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Plant extract enhanced ruminal CLA concentration, *in vitro*

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ABSTRACT. The effect of adding plant extracts to ruminal fluid on the formation of conjugated linoleic acid (CLA) isomers and on fermentation parameters was evaluated. Initially, fifteen plant extracts at two levels (1 and 2 mg · ml^{-1} buffered rumen fluid; BRF) were incubated with BRF and substrate (hay and concentrate, 50:50) at 39°C for 24 h, in a completely randomized block design. In the second experiment, four promising plant extracts affecting the formation of CLA and vaccenic acid (VA) were tested for their influence on basal parameters of rumen fermentation. The first study revealed that extracts of *Azadirachta indica, Allium sativum, Cuminum cyminum* at the lower level (1 mg · ml^{-1}) and extracts of *Terminalia chebula* at the higher level (2 mg · ml^{-1}) enhanced CLA isomer formation by 45.56%, 41.54%, 51.09%, and 15.54%, respectively, and the VA concentration by 10.97%, 10.82%, 14.93%, and 29.61%, respectively, in ruminal fluid when compared with the control ($p < 0.05$). The second experiment documented that the selected plant extracts did not impair nutrient fermentation. Nonetheless, addition of *C. cyminum* extract to ruminal fluid increased the partitioning factor ($p = 0.054$) and the digestibility of nutrients ($p < 0.05$). Furthermore, the polyunsaturated fatty acid concentration was higher when ruminal fluid was incubated with the *C. cyminum* extract ($p = 0.049$). In conclusion, extracts of *Azadirachta indica, Allium sativum, Cuminum cyminum* and *Terminalia chebula* modulate ruminal biohydrogenation and increase the concentrations of CLA isomers, their precursors, and VA without negatively affecting other rumen parameters. *Cuminum cyminum* did, however, improve the pattern of nutrient fermentation and can be considered a valuable supplement in ruminant nutrition to enhance the healthiness of ruminant meat and milk.

Introduction

Conjugated linoleic acid (CLA) isomers are positional and geometric isomers of linoleic acid (LA) with conjugated double bonds. They have been shown to reduce the incidence and severity of animal carcinogenesis (Ip et al., 1991). CLA isomers are synthesized in particular by rumen biohydrogenating bacteria *via* isomerization of dietary linoleic acid (LA; *cis9cis12*C18:2). CLA isomers are also formed endogenously in the mammary glands by the action of Δ-9 desaturase on vaccenic acid (VA; *trans11*C18:1), which is another product of biohydrogenation (BH) of LA and linolenic acid (LNA; *cis9cis12cis15*C18:3) in the rumen (Harfoot and Hazelwood, 1997;

Griinari et al., 2000). CLA and other ethenoic isomers occur as intermediates in the process of ruminal biohydrogenation (Harfoot and Hazelwood, 1997).

Polyunsaturated fatty acids (PUFA) are toxic to ruminal bacteria; in fact, BH is a detoxification mechanism (Maia et al., 2010). The microbial ecology of BH is not completely understood, but the most active species are found in the *Butyrivibrio* group. While a large number of species convert LA to CLA isomers and VA, only *Butyrivibrio proteoclasticus*, formerly known as *Clostridium proteoclasticum* (Wallace et al., 2006; Moon et al., 2008), converts VA to stearic acid (SA; C18:0) (Harfoot and Hazlewood, 1997). Recently, however, uncultured bacteria have been shown to be involved in the biohydrogenation of PUFA (Huws et al., 2011).

Ruminant nutritionists are interested in improving the fatty acid profile of the ruminal outflow. This could be achieved either through the optimization of diet formulations or utilization of feed additives that modify the environment, modulate the competition among different microbial populations, enhance or inhibit specific microbial populations, or target biohydrogenating enzyme(s) (Palmquist et al., 2005; Khiaosa-Ard et al., 2009). Different additives such as plant oils and fish oil, individually or in combination with minerals, have been reported to successfully manipulate the fatty acid metabolism in the rumen (Niedźwiedzka et al., 2008; Czauderna et al., 2012; Cieślak et al., 2013). Furthermore, inclusion of plants containing secondary metabolites into diets is an effective strategy for modifying the fatty acid composition of meat and milk (Lourenco et al., 2007a,b; Khiaosa-Ard et al., 2009; Vasta et al., 2009; Wood et al., 2010). Therefore, this experiment was conducted to screen indigenous tropical herbs for their potential to modulate PUFA biohydrogenation while not impairing fermentation in the rumen.

Material and methods

Plants and extracts

The plant species used in this study are shown in Table 1. Fifteen plants chosen on the basis of their known medicinal properties and antimicrobial effects were tested. Air-dried plant parts were purchased from the local market in Karnal, Haryana (India). Aqueous methanolic extracts of each plant were prepared, freeze-dried and kept at 4°C. The yield of each extract was quantified based on initial weight and weight of the freeze-dried extract.

Animals, digesta sampling and experimental diet

Rumen liquor was collected from three fistulated bulls maintained on a roughage based diet (2.0 kg concentrate mixture in equal proportions at 10.00 a.m. and 16.00 p.m. and wheat straw *ad libitum*) before the morning feeding, mixed and strained through two layers of cheesecloth into pre-warmed insulated containers until it was used in the trial within 20 min after collection.

The substrate was a 50:50 (w/w) concentrate: forage diet consisting of 100 mg hay (*Trifolium alexandrinum*), and 100 mg concentrate mixture. The concentrate mixture contained, on a dry matter (DM) basis: deoiled rice bran (11%), mustard cake (12%), maize (33%), wheat bran (20%), ground nut cake (21%), common salt (1%) and mineral mixture (2%).

Incubation procedures

Screening assay for CLA production. Each batch incubator was a 100 ml serum bottle containing 30 ml of buffered rumen fluid (BRF), forage (*Trifolium alexandrinum)* and concentrate (1:1) as the substrate (200 mg), and 0.5 ml of plant extract. Extracts were dissolved in distilled water (to apply the required dose per bottle in 0.5 ml) and unique volume containing 30 or 60 mg extract injected into the serum bottle. Rumen fluid was strained through four layers of cheesecloth into a pre-warmed thermo-flask. Strained ruminal fluid (SRF) was mixed with the buffer solution of Menke and Steingass (1988) at a ratio of 1:2 (v/v) at 39°C under continuous flushing with CO_2. The bottles were incubated with BRF in triplicate. The bottles were closed by a crimped butyl septum and sealed with an aluminum cap. The final concentration of plant extract in each flask was 1 $mg \cdot ml^{-1}$ or 2 $mg \cdot ml^{-1}$. No extract was added to the control. Bottles were kept in an incubator set to 39°C. Gases produced during the incubation were removed through a needle after 3, 6, 9 and 12 h of incubation, and each time the flasks were mixed manually. At the end of the incubation, the reaction (microbial activity) was stopped by cooling the bottles in ice water. The entire content of the serum bottle was freeze-dried, weighed, ground, homogenized in a ball mill, and kept at –20°C until analysis. The *in vitro* incubations were performed for three runs under similar conditions using samples withdrawn from the same bulls

Table 1. List of the plants used in present study

No.	Botanical name	Common name	Plant part	Yield,%
1	*Acacia concinna*	shikakai	pods	13.04
2	*Allium sativum*	garlic	bulb	37.90
3	*Azadirachta indica*	Indian lilac	leaf	11.15
4	*Cuminum cyminum*	cumin	seed	18.39
5	*Cynodon dactylon*	bermuda grass	aerial	23.39
6	*Ocimum sanctum*	holy basil	aerial	7.05
7	*Medicago sativa*	lucerne	aerial	13.18
8	*Sapindus mukorossi*	soapnut	fruit	23.42
9	*Syzygium cumini*	black plum	leaf	10.09
10	*Trifolium alexandrinum*	berseem	aerial	10.12
11	*Terminalia arjuna*	white marudah	stem	13.55
12	*Terminalia bellirica.*	beleric myrobalan	fruit	13.34
13	*Terminalia chebula*	chebulic myrobalan	fruit	18.65
14	*Trachyspermum ammi*	ajwain	fruit	5.02
15	*Thymus serpyllum*	wild thyme	fruit	7.33

Fermentation trial

Four promising plant extracts (*A. indica, A. sativum, C. cyminum* at 1 mg · ml^{-1} and *T. chebula* at 2 mg · ml^{-1}) affecting CLA isomer and VA production were selected for the second *in vitro* study. The selected plant extracts were incubated with BRF (30 ml) and substrate (200 mg) in 100 ml capacity glass syringes (Haberle Labortechnik, Lonsee-Ettlenschie, Germany) in triplicate. The control contained BRF, substrate and distilled water with no additive. The experimental conditions were the same as above (screening assay for CLA production). A parallel set of blanks (syringe without substrate containing only BRF) and specific blank (control blank plus respected additive), each in triplicate, was run simultaneously.

Analytical procedures

Fatty acid extraction and analysis. Lipids were extracted according to the method described by O'Fallon et al. (2007). Briefly, 0.5 g of freeze-dried sample was placed into a 16 × 125 mm screw-cap Pyrex culture tube to which 200 µl of the internal standard (C19:0, 0.5 mg of C19:0 · ml^{-1} methanol), 0.7 ml of aqueous 10 N KOH and 5.3 ml of methanol were added. The tube was incubated in a 55°C water bath for 1.5 h with vigorous hand-shaking for 5 s every 20 min to properly permeate, dissolve and hydrolyse the sample. After cooling to below room temperature in a cold tap-water bath, 0.58 ml of aqueous 24 N H_2SO_4 was added. The tube was mixed by inversion and in the presence of the precipitated K_2SO_4 was incubated again in a 55°C water bath for 1.5 h with hand-shaking for 5 s every 20 min.

After the synthesis of fatty acid methyl esters (FAME), the tube was cooled in a cold tap-water bath. Three millilitres of hexane were added, and the tube was vortex-mixed for 5 min. The tube was centrifuged for 5 min in a tabletop centrifuge at 2000 rpm. The solvent was removed by flushing with nitrogen. The FAME were placed into a GC vial, capped and kept at −20°C until GC analysis. An aliquot of 0.5 µl FAME in hexane at a split ratio of 10:1 was subjected to gas liquid chromatography on a Nucon 5700 (Nucon Engineering CO, New Delhi, India) equipped with a capillary column (60 m, 0.25 mm, 0.25 µm; ID-BPX70) and flame-ionization detector (FID). Helium was the carrier gas. The separation was under the following conditions: the injector temperature was maintained at 220°C and the detector temperature set at 250°C. The initial oven temperature was held at 100°C and increased at a rate of 2°C per min. The concentrations of CLA isomers, VA and SA were analysed (Heidarian et al., 2013). Mixtures of fatty acids were analysed in another run only for extracts that had been selected for further study. For this analysis, the initial oven temperature was 70°C.

Chemical analysis

The chemical composition of formulated substrate was estimated as follows: organic matter (OM) was determined by ashing at 550°C for 5 h. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the methods of Van Soest et al. (1991) and AOAC (2006), method 973.18 (A-D), respectively. Crude protein (CP) content (N × 6.25) was determined using a KEL PLUS N analyser (Pelican, India) which is based on the method described by AOAC (2006), method 984.13 (A-D). Ether extract (EE) was estimated using AOAC (2006) method 920.39 (A).

In vitro gas production

Total gas was recorded from the change in piston position and subtracted from the initial reading. CH_4 was analysed using a gas chromatograph (Nucon 5700, Nucon Engineers, New Delhi, India) equipped with a Flame Ionisation Detector (FID) and stainless steel column packed with Porapak-Q (length 1.5 m; o.d. 3.2 mm; i.d. 2 mm; mesh range 80–100). The temperature of the injection port was 150°C, column 60°C and detector 130°C. Total gas and the methane produced from the substrate after 24 h incubation were calculated by correcting for the corresponding blank values.

In vitro rumen fluid characteristics

Ammonia nitrogen and volatile fatty acid (VFA) concentrations were analysed according to the method described by Makkar (2010). Individual VFA in the samples were determined using a gas chromatograph (Nucon 5700, Nucon Engineers, New Delhi, India) equipped with a flame ionization detector and stainless steel column, packed with chromosorb 101 mesh 80−100 (length 1.5 m; o.d 3.175 mm; i.d. 2 mm). The analytical conditions for fractionation of VFA were: injection port temperature 210°C, column 180°C and detector 230°C. The flow rate of the carrier gas (nitrogen) was 30 ml · min^{-1}.

Analysis of plant secondary metabolites

Total saponins (SP) were determined by the method of Hiai et al. (1976) as described by Makkar et al. (2007). For phenols and tannins, 50 mg extract were transferred to a test tube and 5 ml diethyl ether containing 1% acetic acid (v/v) were added to remove pigments. After 5 min the supernatant was carefully discarded and total phenolics and tannins were estimated according to the Folin-Ciocalteu method (Makkar et al., 1993).

Calculations

Following equations were used:

Truly digested substrate (dry matter, TDS) and true organic matter digestibility (TOMD):

$$TDS\ (mg) = ([S - (R - B)]),$$

where: S − the weight of substrate, R − the weight of dried residue in the crucible after treatment with neutral detergent solution, B − the weight of the dried residue in the crucible of representative blanks after treatment with neutral detergent solution.

$$TOMD\ (mg) = ([OMS - (OMR - OMB)]),$$

where: OMS − the weight of OM in the substrate, OMR − the weight of OM the residue in the crucible, OMB − the weight of the OM residue in representative blanks.

The partitioning factor (PF) was estimated as true organic matter digested (mg) per volume of net gas produced. The partitioning factor and microbial biomass production (MBP) were calculated based on truly degraded substrate (TDS) as described by Blümmel et al. (1999) and Blümmel et al. (2005), respectively.

$$PF = TDS\ (mg) / net\ gas\ production\ (ml),$$

where: TDS was calculated by multiplying TOMD (%) by mg OM content of substrate.

Microbial biomass production (MBP) was calculated from TDOM using the equation:

$$MBP\ (mg) = TDS\ (mg) - (2.25 \times net\ gas\ volume),$$

where: the constant 2.25 is the stoichiometric factor and the efficiency of microbial production (EMP) was estimated as:

$$[TDS - (gas\ volume \times 2.20)]/TDS.$$

Statistical analysis

To assess the differences between extracts, data from the screening experiment were analysed by one-way ANOVA using the General Linear Model of Statistical Analysis Systems statistical software package version 8.2 (SAS Institute. Cary, NC, 2002), with an extract as the treatment factor (fifteen extracts plus control) and experimental day as a blocking factor. The number of replicates for each experimental treatment was nine (three experimental days with triplicated observations each day). In this case, the statistical model used was:

$$Y_{ijk} = \mu + \beta_i + a_j + e_{ijk,}$$

where: y_{ijk} − the value for each observation, μ − the overall mean, β_i − the effect of the i^{th} block (day), a_i − represents the effect of j^{th} treatment, e_{ijk} − the random error.

Since fifteen extracts were supplemented at two levels, there were 31 treatments including T_1 (control, no additive), T_2 (extract of plant 1, level 1), T_3 (extract of plant 1, level 2). T_{31} (extract of plant 15, level 2) in triplicate and the experiment was repeated three times. The Duncan test was used for comparison of the treatments.

To evaluate differences between the effect of selected extracts on fermentation pattern and digestibility, ANOVA was performed using a completely randomized design with a model that included treatment and experimental error. The blank represented only BRF and was used for correction of the value in the control. In this case, the statistical model used was:

$$Y_{ij} = \mu + a_j + e_{ij}$$

where: y_{ij} – the value for each observation, μ – the overall mean, a_i – represents the effect of j^{th} treatment, e_{ij} – the random error.

Following a significant F test ($p < 0.05$), differences among means were examined by the Duncan test.

Results

Composition of the substrate

In all of the *in vitro* incubations, the substrate was concentrate plus shade-dried berseem. The FA composition of the substrate is presented in Table 2. Its dry matter content was 91.2% and contained about OM 91.6%, CP 15%, NDF 62.23%, ADF 30.2% and EE 1.65% of DM.

Table 2. Fatty acid composition of the substrate (g · kg^{-1} fatty acid methyl esters) incubated in buffered rumen fluid together with plant extracts

Fatty acids	Substrate [1]
12:0	4.5
14:0	5.8
14:1	1.1
15:0	0.9
15:1	1.6
16:0	206.1
16:1	1.1
18:0	24.0
cis-9-C18:1	168.4
18:2 *n*-6	426.7
18:3*n*-6	4.1
18:3*n*-3	233.1

[1] hay (berseem) plus concentrate mixture [including rice bran deoiled (11%), mustard cake (12%), maize (33%), wheat bran (20%), ground nut cake (21%), common salt (1%) and mineral mixture (2%) on the bases %DM]

Screening assay for CLA production

The result of the first experiment is presented in Figure 1. In the screening experiment, out of 15 plant extracts, *A. indica, A. sativum* and *C. cyminum* (1 mg · ml^{-1}) increased the concentration of rumenic acid (*cis*-9*trans*-11 CLA, RA) by more than 58% (36.23, 35.89, 37.57 µg per vessel, respectively) as compared with the control (22.75 µg per vessel) (P<0.0001). The maximum increase was observed by addition of *C. cyminum* with a 65% increase in RA concentration, followed by *A. indica* at 1 mg · ml^{-1} (59%) and bulb of *A. sativum* (1 mg · ml^{-1}) (58%; $P < 0.0001$). The extract of *T. chebula* (2 mg·ml^{-1}) increased the VA concentration by 29% as compared with the control (158.8 vs 122.6 µg per vessel, respectively; $P = 0.0001$).

A higher value for total CLA plus VA was observed when buffered ruminal fluid was incubated with the substrate plus extract of *C. cyminum, A. indica, A. sativum* (1 mg · ml^{-1}), *T. chebula* (2 mg · ml^{-1}) as compared with other extracts ($P < 0.0001$). Likewise, the ratio of CLA + VA to SA was higher in *C. cyminum, A. indica, A. sativum* (1 mg · ml^{-1}) and *T. chebula* (2 mg · ml^{-1}; $P < 0.0001$), indicating a favourable effect on biohydrogenation processes in the ruminal fluid. These extracts were, therefore, selected for further investigation of their effects on ruminal FA profile and fermentation characteristics.

Fatty acid profile of ruminal fluid

The fatty acid profiles in incubators supplemented with extracts of *A. indica, A. sativum, C. cyminum* (1 mg · ml^{-1}), and *T. chebula* (2 mg · ml^{-1}) are presented in Figure 2. There were no differences in the concentrations of C18:1n9t, C18:1n9c, C18:2n6t, C18:2n6c, and C18:3n6 with the selected extracts, as compared with the control, but *C. cyminum* gave the highest concentration of C18:3n3 (4.21 mg · 100 mg^{-1} of total FAME vs 1.43 mg · 100 mg^{-1} of total FAME in the control $p = 0.0002$) and PUFA as compared with the control (9.14 vs 5.25 mg · 100 mg^{-1} of total FA; $p = 0.04$). The effect of *T. chebula* (2 mg · ml^{-1}) on C18:1n9c and MUFA and of *A. sativum, C. cyminum* (1 mg · ml^{-1}) on reducing the concentrations of saturated fatty acids (SFA), only tended towards significance ($p = 0.088$, $p = 0.086$, $p = 0.078$), however.

Effect on ruminal fermentation pattern

The effect of *A. indica, A. sativum, C. cyminum* (1 mg · ml^{-1}) and *T. chebula* (2 mg · ml^{-1}) extracts on fermentation parameters are given in Table 3. Total net gas production (ml · 24 h^{-1}) was not affected ($p = 0.52$) by addition of extracts. Net methane production was not significantly influenced ($p = 0.08$), by extracts compared with the control (Table 3), however, when expressed per 100 units of gas produced, the estimated methane percentage decreased with *T. chebula* and *A. sativum* (16.7% and 10.5% decrease, respectively; $P = 0.008$). Expressed per gram of TDS, net methane production was reduced ($p = 0.054$) when extracts were included.

Microbial biomass production (MBP) from 200 mg of substrate and efficiency of microbial production (EMP) were not affected by inclusion of extracts ($p > 0.05$). Addition of *C. cyminum* resulted in higher TDS ($P < 0.001$) and TOMD ($P = 0.0001$) levels compared with controls.

The partitioning factor (PF; ratio of substrate truly degraded to gas volume produced at 24 h of incubation) had a higher value ($p = 0.054$) with *C. cyminum* (Table 3).

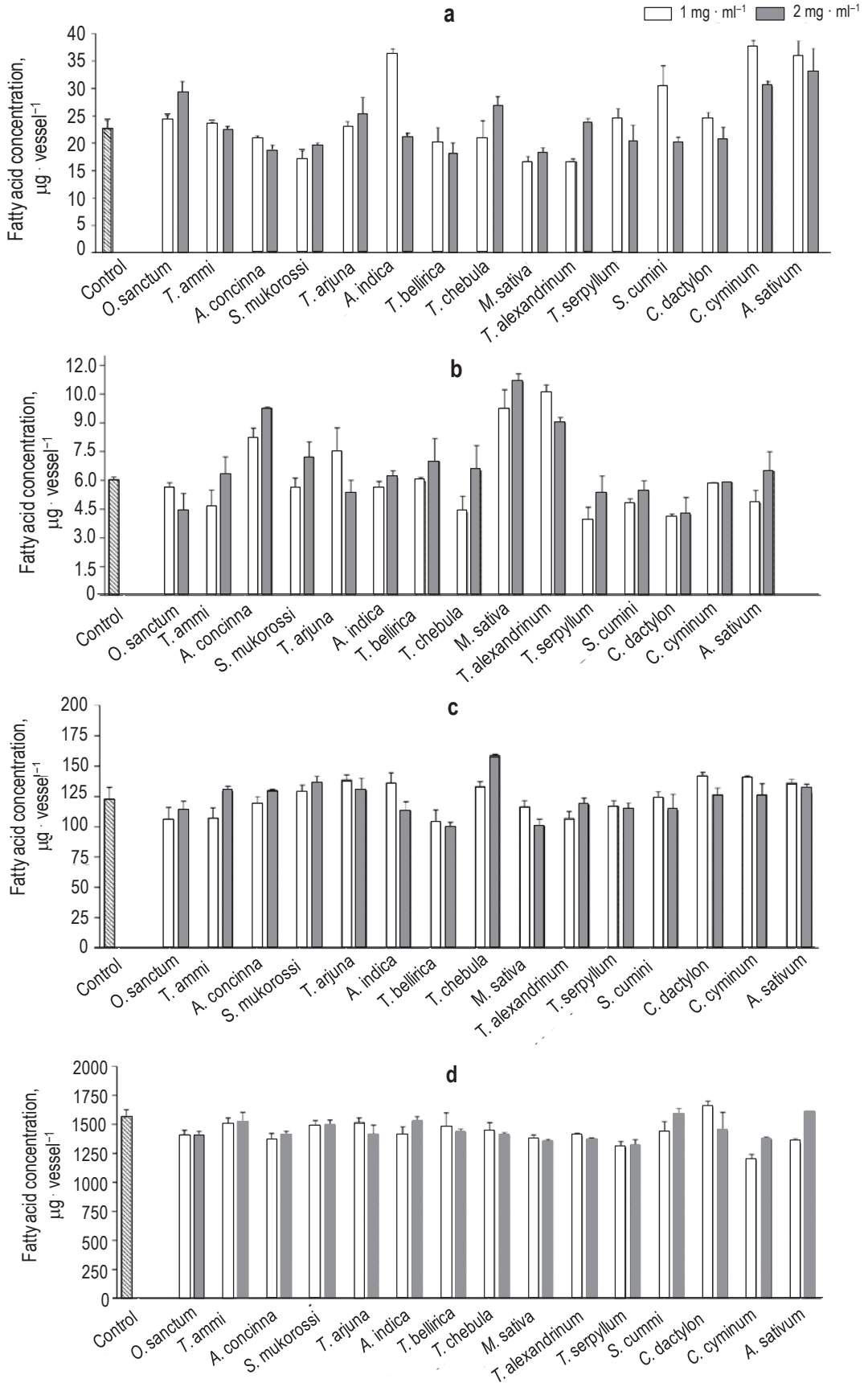

Figure 1. Concentration of *cis*-9*trans*-11 CLA (a), *trans*-10*cis*-12 CLA (b), vaccenic acid (c) and stearic acid (d) in 30 ml BRF after 24 h incubation in the presence of plant extracts, (note: 'Control' refers to the buffered rumen liquor + substrate, plant extracts were used at 1 and 2 mg per ml of buffered rumen liquor in treatments

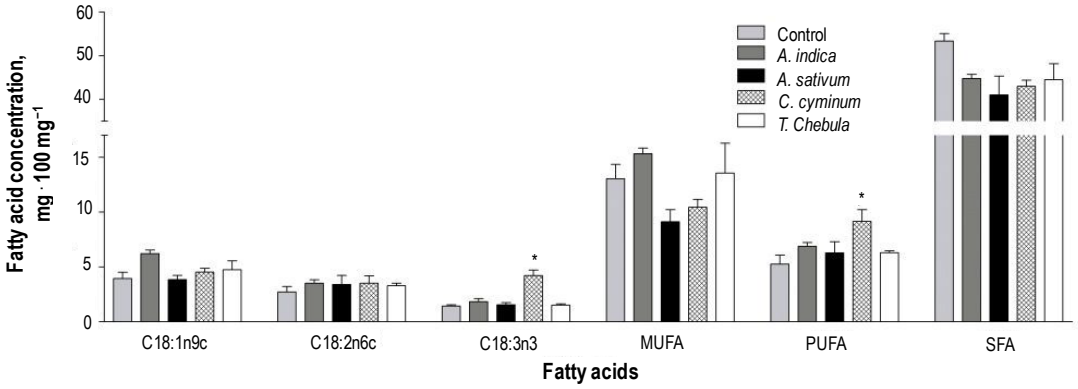

Figure 2. Ruminal fatty acid concentration (mg · 100 mg^{-1} fatty acid methyl ester) in incubators either supplemented with *Allium sativum*, *Azadirachta indica*, *Cuminium cyminum* (1 mg · ml^{-1}), *Terminalia chebula* (2 mg · ml^{-1}), or unsupplemented (control) with plant extract after 24 h of incubation *in vitro.* Standard error is indicated by bars over each column. For C18:1n9c p = 0.088 for C18:2n6c, p = 0.827 for C18:3n3, P = 0.0002 for MUFA (monounsaturated fatty acids = C14:1 + C16:1 + C18:*1 cis*-9 + C18:1 *trans*-11), p = 0.086 for PUFA (polyunsaturated fatty acids = C18:2 *cis*-9, *cis*-12 + C18:2 *trans*-9, *trans*-11 + C18:3n-3 + C18:3n-6), p = 0.049 and for SFA (saturated fatty acids = C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0), p = 0.078

Table 3. Effect of extract of *Azadirachta indica, Allium sativum, Cuminium cyminum* (1 mg · ml^{-1}) and *Terminalia chebula* (2 mg · ml^{-1}), on *in vitro* rumen fermentation parameters after 24 h incubation with hay and concentrate (1:1) as substrate (n=9)

Parameter	Control	*A. indica*	*A. sativum*	*C. cyminum*	*T. chebula*	*P* value	SEM
Net gas production, ml	41.17	41.17	44.17	38.50	38.17	0.52	1.522
Net CH_4, ml	8.424	7.854	7.774	7.469	6.507	0.087	0.243
CH_4,%	23.59^{a}	22.26^{ab}	21.11^{bc}	23.27^{a}	19.63^{c}	0.008	0.374
TDS, mg	150.4^{b}	151.3^{b}	155.1^{b}	165.0^{a}	150.6^{b}	0.001	1.032
TOMD, mg	125.7^{bc}	124.6^{bc}	131.9^{b}	151.3^{a}	120.2^{c}	0.0001	1.517
PF, mg· ml^{-1}	3.076	3.069	3.018	3.960	3.156	0.054	0.125
Net gas, ml ·TDS g^{-1}	283.2	272.2	274.5	233.2	253.6	0.32	9.969
Net CH_4, ml ·TDS g^{-1}	57.99^{a}	51.99^{ab}	48.37^{ab}	45.24^{b}	43.23^{b}	0.054	1.837
MBP, mg	59.80	60.70	57.90	80.33	66.6	0.169	3.718
EMP	0.397	0.401	0.373	0.487	0.442	0.347	0.021
NH_3-N, mg ·100 ml^{-1}	30.22^{a}	22.17^{bc}	24.85^{ab}	26.37^{ab}	17.50^{c}	0.005	1.013

^{a,b,c} mean values within a row with unlike superscript letters were significantly different; * SEM – standard error of the mean; TDS – truly degraded substrate; TOMD – true organic matter digested; PF – partitioning factor was estimated as true organic matter digested per volume of net gas produced; MBP – microbial biomass production was estimated as [TDS (net gas volume × 2.20)]; EMP – efficiency of microbial production was estimated as [TDS − (gas volume × 2.20)]/TDS

The ammonia nitrogen concentration (mg·100 ml^{-1}) after 24 h incubation with *T. chebula* was significantly lower than with the other extracts (P=0.005).

The VFA, acetate, propionate, *iso*-butyrate and valerate concentrations were not affected by inclusion of extracts. Nevertheless, butyrate and *iso*-valerate were found at higher concentrations (P = 0.0003, P = 0.002, respectively) in the syringes incubated with substrate plus extract of *A. sativum* and lower with *A. indica*, when compared with the control (Table 4). The acetate-to-propionate ratio was not significantly affected (p = 0.19) by different extracts (Table 4).

Chemical composition and plant secondary compounds of the selected extracts

The chemical composition and secondary metabolites present in *A. indica, A. sativum, C. cyminum* and *T. chebula* are presented in Table 5. The OM, DM, CP, NDF and ADF contents of the extracts did not differ. The *C. cyminum* extract contained high amounts of EE (18% of DM), that of *T. chebula* had substantial quantities of total phenols and total tannins (45.3% and 40.1%, respectively). *C. cyminum* and *A. indica* extracts had both total phenols (26.3% and 20.2%, respectively) and total tannins (15.9% and 13.5%, respectively) in smaller amounts. The *A. sativum* extract was a poor source of phenolic compounds. All of the extracts were poor in saponin contents.

Table 4. Effect of extract of *Azadirachta indica, Allium sativum, Cuminium cyminum* (1 mg · ml^{-1}) and *Terminalia chebula* (2 mg · ml^{-1}) on *in vitro* ruminal VFA concentration (mM) after 24 h incubation with hay and concentrate (1:1) as substrate (n=9)

Parameter	Control	*A. indica*	*A. sativum*	*C. cyminum*	*T. chebula*	*P* value	SEM
Total VFA	19.58	18.54	20.07	19.16	19.19	0.07	0.186
Acetate	11.91	11.53	12.07	11.48	11.35	0.27	0.145
Propionate	4.369	4.136	4.629	4.462	4.768	0.15	0.306
iso-butyrate	0.146	0.138	0.142	0.139	0.101	0.28	0.009
Butyrate	2.702^{b}	2.447^{c}	2.835^{a}	2.745^{ab}	2.679^{b}	0.0003	0.021
iso-valerate	0.197^{a}	0.073^{c}	0.171^{ab}	0.130^{b}	0.121^{b}	0.002	0.009
Valerate	0.252	0.222	0.219	0.207	0.179	0.12	0.011
Acetate:propionate	2.744	2.792	2.618	2.574	2.384	0.19	0.067

^{a,b,c} – as in Table 3; SEM – see Table 3

Table 5. Chemical composition of aqueous methanolic extract of *A. indica, A. sativum, C.cyminum* and *T. chebula,* % of DM

Item	*A. indica*	*A. sativum*	*C. cyminum*	*T. chebula*
DM, **%**	91.1	94.2	93.6	95.2
OM	90.1	96.1	96	96.4
CP	4.9	8.03	7.4	3.8
EE	8.5	10.8	18	1.4
NDF	3.2	2.03	2.3	3.5
ADF	1.1	0.7	0.4	0.65
Hemicelloluse	2.1	1.33	1.9	2.85
Total phenol	20.2	16.3	26.3	45.3
Total tannins	15.9	12.4	13.5	40.1
Saponins	3.8	0.1	5.04	3.3

Discussion

Screening assay

The effects of dietary supplementation of plant secondary compounds on accumulation of biohydrogenation intermediates in the rumen and, consequently, in meat and milk have been reported (Lourenco et al., 2007a,b; Vasta et al., 2009; Rana et al., 2012). This study screened plant extracts for a significant effect on ruminal CLA isomers, their precursors, and VA enhancement *in vitro*. We tried not to add LA to the vessels to avoid potential interaction between oil and plant secondary metabolites on rumen microorganisms. Nonetheless, higher proportions of biohydrogenation intermediates, in particular *cis*-9*trans*-11 CLA, were found in some of the treatments despite the unique substrate provided. These observations suggest that inclusion of the extract affected PUFA biohydrogenation and, as a result, changed CLA isomer and VA concentrations in ruminal fluid *in vitro*. CLA is an intermediate of LA biohydrogenation in the rumen (Harfoot and Hazlewood, 1997). Enzymatic pathways in biohydrogenation of *cis*9*cis*12C18:2 by rumen microbes initially involve isomerization of the *cis*12 double bond, giving *cis*9*trans*11CLA. The produced CLA then is converted to C18:1 and subsequently C18:0 by the action of reductases. While isomerization of linoleic acid to CLA is a spontaneous reaction, the reduction of CLA to VA is an energy-demanding step (Harfoot and Hazlewood, 1997). Factors that impair available energy for reductases consequently accumulate CLA. Previous studies reported that *A. indica, A. sativum* and *T. chebula* have antimicrobial effects (Iacobellis et al., 2005; Amagase et al., 2006; Joshi et al., 2011) that might impair bacterial growth and activity, therefore reduce energy sources, slowing the progression of biohydrogenation. This suggests that the CLA accumulation in our study might be a result of diminished energy availability during the biohydrogenation of PUFA. Experiments on pure cultures of *B. fibrisolvens* showed that inhibitory effects on cell growth significantly affect the reduction steps of biohydrogenation, but not isomerization (Kim, 2003).

C. cyminum extract contains substantial amounts of ether extract. When the fatty acid composition of this extract was analysed, LA and LNA comprised 4% and 3.5% of it, respectively. This amount is equal to the 2.25 mg LA and LNA provided by *C. cyminum* extract per vessel. Troegeler-Meynadier et al. (2006) reported that LA and LNA, by saturation and inhibition, respectively, inhibit the activity of isomereases and reductases. Further studies by Wood et al. (2010) showed that high LNA concentrations inhibit the rate of LA metabolism and increased accumulation of RA and VA as the duration of incubation increased. We assume that the effectiveness of *C. cyminum* might be associated with its high concentration of LNA. On the other hand, the chemical constituents of *C. cyminum* include some aromatic compounds that by themselves, or a synergistic effect with LA and LNA and the secondary metabolites present in *C. cyminum*, could contribute to inhibition of BH.

The fatty acid concentration in vessels showed that PUFA and C18:3n3 concentrations were higher with *C. cyminum* extract. *C. cyminum* has a long

history in traditional medicine. *C. cyminum* decreases phospholipase activity (Azeez, 2008), which is one of the necessary enzymes for hydrolysis of lipids and lipolysis of esterified unsaturated fatty acids is a prerequisite for biohydrogenation, so addition of *C. cyminum* increases ruminal recovery of the PUFA present in the substrate.

Fermentation trial

In the present experiment, net gas (ml) and net methane (ml) production were not affected by inclusion of extracts, however, methane production expressed per 100 units of gas production was reduced with *A. sativum* and *T. chebula*, which is in agreement with what has been reported in the literature (Kamra et al., 2006; García-González et al., 2008; Patra et al., 2010) for *in vitro* systems. Some of the secondary compounds present in *A. sativum* (thymol, eugenol, carvacrol) and *T. chebula* (tannins) were implicated in this effect (Chiquette and Benchaar, 2005; Hess et al., 2005).

C. cyminum improved *in vitro* TDS and TOMD when compared with the control (Table 3), in agreement with another study (Khan and Chadhury, 2010) that found greater digestibility of dry matter and organic matter in forages. The presence in *C. cyminum* of important minerals such as calcium, phosphorous and copper that have positive correlations with forage digestibility (Martinez and Church, 1970; Khan and Chadhury, 2010) might be responsible for the changes observed in substrate digestibility.

The higher partitioning factor with *C. cyminum* points to degradation of the substrate towards microbial biomass production rather than gas and short-chain fatty acids. This suggests that *C. cyminum* changed the ruminal fermentation pattern of dietary organic matter from gaseous end products, which are nutritionally wasteful, to greater microbial protein production.

Ammonia nitrogen concentrations were lower with *A. indica* and *T. chebula*. This decrease following inclusion of these two tannin-containing plants (Joshi et al., 2011; Rana et al., 2012) is associated with the protein binding properties of tannins (Terrill et al., 1992). Although reduction of ammonia nitrogen concentrations after adding tannin-containing plants in continuous culture has been reported (Khiaosa-Ard et al., 2009), other studies have reported no effect of *A. indica* oil on ammonia nitrogen (Yang et al., 2009). *C. cyminum* seeds contain 23% CP, which is a considerable amount (Azeez, 2008). The breakdown of dietary protein produces ammonia in the rumen, and as a result *C. cyminum* did not affect the ammonia concentration.

The known biohydrogenating bacteria in the rumen are cellulolytic bacteria and any reduction in their activity consequently occurs at the expense of fibre degradation. These findings suggest that *C. cyminum* may specifically inhibit the biohydrogenation pathway while improving nutrient digestibility in the rumen.

Conclusions

Evaluation of fifteen plant extracts for their potential to affect ruminal biohydrogenation suggests that *Azadiracta indica*, *Allium sativum*, *Cuminum cyminum* and *Terminalia chebula* have the potential to manipulate ruminal biohydrogenation towards accumulation of health-promoting fatty acids, in particular, of conjugated linoleic acid isomers, their precursors, and vaccenic acid, without negative affecting substrate digestibility. *C. cyminum*, did however, increase partitioning of nutrients towards microbial protein synthesis and affected biohydrogenation, leading to for higher polyunsaturated fatty acids (PUFA) concentrations in ruminal fluid. We hypothesize that *C. cyminum* contains active compounds that affect microbial biohydrogenation, particularly their enzymatic activity that could specifically influence biohydrogenation and not fermentation. Further studies are necessary to investigate the mechanism by which these extracts affect biohydrogenating microorganisms, specific enzymatic pathways in PUFA biohydrogenation, and the active compound(s) responsible for the effect observed in the rumen environment and to explore the validation of these results under practical conditions.

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