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A 22:6 *n-3* RICH SUPPLEMENT AFFECTS THE RUMINAL MICROBIAL COMMUNITY AND FERMENTATION AND ALTERS PLASMA METABOLITES*

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Abstract

This study investigated the effects of a 22:6 *n-3* rich supplement on ruminal fermentation characteristics and microbial changes, and also the effects of a 22:6 *n-3* rich supplement on plasma metabolites by using rumen cannulated wether goats in a 4×4 Latin square design. The 22:6 *n-3* rich supplement was infused into the rumen of the goats twice daily in equal portions at a rate of 0.0, 2.5, 5.0 and 10.0 g/d [corresponding to 0, 0.4%, 0.8% and 1.5% of diets (dry material base), respectively]. The concentration of NH_3-N and total volatile fatty acid (TVFA), and the molar proportions of acetate and butyrate were decreased by the supplement (P<0.001). The supplement decreased diversity of the rumen bacterial community (P<0.01), and reduced the abundance of *Ruminococcus flavefaciens*, *R. albus*, *Fibrobacter succinogenes* and protozoa (P<0.01). However, it increased the abundance of ruminal fungi (P<0.01). The supplement had no influence on the concentration of plasma glucose, HDL-cholesterol, LDL-cholesterol and urea but increased the concentration of triglycerides and total cholesterol (P<0.05). In conclusion, the 22:6 *n-3* rich supplement inhibited ruminal fermentation and this was accompanied by the decrease of the abundance of ruminal microbes, and also affected plasma metabolites.

Key words: 22:6 *n-3*, goat, ruminal fermentation, ruminal microbes

Ruminant-derived foods are a significant source of fat in the human diet and the potential of increasing 20:5 (*n-3*) and 22:6 (*n-3*) in milk and meat could improve the (*n-3*) polyunsaturated fatty acids (PUFA) status of human populations (Shingfield et al., 2012). Ruminant diets supplemented with marine products, such as fish oil or algae, rich in 20:5 *n-3* and/or 22:6 *n-3* have been shown to be effective in inhibiting the

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complete biohydrogenation of 18 carbon unsaturated fatty acids in the rumen, and increasing the flow of trans 18:1 [the precursor of cis-9, trans-11 conjugated linoleic acid (CLA)], 20:5 *n-3* and 22:6 *n-3*, and trans 18:2 leaving the rumen (Boeckaert et al., 2008; Duckett and Gillis, 2010; Shingfield et al., 2012), and finally increasing the amount of cis-9, trans-11 conjugated linoleic acid, 20:5 *n-3* and 22:6 *n-3* in ruminant products (Shingfield et al., 2003; Cooper et al., 2004).

Nevertheless, recent studies showed that fish oil and algae can alter ruminal fermentation pathways, cause shifts in fermentation patterns, and, sometimes, inhibit ruminal metabolism. Algae (6.66 mg/ml) were found to be able to inhibit rumen fermentation *in vitro* (Fievez et al., 2007). Diets supplemented with 2% (Mirzaei et al., 2009) or 4% (Fievez et al., 2003) fish oil could decrease acetate and butyrate concentration, and increase propionate concentration in sheep. The effects of PUFA on rumen fermentation were linked to the influence of PUFA on rumen microorganisms. Lipids in fish oil and algae are extensively hydrolysed in the rumen, releasing fatty acids. Among them, unsaturated fatty acids show more antimicrobial activity than saturated ones (Harfoot and Hazlewood, 1997). Limited *in vitro* studies showed that 50 μM 22:6 *n-3* inhibited the growth of cellulolytic bacteria, and 50 μM 18:2 *n-3* inhibited the growth of ruminal fungi (Maia et al., 2007). Dietary supplementation with 1% fish oil (Belenguer, et al., 2010) or 0.8% algae (Castro-Carrera et al., 2014) was found to be able to alter rumen bacterial community structure in sheep. However, long-chain unsaturated fatty acids in marine products are a complex mixture, and the proportion of 20:5 *n-3* or 22:6 *n-3* in total fatty acids is usually less than 20% in fish oil and less than 40% in algae. Thus, the *in vivo* direct effects of 22:6 *n-3* on ruminal fermentation and the ruminal microbial community are not clear. In addition, rumen fungi and rumen cellulolytic bacteria species *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Fibrobacter succinogenes* and *Butyrivibrio flavefaciens* (Wanapat and Cherdthong, 2009) play key roles in the digestion of fibre in the rumen fibre metabolism, changes in their amounts also could potentially affect ruminal fermentation. This study evaluates the effects of 22:6 *n-3* on rumen fermentation and the important microorganisms *in vivo*.

Diets supplemented with fish oil or algae could increase available energy and 20:5 *n-3* and/or 22:6 *n-3* for ruminants (Kupczyński et al., 2011). *In vitro* study indicated that 20:5 *n-3* and/or 22:6 *n-3* have the ability to modulate lipid metabolism, and result in a decrease of triglycerides and cholesterol in bovine hepatocytes (Mashek et al., 2002). Dietary supplementation with 2% (Mirzaei et al., 2009) or 4% (Childs et al., 2008) fish oil was found to be able to increase the plasma concentration of triglycerides and cholesterol in sheep. The inconsistent results of *in vitro* study and *in vivo* study raise the question of how 22:6 *n-3* affects the lipid metabolism *in vivo*. Based on the results of previous *in vitro* studies, we hypothesise that 22:6 *n-3* rich supplementation will reduce plasma triglycerides and cholesterol and alter plasma metabolic profile. Another aim of the present study was to use a 22:6 *n-3* rich supplement to investigate the effects of 22:6 *n-3* on rumen fermentation patterns.

Material and methods

Experimental design and diets

Four rumen-cannulated Boer crossbred wether goats (24.49±1.85 kg body weight) were used in this study. The goats were randomly distributed in a 4×4 Latin square experiment. The trial consisted of four 21-day periods, with the first 18 days for diet adaptation, followed by 4 days for sample collection per period. The goats were housed in individual pens and had free access to water. The four treatments included administration of 0.0, 2.5, 5.0 or 10.0 g/d of a 22:6 *n-3* rich supplement (75% DHA oil, Hebei Haiyuan Health Biological Science and Technology Co., Cangzhou, China) by infusion through the ruminal cannula, corresponding to 0, 0.4%, 0.8% and 1.5% of diets (dry material basis), respectively. The supplement was an esterified oil, containing 76% of 22:6 *n-3*, in addition to 6% of 20:5 *n-3* and some other minor saturated (14:0, 0.28%; 16:0, 1.66%; 18:0, 0.82%; 20:0, 0.36%), monounsaturated (16:1 *n-7*, 0.26%; 18:1, 5.56%; 20:1 *n-9*, 0.09%; 22:1 *n-9*, 0.02%; 24:1 *n-9*, 0.05%) and polyunsaturated (18:2 *n-6*, 0.91%; 18:3 *n-3*, 0.35%; 20:4 *n-6*, 0.68%; 22:3 *n-6*, 0.19%; 22:5 *n-3*, 0.15%) fatty acids. The 22:6 *n-3* rich supplement was administered in two equal portions at 0630 h and 1830 h immediately before feeding.

The diets were formulated according to the Feeding Standard of Meat-producing Sheep and Goats (NY/Y 816-2004; Ministry of Agriculture, China, 2004) to meet the maintenance requirements for metabolisable energy and crude protein, with a forage:concentrate ratio of 60:40 (dry matter basis). To ensure that the animals received isonitrogenous and isoenergetic nutrients, the values of dry matter and energy in the 22:6 *n-3* rich supplement were calculated as a part of the concentrate, the supplement replaced concentrate ingredients on a proportional basis. The goats were offered the diet (660 g DM per goat) in equal amounts twice daily (0630 h and 1830 h). Amount of feed offered daily was to ensure full consumption of the diet. The ingredients and the chemical composition of the diets are shown in Table 1.

Sampling

About 50 mL ruminal contents was collected on days 18, 19 and 20 from multiple sites within the rumen of each animal via the ruminal cannula by suction before (0 h) and 2, 4, 6, 8, 10 and 12 h after the morning feeding. The pH of the ruminal content was recorded immediately using a pH meter (HI 9024C; HANNA Instruments, Woonsocket, Rhode Island, USA). The ruminal content samples were not pooled but homogenised and analysed individually. After homogenising, about 20 mL of the ruminal content was strained through four layers of cheesecloth. For NH_3-N analysis, a portion of 4 mL of the strained ruminal fluid was acidified with 400 μL of 2 M HCl and stored at –20°C until analysis (Weatherburn, 1967). For the analysis of volatile fatty acid (VFA), a portion of 4 mL of the strained ruminal fluid was mixed with 1 mL of freshly prepared 25% (w/v) metaphosphoric acid and stored at –20°C until analysis (Mao et al., 2007). Five millimeters of ruminal contents sample, collected at 0 h on 18 d from each animal, was stored immediately at –80°C until DNA extraction. Ten millimeters of ruminal contents collected at 0 h on 18 d from each animal was squeezed through two layers of cheesecloth, and 3 mL of filtrate was then fixed

with 3 mL of 50% formalin solution in normal saline (0.9% NaCl) and stored at 4°C for protozoa counting (Dehority, 1984). About 10 mL of blood was collected on 21 d at 0 h. The blood sample was collected in a heparin-containing tube through the jugular vein and centrifuged (3000 × g, 15 min) at 4°C immediately. The plasma was then harvested and stored at –20°C until analysis.

Chemical analysis

Feed samples were analysed for DM (method 934.01), acid detergent fibre (ADF, method 973.18), ash (method 942.05) and N (method 984.13) according to Association of Official Analytical Chemists (AOAC) methods. Analysis of neutral detergent fibre (NDF) was conducted according to Van Soest et al. (1991) by an Ankom 220 Fibre Analyser (Ankom Technology Corp., Fairport, NY, USA) with addition of sodium sulfite and α-amylase, the results were expressed inclusive of residual ash.

DNA extraction

The DNA from pure species *R. flavefaciens*, *R. albus*, *F. succinogenes* and *B. fibrisolvens* species (from Aberystwyth University, UK), ruminal anaerobic fungi isolated by Cheng et al. (2006), and total DNA of the ruminal content were extracted according to respective methods as described by Denman and McSweeney (2006). Thereafter, the DNA was checked by electrophoresis on a 1.2% agarose gel, and the concentrations of DNA were determined using an ND-1000 UV-Vis (Thermo Fisher Scientific, Inc., Madison, Wisconsin, USA) nano-drop spectrophotometer.

PCR-denaturing gradient gel electrophoresis (PCR-DGGE)

The V6-V8 regions of the bacterial 16S ribosomal RNA (rRNA) gene were amplified with primers U968-GC (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC 3′) and L1401 (5′ CGG TGT GTA CAA GAC CC 3′) (Nübel et al., 1996). PCR for DGGE was conducted with the Taq DNA polymerase kit from Promega (Madison, WI, USA) as described by Sun et al. (2008). The amplifications were analysed by electrophoresis on a 1.2% agarose gel to check the size and amount of the amplicons.

The amplicons of V6–V8 regions of the 16S rRNA gene were used for sequence-specific separation by DGGE, following the method of Sun et al. (2008), with a slight modification by using a gradient of 38–60% of urea. Similarities of DGGE profiles were analysed by calculating a band similarity (Dice) coefficient (Konstantinov et al., 2003). The Shannon index of general diversity, H', was calculated as a parameter for the structural diversity of the bacterial community (Shannon and Weaver, 1963; Konstantinov et al., 2003).

Quantification of ruminant bacteria and fungi by real-time PCR

Standard curves of real-time PCR for ruminant bacteria were prepared with PCR products corresponding to almost complete bacterial 16S rRNA genes amplified from *R. flavefaciens*, *R. albus*, *R. flavefaciens*, *F. succinogenes* and *B. fibrisolvens,* with primers 8f (CAC GGA TCC AGA GTT TGA T(C/T)(A/C) TGG CTC AG) and 1510r (GTG AAG CTT ACG G(C/T) TAC CTT GTT A CG ACT T) (Lane, 1991)

using the Taq DNA polymerase kit from Promega (Madison, WI, USA). Standard curves for fungi qPCR were prepared with the PCR products of the 18S rRNA gene amplified from the ruminal fungi isolated by Cheng et al. (2006). The primers used for the fungi 18S rRNA gene amplification are listed in Table 2, and the PCR reaction was conducted with the Taq DNA polymerase kit from Promega, according to the method described by Yang et al. (2012). The PCR products were checked by electrophoresis on a 1.2% agarose gel. The concentration of the PCR products was then quantified using an ND-1000 UV-Vis (Thermo Fisher Scientific, Inc., Madison, Wisconsin, USA) nano-drop spectrophotometer before dilution. Thereafter, serial dilutions of purified PCR products from each strain were used to construct standard curves.

Table 1. Ingredients and chemical composition of diets

	Diet group			
	S 0.0	S 2.5	S 5.0	S 10.0
Ingredients (g kg^{-1} DM):				
Leymus chinensis hay	600	600	600	600
corn grain	275	260	245	225
soybean meal	105	107	105	105
wheat bran	15	24	37	49
22:6 *n-3* rich supplement	0	4	8	15
salt	5	5	5	5
Chemical composition (g kg^{-1} DM):				
DM	890	886	883	878
crude protein	115	115	114	113
NDF	380	376	382	383
ADF	251	254	254	256
ether extract	38	42	45	52

Diet group: rumen infusion of 0.0 g/d 22:6 *n-3* rich supplement (S 0.0); rumen infusion of 2.5 g/d 22:6 *n-3* rich supplement (S 2.5); rumen infusion of 5.0 g/d 22:6 *n-3* rich supplement (S 5.0); rumen infusion of 10.0 g/d 22:6 *n-3* rich supplement (S 10.0).

Real-time PCR was carried out with an ABI Prism 7300 Sequence Detection System with Sequence Detection Software V1.2 (Applied Biosystems, Foster, California, USA). The final volume of the reaction was 20 μL. Each reaction mixture was done in triplicate. The amplification reaction mixture consisted of 10 μL of FastStart Universal SYBR Green Master (Roche Applied Science, Rotkreuz, Switzerland), 0.3 mM of each primer and 2 μL of the template DNA. These replicates were considered as analytical replicates, and the mean values were calculated for statistical analysis. The primers for quantification of total bacteria, *R. flavefaciens*, *R. albus*, *F. succinogenes*, *Butyrivibrio* spp. and ruminal fungi are listed in Table 2.

The qPCR analysis of total bacteria was performed as the method described by Suzuki et al. (2000). The qPCR analysis of *R. flavefaciens*, *F. succinogenes* and the ruminal fungi was performed according to the method described by Denman and McSweeney (2006). The qPCR analysis of *R. albus* was conducted as described by Mosoni et al. (2007), and the qPCR analysis of *Butyrivibrio* spp was conducted according to the method of Boeckaert et al. (2008).

Table 2. Primers for real time PCR analysis

Target strain	Primer		Size (bp)	Reference
Total bacteria	Forward	CGGTGA ATACGTTCYCGG	96	Suzuki et al. (2000)
	Reverse	GGWTACCTTGTTACGACTT		
Ruminococcus flavefaciens	Forward	CGAACGGAGATAATTTGAGTTTACTTAGG	132	Denman and McSweeney (2006)
	Reverse	CGGTCTCTGTATGTTATGAGGTATTACC		
Ruminococcus albus	Forward	CCCTAAAAGCAGTCTTAGTTCG	175	Mosoni et al. (2007)
	Reverse	GGTATGGGATGAGCTTGC		
Fibrobacter succinogenes	Forward	GTTCGGAATTACTGGGCGTAAA	121	Denman and McSweeney (2006)
	Reverse	CGCCTGCCCCTGAACTATC		
Butyrivibrio flavefaciens	Forward	GYGAAGAAGTATTTCGGTAT	417	Boeckaert et al. (2008)
	Reverse	CCAACACCTAGTATTCATC		
Fungi	Forward	GAGGAAGTAAAAGTCGTAACAAGGTTTC	120	Denman and McSweeney (2006)
	Reverse	CAAATTCACAAAGGGTAGGATGATT		

The amplification efficiency of all the quantitative PCR data was calculated as follows: efficiency = $10^{(-1/slope)} - 1$. The amplification efficiencies for the qPCR were as follows: total bacteria: 1.03 (slope = –3.36, $R^2 = 0.99$), *R. flavefaciens*: 0.92 (slope = –3.52, $R^2 = 0.99$); *R. albus*: 1.04 (slope = –3.24, R2 = 0.99); *F. succinogenes*: 1.02 (slope = –3.28, $R^2 = 0.99$); *Butyrivibrio* spp: 0.99 (slope = –3.34, slope = –3.36, $R^2 = 0.99$); and ruminal fungi: 0.94 (slope = –3.48, $R^2 = 0.98$).

Protozoa counts

The protozoa cells were counted as the method described by Dehority (1984).

Analysis of plasma metabolites

The analysis was performed according to the instruction of the test kits (Including Glucose GOD FS, Triglycerides FS, Cholesterol FS, HDL-C Immuno FS, LDL-C Select FS and Urea FS; Diasys Diagnostic Systems, Shanghai Co. Ltd, China) using an automatic biochemistry analyser (Hitachi 7020; Hitachi, Tokyo, Japan).

Statistical analysis

Data of qPCR, protozoa counting, Shannon index and plasma metabolites were analysed as a 4 × 4 Latin square design using the PROC MIXED procedure of SAS (version 9.1, SAS Inst. Inc., Cary, NC, USA). The model used was as follows:

$$Y_{ijm} = \mu + A_i + P_j + T_m + e_{ijm}$$

where:

Y_{ijm} is the dependent variable,
μ is the overall mean,
A_i is the random effect of the animal,
P_j is the random effect of the period,
T_m is the fixed effect of the treatment,
e_{ijm} is the random residual error.

Treatment means were compared using Tukey's multiple comparison test. The dose-dependent effect of the 22:6 *n-3* rich supplement was determined by evaluation of linear and quadratic effects using orthogonal contrasts with the CONTRAST statement.

The ruminal fermentation data were analysed as a 4×4 Latin square design using the PROC MIXED procedure of SAS (version 9.1, SAS Inst. Inc., Cary, NC, USA). The model used was as follows:

$$Y_{ijkm} = \mu + A_i + P_j + S_k + T_m + S_k \times T_m + e_{ijkm}$$

where:

Y_{ijkm} is the dependent variable,
μ is the overall mean,
A_i is the random effect of the animal,

P_j is the random effect of the period,
S_k is the fixed effect of the sampling time,
T_m is the fixed effect of the treatment,
$S_k \times T_m$ is the sampling time by treatment interaction,
e_{ijkm} is the random residual error.

The first order autoregressive covariance structure was selected for the repeated term based on the Akaike information criterion of the mixed models of SAS. The treatment means were compared using Tukey's multiple comparison test. The dose-dependent effect of the 22:6 *n-3* rich supplement was determined by evaluation of linear and quadratic effects using orthogonal contrasts with the CONTRAST statement.

Linear regression was performed to investigate the relationship between the log copies of qPCR of *R. flavefaciens*, *R. albus*, *F. succinogenes* and the concentration of total volatile fatty acids (TVFA). Further, a bootstrap analysis (100 bootstrap measures) was performed to study the correlation between the log copies of qPCR of *R. flavefaciens*, *R. albus*, *F. succinogenes* and the concentration of total fatty acids through the R programming language (2.12.0 for Windows).

Statistical significance was accepted at $P<0.05$, and trends were considered at $P<0.10$.

Results

The impact of 22:6 *n-3* rich supplement on rumen fermentation characteristics

The influence of the 22:6 *n-3* rich supplement on the ruminal fermentation characteristics is shown in Table 3. The pH value increased when the 22:6 *n-3* rich supplementation rate increased ($P<0.001$). The 22:6 *n-3* rich supplement infusion reduced the concentration of NH_3-N ($P<0.001$), and it reduced the concentration of TVFA ($P<0.001$). The concentration of NH_3-N was reduced 13.68% (10 g/d) by the supplement; the concentration of TVFA was reduced 10.87% (5 g/d) and 22.82% (10 g/d), respectively. The composition of VFA changed following the infusion of the 22:6 *n-3* rich supplement. Compared with the non-supplement control, the molar proportions of acetate decreased ($P<0.01$) 6.59% (10.0 g/d), of butyrate decreased 44.72% (10.0 g/d) ($P<0.001$), of propionate increased 50.97% (10.0 g/d) ($P<0.001$).

Change in the ruminal microbe community after infusion of the 22:6 *n-3* rich supplement

DGGE technology based on 16S rRNA genes is usually used to analyse global changes in the dominant bacterial communities. The results of the DGGE analysis showed that infusion of the 22:6 *n-3* rich supplement caused some changes in the bacterial community (Figure 1a). Dice similarity coefficient and cluster analysis resulted in two distinct clusters (Figure 1b). The first cluster contained the samples

from the non-supplement control goats and the goats receiving 2.5 and 5.0 g/d 22:6 *n-3* rich supplement, and the second cluster contained the samples from the goats receiving 10.0 g/d 22:6 *n-3* rich supplement. The similarity between the two clusters was 56%. In the first cluster, the similarity between the samples from the control goats and the goats receiving 2.5 and 5.0 g/d 22:6 *n-3* rich supplement was below 70%. The results of Shannon index analysis indicated that the 22:6 *n-3* rich supplement reduced the diversity of the bacteria community (P<0.05) (Table 4).

Table 3. Effect of rumen infusion of a 22:6 *n-3* rich supplement on rumen fermentation characteristics

	Treatments				SEM	P-value		
	S 0.0	S 2.5	S 5.0	S 10.0		T	linear	quadratic
pH	6.54 c	6.60 b	6.67 a	6.61 b	0.015	xxx	xxx	xxx
NH_3-N (mM)	9.72 a	10.48 a	10.00 a	8.39 b	0.278	xxx	xxx	xxx
TVFA (mM)	65.68 a	60.42 ab	58.54 b	50.69 c	1.922	xxx	xxx	ns
Molar proportion (%)								
Acetate	69.18 a	67.67 a	66.77 a	64.62 b	0.790	xx	xxx	ns
Propionate	19.17 b	20.13 b	20.22 b	28.94 a	0.827	xxx	xxx	xxx
Butyrate	11.65 b	12.20 ab	13.01 a	6.44 c	0.378	xxx	xxx	xxx

Treatments: rumen infusion of 0.0 g/d 22:6 *n-3* rich supplement (S 0.0); rumen infusion of 2.5 g/d 22:6 *n-3* rich supplement (S 2.5); rumen infusion of 5.0 g/d 22:6 *n-3* rich supplement (S 5.0); rumen infusion of 10.0 g/d 22:6 *n-3* rich supplement (S 10.0).

P-value: T = treatment effect of rumen infusion of 20:5 *n-3* rich supplement; linear, quadratic = linear or quadratic effect of rumen infusion of 20:5 *n-3* rich supplement; ns = non-significant (P>0.05); x – P<0.05; xx – P<0.01; xxx – P<0.001.

a, b, c – values within a row with different letters differ significantly at P<0.05.

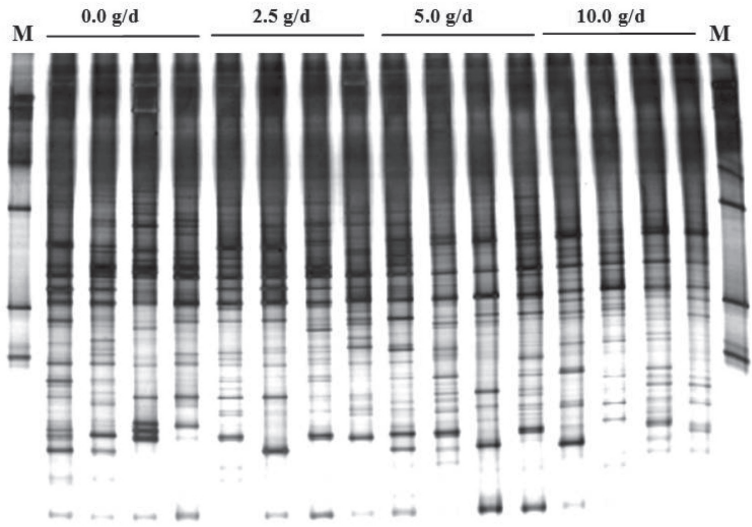

Figure 1. (a) DGGE profiles of rumen bacteria of goats receiving various doses of a 22:6 *n-3* rich supplement (4 goats per dose). M, Marker

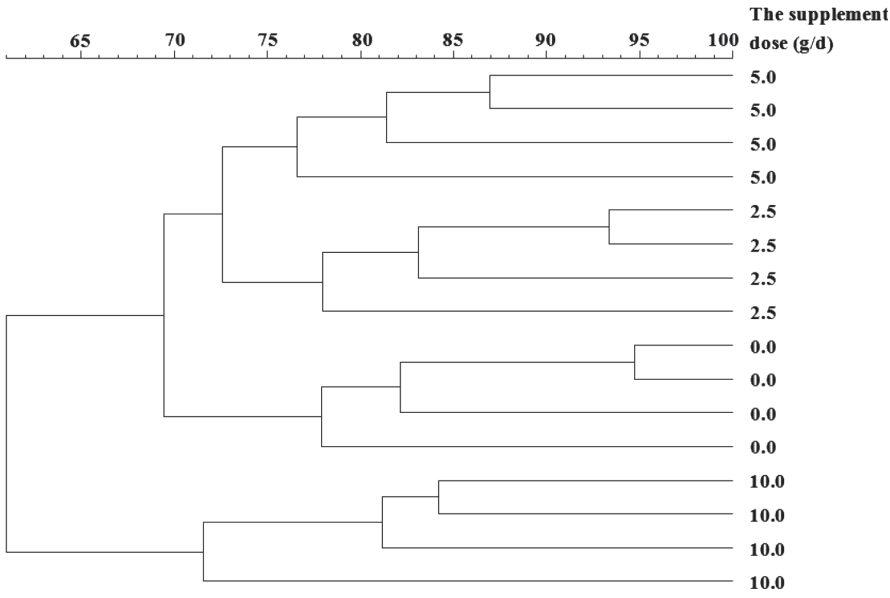

Figure 1. (b) Cluster analysis of DGGE profiles obtained from rumen microflora of goats receiving various doses of a 22:6 *n-3* rich supplement (4 goats per dose)

Table 4. Effect of rumen infusion of a 22:6 *n-3* rich supplement on the abundance and diversity of rumen microbe

	Treatments				SEM	P-value		
	S 0.0	S 2.5	S 5.0	S 10.0		T	linear	quadratic
Total bacteria[1]	9.56 a	9.50 a	9.08 b	8.41 c	0.105	xxx	xxx	xx
Ruminococcus flavefaciens[1]	8.24 a	8.25 a	7.89 b	7.78 b	0.079	xxx	xxx	ns
Ruminococcus albus[1]	6.72 a	6.69 a	6.45 b	6.14 c	0.056	xxx	xxx	x
Butyrivibrio spp.[1]	6.91	6.94	6.82	6.81	0.098	ns	ns	ns
Fibrobacter succinogenes[1]	7.64 a	7.66 a	7.58 ab	7.27 b	0.088	x	xx	0.071
Protozoa[1]	5.99 ab	6.06 a	6.03 ab	5.95 b	0.031	x	ns	xxx
Fungi[1]	4.20 b	4.12 b	4.29 b	4.80 a	0.152	x	xx	ns
Shannon index[2]	2.82	3.03	2.83	2.98	0.168	x	ns	xx

Treatments: rumen infusion of 0.0 g/d 22:6 *n-3* rich supplement (S 0.0); rumen infusion of 2.5 g/d 22:6 *n-3* rich supplement (S 2.5); rumen infusion of 5.0 g/d 22:6 *n-3* rich supplement (S 5.0); rumen infusion of 10.0 g/d 22:6 *n-3* rich supplement (S 10.0).

[1]Values were expressed in $\log_{10}$ abundance of 16S rRNA gene copies/mL rumen contents.

[2]Shannon index of rumen bacteria community.

P-value: T = treatment effect of rumen infusion of 20:5 *n-3* rich supplement; linear, quadratic = linear or quadratic effect of rumen infusion of 20:5 *n-3* rich supplement; ns = non-significant ($P>0.05$); x – $P<0.05$; xx – $P<0.01$; xxx – $P<0.001$.

a, b, c – values within a row with different letters differ significantly at $P<0.05$.

Bacterial abundance in the ruminal contents and its relation to TVFA

The results of the qPCR are shown in Table 4. The infusion of the 22:6 *n-3* rich supplement had no effect on the abundance of *Butyrivibrio* spp., but it decreased the abundance of total bacteria, *R. flavefaciens*, *R. albus* and *F. succinogenes* in a dose-dependent manner ($P<0.05$). The protozoal abundance is reduced with infusion of the 22:6 *n-3* rich supplement ($P<0.05$). The abundance of total fungi increased ($P<0.05$) when infusion of the 22:6 *n-3* rich supplement rate increased. The results of linear regression analysis revealed positive linear relationships between the concentration of TVFA and the abundance of *R. flavefacien*s ($P<0.05$, $R^2 = 0.34$; Figure 2a), between the concentration of TVFA and the abundance of *R. albus* ($P<0.05$, $R^2 = 0.60$; Figure 2b) and between the concentration of TVFA and the abundance of *F. succinogenes* ($P<0.05$, $R^2 = 0.22$; Figure 2c).

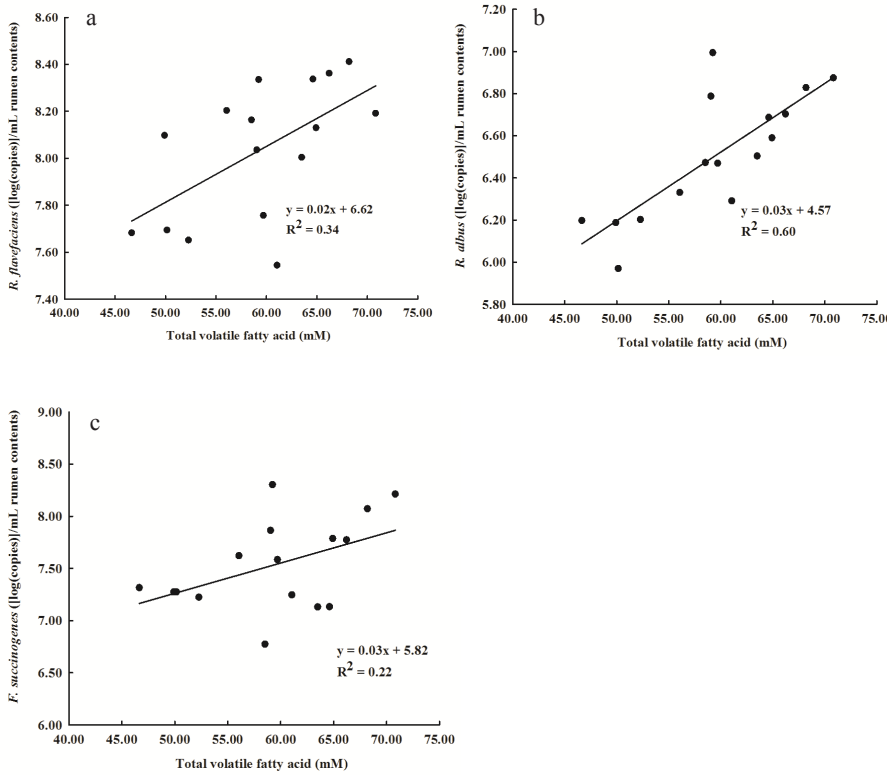

Figure 2. Correlations between the concentration of total volatile fatty acid and the abundance of *Ruminococcus flavefaciens* (a) and the abundance of *R. Albus* (b) and the abundance of *Fibrobacter succinogenes* (c)

Table 5. Effect of rumen infusion of a 22:6 *n-3* rich supplement on the plasma metabolites concentration (mM)

	Treatments				SEM	P-value		
	S 0.0	S 2.5	S 5.0	S 10.0		T	linear	quadratic
Glucose	2.30	2.18	2.55	2.38	0.153	ns	ns	ns
Triglyceride	0.28	0.25	0.35	0.43	0.038	x	x	ns
Total cholesterol	1.31	1.25	1.84	1.63	0.127	x	x	ns
HDL-cholesterol	0.89	0.85	0.81	0.88	0.030	ns	ns	ns
LDL-cholesterol	0.33	0.38	0.46	0.45	0.069	ns	ns	ns
Urea	5.85	6.08	6.40	5.88	0.300	ns	ns	ns

Treatments: rumen infusion of 0.0 g/d 22:6 *n-3* rich supplement (S 0.0); rumen infusion of 2.5 g/d 22:6 *n-3* rich supplement (S 2.5); rumen infusion of 5.0 g/d 22:6 *n-3* rich supplement (S 5.0); rumen infusion of 10.0 g/d 22:6 *n-3* rich supplement (S 10.0).

P-value: T = treatment effect of rumen infusion of 20:5 *n-3* rich supplement; linear, quadratic = linear or quadratic effect of rumen infusion of 20:5 *n-3* rich supplement; ns = non-significant ($P>0.05$); x – $P<0.05$.

Plasma metabolite concentration

The influence of the 22:6 *n-3* rich supplement on the goat plasma metabolite concentration is shown in Table 5. The ruminal infusion of the supplement increased the concentration of triglyceride and total cholesterol in a dose-dependent manner ($P<0.05$), but it had no influence on the concentration of glucose, HDL-cholesterol, LDL-cholesterol and urea.

Discussion

Effect of the 22:6 *n-3* rich supplement on ruminal fermentation characteristics

In the rumen, dietary fats are hydrolysed first, and then free unsaturated fatty acids are dynamically hydrogenated to saturated fatty acids by bacterial biohydrogenation (Harfoot and Hazlewood, 1997; Buccioni et al., 2012). As unsaturated fatty acids are more antimicrobial than saturated fatty acids, biohydrogenation enables ruminal bacteria to protect themselves from the negative effect of unsaturated fatty acids (Harfoot and Hazlewood, 1997). Both lipolysis and hydrogenation can influence ruminal fermentation. In the present study, the results showed that the 22:6 *n-3* rich supplement inhibited ruminal fermentation.

Diets supplemented with material rich in unsaturated fatty acids, such as marine products, can influence the pattern of ruminal fermentation. Fievez et al. (2003) demonstrated that diets supplemented with 4.2% fish oil (EPA, 18.1%; DHA, 11.9%) increased the proportion of propionate, decreased the proportion of acetate and had no influence on the concentration of TVFA in sheep. Mirzaei et al. (2009) found that dietary supplementation with 2% fish oil significantly inhibited ruminal fermentation

in sheep, accompanied with a large decrease of NH_3-N and TVFA concentration, acetate and butyrate proportions, increase of propionate proportions. However, Toral et al. (2010) found that diets supplemented with 3% of a mixture (1:2 weight/weight) of fish oil (EPA, 0.6%; DHA, 1.7%) and sunflower oil did not affect ruminal fermentation in ewes. The inconsistency in these results may be due to the different fatty acid composition of marine products or to the different supplementation rates. Although polyunsaturated fatty acids have long been regarded as key compounds in alterations of ruminal fermentation patterns, only a few researches with *in vitro* experiments of this issue have been conducted (Maia et al., 2007; Paillard et al., 2007). Maia et al. (2007) showed that 22:6 *n-3* and 20:5 *n-3* inhibited the growth and activity of ruminal bacteria *in vitro*. Paillard et al. (2007) reported that 18:2 *n-6* reduced fermentation products of rumen bacteria *in vitro*. The present study investigated the effect of a 22:6 *n-3* rich supplement on ruminal fermentation *in vivo*. The 22:6 *n-3* rich supplement altered ruminal fermentation by reducing the concentrations of TVFA and the proportion of acetate and butyrate, while increasing the proportion of propionate. In this study, the ratio of 22:6 *n-3* in the supplement is 75%. Combing the results from previous study which indicated that the negative effect of polyunsaturated fatty acids (PUFA) on ruminal bacteria growth and fermentation (Maia et al., 2007; Maia et al., 2010; Paillard et al., 2007), it seems that the inhibition of ruminal fermentation can be largely attributed to the effect of the 22:6 *n-3*. However, other fatty acids, such as 18:3 *n-3* and 20:5 *n-3*, although their proportions are very small in the supplement fatty acids, might be also involved in this inhibition. It should be noted that, in this study, in order to ensure that the animals received isonitrogenous and isoenergetic nutrient, the addition of the oil was compensated by a reduction in corn grain and an increase in wheat bran. Although changes of diet composition were very small, these changes in diet formulation may have an effect on the rumen environment.

Effect of the 22:6 *n-3* rich supplement on ruminal microorganisms

Unsaturated fatty acids have a stronger antimicrobial effect than saturated fatty acids (Harfoot and Hazlewood, 1997). Diets supplemented with materials rich in unsaturated fatty acids can lead to a shift in the ruminal microbial population. Studies demonstrated that diets supplemented with 3% fish oil (Kim et al., 2008) or 8.5% algae (Boeckaert et al., 2008) reduced the diversity of the ruminal bacterial community in cows. Our results revealed that the 22:6 *n-3* rich supplementation reduced the total bacteria and altered the bacterial community structure of the rumen in a dose-dependent pattern. This finding suggests that the administration of the 22:6 *n-3* rich supplement substantially changed the ruminal bacteria community, particularly at the level of 10 g/d.

Our results showed that the 22:6 *n-3* rich supplement reduced the abundance of *R. flavefaciens*, *R. albus* and *F. succinogenes*. Recently, Huws et al. (2010) reported that the abundance of *F. succinogenes* decreased following fish oil (2%) supplementation of grass silage-fed steers. Liu et al. (2012) found that dietary supplementation with fish oil (2%) decreased the abundance of *B. fibrisolvens* and *F. succinogenes*, coupled with the amount of acetate and butyrate decreased and the amount of proportion increased in the rumen. *R. flavefaciens*, *R. albus* and *F. succinogenes* are the

major active and dominant cellulolytic bacteria in the rumen, and play an important role in ruminal fermentation. Considering the results of the aforementioned studies and current study, it can be suggested that the reduction in TVFA and the proportion of acetate and butyrate in this study may be due to the negative effects of 22:6 *n-3* on the growth and/or metabolism of those cellulolytic bacteria. The results of the correlation analysis also revealed that the concentration of TVFA was positively associated with the abundance of *R. flavefaciens*, *R. albus* and *F. succinogenes* in the current study. Thus, 22:6 *n-3* rich supplement especially at high level could cause detrimental effects on cellulolytic degradation.

Butyrivibrio group bacteria are known as active bacteria involved in the digestion of fibre and fatty acids in the rumen. Previous *in vitro* experiments confirmed that 22:6 *n-3* effectively inhibited the growth and activity of *B. fibrisolvens* (Wąsowska et al., 2006). Only a few studies have examined the influence of marine products on the abundance of *Butyrivibrio* group bacteria *in vivo* (Boeckaert et al., 2008; Belenguer et al., 2010). These studies showed that dietary supplementation with algae had no effect on the abundance of *Butyrivibrio* group bacteria in cows and sheep. In the present study with goats, the 22:6 *n-3* rich supplement had no effect on the abundance of *Butyrivibrio* group bacteria. Thus, 22:6 *n-3* rich supplements or other long-chain unsaturated fatty acids may have little effect on *Butyrivibrio* group bacteria.

In the current study, the ruminal infusion of the 22:6 *n-3* rich supplement decreased the abundance of protozoa. Similarly, Boeckaert et al. (2007) reported that some ciliates disappeared and that the abundance of ruminal protozoa decreased after algae feeding in cows. Although the magnitude of the antiprotozoal properties of fatty acids may depend on the degree of unsaturation of fatty acids (Hristov et al., 2005), the mechanism behind the effect on rumen protozoa needs to be further investigated. Nocek and Russell (1988) found that ruminal NH_3-N concentrations were much lower in defaunated animals than non-defaunated animals, indicating that ruminal protozoa limit complete degradation of proteins by uptake of peptides or amino acids into protozoa cells. In the present study, the decrease in the NH_3-N concentration may suggest a suppression role of the 22:6 *n-3* rich supplement on rumen protozoa growth. The decrease in the abundance of protozoa in the current study may indicate that 22:6 *n-3* is able to inhibit the growth of protozoa at high concentration.

An interesting finding of the present study was that the abundance of ruminal fungi was increased by rumen infusion of 22:6 *n-3* rich supplement. Maia et al. (2007) observed that no ruminal fungi grew on an agar medium containing 50 μg/ml of 18:2 *n-6*, and suggested an inhibition effect of PUFA on rumen fungi. In another *in vitro* study, 4 μg/ml of 18:3 *n-3* stimulated the growth of ruminal fungi (Orpin and Letcher, 1979). Based on our data and those of earlier studies, PUFA may be able to stimulate the growth of fungi at a low concentration. Nevertheless, further studies are needed to clarify the effects of 22:6 *n-3* on the growth of ruminal fungi.

Effect of 22:6 *n-3* rich supplement on plasma metabolites

In this study, rumen infusion of the supplement increases available energy and 22:6 *n-3* for the goats. Although the effects of 22:6 *n-3* on plasma metabolism in humans and in rats have been reported (Lunn and Theobald, 2006), *in vivo* data on

ruminants are limited. Previous *in vitro* research showed that a decrease of triglycerides and cholesterol in bovine hepatocytes, and indicated that 22:6 *n-3* has the ability to modulate bovine lipid metabolism (Mashek et al., 2002). Janeczek et al. (2011) found that diets supplemented with 1% algae increased the serum concentration of triglycerides and total cholesterol in cows. In agreement with these studies, our results showed that 22:6 *n-3* increased the plasma concentration of triglycerides and total cholesterol. The high digestibility of unsaturated fats may explain this finding (Mirzaei et al., 2009). Further, PUFA comprise the majority of fatty acids upon infusing into the rumen, but can be extensively biohydrogenated in the rumen, and saturated acid comprises most of the fatty acids leaving the rumen and reaching body tissues. Thus, another possible reason may be that the amount of saturated fatty acids entering the intestine was elevated as the 22:6 *n-3* rich supplement rate increased. This would have increased the absorption of fats from the small intestine, finally leading to increased synthesis of cholesterol and triglycerides in tissue (Mirzaei et al., 2009). These results are contrary to the original hypothesis that 22:6 *n-3* would decrease plasma cholesterol and triglycerides content. Therefore, when 22:6 *n-3* rich supplements are added to ruminants diet to increase PUFA content in the meat or milk, care should also be taken on the final influence on animal health.

Conclusion

This study found that the 22:6 *n-3* rich supplement increased the plasma concentrations of triglycerides and total cholesterol. The 22:6 *n-3* rich supplement inhibited ruminal fermentation, resulting in a decrease in the concentrations of NH_3-N and TVFA. This inhibitory effect was associated with a decrease in the abundance of specific groups of ruminal cellulolytic bacteria, including *R. flavefaciens*, *R. albus* and *F. succinogenes*, and a decrease in the populations of protozoa. These findings suggest that 22:6 *n-3* may alter ruminal fermentation through an inhibition effect on rumen cellulolytic bacteria, and imply care should be taken when PUFA products are applied in ruminant feed.

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