Chapter 1.2

Development of Dairy Based Functional Foods Enriched in Rumenic Acid

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1.2.1 Introduction

Conjugated linoleic acid (CLA) is the term used to describe the positional and geometric isomers of linoleic acid (LA) with either one or both of the double bonds in the *cis* (*c*) or *trans* (*t*) conformation and separated by simple carbon-carbon linkage. CLA isomers are naturally found in dairy and meat products of ruminants and in recent years have attracted considerable interest because of the many health promoting activities observed both *in vitro* and *in vivo.* It was first purported that CLA might be a potential anti-carcinogen by Pariza *et al.* (1979) when investigations into mutagenic components of grilled hamburgers provided evidence of a mutagenesis modulator. This component was later identified as CLA and shown to be an effective inhibitor of chemically induced epidermal carcinogenesis in mice (Ha *et al.*, 1987). In addition to being anti-carcinogenic, CLA manifests activities that confer a reduced risk of atherogenesis, adipogenesis, diabetogenesis, inflammation, bone density loss and immune dysfunction (Bhattacharya *et al.*, 2006; Pariza *et al.*, 2004; Roche *et al.*, 2001; Wahle *et al.*, 2004). These activities have been primarily attributed to the two major CLA isomers, rumenic acid (*c*9, *t*11-C18:2) and the *t*10, *c*12 CLA isomer, and stem from their ability to positively influence transcriptional and/or translational control of immunoglobulins, lipids including eicosanoids, cytokines, and cell signalling machinery components.

Given the range of health promoting activities attributed to rumenic acid, ensuring an adequate supply of the isomer would appear desirable. However, in light of the negative perception of milkfat by consumers and prevalence of low-fat nutrition, the concentration of CLA in the diet has fallen in recent years (Lawson *et al.*, 2001). Indeed, it is estimated that the intake of CLA in North America is approximately 212 and 151 mg/d for men and women, respectively (Ritzenthaler *et al.*, 2001), while in Germany intake of CLA is estimated to be approximately 440

and 360 mg/d for men and women (Sieber *et al.*, 2004). These levels are lower than the dietary CLA intake of 3.0 g/d which Ip *et al.* (1994) estimated to be required, spurring interest in the development of CLA and in particular rumenic acid enriched foods. The dietary intake of CLA could be successfully increased through use of rumenic acid enriched dairy products. For example, a meal containing a serving of high rumenic acid whole milk (460 mg CLA), a sandwich with high rumenic acid butter (365 mg CLA) and high rumenic acid cheddar cheese (721 mg CLA) could be used to provide 1.546 g of CLA (Donovan *et al.*, 2000). This intake of rumenic acid would represent more than half the dose recommended by Ip *et al.* (1994) and supplied in a manner which would not require large adjustments to human dietary habits. The potential of rumenic acid has seen much recent attention directed towards identifying strategies for the enrichment of milk with CLA and the development of CLA enriched dairy products. Strategies for naturally enhancing CLA in milk have included manipulation of the diet of lactating ruminants to favour increased rumenic acid production in the milk, and research into the potential offered by CLA producing starter cultures.

1.2.2 Health benefits of CLA

The majority of *in vitro* and *in vivo* studies which have reported the health promoting activities of CLA have employed a mixture of CLA isomers containing approximately equal parts of rumenic acid and the *t*10, *c*12 CLA isomer (Pariza., 2004; Roche *et al*., 2001; Wahle *et al*., 2004). However, the CLA content of milk and dairy products is typically comprised of 85–90% rumenic acid (Lock & Bauman, 2004). Some studies do however address rumenic acid primarily and in this regard the fatty acid has been associated with improving cardiovascular health, anti-carcinogenic properties and improved immune function. (Pariza *et al*., 2004;

Roche *et al*., 2001; Wahle *et al.*, 2004; Lock & Bauman, 2004).

Studies in hamsters have shown that dietary rumenic acid reduced a number of atherosclerotic risk factors including reducing the ratio of non-high density lipoprotein cholesterol to high density lipoprotein (HDL) cholesterol and significantly reducing plasma triglyceride concentrations (Valeille *et al.*, 2004; Valeille *et al.*, 2005; Wilson *et al.*, 2006). Similar results were also observed when hamsters were fed a diet containing high CLA milkfat (2.59% rumenic acid). Here animals on the high CLA milkfat diet displayed 25% less aortic cholesteryl-ester deposition, a lower ratio of low density lipoprotein (LDL) cholesterol to HDL cholesterol, a lower local inflammatory status, and a lower aortic expression of vascular cell adhesion molecule-1 (Valeille *et al.* 2006). It has been proposed that many of the anti-artherogenic properties attributed to rumenic acid may originate from the molecules influence on peroxisome proliferator-activated receptor α (PPARα) and sterol regulatory element-binding protein 1c via their influence on stearoyl-CoA desaturase, cycloxygenase (COX), and fatty acid synthase expression and activity (Bhattacharya *et al.*, 2006).

Much of the data surrounding the anti-carcinogenic activity of rumenic acid has been derived from a number of *in vitro* studies which have assessed the impact of the isomer on a diverse range of cancers. These studies have included investigations into the effect of rumenic acid on prostate cancer (Ochoa *et al.*, 2004; Palombo *et al.*, 2002; Song *et al.*, 2006), colon cancer (Lampen *et al.*, 2005; Miller *et al.*, 2002; Palombo *et al.*, 2002), breast cancer (Chujo *et al.*, 2003; Hubbard *et al.*, 2003; Kim *et al.*, 2005), gastric cancer (Liu *et al.*, 2004; Liu *et al.*, 2002a; Liu *et al.*, 2002b) and leukaemia (Agatha *et al.*, 2004). It has been proposed that the mechanisms for this inhibitory activity include the increased uptake of hydroperoxides into cancer cells, which increase the cells susceptibility to lipid

peroxidation; the reduced accumulation of arachidonic acid in the cell membrane phospholipids, resulting in the reduced eicosanoid production; and through PPAR regulated expression of key genes associated with the proliferation of cancer cells (Bhattacharya *et al.,* 2006; Wahle *et al.*, 2004). O'Shea *et al*. (2000) reported the inhibitory effect that milkfat containing an elevated concentration of rumenic acid had on the growth of MCF-7 mammary cancer cells, reporting up to a 90% reduction in cancer cell numbers and a 15-fold increase in cellular lipid peroxidation following eight days exposure.

It has also been reported that rumenic acid exhibits anti-inflammatory properties in inflammatory airway disease (Jaudszus *et al.*, 2005). This anti-inflammatory activity is mediated through reduced production of the inflammatory cytokine interleukin-8 via the nuclear receptor PPARγ which has been previously shown to regulate inflammatory response (Yu *et al.,* 2002).

Also of interest to this review is the increasing number of reports regarding the anti-carcinogenic activity of the microbially produced *t*9, *t*11 CLA isomer (Beppu *et al.*, 2006; Coakley *et al.,* 2006). Beppu *et al.* (2006) reported the inhibitory effects of this isomer against the Caco-2, HT-29 and DLD-1 colon cancer lines, indicating that exposure of Caco-2 cells to the *t*9, *t*11 isomer resulted in apoptosis due to the uptake of the isomer into the cell which resulted in increased lipid peroxidation. Coakley *et al.* (2006) also reported the anti-proliferative activity of the *t*9, *t*11 isomer on SW480 and HT-29 colon cancer cells with a 55% and 94% reduction in the growth of the SW480 cell line following four days incubation in the presence of 10 μg/ml and 20 μg/ml of *t*9, *t*11 CLA, respectively.

1.2.3 Mechanisms of CLA production in lactating ruminants and starter bacteria

1.2.3.1 Ruminant CLA production

The presence of CLA in the milk of lactating ruminants is a direct result of the action of the ruminal microbiota on dietary linoleic and linolenic acids. In the diet these fatty acids are primarily delivered in the form of glycolipids, phospholipids, and triglycerides (Bauman *et al.*, 1999) and are released by indigenous and endogenously produced lipases following ingestion (Bauman *et al.*, 1999; Dawson *et al.*, 1977; Dawson & Kemp, 1970; Keeney, 1970). These fatty acids subsequently undergo microbial biohydrogenation in the rumen by various ruminant bacteria of which *Butyrivibrio fibrisolvens* is the foremost (Fujimoto *et al.*, 1993; Harfoot & Hazlewood, 1988; Kepler *et al.*, 1966). The biohydrogenation process, which results in the conversion of linoleic and linolenic acids to stearic acid, consists of several steps **(Figure 1.2.1)**. Rumenic acid is formed as the first intermediary in the biohydrogenation of linoleic acid via the activity of the microbial enzyme linoleic acid isomerase (Kepler *et al.*, 1966), which catalyses the isomerisation of the *cis* 12 double bond of linoleic acid (Bauman *et al.*, 1999). A portion of the ruminally produced rumenic acid is absorbed in the intestine, while the majority is further reduced to vaccenic acid and ultimately to stearic acid. As the hydrogenation of vaccenic acid to stearic acid occurs at a slower rate than the previous step, an accumulation of vaccenic acid in the rumen occurs. Much of this accumulated vaccenic acid is absorbed in the intestine passing into the circulatory system (Harfoot *et al.*, 1973; Kellens *et al.*, 1986; Singh & Hawke, 1979; Tanaka & Shigeno, 1976) and transported to the mammary gland, where it may be converted to rumenic acid via the action of the enzyme Δ^9-desaturase (Corl *et al.*, 2001; Griinari *et al.*, 2000; Mahfouz *et al.*, 1980; Pollard *et al.*, 1980) **(Figure 1.2.1)**. This endogenous synthesis of rumenic acid is estimated to account for as much as 75-90% of the total rumenic acid in ruminant milkfat (Griinari *et al.*, 2000; Kay *et*

al., 2004; Lock & Garnsworthy, 2002; Piperova *et al.*, 2002). In addition to rumenic acid, milkfat typically contains a number of other CLA isomers including the *t*7, *c*9 CLA, which may account for up to 3-16% of total milkfat CLA (Yurawecz *et al.*, 1998) and to a lesser extent the *t*10, *c*12 CLA isomer which also has reported biogenic activities. This ruminal and endogenous production of the various CLA isomers gives rise to total milkfat CLA concentrations of 0.2-3.7% in bovine milk (Sebedio *et al.*, 1997), 0.58-1.05% in goats milk and 0.7-2.97% in ovine milk (Parodi, 2002). Variation occurs with animals, lactation number, region, season, stage of lactation, breed and in particular diet. It is therefore through manipulation of these factors that marked increases in milkfat CLA can be achieved.

1.2.3.2 Intestinal and dairy microbiota

In 1984 it was reported that gnotobiotic rats possessed less CLA in their tissues than normal rats when fed free linoleic acid (Chin *et al.*, 1994). This observation led to speculation that some non-ruminant bacteria may convert linoleic acid to CLA. Since that time a number of non-ruminant bacteria have been identified as being capable of converting free linoleic acid to CLA, including strains of *Lactobacillus*, *Propionibacterium*, *Pediococcus*, *Enterococcus*, *Streptococcus*, *Bifidobacterium*, and *Lactococcus*. These bacteria convert free linoleic acid to rumenic acid in a similar manner to ruminant bacteria, via the action of the enzyme linoleic acid isomerase (Lin *et al.*, 2002). This rumenic acid may undergo conformational changes giving rise to the *t*9, *t*11 CLA isomer (Coakley *et al.*, 2003; Kishino *et al.*, 2002), which itself has reported health promoting properties (Beppu *et al.*, 2006; Coakley *et al.*, 2006). The mechanism of microbial production of rumenic acid and the *t*9, *t*11 CLA isomer was characterised using washed cells of the strain *Lb. acidophilus* AKU 1137 and involves the production of hydroxyl fatty acids as a

precursor to formation of both CLA isomers (Ogawa *et al.,* 2001). When isolated and introduced to the washed cells these hydroxyl fatty acids are rapidly transformed to their respective CLA isomers. Thus, CLA formation by *Lb. acidophilus* was found to consist of two distinct steps, *step one:* the hydration of linoleic acid to 10-hydroxy-18:1 and *step two:* the subsequent dehydration and isomerisation of these hydroxy fatty acids to rumenic acid and the *t*9, *t*11 CLA isomer **(Figure 1.2.2)**. The discovery that strains of bacteria commonly used as dairy starters or probiotics can produce rumenic acid has opened a number of avenues for their use. These include the potential for the enrichment of dairy products with rumenic acid as a result of microbial fermentation, or the possibility of incorporation of such strains into dairy products in numbers where they could colonise the gastro-intestinal tract and produce rumenic acid from dietary linoleic acid *in situ.*

1.2.4 Enrichment of milk with CLA through animal feeding and management strategies

Booth *et al.* (1935) first reported the presence of CLA in milkfat and with the subsequent discovery of its health promoting activity, identification of strategies for the enrichment of ruminant milk with this fatty acid have received substantial attention. The rumenic acid content of milkfat is directly affected by a number of factors including the species, breed, lactation number, stage of lactation, season, location, farm management strategies and most importantly animal dietary regime. Of these elements it is dietary manipulation which has been recognised as being the most successful strategy for elevation of the CLA content in milkfat (Booth *et al.*, 1935). In particular this can be achieved through the supplementation of the ruminant diet with fish oils, animal fats, plant oils, and forage.

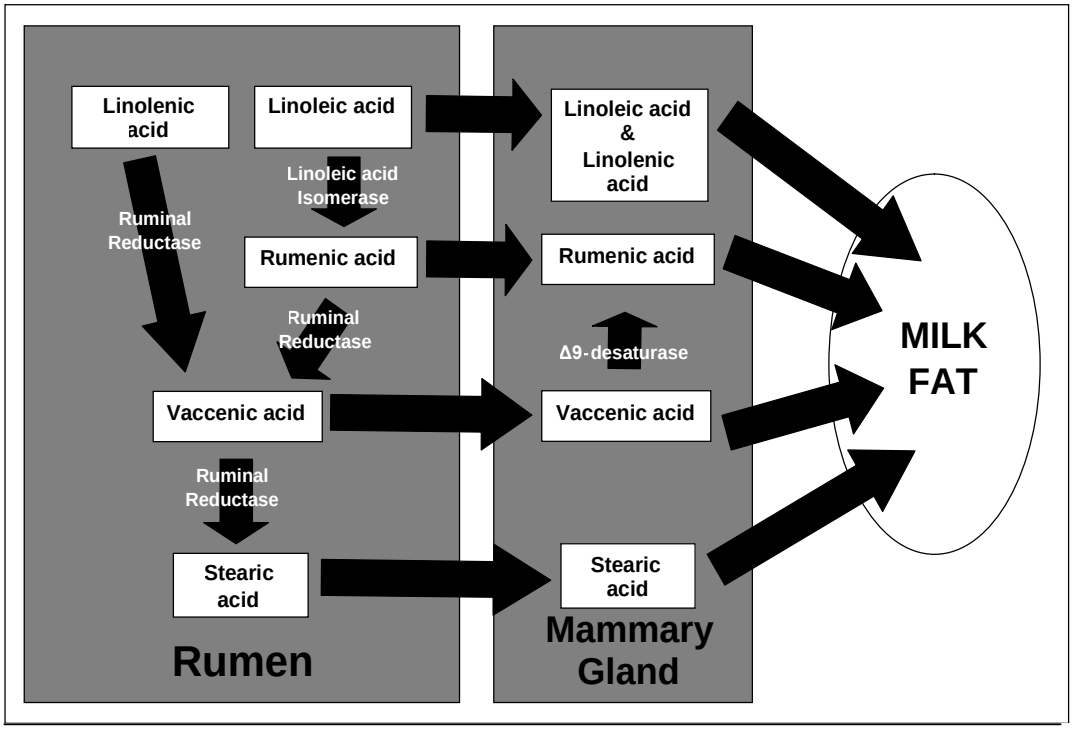

Figure 1.2.1 Formation of rumenic acid by ruminants

1.2.4.1 Plant oils and seeds

A number of different plant oils and seeds derived from a range of different sources such as cottonseed, rapeseed, soybean, corn, sunflower, peanut, safflower, canola and linseed have been fed to ruminants in attempts to elevate CLA production **(Table 1.2.2)**. These oils are rich in linoleic acid (cottonseed, soybean, sunflower, safflower, corn) and α-linolenic acid (linseed), key precursors in the formation of CLA and in particular rumenic acid. The mechanisms by which these oils increase milkfat CLA have been elucidated and are attributed not only to the increased supply of substrate in the form of linoleic and α-linolenic acids, but also as a direct result of inhibition of the reductase enzymes in the rumen responsible for the conversion of vaccenic to stearic acid. This results in the increased accumulation and absorption of vaccenic acid by the animal which can be endogenously converted to rumenic acid in the mammary gland via the Δ^9-desaturase enzyme (Griinari & Bauman, 1999). A large number of studies have addressed the effect of plant oils on milkfat CLA and are of general consensus that the supplementation of a basal diet of concentrates and conserved forage with plant-derived oils can substantially increase total milkfat CLA concentrations through elevated rumenic acid production **(Table 1.2.2)**. Large variations between the effects of the different oils on the extent of the increase in milkfat CLA have been reported. For example, the differences between oils rich in linoleic or α-linolenic acid were assessed by comparing the effect of supplementation of the basal diet with a linoleic acid rich (1% dietary dry matter (dDM) soybean oil) or α-linolenic acid rich (1% (dDM) linseed oil) oil for five weeks. Dietary supplementation increased CLA content of milkfat from 0.50% in the control to 1.45% with soybean oil, and 0.73% with linseed oil (Dhiman *et al.*, 2000). Similarly, Loor & Herbain, (2003a) demonstrated that supplementation of the bovine diet with a high linoleic acid

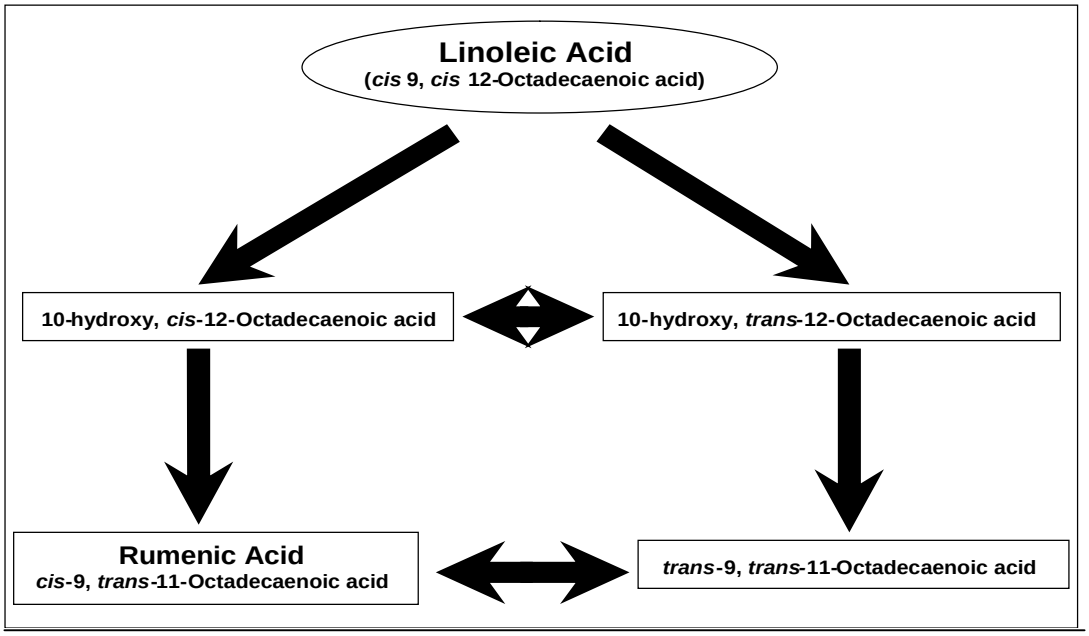

Figure 1.2.2 Formation of rumenic acid and *t*9, *t*11 CLA by *Lactobacillus acidophilus* AKU 1137.

plant oil, i.e. soybean oil (3% (dDM)) led to a greater increase in the milkfat rumenic acid concentrations (0.7%) compared to an oil containing a high oleic acid content (0.5%). Based on these observations it was concluded that plant oils rich in linoleic acid are most effective in increasing milkfat rumenic acid (Dhiman *et al.*, 2000; Loor & Herbein, 2003a). Several methods for the inclusion of plant oils in the ruminant diet have been used, including free oils, protected oils, whole oilseeds, or processed oilseeds (crushed, extruded, ground, roasted, or microionized), with the method by which these plant oils are delivered in the diet substantially influencing the extent of their impact on total milkfat CLA (Chouinard *et al.*, 1997a; Chouinard *et al.*, 1997b; Chouinard *et al.*, 2001; Dhiman *et al.*, 1999b; Dhiman *et al.*, 2000; Loor *et al.*, 2002; Stanton *et al.*, 1997). The variability in effect stems from differences in the availability of the fatty acids from the plant oil for microbial biohydrogenation in the rumen, which is critical to the production of CLA and its endogenous precursor vaccenic acid. A comparison between the effect of diets supplemented with raw linseed or extruded linseed have revealed that the processed linseed by means of extrusion resulted in higher concentrations of milkfat CLA compared to raw linseed (1.90% and 1.40%, respectively) (Gonthier *et al.*, 2005). Similarly, supplementation of the ruminant diet with 14% (dDM) cottonseed hull (control), 14% (dDM) whole cottonseeds or 14% (dDM) small cottonseed pellets demonstrated that the processed cottonseed pellets resulted in the greatest increase in milkfat CLA (Reveneau *et al.*, 2005). Clearly, processing results in the release of the oils held within the seeds allowing the rumen microbiota greater access to the fatty acids and hence increased production of rumenic acid and vaccenic acid in milk. Physical processes such as grinding and crushing break the seed releasing free oils and hence increasing the surface area of the seed exposed to the microbial population, while processes that involve heating can result in the partial hydrolysis

Table 1.2.1 The principal fatty acids found in some of the most common plant oils.

Plant oil seed	14:0	16:0	16:1 (g/100g)	18:0	18:1	18:2	18:3
Cottonseed	0.8	25.3		2.8	17.1	53.2	0.1
Rapeseed		4.3	0.3	1.7	59.1	22.8	8.2
Soybean		10.7	0.3	3.9	22.8	50.8	6.8
Sunflower	0.2	5.5		3.6	21.7	68.5	0.1
Peanut	0.1	11.5		3.0	53.0	26.0	
Safflower		8.0		3.0	13.5	75.0	0.5
Olive		13.0	1.0	2.5	74.0	9.0	
Canola		4.8		1.9	58.5	23.0	7.7
Linseed		6.4		3.1	20.1	18.2	51.4
Corn				17.0	24.0	59.0	

Modified from Stanton *et al*. (2003)

Table 1.2.2 Effect of animal feeding strategies on milkfat CLA concentrations.

	% dietary dry matter	CLA content of Control	Total CLA % of fat	Ref
Plant Seed Oils				
Soybean oil	5.3	N.S.	2.44	1
Soybean oil	2	0.35	0.71	3
Soybean oil	0.5-4	0.5	0.75-2.08	2
Soybean oil	3	N.S.	0.71	4
Soybean oil	3.6	0.39	2.1	2
Soybean oil	3	3	7.14	4
Linseed oil	5.3	N.S.	1.67	1
Linseed oil	1	0.5	0.73	2
Linseed oil	2.2-4.4	0.39	1.58-1.63	2
Peanut oil	5.3	N.S.	1.33	1
Safflower oil	6	0.45	3.36	6
Safflower oil	2.5	N.S.	7	5
Flaxseed oil & Vit E	6	0.68	2.8	6
Cottonseed oil	2	0.35	0.6	3
Corn oil	2	0.35	1.03	3
Canola oil	3	3	5.01	4
Canola oil	3.3	0.5	1.1	8
Sunflower oil	2.5	N.S.	5.2	5
PR infusion Safflower oil	150 g/d	0.59	0.58	7
Processed Plant Seed Oils				
Raw cracked soybean	18	0.39	0.37	2
Raw cracked roasted soybean	18	0.39	0.77	2
Raw ground soybean	17.5	N.S.	0.31	9
Roasted soybean	17.5	N.S.	0.66	9
Microionized soybean	17.5	N.S.	0.7	9
Extruded soybean	17.5	N.S.	0.89	9
Raw flaxseed	12.6	0.9	1.4	10
Extruded flaxseed	12.6	0.9	1.9	10
Cotton seed hull	14	N.S.	0.94	11
Whole cottonseed	14	N.S.	0.97	11
Small cottonseed pellets	14	N.S.	1.47	11
High full fat rapeseed diet	825 kg/d	3.94	7.89	12
Low full fat rapeseed diet	1200 kg/d	3.94	5.23	12
Protected Plant Seed Oils				
Calcium salt of Canola oil	4	0.35	1.32	9
Calcium salt of Soybean oil	4	0.35	2.25	9
Calcium salt of linseed oil	4	0.35	1.95	9
Canolamide	3.3	0.5	0.7	8
Marine Oils				
Fish oil	1.6	0.16	1.55	13
Fish oil	1	0.71	1.71	14
Fish oil	2	0.71	2.53	14
Fish oil	3	0.71	2.12	14
Fish oil	160 g/d	2.25	3.23	15

	% dietary dry matter	CLA content of Control	Total CLA % of fat	Ref
Marine oils contd				
Fish oil	320 g/d	2.25	3.64	15
Soymeal replaced by Fish meal	0%	0.53	0.53	16
Soymeal replaced by Fish meal	25%	0.53	0.63	16
Soymeal replaced by Fish meal	50%	0.53	0.66	16
Soymeal replaced by Fish meal	100%	0.53	1	16
Palm and fish oil	2.7	0.61	1.27	17
Marine Algae Protected	4	0.37	2.31	18
Marine Algae Unprotected	4	0.37	2.62	18
Animal fats				
Tallow (Jersey)	1.10%	N.S.	0.7-1.18	19
Tallow (Holstein)	1.10%	N.S.	0.83-0.84	19
Tallow	3	N.S.	1.1	20
PR infusion with Tallow	150 g/d	0.59	0.61	7
Fresh and conserved forage				
Grass maize silage Day 2	ad libitum	2.43	1.03	21
Grass maize silage Day 6	ad libitum	2.43	0.48	21
Grass maize silage Day 14	ad libitum	2.43	0.44	21
Grass silage early heading	ad libitum	none	1.14	22
Grass silage flowering	ad libitum	none	0.48	22
Grass silage second cutting	ad libitum	none	0.81	22
Pasture	ad libitum	0.44	2.43	21
Pasture	16 Kg/d	none	0.39	12
Pasture	20 Kg/d	none	0.55-0.57	12
Pasture	24 Kg/d	none	0.59-0.68	12
Pasture	50	none	1.57	23
Pasture	65	none	1.61	23
Pasture	80	none	1.9	23
Pasture	ad libitum	0.4	1.1	28
Splega Ryegrass	ad libitum	none	1.54	24
Portstewart Ryegrass	ad libitum	none	1.71	24
Napoleon Ryegrass	ad libitum	none	1.35	24
Millenium Ryegrass	ad libitum	none	1.72	24
Annual Ryegrass	ad libitum	none	1.2-1.43	25
Sulla	ad libitum	none	1.12-1.25	25
Burr medic	ad libitum	none	1.65-2.3	25
Daisy forb	ad libitum	none	2.33-2.35	25
Red clover	ad libitum	0.4	1.3	28
Miscellaneous Strategies				
Monensin	24ppm	0.48	0.56	6
Monensin	380 mg/d	0.8	1.3	26
6 % Safflower oil & Vitamin E	150 IU/kg	4.16	3.54	6
Mixed ration & Vitamin E	10000 U/d	0.71	0.72	27

References: Kelly *et al* (1998a) =1, Dhiman *et al* (2000) =2, Zheng *et al* (2005) =3, Loor & Herbain (2003a) =4, Loor & Herbain (2003b) =5, Bell *et al* (2006) =6, Bell & Kennelly (2003) =7, Loor *et al* (2002) =8, Chouinard *et al* (2001) =9, Gonthier *et al* (2005) =10, Reveneau *et al* (2005) =11, Stanton *et al* (1997) = 12, Offer *et al* (1999) =13, Donavan *et al* (2000) =14, Rego *et al* (2005) =15, Abu-Ghazaleh *et al* (2001) =16, Allred *et al* (2006) =17, Franklin et al (1999) =18, Morales *et al* (2000) =19, Jones *et al* (2000) =20, Elgersma *et al* (2004) =21, Chouinard *et al* (1998) =22, Ward et al (2003) =23, Loyola et al (2002) =24, Addis *et al* (2005) =25, Sauer *et al* (1998) =26, Kay *et al* (2005a) =27 and Benchaar *et al* (2002) =28

of bound fatty acid making them more available to the ruminal microbiota. To assess the effect of different processing strategies Chouinard *et al.* (2001) fed cows 17.5% (dDM) full fat soybeans treated by grinding, extrusion, microionization and roasting. Supplementation of these processed oils led to milkfat CLA concentrations of 0.31%, 0.89%, 0.70%, and 0.66%, with the extruded soybeans found to be the most effective in enhancing milkfat CLA. In another study, cows were fed 18% raw cracked soybeans, 18% roasted cracked soybeans or 3.60% soybean oil (dDM) to assess the effect of processing, resulting in total milkfat CLA contents of 0.37%, 0.77% and 2.1%, respectively, compared to the control (0.39%) (Dhiman *et al.*, 2000). A number of studies have also assessed the effect of dietary supplementation with ruminally protected plant oils, either by formation of calcium salts, fatty acyl amides, a formaldehyde-protein protection matrix, or lipid encapsulation (Chouinard *et al.*, 2001; Loor *et al.*, 2002). These methods of fatty acid protection not only serve to reduce the negative effects that processing and gastric transit can have on the fatty acid composition of the oil, but also protect the oils from ruminal biohydrogenation and as such their impact on the production of vaccenic acid and CLA in the rumen would be anticipated to be minor. Investigations into the effect that feeding cows calcium salts of canola oil, soybean oil and linseed oil (4% (dDM) for four weeks) had on CLA content of milkfat have also been performed. Milkfat CLA concentrations of 1.32%, 2.25%, and 1.95% were reported for the respective fatty acids relative to 0.35% in the control (Chouinard *et al.*, 2001). Based on these results it is evident that calcium salts of plant oils offer little protection from ruminal biohydrogenation. A similar experiment compared the effect of canolamide, a formaldehyde protected form of canola oil, with that of unprotected canola oil (3.3% (dDM) for three weeks). As expected, the free oil resulted in a substantial increase in the concentration of

milkfat rumenic acid (1.1%) relative to that achieved in the control (0.5%) or with the formaldehyde protected oil (0.7%) (Loor *et al.*, 2002). From these studies it is evident that the manner in which oils are ruminally protected has a large bearing on the success of the oil in increasing the CLA concentration of the milkfat. Thus, it would appear that ruminally protecting oils has a negative effect on CLA production compared to free oils.

1.2.4.2 Marine oils

Dietary supplementation of lactating ruminants with fish oils and oils derived from marine sources have been shown to result in an elevation of milkfat CLA concentrations (Allred *et al.*, 2006; Franklin *et al.*, 1999; Jones *et al.*, 1998; Offer *et al.*, 1999; Rego *et al.*, 2005). These oils are rich in long chain polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which inhibit the reductase enzymes that catalyse the conversion of vaccenic acid to stearic acid in the rumen, additionally, modifying the microbial population of the rumen (Chow *et al.*, 2004; Griinari & Bauman, 1999; Loor *et al.*, 2005). These changes lead to an accumulation of vaccenic acid in the rumen. which following its subsequent absorption and bioconversion to rumenic acid in the mammary gland lead to increased total milkfat CLA and vaccenic acid concentrations. This increase in milkfat CLA via supplementation of the ruminant diet with fish oils has been reported in a number of studies including those of Donovan *et al.* (2000) and Rego *et al.* (2005) **(Table 1.2.2)**. Fish oil is not the only marine derived oil which has been assessed for its ability to increase milkfat CLA. Cows fed on diets supplemented with 4% (dDM) marine algae in both protected and unprotected form have also been shown to produce milk containing elevated concentrations of CLA. In this study milkfat rumenic acid concentrations were

increased from 0.37% in the control to 2.31% and 2.62% for the respective diets (Franklin *et al.*, 1999). Thus, it would appear that supplementation with fish oils and marine algae can be an effective strategy for increasing the rumenic acid content of milkfat. The use of processed oils in the form of protected fish oils and fish meal have also been assessed **(Table 1.2.2)**. Allred *et al.* (2006) reported that feeding cows a diet containing 2.7% (dDM) calcium salts of palm and fish oil for six weeks produced a two fold increase in milkfat CLA concentrations. Abu-Ghazaleh *et al.* (2001) assessed the effect of replacing soy meal in the bovine diet with 25%, 50% and 100% fish meal for three weeks reporting milkfat rumenic acid concentrations of 0.44%, 0.66% and 0.72%, respectively, compared to 100% soy meal which resulted in a milkfat CLA concentration of 0.39%.

It has been reported that the fatty acid DHA, found in fish oil may play a substantial role in the elevation of milkfat CLA via increases in the ruminal output of vaccenic acid. A mixed ruminal culture (*in vitro*) supplemented with DHA (5 mg) for 24 h produced 1.3-fold more vaccenic acid than a culture supplemented with soybean oil (30 mg) and 2.5-fold more vaccenic acid than the control (AbuGhazaleh & Jenkins, 2004). As the fatty acid composition of fish oils and marine algae vary with species, season, diet, and location, with DHA and EPA concentrations ranging from 2-25% and 4-32%, respectively, it is likely that fish and marine oils derived from different sources differ substantially in their effect on ruminal biohydrogenation and the resulting milkfat CLA and vaccenic acid concentrations.

1.2.4.3 Animal Fats.

A number of studies have assessed the effect of supplementation of the ruminant diet with animal fats such as tallow and grease. These animal derived fats are

generally rich in saturated fatty acids and usually contain a low concentration of PUFA. Despite their composition, these sources of animal fat have been shown to elevate milkfat CLA concentrations **(Table 1.2.2)**. One such study supplemented the diet of copper deficient Holstein and Jersey cows with 1.1% (dDM) tallow, which led to milkfat CLA concentrations of 0.7-1.18% (Morales *et al.*, 2000). In another study, Jones *et al.* (2000) supplemented the bovine diet with three percent (dDM) tallow leading to milkfat CLA concentrations of 1.1%, while Pantoja *et al.* (1996) found that supplementation with five percent (dDM) tallow elevated vaccenic acid production, a key rumenic acid precursor, from 0.29% to 1.53% of the total milkfat. Onetti *et al.* (2001) on the other hand reported that supplementation of the bovine diet with 0%, 2% and 4% tallow (dDM) did not substantially increase milkfat CLA. Feeding animal fats to ruminants is not permitted in the European Union.

1.2.4.4 Forage.

A two to three fold increase in the CLA content of cows milk is commonly observed when cows are turned out to pasture (Riel, 1963). A number of subsequent studies have confirmed this observation, reporting a dramatic and abrupt increase in the CLA content of milk over the first five days following the transfer of cows from indoor winter feeding to fresh pasture (Dhiman *et al.*, 1999a; Kelly *et al.*, 1998b; Precht & Molkentin, 1997; Stanton *et al.*, 1997; Timmen & Patton, 1988) **(Table 1.2.2)**. Furthermore, Elgersma *et al.* (2004) reported that this increase in milkfat rumenic acid is rapidly reversed on the return of animals to indoor feeding. In this study the rumenic acid content of milkfat fell from 2.30% on day zero when cows were at pasture, to 0.95%, 0.43%, and 0.37%, respectively, after 2, 6 and 14 days on mixed grass-maize silage diet. In temperate countries, the dry matter of fresh grass

is composed of about 1-3% fatty acids of which 48-65% are linolenic acids (Bauchart *et al.*, 1984; Chilliard *et al.*, 2001). Studies have suggested that it is the increased supply of substrate along with the improved growth of the ruminal microbiota (due to higher concentrations of fermentable sugar and soluble fibre), which are responsible for the increased production of rumenic acid in animals at pasture (Dhiman *et al.*, 2005; Griinari & Bauman, 1999; Stanton *et al.*, 2003).

Comparisons between the milkfat CLA content of typical United States milk (where animals are fed indoors on a diet of fresh and conserved forage, along with concentrates year round) and temperate countries such as Ireland, Australia and New Zealand (where animals receive indoor feeding of conserved forage and concentrate, and fresh pasture on a seasonal basis) highlight the effect of forage on milkfat CLA. Indeed, it has been observed that the average milkfat CLA content of US milk is approximately 0.55% while milkfat produced in a temperate climate such as Ireland typically contains approximately 1.6% CLA during access to fresh pasture (Stanton *et al.*, 2003). The factors which effect the impact that fresh pasture has on the CLA content of milkfat have been elucidated and include pasture allowance, forage maturity, forage type, season and the provision of dietary supplements. In relation to pasture allowance, we found that cows on a high (24 Kg/d) or medium (20 Kg/d) pasture allowance had higher milkfat CLA than those on the low pasture allowance (16 Kg/d) (Stanton *et al.*, 1997). Others have reported that increasing pasture allowance resulted in a linear increase in the total CLA content of the milk, in particular the rumenic acid content (Couvreur *et al.*, 2006; Ward *et al.*, 2003). It has been demonstrated that animals fed grass silage which had been cut at three stages of growth, early heading, flowering and second cutting, showed substantial differences in their impact on milkfat CLA content (1.14%, 0.48% and 0.81%, respectively) (Chouinard *et al.*, 1998). The higher CLA in milk

produced by cows grazing early forage is most likely a result of the higher linolenic acid content of the young grass. Investigations into the effect of maturity on the fatty acid composition of a range of traditional and novel forages over three week periods found that in almost all instances the concentration of the key CLA precursors linoleic and linolenic acid decreased with stage of growth (Clapham *et al.*, 2005) substantiating the findings of Chouinard *et al.* (1998). However, in a similar study Griinari *et al.* (1998) found forage maturity did not substantially affect milkfat CLA or rumenic acid **(Table 1.2.2)**.

The composition of forage supplied to animals may be quite variable and include a range of plant types. The fatty acid composition of these plants may differ substantially and as such their effect on the concentration of CLA in milkfat would be expected to differ. Loyola *et al.* (2002) and Addis *et al*. (2005) both assessed the effect of feeding different plants types or cultivars on milkfat CLA concentrations, investigating differences between the ryegrass cultivars Splega, Portstewart, Napoleon, and Millennium; and between annual ryegrass, sulla, burr medic and daisy forb, respectively. The results highlight substantial differences in the impact that different plant types and cultivars have on milkfat CLA **(Table 1.2.2)**.

The effect of season on milkfat rumenic acid and CLA is directly related to the PUFA content of the forage (Bauchart *et al.*, 1984). The total CLA content in milk is found to peak in early spring and autumn and fall in summer in parallel with the linolenic acid concentration of the dietary forage (Chouinard *et al.*, 1998; Mackle *et al.*, 1999; Nudda *et al.*, 2005; Precht & Molkentin, 2000; Thorsdottir *et al.*, 2004). A number of studies have also assessed the effect of supplementation of a forage diet in part with concentrates and grain, a practice which mostly sees the depression of milkfat CLA as a result of the reduced intake of linolenic and linoleic acids (Bargo *et al.*, 2006; Dhiman *et al.*, 1999a; Ward *et al.*, 2003). However, in a

study by Chouinard *et al.* (1998), a low forage high concentrate diet was found to increase the concentration of milkfat CLA in comparison to a high forage low concentrate diet **(Table 1.2.2)**.

Hay and silage (grass and maize) make up a considerable portion of the ruminant feeding strategy and as such, play an important role in milkfat rumenic acid concentrations particularly during indoor feeding. Preserving forage as hay results in a substantial reduction in the concentration of fatty acids and in particular linolenic acid. This effect is seen to a lesser extent with high quality silage but may occur if forage is wilted before ensiling, or under undesirable fermentation conditions (Doreau & Poncet, 2000; Lough & Anderson, 1973). Data as to the effect of hay and grass silage on the concentrations of milkfat rumenic acid are scant. However, as a result of the lower concentrations of linolenic acid in these feeds, their effect on milkfat CLA would be expected to be less profound than animals receiving fresh pasture. Interestingly, Ward *et al.* (2003) showed that feeding cow's fresh forage or hay supplemented with an equivalent concentration of tallow resulted in milkfat CLA contents of 1.07% and 0.93%, respectively. Assuming that the impact of the tallow was the same in both diets it would appear that fresh forage and hay differed only slightly in terms of their effect on milkfat CLA. Chilliard *et al.* (2001) reported that cows fed a diet consisting of over 60% maize silage had a milkfat CLA content of between 0.4% and 0.6%, considerably less than would be expected from fresh forage.

1.2.4.5 Miscellaneous feeding strategies

A number of studies have assessed the effect of the addition of ionophores to the ruminant diet. These compounds are found to inhibit the growth of the gram-positive bacteria and as a result, to directly impact on ruminal biohydrogenation.

Using *in vitro* studies the effect of the ionophores nigericin, monensin and tetronasin on the production of CLA by a mixed ruminal population was investigated **(Table 1.2.2)**. The addition of these ionophores resulted in a two-fold increase in the production of rumenic acid through inhibition of the complete biohydrogenation of linoleic acid (Fellner *et al.*, 1997). Furthermore, in a subsequent study it was shown that the combination of monensin and soybean oil in continuous cultures of mixed ruminal microorganisms resulted in increased production of *t*10 $C_{18:1,}$ a CLA precursor, to a greater extent than either additive alone, but only when supplemented with barley grains (Jenkins *et al.*, 2003). The effect of supplementation of the ruminant diet with these compounds has also been investigated **(Table 1.2.2)**. On supplementation of the bovine diet with 380 mg monensin it was found that the concentration of milkfat CLA increased from 0.8% in the control group to 1.3% in the monensin supplemented group (Sauer *et al.*, 1998). However, this effect has proved less substantial in other studies (Chouinard *et al.*, 1998; Dhiman *et al.*, 1999a). Bell *et al.* (2006) evaluated the effect of safflower oil in combination with monensin on the concentration of rumenic acid in bovine milk. Cows fed a diet supplemented with 24 ppm of monensin, 6% (dDM) safflower oil, or 6% (dDM) safflower oil plus 24 ppm of monensin for 15 days yielded milkfat rumenic acid concentrations of 0.52%, 3.36%, and 5.15% compared to a control value of 0.45%. These data demonstrate that while monensin alone only initiates a small increase in milkfat rumenic acid its use in combination with plant oils such as safflower oil is extremely effective in increasing milkfat rumenic acid.

The effect of feeding ruminally protected synthetic CLA or post ruminal infusion with synthetically produced CLA on milkfat CLA concentrations has also been investigated **(Table 1.2.2)**. Administration of CLA in this manner resulted in a relatively minor increase in milkfat CLA, but also dramatically reduces milk yield

and fat (Bell & Kennelly, 2003; Bernal-Santos *et al.*, 2003; Chouinard *et al.*, 1999; Giesy *et al.*, 2002; Mackle *et al.*, 2003; Perfield *et al.*, 2004).

Bell *et al.* (2006) compared the effects of the dietary consumption of 6% (dDM) safflower oil supplemented with vitamin-E (150 IU/kg of dDM), or 6% (dDM) safflower oil alone, on the concentration of milkfat CLA. Following eight weeks treatment it was found that animals fed safflower oil alone produced a higher milkfat CLA (4.16%) than animals fed safflower oil supplemented with vitamin-E (3.54%). In addition, vitamin-E supplementation was also found to reduce milkfat vaccenic acid concentrations. This suggests that vitamin-E may reduce milkfat CLA by negatively affecting ruminal biohydrogenation. In an attempt to determine if vitamin-E was the component of fresh pasture responsible for elevated milkfat CLA concentrations relative to conserved forage or grains Kay *et al.* (2005a) fed cows either fresh pasture, total mixed ration or total mixed ration with vitamin-E supplementation (10,000 IU/d) for three weeks. These diets yielded milkfat CLA concentrations of 1.84%, 0.71% and 0.72%, respectively, suggesting that vitamin-E does not play a substantial role in the elevation of milkfat CLA which is seen with fresh forage.

1.2.4.6 Combination diets

A number of studies have assessed the effect of diets containing combinations of fish oils, plant oils, animal fats and forage on the rumenic acid and total CLA content of ruminant milkfat **(Table 1.2.2)**. Following three weeks supplementation with tallow or choice white grease (CWG) at 2% and 4% (dDM) in combination with a corn silage based diet, milkfat CLA concentrations from cows fed the animal fat supplemented diet were lower than the control suggesting the use of conserved forage alone is superior to those supplemented with animal fats (Onetti *et al.*, 2001).

Variable effects on milkfat rumenic acid concentrations were produced when plant and fish oils were used in combination. In a large number of these studies, it was found that fish oils and fish meals were superior to combinations of fish and plant oils at increasing milkfat rumenic acid and total CLA concentrations (Abu-Ghazaleh *et al.*, 2001; Ramaswamy *et al.*, 2001; Whitlock *et al.*, 2002). Abu-Ghazaleh *et al.* (2001) investigated the effect of replacing soy meal with three diets containing increasing amounts of fish meal on an isonitrogenous basis (100% soy meal, 50% soy meal and 50% fish meal or 100% fish meal), yielding milkfat CLA contents of 0.53%, 0.66% and 1.0%, respectively. Similarly, feeding cows a diet supplemented with 2% fish oil, 2% extruded soybeans, or a combination of 1% fish oil and 1% extruded soybeans yielded milkfat CLA of 2.07%, 1.18%, 1.86%, and 2.3%, 1.24% 2.17%, respectively (Ramaswamy *et al.*, 2001; Whitlock *et al.*, 2002). More recent studies have produced contrasting results, with both Abu-Ghazaleh *et al.* (2002b, 2003) and Allred *et al.* (2006) showing consistently that combinations of fish oil and plant oils were more effective at increasing total milkfat CLA and rumenic acid than either alone **(Table 1.2.2)**. Supplementation of the bovine diet with 0.5% (dDM) fish oil in the form of fish meal, 2.5% (dDM) soybean oil in the form of extruded soybeans, and a combination of both, for a period of four weeks resulting in milkfat CLA concentrations of 0.56%, 0.91%, and 1.59%, respectively (Abu–Ghazaleh *et al.*, 2002b). In a more recent study, the effect of consumption of 2.7% (dDM) calcium salts of palm and fish oil, a combination diet of 2.7% (dDM) calcium salts of palm and fish oil and 5% (dDM) full fat extruded soybeans, or 2.7% (dDM) calcium salts of palm and fish oil and 0.75% soybean oil, on milkfat CLA concentrations was investigated over six weeks. In this study milkfat CLA concentrations of 1.27%, 1.44%, and 1.82% were obtained, respectively (Allred *et al.*, 2006). Abu-Ghazaleh *et al.* (2003) assessed the effect of supplying fish oil in

combination with different plant oils on total milkfat CLA concentrations in ruminants, by feeding a diet containing 1% (dDM) fish oil supplemented with 2.0% (dDM) high oleic acid sunflower seeds, 2% (dDM) high linoleic acid sunflower seeds, or 2% (dDM) linseed (high α-linolenic acid), (all of which were cracked with rollers) to investigate which plant oil had the greatest synergistic effect on milkfat CLA when used in combination with fish oil over a four week trial period. Total milkfat CLA concentrations of 1.21%, 1.94%, and 1.21%, respectively were achieved, indicating combinations of high linoleic acid oils with fish oil yield the greatest increases in total milkfat CLA.

1.2.4.7 Management strategies, lactation number, breed, and stage of lactation

The effect of ruminant feeding strategies such as, indoors feeding of concentrates and conserved forage year round versus seasonal pasture feeding, restricted versus unrestricted dietary intake, and the effect of supplementation of the ruminant diet with lipid supplements on the total CLA and rumenic acid concentration in ruminant milk have been investigated. In addition, studies have addressed a number of other factors which could potentially have a bearing on the total CLA and rumenic acid content of milkfat such as altitude, farm management strategy, animal breed, lactation number and stage of lactation. Differences in milkfat CLA concentrations in the milk of cows grazing at different altitudes have been observed. In one such study milkfat CLA concentrations of 0.85%, 1.58%, and 2.34% were reported from the milk of cows grazing in the lowlands (600-650 m above sea level), mountains (900-1210 m above sea level), and highlands (1275-2120 m above sea level) of Switzerland, respectively (Collomb *et al.*, 2002). These differences in total milkfat CLA were attributed to variations in the plant species between these regions, although differences in the fatty acid metabolism of the

animals at the different locations were not ruled out (Collomb *et al.*, 2001). In a study investigating the impact of farm management strategies on the rumenic acid content of ruminant milk, Jahreis *et al.* (1997) compared three different farm management strategies. Indoor feeding of conserved forage resulted in a low concentration of milkfat rumenic acid (0.34%), indoors and pasture feeding on a seasonal basis resulted in a milkfat rumenic acid content (0.61%), while an ecological farming practice resulted in the highest milk fat rumenic acid content (0.8%). These differences were attributed to differences in the forage type and fatty acid composition of the different strategies (herd size and elevation differed between the management strategies). Ellis *et al.* (2006) collected bulk-tank milk derived each month from 17 organic and 19 conventional dairy farms in the United Kingdom over a 12 month period, to assess differences in the respective CLA (rumenic acid) content of the milk. The study showed that organically produced milk contained 12% more CLA than milk from conventionally managed farms. The differences between the two were attributed to differences in animal management and nutrition between the two systems. The effect of breed on total milkfat CLA has been investigated in a number of studies, including investigations into the CLA content of milk derived from Holstein-Friesian, Brown Swiss, Jersey, Normande, Montbeliard, Ayrshire and Guernsey cows (Capps *et al.*, 1999; Dhiman *et al.*, 2002; Kelsey *et al.*, 2003; Lawless *et al.*, 1999; Medrano *et al.*, 1999; Morales *et al.*, 2000; Ramaswamy *et al.*, 2001; White *et al.*, 2001; Whitlock *et al.*, 2002). These observations indicate that at pasture Montbeliards produce the highest concentrations of milkfat CLA (1.85%), followed by Normandes (1.64%), Holstein-Friesians (0.72-1.66%), Brown Swiss (1.22%), Jersey (0.59-0.77%), Ayrshire (0.57%), and Guernsey (0.36%) (Data from Table. 2, Dhiman *et al.*, 2005). The difference between the milkfat concentrations of CLA produced by different breeds

of cows has been attributed to differences in the activity of the mammary enzyme Δ^9-desaturase (Medrano *et al.*, 1999; Stanton *et al.*, 2003). The effect of lactation number on total milkfat CLA and rumenic acid has also been reported (Lal & Narayanan, 1984; Stanton *et al.*, 1997). In our centre, we compared the fatty acid composition of milkfat from cows with a lactation number of five to those with a lactation number of between two and four. Following eight weeks of grazing supplemented with grass nuts, milk fat CLA concentrations of 0.59% and 0.41%, respectively, were obtained (Stanton *et al.*, 1997), substantiating previous data indicating that milk of cows of higher lactation number yielded higher milkfat CLA concentrations than low lactation number cows (Lal & Narayanan, 1984). The factors responsible for the higher milkfat CLA levels in higher lactation number cows has not been fully elucidated, but may be associated with changes in the microbial population of the rumen and fatty acid metabolism of the animal (Dhiman *et al.*, 2005). In a recent study, milk fatty acid composition was recorded over sixteen weeks postpartum in both a low merit and high merit bovine genetic line (in terms of milk yield). It was found that milkfat CLA content increased from a low of 0.31% on week one, to 0.46% on week eight to a high of 0.54% on week sixteen. During this period, the activity of the enzyme Δ^9-desaturase remained relatively constant, with the increase in milkfat CLA attributed to an increased supply of vaccenic acid (Kay *et al.*, 2005b). In another study by Lock *et al.* (2005a), changes in milkfat CLA concentrations and Δ^9-desaturase activity from winter to summer were investigated. It was reported that the rumenic acid content of the milk varied from 0.1% to 3.2% over the sampling period, with the authors concluding that under normal conditions stage of lactation had no bearing on milkfat CLA concentrations. Furthermore, it was observed that milk yield, fat content and fat yield did not affect either the CLA content of the milkfat or desaturase activity,

substantiating the earlier observations of Kelsey *et al.* (2003).

1.2.5 CLA producing cultures of dairy significance

In recent years strains of a number of dairy starter and probiotic cultures have been identified as possessing the ability to biosynthesise CLA, including strains of *Lactococcus, Streptococcus, Enterococcus, Lactobacillus, Bifidobacterium,* and *Propionibacterium.* These cultures are of extreme importance to the dairy industry and are essential for the production of a range of traditional and novel dairy products.

1.2.5.1 Lactococci, streptococci, and enterococci

Lactococci, streptococci, and enterococci are some of the most important lactic acid bacteria (LAB) involved in the dairy industry and play a critical role in the manufacture of fermented dairy products like buttermilk, lactic butter, ripened cream, yogurt and cheese. In recent years, these cultures have received substantial attention as a result of their reported probiotic activity and use in alleviation of a gastro-intestinal disease (Benyacoub *et al.*, 2005; Cremonini *et al.*, 2002; Marteau *et al.*, 2001; Steidler *et al.*, 2000). In addition to these properties, a number of recent investigations have indicated the ability of the bacteria to produce CLA and in particular rumenic acid from free linoleic acid (Kishino *et al.*, 2002; Lin *et al.*, 1999) **(Table 1.2.3)**. Lin *et al.* (1999) assessed the CLA producing abilities of *Lc. Lactis* subsp. *cremoris* CCRC12586, and *Lc. lactis* subsp. *lactis* CCRC10791 and the streptococcal strain *S. salivarius* subsp. *thermophilius* CCRC12257 in 12% (w/v) reconstituted skimmed milk (RSM) containing 0.1 or 0.5 mg/ml linoleic acid after 24 h incubation. The fermentation resulted in the production of 0.042 mg/ml, 0.058 mg/ml and 0.049 mg/ml of CLA, respectively, at a linoleic acid concentration

of 0.1 mg/ml, and 0.044 mg/ml, 0.053 mg/ml, and 0.065 mg/ml of CLA, respectively, at a linoleic acid concentration of 0.5 mg/ml. Kishino *et al.* (2002) assessed the CLA producing abilities of a number of LAB including the strain *E. faecium* AKU 1021. Strains were screened for CLA production at 28^{o}C under O_2 limiting conditions for 24-72 h shaking in the presence of 0.6 mg/ml linoleic acid. The products recovered were 0.04 mg/ml rumenic acid and 0.06 mg/ml of the *t*9, *t*11 CLA isomer. Lin *et al.* (1999) and Kishino *et al.* (2002) reported the ability of strains of lactococci, streptococci, and enterococci to produce CLA, however, many other studies have found no such bioconversion **(Table 1.2.3)**. In our centre, we investigated the potential of a number of food cultures including *Lactococcus* to produce CLA in de Man, Rogosa and Sharpe (MRS) medium containing 0.55 mg/ml free linoleic acid, observing that all of the lactococcal strains assayed were negative for CLA production (Coakley *et al.*, 2003). Similar findings were observed in the work of Jiang *et al.* (1998) who assessed the potential for the production of CLA from the lactococcal strains *Lc. lactis* subsp. *lactis* NCFB 176, *Lc. lactis* subsp. *lactis* ATCC 19435, *Lc. lactis* subsp. *cremoris* ATCC 19257, and *Lc. lactis* subsp. *cremoris* NCFB 924, and the streptococcal strains *S. salivarius* subsp. *thermophilus*, and *S. salivarius* subsp. *thermophilus* ATCC 19258 when grown in MRS containing 0.025 mg/ml free linoleic acid.

1.2.5.2 Propionibacteria

Dairy propionibacteria are commonly found in Swiss type cheeses where they produce acetate, propionate, and carbon dioxide, which contribute to flavour and the characteristic eyes of the cheese. However, they have also been isolated from soil, silage, brines for olive fermentation and rum distilleries (Cummins & Johnson, 1986). In addition to their role in the manufacture of dairy products,

Table 1.2.3 CLA production by strains of *Lactococcus, Streptococcus and Enterococcus*

Culture	Culture Type	Medium	Linoleic acid mg/ml	Incubation time	Total CLA mg/ml	Ref
Lc. lactis subsp. *lactis* NCFB 176	Growing culture	MRS	0.025	72 hrs	N.D.	Jiang *et al* (1999)
Lc. lactis subsp. *lactis* ATCC 19435	Growing culture	MRS	0.025	72 hrs	N.D.	Jiang *et al* (1999)
Lc. lactis subsp. *cremoris* ATCC 19257	Growing culture	MRS	0.025	72 hrs	N.D.	Jiang *et al* (1999)
Lc. lactis subsp. *cremoris* NCFB 924	Growing culture	MRS	0.025	72 hrs	N.D.	Jiang *et al* (1999)
Lc. lactis subsp. *lactis* DPC3147	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley *et al* (2003)
Lc. lactis subsp. *lactis* DPC 436	Growing culture	MRS	0.55	24 hrs	N.D.	Coakley *et al* (2003)
Lc. Lactis subsp. *cremoris* CCRC12586	Growing culture	12 % RSM	0.5	24 hrs	0.044	Lin *et al* (1999)
Lc. lactis subsp. *lactis* CCRC10791	Growing culture	12 % RSM	0.1	24 hrs	0.0575	Lin *et al* (1999)
S. salivarius subsp *thermophilus*	Growing culture	MRS	0.025	25 hrs	N.D.	Jiang *et al* (1999)
S. salivarius subsp *thermophilus* ATCC 19258	Growing culture	MRS	0.025	25 hrs	N.D.	Jiang *et al* (1999)
S. salivarius subsp. *thermophilius* CCRC12257	Growing culture	12 % RSM	0.5	24 hrs	0.0645	Lin *et al* (1999)
E. faecium AKU 102	Growing culture	MRS	0.6	24-72 hrs	0.1	Kishino *et al* (2002)

Propionibacterium have been reported to produce B-vitamins (Quesada-Chanto *et al.*, 1994; Roessner *et al.*, 2002), bacteriocins (Brede *et al.*, 2004; Lyon *et al.*, 1993; Van der Merwe *et al.*, 2004), and bifidogenic compounds (Kaneko *et al.*, 1994) spurring increased interest in these cultures. The ability of propionibacteria to produce rumenic acid and other CLA isomers from linoleic acid has been confirmed and resulted in the identification of a large number rumenic acid and *t*9, *t*11 producing strains (Jiang *et al.*, 1998; Kishino *et al.*, 2002; Rainio *et al.*, 2001; Rainio *et al.*, 2002; Verhulst *et al.*, 1987) **(Table 1.2.4)**. Verhulst *et al.* (1987) examined 36 strains of *Propionibacterium* for the production of CLA from 0.02 mg/ml linoleic acid in modified BHI (mBHI) medium resulting in the identification of a large number of CLA producing strains of *P. freudenreichii* subsp. *freudenreichii*, *P. freudenreichii* subsp. *shermanii*, *P. acidi-propionici*, and *P. technicum* with between 50% and 80% of the CLA produced in the form of rumenic acid. Similarly, Kishino *et al.* (2002) reported *P. shermanii* AKU 1254 exhibited linoleic acid isomerase activity when grown in MRS containing 0.6 mg/ml linoleic acid, converting 15% and 3.33% of the linoleic acid to rumenic acid and the *t*9, *t*11 isomer, respectively. Jiang *et al.* (1998) assayed a number of dairy cultures for CLA production, including six strains of propionibacteria, using MRS containing 0.025 mg/ml linoleic acid, finding that strains of *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* were capable of producing rumenic acid **(Table. 1.2.4)**. Using these strains the authors assessed the effect of incrementally increasing the linoleic acid from 0 to 1.5 mg/ml on rumenic acid production showing that each strain had an optimum linoleic acid concentration and that concentrations above this, both growth and CLA production were inhibited. The inhibitory activity of unsaturated fatty acids has been reported in a number of studies with the bioconversion of linoleic acid to CLA suggested as a detoxification

mechanism (Heczko *et al.*, 1979; Kelsey *et al.*, 2006; Lee *et al.*, 2002).

Most of the studies into the production of CLA by strains of propionibacteria have utilised synthetic media or milk containing free linoleic acid emulsified using detergents such as Tween 80 or through the formation of complexes with proteins such as BSA. Vahvaselkä *et al.* (2004) used an alternative approach where a species of oats (*Avena sativa* L.), with a high linoleic acid content and endogenous lipolytic activity was used to prepare a linoleic acid enriched slurry (5% (w/v)) as the substrate for CLA production by *P. freudenreichii* subsp. *shermanii* JS under optimised conditions. Once optimised such slurries were capable of yielding CLA concentrations of up to 0.44 mg/ml, which could be further increased to 0.85 mg/ml by increasing the flour content of the slurry up to 15% (w/v). The CLA produced via this fermentation strategy was concentrated onto the solid phase by acidification and was easily removed from this solid material on centrifugation or filtration. Vahvaselkä *et al.* (2006) repeated this production using the strain *P. freudenreichii* subsp. *shermanii* DSM 2027 reporting the production of 116 mg/g fat of CLA from the oaten flower slurry following 20 h fermentation.

1.2.5.3 Lactobacilli

Lactobacilli are one of the most common dairy starter cultures and are used in the production of a diverse range of products including acidophilus buttermilk, yoghurt, kefir, cheese and koumiss, where they contribute to acid production and flavour through the production of lactic acid, acetic acid or ethanol. In addition to their use as starter bacteria, lactobacilli have been frequently used as probiotics and have been associated with the alleviation of a number of gastro-intestinal diseases (Bergonzelli *et al.*, 2005; Cremonini *et al.*, 2002; Gosselink *et al.*, 2004; O'Mahony *et al.*, 2005; Orrhage *et al.*, 2000; Sartor, 2005; Schultz & Sartor, 2000). Added to

these benefits a large number of studies have also reported that lactobacilli possess the ability to conjugate linoleic acid and produce CLA **(Table 1.2.5)**. Kim & Liu (2002) assayed strains of *Lb. acidophilus*, *Lb. bulgaricus, Lb. heleveticus*, *Lb.*

Table 1.2.4 CLA production by strains of *Propionibacterium*.

Culture	Culture Type	Medium	Linoleic acid mg/ml	Incubation time	Total CLA mg/ml	Ref
P. freudenreichii subsp. *freudenreichii* NCIB 8896	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. freudenreichii subsp. *freudenreichii* NCIB 5959	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. freudenreichii subsp. *shermanii* NCIB 10585	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. freudenreichii subsp. *shermanii* NCIB 5964	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. freudenreichii subsp. *shermanii* NCIB 8099	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acidi-propionici NCIB 8070	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acidi-propionici NCIB 5959	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. technicum NCIB 5965	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acnes ATCC 6919	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acnes ATCC 6921	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acnes no 27	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acnes VPI 163	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acnes VPI 164	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acnes VPI 199	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acnes VPI 186	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acnes VPI 174	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. acnes VPI 170	Growing culture	BHI	0.02	24 h	Detected	Verhulst *et al* (1987)
P. avidum, (VPI 575, VPI 576, VPI 598, VPI 668, VPI 671, ATCC 25557, CN 6976, CN 5888, and CN 6278)	Growing culture	BHI	0.02	24 h	N.D.	Verhulst *et al* (1987)
P. jensenii (NCIB 5960, NCIB 5967, and NCIB 5962)	Growing culture	BHI	0.02	24 h	N.D.	Verhulst *et al* (1987)
P. thoenii (NCIB 8072, and NCIB 5966)	Growing culture	BHI	0.02	24 h	N.D.	Verhulst *et al* (1987)
P. lymphophilum CN 5936	Growing culture	BHI	0.02	24 h	N.D.	Verhulst *et al* (1987)
P. freudenreichii subsp. *freudenreichii* ATCC 6027	Growing culture	MRS	0.1	72 h	0.023	Jiang *et al* (1999)
P. freudenreichii subsp. *freudenreichii* Propioni-6	Growing culture	MRS	0.75	73 h	0.265	Jiang *et al* (1999)
P. freudenreichii subsp. *shermanii* 9093	Growing culture	MRS	0.5	74 h	0.112	Jiang *et al* (1999)
P. shermanii AKU 1254	Growing culture	MRS	0.6	24-72 h	0.11	Kishino *et al* (2002)
P. freudenreichii subsp. *shermanii* JS	Growing culture	WPM	2	N.S.	1.6	Rainio *et al* (2002)
P. freudenreichii subsp. *shermanii* 56	Growing culture	Yoghurt	5	N.S.	Detected	Xu et al (2005)
P. freudenreichii subsp. *shermanii 51*	Growing culture	Yoghurt	5	N.S.	Detected	Xu et al (2005)
P. freudenreichii subsp. *freudenreichii* 23	Growing culture	Yoghurt	5	N.S.	Detected	Xu et al (2005)
P. freudenreichii subsp. *shermanii* strain JS	Growing culture	Hydrolyzed oat flour slurry	12.6 mg/g (DM)	30 h	10.1 mg/g (DM)	Vahvaselka *et al* (2004)
P. freudenreichii subsp. *shermanii* DSM 20270	Growing culture	Hydrolyzed oat flour slurry	30 mg/g (DM)	20 h	11.5 mg/g (DM)	Vahvaselka *et al* (2006)

johnsonii, and *Lb. plantarum* for CLA production in both MRS and in whole milk at a linoleic acid concentration of 0.1 mg/ml. The study identified four strains of CLA producing lactobacilli (*Lb. acidophilus* 96, and *Lb. plantarum* 4191, *Lb. acidophilus* 56, and *Lb. acidophilus* 43121) whose ability to produce the isomer differed substantially with the type of medium used. When grown in MRS only the strains *Lb. acidophilus* 96, and *Lb. plantarum* 4191 proved positive for CLA production, while growth in whole milk resulted in additional CLA production by the strains *Lb. acidophilus* 56, and *Lb. acidophilus* 43121 and improved CLA production by the strain *Lb. plantarum* 4191. In a similar study, Alonso *et al.* (2003) reported that two strains of *Lb. acidophilus* (L1, and O16) and two strains of *Lb. casei* (E5, and E10) exhibited CLA producing capabilities in MRS and milk containing 0.2 mg/ml linoleic acid. Production of CLA in milk was also assessed by Lin *et al.* (1999) using the strains *Lb. acidophilus* CCRC 14079, *Lb. delbrueckii* subsp. *bulgaricus* CCRC 14009, and *Lb. delbrueckii* subsp. *lactis* CCRC 14078 in 12% (w/v) reconstituted skim milk containing 1.0 mg/ml linoleic acid with all four strains proving positive for CLA production. Kishino *et al.* (2002) identified 15 strains of CLA producing lactobacilli using MRS containing 0.6 mg/ml linoleic acid **(Table 1.2.5)**. Of these strains, *Lb. acidophilus* AKU 1137 produced the highest concentration of rumenic acid (0.85 mg/ml), while the strain *Lb. plantarum* AKU 1009a produced the highest total CLA (3.41 mg/ml), which was primarily found in the form of the *t*9, *t*11 isomer. Studies into the production of CLA by lactobacilli have generally found the majority of the CLA to be intimately associated with the cells or located within. Storage of CLA in this manner by the cells increases the potential for the use of lactobacilli as probiotic vectors for the delivery of CLA and in particular rumenic acid to the human gastro-intestinal tract. However, not all studies have demonstrated the CLA producing abilities of lactobacilli, including

Table 1.2.5 CLA production by strains of *Lactobacillus*.

Culture	Culture Type	Medium	Linoleic acid mg/ml	Incubation time	Total CLA mg/ml	Ref
Lb.acidophilus ATCC 4356	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb bulgaricus	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb casei	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb casei F-19	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb fermentum	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb helveticus ATCC 15009	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb reuteri ATCC 23272	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb lactis subsp. *lactis* NCFB 176	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb lactis subsp. lactis ATCC 19435	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb lactis subsp.*cremoris* ATCC 19257	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb lactis subsp.*cremoris* ATCC NCFB 924	Growing culture	MRS	0.025	24 h	N.D.	Jiang *et al* (1999)
Lb. Reuteri NCIMB 11951	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. Reuteri NCIMB 701359	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. Reuteri NCIMB 701089	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. Reuteri NCIMB 702655	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. Reuteri NCIMB 702656	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. heleveticus NCIMB 700257	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. heleveticus ATCC 15009	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. heleveticus NCIMB 701244	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. paracasei UCC 43338	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. paracasei UCC 43364	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. paracasei UCC 42319	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. paracasei UCC 43348	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. paracasei DPC 5336	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. delbreuckii subsp. *lactis* NCIMB 8117	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. delbreuckii subsp. *lactis* NCIMB 8118	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb. salivarius UCC 43310	Growing culture	MRS	0.55	24 h	N.D.	Coakley *et al* (2003)
Lb fermentum	Growing culture	MRS	5	24 h	Detected	Ham *et al* (2002)
Lb. acidophilus 96	Growing culture	MRS	0.1	24 h	< 2mg/g fat	Kim *et al* (2002)
Lb. acidophilus 56	Growing culture	MRS	0.1	24 h	N.D.	Kim *et al* (2002)
Lb. acidophilus ATCC 4356	Growing culture	MRS	0.1	24 h	N.D.	Kim *et al* (2002)
Lb. acidophilus ATCC 43121	Growing culture	MRS	0.1	24 h	N.D.	Kim *et al* (2002)
Lb bulgaricus	Growing culture	MRS	0.1	24 h	N.D.	Kim *et al* (2002)
Lb. heleveticus ATCC 15009	Growing culture	MRS	0.1	24 h	N.D.	Kim *et al* (2002)
Lb. johnsonii 88	Growing culture	MRS	0.1	24 h	N.D.	Kim *et al* (2002)
Lb. plantarum 4191	Growing culture	MRS	0.1	24 h	< 2mg/g fat	Kim *et al* (2002)
Lb. acidophilus 96	Growing culture	Whole milk	0.1	24 h	< 2mg/g fat	Kim *et al* (2002)
Lb. acidophilus 56	Growing culture	Whole milk	0.1	24 h	< 2mg/g fat	Kim *et al* (2002)
Lb. acidophilus ATCC 4356	Growing culture	Whole milk	0.1	24 h	N.D.	Kim *et al* (2002)
Lb. acidophilus ATCC 43121	Growing culture	Whole milk	0.1	24 h	< 2mg/g fat	Kim *et al* (2002)
Lb bulgaricus	Growing culture	Whole milk	0.1	24 h	N.D.	Kim *et al* (2002)
Lb. heleveticus ATCC 15009	Growing culture	Whole milk	0.1	24 h	N.D.	Kim *et al* (2002)
Lb. johnsonii 88	Growing culture	Whole milk	0.1	24 h	N.D.	Kim *et al* (2002)
Lb. plantarum 4191	Growing culture	Whole milk	0.1	24 h	2-4 mg/g fat	Kim *et al* (2002)
Lb. acidophilus L1	Growing culture	MRS	0.2	24 h	0.132	Alonso *et al* (2003)
Lb. acidophilus O16	Growing culture	MRS	0.2	24 h	0.069	Alonso *et al* (2003)
Lb. casei E5	Growing culture	MRS	0.2	24 h	0.111	Alonso *et al* (2003)
Lb. casei E10	Growing culture	MRS	0.2	24 h	0.08	Alonso *et al* (2003)
Lb. acidophilus L1	Growing culture	10% RSM	0.2	24 h	0.116	Alonso *et al* (2003)
Lb. acidophilus O16	Growing culture	10% RSM	0.2	24 h	0.054	Alonso *et al* (2003)
Lb. casei E5	Growing culture	10 % RSM	0.2	24 h	0.01	Alonso *et al* (2003)
Lb. casei E10	Growing culture	10 % RSM	0.2	24 h	0.071	Alonso *et al* (2003)
Lb. acidophilus CCRC 14079	Growing culture	12 % RSM	0.1	24 h	0.105	Lin *et al* (1999)
Lb. delbrueckii subsp. *bulgaricus* CCRC 14009	Growing culture	12 % RSM	0.1	24 h	0.087	Lin *et al* (1999)
Lb. delbrueckii subsp. *lactis* CCRC 14078	Growing culture	12 % RSM	0.1	24 h	0.078	Lin *et al* (1999)
Lb. acidophilus AKU 1137	Growing culture	MRS	0.6	24-72 h	1.5	Kishino *et al* (2002)
Lb. acidophilus IAM10074	Growing culture	MRS	0.6	24-72 h	0.6	Kishino *et al* (2002)

Lactobacilli Contd.

Culture	Culture Type	Medium	Linoleic acid mg/ml	Incubation time	Total CLA mg/ml	Ref
Lb. acidophilus AKU 1122	Growing culture	MRS	0.6	24-72 h	0.12	Kishino *et al* (2002)
Lb brevis IAM 1082	Growing culture	MRS	0.6	24-72 h	0.55	Kishino *et al* (2002)
Lb. paracasei subsp. *paracasei* IFO 12004	Growing culture	MRS	0.6	24-72 h	0.2	Kishino *et al* (2002)
Lb. paracasei subsp. *paracasei* JCM 1109	Growing culture	MRS	0.6	24-72 h	0.07	Kishino *et al* (2002)
Lb. paracasei subsp. *paracasei* AKU 1142	Growing culture	MRS	0.6	24-72 h	0.07	Kishino *et al* (2002)
Lb. paracasei subsp. *paracasei* IFO 3533	Growing culture	MRS	0.6	24-72 h	0.09	Kishino *et al* (2002)
Lb. pentosus AKU 1148	Growing culture	MRS	0.6	24-72 h	0.08	Kishino *et al* (2002)
Lb. pentosus IFO 12011	Growing culture	MRS	0.6	24-72 h	0.13	Kishino *et al* (2002)
Lb. plantarum AKU 1138	Growing culture	MRS	0.6	24-72 h	0.45	Kishino *et al* (2002)
Lb. plantarum AKU 1009a	Growing culture	MRS	0.6	24-72 h	3.41	Kishino *et al* (2002)
Lb. plantarum JCM 8341	Growing culture	MRS	0.6	24-72 h	0.19	Kishino *et al* (2002)
Lb. plantarum JCM 1551	Growing culture	MRS	0.6	24-72 h	1.02	Kishino *et al* (2002)
Lb. rhamnosus AKU 1124	Growing culture	MRS	0.6	24-72 h	1.41	Kishino *et al* (2002)
Lb. acidophilus AKU 1137	Washed cells	KPB	5	96 h	4.9	Ogawa *et al* (2001)
Lb. plantarum AKU 1009a	Washed cells	KPB	20	48 h	8.9	Kishino *et al* (2002)
Lb. Reuteri ATCC 55739	Immobilized cells	Silica gel	0.5	1 h	0.175	Lee *et al* (2003)
Lb. delbrueckii subsp. *bulgaricus* CCRC 14009	Immobilized cells	Polyacrylamide	3	24 h	2.211	Lin *et al* (2004)
Lb. delbrueckii subsp. *bulgaricus* CCRC 14009	Immobilized cells	Chitosan	3	24 h	0.283	Lin *et al* (2004)
Lb. delbrueckii subsp. *bulgaricus* CCRC 14009	Free cells	KPB	3	24 h	0.01	Lin *et al* (2004)
Lb. acidophilus CCRC 14079	Immobilized cells	Polyacrylamide	3	24 h	0.218	Lin *et al* (2004)
Lb. acidophilus CCRC 14079	Immobilized cells	Chitosan	3	24 h	0.055	Lin *et al* (2004)
Lb. acidophilus CCRC 14079	Free cells	KPB	3	24 h	0.022	Lin *et al* (2004)
Lb. rhamnosus	Growing culture	yoghurt	5	N.S.	Detected	Xu et al (2005)

those of Jiang *et al.* (1998), Coakley *et al.* (2003) and Ham *et al.* (2002), where investigations into the production of CLA by lactobacilli proved negative **(Table 1.2.5)**.

The use of growing cells of lactobacilli as a means of rumenic acid and CLA production has proved a very successful strategy, it is not however the only avenue currently being explored. Ogawa *et al*. (2001) used washed cells of *Lb. acidophilus* AKU 1137 which when exposed to 5 mg/ml free linoleic acid complexed to BSA produced 4.9 mg/ml of CLA of which almost all was in or associated intimately with the cells. Kishino *et al.* (2002) assessed the CLA forming abilities of washed cells of *Lb. plantarum* AKU 1009a, varying reaction conditions such as pH, temperature, form of linoleic acid supplied, linoleic acid concentration, oxygen exposure and the ratio of BSA to linoleic acid. Maximum CLA production was achieved by maintaining the pH and temperature at the optimum for the strain's linoleic acid isomerase, observing only the free form of linoleic acid was converted to CLA. The optimum ratio of BSA to linoleic acid was deemed to be 1:5 or 2.5:5 (weight ratio). When exposed to 120 mg/ml free linoleic acid these washed cells produced 40 mg/ml of CLA after 108 h, while reduction of this concentration to 26 mg/ml resulted in a 50% reduction in CLA production. Based on the observations of Ogawa *et al.* (2001) and Kishino *et al.* (2002) the use of washed cells would appear to be an efficient and effective method for the natural production of CLA and in particular rumenic acid on an industrial scale.

The use of immobilised cells for the production of rumenic acid and other CLA isomers was reported by Sun-Ok *et al.* (2003). Immobilised cells of *Lb. reuteri* ATCC 55739 in a silica gel matrix were reported to produce 0.175 mg/ml CLA from 0.5 mg/ml linoleic acid following incubation for one hour under optimised conditions in the presence of 1.0 mM Cu^{2+} **(Table 1.2.5)**. The strategy

proved extremely successful when compared to the production of CLA by washed cells of the same strain, which produced only 0.032 mg/ml CLA under optimised conditions. Furthermore, these immobilised cells could be reused up to five times resulting in the production of over 0.344 mg/ml CLA from 0.5 mg/ml linoleic acid. More recently Lin *et al.* (2005) investigated the use of immobilized cells of *Lb. delbrueckii* subsp. *bulgaricus* CCRC 14009 and *Lb. acidophilus* CCRC 14079 in two different gel matrixes (chitosan, and polyacrylamide) for the production of CLA following incubation in the presence of 3 mg/ml linoleic acid for 24 h. Using this approach *Lb. delbrueckii* subsp. *bulgaricus* CCRC 14009 produced 1.23 mg/ml, and 0.052 mg/ml of rumenic acid in the polyacrylamide and chitosan gel matrices, respectively, compared to washed cells of the same strain which produced only 0.03 mg/ml **(Table 1.2.5)**. Similar results were also obtained with *Lb. acidophilus* CCRC 14079, which produced substantially more CLA in polyacrylamide and chitosan gel matrices compared to washed cells.

Lin *et al.* (2003) and Lin, (2006) used crude enzyme extracts which harbour the enzyme linoleic acid isomerase extracted from lactobacilli as the catalyst for CLA production. Partially purified enzyme extract derived from the strain *Lb. acidophilus* CCRC 14079 was used for the production of CLA from 50 and 75 mg of linoleic acid using increasing concentrations of enzyme extract (25-75 mg) (Lin *et al.*, 2003). The production of CLA increased in parallel with the concentration of enzyme extract supplied resulting in the production of 0.305 mg and 0.439 mg of CLA at linoleic acid concentrations of 50 and 75 mg respectively. Lin (2006) investigated the effect of the exposure of linoleic, linolenic, and oleic acids to an enzyme extract derived from the strain *Lb. delbrueckii* subsp. *bulgaricus* CCRC 14009. In the assay 25 mg of each fatty acid was mixed with 50 mg of the enzyme extract and incubated at 37^{o}C for 108 h, yielding 0.0085 mg, 0.0035 mg and 0.0047

mg of CLA from linoleic, linolenic and oleic acid, respectively. The use of crude enzyme extracts in the production of CLA resulted in the formation of a diverse range of CLA isomers. In the study of Lin *et al.* (2003) eight different CLA isomers were detected, while Lin (2006) isolated six CLA isomers.

1.2.5.4 Bifidobacteria

Bifidobacteria have been used for centuries in the production of bifidus milks and more recently in the production of functional dairy products. As natural inhabitants of the human gastro-intestinal tract bifidobacteria have been associated with a large number of probiotic properties and with the prevention or alleviation of a number of human gastro-intestinal conditions (Cremonini *et al.*, 2002; Gionchetti *et al.*, 2000a; Gionchetti *et al.*, 2000b; O'Mahony *et al.*, 2005; Orrhage *et al.*, 2000; Saavedra, 2000; Sartor, 2004). In addition, a number of recent studies have also reported the production of CLA (primarily rumenic acid and/or the *t*9, *t*11 CLA isomer) from linoleic acid by bifidobacteria **(Table 1.2.6)**.

The production of CLA by bifidobacteria was first reported by Coakley *et al.* (2003) following the screening of strains of *B. adolescentis, B. angulatum, B. bifidum, B. breve, B. dentium, B. infantis, B. lactis, and B. pseudocatenulatum,* for the ability to bioconvert 0.55 mg/ml linoleic acid to CLA **(Table 1.2.6)**. *B. breve, B. dentium,* and *B. psuedocatenulatum* were found to produce the highest concentration of CLA, which was primarily found as rumenic acid. Deok-Khun *et al.* (2003) identified two further strains of CLA producing bifidobacteria (*B. breve* KCTC 10462 and *B. pseudocatenulatum* KCTC 10208) following the screening of faecal samples derived from breast fed infants. Both Coakley *et al.* (2003) and Oh *et al.* (2003) observed that the CLA produced by bifidobacteria was found almost exclusively in the supernatant.

Table 1.2.6 CLA production by strains of *Bifidobacterium.*

Culture	Culture Type	Medium	Linoleic acid mg/ml	Incubation time	Total CLA mg/ml	Ref
B. acodelescentis NCFB 2230	Did not grow	cys-MRS	0.55	48 h	N.D.	Coakley *et al* (2003)
B. acodelescentis NCFB 2204	Growing culture	cys-MRS	0.55	48 h	0.0035	Coakley *et al* (2003)
B. acodelescentis NCFB 2231	Growing culture	cys-MRS	0.55	48 h	0.0028	Coakley *et al* (2003)
B. angulatum NCFB 2236	Growing culture	cys-MRS	0.55	48 h	0.0012	Coakley *et al* (2003)
B. bifidum NCFB 795	Growing culture	cys-MRS	0.55	48 h	0.001	Coakley *et al* (2003)
B. breve NCFB 2257	Growing culture	cys-MRS	0.46	48 h	0.2311	Coakley *et al* (2003)
B. breve NCFB 2258	Growing culture	cys-MRS	0.55	48 h	0.3982	Coakley *et al* (2003)
B. breve NCTC 11815	Growing culture	cys-MRS	0.55	48 h	0.2151	Coakley *et al* (2003)
B. breve NCIMB 8815	Growing culture	cys-MRS	0.55	48 h	0.2281	Coakley *et al* (2003)
B. breve NCIMB 8807	Growing culture	cys-MRS	0.46	48 h	0.1279	Coakley *et al* (2003)
B. dentium NCFB 2243	Growing culture	cys-MRS	0.55	48 h	0.1598	Coakley *et al* (2003)
B. infantis NCFB 2205	Growing culture	cys-MRS	0.55	48 h	0.0036	Coakley *et al* (2003)
B. infantis NCFB 2256	Growing culture	cys-MRS	0.55	48 h	0.0246	Coakley *et al* (2003)
B. lactis Bb12	Growing culture	cys-MRS	0.55	48 h	0.281	Coakley *et al* (2003)
B. psuedocatenulatum NCIMB 8811	Growing culture	cys-MRS	0.55	48 h	0.0233	Coakley *et al* (2003)
B. breve NCFB 2258	Growing culture	cys-MRS	0.5	72 h	36.7%	Rosberg-cody *et al* (2004)
B. breve (PFGE pattern B)	Growing culture	cys-MRS	0.5	72 h	29.0%	Rosberg-cody *et al* (2004)
B. breve (PFGE pattern F2)	Growing culture	cys-MRS	0.5	72 h	27.4%	Rosberg-cody *et al* (2004)
B. bifidum (PFGE pattern A1)	Growing culture	cys-MRS	0.5	72 h	17.9%	Rosberg-cody *et al* (2004)
B. breve KCTC 10462	Growing culture	cys-MRS	0.5	48 h	0.16	Deok-Kun *et al* (2003)
B. psuedocatenulatum KCTC 10208	Growing culture	cys-MRS	0.5	48 h	0.135	Oh *et al* (2003)
B. breve KCTC 3461	Growing culture	cys-MRS	4.0	40 h	0.69	Sonng *et al* (2005)

Bifidobacteria are commonly isolated from the intestine and faeces of adults and infants, which represents a large reservoir for the isolation of rumenic acid producing strains. Rosberg-Cody *et al.* (2004) reported the isolation of novel strains of bifidobacteria from infant faecal material, and the identification of two strains with efficient CLA producing bifidobacteria belonging to the species *B. breve* and one strain belonging to the species *B. bifidum* **(Table 1.2.6)**. This study along with that of Oh *et al.* (2003) have demonstrated that populations of bifidobacteria with ability to produce CLA and in particular rumenic acid develop in the neonate shortly after birth and as such it may be assumed they play an important role in the health of neonates. The anti-carcinogenic activity of CLA (rumenic acid and *t*10, *c*12 isomer) naturally produced by the probiotic mix VSL3 (mixture of CLA producing strains of *Lb. acidophilus*, *Lb. bulgaricus, Lb. casei, Lb. plantarum*, *B. breve, B. infantis, B. longum* and *S. thermophilus*) from 0.5 mg/ml linoleic acid on HT-29 and Caco-2 cell lines was investigated (Ewaschuk *et al*., 2006). Reduced viability and increased cellular apoptosis were reported in both cell lines. Furthermore, in an *ex vivo* assay it was shown that following administration of the probiotic VSL3, murine faeces supplemented with linoleic acid produced 100-fold more CLA than faeces collected prior to VSL3 feeding. These observations suggest the ability of CLA producing probiotic bacteria to produce CLA isomers *in vivo*. In another study, we have recently demonstrated the anti-proliferative effect of the two main CLA isomers formed by *B. breve, i.e.* rumenic acid and *t*9, *t*11 CLA using SW480 and HT-29 human colon cancer cells, which were cultured in the presence of the CLA isomers. This study demonstrated that the *t*9, *t*11 CLA had a more potent anti-proliferative effect than rumenic acid (Coakley *et al.*, 2006) and supports the earlier observations of Beppu *et al*. (2006) who also reported the higher anti-proliferative activity of the *t*9, *t*11 CLA isomer relative to rumenic acid.

1.2.6 Production of rumenic acid enriched dairy products

Our ability to successfully manipulate the CLA content of ruminant milkfat and the identification of CLA producing bacteria have opened up new avenues for the development of CLA enriched dairy products additionally explaining the differences seen previously in the CLA content of dairy products **(Table 1.2.7)**. Using this knowledge a number of studies have proceeded to produce a range of CLA enriched dairy products, including, ultra-high temperature (UHT) treated milk, butter, yoghurt and in particular cheeses.

1.2.6.1 UHT milk

In a recent study high rumenic acid milk (9.1-fold more rumenic acid than control), produced through the supplementation of the basal diet of Friesian cows with sunflower oil and fish oil, was utilised to produce a UHT milk (Jones *et al.*, 2005). Following UHT treatment, the rumenic acid content of the milk was 9.33-fold greater than the control milk, and while sensory characteristics differed compared to the control, a negative impact on the quality of the milk was not found. The results demonstrate the stability of rumenic acid enriched milk to the processing conditions employed in the production of UHT milk.

1.2.6.2 Butter

A number of studies have investigated the fatty acid composition of a range of different butters from a range of locations (Jiang *et al.*, 1997; Lin *et al.*, 1995; Ma *et al.*, 1999; Seckin *et al.*, 2005; Shantha *et al.*, 1995). These studies have highlighted the differences in the rumenic acid content of butters, which can be attributed to factors such as the animals diet or farm management practices. Butter enriched in

rumenic acid has been produced in a number of studies through the use of rumenic acid enriched milkfat (Bauman *et al.*, 2000; Jones *et al.*, 2005; Ramaswamy *et al.*, 2001). The resulting butters had elevated concentrations of both rumenic acid and its precursor vaccenic acid, a direct reflection of the composition of the milk from which they were manufactured. Butters produced from the milk of animals supplemented with plant and fish oils were very similar to the controls in most respects but were found to be less firm (Baer *et al.*, 2001; Jones *et al.*, 2005).

1.2.6.3 Fermented milk, and yoghurt

Boylston & Beitz (2002) produced a high CLA yoghurt using milk derived from animals fed a diet supplemented with 5% (dDM) soybean oil. The CLA content of this yoghurt was almost identical to the CLA content of the raw milk from which it was manufactured. This result suggests that the CLA content of milk remains stable throughout the fermentation process; in addition, during investigations into the stability of CLA over seven days refrigerated storage CLA content of this yoghurt remained stable. As in the case of butter the rumenic acid content of yoghurt can differ substantially, a fact which can in part be attributed to the different dietary and animal management practices employed **(Table 1.2.7).** However, as yoghurt is a fermented product the influence of the previously described rumenic acid producing bacteria cannot be overlooked. The ability of strains to produce CLA from free linoleic acid during the fermentation of milk has been shown in a large number of studies including those by Kim & Liu (2002), Jiang *et al.* (1998), Lin *et al.* (1999) and Alonso *et al.* (2003). As a number of these strains belong to families of starter bacteria it is possible that the starters used in the production of fermented milks and yoghurts could potentially convert linoleic acid naturally found in milk to rumenic acid. Evidence for this can be obtained from fermented dairy products such as Dahi

Table 1.2.7 CLA content of a range of fermented and non-fermented dairy products.

Product	Total Fat (g/100g)	*c* 9, *t*11 isomer (mg/g fat)	Total CLA (mg/g fat)	Ref
Milk and milk powder				
2 % fat milk	N.S.	4.14	N.S	1
Evaporated milk 1	N.S.	3.38	N.S	1
Evaporated milk 2	N.S.	6.39	N.S	1
Whole milk	N.S.	4.49	N.S	1
Skim milk powder	0.1	N.S	1.8	2
Whole milk	3.2	N.S	3.4	2
1 % milk	1	N.S	4.3	2
2 % milk	2.1	N.S	5	2
3 % fat milk	3	5.88	N.S	5
1.5 % fat milk	1.5	5.83	N.S	5
Cheese				
Blue cheese 1	N.S.	4.87	N.S	1
Blue cheese 2	N.S.	7.96	N.S	1
Brie	N.S.	4.75	N.S	1
Medium cheddar	N.S.	4.02	N.S	1
Sharp cheddar	N.S.	4.59	N.S	1
Cougar Gold cheese	N.S.	3.72	N.S	1
Cream cheese	N.S.	4.3	N.S	1
Cottage cheese	N.S.	4.8	N.S	1
Edam cheese	N.S.	5.38	N.S	1
Monteray Jack cheese	N.S.	4.8	N.S	1
Mozzarella cheese	N.S.	4.31	N.S	1
Processed American cheese	N.S.	3.64	N.S	1
Processed cheese spread 1	N.S.	4.26	N.S	1
Processed cheese spread 2	N.S.	4.02	N.S	1
Parmesan cheese	N.S.	4	N.S	1
Swiss cheese	N.S.	5.45	N.S	1
Viking cheese	N.S.	3.59	N.S	1
Buttermilk	N.S.	4.66	N.S	1
Sour cream	N.S.	4.14	N.S	1
Yoghurt	N.S.	3.82	N.S	1
Goat cheese	28.5	N.S	2.7	2
Brie cheese	27.9	N.S	3.8	2
Italian parmesan cheese	28.3	N.S	4.2	2
Mozzarella cheese	24.9	N.S	4.6	2
Cheddar cheese	34.6	N.S	4.2	2
Imperial cheddar cheese	33	N.S	4.7	2
Farmer cheese	28.9	N.S	4.7	2
Cream cheese	33.8	N.S	2.7	2
Yoghurt	5.4	N.S	4.4	2
Butter	91.1	N.S	4.7	2
Cheese Whiz	19.1	N.S	4.9	2
Sour cream	12.6	N.S	5	2
Processed Parmesan cheese	28.5	N.S	5.3	2
Cottage cheese	3.1	N.S	5.9	2
Processed cheese	24.3	N.S	6.2	2
Turkish processed cheese 1	26.5	3.63	N.S	3
Turkish processed cheese 2	20	1.5	N.S	3
Turkish processed cheese 3	31	2.36	N.S	3
Turkish processed cheese 4	18.5	2.1	N.S	3
Turkish processed cheese 5	26	2.72	N.S	3
Turkish processed cheese 6	27	1.98	N.S	3
Cheese (contd.)				
Mozzarella cheese	11.5	4.15	4.96	4
Gouda cheese	30	5.18	5.96	4
Cheddar cheese	32	3.94	5.02	4
Blue cheese	39	6.2	N.S	5
Cheddar cheese	35	5.86	N.S	5
Pratost cheese	31	5.01	N.S	5
Herragardsost cheese	28	5.45	N.S	5
Vasterbottenost cheese	31	6.02	N.S	5
Greve cheese	28	7.06	N.S	5
Butter				
Turkish butter 1	83	4.41	N.S	3
Turkish butter 2	82.5	4.67	N.S	3
Turkish butter 3	82	4.49	N.S	3
Turkish butter 4	83	3.87	N.S	3
Turkish butter 5	82	3.82	N.S	3
Turkish butter 6	82.5	3.88	N.S	3
Turkish butter 7	82.25	4.62	N.S	3
Turkish butter 8	82	2.85	N.S	3
Butter (salted)	80	6.42	8.11	4
Butter (unsalted)	80	6.11	7.82	4
Butter	80	6.19	N.S	5
Cream				
Whipping cream	N.S.	4.24	N.S	1
half/half cream	12.2	N.S	5.5	2
Turkish Cream 1	35	7.94	N.S	3
Turkish Cream 2	35	5.74	N.S	3
Sour cream	19	5.86	7.49	4
Ice milk	5	2.79	3.8	4
Ice cream	10	3.77	4.95	4
Whipping cream	40	6.18	N.S	5
Sour cream	34	6.22	N.S	5
Dairy blend	80	4.32	N.S	5
Low dairy blend	40	2.31	N.S	5
Fermented milk & yoghurt.				
0.05 % fat Yoghurt	0.05	3.73	5.25	4
1 % fat Yoghurt	1	7.37	9.01	4
3.25 % fat Yoghurt	3.25	4.27	5.12	4
Yoghurt 1	3	6.15	N.S	5
Yoghurt 2	0.5	6.22	N.S	5
Fjallfal fermented milk	4.2	6.12	N.S	5
Mellanfil fermented milk	1.5	6.07	N.S	5
Bifilus fermented milk	1.5	4.47	N.S	5
Dofilus fermented milk	0.5	5.16	N.S	5
Halsofil fermented milk	0.5	5.24	N.S	5
Turkish Kaymak				
Turkish Kaymak 1	65	6.09	N.S	3
Turkish Kaymak 2	65	5.28	N.S	3
Turkish Kaymak 3	60	5.83	N.S	3
Turkish Kaymak 4	60	4.33	N.S	3

References: Lin *et al* (1995) =1, Ma *et al* (1999) =2, Seckin *et a*l (2005)=3, Shanta *et al* (1995) =4 & Jiang *et al* (1997) =5.

where milkfat CLA concentrations increased approximately 4.8-fold following fermentation (Aneja & Murthy, 1990), 0.05% fat yoghurt with a 1.19-fold increase in CLA following fermentation (Shantha *et al.*, 1995), 3.0% fat yoghurt with a 1.05-fold increase in CLA following fermentation (Jiang *et al.*, 1997), 1.5% fat Mellanfil with a 1.03-fold increase in CLA following fermentation (Jiang *et al.*, 1997), and 5.4% fat yoghurt with a 1.29-fold increase in CLA compared to the raw whole milk (Ma *et al.*, 1999). Lin (2003) assessed the use of the rumenic acid producing strain, *Lb. acidophilus* CCRC 14079, in co-culture with traditional starters, *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, in the production of non-fat set yoghurt supplemented with 1.0 mg/ml linoleic acid. It was found that the starter strains exhibited some linoleic acid isomerase activity increasing the rumenic acid content of the yoghurt from 1.10 mg/g to 1.63 mg/g. However, when combined with the rumenic acid producing strain *Lb. acidophilus* CCRC 14079 this conversion could be substantially increased compared to the control which contained the yoghurt cultures alone (0.93 mg/g to 2.95 mg/g). In a similar study, Xu *et al.* (2005) investigated the effect of using one of three rumenic acid forming *Propionibacterium* (*P. freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51, and *P. freudenreichii* subsp. *freudenreichii* 23) or the rumenic acid producing strain *Lb. rhamnosus*, on the rumenic acid content of a fermented milk when used alone or in co-culture with the traditional yoghurt cultures *Lb. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* YC-180. In this study 12% (w/v) skimmed milk was supplemented with hydrolyzed soybean oil to give a free linoleic acid concentration of 5.0 mg/ml, fermented, followed by storage at 4^{o}C for 14 days. During storage it was observed that the co-culture of CLA producing propionibacteria with yoghurt cultures resulted in on average a 10.53% increase in the concentration of CLA compared to yoghurt cultures alone, while co-

culture of the starter cultures with the strain of *Lb. rhamnosus* resulted in a 70% increase in the CLA concentration. Importantly, in both the studies by Lin (2003) and Xu *et al.* (2005) the product acceptability of the yoghurt prepared through the use of the CLA producing strain in co-culture with traditional yoghurt cultures was unaffected when compared to the control.

1.2.6.4 Cheese

Milk enriched in rumenic acid, produced through animal dietary supplementation, has been used for the production of a number of rumenic acid enriched cheeses (Addis *et al.*, 2005; Dhiman *et al.*, 1999b; Jones *et al.*, 2005). The rumenic acid content and key sensory properties of these cheeses were found to be unaffected by the cheesemaking process, but the rumenic acid enriched cheese were found to be softer than the control. Studies into the fatty acid compositions of different cheeses have highlighted the often substantial differences in their rumenic acid and CLA content. These differences are for the most part a result of the variation in the rumenic acid and CLA content of raw milk used in the cheesemaking process, but given the existence of CLA producing starter bacteria, their potential impact cannot be overlooked (see above). Strains of *Lactobacillus*, *Lactococcus*, *Streptococcus thermophilius*, *Enterococcus faecium*, and *Propionibacterium* have all been identified as producing CLA and are commonly used in the production of commercial and farmhouse varieties of cheese as starter or adjunct cultures. It has been observed that the CLA content of cheese (manufactured with milk from the same season) increased from 16.1 mg/g fat after 5 months ripening to 17.3 mg/g fat after 1 years ripening (Lavillonniere *et al.*, 1998). In a separate study, hard cheeses which were aged longer had higher CLA content than hard cheeses with a shorter aging time (Zlatanos *et al.*, 2002). Despite this evidence there are studies which

have shown that CLA levels remain unchanged during cheesemaking (Jiang *et al.*, 1997; Luna *et al.*, 2005). Addis *et al.* (2005) found that the fatty acid composition of cheeses produced from the milk of sheep on a diet of Mediterranean forages did not differ after 1 and 60 days ripening, while Shanta *et al.* (1995) reported that the total CLA and rumenic acid concentration of Mozzarella, Gouda, and cheddar cheeses did not change over 32 weeks at 4^{o}C. Both observations suggest inactivity by the culture used in terms of CLA formation.

A number of studies have commented on the influence that the manufacturing conditions employed during the production of cheese can have on its rumenic acid and CLA content. Gnadig *et al.* (2004) reported that neither the type of milk used (raw or thermised milk) nor the cooking process had an effect on the CLA content of cheese. However, the use of low and high lipolytic *Propionibacterium* strains did cause a small elevation in the CLA content of the cheese from 9.5 mg/g fat in the control to 9.9 mg/g fat and 10 mg/g fat for the low and high lipolytic strains, respectively. Previously Garcia-Lopez *et al.* (1994) reported an increase in the total CLA content of cheese following the application of heat during processing. This observation supports an earlier study where it was found that the use of elevated temperatures (80^{o}C) during the manufacture of processed cheese could also increase the concentration of CLA present (Kanner *et al.*, 1987).

While some studies demonstrate the positive influence of processing on CLA and rumenic acid production a number of studies also suggest this is not the case. The effect of manufacturing on the CLA content of processed cheese was examined at four points of manufacture, in the raw material, following cooking, following creaming and in the final product (Luna *et al.*, 2005). In this study only negligible changes in the CLA and linoleic acid concentration of the cheese were

observed throughout manufacture. A similar finding was also made by Jiang *et al.* (1997) who investigated the effect of manufacturing conditions on the production of the hard cheeses, Grevé and Herragårdsost, at various time points during manufacture and ripening. In this study it was found that the CLA concentration remained relatively unchanged in both cheeses. These studies suggest that neither the manufacturing nor ripening of cheese influence the CLA concentration and that in such products the starter or adjunct cultures do not produce substantial amounts of CLA during ripening or storage.

1.2.7 Assessing the safety of CLA enriched foods on human health

While a plethora of data report the health promoting activities of rumenic acid in both animal and *in vitro* studies, reports of a number of negative health effects attributed to the *t*10, *c*12 CLA isomer (Larsen *et al.*, 2003; Pariza, 2004; Wahle *et al.*, 2004) and the technical classification of rumenic acid as a *trans* fatty acid have raised a number of questions as to the safety of consuming rumenic acid enriched foods. Scimeca (1998) assessed the effect of the dietary intake of CLA in Fisher 344 rats receiving either a basal diet or a diet supplemented with 1.5% (dDM) of a CLA mix (42.5% rumenic acid, and 43% *t*10, *c*12 CLA) for 36 weeks, with weekly assessment of food intake, and body weight along with post mortem analysis of 15 organs from 10 random animals from both the test and control group. The study showed that the dietary intake of CLA did not have any toxicological effects on these rats during the trial period. The long term effects of feeding a CLA mix (1.0% dDM of 41.9% rumenic acid, and 43.5% *t*10, *c*12) to Fisher 344 rats was studied by Park *et al.* (2005), who found that rats fed the CLA mix had a lower food intake but no differences in the percentage fat and tissue weight of the CLA fed animal were found. Interestingly, CLA feeding did result in significant reductions in blood

glucose levels, mean corpuscular volume and cholesterol. During the study, animals from both groups developed chronic renal disease, a condition attributed to the high protein content of the feed and characterized by increases in urinary protein concentrations. Interestingly it was observed that dietary CLA intake reduced the amount of protein in the animal's urine suggesting that it may reduce the severity of renal failure. Using Clarinol G80, a product containing a 50:50 mix of the two main CLA isomers, O'Hagan & Menzel, (2003) conducted a 90 day toxicological feeding study in Wistar out bred [Crl:(WI)WU BR] rats. In addition to the toxicological study, the effects of exposure to Clarinol G80 on bacterial mutation and on chromosome aberration in human peripheral blood lymphocytes was examined. The results showed Clarinol G80 to be non-mutagenic and that at a concentration of 5%, not to cause any adverse health effects. However, at the highest dose level (15% w/w) Clarinol G80 was found to initiate hepatocellular hypertrophy, an effect that was reversible upon withdrawal of test material. Whigham *et al.* (2004) assessed the safety of the dietary intake of 6 g/d of Clarinol by obese humans over a 12 month period. The study found that the dietary intake of CLA did not negatively affect serum glucose concentrations, insulin resistance or alkaline phosphatase activity in the participants. Furthermore, the CLA group also reported significantly lower frequencies of skin rash, depression, irritability, hair loss, and infection compared to the control group. Based on these observations, it would appear that the use of CLA in the form of Clarinol in the treatment of obesity for up to one year is safe.

The effect of the dietary intake of a high CLA butter on cholesterol and lipoprotein metabolism compared to a control butter or partially hydrogenated vegetable oil (PHVO) has recently been reported using Golden Syrian Hamsters (Lock *et al.*, 2005b). The high-CLA butter was produced using milk enriched in

CLA and vaccenic acid. This butter was produced through supplementation of the bovine diet with sunflower oil resulting in a product that contained 15.36 g of vaccenic acid per 100g of fat and 3.61g rumenic acid per 100g fat. The hamsters were fed a basal diet supplemented with 0.2% crystalline cholesterol and either 20% fat derived from a control butter, from the high-CLA butter or from PHVO. It was shown that the group fed the high CLA butter had a lower very-low density lipoprotein (VLDL) cholesterol and a reduced ratio of intermediate density lipoprotein (IDL) and LDL to HDL, compared with the control or PHVO groups. A recent report assessed the effect that ingesting butter containing elevated levels of vaccenic acid (3.1g/100g fat) and rumenic acid (1.3g/100g fat) would have in humans compared to a control butter (vaccenic acid 0.4 g/100g fat, rumenic acid CLA 0.3g/100g fat) (Tholstrup *et al.*, 2006). Subjects consumed 115 g/d of the CLA and vaccenic acid enriched butter each day for five weeks. Ingestion of butter with elevated CLA and vaccenic acid did not significantly affect body weight, serum insulin, serum glucose, or inflammatory response, oxidative stress, or haemostatic risk factors. The diet containing elevated CLA and vaccenic acid did however result in a lower total and HDL cholesterol level, which the authors attributed to the greater concentration of monounsaturated fatty acids and lower concentration of saturated fatty acids in the test butter compared to the control. A study by Tricon *et al.* (2006) assessed the effects of ingestion of CLA enriched butter, milk and cheese over six weeks on body weight, blood lipid profile, inflammatory response, serum insulin, serum glucose and the ratio of LDL to HDL cholesterol. During the trial 32 healthy male participants consumed a control diet containing 0.151 g/d CLA and a test diet containing 1.421 g/d CLA delivered in the form of cheese, butter and milk naturally enriched in CLA. The study demonstrated that the high CLA and vaccenic acid diet did not significantly affect body weight,

inflammatory markers, serum glucose and insulin concentrations, triacylglycerols, or the ratio of total LDL to HDL cholesterol. The diet did, however, result in a minor increase in the ratio of LDL to HDL cholesterol and small changes in the fatty acid composition of LDL cholesterol. Overall, the study concluded that the consumption of dairy products naturally enriched in CLA did not have a significant effect on blood lipid profile or pose any increase in cardiovascular disease risk variables. Raff *et al.* (2006) assessed the effect of the dietary intake of 115 g/d fat derived from consumption of milk containing elevated vaccenic acid (23.4 g/100g fat) and rumenic acid (1.3 g/100g fat) by healthy young men with a BMI of 22.5 kg m^2. During the study the authors measured blood pressure and arterial elasticity and found that following consumption of the CLA and vaccenic acid enriched milk no differences in systolic and diastolic blood pressure, pulse pressure, isobaric arterial compliance, distensibility, or volume could be found. These observations led the authors to conclude that the dietary intake of CLA or vaccenic acid through consumption of CLA and vaccenic acid enriched milk has little or no impact on blood pressure or arterial elasticity indices in healthy young men compared with a control diet.

1.2.8 Conclusion

Research into the health promoting activity of CLA and rumenic acid is vast and suggests that these fatty acids may positively impact some of the major conditions affecting human health. Although naturally present in the milk of ruminants through the selection of appropriate animal feeding and management strategies the yield of CLA in ruminant milk can be substantially increased. This CLA enriched milk can subsequently be utilised to produce a range of health promoting CLA enriched dairy products. In addition to our ability to manipulate the CLA content of ruminant milk through animal feeding and management strategies, CLA producing dairy cultures may also be employed to increase the CLA content of fermented dairy products from free linoleic acid. Furthermore, the identification of CLA producing bacteria from the human gastro-intestinal tract is suggestive of the ability of humans to produce health promoting CLA *in vivo* from dietary linoleic acid.

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