Livestock Science 147 (2012) 104–112

Contents lists available at SciVerse ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

# Ruminal disappearance of polyunsaturated fatty acids and appearance of biohydrogenation products when incubating linseed oil with alpine forage plant species *in vitro*

A. Jayanegara, M. Kreuzer, F. Leiber*

*ETH Zurich, Institute of Agricultural Sciences, Universitaetstrasse 2, 8092 Zurich, Switzerland*

ARTICLE INFO

*Article history:*
Received 10 October 2011
Received in revised form
12 April 2012
Accepted 18 April 2012

*Keywords:*
Mountain pasture
Biohydrogenation
Fatty acid
Phenol
Chestnut
Rumen

ABSTRACT

Elevated contents of α-linolenic and rumenic acids in dairy foods originating from alpine areas have been hypothesized to be partially due to the modulatory effect of plant secondary metabolites on ruminal biohydrogenation. The objective of the present study was to investigate the effect of some representatives of typical and abundant alpine forage plant species on ruminal fatty acid (FA) biohydrogenation and its relationship to the polyphenols, a class of plant secondary compounds, in the plants. Overall, two species of grasses, nine dicotyledonous non-leguminous herbs, three herbaceous legumes and leaves or flowers from three trees were collected from sites situated in the Swiss Alps. These plants were incubated *in vitro* at 39 °C for 24 h together with linseed oil as a source rich in polyunsaturated FA (particularly α-linolenic acid) in a rumen fluid:buffer mixture using the Hohenheim gas test method ($n=4$ replicates per plant). The plants were analyzed for their contents of different polyphenol fractions. The FA profiles of the plants, of the linseed oil and of the fermentation fluid after incubation (total syringe content) were determined by gas chromatography. From these data, the disappearances of unsaturated FA and the appearances of rumenic, vaccenic and stearic acids as biohydrogenation products were calculated. There were no differences in the disappearance of α-linolenic and linoleic acids when incubating any of the plant species, except for *Castanea sativa* where the disappearance was lower than for all other plants (both at $P<0.05$). Even though not significantly different from the other plants, the appearance of rumenic and vaccenic acids was high with *C. sativa* which led also to the lowest ($P<0.05$) appearance of stearic acid, the ultimate biohydrogenation product. Total extractable phenols, total tannins and hydrolysable tannins were significantly negatively correlated with the disappearance of α-linolenic and linoleic acids and with the appearance of stearic acid, and positively correlated with the appearance of vaccenic acid. Only hydrolysable tannins showed a clear positive correlation with the appearance of rumenic acid ($P<0.05$). No significant positive or negative correlations were instead established with the non-tannin phenols and the condensed tannins. The above mentioned relationship was, however, almost exclusively the result of including *C. sativa*. This plant might be interesting for strategic feeding aimed at elevating the contents of some beneficial FA but these results do not explain the particularly favorable FA profile of the milk obtained from ruminants fed alpine pastures.



*Abbreviations:* CLA, conjugated linoleic acid; CP, crude protein; CT, condensed tannins; DM, dry matter; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatograph; HT, hydrolysable tannins; NDF, neutral detergent fiber; NTP, non-tannin phenols; PUFA, polyunsaturated fatty acid; TEP, total extractable phenols; TT, total tannins.

* Corresponding author. Tel.: +41 44 632 8942; fax: +41 44 632 1128.
*E-mail address:* florian.leiber@usys.ethz.ch (F. Leiber).

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http://dx.doi.org/10.1016/j.livsci.2012.04.009

## 1. Introduction

Alpha-linolenic acid (C18:3 *n*-3) and rumenic acid (*c*9,*t*11 C18:2), the most abundant among conjugated linoleic acid (CLA) isomers, are considered to be beneficial for human health (Barcelo-Coblijn and Murphy, 2009; Benjamin and Spener, 2009). Milk and cheese originating from cows grazing on alpine areas have been reported to contain higher amounts of these FA, especially when compared to those from intensive lowland production systems (Collomb et al., 2002; Kraft et al., 2003). Also pasture vegetation type (Povolo et al., 2012) and season of mountain grazing (Leiber et al., 2009) may influence the milk FA profiles. Even though a higher intake of C18:3 *n*-3 may increase its occurrence in dairy foods (Dewhurst et al., 2006), Leiber et al. (2004, 2005) found C18:3 *n*-3 levels in milk to be independent from intake when testing forages of alpine origin. One hypothesis to explain this phenomenon was that these biodiverse swards may contain plants with various and elevated levels of plant secondary compounds (Leiber et al., 2005). There is evidence that polyphenols (a group of plant secondary compounds), particularly the tannins, are able to reduce the extent of ruminal biohydrogenation of polyunsaturated FA (PUFA) at various steps of the pathways (Cabiddu et al., 2010; Jayanegara et al., 2011a; Khiaosa-ard et al., 2009; Vasta et al., 2009). This would explain a higher bypass of C18:3 *n*-3 through the rumen, as well as a higher concentration of vaccenic acid (*t*11 C18:1) serving as the precursor for *c*9,*t*11 C18:2 synthesis within the mammary gland. Apparently, the effects are related to the toxicity that certain phenols exhibit on microbial species that are involved in different steps of FA biohydrogenation (Khiaosa-ard et al., 2009; Lourenço et al., 2010; Vasta et al., 2010). Consistent with that, a comparably high content of total extractable phenols (TEP) in fresh buckwheat forage was associated with an increased transfer rate of C18:3 *n*-3 from feed to milk of dairy cows (Kälber et al., 2011).

Only few studies have attempted to investigate the relationships between polyphenols in alpine forage plants and FA biohydrogenation in the rumen. Khiaosa-ard et al. (2011) observed a clear positive effect of grazing alpine pasture on the concentrations of *t*11 C18:1 in the rumen fluid; however effects on ruminal C18:3 *n*-3 concentrations could not be found either *in vivo* or by *in vitro* incubation of alpine and lowland hay. These inconsistencies warrant a closer investigation as animals grazing these pastures may actually select for (some) plants rich in polyphenols which are known to exist on such grasslands (Fraisse et al., 2007). Therefore, individual alpine forage plant species may affect FA biohydrogenation differently from each other, of which the effect could not be observed when they are investigated as a mixture. Identification of alpine plant species that are responsible for elevating beneficial FA in the products and/or the rumen is important to be investigated since the outcome may potentially contribute to the improvement of alpine feeding strategies.

The main objective of the present study was, therefore, to collect specimen from various abundant alpine forage plant species and test their individual effect and, additionally, to establish general relationships between polyphenol fractions and ruminal FA biohydrogenation. The plant species collected included grasses, leguminous herbs, other herbs and trees to ensure the representation of all major functional groups of forage species. Linseed oil was added to the incubations as a PUFA source. Emphasis was put on the disappearance of the unsaturated FA C18:1 *n*-9, C18:2 *n*-6 and C18:3 *n*-3, and the appearance of important biohydrogenation products (*c*9,*t*11 C18:2, *t*11 C18:1 and C18:0).

## 2. Materials and methods

### 2.1. Experimental plants

Samples from 18 different plant species (Table 1) were collected in early July 2009 from Misox valley, Rhine forest (Sufers) and Albula valley located in the canton of Grisons, south-eastern Switzerland, at altitudes of 800, 1400 and 1800–2300 m above sea level, respectively. The time point was chosen in order to evaluate the plants when the majority was in the flowering stage, because it is a typical characteristic of alpine pastures, that they are grazed in the phenological stage of flowering, other than intensively managed lowland pastures (Leiber et al., 2005). The plants were classified into four functional groups, i.e. grasses, dicotyledonous non-leguminous herbs, leguminous herbs and trees. Leaves or flowers of the tree species were included because they had been commonly fed to domestic ruminants in the alpine region in former times and were supposed to contain high levels of phenolic compounds. Grasses and herbs were cut at 1 cm above ground; from trees only leaves or flowers were harvested. Approximately 0.5 kg of fresh matter was collected from each plant species. All plants were sampled from at least five plots within areas of at least 1 ha at the respective sites. After cutting, plants were pooled into one sample per species. After collection, all plant samples were stored at 4 °C overnight, oven dried at 60 °C for 24 h and ground to pass a 1-mm sieve. The material was the same as that used for the 2009 data in Jayanegara et al. (2011b).

### 2.2. In vitro incubation

Amounts of 200 mg of dry matter (DM) of the plants were subjected to *in vitro* incubation in 30 ml rumen fluid:buffer mixture (1:2 v/v) in a special glass syringe by following the protocol of Menke and Steingass (1988), and kept at 39 °C for 24 h. Additionally, always 50 mg linseed oil per g plant DM, emulsified in 1:99 v/v aqueous solution of Tween 80 (Sigma-Aldrich, Inc., St. Louis, Missouri, USA; Khiaosa-ard et al., 2010) was added. The incubation was done in four subsequent runs for each species, represented by one syringe per run. The donor of the rumen fluid was a rumen-fistulated Brown Swiss cow (maintained according to Swiss guidelines for animal welfare) which consumed white clover-ryegrass hay (*ad libitum*) as well as 0.5 kg/day of dairy concentrate (UFA 149; containing 7.35 MJ net energy for lactation and 390 g crude protein per kg; UFA AG, Herzogenbuchsee, Switzerland). Prior to use, the rumen fluid (containing solid particles from the feeds consumed) had been strained

**Table 1**
Contents (mg/g dry matter) of crude protein, neutral detergent fiber and polyphenols of the experimental plants[a].
*Source*: Jayanegara et al. (2011b)

| No. | Plant species | Site | Phenological stage | Crude protein | NDF | Ether extract | Total phenols | Non-tannin phenols | Total tannins | Condensed tannins | Hydrolysable tannins |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Grasses | | | | | | | | | | |
| 1 | *Nardus stricta* | Albula | Bu | 118 | 741 | 3 | 7 | 7 | nd | nd | nd |
| 2 | *Poa alpina* | Albula | Fl | 130 | 501 | 16 | 18 | 14 | 4 | nd | 4 |
| | Dicotyledonous non-leguminous herbs | | | | | | | | | | |
| 3 | *Achillea millefolium* | Misox | Bu | 215 | 393 | 15 | 12 | 9 | 3 | nd | 3 |
| 4 | *Alchemilla xanthochlora* | Albula | Fr | 162 | 230 | 19 | 55 | 21 | 34 | 1 | 33 |
| 5 | *Capsella bursa-pastoris* | Albula | Fl/Fr | 224 | 293 | 18 | 18 | 15 | 3 | nd | 3 |
| 6 | *Carum carvi* | Sufers | Fr | 128 | 375 | 16 | 18 | 12 | 6 | nd | 6 |
| 7 | *Chrysanthemum adustum* | Albula | Fl | 89 | 377 | 9 | 17 | 12 | 5 | nd | 5 |
| 8 | *Crepis aurea* | Albula | Fl | 136 | 324 | 37 | 19 | 14 | 5 | 1 | 4 |
| 9 | *Plantago atrata* | Albula | Fl | 117 | 463 | 9 | 21 | 13 | 8 | nd | 8 |
| 10 | *Rhinanthus alectorolophus* | Albula | Fl | 151 | 291 | 17 | 29 | 19 | 10 | 1 | 9 |
| 11 | *Rumex arifolius* | Albula | Fr | 121 | 387 | 17 | 33 | 15 | 18 | 5 | 13 |
| | Leguminous herbs | | | | | | | | | | |
| 12 | *Anthyllis vulneraria* | Albula | Fl | 129 | 365 | 11 | 26 | 16 | 10 | 2 | 8 |
| 13 | *Hedysarum hedysaroides* | Albula | Fl | 207 | 317 | 17 | 67 | 32 | 35 | 9 | 26 |
| 14 | *Trifolium badium* | Albula | Fl | 139 | 315 | 11 | 41 | 21 | 20 | 5 | 15 |
| | Trees | | | | | | | | | | |
| 15 | *Castanea sativa* (leaves) | Misox | | 129 | 359 | 22 | 92 | 13 | 79 | 1 | 78 |
| 16 | *Fraxinus excelsior* (leaves) | Misox | | 179 | 442 | 17 | 13 | 12 | 1 | nd | 1 |
| 17 | *Sambucus nigra* (leaves) | Sufers | | 234 | 245 | 33 | 22 | 22 | nd | nd | nd |
| 18 | *Sambucus nigra* (flowers) | Sufers | | 257 | 263 | 35 | 40 | 28 | 12 | 1 | 11 |

Bu, budding; Fl, flowering; Fr, fruiting; nd, not detected; NDF, neutral detergent fiber.
[a] The analyses were done in duplicate except for that of NDF which was done in triplicate.

through four layers of gauze (1 mm pore size, Type 17 MedPro; Novamed AG, Flawil, Switzerland). Parallel to the incubation of the plant samples, 200 mg DM of standard hay and standard concentrate each (obtained from Institute of Animal Nutrition, University of Hohenheim, Stuttgart, Germany) with known amounts of gas production was incubated in triplicate in each run for controlling that incubation was successful (Menke and Steingass, 1988). After terminating the incubation, the total syringe content (fermentation fluid and feed residues) was collected and stored at −20 °C until use. Additionally, the buffered rumen fluid was sampled before incubation (0 h) to determine the initial FA profiles which later were used for calculating the 'disappearance' and 'appearance' of FA. The pH was monitored in all samples with a potentiometer (model 632; Metrohm, Herisau, Switzerland) in order to ensure that it remained in a range where ruminal conditions were optimal.

### 2.3. Chemical analyses of plants

Plant samples were analyzed for their crude protein (CP; AOAC, 1997; AOAC no. 977.02) contents using a C/N analyzer (Leco-Analysator Typ FP-2000, Leco Instrumente GmbH, Kirchheim, Germany). Analysis of neutral detergent fiber (NDF) was done according to Van Soest et al. (1991) using the Fibertec apparatus (Fibertec System M, Tecator, 1020 Hot Extraction, Flawil, Switzerland). For that purpose α-amylase (Termamyl 120L, type S, Novo Nodirsk A/S, Bagsvaerd, Denmark) but no sodium sulfite was added, and the result was expressed exclusive of residual ash.

Prior to the determinations, polyphenols in the plants 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) as well as the polyphenol fractions non-tannin phenols (NTP), total tannins (TT) and condensed tannins (CT) were then analyzed based on standard protocols (Makkar, 2003). Accordingly, a modified Folin-Ciocalteu method was applied for TEP, NTP and TT (expressed as gallic acid equivalents). In brief, from 0.02 to 0.1 ml of the extract (depending on the concentrations of TEP and TT in the sample) was put in a test tube, and supplemented with distilled water to 0.5 ml. Then 0.25 ml of Folin reagent

(1 N; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 1.25 ml of sodium carbonate solution (40 g $Na_2CO_3 \cdot 10H_2O$) were added. The tube was vortexed and kept in the dark for 40 min. The absorbance was read at 725 nm using a UV–vis spectrophotometer (Shimadzu UV-160, Shimadzu Corporation, Kyoto, Japan). The butanol-HCl-iron method was used to analyze CT (expressed as leucocyanidin equivalents; Makkar, 2003). Briefly, a total of 0.25 ml of the extract was mixed with aqueous acetone (7:3 v/v) in a glass tube, where the ratio of extract to acetone depended on CT concentration in the sample. An amount of 50 μl of ferric reagent (2.0 g ferric ammonium sulfate in 100 ml of 2 N 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. All chemical analyses were conducted in duplicate except for NDF (in triplicate) and the results are presented in Table 1.

### 2.4. Fatty acid determination

The FA in the plant samples were analyzed after being extracted (in duplicate) with hexane:propan-2-ol (3:2 v/v) using an accelerated solvent extraction apparatus (ASE 200, Dionex Corporation, Sunnyvale, CA, USA). An internal standard solution (1 mg C19:0/ml dichloromethane; Sigma-Aldrich GmbH, Buchs, Switzerland) was used to collect the ASE extract. The solvent was evaporated under nitrogen stream, and the extract was collected in 2 ml dichloromethane. The extract was then mixed with distilled water (1:10). Fatty acids present in the samples obtained from the syringes (fermentation fluid and the feed residues) were mixed with the same internal standard solution as used for the plant extracts (10:1). Ten mg of linseed oil were diluted in 1 ml of internal standard solution. Subsequently all these samples were extracted and transesterified to FA methyl esters (FAME) using a non-chlorinated technique as described by Khiaosa-ard et al. (2009). Briefly, the samples were hydrolyzed with 10 M NaOH, acidified by 6 M HCl to release free FA, and then extracted by propan-2-ol and cyclohexane twice. The methylation step to generate FAME was carried out by adding 2 M trimethylsilyl-diazomethane dissolved in hexane, followed by purification using thin-layer chromatography. Separation of the FAME from plants, linseed oil and fermentation fluid lipids was done using a gas chromatograph (GC, 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 ionization detector. The GC settings were the same as described by Khiaosa-ard et al. (2009). A mixed FAME standard (Supelco 37 Component, Supelco Inc., Bellefonte, PA, USA) was used for the identification of individual FA. Additionally, identification of the peaks not present in the standard was conducted with GC–mass spectroscopy, and confirmed by comparison with the chromatograms published by Kramer et al. (2002). It is likely that the peak of *t*11 C18:1 also contained *t*10 and *t*12 C18:1 due to similar retention times even on a 60 m Supelcowax capillary column (Kramer et al., 2002). Further, the peak of *c*9,*t*11 C18:2 likely contained *t*7,*c*9 C18:2 and *t*8,*c*10 C18:2 (Kraft et al., 2003). However, the amounts of these additional isomers can be assumed to be much lower than the main ones (Jouany et al., 2007). It has to be mentioned that many other intermediates of ruminal biohydrogenation are likely to occur (Jouany et al., 2007; Lee and Jenkins, 2011), which could not be clearly enough separated and identified with the column used in the current study. The focus of the current study had rather been put on the main relevant isomers for milk production on alpine pastures (Collomb et al., 2002; Kraft et al., 2003).

### 2.5. Calculations and statistical analysis

Data of FA in the syringes are presented in the tables as the relative differences between the corresponding amounts before (0 h) and after incubation (24 h; adjusted by the recovery of total FA). These calculations considered all FA sources in the syringes, i.e. the experimental plants, linseed oil and incubation medium at 0 h. The proportionate 'disappearances' (*D*) of C18:3 *n*-3, C18:2 *n*-6 and C18:1 *n*-9 FA during the incubation were calculated with the following equations:

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

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

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

Opposite to these FA, the proportionate 'appearances' (*A*) of *c*9,*t*11 C18:2, *t*11 C18:1 and C18:0 during the incubation were computed as:

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

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

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

A mixed model of analysis of variance was employed as the statistical model to the data obtained. The experimental plants ($n=18$) were treated as fixed effects while the different incubation runs ($n=4$) were considered as random effects and served as blocks. Difference among means of the fixed effects were further assessed by a post-hoc test, i.e. Tukey's test at $P<0.05$. Correlation and regression analyses, based on data averaged per plant, were performed with the chemical composition of the plants and the disappearance and appearance of the FA. All these statistical analyses were performed using SPSS statistical software version 17.0 (SPSS Inc., 2008).

## 3. Results

### 3.1. Chemical composition and fatty acid profile of the alpine plants

Amongst the plants investigated, *Sambucus nigra* (both leaves and flowers), *Achillea millefolium*, *Capsella bursa-pastoris* and *Hedysarum hedysaroides* contained high CP contents, i.e. >200 mg/g DM (Table 1). The two grass species, i.e. *Nardus stricta* and *Poa alpina* were particularly high in NDF. With regard to polyphenol contents, the highest concentration of TEP was obtained in *Castanea sativa*, and the value was much higher than for the other plant species. Hydrolysable tannins were the predominant fraction in *C. sativa*, comprising of 85% of the TEP. Appreciable amounts of TEP were also found in *H. hedysaroides* and *Alchemilla xanthochlora*. None of the species contained substantial amounts of CT.

Within grass species, *P. alpina* contained more total FA than *N. stricta* (Table 2). The former species had a higher PUFA proportion (both C18:3 *n*-3 and C18:2 *n*-6) and a lower proportion of saturated fatty acid (SFA) compared to the latter. Among the dicotyledonous non-leguminous and leguminous herbs, total FA ranged from 10.7 (in *Chrysanthemum adustum*) to 21.8 mg/g DM (in *C. bursa-pastoris*). Among the herbs, the highest PUFA proportion was found in *A. xanthochlora*; this plant had also the highest C18:3 *n*-3 proportion and the lowest SFA proportion. *Crepis aurea* had the highest proportions of C18:0 and C16:0 among the herbs. Within the tree species investigated, *Fraxinus excelsior* was found to possess the highest PUFA and C18:3 *n*-3 proportions and the lowest SFA proportion. Some FA, i.e. C12:0, 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 were classified as "others" in Table 2. Linseed oil contained 48, 32, 164, 153 and 585 mg/g total FAME of C16:0, C18:0, C18:1 *n*-9, C18:2 *n*-6 and C18:3 *n*-3, respectively.

### 3.2. Disappearance and appearance of fatty acids during incubation

The C18:3 *n*-3, mainly originating from the linseed oil, disappeared during 24 h incubation at proportions being almost always above 900 mg/g (Table 3). This was not different among plant species even when they were from different functional groups. One exception were the *C. sativa* leaves where the C18:3 *n*-3 disappearance was lower ($P<0.05$) than with the other plants. A similar pattern was observed for the disappearance of C18:2 *n*-6 as it was also only reduced by the incubation of *C. sativa* ($P<0.05$). This pattern was not found in the case of C18:1 *n*-9. Here, incubation with *Carum carvi* resulted in a disappearance which was lower ($P<0.05$) than with *C. sativa* leaves and *S. nigra* flower. The appearances of *c*9,*t*11 C18:2 and *t*11 C18:1 were not different among plants incubated. The lowest ($P<0.05$) appearance of C18:0, the ultimate biohydrogenation product, was found when incubating with *C. sativa*.

**Table 2**
Composition of fatty acids of the experimental plants[a].

| No. | Plant species | Total fatty acids | C16:0 | C16:1 *n*-9 | C18:0 | C18:1 *n*-9 | C18:2 *n*-6 | C18:3 *n*-3 | C20:0 | Others[b] | SFA | MUFA | PUFA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (mg/g DM) | (mg/g total fatty acid methyl esters) | | | | | | | | | | |
| | Grasses | | | | | | | | | | | | |
| 1 | *Nardus stricta* | 4.6 | 305 | 35 | 36 | 62 | 106 | 202 | 33 | 201 | 558 | 130 | 312 |
| 2 | *Poa alpina* | 12.4 | 168 | 21 | 24 | 29 | 152 | 489 | 12 | 83 | 270 | 85 | 645 |
| | Dicotyledonous non-leguminous herbs | | | | | | | | | | | | |
| 3 | *Achillea millefolium* | 14.3 | 179 | 36 | 14 | 37 | 185 | 462 | 4 | 55 | 239 | 108 | 653 |
| 4 | *Alchemilla xanthochlora* | 18.6 | 110 | 29 | 23 | 16 | 106 | 620 | 10 | 57 | 184 | 83 | 733 |
| 5 | *Capsella bursa-pastoris* | 21.8 | 162 | 7 | 31 | 30 | 167 | 453 | 7 | 122 | 237 | 136 | 627 |
| 6 | *Carum carvi* | 17.0 | 190 | 14 | 14 | 24 | 365 | 260 | 8 | 109 | 264 | 104 | 632 |
| 7 | *Chrysanthemum adustum* | 10.7 | 165 | 15 | 20 | 36 | 286 | 392 | 8 | 68 | 246 | 72 | 682 |
| 8 | *Crepis aurea* | 19.4 | 207 | 21 | 42 | 23 | 261 | 278 | 13 | 139 | 390 | 67 | 543 |
| 9 | *Plantago atrata* | 10.9 | 158 | 15 | 24 | 25 | 178 | 503 | 10 | 68 | 239 | 74 | 687 |
| 10 | *Rhinanthus alectorolophus* | 18.3 | 171 | 16 | 22 | 18 | 145 | 548 | 7 | 53 | 237 | 61 | 702 |
| 11 | *Rumex arifolius* | 19.7 | 149 | 22 | 12 | 62 | 250 | 436 | 4 | 48 | 198 | 107 | 695 |
| | Leguminous herbs | | | | | | | | | | | | |
| 12 | *Anthyllis vulneraria* | 14.2 | 186 | 18 | 32 | 36 | 170 | 414 | 13 | 114 | 333 | 78 | 589 |
| 13 | *Hedysarum hedysaroides* | 15.4 | 159 | 21 | 31 | 15 | 145 | 529 | 21 | 65 | 267 | 55 | 678 |
| 14 | *Trifolium badium* | 10.8 | 174 | 21 | 36 | 31 | 189 | 401 | 30 | 103 | 327 | 76 | 597 |
| | Trees | | | | | | | | | | | | |
| 15 | *Castanea sativa* (leaves) | 13.9 | 162 | 46 | 17 | 56 | 111 | 472 | 26 | 86 | 276 | 137 | 587 |
| 16 | *Fraxinus excelsior* (leaves) | 21.7 | 131 | 34 | 19 | 76 | 62 | 623 | 4 | 32 | 174 | 135 | 691 |
| 17 | *Sambucus nigra* (leaves) | 23.5 | 151 | 38 | 14 | 20 | 103 | 571 | 7 | 68 | 204 | 117 | 679 |
| 18 | *Sambucus nigra* (flowers) | 17.2 | 218 | 3 | 13 | 126 | 202 | 285 | 8 | 131 | 295 | 214 | 491 |

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
[a] Analysis was done in duplicate.
[b] The sum of C12:0, C14:0, C15:0, 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.

**Table 3**
Disappearance and appearance of C18 fatty acids (mg/g) in the fermentation fluid after 24 h *in vitro* incubation of the experimental plants ($n=4$).

| No. | Plant species | Disappearance | | | Appearance | | |
|---|---|---|---|---|---|---|---|
| | | C18:3 *n*-3 | C18:2 *n*-6 | C18:1 *n*-9 | *c*9,*t*11 C18:2[1] | *t*11 C18:1[2] | C18:0 |
| | Grasses | | | | | | |
| 1 | *Nardus stricta* | 982$^{b}$ | 751$^{b}$ | 387$^{abc}$ | 17.3 | 118 | 430$^{bc}$ |
| 2 | *Poa alpina* | 980$^{b}$ | 753$^{b}$ | 118$^{ab}$ | 14.7 | 91 | 465$^{c}$ |
| | Dicotyledonous non-leguminous herbs | | | | | | |
| 3 | *Achillea millefolium* | 969$^{b}$ | 720$^{b}$ | 180$^{abc}$ | 25.2 | 130 | 403$^{bc}$ |
| 4 | *Alchemilla xanthochlora* | 967$^{b}$ | 711$^{b}$ | 156$^{abc}$ | 39.3 | 161 | 356$^{bc}$ |
| 5 | *Capsella bursa-pastoris* | 964$^{b}$ | 687$^{b}$ | 256$^{abc}$ | 16.4 | 164 | 231$^{ab}$ |
| 6 | *Carum carvi* | 981$^{b}$ | 765$^{b}$ | 92$^{a}$ | 16.5 | 127 | 382$^{bc}$ |
| 7 | *Chrysanthemum adustum* | 977$^{b}$ | 703$^{b}$ | 298$^{abc}$ | 25.0 | 122 | 315$^{bc}$ |
| 8 | *Crepis aurea* | 976$^{b}$ | 768$^{b}$ | 223$^{abc}$ | 10.8 | 136 | 409$^{bc}$ |
| 9 | *Plantago atrata* | 969$^{b}$ | 706$^{b}$ | 227$^{abc}$ | 20.7 | 112 | 420$^{bc}$ |
| 10 | *Rhinanthus alectorolophus* | 972$^{b}$ | 699$^{b}$ | 171$^{abc}$ | 15.5 | 105 | 447$^{c}$ |
| 11 | *Rumex arifolius* | 979$^{b}$ | 764$^{b}$ | 165$^{abc}$ | 21.8 | 165 | 410$^{bc}$ |
| | Leguminous herbs | | | | | | |
| 12 | *Anthyllis vulneraria* | 975$^{b}$ | 748$^{b}$ | 238$^{abc}$ | 24.0 | 155 | 438$^{c}$ |
| 13 | *Hedysarum hedysaroides* | 960$^{b}$ | 692$^{b}$ | 215$^{abc}$ | 29.5 | 166 | 380$^{bc}$ |
| 14 | *Trifolium badium* | 975$^{b}$ | 730$^{b}$ | 226$^{abc}$ | 21.7 | 166 | 411$^{bc}$ |
| | Trees | | | | | | |
| 15 | *Castanea sativa* (leaves) | 791$^{a}$ | 454$^{a}$ | 434$^{c}$ | 37.3 | 227 | 92$^{a}$ |
| 16 | *Fraxinus excelsior* (leaves) | 949$^{b}$ | 684$^{b}$ | 112$^{ab}$ | 34.6 | 124 | 396$^{bc}$ |
| 17 | *Sambucus nigra* (leaves) | 969$^{b}$ | 712$^{b}$ | 114$^{ab}$ | 40.7 | 127 | 409$^{bc}$ |
| 18 | *Sambucus nigra* (flowers) | 970$^{b}$ | 744$^{b}$ | 421$^{bc}$ | 14.6 | 161 | 439$^{c}$ |
| SEM | | 6.1 | 10.5 | 17.4 | 1.93 | 9.5 | 13.4 |
| *P*-value | | $<0.001$ | $<0.001$ | 0.001 | 0.085 | 0.580 | $<0.001$ |

SEM, standard error of the mean. $^{a,b,c}$ Numbers in one column, carrying different superscripts, are significantly different at $P<0.05$.
[1] May contain *t*7,*c*9 C18:2 and *t*8,*c*10 C18:2.
[2] May contain other *t*10 and *t*12 C18:1 isomers.

**Table 4**
Pearson correlation coefficients between plant chemical composition and disappearance as well as appearance of C18 fatty acids ($n=18$).

| Fatty acid | Crude protein | NDF | Total phenols | Non-tannin phenols | Total tannins | Condensed tannins | Hydrolysable tannins |
|---|---|---|---|---|---|---|---|
| Disappearance | | | | | | | |
| C18:3 *n*-3 | 0.05 | 0.09 | −0.72$^{**}$ | 0.07 | −0.84$^{***}$ | 0.02 | −0.88$^{***}$ |
| C18:2 *n*-6 | 0.00 | 0.13 | −0.70$^{**}$ | 0.03 | −0.80$^{***}$ | 0.03 | −0.83$^{***}$ |
| C18:1 *n*-9 | −0.01 | 0.19 | 0.37 | 0.02 | 0.41 | −0.01 | 0.43 |
| Appearance | | | | | | | |
| *c*9,*t*11 C18:2[a] | 0.18 | −0.27 | 0.46 | 0.18 | 0.46 | 0.09 | 0.47$^{*}$ |
| *t*11 C18:1[b] | 0.16 | −0.39 | 0.81$^{***}$ | 0.30 | 0.81$^{***}$ | 0.42 | 0.79$^{***}$ |
| C18:0 | 0.03 | 0.19 | −0.57$^{*}$ | 0.14 | −0.69$^{**}$ | 0.07 | −0.73$^{**}$ |

NDF, neutral detergent fiber.
[a] May contain *t*7,*c*9 C18:2 and *t*8,*c*10 C18:2.
[b] May contain other *t*10 and *t*12 C18:1 isomers.
$^{*}$ $P<0.05$.
$^{**}$ $P<0.01$.
$^{***}$ $P<0.001$.

### 3.3. Relationships between phenols in alpine plants and ruminal biohydrogenation of unsaturated fatty acids

Crude protein and NDF were not significantly correlated with any of the considered C18 FA (Table 4). Among the phenol fractions, the TEP were negatively correlated with the disappearance of C18:3 *n*-3 and C18:2 *n*-6 (both at $P<0.01$), but not with that of C18:1 *n*-9. The TEP were positively correlated with the appearance of *t*11 C18:1 ($P<0.001$) but negatively with C18:0 appearance ($P<0.05$). When looking at the phenol fractions it became clear that TT and, within TT, the HT explained these relationships. It appeared that HT demonstrated a similar pattern of correlation to TT. Additionally, the positive correlation with the appearance of *c*9,*t*11 C18:2 was significant with HT ($P<0.05$). Different from that, there were no significant correlations with the disappearance and appearance of the respective FA with NTP and CT. The regression between TT and C18:3 *n*-3 disappearance followed a quadratic pattern rather than a linear one, and this was also the case for that of C18:2 *n*-6 (Fig. 1). The figure also shows that *C. sativa* was the plant determining most of the correlations.

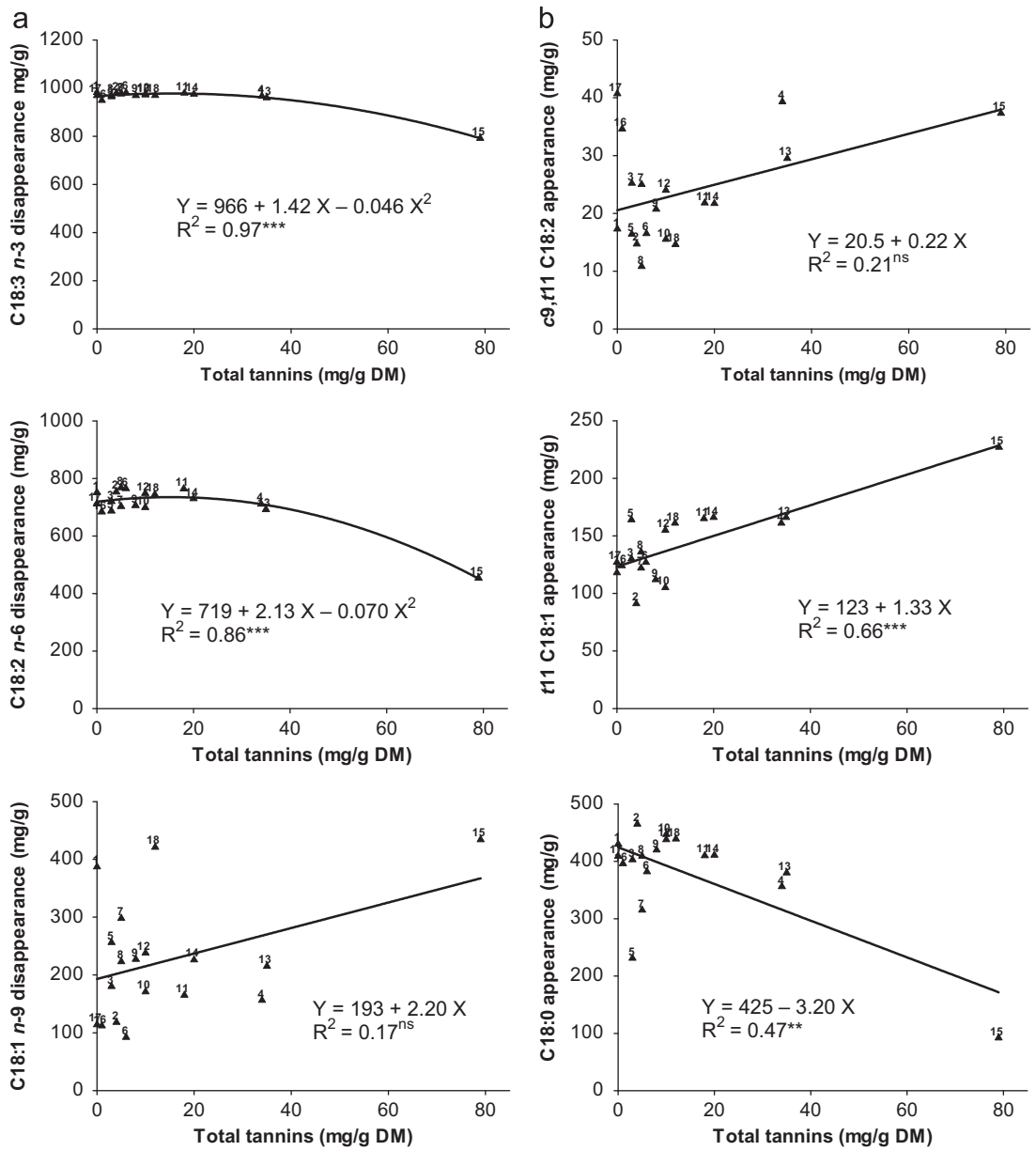


**Fig. 1.** Regressions between total tannins in experimental plants and (a) disappearance of C18:3 *n*-3, C18:2 *n*-6 and C18:1 *n*-9, as well as (b) appearance of *c*9,*t*11 C18:2, *t*11 C18:1 and C18:0 in fermentation fluid after 24 h incubation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. *t*11 C18:1 may contain *t*10 and *t*12 C18:1 isomers. *c*9,*t*11 C18:2 may contain *t*7,*c*9 C18:2 and *t*8,*c*10 C18:2. The number codes refer to plant species (see Table 1).

## 4. Discussion

Since it has been hypothesized that plant secondary compounds may play a role for the elevated contents of C18:3 *n*-3 and *c*9,*t*11 C18:2 occurring in the alpine dairy foods (Falchero et al., 2010; Leiber et al., 2005), no direct evidence for that has evolved. The present study attempted to provide such evidence by collecting various alpine forage plant species from different functional botanical groups, measuring their polyphenol contents (one of the most important groups of plant secondary compounds) and incubating them with buffered-rumen fluid and linseed oil *in vitro*.

The variation in the polyphenol contents of the alpine plants investigated was smaller than what had been expected from the literature (Fraisse et al., 2007). This was also the case for the resulting disappearance and appearance of FA during incubation. Only *A. xanthochlora*, *H. hedysaroides* and *C. sativa* (chestnut) leaves contained more than 50 g TEP/kg DM. Regarding the FA biohydrogenation, *C. sativa* was the only plant with a distinct property, which got particularly obvious in the lower disappearance of C18:3 *n*-3 and C18:2 *n*-6 and appearance of C18:0. It is likely that this can be attributed to the high polyphenol contents of this plant, especially with respect to the HT fraction. Although *C. sativa* contained

considerably less TEP and also HT than several tropical plants (cf. Jayanegara et al., 2011a), it exhibited a comparably pronounced effect like the most effective of these plants in terms of protection of C18:3 *n*-3 and C18:2 *n*-6 from biohydrogenation. This suggests that the compounds present in *C. sativa* are particularly bioactive. The main phenolic compound in *C. sativa* was HT, which, according to Jayanegara et al. (2011a) is sometimes associated with low C18:3 *n*-3 and C18:2 *n*-6 disappearance and sometimes not. It therefore remains open, whether HT or a certain interaction with other compounds or a non-phenolic (and therefore not analyzed) compound are responsible for the given effect. In agreement with the present results, Cabiddu et al. (2010) observed a negative relationship between polyphenols present in *Vicia sativa* as well as *Trifolium incarnatum* and the biohydrogenation of C18:3 *n*-3. Also Jayanegara et al. (2011a), using rumen fluid from the same donor animal as in the present study, found a clear negative correlation of TEP concentration with biohydrogenation when incubating tropical feedstuffs, which, however, contained clearly higher amounts of these secondary compounds. Toxicity of polyphenols to some bacteria species involved in FA biohydrogenation may explain the reduced disappearance of PUFA (Vasta et al., 2010). However, supplementation of commercially available extracts of tannins, i.e. a 1:1 mixture of *C. sativa* and *Schinopsis lorentzii* (quebracho, a source of CT) at a level of 10 mg/g DM to a diet containing sunflower oil did not alter the proportions of the major FA classes in milk (PUFA, MUFA and SFA) as well as the proportions of *c*9,*t*11 C18:2 and *t*11 C18:1 in milk (Toral et al., 2011). The low dose of the tannin mixture in that study was presumed to have been the reason for the lack of change in the FA measured by Toral et al. (2011). It appears that the levels of polyphenols in diets as well as their forms and the sources from where they were obtained are among the factors of influence affecting the biohydrogenation of FA and general rumen fermentation as the example of *C. sativa* in comparison with the tropical sample plants (Jayanegara et al., 2011a) shows.

Many of the studies investigating the influence of plant polyphenols on FA composition, either in the rumen or in the products of animals, *in vitro* or *in vivo*, have been focusing on CT rather than HT or other phenol fractions (Cabiddu et al., 2009; Jeronimo et al., 2010; Khiaosa-ard et al., 2009; Kronberg et al., 2007; Vasta et al., 2009). In the present study, no relationship of CT and biohydrogenation was observed. But this is rather due to the low amounts of CT in the plants investigated ( $< 10$ mg/g DM). On the other hand, the present study indicated that a certain inhibition of different steps in the biohydrogenation cascade might also be possible by HT as was also indicated in the study of Khiaosa-ard et al. (2011). Using 27 different tropical plant species and incubating them *in vitro* with linseed oil, Jayanegara et al. (2011a) found that both HT and CT fractions contributed to the inhibition of FA biohydrogenation but at different steps. The former inhibited biohydrogenation more in an earlier step as shown by the negative correlations between HT and C18:3 *n*-3 or C18:2 *n*-6, whereas the latter was effective in a later step of biohydrogenation as indicated by the positive correlation between CT and appearance of *c*9,*t*11 C18:2 as well as the negative correlation with the appearance of C18:0. However, summing up the results of the current and the previous study (Jayanegara et al., 2011a), it seems clear, that a simple and direct influence of phenolic fractions on ruminal biohydrogenation is difficult to establish, and, although phenolics are clearly shown to play a role, the whole mechanism appears to be more complex and involving only very specific phenolic molecules (Lourenço et al., 2008) and also other compounds and interactions.

One further finding of the current study was, that the effect of *C. sativa* on *c*9 C18:1 disappearance was opposite to that on C18:3 *n*-3 and C18:2 *n*-6. This is different from what was found with the tropical plants (Jayanegara et al., 2011a) but similar to the effect of sainfoin forage (Khiaosa-ard et al., 2009). The reason for these contrasting effects is not clear.

The current study revealed less *in vitro* influence of individual alpine forage plants on biohydrogenation than had been expected. Thus it was not possible to provide evidence for a ruminal effect of alpine forages (Leiber et al., 2005) which would explain the particularly high C18:3 *n*-3 concentrations in milk fat originating from such forages, which is however repeatedly proven (Falchero et al., 2010; Ferlay et al., 2006; Kraft et al., 2003 and many others). This might mean that other plants or interactions among plant constituents and physiological processes are involved which have not been taken into consideration so far.

It would be also of interest to conduct further analysis on longer GC-columns in order to distinguish between more different C18-biohydrogenation intermediates (Lee and Jenkins, 2011) in order to establish a more complete picture of the changes in ruminal FA metabolism by alpine forage plants. However, since *trans*-monoenes other than *t*11 C18:2 and CLA other than *c*9,*t*11 C18:2 occur only in comparably small concentrations in pasture-based milk (Kraft et al., 2003), the main focus of the current study had been put on these two intermediates and the native plant FA C18:3 *n*-3 and C18:2 *n*-6.

## 5. Conclusion

The ability of different alpine plants in modifying ruminal FA biohydrogenation varied less in the present study than expected. With the exception of one plant species, the plants showed no great difference in their effect on the disappearance of C18:3 *n*-3 and C18:2 *n*-6. Low contents of polyphenols in almost all plants appeared to be a main explanation behind this finding. Being rich in HT, *C. sativa* was demonstrated to reduce disappearance of C18:3 *n*-3 and C18:2 *n*-6 as well as the appearance of C18:0 and, at the same time promote the generation of *c*9,*t*11 C18:2 and *t*11 C18:1. This could make leaves of this plant interesting for strategic feeding of milk-producing ruminants in order to improve the FA profile of the milk towards FA considered beneficial for human health.

## Conflict of interest statement

The authors state, that there is *no* conflict of interests concerning this manuscript.

## Acknowledgments

The first author is grateful to the Indonesian Department of National Education for providing a DIKTI scholarship enabling him to carry out a Ph.D. study at ETH Zurich.

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