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Published in:
Journal of Nutrition

Publication date:
2008

Citation for published version (APA):

Kim, E. J., Huws, S. A., Lee, M. R., Wood, J. D., Muetzel, S. M., Wallace, R. J., & Scollan, N. D. (2008). Fish Oil Increases the Duodenal Flow of Long Chain Polyunsaturated Fatty Acids and trans-11 18:1 and Decreases 18:0 in Steers via Changes in the Rumen Bacterial Community. *Journal of Nutrition*, 138(5), 889-896. <http://hdl.handle.net/2160/2319>

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Fish Oil Increases the Duodenal Flow of Long Chain Polyunsaturated Fatty Acids and *trans*-11 18:1 and Decreases 18:0 in Steers via Changes in the Rumen Bacterial Community^{1,2}

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Abstract

Ruminant fat is rich in SFA, partly due to the biohydrogenation of dietary PUFA to SFA in the rumen. This process can be inhibited by the dietary inclusion of fish oil. The only bacteria isolated from the rumen capable of converting PUFA to SFA are closely related to *Clostridium proteoclasticum*. The aim of this study was to investigate if a correlation could be found in vivo between dietary fish oil inclusions and the composition of the ruminal bacterial community and specifically of *C. proteoclasticum*. Six Hereford × Friesian steers, prepared with ruminal and duodenal cannulae, received grass silage plus 1 of 3 concentrates resulting in total dietary fish oil contents of 0, 1, or 3% of dry matter. A dual flow marker technique was employed to estimate the relative flow of fatty acids. Steers fed the 3% fish oil diet had 100% increases in *trans* 18:1 flow, whereas 18:0 flow declined to 39% of steers fed the control diet. 16S ribosomal RNA-based denaturing gradient gel electrophoresis profiles obtained from ruminal digesta showed major changes in the bacterial community within steers fed the 3% fish oil diet. Quantitative PCR indicated only a weak relation between numbers of *C. proteoclasticum* and 18:0 flow between treatments and in individual steers ($P < 0.05$, but the percentage variance accounted for only 22.8) and did not provide unambiguous evidence that numbers of *C. proteoclasticum* in the rumen dictate the ratios of SFA:PUFA available for absorption by the animal. Understanding which microbes biohydrogenate PUFA in the rumen is key to developing novel strategies to improve the quality of ruminant products. J. Nutr. 138: 889–896, 2008.

Introduction

Recent nutritional advice has emphasized the need to decrease intake of SFA and increase intakes of beneficial PUFA, in particular α -linolenic acid [18:3(n-3)] and the longer chain (n-3) PUFA [20:5(n-3) and 22:6(n-3)], in the diet (1). Ruminant products make an important contribution to the human diet but have caused concern due to their enriched SFA content (2). This is largely due to microbial biohydrogenation of dietary unsaturated fatty acids in the rumen, although some intermediates of biohydrogenation such as conjugated linolenic acid (*cis*-9, *trans*-11 CLA)⁷ and

trans-11 18:1 could be important in human health (3). Previous studies have shown that including fish oil in the diet of beef cattle resulted in increased long chain (longer than 20 C) PUFA (LCPUFA) in muscle resulting in a lower (n-6):(n-3) fatty acid ratio (4). Fish oil has also been shown to interrupt the complete biohydrogenation of C18 PUFA, resulting in increased production of *trans*-11 18:1 (5–7), the precursor for CLA (*cis*-9, *trans*-11) in the mammary gland (8).

The bacteria involved in the different steps of the biohydrogenation pathway have been categorized as Group A and B (9): group A bacteria hydrogenate 18:2(n-6) and 18:3(n-3) to *trans*-11 18:1; in contrast, group B bacteria convert the same fatty acids to 18:0. The only Group B bacteria identified for many years was '*Fusocillus*' spp. (10,11). Modern phylogenetic analysis of recent isolates has now shown that 18:0-forming bacteria, like the most active Group A bacteria, are part of the *Butyrivibrio fibrisolvens* group, an ill-defined taxon that includes the genera *Butyrivibrio* and *Pseudobutyrvibrio* and the species *Clostridium proteoclasticum* (12,13). Group B bacteria (18:0 producers) form a tight grouping in which strains cluster together close to *C. proteoclasticum* (14,15). For this reason, in this article, the 18:0 producers are described as *C. proteoclasticum*.

¹ Supported by the Department for Environment, Food and Rural Affairs, the Biotechnology and Biological Sciences Research Council, and the Scottish Executive Environment and Rural Affairs Department, UK.

² Author disclosures: E. J. Kim, S. A. Huws, M. R. F. Lee, J. D. Wood, S. M. Muetzel, R. J. Wallace, and N. D. Scollan, no conflicts of interest.

⁶ These authors contributed equally to this study.

⁷ Abbreviations used: CLA, conjugated linoleic acid; DGGE, denaturing gradient gel electrophoresis; DM, dry matter; LCPUFA, long chain PUFA; NDF, neutral detergent fiber; OM, organic matter; QPCR, quantitative PCR; rRNA, ribosomal RNA; VFA, volatile fatty acid.

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L346616, so it should also be noted that this PCR-DGGE may amplify *Eubacterium* spp. The reverse B fib primer does have one and sometimes 2 mismatches in the middle of the primer for sequences deposited for cultivable *Eubacterium* spp. and in the presence of eubacterial 16S rRNA having a 100% match with this primer, amplification of *Eubacterium* spp. should be minimal.

All PCR amplifications were performed using a 2720 thermal cycler (Applied Biosystems) in 50- μ L volumes containing 1 \times PCR buffer (20 mmol \cdot L⁻¹ Tris HCl, pH 8.4, 50 mmol \cdot L⁻¹ KCl), 3 mmol \cdot L⁻¹ MgCl₂, 200 μ mol/L deoxyribonucleotide triphosphate mix, 500 mmol \cdot L⁻¹ each primer, and 1.25 U of iTaq DNA polymerase (Bio-Rad Laboratories) with \sim 100 ng of DNA template. Amplification conditions were: an initial denaturation of 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 56°C (total eubacteria) or 58°C (*Butyrivibrio* group) for 30 s and 72°C for 1 min, and then a final extension of 72°C for 5 min. Amplification of products was verified by agarose gel electrophoresis.

Amplicons were loaded onto 6% polyacrylamide gels with a 35–60% (total eubacteria) or a 35–65% (*Butyrivibrio* group) denaturing gradient [100% denaturant consisting of 40% (v:v) deionized formamide and 7 mol/L urea] and electrophoresis performed in a D-Code system (Bio-Rad Laboratories) as described previously (29). Gels were then stained with AgNO₃ (30). Gels were scanned using a GS-710 calibrated imaging densitometer (Bio-Rad Laboratories) and the saved image imported into the software package Fingerpringing (Bio-Rad Laboratories) for analysis. DGGE banding patterns were analyzed based on the presence and absence of the bands and resultant binary matrices were translated into distance matrices using the Dice similarity coefficient, with a position tolerance of 0.5% and optimization parameter of 1%. Finally, clusters were constructed using the method of unweighted pair group method with arithmetic mean analysis. The binary data generated were used to calculate band number and the Shannon-Weiner diversity index (24,31) using the Fingerprint Analysis with Missing Data software (32).

QPCR analysis. Total eubacterial amplification was carried out on ruminal digesta samples in a final volume of 25 μ L containing 12.5 μ L SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), 250 nmol \cdot L⁻¹ each of EubF1 5'-GTG STG CAY GGY TGT CGT CA-3' and Eub R1 5'-GAG GAA GGT GKG GAY GAC GT-3' (33), and 2 μ L of a 1:100 dilution of extracted genomic DNA. The thermal cycling program was 30 cycles of 94°C for 30 s and 61°C for 30 s with an initial cycle of 94°C for 5 min. After PCR, a dissociation curve (melting curve) was constructed in the range of 55°C to 95°C. All samples were run in triplicate. A bacterial standard was prepared with equal amounts of genomic DNA from 8 different pure cultures of bacteria: *Clostridium aminophilum* (ATCC 49906), *Peptostreptococcus anaerobius* (ATCC 27337), *Prevotella ruminicola* (ATCC 19189), *Fibrobacter succinogenes* (ATCC 19169), *B. fibrisolvens* [JW11; Rowett Research Institute (RRI)], *Ruminococcus albus* (SY3; RRI), *Selenomonas ruminantium* (Z108; RRI), and *Streptococcus bovis* (ES1; RRI).

QPCR analysis of 18:0-producing bacteria was conducted according to the method by Paillard et al. (34) on rumen-derived samples. Dilutions of purified genomic DNA from the control strain *Clostridium proteoclasticum* P-18 (RRI) were used to construct specific calibration curves. All samples were run in triplicate. Amplification was carried out in a final volume of 25 μ L containing 12.5 μ L of JumpStart Taq ReadyMix (Bio-Rad Laboratories), 400 and 800 nmol \cdot L⁻¹ of forward (SA-FW; 5'-TCC GGT GGT ATG AGA TGG GC) and reverse primers (SA-RV; 5'-GTC GCT GCA TCA GAG TTT CCT-3'), respectively, 250 nmol \cdot L⁻¹ of molecular beacon (5'-6 FAM-CCG CTT GGC CGT CCG ACC TCT CAG TCC GAG CGG-DABCYL-3'), and 2 μ L of a 1:10 dilution of extracted genomic DNA. The thermal cycling program was 40 cycles of 30 s at 95°C, 1 min at 55°C, and 30 s at 72°C with an initial cycle of 95°C for 10 min. Fluorescence data were collected at the end of the hybridization step at excitation and emission wavelengths of 490 and 530 nm, respectively.

All QPCR were performed using an iCycler iQ thermal cycler (Bio-Rad Laboratories) and results were analyzed using the iCycler iQ detection system software (Bio-Rad Laboratories).

Calculations and statistical analysis. Digesta flows were estimated after mathematical reconstitution of true digesta as described by

Faichney (18). Biohydrogenation of PUFA and LCPUFA was assessed as the difference between daily intake and duodenal flow ($g \cdot d^{-1}$) as a proportion of daily intake. Data for intakes and flows of nutrients [organic matter (OM), total N, neutral detergent fiber (NDF), and fatty acids], for rumen fermentation characteristics (pH, ammonia-N, VFA), and for eubacterial QPCR quantification were subjected to Restricted maximum likelihood analysis using GenStat [release 9.1, (35)]. The model included carryover and diet (treatment) as the fixed effect, and steer, period, and their interactions as the random effect. Treatment effects were further partitioned using a polynomial contrast to evaluate the significance of linear and quadratic components of the response to treatments. The level treated as not significant was $P > 0.05$, but trends were also expressed ($P < 0.1$). For band number and Shannon-Weiner diversity index data, difference was also inferred by using a Student's *t* test (32,35). For the correlation between the flow of 18:0 and the DNA concentration of 18:0-producing bacteria, bootstrap analysis (100 bootstrap measures) (35) was conducted to characterize the relationship more accurately, because linear regression demonstrated a few data points with high leverages.

Results

Chemical composition of the experimental diets. Concentrates were of typical composition with respect to barley and molasses sugar beet pulp with differences in the oil composition only (Table 1). The grass silage was of good quality with a low pH, and had comparatively low ammonia-N $\cdot g^{-1}$ total-N with 30.1 g total-N $\cdot kg^{-1}$ DM (Table 2). More than 50% of the fatty acid existed in the form of 18:3(n-3) in the grass silage. LCPUFA such as 20:5(n-3), 22:5(n-3), and 22:6(n-3) were undetectable in F0. Supplementing the concentrate with fish oil increased the LCPUFA concentration roughly proportionally to the dietary concentration of fish oil (Table 2).

TABLE 2 Chemical composition and fatty acid profile of the experimental diets¹

	Silage	Concentrate		
		F0	F1	F3
		<i>g \cdot kg^{-1} DM</i>		
DM	249	872	873	876
Crude protein	188	161	164	163
NDF	500	274	271	286
Ammonia-N	2.89	N/A ²	N/A ²	N/A ²
pH	3.72	N/A ²	N/A ²	N/A ²
Lactic acid	123	N/A ²	N/A ²	N/A ²
Fatty acid composition				
12:0	0.06	0.05	0.11	0.04
14:0	0.17	0.83	1.84	3.37
16:0	4.75	32.89	26.2	14.4
16:1	0.06	0.12	1.08	3.49
18:0	0.40	2.58	2.34	1.92
18:1(n-9)	0.53	22.42	18.94	12.11
18:2(n-6)	3.70	15.56	14.28	13.48
18:3(n-3)	16.29	1.29	1.42	1.93
20:0	0.12	0.20	0.19	0.17
20:5(n-3)	ND ³	ND ³	0.94	3.87
22:5(n-3)	ND ³	ND ³	0.25	1.00
22:6(n-3)	ND ³	ND ³	1.32	5.60
Total fatty acids	27.1	77.9	74.5	72.7

¹ Values are means; *n* = 3.

² N/A, Not applicable.

³ ND, Not detected.

Nutrient intake, duodenal flow, and rumen fermentation characteristics. As indicated in the experimental design, DM intake was controlled, with the mean intake of 7.42 kg · d⁻¹ with the targeted forage:concentrate ratio (60:40, DM basis) throughout the experiment. Duodenal flow of total-N indicated a net gain in steers fed diet F0 and F1 compared with the intakes and was not different between treatments (Table 3). Apparently digested NDF in the rumen ranged from 65 to 80% of intakes in all treatments. There were very few changes in rumen pH and the concentrations of ammonia-N and total VFA in the rumen (Table 3).

Fatty acids intake, duodenal flow, and biohydrogenation. Total fatty acid intake was different ($P < 0.001$) across treatments with steers fed F3 consuming 25 g · d⁻¹ less than steers offered the F0 diets, although the difference was marginal (just 7% of F0) (Table 4). Duodenal flow of total fatty acids indicated a net gain of 52 and 73 g · d⁻¹ in steers fed diets F0 and F1, respectively, but a net loss of 14 g · d⁻¹ in steers fed diet F3 compared with the intakes. Increasing the concentration of fish oil culminated in greater intakes and flows of major LCPUFA, including 20:5(n-3), 22:5(n-3), and 22:6(n-3). Duodenal flow of 18:0 was much higher than intake in steers receiving all diets and decreased extensively with increasing concentration of fish oil from F0 to F3 ($P < 0.001$). Flow of total *trans* 18:1 increased 2.0-fold in steers fed diet F3 relative to steers fed diet F0 ($P < 0.001$) and this was largely due to increased *trans*-10 and *trans*-11, with these isomers contributing >50% of total *trans* 18:1 in digesta (Table 5). Further fractionation of total *cis* 18:1 showed that the *cis*-9 was largely responsible for the decrease in the duodenal flow of this fraction. The most commonly reported CLA isomers, namely *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA, combined flow to the duodenum accounted for 52 and 50% of total CLA flow in

TABLE 3 Intake and duodenal flow of OM, total-N, NDF, and rumen fermentation parameters in steers receiving F0, F1, or F3 diets¹

	Diets			SED	Significance ²	
	F0	F1	F3		L	Q
Intake, kg · d ⁻¹						
OM	6.61	6.61	6.70	0.074	NS	NS
Total N	0.21	0.21	0.21	0.002	*	NS
NDF	3.08	2.92	3.10	0.034	NS	‡
Duodenal flow, kg · d ⁻¹						
OM	3.83	3.62	3.46	0.317	NS	NS
Total N	0.24	0.23	0.22	0.022	NS	NS
NDF	1.07	0.77	0.61	0.158	†	NS
Fermentation parameters						
pH	6.46	6.58	6.59	0.127	NS	NS
Ammonia-N, mmol · L ⁻¹	9.69	10.25	10.03	0.714	NS	NS
VFA (molar proportion)						
Acetate	58.9	57.8	57.1	0.81	*	NS
Propionate	22.6	22.9	23.3	0.66	NS	NS
Iso-Butyrate	1.40	1.46	1.45	0.127	NS	NS
n-Butyrate	13.2	13.5	14.1	0.665	NS	NS
Iso-Valerate	1.93	2.22	2.57	0.140	‡	NS
n-Valerate	1.73	1.70	1.70	0.117	NS	NS
Total VFA, mmol · L ⁻¹	78.9	77.7	79.8	4.81	NS	NS

¹ Values are means, n = 6.

² L, Linear response; Q, quadratic response; NS, not significant, $P \geq 0.05$; * $P < 0.1$; † $P < 0.05$; ‡ $P < 0.01$.

TABLE 4 Fatty acid intake and duodenal flows in steers receiving F0, F1, or F3 diets¹

	Diets			SED	Significance ²	
	F0	F1	F3		L	Q
Intake	g · d ⁻¹					
12:0	0.45	4.07	2.94	0.108	§	§
14:0	2.92	5.80	10.13	0.157	§	†
16:0	116.2	94.0	57.6	1.29	§	*
18:0	9.23	8.28	6.81	0.091	§	NS
18:1(n-9)	66.3	54.7	33.3	0.75	§	NS
18:2(n-6)	59.4	56.9	54.3	0.60	§	NS
18:3(n-3)	78.4	78.8	81.0	0.94	†	NS
20:4	0.07	0.27	0.77	0.013	§	†
20:5(n-3)	ND ³	2.87	10.65	0.169	§	N/A
22:5(n-3)	ND ³	0.72	2.73	0.043	§	N/A
22:6(n-3)	ND ³	3.88	15.29	0.241	§	N/A
Total fatty acids	344	331	319	3.6	§	NS
Duodenal flow						
12:0	0.58	1.08	0.50	0.092	§	§
14:0	2.93	4.67	4.70	0.275	§	§
16:0	118.8	112.2	65.3	9.50	§	NS
16:1	0.30	0.75	1.58	0.262	§	NS
18:0	152.7	115.1	58.9	19.09	§	NS
18:1 <i>trans</i> (total)	42.5	73.2	83.4	9.96	‡	*
18:1(n-9)	20.4	21.2	14.4	2.89	†	NS
18:2(n-6)	7.40	7.64	3.40	1.344	‡	NS
18:3(n-3)	3.32	3.71	2.08	0.734	*	NS
18:2 <i>cis</i> -9, <i>trans</i> -11	0.21	0.28	0.13	0.058	NS	*
20:4	0.34	0.45	0.24	0.127	NS	NS
20:5(n-3)	0.27	0.48	0.83	0.199	†	NS
22:5(n-3)	0.25	0.40	0.88	0.294	*	NS
22:6(n-3)	0.14	0.39	1.01	0.249	‡	NS
Total fatty acids	396	404	305	34.0	†	NS

¹ Values are means; n = 6.

² L, linear response; Q, quadratic response; N/A, not applicable; NS, not significant, $P \geq 0.05$; * $P < 0.1$; † $P < 0.05$; ‡ $P < 0.01$; § $P < 0.001$.

³ ND = not detected.

steers fed the F1 and F3 diets, respectively (Table 5). Fish oil did not affect the duodenal flow of the *cis*-9, *trans*-11 isomer, while increasing fish oil had an elevatory effect on *trans*-10, *cis*-12 flow ($P < 0.001$). However, total CLA flow to the duodenum did not change, with *trans*-11 *trans*-13 most abundant in steers fed all diets. Biohydrogenation of 18:1(n-9), 18:2(n-6), and 18:3(n-3) ranged between 0.58 and 0.97 among treatments. Likewise, the majority of 20:5(n-3) and 22:6(n-3) was hydrogenated in the rumen and biohydrogenation of 22:5(n-3) was lower than the other LCPUFA (Table 6).

Microbial population profiling. PCR-DGGE analysis of 16S rRNA genes using primers universal for eubacteria indicated complex communities in all samples, with many similarities between steers receiving the F0 and F1 diets (Fig. 1A). Diet F3 substantially altered the bacterial composition in the rumen of steers fed this diet. Cluster analysis separated steers fed diet F3 from those fed diets F0 and F1, with 77% of the band positions being the same (Fig. 1A). Band number also decreased ($P < 0.05$) in rumen samples taken from steers fed diet F3 compared to those fed diets F0 and F1 (Table 7). Similar analysis for members of the *Butyrivibrio* group showed a less complex pattern than eubacteria (Fig. 1B). Two main clusters were evident, separating

TABLE 5 Duodenal flow of 18:1 and CLA isomers in steers receiving F0, F1, or F3 diets¹

	Diets			SEM	Significance ²	
	F0	F1	F3		L	Q
<i>g · d⁻¹</i>						
Fatty acid flow						
18:1 <i>trans</i>						
4	0.38	0.35	0.17	0.017	§	†
5	0.16	0.21	0.18	0.024	NS	NS
6 + 7 + 8	2.74	4.13	3.64	0.641	NS	*
9	1.60	3.01	2.99	0.409	†	†
10	2.69	4.41	15.40	2.910	§	NS
11	19.1	40.4	40.9	6.705	†	†
12	3.01	5.72	6.00	0.515	§	‡
13 + 14 and <i>cis</i> -6	4.47	5.07	3.55	0.770	NS	NS
15 and <i>cis</i> -10	3.93	5.09	5.00	0.680	NS	NS
16	4.17	5.15	5.72	0.766	*	NS
Total 18:1 <i>trans</i>	42.5	73.2	83.4	9.96	‡	*
18:1 <i>cis</i>						
9	20.4	21.2	14.4	2.89	†	NS
11	0.98	1.72	2.78	0.408	§	NS
12	1.00	0.78	0.65	0.141	†	NS
13	0.04	0.58	0.75	0.222	†	NS
14	0	0.44	1.10	0.240	§	NS
15	1.02	1.07	2.02	0.201	§	NS
Total 18:1 <i>cis</i>	23.5	25.8	21.6	3.14	NS	NS
CLA ³						
<i>cis</i> -9, <i>trans</i> -11	0.21	0.28	0.13	0.058	NS	*
<i>trans</i> -10, <i>cis</i> -12	0.09	0.26	0.49	0.070	§	NS
<i>trans</i> -11, <i>trans</i> -13 + <i>trans</i> -13, <i>trans</i> -15	0.62	0.44	0.54	0.094	NS	*
<i>trans</i> -7, <i>trans</i> -9 to <i>trans</i> -10, <i>trans</i> -12	0.06	0.07	0.05	0.033	NS	NS
Total CLA	0.96	1.03	1.23	0.180	NS	NS

¹ Values are means; *n* = 6.

² L, linear response; Q, quadratic response; N/A, not applicable; NS, not significant, *P* ≥ 0.05; **P* < 0.1; †*P* < 0.05; ‡*P* < 0.01; §*P* < 0.001.

³ CLA isomers of double bond positions were identified according to Delmonte et al. (55).

steers fed diet F3 from those fed the other 2 diets, with 66% of the band positions being similar (Fig. 1B), and again band numbers were lower (*P* < 0.05) in F3 (Table 7). Subclusters between rumen samples derived from steers fed diets F0 and F1 were not clear in either analysis, indicating that supplementing fish oil at 1% of DM did not substantially alter microbial

TABLE 6 Biohydrogenation of unsaturated fatty acids in the rumen of steers receiving F0, F1, or F3 diets¹

	Diets			SED	Significance ²	
	F0	F1	F3		L	Q
<i>g · g⁻¹</i>						
18:1(n-9)	0.69	0.61	0.58	0.056	*	NS
18:2(n-6)	0.88	0.88	0.94	0.024	†	NS
18:3(n-3)	0.96	0.95	0.97	0.010	NS	NS
20:5(n-3)	N/A ²	0.82	0.93	0.021	§	N/A
22:5(n-3)	N/A ²	0.48	0.70	0.223	NS	N/A
22:6(n-3)	N/A ²	0.90	0.94	0.031	NS	N/A

¹ Values are means; *n* = 6.

² L, linear response; Q, quadratic response; N/A, not applicable; NS, not significant, *P* ≥ 0.05; **P* < 0.1; †*P* < 0.05; §*P* < 0.001.

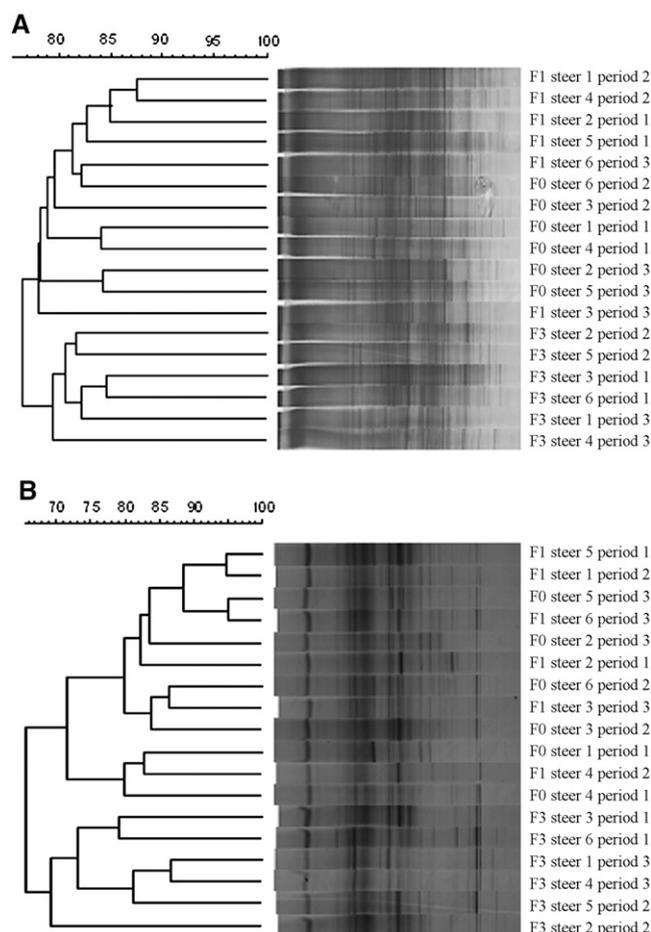


FIGURE 1 PCR-DGGE-derived unweighted pair group method with arithmetic mean dendrogram showing the effect of fish oil on the total eubacterial (A) and *Butyrivibrio* (B) populations in the rumen. F0, F1, and F3 relate to diets consisting of 0, 1, and 3% fish oil intake expressed in terms of DM intake. Scale relates to percent similarity.

population in the rumen. The Shannon-Weiner diversity index showed only minor differences in bacterial diversity for steers fed all diets with respect to both total eubacteria and the *Butyrivibrio* population (Table 7).

QPCR of 16S rRNA genes indicated that total eubacterial DNA concentration was not significantly different as fish oil increased (data not shown). The same was true of *C. proteoclasticum* 16S rRNA gene concentration (Fig. 2A). When *C. proteoclasticum* numbers were expressed as a proportion of total eubacteria, there was no significant differences as fish oil increased (data not shown). A regression approach to the relationship between fish oil supplementation in the diets of the steers with the number of *C. proteoclasticum* in the rumen showed a significant relationship (*P* < 0.05), but the percentage variance accounted for (equivalent to adjusted *r*²) was only 22.8, which was not sufficient to provide a reliable interpretation of the relationship (Fig. 2B). Therefore, bootstrap estimates from 100 bootstrap samples were performed to examine the correlation more accurately and the results were not fully supportive of a clear association between the 2 variables (Fig. 2B).

Discussion

Numerous reports have appeared in the literature demonstrating the effects of fish oil on ruminal biohydrogenation. The addition

TABLE 7 Band number and Shannon-Weiner diversity index calculated from the total eubacterial and the *Butyrivibrio* group-specific DGGE profiles obtained from rumen samples of steers receiving F0, F1, or F3 diets¹

	Diets		
	F0	F1	F3
Total eubacteria			
Band number	69 ± 3.4 ^a	66 ± 3.9 ^a	58 ± 4.6 ^b
Shannon-Weiner diversity index	6.56 (0.005) ^a	6.36 (0.007) ^b	6.61 (0.005) ^b
<i>Butyrivibrio</i> group specific			
Band number	69 ± 2.7 ^a	67 ± 6.4 ^a	61 ± 3.9 ^b
Shannon-Weiner diversity index	4.37 (0.041)	4.18 (0.054)	4.51 (0.035)

¹ Values are means ± SEM, n = 6 or index (variance). Means in a row with super-scripts without a common letter differ, P < 0.05.

of fish oil to ruminant rations has been used to manipulate LCPUFA concentrations of meat (2,4) and milk (36,37). Upon ingestion by ruminants, dietary lipid undergoes substantial lipolysis followed by microbial biohydrogenation to produce a high concentration of SFA in the rumen. One of the major pathways of biohydrogenation in the rumen is simplified as the conversion of free C18 PUFA to conjugated dienes or trienes, and then to 18:1 and finally by reduction to form 18:0 (11). The results of the present experiment were consistent with earlier findings in that the flow of 18:0 to the duodenum was significantly lower when fish oil was incorporated into the diet

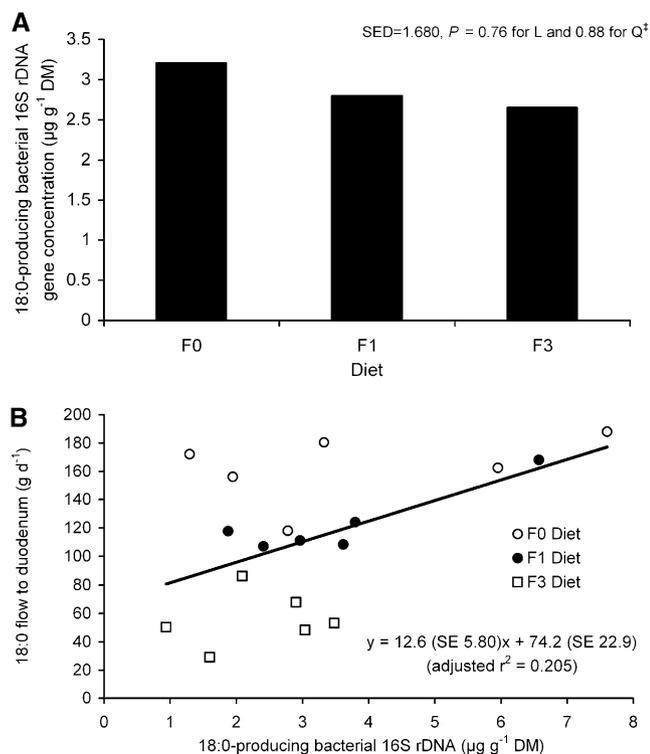


FIGURE 2 18:0-producing bacterial 16S rDNA gene concentration obtained from strained rumen fluid of steers given the experimental diets (A), and correlation between 18:0-producing bacterial 16S rDNA gene concentration and 18:0 flow to the duodenum (B). F0, F1, and F3 relate to diets consisting of 0, 1, and 3% fish oil intake expressed in terms of DM intake, n = 6. L, Linear response; Q, quadratic response.

of steers. The effect was also dependent on the concentration of the fish oil, as observed in other studies too (5,6,36). Several researchers reported a similar phenomenon with fish oil-supplemented diets (5–7,36) and suggested that the fish oil effect might involve an inhibition of the enzyme that catalyzes the final biohydrogenation step in the rumen. Alternatively, the proliferation of bacteria capable of conversion of 18:3 and 18:2 through to 18:0 might be inhibited by the LCPUFA in fish oil, resulting in increased *trans*-11 18:1. A recent study by Wasowska et al. (38) showed that the addition of 20:5(n-3) or 22:6(n-3) ($50 \text{ mg} \cdot \text{L}^{-1}$) to pure cultures inhibited the growth and isomerase activity of *B. fibrisolvens*, whereas fish oil, in which the fatty acids are present as triacylglycerol, had no effect. Other reports (39,40) suggested that the accumulation of 18:2(n-6) in the rumen [by feeding more 18:2(n-6) to the ruminant] causes incomplete biohydrogenation. However, in the present study, the intake of 18:2(n-6) was similar with increasing concentrations of fish oil, excluding any possibility that 18:2(n-6) in the diets was responsible for the observed effect on biohydrogenation. Our data showed that fish oil at 3% of DM intake decreased the 18:0 flow and also increased the flow of *trans*-11 18:1 to the duodenum.

Increased flows of 20:5(n-3), 22:5(n-3), and 22:6(n-3) to the duodenum occurred in steers fed fish oil diets. The increased duodenal flow of these fatty acids in steers was lower than the increased dietary intake between diets F1 and F3. For example, the intake of 22:6(n-3) in steers fed diet F3 was 2.9-fold higher than that of steers fed diet F1 while only 1.6-fold higher in the duodenal flow. Biohydrogenation was presumably a major reason for this difference (5–7), although the degree to which these LCPUFA are biohydrogenated and the factors affecting biohydrogenation of LCPUFA in the rumen are still not well understood. Several studies have indicated that 20:5(n-3) and 22:6(n-3) are extensively metabolized in the rumen in vivo (5,6,41). In vitro studies have been less clear with some studies showing limited biohydrogenation (42), while others showed a decrease (43,44) or an increase in the extent of 20:5(n-3) and 22:6(n-3) biohydrogenation in relation to fish oil addition (45). It is also notable that although there was no intake of LCPUFA in the present study for the F0 diet, there were still measurable flows of these LCPUFA at the duodenum, which may reflect endogenous lipid from cell desquamation during digestive processes.

In terms of CLA, *cis*-9, *trans*-11 CLA was not the major isomer in duodenal digesta; *trans*-11, *trans*-13 CLA isomer accounted for ~50% of total CLA. Increased flow of *trans-trans* CLA leaving the rumen is consistent with previous reports of diets supplemented with fish oil (5,6,46). In addition, Lee et al. (7) reported that diets with high levels of 18:3(n-3), such as those based on forage or supplemented with oil rich in 18:3(n-3), produced *trans*-11, *trans*-13 as the predominant CLA isomer, possibly as a consequence of its involvement in the 18:3(n-3) biohydrogenation pathway. Duodenal flow of *trans*-10, *cis*-12 CLA, which is associated with modulating fat deposition (47) and milk fat depression (48), was significantly higher in steers fed the F3 diet. Several bacteria have been reported to convert 18:2(n-6) to *trans*-10, *cis*-12 CLA, including *Lactobacillus* spp. (49), *Propionibacterium acnes* (50), and *Megasphaera elsdenii* (51). However, to what extent these particular microorganisms play a role in ruminal biohydrogenation with the diet supplemented with fish oil remains unclear.

The microbial ecology of the rumen changed substantially with the addition of fish oil to the diet of steers, particularly at the higher inclusion rate. Many fewer bands were present in DGGE of 16S rDNA genes amplified by universal eubacterial primers. This reflects the toxicity of unsaturated fatty acids to

ruminal bacteria, which is particularly severe for cellulolytic species and also butyrate producers (52), although no difference in the concentration of ruminal butyrate was observed in the present study. The altered banding pattern presumably reflects the loss of the most sensitive species. Biohydrogenation occurs to detoxify the fatty acids. DGGE was also used to analyze the impact of fish oil on the *Butyrivibrio* population. These bacteria are believed to be the most active species involved in fatty acid biohydrogenation (39,53). Their sensitivity to PUFA is highly variable, with some members of the group sensitive to growth inhibition at PUFA concentrations of 5 mg/L (13) while others tolerate concentrations many times higher. The persistence of some bands but not others in the *Butyrivibrio* DGGE is consistent with this range of sensitivity.

Bacteria forming a small branch of the *Butyrivibrio* phylogenetic tree, clustering around *C. proteoclasticum*, are among the most sensitive ruminal species to the toxic effects of PUFA (13,15). They are also the only known ruminal species to convert *trans*-11 18:1 to 18:0 (10,15). *C. proteoclasticum* was originally isolated as a proteolytic species (54). Its name does not reflect its taxonomic position accurately, because it is not a spore former and is distantly related to true *Clostridium* species (15) and its name is in need of revision. Whether *C. proteoclasticum* truly represents the predominant 18:0 producers in the rumen is by no means certain and it is possible that other 18:0 producers have not yet been cultivated; indeed, some may not be cultivable at all using present culture techniques. If *C. proteoclasticum* is indeed the main 18:0 producer, a strong correlation might be expected between their numbers and the extent of biohydrogenation to 18:0 in digesta leaving the rumen. Primers and a probe designed to detect the *C. proteoclasticum* group and to exclude related *Butyrivibrio* that do not form 18:0 (34) were used in QPCR to assess the influence of fish oil on *C. proteoclasticum* numbers. The results were equivocal in the sense that a correlation was found, but it was rather weak, particularly when comparing different steers. It may be that other microbial species are involved, although no other ruminal bacteria, protozoa, or fungi are known to carry out the reaction to date. Alternatively, metabolic factors may be involved; for example, 18:0 formation occurs during the growth phase of *C. proteoclasticum*, not when it reaches stationary phase (15). Thus, the metabolic activity of *C. proteoclasticum* may not be proportional to 16S rRNA gene concentration and RNA may be a better marker.

In conclusion, these results are consistent with the hypothesis that fish oil has an inhibitory effect on the biohydrogenation of fatty acids in the rumen via its influence on microbial ecology. Total bacteria and *Butyrivibrio* populations were changed, consistent with the elimination of a number of species by fish oil. Numbers of *C. proteoclasticum* and duodenal fatty acid composition gave a weak correlation that neither offers strong support for nor against its predominant role in the process vis-à-vis other unknown bacteria that may convert *trans*-11 18:0 to 18:0. We are now attempting to link microbial changes to differences in fatty acid flow to the duodenum, using multivariate statistical approaches and sequencing of key bands, to identify other key bacteria that may be involved at various points of the biohydrogenation sequence. This information could potentially allow the development of novel strategies for manipulating this process, leading to the beneficial enhancement in nutritional value of ruminant products.

Acknowledgments

We thank Martin Leyland at Institute of Grassland and Environmental Research, Trawsgoed research farm for the care of the

steers, Delma Jones and colleagues in the Analytical Chemistry Department, John Tweed for the skilled GC analysis of lipid samples, and Mark Scott for help with the microbial analysis. We are also thankful for the statistical support of Graham Horgan.

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