



## The Effect of Forage Level and Oil Supplement on *Butyrivibrio fibrisolvens* and *Anaerovibrio lipolytica* in Continuous Culture Fermenters

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**ABSTRACT :** The objective of this study was to evaluate the effects of forage level and oil supplement on selected strains of rumen bacteria believed to be involved in biohydrogenation (BH). A continuous culture system consisting of four fermenters was used in a 4×4 Latin square design with a factorial arrangement of treatments, with four 10 d consecutive periods. Treatment diets were: i) high forage diet (70:30 forage to concentrate (dry matter basis); HFC), ii) high forage plus oil supplement (HFO), iii) low forage diet (30:70 forage to concentrate; LFC), and iv) low forage plus oil supplement (LFO). The oil supplement was a blend of fish oil and soybean oil added at 1 and 2 g/100 g dry matter, respectively. Treatment diets were fed for 10 days and samples were collected from each fermenter on the last day of each period 3 h post morning feeding. The concentrations of vaccenic acid (*t*11C18:1; VA) and *c*9*t*11 conjugated linoleic acid (CLA) were greater with the high forage diet while the concentrations of *t*10 C18:1 and *t*10*c*12 CLA were greater with the low forage diet and addition of oil supplement increased their concentrations at both forage levels. The DNA abundance of *Anaerovibrio lipolytica*, and *Butyrivibrio fibrisolvens* vaccenic acid subgroup (*Butyrivibrio* VA) were lower with the low forage diets but not affected by oil supplement. The DNA abundance of *Butyrivibrio fibrisolvens* stearic acid producer subgroup (*Butyrivibrio* SA) was not affected by forage level or oil supplement. In conclusion, oil supplement had no effects on the tested rumen bacteria and forage level affected *Anaerovibrio lipolytica* and *Butyrivibrio* VA. (**Key Words :** Oil, Forage Level, Biohydrogenation, *Trans* Fatty Acids, Fermenters)

### INTRODUCTION

*Trans* C18:1 fatty acids (FA) and conjugated linoleic acid (CLA) are produced by the incomplete biohydrogenation (BH) of long chain unsaturated FA in the rumen (Harfoot and Hazlewood, 1997), and are subsequently incorporated into milk and meat of ruminant animals. The different positional and geometric isomers of CLA confer different health effects on mammals (Bhattacharya et al., 2006; Kelly et al., 2007). The *c*9*t*11 CLA isomer has been shown to be anticarcinogenic (Amarù and Field, 2009; Huot et al., 2010), while the *t*10*c*12 CLA isomer has been shown capable of decreasing body fat and increasing lean body mass (Miranda et al., 2009; Jiang et al., 2010). The *t*10*c*12 CLA isomer also decreases fat concentration in dairy cow's milk in a dose dependent fashion (Peterson et al., 2002).

Formation of *trans* C18:1 FA and CLA in the rumen are

known to be influenced by dietary supplementation with unsaturated plant oils (Varadyova et al., 2007; Doreau et al., 2009; Duckett and Gillis, 2010), fish oil (Jin et al., 2008; Lee et al., 2008) or rumen pH (Sackmann et al., 2003; AbuGhazaleh and Jacobson, 2007; Alzahal et al., 2009). Feeding low forage diets supplemented with plant oils, fish oil or their blend (Loor et al., 2004; AbuGhazaleh and Jacobson, 2007; Belenguer et al., 2010) altered ruminal BH resulting in *t*10 rather than VA being the predominant *trans* C18:1 intermediates. Similarly, Fuentes et al. (2009) showed that the flow of VA and *c*9*t*11 CLA from fermenters decreased while the flow of *t*10 C18:1 and *t*10*c*12 CLA increased when high concentrate diet replaced high forage diet. Although the effects of lipid supplements and forage level on ruminal *trans* FA formation are well documented, little information is currently available about the effects of feeding such diets on rumen microbial ecology, particularly, bacterial species believed to be involved in the BH process.

Harfoot and Hazlewood (1997) categorized the bacteria involved in the different steps of the BH pathway into two groups. Group A bacteria hydrogenate linoleic acid

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(C18:2n6) and linolenic acid (C18:3n3) to VA; in contrast, group B bacteria convert the same FA to 18:0. Using 16S rDNA sequence data, Paillard et al. (2007) constructed a phylogenetic tree that contains two main groups of rumen *Butyrivibrio* bacteria called vaccenic acid-producing (*Butyrivibrio* VA) and stearic acid-producing (*Butyrivibrio* SA). Although both groups formed VA from C18:2n6, only SA bacteria produced C18:0 (Paillard et al., 2007). The main objective of this study was to evaluate the effects of forage level and lipid supplement on *Butyrivibrio* VA and SA and *Anaerovibrio lipolytica* using continuous culture fermenters.

## MATERIALS AND METHODS

### Experimental design and treatments

Four single flow continuous fermenters (800 ml) were used in 4×4 Latin square design with 4 periods of 10 d each. Treatment diets were fed (45 g/d dry matter basis) in three equal portions during the day at 0700, 1500 and 2300 h. Treatment diets were: i) high forage diet (70:30 forage to concentrate (dry matter basis); HFC), ii) high forage plus oil supplement (HFO), iii) low forage diet (30:70 forage to concentrate; LFC), and iv) low forage plus oil supplement (LFO). The forage source was alfalfa pellets. The oil supplement was a blend of fish oil (C20:5n3 = 14.6 g/100 g FA; C22:6n3 = 9.8 g/100 g FA) and soybean oil (C18:2n6 = 53.8 g/100 g FA) added at 1 and 2 g/100g DM, respectively. Menhaden fish oil (Omega Protein Inc., Hammond, LA, USA) was used in this study. Corn, soybean meal, and minerals made up the concentrate mix (Table 1).

Whole ruminal contents were collected 4 h after the morning feeding from two ruminally fistulated Holstein cows fed a 50:50 forage:concentrate diet. After collection,

ruminal contents were transferred to the laboratory in sealed bags, mixed, and then strained using a double-layered cheese cloth. Approximately 700 ml of the ruminal fluid was added to each of the four fermenters, containing 100 ml of prewarmed buffer. Anaerobic conditions in fermenters were maintained by infusing CO<sub>2</sub> at 45 ml/min. Cultures were stirred continuously at 45 rpm and fermenter pH was measured daily before feeding using a portable pH meter. Fermenter temperature was maintained at 39°C using a circulating water bath. Buffer was delivered continuously at a flow rate of 1.25 ml/min (11%/h liquid dilution rate), using a precision pump. Flow rate of each fermenter was recorded every day at 07:00.

### Sample collection and analyses

On day 10 of each period, two 10-ml samples were collected from each fermenter, using a wide-bore pipette, at 3 h post morning feeding for FA analysis (AbuGhazaleh and Jacobson, 2007) and bacterial analysis (AbuGhazaleh et al., 2011; Potu et al., 2011). Briefly, bacterial samples collected from fermenters were frozen immediately in liquid nitrogen and stored at -80°C until bacterial analysis. Samples were thawed, thoroughly mixed and 1 ml subsample was then used for DNA extraction using the MO BIO Ultraclean™ Microbial DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Purified cultures of *Butyrivibrio* SA (DSMZ 10302), *Butyrivibrio* VA (DSMZ 10316), and *Anaerovibrio lipolytica* (DSMZ 3074) were obtained from DSMZ (German resource center for biological material, Braunschweig, Germany) and grown in our laboratory in sealed Hungate tubes as specified by DSMZ. The three bacteria were used to generate standard curves for the relative quantitation analyses (AbuGhazaleh et al., 2011; Potu et al., 2011). The slopes and coefficient of determination (R<sup>2</sup>) for the standard curves were -3.456 and 0.995 for *Butyrivibrio* SA, -3.654 and 0.995 for *Butyrivibrio* VA, and -3.635 and 0.989 for *Anaerovibrio lipolytica*, respectively. The primer pairs for *Anaerovibrio lipolytica* was described by Tajima et al. (2001). The primer pairs for *Butyrivibrio* SA and *Butyrivibrio* VA were described by Fuentes et al. (2009). The specificity of primers was confirmed using the BLAST program in the GeneBank Database. The PCR products for all tested bacteria were sent also to laboratory (GENEWIZ, South Plainfield, NJ, USA) for sequence validation (> 98%).

Individual species-specific real time quantitative PCR (qPCR) was performed using Bio-Rad iCycler MyiQ single color real-time PCR detection system (Bio-Rad laboratories, Inc, Hercules, CA, USA), using fluorescence detection of SYBR green mix (Bio-Rad laboratories, Inc, Hercules, CA, USA) as described by AbuGhazaleh et al. (2011) and Potu et al. (2011). Briefly 12.5 µl SYBR green mix, 2 µl of each primer, sample DNA (starting concentration; Table 2), and

**Table 1.** Ingredients of treatment diets

Ingredient (g/kg DM)	Treatment			
	HFC <sup>a</sup>	HFO <sup>b</sup>	LFC <sup>c</sup>	LFO <sup>d</sup>
Alfalfa hay	700.00	700.00	300.00	300.00
Soybean meal	45.00	45.00	113.00	113.00
Ground corn	250.00	220.00	582.00	552.00
Soybean oil	0.00	20.00	0.00	20.00
Fish oil	0.00	10.00	0.00	10.00
Minerals mix <sup>e</sup>	5.00	5.00	5.00	5.00

<sup>a</sup> HFC = High forage control diet; alfalfa pellets at 700 g/kg (diet DM) and no oil supplementation.

<sup>b</sup> HFO = High forage oil diet; control diet with 20 g/kg (diet DM) soybean oil and 10 g/kg (diet DM) fish oil.

<sup>c</sup> LFC = Low forage control diet; alfalfa pellets at 300 g/kg (diet DM) and no oil supplementation.

<sup>d</sup> LFO = Low forage oil diet; control diet with 20 g/kg (diet DM) soybean oil and 10 g/kg (diet DM) fish oil.

<sup>e</sup> Contained (g/kg): NaCl (955 to 9.8), Zn (10.0), Mn (7.5), Fe (6.0), Mg (0.5), Cu (0.32), I (0.28), and Co (0.11).

**Table 2.** PCR primers and starting DNA concentration for detection of selected ruminal bacteria

Target bacterium	Primer sequences (5'-3')	Starting DNA concentration (ng)/25 µl	Annealing temp (°C)	Product size (bp)
<i>Butyrivibrio</i> SA	F: TCCGGTGGTATGAGATGGGC	18	60	126
	R: GTCGCTGCATCAGAGTTTCCT			
<i>Butyrivibrio</i> VA	F: GCCTCAGCGTCAGTAATCG	18	60	124
	R: GGAGCGTAGGCGTTTAC			
<i>Anaerovibrio lipolytica</i>	F: TGGGTGTTAGAAATGGATTCC	60	57	597
	R: CTCTCCTGCACTCAAGAATT			

RNAse free water were added to make a total volume of 25 µl. Amplifications for *Anaerovibrio lipolytica* and *Butyrivibrio* SA and *Butyrivibrio* VA were described by Potu et al. (2011) and Fuentes et al. (2009), respectively. Standard curves, DNA sample quantification and melting curve analyses were obtained using iQ5 Optical System Software (version 2.1, Bio-Rad laboratories, Inc, Hercules, CA, USA). Melting curve analysis was performed after each amplification step to determine the specificity of PCR product. Samples were amplified in triplicate along with dilution standards of known bacterial DNA concentrations. Samples and standards were assayed on the same plate to allow for the relative quantification of bacterial DNA present in sample.

#### Statistical analysis

Data were analyzed as a 4×4 Latin square design in a 2×2 factorial design using the PROC MIXED of SAS (SAS Institute, Inc., Cary, NC). The statistical model included: forage effect, oil effect, and their interactions. Fixed effects were treatment diets and period. Random effect was fermenter. Results were expressed as least square means with standard error of the means. The significance threshold

was set at p<0.05. The statistical model was:

$$Y_{ijk} = \mu + F_i + O_j + P_{ij} + e_{ij}$$

where  $Y_{ijk}$  = the observation;  $\mu$  = overall mean;  $F_i$  = forage effect (forage level; HF and LF);  $O_j$  = oil effect;  $P_{ij}$  = forage and oil interaction and  $e_{ij}$  = residual error.

## RESULTS AND DISCUSSION

The fermenters pH for the high and low forage diets averaged 6.58 and 5.53, respectively and was not affected by oil supplement. The effects of forage level and oil supplement on selected BH intermediates are presented in Table 3. The greater concentration of *trans* C18:1 along with the lower concentration of C18:0 with the LFC relative to the HFC suggest an incomplete BH of unsaturated FA with the LFC. Low ruminal pH, caused by a high-concentrate diet, increased the accumulation of *trans* C18:1 in other studies (Piperova et al., 2002; Loor et al., 2004; AbuGhazaleh and Jacobson, 2007). Although the cause of the accumulation of *trans* C18:1 under low rumen pH conditions is still unknown, alteration of the rumen

**Table 3.** The effect of forage level and oil supplements on fermenter fatty acid (mg/g DM)

Fatty acid	Treatment				MSE	p value		
	HFC <sup>a</sup>	HFO <sup>b</sup>	LFC <sup>c</sup>	LFO <sup>d</sup>		Forage	Oil	Forage×oil
C18:0	9.47	3.81	0.98	3.03	0.612	0.01	0.02	0.01
C18:1 <i>total trans</i>	2.75	17.71	5.63	12.33	0.887	0.20	0.07	0.57
<i>t</i> 10 C18:1	0.24	0.79	5.08	9.09	0.462	0.01	0.01	0.01
<i>t</i> 11 C18:1 (VA) <sup>e</sup>	2.05	15.78	0.41	2.02	0.886	0.01	0.01	0.01
C18:1n9	1.99	5.14	7.15	7.08	1.012	0.01	0.18	0.17
C18:2n6	2.43	4.56	7.07	10.67	0.559	0.01	0.01	0.24
C18:3n3	0.84	1.12	0.81	1.26	0.101	0.61	0.01	0.44
<i>c</i> 9 <i>t</i> 11 CLA <sup>f</sup>	0.18	0.58	nd <sup>g</sup>	0.06	0.054	0.01	0.01	0.01
<i>t</i> 10 <i>c</i> 12 CLA	nd	0.02	0.09	0.48	0.061	0.01	0.01	0.02

<sup>a</sup> HFC = High forage control diet; alfalfa pellets at 700 g/kg (diet DM) and no oil supplementation.

<sup>b</sup> HFO = High forage oil diet; control diet with 20 g/kg (diet DM) soybean oil and 10 g/kg (diet DM) fish oil.

<sup>c</sup> LFC = Low forage control diet; alfalfa pellets at 300 g/kg (diet DM) and no oil supplementation.

<sup>d</sup> LFO = Low forage oil diet; control diet with 20 g/kg (diet DM) soybean oil and 10 g/kg (diet DM) fish oil.

<sup>e</sup> VA = Vaccenic acid. <sup>f</sup> CLA = Conjugated linoleic acid. <sup>g</sup> nd = Not detected.

ecosystem and/or inhibition of the reductase activity of ruminal microorganisms responsible for the reduction of *trans* C18:1 to C18:0 have been suggested (Loor et al., 2004; AbuGhazaleh and Jacobson, 2007). Despite the greater concentration of *trans* C18:1 with the LFC diet, the greater concentrations of BH products (the sum of *trans* C18:1 and C18:0) seen with the HFC (12.22 mg/g of DM) compared with the LFC (6.61 mg/g of DM) suggest greater microbial BH activities with the high forage diets. The increase in the concentration of *trans* C18:1 with oil supplement was expected as inputs of dietary C18 unsaturated FA, the precursors for *trans* C18:1, were increased with the oil supplement. Other studies also reported similar increases in *trans* C18:1 concentration in rumen (Varadyova et al., 2007; Belenguer et al., 2010) and duodenum (Kucuk et al., 2008) when oils were included in the diets of ruminant animals. The greater increase in *trans* C18:1 concentration with oils addition to the high forage diet further support the findings that greater BH activities occurred in fermenters fed the high forage diets (discussed below).

The concentration of VA was greater ( $p < 0.05$ ) with the high forage diets while the concentration of *t10* C18:1 was greater ( $p < 0.05$ ) with the low forage diets and both increased ( $p < 0.05$ ) further with oil supplementation (Table 3). Similarly, the concentration of *c9t11* CLA was greater ( $p < 0.05$ ) with the high forage diets while the concentration of *t10c12* CLA was greater ( $p < 0.05$ ) with the low forage diets and both increased ( $p < 0.05$ ) further with oil supplementation. The lower concentrations of VA and *c9t11* CLA with the low forage diet are consistent with previous studies that showed a shift toward *t10* C18:1 and *t10c12* CLA formation with decreasing dietary forage level (Piperova et al., 2002; Sackmann et al., 2003; AbuGhazaleh and Jacobson, 2007) or low ruminal pH (Choi et al., 2005; Fuentes et al., 2009). At a constant dietary oil level, AbuGhazaleh and Jacobson (2007) showed that decreasing dietary forage levels resulted in *t10* C18:1 and *t10c12* CLA replacing VA and *c9t11* CLA, respectively as predominate *trans* C18:1 and CLA isomers in fermenters. Additionally,

*t10* C18:1 replaced VA as the predominant *trans* C18:1 isomer in the rumen when high concentrate-low fiber diets were fed to cows (Loor et al., 2004) and steers (Sackmann et al., 2003). Relative to *t10* C18:1, the increase in VA concentrations with oil supplements were more markedly with the high forage diet (forage $\times$ oil interaction,  $p < 0.01$ ) possibly indicating more active BH.

The effects of treatment diets on the DNA abundance of the selected ruminal bacteria are presented in Table 4. Dietary oil supplement had no effects ( $p > 0.05$ ) on the DNA abundance of *Anaerovibrio lipolytica* and *Butyrivibrio* VA-producing bacteria at both forage levels. Potu et al. (2011) reported no effects for fish oil or soybean oil supplementation at 3% of diet DM on *Anaerovibrio lipolytica*. Feeding fish oil, sunflower oil or their combination to dairy sheep had no effect on the abundance of *Butyrivibrio* VA-producing bacteria (Belenguer et al., 2010). Maia et al. (2007) also reported no change in the growth of *Anaerovibrio lipolytica* upon incubating with PUFA at 50  $\mu\text{g/ml}$ ; demonstrating the low sensitivity of *Anaerovibrio lipolytica* to oil supplements. The DNA abundances for *Anaerovibrio lipolytica* and *Butyrivibrio* VA-producing bacteria were significantly lower with the low forage diets suggesting that these bacteria are sensitive to low pH conditions. This is in agreement with Fuentes et al. (2009) who also reported similar decreases in the DNA abundance for *Anaerovibrio lipolytica* and *Butyrivibrio* VA-producing bacteria when fermenters pH was reduced from 6.4 to 5.6. Hobson (1965) and Henderson et al. (1969) also reported lower growth and lipase activity for *Anaerovibrio lipolytica* under low pH conditions. The decrease in *Anaerovibrio lipolytica* and *Butyrivibrio* VA-producing bacteria with low forage diets may, in part, explain the greater concentrations of C18:2n6 and C18:1n9 and the lower concentrations of BH end-products (*trans* C18:1 and C18:0) with the low forage diets relative to the high forage diets. Switching lactating dairy cows from a high to a low forage diet, which promotes low rumen pH, resulted in lower levels of lipolytic activity and BH of unsaturated FA in ruminal fluid as measured by *in vitro* experiment (Van

**Table 4.** The effect of forage level and oil supplements on the DNA abundance (pg) of selected rumen bacteria at a given starting concentration

Bacteria	Treatment				MSE	p value		
	HFC <sup>a</sup>	HFO <sup>b</sup>	LFC <sup>c</sup>	LFO <sup>d</sup>		Forage	Oil	Forage $\times$ oil
<i>Butyrivibrio</i> SA, 18ng*	14.93	13.61	12.34	15.82	1.105	0.87	0.36	0.07
<i>Butyrivibrio</i> VA, 18ng*	0.33	0.29	0.01	0.01	0.044	0.01	0.77	0.72
<i>Anaerovibrio lipolytica</i> , 60 ng*	1.67	2.6	0.05	0.09	0.449	0.01	0.32	0.36

<sup>a</sup> HFC = High forage control diet; alfalfa pellets at 700 g/kg (diet DM) and no oil supplementation.

<sup>b</sup> HFO = High forage oil diet; control diet with 20 g/kg (diet DM) soybean oil and 10 g/kg (diet DM) fish oil.

<sup>c</sup> LFC = Low forage control diet; alfalfa pellets at 300 g/kg (diet DM) and no oil supplementation.

<sup>d</sup> LFO = Low forage oil diet; control diet with 20 g/kg (diet DM) soybean oil and 10 g/kg (diet DM) fish oil.

\* Starting concentration.

Nevel and Demeyer, 1996; AbuGhazaleh and Jacobson, 2007).

Interestingly, dietary oil supplement and forage level had no effects on the DNA abundance of *Butyrivibrio* SA-producing bacteria in spite of the decrease in C18:0 concentrations with oil supplementation and low forage diets (Table 3). *Butyrivibrio* SA-producing bacteria was identified to be the ruminal species responsible for the conversion of *trans* C18:1 into C18:0 (Paillard et al., 2007). The lack of significant effect of fish oil and low pH on *Butyrivibrio* SA-producing bacteria has been also reported by Belenguer et al. (2010) and Fuentes et al. (2009), respectively. Kim et al. (2008) and Huws et al. (2010) also reported that DNA abundance from the *Butyrivibrio* SA-producing group did not correlate with the C18:0 concentrations suggesting that *Butyrivibrio* SA-producing group may not indeed be the main C18:0 producer in the rumen. These results, therefore, would suggest that other, yet-uncultivated microbial species might be involved in C18:0 production and might fulfill a more important role in the final step of the BH process. Additionally, it's still also possible that oil supplement and low forage diets decreased the capacity of these bacteria to hydrogenate *trans* C18:1 rather than the bacteria as such. A recent study by Maia et al. (2010) reported that the inhibitory-growth effects of unsaturated FA on bacteria may be mediated via metabolic means rather than disruption of cell membrane.

## CONCLUSION

The concentrations of VA and *c9t11* CLA were greater with the high forage diets while the concentrations of *t10* C18:1 and *t10c12* CLA were greater with the low forage diets and concentrations of these *trans* FA were further increased with oil supplementation. The lower concentrations of BH products (CLA, *trans* C18:1 and C18:0) seen with the low forage diets was in parallel with the lower DNA abundance for *Butyrivibrio* VA suggesting a role of these bacteria in *trans* FA formation particularly under high ruminal pH conditions. The nonresponsive effects of *Butyrivibrio* SA to oil supplementation and forage levels may indicate that these bacteria play a minor role in the production of C18:0 from *trans* C18:1.

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