

EFFECT OF ESSENTIAL ORANGE (*CITRUS SINENSIS* L.) OIL ON RUMEN MICROBIAL FERMENTATION USING *IN VITRO* GAS PRODUCTION TECHNIQUE

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ABSTRACT

The aim of the current study was to evaluate the effect of increasing doses of essential orange oil *in vitro* gas production, volatile fatty acid (VFA), methane, ammonia production, metabolisable energy (ME), organic matter digestibility (OMD), true dry matter digestibility (TDMD) and neutral detergent fiber digestibility (NDFD). Gas productions of soybean meal were measured at 0, 3, 6, 12, 24, 48, 72 and 96 h incubation times in the presence (100, 200, 400, 800 and 1200 mg/L) and in the absence of essential orange oil. The gas production kinetics were estimated using the non-linear exponential equation $y = A(1 - \exp^{-ct})$. The supplementation of essential orange oil significantly ($P < 0.001$) decreased fermentation parameters and estimated parameters such as the potential gas production (A), ME, TDMD, OMD, NDFD, total VFA, methane and ammonia productions. The mean reductions in A, ME, TDMD, NDFD, OMD, total VFA, methane and ammonia productions were 0.022, 0.033, 0.00209, 0.0197, 0.0213, 0.0414, 0.0207, 0.0093 units per mg essential orange oil supplementation respectively. The current study revealed that essential orange oil had significant anti-microbial activity causing an inhibition of the overall fermentation process. Therefore, before large scale implementation, further investigations are required to determine the effect of essential orange oil on voluntary food intake, animal performance and the profitability of the supplementation *in vivo*.

Key words: Orange, *Citrus sinensis* (L) Osbeck, Essential oil, *In vitro* gas production, Digestibility, Ammonia, Methane, Volatile fatty acids.

INTRODUCTION

An antibiotic has been used to improve the nutritional efficiency through reducing the loss of energy as methane and N as ammonia from rumen (Tamminga, 1996, Dinius et al. 1976, van Nevel and Demeyer, 1977). However the use of antibiotics in European Union was banned in 2006 with the regulation (1831/2003/EC) due to risk of transferring residue into animal products.

The prohibition of the use of antibiotics as a growth promoter in animal feeds led to intensified efforts in natural products as alternative means of manipulating ruminal fermentation of nutrients. Essential oils which are obtained from plants are natural products and have antibacterial, antifungal and antioxidant properties (Cowan, 1999). Recently different essential oils and their derivatives were used to manipulate the rumen metabolism (McIntosh et al. 2003, Wallace et al. 2003, Newbold et al. 2004, Castillejos et al. 2005, Benchaar et al. 2006, Garcia et al. 2007, Kamalak et al. 2011). However, the effect of essential oils and their derivatives on rumen bacteria and ruminal fermentation are not consistent and are variable (Busquet et al. 2006, Castillejos et al. 2006). There are discrepancies among studies which were attributed to difference in type and doses of essential oils (Busquet et al. 2006) and differences in technique used in these experiments (Fraser et al. 2007).

Recently several researchers carried out some experiments to determine the effect of orange essential oil from *Citrus sinensis* and *Citrus reticulata* on *in vitro* gas production and fermentation end products (Benchaar et al. 2007; Sobhy and Samir, 2010). However there is still limited research related to the effects of orange essential oil on *in vitro* gas production and fermentation end products. Therefore the aim of the current experiment was to determine the effect of essential orange oil on *in vitro* gas production, total VFA, methane, ammonia production, ME, OMD, TDMD and NDFD.

MATERIALS AND METHODS

Chemical analysis: Dry matter content of soybean meal sample milled through a 1 mm sieve was determined by drying the samples at 105°C overnight and organic matter was determined by igniting the samples in muffle furnace at 525°C for 8 h. Nitrogen (N) content was measured by the Kjeldahl method (AOAC, 1990). Crude protein was calculated as $N \times 6.25$. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) of soybean meal sample were analyzed with the ANKOM fiber analyzer using reagents described by van Soest and Wine (1967) and van Soest (1963) respectively. All chemical analyses were carried out in triplicate.

In Vitro Gas Production: Soybean meal samples milled through a 1 mm sieve were incubated *in vitro* rumen fluid

in calibrated glass syringes following the procedures of Menke and Steingass (1988). Rumen fluid was obtained from three fistulated sheep fed twice daily with a diet containing alfalfa hay (60%) and concentrate (40%). 0.200 gram dry weight of samples was weighed in triplicate into calibrated glass syringes of 100 mL in the presence (100, 200, 400, 800 and 1200 mg/L) and in the absence of essential orange (*Citrus sinensis* L.) oil from Sigma-Aldrich. The syringes were prewarmed at 39°C before the injection of 30 mL rumen fluid-buffer mixture into each syringe followed by incubation in a water bath at 39°C. Gas production was recorded before incubation (0) and 3, 6, 12, 24, 48, 72 and 96 h after incubation. Total gas values were corrected for blank incubation. Cumulative gas production data were fitted to non-linear exponential equation as follows: $Y = A(1 - \exp^{-ct})$

Where Y is gas production at time 't', A is the potential gas production (ml/200 mg DM), c is the gas production rate (h^{-1}) and t is the incubation time (h).

Metabolisable energy (ME, MJ/kg DM) and organic matter digestibility (OMD, %) of soybean meal were estimated using equations of Menke and Steingass (1988) as follows:

$$\text{ME (MJ/kg DM)} = 1.06 + 0.9991\text{GP} + 0.0595\text{CP} + 0.081\text{XA}$$

$$\text{OMD (\%)} = 1.06 + 0.157\text{GP} + 0.0084\text{CP} + 0.0220\text{EE} - 0.081\text{XA}$$

Where GP = 24 h net gas production (ml/200 mg); CP = Crude protein, XA = Ash content (%), EE = Ether extract (%)

At the end of incubation period, serum bottles were opened. The pH was determined in culture fluid and samples for VFA analysis were collected. The VFA contents of cultured fluid were determined using a gas chromatograph with a semi-capillary FFAP column (Hewlett-Packard, Wardbronn, Germany), over a temperature range of 45–230°C. Ammonia content of rumen fluid was determined by the spectrophotometric method described by Broderick and Kang (1980). Methane production was determined by equations suggested by Wolin (1960) using the VFA concentration estimated by gas chromatograph as follows:

$$\text{CH}_4 \text{ (mmol)} = (\text{A} + 2\text{B}) - \text{CO}_2, \text{CO}_2 \text{ (mmol)} = \text{A}/2 + \text{P}/4 + 1.5\text{B}$$

Where A = Acetate (mmol), P = Propionate (mmol), B = Butyrate (mmol).

In Vitro Digestibility: *In vitro* digestibility with the DAISY Incubator was carried out using rumen fluid obtained from the same fistulated sheep used in *in vitro* gas production experiment. Rumen fluid (400 ml) was transferred to digestion jar of Daisy^{II} Incubator containing 1600 ml buffer solution and heat-sealed bags containing soybean meal samples. Buffer solution was made from two other solutions (A and B) in a ratio 1:5 to obtain a final pH of 6.8 at 39°C. The soybean meal samples were incubated in triplicate at 39.5°C for 48 h in the presence (100, 200, 400, 800 and 1200 mg/L) and in the absence of essential orange oil from Sigma-Aldrich. At the end of 48 h incubation period, the bags were rinsed

with cold tap water until water is clear. The rinsed bags were dried at 105°C overnight and weighed to determine TDMD of soybean meal samples. The dried bags were placed into the ANKOM200/220 Fiber Analyzer and subjected to the normal procedure for determining NDF content of the residue in the bags to determine NDFD of soybean meal.

Statistical Analysis: One-way analysis of variance (ANOVA) was carried out to determine the effect of essential orange oil on *in vitro* gas production kinetics, total VFA, methane, ammonia production, ME, OMD, TDMD and NDFD using General Linear Model (GLM) of Statistica for windows. Significance between individual means was identified using the Duncan multiple range test. Mean differences were considered significant at $P < 0.05$. Standard errors of means were calculated from the residual mean square in the analysis of variance.

RESULTS AND DISCUSSION

The chemical composition of soybean meal used in the current study is given in Table 1.

Table 1. The chemical composition of soybean meal

Composition	%
Organic matter	92.59
Crude protein	45.01
Ether extract	1.93
Neutral detergent fiber	15.21
Acid detergent fiber	7.83

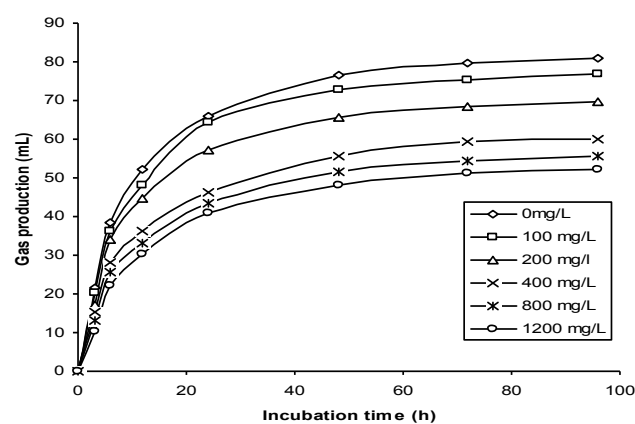


Figure 1. The effect of essential orange oil on *in vitro* gas production.

As can be seen from Figure 1 addition of essential orange oil decreased the gas production of soybean meal at all incubation times. The effect of essential orange oil on *in vitro* gas production kinetics, ME, OMD, TDMD and NDFD is given in Table 2. The

essential orange oil supplementation had significant ($P<0.001$) effect on the gas production kinetics, ME, OMD, TDMD and NDFD. There were significant

($P<0.001$) reductions in A, ME, OMD, TDMD and NDFD with increasing level of essential orange oil.

Table 2. The effect of essential orange oil on *in vitro* gas production kinetics, metabolisable energy and organic matter digestibility of soybean meal.

Parameters	Treatment (mg/L)						SEM	Sig.
	0	100	200	400	800	1200		
A	78.35a	74.60b	67.44c	57.81d	53.64e	50.60f	1.050	***
c	0.096a	0.095ab	0.097a	0.087a	0.085cd	0.071d	0.034	***
ME	11.24a	11.01a	9.88b	8.15c	7.71d	7.32e	0.119	***
OMD	77.65a	76.15a	68.99b	58.03c	55.17d	52.68e	0.757	***
TDMD	81.01a	79.57a	73.08b	67.33b	58.49d	57.37d	1.052	***
NDFD	71.73a	69.14b	61.97c	55.62d	51.37e	47.41f	0.921	***

^{a b c} Row means with common superscripts do not differ ($P>0.05$); SEM. – standard error mean; Sig. – significance level; A – potential gas production (mL), c – gas production rate (%); ME - Metabolisable energy (MJ /Kg DM); OMD - Organic matter digestibility(%), TDMD - True dry matter digestibility(%), NDFD – neutral detergent fiber analysis (%), *** $P<0.001$.

This result is in agreement with finding of Sobhy and Samir (2010) who reported that addition of essential orange oil or limonene decreased the gas production. Blummel and Orskov (1993) reported that the gas production is a sum of direct gas production as a result of fermentation (CO_2 and methane) and the in direct gas production from buffering of VFA (CO_2). Gas production is associated with volatile fatty acid (VFA) production following fermentation of substrate so the more fermentation of a substrate the greater the gas production, although the fermentation end products do influence more closely with gas production. In this experiment the decrease in total gas productions with increasing level of essential orange oil supplementation could be explained by the reduction in total VFA production. Benchaar *et al.* (2007) reported that the decrease in VFA production in rumen may yield adverse nutritional consequences if this same effect was obtained *in vivo* conditions since VFA produced through carbohydrate fermentation in the rumen is the main energy source for ruminant animals. As can be seen from Table 2 the estimated ME content of soybean meal were decreased with increasing level of essential orange oil supplementation *in vitro* condition. This result consistent with that a decrease in VFA production in rumen may yield adverse nutritional consequences.

The relationship between dose of essential orange oil supplementation and potential gas production is given in Figure 2. The mean decrease in potential gas production (A) was 0.0227 ml per mg essential orange oil supplementation. The relationship between dose of essential orange oil supplementation and metabolisable energy is given in Figure 3. The mean decrease in ME was 0.0033 units per mg essential orange oil supplementation.

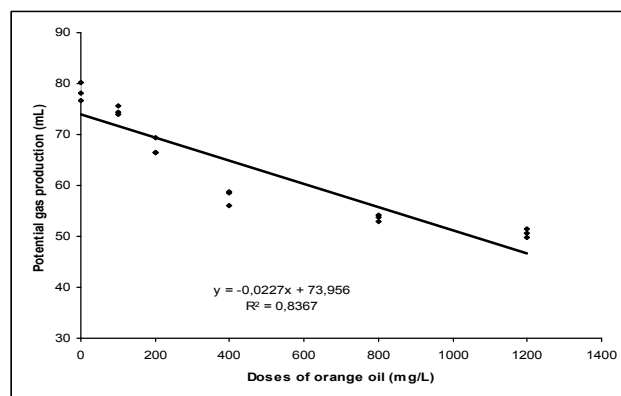


Figure 2. The relationship between dose of essential orange oil supplementation and potential gas production

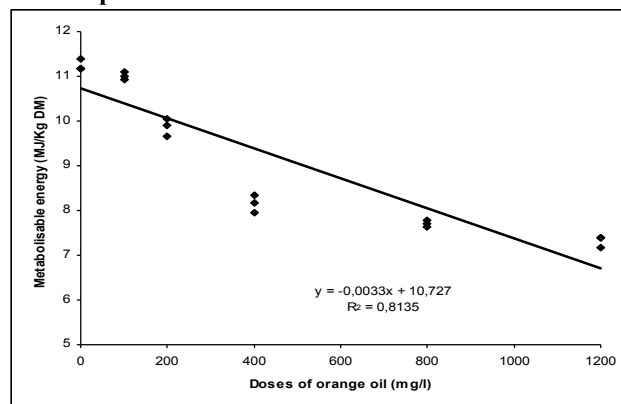


Figure 3. The relationship between dose of essential orange oil supplementation and metabolisable energy

The relationships between dose of essential orange oil supplementation and digestibility values are given in Figure 4. The mean decreases in TDMD, NDFD

and OMD were 0.00209, 0.0197 and 0.0213 digestibility units per mg essential orange oil supplementation respectively. This result is in agreement with finding of Sobhy and Samir (2010) who showed that essential orange oil supplementation significantly reduced the dry matter and organic matter digestibility. On the other hand, this result obtained in the current study is not in agreement with finding of Benchaar *et al.* (2007) who found that supplementation of essential sweet orange oil at 200 mg/L did not affect the *in vitro* dry matter digestibility. The differences among two studies can be attributed to the techniques used. In the current experiment *in vitro* gas production technique was used whereas Benchaar *et al.* (2007) used dual-flow continuous culture system. Fraser *et al.* (2007) suggested that differences in techniques used in different experiments may result in discrepancies among studies. However Benchaar *et al.* (2007) reported that supplementation of essential sweet orange oil at 200 mg/L decreased the NDFD.

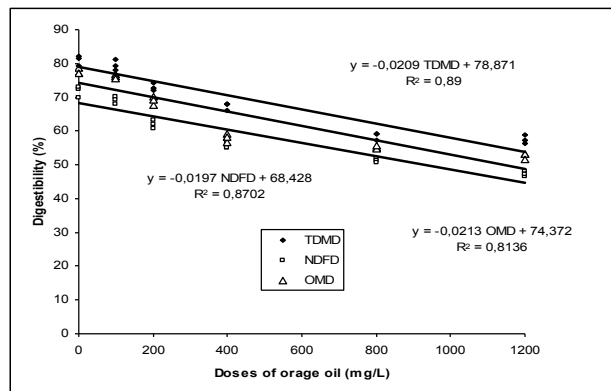


Figure 4. The relationships between dose of essential orange oil supplementation and digestibility of dry matter, neutral detergent fiber and organic matter

The effect of essential orange oil on rumen fermentation parameters is given in Table 3. The essential orange oil inclusion had significant ($P < 0.001$) effect on pH, ammonia, methane, total VFA production and molar proportion of VFA. The ammonia, methane and total VFA production significantly ($P < 0.001$) decreased with increasing level of essential orange oil whereas final pH was decreased. This result is in agreement with finding of Sobhy and Samir (2010) who showed that essential orange oil supplementation significantly reduced the ammonia, methane and total VFA productions. On the other hand, this result obtained in the current study is not in agreement with finding of Benchaar *et al.* (2007) who found that supplementation of essential sweet orange oil at 200 mg/L did not effect the ammonia, methane and total VFA productions. As mentioned before, the differences among two studies can be attributed to the techniques used.

The inclusion of essential orange oil also had a significant ($P < 0.001$) effect on the molar proportion of VFA. The molar proportion of acetate significantly ($P < 0.001$) increased with increasing level of essential orange oil whereas the molar proportion of propionate, butyrate, iso-butyrate, valerate and isovalerate significantly ($P < 0.001$) decreased. When methane production is inhibited, a decrease in acetate and a concomitant increase in propionate production are expected (van Nevel and Demeyer, 1996). The methane synthesis is usually associated with increased propionate production and reduced acetate to propionate ratio (Russell, 1998) but in the current study the acetate to propionate ratio increased with increasing level of essential orange oil, possibly due to accumulation of molecular hydrogen. Gottschalk (1986) reported that the increasing level of essential orange oil supplementation into reaction mixture reduced the efficiency of hydrogen utilization for VFA and methane synthesis. However, in the current study, it is not possible to explain why the efficiency of hydrogen utilization for VFA and methane synthesis is low. Burt (2004) suggested that the antimicrobial activity of these phenolic compounds is through the disturbance of cytoplasmic membrane, disrupting the proton motive force, electron flow active transport, and coagulation of cell contents. It was also shown that two phenolic derivatives, carvacol and thymol, reduced the intracellular ATP pool and increased the extracellular ATP concentration of *E. coli* through the disruptions of the cytoplasmic membrane (Halender *et al.* 1998).

The relationship between dose of essential orange oil supplementation and digestibility ammonia or methane production is given in Figure 5. The mean decreases in ammonia and methane production were 0.0207 and 0.0093 units per mg essential orange oil supplementation respectively.

The relationship between dose of essential orange oil supplementation and total VFA production is given in Figure 6. The mean decreases in total VFA production was 0.0414 units per mg essential orange oil supplementation. The relationship between dose of essential orange oil supplementation and molar proportion of individual VFA production is given in Figure 7. The mean decreases in molar proportion of acetate, propionate and butyrate production were 0.006, 0.0018 and 0.0021 units per mg essential orange oil supplementation respectively. As can be seen from Table 2 and 3 essential orange oil has a potential to modify rumen N metabolism by reducing ammonia and methane production. However, these beneficial effects of essential orange oil has been offset by a decrease of total VFA and digestibility of feed. Similar results were obtained by Sobhy and Samir (2010). The current study revealed that essential orange oil had significant anti-microbial activity

causing an inhibition of the overall fermentation process of soybean meal. Therefore, before large scale implementation, further investigations are required to

determine the effect of essential orange oil on voluntary food intake, animal performance and the profitability of the supplementation *in vivo*.

Table 3. The effect of different doses of essential orange oil on rumen fermentation parameters of soybean meal

Parameters	Treatment (mg/L)						SEM	Sig
	0	100	200	400	800	1200		
pH	6.21d	6.24d	6.35c	6.43c	6.53b	6.64a	0.036	***
Ammonia(mg/dL)	47.58a	39.17b	36.85c	33.44d	26.28e	19.40f	1.037	***
Methane(mmol)	30.07a	27.46b	25.97c	23.47d	20.68e	18.08f	0.578	***
Total VFA(mmol/L)	114.58a	106.57b	95.47c	84.85d	71.64e	63.12f	2.015	***
Molar proportion of volatile acids (% of Total VFA)								
Acetate	45.38c	45.23c	48.09b	48.12b	51.05a	52.33a	0.697	***
Propionate	25.79a	26.46a	25.50a	25.47a	23.98b	24.23b	0.475	***
Butyrate	20.00a	19.51ab	19.05ab	19.92a	18.70b	17.03c	0.510	***
Izo-butyrate	2.81a	2.88a	2.30b	2.17bc	2.06c	2.04c	0.103	***
Valerate	2.67a	2.65a	2.17b	2.12b	2.34b	2.30b	0.108	***
Izo-valerate	3.23a	3.24a	2.86a	2.18b	1.84b	1.99b	0.284	***
A:P Ratio	1.76c	1.71c	1.88b	1.89b	2.13a	2.15a	0.053	***

^{a b c} Row means with common superscripts do not differ ($P>0.05$); s.e.m. – standard error mean; Sig. – significance level; c – gas production rate (%), *** $P<0.001$.

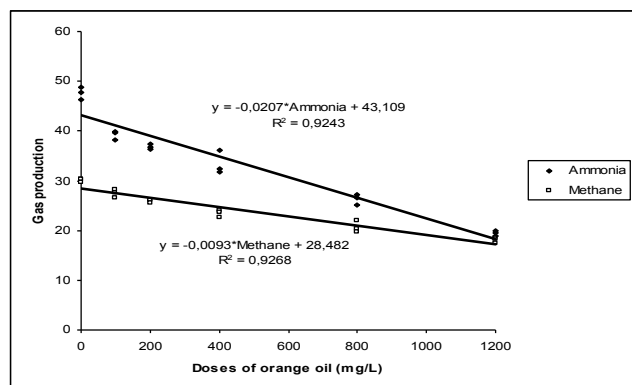


Figure 5. The relationship between dose of essential orange oil supplementation and digestibility ammonia or methane production

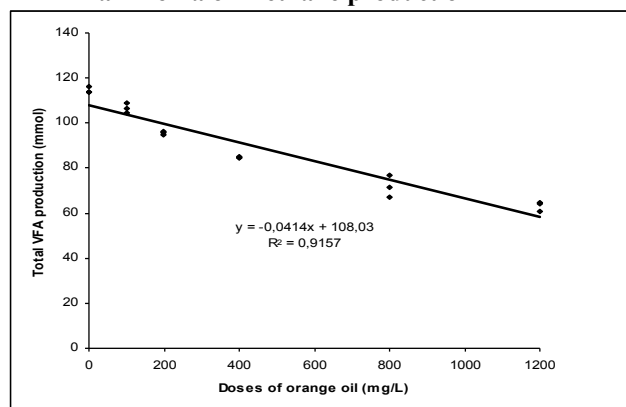


Figure 6. The relationship between dose of essential orange oil supplementation and total VFA production

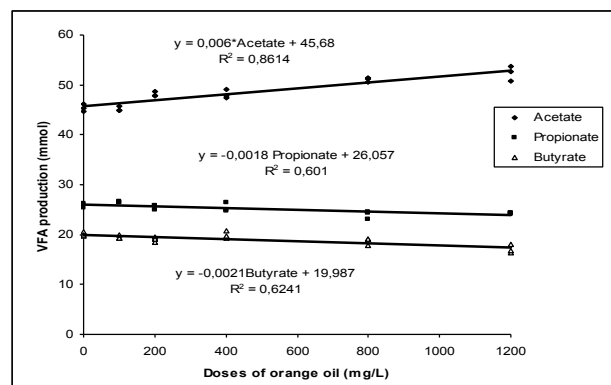


Figure 7. The relationship between dose of essential orange oil supplementation and molar proportion of individual VFA production

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