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ORIGINAL ARTICLE

Effects of incremental amounts of fish oil on *trans* fatty acids and *Butyrivibrio* bacteria in continuous culture fermenters

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Summary

Previous studies have shown that adding fish oil (FO) to ruminant animal diets increased vaccenic acid (VA; *t*11 C18:1) accumulation in the rumen. Therefore, the objective of this study was to evaluate the effect of dietary FO amounts on selected strains of rumen bacteria involved in biohydrogenation. A single-flow continuous culture system consisting of four fermenters was used in a 4 × 4 Latin square design with four 9 days consecutive periods. Treatment diets were as follows: (i) control diet (53:47 forage to concentrate; CON), (ii) control plus FO at 0.5% (DM basis; FOL), (iii) control plus FO at 2% (DM basis; FOM) and (iv) control plus FO at 3.5% (DM basis; FOH). Fermenters were fed treatment diets three times daily at 120 g/day. Samples were collected from each fermenter on day 9 of each period at 1.5, 3 and 6 h post-morning feeding and then composited into one sample per fermenter. Increasing dietary FO amounts resulted in a linear decrease in acetate and isobutyrate concentrations and a linear decrease in acetate-to-propionate ratio. Propionate, butyrate, valerate and isovalerate concentrations were not affected by FO supplementation. Concentrations of C18:0 in fermenters linearly decreased, while concentrations of *t*10 C18:1 and VA linearly increased as dietary FO amounts increased. The concentrations of *c9t*11 and *t*10*c*12 conjugated linoleic acid were not affected by FO supplementation. The DNA abundance for *Butyrivibrio fibrisolvens, Butyrivibrio* vaccenic acid subgroup, *Butyrivibrio* stearic acid subgroup and *Butyrivibrio proteoclasticus* linearly decreased as dietary FO amounts increased. In conclusion, FO effects on trans fatty acid accumulation in the rumen may be explained in part by FO influence on *Butyrivibrio* group.

Keywords fish oil, trans fatty acids, bacteria, fermenters

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Introduction

Trans C18:1 fatty acids and conjugated linoleic acid (CLA) are produced by the incomplete biohydrogenation of dietary long-chain unsaturated fatty acids in the rumen (Harfoot and Hazlewood, 1997) and are subsequently incorporated into milk and meat of ruminant animals. Among the different microbes present in the rumen, bacterial species identified as being most involved in the biohydrogenation of dietary unsaturated fatty acids belong to the *Butyrivibrio* group (Paillard et al., 2007a). Harfoot and Hazlewood (1997) divided the rumen bacteria into two groups where Group A bacteria hydrogenate C18 unsaturated fatty acids to *trans* C18:1 and C18:2 isomers, whereas Group B bacteria convert *trans* C18:1 to C18:0. Using 16S rDNA sequence data, Paillard et al. (2007a) 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 *Butyrivibrio* SA bacteria produced C18:0 (Paillard et al., 2007b).

Conjugated fatty acids have recently attracted significant attention because of their health benefits in a variety of models of metabolic and chronic inflammatory diseases (Kennedy et al., 2010; Crumb, 2011). Among the many CLA isomers, *c9t*11 CLA is the most abundant in ruminant-derived foods. Although some of the *c9t*11 CLA can be produced directly by microbial hydrogenation in the rumen, the vast majority is produced in animal tissues via Δ^9 desaturase using vaccenic acid (VA, *t*11 C18:1) as its substrate (Griinari et al., 2000). Increasing the formation and flow of VA from the rumen is therefore desirable as it would aid in the process of developing healthier ruminant-derived food products.

Supplement ruminant animal diets with plant oils (Varadyova et al., 2007; Doreau et al., 2009) and fish oil (FO; Lee et al., 2008; Kim et al., 2008) resulted in an increase in the formation and flow of VA from the rumen. Fish oil supplementation to ruminant animal diets has been shown to inhibit the complete biohydrogenation of C18 unsaturated fatty acids in the rumen, resulting in the accumulation of *trans* C18:1, VA in particular (AbuGhazaleh and Jenkins, 2004; Kim et al., 2008; Lee et al., 2008; Potu et al., 2011). Although the effects of FO on ruminal *trans* FA formation are well documented, little information is currently available on the effects of FO on rumen microbial ecology, particularly bacterial species believed to be involved in the biohydrogenation process. In recent years, there has been great interest in identifying the bacteria most involved in VA formation in the rumen as such identification is considered an important component in developing novel strategies to increase VA availability for desaturation in animal body tissues. Therefore, the main objective of this study was to evaluate the effects of incremental amounts of FO on *Butyrivibrio* group using continuous culture fermenters.

Materials and methods

Experimental protocol

Four single-flow continuous culture apparatus were used in a 4 × 4 Latin square over four periods of 9 days each. Treatments used in this study were as follows: (i) control diet (53:47 forage to concentrate; CON), (ii) control plus FO at 0.5% (DM basis; FOL), (iii) control plus FO at 2% (DM basis; FOM) and (iv) control plus FO at 3.5% (DM basis; FOH). Alfalfa hay and grass hay (approximately 3:1 DM basis) were used as forage source. The nutrient composition of CON diet averaged 13.4 g/100 DM for crude protein (CP), 22.2 g/100 g DM for acid detergent fibre (ADF), 33.9 g/100 g DM for neutral detergent fibre (NDF) and 6.5 g/100 g DM for ash. Treatment diets (Table 1) were fed at 120 g/day DM in three equal portions at 08:00, 15:00 and 21:00 h.

Whole ruminal contents were taken from two ruminally fistulated Holstein cows fed a 60:40 forage/concentrate diet. At each collection time, approximately 3.5 kg of ruminal content was taken from each cow 4 h after feeding. The rumen samples were then composited into one sample, strained through two layers of cheesecloth and transported to the laboratory in a sealed container and used within 20 min. Fermenter canisters were filled with approximately 1300 ml of rumen fluid and 400 ml of pre-warmed buffer (Weller and Pilgrim, 1974). Buffer was delivered continuously at a flow rate of 0.10 per h using a precision pump. Flow rate of each fermenter was recorded every day at 08:00. Fermenters were constantly mixed at 120 rpm via a magnetic impeller stirrer unit, purged with N^2 gas (80 ml/min), and temperature was maintained at 39 °C. The pH was measured daily at 08:00, 15:00 and 21:00 h using a portable pH meter (Accumet* AP85 Portable, Fisher Scientific, Pittsburgh, PA, USA).

Table 1 Ingredient and fatty acid composition of treatment diets

	Treatment			
	CON	FOL	FOM	FOH
Fish oil supplement (% of diet DM)	0.00	0.50	2.00	3.50
Ingredient (g/100 g of DM)				
Alfalfa hay	40.00	40.00	40.00	40.00
Grass hay	13.00	13.00	13.00	13.00
Corn	30.00	30.00	30.00	30.00
Soybean meal, 44% CP	7.50	7.50	7.50	7.50
Corn distillers	3.30	3.30	3.30	3.30
Soybean hulls	5.50	5.50	5.50	5.50
Corn oil	0.50	0.50	0.50	0.50
Mineral mix*	0.20	0.20	0.20	0.20
Fatty acid composition (mg/g DM)				
C16:0	3.35	4.06	6.36	8.85
C18:0	0.55	0.67	1.04	1.43
C18:1 *c*9	4.75	5.34	5.38	5.69
C18:2n6	10.49	11.39	11.78	11.71
C18:3n3	1.46	1.42	1.78	1.89
C20:5n3	nd	0.76	2.55	3.24
C22:6n3	nd	0.48	1.81	2.72
Total fatty acids	23.79	27.76	38.59	49.66

CON, control diet (53:47 forage to concentrate); FOL, control plus fish oil at 0.5% (diet DM); FOM, control plus fish oil at 2% (diet DM); FOH, control plus fish oil at 3.5% (diet DM); nd, not detected or detected at <0.01.
*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).

Sample collection and analysis

On day 9 of each period, three-10 samples were collected from each fermenter at 1.5 and 6 h post-morning feeding, composited into one sample per fermenter and then analysed for volatile fatty acids (VFA), fatty acids and bacterial DNA. Samples for VFA and fatty acids were analysed as described by Potu et al. (2011). Bacterial samples collected from fermenters were frozen immediately in liquid nitrogen and stored at −80 °C until bacterial analysis (AbuGhazaleh et al., 2011; Potu et al., 2011).

The frozen microbial samples were thawed at room temperature and then centrifuged for 10 min at 1000 *g* (Marathon Micro-A, Fisher Scientific), and the DNA from pellets was then extracted using the MO BIO Ultraclean™ microbial DNA isolation kit (MO BIO

Laboratories, Carlsbad, CA, USA). Concentration of DNA was measured by BioPhotometer (Eppendorf Scientific, Westbury, NY, USA). The purity of the DNA was assessed from the 260:280 nm ratio. Purity of DNA was considered acceptable with ratios >1.8.

Purified cultures of *Butyrivibrio fibrisolvens* (DSMZ No 3071), *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). *Butyrivibrio proteoclasticus* (ATCC 51982) was obtained from ATCC (The Global Bioresource Center, Manassas, VA, USA). All bacteria were grown in our laboratory in sealed Hungate tubes as specified by DSMZ and ATCC and used to generate standard curves for the relative quantitative analyses (AbuGhazaleh et al., 2011; Potu et al., 2011). The slopes and coefficient of determination (R^2) for the standard curves were −3.837 and 0.98 for *B. fibrisolvens*, −3.456 and 0.995 for *Butyrivibrio* SA, −3.654 and 0.995 for *Butyrivibrio* VA, −3.569 and 0.996 for *B. proteoclasticus* and −3.635 and 0.989 for *A. lipolytica* respectively. The primer pairs for *Butyrivibrio* SA and *Butyrivibrio* VA were described by Gudla et al. (2012). The primer pairs for *B. fibrisolvens, A. lipolytica* and *B. proteoclasticus* were described by Potu et al. (2011). The specificity of primers was confirmed using the BLAST program in the GenBank Database. The PCR products for all tested bacteria were sent to a DNA sequencing laboratory (GENEWIZ, South Plainfield, NJ, USA) for validation (>97% homology).

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, Hercules, CA, USA), using fluorescence detection of SYBR Green mix (Bio-Rad Laboratories) as described by AbuGhazaleh et al. (2011) and Potu et al. (2011). The PCR conditions for *Butyrivibrio* SA and *Butyrivibrio* VA were described by Fuentes et al. (2009). The PCR conditions *B. fibrisolvens, A. lipolytica* and *B. proteoclasticus* were described by Potu et al. (2011). Standard curves, DNA sample quantification and melting curve analyses were obtained using iQ5 Optical System Software (version 2.1, Bio-Rad Laboratories). 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.

The DM of treatment diets samples was determined by drying at 105 °C for 48 h (AOAC, 1990, 2000; Method 930.15). The NDF was determined using the Van Soest et al. (1991) procedure. The heat-stable amylase and sodium sulphite were used to determine NDF. Samples were analysed for CP (method 976.05) and ash (method 942.05) according to AOAC (2000) methods. The ADF content was determined according to AOAC (1990); Method 973.18).

Statistical analysis

Data were analysed as a 4 × 4 Latin square design using the PROC MIXED of SAS (SAS Institute, Cary, NC, USA) using the following model:

$$Y_{ijk} = \mu + T_i + P_j + F_k + e_{ijk}$$

where μ is the overall mean, T is the fish oil amount, P is the period effect, F is the fermenter effect, and e is the residual error. ~~The random effect was fermenter~~. Fixed effects were period and diet, while fermenter was the random effect. Preplanned comparisons were linear and quadratic effects. Significance was declared at $p < 0.05$.

Results

The effects of dietary FO amounts on fermenters VFA and fatty acids are presented in Tables 2 and 3. The concentrations of acetate and isobutyrate and the acetate-to-propionate ratio decreased in a linear manner ($p < 0.05$) as dietary FO amounts increased. Relative to CON, propionate concentration tended to decrease ($p < 0.10$) with dietary FO. The inclusion of FO in the diet had no effects ($p > 0.05$) on the concentrations of

Table 2 Effect of incremental amounts of fish oil on fermenters volatile fatty acids (mM) and pH

	Treatment					p-value	
	CON	FOL	FOM	FOH	SEM	Lin	Quad
Acetate	23.42	19.57	15.64	14.53	1.422	0.01	0.24
Propionate	10.87	10.38	12.41	14.47	1.141	0.08	0.47
Butyrate	8.84	9.48	7.27	7.69	1.035	0.35	0.92
A/P	2.15	1.92	1.21	1.05	0.155	0.01	0.79
Isobutyrate	0.62	0.54	0.41	0.46	0.028	0.01	0.12
Isovalerate	1.88	1.62	1.37	1.61	0.182	0.32	0.26
Valerate	1.67	1.49	1.35	1.67	0.147	0.88	0.18
Total VFA	47.29	43.05	37.98	40.54	3.165	0.13	0.29
pH	6.42	6.51	6.48	6.53	0.129	0.82	0.91

CON, control diet (53:47 forage to concentrate); FOL, control plus fish oil at 0.5% (diet DM); FOM, control plus fish oil at 2% (diet DM); FOH, control plus fish oil at 3.5% (diet DM); Lin, linear effect; Quad, quadratic; A/P, Acetate-to-propionate ratio.

Table 3 Effect of incremental amounts of fish oil on fermenters fatty acid (mg/g of DM)

	Treatment					p-value	
	CON	FOL	FOM	FOH	SEM	Lin	Quad
C14:0	0.27	0.38	0.77	1.26	0.031	0.01	0.01
C16:0	3.22	4.51	4.9	6.55	0.311	0.01	0.37
C18:0	5.97	5.22	1.00	1.46	0.229	0.01	0.04
C18:1 *t*6/8	0.03	0.04	0.02	0.03	0.011	0.84	0.93
C18:1 *t*9	0.06	0.11	0.07	0.08	0.019	0.44	0.05
C18:1 *t*10	0.11	0.40	0.59	0.89	0.131	0.01	0.96
C18:1 *t*11	1.31	1.97	4.48	4.47	0.269	0.01	0.33
C18:1 *t*12	0.11	0.21	0.33	0.43	0.063	0.02	0.96
C18:1 *t*16	0.25	0.37	0.58	0.84	0.044	0.01	0.09
C18:1 total *trans*	1.86	3.35	6.06	6.74	0.527	0.01	0.29
C18:1*c*9	1.64	2.36	2.24	2.37	0.178	0.04	0.17
C18:2n6	2.32	2.82	2.17	2.31	0.235	0.49	0.47
C18:3n3	0.38	0.44	0.38	0.48	0.022	0.09	0.51
CLA, *c*9*t*11	0.04	0.04	0.07	0.06	0.013	0.22	0.36
CLA, *t*10*c*12	0.01	0.01	0.03	0.01	0.012	0.82	0.49
C20:5n3	nd	0.02	0.65	0.80	0.067	0.01	0.45
C22:6n3	nd	0.03	0.78	0.97	0.073	0.01	0.16
Total fatty acids	22.57	26.42	28.55	34.57	1.413	0.01	0.21

CON, control diet (53:47 forage to concentrate); FOL, control plus fish oil at 0.5% (diet DM); FOM, control plus fish oil at 2% (diet DM); FOH, control plus fish oil at 3.5% (diet DM); Lin, linear effect; Quad, quadratic; nd, not detected or detected at <0.01.

total VFA, butyrate, valerate and isovalerate or fermenters pH.

The concentrations of C18:0 decreased, while *trans* C18:1 increased ($p < 0.05$) in a linear manner as dietary FO amounts increased. Vaccenic acid was the predominant *trans* C18:1 isomer, and its concentration linearly increased ($p < 0.01$) with FO supplementation. Fish oil inclusion also resulted in a linear increase ($p < 0.02$) in the concentrations of *t*10, *t*12 and *t*16 C18:1 and total *trans* C18:1. Dietary FO amounts had no effects on the concentrations of *c*9*t*11 and *t*10*c*12 CLA. The quadratic effect was significant ($p < 0.05$) only with C18:0 and *t*9 C18:1.

The effect of dietary FO amounts on rumen bacteria is presented in Table 4. The DNA abundance for *B. fibrisolvens*, *Butyrivibrio* SA, *Butyrivibrio* VA and *B. proteoclasticus* decreased in a linear manner ($p < 0.01$) as dietary FO amounts increased. Fish oil supplementation had also quadratic effects ($p < 0.04$) on both *A. lipolytica* and *B. fibrisolvens*.

Discussion

Supplementing the diet with FO had no effect on fermenters pH but shifted fermentation towards propionate at the expense of acetate consistent with the

Table 4 Effect of incremental amounts of fish oil on the DNA abundance (pg) of selected rumen bacteria at a given starting DNA concentration

	Treatment					p-value	
	CON	FOL	FOM	FOH	SEM	Lin	Quad
Butyrivibrio fibrisolvens, 36 ng*	64.63	32.2	18.54	27.04	7.592	0.01	0.02
Butyrivibrio VA, 24 ng*	1.54	1.34	0.73	0.50	0.112	0.01	0.12
Butyrivibrio SA, 18 ng*	39.79	38.97	19.16	18.89	4.753	0.01	0.95
Butyrivibrio proteoclasticus, 20 ng*	1.46	1.25	0.60	0.40	0.225	0.01	0.46
Anaerovibrio lipolytica, 60 ng*	5.45	3.83	4.73	5.82	0.937	0.84	0.04

CON, control diet (53:47 forage to concentrate); FOL, control plus fish oil at 0.5% (diet DM); FOM, control plus fish oil at 2% (diet DM); FOH, control plus fish oil at 3.5% (diet DM); Lin, linear effect; Quad, quadratic.

findings of others (Doreau and Chilliard, 1997; Shingfield et al., 2003, 2011), and these changes may be linked to the alterations in the bacterial community with the FO supplementation (Huws et al., 2011; Potu et al., 2011). Consistent with our results, Lee et al. (2008) and Kim et al. (2008) reported no effects of incremental dietary FO supplementation on total VFA and butyrate concentrations. The relatively greater molar proportions of butyrate in fermenters are consistent with others' continuous fermenter studies (Miller-Webster et al., 2002; Vallimont et al., 2004; Gregorini et al., 2010; Potu et al., 2011; Gudla et al., 2012). Under *in vivo* condition, the rate of VFA absorption in the rumen is highest for butyrate and least for acetate (Bergman, 1990). Additionally, metabolized butyrate by the ruminal epithelium (Aschenbach et al., 2011) may also explain the greater butyrate values typically seen in continuous fermenters.

Increasing VA formation and flow from the rumen is the most limiting factor in enhancing milk *c9t11* CLA content as the majority of *c*9*t*11 CLA in milk is a result of endogenous synthesis by Δ-9 desaturase, with VA as the substrate (Griinari et al., 2000). The most noticeable changes in fermenters fatty acid concentrations with increasing FO supplementation were seen with respect to VA and C18:0. Fish oil supplementation interrupted the complete biohydrogenation of C18 *trans* fatty acids leading to a decrease in the concentration of C18:0 and increase in *trans* C18:1, VA in particular. Supplementing the diet with FO resulted in quadratic ~~increases~~ in C18:0 with concentration reaching a minimum with FOM consistent

with the findings of Lee et al. (2008) and Toral et al. (2012). However, other studies (Shingfield et al., 2011, 2012) reported only linear increases in C18:0 when incremental amounts of FO were added to animal diet. Speculating on reasons for the quadratic effects in this experiment is difficult as samples were collected from fermenters within the first six hours after feeding and quadratic effects were not seen with the other fatty acids. It is tempting, however, to speculate that the slight increases in C18:0 concentration with the FOH diet may have resulted from the increase in C18:0 intake with the HFO diet (Table 1). Alternation in ruminal biohydrogenation pathways leading to an increase in rumen or duodenal VA accumulation and a marked decrease in C18:0 formation are known to occur with FO supplementation (AbuGhazaleh and Jenkins, 2004; Wasowska et al., 2006; Kim et al., 2008; Lee et al., 2008; Potu et al., 2011). Previously, AbuGhazaleh and Jenkins (2004) demonstrated that the docosahexaenoic acid (DHA; C22:6n3), in FO or its derivatives are responsible for inhibiting the terminal hydrogenation of *trans* C18:1 to C18:0, causing the accumulation of *trans* C18:1 in the rumen. Although the mode of action could not be determined, they speculated that FO may alter the rumen ecosystem and/or inhibits the reductase activity of ruminal micro-organisms responsible for the reduction of *trans* C18:1 to C18:0.

Fish oil supplementation also induced a linear increase in the concentration of *t*10 C18:1, the second main *trans* C18:1 isomer after VA, suggesting a shift in the ruminal biohydrogenation pathway with the FO diets. The formation of *t*10 C18:1 is known to increase under low rumen pH conditions usually seen with feeding low-forage, high-concentrate diets (AbuGhazaleh and Jacobson, 2007; Fuentes et al., 2009; Gudla et al., 2012). Previously, Bauman et al. (1999) proposed a putative pathway for the production of *t*10 C18:1 under low rumen pH conditions where the *t*10*c*12 CLA-producing bacteria become predominant in the rumen resulting in formation of *t*10*c*12 CLA as the first intermediate during C18:2n6 biohydrogenation. Hydrogenation of the *c*12 bond would then result in the formation of *t*10, analogous to the production of VA from *c*9*t*11 CLA. The increases in *t*10C18:1 concentration in this study were not accompanied by greater concentrations of *t*10*c*12 CLA or decreases in fermenters pH consistent with the findings of others (Kim et al., 2008; Shingfield et al., 2011, 2012; Toral et al., 2012). Although the rapid reduction of *t*10*c*12 CLA to *t*10 C18:1 may explain the lack of *t*10*c*12 CLA accumulation in fermenters, these results may also suggest that *t*10*c*12 CLA may not be the only precursor for *t*10 C18:1 and other CLA isomers (*t*8*c*10, *t*8*t*10, *t*10*t*12) may also serve as precursors for *t*10 C18:1. Recently, Shingfield et al. (2011, 2012) reported an increase in the ruminal flow of *t*8*c*12 and *t*8*t*10 CLA when dairy cows and steer diets were supplemented with FO. The shift towards *t*10 C18:1 formation with FO supplementation may also indicate that FO increased the proliferation of certain bacteria that possess high *t*10 isomerase activity. Using the PCR-DGGE analysis of 16S rRNA genes, Kim et al. (2008) reported that only 77% of the band positions were similar between steers fed the control diet and the FO diet (30 g FO/kg; DM basis). Consistent with the findings of others (Kim et al., 2008; Shingfield et al., 2012; Toral et al., 2012), FO supplementation had no effect on the concentration of *c*9*t*11 CLA in the present experiment.

It is generally recognized that bacteria in the rumen are responsible for most of the biohydrogenation process in the rumen (Or-Rashid and Alzahal, 2008) and the main bacterial species identified as being most involved belong to the *Butyrivibrio* group (Paillard et al., 2007a). Using 16S rDNA sequence data, Paillard et al. (2007a) constructed a phylogenetic tree that contains two main groups of rumen *Butyrivibrio* bacteria called *Butyrivibrio* VA and *Butyrivibrio* SA. With this group, the only cultured *Butyrivibrio* SA producing bacteria are *B. proteoclasticus*. Lourenço et al. (2010) and McKain et al. (2010) identified *B. proteoclasticus* to be the only known ruminal species to convert *trans* C18:1 to C18:0.

The alternations in ruminal biohydrogenation to FO supplementation in this study were associated with changes in the DNA abundance of selected bacteria (Table 4). Dietary FO supplementation significantly lowered the DNA abundance of *B. fibrisolvens* indicating that FO inhibited their growth. This decrease in *B. fibrisolvens* is consistent with several observations reporting a decline in *B. fibrisolvens* with FO or DHA supplementations (Wallace et al., 2006; Potu et al., 2011; Toral et al., 2012) and may in part explain the reductions in acetate concentration with FO diets as it is generally agreed that *B. fibrisolvens* play fundamental role in fibre digestion (Harfoot and Hazlewood, 1997). Fish oil supplementations also significantly lowered the DNA abundance for *Butyrivibrio* VA. Belenguer et al. (2010) also reported that *Butyrivibrio* VA tended to be lower in sheep fed diets supplemented with FO. Additionally, results from Fuentes et al. (2009) and Gudla et al. (2012) showed that the increase in *t*10 C18:1 formation under low rumen pH conditions was also associated with a reduction in the abundance of *Butyrivibrio* VA. Whether the decrease in *Butyrivibrio* VA with the FO supplement is responsible

for the shift towards *t*10 C18:1 formation is by no means certain as other bacteria may also be involved.

Supplementing the diet with FO also linearly decreased the DNA abundance for *Butyrivibrio* SA and *B. proteoclasticus*. Belenguer et al. (2010) also reported a reduction in *Butyrivibrio* SA in steers fed FO diets. Because *B. proteoclasticus* is the only known cultured bacterium known to convert *trans* C18:1 to C18:0 (Lourenço et al., 2010; McKain et al., 2010), it is tempting to correlate the reductions in C18:0 formation usually seen with FO diets with the decreases in the abundance of this bacterium. However, inconsistent with our findings, other studies have failed to establish a strong correlation between the reductions in C18:0 flow with FO and marine algae diets and *B. proteoclasticus* (Kim et al., 2008; Potu et al., 2011; Shingfield et al., 2012; Toral et al., 2012). Additionally, Fuentes et al. (2009) and Gudla et al. (2012) found no correlations between *Butyrivibrio* SA and C18:0 flow under low rumen pH conditions. Studies that reported weak correlations between *Butyrivibrio* SA and *B. proteoclasticus* and C18:0 formation had suggested that other, yet-uncultivated bacteria might fulfil a more important role in the final step of the biohydrogenation process (Boeckaert et al., 2008; Huws et al., 2010) or the activity of these bacteria may not be proportional to DNA concentration (Huws et al., 2011). Recently, Huws et al. (2011) suggested that *B. proteoclasticus* may not play the dominant role in C18:0 formation and other yet-uncultivated bacteria, particularly those belonging to the genera *Prevotella* and *Anaerovoax*, and unclassified *Clostridiales* and *Ruminococcaceae* may play a role in biohydrogenation.

Anaerovibrio lipolytica is the bacterium most involved in lipolysis in the rumen (Prins et al., 1975). In this study, FO supplementation had quadratic effects on the DNA abundance of *A. lipolytica* with the least DNA abundance was seen with the FOL diet possibly suggesting that above a certain threshold, *A. lipolytica* becomes more active in hydrolysing ingested lipids. Results from Maia et al. (2007) reported no changes in the growth of *A. lipolytica* upon incubating with polyunsaturated fatty acids at 50 μg/ml, demonstrating the low sensitivity of this bacterium to high levels of oils. Recently, Potu et al. (2011) reported no changes in the DNA abundance of *A. lipolytica* when control diet was supplemented with FO and soybean oil at 3% of diet DM.

Conclusion

Supplementing the diet with incremental amounts of FO altered the fatty acid and specific bacteria composition in fermenters. Incremental inclusion of FO in the diet caused dose-dependent increases in VA and *t*10 C18:1 and a concomitant decrease in C18:0 formation, an effect that was accompanied by significant reductions in the DNA abundance of *Butyrivibrio* group. The dose-dependent reductions in the DNA abundance of *Butyrivibrio* VA, *Butyrivibrio* SA and *B. proteoclasticus* with FO may in part explain FO effects on the biohydrogenation of unsaturated fatty acids in the rumen.

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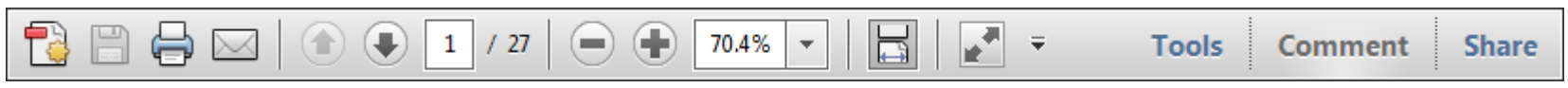

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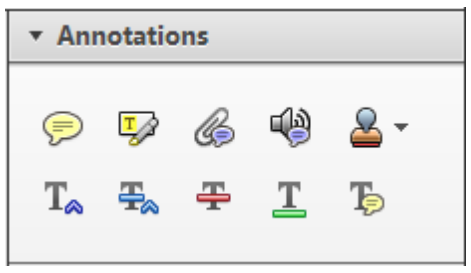

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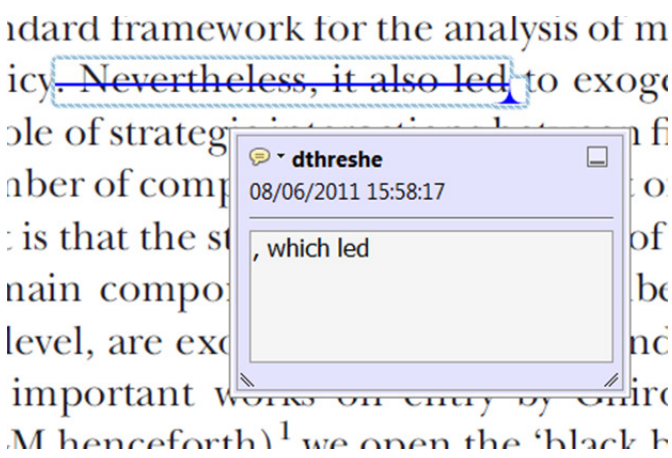

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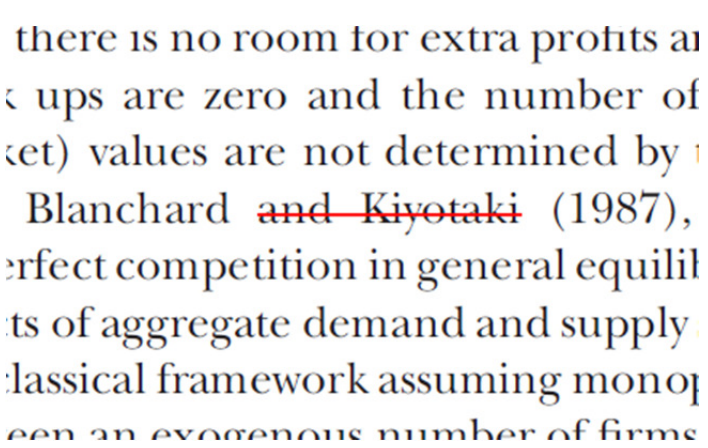

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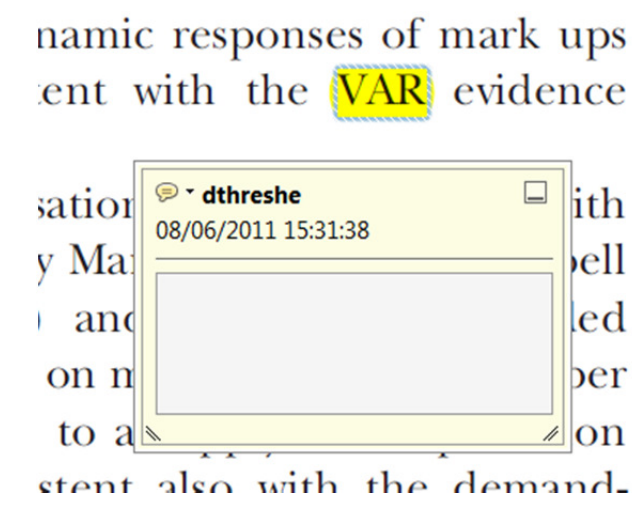

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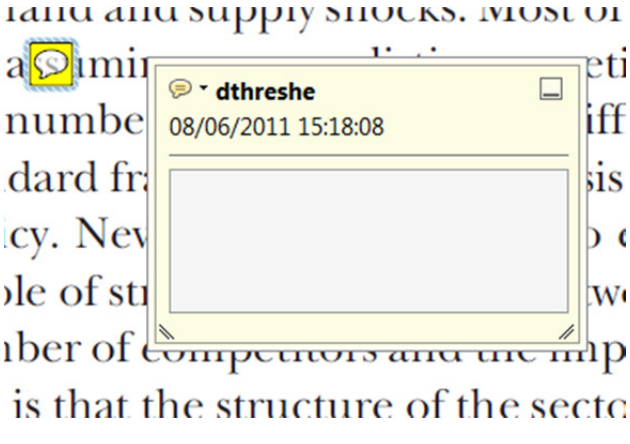

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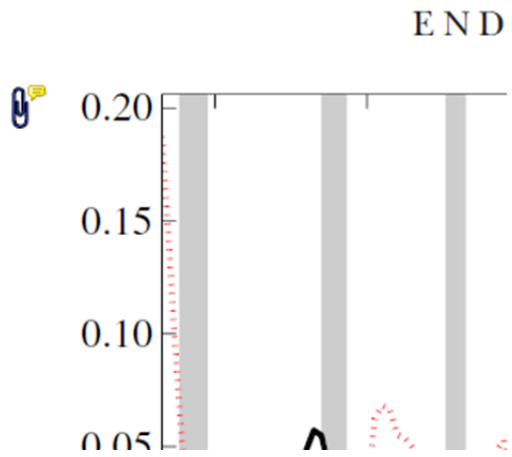

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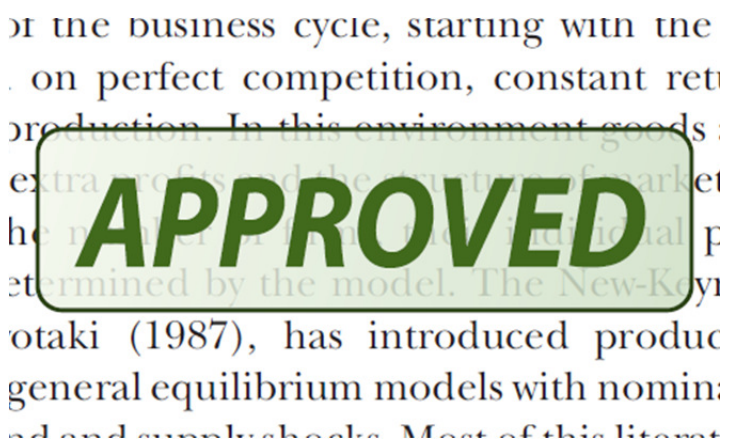

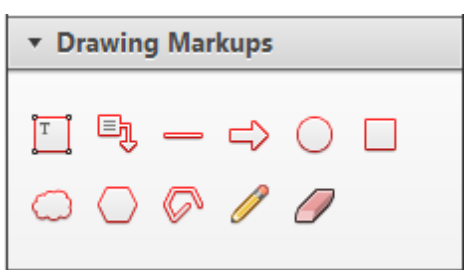

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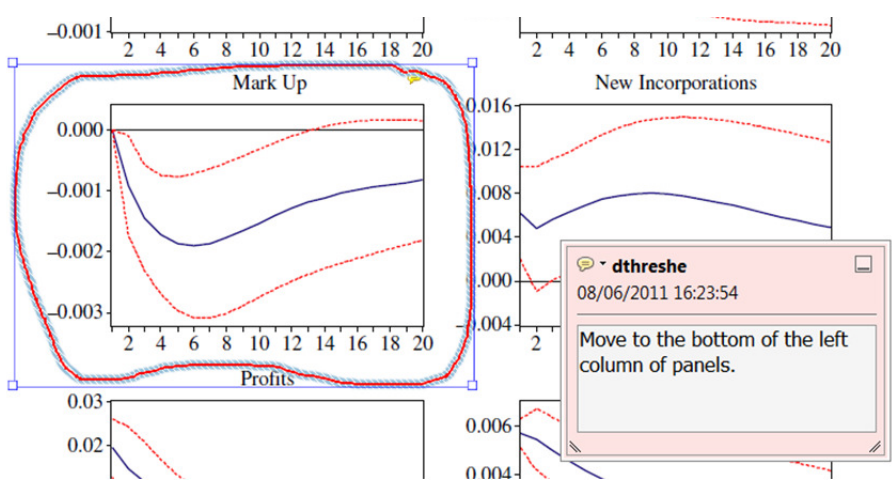

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