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Significance of phenolic compounds in tropical forages for the ruminal bypass of polyunsaturated fatty acids and the appearance of biohydrogenation intermediates as examined *in vitro*

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Abstract. The purpose of the present study was to assess the influence of phenol-rich tropical ruminant feeds on the extent of ruminal biohydrogenation (BH) of polyunsaturated fatty acids (PUFA). Samples of 27 tropical forages (mainly tree and shrub leaves), characterised by different phenolic profiles, were incubated *in vitro* ($n = 4$ replicates) with buffered rumen fluid for 24 h using the Hohenheim gas test method. Linseed oil was added as a rich source of PUFA. In the plants, total extractable phenols (TEP), non-tannin phenols, condensed tannins, and fatty acids were determined. After terminating incubation, the fatty acid profile present in fermentation fluid (total syringe content) was analysed by gas chromatography. The relationship between TEP and the disappearance of α-linolenic acid from the incubation fluid was negative ($R^2 = 0.48$, $P < 0.001$), indicating that TEP reduced the ruminal BH of this PUFA. Similarly, TEP were negatively related with the disappearances of linoleic acid ($R^2 = 0.52$, $P < 0.001$) and oleic acid ($R^2 = 0.58$, $P < 0.001$). The appearance of rumenic acid, an important conjugated linoleic acid isomer, was positively correlated with TEP ($R^2 = 0.30$, $P < 0.01$), while the opposite result was seen with stearic acid ($R^2 = 0.22$, $P < 0.05$). Leaves of avocado (*Persea americana*) were particularly interesting, because they changed the BH pattern at a moderate TEP content of 73 g/kg DM. It is concluded that, in the tropical feedstuffs investigated, TEP have an impact on ruminal fatty acid BH and are associated with an increased bypass of PUFA and the generation of conjugated linoleic acid.

Additional keywords: biohydrogenation, *n*-3 fatty acids, plant secondary compounds, ruminant.

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Introduction

The production of healthy food is an important issue in current ruminant nutrition research. Health promoting properties have been assigned to polyunsaturated fatty acids (PUFA), particularly to *n*-3 fatty acids (for instance α-linolenic acid, C18:3 *n*-3; see Barceló-Coblijn and Murphy 2009) and conjugated linoleic acids (CLA; especially rumenic acid, *c*9,*t*11-C18:2; see Benjamin and Spener 2009). Although the concentration of these fatty acids (FA) is limited in dairy products, the supply to the human diet is constant and therefore changes in the FA profiles in milk are of considerable nutritional relevance (Jutzeler van Wijlen and Colombani 2010). Large amounts of C18:3 *n*-3, and of other PUFA, originally present in feed, disappear in the rumen by microbial biohydrogenation (BH) (Chilliard *et al*. 2007; Doreau *et al*. 2011). Together with different *trans*- and *cis*-C18:1 isomers (especially vaccenic acid, *t*11-C18:1) and stearic acid (C18:0; the ultimate product), *c*9,*t*11-C18:2 is a product of the same process (Chilliard *et al*. 2007; Jenkins *et al*. 2008). Reducing the extent of ruminal BH of plant PUFA on the one hand and maximising the synthesis of *c*9,*t*11-C18:2 on the other hand are therefore desirable, though partly contrasting, goals of ruminant nutrition.

The BH process is largely influenced by forage species and the composition of the plants (Dewhurst *et al*. 2006; Durmic *et al*. 2008; Lourenço *et al*. 2008), including plant secondary metabolites like phenolic compounds (Khiaosa-ard *et al*. 2009; Cabiddu *et al*. 2010). The latter are considered to have both adverse and beneficial effects, depending on their concentration and nature, as well as other factors, such as animal species, physiological state of the animal, and composition of the diet (Mueller-Harvey 2006). Some phenols from plant sources have been tested for their effect on ruminal BH. Occasionally there were significant effects of extracted phenols or certain plant species on ruminal BH, including a reduced PUFA BH, an accumulation of *t*11-C18:1, and/or a decreased concentration

of C18:0 (Durmic *et al.* 2008; Khiaosa-ard *et al.* 2009; Vasta *et al.* 2009*a*; Cabiddu *et al.* 2010; Jerónimo *et al.* 2010). However, treatments seldom resulted in an efficient inhibition of the first step of C18:3 *n*-3 BH, which is necessary to increase the ruminal bypass of this FA.

The hypothesis tested in the present study was that plant species characterised by elevated concentrations of phenolic compounds will lead to a decreased disappearance of C18:3 *n*-3 during the ruminal fermentation process, or to a higher accumulation of *t*11-C18:1 and *c*9,*t*11-C18:2, or both, by inhibiting different steps of the microbial BH pathway in the rumen. This was assessed *in vitro* by following the effect on BH of FA from linseed oil when incubating a wide range of tropical forages containing different amounts and profiles of phenols. To our knowledge, the potential of tropical plant forages to modify ruminal BH of FA has not previously been studied.

Materials and methods

Experimental plants and their chemical analyses

Samples from 27 plant species (Table 1) were collected from the area around Bogor Agricultural University, Darmaga, Indonesia (180 m elevation), and the Indonesian Research Institute for Animal Production, Ciawi (350 m elevation). These plant species have been used previously to study the relationships between phenolic fractions in tropical plants and their methanogenic potential (Jayanegara *et al.* 2011*b*). The plant species considered are commonly used either as ruminant feeds or as traditional veterinary medicinal plants in Indonesia. They consisted of one grass, four herb, nine shrub, and 13 tree species. Approximately 3-kg fresh biomass of leafy material of several individual plants was collected from each species in November 2008. After being stored indoors for 2 days, the samples were dried at 50°C and ground to pass through a 1-mm sieve. This procedure was chosen, as own preliminary investigation (data not shown) did not reveal systematic differences between oven drying and freeze drying regarding the phenol contents of the sample. Also Muetzel and Becker (2006) observed that oven drying at 60°C did not lead to a general decrease in the extractability of tannin phenols and confirmed an intact biological activity of the compounds compared with the freeze-dried and freshly frozen samples.

Analysis of crude protein content followed the standard procedure of AOAC (1997) using a C/N analyser (Leco-Analyser Type FP-2000, Leco Instruments GmbH, Kirchheim,

Table 1. Contents (g/kg DM) of crude protein, neutral detergent fibre and phenolic compounds of the experimental plants (n = 2)
Source: Jayanegara *et al.* (2011*b*); NDF, neutral detergent fibre; s.e.m., standard error of the mean

No.	Plant species	Common name	Sample type	Crude protein	NDF	Total phenols[A]	Non-tannin phenols[A]	Total tannins[A]	Condensed tannins[B]	Hydrolysable tannins[C]
1	*Acacia mangium*	Black wattle	Shrub leaves	162	436	105	14	91	42	49
2	*Acacia villosa*	Redwood	Shrub leaves	311	246	236	16	220	14	206
3	*Albizia falcataria*	Albisia	Tree leaves	223	310	113	49	64	46	18
4	*Artocarpus heterophyllus*	Jackfruit	Tree leaves	142	339	96	16	80	47	33
5	*Calliandra calothyrsus*	Calliandra	Shrub leaves	286	326	127	46	81	22	59
6	*Canna indica*	Indian shot	Herb	158	581	14	6	8	2	6
7	*Carica papaya*	Papaya	Tree leaves	386	155	25	17	8	0	8
8	*Clidemia hirta*	Soapbush	Shrub leaves	129	232	216	4	212	10	202
9	*Cycas rumphii*	Queen sago	Tree leaves	209	509	26	7	19	11	8
10	*Erythrina orientalis*	Coral tree	Tree leaves	343	447	20	16	4	1	3
11	*Eugenia aquea*	Water apple	Tree leaves	100	479	169	102	67	40	27
12	*Hibiscus tiliaceus*	Sea hibiscus	Shrub leaves	168	541	55	13	42	23	19
13	*Ipomoea batatas*	Sweet potato	Herb	236	334	29	13	16	0	16
14	*Lantana camara*	Lantana	Herb	186	382	47	28	19	2	17
15	*Leucaena diversifolia*	Wild tamarind	Shrub leaves	336	249	114	56	58	34	24
16	*Leucaena leucocephala*	Leucaena	Shrub leaves	306	263	96	29	67	18	49
17	*Manihot esculenta*	Cassava	Shrub leaves	377	211	32	16	16	4	12
18	*Melia azedarach*	China berry	Tree leaves	253	253	30	17	13	4	9
19	*Mimosa invisa*	Nila grass	Herb	152	555	45	15	30	17	13
20	*Morinda citrifolia*	Noni	Tree leaves	228	223	25	15	10	3	7
21	*Myristica fragrans*	Nutmeg	Tree leaves	101	380	181	6	175	72	103
22	*Paspalum dilatatum*	Dallis grass	Grass	79	710	25	11	14	1	13
23	*Persea americana*	Avocado	Tree leaves	149	480	73	27	46	34	12
24	*Pithecellobium jiringa*	Blackbead	Tree leaves	215	472	193	8	185	10	175
25	*Psidium guajava*	Guava	Tree leaves	146	385	132	62	70	34	36
26	*Sesbania grandiflora*	Swamp pea	Shrub leaves	312	251	19	17	2	0	2
27	*Swietenia mahagoni*	Mahagony	Tree leaves	112	281	207	69	138	86	52
	s.e.m.	–	–	17.3	26.3	13.6	4.5	12.7	4.4	11.3

[A]As gallic acid equivalents.
[B]As leucocyanidin equivalents.
[C]Estimated by difference between total tannins and condensed tannins.

Germany), and crude protein was calculated as N × 6.25. Determination of neutral detergent fibre was performed according to Van Soest *et al*. (1991) using the Fibretec apparatus (Fibretec System M, Tecator, 1020 Hot Extraction, Flawil, Switzerland). This was done with α-amylase (Termamyl 120 L, type S, Novo Nodirsk A/S, Bagsværd, Denmark) but without sodium sulfite addition, and the value is expressed exclusive of residual ash.

Determinations of phenolic contents were conducted by following the protocols of Makkar (2003). Prior to the determinations, the plant phenols were extracted twice with aqueous acetone (700 : 300 v/v) under ultrasonic treatment for 20 min at 25°C in an ultrasonic water bath (TEC-25, Telsonic AG, Bronschhofen, Switzerland). Total extractable phenols (TEP), non-tannin phenols (NTP) and total tannins (TT) were determined by a modified Folin–Ciocalteu method using polyvinyl-polypyrrolidone (Sigma-Aldrich GmbH, Steinheim, Germany) to separate tannin phenols from NTP; the values were calibrated against a gallic acid standard (Sigma-Aldrich GmbH) and expressed as gallic acid equivalents. Briefly, an amount of 0.02–0.1 mL of the extract (depending on the concentration of TEP and TT in the sample) was put in a test tube, and complemented with distilled water until accounting for 0.5 ml. Then 0.25 mL of Folin reagent (1 N; Sigma-Aldrich GmbH) and 1.25 mL of sodium carbonate solution (40 g $Na_2CO_3.10H_2O$) were added. The tube was vortexed and kept in the dark thereafter for 40 min. The absorbance was read at 725 nm using a UV-vis spectrophotometer (Shimadzu UV-160, Shimadzu Corporation, Kyoto, Japan). Condensed tannins (CT) were determined by the butanol-HCl-iron method (Porter *et al.* 1986) and expressed as leucocyanidin equivalents. In brief, a total of 0.25 mL of the extract was mixed with aqueous acetone (7 : 3 v/v) in a glass tube, in which the ratio of extract to acetone depended on the expected CT concentration in the sample. An amount of 50 µl of ferric reagent (2.0 g ferric ammonium sulfate in 100 mL of 2 M HCl) was added, followed by 1.5 mL butanol-HCl (95 : 5 v/v), and the tube was vortexed. Subsequently, the tube was heated in a boiling water bath at 100°C for 60 min. The absorbance was read at 550 nm. Hydrolysable tannins (HT) were estimated as the difference between TT and CT (Singh *et al.* 2005). An underestimation of CT and, consequently, an overestimation of HT cannot be completely excluded but, based on Schofield *et al*. (2001), this is one method of choice and the error is assumed to be low. The chemical analyses were conducted in duplicate except for neutral detergent fibre (in triplicate). These analytical results formed the basis for relating *in vitro* results to feed characteristics, and are displayed in Table 1.

In vitro *incubation*

Plant samples were incubated *in vitro* using the Hohenheim gas test method (Menke and Steingass 1988) as modified by Soliva and Hess (2007), carried out at ETH Zurich, Zurich, Switzerland. This *in vitro* batch technique has previously been used to investigate the BH of dietary FA (Vasta *et al*. 2009*a*; Khiaosa-ard *et al*. 2011). Briefly, ~200 mg DM of each plant sample was incubated in four replicates (represented by one syringe per replicate) with 30 mL rumen fluid and Hohenheim gas test buffer solution (1 : 2 v/v), and kept at 39°C for 24 h. The composition of the rumen-fluid buffer mixture was as follows: 323 mL rumen fluid, 5.64 g $NaHCO_3$, 0.64 g NH_4HCO_3, 1.00 g KH_2PO_4, 0.92 g Na_2HPO_4, 0.097 g $MgSO_4 \times 7\ H_2O$, 7.99 mg $MnCl_2 \times 4H_2O$, 10.55 mg $CaCl_2 \times 2\ H_2O$, 0.80 mg $CoCl_2 \times 6\ H_2O$, 6.39 mg $FeCl_3 \times 6\ H_2O$, 0.80 mg resazurine, 0.31 mg $Na_2S.9H_2O$, 0.074 mg NaOH, made up to 1 L by distilled water. The incubation was done after adding 50 mg linseed oil per g plant DM (emulsified in 1 : 99 v/v aqueous solution of Tween 80, Sigma-Aldrich, Inc., St Louis, MO, USA). Rumen fluid, containing solid particles from the feeds consumed, was drawn before morning feeding from a fistulated lactating Brown Swiss cow (the cow was cared for according to Swiss guidelines for animal welfare) fed *ad libitum* (~17 kg/day of hay with 880 g DM /kg was consumed) with a hay of white clover (*Trifolium repens*) and ryegrass (*Lolium multiflorum*), as well as 0.5 kg/day concentrate (850 g DM/kg; UFA 149, UFA AG, Herzogenbuchsee, Switzerland). Amounts of 200 mg DM each of standard hay and standard concentrate (obtained from the Institute for Animal Nutrition, University of Hohenheim, Stuttgart, Germany) with a known amount of gas production was incubated in triplicate in each run for control (Menke and Steingass 1988). After 24 h of incubation, the fermentation fluid containing feed residues (total syringe content) was removed from the syringe and stored at −20°C until use. Additionally, a sample of the buffered rumen fluid sample was taken before the incubation (0 h) in order to obtain the initial FA profiles and to be used for calculating the 'disappearance' and 'appearance' of FA (described later).

Fatty acid determination

The plant FA were extracted (in duplicate) using accelerated solvent extraction (ASE 200, Dionex Corporation, Sunnyvale, CA, USA) with hexane : propan-2-ol (3 : 2 v/v) as described by Khiaosa-ard *et al*. (2009). The C19:0 was added (1 mg C19:0/mL dichloromethane; Sigma-Aldrich GmbH, Buchs, Switzerland), serving as an internal standard. The solvent was evaporated in a nitrogen stream, and the extract was collected with 2 mL of dichloromethane. An amount of 1 mL of the extract was then mixed with 10 mL of distilled water. Fatty acids from the fermentation fluid (total syringe content, including the feed residues) samples were extracted and transesterified to FA methyl esters (FAME) as also described in Khiaosa-ard *et al*. (2009). In brief, the samples were hydrolysed by 10 M NaOH, followed by acidification using 6 M HCl to release free FA. The free FA were extracted twice by propan-2-ol and cyclohexane. After evaporating the organic solvent, a methylation step was conducted by adding trimethylsilyl-diazomethane (2 M in hexane) to the resultant FAME, followed by a purification step employing a thin-layer chromatography technique. The FAME were then separated using a gas chromatograph (model HP 6890, Agilent Technologies Inc., Wilmington, DE, USA), equipped with a 30 m × 0.32 mm Supelcowax-10 capillary column (Supelco Inc., Bellefonte, PA, USA) and a flame ionisation detector, following the settings of Khiaosa-ard *et al*. (2009). Identification of individual FA was performed by running a mixed FAME standard (Supelco 37 Component, Supelco Inc.) and by identifying individual peaks with GC/MS. The chromatograms published by Kramer *et al*. (2002)

were used as a reference for checking the results obtained. The individual *trans*- and *cis*-isomers of C18:1 could not be fully separated with the column used. For the results presented here, in addition to C18:1 *n*-9 (oleic acid), only one further C18:1-peak was separately displayed and used for calculations. It is this peak that contains *t*10 and *t*11-C18:1, and probably also the respective *cis*-isomers (Kramer *et al.* 2002). Since *t*11-C18:1 is by far the most prominent isomer in forage and α-linolenic acid-based rumen environments (Jouany *et al*. 2007; Lourenço *et al*. 2008), in the present experiment this peak was considered to represent mainly *t*11-C18:1, although it likely contained some other C18:1 isomers as well. The recovery of the FA measured after 24 h of incubation was 82.4 ± 19.8% for total FA and 77.3 ± 21.1% for total C18 FA.

Calculations and data analyses

The FA composition of the plant species was expressed as g/kg total FAME, and total FAME was related to DM to describe total FA content. Since the origin of the FA in the syringes was from plants, linseed oil, and incubation medium at 0 h, all of these different sources were included in the calculations concerning BH. The FA data in the syringes are presented as the difference between the corresponding amounts before (0 h) and after incubation (24 h; adjusted by the recovery of total FA) in relation to the corresponding FA substrates at 0 h (in g/kg). Amounts of substrate FA, which exclusively originated from the feed, which were not recovered, were supposed to have 'disappeared' (D) and were calculated as follows:

$$D_{C18:3\,n\text{-}3} = (C18:3\,n\text{-}3_{0\,h} - C18:3\,n\text{-}3_{24\,h})/C18:3\,n\text{-}3_{0\,h}$$

$$D_{C18:2\,n\text{-}6} = (C18:2\,n\text{-}6_{0\,h} - C18:2\,n\text{-}6_{24\,h})/C18:2\,n\text{-}6_{0\,h}$$

$$D_{C18:1\,n\text{-}9} = (C18:1\,n\text{-}9_{0\,h} - C18:1\,n\text{-}9_{24\,h})/C18:1\,n\text{-}9_{0\,h}$$

Conversely, the extra amounts of BH intermediates found were defined as to have 'appeared' (A) because they have been produced during incubation:

$$A_{c9,t11\text{-}C18:2} = (c9,t11\text{-}C18:2_{24h} - c9,t11\text{-}C18:2_{0\,h}) / C18:2\,n\text{-}6_{0\,h}$$

$$A_{t11\text{-}C18:1} = (t11\text{-}C18:1_{24h} - t11\text{-}C18:1_{0\,h}) / (C18:3\,n\text{-}3 + C18:2\,n\text{-}6 + C18:1\,n\text{-}9)_{0\,h}$$

$$A_{C18:0} = (C18:0_{24\,h} - C18:0_{0\,h}) / (C18:3\,n\text{-}3 + C18:2\,n\text{-}6 + C18:1\,n\text{-}9)_{0\,h}$$

In the cases of $A_{c9,t11\text{-}C18:2}$ and $A_{t11\text{-}C18:1}$, these are net appearances, because these values depend on both appearance and disappearance of the FA in the BH pathway. Data generated from the *in vitro* incubations were subjected to ANOVA using the procedure MIXED of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). A randomised complete block design was employed as the statistical model. The experimental plant species (n = 27) were treated as fixed effects, while different incubation runs (n = 4) were considered as random effects and served as blocks. Least significant difference (l.s.d.) values were generated with the mixed model as the basis for multiple comparisons among means. Linear regression and correlation analyses, based on data averaged per plant species and the corresponding graphs, were performed using SigmaPlot version 11.0 (Systat Software Inc., San Jose, Chicago, IL, USA). Model statistics used were Pearson's correlation coefficient (r) or coefficient of determination (R^2) and P-value.

Results

Fatty acid profiles of the experimental plant species

The total FA in the experimental plant species ranged from 3.4 (in *Persea americana*) to 24.3 g/kg DM (in *Calliandra calothyrsus*; see Table 2). Aside from *C. calothyrsus*, only *Artocarpus heterophyllus* and *Acacia mangium* contained >20 g total FA/kg DM. Within total FAME, *Carica papaya* lipids contained the highest proportion of C18:3 *n*-3, followed by those of *Leucaena diversifolia* and *Morinda citrifolia*. The proportions of total PUFA in these plant species were also high (>500 g/kg FA). Low proportions of C18:3 *n*-3 were found in *Hibiscus tiliaceus*, *Myristica fragrans*, and *Mimosa invisa*. *Hibiscus tiliaceus* was also low in PUFA proportion. The highest proportions of C18:2 *n*-6 and C18:1 *n*-9 were found in *Canna indica* and *Cycas rumphii*, respectively, whereas the lowest were found in *A. mangium* and *Acacia villosa*, respectively. Proportions of saturated fatty acids were lowest in *C. calothyrsus* and high in *H. tiliaceus*, *M. invisa*, *Erythrina orientalis*, and *Ipomoea batatas*. Some FA that were not included in Table 2 due to their small proportions are: C6:0, C8:0, C10:0, C12:0, C13:0, C14:0, C15:0, C15:1, C17:0, C17:1, C18:1 *n*-5, C18:1 *n*-7, C18:3 *n*-6, the unsaturated C20 FA, and other FA with chain length >C20.

Disappearance and appearance of fatty acids during incubation

Across all plant species investigated, the proportions of C18:3 *n*-3, C18:2 *n*-6, and C18:1 *n*-9 that had disappeared during 24 h of incubation were in the ranges of 833–987 (average of 956 ± 31), 396–760 (683 ± 75), and 100–614 (410 ± 154) mg/g, respectively (Table 3), whereas the levels of appearance of *c*9,*t*11-C18:2, *t*11-C18:1 and C18:0 ranged from 1.8 to 31.2 (11.6 ± 8.4), 64 to 181 (109 ± 29) and 177 to 694 (527 ± 112) mg/g, respectively. With most of the plants, disappearance of C18:3 *n*-3 was higher than 90%, except in the incubation of *A. villosa*, which differed significantly from the other plant species based on l.s.d. $P < 0.05$. Incubation of *A. villosa* also led to the lowest disappearance of C18:2 *n*-6, and this was different ($P < 0.05$) from the next lowest plant species, i.e. *C. calothyrsus*. The lowest disappearance of C18:1 *n*-9 was found in the incubation of *P. americana*, although the value was not significantly different from those of *M. fragrans*, *A. villosa*, *Swietenia mahagoni*, *C. calothyrsus*, and *Psidium guajava*. No significant difference across plant species was found for the appearance of *c*9,*t*11-C18:2. Appearances of the peak containing *t*11-C18:1 were found to be high in the incubations of *C. papaya*, *P. americana*, and *S. mahagoni*. Incubation of *P. americana* and *A. villosa* resulted in extremely low appearances of C18:0, the ultimate BH product of C18 FA.

Table 2. Fatty acid composition of the experimental plants (n = 2)
MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; s.e.m., standard error of the mean; SFA, saturated fatty acids

No.	Plant species	Total fatty acids (g/kg DM)	C16:0	C16:1	C18:0	C18:1 *n*-9	C18:2 *n*-6	C18:3 *n*-3	C20:0	SFA	MUFA	PUFA
			(g/kg total fatty acid methyl esters)									
1	*Acacia mangium*	23.6	228	3	61	54	61	186	38	481	94	424
2	*Acacia villosa*	16.4	177	2	80	22	114	409	34	390	62	549
3	*Albizia falcataria*	9.0	219	3	68	33	110	298	59	505	62	434
4	*Artocarpus heterophyllus*	23.8	357	2	48	55	137	234	9	523	82	395
5	*Calliandra calothyrsus*	24.3	51	20	11	30	162	376	31	167	282	551
6	*Canna indica*	10.3	348	2	30	42	214	142	16	554	77	369
7	*Carica papaya*	8.0	196	4	26	41	122	499	4	283	79	638
8	*Clidemia hirta*	12.3	215	4	37	124	168	278	23	373	159	467
9	*Cycas rumphii*	10.5	403	3	42	139	109	115	12	567	182	250
10	*Erythrina orientalis*	15.8	357	5	135	74	117	97	17	640	133	227
11	*Eugenia aquea*	10.0	281	2	76	66	186	216	19	469	101	430
12	*Hibiscus tiliaceus*	7.6	424	5	89	65	95	61	29	695	115	189
13	*Ipomoea batatas*	8.5	246	2	84	22	169	143	36	629	37	333
14	*Lantana camara*	7.3	277	3	41	46	93	305	23	459	84	457
15	*Leucaena diversifolia*	19.6	149	2	70	34	130	483	21	300	64	636
16	*Leucaena leucocephala*	8.8	212	5	96	49	85	367	33	436	83	480
17	*Manihot esculenta*	9.4	263	7	73	52	89	380	10	407	104	489
18	*Melia azedarach*	8.0	259	1	40	31	87	397	20	427	58	515
19	*Mimosa invisa*	5.6	268	5	117	69	155	80	27	642	99	259
20	*Morinda citrifolia*	4.6	290	4	32	40	97	414	29	395	66	539
21	*Myristica fragrans*	7.2	134	3	38	132	73	74	13	361	335	304
22	*Paspalum dilatatum*	5.5	275	1	47	30	188	278	17	470	51	479
23	*Persea americana*	3.4	332	6	27	55	147	184	9	457	95	448
24	*Pithecellobium jiringa*	4.3	231	1	76	131	86	193	14	402	159	439
25	*Psidium guajava*	7.3	303	3	72	65	139	184	18	540	117	343
26	*Sesbania grandiflora*	4.4	242	2	86	49	146	287	26	456	76	468
27	*Swietenia mahagoni*	6.4	281	2	57	51	148	269	13	466	88	447
	s.e.m.	1.18	15.8	0.7	5.6	6.5	7.5	24.5	2.3	22.6	12.9	22.2

Relationships between dietary phenolic fractions and ruminal fatty acid biohydrogenation

For the TEP fraction, there were clear negative relationships between TEP in the plants and the disappearance of C18:3 *n*-3, C18:2 *n*-6, and C18:1 *n*-9 (R^2 = 0.48, 0.52, and 0.58, respectively; $P < 0.001$: Fig. 1*a*). The slope of the linear regression line was highest for the disappearance of C18:1 *n*-9, followed by that of C18:2 *n*-6, and then C18:3 *n*-3. The appearance of *c*9,*t*11-C18:2 was positively correlated with TEP (R^2 = 0.30, $P < 0.01$; Fig. 1*b*), while the appearance of C18:0 was negatively correlated with TEP (R^2 = 0.22, $P < 0.05$). No significant relationship was found between TEP and the appearance of the *t*11-C18:1 peak. Avocado leaf (*P. americana*; no. 23) was notable because it resulted in the lowest C18:1 *n*-9 disappearance, and had among the highest *c*9, *t*11-C18:2 appearance and the lowest C18:0 appearance after 24 h incubation at a quite moderate TEP content.

The relationships between TT and FA in fermentation fluid revealed the same patterns as the TEP, i.e. negative correlations with the disappearances of C18:3 *n*-3, C18:2 *n*-6, and C18:1 *n*-9 (R^2 of 0.42, 0.48 and 0.44, respectively; all at $P < 0.001$), and the appearance of C18:0 (R^2 = 0.18; $P < 0.05$), but a positive correlation with the appearance of *c*9,*t*11-C18:2 (R^2 = 0.27; $P < 0.01$; Table 4). A negative correlation occurred between NTP and the disappearance of C18:1 *n*-9 ($P < 0.05$), but for other FA no influence of NTP could be found. A strong positive correlation was found between CT and the appearance of *c*9,*t*11-C18:2 ($P < 0.001$). Different from CT, no significant correlation was found between HT and *c*9,*t*11-C18:2. Instead, HT were negatively correlated with the disappearance of C18:3 *n*-3 and C18:2 *n*-6 (both at $P < 0.001$) with R^2 of 0.40 and 0.41, respectively.

Discussion

Increasing *n*-3 PUFA and/or CLA contents in ruminant products through nutritionally controlling BH has attracted more attention in recent years. Recommended nutritional strategies include feeding forage-based diets (Dewhurst *et al*. 2006; Hilario *et al*. 2010), adding various PUFA sources (Scollan *et al*. 2001; Kitessa *et al*. 2010), using protected lipids (Fievez *et al*. 2007; Doreau *et al*. 2011), and supplementing essential oils (Benchaar *et al*. 2008). Phenolic compounds seem to play a major role in this respect (Khiaosa-ard *et al*. 2009; Vasta *et al*. 2009*a*). The results of the present study provide conclusive evidence for the influence of phenolic compounds, regardless of their specific nature.

Extent of fatty acid biohydrogenation

The present findings confirmed the higher disappearance of C18:3 *n*-3 compared with that of C18:2 *n*-6, suggesting that

Table 3. Disappearance and appearance of C18 fatty acids in the fermentation fluid after 24 h *in vitro* incubation of the experimental plants ($n = 4$)
l.s.d., least significant difference; n.a., not applicable; s.e.m., standard error of the mean

No.	Plant species	Disappearance (g FA/kg fermentation fluid)			Appearance (g FA/kg fermentation fluid)		
		C18:3 *n*-3	C18:2 *n*-6	C18:1 *n*-9	*c*9,*t*11-C18:2	*t*11-C18:1[A]	C18:0
1	*Acacia mangium*	953	643	301	20.4	139	544
2	*Acacia villosa*	833	396	209	12.0	100	244
3	*Albizia falcataria*	954	657	343	4.9	74	591
4	*Artocarpus heterophyllus*	965	688	412	14.8	108	555
5	*Calliandra calothyrsus*	910	585	223	16.1	131	510
6	*Canna indica*	987	760	593	9.1	73	694
7	*Carica papaya*	976	732	577	1.8	181	543
8	*Clidemia hirta*	934	649	324	19.4	66	541
9	*Cycas rumphii*	983	756	500	5.3	90	532
10	*Erythrina orientalis*	980	708	577	4.0	101	542
11	*Eugenia aquea*	931	632	238	16.6	64	456
12	*Hibiscus tiliaceus*	982	718	399	15.1	115	523
13	*Ipomoea batatas*	976	699	591	7.0	109	605
14	*Lantana camara*	954	688	583	3.2	83	564
15	*Leucaena diversifolia*	954	716	440	5.2	109	594
16	*Leucaena leucocephala*	957	711	490	4.9	108	579
17	*Manihot esculenta*	954	713	519	6.6	115	564
18	*Melia azedarach*	966	731	476	7.3	98	587
19	*Mimosa invisa*	979	683	413	10.2	112	531
20	*Morinda citrifolia*	968	703	523	6.7	124	573
21	*Myristica fragrans*	932	610	185	31.2	106	407
22	*Paspalum dilatatum*	978	753	595	5.0	87	613
23	*Persea americana*	962	751	100	26.5	165	177
24	*Pithecellobium jiringa*	978	718	391	9.7	115	624
25	*Psidium guajava*	947	643	234	30.9	121	474
26	*Sesbania grandiflora*	967	755	614	3.3	78	644
27	*Swietenia mahagoni*	950	635	215	17.3	161	412
	s.e.m.	4.2	10.2	17.7	1.58	4.6	13.9
	P-value	<0.001	<0.001	<0.001	0.363	<0.001	<0.001
	l.s.d.	44.7	102.1	136.7	n.a.	43.5	111.5

[A]May contain other C18:1 isomers.

the extent of disappearance is higher at higher desaturation degrees of C18 FA. Similarly, employing a factorial design with two species (*Vicia sativa* and *Trifolium incarnatum*) at two phenological stages (vegetative and reproductive), Cabiddu *et al*. (2010) showed that the BH of C18:3 *n*-3 and C18:2 *n*-2 ranged from 41.5 to 80.3% and 17.9 to 57.5%, respectively, when using an *in vitro* batch culture technique. This response may be due to a preferential uptake of C18:2 *n*-6 by rumen bacteria compared with that of C18:3 *n*-3, which protects the former FA against BH, or due to differences in microbial isomerase or saturase affinity between the two FA (Jouany *et al*. 2007). The present findings also showed that the appearances of *c*9,*t*11-C18:2 and *t*11-C18:1 were relatively minor as compared with C18:0 formation. Accordingly, Fievez *et al*. (2007) reported that 66.6 and 59.9% of C18 PUFA were transformed to C18:0 under *in vitro* and *in vivo* conditions, respectively. Comparing the appearance of the BH intermediates *c*9,*t*11-C18:2 and (the peak containing) *t*11-C18:1, the extent of the former was much lower than that of the latter. This may be related to the BH pathway, i.e. *c*9,*t*11-C18:2 is formed by C18:2 *n*-6 through isomerisation whereas *t*11-C18:1 is formed not only from C18:2 *n*-6 but also from C18:3 *n*-3 BH (Jenkins *et al*. 2008). Additionally, BH of *t*11-C18:1 to C18:0 is considered to be the limiting step of the entire BH process, because fewer species of bacteria seem to be involved than in the preceding steps (Khiaosa-ard *et al*. 2009; Lourenço *et al.* 2010).

Significance of phenolic compounds for the extent of biohydrogenation

The TEP contents of tropical plants, such as those investigated here, are typically higher than those of temperate-climate plants (Fraisse *et al*. 2007; Jayanegara *et al*. 2011*a*). This is unsurprising, since phenols, like other plant secondary metabolites, are at least partly formed as a response to environmental stress (Vogt 2010), including solar ultraviolet radiation (Berli *et al.* 2011), which is more prevalent in the tropics.

The significant negative correlations between TEP and the disappearances of C18:3 *n*-3, C18:2 *n*-6, and C18:1 *n*-9 suggest that phenols may reduce FA BH right from the first step. This is in agreement with Cabiddu *et al*. (2010) who observed a negative relationship between tannins, present in *V. sativa* and *T. incarnatum*, and C18:3 *n*-3 BH, even though the tannin contents in that study were much lower (ranging between 3.6

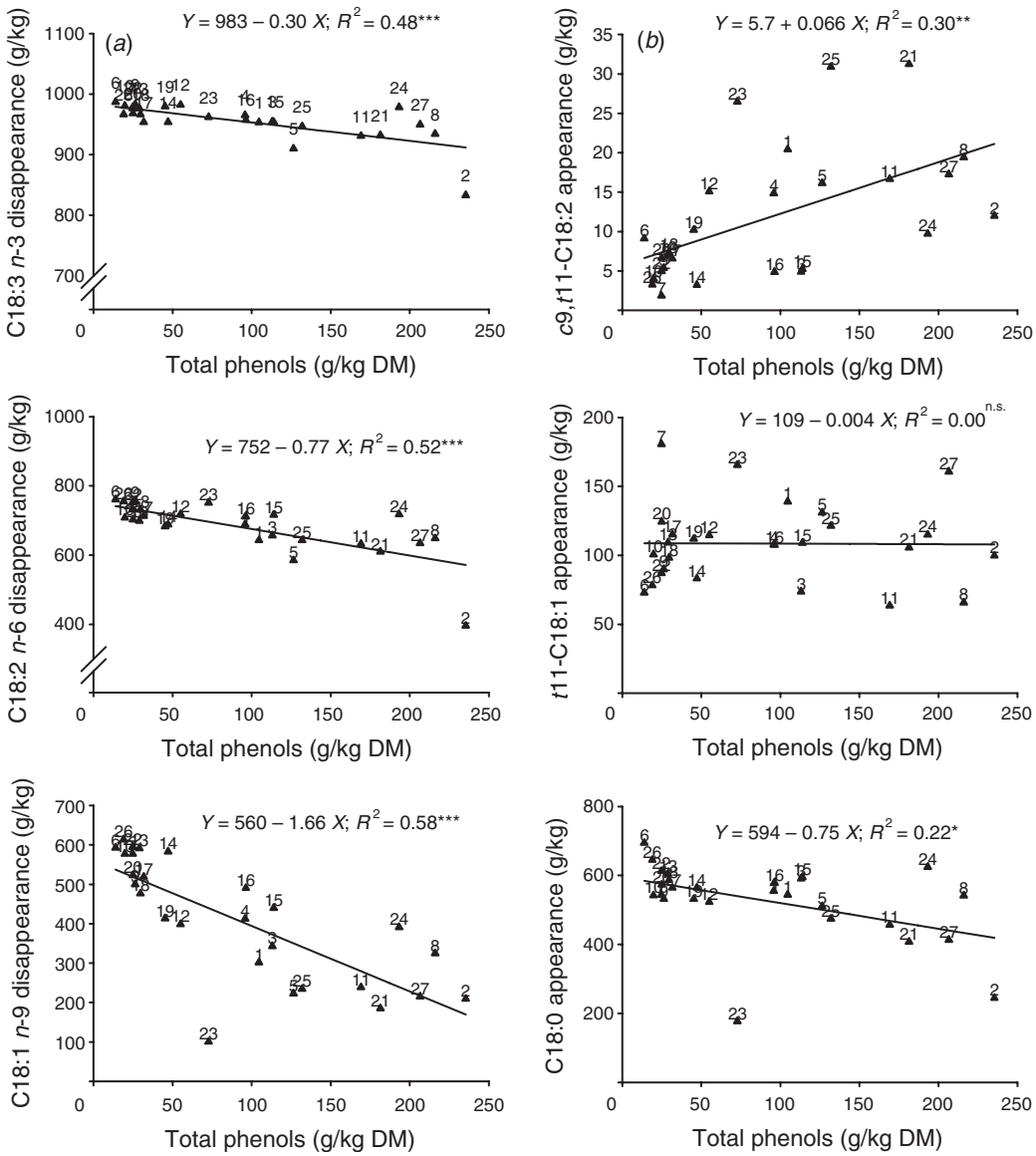

Fig. 1. Linear regressions between total phenols in plants and (*a*) disappearance of C18:3 *n*-3, C18:2 *n*-6, C18:1 *n*-9, and (*b*) appearance of *c*9,*t*11-C18:2, *t*11-C18:1, C18:0 in total fermentation fluid after 24 h incubation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant. *t*11-C18:1 may contain other *trans*-C18:1 isomers.

Table 4. Pearson correlation coefficients between plant chemical composition and disappearance and appearance of C18 fatty acids (*n* = 27)
NDF, neutral detergent fibre; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Fatty acid	Crude protein	NDF	Total phenols	Non-tannin phenols	Total tannins	Condensed tannins	Hydrolysable tannins
			Disappearance				
C18:3 *n*-3	–0.09	0.36	–0.69***	–0.25	–0.65***	–0.24	–0.63***
C18:2 *n*-6	0.02	0.26	–0.72***	–0.23	–0.69***	–0.34	–0.64***
C18:1 *n*-9	0.40*	0.01	–0.76***	–0.42*	–0.66***	–0.73***	–0.46*
			Appearance				
*c*9,*t*11-C18:2	–0.59**	0.15	0.55**	0.21	0.52**	0.64***	0.33
*t*11-C18:1[A]	0.19	–0.22	–0.01	0.02	–0.02	0.26	–0.12
C18:0	0.17	0.04	–0.47*	–0.22	–0.43*	–0.44*	–0.31

[A]May contain other C18:1 isomers.

and 4.7 g/kg DM) than those in the present study. Kälber *et al.* (2011) also hypothesised that buckwheat phenols (to a large proportion represented by HT) were responsible for the increase found in the transfer of C18:3 *n*-3 from feed to milk. This effect might be related to phenolic toxicity on bacterial species involved in FA BH, through selective inhibition of cell wall synthesis (Smith *et al.* 2005), interaction of phenols with microbial proteins (Silanikove *et al.* 2001), and direct interaction between phenols and lipids (He *et al.* 2006). The main class of phenols which prevented C18:3 *n*-3 and C18:2 *n*-6 from BH was that of the HT, while the appearance of *c*9,*t*11-C18:2 was closer correlated with the CT. This illustrates that both types of tannins are involved in the inhibition of biohydrogenation but in different steps, namely HT in the first and CT in the second step. By contrast, it has been observed *in vitro* (Khiaosa-ard *et al.* 2009) and *in vivo* (Vasta *et al.* 2009*b*) that the addition of CT extracts to the diet caused no difference in the concentration of conjugated C18:2 in the ruminal fluid, but led to a considerable increase of *t*11-C18:1 at the expense of C18:0 instead. This indicates that inhibition of the third step of BH took place. Thus, a differentiated and still not always coherent influence of different tannins on the BH pathway can be observed. For the present study, this is different from the effects of the same plants on methanogenesis, which had been similar between representatives of both types of tannins (Jayanegara *et al.* 2011*b*).

The hypothesis of Kälber *et al.* (2011) that (hydrolysable) tannins present in forages may increase the C18:3 *n*-3 transfer from feed to milk by inhibiting BH, is clearly supported by the results of the present study. However, the correlations were strongest with TEP, as also has been true for variables describing ruminal methanogenesis (Jayanegara *et al.* 2011*b*). This indicates that also NTP are influencing ruminal fermentation parameters. It has to be noted that such strong relationships cannot be found in every study (Abbeddou *et al.* 2011) and it appears that the phenolic effects are minor when the TEP concentration is below a certain threshold (Jayanegara *et al.* 2011*a*; Khiaosa-ard *et al.* 2011).

The slope between TEP and C18:3 *n*-3 does not appear to be steep, but considering the low proportion of C18:3 *n*-3 able to escape the rumen at all (Chilliard *et al.* 2007), it is nevertheless noteworthy. The finding that relationships were clearer for the substrate FA than for the BH intermediates follows the logic of BH (Jenkins *et al.* 2008), because the disappearance of the former only depends on the first BH step, while the intermediates are generated at first and subsequently partially isomerised, at which point they disappear. Against the background of decreased activity in the first step of BH, the accumulation of *c*9,*t*11-C18:2 can only be explained by an even stronger inhibition of the second step.

In addition to the general effect of phenols on FA BH, there appeared to be differences in their effect between plant species, as can be seen from the variation around the regression lines in Fig. 1*a* and Fig. 1*b*. This might be due to the difference in phenol composition and interactions with other plant compounds, leading to different digestive availability and activity among individual plant species (Mueller-Harvey 2006). Plant species that inhibited disappearance of C18:3 *n*-3 and C18:2 *n*-6 in the present study such as *A. villosa*, *Eugenia aquea* and *M. fragrans* contained substantial amounts of TEP. These plant species were also found to possess an anti-methanogenic potential (Jayanegara *et al.* 2011*b*). The results suggest that phenols are able to modulate BH and methanogenesis processes simultaneously and both towards a desirable direction. Apparently, a decrease in H_2 flow in the presence of substantial amounts of phenols to PUFA and to CO_2 may explain the respective results since H_2 is a common factor for linking both BH and methanogenesis processes (Lourenço *et al.* 2010). It would be of particular interest to identify plant species with low to moderate TEP contents but with a high efficiency in modulating FA BH. Across all plant species investigated in the present study, only *P. americana* seemed to have this property. In addition to the TEP contents, *P. americana* contains various kinds of essential oils such as β-caryophyllene, valencene and methyl eugenol (Ogunbinu *et al.* 2007; Larijani *et al.* 2010). These compounds may potentially alter FA BH by influencing microbial species involved in the respective process as well (Benchaar and Chouinard 2009; Lourenço *et al.* 2010). Although it is not possible to differentiate such effects from the effects of CT in the present study, this may explain why this plant appears as an outlier in the present collection of forage species.

This study had been designed to explore fundamentally the general relationship between plant-intrinsic phenolic compounds and the degree of BH. Every tested plant species is generally used as a forage or medical plant for ruminants in Indonesia. However, in practical feeding these plants make up only part of the diet, and effects are likely to be correspondingly smaller.

Conclusions

Phenols as constituents of a selection of tropical plant species were demonstrated to have the potential to modify ruminal FA BH towards lowering the disappearance of PUFA and the appearance of C18:0. In addition, an increase in the production of the major CLA isomer can also be expected. If this can also be confirmed *in vivo*, using such plants may have a considerable potential to improve the nutritional quality of products. The *in vitro* rumen fermentation technique applied in this study proved to be useful for identifying plant species that have a potential to inhibit ruminal BH of FA. Moreover, the concentration of TEP in tropical plant species was demonstrated to be a suitable indicator of the potential of these species to inhibit ruminal BH.

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