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Factors Influencing Biohydrogenation and Conjugated Linoleic Acid Production by Mixed Rumen Fungi

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The objective of this study was to evaluate the effect of soluble carbohydrates (glucose, cellobiose), pH (6.0, 6.5, 7.0), and rumen microbial growth factors (VFA, vitamins) on biohydrogenation of linoleic acid (LA) by mixed rumen fungi. Addition of glucose or cellobiose to culture media slowed the rate of biohydrogenation; only 35-40% of LA was converted to conjugated linoleic acid (CLA) or vaccenic acid (VA) within 24 h of incubation, whereas in the control treatment, 100% of LA was converted within 24 h. Addition of VFA or vitamins did not affect biohydrogenation activity or CLA production. Culturing rumen fungi at pH 6.0 slowed biohydrogenation compared with pH 6.5 or 7.0. CLA production was reduced by pH 6.0 compared with control (pH 6.5), but was higher with pH 7.0. Biohydrogenation of LA to VA was complete within 72 h at pH 6.0, 24 h at pH 6.5, and 48 h at pH 7.0. It is concluded that optimum conditions for biohydrogenation of LA and for CLA production by rumen fungi were provided without addition of soluble carbohydrates, VFA or vitamins to the culture medium; optimum pH was 6.5 for biohydrogenation and 7.0 for CLA production.

Keywords: rumen fungi, biohydrogenation, linoleic acid, CLA

Changes in diets offered to ruminants can have profound effects on rumen fermentation and yield of rumen microorganisms. The balance of substrates available for fermentation determines the balance of species in the microbial population and overall level of microbial activity. In addition to substrate supply, associated changes in rumen environmental factors, such as pH, microbial growth factors and microbial inhibitors, have differential effects on individual microbial species (Hungate, 1966; Church, 1969).

Diets rich in forage such as hay and silage, with a long rumen residence time, encourage high population densities of fungi (Grenet *et al.*, 1989a; Fonty and Grenet, 1994) except when hay is finely milled and fed as pellets (Bauchop, 1979) or supplemented with soluble-carbohydrate sources. In contrast, diets rich in soluble-carbohydrates, such as young pasture, whey and fodder beet, result in a relatively low population density of rumen fungi (Grenet *et al.*, 1989b; Fonty and Grenet, 1994). Concentrates added to an all hay diet also reduced fungal concentrations (Obispo and Dehority, 1992). The explanation for these effects is that long forage provides substrates for fungal growth, but low rumen pH induced by rapid fermentation of soluble carbohydrates inhibits fungal growth.

The normal range of rumen pH is between 5.5 and 7.0 and outer limits for rumen pH lie between 4.5 and 7.5 (Dohority, 2003). Fungal growth is influenced by rumen pH; maximum development of fungal flagellates occurs at pH 6.5 (Orpin, 1976); fungal growth and zoospore production are reduced when pH is below 6.5 (Orpin, 1977).

Polyunsaturated fatty acids are toxic to rumen microorganisms. To protect themselves against these toxic effects, rumen microorganisms have evolved mechanisms to hydrolyse and biohydrogenate dietary lipids. Biohydrogenation is particularly important for diets containing a high proportion of forage because forage lipids have a high proportion of polyunsaturated fatty acids. Many strains of forage-digesting (cellulolytic) bacteria have been shown to be capable of biohydrogenation (see review by Harfoot and Hazlewood, 1997). We have recently shown that rumen fungi also can biohydrogenate long-chain fatty acids, although the rate of biohydrogenation is considerably slower than that for bacteria (Nam and Garnsworthy, 2007).

It is well documented that rumen pH, soluble carbohydrates and fatty acids affect the rate and extent of biohydrogenation by rumen bacteria (Henderson, 1973; Martin and Jenkins, 2002; Troegler-Meynadier *et al.*, 2003, 2006). Because nutrient requirements and optimum environmental conditions vary between rumen bacteria and fungi, effects of pH and nutrient sources on fungal biohydrogenation activity cannot be predicted. Therefore, the objective of this study was to evaluate the effects of soluble carbohydrates, pH, and rumen microbial growth factors and inhibitors [volatile fatty acids (VFA) and vitamins] on biohydrogenation of linoleic acid by mixed rumen fungi. Of particular interest was the rate of production of *cis*-9, *trans*-11 conjugated linoleic acid (CLA), a biohydrogenation intermediate that has potential benefits for human health (Parodi, 2004).

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Materials and Methods

Animals, feeding and rumen fluid

Two non-lactating Holstein-Friesian cows, fitted with rumen cannulae, were used as a source of rumen fluid. Each cow consumed a daily allowance of 3 kg DM grass silage and 2.7 kg DM concentrate (91.5% wheat, 2.5% molasses, 0.5% soya oil, 5.5% minerals and vitamins) in two equal meals, with free access to water. Rumen fluid was collected anaerobically via rumen cannulae, using a 1 cm internal diameter polyethylene pipe connected to a vacuum pump.

Culture conditions

All cultures were grown anaerobically under O_2-free CO_2. Twelve ml tubes at 39°C were used for maintenance and subculturing of fungal cultures, as described by Joblin (1981). Rumen fluid was homogenised (30 s) under O_2-free CO_2 to separate rumen fungi from the surface of feed particles and filtered through two layers of cheesecloth. Aliquots of 1 ml filtered rumen fluid were transferred into 12 ml tubes containing 9 m of autoclaved (121°C, 20 min) Hay Sloppy (HS) medium (Nam and Garnsworthy, 2007; Table 1) with 0.5 ml antibiotic solution (2×10^4 IU/ml of benzylpenicillin and 2 mg/ml of streptomycin sulphate, Sigma, UK). HS medium was modified from Bauchop (1979) by the replacement of cellulose with grass hay, as described by Nam and Garnsworthy (2007). Each culture was incubated at 39°C for 3 days, and then 10% of cultured sample was transferred to fresh HS medium without antibiotic solution. This transfer procedure was repeated every three days throughout the study to maintain cultures. Absence of bacteria and protozoa was confirmed by microscopy. In our previous study (Nam and Garnsworthy, 2007), which used the same culture methods, absence of bacteria was confirmed also by examination of genomic DNA.

Linoleic acid (LA) solution for *in vitro* biohydrogenation tests was prepared by the method of Kim *et al.* (2000).

Table 1. Composition (per litre) of Hay Sloppy medium used for culture of rumen fungi

Component	Hay Sloppy medium
Solution A[1], ml	165
Solution B[2], ml	165
Rumen fluid[3], ml	170
Resazurin[4], ml	2
$NaHCO_3$, g	5
Yeast extract, g	2
Peptone, g	2
Cys-HCl.H_2O, g	0.2
Agar, g	1
Hay[5], g	3
H_2O[6], ml	≈485

[1] Solution A contained (g/ L ultrapure water): KH_2PO_4, 3; NaCl, 6; $(NH_4)_2SO_4$, 3; $CaCl_2$, 0.3; and $MgSO_4$, 0.3.
[2] Solution B contained 3 g K_2HPO_4 /L ultrapure water.
[3] Rumen fluid was filtered through 4 layers of cheese cloth, centrifuged for 30 min at 11,000 *g*, autoclaved at 125°C for 15 min, centrifuged again for 30 min at 11,000 *g*, gently bubbled with O_2 free-CO_2 gas, and stored at -80°C until used.
[4] 0.1 % resazurin solution
[5] Field-cured grass hay (mostly *Lolium perenne, Lolium multiflorum* and *Phleum pretense*); oven-dried, then ground using a blender for 5 min.
[6] Ultrapure water with 0.2 micron filtration (Purite Ltd, Oxford, UK); sufficient to make up to 1 L

Culturing with soluble carbohydrates

Solutions of glucose or cellobiose (3 g/L) were prepared in ultra-pure water (Purite Ltd, UK) and sterilely filtered (pore size, 0.25 μm, Millipore). These solutions were added anaerobically to autoclaved HS medium. One milliliter of HS medium, containing mixed rumen fungi, was anaerobically added to 9 ml of medium containing 3 g/L of glucose or cellobiose in 12 ml tubes. The tubes were pre-incubated at 39°C for 24 h. After pre-incubation, 0.1 ml LA stock solution was added anaerobically to produce a LA concentration of 700 μM. The final pH of the culture medium was pH 6.5. Tubes were then incubated for 1, 3, 6, 9, 12, 24, 48, and 72 h. The biohydrogenation reaction was stopped by putting tubes into ice water and tube contents were stored at -80°C until analysis for fatty acid contents.

Culturing with VFA and vitamins

A VFA solution was prepared containing 6.85 ml acetate, 3 ml propionate, 1.48 ml butyrate, 0.55 ml isovalerate, 0.47 ml isobutyrate, 0.55 ml DL-α-methylbutyrate and 0.55 ml n-valerate in 700 ml of 0.2 M NaOH, adjusted to pH 7.0 with 1 M NaOH, and made up to 1 L with ultra-pure water. A vitamin solution was prepared containing 0.2 g pyridoxine HCl, 0.2 g riboflavin, 0.2 g thiamin HCl, 0.01 g para-aminobenzoic acid and 0.1 ml stock solution (125 mg folic acid, 125 mg biotin and 12.5 mg cobalamin in 25 ml ultra-pure water) in 700 ml ultra-pure water, adjusted to pH 7.0 with 1 M NaOH and made up to 1 L with ultra-pure water. VFA and vitamin solutions were filtered through a Millipore filter (pore size, 0.25 μm). One milliliter of HS medium, containing mixed rumen fungi, was transferred anaerobically to 9 ml of medium containing 1 ml of VFA or vitamin solution in 12 ml tubes. The tubes were then pre-incubated at 39°C for 24 h. After pre-incubation, 0.1 ml LA stock solution was added anaerobically to produce a LA concentration of 700 μM. The final pH of culture medium was pH 6.5. Tubes were then incubated for 1, 3, 6, 9, 12, 24, 48, and 72 h. The biohydrogenation reaction was stopped by putting tubes into ice water and tube contents were stored at -80°C until analysis for fatty acid contents.

Culturing with pH-adjusted HS medium

After bubbling with O_2-free CO_2 gas, pH was adjusted with 7 N HCl to 6.0, 6.5 or 7.0. Aliquots (9.5 ml) of pH-adjusted media were dispensed into 12 ml tubes that were then capped with butyl rubber stoppers and seals. Tubes were then autoclaved at 121°C, for 20 min. 0.5 ml of HS medium, containing mixed rumen fungi, was transferred anaerobically to 9.5 ml of pH-adjusted HS medium in 12 ml tubes. The tubes were then pre-incubated at 39°C for 24 h. After pre-incubation, 0.1 ml LA stock solution was added anaerobically to produce a LA concentration of 700 μM. Tubes were then incubated for 1, 3, 6, 9, 12, 24, 48, and 72 h. The biohydrogenation

reaction was stopped by putting tubes into ice water and tube contents were stored at -80°C until analysis for fatty acid contents.

Fatty acid analysis

Fatty acids were extracted from culture fluid using the hexane-isopropanol method of Hara and Radin (1978). Methyl esters of fatty acids (FAME) were prepared by the method of Christie (1982), as modified by Chouinard *et al.* (1999). FAME were quantified using a gas chromatograph (6890; Agilent Technologies, Stockport, UK) equipped with a flame-ionization detector (7673 FID), autosampler (7683), automatic injector (7683), split injection port and a 100 m fused silica capillary column (i.d., 0.25 mm) coated with 0.2 μm film of cyanopropylpolysiloxane (CP-SIL 88; Varian). Hydrogen was used as the carrier (2.1 ml/min) and fuel gas (32 ml/min). All gases were passed through 7 μm inline filters (Nupro Co., Willoughby, OH). Injector temperature was 225°C and detector temperature was 255°C. Injection volume was 2 μl, with a split ratio of 1:100. Column temperature was held at 70°C for 4 min post-injection, increased to 110°C (8°C/min), increased to 170°C (5°C/min), held at 170°C for 10 min, raised to 225°C (4°C/min), raised to 240°C (20°C/min), and held for 5 min. Total run time was 50.5 min. Heptadecanoic acid (Sigma, UK) was used as an internal standard. Peaks were identified using pure methyl ester standards (FIM-FAME-7 mixture; Matreya Inc., Pleasant Gap, USA; and CLA standard, Sigma, UK).

Statistical analysis

Mean values and standard deviation of the mean are shown. All incubations were performed in triplicate.

Results

Effects of soluble carbohydrates on biohydrogenation

Both glucose and cellobiose inhibited biohydrogenation compared with the control, although biohydrogenation patterns for glucose and cellobiose cultures were similar. Less than 20% of LA was biohydrogenated when rumen fungi were incubated with glucose or cellobiose for 12 h (Fig. 1A); after 24 h incubation, only 35% of LA had been converted to CLA or *trans*-11 vaccenic acid (VA), but 100% had been converted in the control culture (Fig. 1B and C). Moreover, only 60% of LA had disappeared, in the glucose and cellobiose treated cultures, after 72 h of incubation. CLA started to accumulate from 1 h incubation and a small concentration of VA was detected from the beginning of incubation. LA was converted to VA via CLA, but the conversion rate was higher for VA than for CLA. There was no significant effect of carbohydrates on LA reduction at 1 h incubation. From 3 h incubation, significant decreases ($p<0.05$) in LA were measured in control culture. This continued until the end of incubation (72 h). CLA production was significantly higher ($p<0.05$) for control culture by 12 h. From 24 h incubation, glucose and cellobiose supplemented cultures showed higher ($p<0.05$) CLA production until the end of incubation. VA production was higher ($p<0.05$) for control culture until the end of incubation. There was no significant difference ($p>0.05$) between glucose and cellobiose supplemented cultures for LA, CLA, and VA concentrations. Even though fungal biohydrogenation was inhibited by glucose and cellobiose supplementation, VA was the major end-product in this study. Concentration of stearic acid (SA), the end-product of biohydrogenation by mixed rumen fungi, varied between 0.5 and 1.5% of total fatty acids in treatment and blank cultures. CLA and VA concentrations were still increasing at 72 h and might have reached higher values if incubation had been continued.

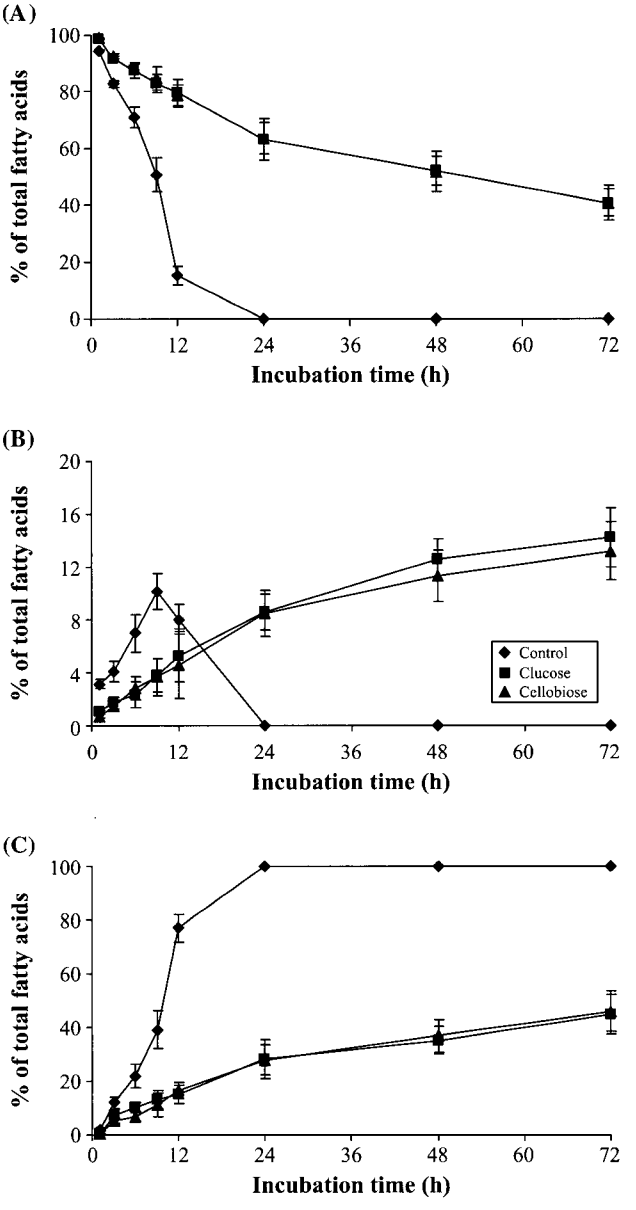

Fig. 1. Biohydrogenation of linoleic acid by mixed rumen fungi grown in 12 ml tubes with or without soluble carbohydrate (glucose and cellobiose) supplemented HS medium. (A) LA (B) CLA (C) VA ◆ Control (no added glucose or cellobiose), ■ Glucose, ▲ Cellobiose (3 g/L).

Effects of VFA and vitamins on biohydrogenation

Addition of VFA or vitamins to culture media did not affect

the pattern or extent of biohydrogenation (Fig. 2). All cultures completely converted LA within 24 h incubation. CLA was detected in all cultures until the concentration of linoleic acid was low. VA increased immediately after CLA concentration started to increase.

Effects of pH on biohydrogenation

When LA was added to pH 6.0 HS medium culture, biohydrogenation was slower than for the control (pH 6.5) or pH 7.0. At pH 7.0, less then 20% LA was found after 24 h incubation. At pH 6.5, LA was slowly converted to CLA and VA over 48 h. However, no LA was found in control culture after 24 h incubation (Fig. 3). CLA was detected in all cultures from 1 h incubation until the concentration of

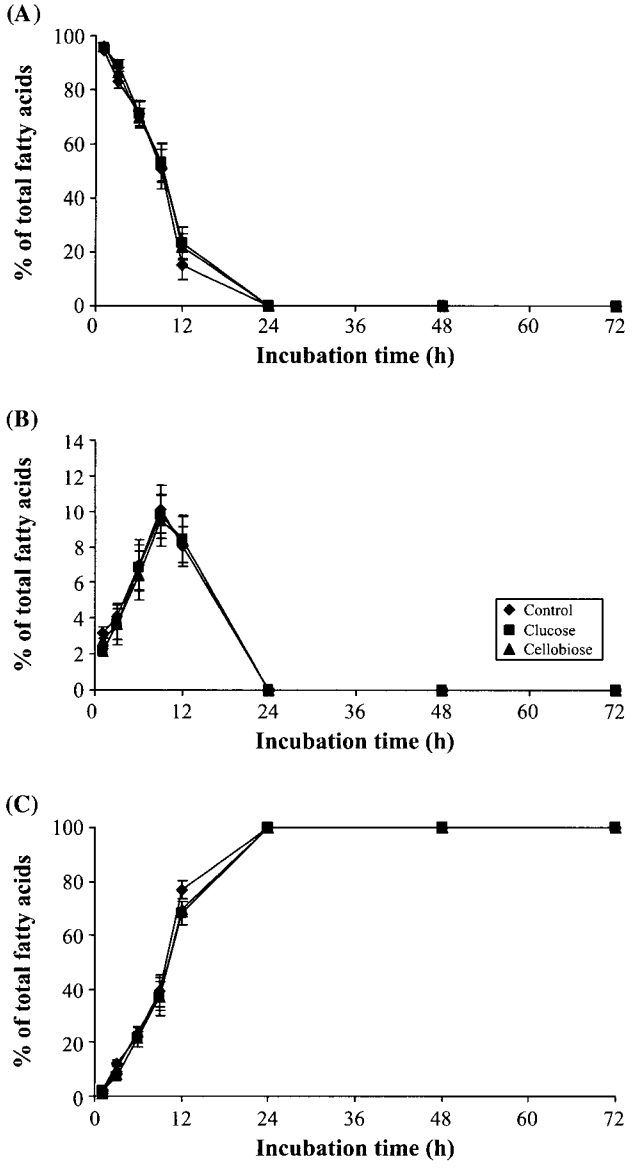

Fig. 2. Effect of VFA solution or vitamin supplementation on biohydrogenation of linoleic acid by mixed rumen fungi grown in 12 ml tubes. (A) LA (B) CLA (C) VA, ◆ Control (no added VFA or vitamin), ■ VFA mixture, ▲ Vitamin solution (1 ml per 100 ml).

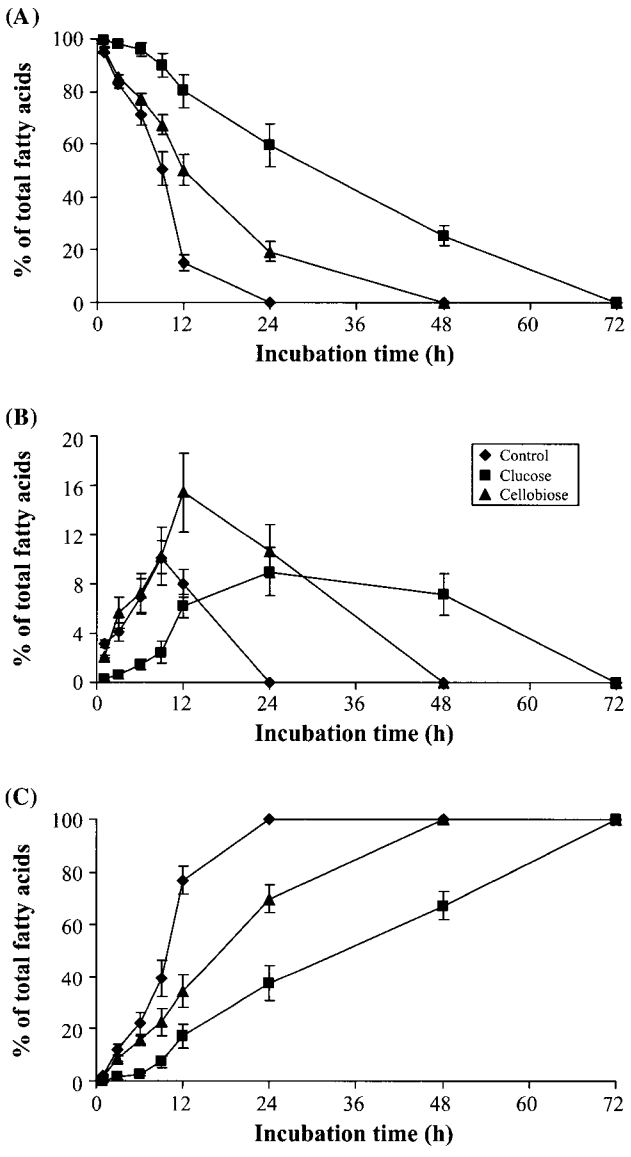

Fig. 3. Effect of pH of HS medium on biohydrogenation of linoleic acid by mixed rumen fungi grown in 12 ml tubes. (A) LA (B) CLA (C) VA ◆ pH 6.5, ■ pH 6.0, ▲ pH 7.0.

LA was low. CLA production at pH 6.0, however, was lower than at pH 6.5 or pH 7.0. A significant reduction ($p<0.05$) in LA was observed at 1 h and 3 h incubation in both pH 6.5 and pH 7.0 cultures. After 6 h, however, LA concentration in the pH 6.5 culture was significantly reduced ($p<0.05$). Highest CLA production ($p<0.05$) was found after 12 h incubation in pH 7.0 culture. There was a significant effect of pH on VA production at the beginning of incubation (1 h) but, after 3 h incubation, VA production was always lower ($p<0.05$) at pH 6.0 than at pH 6.5 or pH 7.0 until the end of incubation. VA was significantly higher ($p<0.05$) at pH 6.5 than at pH 7.0 for every incubation time after 3 h incubation. Biohydrogenation was completely finished within 24 h at pH 6.5, 48 h at pH 7.0 and 72 h at pH 6.0.

Discussion

The rate and products of biohydrogenation in control cultures were similar to those observed in our previous study (Nam and Garnsworthy, 2007); biohydrogenation was finished within 24 h, with VA as the end product. The small amount of SA present in all cultures originated from the rumen fluid contained in HS medium. Bauchop (1979) and Orpin (1976) routinely used media with glucose or cellobiose concentrations of 2-3 g/L. In the current study we used a concentration of 3 g/L for glucose and cellobiose cultures, compared with zero for control, to determine if soluble-carbohydrate sources affect fungal biohydrogenation. Mountfort and Asher (1983) reported utilization of soluble-carbohydrate by rumen fungi. Glucose was the preferred substrate when compared with fructose and xylose, and cellobiose was preferentially utilized compared with fructose. When a culture medium was supplemented with glucose, fungal zoospores rapidly developed (Mountfort and Asher, 1983). It is possible that biohydrogenation capacity is reduced during the zoospore-production stage of the fungal life cycle. The major products of cellobiose fermentation by rumen fungi are lactate, acetate, hydrogen and carbon dioxide (Orpin and Munn, 1986). It is possible that accumulation of one or more of these products might inhibit biohydrogenation. CLA production and biohydrogenation were inhibited by supplementing with glucose or cellobiose compared with control conditions at 9 h of incubation (the time of highest CLA production in control conditions). To explain how glucose or cellobiose affects CLA production and biohydrogenation, a continuous-culture *in vitro* system with pH control could be useful.

Some vitamins and VFAs have been evaluated as essential growth factors for rumen microorganisms. Supplementation with vitamin solution increased enzyme activity in rumen bacteria, produced higher propionic acid and increased the efficiency of microbial synthesis in *in vitro* incubation with different substrates (Schussler *et al.*, 1978; Shields *et al.*, 1983). Iso-acids or four-five carbon VFAs are required to stimulate growth of fibrolytic microorganisms (Bryant, 1973). Supplementation of mixed cultures with VFAs did not affect fibre digestibility or microbial protein production, but VFAs could influence intermediary metabolism (Andries *et al.*, 1987). No information has been reported for effects of VFA on rumen fungi. Hence, we hypothesised that increasing the supply of growth factors (vitamins and VFA) above concentrations found in the control culture might affect fungal biohydrogenation. The reasoning behind this hypothesis is that although rumen fluid was a component of HS medium, thus supplying some VFA and vitamins, it was diluted to 170 ml/L. Therefore, concentrations of VFA and vitamins in HS medium were only 17% of those found in rumen fluid. Furthermore, it was likely that some vitamins were denatured by autoclaving. Addition of VFA solution was calculated to increase molar concentrations of VFA in HS medium by 50 to 60%; concentrations of vitamins in the rumen fluid were not measured, but vitamins were added at a level that has produced responses in rumen bacteria (Schussler *et al.*, 1978; Shields *et al.*, 1983). The results indicated that neither additional vitamins nor additional VFA affected fungal biohydrogenation under the conditions used in this study, probably because these growth factors were not limiting in the control medium. We know that some growth factors provided by rumen fluid are essential for fungal growth, however, because no growth occurred in cultures without rumen fluid. Further work is required to identify limiting concentrations of vitamins and VFA.

Martin and Jenkins (2002) studied the effects of pH on biohydrogenation of LA and linolenic acid (LE), and production of intermediates by rumen bacteria. Their results indicated that high pH (6.7) in media produced more intermediates than low pH (5.5). Choi *et al.* (2005) also observed a tendency for *cis*-9, *trans*-11 CLA concentration to be higher at high pH than at low pH, but *trans*-10, *cis*-12 CLA was higher at low pH. Our results show that biohydrogenation of LA at high pH (pH 7.0) increased CLA (*cis*-9, *trans*-11) production by fungi and low pH (pH 6.0) inhibited CLA production. Troegeler-Meynadier *et al.* (2003) reported that high pH (over 6.5) and low amounts of fatty acids (less than 1.0 mg fatty acids per milliliter of rumen contents) should produce the highest concentrations of intermediates such as CLA and VA by rumen bacteria. In a later report (Troegeler-Meynadier *et al.*, 2006), these authors calculated that low pH reduced efficiency of biohydrogenation mainly by inhibition of isomerisation, which is the first step in the biohydrogenation pathway. Martin and Jenkins (2002) reported higher LA reduction by mixed rumen bacteria at pH 6.7 than at pH 5.5; the same effect as in the current study of fungal biohydrogenation. In the current study, however, biohydrogenation time at pH 7.0 was extended compared with pH 6.5. Orpin (1976) found maximum fungal activity occurred at pH 6.5 and 39°C, in the presence of CO_2 gas. Optimum pH for isomerase activiy in *Butyrivibrio fibrisolvens* was found to be pH 7.0-7.2 (Kepler and Tove, 1967; Hughes *et al.*, 1982). In the current study, one of the reasons why high pH (7.0) produced the highest CLA could be greater activity of isomerase.

It is concluded that under the conditions examined in this study, optimum biohydrogenation of LA and for CLA production by rumen fungi was in cultures without addition of soluble carbohydrates, VFA or vitamins to the culture medium; optimum pH was 6.5 for biohydrogenation and 7.0 for CLA production.

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