Effect of Concentrate Level on the Formation of Conjugated Linoleic Acid and Trans-octadecenoic Acid by Ruminal Bacteria when Incubated with Oilseeds

In Vitro*

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ABSTRACT : An in vitro study was conducted to examine the effect of addition level of concentrate on fermentation characteristics and long-chain unsaturated fatty acids composition, especially conjugated linoleic acid (CLA) and trans-octadecenoic acid (t-FA) by mixed ruminal bacteria when incubated with linseed or rapeseed. Four levels (0.83, 1.25, 1.67 and 2.08%, w/v) of concentrate and ground oilseeds (linseed or rapeseed: 0.83%, w/v) were added to mixed solution of strained rumen fluid with artificial saliva (1:1, v/v) in the glass jar with a glass lid equipped with stirrer, and was incubated anaerobically for 24 h at 39°C. Addition level of concentrate slightly reflect on pH and ammonia concentration of the culture solution at the various incubation times when incubated with both linseed and rapeseed. Total VFA concentration slightly increased with incubation times and concentrate levels for incubations with oilseeds. While CLA composition had a clearly increasing trend with incubation time when incubated with linseed, percent CLA was relatively stable when incubated with rapeseed. Percent CLA, however, had a clearly decreasing trend with concentrate level throughout incubation times with significances at 3 h incubations when incubated with linseed (p<0.038) and rapeseed (p<0.0009). The differences in compositions of t-FA were relatively small among concentrate levels for both incubations with linseed and rapeseed. The ratios of t-FA to CLA were lower for linseed with increased proportion of CLA than for rapeseed. (Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 5 : 687-694)

Key Words : Concentrate Level, Oilseed, Bio-Hydrogenation, CLA, Octadecenoic Acid, In Vitro, Mixed Ruminal Bacteria

INTRODUCTION

The bio-hydrogenation activity of ruminal microbes is very high (Wu et al., 1991; Huang et al., 1999; Wang and Song, 2001) although fatty acid compositions in beef and milk have been affected, to some extent, by feeding vegetable oils or oilseeds (Kennelly, 1996; Chouinard et al., 1998a). Conjugated linoleic acid (CLA) and trans-C18:1 fatty acid (t-FA) are formed as a result of incomplete bio-hydrogenation of unsaturated fatty acids in the rumen (Wu et al., 1991). Limited number of bacterial spp were reported to be able to produce CLA from free linoleic acid (C18:2) in vitro (Jiang et al., 1998).

While the CLA exhibits promising beneficial health effects (NRC, 1996) the t-FA has the negative effects on animal performance (Kennelly, 1996) and human health (Erasmus, 1993). The contents of CLA and t-FAs, however, tend to be positively correlated in rumen contents (Kennelly et al., 1998a). Conjugated linoleic acid (CLA) and trans-C18:1 fatty acid (t-FA) are formed as a result of incomplete bio-hydrogenation of unsaturated fatty acids in the rumen (Wu et al., 1991). Limited number of bacterial spp were reported to be able to produce CLA from free linoleic acid (C18:2) in vitro (Jiang et al., 1998).

Thus, it might be important to manipulate the ruminal bio-hydrogenation in order to increase CLA output with a low t-FA/CLA ratio. Because the extent of bio-hydrogenation by ruminal bacteria could be affected by concentrate to roughage ratio (Leat, 1977) and ruminal pH (Song and Choi, 1998; Wang and Song, 1999) demand for the dietary manipulation relating to the concentrate levels and lipid sources has been increased in the examination of the transformation of C18-unsaturated fatty acids by rumen microbes.

Therefore, the present in vitro study was conducted to examine the effect of addition level of concentrate on fermentation characteristics and C18-long chain fatty acids composition, especially CLA and t-FA by ruminal bacteria when used two lipid sources consisting of different fatty acids.

MATERIALS AND METHODS

Preparation of rumen fluid

Rumen contents were collected at 3 h after morning feeding (06:00) from the ruminally cannulated Holstein cow fed 5 kg of corn silage (60%) and concentrate (40%) on a
dry matter (DM) basis twice daily. The rumen contents were brought to the laboratory and were blended in a Waring blender (Fisher 14-509-1) for 20 seconds at high speed to detach the bacteria from the feed particles, and were strained through 12 layers of cheesecloth to remove the feed particles and large protozoa. CO₂ was flushed into the strained rumen fluid.

Preparation and incubation of culture
Strained rumen fluid was mixed with McDougall’s artificial saliva (1948) at the ratio of 1:1 under flushing of CO₂. Four levels (0.83, 1.25, 1.67 and 2.08%, w/v) of concentrates and ground (1 mm) linseed (*Linum usitatissimus*) or rapeseed (*Brassica napus*) at the level of 0.83% (w/v) were added to 1,200 ml mixed solution in the glass culture jar, and CO₂ was flushed into the culture solution for 3 minutes. The culture jar was covered with a glass lid equipped with stirrer and was placed into a waterbath (39°C). Culture solution was again flushed with CO₂ through glass tube connected to the jars for the infusion purpose for 3 min., and was incubated up to 24 h. Stirring speed during incubation was adjusted to 120 times/min. The incubation of culture solution was done three times under similar condition.

Enumeration of total viable bacteria
The number of viable bacteria in culture solution was determined at 12 h and 24 h incubation by the anaerobic culture techniques of Hungate (1966). One ml of culture solution from each treatment was taken and diluted to 10⁻⁵-10⁻⁷ using the Bryant’s diluting solution (1961). Then, 1ml diluted solution was inoculated into the roll tube containing non-selective artificial medium (Scott and Dehority, 1965). The number of colony was counted after the roll tubes were incubated at 39°C for 5 days.

Sampling and analysis
pH of culture solution was measured at the incubation times of 3, 6, 12 and 24 h, and 5 ml culture solution was collected for ammonia and volatile fatty acid (VFA) analysis. All samples collected were kept frozen at -20°C until analyzed. Ammonia concentration was determined by the method of Fawcett and Scott (1960) using the spectrophotometer (DU-650). Four ml culture solution was mixed with 1 ml 25% phosphoric acid and 0.5 ml pivalic acid solution (2%, w/v) as an internal standard. The mixed solution was centrifuged at 15,000g for 15 min., and the supernatant was used to determine the concentration and composition of VFA using gas chromatograph (GC, HP 5890ll, Hewlett Packard Co.). Two hundred ml incubation solution was also collected at the incubation times of 3, 6, 12 and 24 h, and freeze dried and lipids were extracted using Folch’s solution (Folch et al., 1957). Methylation of the lipids followed the method of Lepage and Roy (1986) prior to injecting into the GC, using a fused silica capillary column (100 m×0.25 mm, i.d.×0.20 μm thickness, Supelco, SPTM-2560; USA).

Statistical analysis
The results obtained were subjected to least squares analysis of variance according to the general linear models procedure of SAS (1985) and significances were compared by S-N-K Test (Steel and Torrie, 1980).

RESULTS

Compositions of C₁₈-fatty acids in oilseeds and concentration were presented in table 1. Composition of linolenic acid (C₁₈:₃) in both linseed and rapeseed was highest as 59.2% and 34.0%, respectively, and followed by oleic acid (C₁₈:₁) and C₁₈:₂. But C₁₈:₂ composition was highest in concentrate. Total lipids in culture solution were shown in table 2. Concentrate additions increased the amount of lipids at the ranges of 0.64 to 1.61 g in culture solutions containing both linseed and rapeseed.

Addition level of concentrate did not clearly reflect on pH of the culture solution at the various incubation times but higher concentrate levels had the trends of low pH than lower levels of concentrate when incubated with both linseed and rapeseed (figure 1). No differences were observed in ammonia concentration among concentrate levels although it tended to decrease with the level of concentrate for incubations with both oilseeds (figure 2). There were increasing trends in total VFA concentration with incubation time and concentrate level for both incubations with linseed (table 3) and rapeseed (table 4).

| Feed Components | Lipid (%, DM) | Stearic acid (C₁₈:₀) | Oleic acid (C₁₈:₁) | Linoleic acid (C₁₈:₂) | Linolenic acid (C₁₈:₃) |
|-----------------|---------------|----------------------|-------------------|---------------------|----------------------|
| Linseed         | 34.04         | 2.75                 | 16.9              | 13.9                | 59.2                 |
| Rapeseed        | 43.83         | 3.22                 | 31.1              | 22.3                | 34.0                 |
| Concentrate     | 6.42          | 4.77                 | 23.5              | 43.1                | 4.5                  |

Table 1. Lipid contents and C₁₈-fatty acid composition of oilseeds and concentrate.
and higher levels of concentrate addition significantly increased total VFA concentration in culture solution incubated with linseed at 6 h (p<0.022, table 3) and with rapeseed at 24 h (p<0.030, table 4). As expected, molar percent of acetate (C2) had a decreasing trend while that of propionate (C3) tended to increase with incubation times and concentrate level for both incubations with linseed (table 3) and rapeseed (table 4). Although no clear differences among concentrate levels were observed in molar percent of C2 for both incubations with oilseeds, that of C3 was increased by the highest addition level of concentrate 12 h (p<0.029) and 24 h (p<0.030) when incubated with linseed. Bacterial number in culture solution had an increasing trend with concentrate level at incubations of 24 h (linseed, table 3) and 12 h (rapeseed, table 4).

Decreasing trends in the compositions of oleic acid (C18:1), C18:2 and C18:3 but increasing trends of stearic acid (C18:0) and t-FA compositions were found from culture contents with incubation time when incubated with both linseed (table 5) and rapeseed (table 6) in the all concentrate levels. But while CLA composition had a clearly increasing trend with incubation time when incubated with linseed (table 5) percent CLA was relatively stable when incubated with rapeseed (table 6). When incubated with linseed, the concentrate level did not affect compositions of C18:0 and C18:1, but that of C18:2 tended to increase with concentrate level for all incubation times while C18:3 tended to decrease up to 6 h, thereafter increased with concentrate level (table 5). But when incubated with rapeseed, both C18:2 and C18:3 tended to increase with concentrate level from 6h incubation (table 6). The differences in compositions of t-FA were relatively small among concentrate levels up to 12 h incubation but decreasing trend was observed with concentrate level at 24 h when incubated with linseed (table 5). Decreasing trends also were found in t-FA composition as incubation proceeded when incubated with rapeseed (table 6). The ratio was lower for linseed with increased proportion of CLA than for rapeseed although the ratio tended to increase with the concentrate level.

**DISCUSSION**

The degradations of concentrate and oilseeds, and the bio-hydrogenation of C18-unsaturated fatty acids are mostly considered to be the results of bacterial activity in the present study since protozoa were seldom found from the strained rumen fluid under the microscope due to the straining of rumen contents through 12 layers of cheesecloth. Based on the pH (figure 1), ammonia...
concentration (figure 2) and VFA production (tables 3 and 4), fermentation characteristics were reflected by the concentrate levels in both incubations with oilseeds, but VFA concentrations with incubation times were slightly higher for linseed addition than for rapeseed, indicating greater fermentation of culture solution containing linseed. Trans-11 C18:1 was the major isomer of oleic acid, and trans-7 and trans-9 isomers were detected a little in the present study, thus they were combined with the trans-11 isomer as a total t-FA of C18:1. Only cis-9, trans-11 isomer was detected in the present study and it has been the major type of CLA (Parodi, 1997; Sehat et al., 1998).

The changes in t-FA and CLA production with incubation times indicated that the initial step occurred rapidly, but higher concentrate level reduced the extent of bio-hydrogenation of C18:2 and C18:3 and isomerization of C18:2 in culture solution containing oilseeds, resulting in reduced proportions of t-FA and CLA (tables 5 and 6). The reduced proportions of t-FA and CLA might be caused by the lowered pH (figure 1) as addition level of concentrate increased. The possible pH effect on CLA production was released by Hughes et al. (1982) as cis-9, trans-11 octadecadienoate reductase activity of Butirivibrio fibrisolvens was stimulated at the higher pH and had a maximum activity for pH between 7.2 and 8.2. Similar result was found from the in vivo study of Kelly and Bauman (1996) where the CLA levels in milk were halved when the forage to concentrate ratio was changed from 50:50 to 20:80. Grimari et al. (1996) also reported that relatively increased forage feeding to concentrate increased milk fat concentrations of CLA.

The bio-hydrogenation pathway of C18:2 has involved an

### Table 3. Concentration, molar proportion of VFA and number of viable bacteria in culture solution when incubated with linseed

| Items | Concentrate | SEM | Pr>F |
|-------|-------------|-----|------|
|       | 0.83 | 1.25 | 1.67 | 2.08 | 3 h |
| Total VFA (mmoles/100 ml) | 62.05 | 63.28 | 69.99 | 69.34 | 3.922 | 0.503 |
| Molar proportion (mmoles/100 mmoles) | | | | | |
| Acetate (C2) | 48.62 | 47.87 | 47.34 | 46.41 | 1.155 | 0.628 |
| Propionate (C3) | 26.85 | 28.05 | 28.96 | 29.96 | 0.816 | 0.189 |
| C2/C3 | 1.81 | 1.71 | 1.64 | 1.55 | 0.088 | 0.325 |
| 6 h | | | | | |
| Total VFA (mmoles/100 ml) | 66.71b | 74.03ab | 81.14a | 83.60a | 9.314 | 0.022 |
| Molar proportion (mmoles/100 mmoles) | | | | | |
| Acetate (C2) | 47.85 | 46.6 | 45.60 | 44.37 | 1.409 | 0.443 |
| Propionate (C3) | 26.79 | 28.02 | 29.09 | 30.10 | 0.683 | 0.096 |
| C2/C3 | 1.79 | 1.67 | 1.57 | 1.48 | 0.088 | 0.221 |
| 12 h | | | | | |
| Total VFA (mmoles/100 ml) | 85.1 | 92.97 | 97.08 | 102.67 | 6.140 | 0.354 |
| Molar proportion (mmoles/100 mmoles) | | | | | |
| Acetate (C2) | 46.79 | 45.74 | 44.44 | 42.92 | 1.070 | 0.203 |
| Propionate (C3) | 26.58b | 27.57ab | 28.50ab | 29.35a | 0.393 | 0.029 |
| C2/C3 | 1.76 | 1.66 | 1.56 | 1.46 | 0.059 | 0.087 |
| Bacteria (×10⁷) | 56.75 | 33.65 | 46.75 | 41.85 | 21.200 | 0.886 |
| 24 h | | | | | |
| Total VFA (mmoles/100 ml) | 99.69 | 108.13 | 113.69 | 121.06 | 5.911 | 0.216 |
| Molar proportion (mmoles/100 mmoles) | | | | | |
| Acetate (C2) | 45.17 | 43.80 | 41.81 | 40.13 | 1.169 | 0.124 |
| Propionate (C3) | 26.25b | 27.18ab | 27.51ab | 28.58a | 0.323 | 0.031 |
| C2/C3 | 1.72a | 1.61ab | 1.52ab | 1.40b | 0.053 | 0.054 |
| Bacteria (×10⁷) | 12.65 | 22.25 | 44.15 | 48.90 | 13.440 | 0.309 |

1) Addition levels of concentrate (% w/v) into 1,200 ml culture solution.
2) Means in the same row with different superscripts differ.
3) Standard error of the mean.
4) Probability levels.
initial isomerization step, resulting in the formation of cis-9, trans-11 CLA, which undergoes sequential reduction steps yielding trans-11-octadecenoic acid and then C_{18:0} (Harfoot and Hazlewood, 1988). This sequential reduction steps of C_{18:3} bio-hydrogenation pathway did not consider CLA as a intermediate but produce trans-11 C_{18:1} as one of the final products. Chouinard et al. (1998b) and Kelly et al. (1998) also indicated that CLA was mostly derived from the dietary C_{18:2}. The results of the present study, however, revealed greater proportions of CLA from linseed incubation (table 5) than from rapeseed (table 6). Linseed had a higher C_{18:3} proportion than rapeseed (table 1). This indicates that an alternative pathway may exist in the bio-hydrogenation of C_{18:3} to produce the CLA. Bessa et al. (2000) also postulated the possibility that alternative pathways may exist in the production of CLA from C_{18:3} due to the extreme microbial diversity in the reticulo-rumen.

Both CLA and t-FA are all derived from rumen bio-hydrogenation of C_{18-} poly USFA, and the contents of CLA and t-FA are positively correlated in rumen contents, fat depots and milk (Solomon et al., 2000). But due to the impacts of both bio-hydrogenation intermediates on the human health, it may be important to manipulate rumen bio-hydrogenation toward the CLA increase with a low t-FA/CLA ratio. The t-FA/CLA ratios between oilseeds differed with incubation times and with concentrate levels (tables 5 and 6). The ratio was lower for linseed with increased proportion of CLA than for rapeseed although the ratio tended to increase with the concentrate level. This trend might be related to the rate of fermentation and in turn,
CONCLUSION

The fermentation characteristics and the extent of biohydrogenation of C_{18}-PUFA, especially in the production of t-FA and CLA were influenced, to some extent, by both oilseeds and concentrate level. Greater CLA production from linseed which contains more C18:3 than rapeseed may indicate that CLA could also be derived from the C18:3.

Table 5. Composition (%) of C_{18}-fatty acids in culture solution when incubated with linseed

| Fatty acids | Concentrate | SEM | Pr>F |
|-------------|-------------|-----|------|
|             | 0.83        | 1.25| 1.67 | 2.08 |
| C_{18:0}    |             |     |      |      |
| C_{18:1}    |             |     |      |      |
| t-C_{18:1}  |             |     |      |      |
| CLA         |             |     |      |      |
| t-FA/CLA    |             |     |      |      |
| C_{18:2}    |             |     |      |      |
| C_{18:3}    |             |     |      |      |
| C_{18:0}    |             |     |      |      |
| C_{18:1}    |             |     |      |      |
| t-C_{18:1}  |             |     |      |      |
| CLA         |             |     |      |      |
| t-FA/CLA    |             |     |      |      |
| C_{18:2}    |             |     |      |      |
| C_{18:3}    |             |     |      |      |

1) Addition levels of concentrate (% w/v) into 1,200 ml culture solution.
2) Means in the same row with different superscripts differ.
3) Standard error of the mean.
4) Probability levels.
5) Total trans-C_{18:1} isomers.
6) Conjugated linoleic acid (cis-9, trans-11 isomer of linoleic acid).
7) The ratio of total trans-C_{18:1} isomers to Conjugated linoleic acid.
Table 6. Composition (%) of C18-fatty acids in culture solution when incubated with rapeseed

| Fatty acids | Concentrate (g/l) | SEM (g/l) | Pr>F (g/l) |
|------------|------------------|-----------|------------|
|            | 0.83             | 1.25      | 1.67       | 2.08       |
|            | 3 h               | 6 h       | 12 h       | 24 h       |

| C18:0       | 27.91            | 27.69     | 30.56      | 30.47      | 2.654 | 0.793 |
| C18:1       | 21.29            | 20.78     | 19.35      | 17.48      | 2.675 | 0.757 |
| t-C18:1     | 3.67             | 3.69      | 5.02       | 6.34       | 0.582 | 0.081 |
| CLA         | 0.81             | 0.61      | 0.71       | 0.70       | 0.011 | 0.0009 |
| t-FA/CLA    | 5.99             | 4.54      | 7.09       | 9.01       | 0.859 | 0.082 |
| C18:2       | 15.65            | 16.09     | 14.78      | 14.30      | 1.161 | 0.707 |
| C18:3       | 13.30            | 12.77     | 10.63      | 12.50      | 2.325 | 0.857 |
| C18:0       | 32.45            | 32.57     | 30.41      | 26.03      | 2.045 | 0.226 |
| C18:1       | 21.06            | 19.05     | 19.55      | 20.78      | 1.965 | 0.864 |
| t-C18:1     | 7.11             | 7.58      | 7.42       | 5.95       | 1.215 | 0.780 |
| CLA         | 0.87             | 0.52      | 0.43       | 0.51       | 0.189 | 0.456 |
| t-FA/CLA    | 9.75             | 14.68     | 17.85      | 11.95      | 3.189 | 0.415 |
| C18:2       | 10.88            | 11.46     | 12.71      | 16.22      | 1.046 | 0.071 |
| C18:3       | 10.49            | 10.06     | 10.12      | 12.08      | 3.323 | 0.967 |
| C18:0       | 32.61            | 33.59     | 32.65      | 32.00      | 1.330 | 0.863 |
| C18:1       | 19.52            | 19.01     | 19.19      | 17.95      | 1.109 | 0.779 |
| t-C18:1     | 12.33            | 9.84      | 9.20       | 7.83       | 1.968 | 0.509 |
| CLA         | 0.82             | 0.66      | 0.60       | 0.54       | 0.092 | 0.298 |
| t-FA/CLA    | 14.94            | 14.80     | 15.19      | 14.54      | 1.504 | 0.991 |
| C18:2       | 7.33             | 9.18      | 10.98      | 11.83      | 1.06  | 0.128 |
| C18:3       | 8.16             | 8.76      | 9.22       | 12.00      | 3.224 | 0.772 |
| C18:0       | 36.64            | 36.66     | 36.51      | 33.85      | 1.258 | 0.417 |
| C18:1       | 16.12            | 16.36     | 17.74      | 17.05      | 0.869 | 0.597 |
| t-C18:1     | 14.06            | 12.08     | 11.56      | 10.00      | 1.528 | 0.416 |
| CLA         | 0.64             | 0.67      | 0.61       | 0.52       | 0.072 | 0.587 |
| t-FA/CLA    | 21.92            | 18.06     | 18.98      | 19.16      | 1.286 | 0.308 |
| C18:2       | 2.80             | 5.33      | 7.23       | 9.13       | 1.211 | 0.077 |
| C18:3       | 7.17             | 6.96      | 7.41       | 11.60      | 0.919 | 0.469 |

1) Addition levels of concentrate (% w/v) into 1,200 ml culture solution.
2) Means in the same row with different superscripts differ.
3) Standard error of the mean.
4) Probability levels.
5) Total trans-C18:1 isomers.
6) Conjugated linoleic acid (cis-9, trans-11 isomer of linoleic acid).
7) The ratio of total trans-C18:1 isomers to Conjugated linoleic acid.

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