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Dietary Transformation of Lipid in the Rumen Microbial Ecosystem*

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ABSTRACT : Dietary lipids are rapidly hydrolysed and biohydrogenated in the rumen resulting in meat and milk characterised by a high content of saturated fatty acids and low polyunsaturated fatty acids (PUFA), which contributes to increases in the risk of diseases including cardiovascular disease and cancer. There has been considerable interest in altering the fatty acid composition of ruminant products with the overall aim of improving the long-term health of consumers. Metabolism of dietary lipids in the rumen (lipolysis and biohydrogenation) is a major critical control point in determining the fatty acid composition of ruminant lipids. Our understanding of the pathways involved and metabolically important intermediates has advanced considerably in recent years. Advances in molecular microbial technology based on 16S rRNA genes have helped to further advance our knowledge of the key organisms responsible for ruminal lipid transformation. Attention has focused on ruminal biohydrogenation of lipids in forages, plant oils and oilseeds, fish oil, marine algae and fat supplements as important dietary strategies which impact on fatty acid composition of ruminant lipids. Forages, such as grass and legumes, are rich in omega-3 PUFA and are a useful natural strategy in improving nutritional value of ruminant products. Specifically this review targets two key areas in relation to forages: i) what is the fate of the lipid-rich plant chloroplast in the rumen and ii) the role of the enzyme polyphenol oxidase in red clover as a natural plant-based protection mechanism of dietary lipids in the rumen. The review also addresses major pathways and micro-organisms involved in lipolysis and biohydrogenation. (**Key Words :** Lipid, Biohydrogenation, Rumen, Microbial Ecosystem, Chloroplast, PPO)

INTRODUCTION

Fatty acid (FA) metabolism of dietary lipids in the rumen has a large effect on the FA composition of ruminant tissue and milk lipids. Ruminant lipids are recognised to be highly saturated in nature relative to non-ruminants and consumption of ruminant fats has been linked to increased incidence of coronary heart disease (Williams, 2000; Givens, 2005). Hence, research has focused on optimising the fatty acid composition of ruminant lipids by decreasing saturated FA content and increasing beneficial *n*-3 polyunsaturated fatty acids (PUFA) (Scollan et al., 2006; Shingfield and Griinari, 2007). Dietary lipids are hydrolysed to constituent FA and then PUFA are rapidly hydrogenated by the rumen microbes resulting in the production of saturated FA (principally stearic acid; 18:0). This is one of the principal reasons for the highly saturated nature of ruminant lipids. This process also results in the formation of conjugated linoleic acid (CLA) and *trans* monoene intermediates, including *cis*-9, *trans*-11 CLA and vaccenic acid (VA; *trans*-11 18:1; Figure 1). CLA is a collective term used to refer to positional and geometric isomers of linoleic acid (18:2*n*-6) with a conjugated double bond. The two predominant isomers of CLA, *cis*-9, *trans*-11 and *trans*-10, *cis*-12, are naturally found in dairy products and ruminant meats, with *cis*-9, *trans*-11 CLA being the most abundant isomer (~75 to 90% *cis*-9, *trans*-11 CLA, 10 to 25% *trans*-10, *cis*-12 CLA). Since the majority of *cis*-9, *trans*-11 CLA in animal tissues is synthesised by delta-9 desaturase from ruminally derived VA, factors influencing the production of VA in the rumen are of interest. Dietary CLA has been shown in many animal models to be associated with cancer prevention, reduced atherosclerosis, improved immune response and reduced fat and increased lean deposition (i.e. Park et al., 1999). Research has focused on developing nutritional strategies which influence biohydrogenation and identification of the major micro-

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organisms involved with a view to improving the healthiness of ruminant products. This paper is focused on the importance of forages, grasses and legumes, and the identification of micro-organisms and metabolically important intermediates involved in biohydrogenation.

PATHWAYS OF LIPOLYSIS AND BIOHYDROGENATION IN THE RUMEN

The pathways of biohydrogenation of the major dietary PUFA, linoleic acid (18:2*n*-6) and linolenic (18:3*n*-3) acids, were established in classical studies conducted during the 1960s through to 1980s (see Harfoot and Hazlewood, 1997 and reviews by Palmquist et al., 2005; Bauman and Lock, 2006 and Jenkins et al., 2008). Dietary lipids entering the rumen are usually triglycerides, phospholipids and galactolipids. The first transformation is lipolysis, the hydrolysis of the ester linkages releasing free FA. The establishment of a free carboxyl group is a critical first step before hydrogenation may proceed. The first stage in the saturation of both 18:2*n*-6 and 18:3*n*-3 involves an isomerisation reaction that converts the *cis*-12 double bond to a *trans*-11 isomer, resulting in CLA and CALA (conjugated linolenic acid) respectively (Figure 1). A recent report suggests that isomers of 10,12 CLA are synthesised by a different mechanism compared to the synthesis of 9,11 isomers (Wallace et al., 2007). Hydrogenation of the *cis*-9 bond can then proceed by a microbial reductase, with the formation of VA. The final step in the ruminal biohydrogenation pathway involves a further hydrogenation of the *trans*-11 double bond producing 18:0 (18:2*n*-6 pathway; Figure 1) or *trans*-15 18:1 (18:3*n*-3 pathway; Figure 1). Recent advances in chromatographic characterisation of these isomers combined with interest in their potential bioactive properties in human health and animal performance has stimulated interest in biohydrogenation in the rumen (Jenkins et al., 2008).

MICRO-ORGANISMS INVOLVED IN LIPOLYSIS AND BIOHYDROGENATION

Evidence suggests that the saturation pathway in biohydrogenation is carried out almost exclusively by rumen bacteria. Nonetheless, biohydrogenation was observed by Wright (1959, 1960) in both bacterial and protozoal fractions of rumen contents. The rumen protozoa also had associated bacteria, which led Dawson and Kemp (1969) to question this hypothesis. They measured rates of biohydrogenation in faunated and defaunated sheep and found no differences, concluding that protozoa are not essential for biohydrogenation. Recent *in vitro* experiments carried out at the Rowett Research Institute (Devillard et al., 2006) showed that the protozoa have much higher CLA concentrations than bacteria, but did not possess delta-9 desaturase activity suggesting that protozoa preferentially incorporate CLA and VA formed by bacteria. *In vivo* data also showed that protozoa were proportionally high in PUFA and CLA compared with bacteria (Or-Rashid et al., 2007). The anaerobic rumen fungi have the capacity to biohydrogenate (Nam and Garnsworthy, 2007) but at a much slower rate than bacteria.

The saturation pathways require initial hydrolysis of ingested plant esterified lipid by the plants own lipases (Lee et al., 2004) and microbial lipases (Harfoot, 1978), causing the release of constituent FA. *Anaerovibrio lipolytica* is a well known rumen lipolytic bacterium (Hungate 1966); the lipase produced by this bacterium is extracellular and it has the capacity to hydrolyse diglycerides more readily than triglycerides. The lipase does not however, attack phospholipids and galactolipids. Bacteria that were

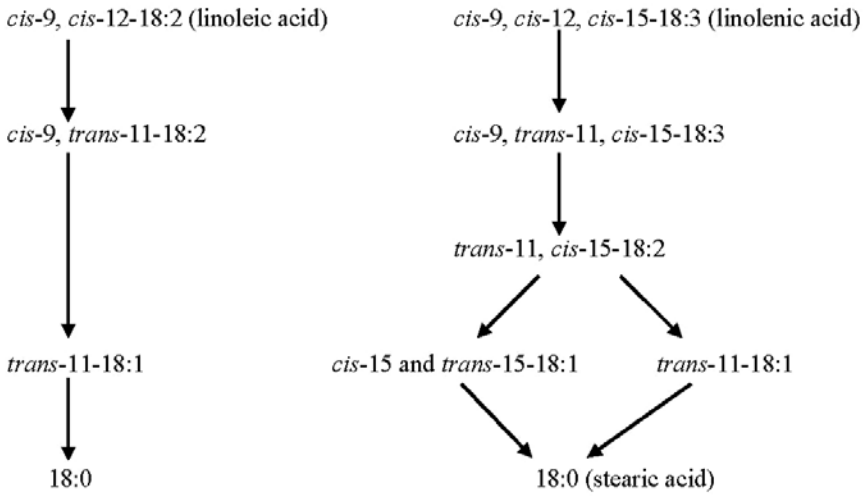

Figure 1. Biohydrogenation pathways of linoleic acid and linolenic acid (adapted from Harfoot and Hazlewood (1997)).

morphologically similar to the genus *Butyrivibrio* with the capacity to readily hydrolyse triglycerides have been isolated (Latham et al., 1972). Phospholipase activity has also been assigned to *Butyrivibrio fibrisolvens* and a *Butyrivibrio* strain named LM8/1B (Hazlewood and Dawson, 1975). Evidence suggests that ciliated rumen protozoa also cause hydrolysis of dietary esterified lipid, but again the presence of strongly-associated bacteria makes these observations less convincing (Hobson and Stewart, 1997). There is no evidence linking the fungi to hydrolysis of dietary esterified lipid in the rumen. Regardless, before biohydrogenation of released PUFA proceeds, the FA must be unesterified (i.e. having a free carboxyl group).

The released PUFA absorbs onto the surface of plant matter through hydrophobic interactions (Lough, 1970), so it is believed that surface-associated micro-organisms are mainly responsible for biohydrogenation (Harfoot et al., 1973). Kemp and Lander (1984) grouped the bacteria involved in the biohydrogenation pathways into Group A and B; Group A bacteria were classified based on their ability to hydrogenate PUFA to VA whereas Group B bacteria were categorised based on the ability to hydrogenate PUFA through to 18:0 (Figure 1). The main Group A bacterium is believed to be *B. fibrisolvens* (Harfoot and Hazlewood, 1997), whereas the main Group B organism identified to date is '*Fusocillus*' sp. (Kemp et al., 1975). The latter is a genus that no longer exists in modern taxonomy, and stored isolates of this organism are no longer viable (Wallace et al., 2006). Nonetheless, recent attempts using culture-dependent techniques have been made to re-isolate this '*Fusocillus*' or any bacteria capable of biohydrogenating PUFA to 18:0 with some success. Modern phylogenetic analysis, using 16S rDNA sequencing, of recent isolates has now shown that re-isolated 18:0-forming bacteria, like the most active Group A bacteria, are part of the *B. fibrisolvens* group, an ill defined taxon that includes the genera *Butyrivibrio* and *Pseudobutyrivibrio* and the species *Clostridium proteoclasticum* (Kopečný et al., 2003; Paillard et al., 2007). Group B bacteria (18:0 producers) form a tight grouping in which strains cluster together close to *C. proteoclasticum* (van de Vossenberg and Joblin, 2003; Wallace et al., 2006). Other workers have also demonstrated that the rumen bacterium, *Streptococcus bovis*, has the capacity to hydrate 18:2*n*-6 to 13-hydroxy-9-octadecenoic acid, thus diverting it away from the biohydrogenation pathway (Hudson et al., 1998). This provides evidence that other facultative anaerobic bacteria that also reside within the rumen, namely *Staphyloccous*, *Enterococcus*, *Lactobacillus* and *Pediococcus*, also have the capacity to hydrate 18:2*n*-6 (Hudson et al., 2000). Furthermore, following concentrate feeding it has also been demonstrated that a concomitant rise in *Megasphaera elsdenii* occurs within the rumen (Counotte et al., 1981). *Megasphaera elsdenii* causes the biohydrogenation of 18:2*n*-6 to the *trans*-10, *cis*-12 CLA (Kim et al., 2002), which explains the increase in this isomer following concentrate feeding. There may be many more bacteria involved in the biohydrogenation pathways but data is limited due to the fact that isolating such organisms is time-consuming, as biohydrogenators cannot be isolated by using specific selective media. A wide range of culturable bacteria have to first be isolated from the rumen, then pure cultures screened for their ability to biohydrogenate unsaturated FA. Kemp et al. (1975), for example, screened approximately 200 isolates in order to obtain 5 strains that had biohydrogenation capabilities. Recent data from our group using 16S rRNA based bacterial diversity profiling coupled with multivariate statistical techniques has shown that other bacteria, and potentially as yet unculturable bacteria, also play a role in these saturation events (Huws et al., 2006).

The reasoning's for biohydrogenation have included the fact that PUFA are believed to be more toxic than saturated FA to the Group A and B bacteria hence their saturation within the rumen (Henderson, 1973). Indeed recent data has indicated that the severity of toxicity of FA to ruminal bacteria was related mainly to the number of unsaturated bonds (Maia et al., 2007). Within this study they found that the *Butyrivibrio* group was exceptionally sensitive to the toxic effects, in particular *C. proteoclasticum* which may explain why biohydrogenation occurs. Regardless, there is now increasing research into the identification of other microbial species, using both culture-dependant and culture-independent techniques that may be involved in biohydrogenation and how they are influenced by diet. This will aid the understanding of methods that can be used to manipulate the biohydrogenation pathways to improve the nutritional properties of ruminant products.

DIETARY EFFECTS ON RUMINAL LIPOLYSIS AND BIOHYDROGENATION

Major sources of lipid in common diets of ruminants can be subdivided into marine (i.e. fish oil and algae) and plant sources (i.e. oil seed extracts). The extent of lipolysis of unprotected oils has been estimated to be in the range of 0.85-0.95, while the hydrolysis of structural plant lipids is thought to be lower due to the need to remove surrounding cellular matrices before lipolysis can occur (Doreau and Ferlay, 1994; Dewhurst et al., 2006). Lipolysis is considered to be rate limiting for biohydrogenation (Harfoot and Hazlewood, 1997). The rate of lipolysis *in vitro* is altered by diet composition (Gerson et al., 1983; Gerson et al., 1985), including forage maturity (Gerson et al., 1986) and particle size (Gerson et al., 1988). Atkinson et al. (2006) reported that the extent of ruminal lipolysis was decreased

in response to an increase in dietary high-linoleate safflower oil using multi-cannulated sheep.

Across a wide range of diet types, biohydrogenation of 18:3*n*-3 and 18:2*n*-6 in the rumen averages 0.93 and 0.85, respectively (Doreau and Ferlay, 1994; Dewhurst et al., 2006; Fievez et al., 2007; Jenkins et al., 2008). A recent study by Glasser et al. (2008) conducted a meta-analysis of the digestion of C18 fatty acids in the rumen with a database of 77 experiments (294 treatments). The major factors which influenced biohydrogenation included: ruminal pH, forage:concentrate ratio, level of intake and fish oil supplementation. These workers found a significant protective effect of low ruminal pH on cis-9, cis-12 18:2*n*-6 and 18:3*n*-3. Likewise a high proportion of forage in the diet had a negative effect on the flows of 18:2*n*-6 and 18:3*n*-3 to duodenum, suggesting increases in biohydrogenation. High levels of intake increased 18:3*n*-3 biohydrogenation and fish oil appeared to act on 18:3*n*-3 and *trans*-18:1, decreasing the proportion of 18:0 in total C18 duodenal flows. Another dietary strategy to alter biohydrogenation has included the use of copper supplementation (Engle et al., 2001) where copper decreased the concentrations of the 18:1 *trans* isomer and the C18-conjugated dienes in milk.

As presented in Table 1, almost all sources of long-chain PUFA (C20+) are from fish oil, but reports on the level of ruminal biohydrogenation of 20:5*n*-3 and 22:6*n*-3 are variable. Studies conducted *in vitro* and *in vivo* suggest that the response to fish oil is dependent on level of supplemented oil, the degree of accumulated unesterified FA and period of microbial adaptation (Dohme et al., 2003; Sinclair, 2007).

However, the use of fish oil has been very successful at inhibiting the final biohydrogenation step from *trans*-11 18:1 to 18:0 by its toxic effect on certain bacterial species (Kim et al., 2008). Other approaches have adopted protection technologies to by-pass the action of the rumen micro-organisms, including the use of calcium salts (Schauff and Clark, 1989), fatty acid acyl amides (Fotouhi and Jenkins, 1992) and encapsulation of lipid in a formaldehyde treated protein matrix (Scollan et al., 2003).

Considerable attention has also been given to beneficial increases in some FA, including CLA and *n*-3 PUFA in tissue and milk lipids of animals grazing 'species-rich' grassland relative to improved lowland grass swards (Collomb et al., 2002). These are thought to be related to alterations in rumen lipid metabolism as a result of changes in ruminal microbial population reducing the extent of PUFA biohydrogenation or the action of secondary plant metabolites produced in many 'weed' species common in 'species-rich' grassland. Such compounds include, polyphenol oxidase (see below) but also essential oils (Wallace, 2004), saponins (Shi et al., 2004; Wallace, 2004) and catecholamines (Lafontan et al., 2002) which inhibit lipases and possess anti-microbial properties. Condensed tannins found in certain leguminous species inhibited several strains of *Butyrivibrio fibrisolvens* (Min et al., 2003).

In our studies at Aberystwyth, emphasis is placed on maximising use of forages to increase the nutritional properties of ruminant lipids. The following sections review activities at our Institute in relation to i) the fate of the *n*-3 PUFA-rich chloroplast within the rumen ii) the relevance of polyphenol oxidase in red clover as a plant based mechanism to protect dietary lipids in the rumen. The thylakoid membranes of plant chloroplasts are rich in PUFA (Hawke, 1973), in particular 18:2*n*-6 and 18:3*n*-3, due to *de novo* synthesis of FA by fatty acid synthetase in the plastids (Murphy, 1999). Understanding what happens to the plant chloroplast post-ingestion especially, in grazing animals is central to improving food quality for human health. Feeding red clover relative to grass resulted in reduced biohydrogenation of dietary PUFA resulting in increased PUFA in meat and milk (Lee et al., 2003). This we believe relates to the activity of polyphenol oxidase in the red clover plant.

THE FATE OF CHLOROPLASTS WITHIN THE RUMEN

It has been estimated by image analysis following vital staining that approximately 50% of the plant cells in ingested plant fragments are viable on entry to the rumen (A Gay, E. J. Kim and A. H. Kingston-Smith, unpublished results). Thus 50% of chloroplast will remain within the

Table 1. Dietary effects on biohydrogenation of long-chain polyunsaturated fatty acid (C20+) in the rumen

Dietary treatments	Experiment type	Biohydrogenation	References
Fish oil	*In vitro*	Minimal	(Ashes et al., 1992)
Fish oil	*In vitro*	Variable	(Dohme et al., 2003)
Fish oil/marine algae	*In vitro*	Variable	(Sinclair et al., 2005)
Commercial fat containing fish oil (protected)	*In vitro*	Minimal	(Carriquiry et al., 2008)
Fish oil	*In vivo* (dairy cow)	Extensive	(Doreau and Chilliard, 1997)
Fish oil	*In vivo* (sheep)	Extensive (72-79%)	(Wachira et al., 2000)
Fish oil	*In vivo* (steer)	Extensive	(Scollan et al., 2001)
Fish oil	*In vivo* (steer)	Variable (48-94%)	(Kim et al., 2008)

plant tissue and 50% will enter the rumen milieu whereby some are lysed or damaged by the rumination process and others remain intact within the liquid fraction. In this respect some forage-derived PUFA will be released either attached to glycerol or free within the liquid fraction following mastication and rumination although there is evidence to suggest that released PUFA are absorbed onto the surface of plant matter post-release through hydrophobic interactions (Lough, 1970). Microbial lipolysis and biohydrogenation of esterified free PUFA can then be undertaken.

The rumen microbiota may be able to access and utilise FA which are free within the planktonic phase or attached extracellularly to forage more easily than those that are less accessible within chloroplasts that are free-floating or within the forage itself. Irrespective of saturation the FA can be directly incorporated into cellular membranes of rumen bacteria (Hawke, 1971) and protozoa (Williams et al., 1963; Girard and Hawke, 1978; Or-Rashid et al., 2007). It is nonetheless uncommon to find PUFA within rumen bacteria which are predominantly composed of *de novo* synthesised branched and odd-chain fatty acids (Goldfine, 1982) suggesting that cellular incorporation is minimal within rumen bacteria. Rumen protozoa, on the other hand, are rich in PUFA which has recently been suggested to be mainly due to their ingestion of chloroplasts as opposed to direct incorporation into cellular membranes (Huws et al., 2009). Recent work from our group has revealed that *Epidinium spp.* are commonly saturated with intracellular chloroplasts (Figure 2). *Epidinium spp.* are unique among the rumen ciliates in that they attach to plant material using an anterior pleated zone which is distinct from the mouth before actively invading plant tissues and breaking them down (Williams and Coleman, 1992). Whether they actively seek the chloroplast or take them up accidentally during grazing is unclear. Irrespective, we have evidence using chlorophyll and 18:3*n*-3 as markers that rumen protozoa are rich in PUFA mainly as a consequence of chloroplast uptake as opposed to cellular uptake (Huws et al., 2009). Nonetheless ciliates are selectively retained within the rumen by a migration/sequestration mechanism that depends on chemotaxis (Abde et al., 1981). As a consequence protozoal biomass reaching the duodenum is proportionally less than would be expected if they were to flow with the rest of the ruminal digesta (Hungate et al., 1971). Thus enhancing the flow of protozoa to the duodenum, whilst still maintaining density within the rumen, may be beneficial to enhancing the PUFA content of meat and milk (Or-Rashid et al., 2007). Work in our group is now concentrated on quantifying ingestion and egestion rates and also evaluating what happens to the chloroplast post-ingestion by ruminal protozoa as well as evaluating strategies to increase protozoal flow to the duodenum in grazing ruminants.

EFFECT OF FORAGE - POLYPHENOL OXIDASE

Unlike other common dietary feeds for the ruminant, biohydrogenation of 18:2*n*-6 and 18:3*n*-3 is lower in red clover (*Trifolium pratense*) relative to grass silage thus delivering more PUFA through to milk (Al-Mabruk et al., 2004; Van Dorland et al., 2008) and meat (Lee et al., 2009; Richardson et al., 2005). This relates to increased flow of dietary *n*-3 PUFA into the small intestine (Lee et al., 2003). This response to red clover feeding appears to be associated with the enzyme polyphenol oxidase (PPO) and a reduction in lipolysis of esterified lipid in the rumen.

PPO of plants and fungi are copper metalloenzymes which carry out *o*-hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones using molecular oxygen. These PPO generated quinones are highly reactive and can readily bind covalently with nucleophilic sites, e.g. on amino acids, resulting in the

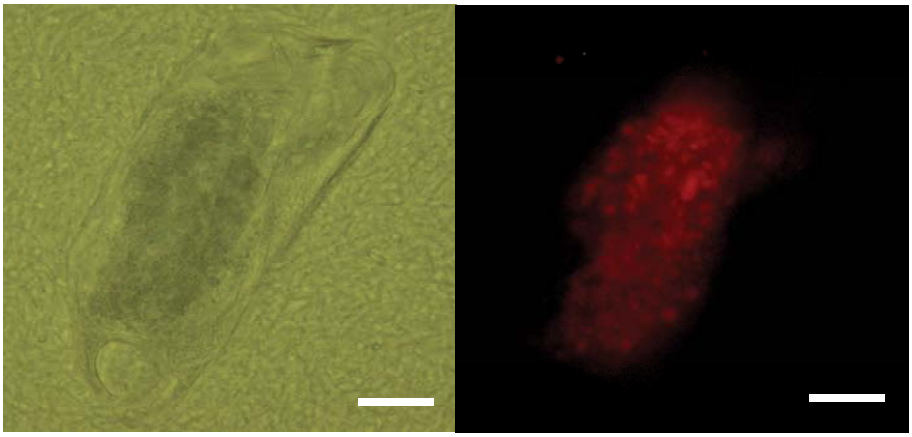

Figure 2. Confocal microscopy image of an *Epidinium sp.* fractionated from a steer 2 h post-grass feeding saturated with intracellular auto-fluorescing chloroplasts (Scale-20 μm).

formation of cross-linked protein polymers (Igarashi and Yasui, 1985). The role of PPO in the host organism is proposed to be a defence mechanism against invasive pathogens (Li and Steffens, 2002; Thipyapong et al., 2004) animal herbivory (Wang and Constabel, 2004) or physiological stress (e.g. drought resistance; Thipyapong et al., 2004).

The protection of esterified lipids against lipolysis by the action of PPO was first reported as a plant-mediated response (Lee et al., 2004; Lourenco et al., 2005) suggesting a deactivation of lipase protein, although an inhibitory action of grape seed extract on lipases was previously reported (Moreno et al., 2003). Later work showed that lipid was also protected in the presence of rumen micro-organisms suggesting something other than deactivation of plant lipases (Lee et al., 2007). Further, since PPO cannot function in the anaerobic conditions of the rumen, deactivation of microbial lipases is unlikely. Huws et al. (2006) reported that switching from grass to red clover silage feeding altered the ruminal microbial population and that this may partly explain the difference in lipolytic activity during red clover feeding (Figure 3). However it has recently been shown that the rumen microbial population are unable to adapt to utilise PPO protected red clover protein and esterified lipid and the associated ecological shift does not correlate with an increase in proteolytic or lipolytic activity (Lee et al., 2008d). This alludes to an active protection mechanism of the esterified lipid through the PPO induced chemical cascade. Proposed protection mechanisms include lipid-phenol complex formation either through polar head group binding or carboxyl binding or as recently suggested lipid micelle formation within protein-phenol complexes (Lee et al., 2008c).

PPO in red clover exists in either an active or a latent state. The former is active at neutral pH while the latter only shows activity at neutral pH following activation by treatments including acid/base shock, anionic detergents and proteases (Moore and Flurkey, 1990; Nozue et al., 1999; Stewart et al., 2001). More recently it has been demonstrated that latent red clover PPO is activated by its endogenous phenolic substrates, phaselic acid and clovamide (Winters et al., 2008). However while constitutive red clover PPO is predominantly in the latent form, *in vivo* substrate activation is prevented in healthy tissue by the separate subcellular compartmentation of the enzyme, which resides in the chloroplast (Mayer, 2006), and its substrates which reside in the vacuole. Therefore for PPO to elicit a reduction in lipolytic activity it requires both cell damage to activate the latent PPO and oxygen to oxidise *o*-diphenols. As a consequence studies on the potential benefits of high PPO forage crops such as red

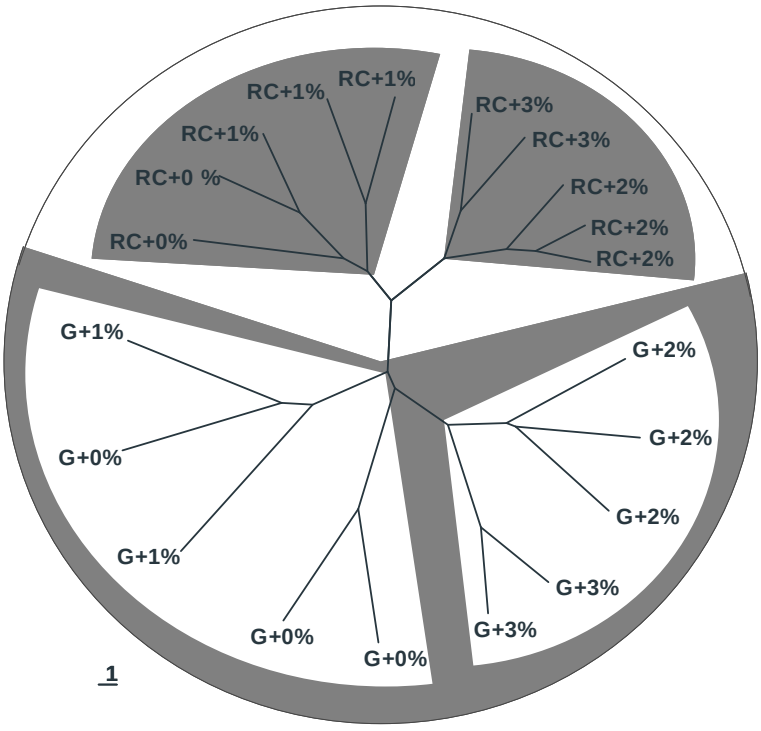

Figure 3. Bacterial DGGE (denaturing gradient gel electrophoresis) profiles of rumen solid from steers fed either red clover silage (RC) or grass silage (G) supplemented with fish oil at either 0, 1, 2 and 3% of dry matter intake (i.e. +0%, +1%, +2%, +3%) were compared by cluster analysis. Profiles clustered distinctly due to silage type, with sub-clusters evident according to the fish oil concentration (Huws et al., 2006).

clover have predominately concentrated on conserved feeds such as silage where there is adequate cell damage and exposure to air following harvesting. Studies at our institute (Lee et al., 2008b) have observed activation of 'grazed' red clover PPO during mastication which provides a critical 'window of opportunity' of cell damage and aeration. The extent of activation was related to an optimal fibre content of the forage in that too little fibre resulted in minimal mastication and a rapid swallow with little cell damage and too much fibre often corresponded to more mature red clover with lower PPO contents. They also reported that despite the activation of 'grazed' red clover during mastication a greater PPO activation and therefore retardation of lipolysis was achieved when the clover had received a prior 24 h wilt. In contrast further investigation showed that activated PPO in frozen and thawed red clover did not enhance the PUFA milk content over cut and carried ('grazed') red clover which had a lower activated PPO content when fed to dairy cows (Lee et al., 2008a). It is therefore likely that the activation procedure altered the way PPO protected the lipid as little is known about how maceration alters PPO action. For example a slow cell damage procedure such as wilting may result in a gradual *o*-diphenol oxidation which could favour nucleophilic coupling of *o*-quinones to proteins followed by oxidative cross linking of the *o*-diphenol-protein-lipid complexes. Such cross-linking would protect proteins and lipids from plant and microbial protease and lipase action (Winters and Minchin, 2001). By contrast a more severe cell damage such as freezing and thawing may result in a more rapid oxidation of *o*-diphenols favouring coupling of *o*-quinones with proteins at the expense of continued coupling reactions to cross link *o*-diphenol-protein complexes (Grabber, 2008). Under this scenario the protein and lipid would be protected from plant proteases and lipases but only partially from microbial proteases and lipases. Such questions regarding mechanism highlight the need for further research into the action of red clover in improving C18 PUFA in ruminant products. Research in the United States has focused on the transgenic incorporation of the PPO gene into other forage crops such as alfalfa (Sullivan et al., 2004). Other research is investigating other forages with PPO activity in particular grass species such as cocksfoot which has been shown to have activity comparable to red clover (Lee et al., 2006).

CONCLUSIONS

Ruminal transformation of dietary lipid plays an important part in determining the FA composition of ruminant products. Our understanding of the complexity of ruminal biohydrogenation has advanced considerably in recent years benefiting from advances in the chemical analysis of lipids using gas-chromatography (and/or with mass spectrometry) and molecular profiling tools to help identify micro-organisms involved. Forages are an important source of nutrients for ruminants and despite their low lipid content (2-5% dry matter) this lipid is rich in 18:3*n*-3 and is an important strategy for increasing *n*-3 PUFA in ruminant products. A high proportion of lipid in forages is located within the chloroplast and recent studies in our institute have focused on gaining an increased understanding of what happens to the chloroplast within the rumen and in particular their interaction with rumen protozoa. The enzyme PPO in red clover helps to reduce lipolysis (and hence biohydrogenation) of dietary lipids resulting in increased delivery of *n*-3 PUFA through into meat and milk. This is an important "natural" plant-based strategy to help protect dietary lipids from ruminal biohydrogenation. Increased understanding of micro-organisms involved in biohydrogenation and development of methods to regulate this process will help in the delivery of ruminant foods with higher nutritional and health benefits for consumers.

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