for *in vivo* rumen fermentation they can and have been used extensively to elucidate the processes performed by rumen microorganisms and the factors that affect them. It is evident that fumarate increases propionate concentrations, while nitrate reduces individual and total VFA concentrations indicating a suppression of fermentation. Therefore it was hypothesized that a nitrate-fumarate combination would increase propionate production and reduces the potential negative impact of nitrate alone on VFA production. The current study was conducted to quantitatively and qualitatively determine the effects of addition of nitrate, fumarate and the nitrate-fumarate combination on *in vitro* VFA production.

Materials and Methods

Animal, Sampling Method and in vitro Techniques Used in the Study

The experiment was approved by the North Carolina Agriculture and Technical State University Institutional Review Board. The experiment consisted of two rumen-cannulated Holstein-Friesian cattle (steer and dry cow) average BW = 650 kg fed a daily feed allowance of 11.4 kg/d per animal of equal amounts of soybean meal, whole cottonseed and ground corn once a day and offered free grass hay ad libitum when grass was not available in the winter and grazed on forage during the spring and summer months. The diet was formulated to meet the nutrient requirements of both animals.

Rumen contents were obtained by direct sampling from the rumen (Bar Diamond, Idaho, US) at 09.00 h prior to feeding. The experiment was conducted from January 2012-August 2012. The mixed rumen contents were removed and strained through eight layers of cheesecloth into a pre-warmed thermos to a volume (1L) to minimize oxygen in the headspace and primed with CO_2; sealed tightly and transported to the university laboratory for analysis. Prior to start of the experiment feed samples for the experiment were collected from the feed trough and oven dried at 70°C for 24 h for lab analysis. The *in vitro* method for the determination of VFA production was carried out according to the first stage *in vitro* digestibility procedure of Tilley and Terry (1963). The artificial saliva was prepared overnight at 39°C according to the procedures McDougall (1948).

Chemical Analysis of Feed

Table 1 shows the chemical composition of feed. The chemical composition was determined after samples were oven dried 70°C for 24 h. Feed samples were ground into 1-mm screen before analysis. Subsequently, feed samples Dry Matter (DM) was determined in duplicate by oven drying at 100°C for 4 h. The organic matter (ash) content was determined by incineration in a muffle-furnace at 550°C for 1 h, weighed and percent ash was calculated. Neutral Detergent Fiber (aNDF) was determined with the filter bag method using α-amylase and sodium sulfite and Acid Detergent Fiber analysis (ADF) was also determined according to Van Soest *et al*. (1991) (ANKOM Technology, USA). Crude Protein was determined on the TruSpec CN (Leco Corporation, Michigan, US).

Determination of Volatile Fatty Acid Concentrations

Sample preparation included collecting 30mL aliquots of rumen fluid from *in vitro* fermentation flasks in 50 mL conical tubes, centrifuged at 4000 rpm for 15 minu at 4°C and stored at -20°C until analysis.

Table 1. Chemical composition of experimental diet

Dry matter (%)	94.7
Total Ash (%)	12.5
Crude protein[a] (%)	12.4
aNDF %	56.3
ADF%	29.7
Ca (ppm)	23.4
K (ppm)	46.3
Mg (ppm)	10.0
P (ppm)	23.0

Samples were thawed at room temperature and 5.0 mL of rumen fluid from each vial was transferred by pipette into a 15 mL centrifuge tube. Subsequently, 1 mL of a 25% aqueous meta-phosphoric acid was added to each vial, vortexed to precipitate any proteins present, than allowed to stand for 30 min at room temperature (Cottyn and Boucque, 1968). Next, 1 mL of the mixture was transferred by pipette from each 15 mL tube into separately labeled GC vials for analysis.

Standards for the VFAs were prepared using reagent grade acids (Sigma-Aldrich, St. Louis, US). Individual acid retention times (rt) were determined by GC. Next, 0.50 mL of each acid (6) was transferred by pipette into 10 mL vials for a total volume of 3.0 mL. The weight of each acid was determined using their specific gravity and volume. The concentration of each acid was calculated by dividing each weight by the 3.0 mL volume (after pre-adjusting the volume to 100 mL basis) and then converted to ppm (mg/L). The VFA profiles and concentrations of acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate were determined on a Thermo Fisher Trace Ultra gas chromatograph with a Tri-Plus auto sampler coupled to a flame ionization detector. The GC parameters were: Column: Nukol, 15 m, 0.53 mm id, 0.50 um film thickness, Injector and detector temperature: 210°C, Carrier: Helium, constant flow 1.0 mL min^{-1}, Gas flows: Air 350 mL min^{-1}, Hydrogen 35 mL min^{-1} and Nitrogen 30 mL min^{-1}, Oven parameters: start 70°C hold 4 min, ramp from 70°C at 6°C/min to 200°C hold 1 min. The VFAs and their concentrations were identified by comparing the sample rt with the standard rt and quantified using peak area measurements.

Statistical Analysis

Data for the *in vitro* VFA production was analyzed using SAS (SAS version 9.3, SAS Institute Inc., Cary, NC). The analysis was conducted in a randomized complete block design with animals as blocks and 3 replications of the feed additive treatments per block. Volatile fatty acid production data was analyzed with PROC MIXED of SAS, using a mixed model with animals as a random effect and feed additive treatments as fixed effects (Littell *et al*., 2006; SASI, 2012). All treatment effects were declared significant at ($p<0.05$). All treatment effects ($p>0.06$) and ($p<0.10$) were declared trends.

Results

Effect of Feed Additives on the Acetate to Propionate Ratios

The addition of nitrate increased ($p<0.05$) acetate to propionate ratios compared to the control, fumarate and nitrate-fumarate combination (Fig. 1). The addition of fumarate and the nitrate-fumarate combination had no effect on the acetate to propionate ratios compared to the control. There was no significant difference between the addition of fumarate and the nitrate-fumarate combination. Therefore only the addition of nitrate had an effect by increasing the acetate to propionate ratios.

Effect of Feed Additives on Volatile Fatty Acid Production

Figure 2 shows the effects of feed additives on individual VFA production. The addition of nitrate reduced ($p<0.05$) individual VFAs compared to the control, fumarate and nitrate-fumarate combination. The addition of fumarate and the nitrate-fumarate combination had no effect on acetate production compared to the control and were not statistically different when compared to each other. The addition of nitrate reduced ($p<0.05$) propionate, while the addition of fumarate and nitrate-fumarate combination had no effect on propionate production compared to the control. The addition of nitrate and the nitrate-fumarate combination also reduced ($p<0.05$) butyrate, while the addition of fumarate had no effect on butyrate production compared to the control. The addition of nitrate and the nitrate-fumarate combination decreased ($p<0.05$) iso-butyrate compared to fumarate and the control. Nitrate and the nitrate-fumarate combination decreased ($p<0.05$) valerate and iso-valerate production compared to the control. Fumarate had no effect on valerate and iso-valerate production. Therefore, while the addition of nitrate decreased all VFA production, addition of fumarate had no effect on individual VFA production compared to the control. However the nitrate-fumarate combination numerically increased propionate production ($p<0.10$). The addition of nitrate-fumarate also had no effect on acetate, valerate and iso-valerate concentrations.

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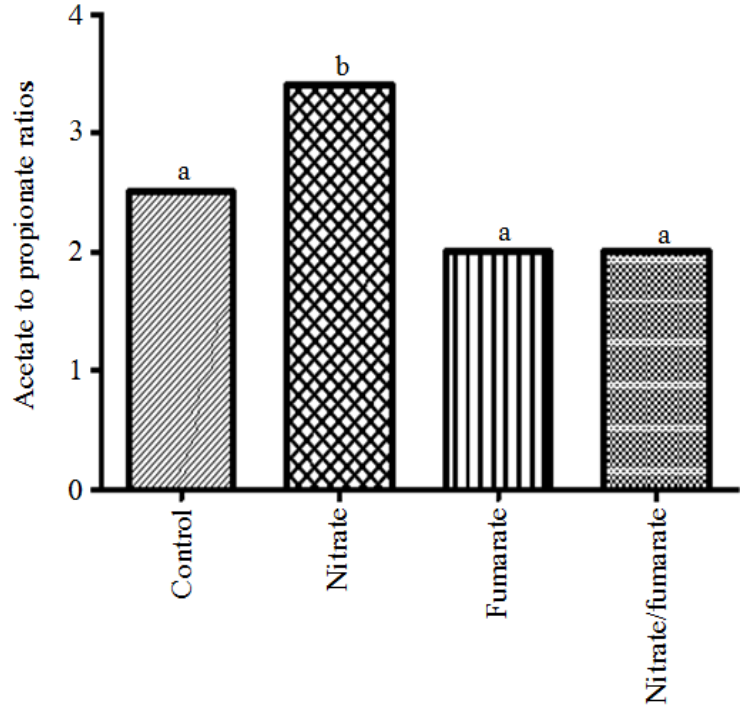

Fig. 1. Acetate to Propionate Ratios (ppm). Bars with different superscripts differ ($p<0.05$)

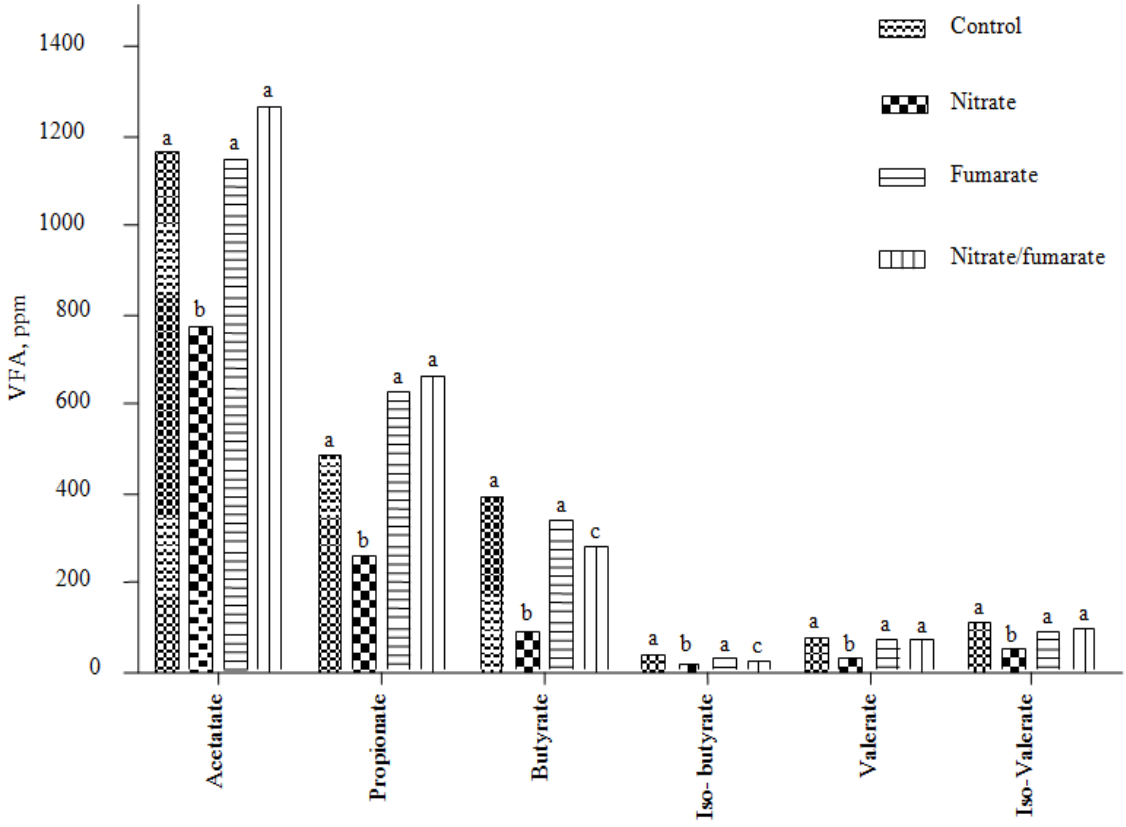

Fig. 2. *in vitro* VFA production (ppm). Bars with different superscripts differ ($p<0.05$)

Discussion

The main focus of this study was to investigate the individual and combined effects of nitrate and fumarate on *in vitro* VFA production. In the current study the addition of nitrate significantly reduced *in vitro* VFA production. Similarly, (Iwamoto *et al*., 1999; Zhou *et al*., 2011) reported that nitrate reduced total *in vitro* VFA production and indicated that fermentation was possibly suppressed. The addition of fumarate in the current study had no effect on individual VFA production or the acetate to propionate ratios. These findings contradict with reports from (Bayaru *et al*., 2000; Carro and Ranilla, 2003; Beauchemin and McGinn, 2006a; 2006b; Yu *et al*., 2010) who indicated that the addition of fumarate increased total VFAs concentrations. However, others (Abdl-Rahman *et al*., 2010; Mao *et al*., 2008) reported fumarate had no effect on total VFA concentrations. The addition of fumarate in the current study also had no effect on acetate production and this could possibly be explained by the fact that fumarate, even at low concentrations is thermodynamically favored to be reduced to acetate (Ungerfeld *et al*., 2007) and in our current study it could possibly be reduced by fumarate-reducing bacteria to succinate (Iwamoto *et al*., 1999) and then to propionate and acetate. It was also observed in the current study that addition of nitrate increased the acetate to propionate ratios. This increased acetate to propionate ratio can best be explained by the fact that nitrate effectively reduced propionate by as much as 46% and reduced acetate by only 34%. Therefore, although nitrate reduced acetate, reduction of propionate was to a much greater extent. The decrease in VFA concentrations by the addition of nitrate in the present study could most likely be due to suppression of microbial fermentation (Iwamoto *et al*., 1999). On the other hand, fumarate has been shown to minimize the suppression of microbial fermentation and enhance nitrate/nitrite reduction (Iwamoto *et al*., 1999) when combined with nitrate, which in effect reduces the adverse effects caused by nitrate on microbial fermentation. The combination of nitrate with fumarate in the present study had variable effects on individual VFA production for example only butyrate and iso-butyrate concentrations were reduced and there was no effect on all other individual VFAs. Iwamoto *et al*. (1999) reported that the addition of fumarate and nitrate increased propionate production. In the current study the addition of nitrate and fumarate had no effect on propionate production, which contradicts with Iwamoto *et al*. (1999). However, in the current study we used a 1:1 nitrate to fumarate ratio where as Iwamoto *et al*. (1999) used 1:1.5 and 1:3 ratios. The addition of the nitrate-fumarate combination had no effect on the acetate to propionate ratios. This result could possibly be due to the fact that the nitrate-fumarate combination had no effect on the production of acetate and propionate. Therefore, the results from the experiment could explain why the nitrate-fumarate combination had no effect on the acetate to propionate ratios. The effect of the addition of the nitrate-fumarate combination on VFA production in the current study shows that there was no effect on propionogenesis. The fact that the addition of nitrate consistently reduced individual and total VFA may indicate that the addition of nitrate led to the accumulation of nitrite that inhibits microbial growth (Iwamoto *et al*., 1999; Zhou *et al*., 2011). Nitrate reducing bacteria like *Selenomonas ruminantium*, *Veillonella parvula* and *Wollinella succinogenes* can also reduce fumarate (Iwamoto *et al*., 1999) as an energy source. Therefore, it is assumed that the addition of nitrate may have placed these bacteria in unfavorable conditions due to the accumulation of nitrite (Iwamoto *et al*., 1999) whereas the addition of fumarate could possibly provide these bacteria with sources of energy for growth.

Conclusion

The current study suggests that nitrate alone may reduce *in vitro* VFA production, while the additive effect of nitrate and fumarate reduced the potential negative effect of nitrate on VFA production. The feed additive combination was also effective in reducing the availability of a precursor for methane production, butyrate in particular. The use of these feed additives has the potential to improve animal efficiency through the reduction of energy loss from methane production and may not negatively impact rumen fermentation. However, further study is required prior to using this feed additive combination as a dietary strategy to shift VFA production in favor of lower methane production and improved animal productivity.

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Author's Contributions

A'ja V. Duncan: A doctoral USDA National Needs Fellow completed this study as a partial fulfillment towards her degree.

Abraham Woldeghebriel: Assisted with the study design and served as the research advisor for this research study.

Mulumebet Worku: Served as the Principle Investigator for USDA National Needs Funding and also assisted with the study design.

Courtney Privott: Was an undergraduate research student who assisted with sample preparation and collection.

John Carver and Bryce Holmes: Assisted with sample analysis.

Jim Henson: Assisted with all statistical analysis.

Ethics

The authors have declared there are no ethical issues that may arise after the publication of this manuscript.

References

Anderson, R.C. and M.A. Rasmussen, 1998. Use of a novel nitrotoxin-metabolizing bacterium to reduce ruminal methane production. Biores. Technol., 64: 89-95. DOI: 10.1016/s0960-8524(97)00184-3

Abdl-Rahman, M.A., Sawiress, F.A.R. and A.M. Abd El-Aty, 2010. Effect of sodium lauryl sulfate-fumaric acid coupled addition on the *in vitro* rumen fermentation with special regard to methanogenesis. Vet. Med. Int., 2010: 7-7. DOI: 10.4061/2010/858474

Araújo, W.L., A. Nunes-Nesi and A.R. Fernie, 2011. Fumarate: Multiple functions of a simple metabolite. Phytochemistry, 72: 838-843. DOI: 10.1016/j.phytochem.2011.02.028

Bayaru, E., S. Kanda, T. Kamada, H. Itabashi and S. Andoh *et al*., 2000. Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle roughage alone. Anim. Sci. J., 72: 139-146.

Boadi, D., C. Benchaar, J. Chiquette and D. Masse, 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Canad. J. Anim. Sci., 84: 319-335. DOI: 10.4141/A03-109

Beauchemin, K.A. and S.M. McGinn, 2006a. Effects of various feed additives on the methane emissions from beef cattle. Int. Cong. Series, 1293: 152-155. DOI: 10.1016/j.ics.2006.01.042

Beauchemin, K.A. and S.M. McGinn, 2006b. Methane emissions from beef cattle: Effects of fumaric acid, essential oil and canola oil. J. Anim. Sci., 84: 1489-1496. PMID: 16699105

Bozic, A.K., R.C. Anderson, G.E. Carstens, S.C. Ricke and T.R. Callaway *et al*., 2009. Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, Lauricidin® and the Hawaiian marine algae Chaetoceros on ruminal fermentation *in vitro*. Biores. Technol., 100: 4017-4025. DOI: 10.1016/j.biortech.2008.12.061

Cottyn, B.G. and C.V. Boucque, 1968. Rapid method for the gas-chromatographic determination of volatile fatty acids in rumen fluid. J. Agric. Food Chem., 16: 105-107. DOI: 10.1021/jf60155a002

Carro, M.D. and M.J. Ranilla, 2003. Influence of different concentrations of disodium fumarate on methane production and fermentation of concentrate feeds by rumen micro-organisms *in vitro*. Brit. J. Nutrit., 90: 617-623. DOI: 10.1079/BJN2003935

Castro-Montoya, J., S. De Campeneere, G. Van Ranst and V. Fievez, 2012. Interactions between methane mitigation additives and basal substrates on *in vitro* methane and VFA production. Animal Feed Sci. Technol., 176: 47-60. DOI: 10.1016/j.anifeedsci.2012.07.007

Hulshof, R.B.A., A. Berndt, W.J.J. Gerrits, J. Dijkstra and S.M. Van Zijderveld *et al*., 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. J. Anim. Sci., DOI: 10.2527/jas.2011-4209

Iwamoto, M., N. Asanuma and T. Hino, 1999. Effects of nitrate combined with fumarate on methanogenesis, fermentation and cellulose digestion by mixed ruminal microbes *in vitro*. Japanese Soc. Anim. Sci., 70: 471-478. DOI: 10.2508/chikusan.70.471

Littell, R.C., G.A. Milliken, R.D. Wolfinger and O. Schabenberger, 2006. SAS for Mixed Models. 2nd Edn., SAS Institute, Cary, N.C., ISBN-10: 1599940787, pp: 828.

Mcdougall, E.I., 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J., 43: 99-109. PMID: 1274641

Mao, S.Y., G. Zhang and W.Y. Zhu, 2008. Effect of disodium fumarate on ruminal metabolism and rumen bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. Anim. Feed Sci. Technol., 140: 293-306. DOI: 10.1016/j.anifeedsci.2007.04.001

Morvay, Y., A. Bannink, J. France, E. Kebreab and J. Dijkstra, 2011. Evaluation of models to predict the stoichiometry of volatile fatty acid profiles in rumen fluid of lactating Holstein cows. J. Dairy Sci., 94: 3063-3080. DOI: 10.3168/jds.2010-3995

Sar, C., B. Santoso, B. Mwenya, Y. Gamo and T. Kobayashi *et al*., 2004. Manipulation of rumen methanogenesis by the combination of nitrate with β1-4 galacto-oligosaccharides or nisin in sheep. Anim. Feed Sci. Technol., 115: 129-142. DOI: 10.1016/j.anifeedsci.2004.01.006

SASI, 2012. SAS/STAT 12.1 User's Guide. 1st Edn., SAS Institute, North Carolina, ISBN-10: 1612905099, pp: 772.

Tilley, J.M.A. and R.A. Terry, 1963. A two-stage technique for the *in vitro* digestion of forage crops. Grass Forage Sci., 18: 104-111. DOI: 10.1111/j.1365-2494.1963.tb00335.x

Ungerfeld, E.M., R.A. Kohn, R.J Wallace and C.J. Newbold, 2007. A meta-analysis of fumarate effects on methane production in ruminal batch cultures. J. Animal Sci., 85: 2556-2563. DOI: 10.2527/jas.2006-674

Van Soest, P.J., J.B. Robertson and B.A. Lewis, 1991. Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci., 74: 3583-3597. DOI: 10.3168/jds.S0022-0302(91)78551-2

Van Zijderveld, S.M., W.J.J. Gerrits, J. Dijkstra, J.R. Newbold and R.B.A. Hulshof *et al*., 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. J. Dairy Sci., 94: 4028-4038. DOI: 10.3168/jds.2011-4236

Van Zijderveld, S.M., W.J.J. Gerrits, J.A. Apajalahti, J.R. Newbold and J. Dijkstra *et al*., 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J. Dairy Sci., 93: 5856-5866. DOI: 10.3168/jds.2010-3281

Wood, T.A., R.J. Wallace, A. Rowe, J. Price and D.R. Yáñez-Ruiz *et al*., 2009. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. Animal Feed Sci. Technol., 152: 62-71. DOI: 10.1016/j.anifeedsci.2009.03.006

Yu, C.W., Y.S. Chen, Y.H. Cheng, Y.S. Cheng and C.M.J. Yang *et al*., 2010. Effects of fumarate on ruminal ammonia accumulation and fiber digestion *in vitro* and nutrient utilization in dairy does. J. Dairy Sci., 93: 701-710. DOI: 10.3168/jds.2009-2494

Zhou, Z., Z. Yu and Q. Meng, 2011. Effects of nitrate on methane production, fermentation and microbial populations in *in vitro* ruminal cultures. Biores. Technol., 103: 173-179. DOI: 10.1016/j.biortech.2011.10.013