In vitro Biohydrogenation of Linolenic and Linoleic Acids by Microorganisms of Rumen Fluid

S. W. Lee*, Yvan Chouinard** and Binh N. Van***,

Faculty of Life Science & Technology, Sungkyunkwan University, Korea*
Laboratory of Animal Science, Laval University, Quebec, Canada**
Central Laboratory of Thai Nguyen University of Agriculture and Forestry, Vietnam***

반추위액의 미생물에 의한 *In vitro* 상에서의 리놀렌산과 리놀산의 Biohydrogenation

이수원*, Yvan Chouinard**, Binh N. Van***,

성균관대학교 생명공학부*, Laval 대학교, 캐나다**, Thai Nguyen 대학교, 베트남***

ABSTRACT

리놀렌산(C18:3)과 리놀산(C18:2)의 biohydrogenation 과 반추위 내에서의 이들 지방산의 bypass를 검토하기 위하여 3 구로 나누어 티모시 조사료를 반추위액과 함께 *in vitro* 상에서 39℃, 0, 3, 6, 9, 24, 36 시간 동안 혐기 배양 시험을 하였다. 첫번째 시험의 목적은 C18:2과 C18:3의 *in vitro* biohydrogenation에 대한 조사료의 성장 단계(stem elongation, early heading, late heading, early flowering)와 질소 시비(0 and 120 kg N ha^{-1})의 영향을 시험하는 것이었다. 수소첨가가능분획(hydrogenable fraction), C18:2과 C18:3의 효과적인 소실과 bypass는 stem elongation 시 수확된 티모시에서 높았고, 성숙함에 따라 일정하게 감소하였다. 질소 시비구는 C18:3의 수소첨가가능분획, C18:2과 C18:3의 효과적인 소실과 bypass가 증가하였다. 그러나 C18:2과 C18:3의 소실율은 성숙과 질소시비(P＞0.1)에 의해 영향을 받지 않았다. 2번째 시험에서는 *in vitro* C18:2과 C18:3 biohydrogenation에 대한 티모시 보존 방법의 영향을 보았다. 사일리지는 C18:2과 C18:3를 가장 효과적으로 소실시켰으며, 건초는 가장 효과가 낮았다. C18:2과 C18:3의 biohydrogenation 된 양은 건초에서 보다 헤일리지와 사일리지에서 더 높았다. 티모시 헤일리지와 비교하였을 때 C18:3의 bypass는 신선 목초, 시든풀, 건초에서 더 높았다. C18:2의 bypass는 건초와 헤일리지에 비해 신선목초와 사일리지가 더 높았다. 3번째 시험에서는 C18:2과 C18:3의 소거와 bypass에 대한 티모시 헤일리지와 사일리지에 대한 개미산 첨가와 *Lactobacillus plantarum* 접종의 효과를 검토하였다. 개미산의 첨가는 헤일리지와 사일리지에 있어서 C18:3의 biohydrogenation 비율을 증가시켰으나 사일리지에 있어서 C18:2의 수소첨가 가능분획을 감소시켰다. 이러한 3가지 배양구의 결과는 티모시에 있어 C18:2과 C18:3의 수소첨가 가능분획과 bypass가 성숙도에 따라 감소하였고 질소시비에 따라 증가하였음을 보여 준다. 헤일리지와 사일리지에서 건초에서 보다 C18:2과 C18:3의 더 많은 양이 biohydrogenation 되었으며 C18:3의 반추위 소실은 헤일리지에서 보다 신선목초, 시든풀, 건초에서 더 높았다.

(**Key words** : Biohydrogenation, Conjugated Linoleic Acid, Isomerization, Linoleic acid)

Ⅰ. INTRODUCTION

Forage lipids are mainly galactosyglycerides and phospholipids(Weenink, 1964), and these complex lipids are rapidly hydrolysed in the rumen by bacterial extracellular lipases. Complex lipids contain large proportion of α－linolenic (C18:3) and linoleic (C18:2) acids in their constituent fatty

Corresponding author : Binh, N. Van, Central Laboratory of Thai Nguyen University of Agriculture and Forestry, Vietnam.
Tel：+84-280-852884, Fax：+84-280-852921, E-mail：ngbinh2002@yahoo.com

acids (Weenink, 1961). These two polyunsaturated fatty acids (PUFA) liberated by lipolysis are adsorbed onto feed particles where they are hydrogenated by rumen microorganisms (Demeyer and Doreau, 1999). The initial step in the biohydrogenation of α-linolenic (*cis*-9, *cis*-12, *cis*-15 C18:3), and linoleic acid (*cis*-9, *cis*-12 C18:2) is the conjugation of the double bond *cis*-9, *cis*-12 by the action of *cis*-12, *trans*-1 isomerase (Kepler et al., 1970). This isomerization yields to the formation of *cis*-9, *trans*-1 C18 conjugated acids, and is followed by the hydrogenation of *cis*-9 double bond. The products from C18:3, *trans*-11, *cis*-15 C18:2 are further hydrogenated to *trans*-1 C18:1 (vaccenic acid), and finally to C18:0 by mixed rumen bacteria (Kemp and Lander, 1983). Studies with pure strains of rumen bacteria had show that most bacteria are capable to hydrogenate C18:2 to *trans*-C18:1 and related isomers, but only a few bacteria had the ability to hydrogenate C18:2 not only to *trans*-C18:1, but also to stearic acid (C18:0) (Fujimoto et al., 1993).

Biohydrogenation of PUFA in the rumen is affected by many factors such as the type and amount of fatty acid substrate (Noble et al. 1974), N content of the diet (Gerson et al., 1983), forage to concentrate ratio in the diet (Gerson et al., 1985), age of the mixed culture, type of the inoculum (Kellens et al., 1986), and the pH of rumen fluid (Van Nevel and Demeyer, 1996). The concentration of C18:2 present in the incubation mixture is a major factor affecting the pattern of hydrogenation (Harfoot et al., 1973).

Conjugated linoleic acid, *cis*-9, *trans*-11 C18:2 (CLA), is also an intermediate product of biohydrogenation of C18:2 by the rumen bacterium *Butyrivibrio fibriosolvens* (Kepler and Tove, 1967). Fujimoto et al. (1993) reported that a *Selenomonas ruminantium and Butyrivibrio fibriosolvens* could isomerize C18:2 to *trans*- 9 C18:1 (eladic acid). Normally, the *trans*-11 C18:1 is the major isomer found in rumen (Harfoot, 1981; Kemp and Lander, 1984) since it represents at least 80% of the total rumen *trans* C18:1 (Demeyer and Doreau, 1999). It appears to be the penultimate end-product in the biohydrogenation of C18:2 and C18:3 that is slowly hydrogenated to C18:0 compared with *cis*-9 C18:1 (oleic acid) (Kemp and Lander, 1984).

Recently, attention had been given to omega-3 PUFA, such as C18:3 which are recognized as healthy fatty acids for human by their beneficial cardiovascular and anti-inflammatory properties (Williams, 2000). CLA possess anticarcinogenic properties as well as anti-lipogenic and immuosuppressive effects (Williams, 2000). Ruminant meat and dairy products are significant sources of CLA (Dhiman et al., 1999). However, they contain approximately only 1% of C18:3 despite the high content of C18:3 in lipids of pasture grazed by ruminants and in oilseeds such as canola in their diets (Ashes et al., 1997). The low content of C18:3 in milk is due to an extensive biohydrogenation of PUFA in the rumen with consequently the formation of *trans*-C18:1 and C18:0 (Gulati et al., 1997).

The FA composition of total lipids of various forage plants is dominated by high proportions of unsaturated α-linolenic (C18:3) and linoleic (C18:2) acids (Harfoot and Hazlewood, 1988), but small amounts of oleic acid (C18:1) are also present (Harfoot, 1981). In fresh grasses, C18:3 represent between 50 and 75% of total FA (**TFA**) (Hawke, 1973), C18:2 and palmitic acid (C16:0) acids are the next most abundant (McDonald et al., 1988). Lough and Aderson have also found that C18:3 was the major component of the esterified FA (simple and complex lipids) of mixed pasture grasses including ryegrass, timothy and meadow fescue. However, in ensiled grasses, C18:3 was present in small proportion in the esterified FA and absent from complex lipids of silage. The FA concentration in forages varied

with many factors such as species and senescence (Harfoot and Hazlewood, 1988; Harwood, 1980), growth stage (Bauchart et al., 1984), conservation methods (Lough and Anderson, 1973; Yang and Fujita, 1997), as well as wilting, shading and silage additives (Dewhurst and King, 1998).

The addition of formic acid to fresh or wilted ryegrass leads to a decrease in silage fermentation and consequently a small production of acetic, lactic and butyric acids (Dewhurst and King, 1998). However, we ignore the effect of silage additives on their isomerization and biohydrogenation of forage unsaturated fatty acids in the rumen and the amount of these fatty acids that could pass out the rumen. One way to increase milk fat content of C18:3 could be to increase the rumen outflow of this fatty acid. The objectives of the present research was to study the influence of the growth stage, N-fertilization, conservation methods, and silage additives, such as formic acid and lactic acid bacteria (LAB), on *in vitro* biohydrogenation of C18:2 and C18:3 fatty acids and to evaluate their ruminal bypass.

Ⅱ. MATERIALS AND METHODS

1. Experiment trials

(1) Trial 1

Sixty four plots (3.25 m^2) of timothy, cultivar Champ, were used in a 2 × 4 factorial design; two levels of N- fertilization (0 and 120 kg ha^{-1}) and four growth stages (stem elongation, **SE**; early heading, **EH**; Late heading, **LH**; and early flowering, **EF**). The harvest interval is one week between each growth stage. A forage sample with the parts can be used as feed for cows (c.a 250 g) was taken from each plot at each harvest, dried at 55 ℃ in a forced-draft oven for 3 d, and ground using a Wiley mill (Standard Model 3, Arthur H. Thomas Co., Philadelphia, PA) fitted with a 1－mm screen.

(2) Trial 2

In this trial, different conservation treatments of timothy harvested at early heading stage were studied. A part of a timothy field was harvested at 5－cm height with a hand clipper. The fresh grass (**FG**) material (230 g DM kg^{-1}) was separated into two parts. One part was spread out uniformly on wire netting and was either field-wilted for few h to reach 400 g DM kg^{-1} (wilted grass, **WG**), or for 3 d until 850 g DM kg^{-1} (grass hay, **GH**). The WG and the second part of the fresh material were used to make mini silos. After 60 d of fermentation, silos were opened and emptied. Samples of FG, WG, GH, haylage (400 g DM kg^{-1}, **H**), and silage (230 g kg^{-}1, **S**) were frozen and lyophilized. All lyophilized samples were ground at 1mm (Standard Model 3, Arthur H. Thomas Co., Philadelphia, PA).

(3) Trial 3

Timothy haylage and silage were made as described in the previous section but LAB (*Lactobacillus plantarum*) inoculant or formic acid was added to the forage before making silage. There were a total of 6 treatments (Table 1).

Table 1. Treatments applied to timothy haylage and silage (Trial 3)

Treatment	Dry matter (g kg^{-1})	Additive	Level
Haylage, H	40	－	－
Haylage, Hi	40	LAB	10^6 CFUg^{-1} FM
Haylage, Hfa	40	Formic acid	6 L t^{-1} FM
Silage, S	23	－	－
Silage, Si	23	LAB	10^6 CFUg^{-1} FM
Silage, Sfa	23	Formic acid	6 L t^{-1} FM

LAB : *Lactobacillus plantarum* inoculant.
CFU : Colony forming units.
FM : Fresh matter.

2. Rumen fluid collection

A rumen fistulated Holstein cow in early lactation was used as a donor of rumen content. This cow was fed trefoil-fescue and orchard grass-timothy silages (12 meals day^{-1}), timothy hay (3 kg fresh matter (FM) day^{-1}), commercial concentrate (Purina 1233 Lactena B16), and mineral (150 g FM day^{-1}) and vitamin (300 g FM day^{-1}) supplements. The nutrient composition of dietary ingredients is presented in Table 2. Two L of rumen fluid was collected and was immediately poured into a 39 ℃ pre-warmed thermos. Two handles of fibrous material from the rumen content were added in the thermos, which was the completely filled with rumen fluid in order to assure anaerobic conditions during transportation. The thermos content was emptied in a 39 ℃ pre-warmed and $C0_2$ gassed blender (Warning Commercial Blender) and mixed at a high speed during 30 s in order to dislodge microorganismss associated to solid particles of the rumen content. The mixed rumen fluid was strained through four layers of cheesecloth and the inoculum was mixed with a magnetic stirrer and maintained at 39 ℃ under anaerobic conditions.

Table 2. Nutrient composition of dietary ingredients

Nutrient	Trefoil-fescue Silage	Orchard grass-timothy silage	Timothy hay	Concentrate
DM	94.8	94.4	95.5	95.2
CP	12.9	11.8	11.6	20.1
ADF	30.7	32.1	34.2	6.4
NDF	51.9	50.7	58.4	15.5
P	0.2	0.2	0.3	0.7
EE	3.1	2.7	2.7	8.4

DM, CP, ADF, NDF, P and EE = dry matter, crude protein, acid detergent fiber, neutral detergent fiber, phosphorus and ether extract.

3. *In vitro* biohydrogenation method

Three different incubations were done separately, one for each trial. Timothy samples (250 mg) were incubated in duplicate in 50 mL glass tube with 25 mL of McDougall buffer (pH 6.9) and 5 mL of rumen inoculum. A standard timothy sample was used as control in all incubation. Blank tubes containing only the inoculum and McDougall buffer were also included in all incubations. All tubes were gassed with CO_2, tightly fitted with rubber stopper equipped with a gas release valve, and incubated at 39 ℃ for 0, 3, 6, 9, 24, or 36h. The rotating platform of the incubator (Lab-line, Environ-Melrose Park, IL) was adjusted at 175 turns min^{-1}. At the end of each incubation period, tubes were withdrawn and immediately frozen. Frozen tubes were lyophilized during five to seven days for further fatty acid analysis.

4. Fatty acid analysis

Fatty acids in lyophilized incubation mixture were extracted and methylated following a one-step procedure (Sukhija and Palmquist, 1988) using toluene as solvent. Methyl nonadecanoate was used as an internal standard. Fatty acid methyl esters were quantified by gas chromatography using a HP 5890 chromatograph (Hewlett Packard Co.) under the following condition: 600m × 0.32mm DB-23 capillary column, 0.25 μm film thickness, H_2 carrier gas, 2.8 cm^3 min^{-1} volumetric flow rate, injector split 1/100 at 240 ℃, septum purge vent at 2 ml min^{-1}, flame ionization detector at 250℃, and 15 kPa of heat pressure. The initial temperature was 150 ℃, which was increased 5 ℃ min^{-1} up to 200 ℃. Fatty acid methyl esters in toluene were directly injected through the split injection port. Peak area of each fatty acid was measured using a Turbochrom 3 analytical system (Version 3.3 ; PE Nelson). Each peak was identified and quantified using pure methyl ester standards (Alletch, Deer-

field, IL).

5. Statistical analyses

PUFA are not extensively metabolized by ruminal microorganisms. The amount of C18:2 and C18:3 disappearing from the incubation mixture were therefore considered to be biohydrogenated. The disappearance of C18:2 and C18:3 in timothy samples during the *in vitro* incubation was described according to the following equation:

$$Pt = x + y^* e^{-zt}$$

where

Pt = amount (mg g^{-1} DM) of timothy C18:2 and C18:3 present in the incubation mixture at time t (h),

x = non-hydrogenable fraction of C18:2 and C18:3 in timothy (mg g^{-1} DM),

y = hydrogenable fraction of C18:2 and C18:3,

z = rate of disappearance of fraction y (% h^{-1})

t = incubation time (h)

Effective disappearance (ED) of C18:2 and 18:3 were calculated using a rate of passage (k) of 0.06 h^{-1} as followed:

$$ED = y(z/(z+k))$$

The bypass (BP) of C18:2 and C18:3, defined as the amount of C18:2 and C18:3 that potentially escape from the rumen biohydrogenation, was estimated using this equation:

$$BP = x + y - ED.$$

The appearance of C18:0 in timothy during the biohydrogenation process was estimated by the model of Orskov and MacDonald (1979):

$$Pt = a + b(1-e^{-ct}).$$

where

Pt = amount (mg g^{-1} DM) of timothy C18:0 present in the incubation mixture at time t (h),

a = amount of C18:0 (mg g^{-1} DM) present in the incubation mixture at time 0,

b = amount of C18:0 (mg g^{-1} DM) appearing during the biohydrogenation process,

c = rate of appearance of fraction b (% h^{-1}),

t = incubation time (h).

Effective appearance (EA) was estimated, with a rate of passage (k) of 0.06 h^{-1} as followed:

$$BP = a + EA.$$

The parameters x, y, z, a, b, c, ED, EA, and BP for each fatty acid and each treatment were calculated according to the nonlinear Marquardt procedure of SAS (SAS Institute, Inc. 1985). In trial 1, data for parameters x, y, z, a, b, c, ED, EA, and BP were subjected to ANOVA by the GLM procedure of SAS (SAS Institute, Inc. 1985) according to a split plot factorial design. Growth stages were the main plot, and N-fertilization was the subplot with four replications per treatment. Quantitative contrasts were used to test linear, quadratic, and cubic effects of growth stage and the interactions between growth stage and N-fertilization. In trial 2 and 3, data were analyzed using the GLM procedure of SAS (SAS Institute, Inc. 1985) as a randomized design with four replications per treatment. In trial 2, the parameters for each fatty acid were compared using the LSD test (SAS Institute, Inc. 1985). In trial 3, simple contrasts were used to test differences between treatments.

Ⅲ. RESULTS AND DISCUSSION

1. Effect of growth stage

The curves of kinetic disappearance of C18:2 and C18:3, an appearance of C18:0 during the *in vitro* incubation of timothy harvested at different growth stages is presented in Figure 1. The disappearance of the hydrogenable fraction (y) of C18:2 and C18:3 were almost completed after 9 h of incubation. However, a portion of the initial concentration of C18:2 and C18:3 remained in the incubation mixture after 36h (fraction x).

The hydrogenable fraction of C18:2 and C18:3 as well as the amount of C18:0 appearing during the biohydrogenation process decreased linearly

with maturity (Table 3 and Figure 1). In contrast, the non-hydrogenable fractions of C18:2 and C18:3 were not significantly affected by growth stage (P>0.1). The ED of C18:3 and C18:2, and the EA of C18:0 were high in timothy harvested at SE, and decreased linearly with maturity

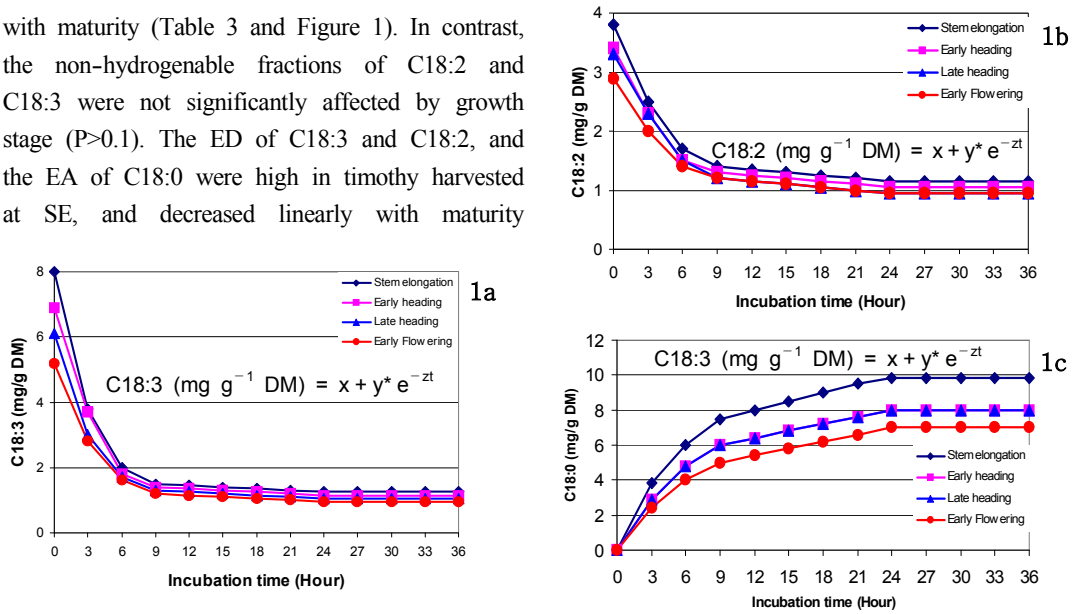

Fig. 1. Curves of kinetic disappearance of C18:3(1a) and C18:2(1b) and appearance of C18:0(1c) during the *in vitro* incubation of timothy harvested at different growth stages, x = non hydrogenable fraction of C18:2 and C18:3 in timothy (mg g^{-1} DM), y = hydrogenable of C18:2 and C18:3 in timothy (mg g^{-1} DM), z = rate of disappearance of fraction y (% h^{-1}), a = amount of C18:0 (mg g^{-1} DM) in th incubation mixture at time 0, b = amount of C18:0 (mg g^{-1} DM) appearing during the biohydrogenation process, C18:2 and C18:3 = rate of appearance of fraction b (% h^{-1}) and t = incubation time (h).

Table 3. Biohydrogenation of C18:2 and C18:3 and appearance of C18:0 during *in vitro* incubation of timothy at four growth stages and two levels of N-fertilization (Trial 1)

Fatty acid parameter[1]		Treatment								Probability[2] of treatment and contrast[3] effects						
		0 kg N ha^{-1}				120 kg N ha^{-1}										
		SE	EH	LH	EF	SE	EH	LH	EF	GS_L	GS_Q	GS_C	N	GS_LxN	GS_QxN	GS_CxN
C18 :2	x	0.93	0.80	0.64	0.72	0.90	0.85	1.00	0.99	NS	NS	NS	**	**	NS	NS
	y	2.74	2.25	2.25	2.01	3.00	2.75	2.50	1.98	**	NS	NS	NS	NS	NS	NS
	z	0.23	0.20	0.18	0.20	0.20	0.20	0.20	0.20	NS	NS	NS	NS	NS	NS	NS
	ED	2.16	1.73	1.62	1.55	2.31	2.12	1.92	1.52	**	NS	NS	#	NS	NS	NS
	BP	1.51	1.32	1.27	1.18	1.59	1.48	1.58	1.45	**	NS	NS	**	#	NS	NS
C18 :3	x	0.91	0.73	0.69	0.76	1.06	1.16	0.95	0.92	NS	NS	NS	**	NS	*	NS
	y	6.25	4.99	4.25	3.75	7.76	7.02	6.25	5.00	**	NS	NS	**	NS	NS	NS
	z	0.33	0.28	0.30	0.33	0.32	0.30	0.30	0.30	NS	NS	NS	NS	NS	NS	NS
	ED	5.28	4.09	3.54	3.17	6.53	5.85	5.21	4.17	**	NS	NS	**	NS	NS	NS
	BP	1.88	1.64	1.40	1.35	2.28	2.33	1.99	1.76	**	NS	NS	**	NS	#	NS
C18 :0	a	0.04	0.31	-0.15	0.06	0.49	-0.48	-0.15	-0.26	NS	NS	NS	NS	NS	NS	#
	b	9.04	7.46	7.19	7.21	10.40	10.14	8.97	8.34	**	NS	NS	**	NS	NS	NS
	c	0.16	0.13	0.13	0.12	0.13	0.14	0.15	0.13	NS	NS	NS	NS	NS	NS	NS
	EA	6.58	5.46	4.74	4.85	7.52	6.58	6.14	5.40	**	NS	NS	**	NS	NS	NS
	BP	6.62	5.77	4.59	4.91	8.01	6.10	5.99	5.15	**	#	NS	**	NS	NS	*

[1] The hydrogenation of C18:2 and C18:3 was described according to the equation Pt = x + y e^{-zt}, The appearance of C18:0 was described according to the equation Pt = a + b (1 − e^{-ct}),

[2] NS, #, *, **, non significant, and significant at P<0.1, P<0.05, and P<0.01, respectively.

[3] L, Q, C = Linear, quadratic, and cubic effect of growth stage (GS), respectively.

SE, EH, LH and EF = Stem elongation, early heading, late heading and early flowering.

(Table 3). We previously observed that concentrations of C18:2 and C18:3 in timothy decreased linearly with maturity. This result explains the linear decrease of the hydrogenable fraction of C18:2 and C18:3 in timothy observed with advance in maturity.

The rate of disappearance of C18:2 and C18:3 and the rate of appearance of C18:0 were not affected by maturity (P>0.1). The bypass of C18:2, C18:3, and C18:0 were higher at SE and decreased linearly with maturity. The amount of C18:2 and C18:3 potentially escaping the ruminal biohydrogenation (BP) were 14 and 25% higher in timothy harvested at SE as compared with EF. The amount of C18:0 potentially leaving the rumen was 31% higher at SE than at EF.

No *trans*-C18:1 was detected in timothy grass harvested at different growth stages. However, the concentration of *trans*-C18:1 in the incubation mixture increased gradually during the first 9 h of incubation, then remained at a constant level until the end of incubating period (Figure 2, 2a). This kinetic of *trans*-C18:1 concentration in the incubation mixture did not fit in the models used for C18:2, C18:3, and C18:0. The average concentrations of *trans*-C18:1 between 9 and 36 h of incubation were therefore used for statistical analysis (Table 4).

Trans-C18:1 are among intermediate produced during ruminal biohydrogenation of C18:2 and C18:3. Many isomers have been identified, including *trans*-9 C18:1 (Fujimoto et al., 1993), as well as *trans*-15 and *trans*-11 C18:1 (Harfoot and Hazlewood, 1988). Among those intermediates, *trans*-11 C18:1 is the most abundant *trans* isomer of C18:1 produced in the rumen under the normal feeding conditions (Harfoot and Hazlewood, 1988). However, the method used to determine

Table 4. Concentrations of *cis*-C18:1 and *trans*-18:1 in timothy at growth stages and two levels of N-fertilization after different incubation periods (Trial 1)

	Fatty acid (mg g^{-1} DM)		
	trans-C18:1	cis_i-18:1	cis_f-18:1
Growth stage			
Stem elongation	1.97	0.45	0.04
Early heading	1.50	0.45	−0.10
Late heading	1.50	0.33	0.01
Early flowering	1.41	0.22	−0.04
SEM	0.11	0.07	0.17
N−fertilization (kg ha^{-1})			
0	1.32	0.25	−0.17
120	1.87	0.47	0.12
SEM	0.15	0.10	0.23
Source of variation	Probability[a)]		
Growth stage (GS)	**	*	NS
Linear effect (GL_L)	*	**	NS
Quadratic effect (GS_Q)	NS	NS	NS
Cubic effect (GS_C)	NS	NS	NS
N-fertilization (N)	**	**	*
Growth stage × N-fertilization	NS	NS	NS
GS_L × N	NS	NS	NS
GS_Q × N	#	#	NS
GS_C × N	NS	NS	NS

[a)] NS, #, *, ** = non significant, and significant at P<0.1, P<0.05, and P<0.01, respectively.

the FA profile did not allow for a complete separation of different *trans* isomers of C18:1. The sum of *trans* −C18:1 is therefore reported (Figure 2, 2a and Table 4). The average concentration of *trans*-C18:1 after 9, 24, and 36 h of incubation was higher in SE samples, and it decreased linearly with maturity of timothy (Table 4). The linear decrease of *trans*-C18:1 concentration observed with the advance in maturity of timothy was probably related to the decreased hydrogenable fraction of either C18:2 and C18:3 (Table 3).

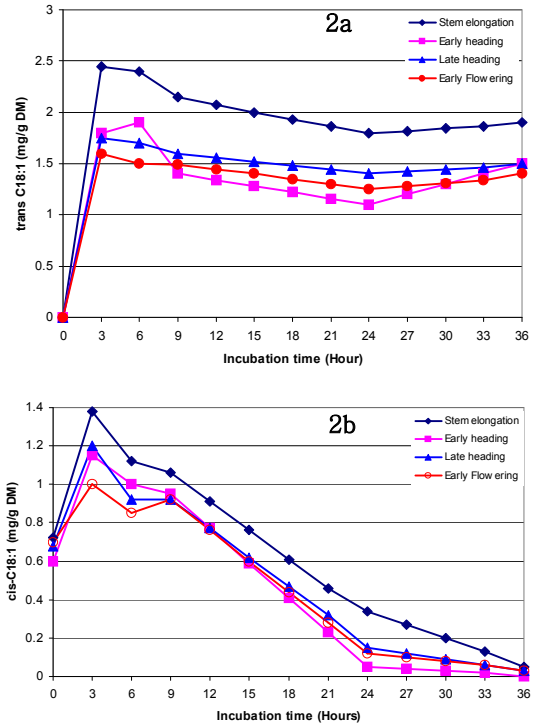

Fig. 2. Concentration of *trans* - C18:1 (2a) and *cis* - C18:1 (2b) during the *in vitro* incubation of timothy harvested at different growth stages.

The concentration of *cis*-C18:1 in timothy grass was not affected by growth stages. The concentration of *cis*-C18:1 increased rapidly during the first 3 h of incubation (Figure 2, 1b) for all treatments. According to the metabolic biohydrogenation pathway of C18:3 described by Harfoot (1981), *cis*-9, cis-12, *cis*-15 C18:3 are metabolized to *cis*-12, *cis*-15 C18:2 isomer. The *cis*-12 double bond of this C18:2 isomer is then reduced to form *cis*-15 C18:1 which is further hydrogenated to C18:0. The formation of *cis*-15 C18:1 as an intermediate during the biohydrogenation of C18:3 may explain the temporary increase in the concentration of *cis*-C18:1 during the first 3 h of incubation. The initial production of *cis*-C18:1 observed during this period decreased linearly with the advance in maturity of timothy (Table 4). This effect is again probably related to the decreased concentration of C18:2 and C18:3 at the beginning of incubation with maturity of timothy. The concentration of *cis*-C18:1 started to decrease after 9 h of incubation, and the hydrogenation was almost completed at the end of incubation period (Figure 2, 1b).

In other study we also found that the concentrations of C16:0, C16:1, C18:0, C18:2, C18:3, and TFA decreased linearly with growth stage. However, concentration of C14:0, C18:0 and C18:1 were not affected by the maturity of timothy. Gervais and St-Pierre (1979) also found a reduction in ether extraction with advance in maturity. They reported that the concentration of timothy ether extract was respectively 4.6, 3.3, 2.3, 2.0, 1.8 and 1.3% at vegetative stage, stem elongation, boot stage, heading stage, blooming and seed ripening stage.

2. Effect of N fertilization

Fertilized timothy had a higher non-hydrogenable fraction (x) of C18:2 and C18:3, as well as a higher hydrogenable fraction (y) of C18:3 as compared to non fertilized timothy (Table 3). The amount of C18:0 formed during the biohydrogenation process was higher in fertilized than non-fertilized timothy (Table 3).

N-fertilization had no effect (P>0.1) on the rate of disappearance of C18:2 and C18:3 as well as the rate of appearance of C18:0. However, N

treatment increased the ED of C18:2 and C18:3, the EA of C18:0, and the BP of C18:2, C18:3 and C18:0 (Table 3). The increased ED of C18:2 and C18:3 in fertilized timothy was reflect of higher amount of C18:2 and C18:3 in fertilized timothy in the incubation mixture. In particular, N-fertilization (120 kg ha^{-1}) increased crude protein concentration (104.4 vs 161.3 mg g^{-1} DM) and decreased NDF concentration (62.7 vs 60.3 mg g^{-1} DM) in timothy grass. Based on these results, we could assume that the metabolic component of forage biomass, which is made of cellular contents increased, whereas the structural component, which is made essentially of cell walls decreased with N-fertilization. Belanger and McQueen (1998) also hypothesized that N deficiency increase the lignifications of grasses. The increase in the metabolic component, including chloroplasts, could cause a greater synthesis and accumulation of lipids and FA in the plant.

The average concentration of *trans*-C18:1 after 9, 24, and 36 h, and the initial increase of *cis*-C18:1 at the beginning of the incubation was higher in fertilized timothy as compared with non fertilized timothy (Table 4). These results were probably related to the higher amount of C18:2 and C18:3 in fertilized timothy at the beginning of the incubation.

Study the effect of nitrogen fertilization on the FA concentrations of timothy, we also found that nitrogen fertilization increased the concentration of C14:0, C16:0, C16:1, C18:1, C18:2, C18:3 and TFA. But the concentrations of C12:0 and C18:0 were not affected by nitrogen fertilization.

In conclusion for this first trial, timothy grass should be harvested at an early growth stage, and fertilized adequately with N in order to maximize the amount of PUFA escaping ruminal biohydrogenation. Most of the effects observed were related to the initial concentration of C18:2 and C18:3 in timothy and no major difference has been noted on the rate of disappearance of PUFA during incubation period. In the current experiment, the apparent BP of C18:2 and C18:3 were calculated assuming a constant ruminal rate of passage (k) of 0.06 h^{-1}. However, Fredickson et al. (1993) reported that ruminal retention time increased, and passage rate of indigestible NDF decreased as the grazing season progressed in crossbred steers. If more mature forage, containing less PUFA, remains in the rumen for a longer period of time, this will exacerbate the effect of growth stage on the apparent ruminal BP of C18:3 and C18:3.

3. Effect of conservation method

The conservation treatment applied to timothy after the harvest had a significant effect ($P<0.01$) on the hydrogenable (y) and the non-hydrogenable (x) fraction of C18:2 and C18:3 (Table 5). Fresh grass had a higher non-hydrogenable fraction of C18:2 and C18:3 as compared to GH, H, and S. The amount of C18:2 and C18:3 biohydrogenated were higher in S than in H, GH, WG, and FG. The amount of C18:2 and C18:3 biohydrogenated were also higher in H and S than in GH. However, the amount of C18:0 produced during the biohydrogenation process was lower in H than in S, GH, and FG. In FG, the hydrogenable fraction of C18:3 disappeared at a rate of 25, 37, 38, and 43% h^{-1}, respectively. Grass hay had the lowest rate of disappearance of C18:3 and a lower rate of disappearance of C18:2 comparatively to H and S. These results show that C18:2 and C18:3 were more extensively hydrogenated by rumen fluid microorganisms when timothy was incubated as H and S instead of GH and/or WG.

The ED and BP of C18:2 and C18:3, as well as the EA and BP of C18:0 were significantly affected by timothy conservation treatment ($P<0.01$). Among all treatments, S had the highest ED of C18:2 and C18:3 as well as highest EA of C18:0. On the other hand, GH had the lowest

Table 5. Biohydrogenation of C18:2 and C18:3 and appearance of C18:0 during *in vitro* incubation of timothy harvested following different methods (Trial 2)

Fatty acid	Parameter[1]	Treatment[2]					SEM	Probably[3] of treatment effect
		FG	WG	GH	H	S		
C18:2	x	1.20^{a}	1.00^{b}	0.68^{c}	0.82^{bc}	0.86^{bc}	0.06	**
	y	2.25^{bc}	1.97^{c}	2.00^{c}	2.75^{b}	3.77^{a}	0.18	**
	z	0.20^{ab}	0.20^{ab}	0.15^{b}	0.26^{a}	0.25^{a}	0.02	*
	ED	1.74^{bc}	1.52^{c}	1.38^{c}	2.20^{b}	3.04^{a}	0.15	**
	BP	1.72^{a}	1.45^{bc}	1.30^{c}	1.36^{c}	1.60^{ab}	0.05	**
C18:3	x	1.05^{a}	0.85^{ab}	0.75^{bc}	0.55^{c}	0.63^{bc}	0.07	**
	y	5.25^{bc}	5.25^{bc}	4.75^{c}	5.50^{b}	7.00^{a}	0.21	**
	z	0.52^{a}	0.37^{b}	0.25^{c}	0.38^{b}	0.43^{b}	0.02	**
	ED	4.71^{b}	4.52^{b}	3.81^{c}	4.73^{b}	6.13^{a}	0.19	**
	BP	1.59^{ab}	1.58^{ab}	1.69^{a}	1.32^{c}	1.50^{bc}	0.05	**
C18:0	a	0.73^{a}	1.08^{a}	0.78^{a}	0.97^{a}	1.08^{a}	0.21	NS
	b	10.04^{a}	9.11^{ab}	10.22^{a}	8.27^{b}	10.59^{a}	0.52	#
	c	0.15^{ab}	0.09^{bc}	0.08^{c}	0.18^{a}	0.17^{a}	0.02	*
	EA	7.84^{b}	6.60^{c}	6.53^{c}	7.04^{c}	8.66^{a}	0.24	**
	BP	8.58^{ab}	7.68^{bc}	7.31^{c}	8.01^{bc}	9.74^{a}	0.35	**

[1] The hydrogenation of C18:2 and C18:3 was described according to the equation $Pt = x + y\,e^{-zt}$, The appearance of C18:0 was described according to the equation $Pt = a + b\,(1-e^{-ct})$.

[2] Means within a row followed by the same letter are not different at a 0.05 level of significance by LSD multiple range test.

[3] NS, #, *, ** =non significant, and significant at P<0.1, P<0.05, and P<0.01, respectively.
FG, WG, GH, H, and S = fresh grass, wilted grass, grass hay, haylage and silage, respectively.

ED of C18:2 and C18:3 as well as lowest EA of C18:0. In H and S, the ED of 18:2 was higher as compared to GH and WG, and the ED of C18:3 were higher than GH. The amount of C18:3 which bypass the rumen was higher in FG, WG, and GH in comparison to H. Grass hay also had a higher BP of C18:3 than S. The BP of C18:2 and C18:0 were higher in FG and S in comparison to GH and H. The lower BP of C18:0 in GH was related to lower concentrations of C18:2 and C18:3 as well as a lower hydrogenation rate of these PUFA in GH.

The concentration of C18:2 and C18:3 were higher in S as compared to FG or GH. This effect was probably due to a loss of some components during fermentation (such as volatile FA and CO_2) thus causing an increase in the concentration of the other components. Mayne and Gordon(1986) measured the in silo DM losses during the ensilage of unwilted and wilted perennial ryegrass silages stored in 100 tone capacity bunker silos. In unwilted silages, the DM loss present 13.4% of DM ensiled, including 9.9% arising from fermentation, plant respiration and gaseous losses, 2.5% from effluent and 1.0% from surface waste material production. Whereas, only 6.0% DM was lost during the ensilage of wilted silages, distributed as 4.8% DM loss during fermentation and oxidation, 0% in effluent and 1.2% in surface waste (Mayne and Gordon, 1986). Losses of either DM during S fermentation, or soluble components in S effluent, which contains soluble carbohydrates and soluble nitrogenous compounds (McDonald et al., 1991) could have contributed to an increase in the C18:2 and C18:3 concentration of S. The DM content of typical silage effluent varies between 20 and 100 g kg^{-1} DM and contains organic N, potassium,

ammonia, and phosphorus (Graves and Vanderstappen, 1993).

Collins (1995) observed that *in vitro* DM digestibility was lower in H as compared with standing herbage (FG). Sniffen et al. (1992) also reported lower digestion rate constants for carbohydrate and protein fractions in GH as compared with S. A lower degradability of DM might have physically protected PUFA from ruminal hydrogenation; which may explain the lower rate of disappearance, and the increased BP of C18:3 in GH as compared with FG or S.

The average concentration of *trans*-11 C18:1 in timothy after 9, 24, and 36 h of incubation was significantly affected (P<0.01) by the conservation treatment (Table 6). More *trans*-C18:1 was produced with H and S as compared with FG, WG, and GH. Lough and Anderson (1973) have found higher proportion of C18:1 with trans unsaturation in the esterified fatty acids of silage than of fresh mixed grasses (ryegrass, timothy, and meadow fescue). According to these authors, the presence of *trans*-C18:1 in silage was a consequence of the isomerization and hy drogenation of PUFA occurring in silage. The analysis of FA composition of timothy conducted previously before the *in vitro* biohydrogenation experiment, had not revealed the existence of *trans*-C18:1 isomer in S. Therefore, the presence of *trans*-18:1 in S measured after 9, 24, and 36 h of incubation was not produced during silage fermentation.

Trans-C18:1 is intermediates during ruminal biohydrogenation of PUFA. As reported previously, the amount of C18:2 and C18:3 biohydrogenated, and the rate of disappearance of these FA were higher in S as compared with GH. Such effect on the level of PUFA and the rate of biohydrogenation may explain the higher production of *trans*-C18:1 during the *in vitro* incubation of S and H as compared with GH. The initial production of *cis*-C18:1 during the first 9 h of incubation was higher in S and FG than in WG and GH. This increase in the production of *cis*-18:1 at the beginning of the incubation period may also be a consequence of the higher levels of PUFA as well as the higher rate of biohydrogenation in S as compared with GH. The concentration of *cis*-C18:1 after 36 h of incubation was higher in S than in WG and GH. However, the level of *cis*-C18:1 remained higher at the end of the incubation period when compared with the first trial where the biohydrogenation of *cis*-C18:1 was almost complete (Table

Table 6. Concentrations of *cis* - C18:1 and *trans* - C18:1 in timothy at different conservation treatments after different incubation periods (Trial 2)

Treatments	Fatty acid [y] (mg g^{-1} DM)		
	trans-C18:1	cis_i -C18:1	cis_f -C18:1
Fresh grass (230 g DM kg^{-1})	1.54^{d}	0.73^{ab}	0.66
Wilted grass (400 g DM kg^{-1})	1.93^{c}	0.32^{d}	0.39
Grass hay (850 g DM kg^{-1})	1.72^{cd}	0.41^{cd}	0.43
Haylage (400 g DM kg^{-1})	2.75^{b}	0.54^{bc}	0.71
Silage (230 g DM kg^{-1})	3.31^{a}	0.83^{a}	0.98
SEM	0.07	0.06	0.13
Source of variation		Probability[x]	
Treatments	**	**	#

[y] Means within acolumn follwed by the same latter are not different at a 0.05 level of significance by LSD multiple rage test.

[x] #, *, ** = significant at P<0.1, P<0.05, and P<0.01, respectively.

4). Kemp et al. (1975) reported that Fusocillus organisms, strictly anaerobic bacteria, isolated from the rumen of sheep were able to hydrogenate C18:3 to *cis*-15 C18:1 which appears not to be further hydrogenated either by pure cultures or by mixed rumen microorganisms. The inability of rumen bacteria to hydrogenated *cis*-15 C18:1 may explain the high level of *cis*-C18:1 observed in the incubation mixture after 36 h of incubation. It is not known however why such phenomenon was not observed in the first trial.

In other study, we also found that the conservation treatment applied to timothy after harvest (fresh grass, wilting, hay, haylage and silage) had a significant effect on the proportion of TFA, C18:3, C18:1, C16:1, C16:0 and C12:0. However, the concentration of C18:0 was not affected by conservation treatments. Fresh grass had higher concentration of TFA, C18:3, C18:2, C18:1, C18:0, C16:1, C16:0, and C14:0 than wilted grass and grass hay. Haylage contained higher concentration of TFA, C18:3, C18:2, C16:0, and C14:0 as compared to wilted grass and grass hay, and lower concentrations of TFA, C18:3, C18:2, C16:1, and C16:0 than silage.

4. Effect of haylage and silage additives

Silage had higher hydrogenable fraction (y) of C18:2 and C18:3 than H. The ED of C18:3, the BP of C18:2, and the EA of C18:0 were higher in S than in H (Table 7). The initial production of *cis*-C18:1 at the beginning of the incubation period was also higher in S than in H (Table 8).

Table 7. Biohydrogenation of C18:2 and C18:3 and appearance of C18:0 during *in vitro* incubation of timothy silage and haylage with and without additives (Trial 3)

Fatty acid Parameter[1]		Treatment[2]						SEM	Probability[3]					
									Treatment effect	Constrasts				
		H	Hi	Hfa	S	Si	Sfa			H vs Hi	H vs Hfa	H vs S	S vs Si	S vs Sfa
C18:2	x	0.90	0.92	1.03	1.10	0.90	1.15	0.08	NS	NS	NS	NS	NS	NS
	y	3.01	2.76	2.52	3.74	3.50	2.99	0.23	#	NS	NS	#	NS	#
	z	0.25	0.25	0.27	0.25	0.20	0.30	0.03	NS	NS	NS	NS	NS	NS
	ED	2.41	2.19	2.05	3.01	2.69	2.47	0.20	NS	NS	NS	NS	NS	NS
	BP	1.50	1.50	1.50	1.83	1.71	1.67	0.06	*	NS	NS	**	NS	NS
C18:3	x	0.75	0.60	0.84	0.55	0.60	0.66	0.06	#	NS	NS	#	NS	NS
	y	6.01	5.75	5.48	7.25	7.00	7.26	0.32	*	NS	NS	*	NS	NS
	z	0.43	0.36	0.53	0.40	0.40	0.55	0.13	**	NS	*	NS	NS	**
	ED	5.26	4.91	4.91	6.30	6.09	6.54	0.27	**	NS	NS	*	NS	NS
	BP	1.50	1.44	1.40	1.50	1.51	1.37	0.06	NS	NS	NS	NS	NS	NS
C18:0	a	0.95	0.50	0.07	0.36	0.19	0.72	0.28	NS	NS	#	*	NS	#
	b	8.58	7.76	9.87	11.24	9.73	11.63	0.78	#	NS	NS	#	NS	NS
	c	0.11	0.24	0.14	0.15	0.26	0.10	0.04	NS	#	NS	NS	NS	NS
	EA	6.52	6.57	6.88	7.71	7.47	7.55	0.32	NS	NS	NS	#	NS	NS
	BP	7.47	7.07	6.95	7.35	7.66	8.27	0.43	NS	NS	NS	NS	NS	NS

[1] The hydrogenation of C18:2 and C18:3 was described according to the equation $Pt = x + y\ e^{-zt}$,
The appearance of C18:0 was described according to the equation $Pt = a + b\ (1 - e^{-ct})$.

[2] H = haylage, Hi = Haylage with *Lactobacillus plantarum* inoculant, Hfa = Haylage with formic acid, S = Silage, Si = silage with *Lactobacillus plantarum* inoculant.

[3] NS, #, *, ** =non significant, and significant at P<0.1, P<0.05, and P<0.01, respectively.

These results confirm the observations made in the previous trial, and may be explained by the higher concentrations of C18:2 and C18:3 in S than in H.

The addition of 6 L of formic acid t^{-1} FM increased the rate of biohydrogenation of C18:3 by 23% in H and by 38% in S, but it decreased the hydrogenable fraction (y) of C18:2 from 3.74 to 2.99 mg g^{-1} DM in S (Table 7). We previously observed a lower C18:2 concentration in Sfa than in S, which may explain this decrease of the hydrogenable fraction of C18:2 in Sfa. The LAB inoculant added to H and S had no significant effect on all parameters of kinetic disappearance of C18:2 and C18:3, and appearance of C18:0 during the *in vitro* incubation, except for the rate of appearance of C18:0 which was higher in Hi than H.

The addition of formic acid and LAB inoculant in H and S had no effect on the average concentration of *trans*-C18:1 after 9, 24, and 36 h of incubation. However, the initial production of *cis*-C18:1 at the beginning of the incubation period was increased with the addition of LAB inoculant and formic acid in timothy H, but was decreased by the same additives in timothy S (Table 8). The addition of LAB inoculant in silage allows this microbial population to dominate fermentation and reduce the time until rapid lactic acid production begins (Jasted, 1995). The addition of formic acid in ensiled forage decreases silage pH, which reduces the extent of fermentation. Dewhurst and King (1998) reported that the use of formic acid as additive in wilted and non-wilted perennial ryegrass silage decreased silage fermentation, and consequently the levels of acetic, lactic and butyric acids produced. These results suggest that formic acid have an inhibiting effect on the activity of cellulolytic and proteolytic microorganisms responsible of carbohydrates and protein fermentation in silage. The levels of volatile FA produced during silage

Table 8. Concentrations of *cis* - C18:1 and *trans* - C18:1 in timothy silage and haylage at different incubation periods with or without additives (Trial 2)

Treatments	Fatty acid [x] (mg g^{-1} DM)		
	trans-C18:1	cis_i -C18:1	cis_f -C18:1
Haylage (400 g DM kg^{-1}) (H)	3.12	0.38	0.44
Haylage with LAB inoculum (10^6 CFU g^{-1} DM) (Hi)	3.05	0.67	0.46
Haylage with formic acid (6 L t^{-1} FM) (Hfa)	3.18	0.64	0.55
Silage (230 g DM kg^{-1}) (S)	3.92	1.07	1.09
Silage with LAB inoculum (10^6 CFU g^{-1} DM) (Si)	3.88	0.80	0.60
Silage with formic acid (6 L t^{-1} FM) (Sfa)	3.65	0.61	0.52
SEM	0.12	0.07	0.14
Source of variation	Probability[z]		
Treatments	**	**	NS
H vs Hi	NS	*	NS
H vs Hfa	NS	#	NS
H vs S	**	**	*
S vs Si	NS	#	#
S vs Sfa	NS	**	*

[x] *trans*-C18:1 = average concentration of *trans*-C18:1 at 9, 24, 36 h of incubation.
cis_i -C18:1 = initial production of *cis*-C18:1 during the first 9 h of incubation, and.
cis_f -C18:1 = final concentration of *cis*-C18:1 after 36 h of incubation.
[z] NS, #, *, ** =non significant, and significant at $P<0.1$, $P<0.05$, and $P<0.01$, respectively.

fermentation were not determined in the current experiment. Nevertheless, the addition of formic acid in S and H increased the rate of biohydrogenation of C18:3. However, this increase in the rate of biohydrogenation had no major effect on the apparent BP of C18:3.

When study the effect of haylage and silage additives, our results showed that the dose of LAB inoculant (10^5 vs. 10^6 CFU g^{-1} FM) had no significant effect on all fatty acid concentrations in hay and silage except for C18:2 which tended to be lower with the highest dose of inoculant. However, the dose of formic acid (2 L vs. 6 L t^{-1} FM) had no significant effect on the concentration of all fatty acids in hay and silage ($P>0.1$).

Ⅳ. CONCLUSIONS

This series of experiments indicate that the BP of C18:2 and C18:3 in timothy decreased with maturity, and increased with N-fertilization. Most of the effects observed were related to the initial concentration of C18:2 and C18:3 in timothy, and no major difference has been noted on the rate of disappearance of PUFA during incubation period. The concentration of C18:3 was higher in timothy S and H as compared with GH at the beginning of the incubation period. However, the rate of biohydrogenation of C18:3 was higher in S and H as compared with GH. As a consequence, the BP of C18:3 was higher when timothy was harvested as GH. Moreover, the rates of C18:2 and C18:3 biohydrogenation and the amount of C18:2 and C18:3 escaping ruminal biohydrogenation were not affect by the addition of formic acid or LAB inoculant in H and S. It is therefore recommended to harvest timothy at an early growth stage, fertilize it with N, and conserve it as grass hay in order to maximize the amount of C18:3 that BP the ruminal biohydrogenation.

Ⅴ. ABSTRACT

In vitro anaerobic incubations of timothy (Phleum pretense L.) forage with bovine rumen fluid were conducted at 39℃ for 0, 3, 6, 9, 24, and 36 h in three trials to examine the biohydrogenation of linolenic (C18:3) and linoleic acids (C18:2) and their bypass from the rumen. The objectives of the first trial was to study the effect of growth stage (stem elongation, early heading, late heading, and early flowering) and N-fertilization (0 and 120 kg N ha^{-1}) on *in vitro* biohydrogenation of C18:2 and C18:3. The hydrogenable fraction, the effective disappearance and the bypass of C18:2 and C18:3 were high in timothy harvested at stem elongation, and decrease linearly with maturity. The N-fertilization increased the hydrogenable fraction of C18:3, the effective disappearance and the bypass of C18:2 and C18:3. However, the rate of disappearance of C18:2 and C18:3 were not affected by maturity and N-fertilization ($P>0.1$). In trial 2, the effect of timothy conservation method on *in vitro* C18:2 and C18:3 biohydrogenation was determined. Silage had the highest effective disappearance of C18:2 and C18:3, and grass hay had lowest one. The amounts of C18:2 and C18:3 biohydrogenated were higher in haylage and silage than in grass hay. Comparative to haylage timothy, the bypass of C18:3 was higher in fresh grass, wilted grass and grass hay. The bypass of C18:2 was higher in fresh grass and silage in comparison to grass hay and haylage. In trial 3, the effects of formic acid and *Lactobacillus plantarum* inoculum addition to timothy haylage and silage on C18:2 and C18:3 disappearance and bypass were studied. Haylage and silage additives had no effect ($P>0.1$) on effective disappearance and bypass of C18:2 and C18:3. The addition of formic acid increased the rate of biohydrogenation of C18:3 in haylage and silage, but it decreased the hydrogenable fraction of C18:2 in silage. The

results of these three incubation trials show that the hydrogenable fraction and the bypass of C18:2 and C18:3 in timothy decreased with maturity and increased with N-fertilization. Higher amount of C18:2 and C18:3 were biohydrogenated in haylage and silage than in grass hay, and C18:3 ruminal disappearance was higher in fresh grass, wilted grass and grass hay than in haylage.

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