Chapter 9
Manipulating Ruminal Biohydrogenation by the Use of Plants Bioactive Compounds

Valentina Vasta and Rui J.B. Bessa

Abstract Ruminal microbial community is responsible for the biohydrogenation (BH) of the dietary unsaturated fatty acids ingested by ruminants. This process results in the production of saturated fatty acids (SFA) at the expenses of the unsaturated fatty acids (UFA). Animal scientists are attempting different possible strategies to manipulate ruminal BH process, in order to obtain meats and milk with a lower SFA content, which would be of great value for consumers' health. To avoid the use of synthetic molecules, such as some drugs or additives in livestock farming, animal scientists are focusing on the use of plant bioactive compounds (PBC) as modulators of ruminal BH. This manipulation is performed through a direct action of PBC on the bacterial and protozoa community involved in the BH process directly or indirectly. In this chapter, we report the effects of tannins, saponins and essential oils on ruminal BH with emphasis to their effects on the microbial ecosystem. A brief description of the impact of PBC on meat and milk fatty acid profile is given.

Keywords Ruminal biohydrogenation • Conjugated linoleic acid • Phytochemicals • Saponins • Tannins • Essential oils

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9.1 Introduction

The meat and milk from ruminant are often blamed for their high content of saturated fatty acids (SFA), intake of which is correlated to an increase in the risk of chronic diseases in humans (Burlingame et al. 1999) and for the relatively poor content of the health promoting polyunsaturated fatty acids (PUFA). At a first glimpse this would seem a paradox, as ruminants' diet is typically based on forages and grains which contain much greater proportions of PUFA than SFA (Morand-Fehr and Tran 2001).

The first explanation for this ruminant paradox was that animal tissues cause either a preferential oxidation or a direct saturation of dietary PUFA (Banks and Hilditch 1931). The evidence that dietary C18 PUFA are extensively hydrogenated in the rumen became available in the 1950s (Reiser 1951; Reiser and Reddy 1956). In late 1950s Shorland and co-workers demonstrated that the incubation of linoleic and linolenic acids (C18:2 *cis*-9 *cis*-12 [LA] and C18:3 *cis*-9 *cis*-12 *cis*-15 [LNA], respectively) with sheep rumen content led to the disappearance of LA and LNA and resulted in the production of stearic acid (C18:0, SA) and of a number of C18:1 isomers with *trans* double bond configuration (Shorland et al. 1957). These results have been attributed to the activity of ruminal bacteria and this pathway is currently named as ruminal BH of PUFA. In 1967, Kepler and Tove showed that the incubation of *Butyrivibrio fibrisolvens*, a bacterial species present in the rumen, with LA produced a conjugated dienoic fatty acid, identified as C18:2 *cis*-9 *trans*-11 and that the conversion of LA to the conjugated diene was carried out by LA isomerase enzyme (Kepler and Tove 1967). Although the presence of conjugated dienes in the ruminant edible fat was reported a long ago (Riel 1963; Parodi 1977), it was only in the late 1980s that conjugated linoleic acid (CLA) was identified as a potent anticarcinogenic agent (Ha et al. 1987). In 1996, the National Academy of Sciences (NRC 1996) concluded that ". . . conjugated linoleic acid is the only fatty acid shown unequivocally to inhibit carcinogenesis in experimental animals." Therefore, a further paradox of ruminants' meats and milk fatty acid profile is that, despite its high SFA content, it contains a nutraceutical component such as CLA.

The C18:2 *cis*-9 *trans*-11 is the major naturally occurring conjugated isomer of LA. The common name of this fatty acid, i.e., rumenic acid (RA) (Kramer et al. 1998) suggests that this is mainly produced in the rumen, however RA is also produced endogenously in the intestinal mucosa, in the muscle and in the mammary gland of mammals from the desaturation of C18:1 *trans*-11 (vaccenic acid, VA) (Corl et al. 2003), a fatty acid which arises from the BH of both LA and LNA.

While during the 1960s and 1970s the scientific community showed an interest in elucidating BH pathways, later on it was recognized that the best approach for improving the productivity of ruminants and increasing PUFA concentrations in meat and milk is minimizing the interactions between lipids and rumen microorganisms, thus avoiding any BH activity. This led to the development of protected fat sources which are extensively used in ruminant nutrition. A new surge of interest on BH and on its modulation started in the mid 1990s after the recognition of RA as a potent bioactive fatty acid, as shown by a large number of biomedical studies on its

mechanisms of actions. Recent advances on ruminal BH aim at unravelling the complexity of the microorganisms responsible of this pathway and identifying the BH intermediates.

The first animal production study aimed at identifying factors that affect RA concentrations in ruminant products (milk) was conducted by Jiang et al. (1996). Since then, the research on dietary factors that modulate the BH process and thus fatty acid composition of milk and meat has greatly increased. The exploration of plants secondary metabolites as dietary components capable to manipulate ruminal BH is very recent and will be reviewed here.

9.2 Fatty Acid Metabolism in Ruminants

9.2.1 The Fate of Dietary Fatty Acids in the Rumen: Lipolysis and Biohydrogenation

The BH process takes place in the rumen after the lipolysis of dietary triacylglycerols (present mostly in cereals) and of sulfo-, galacto- and phospholipids (predominating in green forages) (Dawson et al. 1977). In the rumen, the hydrolysis of ester linkages of dietary acyl lipids to non-esterified FA, or lipolysis, occurs rapidly (Garton et al. 1958; Dawson and Hemington 1974; Dawson et al. 1977). The presence of the free carboxylic group at the Δ-end of the fatty acids is an absolute requirement for the proceeding of the BH (Harfoot and Hazlewood 1997), so that the extent of lipolysis in the rumen is a major determinant of the extension of BH. Therefore, the partial inhibition of lipolysis in the rumen can be an effective approach to modulate both the amount of PUFA that escape BH and the pattern of biohydrogenation intermediates produced (Lourenço et al. 2010).

The lipases operating this process are both of plant (feed) and microbial origin, the contribution of each being still controversial (Lourenço et al. 2010). Fay et al. (1990) reported that 74 strains of ruminal bacteria showed lipolyzing capability, although the level of activity varied largely between the strains. The lipolytic activity in *Anaerovibrio lipolytica* (Harfoot 1978) and *Butyrivibrio fibrisolvens* (Hespell and O'Bryan-Shah 1988) was deeply investigated. Henderson (1971) reported that *Anaerovibrio lipolytica* did not hydrolyze phospholipids and galactolipids, while they hydrolyzed diglycerides more rapidly than triglycerides. These results suggest that *Anaerovibrio lipolytica* could play a minor role in lipolysis in forage-fed ruminants. Hespell and O'Bryan-Shah (1988) reported that among 30 strains of *B. fibrisolvens* tested the lipolytic activity largely varied and in some strains this activity increased with cell growth until the stationary growth phase was reached, after which the lipolytic activity remained constant.

Early studies also reported that protozoa might possess lipolytic activity (Wright 1961; Latham et al. 1972). When studying protozoa extracts, Wright (1961) observed that treating the cultures with penicillin reduced lipolysis and hypothesized that protozoa (mainly *Epidinium* spp.) could contribute up to 40% of ruminal lipolysis.

It has been also suggested that the protozoa lipolytic activity could be due to the feed lipases present in the chloroplasts engulfed by protozoa (Harfoot and Hazelwood 1988). Further studies supported by the recently available analytical techniques are needed to better understand the role of protozoa in ruminal lipolysis.

After lipolysis, the non-esterified unsaturated fatty acids liberated are mainly adsorbed into rumen particulate matter including bacterial surfaces (Keeney 1970), where PUFA can undergo through extensive BH. The reasons why rumen microbial ecosystem hydrogenate the unsaturated FA are not well understood. However, as pointed out by Jenkins et al. (2008), understanding why bacteria developed the enzymatic capacity to conduct BH may hold the key to find methods to manipulate BH in a predictable manner. Several hypotheses have been proposed so far. Lennarz (1966) hypothesized that the BH could serve as a hydrogen acceptor pathway to save the hydrogen to be rechanneled to other processes. Nevertheless, already at the beginning of the 1970, Czerkawski (1972) estimated that through the BH only small proportions of hydrogen could be saved. The hypothesis that BH is a detoxification mechanism was advanced by Kemp and Lander (1984), and it is based on the toxic effects of PUFA to most rumen bacteria. Recently, Maia et al. (2007, 2010) reported that the PUFA inhibited the growth of pure bacterial strains with BH activity, particularly the stearic acid producing bacteria.

Bessa et al. (2000) proposed that the generation of *trans* C18:1 isomers through incomplete BH could be an adaptive strategy of rumen ecosystem to deal with stressor stimuli including rumen lipid overload. This is because some BH intermediates, such as *trans* C18:1 isomers could have a protective role to rumen bacteria in certain environmental conditions as reported for other microbial systems (Keweloh and Heipieper 1996). In fact, *trans* C18:1 isomers are ubiquitous in the rumen ecosystem whatever the dietary conditions are.

At the end of the 1960s a scheme of the biohydrogenation pathway of LA was proposed by Kepler et al. (1966). From the very beginning of the studies of ruminal biohydrogenation it was clear that the scheme proposed was only one among the possible BH pathways, as a number of intermediary trienes and dienes (both conjugated and not conjugated) and monoenes isomers with 18 carbon chain were shown to arise from the BH of C18 PUFA (Kepler et al. 1966). In more recent studies some biohydrogenative pathways have been proposed and several intermediates of ruminal BH have been identified (Mosley et al. 2002; Buccioni et al. 2006; Bessa et al. 2007) and are listed in Table 9.1. Among all the possible BH pathways, it is noteworthy to describe in detail two mechanisms involving LA and LNA (Fig. 9.1).

1. the LA is first converted by LA-isomerase enzyme to RA, which is then hydrogenated to form VA by RA reductase enzyme. Then, VA is hydrogenated to form SA.
2. the LNA is isomerised to C18:3 *cis*9 *trans*11 *cis*15, which is then hydrogenated to C18:2 *trans*11 *cis*15. This fatty acid can be either directly hydrogenated to VA and C18:1 *cis*-15 or can be isomerised to form C18:2 *trans*11 *cis*13, which in a following step is hydrogenated to VA. The last step of the BH is the hydrogenation of VA to SA.

Table 9.1 Intermediates of ruminal biohydrogenation

	C18:2	C18:2	
C18:1	Non conjugated	Conjugated	C18:3
Trans-6	Trans-8, cis-13	Trans-6, trans-8	Cis-9, trans-11, cis-15
Trans-7	Cis-9, trans-12	Cis-7, trans-9	
Trans-9	Cis-9, trans-13	Trans-7, trans-9	
Trans-10	Trans-9, trans-12	Trans-7, cis-9	
Trans-11	Trans-9, trans-13	Trans-8, cis-10	
Trans-12	Cis-9, cis-15	Trans-8, trans-10	
Trans-13	Trans-12, trans-14	Cis-9, cis-11	
Trans-14	Cis-12, cis 15	Cis-9, trans-11	
Trans-15		Trans-9, trans-11	
Trans-16		Trans-10, trans-12	
Cis-11		Trans-10, cis-12	
Cis-12		Trans-11, trans-13	
Cis-13		Cis-11, trans-13	
Cis-14		Trans-11, cis-13	

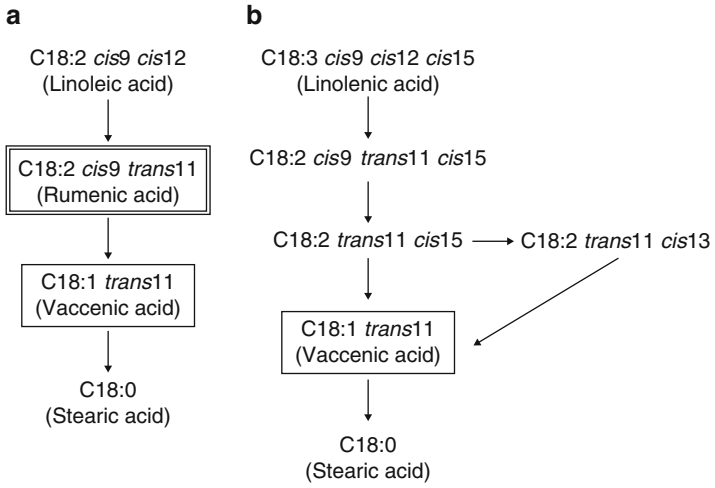

Fig. 9.1 The LA and LNA biohydrogenation pathways

While RA present in the rumen is generated only during the BH of LA, VA is synthesized during the hydrogenation of both LA and LNA. This process has major implications. In fact, Santora et al. (2000) and Piperova et al. (2002) shown that up to the 90% of RA detected in meat and milk is originated by the desaturation of VA operated in the muscle or in the mammary gland by Δ^9-desaturase enzyme; as a consequence, the RA synthesized during ruminal BH contributes a little to the overall RA content in meats and in dairy products. Therefore, it is now clear that, when attempting to increase RA content in meat and milk, the best strategy is enhancing VA formation in the rumen and its uptake in the duodenum, rather than increasing RA post-ruminal absorption.

9.2.2 Ruminal Microorganisms Participating to the Biohydrogenation

From the very beginning of the studies concerning the BH it was found that the bacteria that mainly carry out the BH are the cellulolytic bacteria belonging to the *Butyrivibrio* group (Kepler and Tove 1967) including the genera *Butyrivibrio* and *Pseudobutyrivibrio* (Paillard et al. 2007) and it is now largely agreed that the BH is undertaken by a consortium of bacteria (Lourenço et al. 2010). In 1984, Kemp and Lander (1984) proposed a division of the bacteria based on their participation to the different steps of the BH: group A bacteria -the *Butyrivibrio* group- that are able to hydrogenate linoleic and linolenic acid to VA, and group B: bacteria that are also able to saturate VA to form SA. The SA producing bacteria were identified as *Fusocillus* spp. (Kemp et al. 1975), which corresponds to *Clostridium proteoclasticum* isolated by Wallace et al. (2006), recently reclassified as *Butyrivibrio proteoclasticus* (Moon et al. 2008). Huws et al. (2011) have suggested that uncultured bacteria classified as *Prevotella*, *Anaerovoax* and unclassified Clostridiales and Ruminococcaceae may play a major role in the BH process.

The role of protozoa community in ruminal BH has been only little investigated, when compared to the large number of studies concerning bacteria, and so far there is an open debate on the role played by ruminal protozoa in BH. Devillard et al. (2006) reported that protozoa incorporate both RA and VA through the engulfment of ruminal bacteria. Yáñez-Ruiz et al. (2006) calculated that protozoa could contribute up to the 40% of RA and VA flow from the rumen to the duodenum. Controversial results are reported regarding an active role of protozoa in the BH pathway. A study conducted by Devillard et al. (2006) failed to detect the ability of protozoa from sheep to convert LA to RA or VA. On the contrary, Or-Rashid et al. (2008) reported that mixed rumen protozoa can convert LA to RA but cannot undertake the successive steps of the BH pathway. Boeckaert et al. (2009) evaluated *in vitro* the capability of *Isotricha prostoma* (a ciliate protozoon) to hydrogenate LA and its intermediates, and found that *I. prostoma* converted only little amount of LA to RA (less than 1%) and to C18:1 isomers. These authors concluded that *I. prostoma* cannot operate the BH of LA, while the bacteria associated (endo- and ectosymbionts) with this protozoan or engulfed by *I. prostoma* could be responsible for the observed results.

Little information is available with regards to the capacity of fungi in the ruminal BH process. Nam and Garnsworthy (2007) reported that rumen fungi can hydrogenate fatty acids *in vitro* until the formation of VA but the BH is slower compared to rumen bacteria.

The type of intermediate products and the completeness of the BH (leading to more saturated products) are strongly dependent on the diets offered to animals (Bessa et al. 2007). A large number of studies have investigated the effects of feeding green grass, silage or concentrates (French et al. 2000; Santos-Silva et al. 2002;

Aurousseau et al. 2004; for a review see Scollan et al. 2006) or the effects of lipid-enriched diets (Mele et al. 2007; Wąsowska et al. 2006; Noci et al. 2007) on ruminal BH and consequently on meat and milk fatty acid profile. However, this extensive topic is out of the scope of the present chapter and will not be further treated.

9.2.3 The Enzymatic Complex Operating Ruminal Biohydrogenation

The LA-isomerase (LA-I) enzyme catalyses the isomerization of LA to form RA. This enzyme was characterized in the late 1960s by Kepler and Tove (1969). So far, the activity of LA-I has been studied mostly with pure bacterial species, such as: *Butyrivibrio fibrisolvens* (Fukuda et al. 2006; Kepler and Tove 1967; Wąsowska et al. 2006), *Troponema* $B_2$5 strain (Yokoama and Davis 1971), *Clostridium sporogenes* (Peng et al. 2007), *Lacobacillus delbrueckii* (Lin 2006) and *Eubacterium lentum* (Verhulst et al. 1986). In an earlier study on the biohydrogenation activity in pure culture strains of *Butyrivibrio fibrisolvens*, Hunter et al. (1976) reported that the biohydrogenation *in vitro* of punicic acid (C18:3 *cis*9 *trans*11 *cis*13) to VA was higher with the incubation of both the cell free extract and the insoluble particulate fraction (which comprises bacterial cell walls) compared with the incubation of endocellular soluble fraction only. This happened because LA-I (Kepler and Tove 1967; Peng et al. 2007) and CLA reductase (Huges and Tove 1982) are membrane-bound enzymes. In another study (Polan et al. 1964), it had been found that the addition of boiled ruminal fluid to a reaction mixture of pure ruminal bacterial strains enhanced LA-I activity. As suggested by Polan et al. (1964), it is likely that some cofactor(s) present in the insoluble particulate fraction or in ruminal fluid might play a role in the reaction. However, a cell extract deriving from a pure culture (Kepler and Tove 1969; Verhulst et al. 1986), is a much simpler system compared with the whole ruminal environment in which the microorganisms responsible for the BH of fatty acids account only to a small proportion of the entire ruminal population. Vasta et al. (2009a) attempted to measure LA-I activity in cows ruminal fluid. The LA-I activity was observed only when the substrate was incubated in the presence of a lysate of ruminal microbial pellet (containing both the endocellular and the membrane fractions), while in the presence of the sole endocellular fraction it was not possible to measure LA-I activity.

9.2.4 Fatty Acids Metabolic Fate

The ruminal content comprising of bacteria and protozoa transits to the duodenum for the following digestive processes. In the lower tract of the small intestine fatty

acids, deriving either from feeds, or from free fatty acids or from digested protozoa and bacteria, are absorbed, re-arranged in the chylomicrons and transferred through lymph and blood to the liver and the tissues and become available for endogenous metabolism, including oxidation, transfer into milk and deposition in tissues. Some fatty acids generated in the rumen might undergo structural changes, particularly due to delta-9 desaturase enzyme action. Saturated fatty acids, like 18:0, are the major substrates for delta-9 desaturase enzyme, but some *trans* octadecenoates, namely those with double bonds from Δ-4 to Δ-13, except those with double bonds located at Δ-8, -9, and -10, served as substrates, originating 18:2 *trans*, *cis*-9 isomers, with the rate of desaturation being higher as the distance of the trans double bond from the Δ-9 position increased (Shingfield et al. 2008). Thus, accounting these predictable structural changes, the pattern of biohydrogenation intermediates (BI) observed in tissues is expected to reflect the BI rumen outflow pattern allowing the indirect monitoring of *in vivo* ruminal biohydrogenation. This is reinforced by the close relationship between duodenal flows and the biohydrogenation intermediates present in milk, as reviewed by Chilliard et al. (2007). This indirect evaluation of *in vivo* rumen biohydrogenation is particularly useful when no direct rumen fatty acid balance data are available, as in most of the studies on the role of PBC on rumen biohydrogenation.

9.3 The Impact of Plants Bioactive Compounds on Ruminal Biohydrogenation

The studies concerning the effects of plants bioactive compounds on ruminal biohydrogenation are quite recent. Most of the early studies focusing on plants (bushes, trees or forages) rich in secondary compounds aimed at evaluating the suitability of these feeds for livestock farming with respect to feed digestibility and animal growth performances, health and reproduction. The impact of dietary PBC on meat and milk fatty acid composition has been investigated only in the last few years (Vasta et al. 2008).

Most researches regarding PBC and ruminal BH have been conducted through *in vitro* studies, while the *in vivo* works available in literature are a few. In most of the studies here reviewed a detailed fatty acid profile of the ruminal content or of the fermentation medium is reported; nevertheless, so far the impact of PBC on the microorganisms responsible of the BH process has been seldom investigated. The following sub-sections will deal with the effect of tannins and phenolic compounds (Sect. 9.3.1), saponins (Sect. 9.3.2) and essential oils (Sect. 9.3.3) on ruminal biohydrogenation. Apart from the impact of PBC on ruminal microorganisms and BH, also a brief description of meat and milk fatty acid profile as affected by PBC will be given.

9.3.1 Tannins and Phenolic Compounds

9.3.1.1 Impact on Ruminal Microbial Community and Biohydrogenation

In vitro and *in vivo* studies conducted between 2003 and 2010 have shown that red clover (RC) (*Trifolium pratense*) reduced ruminal BH of PUFA and that the extent of lipolysis in RC was also hampered compared to other forages (for a review, see Van Ranst et al. 2011). These results were attributed to the interaction between the phenolic compounds from RC leaves and the polyphenol oxidase enzyme (PPO) (Loor et al. 2003), an enzyme which is highly active in RC. It was at first hypothesized that lipolysis could have been reduced by a stable binding between the quinones (produced by PPO through the oxidation of simple phenolics) and plant lipase enzyme (Lee et al. 2004, 2007; Van Ranst et al. 2009). Lourenço et al. (2008b) suggested that lipolytic enzymes from rumen bacteria could also be inhibited by quinones. However, this hypothesis was disproved: Van Ranst et al. (2009) reported no relation between measured lipase activity and lipolysis in red clover silages. Moreover, during ruminal fermentation the lipase of microbial origin seems to play a major role in lipolysis (Lee et al. 2007); therefore, the sole inhibition of plant lipase would not justify the reduced biohydrogenation observed *in vitro* and *in vivo*; Lee et al. (2010), by the use of immunogold labelling technique, concluded that the lipolysis of RC membrane lipids could be reduced through an entrapment of lipids within protein-phenol complexes. This means, in other words, that lipolysis is reduced because the substrate (membrane lipids) is unavailable to lipase enzyme (both of vegetal and of microbial origin). Considering that the lipolysis is a prerequisite for the biohydrogenation, it seems consequential that an impaired lipolysis also results in reduced BH of PUFA. In fact, Lee et al. (2007) and Cabiddu et al. (2010) found that the biohydrogenation of LA and LNA (calculated as the proportional loss of C18 PUFA during the incubation) was reduced by phenols, suggesting that the BH was inhibited. In addition, Cabiddu et al. (2010) also found that, compared to proteins-bound phenols, the tannic polyphenols had a stronger inhibitory effect on biohydrogenation probably because tannins reduced the BH depressing both lipolysis and biohydrogenation.

Khiaosa-Ard et al. (2009) and Vasta et al. (2009a) conducted *in vitro* studies aiming at elucidating the effect of tannins on ruminal BH. Incubating ruminal fluid with the CT extracted from *Acacia mearnsii* (Khiaosa-Ard et al. 2009) or *Schinopsis lorentii* (quebracho) (Vasta et al. 2009a) inhibited the last step of the BH, thus leading to the accumulation of VA at the cost of SA production. Nevertheless, in these studies the accumulation of RA was unaffected by the presence of tannins in the fermenter systems. Also Durmic et al. (2008) reported that when extracts from *Acacia iteaphylla* -which contains condensed tannins (Al-Soqeer 2008)- were incubated *in vitro* with sheep ruminal fluid the production of VA increased while SA decreased. This trend of fatty acids in the ruminal fluid was also observed in two *in vivo* studies (Vasta et al. 2009b, 2010) with lambs supplemented with quebracho tannins. An issue to be solved was whether tannins interfere with the BH through

depressing ruminal microorganisms proliferation or through a direct binding between tannins and the enzymatic system responsible for the BH of fatty acids. Khiaosa-Ard et al. (2009) and Vasta et al. (2009a) reported that the acetate: propionate ratio was reduced by the inclusion of tannins in the fermenters. Considering that the acetate is produced mostly by cellulolytic bacteria (which also operate the BH), the lower acetate: propionate ratio could indicate that the activity of cellulolytic bacteria was impaired by CT. Khiaosa-Ard et al. (2009) also reported that the CT extract increased the bacterial community, while reduced the total protozoa population compared to the control fermenters. Vasta et al. (2009a) investigated LA-isomerase (LA-I) activity in fermented ruminal fluid in the presence of CT. These authors observed that although the CLAs – measured by the unspecific absorbance of dienes bonds at 233 nm wavelength- produced in the LA-I assay were synthesized at lower extent in the presence of tannins, nevertheless LA-I activity (nmol CLAs/mg protein/min) was not affected by tannins because together with CLA the microbial proteins in the reaction mixture also decreased. This result suggested that tannins did not interfere with LA-I *per se*, but interfered with microbial activity, accordingly with the lower VFA production and microbial protein production observed in that study. This would be consistent with earlier observation of Jones et al. (1994), who reported that tannins form *Onobrichis viciifolia* induced morphological changes and reduced the growth in *Butyrivibrio fibrisolvens*.

Converse results were found *in vivo* in the rumen of lambs fed a basal diet (barley grains-based concentrate) with or without the supplementation of quebracho tannins (Vasta et al. 2010). In this study, the LA-I specific activity was lower for the tannins-receiving lambs while the concentration of total CLAs produced in the assay was not affected by tannins supplementation. In the same study, RA accumulated in the rumen of the tannins-fed lambs, while it was absent in the rumen content of the lambs not supplemented with tannins (Fig. 9.2), suggesting that tannins hampered the conversion of RA to VA.

The effect of tannins on ruminal microbial community is still controversial, and in addition, there is only scarce information regarding the interaction between tannins and the bacterial groups responsible for the BH of fatty acids. Vasta et al. (2010) found in an *in vivo* study that quebracho tannins strongly impacted the microbial community. The dendrogram in Fig. 9.3 obtained from clustering analysis of banding profile of rumen bacterial 16S rRNA showed that bacteria from the lambs fed the concentrate with (TY) or without (TN) tannins clustered separately. Concerning the bacteria involved in the BH, Vasta et al. (2010) reported that tannins increased the total protozoa and the relative abundance of *B. fibrisolvens* and decreased the relative abundance of *B. proteoclasticus* in the rumen. Previous studies have shown that tannins from *Lotus corniculatus* (Min et al. 2002) or from *Acacia* spp. (Durmic et al. 2008) reduce the proliferation of *C. proteoclasticum* B316^T and *C. proteoclasticum* P18, respectively. Therefore, it is likely that different bacterial strains are differently sensitive to tannins. Indeed Durmic et al. (2008) tested the inhibitory power upon biohydrogenating bacteria of large number of plants containing secondary compounds, most of which also contained tannins. They found that the minimum dose of plants needed to inhibit the proliferation of *B. fibrisolvens* JW11 (which forms RA)

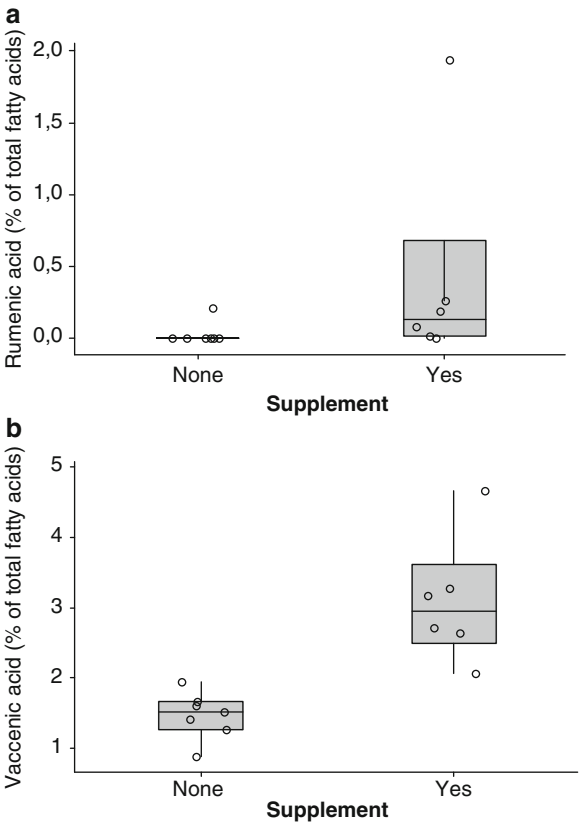

Fig. 9.2 Box plot of the distribution of RA (**a**) and VA (**b**) in the ruminal fluid of lambs fed concentrate with or without tannin supplementation. *Circles* represent individual animals

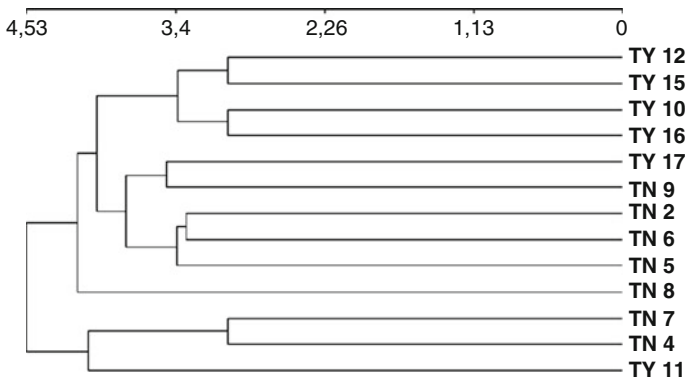

Fig. 9.3 Dendrogram derived from clustering analysis of denaturating gel electrophoresis banding profile of rumen bacterial 16S rRNA from lambs fed concentrate with (*TY*) or without (*TN*) tannins

was much greater than the dose needed to inhibit *Cl. proteoclasticum* P18, suggesting that *Cl. proteoclasticum* P18 is more sensitive to tannins than *B. fibrisolvens* JW11 (Durmic et al. 2008).

The effect of tannins on ruminal bacteria is also strongly dependent on the chemical structure of these compounds. In a study on the susceptibility of *B. fibrisolvens* and *C. proteoclasticum* to tannins, Sivakumaran et al. (2004) found that proanthocyanidins fractions from *Dorycnium rectum* with high molecular weight (HMW) inhibited the growth of *B. fibrisolvens* CF3, while low molecular weight (LMW) fractions did not interfere with bacterial growth. Conversely, *C. proteoclasticum* did not initiate the growth when incubated in the presence of LMW proanthocyanidins, while grew normally in the presence of HMW tannins. In the both cases, these results were also dose-dependent.

9.3.1.2 Tannins and Meat and Milk Fatty Acid Profile

The first study, in which an interaction between dietary condensed tannins (CT) and ruminal BH was hypothesized, was performed by Priolo et al. (2005). In that *in vivo* study, a group of lambs was fed sulla (*Hedysarum coronarium*; CT: 1.8% DM), while another group of lambs received sulla plus polyethylene glycol (PEG; a tannins binding agent). The supplementation of PEG did not affect the fatty acid profile of *longissimus dorsi* muscle: probably the low concentration of tannins in sulla was not enough to inhibit bacterial proliferation. Conversely, Vasta et al. (2007) found that the intramuscular fat of lambs receiving carob (CT: 2.7% feed DM) contained lower RA and VA, compared to lambs receiving the same diet but supplemented with PEG. In a study conducted by Vasta et al. (2009b) ruminal and muscle fatty acid profile of lambs supplemented with tannins were studied. The inclusion of quebracho tannins into concentrate diets resulted in greater amounts of VA, RA and total *trans*-C18:1 and in a reduction of C18:0 in meat (Vasta et al. 2009b). When quebracho was included in herbage, lamb meat contained greater amounts of PUFA compared to lambs fed the same herbage without added quebracho. These results observed in muscle reflected the fatty acid profile of rumen content. Jeronimo et al. (2010) reported that the supplementation of grape seed tannins in the diet of lambs did not affect abomasum or meat fatty acid profile. Feeding lambs a diet containing *Cistus ladanifer* (a tanniniferous bush) and linseed oil enriched meat with VA and RA, compared to the meat of lambs receiving the oil but not the *Cistus* (Jeronimo et al. 2010). These results quite encourage adopting the use of tannins-containing feed for improving meat fatty acid profile.

Cabiddu et al. (2009) analysed the fatty acid profile of milk from ewes grazing sulla (CT: 2.6% DM, average of three sampling dates) with or without supplementation of PEG. They noted that the RA and VA were lower, while C18:2 n-6 and C18:3 n-3 were higher, in the milk from the sulla-fed ewes compared to the milk from ewes receiving sulla + PEG. The authors concluded that tannins had impaired ruminal BH (Cabiddu et al. 2009). Benchaar and Chouinard (2009) reported that supplementing tannins from *Schinopsis balansae* did not affect the overall fatty acid profile

in cow milk. The ineffectiveness of tannins as modifiers of the fatty acid profile could be due to the low level of quebracho inclusion in the diet (0.45% of DMI) chosen for this study. Feeding cows *Lotus corniculatus* increased RA, LA and LNA and reduced VA and C18:0 in milk compared to *Lotus* + PEG (Turner et al. 2005).

It is sometimes doubted about the usefulness of dietary tannins for manipulating meat and milk fatty acid profile because of the generally reported detrimental effects of tannins on animal performances. However, the results reported in literature show converse effect of tannins on animal performance. In the studies from Priolo et al. (2000) and Vasta et al. (2007) lambs fed a diet containing carob pods (condensed tannins = 2.5% and 2.7% of feed DM, respectively) showed dramatically lower growth performance and carcass yield compared to lambs receiving the same diet, but with the addition of PEG. Also the inclusion of quebracho tannins (4.0% DM) in lamb's diets reduced animal growth performances compared to lambs not receiving the supplement (Vasta et al. 2009b). However, in the study of Vasta et al. (2009b), although the performances of the quebracho-supplemented lambs were reduced, animal health and carcass traits were still acceptable for Comisana breed lambs, while ruminal BH and meat fatty acid profile where strongly affected by tannins. As reported in an *in vitro* study by Vasta et al. (2009a), quebracho tannins have a "milder" inhibitory effect on ruminal microflora compared to carob tannins; this could explain the more detrimental impact of carob (2.7% DM) than quebracho (4.0% DM) on animal performance observed in the two studies form Vasta et al. (2007, 2009b). Jeronimo et al. (2010) found that tannins from *Cistus ladanifer* or from grape seed (2.5% DM) modulated fatty acid metabolism in the digestive tract, but they did not affect the average daily gain (ADG) and final live weight (LW) in lambs compared to the lambs fed a control diet. The effects of sulla on productivity were tested in dairy ewes (Molle et al. 2009; Cabiddu et al. 2009) and lamb (Priolo et al. 2005), and in both cases animal performances (milk yield and body condition score in ewes, and ADG and final LW in lambs) were similar to that of animals supplemented with PEG. Therefore, the use of tannins-containing feed seems to be promising. Further studies are needed to assess the opportune level of inclusion of each type of tannin in the diet, in order to manipulate ruminal biohydrogenation avoiding detrimental effects on animal productivity.

9.3.2 Saponins

9.3.2.1 Impact on Ruminal Microbial Community and Biohydrogenation

The impact of saponins upon ruminal BH has been studied only very recently. Similar to tannins, saponins have been shown to possess antimicrobial effects (Patra and Saxena 2009; Wallace et al. 2004). Makkar and Becker (1997) found that until 6 h incubation of *Quillaja s*aponins with ruminal fluid, saponins were not degraded, while saponins content in the fermenters decreased after 9 h incubation, suggesting that ruminal mixed cultures after an adaptation period are capable of degrading

Quillaja saponins. Wina et al. (2005) found that different bacterial groups within the fibrolytic bacteria responded differently to saponins exposition. With regards to the microorganisms mainly involved in ruminal BH, some early studies reported that *B. fibrisolvens* strains isolated from cattle rumen were able to degrade alfalfa saponins (Gutierrez et al. 1959). Conversely, Wallace et al. (2004) found that saponins from *Yucca schidigera* suppressed the growth of pure strains of *Butyrivibrio fibrisolvens* SH13, while prolonged the lag phase of *Streptococcus bovis* and enhanced the proliferation of *Prevotella ruminicola*. These results suggest that *Butyrivibrio* spp. might be more sensitive to saponins compared to other bacterial strains. Lourenço et al. (2008a) reported that incubating cow rumen content in continuous fermenters with added *Quillaja* saponins (up to a concentration level of 1 ppm) did not affect the production of fatty acids arising from the BH as compared to the control fermenters. In that study, VFA profile and the branched chain fatty acids, which can be considered as indicators of microbial growth (Vlaeminck et al. 2004) were unchanged by saponins, suggesting that the microbial activity was unaffected by saponins. However, it should be considered that changes in rumen microbial community caused by PBC do not necessarily imply changes in the biochemical pathways and *vice versa* (Goel et al. 2008). Khiaosa-Ard et al. (2009) found no effect of *Yucca schidigera* on ruminal BH *in vitro*. Moreover, in that study total rumen bacteria were not affected by saponins, while the protozoa community even increased. *In vivo* studies reported that feeding sheep 30 g/day *Yucca schidigera* saponins (Eryavuz and Dehority 2004) or cows 60 g/day (Benchaar et al. 2008) did not affect ruminal protozoa community. Nevertheless, *B. fibrisolvens* isolates and protozoa have been shown to be sensitive to saponins *in vitro* (Wallace et al. 2004). It is likely that saponins are less toxic to these microorganisms when added to mixed rumen bacteria or when fed to animals because in the consortium of the ruminal content some bacterial strains can degrade the saponins (Makkar and Becker 1997). Moreover, if some bacteria strains are inhibited by saponins, some other strains could take advantage, thus explaining the results from Khiaosa-Ard et al. (2009) and Benchaar et al. (2008). Nevertheless, it is mentioned that the response of ruminal microbial community to saponins seems to be dose-dependent (Patra and Saxena 2009).

9.3.2.2 Dietary Saponins and Meat and Milk Fatty Acid Profile

The studies aiming at investigating the effects of saponins-containing feed on meat or milk fatty acid profile are still very few. Ben Salem et al. (submitted) noted that lambs fed on diets containing up to 45 g (equal to 1.35 g diosgenin) of fenugreek seed (*Trigonella foenum-graecum* L.) and found no effect of saponin on longissimus muscle fatty acid profile. Similarly, Brogna et al. (2011) reported that the inclusion of *Quillaja saponaria* (level of inclusion in the diet: 30, 60 or 90 ppm) in the diets of lambs had no effects on the concentration in meat of those fatty acids arising during ruminal biohydrogenation (C18:1 *trans/cis* and C18:2 *trans/cis* isomers). Similarly, saponins did not modify cow milk fatty acid composition (Benchaar and Chouinard 2009).

9.3.3 Essential Oils

9.3.3.1 Impact on Ruminal Microbial Community and Biohydrogenation

The use of essential oils (EO) as modifiers of rumen fermentation and their impact on ruminal ecosystem has been largely investigated (Busquet et al. 2005; Duval et al. 2007; Calsamiglia et al. 2007). The term "essential oils" refers to a vast number of chemical compounds that can be classified as terpenoids (mono- and sesquiterpenes) and phenylpropanoids. These compounds possess potent anti-microbial properties. They adsorb on bacteria cell membranes causing conformational changes, membrane fluidification and leakage of ions across the membrane; and they also bind with enzymes, thus, hampering bacterial growth and activity (Calsamiglia et al. 2007). The sensitivity of bacteria to EO can vary depending on the: (i) chemical structure of the compound: molecules with carbonyl and hydroxyl groups are more toxic (Griffin et al. 1999), (ii) the type of bacteria: gram-positive bacteria seem to be more sensitive than gram-negative (Cox et al. 2001), and (iii) the concentration in the medium. Some mono- and sesquiterpenes can be degraded by rumen microorganisms. This has been shown *in vitro* by Broudiscou et al. (2007) who found that α-copaene, myrcene, β-ocimene, α-pinene and sabinene were degraded by caprine rumen micro-organisms, while camphene and thymol were not degraded. According to Busquet et al. (2005), ruminal microbial populations can adapt to the presence of EO. This implies that results on ruminal fermentation obtained in a short-term exposure of ruminal bacteria to EO might be only a partial view of the real impact of EO on ruminal microorganisms.

McIntosh et al. (2003) screened *in vitro* the sensitivity of some ruminal bacterial strains to a blend of EO (containing thymol, eugenol, vanillin and limonene) and they found that *B. fibrisolvens* SH13 was more sensitive to the exposure to EO among the 21 bacterial strains studied. Durmic et al. (2008) tested *in vitro* the resistance of *B. fibrisolvens* JW11 and *Cl. proteoclasticum* P18 to plant EO. The results of this study showed that the EO extracted from *Lavandula intermedia*, *Agonis fragrans*, *Malaleuca capreolata*, *Santalum spicatum*, *Eucalyptus plenissima*, *Eucalyptus staigeriana*, *Leptospermum petersonii* and *Malaleuca ericifolia* inhibited the growth of *B. fibrisolvens* JW11 and *Cl. proteoclasticum* P18 and that the minimal inhibitory concentration of these EO was different between the two bacterial strains. Lourenço et al. (2008a) found that the inclusion of eugenol (250 mg/l) in fermenter systems induced some minor inhibition of the BH process, while the inclusion of cinnamaldehyde (500 mg/l) had dramatically hampered the BH and bacterial activity, leading to a great accumulation of those fatty acids that are intermediate products of the BH process. From this study it is not easy to conclude whether the type of molecule (eugenol vs. cinnamaldehyde) or their concentrations in the fermenter (250 vs. 500 mg/l) were responsible for the different results. Supplementing dairy cows with 1 g/day of cinnamaldehyde did not modify ruminal volatile fatty acid concentrations (which is an indicator of microbial activity) and protozoa count (Benchaar et al. 2008).

9.3.3.2 Dietary Essential Oils and Milk Fatty Acid Profile

The above stated dramatic effect of cinnamaldehyde on the biohydrogenation observed *in vitro* by Lourenço et al. (2008a) was not confirmed *in vivo*: the inclusion of 1 g/day of cinnamaldehyde in the diet did not change milk fatty acid composition (Benchaar and Chouinard 2009). With regards to other terpenoids, the administration of 750 mg/day of EO to dairy cows did not alter the total counts of cellulolytic bacteria and protozoa and milk fatty acid profile compared to milk from cows not receiving the EO (Benchaar et al. 2007). In this study, the animals were subjected to the experimental treatment for 28 days, which probably allowed ruminal population to adapt to the presence of the EO blend. Similar results were reported by Malecky et al. (2009) in a study conducted with dairy goats receiving a monoterpene blend (linalool, p-cymene, α-pinene and β pinene; 0.43 g/kg DMI) through rumen cannula. The *in vivo* results do not encourage using the EO as modifiers of ruminal biohydrogenation.

9.4 Implications

Modulating ruminal biohydrogenation is a key point to improve meat and milk fatty acid profile. Increasing RA and PUFA and reducing SFA in ruminants' products would have a major impact on consumers' health. Among the possible strategies to manipulate ruminal BH, the use of some plant bioactive compounds seem to be effective in reducing the BH. Condensed tannins have been shown to impair the last step of the biohydrogenation. Saponins and essential oils seem to be less effective than tannins in modifying the BH pattern. The research should be extended also to other PBC such as simple phenols, alkaloids and oxalates. Nevertheless, more research is needed to unravel the complexity of the interaction between PBC and the BH. It is noteworthy to encourage the collaboration between nutritionists, ruminal ecologists and meat and dairy scientists. Research should be conducted at two different and subsequent levels: (i) assessing *in vitro* the effects of purified PBC and of feedstuffs containing PSC on the BH pattern and the microorganisms involved in the BH; and (ii) testing the PBC, both present in feed and purified in *in vivo* trials, in which ruminal ecosystem, functionality, BH process and meat/milk fatty acid profile are contextually analysed. In addition to ruminal fatty acid metabolism, it is worthy to study also abomasal and duodenal fatty acid composition and net flow, which would help to predict the type and amount of fatty acids that will be absorbed and transferred in animal tissue. It is also of paramount importance to evaluate the dose-response effect of PBC not only on ruminal BH and products fatty acid composition, but also on animal performances.

Under the economical and social sustainability, the use of PBC as modulators of ruminal BH is more desirable than other supplements such as oils or oil-rich grains. Through the modification of the BH pattern we do not claim to get "health-promoter meat/milk" but to obtain products with a better fatty acid profile compared to those products deriving from animals raised under intensive production systems.

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