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Effect of different plant extracts, fatty acid and oils on conjugated linoleic acid (CLA) production by *Butyrivibrio fibrisolvens*

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ABSTRACT

Present study evaluated the effect of added free fatty acids, dietary oils and plant extracts on conjugated linoleic acid (CLA) production potential of anaerobic *Butyrivibrio fibrisolvens* bacteria. Different concentration of plant extracts of *Solanum nigrum, Rumex dentatus*, *Boerhaavia diffusa* Linn.*, Amaranthus nlitum* Linn.*, Peristrophe bicalyculata, Phyllanthus* sp. *Leucas aspera* and *Cuminum cyminum* (0.50, 1.0, 1.5%, 2.0, 4.0, 6.0, 8 and 10 mg/ml of media); pure linoleic and linolenic acid (50, 100, 150, 200, 250 µg/ml of media) and dietary oils (50, 100, 150, 200, 250, 300 µg/ml of media) were used in media. The results revealed that an increase in the concentration of fatty acids suppressed growth of *B. fibrisolvens* and its cell density reached maximum (1.58 OD; 600 nm) at 18 h of incubation. A gradual rise in CLA production by *B. fibrisolvens* was observed parallel to increasing concentration of dietary oils from 50 to 250 µg/ml followed with slight decrease at level of 300 µg. Supplementation of sunflower oil resulted in highest increment in CLA production among the treatments. Extracts of different plants at different concentrations showed significant changes in CLA production potential by *B. fibrisolvens*. *Cuminum cyminum* with 457% increase in CLA concentration was the most efficient extract. Study established that careful optimization of dietary supplementation results in increased activity of *B. fibrisolvens* thereby facilitating higher CLA production.

Key words: *Butyrivibrio fibrisolvens*, Conjugated linoleic acid, Free fatty acids, Plant extracts

Enhancement of conjugated linoleic acid (CLA) content and reduction of saturated fatty acid (SFA) levels, recommended by EFSA (2010), are 2 crucial approaches for designing animal products having numerous potential humane health benefits, including potent cancer-fighting properties, anti-inflammatory, anti-diabetic, antiatheroschlerotic and immuno-modulatory effects (Whigham *et al*. 2000, Roche *et al.* 2001). CLA is an intermediate product of rumen bacterial bio-hydrogenation of dietary lipids (Roche *et al.* 2001). Diverse bacteria relating to rumen bio-hydrogenation were isolated on them; *B. fibrisolvens* was proved to be the most active bacteria involved in the production of CLA (Kepler *et al*. 1966, Wang *et al*. 2002). Inclusion of diet rich in linoleic acid ($C_{18:2,}$ LA) and linolenic acid ($C_{18:3,}$ LNA) in ruminant is one of the effective feeding strategies for increasing CLA production (Dhiman *et al*. 2005). Apart from LA and LNA rich sources, studies have demonstrated potential application of plant extract on manipulation of rumen bio-hydrogenation (Lourenco *et al*. 2008, Vasta *et al*. 2010, Rana *et al*. 2011). Plant extracts rich in polyphenols or tannins favours the synthesis of CLA by altering rumen bio-hydrogenation (Min *et al.* 2005, Durmic *et al.* 2008). The cumulative effect of increased availability of poly unsaturated fatty acids (PUFAs) as precursors and plant extracts as supplements, not withstanding their negative effect on rumen bacterial growth, remains to be explored for effect on CLA production in ruminants. Understanding these effects could have important indirect implications for human health by enabling rumen bio-hydrogenation of dietary PUFA.

In view of the above, the study amied to analyze the effect of pure free fatty acids, dietary oils and plant extracts supplementation on CLA production *in vitro*.

MATERIALS AND METHODS

Plant extracts preparation: In present study, effects of different plant extracts, viz. *Solanum nigrum, Rumex dentatus*, *Boerhaavia diffusa* Linn.*, Amaranthus nlitum* Linn., *Peristrophe bicalyculata, Phyllanthus* sp.*, Leucas aspera* and *Cuminum cyminum* were used to see the effect on CLA producing potential of *B. fibrisolvens.* The collected plant material was botanically identified. The species

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conformation was engaged at National Botanical Research Institute, Lucknow, India. The collected materials were shade dried and coarsely powdered using a pulverizer. The coarse powder was subjected to extraction with ethanol (95%) at room temperature by Soxhlet method for 3 days. The collected extracts were filtered and were concentrated under reduced pressure using a rotary vacuum evaporator. Finally, the concentrated extract was lyophilized by using freeze dry system.

Direct fatty acid methyl ester (FAME) synthesis: Different dietary oils (sunflower oil, soybean oil, mustard oil) were subjected to fatty acid analysis. Forty µl of each sample was taken in a tube to which 0.7 ml of 10 N KOH, and 5.3 ml of methanol were added. The tube was incubated in a 55°C shaking water bath for 1.5 h to properly permeate, dissolve, and hydrolyze the sample. FAME was synthesized by adding 0.58 ml of 24 N H_2SO_4 followed with incubation in water bath at 55°C for 1.5 h. After FAME synthesis, the tube was cooled in a cold tap water bath. Three milliliters of hexane was added, and the tube was vortex-mixed for 5 min followed with centrifugation for 5 min at 2,000 rpm. The solvent was evaporated from FAME by flushing of nitrogen. The vial was capped and stored at –20°C until GC analysis. Dietary oils (soybean oil, sun flower oil and mustard oil) purchased from local markets were methylated for estimation of their fatty acid composition (Sukhija and Palmquist 1988).

Fatty acid composition: The fatty acid composition of the FAME was determined by capillary GC on a BPX-70, 60 m × 0.25 mm × 0.25 µm capillary column installed on a gas chromatography equipped with a flame ionization detector and split injection. The initial oven temperature was 70°C which was increased to 120°C immediately after injection. The injector temperature was 220°C while detector temperature was 230°C. After attaining 120°C, 2°C/min increase program was set until 230°C. For CLA estimation injector temperature was 240°C while detector temperature was set to 260°C, initial oven temperature was 150°C which was increased to 240°C at 2°C/min. Helium was used as the carrier gas at a flow rate of 30 ml/min, and the column head pressure was 280 kPa. Fatty acids were identified by comparing their retention times with the fatty acid methyl standards. The identification of peaks was based on commercial standard mixtures.

Isolation and identification of B. fibrisolvens*:* The modified anaerobic technique was used to isolate *B. fibrisolvens*. Rumen liquor was collected from crossbred (Alpine × Beetal) early lactating goats before feeding in a thermos flask preflushed with carbon dioxide using a pipe and strained through four layers of muslin cloth. Crossbred goats were maintained on concentrate and forage (50:50). Collected rumen liquor was centrifuged at 2,000×g for 10 min at 4ºC to remove the feed particles, protozoa, fungi and bacteria. The clear supernatant was used as a part of media. Rumen liquor (without pre-treatment) was inoculated into the media and incubated at 39°C for 48 h. Colonies based on morphology (translucent, creamy and light tan filamentous) were picked up after plating rumen liquor on *B. fibrisolvens* selective ATCC media. Gram's staining was followed up with molecular confirmation with PCR. A 505 bp product confirmed *B. fibrisolvens* sp. The genus and species identity of isolates as *B. fibrisolvens* was confirmed by PCR using 16s rRNA sequence specific primers.

In-vitro *experiment: B. fibrisolvens* was grown on ATCC selective media (10 ml) with strained and centrifuged rumen liquor. Pure free fatty acids, oils and plant extracts were dissolved in distilled water by sonication. Varying concentrations of pure LA, LNA (50, 100, 150, 200, 250 µg/ml of media), dietary oils (50, 100, 150, 200, 250, 300 µg/ml of media), extract of plants (0.50, 1.0, 1.5%, 2.0, 4.0, 6.0, 8 and 10 mg/ml of media) were added to the medium before dispensing and autoclaving. Media without added free fatty acids, oils and plant extracts was used as a control. Percentage change value of each sample at different incubation period i.e. 0, 8, 12, 18, 24, 48 and 72 h was compared with control. Screening of bacterial growth and CLA production was performed at different incubation periods. CLA production at different concentrations was estimated by FAME analysis.

Bacterial growth: Growth of bacteria was monitored via changes in optical density (600 nm) at different period of time (0, 8, 12, 18, 24 h). The growth rate was estimated from differences in natural logarithms of optical density in triplicates.

RESULTS AND DISCUSSION

Pure isolates based on colonial morphology (translucent, creamy and light tan filamentous) were picked up from a number of colonies after plating rumen liquor (anaerobic diluent) collected from cattle, buffalo, goat and sheep. The isolates were pink, colored Gram-positive (Sometimes though appeared as Gram-negative), single and short chain rods of varying size under microscope using oil immersion objective technique. These characteristics of the isolates matched with that of *Butyrivibrio* spp (Holt *et al*. 1994). Identification of genus and species comprising a number of morphological, physiological, biochemical and molecular tests were done to confirm the isolates as *Butyrivibrio fibrisolvens* (data not shown here).

Apparent differences were identified between the mechanisms underlying the synthesis of CLA from LA (Wallace *et al.* 2007), and also in the bacterial species that carry out the reactions. Here we provide analogous information on differences in CLA production with fatty acids (LA and LNA), plant extract and dietary oils supplementation. The experiments of present study were carried out with growing cultures of bacteria rather than cell suspensions because for bio-hydrogenation process active growth of bacteria is required (Wallace *et al.* 2006).

An increase in the concentration of fatty acids suppressed growth of *B. fibrisolvens* and negative effect of longer incubation time was more significant ($P<0.05$). The *B. fibrisolvens* cell density reached maximum at 24 h of incubation. The OD declined to 1.48, 1.38, 1.24, 1.07, 0.68

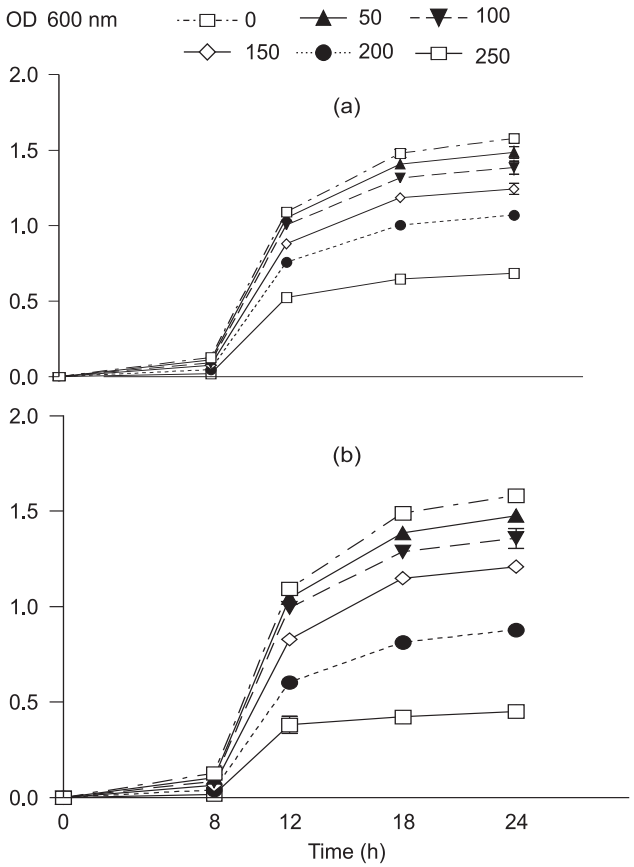

Fig 1. Effect of (a) linolenic acid and (b) linoleic acid concentration (µg/ml of media) on growth of *Butyrivibrio fibrisolvens* (P < 0.05). The decline in growth is more prominent (70%) in LNA at 250µg/ml compared to LA (56%) at the same concentration.

and 1.48, 1.36, 1.21, 0.88, 0.45 with increasing concentration (50, 100, 150, 200, 250 µg/ml) of LA and LNA (Fig. 1). The bacterial growth in present study was recorded lowest from 0 to 8 h during LA and dietary oils supplementation i.e. a prolonged lag phase during bacterial growth in the initial stages, which might be the contributing factor in increased CLA production (Maia *et al*. 2007). Furthermore, metabolism occurs in the exponential phase, but no further transformations occur during the stationary phase. Concentrations of more than 200 mg/l of 18: 2 (LA) and 18:3 (LNA) fatty acids inhibited biohydrogenation (Beam *et al.* 2000, Fievez *et al.* 2007). Such concentrations of LA and LNA would be common in grazing animals (Dawson and Hemington 1974). Different concentrations ranging from 50 mg/l to 300 mg/l were used here. It was also observed that growth of the *B. fibrisolvens* was affected more in LNA than in LA supplemented media. Supplementation of 200 and 250 µg/ml LNA resulted in 13% and 15% more reduction in cell density of *B. fibrisolvens* as compared to LA (P<0.05) (32%, 56% vs. 45% and 71% reduction for LA and LNA, respectively). This may be attributed to more number of double bonds in LNA than in LA as number of double bonds is considered as measure of unsaturated fatty acids toxicity i.e.

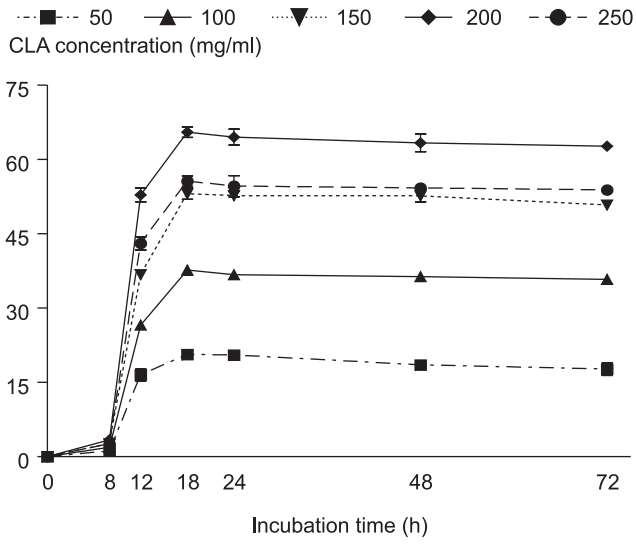

Fig. 2. CLA production by *Butyrivibrio fibrisolvens* inoculated with different concentration of linoleic acid. (P < 0.05). Overall average total CLA production increased significantly after LA supplementation until 200µg/ml concentration but a decline was observed in 250 µg LA supplemented group.

DHA>EPA>LNA>LA. Production of CLA in culture was parallel to the increasing concentration of LA up to 200 µg. However any increment beyond that did not increase CLA concentration in media and even resulted in decline in its production (Fig. 2). No significant changes in CLA production were with addition of 150 and 250 µg of LA.

Fatty acid composition of sunflower, soybean and mustard oil was estimated prior to their supplementation (Table 1). Addition of oils in culture of *B. fibrisolvens* bacteria resulted in higher average total CLA production (Fig 3). An incubation period of 18 h was optimal with respect to CLA production. A gradual rise in CLA production was observed parallel to increasing concentration of dietary oils from 50 to 250 µg/ml followed with slight decrease at level of 300 µg. As the level of supplemented oil increased a decrease in *B. fibrisolvens* bacterial cell density was observed. Higher concentration of supplemented oil at early incubation, resulted in significant reduction in bacterial cell density (P<0.05). At 18 h of incubation, the growth OD was 1.48, 1.38, 1.28, 1.14, 0.98, 0.66 in sunflower oil, 1.48, 1.40, 1.29, 1.15, 1.01, 0.69 in soybean oil and 1.49, 1.41, 1.30, 1.16, 1.02, 0.71 in mustard oil at 50, 100, 150, 200, 250 and 300 µg/ml

Table 1. Fatty acids composition in different dietary oils as a proportion of total fatty acids (mg/100mg of FAME)

Attributes	C18:1	C18:2	C18:3	Total PUFA
Sunflower oil	22.3	65	0.5	87.8
Soybean oil	19.5	53.2	9.1	81.8
Mustard oil	37.2	36.4	13.4	87.0
Berseem	21.58	14.62	0.58	36.78
Concentrate mixture	10.81	30.45	0.46	41.72

Number of replicate for each attributes, 3.

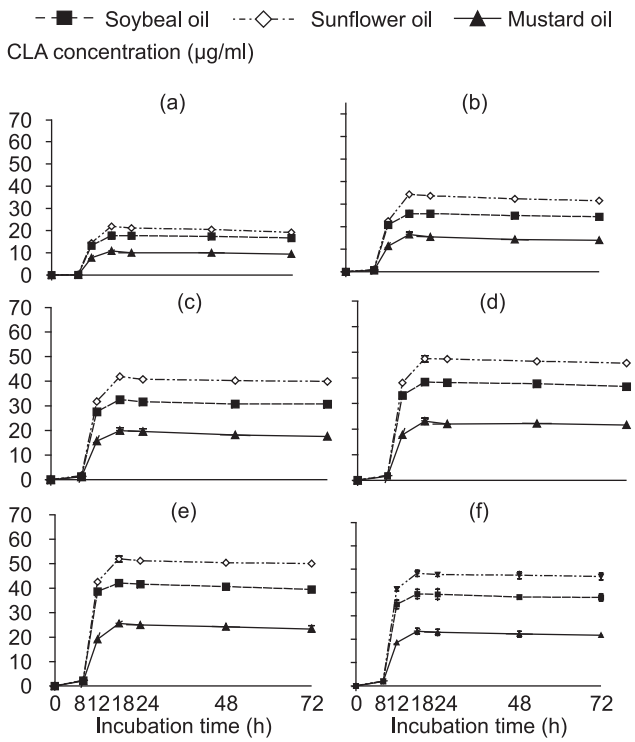

Fig. 3. CLA production of *Butyrivibrio fibrisolvens* at presence of (a) 50, (b) 100, (c) 150, (d) 200, (e) 250, (f) 300 µg/ml for 3 dietary oils. (P < 0.05). CLA production reached its peak after 18 h of incubation at every concentration.

concentration of oils respectively. Proportionately, highest drop in *B. fibrisolvens* growth was observed beyond 250 µg/ml of oil supplementation. Supplementation of sunflower oil resulted in highest percentage increment in CLA production among the treatments (Fig. 3). Mustard oil was least efficient in promoting CLA production (P<0.001).

Increased CLA accumulation at inhibitory conditions for bacterial growth (high LA) may also be due to conversion of LA to CLA by isomerase enzyme, which is membrane bound and is active even after lysis or death of bacteria as these conditions are less effective on the isomerization step than reduction step during biohydrogenation (Kim *et al.* 2002). Supplementation with sunflower oil revealed highest percentage increment in CLA production among the treatments and mustard oil was least efficient to promote CLA production. Also CLA production during treatments was positively correlated with the proportion of LA in oils and negatively related to ratio of LNA: LA in oil as LNA has more negative effect on the growth of bacteria.

Screening of plant extracts was carried out to evaluate their effect on CLA producing ability of *B. fibrisolvens* isolate. Extracts of *Solanum nigrum, Rumex dentatus*, *Boerhaavia diffusa* Linn., *Amaranthus nlitum* Linn., *Peristrophe bicalyculata, Phyllanthus* sps. *Leucas aspera* and *Cuminum cyminum* showed significant (P≤0.001) changes in CLA production at different concentrations (Fig. 4). *Cuminum cyminum* with 457% increase in CLA concentration was the most efficient extract followed by *Leucas aspera* plant extract as compare to control.

In some previous studies plant extracts have been successfully used to modify rumen fermentation (Hart *et al*. 2008, Alexander *et al*. 2008, Busquet *et al.* 2006, Grainger *et al.* 2009). Some of the plants used in the present study contain FAs such as LA, LNA and presence of these FAs in the aqueous methanolic extract is likely as they are soluble in methanol and provided additional substrate for biohydrogenation. Inclusion of *C. cyminum* extract increased CLA content and accumulated higher VA, act as the modulator of biohydrogenation pathway. High concentration of LNA in the extract caused accumulation of CLA and VA in strained rumen fliud *in vitro* (Wood *et al.* 2010). *C. cyminum* contains essential oil compounds, tannins and saponins that have potential in controlling the biohydrogenation (Durmic *et al*. 2008, Lourenco *et al.* 2008, Vasta 2008, Khiaosa *et al.* 2009). Supplementation with *Leucas aspera* extract resulted in increase in CLA production, which may be due to the effect of the bioactive compounds, including polyphenols, on CLA producing bacteria (Rana *et al.* 2011).

Present study established that optimized dietary supplements increased the efficiency of *B. fibrisolvens* for

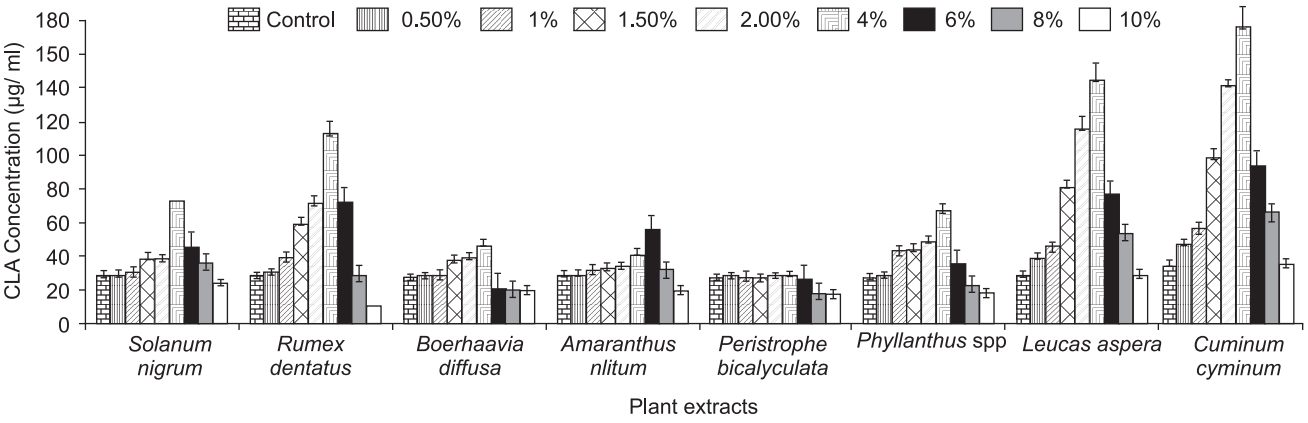

Fig. 4. Effect of weed extract supplementation on CLA production potential of highest CLA producing *Butyrivibrio fibrisolvens* isolate.

the higher CLA production. Supplementation with free fatty acids, dietary oils and plant extracts altered CLA production potential of *B. fibrisolvens*. This is a preliminary study and further investigations are required to check the utility of these plant extracts *in-vivo* to increase the activity of CLA producing bacteria.

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REFERENCES

Alexander G, Singh B, Sahoo A and Bhat T K. 2008. *In vitro* screening of plant extracts to enhance the efficiency of utilization of energy and nitrogen in ruminant diets. *Animal Feed Science and Technology* **145**: 229–44.

Beam T M, Jenkins T C, Moate P J, Kohn R A and Palmquist D L. 2000. Effects of amount and source of fat on the rates of lipolysis and biohydrogenation of fatty acids in ruminal contents. *Journal of Dairy Science* **83**: 2564–73.

Busquet M, Calsamiglia S, Ferret A and Kamel C. 2006. Plant extracts affect *in vitro* rumen microbial fermentation. *Journal of Dairy Science* **89**: 761–71.

Dawson R M and Hemington N. 1974. Digestion of grass lipids and pigments in the sheep rumen. *British Journal of Nutrition* **32**: 327–40.

Dhiman T R, Nam S H and Ure A L. 2005. Factors affecting conjugated linoleic acid content in milk and meat. *Critical Reviews in Food Science and Nutrition* **45**: 463–82.

Durmic Z, Mc Sweeney C S, Kemp G W, Hutton P, Wallace R J and Vercoe P E. 2008. Australian plants with potential to inhibit bacteria and processes involved in ruminal biohydrogenation of fatty acids. *Animal Feed Science and Technology* **145**: 271–84.

European Food Safety Authority (EFSA). 2010. Scientific opinion on dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, *trans* fatty acids, and cholesterol. *EFSA Journal* **8**: 1461–67.

Fievez V, Vlaeminck B, Jenkins T, Enjalbert F and Doreau M. 2007. Assessing rumen biohydrogenation and its manipulation *in vivo, in vitro* and *in situ*. *European Journal of Lipid Science and Technology* **109**: 740–56.

Grainger C, Clarke T, Auldist M J, Beauchemin K A, Mc Ginn S M, Waghorn G C and Eckard R J. 2009. Mitigation of greenhouse gas emissions from dairy cows fed pasture and grain through supplementation with *Acacia mearnsii* tannins. *Canadian Journal of Animal Science* **89**: 241–51.

Hart K J, Yanez-Ruiz D R, Duval S M, McEwan N R and Newbold C J. 2008. Plant extracts to manipulate rumen fermentation. *Animal Feed Science and Technology* **147**: 8–35.

Holt J G, Kreig N R, Sneath P H A, Staley J T and William S T. 1994. *Bergey's manual of Determinative Bacteriology*, 9th edn. Williams and Wilkins, Baltimore.

Kepler C R, Hirons K P, McNeil J J and Tove S B. 1966. Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. *Journal of Biological Chemistry* **241**: 1350–54.

Khiaosa-Ard R, Bryner S F, Scheeder M R L, Wettstein H R, Leiber F, Kreuzer M and Soliva C R. 2009. Evidence for the inhibition of the terminal step of ruminal alpha linolenic acid biohydrogenation by condensed tannins. *Journal of Dairy Science* **92**: 177–88.

Kim M R, Park Y, Albright K J. 2002. Differential responses of hamsters and rats fed high fat or low fat diets supplemented with conjugated linoleic acid. *Nutrition Research* **22**: 715–22.

Lourenco M, VanRanst G, Vlaeminck B, De Smet S and Fieve V. 2008. Influence of different dietary forages on the fatty acid composition of rumen digesta as well as ruminant meat and milk. *Animal Feed Science and Technology* **145**: 418–37.

Maia M R G, Chaudhary L C, Fiueres L and Wallace R J. 2007. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. *Antonie van Leeuwenhoek* **91**: 303–14.

Min B R, Attwood G T, Mc Nabb W C, Molan A L and Barry T N. 2005. The effect of condensed tannins from *Lotus corniculatus* on the proteolytic activities and growth of rumen bacteria. *Animal Feed Science and Technology* **121**: 45–58.

Rana M S, Tyagi A, Hossain A and Tyagi A K. 2011. Effect of tanniniferous *Terminalia chebula* extract on rumen biohydrogenation, Ä9-desaturase activity, CLA content and fatty acid composition in *longissimus dorsi* muscle of kids. *Meat Science* **90**: 558–63.

Roche H M, Noone E, Nugent A P and Gibney M J. 2001. Conjugated linoleic acid: a novel therapeutic nutrient. *Nutrition Research Reviews* **14**: 173–87.

Sukhija P S and Palmquist D L. 1988. Rapid method for determination of total fatty acid content and composition of feed stuffs and feces. *Journal of Agricultural and Food Chemistry* **36**: 1202–06.

Vasta V, Makkar H P S, Marcello M and Alessandro P. 2008. Ruminal biohydrogenation as affected by tannins *in vitro*. *British Journal of Nutrition* **102**: 82–92.

Vasta V, Yanez-Ruiz D R, Mele M, Serra A, Luciano G, Lanza M, Biondi L and Priolo A. 2010. Bacterial and protozoal communities and fatty acid profile in the rumen of sheep fed a diet containing added tannins. *Applied and Environmental Microbiology* **76**: 2549–55.

Wallace R J, Chaudhary L C, McKain N, Mc Ewan N R, Richardson A J, Vercoe P E, Walker N D and Paillard D. 2006. *Clostridium proteoclasticum*: a ruminal bacterium that forms stearic acid from linoleic acid. *FEMS Microbiology Letters* **265**: 195–201.

Wallace R J, Mc Kain N, Shingfield K J and Devillard E. 2007. Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. *Journal of Lipid Research* **48**: 2247–54.

Wang J H, Song M K, Son Y S and Chang M B. 2002. Addition effect of seed-associated or free linseed oil on the formation of cis-9, trans–11 conjugated linoleic acid and octadecenoic acid by ruminal bacteria in vitro. *Asian-Australasian Journal of Animal of Science* **15**: 1115–20.

Whigham L D, Cook M E, Atkinson R L. 2000. Conjugated linoleic acid: Implications for human health. *Pharmacological Research* **42**: 503–10.

Wood T A, Ramos-Morales E, Mc Kain N, Shen X, Atasoglu C and Wallace R J. 2010. Chrysanthemum coronarium as a modulator of fatty acid biohydrogenation in the rumen. *Animal Feed Science and Technology* **161**: 28–37.