Short Communication: Eicosatrienoic Acid and Docosatrienoic Acid Do Not Promote Vaccenic Acid Accumulation in Mixed Ruminal Cultures

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ABSTRACT

Previous research found that docosahexaenoic acid (C22:6n-3) was a component of fish oil that promotes *trans*-C18:1 accumulation in ruminal cultures when incubated with linoleic acid. The objective of this study was to determine if eicosatrienoic acid (C20:3n-3) and docosatrienoic acid (C22:3n-3), n-3 fatty acids in fish oil, promote accumulation of *trans*-C18:1, vaccenic acid (VA) in particular, using cultures of mixed ruminal microorganisms. Treatments consisted of control, control plus 5 mg of C20:3n-3 (ETA), control plus 5 mg of C22:3n-3 (DTA), control plus 15 mg of linoleic acid (LA), control plus 5 mg of C20:3n-3 and 15 mg of linoleic acid (ETALA), and control plus 5 mg of C22:3n-3 and 15 mg of linoleic acid (DTALA). Treatments were incubated in triplicate in 125-mL flasks, and 5 mL of culture contents was taken at 0 and 24 h for fatty acid analysis by gas-liquid chromatography. After 24 h of incubation, the concentrations of *trans*-C18:1 (0.87, 0.88, and 0.99 mg/culture), and VA (0.52, 0.56, and 0.62 mg/culture) were similar for the control, ETA, and DTA cultures, respectively. The concentrations of *trans*-C18:1 (5.51, 5.41, and 5.36 mg/culture), and VA (4.78, 4.62, and 4.59 mg/culture) were also similar between LA, ETALA, and DTALA cultures, respectively. These data suggest that C20:3n-3 and C22:3n-3 are not the active components in fish oil that promote VA accumulation when incubated with linoleic acid.

Key words: docosatrienoic acid, eicosatrienoic acid, vaccenic acid

Conjugated linoleic acid (CLA) refers to a collection of positional and geometric isomers of octadecadienoic acid with conjugated double bonds. The *cis*-9,*trans*-11 CLA can be synthesized in the rumen as an intermediate in the biohydrogenation of linoleic acid and in animal tissues by Δ^9 -desaturase from vaccenic acid

(*trans*-11 C18:1, VA), another intermediate in ruminal biohydrogenation (Griinari and Bauman, 1999). In animal models, *cis*-9,*trans*-11 CLA reduced the incidence and growth of tumors, enhanced immune function, and prevented diabetes (Belury, 1995; Parodi, 1997). Recent studies (Corl et al., 2001; Piperova et al., 2002) have indicated that the majority of milk *cis*-9,*trans*-11 CLA (78 to 93%) is derived from Δ^9 -desaturation of VA. Therefore, increasing VA production in the rumen would be the most feasible approach to enhance milk fat *cis*-9,*trans*-11 CLA.

Production of VA in the rumen can be enhanced by changes in the diet, especially through utilization of diets with high concentrations of unsaturated fat. When fed at similar concentrations, fish oil was more efficient than polyunsaturated plant oils in increasing milk VA concentration, even though fish oil contains only small amounts of the primary precursors (linoleic acid, linolenic acid) of VA (Offer et al., 1999; Whitlock et al., 2002). These findings led AbuGhazaleh et al. (2001) to hypothesize that a component in fish oil may have stimulated ruminal VA production from other sources of unsaturated fatty acids. To test this hypothesis, AbuGhazaleh et al. (2002) fed fish oil from fish meal with or without extruded soybeans (linoleic acid source) to dairy cows. They observed that feeding the fish meal and extruded soybeans diet increased the concentration of milk *cis*-9,*trans*-11 CLA and VA more than feeding the fish meal or extruded soybeans separately. Recently, AbuGhazaleh and Jenkins (2004a) identified docosahexaenoic acid (C22:6n-3; DHA) as an active component in fish oil that promotes VA accumulation in ruminal cultures. Because fish oil contains additional n-3 fatty acids suspected of playing a role in ruminal *trans*-C18:1 production, in particular VA, we hypothesize that eicosatrienoic (C20:3n-3) and docosatrienoic acid (C22:3n-3), n-3 fatty acids found in fish oil (approximately 2% of total fatty acids), also promote VA accumulation when incubated with linoleic acid.

Eicosatrienoic acid, C22:3n-3, and linoleic acid (>99% purity) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO), dissolved in etha-

Received March 13, 2006.

Accepted May 22, 2006.

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nol, and stored at -20°C until used. Ruminal contents were collected 3 h after morning feeding (0600 h) from a ruminally fistulated Holstein cow fed a TMR composed of 50% concentrate mix, 25% corn silage, 12.5% alfalfa haylage, and 12.5% alfalfa hay (DM basis). The rumen contents were brought to the laboratory in a plastic bag under anaerobic conditions, strained through 2 layers of cheesecloth, and used within 15 min. Treatments consisted of control (no added fatty acids; **CONT**), control plus 5 mg of C20:3n-3 (**ETA**), control plus 5 mg of C22:3n-3 (**DTA**), control plus 15 mg of linoleic acid (**LA**), control plus 5 mg of C20:3n-3 and 15 mg of LA (**ETALA**), and control plus 5 mg of C22:3n-3 and 15 mg of LA (**DTALA**). Eicosatrienoic, C22:3n-3, and LA in 100 μL of ethanol were added directly into the treatment cultures.

Cultures were maintained in 125-mL Erlenmeyer flasks containing 500 mg of the cow TMR diet dried and ground through a 2-mm screen, 10 mL of strained ruminal fluid, 40 mL of media, and 2 mL of reducing solution according to Goering and VanSoest (1970). All buffer solutions were prewarmed at 39°C and flushed with CO_2 . Cultures were run in triplicate at 39°C under anaerobic conditions. A 5-mL sample was taken from each culture flask at 0 and 24 h while being stirred with a magnetic bar under a stream of CO_2 , placed immediately in an ice bath, and then stored at -20°C .

Samples were freeze-dried and then methylated according to Kramer et al. (1997) and analyzed for fatty acids by GLC. Methylated fatty acids were separated on a Shimadzu GC-2010 gas chromatograph equipped with a flame-ionization detector and a Supelco 100-m SP-2560 fused-silica capillary column (0.25 mm i.d. $\times$ 0.2- μm film thickness, Supelco, Bellefonte, PA). The helium carrier gas was maintained at a linear velocity of 23 cm/s. The oven temperature was programmed for 170°C for 50 min, then increased at $5^{\circ}\text{C}/\text{min}$ to 249°C and held for 10 min. The injector and detector temperatures were set at 255°C . Heptadecanoic acid (C17:0) was added to all samples as an internal standard. Peaks were identified by comparing the retention times with those of the corresponding standards (Nuchek Prep, Elysian, MN; Supelco; and Larodan Fine Chemicals, Malmo, Sweden).

Data were analyzed using the mixed model procedure of SAS (SAS Inst., Inc., Cary, NC) using treatment as the fixed effect, and replicate as the random effect. All results were expressed as least squares means. Preplanned contrasts were 1) CONT vs. ETA, 2) CONT vs. DTA, 3) LA vs. ETALA, and 4) LA vs. DTALA. Significance was declared at $P < 0.05$.

If C20:3n-3 or C22:3n-3 were the components in fish oil that promote *trans*-C18:1 accumulation when incubated with other unsaturated fat sources, combining these 2 fatty acids with linoleic acid would maximize *trans*-C18:1 concentration, VA in particular. After 24 h of incubation, the concentration of *trans*-C18:1 was similar ($P > 0.05$), with CONT, ETA, and DTA cultures averaging 0.87, 0.88, and 0.99 mg/culture, respectively (Table 1). Additions of C20:3n-3 or C22:3n-3 to ruminal cultures did not increase VA (0.56 and 0.62 mg/culture, respectively) concentration compared with CONT cultures (0.52 mg/culture). As expected, addition of linoleic acid to ruminal cultures increased *trans*-C18:1 and VA concentrations approximately 5- and 8-fold respectively, compared with CONT. However, combining C20:3n-3 or C22:3n-3 with linoleic acid did not further increase ($P > 0.05$) *trans*-C18:1 and VA concentrations compared with LA cultures (Table 1). The concentrations of *trans*-C18:1 (5.51, 5.41, and 4.91 mg/culture) and VA (4.78, 4.62, and 4.19 mg/culture) were similar ($P > 0.05$) for LA and ETALA, and DTALA, respectively. Previously, AbuGhazaleh and Jenkins (2004b) showed that combining DHA with a linoleic acid source resulted in greater *trans*-C18:1 and VA concentrations and VA:C18:0 ratio than when they were added separately in cultures. AbuGhazaleh and Jenkins (2004a) concluded that DHA or its derivatives promoted *trans*-C18:1 and VA accumulations, possibly by inhibiting the reductase enzyme in ruminal microorganisms that is responsible for the terminal hydrogenation of *trans*-C18:1 to C18:0. In the current study, addition of C20:3n-3 or C22:3n-3 to ruminal cultures had no effect on the VA:C18:0 ratio (Table 1).

Both ETA and DTA disappeared from cultures after 24 h of incubation and the disappearance of ETA was greater (47 to 60%) than that of DTA (23 to 25%). Disappearance of ETA and DTA from cultures can be caused by their transformation into other C20:3 and C22:3 isomers as a result of enzymatic isomerization; hydrogenation of their double bond; or by shortening of their carbon chain. Despite ETA and DTA disappearance from cultures, there were no increases in any of the identified fatty acids (Table 1) when compared with CONT, indicating that EPA and DTA were not converted into any of these fatty acids. However, some unidentified peaks were detected around the ETA and DTA peaks in cultures. Similar unidentified peaks around DHA and eicosapentaenoic acid (C20:5) peaks were seen previously (AbuGhazaleh and Jenkins, 2004b).

The lack of effect of C20:3n-3 and C22:3n-3 on accumulation of *trans*-C18:1 and VA and on VA:C18:0 ratio

Table 1. Effect of treatments on milligrams of fatty acids per culture

Fatty acid	Time, h	Treatment ¹						SE	Contrast ² (<i>P</i> -value)			
		CONT	ETA	DTA	LA	ETALA	DTALA		1	2	3	4
		(mg/culture)										
C16:0	0	3.69	4.01	4.18	3.72	3.93	4.01	0.16	0.18	0.07	0.47	0.35
	24	4.49	4.77	4.74	4.70	4.82	4.59	0.10	0.06	0.06	0.32	0.36
C18:0	0	6.01	6.29	6.38	5.97	6.16	6.31	0.11	0.12	0.17	0.34	0.16
	24	7.90	7.99	8.30	12.33	12.96	12.36	0.43	0.88	0.44	0.23	0.14
C18:1 <i>trans</i> 6-8	0	0.07	0.06	0.06	0.06	0.06	0.07	0.004	0.49	0.16	0.52	0.18
	24	0.09	0.08	0.09	0.22	0.23	0.24	0.01	0.55	0.76	0.41	0.56
C18:1 <i>trans</i> 9	0	0.04	0.05	0.04	0.04	0.05	0.04	0.004	0.11	0.17	0.13	0.27
	24	0.05	0.05	0.06	0.15	0.16	0.17	0.008	0.84	0.27	0.25	0.94
C18:1 <i>trans</i> 10	0	0.08	0.09	0.08	0.09	0.08	0.08	0.003	0.27	0.88	0.21	0.14
	24	0.09	0.08	0.09	ND ³	ND	ND	0.008	0.26	0.44	1.00	1.00
C18:1 <i>trans</i> 11 (vaccenic acid)	0	0.22	0.22	0.24	0.22	0.22	0.22	0.02	0.94	0.47	0.99	0.94
	24	0.52	0.56	0.62	4.78	4.62	4.59	0.09	0.56	0.13	0.12	0.11
C18:1 <i>trans</i> 12	0	0.11	0.11	0.11	0.11	0.11	0.11	0.007	0.54	0.80	1.00	0.59
	24	0.14	0.13	0.15	0.38	0.40	0.42	0.02	0.74	0.67	0.08	0.36
C18:1 <i>trans</i>	0	0.51	0.51	0.54	0.49	0.50	0.51	0.04	0.93	0.46	0.91	0.71
	24	0.87	0.88	0.99	5.51	5.41	5.32	0.09	0.95	0.25	0.37	0.15
C18:1 <i>cis</i> 9	0	0.72	0.74	0.78	0.74	0.73	0.78	0.03	0.73	0.19	0.78	0.49
	24	0.55	0.58	0.60	0.99	1.07	1.01	0.05	0.48	0.12	0.09	0.11
C18:2 <i>cis</i> 9, <i>cis</i> 12	0	1.27	1.38	1.58	14.49	14.81	14.43	0.08	0.29	0.13	0.22	0.49
	24	0.45	0.51	0.53	2.52	2.39	2.36	0.26	0.89	0.80	0.55	0.49
C18:3 <i>cis</i> 9, <i>cis</i> 12, <i>cis</i> 15	0	1.23	1.36	1.37	1.25	1.27	1.30	0.06	0.16	0.20	0.84	0.64
	24	0.52	0.54	0.50	0.49	0.55	0.49	0.02	0.35	0.53	0.04	0.89
CLA ⁴	0	ND	ND	ND	ND	ND	ND					
	24	0.01	0.06	0.01	0.96	0.78	0.75	0.05	0.34	0.85	0.01	0.01
C20:3	0	ND	4.74	ND	ND	4.79	ND	0.20	0.01	1.00	0.01	1.00
	24	0.01	2.49	0.03	0.01	1.89	0.04	0.03	0.01	0.54	0.01	0.46
C22:3	0	ND	ND	5.20	ND	ND	5.14	0.13	1.00	0.01	1.00	0.01
	24	ND	ND	3.90	ND	ND	3.97	0.06	0.99	0.01	0.99	0.01
Vaccenic acid:C18:0	0	0.04	0.04	0.04	0.04	0.04	0.03	0.01	0.72	0.67	0.76	0.55
	24	0.07	0.07	0.07	0.39	0.36	0.37	0.02	0.82	0.56	0.15	0.18
Total	0	21.4	28.27	29.12	34.75	39.64	40.25	0.95	0.01	0.01	0.02	0.01
	24	31.3	36.99	37.42	46.39	51.8	50.97	1.06	0.01	0.01	0.01	0.01

¹CONT = control; ETA = control plus 5 mg of C20:3n-3; DTA = control plus 5 mg of C22:3n-3; LA = control plus 15 mg of linoleic acid; ETALA = control plus 5 mg of C20:3n-3 and 15 mg of linoleic acid; and DTALA = control plus 5 mg of C22:3n-3 and 15 mg of linoleic acid.

²Contrasts: 1 = CONT vs. ETA; 2 = CONT vs. DTA; 3 = LA vs. ETALA; and 4 = LA vs. DTALA.

³ND = Not detected or detected at < 0.01.

⁴CLA = Conjugated linoleic acid.

in ETA, DTA, ETALA, and DTALA cultures compared with CONT and LA cultures, respectively, indicates that C20:3n-3 and C22:3n-3 are not the active components in fish oil that promote VA accumulation when incubated with linoleic acid.

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