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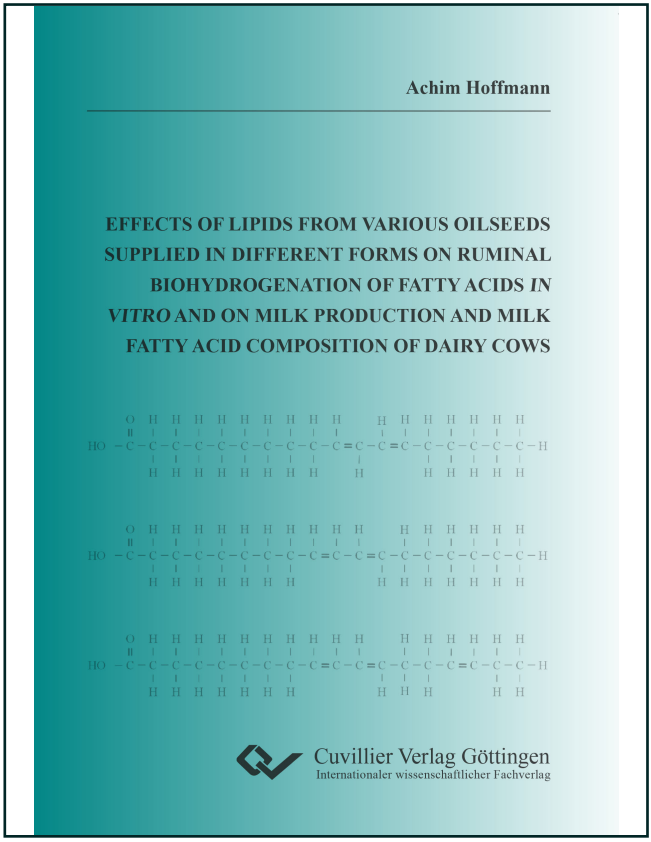
Effects of lipids from various oilseeds supplied in different forms on ruminal biohydrogenation of fatty acids in vitro and on milk production and milk fatty acid composition of dairy cows



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1. GENERAL INTRODUCTION AND WORK HYPOTHESIS

1.1. DIETARY LIPID SUPPLEMENTS IN RUMINANT DIETS - INTENTION AND LIMITATION

Dietary lipid supplements in ruminant diets, whether from various natural sources or supplements such as rumen protected lipids, have a long history and are widely used. The main reasons for using these supplements include an increased energy density of dairy cow diets, e.g. in the early stage of lactation (Clapperton and Steele, 1983). Moreover, reproductive parameters might are affected as well by using lipid supplements, either indirectly by changes in the energy balance of cows or directly due to the effects of certain fatty acids (FA) on reproductive organs and processes (Leroy et al., 2014). Furthermore, lipid supplementation focuses on altering the FA composition of products of ruminant origin such as milk, meat, and their processed products (Woods and Fearon, 2009). Recommendations for the use of lipid supplements in ruminant diets should be classified by their availability in the rumen and degree of rumen protection. Therefore, the classes of lipids and supplements that need to be considered when formulating dairy cow diets are unprotected lipids such as vegetable oils or partly natural protected sources such as oilseeds, intact or mechanically treated. Moreover, special protected lipid supplements are available, e.g. coated or saponified lipids and certain protected FA mixtures.

Diets for lactating dairy cows typically contain up to 5% lipids in dry matter (DM) (Bauman et al., 2003). According to Jilg et al. (1988), the dosage of lipid supplements in the diets is limited. These limitations comprise potential negative effects on DM intake (DMI), when exceeding the physiological level. Moreover, changes in rumen fermentation result in a potential reduction of nutrient digestibility, especially reduced fibre digestion (Jilg et al., 1988). A reduced digestion of fibre interferes with the production of short-chain FA (SCFA), which was supported by *in vitro* results, when using 5% of vegetable oils (Szumacher-Strabel et al., 2004). Similar results are reported in a review on feeding high lipid levels (8-9% of DM) to ruminants by Schroeder et al. (2004), who analysed the effects of lipid supplementation in pasture-based dairy cow diets. Therefore, a certain dietary lipid level should not be exceeded, because of potential detrimental effects of lipid supplements on rumen metabolism. Protected lipids are less restricted in their supplementation level compared to unprotected lipids. According to Palmquist and Jenkins (1980), up to 5% (DM) of lipids can be added to dairy cow diets without negative effects. The National Research Council (NRC, 2001) recommends that the total dietary lipid intake should not exceed 6 to 7% of DM, to avoid negative effects on the DMI and rumen fermentation (Jenkins, 1993).

Since there are different recommendations regarding the total dietary lipid level in dairy cow diets, it is mandatory to consider the lipid content of the basic diet and the nature of the lipid supplements. Depending on their amount and nature, different effects on feed intake and digestibility can be observed. A reduced DMI and a reduction in ruminal fibre digestion have been reported, if the total lipid intake exceeds a physiologically acceptable level (Jenkins, 1993). Recommendations by Männer (2002) distinguish between the intake of unprotected and protected lipids, and consider the diet´s total lipid level. The author recommends a range between 0.2 to 0.4 and 0.4 to 1 kg per cow and day for rumen unprotected and protected lipid supplements, respectively. Therefore, a practical implication can be that the total daily lipid intake should not exceed 1 kg when using unprotected sources or 1.5 kg when using protected sources or a combination of both. Calculating a DMI of 20 kg this results in a lipid content of 5% and 7.5% of DM in the diet, respectively.

1.2. DIETARY LIPID SOURCES AND FATTY ACIDS

Dietary lipids in ruminant diets are derived from roughage, cereals, and oilseeds as well as their by-products from oil extraction (Harfoot and Hazlewood, 1997). Fresh roughage has a total lipid content typically ranging between 3 to 8% of DM (Schroeder et al., 2004). About 50% of the total lipids in roughage are FA (mainly galacto- and phospholipids), and the rest consists of cuticular waxes, pigments (e.g. chlorophyll) and other unsaponifiable substances (Schroeder et al., 2004; Harfoot and Hazlewood, 1997; Palmquist and Jenkins, 1980). The lipid content of roughage sources such as grass or maize is affected by the processes for conservation and storage (Khan et al., 2012; Alves et al., 2011; Kalač and Samková, 2010). Losses of FA, especially unsaturated FA, from the roughage source might occur due to the oxidation and lipolysis of membrane lipids (Khan et al., 2012; Alves et al., 2011; Kalač and Samková, 2010). These effects need to be considered in calculating the contribution of the roughage lipid content to the total dietary lipid content. The FA composition of roughage is highly unsaturated, with linolenic acid (C18:3c9,12,15; LNA), linoleic acid (C18:2c9,12; LA), oleic acid (C18:1c9, OA) and palmitic acid (C16:0) as the dominant FA (Kalač and Samková, 2010) (Table 1).

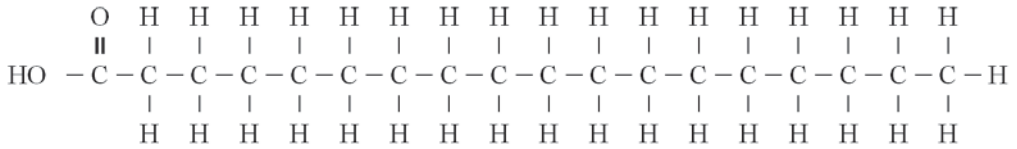
Table 1. Fatty acid (FA) composition of common roughage, cereals, and oilseeds.

	Ether extract* (% DM)	FA* (% DM)	FA composition (g/ 100g FA)							
			C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0
Roughage										
Perennial ryegrass	3.6	1.8	<0.7	12.9	1.7	3.0	3.2	14.6	65.2	1.9
Red clover	5.1	2.3	1.5	14.2	-	3.7	-	5.6	72.3	-
White clover	5.0	2.2	1.1	6.5	2.5	0.5	6.6	18.5	60.7	2.0
Alfalfa	-	3.9	-	16.9	-	1.9	1.8	16.0	63.9	-
Small meadow fescue	3.2	1.6	-	17.7	-	1.5	4.4	15.9	43.4	-
Cocksfoot	3.6	1.9	-	20.9	-	4.9	2.4	15.3	56.5	-
Grass, cool season	3.3	1.7	2.1	17.0	2.5	2.2	3.9	13.5	60.3	<0.5
Grass, silage	4.1 ± 0.6	1.9 ± 0.5	-	17.9	-	1.7	2.5	17.1	58.3	-
Maize, silage	3.5 ± 0.3	2.0 ± 0.3	-	15.2	-	2.3	22.6	52.1	5.5	-
Cereal grains										
Corn	4.3 ± 0.7	3.2	<0.5	16.3	-	2.6	30.9	47.8	2.3	-
Wheat	2.3 ± 1.1	1.0	<0.5	20.0	0.7	1.3	17.5	55.8	4.5	-
Barley	2.2 ± 0.6	1.6	-	27.6	0.9	1.5	20.5	43.3	4.3	-
Oilseeds										
Sunflower	41.9 ± 3.5	34.7	<0.5	5.5	-	3.6	21.7	68.5	<0.5	<0.5
Cottonseed	19.3 ± 1.4	18.6	0.8	25.3	-	2.8	17.1	53.2	0.1	0.1
Soybeans	20.1 ± 4.5	19.0	<0.5	10.7	<0.5	3.9	22.8	50.8	6.8	<0.5
Canola/Rapeseed	40.5 ± 5.3	38.0	-	4.3	<0.5	1.7	59.1	22.8	8.2	1.0
Linseed	44.8	-	-	5.7	<0.5	3.8	17.2	15.5	57.3	<0.5

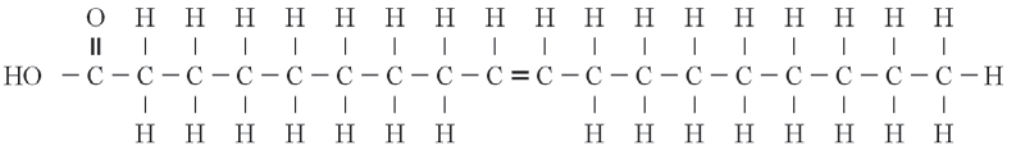
Adapted from Schroeder et al. (2004), Khan et al. (2012), Kalač and Samková (2010), completed with own data.

*Values are presented as means ± standard deviation (given, if available in the used literature).

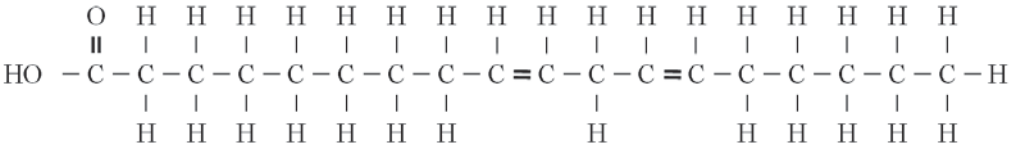
Linolenic acid is dominant in roughage components based on grass, while LA and OA are the dominant FA in maize-based roughage (Table 1). The main FA in commonly used cereals such as wheat, barley and corn are LA and OA (Table 1) (Becker, 2007). In most oilseeds, LA is the dominant FA, except in linseed and rapeseed/canola, in which LNA and OA are the predominant FA, respectively. The FA composition of common diets and lipid supplements mainly consist of long-chain FA (≥ C18), including saturated FA (SFA), monounsaturated FA(MUFA), and polyunsaturated FA (PUFA) (Table 1). Moreover, the almost exclusive *cis* configuration of their DB is typical for naturally dietary FA (Bauman et al., 2003). An overview of the main dietary FA and their DB configurations is given in Figure 1.



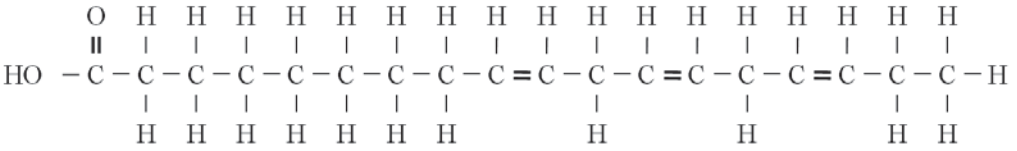
C18:0; Stearic Acid (SA)



C18:1 *cis*9; Oleic Acid (OA)



C18:2 *cis*9, *cis*12; Linoleic Acid (LA)



C18:3 *cis*9, *cis*12, *cis*15; Linolenic Acid (LNA)

Figure 1. C18 fatty acids, C18:0 stearic acid (SA), C18:1*cis*9 oleic acid (OA), C18:2*cis*9,*cis*12 linoleic acid (LA), C18:3*cis*9,*cis*12,*cis*15 linolenic acid (LNA), including natural *cis* double bonds (DB) configuration, in which the hydrogen atoms are locked on the same side of the DB.

Roughage is the major component, and therefore, a primary source of lipids in ruminant diets, along with cereal containing concentrates (Bauman et al., 2003). Most lipids from cereals and

oilseeds are present in the form of triacylglycerols (TAG), which contain three FA bound by ester linkages on a glycerol back bone (Harwood, 1980) (see Chapter 1.3, Figure 2).

Protection technologies for lipids to resist rumen microbial enzymes include either encapsulation inside a microbial-resistant shell or alteration of the FA structure (Jenkins and Bridges, 2007). The possible procedures comprise, for example the encapsulation of PUFA within a layer of formaldehyde-treated casein, and the production of calcium salts of FA or FA amides (Jenkins and Bridges, 2007). These technologies might protect FA from rumen metabolism as well as reduce the potential negative effects of lipids on rumen metabolism (Jenkins and Bridges, 2007). Besides technological protection, a kind of natural protection of lipids can be provided by using oilseeds in an intact or crushed form. This natural protection is assumed to be attributed to the shielding of the lipids by plant cell wall structures, resulting in a slower release of the FA or simply not being available for rumen microbes as compared to free oils (Grummer, 1991). The comparison between this natural protection of oilseeds and their pure oils, as well as their effects on changes in the FA composition *in vitro* in rumen fluid and *in vivo* in milk fat is main focus of the present thesis.

1.3. CHARACTERISTICS OF RUMEN METABOLISM OF DIETARY LIPIDS

The dominating lipid classes in ruminant diets are TAG, phospholipids and galactolipids (Jenkins et al., 2008). The FA in TAG frequently differ in their carbon chain-length. In animal lipids, TAG are predominant. Since the use of animal products in ruminant nutrition is banned in European countries, dietary lipids in ruminant diets are exclusively of vegetable origin.

Non-protected lipids entering the rumen are usually transformed during the rumen passage, and three fundamental processes are involved in this transformation (Jenkins and Bridges, 2007). The first process is the hydrolysis of the esterified plant lipids (see Chapter 1.3.1). The hydrolysis is followed by the isomerisation of DB, which comprises changes in the *cis-trans* configuration of DB (see Chapter 1.3.2). The final step in FA transformation in the rumen is the biohydrogenation (BH) of the DB (Harfoot and Hazlewood, 1997) (see Chapter 1.3.3). In literature, the term BH includes isomerisation and hydrogenation. For clarification purposes, these processes are described in separate chapters, followed by another chapter, in which the complex process of BH comprising both isomerisation and hydrogenation will be further elaborated.

A general overview of the processes that affect the lipids entering the rumen is shown in Figure 2. Accordingly, the rumen provides a potent barrier, preventing the delivery of unsaturated FA to the small intestine for later digestion and absorption (Beam et al., 2000). In case non-rumen protected lipids are ingested, a more saturated FA composition, containing higher amounts of *trans* FA leaves the rumen, compared to the initial dietary FA composition.

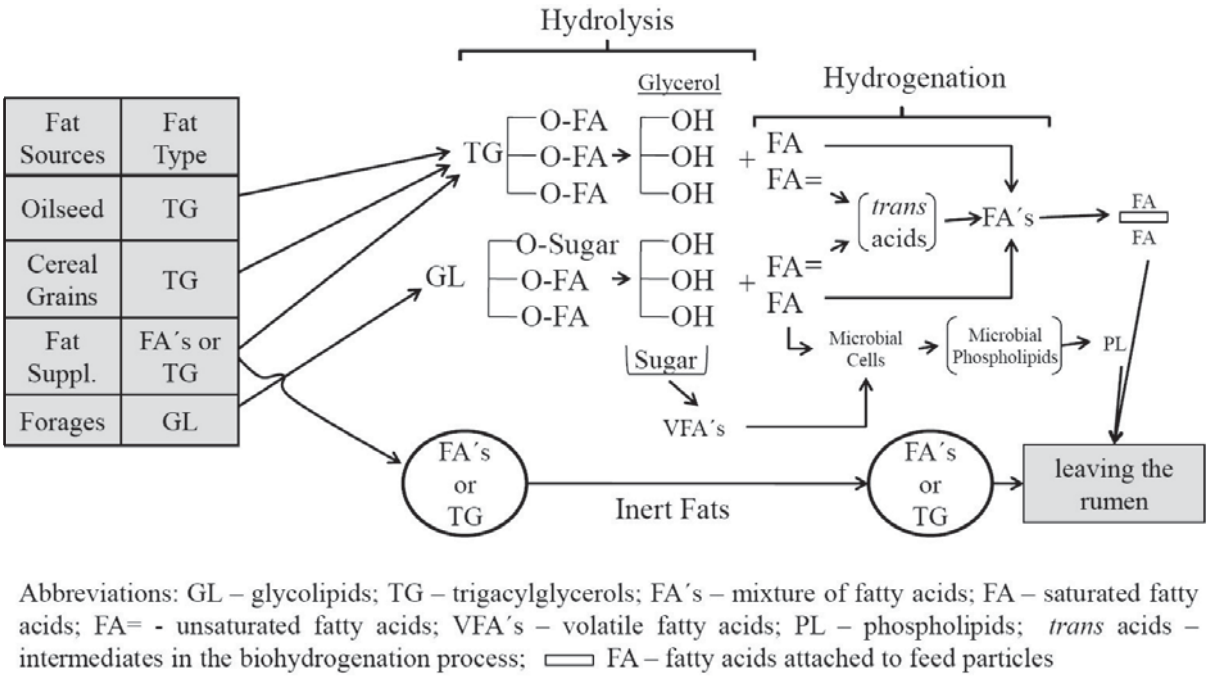


Figure 2. Intake of major fat types, comprising triacylglycerols, glycolipids, and fatty acids in ruminant diets, and their metabolism in the rumen. Protected lipid supplements leave the rumen unaffected, while the other sources are metabolised to glycerol and the different fatty acids leave the rumen. Adapted from Davis (1990).

1.3.1. HYDROLYSIS

The initial process in rumen lipid metabolism is the hydrolysis of the ester linkages of the lipids, resulting in free FA and glycerol backbones (Figure 3, Bauman et al., 2003). Additionally, sugar and phosphoric acids originate from the hydrolysis of glycolipids and phospholipids. The esterified lipids are hydrolysed in the rumen by endogenous plant and microbial lipases (Kim et al., 2009). According to Jenkins et al. (2008) and supported by the results of Lourenço et al. (2010), the hydrolysis of the esterified plant lipids in the rumen is partially promoted by inherent plant lipases. However, the lipases produced by rumen

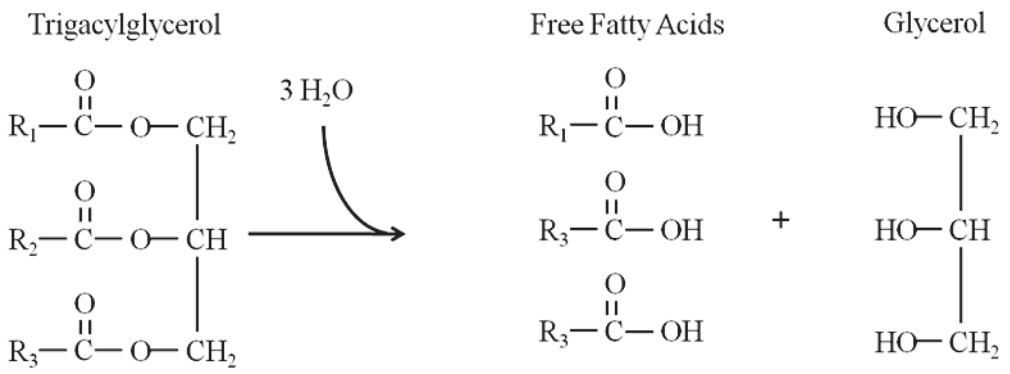


Figure 3. Process of hydrolysis of triacylglycerol, resulting in free fatty acids and glycerol as final products.

microorganisms are most important for the hydrolysis of dietary lipids (Jenkins et al., 2008). Further studies are crucial for assessing the role of endogenous plant lipases in the rumen, by focussing on the comparison and importance of plant and microbial lipases (Jenkins et al., 2008; Lourenço et al., 2010). Among rumen microorganisms, anaerobic bacteria have great relevance in hydrolytic activity (Lourenço et al., 2010). Rumen protozoa are of low importance, and rumen fungi do not contribute to hydrolytic activity at all (Harfoot and Hazlewood, 1997). However, the results for protozoa and fungi are derived from a considerable number of older studies, thus further investigations using latest methodology should be carried out (Lourenço et al., 2010). Bacterial lipases are often extracellular enzymes and classified regarding their reaction specificity, which are non-specific, 1,3-specific and FA specific (Mackie et al., 1991). Bacterial species and their enzymes differ in their affinity to hydrolyse different lipids. Phospho- and galactolipids seem to be hydrolysed by lipases of bacteria of *Butyrivibrio*-like species (Lourenço et al., 2010; Harfoot and Hazlewood, 1997). These *Butyrivibrio* subspecies cannot break down TAG (Lourenço et al., 2010) which is the main lipid class in cereals and oilseed lipids, predominant in diets that are high in concentrates and lipid supplements. The TAG are mostly hydrolysed by the lipase of *Anaerovibrio lipolytica*, the most active bacterial species considering TAG as substrate, but these bacteria do not hydrolyse phospho- and galactolipids (Lourenço et al., 2010; Mackie et al., 1991). According to Noble et al. (1974), the hydrolysis is a stepwise process, in which TAG are transformed to di- and monoacylglycerols, and finally to non-esterified FA (NEFA) and glycerol (Figure 3). The glycerol is further metabolised to SCFA in the metabolic pathway of carbohydrates, including glycolysis (Jeroch et al., 1999) (Figure 2). The hydrolysis is believed to be rate limiting for the BH, since the accumulation of free FA available for the BH process depends on hydrolysis (Palmquist et al., 2005; Harfoot and Hazlewood, 1997).

Although high rates of hydrolysis have been reported, several rumen environmental factors affect the extent of hydrolysis (Palmquist et al., 2005) which increases with increasing contents of fibre and nitrogen in the diet (van Nevel and Demeyer, 1996). A reduction in hydrolysis is reported at pH levels ≤ 6.0 (inhibition of 38% at pH 5.92) as compared to pH level > 6.0, but it further increases with decreasing pH (inhibition of 76% at pH 5.25) (van Nevel and Demeyer, 1996). Rumen hydrolysis is very efficient as reported by Bauchart et al. (1990), who observed apparent rumen hydrolysis of TAG greater than 85% and even higher (> 94%) in lipid-rich diets containing rapeseed oil, crushed or extruded full-fat rapeseeds. Similar levels of hydrolysis are reported for unprotected oil, considering that in general, the hydrolysis of lipids bound to plant matrices, e.g. intact or crushed seeds, is thought to be lower (Dewhurst et al., 2006; Doreau and Ferlay, 1994). On the contrary, Beam et al. (2000) reported a decrease in the hydrolysis *in vitro*, when the level of supplemented soybean oil in the diet increased from 2 to 10% (w/w of the substrate). Obviously, the hydrolytic process is restricted by a maximum lipid level in the diet. In an earlier study by Clarke and Hawke (1970), who incubated peanut oil and synthetic glyceryltri(oleate-1-^{14}C), the optimum for hydrolysis was found to be 1 mg TAG per ml of strained rumen fluid. Under physiological conditions, the extent of hydrolysis is sufficient to allow a considerable level of isomerisation, and the BH of released FA by rumen microorganisms (Hawke and Silcock, 1970). In conclusion, it seems that the major factors determining the extent of hydrolysis are the rumen ecosystem, especially the pH level and the amount of lipids in the rumen.

1.3.2. ISOMERISATION

The second step in the transformation of dietary lipids in the rumen is isomerisation, comprising positional change in the DB configuration. The change from *cis* to *trans* configuration depends on bacterial isomerase activity (Bauman et al., 2003). The *trans* configuration means that the hydrogen atoms are located on the opposite side of the DB (see Figure 4). In the FA formula syntax, the DB configurations of *trans* and *cis* are abbreviated as t and c, respectively, followed by the number indicating where the DB is located in the FA. The isomerised DB is separated by one single bond, and occurs in a conjugated configuration. Therefore, the FA resulting from the isomerisation of LA is called conjugated LA (CLA), and a conjugated triene occurs for LNA (see Figure 5).

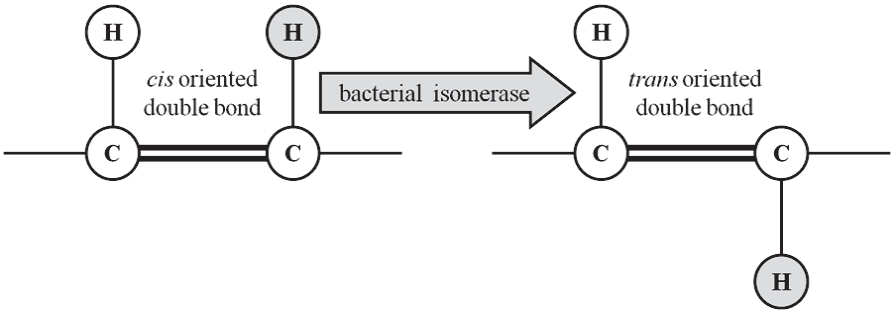
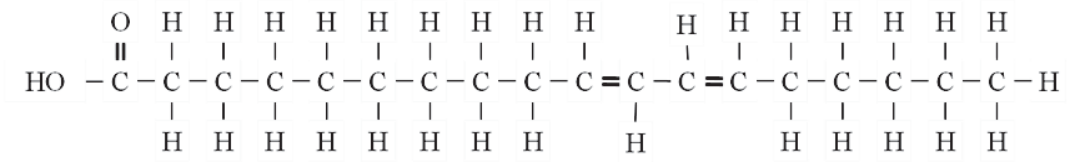
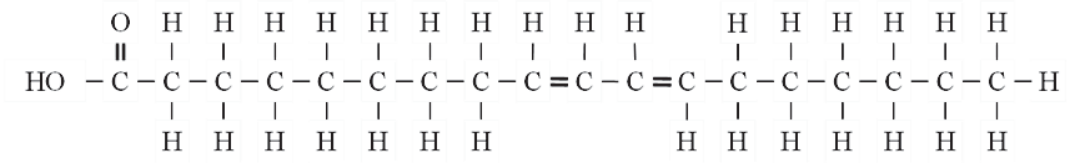


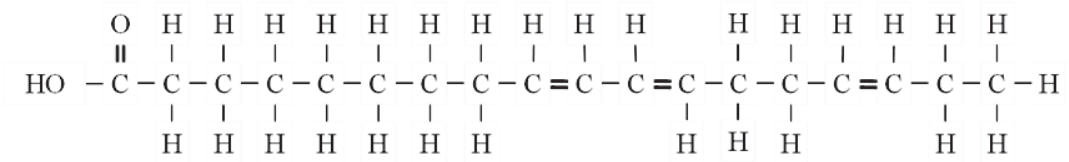
Figure 4. Isomerisation process of the change of a double bond configuration from *cis* to *trans* configuration within an unsaturated fatty acid.



C18:2 *trans*10, *cis*12; Conjugated Linoleic Acid (CLA) isomer, conjugated diene



C18:2 *cis9*, *trans*11; Conjugated Linoleic Acid (CLA) isomer, conjugated diene



C18:3 *cis9*, *trans*11, *cis*15; Conjugated triene

Figure 5. C18 fatty acids after isomerisation, C18:2*trans*10,*cis*12 conjugated linoleic acid, C18:2*cis*9,*trans*11 conjugated linoleic acid, C18:3*cis*9,*trans*11,*cis*15 conjugated triene of linolenic acid, including *cis* and *trans* double bond configurations.

The most common CLA isomer is C18:2c9t11, rumenic acid (RA) (Palmquist et al., 2005). According to Table 2 (Chapter 1.3.3), RA is predominant in ruminal outflow as compared to other conjugated isomers. Dietary changes and changes in rumen environment may shift isomerisation pathways, resulting in the appearance of other intermediates. Increased amounts of the CLA isomer C18:2t10c12 are found in rumen fluid and milk fat of cows showing milk fat depression. The latter is associated with low rumen pH, which typically occurs when cows are fed diets with high amounts of rapidly fermentable carbohydrates (Bauman and Griinari, 2003).

1.3.3. BIOHYDROGENATION

Unsaturated FA are reported to be toxic for many rumen bacteria, and interfere in rumen metabolism (Maia et al., 2007). A strategy to reduce these toxic effects is the hydrogenation of the DB of unsaturated FA by rumen microbes to saturate these FA (Harfoot and Hazlewood, 1997). This process is known as ruminal BH, which is the final step of the ruminal FA transformation (Bauman and Lock, 2006). Major BH pathways for LA and LNA have been described by Harfoot and Hazlewood (1997), including several intermediates and the end product, C18:0 stearic acid (SA) (Figures 6 and 7).

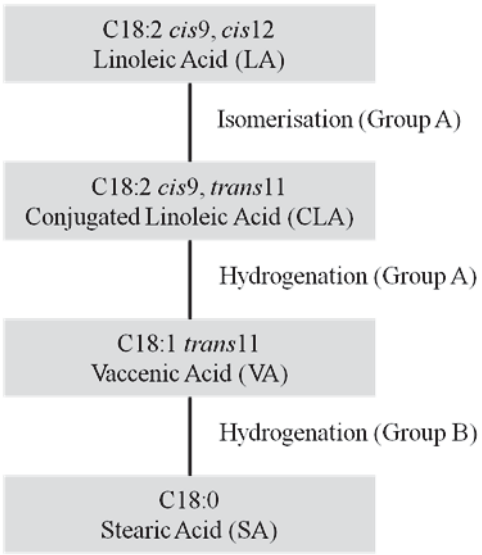


Figure 6. Main biohydrogenation pathway of linoleic acid; Group A and Group B refer to the classes of biohydrogenating bacteria. Adapted from Harfoot and Hazlewood (1997).

Figure 7. Main biohydrogenation pathways of linolenic acid; Group A and Group B refer to the classes of biohydrogenating bacteria. "Some poorly" means not all the bacteria are as efficient as others. Adapted from Harfoot and Hazlewood (1997).