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Influence of docosahexaenoic acid on the concentration of fatty acids and volatile fatty acids in rumen fluid analysed by a rumen-simulation techniques*

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ABSTRACT

Changes in the composition and content of fatty acids (FA), including volatile fatty acids (VFA), in rumen fluid were analysed by a rumen-simulation technique (RUSITEC) following dietary supplementation with docosahexaenoic acid ($C_{22:6}$; DHA). Three different diets were tested: basal diet (CON, 60 : 40 forage to concentrate), basal diet plus 0.65% DHA (Trt1) and basal diet plus 1.30% DHA (Trt2). The experiment lasted 7 days (6 days for adaptation and 1 day for sampling). Culture fluid was collected every 2 h over a 12-h period on the last day of the experimental period. Compared to CON, the stearic ($C_{18:0}$) concentration decreased by 76.93 and 80.35% when Trt1 and Trt2 were administered, respectively ($P<0.01$). Whereas the *trans*-vaccenic acid (*trans-11*$C_{18:1}$; TVA) concentration increased by 185 and 126% compared to CON when Trt1 and Trt2 were administered, respectively, the *cis-9, trans-11* conjugated linoleic acid (CLA) concentration increased by 111 and 142%. Compared to CON, addition of DHA changed the profiles of volatile fatty acids (VFA) in culture fluid, in which propionate content increase in replacement of acetate decrease. The concentrations of volatile fatty acids (VFA), TVA, and *cis-9, trans-11* CLA were affected by the sampling time. These data indicate that dietary supplementation with DHA alters the VFA and FA content of culture fluid; however, these data should be replicated *in vivo*.

KEY WORDS: docosahexaenoic acid, *trans*-vaccenic acid, *cis-9*, *trans-11* conjugated linoleic acid, volatile fatty acids, rumen-simulation technique

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INTRODUCTION

Conjugated linoleic acid (CLA) is a mixture of positional and geometrical isomers of linoleic acid with conjugated double bonds (Ip et al., 1999). The *cis-9, trans-11* isomer of CLA has received considerable attention in recent years because of numerous potential health benefits (Belury, 1995). Recent studies have indicated that most of the *cis-9, trans-11* CLA (78-93%) in milk fat is derived from *trans-11*C18:1 (TVA) in the mammary gland by delta-9 desaturase (Piperova et al., 2002). Therefore, increasing TVA production in the rumen would be the most feasible approach to enhance the milk fat *cis-9*, *trans-11* CLA content.

Previous reports have shown that the level of TVA in rumen fluid can be increased significantly by adding fish oil to the diet (Whitlock et al., 2002; AbuGhazaleh et al., 2003), and these findings led AbuGhazaleh et al. (2001) to hypothesize that a component in fish oil may have stimulated ruminal TVA production from other sources of unsaturated fatty acids (FA). Subsequently, AbuGhazaleh and Jenkins (2004) discovered that the TVA content was significantly increased by adding highly purified DHA to a mixed ruminal culture after 24 h of cultivation, and they suggested that the omega-3 FA (e.g., DHA) in fish oil played a key role in enhancing *trans*-C18:1 production in the rumen, especially TVA. Nevertheless, it was reported that DHA could not form CLA and TVA directly in the rumen (Wonsil et al., 1994). There existed some studies about the effect of fish oil or DHA on FA profiles in mixed culture, but these results were not consistent (Whitlock et al., 2002; AbuGhazaleh et al., 2003; Wasowska et al., 2006). In the meanwhile, most of these studies involved the short-term addition of oil to a culture flask, and few considered changes in the parameters of ruminal fermentation when a high concentration of DHA was used. Thus, the objectives of this study were to examine the FA profile and volatile fatty acid (VFA) content in culture fluid following dietary supplementation with DHA by a rumen-simulation technique (RUSITEC).

MATERIAL AND METHODS

Docosahexaenoic acid was purchased from Hebei Haiyuan Chemical Company (85% purity; Hebei Province, P.R. China). The RUSITEC apparatus consisted of nine vessels with an effective volume of 2 l, and the incubation procedure was as previously described (Mohammed et al., 2004). Inoculum fluid was obtained 3 h after morning feeding from four ruminally fistulated lactating Holstein dairy cows fed a TMR composed of 60% concentrate mixture, 20% maize silage and 20% lucerne hay. The fermenters were randomly assigned to the control (basal

Table 1. Ingredients and chemical composition of the basal diet and its derivatives

Ingredients	Treatment[1]		
	CON	Trt1	Trt2
% of DM basis			
lucerne hay	20	20	20
chinese wild rye	40	40	40
maize	17.5	15.5	12.2
wheat bran	3	3	3
soyabean meal	10	11	12
cotton seed meal	8	9.35	10
calcium carbonate	0.6	0.6	0.6
calcium phosphate	0.6	0.6	0.6
sodium chloride	0.3	0.3	0.3
DHA	nd[2]	0.65	1.30
Chemical composition, % DM			
DM	91.13	91.24	91.31
crude protein	13.89	13.73	13.90
ether extract	2.79	4.71	6.60
NDF	39.97	39.72	39.45
ADF	24.80	24.73	24.70
Ca	0.81	0.81	0.82
P	0.42	0.41	0.41

[1] CON - basal diet; Trt1 - CON with 0.65% DHA; Trt2 - CON with 1.3% DHA; [2] nd - not detected

diet without DHA; CON; Table 1); CON with 0.65% DHA (Trt1), and CON with 1.3% DHA (Trt2), three fermenters were planned for each treatment as replicates. The experimental period consisted of 6 d for adaptation, followed by 1 d for sampling.

Approximately 2000 ml of rumen fluid were collected from each cow 3 h after morning feeding. At the same time about 400 g rumen digesta was collect from four different positions. Rumen fluid and digesta were mixed into one bottle and transferred to laboratory under anaerobic condition at 39°C. The mixture was strained through four layers of cheesecloth before distributed to each fermenter. Each fermenter was filled with 800 ml of rumen fluid and 800 ml of prewarmed McDougall buffer (McDougall, 1948). The fermenters, which were maintained at 39°C, were mixed constantly at 15 rpm *via* an impeller stirrer and purged with CO_2 gas. The pH in each fermenter was regulated by the addition of buffer through a tube connected to a pump. Each fermenter was replenished daily with the respective treatment diet at 08.00 and 20.00 h.

On the last day of the experimental period, culture fluid was collected using an injection syringe from the CO_2 airscoop at time 0 h (before morning replenishing),

and subsequent samples were collected 2, 4, 6, 8 and 10 h later. A pH meter (model 370, Jenway, Essex, UK) was used to determine the pH of the samples, which were then stored at -20°C until further analysis.

The culture fluid was thawed and then centrifuged at 1,000 *g* for 10 min, and 1 ml of the supernatant was transferred into an Eppendorf tube. The fluid was then mixed with 0.2 ml of 25% metaphosphoric acid and incubated for 30 min. The concentration of VFA was subsequently analysed by gas chromatography (model 6890, Series II; Hewlett Packard Co., Avondale, PA) using the method of Mohammed et al. (2004). For culture fluid FA analysis, chloroform/methanol (v/v; 2:1) was prepared to extracted the rumen fluid FA (Kramer et al., 2001), chloroform which contained FA was dried by nitrogen gas, and then the FA in the culture fluid was methylated according to Kramer et al. (1997). Separation of the individual FA in the samples was achieved using a gas chromatograph (model 6890, Series II; Hewlett Packard Co.) fitted with a flame-ionization detector and a fused-silica HP-88 capillary column (100 m, 0.25 mm i.d., 0.20 μm film; Supelco Inc., Bellefonte, PA). A sample containing FA methyl esters in hexane (1 μl) was injected by automatic sampling with split injection. The oven was initially kept at 120°C for 10 min, then heated at 3.2°C/min to 230°C and held for 35 min. The injector temperature was maintained at 250°C, while the detector was kept at 300°C; the total running time was approximately 80 min. Heptadecanoic acid ($C_{17:0}$) was used as a quantitative internal standard. Each peak was identified using FA methyl esters (Nu-Chek Prep, Elysian, MN; Matreya, Pleasant Gap, PA; and 37 Component FAME mix, Supelco). The percentage of each FA was calculated by dividing the area under the FA peak (minus the area under the peak for heptadecanoic acid) by the sum of the areas under all of the reported peaks. All results are reported as g/100 g of FA methyl esters.

The data were analysed using the PROC MIXED procedure of SAS 8.2 (SAS Inst. Inc. Cary, NC). Each fermenter was defined as a random effect, while the treatment and sampling times were defined as fixed effects. For statistical analysis of the rumen fluid, the pH, sampling time, and sampling time by treatment interaction were defined as fixed effects and analysed using repeated measures. Least squares means and pooled SEM are reported for all data. Significance was declared at $P<0.05$.

RESULTS

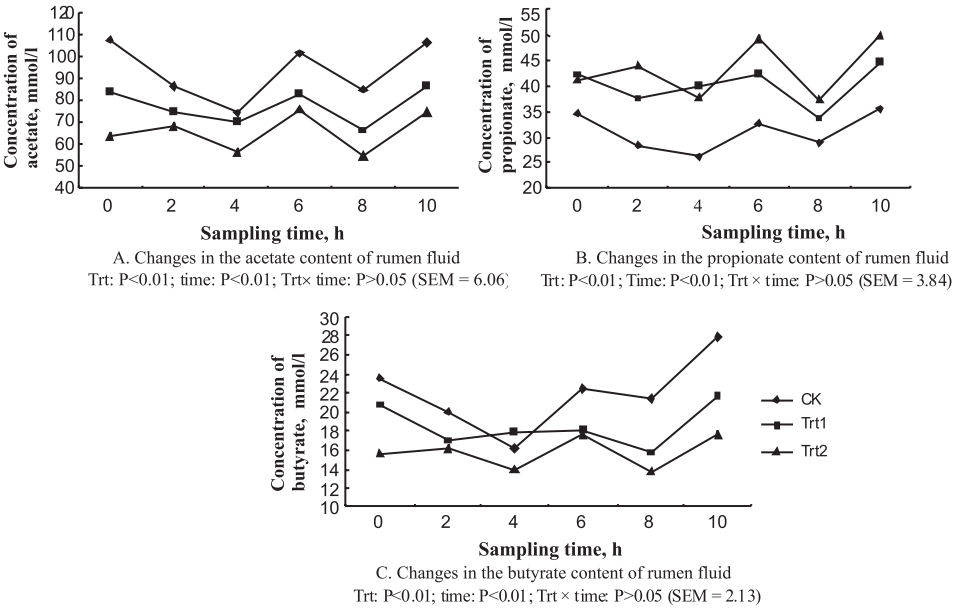
As is shown in Table 2, pH value increased as the DHA addition level ascended ($P<0.01$). The concentration of acetate and butyrate was lowest for Trt2, middle for Trt1 and highest for CON ($P<0.01$). On the contrary, the

Table 2. The effects of adding DHA on rumen fermentation

Item	Treatment[1]			SEM	P		
	CON	Trt1	Trt2		Trt[2]	samT[3]	Trt*samT[4]
pH	6.13[b]	6.24[b]	6.48[a]	0.06	<0.0001	0.0634	0.2420
Acetate, mM/l	93.27[a]	78.84[b]	65.55[c]	4.34	<0.0001	<0.0001	0.1108
Propionate, mM/l	31.08[b]	40.06[a]	43.25[a]	2.51	<0.0001	<0.0001	0.1865
Butyrate, mM/l	21.92[a]	18.50[ab]	15.79[b]	1.79	0.0071	0.0011	0.3393
TVFA[5], mM/l	148.72	140.76	129.32	11.15	0.0808	0.0034	0.5802
Acetate : propionate	3.01[a]	1.97[b]	1.52[b]	0.10	<0.0001	0.1528	0.3124

note: the data are average of three fermenters for each treatment and carried out after 6 d adaptation using RUSITEC; [abc] means in the same row with different superscripts within each group differ significantly; [1] CON - basal diet; Trt1 - CON with 0.65% DHA; Trt2 - CON with 1.3% DHA; [2] Trt - experimental treatment; [3] samT - sampling time; [4] Trt*samT - coeffect of treatment and sampling time; [5] TVFA - total volatile fatty acids

concentration of propionate was highest for Trt2, middle for Trt1, lowest for CON (P<0.01). In the meanwhile, the concentration of each VFA in Trt1 and Trt2 was the lowest 4 h and 8 h after replenished and then increased (P<0.05) (Figure 1). However, the total production of VFA was unchanged (P>0.05) by the addition of DHA.



A. Changes in the acetate content of rumen fluid
Trt: P<0.01; time: P<0.01; Trt× time: P>0.05 (SEM = 6.06)

B. Changes in the propionate content of rumen fluid
Trt: P<0.01; Time: P<0.01; Trt × time: P>0.05 (SEM = 3.84)

C. Changes in the butyrate content of rumen fluid
Trt: P<0.01; time: P<0.01; Trt × time: P>0.05 (SEM = 2.13)

Figure 1. Changes in the concentration of three volatile fatty acids (acetate (A), propionate (B), and butyrate (C) in culture fluid collected from before morning feeding to 10 h after feeding

Dietary intake of DHA affected the concentration of FA in the culture fluid (Table 3 and Figure 2). The content of TVA in culture fluid was higher (P<0.01)

Table 3. Effect of adding DHA on the fatty acid profile of the culture fluid

Fatty acids	Treatment[1]			SEM	p		
	CON	Trt1	Trt2		Trt[2]	Sam[3]	Trt*samT[4]
	—— g/100 g FAME[5] ——						
C6:0	0.03	0.02	0.02	…	0.0223	<0.0001	0.1013
C14:0	0.04[b]	0.04[b]	0.06[a]	…	0.0102	<0.0001	0.3125
C14:1	nd[6]	0.01	0.01	…	<0.0001	0.0070	0.0701
C16:0	21.76	23.68	22.83	0.56	<0.0451	0.0826	0.1020
C18:0	42.44[a]	9.79[b]	8.34[b]	0.84	<0.0001	0.2441	0.0146
*t11*C18:1 (TVA)	9.72[c]	27.71[a]	21.98[b]	1.79	<0.0001	0.0301	0.1252
*c9*C18:1	9.97[b]	11.72[a]	9.26[b]	0.25	<0.0001	<0.0001	0.6004
C18:2	0.04	0.04	0.03	…	0.0832	<0.0001	0.3032
*c9t11*C18:2 (CLA)	0.62[c]	1.31[ab]	1.50[a]	0.12	<0.0001	<0.0001	0.5024
C20:5 (EPA)	0.08[c]	0.77[ab]	0.79[a]	0.11	<0.0001	0.7671	0.4518
C22:6 (DHA)	0.14[c]	1.75[b]	2.56[a]	0.15	<0.0001	0.0001	0.0801

note: the data are average of three fermenters for each treatment and carried out after 6 d adaptation using RUSITEC; [abc] means in the same row with different superscripts within each group differ significantly; [1] CON - basal diet; Trt1 - CON with 0.65% DHA; Trt2 - CON with 1.3% DHA; [2] Trt - experimental treatment; [3] samT - sampling time; [4] Trt*samT - coeffect of treatment and sampling time; [5] FAME - fatty acid methyl ester; [6] nd - not detected

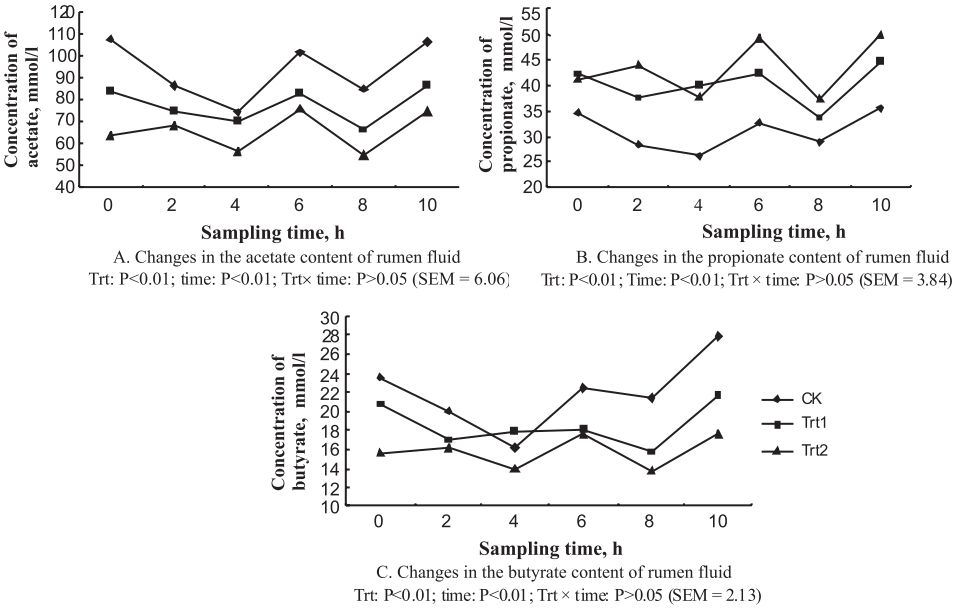


Figure 2. Changes in the concentration of selected fatty acids (C18:0, *t11*C18:1, and *c9t11* CLA) in culture fluid collected from before morning feeding to 10 h after feeding

by Trt1 and Trt2 compared with CON. Within each treatment, the content of TVA ascended slowly after feeding. On the contrary, the content of $C_{18:0}$ in culture fluid was higher ($P<0.01$) by CON compared with Trt1 and Trt2, and within each treatment the change trend was not significant ($P>0.05$). The *cis-9, trans-11* CLA content was highest for Trt2 ($P<0.01$), middle for Trt1, and lowest for CON. Within either DHA treatment, the content of this CLA isomer was highest 3 h after feeding and then decreased. No interactions between the oil supplement and time after feeding were detected in the concentration of palmitic acid, oleic acid, and linoleic acid in the culture fluid.

DISCUSSION

In present study, dietary supplementation with DHA affected the pH of the culture fluid in an incremental fashion. This result is in agreement with those of Huhtanen and Kukkonen (1995) and Shingfield et al. (2003), which showed the effects of oil supplementation. A decrease in dry matter (DM) intake may be one cause of the increase in rumen fluid pH in response to oil seed consumption (Huhtanen et al., 1995). However, in our study, DM intake was held constant, hence, the observed increase in pH was not related to a decrease in DM intake. *Lactobacillus* in the rumen play a key role in pH change, thus, the addition of DHA to the diet may have negatively affected *Lactobacillus* growth, resulting in an increase in pH. Since we did not measure *Lactobacillus*, additional studies are necessary to determine the effect of DHA on *Lactobacillus*.

Our data indicate that dietary supplementation with DHA affects the concentration of VFA in culture fluid. As the level of DHA was increased, the concentrations of acetate and butyrate decreased markedly, whereas the concentration of propionate increased. Dietary supplementation with DHA resulted in significantly different ($P<0.01$) proportions of acetate to propionate depending on the treatment, while no effect on the total VFA content was observed. This result is in agreement with that of Doreau and Chilliard (1997); however, Shingfield et al. (2003) reported that the propionate and butyrate contents increased following dietary fish oil supplementation, whereas the acetate content decreased. Such disparate data may result from different fish oil contents in the diet. Margarida et al. (2007) suggested that lipid supplementation might negatively affect microorganism growth in the rumen, leading to a decrease in fibre digestion. This might affect carbohydrate fermentation in the rumen, thereby changing the dynamics of rumen fermentation. Accordingly, the measured concentration of VFA in the rumen was altered.

*Trans-11*C18:1 concentration increases following dietary supplementation with fish oil (Shingfield et al., 2003; AbuGhazaleh et al., 2004), which is rich in long-

chain unsaturated FA (e.g., DHA). In this study, the addition of DHA significantly increased the TVA and *cis-9*, *trans-11* CLA content, in agreement with previous studies (AbuGhazaleh et al., 2002; Szolloskei et al., 2005). Moreover, Jalc et al. (2005) found that DHA significantly decreased the $C_{18:0}$ content, which is in agreement with our findings.

Fish oil can inhibit the biohydrogenation of unsaturated FA, thereby increasing the flow of *trans*-$C_{18:1}$ (Lee et al., 2005) and *trans*-$C_{18:2}$ (Loor et al., 2005) into the duodenum. Several *in vitro* studies have explored the mechanism underlying the effects of fish oil on the biohydrogenation of polyunsaturated FA in the rumen (Dohme et al., 2001). According to their results, DHA in fish oil inhibit the hydrogenation of unsaturated FA. In our study, we also found that the $C_{18:0}$ content in Trt1 and Trt2 was reduced by increases in DHA. This result is in agreement with that of Jalc et al. (2005), who reported that fish oil significantly lowered the $C_{18:0}$ content. AbuGhazaleh and Jenkins (2004) indicated that DHA in fish oil promotes TVA accumulation when incubated with linoleic acid. It has been suggested that DHA may restrain rumen bacterial growth, thereby decreasing the hydrogenation of unsaturated FA, resulting in an increased level of TVA and a decreased level of $C_{18:0}$ (Griinari and Bauman, 1999). Other researchers have reported that the accumulation of TVA caused by fish oil supplementation in the diet results from inhibition of a reductase in the rumen microorganisms that are responsible for the terminal hydrogenation of TVA to $C_{18:0}$ (Wasowska et al., 2006; AbuGhazaleh et al., 2007). Interestingly, we found that the TVA and *cis-9*, *tran-11* CLA content was affected by sampling time. The *cis-9*, *trans-11* CLA content initially increased and then decreased over time, whereas the TVA concentration increased linearly. These data imply that DHA in the diet may promote CLA reductase activity in the rumen, allowing TVA to accumulate as the level of *cis-9*, *trans-11* CLA is reduced (Fukuda et al., 2005). The amount of DHA in the Trt2 culture fluid was higher than that in the Trt1 culture fluid (data not shown), although AbuGhazaleh et al. (2004) showed that in Trt2 more DHA was transformed into other FA.

CONCLUSIONS

Our results show that adding DHA to the diet alters the VFA concentration, such that the amount of acetate and butyrate is decreased, while the level of propionate is increased. In addition, the pH of rumen fluid is increased by DHA. Adding DHA to a continuous culture of rumen fluid increased the concentration of TVA and *cis-9*, *trans-11* CLA, and significantly lowered the $C_{18:0}$ content. DHA supplementation markedly affected the VFA and FA profile of rumen fluid; however, these data should be replicated *in vivo*.

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