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Ruminal biohydrogenation as affected by tannins *in vitro*

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The aim of the present work was to study the effects of tannins from carob (CT; *Ceratonia siliqua*), acacia leaves (AT; *Acacia cyanophylla*) and quebracho (QT; *Schinopsis lorentzii*) on ruminal biohydrogenation *in vitro*. The tannins extracted from CT, AT and QT were incubated for 12 h in glass syringes in cow buffered ruminal fluid (BRF) with hay or hay plus concentrate as a substrate. Within each feed, three concentrations of tannins were used (0·0, 0·6 and 1·0 mg/ml BRF). The branched-chain volatile fatty acids, the branched-chain fatty acids and the microbial protein concentration were reduced ($P<0·05$) by tannins. In the tannin-containing fermenters, vaccenic acid was accumulated (+23 %, $P<0·01$) while stearic acid was reduced (−16 %, $P<0·0005$). The concentration of total conjugated linoleic acid (CLA) isomers in the BRF was not affected by tannins. The assay on linoleic acid isomerase (LA-I) showed that the enzyme activity (nmol CLA produced/min per mg protein) was unaffected by the inclusion of tannins in the fermenters. However, the CLA produced by LA-I (nmol/ml per min) was lower in the presence of tannins. These results suggest that tannins reduce ruminal biohydrogenation through the inhibition of the activity of ruminal micro-organisms.

Tannins: Rumen: Biohydrogenation: Conjugated linoleic acid: Fatty acids

In recent years, research has focused on the fatty acid composition of ruminant products for the effects of their consumption on human health. In particular, *cis*-9,*trans*-11-C18 : 2 (conjugated linoleic acid, CLA) is active in the prevention of cancer[1] and atherosclerosis in mammals[2]. CLA is partially synthesised in the rumen by cellulolytic bacteria, and mainly by *Butyrivibrio* spp.[3], during the biohydrogenation of linoleic acid (*cis*-9,*cis*-12-C18 : 2, LA). However, the largest proportion of CLA present in meat and milk is endogenously produced in the tissues by the action of the enzyme Δ^9-desaturase on vaccenic acid (*trans*-11-C18 : 1, VA)[4,5], which is another product of the ruminal biohydrogenation of LA and linolenic acid (*cis*-9,*cis*-12,*cis*-15-C18 : 3, LNA)[6].

Recently, Vasta *et al.* [7] analysed the intramuscular fatty acid composition of lambs fed a diet containing tannins from carob (CT; *Ceratonia siliqua*) pulp. They noted that the meat from lambs receiving the tanniniferous diet contained lower amounts of CLA and VA when compared with the meat from those that received the same diet but with the supplementation of polyethylene glycol (PEG), a tannin-inactivating agent[8]. Vasta *et al.* [7] suggested that, perhaps, dietary tannins had inhibited the activity of ruminal micro-organisms responsible for the biohydrogenation. It is widely known and accepted that tannins are able to bind proteins[9] and inhibit the growth of ruminal bacteria[10].

The objectives of the present study were to verify whether the tannins from three different sources (CT; acacia, AT; *Acacia cyanophylla*; quebracho, QT; *Schinopsis lorentzii*) inhibit the ruminal biohydrogenation *in vitro* and whether there is a dose-dependent effect of tannins.

Materials and methods

Optimisation of linoleic acid isomerase assay

Linoleic acid isomerase (LA-I) is the enzyme responsible for the conversion of LA to CLA[3]. So far, the activity of LA-I has been studied only with pure bacterial strains[3,11–16]. Therefore, before conducting the main experiment, we have optimised the method for measuring the LA-I activity in the whole ruminal fluid in order to operate in the conditions of linearity of this assay.

Ruminal content was collected before the morning feeding from one fistulated cow and filtered through two layers of cheesecloth to eliminate raw feed particles. The cow was fed a diet containing 2 kg of concentrate mixture and offered *ad libitum* roughage, with free access to drinking-water. The concentrate mixture comprised (in g/kg) rapeseed cake (360), maize (200), wheat (200), molasses (200) and vitamin and mineral mixtures (40). The filtered fluid was centrifuged

Abbreviations: AT, acacia leaves; BRF, buffered ruminal fluid; CLA, conjugated linoleic acid; CT, carob; H, hay; H + C, hay plus concentrate; LA, linoleic acid; LA-I, linoleic acid isomerase; LNA, linolenic acid; PEG, polyethylene glycol; QT, quebracho; SA, stearic acid; VA, vaccenic acid; VFA, volatile fatty acid; WCE, whole-cell extract.

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at 20 000 ***g*** (20 min at 4°C), and the pellet was then washed with 0·05 M-potassium phosphate buffer (pH 6·8). The washed pellet was then suspended in 3 ml of 0·05 M-potassium phosphate buffer (pH 6·8) and sonicated for three cycles of 60 s each, with 90 s of intervals between each cycle. The power of the sonicator (Vibra Cell, Bioblock Scientific, Illkirch, France) was set to 240 W and the vessel containing the suspended pellet was kept on an ice-bath to keep the temperature of the medium below 4°C. Initially, for the setting up of this method, the cellular membranes (insoluble particulate fraction) obtained after centrifugation (20 000 ***g*** for 20 min) were discarded and the soluble endocellular fraction was used for the assay, as described in earlier studies with pure bacterial strains[3,12,13]. In a second assay, the sonicated pellet (subsequently addressed as whole-cell extract, WCE), containing both microbial cell membranes (insoluble particulate fraction) and endocellular organules, was used for testing the activity of LA-I. The WCE was kept at −80°C until the determination of the enzyme activity. The protein content of the WCE was determined according to Lowry's method[17]. In a third assay (to countercheck the necessity of the presence of the insoluble particulate fraction), the insoluble particulate fraction was separated from the endocellular fraction by centrifuging at 20 000 ***g*** (20 min at 4°C) and the assay (illustrated later) was repeated first on the latter fraction only and successively on the same endocellular extract re-added to its cell-insoluble particulate fraction (reconstituted WCE).

Pure LA was dissolved in 1,3-propanediol by 60 s exposure to sonic oscillation (240 W) to obtain the stock solution of LA (0·72 mM) used as a substrate for the enzyme assay. The stock solution was stored at −80°C until needed. For the enzyme assay, we tested three volumes of the WCE (40, 60 and 80 μl, containing, respectively, 104, 156 and 208 μg proteins), and for each volume of the extract the incubation time was 2, 4 and 6 min. The reaction was started by adding 100 μl LA stock solution to a glass tube containing the WCE, 300 μl of 1,3-propanediol and the 0·1 M-potassium phosphate buffer (pH 7·0), to give a total assay volume of 2·0 ml. After 2, 4 or 6 min of incubation at 25°C, the reaction was stopped by adding 2·5 ml of 2·0 N isopropanol–isooctane–H_2SO_4 (40:10:1, v/v/v). Then, 1·0 ml of isooctane and 1·0 ml of distilled water were added. The reaction mixture was mixed thoroughly by vortexing for 1 min and the isooctane layer containing fatty acids was collected. The absorbance of the isooctane layer was recorded at 233 nm wavelength (λ_{max} of conjugated diene bonds[18]) by a spectrophotometer. The absorption of CLA was read against a blank, in which LA was added after the isopropanol–isooctane–H_2SO_4 solution. An extinction coefficient of $2{\cdot}4 \times 10^4$ M/cm was used[3]. Therefore, it may be noted that all the comments made in the present study refer to the unspecific formation of CLA.

Tannins extraction

Tannins from CT, AT and QT were extracted with aqueous acetone 70 % (v/v) and purified using Sephadex LH-20 (Sigma, St Louis, MO, USA), as described by Makkar & Becker[19]. Tannins were added into the syringes as dissolved in a water solution, freshly prepared in the morning just before starting the incubations. In order to enhance the solubilisation of tannins in distilled water, the solutions were sonicated for 15 min at room temperature in a sonication bath.

In vitro *incubations with ruminal fluid*

The rumen liquor from one rumen-fistulated non-lactating Friesian–Holstein cow was collected manually by squeezing the feed material into pre-warmed (approximately 39°C) thermos flasks. The cow was maintained on a diet containing 2 kg of concentrate mixture and fed *ad libitum* roughage, with free access to drinking-water. The concentrate mixture (in g/kg) comprised rapeseed cake (360), maize (200), wheat (200), molasses (200) and vitamin and mineral mixtures (40). The rumen liquor was collected from the cow just before the morning feeding and transported in warm (approximately 39°C) insulated flasks, strained through two layers of cheesecloth and used as the source of inoculum. Strained ruminal fluid was added to an *in vitro* incubation medium, according to Menke *et al.*[20], in a proportion of 1:2 (v/v), under constant CO_2 flux. In each graduated 100 ml capacity glass syringe, 190 mg DM of hay (H) or hay plus concentrate (H + C, being the two feeds in a proportion of 1:1 on a DM basis) were incubated with 30 ml of the *in vitro* medium containing strained ruminal fluid. The ingredients of the concentrate were as follows: barley (470 g/kg); wheat bran (350 g/kg); soyabean meal (150 g/kg); mineral premix (30 g/kg); soyabean oil (1 g/kg). For each tannin source, the tannin solution was added at two different levels (0·6 and 1·0 mg/ml of buffered ruminal fluid, BRF) into the syringes containing H or H + C. For each type of tannin and at each concentration, two replicates were run. The control syringes, containing either H (*n* 3) or H + C (*n* 3), were tannin free. All the syringes were incubated in a water-bath set to 39°C. At time 0 and 12 h, the position of the piston on each syringe was recorded for net gas production measurement. During the first 6 h and the following 6 h of incubation, the syringes were gently shaken every 2 and 3 h, respectively. After 12 h incubation, the syringe content was immediately handled for the following analyses.

Extraction of fatty acids

The extraction of fatty acids was performed as described by Wąsowska *et al.*[12]. Briefly, 2·4 ml of the syringe content was mixed with 1·5 ml of acidified salt solution (17 mM-NaCl in 1 mM-H_2SO_4). An aliquot of 200 μl of heptadecanoic acid in methanol (200 mM) was added as an internal standard, followed by 5·0 ml of methanol, and the mixture was vortexed for 1 min. Then, 5·0 ml of chloroform containing 0·2 mg/ml butylated hydroxytoluene was added and the mixture was vortexed again for 2 min. The upper layer was removed by aspiration. The lower layer was dried by passing through anhydrous sodium sulphate, and the solvent was removed by fluxing nitrogen for 20 min. The dried lipid extract was suspended in toluene and stored at −30°C until methylation. Extracted fatty acids were converted to fatty acid methyl esters by the addition of 2 ml of 0·5 M-sodium methoxide (10 min at 50°C) and 3 ml of 5 % methanolic HCl (v/v; 10 min at 80°C), as described by Kramer *et al.*[21]. Methyl esters were separated by using a GC 8000 TOP gas chromatograph (Thermo Fisher Scientific Inc., Milan, Italy)

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fitted with a fused silica capillary column (WCOT Select fatty acid methyl esters, 100 m × 0·25 mm internal diameter, × 0·2 μm film thickness; Varian Inc., Middelburg, The Netherlands). Helium was used as a carrier gas. The injector temperature was 270°C, while the detector temperature was 300°C, and the split ratio was 80:1. The oven temperature was 60°C for 5 min, increased by 15°C/min to 170°C, for 44 min, increased by 1°C/min to 202°C, for 1 min, and increased by 3°C/min to 230°C, for 9 min. The identification of 18 : 1 and CLA isomers was based on commercial standard mixtures (Supelco, Bellefonte PA, USA) and published isomeric profiles[22].

Feed analyses

Feed used as a substrate for the incubations were analysed for fibre fractions[23], crude protein (AOAC, 1995; method 984.13)[24], diethyl ether extract (AOAC, 1995; method 39.1.05)[24] and ash (AOAC, 1995; method 942.04)[24].

Linoleic acid isomerase assay and volatile fatty acid

For testing the effects of tannins on the LA-I activity and volatile fatty acid (VFA) production, 18·0 ml of the syringe content were centrifuged at 20 000 ***g*** for 20 min at 4°C. An aliquot of the supernatant (2·0 ml) was collected for the VFA analysis. Then, an aliquot of this solution was sonicated to obtain the WCE for the LA-I assay. Also, 2·0 ml of the WCE were centrifuged (20 000 ***g*** for 15 min at 4°C) and the supernatant was used for protein determination, according to the method described by Lowry *et al.*[17]. This protein content was termed as microbial protein. The WCE was stored at −80°C until the LA-I assay, which was conducted using 60 μl of the WCE, 300 μl of 1,3-propanediol, and 1540 μl of 0·1 M-potassium phosphate buffer (pH 7·0). The reaction was started by the addition of 100 μl of a solution of 0·72 mM-LA in 1,3-propanediol and stopped after 4 min of incubation at room temperature. For each syringe, the assay was run in triplicate.

Statistical analysis

The data were analysed by a two-way ANOVA with a model including two types of feed (H or H + C), three levels of concentration of tannins in the fermenters (0·0, 0·6 and 1·0 mg/ml BRF) and the interaction between the type of feed and the level of concentration. When the interaction was not significant ($P > 0{\cdot}05$), it was excluded from the model. A second two-way ANOVA with a model including two types of feed (H or H + C) and three types of tannin source (CT, AT and QT) was then applied only for those fermenters containing tannins (i.e. 0·6 and 1·0 mg/ml of the *in vitro* system). The interaction between the two factors (type of feed × type of tannin source) was included in the model when it was significant ($P < 0{\cdot}05$). For both analyses, when the ANOVA was significant ($P < 0{\cdot}05$), the differences between means were separated using pairwise Tukey's comparison test. The data were analysed by the software MINITAB version 14.0.

Results

Optimisation of the linoleic acid isomerase assay

When the LA-I activity was measured on the sole soluble endocellular fraction, no increase in the absorption at 233 nm was observed. Conversely, when the WCE was used for the assay, we observed the enzymatic activity. To confirm these observations, the assay was repeated using the endocellular fraction only and no increase in the absorption at 233 nm was observed. However, when the insoluble particulate fraction was added to the endocellular fraction, an increase in the absorption at 233 nm was recorded. The production of CLA increased proportionally by the increase in the WCE (40, 60 or 80 μl; (Fig. 1(A)). When 40 μl of the WCE (which contained 0·104 mg protein) and a short incubation time (2 min) were used, the specific activity of LA-I was not reproducible (Fig. 1(B)).

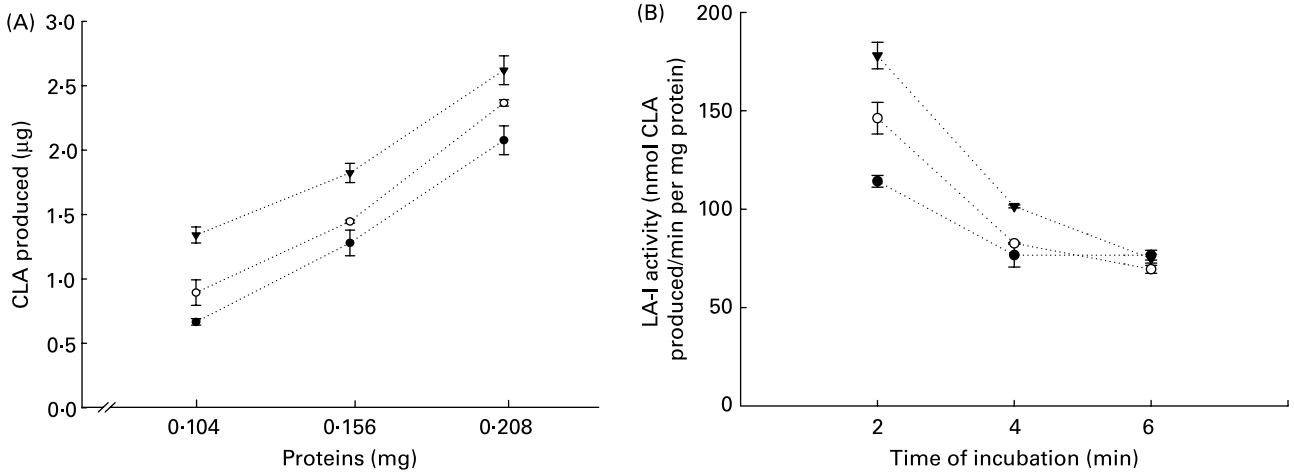

Fig. 1. (A) The effect of the amount of proteins (mg) at different times of incubation (2 min, ●; 4 min, ○; 6 min, ▼) on conjugated linoleic acid (CLA) production (μg) in the ruminal whole-cell extract. (B) The effect of the time of incubation (2, 4 and 6 min) using 40 μl (●), 60 μl (○) or 80 μl (▼) of the whole-cell extract containing respectively 104, 156 and 208 μg of proteins on the linoleic acid isomerase (LA-I) activity (nmol CLA produced/min per mg protein). The results are the mean values for three replicates, with the standard errors represented by vertical bars.

Feed composition

Feed used as the substrates of the fermentation are shown in Table 1. H had a higher content of neutral-detergent fibre (613 *v.* 234 g/kg DM) and of diethyl ether extract (25 *v.* 18 g/kg DM) when compared with the concentrate (Table 1). Lauric acid (12 : 0), α-linolenic (18 : 3*n*-3) and γ-LNA (18 : 3*n*-6) were present at higher percentages in the H diet when compared with the H + C diet. The H + C feed contained higher amounts of stearic acid (18 : 0, SA; +43 %), oleic acid (18 : 1; + 88 %) and LA (18 : 2*n*-6; + 65 %) when compared with the H diet (Table 2).

Net production of gas and volatile fatty acid and microbial protein synthesis

Before starting this experiment, we performed another fermentation using the same experimental conditions as in the present study. In the pilot study, we observed that up to 12 h of fermentation, the inclusion of tannins reduced the gas production, while in the following 12 h this effect was not observed, suggesting that tannins had major effects on the fermentation within 12 h of incubation. Therefore, we have chosen to run the fermentation over a period of 12 h. The net production of gas in the syringes after 12 h of fermentation was higher ($P<0·0005$) in the fermenters containing H + C as a substrate when compared with the syringes incubated with H (43·3 *v.* 30·7 ml, respectively). No tannin dose–response effect was observed on the net gas production (Table 3). However, the gas produced in the syringes containing CT or AT was significantly ($P<0·0005$) lower compared with that in the syringes incubated with tannins from QT (Table 4).

The VFA, *iso*-butyrate, butyrate and valerate were found at a higher concentration ($P<0·05$) in the syringes incubated with H + C when compared with those incubated with H (Table 3). The presence of tannins decreased the production of *iso*-butyrate, *iso*-valerate and valerate ($P<0·005$) when compared with the tannin-free syringes. Also, the VFA production was not significantly affected by the different types of tannins used in the fermenters (Table 4).

The microbial protein concentration was higher ($P=0·002$) in the fermenters incubated with H + C than with H. When tannins were present at 1·0 mg/ml of the BRF, the concentration of soluble proteins was lower ($P=0·014$) compared with the tannin-free syringes (1·98 *v.* 2·44 mg/ml, respectively), while with 0·6 mg/ml of tannins, the microbial protein concentration was not different ($P>0·05$) when compared with the control fermenters. When the effect of the source of tannins was tested, it was found that the microbial protein concentration was lower in the fermenters incubated with CT, while QT tannins seemed not to affect the microbial protein concentration.

Fatty acid profile

Table 5 shows the effect of the feed and concentration of tannins on the fatty acid profile of the fermented ruminal digesta. The syringes incubated with H contained higher amounts of 12 : 0, 14 : 0, *trans*-11,*cis*-15-C18 : 2 and 18 : 3*n*-6 and lower amounts of 18 : 0 and 18 : 2*n*-6 when compared with the syringes incubated with H + C ($P<0·05$). When the fermenters contained 1·0 mg/ml of tannins, the concentrations of 15 : 0 *iso*-, 15 : 0 *anteiso*-, 16 : 0 *iso*-, 17 : 0 and 17 : 0 *anteiso*-fatty acids were significantly lower ($P<0·005$), compared with the control syringes that did not contain tannins. In particular, when H was used as a substrate, 15 : 0 *iso* and 15 : 0 *anteiso* were reduced ($P<0·05$) by 2-fold and 16 : 0 *iso* was reduced ($P<0·05$) by 3-fold by the addition of 1·0 mg/ml tannins, compared with the systems containing 0·6 or 0·0 mg/ml tannins. These results could not be observed in the fermenters incubated with H + C. Among the eighteen carbon fatty acids, SA was found at significantly higher ($P<0·0005$) amounts in the syringes containing 0·0 and 0·6 mg/ml tannins compared with the 1·0 mg/ml fermenters (respectively, 30·1 and 30·7 *v.* 25·3 mg/100 mg total fatty acids). At 0·6 mg/ml tannins, VA (*trans*-11-C18 : 1) was 1·4-fold higher compared with that in the tannin-free syringes ($P<0·05$), while at 1·0 mg/ml, VA acid was intermediate when compared with the tannin-free fermenters and those containing 0·6 mg/ml tannins. The *trans*-11,*cis*-15-C18 : 2 and 18 : 3*n*-3 were lowered ($P=0·026$ and 0·001, respectively) by the incubation with tannins at a level of 1·0 mg/ml compared with 0·6 mg/ml, while the amounts of these fatty acids found in the control syringes were intermediate compared with the tannin-containing fermenters. Among the CLA, the isomer *cis*-11,*trans*-13 was present at significantly ($P=0·015$) lower levels in the syringes containing tannins when compared with the control syringes, while the isomer *trans*-10,*cis*-12-C18 : 2 was reduced ($P=0·015$) when tannins in the syringes amounted to 1·0 mg/ml when compared with the fermenters not containing tannins (0·099 *v.* 0·169 mg/100 mg total fatty acids, respectively). The isomers *cis*-9,*trans*-11, *trans*-7,*cis*-9 and

Table 1. Chemical composition (g/kg DM) of feed used as the substrate for the *in vitro* incubations

	Hay	Concentrate
DM (g/kg)	914	904
Crude protein	112	191
Neutral-detergent fibre	613	234
Acid-detergent fibre	318	50
Acid-detergent lignin	99	22
Diethyl ether extract	25	18
Ash	107	48

Table 2. Fatty acid composition of feed used as the substrate for *in vitro* incubations (mg/100 g of total fatty acids)

Fatty acids	H	H + C
12 : 0	3·52	0·39
14 : 0	0·76	0·53
14 : 1	1·04	0·13
15 : 0	0·15	0·13
15 : 1	0·00	0·18
16 : 0	14·52	23·83
16 : 1	1·35	0·13
18 : 0	1·82	3·20
18 : 1	2·19	18·62
18 : 2*n*-6	16·62	47·30
18 : 3*n*-6	11·26	0·44
18 : 3*n*-3	46·78	5·11

H, hay; H + C, hay plus concentrate.

Table 3. Effect of tannin concentration on microbial protein content and net gas and volatile fatty acid production in ruminal fluid after 12 h of incubation
(Mean values with their standard errors)

	Feed		Tannin concentration (mg/ml)			*P* value			
	H	H+C	0·0	0·6	1·0	Feed	Tannin concentration	Feed × tannin concentration	SEM
Replicates (*n*)	15	15	6	12	12				
Microbial proteins (mg/ml)	1·95	2·29	2·44^{a}	2·13^{ab}	1·98^{b}	0·002	0·014	NS	0·061
Net gas production (ml)	30·67	43·25	36·67	31·88	28·69	<0·0005	0·134	NS	0·971
VFA (mM)									
Acetate	7·67	11·82	13·30	11·70	6·48	0·145	0·124	NS	1·390
Propionate	3·12	4·51	4·53	3·32	4·53	0·071	0·494	NS	0·372
iso-Butyrate	0·05	0·13	0·23^{a}	0·11^{b}	0·01^{c}	0·029	<0·0005	0·054	0·021
Butyrate	1·45	2·93	2·63	2·74	1·51	0·013	0·130	NS	0·313
iso-Valerate	0·08	0·18	0·39^{a}	0·14^{b}	0·03^{c}	0·052	<0·0005	NS	0·032
Valerate	0·19	0·28	0·37^{a}	0·25^{ab}	0·16^{b}	0·047	0·002	NS	0·025
Acetate/propionate	2·21	2·44	2·91^{a}	2·71^{a}	1·71^{b}	0·409	0·001	NS	0·151
Total	12·55	19·85	21·45	19·02	11·50	0·087	0·145	NS	2·110

H, hay; H + C, hay plus concentrate; VFA, volatile fatty acid.
^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

cis-8,*trans*-10 of CLA (which co-eluted) were not affected by the presence of tannins in the fermenters. As shown in Table 6, the fatty acid profile of the fermented ruminal fluid was not influenced by the type of tannins included in the syringes.

Effects of tannins on the linoleic acid isomerase activity

The production of CLA by LA-I was almost doubled ($P<0·05$) using H as feed in the fermenters compared with H + C (2·7 *v.* 1·5 nmol/ml per min, respectively), and this result was displayed in tendency ($P=0·083$) when only the tannin-containing syringes were considered (5·23 *v.* 2·05 nmol/ml per min, respectively). Fig. 2 shows the production of CLA (nmol/ml per min) by the enzyme LA-I as affected by tannins. When rumen fluid was incubated with tannins, at either 0·6 or 1·0 mg/ml, the CLA produced were significantly lower ($P<0·05$) when compared with the control syringes. However, compared with the control syringes, the specific activity of LA-I (nmol CLA produced/min per mg protein) was unaffected ($P>0·05$) by the inclusion of tannins in the fermenters, but it was higher ($P=0·024$) in the fermenters incubated with H (5·73 nmol/mg protein per min) when compared with H + C (2·67 nmol/mg protein per min) (data not shown). As observed for the whole fatty acid profile, the diverse sources of tannins did not affect the production of CLA by LA-I.

Discussion

While optimising the enzymatic assay for the LA-I activity, it was observed that the WCE of the ruminal fluid, comprising both the soluble and insoluble particulate fractions, is needed for the measurement of the enzyme activity. This is consistent with an earlier study on the *in vitro* biohydrogenation activity in the pure culture strains of *Butyrivibrio fibrisolvens* [25], in which it was reported that the biohydrogenation of punicic acid (*cis*-9,*trans*-11,*cis*-13-C18 : 3) to VA (*trans*-11-C18 : 1) was higher when the substrate was incubated with

Table 4. Effect of the source of tannins on microbial protein content and net gas and volatile fatty acid production in ruminal fluid after 12 h of incubation
(Mean values with their standard errors)

	Feed		Tannin source			*P* value			
	H	H+C	Carob	Acacia	Quebracho	Feed	Tannin source	Feed × tannin source	SEM
Replicates (*n*)	12	12	8	8	8				
Microbial proteins (mg/ml)	1·90	2·22	1·88^{b}	2·04^{ab}	2·26^{a}	0·001	0·005	NS	0·060
Net gas production (ml)	24·54	27·38	25·38^{b}	27·38^{b}	30·00^{a}	<0·0005	<0·0005	NS	1·010
VFA (mM)									
Acetate	6·34	11·83	8·52	6·81	11·94	0·094	0·410	NS	1·620
Propionate	2·74	4·65	3·78	3·02	4·30	0·028	0·440	NS	0·438
iso-Butyrate	0·02	0·10	0·03	0·05	0·11	0·051	0·233	NS	0·021
Butyrate	1·21	3·04	1·82	1·56	3·00	0·007	0·148	NS	0·368
iso-Valerate	0·03	0·13	0·05	0·04	0·15	0·073	0·148	NS	0·028
Valerate	0·16	0·25	0·19	0·16	0·26	0·079	0·220	NS	0·026
Acetate/propionate	2·09	2·33	2·25	2·16	2·21	0·517	0·981	NS	0·171
Total	10·51	20·01	14·38	11·67	19·76	0·054	0·365	NS	2·480

H, hay; H + C, hay plus concentrate; VFA, volatile fatty acid.
^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

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Table 5. Influence of the concentration of tannins on the ruminal fluid fatty acid profile after 12 h of incubation
(Mean values with their standard errors)

	Feed		Tannin concentration (mg/ml)			*P* value			
	H	H+C	0·0	0·6	1·0	Feed	Tannin concentration	Feed × tannin concentration	SEM
Replicates (*n*)	15	15	6	12	12				
Fatty acids (mg/100 mg of total fatty acids)									
12 : 0	2·609	2·028	2·182^{b}	1·778^{b}	2·883^{a}	0·042	0·004	NS	0·167
13 : 0	0·168	0·151	0·158	0·157	0·162	0·333	0·942	0·036	0·007
14 : 0	9·174	6·807	7·690^{b}	6·743^{b}	9·265^{a}	0·011	0·044	NS	0·513
15 : 0	2·336	2·245	2·420^{a}	2·409^{a}	2·114^{b}	0·056	0·002	0·001	0·056
15 : 0 *anteiso*	1·668	1·777	1·842^{a}	1·956^{a}	1·444^{b}	0·970	<0·0005	0·001	0·073
15 : 0 *iso*	0·909	1·085	1·144^{a}	1·153^{a}	0·786^{b}	0·114	<0·0005	0·006	0·052
16 : 0	31·063	29·515	31·73^{a}	28·184^{b}	31·73^{a}	0·080	0·003	NS	0·562
16 : 0 *iso*	0·667	0·725	0·798^{a}	0·779^{a}	0·573^{b}	0·600	<0·0005	0·008	0·029
17 : 0	3·809	4·521	4·226^{ab}	4·991^{a}	3·343^{b}	0·143	0·001	NS	0·222
17 : 0 *anteiso*	0·892	0·919	1·056^{a}	0·972^{a}	0·778^{b}	0·704	<0·0005	NS	0·030
17 : 0 *iso*	0·639	0·381	0·288	0·498	0·603	0·029	0·175	NS	0·059
18 : 0	26·760	29·990	30·114^{a}	30·708^{a}	25·350^{b}	0·038	<0·0005	0·026	0·762
cis-12-C18 : 1	0·518	0·693	0·522	0·598	0·655	0·084	0·360	0·026	0·036
cis-11-C18 : 1	0·422	0·213	0·220	0·391	0·276	0·063	0·495	NS	0·055
trans-12-C18 : 1	0·321	0·214	0·226	0·262	0·286	0·001	0·439	NS	0·017
trans-13 + *trans*-14-C18 : 1	0·310	0·209	0·176^{b}	0·221^{b}	0·329^{a}	0·028	0·025	NS	0·024
trans-9-C18 : 1	0·245	0·219	0·190	0·223	0·257	0·347	0·155	NS	0·012
trans-11-C18 : 1	3·249	3·541	2·692^{b}	3·768^{a}	3·327^{ab}	0·130	0·006	NS	0·130
trans-10-C18 : 1	0·340	0·328	0·282	0·339	0·350	0·714	0·134	NS	0·018
trans-6-8-C18 : 1	0·268	0·284	0·226	0·297	0·276	0·497	0·220	NS	0·014
cis-9 + *trans*-15-C18 : 1	4·856	4·963	3·516^{b}	4·020^{b}	6·384^{a}	0·901	<0·0005	0·006	0·362
Total *trans*-18 : 1	4·735	4·797	3·794^{b}	5·112^{a}	4·828^{a}	0·636	0·008	NS	0·156
trans-11,*cis*-15-C18 : 2	0·757	0·593	0·628^{ab}	0·755^{a}	0·607^{b}	0·002	0·026	NS	0·030
trans-9,*trans*-12-C18 : 2*n*-6	0·257	0·319	0·218	0·273	0·336	0·220	0·239	NS	0·027
cis-9,*cis*-12-C18 : 2*n*-6	1·585	3·931	2·608	2·938	2·738	<0·0005	0·364	NS	0·259
trans-10,*cis*-12 CLA	0·133	0·143	0·160^{ab}	0·168^{a}	0·099^{b}	0·668	0·015	NS	0·012
cis-11,*trans*-13 CLA	0·114	0·024	0·116^{a}	0·058^{b}	0·057^{b}	<0·0005	0·015	0·008	0·012
cis-9,*trans*-11 + *trans*-7,*cis*-9 + *cis*-8,*trans*-10 CLA	0·224	0·317	0·280	0·245	0·297	0·009	0·367	NS	0·018
18 : 3*n*-6	0·697	0·394	0·572	0·642	0·426	0·003	0·116	NS	0·056
18 : 3*n*-3	1·610	1·767	1·524^{b}	2·102^{a}	1·350^{b}	0·283	0·001	NS	0·100
20 : 0	0·934	1·023	0·944	1·079	0·896	0·318	0·179	NS	0·046
Other *trans*-18 : 1 and *trans*-18 : 2	1·732	1·214	1·360	1·277	1·695	0·046	0·305	NS	0·130
Total CLA	0·471	0·486	0·556	0·473	0·453	0·864	0·423	NS	0·026

H, hay; H + C, hay plus concentrate; CLA, conjugated linoleic acid.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Table 6. Influence of the source of tannins on the ruminal fluid fatty acid profile after 12 h of incubation

(Mean values with their standard errors)

	Feed		Tannin source			*P* value			
	H	H+C	Carob	Acacia	Quebracho	Feed	Tannin source	Feed × tannin source	SEM
Replicates (*n*)	12	12	8	8	8				
Fatty acids (mg/100 mg of total fatty acids)									
12 : 0	2·602	2·068	2·694	2·254	2·058	0·179	0·399	NS	0·195
13 : 0	0·169	0·149	0·151	0·171	0·155	0·232	0·573	NS	0·008
14 : 0	9·452	6·556	8·790	7·771	7·513	0·011	0·565	NS	0·580
15 : 0	2·277	2·247	2·226	2·255	2·304	0·818	0·885	NS	0·061
15 : 0 *anteiso*	1·600	1·800	1·623	1·746	1·731	0·255	0·813	NS	0·083
15 : 0 *iso*	0·862	1·078	0·885	0·978	1·045	0·074	0·541	NS	0·060
16 : 0	30·877	29·038	30·360	29·770	29·740	0·165	0·905	NS	0·629
16 : 0 *iso*	0·636	0·717	0·631	0·668	0·730	0·218	0·453	NS	0·032
17 : 0	3·722	4·613	3·633	4·421	4·448	0·091	0·341	NS	0·264
17 : 0 *anteiso*	0·850	0·900	0·813	0·909	0·904	0·404	0·341	NS	0·029
17 : 0 *iso*	0·693	0·409	0·488	0·489	0·676	0·032	0·372	NS	0·067
18 : 0	26·150	29·907	26·810	28·290	28·990	0·039	0·571	NS	0·903
cis-12-C18 : 1	0·501	0·753	0·583	0·651	0·646	0·001	0·656	NS	0·042
cis-11-C18 : 1	0·444	0·753	0·468	0·226	0·306	0·086	0·285	NS	0·065
trans-12-C18 : 1	0·329	0·218	0·315	0·271	0·235	0·001	0·105	NS	0·019
trans-13 + *trans*-14-C18 : 1	0·334	0·216	0·276	0·255	0·294	0·038	0·839	NS	0·028
trans-9-C18 : 1	0·250	0·231	0·226	0·254	0·241	0·520	0·748	NS	0·014
trans-11-C18 : 1	3·396	3·699	3·276	3·741	3·625	0·265	0·347	NS	0·134
trans-10-C18 : 1	0·347	0·343	0·330	0·376	0·328	0·869	0·223	NS	0·013
trans-6-8-C18 : 1	0·276	0·298	0·273	0·308	0·280	0·531	0·681	NS	0·016
cis-9 + *trans*-15-C18 : 1	5·032	5·373	6·241	5·111	4·254	0·675	0·153	NS	0·412
Total *trans*-18 : 1	4·934	5·006	4·701	5·203	5·006	0·823	0·441	NS	0·154
trans-11,*cis*-15-C18 : 2	0·761	0·602	0·665	0·663	0·716	0·020	0·738	NS	0·034
trans-9,*trans*-12-C18 : 2*n*-6	0·257	0·353	0·235	0·346	0·332	0·113	0·255	NS	0·031
cis-9,*cis*-12-C18 : 2*n*-6	1·548	4·128	2·768	2·628	3·120	<0·0005	0·387	NS	0·068
trans-10,*cis*-12 CLA	0·128	0·140	0·121	0·135	0·145	0·617	0·734	NS	0·012
cis-11,*trans*-13 CLA	0·115	0·000	0·071	0·048	0·054	<0·0005	0·269	NS	0·013
cis-9,*trans*-11 + *trans*-7,*cis*-9 + *cis*-8,*trans*-10 CLA	0·229	0·313	0·249	0·303	0·261	0·027	0·436	NS	0·019
18 : 3*n*-6	0·713	0·354	0·698	0·494	0·410	0·001	0·065	0·043	0·068
18 : 3*n*-3	1·622	1·831	1·581	1·693	1·905	0·402	0·556	NS	0·119
20 : 0	0·946	1·029	0·856	0·975	1·131	0·428	0·117	NS	0·054
Other *trans*-18 : 1 and *trans*-18 : 2	1·778	1·193	1·629	1·451	1·378	0·068	0·787	NS	0·156
Total CLA	0·472	0·454	0·441	0·488	0·460	0·715	0·728	NS	0·023

H, hay; H + C, hay plus concentrate; CLA, conjugated linoleic acid.

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

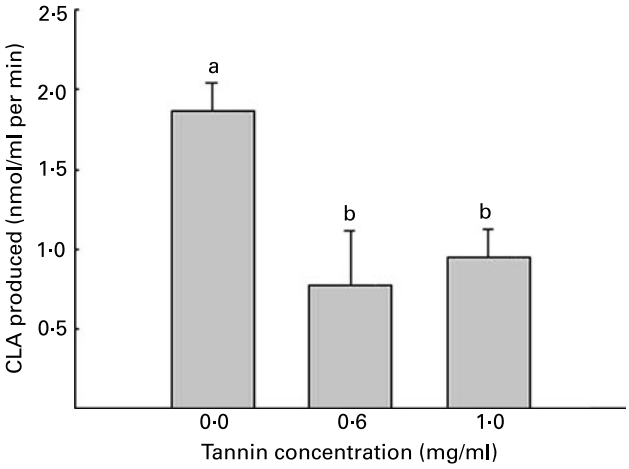

Fig. 2. The effect of different concentrations of tannins (0·0, 0·6 or 1·0 mg/ml of incubation medium) on the *in vitro* production of conjugated linoleic acid (CLA) by linoleic acid isomerase in ruminal fluid. Values are means, with standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P < 0·05$).

both the cell-free extract and the insoluble particulate fraction compared with when only the soluble endocellular fraction was incubated. In a study on the biohydrogenation of unsaturated fatty acids by *Treponema*, it has been reported that the addition of the cell-insoluble particulate fraction to the enzyme preparation obtained by purification through Sephadex G-25 and G-200 did not affect the LA-I activity[13]. In another study[26], it had been found that the addition of boiled ruminal fluid to a reaction mixture of pure ruminal bacterial strains enhanced the LA-I activity. In agreement with Polan *et al.* [26], the present results suggest that some cofactor(s) present in the insoluble particulate fraction or in the ruminal fluid play a key role in the reaction.

Fig. 1(B) shows that at 4 and 6 min of incubation and using 40 and 60 μl of the WCE, the LA-I activity can be measured in a reliable way in the ruminal liquor. Therefore, from these data, the operative condition for this assay at 25°C should be a volume of the WCE between 40 and 60 μl (corresponding to an amount of protein ranging from 0·105 to 0·150 mg) with an incubation time between 4 and 6 min.

The effects of tannins on *in vitro* gas production, protein and DM digestibility have been largely investigated[27,28]. However, so far, the biohydrogenation of fatty acids in the rumen as affected by tannins has not been studied. Only few *in vivo* experiments have focused on the effects of tannins from CT pods[7] or sulla (*Hedysarum coronarium*)[29] on the intramuscular fatty acid composition of lambs, but in these studies, the ruminal fatty acid metabolism was not investigated. When we planned the present study, we decided to evaluate the effects of tannins from AT and CT pods because they are commonly used as feed in small ruminant diets in several Mediterranean countries. QT was chosen because its extract could be used as an additive for animal diets. Most of the studies on the *in vitro* production of CLA in the ruminal fluid were conducted using fat-enriched diets[30,31] as substrates in order to highlight and enhance the effects of dietary fatty acids on the biohydrogenation. However, for the present work, we have chosen to study the interaction of tannins with conventional feed as H and a commercial concentrate in order to simulate a common tanniniferous diet offered to animals, and to avoid potential interactions between oil and tannins on rumen micro-organisms.

The type of substrate included in the fermenters strongly affected the net gas development ($P < 0·0005$), being 43·25 ml in the H + C syringes and 30·67 ml in the H syringes. In the present study, the net gas production was reduced, but only marginally ($P = 0·134$), by the inclusion of tannins to the fermenters. Some other studies reported that the *in vitro* gas production was strongly inhibited by QT tannins[32,33] or *Acacia* spp. tannins[28] in the BRF. In the present study, when compared with AT or CT, QT tannins had the lower relative inhibitory effect on the gas production. A similar trend was also found for the microbial protein production, thus suggesting that QT tannins have a 'milder' inhibitory effect on the ruminal microbial activity when compared with AT or CT. QT tannins also have low protein precipitation capacity and low activity of these tannins on cellulose digestion, which could be attributed to its branched-chain structure[34]. In the literature, there are no studies comparing the effects of CT, AT and QT on ruminal metabolism *in vitro*. It has been reported that tannins from *Acacia angustissima* and *Acacia salicina* [28] reduced the microbial protein synthesis in BRF or in pure ruminal bacteria strains, respectively. Similarly, it has been reported that the *in vitro* protein synthesis was reduced by tannins from QT[33] or mimosa[35] when compared with a tannin-free system or PEG-containing fermenters, respectively.

The total VFA and propionate production was higher ($P = 0·087$ and 0·071, respectively) in the fermenters incubated with H + C than those fermented with H. It is known that low-fibre and high-starch feed, such as concentrate-containing diets, shift ruminal fermentations to high propionate production[36]. Surprisingly, the acetate/propionate ratio was not affected by the type of feed (Table 3). However, it should be considered that in the H + C fermenters, the two feeds were mixed at a ratio of 1:1, and therefore the resulting diet can be considered as less fibrous when compared with the H substrate. Moreover, the fermentation took place for a 12 h period, and this short time was probably insufficient to observe significant differences in the acetate production between the two sets of fermenters (H and H + C). *iso*-Butyrate, butyrate, *iso*-valerate and valerate were found at lower levels in the H fermenters compared with the H + C fermenters, suggesting that the microbial activity was lower when using H as the only substrate. In the present study, the total VFA production was not affected by the concentration of tannins in the medium. However, *iso*-butyrate and *iso*-valerate, which arise from the degradation of feed amino acids by ruminal bacteria[37], were markedly reduced, in a dose-dependent manner, by the inclusion of tannins in the BRF. This result is in agreement with a previous study on the dose–response effect of tannins[33]. Frutos *et al.* [32] reported that the total VFA production *in vitro* was increased when QT tannins were deactivated by the inclusion of PEG in the fermenters and, similarly, Makkar *et al.* [27] found that QT tannins decreased the VFA production *in vitro*. The effect of tannins from CT on ruminal fermentation was investigated *in vivo* [8], showing that a diet based on CT leaves (tannins 50 g/kg DM) decreased the VFA production and impaired ruminal metabolism in goats. Subsequent *in vivo* studies on sheep[38]

and goats[39] reported that tannins from CT pods or leaves did not affect the VFA production in the rumen compared with animals receiving the same diets but supplemented with PEG.

The branched-chain *iso*- and *anteiso*-fatty acids and odd-chain fatty acids arise from a peculiar metabolism of ruminal bacteria (for a review, see Vlaeminck *et al.* [37]). Among ruminal bacteria, those classified as cellulolytic contain high amounts of *iso*- and *anteiso*-fatty acids compared with amylolytic bacteria, while this latter group of micro-organisms contains higher levels of linear odd-chain fatty acids compared with the cellulolytic ones. In the present study, we have found that in the H-containing fermenters, 17 : 0 *iso* increased by 2-fold and 15 : 0 increased slightly when compared with the H + C syringes. The major effects on the branched-chain fatty acids were exerted by tannins, which inhibited the production of 15 : 0 *iso*, 15 : 0 *anteiso*, 16 : 0 *iso* and 17 : 0 *anteiso*; these effects being more evident ($P<0{\cdot}0005$) when H was used as a substrate for the fermentation. This result is consistent with the lower production of *iso*-valerate and *iso*-butyrate mentioned previously, as these two VFA are the precursors of branched-chain fatty acids. Therefore, tannins have reduced the production of *iso*-, *anteiso*- and odd-chain fatty acids compared with the control fermenters through a reduced activity of ruminal micro-organisms and, especially, of cellulolytic bacteria.

The feed fatty acid composition influenced the fatty acid profile of C18 fatty acids in the fermenters. The higher levels of linoleic and SA in the H + C fermenters compared with the syringes incubated with H reflect the lipid profile of the two feeds (Table 2), the H + C feed being richer in these fatty acids. On the contrary, LNA, which was present at higher amounts in the H, did not differ significantly between the H and the H + C fermenters, but *trans*-11,*cis*-15-C18 : 2, which derives from the biohydrogenation of LNA, was found at higher percentages ($P<0{\cdot}05$) in the BRF fermented with H. Also, *cis*-9,*trans*-11 CLA was found at higher percentages in the H + C fermenters compared with those containing H. It is well known that biohydrogenation is enhanced by fibre-rich diets[40], but in the present study, it seems that the substrate availability (e.g. LA and LNA) had the major influence on the biohydrogenation.

When we have planned the present experiment, we hypothesised that ruminal biohydrogenation could be affected by tannins because of a reduction in the activity of ruminal micro-organisms. The ruminal fatty acid profile (Table 5) shows that the inclusion of tannins in the fermenters did not affect total CLA isomers but increased the accumulation of VA. In the present study, SA was strongly reduced (−16 %) by the inclusion of 1·0 mg/ml tannins compared with the tannin-free system. These results suggest that the last step of biohydrogenation, which is the conversion of VA to SA, was affected by tannins to a larger extent compared with the previous reaction, which leads to the formation of CLA. This can also be supported by the accumulation of total *trans*-18 : 1 fatty acids in the presence of tannins. For the same reason, the higher content of oleic acid + *trans*-15-C18 : 1 in the tannin-containing syringes compared with the tannin-free ones could be due to an increased accumulation of *trans*-15-C18 : 1 rather than to oleic acid. Recent studies have shown that *trans*-fatty acids arise from the successive isomerisation of *cis*-9-C18 : 1 operated by ruminal micro-organisms[30], and they are hydrogenated to SA. It is well known that the rate-limiting step of LA biohydrogenation is the saturation of VA[6], while Wąsovska *et al.* [12] and Buccioni *et al.* [41] have shown that the rate of appearance of *cis*-9,*trans*-11 CLA from LA *in vitro* is higher and occurs in shorter time compared with VA and then it reaches a plateau. Probably, if in the present study, the ruminal fatty acid profile was monitored after 6 h (instead of 12 h) of fermentation with tannins, we would have found that the CLA production was inhibited by tannins. This hypothesis is strongly supported by the present results, which clearly show that tannins reduce the CLA production by LA-I (Fig. 2). Although CLA were synthesised at a lower extent by LA-I in the presence of tannins, the LA-I activity (nmol CLA/mg protein/min) was not affected by tannins because, together with CLA, the microbial proteins in the reaction mixture also decreased. This result suggests that tannins did not interfere with LA-I *per se*, which is a cell-bound enzyme[3], but interfered with microbial proliferation, as it is evident from the results on the VFA production and microbial protein concentration, as mentioned previously. Jones *et al.* [42] have reported that tannins from *Onobrychis viciifolia* induced morphological changes and reduced the growth in *B. fibrisolvens*, which is one of the cellulolytic strains mainly involved in ruminal biohydrogenation[3]. In a recent *in vivo* study[7], it was found that CLA and VA in the meat of lambs fed tannins from CT were at a lower amount compared with the meat of lambs receiving the same diet but supplemented with PEG. Also, other studies have found that treating flaxseed with QT tannins reduced the biohydrogenation of LNA *in vitro* [43].

The method developed in the present study for measuring the LA-I activity in the ruminal fluid enables to evaluate the effect of a specific feed or an additive on the ruminal biosynthesis of CLA. However, biohydrogenation is a complex process and the two successive steps, the reduction of CLA to VA and the saturation of VA to SA, need to be accurately monitored as well. By the application of the new method, it was concluded that tannins reduce the biohydrogenation *in vitro* through an inhibition of the activity of ruminal micro-organisms rather than through an inhibition of the enzyme activity *per se*. The different steps of the biohydrogenation seem to be influenced by tannins at different rates, the hydrogenation of VA to SA being affected to a larger extent than the conversion of LA to CLA. Further research is needed to study the evolution of biohydrogenation in the presence of tannins at different times of incubation. *In vivo* studies are needed to better understand if the accumulation of VA caused by tannins could be an effective strategy for increasing the endogenous biosynthesis of CLA in the mammary gland and in the muscle by the action of the enzyme Δ^9-desaturase, thus increasing the health value of milk and meat.

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The experiment was undertaken at the Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim (Germany). A. P. performed the data analysis. M. M. performed the fatty acid analysis at the University of Pisa (Italy). All the authors contributed to the interpretation and the discussion of the results. V. V. and A. P. wish to thank Goffredo Petrone and Ivana Puglisi for their precious suggestions before starting the experiment. We thank Manuel Scerra for feed analysis and Hermann Baumgärtner for technical help and expertise. The authors declare no conflict of interest.

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