Effects of Grass Lipid and Its Fatty Acids on Ruminal Fermentation and Microbial Growth *In Vitro*

U. M. Yang*, H. Fujita[1] and T. Y. Chung

Animal Resources Research Center, College of Animal Husbandry, Konkuk University, Seoul 143-701, Korea

ABSTRACT : In order to clarify the inhibitory effects of orchardgrass (*Dactylis glomerata* L.) lipids on ruminal fermentation and digestion, two experiments were carried out *in vitro*. Experiment 1 was carried out using residues of grass hay from which the lipid fraction was removed by ether extraction. To ground grass samples were added 0, 1.5, 3.0, 4.5 and 6.0% lipids and incubated anaerobically at 39℃ for 24 h, with the mixtures of artificial saliva and rumen fluid. Increasing grass lipid levels remarkably reduced DM and NDF disappearances. Volatile fatty acid concentration was significantly reduced at 3.0, 4.5 and 6.0% lipid levels. Microbial nitrogen proportion to total nitrogen tended to decrease by the addition of the lipids. These results indicated that grass lipids have a marked inhibitory effect on ruminal fermentation and digestion, especially when to the substrate was added 3% or more grass lipids as ether extracts. Experiment 2 was conducted to study the relationship between changes in the free fatty acids and changes in the fermentation traits. Samples were incubated for 3, 6, 9, 12, 15, 18, 21 and 24 h as a sole substrate. The polyunsaturated fatty acids steadily decreased during incubation, whereas the saturated fatty acid ($C_{18:0}$) increased. It was suggested that the hydrogenation was extended during the initial stage of incubation. The unsaturated fatty acids ($C_{18:2}$, $C_{18:3}$) produced at the initial stage of incubation were negatively correlated with the amount of microbial N and DM disappearance, indicating that polyunsaturated fatty acids had the possibility to show an inhibiting effect on ruminal fermentation and digestion. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 2 : 176-181*)

Key Words : Grass Lipid, Fermentation and Digestion, Rumen Microbial N, Free Fatty Acids

INTRODUCTION

It has been well known that a high level of dietary fat has an inhibitory effect on ruminal fermentation and digestion of the diet (Palmquist and Jenkins, 1980; Ikwuegbu and Sutton, 1982; Sutton et al., 1983; Palmquist, 1984; Doreau et al., 1991; Ameny et al., 1995). Recently, it has also been found that the lipid content in grass correlates with ruminal protein degradability as a negative factor (Fujita et al., 1991), and that grass lipids have an inhibitory effect on the growth and activity of rumen fibrolytic bacteria (Hino and Nagatake, 1993). It would appear that these effects are probably brought about by free fatty acids (FFA) produced during fermentation in the rumen (Henderson, 1973; Maczulak et al., 1981; Chalupa et al., 1984).

However, little information is available on the biochemical properties of grass lipids, including their fatty acid composition, and there has been little work on the changes in fatty acid composition during ruminal incubation.

As a fundamental step for studying the inhibitory effect of grass lipids on ruminal fermentation and digestion, this experiment was carried out 1) to clarify the effect of grass lipids on ruminal fermentation, digestion and 2) to study the relationship between changes in the fatty acid composition during ruminal incubation and changes in the general fermentation traits in the rumen.

MATERIALS AND METHODS

Experiment 1

1) Forage material and incubation substrate preparation

The first cut grass, consisting mainly of orchardgrass (*Dactylis glomerata* L.) at the early heading stage, was prepared to make the sun-cured hay. The hay was ground to 1 mm by a Wiley mill. The ground hay sample was extracted for 20 h with diethyl ether. The ether extracts and ether-extracted residue of grass hay were used for the incubation trial as lipid supplements and basal substrates, respectively. The lipid supplements and basal substrates were added at the weight ratio of 0:100, 1.5:98.5, 3:97, 4.5:95.5 and 6:94 in each treatment as an incubation substrate. Chemical composition of orchardgrass hay used in the incubation trial, and diets fed to the wethers for rumen fluid collection are shown in table 1.

* Address reprint request to U. M. Yang. Tel: +82-2-450-3673, Fax: +82-2-455-5305, E-mail: yangum@hotmail.com.

[1] Department of Animal Production, Obihiro University of Agriculture and Veterinary Medicine, Obihiro-shi 080, Japan; Retired.

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Table 1. Chemical composition of orchardgrass hay used for incubation trial as a substrate, and diets fed to wethers for rumen fluid collection (Experiment 1)

	Orchardgrass[1]		Diets	
	Original	Ether extracted	Alfalfa hay cube	Grass hay[2]
Dry matter (%)	88.0	89.5	87.4	87.1
	On dry matter basis (%)			
Organic matter	91.9	91.8	90.0	87.3
Crude protein	12.1	11.5	18.5	13.1
True protein	9.0	8.8	13.9	10.9
NPN	0.50	0.43	0.74	0.35
ADIN	0.30	0.31	0.40	0.49
Ether extracts	2.7	0.06	2.4	2.4
NFE	45.7	48.3	38.0	38.1
Crude fiber	31.4	32.0	31.1	33.7
NDF	66.9	66.0	56.6	72.3
ADF	36.6	38.1	38.6	43.8
Crude ash	8.1	8.2	10.0	12.7

[1] First-cut orchardgrass harvested at the stage of early heading.
[2] Orchardgrass predominated.
ADIN: Acid-detergent insoluble nitrogen, NFE: Nitrogen free extract, NDF: Neutral detergent fiber, ADF: Acid detergent fiber.

2) Rumen fluid collection and incubation

The rumen fluids were taken from three rumen-fistulated Suffolk wethers by an aspirator equipped with a nylon bag filter just before morning feeding. The animals were kept individually in metabolism cages and fed on orchardgrass hay (35%) and alfalfa hay cubes (65%) at a maintenance level (55 g DM kg body weight$^{-0.75}$ day^{-1}) with free access to water and mineral block throughout the experimental period. The diets were fed twice daily at 0800 and 1700 h.

In 3-neck flasks (300 ml volume) containing 200 ml of artificial saliva (McDougall, 1948) and rumen fluid (1:1), 2.5 g of substrates were incubated anaerobically at 39℃ for 24 h. After incubation, the contents of the incubation flasks were centrifuged at 18000×g for 30 min at 4℃ to isolate solid matter and supernatant. Solid matter lyophilized in a freeze dryer was analyzed for dry matter (DM), neutral detergent fiber (NDF) and microbial nitrogen. The supernatant portion was analyzed for volatile fatty acids (VFA), NH_3-N and soluble-N concentration. All the samples were assayed in triplicate.

3) Chemical analyses

Proximate composition of diets and hay samples were determined on air-dried matter basis using the procedures approved by the National Institute of Animal Industry (Horii et al., 1971). Acid detergent fiber (ADF) and NDF were analyzed according to Van Soest et al. (1991). The amount of microbial nitrogen was determined using purines as a microbial marker according to the Zinn and Owens method (1986). Ammonia nitrogen (NH_3-N) concentrations were determined by micro-diffusion as described by Conway and O'Malley (1942). For VFA determination, 4 ml of the supernatant portion was mixed with 1 ml of 25% metaphosphoric acid and then frozen overnight. After melting at room temperature the mixed sample was centrifuged at 8000×g for 5 min. As an internal standard 1 ml of 10 mM-2-ethyl-n-butyric acid was mixed to 1 ml of the supernatant fraction. The mixture was analyzed by gas-liquid chromatography (GC-14A; Shimadzu Corporation, Kyoto, Japan) with a urbon capillary column HR-52 (30 m×0.53 mm i.d.) (Shinwa Chemical Industries, Ltd., Tokyo, Japan). The column temperature was programmed as follows: initially the temperature was at 70℃ for 30 sec and then increased to 90℃ at 4℃ min^{-1}. After holding at 90℃ for 30 sec the temperature was increased to 180℃ at 30℃ min^{-1} to clean the column. The injector and detector temperatures were fixed at 190℃ and 280℃, respectively. The values were processed automatically using a Chromatopac data processing system (C-R4A; Shimadzu Corporation, Kyoto, Japan).

4) Statistical analysis

Statistical analysis was performed by one-way analysis of variance and differences between treatments were determined by Fisher's protected least significant difference (PLSD) test at $p<0.05$ using StatView statistical analysis program (Nagata, 1994).

Experiment 2

Table 2 shows the chemical composition and major fatty acid composition of orchardgrass hay used for incubation trial as a sole substrate. Major fatty acids of rumen fluid (as an inoculum) are also shown. The grass was first cut hay at the stage of late heading, containing crude protein 8.4, crude fat 2.9 and NDF 66.5%. Although the procedures for incubation were similar to those described for Experiment 1, the 1-mm ground hay (2.5 g) was incubated for 3, 6, 9, 12, 15, 18, 21 and 24 h as a sole substrate. Cultures were centrifuged to isolate solid matter and supernatant, and the solid matter was lyophilized, ground, and analyzed for DM, NDF, microbial N and fatty acids. The supernatant was analyzed for VFA and pH.

Analytical methods were similar to those described for Experiment 1 except lipid analysis. The lipids were completely extracted from the freeze-dried sample with chloroform-methanol (2:1, v/v) according to the method of Folch et al. (1957). Extracted lipids were separated

by thin layer chromatography (TLC) on Silica-Gel B5 (Wako Pure Chemical Industries, Osaka, Japan) plates (20×20 cm) with a developing solvent of hexane-diethyl ether-acetic acid (80:30:1, by vol.). The band of FFA fraction was detected under UV light after spraying with a primulin reagent (Sigma Chem. Co., St. Louis, USA), and scraped from the plate into a glass centrifuge tube with screw cap. Pentadecanoic acid solution ($C_{15:0}$; dissolved in benzene solution, 1 μmol/ml) was added into the FFA fraction as an internal standard, and methylated with 5% methanol-HCl (95:5, v/v) solution by boiling at 95℃ for 2 h. The methyl esters thus obtained were extracted 3 times with hexane, and solvents in the pooled extracts were evaporated under N_2 at 30℃. Fatty acids dissolved with hexane were analyzed by gas-liquid chromatography (GC-4C; Shimadzu Corporation, Kyoto, Japan) with FID and equipped with a 10% DEGS column (2 m×3 mm i.d.) using N_2 as the carrier gas. The gas chromatograph was operated isothermally at 170℃ for column and 210℃ for injector and detector. Fatty acid composition was determined directly from the ratios of the peak areas, calculated by triangulation using a data processing system (D-2500 Chromato-Integrator; Hitachi Ltd., Tokyo, Japan).

Table 2. Chemical composition and major fatty acid composition of orchardgrass hay and rumen fluid used for incubation trial (Experiment 2)

	Orchard-grass hay[1]	Rumen fluid[2]
Chemical composition		
Dry matter (%)	91.9	-
Crude protein (%DM)	8.4	-
Crude fat (%DM)	2.9	-
NFE (%DM)	45.5	-
Crude fiber (%DM)	35.6	-
ADF (%DM)	42.5	-
NDF (%DM)	66.5	-
Crude ash (%DM)	7.6	-
Gross energy (kcal/g DM)	4.8	-
Fatty acid composition[3]		
$C_{16:0}$ (mol%)	32.2±1.0	29.2±0.5
$C_{18:0}$ (mol%)	2.9±0.1	66.3±1.5
$C_{18:1}$ (mol%)	4.2±0.3	3.6±0.7
$C_{18:2}$ (mol%)	21.6±0.7	0.6±0.2
$C_{18:3}$ (mol%)	39.1±1.0	0.3±0.2

[1] Sun-cured hay made from primary growth of an orchardgrass sward at the stage of late heading. Used as a sole substrate.

[2] Used as an inoculum.

[3] Each value represents mean±SD for three determinations.

NFE : Nitrogen free extracts, ADF : Acid detergent fiber, NDF : Neutral detergent fiber.

RESULTS AND DISCUSSION

Experiment 1

There were no remarkable differences in chemical composition between original and ether extracted orchardgrass hay, except crude fat content (table 1).

DM and NDF disappearances and VFA concentration after incubation are shown in figure 1. Increasing lipid levels significantly decreased NDF disappearance ($p<0.05$), and tended to decrease DM disappearance with additions of 3.0, 4.5 and 6.0% of lipids compared to control (0%). However, there were no differences among 3.0, 4.5 and 6.0% levels, showing that inhibitory effects of grass lipids on DM and NDF digestibilities did not continue above 3.0% level. Concentration of VFA was significantly lower ($p<0.05$) at and above 3.0% of lipids as compared to control and 1.5% addition.

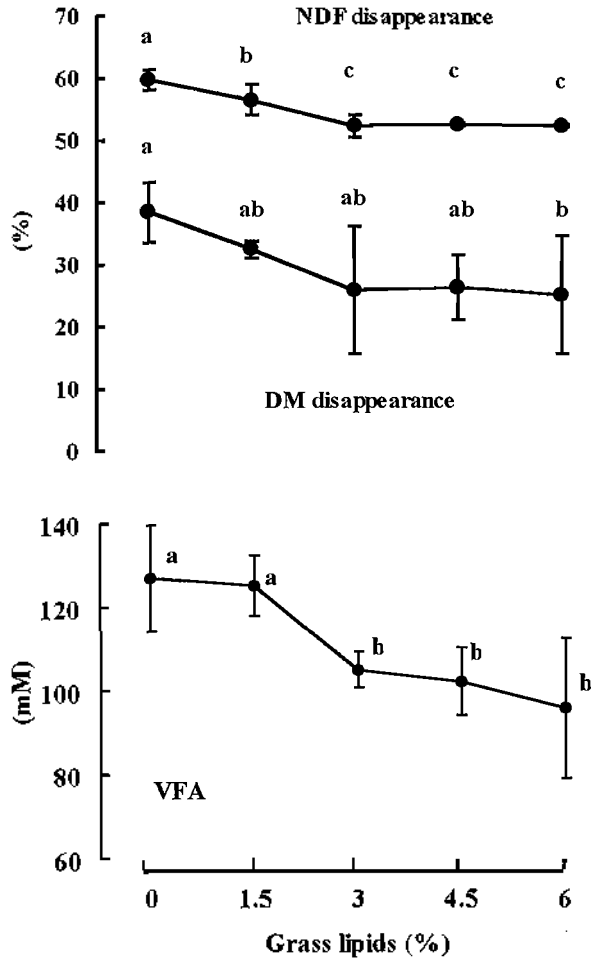

Figure 1. Dry matter and NDF disappearances, and VFA concentration according to the amount of lipid added to ether-extracted grass (Each point indicates a mean with SD as vertical bars. [a,b,c] Means with different letters differ significantly at $p<0.05$)

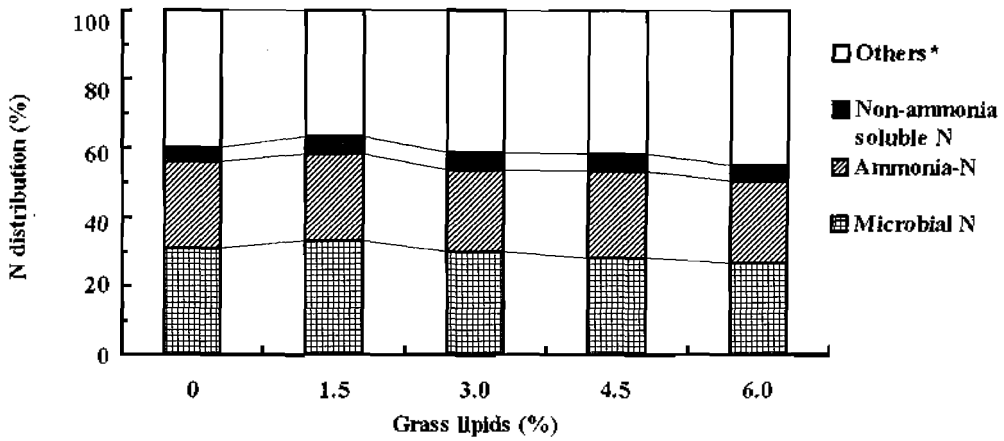

Figure 2. Distribution of nitrogen fractions after incubation * Others=total N minus (non-ammonia soluble N+ ammonia-N+microbial N)

Table 3. Changes of the fermentation traits during incubation when orchardgrass hay was used as a sole substrate (Experiment 2)

	Incubation time (h)								
	0	3	6	9	12	15	18	21	24
DM disapperance (%)	-	6.5^{a}	15.5^{b}	18.1^{bd}	21.2^{bc}	20.8^{bc}	24.9^{cd}	26.9^{c}	22.9^{bc}
pH	8.0^{a}	6.8^{b}	6.8^{b}	6.7^{b}	6.6^{c}	6.6^{cd}	6.6^{cd}	6.5^{d}	6.5^{d}
Total VFA (mM)	36.5^{a}	42.1^{a}	48.7^{ab}	38.3^{a}	52.0^{ab}	66.1^{bc}	78.9^{c}	78.5^{c}	75.0^{c}
Microbial N (mg/vessel)	17.9^{a}	22.4^{ab}	22.9^{ab}	24.9^{b}	27.1^{b}	24.2^{b}	26.2^{b}	23.9^{b}	23.1^{b}

Values are means for three determinations.
^{a,b,c,d} Means in the same row with the different superscripts differ significantly ($p<0.05$).

Distribution of nitrogen after incubation is shown in figure 2. The amount of microbial nitrogen in total nitrogen was slightly increased at 1.5% and decreased thereafter by the addition of 3.0% or more lipids, but these changes were not significant. Ammonia nitrogen and total water-soluble nitrogen were not changed markedly among treatments. Others [Total N minus Non-ammonia soluble N+Ammonia-N+Microbial N] showed a tendency to increase by the addition of 3% or more lipids. These results indicate that grass lipids decrease the ruminal degradation of dietary protein.

Hino and Nagatake (1993) reported that the removal of lipids from grass powder significantly increased NDF digestibility of grass by mixed rumen microorganisms, and that when the lipids from the grass were adsorbed to the grass residue (lipids were removed by extraction with chloroform-methanol), NDF digestion decreased to a value less than that of the original grass powders. The results indicated that grass lipids have an inhibitory effect on the growth and activity of fibrolytic bacteria in the rumen. Our experiment agrees with those results and confirms that grass lipids have an inhibitory effect on ruminal fermentation, especially the digestibility of fiber. However, the inhibitory effect of grass lipids was not necessarily linear when lipids were added above the 3.0% level. It suggested that the inhibitory effect was not enhanced above a certain level.

It was suggested by Hino and Nagatake (1993) that grass lipids also contained a substance stimulating fiber digestion, which was inferred to be β-carotene. Furthermore, β-carotene greatly alleviated the inhibition of NDF digestion by grass lipids, while the effect of β-carotene was smaller when lipids were not present. Although this experiment used the method of ether extraction, ether extracts presumably contained β-carotene, because carotenoids also are extracted by diethyl ether. Thus, the effect of lipid on ruminal fermentation may possibly be influenced by the interaction between negative and positive substances. Further investigation will be needed to specify the amount and fermentable inhibiting effect of lipid fractions on fermentation.

Experiment 2

The changes of fermentation traits during incubation are shown in table 3. DM disappearance increased linearly from 0 to 21 h, and then decreased from 21 to 24 h. The VFA concentration gradually increased over the period of incubation, except at 9 h, and was not different among 18, 21 and 24 h; pH value decreased with time. The amount of microbial nitrogen

increased from 0 to 12 h, and tended to decrease thereafter until 24 h incubation.

Figure 3 shows the changes in FFA composition during incubation. Proportions of polyunsaturated fatty acids such as linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids steadily decreased as incubation time proceeded, while stearic acid ($C_{18:0}$) increased. Proportion of oleic acid ($C_{18:1}$) increased from 0 to 6 h, and then slightly decreased from 9 to 24 h. Proportion of palmitic acid ($C_{16:0}$) tended to decrease from 0 to 12 h, and increased slightly until 24 h incubation. These changes in FFA composition may have been influenced by the changes of FFA in the rumen fluid used for inoculum. The contents of total FFA did not differ between hay itself and mixtures of rumen fluid containing hay, however, $C_{18:2}$ and $C_{18:3}$ were converted to $C_{18:0}$ when mixtures of rumen fluid and hay were incubated. This suggested that little FFA was contributed by rumen fluid.

Table 4 shows the partial correlations between fermentation traits and individual FFA amounts when incubation time is held constant. It was shown that the polyunsaturated fatty acids such as $C_{18:2}$ and $C_{18:3}$ correlated positively with pH, and negatively with microbial nitrogen. However, $C_{18:0}$ showed a reverse relationship. The disappearance of DM correlated negatively with $C_{18:2}$ and $C_{18:3}$, but correlated positively with $C_{18:0}$ and $C_{18:1}$. The concentration of VFA was not correlated with any fatty acids. The partial correlation among FFA showed that $C_{18:2}$ and $C_{18:3}$ correlated with $C_{18:0}$ and $C_{18:1}$.

The polyunsaturated fatty acids ($C_{18:2}$, $C_{18:3}$) decreased during incubation as $C_{18:0}$ increased significantly, and $C_{18:1}$ steadily increased at the initial stage of incubation but thereafter, decreased gradually. These changes suggested that biohydrogenation to $C_{18:2}$ and $C_{18:3}$ occurred relatively early in incubation (3, 6 and 9 h).

Harfoot and Hazlewood (1988) summarized the role of microorganisms in ruminant lipid metabolism mainly with respect to hydrolysis and biohydrogenation. On entering the rumen, dietary acyl lipids are subject to

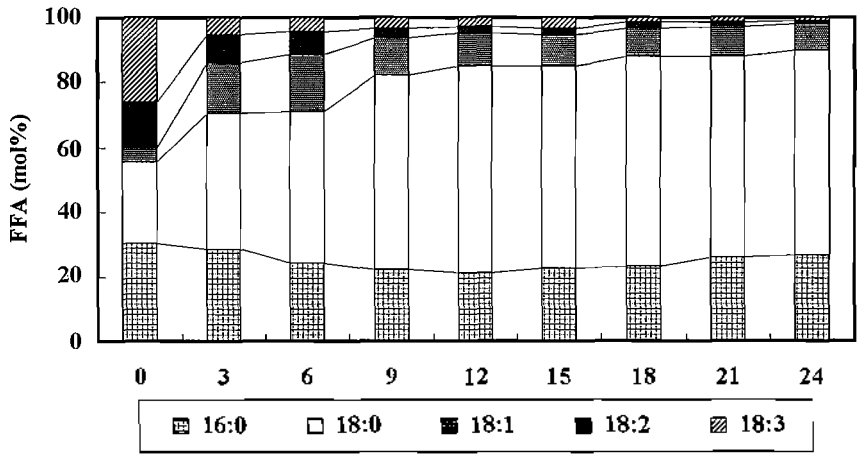

Figure 3. Changes in free fatty acid contents during incubation (16:0 palmitic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic aicd, 18:3 linolenic acid)

Table 4. Partial correlations between fermentation traits and free fatty acid amounts when incubation time is held constant (Experiment 2)

	Palmitic $C_{16:0}$	Stearic $C_{18:0}$	Oleic $C_{18:1}$	Linoleic $C_{18:2}$	Linolenic $C_{18:3}$
DM disappearance (%)	- 0.318	0.408*	0.421*	- 0.394*	- 0.422*
pH	0.169	- 0.612**	- 0.644**	0.577**	0.936**
Total VFA (mM)	0.176	- 0.033	- 0.090	0.207	0.042
Microbial N (mg/vessel)	- 0.375	0.740**	0.155	- 0.441*	- 0.401*
Palmitic acid (mg/vessel)		- 0.047	0.044	0.191	0.188
Stearic acid (mg/vessel)			0.295	- 0.621**	- 0.497*
Oleic acid (mg/vessel)				- 0.415*	- 0.689**
Linoleic acid (mg/vessel)					0.677**

n=27, d.f.=24, * $p<0.05$, ** $p<0.01$.

hydrolysis by microbial lipases. Once liberated as free fatty acids, any unsaturated fatty acids are subject to biohydrogenation by rumen bacteria, the end product of this hydrogenation being $C_{18:0}$. Synthesis *de novo* of microbial lipids also takes place in the rumen, and free acids, both saturated and unsaturated, may be incorporated into microorganisms during cell synthesis. As a result of these microbial transformations, the fatty acids contained in rumen lipids and post-ruminal digesta differ from those present in the diet, being markedly enriched in $C_{18:0}$ at the expense of dietary $C_{18:2}$ and $C_{18:3}$. These effects were supported by the present study using grass hay as the substrate of incubation.

The extent of hydrolysis is very high for most unprotected lipids, 85 to 95% according to Bauchart et al. (1990). This percentage is higher for diets that are rich in fat rather than for classical diets, in which most lipids are in the cellular structures. On the extent of hydrogenation, $C_{18:3}$ n-3 is often completely hydrogenated to $C_{18:0}$. The hydrogenation of $C_{18:2}$ n-6 is not complete. It provides $C_{18:0}$ and different isomers of $C_{18:1}$, of which *trans* vaccenic acid ($C_{18:1}$ n-7) is characteristic of ruminal metabolism (Tamminga and Doreau, 1991). In this study, it was shown that $C_{18:1}$ and $C_{18:0}$ increased during the initial time of incubation, suggesting that hydrogenation from $C_{18:3}$ and $C_{18:2}$ progressed steadily or that hydrogenation of $C_{18:2}$ was incomplete. Polyunsaturated fatty acids ($C_{18:2}$, $C_{18:3}$) correlated negatively with the amount of microbial nitrogen and DM disappearance, while the saturated fatty acid (especially $C_{18:0}$) showed a reverse relationship. The inhibitory effect on ruminal fermentation and digestion, therefore, presumably was alleviated by saturation with hydrogenation.

The unsaturated fatty acids (especially $C_{18:2}$, $C_{18:3}$) produced at initial stages of incubation were negatively correlated with the microbial growth and DM disappearance, indicating that polyunsaturated fatty acids had the possibility to show an inhibiting effect on ruminal fermentation and digestion.

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