Biological Production of CLA and Investigation of Anticarcinogenic Activity

M Sc Thesis

by

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June 2003

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science is entirely my own work and has not been taken from the work of others and to the extent that such work has been cited and acknowledged within the text of my work.

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ABSTRACT

This study investigated a potential mechanism of action underlying the anticancer activity of conjugated linoleic acid (CLA), referring to a mixture of positional and geometric conjugated isomers of linoleic acid *Ras*, an oncogene necessary for normal cell function, when mutated results in uncontrolled cell proliferation and consequently tumour formation Western blot analysis suggested that *c*9, *t*11 and *t*10, *c*12 CLA isomers inhibit (P<0 05) expression and activation of mutated ras compared with ethanol control in SW480 cells, a human colon cancer cell line Linoleic acid stimulated cell growth, however it reduced ras expression, suggesting that events downstream of ras may be more critical targets for modulating growth by these fatty acids A second objective was to investigate the cytotoxic effect of CLA-rich oils produced by the fermentation of linoleic acid by selected CLA-producing strains Linoleic acid-rich oil fermented by *B breve* NCFB 2258 reduced growth of SW480 cells in a time dependant manner at a concentration of 84 μg fat/ml This oil contained 20 μg c9, *t*11 CLA/ml When pure fatty acids were combined to mimic the fatty acid profiles of the microbially fermented oils, the growth inhibitory effect observed was only partly attributable to the CLA content of the oil This study also examined a nutritional approach to the enrichment of CLA in milk fat by dietary supplementation of pre-selected cows on pasture with full fat rapeseeds Surprisingly, there was no significant effect on milk fat *c*9, *t*11 CLA due to rapeseed supplementation In addition, elaidic acid, a fatty acid not previously demonstrated to be influenced by rapeseed supplementation, increased significantly in the milk of cows on the supplemented diet These unexpected results may be attributed to excessive rainfall throughout the period of the trial affecting pasture quality and possibly an altered biohydrogenation process

PUBLICATIONS

Publications

Stanton, C , Murphy, J , **McGrath, E** and Devery, R (2003) Animal feeding strategies for conjugated linoleic acid enrichment of milk In Advances in Conjugated Linoleic Acid Research, Vol 2, AOCS Press (in press)

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McGrath, E., Stanton, C and Devery, R (2002) Conjugated linoleic acid isomers inhibit ras expression in the SW480 colon cancer cell line Irish J Agric Food Res. 41 144 (Abstract)

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Oral Communication

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ABBREVIATIONS

AA	Arachidonic acid
Ag^+ HPLC	Silver ion high performance liquid chromatography
BHB	β-hydroxybutyrate
CLA	Conjugated linoleic acid
DHA	Docosahexaenoic acid
DM	Dry matter
DMEM	Dulbecco's Modified Essential Media
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FAMEs	Fatty acid methyl esters
FFR	Full fat rapeseed
FID	Flame ionization detector
FPP	Farnesyl pyrophosphate
GAPs	GTPase activating proteins
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectroscopy
GDP	Guanine diphosphate
GEFs	Guanine nucleotide exchange factors
Grb2	Growth factor receptor bound protein 2
GTP	Guanine triphosphate
GTPase	Guanine triphosphatase
HMG-CoA	Hydroxymethylglutaryl CoA
HRP	Horse radish peroxidase
LA	Linoleic acid

MAPK	Mitogen activated protein kinase
MEK	Mitogen extracellular signal regulated kinase
NEFA	Non-esterified fatty acids
NS	Not significant
OA	Oleic acid
ODC	Ornithine decarboxylase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween-20
PC	Phosphatidylcholine
PFTase	Protein farnesyltransferase
PI3K	Phosphatidylinositol-3-kinase
PPAR	Peroxisome proliferator activated receptor
PUFAs	Polyunsaturated fatty acids
S_H2	Src homology 2
SOS	Son of sevenless
Syn Rep	Synthetic representation
TEBs	Terminal end buds
TFA	Trans fatty acid
TMG	Tetramethylguanidine
VA	Vaccenic acid

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CHAPTER 1

INTRODUCTION

1.1 Conjugated Linoleic Acid (CLA)

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid, C18:2 (LA).

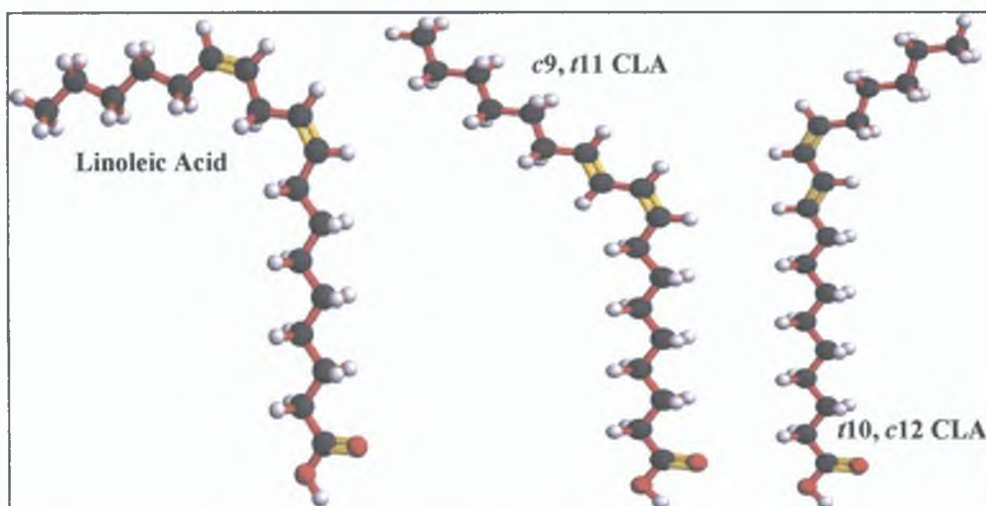

Figure 1.1 Structure of linoleic acid (LA), *c*9, *t*11 CLA and *t*10, *c*12 CLA

Linoleic acid has double bonds at positions 9 and 12 (**Figure 1.1**). CLA differs from LA in that the double bonds are conjugated and not separated by a methylene group. Therefore, there are a number of possible positions for the double bond i.e. C positions 8 and 10, 9 and 11, 10 and 12 and 11 and 13. **Table 1.1** presents the primary sources of CLA in the human diet. CLA is found in the lipid fraction of milk, dairy products and the meat of ruminants (Lin, *et al.*, 1995). Dairy products are the major source of CLA with the *c*9, *t*11 CLA isomer accounting for 80-90% of total CLA in dairy products (Chin, *et al.*, 1992).

Data regarding the dietary intake of CLA in humans is limited. However, it has been estimated that human intake of CLA is below the levels required for optimal physiological benefits (McGuire, *et al.*, 1999; Santora, *et al.*, 2000; Ritzenthaler, *et al.*, 1998).

Table 1 1 Dietary sources of CLA (from Roche, *et al*, 2001)

Source	CLA Content (g/100g fat)
Whole milk	0 55
Low fat milk (2%)	0 41
Condensed milk	0 70
Butter	0 47
Plain yoghurt	0 48
Low fat yoghurt	0 44
Frozen yoghurt	0 28
Cheddar cheese	0 41
Processed cheese	0 50
Beef (uncooked)	0 43
Lamb (uncooked)	0 58
Veal (uncooked)	0 27

Consumption of diets rich in CLA has been shown to enrich blood and tissue CLA in humans (Huang, *et al*, 1994, Jiang, *et al*, 1999) In addition, humans have been shown to be capable of synthesising CLA in the body (Salminen, *et al*, 1998) Fritsche and Steinhart, (1998) reported a daily intake of 0 5 to 1 5 g of CLA in the human diet However, a Swedish study reported an average daily CLA intake of 0 16 g (Jiang, *et al*, 1999) This study also suggested that CLA intake today is lower than that in the 1960s, most probably due to the reduced consumption of animal fat in the diet compared to the 1960s

1.2 Analysis of CLA

Trans fatty acids (TFAs) can be analysed using varying techniques such as infrared spectroscopy (IR), gas chromatography (GC), silver-ion (Ag^+) chromatography, gas chromatograpgy-*Fourier* transform infrared spectrospectory (GC-FTIR) and gas chromatography-mass spectroscopy (GC-MS) (reviewed in Fritsche and Steinhart, 1998)

In the analysis of CLA it is important that the different isomers are separated This is typically done by GC Unlike industrially synthesised CLA, naturally produced CLA is present at very low levels and in esterified form in foodstuffs making analysis of natural CLA more difficult

GC separates components of a volatile sample To use GC, a sample must be in a volatile state Therefore, lipids must undergo trans-esterification to form fatty acid methyl esters (FAMEs) To produce FAMEs, acid- or base-catalysed methylation of the sample must first be carried out CLA is present as both free and esterified fatty acids in foodstuffs In addition, the quantity and type of isomers present varies from one food to another Acid catalysed methylation is used to prepare both free fatty acids and esterified fatty acids for GC analysis Typical catalysts for this reaction are H_2SO_4/methanol, anhydrous HCl/methanol or BF_3/methanol Earlier forms of this method led to the formation of excessive amounts of *trans/trans* CLA isomers and/or allylmethoxy derivatives (AMD) (Yurawecz, *et al*, 1999) This problem was overcome by reducing the temperature and methylation time to 60°C for 20 minutes using 4% methanolic HCl or 14% BF_3/methanol at room temperature for a longer time period (Kramer, *et al*, 1997, Yurawecz, *et al*, 1999) However, any CLA esterified in triglyceride or phosopholipid may not undergo full methylation Using compounds such as tetramethylguanidine (TMG) or $NaOCH_3$ (Shantha, *et al*, 1993) as catalysts to methylate esterified CLA can overcome this problem However, these catalysts are not suitable for free CLA Base catalysed methylation derivatises esterified fatty acids only One of the most popular catalysts for this reaction is 0 5N sodium methoxide in anhydrous methanol, although in some cases potassium methoxide or hydroxide can be used in place of sodium methoxide (reviewed in Christie, 2001)

Park, *et al* (2002) studied the optimum methylation conditions required by a number of CLA samples (**Table 1 2**)

Table 1.2 Example of optimum methylation conditions (Park, *et al* , 2002)

Sample	Methylation Agent	Temperature (°C)	Time (mins)
Free CLA	1 0N H_2SO_4	55	5
CLA esterified in safflower oil	20% TMG	100	5
CLA esterified in phospholipid	20% TMG	100	10
	AND		
	1 0N H_2SO_4	55	5
CLA esterified in egg yolk lipid	Base hydrolysis		
	AND		
	1 0N H_2SO_4	55	5

In general Carbowax capillary columns are used in GC analysis of fatty acids Long retention times are associated with GC analysis of CLA as long columns are required for adequate separation Despite the main CLA isomers (*c*9, *t*11 and *t*10, *c*12) being well separated on this type of column, they are not separated from other positional CLA isomers For example, *c*9, *t*11 and *c*8, *t*10 CLA overlap The isomers elute in the order *cis/trans*, *cis/cis*, and *trans/trans* (Christie, 2001) However, it is still possible to quantify total CLA content

In foodstuffs other fatty acids such as C21 0 and C20 2 elute in the same region as the *cis/cis*, and *trans/trans* CLA isomers In such instances it is beneficial to use GC-MS for analysis The advantage of this system is that it is possible to identify the presence of non-conjugated double bonds and to locate the double bonds

Thin layer chromatography (TLC) poses the problem of elution of conjugated dienes with monoenes Therefore, it is more beneficial to use silver ion high performance liquid chromatography (Ag^+ HPLC) A method devised by Adlof and Lamm (1998) has since been improved upon (reviewed in Yurawecz, *et al*, 1999) In this instance hexane is used as the mobile phase containing a small quantity of acetonitrile, with an ultraviolet detector at 234 nm Remarkable separation can be achieved by using six columns in series This method can also be used to analyse free CLA without the need to methylate prior to analysis (Cross, *et al*, 2000)

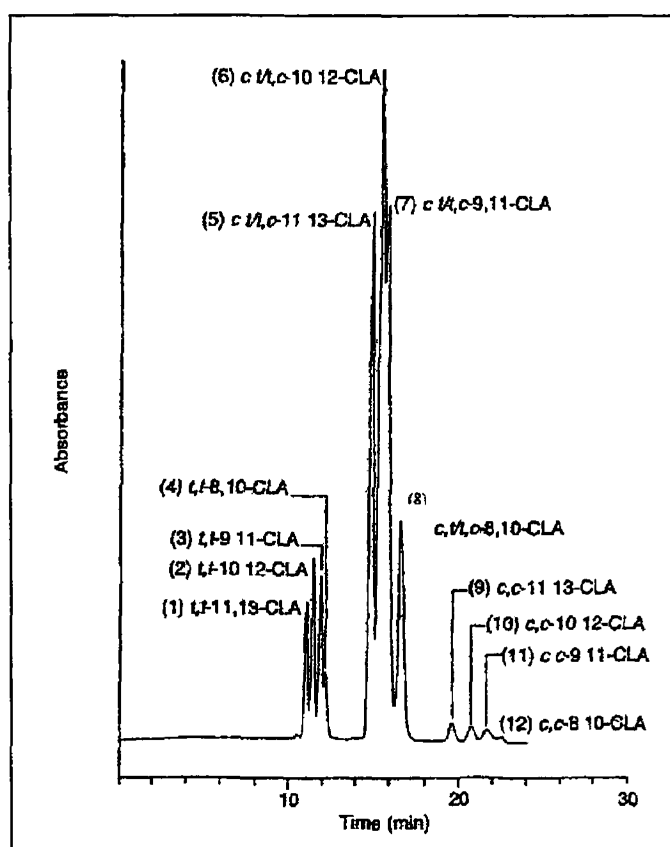

Figure 1 2 Separation of CLA isomers using a single Ag^+ HPLC column (Sehat, *et al*, 1998)

Figure 1.2 shows the separation of CLA isomers achieved by using a single Ag^+ HPLC column whereas **Figure 1 3** shows the separation of CLA isomers achieved with Ag^+ HPLC columns in series

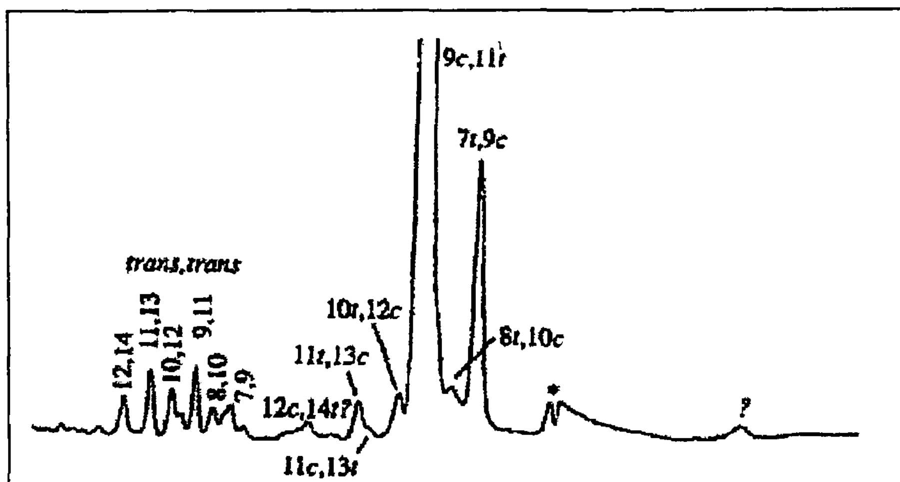

Figure 1 3 Separation of CLA isomers using Ag^+ columns in series (Kramer, *et al*, 1999)

With Ag^+ chromatography, it is also possible to analyse CLA in the form of *p*-methoxyphenacyl esters using a mobile phase which consists of dichloromethane/hexane/acetonitrile (Nikolova-Damyanova, *et al*, 2000) All fatty acids present can be detected via the *p*-methoxyphenacyl moiety

As CLA is often present in foodstuffs at very low concentrations it is sometimes necessary to concentrate the CLA prior to quantification This is usually carried out via reverse-phase chromatography or Ag^+ chromatography, although better results are achieved when these methods are used in series Reverse-phase chromatography can be used to obtain a C18 fraction This fraction is then applied to the reverse-phase Ag^+ HPLC column resulting in a fraction enriched in CLA (reviewed in Christie, 2001)

As individual CLA isomers have been associated with different health benefits, accurate quantification is necessary It is clear that CLA is sensitive to methylation conditions, stressing the importance of the optimum conditions of analysis

1 3 Health Benefits Associated with CLA

A variety of health benefits have been attributed to CLA However, the majority of these health benefits have been observed in animal models Therefore, it is not certain if CLA would benefit humans in the same way or to the same extent CLA has been associated with a number of health benefits including an anti-carcinogenic effect, an anti-atherosclerotic effect, improvements in bone metabolism, an anti-thromobotic effect, a reduction in body fat, enhancement of immune function and improvements in diabetes status (reviewed in Belury, 2002) This study focuses on the anticarcinogenic effects of CLA and this particular health benefit is discussed in detail below

1 4 Effect of CLA on Carcinogenesis

The anticancer effect of CLA is probably the most studied health benefit associated with the fatty acid During a study of the formation of mutagenic compounds during cooking it was discovered that fried ground beef possessed anticarcinogenic properties (Pariza *et al* , 1979) In 1983, Pariza, *et al* isolated the component responsible for the antimutagenic effect and named it a mutagenesis modulator It was in 1987 that this compound was identified as a mixture of four CLA isomers – *c*9,*t*11, *t*9,*t*11, *t*10,*c*12 and *t*10,*t*12 CLA (Ha, *et al* , 1987) Compared with LA, which has been implicated with the incidence of prostate and colon carcinogenesis in humans (Erickson, 1998, Zock and Katan, 1998), it has now been demonstrated that CLA has an ability to antagonise mammary cancer (Ip, *et al* , 1991, Durgam and Fernandes, 1997, Cunningham, *et al* , 1997, Ip and Scimeca, 1997), stomach cancer (Ha, *et al* , 1990), skin cancer (Belury, *et al* , 1996) and prostate cancer (Cesano, *et al* , 1998) It has been demonstrated that CLA can inhibit cancer initiation (Ha, *et al* , 1987, Liew, *et al* , 1995), promotion (Ip, *et al* , 1994a, 1996) and, progression and metastasis (Cesano, *et*

al , 1998) Visonneau, *et al* (1997) attributed an anti-mutagenic effect when the spread of cancer cells to lungs, peripheral blood and bone marrow in SCID (severe combined immuno-deficiency) mice was prevented The ability of CLA to inhibit angiogenesis may contribute to its efficiency as an anti-metastatic agent (Masso-Welch, *et al* , 2002)

Data suggest that there is a concentration at which CLA optimally inhibits carcinogenesis In one particular study it was observed that a concentration of 1% CLA reduced the incidence of mammary tumours, however increasing the concentration did not further reduce tumour incidence (Ip, *et al* , 1991) It has been demonstrated that better results are obtained when CLA is administered prior to tumour initiation (Ip, *et al* , 1995)

It is of interest that the majority of the studies regarding the anticarcinogenic activity of CLA have involved the use of free CLA isomers Ip, *et al* (1999) found that butter rich in CLA and free CLA were effective in reducing terminal end buds (TEBs) to a similar extent TEBs are the primary site for mammary carcinogenesis induction in both rat and human mammary cancer Ip, *et al* (2001) demonstrated that *c*9, *t*11 CLA in a triglyceride was as effective as free *c*9, *t*11 CLA in controlling rat mammary epithelium proliferation The anticancer activity of CLA has been demonstrated despite the type or level of dietary fat present (Ip, *et al* , 1996, Ip and Scimeca, 1997) Ip, *et al* (1996) compared the anticancer effect of 1% CLA in a diet rich in corn oil compared to the same treatment in a diet rich in lard Although the corn oil and lard differed greatly in their linoleate content, the anticancer effect of CLA was independent of the type of fat present Linoleate is a precursor of arachadonic acid (AA) and in turn

eicosanoids A positive relationship between derivatives of eicosanoids and cell proliferation and inflammation in mammary, colon and skin tumours has been reported (Belury, 1995) Unlike CLA, LA has been shown to stimulate the development of tumours (Cesano, *et al* , 1998, Ip, *et al* , 1985)

Differences in species are of great importance when extrapolating rodent data to humans For example, CLA has been linked with an increase in the development of hepatic cancer in a specific mouse model due to an ability to enhance peroxisome proliferation (Belury, *et al* , 1997, Houseknecht, *et al* , 1998) Enhanced peroxisome proliferation has been associated with development of hepatocarcinogenesis (Kraupp, *et al* , 1990) However, CLA did not induce hepatic peroxisome proliferation in Sprague-Dawley rats (Moya-Camarena, *et al* , 1999a)

Data relating to the effect of CLA in humans is limited, but one of the more promising results comes from a study which demonstrated that there was an inverse relationship between the level of CLA in the mammary tissue and the risk of breast cancer (Bougnoux, *et al* , 1999) Both dietary CLA and serum CLA were reported to be lower in Finnish breast cancer patients than in control subjects (Aro, *et al* , 2000) However, a recent epidemiological study showed no relationship between breast cancer risk and CLA intake (Voorrips, *et al* , 2002) while CLA content in breast adipose tissue in a population of French patients was not associated with relative risk of breast cancer (Chajes, *et al* , 2002) It is therefore not yet certain if the suggested anticarcinogenic property of CLA in animal and tissue culture models can benefit humans

1 5 Anticancer Mechanisms of CLA

Despite the abundance of evidence suggesting that CLA inhibits tumour progression, its mechanism of action is not so clearly established Due to the differential effects of CLA isomers it is possible that anticarcinogenic activity may be the result of a number of biochemical mechanisms It may be possible that the combined effect of different CLA isomers leads to the anticarcinogenic property of CLA, however individual isomers have been shown to possess anticarcinogenic ability (O'Shea, 2000) Schønberg and Krokan (1995) demonstrated that the anticancer affect of CLA was specific to a number of cell lines Therefore, it is clear that differing experimental conditions regarding the CLA isomers analysed or experimental model used can lead to varying results

Initially it was believed that the anticarcinogenic activity of CLA was due to an antioxidant property Studies by Ha, *et al* (1990), Ip, *et al* (1991, 1996) and Pariza, *et al* (1991) have all suggested this However this evidence is not explicit Such studies have involved experimental conditions which do not replicate biological systems (Ha, *et al* , 1990) or used measurements of oxidative damage which are indirect and not specific (Ip, *et al* , 1991) In contrast, a study by van den Berg, *et al* , (1995) demonstrated that CLA was not an effective antioxidant, having only a slight effect after 1 hr of oxidative stress In addition, CLA was found to interfere with the metabolism of α-tocopherol (a potent antioxidant) (Nicolosi, *et al* , 1997) CLA has been shown to induce lipid peroxidation in the MCF-7 human breast cancer cell line (O'Shea, *et al* , 1999) The data suggested that accumulation of lipid peroxidation products such as conjugated diene hydroperoxides may lead to internal cellular pro-oxidation However, Wong, *et al* (1997) found that CLA did not increase lipid

peroxidation in mice with transplanted metastatic murine mammary tumours The difference between these two studies may be due to species differences Elevated levels of 8-iso-PGF$_{2\alpha}$ (a biomarker of non-enzymatic lipid peroxidation) and 15-keto-dihydro-PGF$_{2\alpha}$ (a biomarker of enzymatic lipid peroxidation) in the urine and plasma of humans after administration of CLA have been demonstrated by Basu, *et al* (2000) Similar results were observed by Miller, *et al* (2001) in human breast and colon cancer cell lines

Another possibility is the interference of CLA in the pro-oxidant/antioxidant balance via interference with the expression of antioxidant defense enzymes This was demonstrated in studies by O'Shea, *et al* (1999) and O'Shea, *et al* (2000), in which the activity of three such enzymes was increased after CLA treatment The presence of CLA may lead to enhanced oxidative stress due to its conjugated double bonds (Belury, 1995) resulting in increased levels of these enzymes It is widely accepted that peroxides and superoxides generated from lipid peroxidation damage DNA and other cellular functions in pathological conditions Hawkins, *et al*, (1998) demonstrated that the incidence of apoptotic cells in leukaemia and pancreatic cells treated with polyunsaturated fatty acids (PUFAs) was correlated with the degree of lipid peroxidation induced by PUFAs and the number of double bonds present in the PUFAs Further analysis involving vitamin E, an antioxidant, demonstrated that apoptosis induced by PUFAs was oxidative (Tyurina, *et al*, 2000)

Prostaglandins are derived from AA metabolism and fall under the category of eicosanoids along with leukotrienes and thromboxanes **Figure 1 4** shows the pathway involved in the synthesis of eicosanoids and the implications involved with the

presence of prostaglandins in the body. Li and Watkins (1998) have shown that CLA affects prostaglandin synthesis.

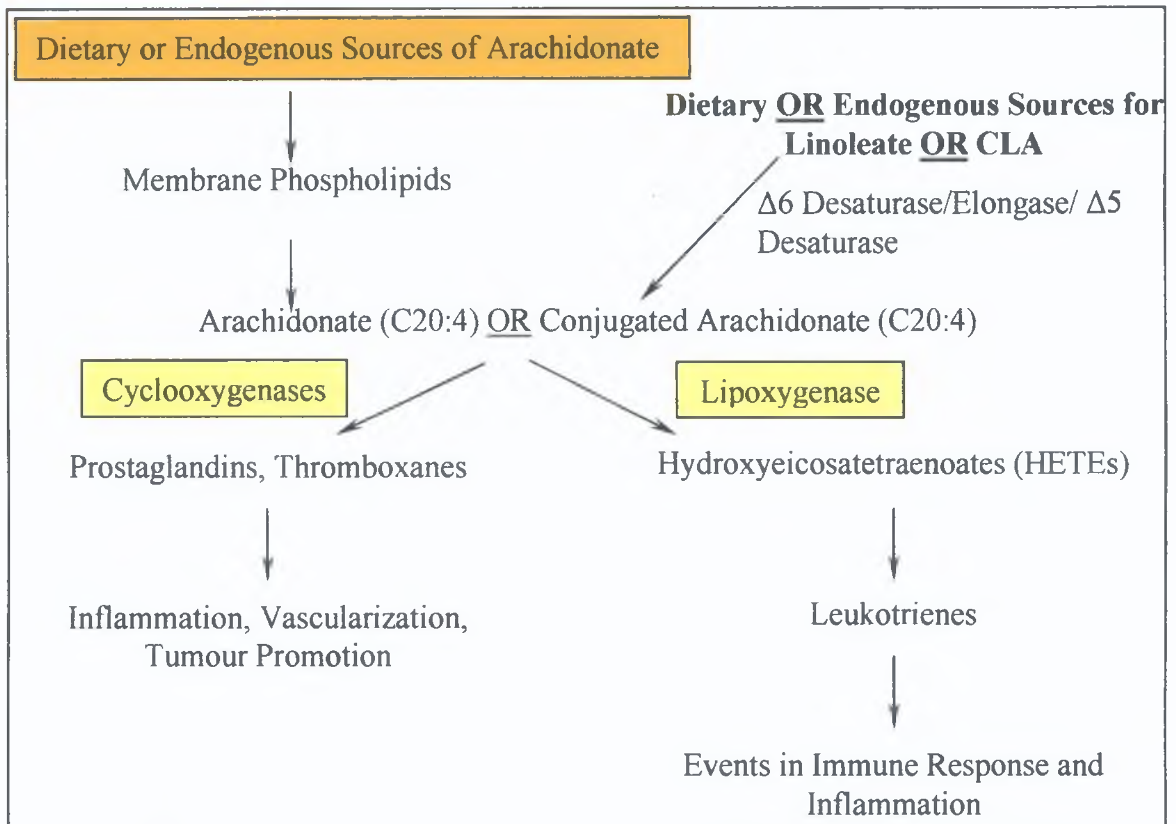

Figure 1.4 Pathway of eicosanoid synthesis from arachidonate (taken from Belury, 2002)

CLA also interferes with prostaglandin metabolism (Liew, *et al.*, 1995; Pariza, *et al.*, 1999) and has been shown to reduce AA production in mice (Banni, *et al.*, 1999; Belury and Kempa-Steczko, 1997). CLA was found to displace AA and LA in membrane phospholipids (Belury and Kempo-Steczko, 1997; Liu and Belury, 1998; Banni, *et al.*, 1999; O'Shea, *et al.*, 2000); therefore it may be possible that this displacement due to CLA may interfere with eicosanoid synthesis and other signalling pathways. Prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and 5-hydroxy-eicosatetraenoic acid have been associated with the stimulation of both prostate and colon tumour proliferation (Qiao, *et al.*, 1995; Bortuzo, *et al.*, 1996; Ghosh and Myers, 1998). AA is formed due to the desaturation of LA. It has been suggested that if CLA competes for desaturation with LA the result would be a reduction in the synthesis of

eicosanoids and in turn a reduction in eicosanoid stimulation of cancer cell proliferation (Belury, 1995, Parodi, 1997, Liu and Belury, 1998, Banni, *et al* , 1999) A study by Miller, *et al* (2001) demonstrated that the *c*9, *t*11 CLA isomer reduced uptake of AA into phosphotidylcholine (PC) in MCF-7 breast cancer cells PC is the phospholipid which is preferentially hydrolysed to synthesise eicosanoids (Hanel, *et al* , 1993)

It has been suggested that CLA may act as a ligand for nuclear hormone receptors, peroxisome proliferator-activated receptors (PPARs) (Kliewer, *et al* , 1997, Moya-Camarena, *et al* , 1999b, Vanden Heuvel, 1999) PPARs are responsible for activation of genes involved in the regulation of lipid and carbohydrate metabolism PPAR-α and γ are known to be expressed in cancer cells and CLA has been found to be a ligand for these two isoforms of PPARs (Kliewer, *et al* , 1997, Moya-Camarena, *et al* , 1999b) Activation of PPAR-γ in human prostate and colon cells results in growth arrest and the induction of cellular differentiation (Sarraf, *et al* , 1998, Kitamura, *et al* , 1999, Mueller, *et al* , 2000) Despite both *c*9, *t*11 and *t*10, *c*12 CLA possessing an ability to activate PPAR-α (Moya-Camarena, *et al* , 1999b), this isoform does not induce differentiation in human colon cancer (Sarraf, *et al* , 1998)

Apoptosis (programmed cell death) due to oxidative stress has been shown to involve the oxidation of membrane phospholipids as part of a final common pathway (Fabisiak, *et al* , 1998) Palombo, *et al* (2002) reported an increase in caspase activity due to treatment of MIP-101 (colorectal cancer) and PC-3 (prostate cancer) cells with 100 μM *t*10, *c*12 CLA, indicative of the induction of apoptosis by this CLA isomer Ip,

et al (2000) demonstrated that a mixture of CLA isomers led to induction of apoptosis m mammary cancer cells via downregulation of *bcl-2*, a membrane protein

Ip, *et al*, (2001) found that CLA suppression of mammary epithelium proliferation was partly due to a decrease in components (cyclin D1 and cyclin A), which play important roles in the cell cycle Combined with the data from Ip, *et al* (1999) a theory for CLA's anticancer activity in mammary carcinogenesis has been suggested administration of CLA during pubesence suppresses cyclm expression Reduced cyclin levels results in a decrease in proliferation of TEBs and in turn a reduction in ductal branching of the mammary tree Reduced ductal branching decreases susceptibility to mammary carcinogenesis

Another possibility is that CLA may interfere with cell signalling via inhibition of ras, an oncogene which regulates cell proliferation – however when mutated, ras can lead to uncontrolled cellular proliferation and cancer development (discussed in **Chapter 2**) Data regarding the effect of CLA on ras is limited Ip, *et al* (1997) suggested that the inhibitory effect of CLA on mammary cancer development is independent of the presence or absence of the ras gene However, O'Shea (2000) demonstrated that when incubated with human colon cancer cells, CLA inhibited the activity of protein farnesyltransferase (PFTase) – an enzyme necessary for the activation of ras The reaction between PFTase and ras leads to localisation of ras to the plasma membrane Ras must be bound to the membrane to be in its active state, therefore inhibition of membrane bound mutated ras renders it inactive and prevents undesirable cell proliferation Davidson, *et al* (1999) demonstrated that PUFAs inhibited membrane

association of colonic ras in rats without affecting farnesylation (the reaction between ras and PFTase) Singh, *et al* (1997a) found that a diet high in corn oil promoted colon carcinogenesis in male F344 rats with a concomitant increase in ras expression, whereas a diet high in fish oil reduced colon carcinogenesis, in addition to a reduction in membrane-bound ras These data suggest that it may be possible that CLA interferes with ras signalling, however much study is required in this area due to the complexities involved as suggested by the varying results discussed above and the many factors involved in ras signalling (discussed in **Chapter 2**)

1.6 Objectives of the Study

Due to the many health benefits associated with CLA the objective of this study was to investigate methods resulting in the availability of enhanced levels of CLA in the human diet The first objective of this study was to investigate one possible anticancer mechanism of CLA – interference with ras expression The second objective was to investigate the ability of two probiotic strains of Bifidobacterium to produce CLA and to analyse the anticancer activity of the CLA produced The third objective was to enhance milk fat CLA via selection of high CLA-producing cows and dietary supplementation with rapeseed

CHAPTER 2

CLA ISOMERS INHIBIT RAS EXPRESSION IN SW480 CELLS

2.1 Introduction

2.1.1 Cancer

Cancer growth can be the result of a mutation in genes, which promote cellular proliferation, or a mutation in genes which suppress cell division. Uncontrolled cell proliferation or inadequate suppression of cell division leads to formation of a tumour.

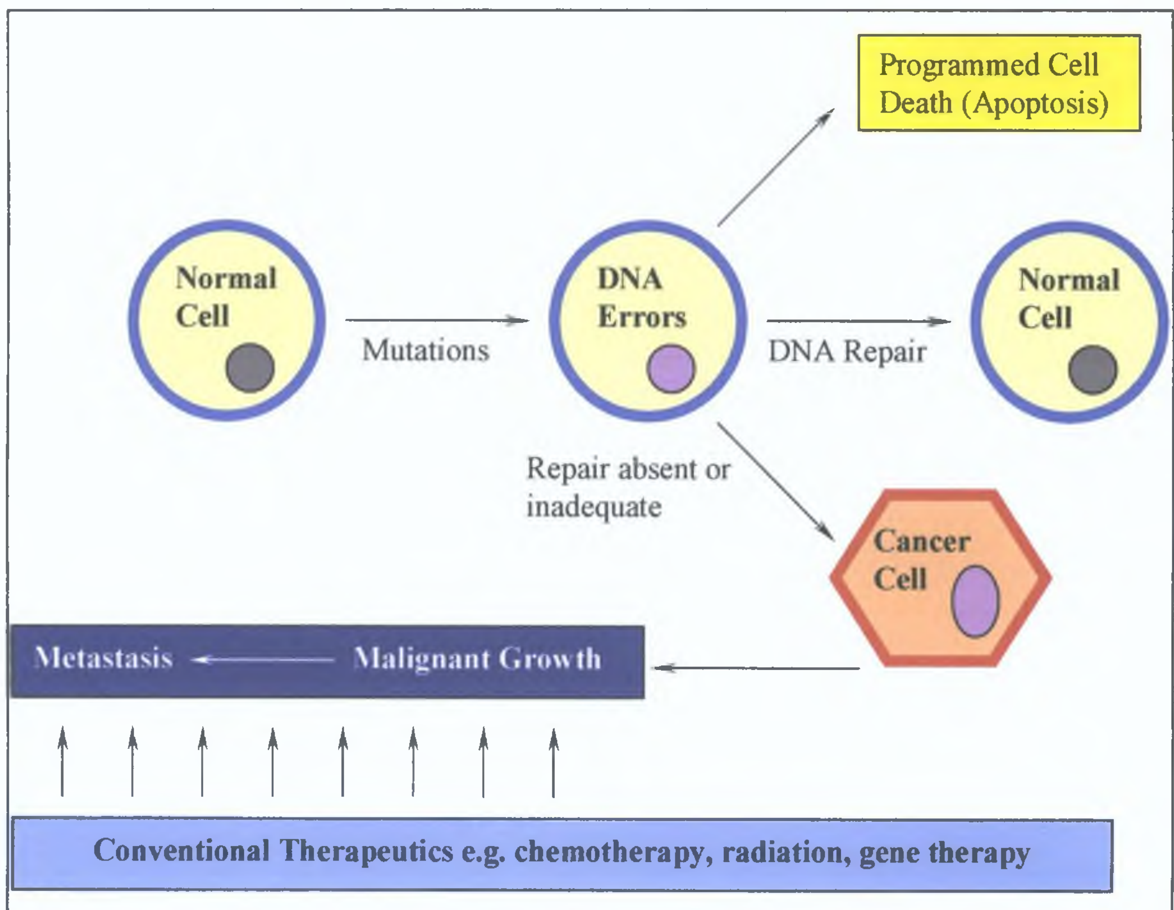

Figure 2.1 Development of cancer cells

If a mutated cell is not repaired by the body's DNA repair system or eliminated via apoptosis, a cancer cell develops.

Figure 2.1 summarises the development of cancer cells. Irreversible damage of DNA leads to the formation of a cell containing mutated DNA which can multiply to form additional mutated cells. Under normal conditions DNA repair may revert the cell back to a normal cell. Another possibility is the occurrence of apoptosis or programmed cell death.

Apoptosis is a normal cellular process that involves eliminating unwanted or useless cells Without DNA repair or apoptosis, malignant growth of the cancer cell takes place with the possibility of metastasis (spread) of the cancer to other parts of the body Research is ongoing to find new and better methods of cancer treatment At present such methods include chemotherapy, radiation and gene therapy There are three stages of cancer initiation, promotion and progression Initiation occurs due to the presence of a chemical, biological or physical agent The promotion stage is not as well understood, but involves the interaction of promoters with cell receptors Tumour progression is the result of molecular genetic mechanisms such as chromosomal rearrangement or further mutations

2 1 2 Cancer and Oncogenes

Oncogenes are genes which, when mutated, deleted, or abnormally expressed can cause growth of cancer cells When normally expressed these genes play an important role in normal physiological activities and are known as protooncogenes One example of a protooncogene is the *ras* gene Ras is the collective term for the three mammalian *ras* genes H-*ras*, K-*ras* (K-*ras*4A and K-*ras*4B) and N-*ras* This gene is necessary for the regulation of cell proliferation and differentiation as it encodes a monomeric guanine triphosphate (GTP) binding protein that is involved in signal transduction Mutations in the dominant oncogene *ras* represent the most commonly found gene mutations in human cancer cells The most common mutations of this gene occur at codons gly-12, gly-13 or gln-61 giving the gene the ability to morphologically transform established cells in culture These mutations all lead to the same defect in ras (described in **Section 2 1 5**) The ras mutation has been detected in 15% of all human tumours and is most commonly

found in tumours of the colon (50%) and pancreas (90%) (reviewed in Bos, 1989) The high frequency of this mutation in human cancers is witness to the central role played by ras-encoded proteins in membrane-to-nucleus signal transduction (Ames, *et al* , 1995)

2 1 3 Ras

Ras is an oncoprotein required for a variety of signal transduction pathways involved in cellular proliferation and differentiation More than 50 low molecular weight proteins categorised into the Ras-related superfamily act positively to stimulate a cascade of kinase-driven phosphorylation events that culminate in the activation of nuclear transcription (Ames, *et al* , 1995) Because of its central role in membrane-to-nucleus signal transduction, it has been proposed that modulation of the Ras superfamily of proteins may provide a novel means by which growth of cancer cells can be inhibited (Gibbs, *et al* , 1994)

Ras begins as a cytosolic precursor, which after post-translational modification, localises to the plasma membrane Farnesylation of ras proteins by PFTase is an essential step for their binding to the inner leaflet of plasma membranes and for activation of ras (Der and Cox, 1991) and downstream effectors leading to cell proliferation (reviewed in Adjei, 2001)

2 1 4 Post-Translational Modification of Ras

Post-translational modification involves the attachment of a farnesyl or geranylgeranyl group (**Figure 2 2**) to the carboxy terminal cysteine residue (Goldstein and Brown, 1990)

The substrate for the reaction is farnesyl pyrophosphate (FPP) from which a farnesyl group is cleaved and transferred to the carboxyl end of the target protein e.g. ras by PFTase. The enzyme is specific for a CAAX sequence where C = cysteine, A = an aliphatic amino acid e.g. leucine, isoleucine or valine and X = methionine, serine or leucine. In K-ras, X is usually methionine while in H-ras X is usually serine (reviewed in Eskens, *et al.*, 2000).

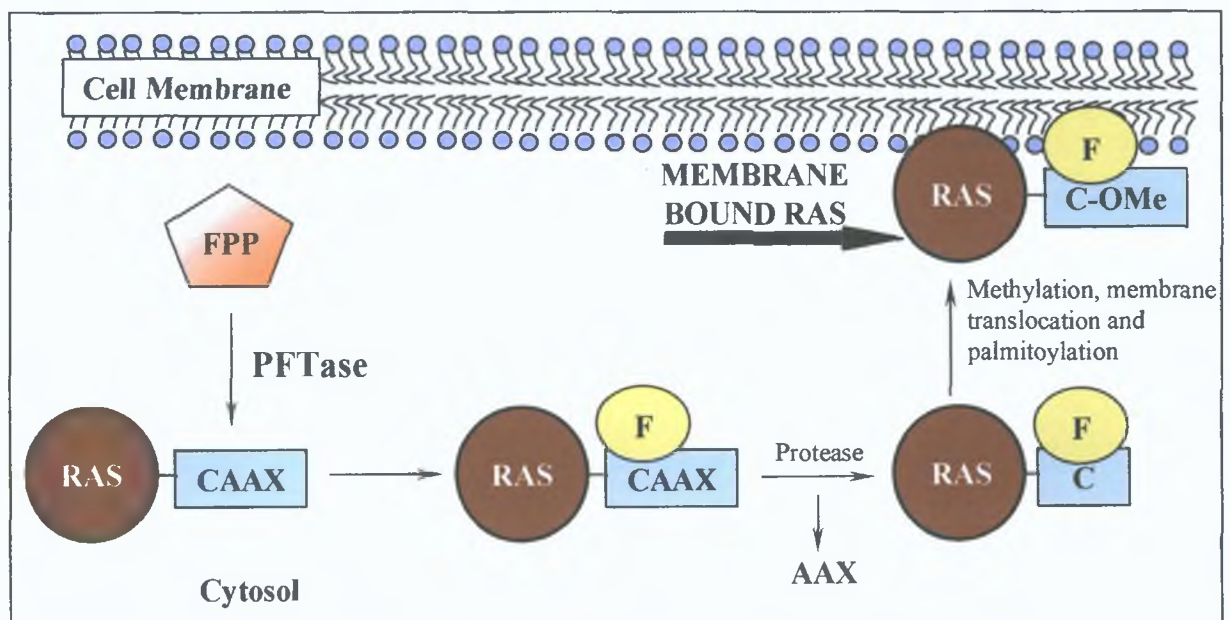

Figure 2.2 Post-translational modification of ras

FPP=farnesyl pyrophosphate, PFTase=protein farnesyl transferase, C=cysteine, A=leucine, isoleucine or valine, X=methionine (in K-ras) or serine (in H-ras), F=farnesyl group, AAX=three cleaved amino acids, C=carboxy-terminal cysteine, C-OMe=methylated cysteine

Following attachment of the farnesyl group, proteolytic cleavage of the AAX residues takes place, followed by carboxymethylation (Clarke, 1992). Due to the presence of a cysteine residue upstream from the CAAX moiety, some ras proteins (H-ras, N-ras and K-ras4A) undergo a further step in that a –SH group on the penultimate cysteine residue is palmitoylated, this reaction being reversible. K-ras4B has a lysine rich sequence which

allows it to complete its attachment to the plasma membrane (reviewed in Adjei, 2001). The ras protein may alternatively undergo geranylgeranylation. It is the amino acid in the X position which determines whether the protein undergoes farnesylation or geranylgeranylation (Casey, *et al.*, 1991; Yokoyama, *et al.*, 1991). When leucine is in the X position the protein will undergo geranylgeranylation (reviewed in Sinensky, 2000).

2.1.5 Activation of Ras

Ras is a membrane-bound GTPase that is required for connecting growth factor signaling to the intracellular phosphorylation cascade. **Figure 2.3** shows that the active form of ras has a molecule of GTP bound in the nucleotide binding site, whereas the inactive form of ras contains a guanine diphosphate (GDP) molecule in that site.

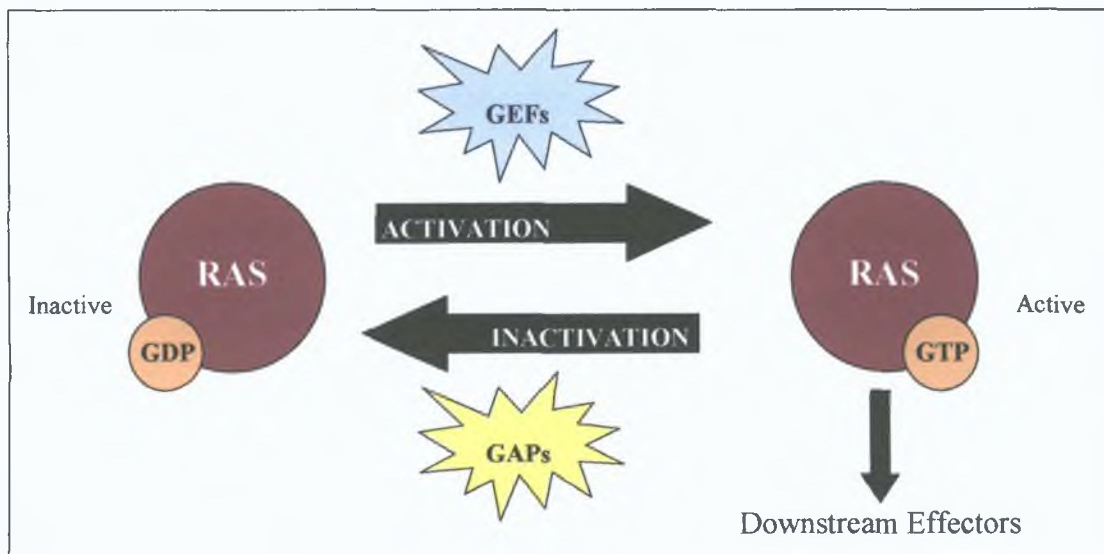

Figure 2.3 Activation of ras

Ras acts as a molecular switch, alternating between its active and inactive state as required. When activated, ras plays an important role in a number of pathways necessary for cell growth and maintenance.

The integral GTPase activity of ras therefore converts active ras-GTP into inactive ras-GDP The switch is controlled by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) The binding of GTP to ras is promoted by GEFs (Quilliam, *et al*, 1995), whereas, GTP hydrolysis and hence ras-GDP is promoted by GAPs (Bollag and McCormick, 1992) GEFs and GAPs are themselves controlled by cell surface receptors such as epidermal growth factor receptor (EGFR)

Ras is a membrane-bound GTPase As mentioned above the most prevalent dominant mutations in ras in codons 12, 13, and 61 cause a defect in the active ras-GTPase activity such that it is always in the active ras-GTP form This gives a false signal to activate growth-promoting genes and undesired cell proliferation takes place even when growth factors are not present

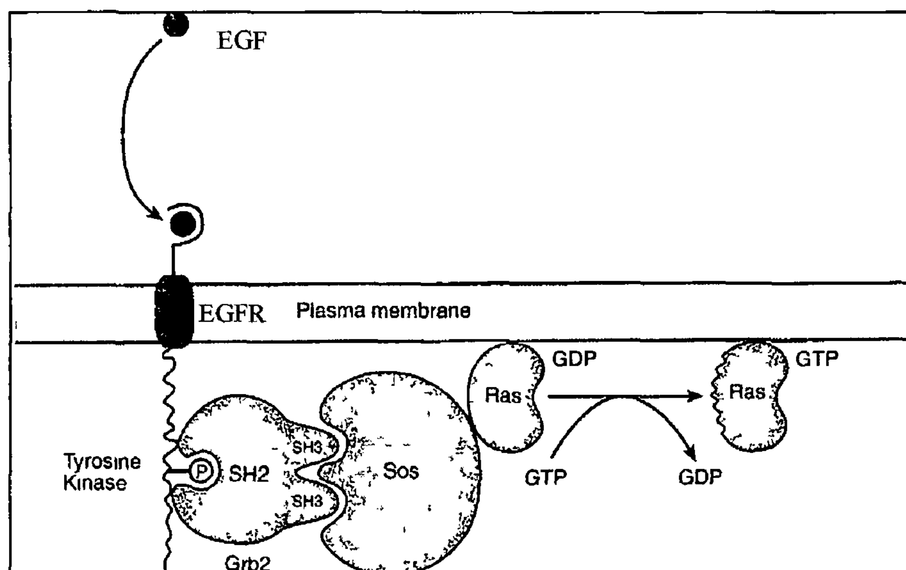

Figure 2 4 Role of adaptor proteins in ras regulation, leading to the activation of ras (taken from Cooper, 1995) For ras to become active it must become localized at the plasma membrane Adaptor proteins play an important role in this process

Growth factor receptors such as EGFR are membrane-bound tyrosine kinases which are upstream of ras in the signal transduction pathway For ras to be activated, EGF (epidermal growth factor) must bind to EGFR (**Figure 2 4**) When EGF and EGFR bind, EGFR's tyrosine-kinase activity is stimulated, the cytoplasmic tail of EGFR is autophosphorylated on tyrosine residues, the result being a S_H2 (src homology 2) protein binding site The S_H2 domain of the adaptor protein Grb2 binds to the phosphorylated EGFR tail, while the two S_H3 domains of Grb2 bind to the S_H3 binding sites on SOS (son of sevenless) which is a GEF The activation of EGFR results in Grb2 bringing SOS to the membrane to activate ras SOS promotes the transformation of ras-GDP to ras-GTP via changes in the conformation of ras The result of these conformational changes is the dissociation of ras from the GDP molecule (reviewed in Adjei, 2001) Cells that express constitutive mutants of EGFR constitutively activate ras through the Grb2-SOS adaptor complex Once ras is bound to a GTP molecule it is in its active state, and becomes involved with a number of downstream effectors leading to cell growth

2 1 6 Downstream Effectors of Ras

Figure 2 5 presents the three most understood signal transduction pathways These involve mitogen activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and RalGDS (involving Rac and Rho) The MAPK pathway leads to the transmittance of signals into the cell, the result being cell proliferation Active ras binds to the Ras Binding Domain (RBD) of raf There are three forms of raf A-raf, B-raf, C-raf (Gunter, *et al* , 1994) with studies suggesting that C-raf-1 plays an important role in ras signalling (Morrison, 1990, Koide, *et al* , 1993) This binding localises raf to the membrane At this

stage of the process raf's serine/threonine specific kinase activity is triggered. Raf phosphorylates mitogen extracellular signal regulated kinase (MEK protein kinase). MEK then phosphorylates cytosolic MAP kinases on threonine and tyrosine residues. Once activated these kinases localise to the nucleus where phosphorylation of transcription factors can take place, leading to the induction of immediate-early genes e.g. Fos and Jun. Raf can activate other kinases that are also involved with additional transcription factors (reviewed in Campbell, *et al.*, 1998).

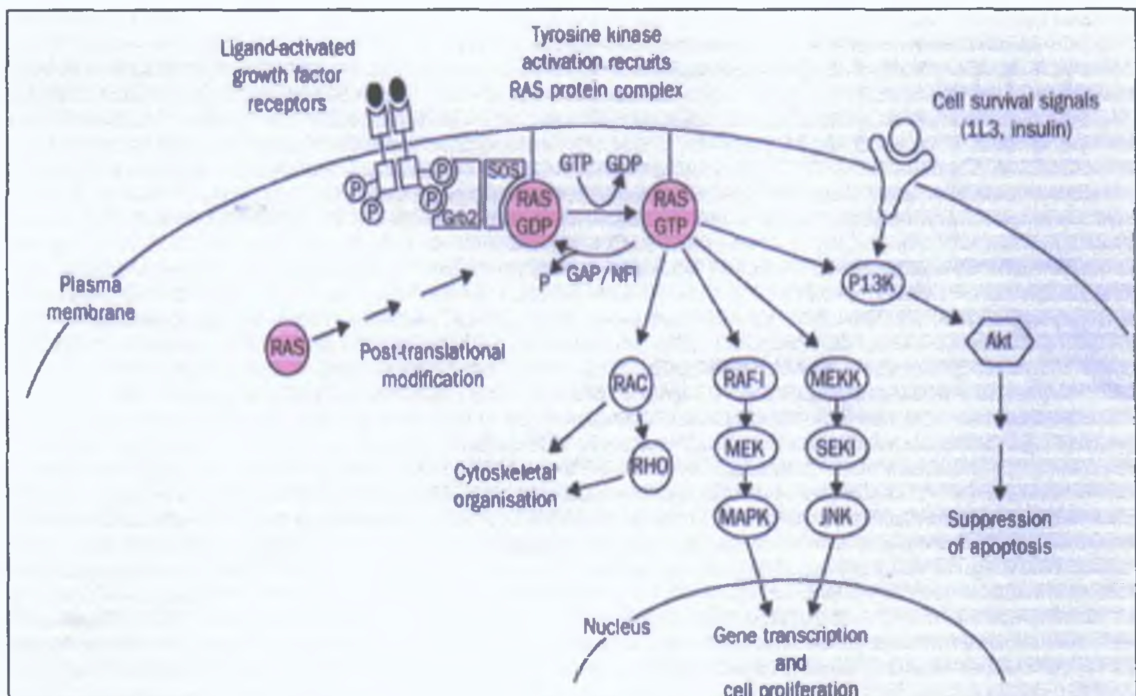

Figure 2.5 Downstream targets of ras (taken from Johnston, 2001). This diagram brings together all the processes involved in cellular signaling of ras, beginning with localization of ras to the membrane, post-translational modification of ras, and signal transduction leading to regulation of cell proliferation.

RalGDS also plays a part in the proliferative role of ras (reviewed in Shields, *et al.*, 2000). Rac and Rho play a key part in cytoskeletal organisation and when activated by mutated ras lead to the invasiveness of transformed cells (reviewed in Adjei, 2001).

PI3K inhibits activation of Bad, Caspase 9 and Bcl-x_L all of which are involved in inducing apoptosis of the cell Both AKT (a serine-threonine kinase and a downstream effector of PI3K) and PI3K can lead to the activation of NF-κB which plays an anti-apoptotic role therefore having implications in cell survival

2 1 7 Strategies to Target Cancer via the Ras Pathway

Many strategies have been devised to impair the dominant activity of *ras* and are reviewed in detail in Kloog and Cox (2000) These include dislodging ras from its membrane anchorage sites (Marom, *et al*, 1995), restoration of ras GTPase activity (Ahmadian, *et al*, 1999), interference with interactions between ras and its downstream effectors (Hermann, *et al*, 1998), use of *ras* antisense oligonucleotides (Aoki, *et al*, 1995), use of a reovirus (Coffey, *et al*, 1998) or use of PFTase inhibitors (Gibbs, *et al*, 1994, Cox and Der, 1997, Kohl, *et al*, 1993, James, *et al*, 1993) The latter have been shown not to interfere with normal cell proliferation and are relatively non-toxic (James, *et al*, 1994) As discussed above farnesylation of ras proteins by PFTase is an essential step for their binding to internal plasma membranes and for activation of events downstream of ras (i e activation of Raf kinase, MAPK kinase, MAP kinase and phosphorylation of *fos* and *jun*) leading to cell proliferation (Stacey, *et al*, 1991) Non-farnesylated mutants of oncogenic ras that cannot appropriately localise into the plasma membrane remain no longer transforming and have been shown to display a dominant inhibitory phenotype to antagonise the activity of membrane-bound oncogenic ras (Stacey, *et al*, 1991)

2 1.8 Effect of Dietary Fat on Ras

Components of dietary fat have been shown to differentially modulate oncogenic ras expression Several studies demonstrated that vegetable oils high in LA enhanced colon carcinogenesis in rats (Bull, *et al*, 1981, Deschner, *et al*, 1990, Reddy, *et al*, 1991, Reddy, 1994) On the other hand, dietary fish oil supplementation reduced growth of colonic polyps in a phase II clinical trial (Anti, *et al*, 1992) In an experimental model of colon carcinogenesis, ras levels were higher in rats fed a high fat diet composed of corn oil (59% omega-6 and 1 2% omega-3 fatty acids) compared to rats fed a high fat fish oil diet (8% omega-6 and 31% omega-3 fatty acids) Further analysis of the occurrence of tumours showed that higher expression of ras correlated with increased incidence and multiplicity of grossly visible colon tumours whereas, in animals fed the high fish oil diet lower expression of ras correlated with decreased incidence and a lower multiplicity of colon tumours (Singh, *et al*, 1997a) Dietary fish oil also resulted in increased accumulation of ras in the cytoplasm with a simultaneous decrease in levels of membrane-bound ras

Subsequently, Singh, *et al* (1998) demonstrated farnesylation of ras could be influenced by the type and amount of dietary fat and that this influenced the promotion and progression stages of colon cancer Their study demonstrated that a diet containing fish oil reduced PFTase activity in the azoxymethane-induced rat colon cancer model, whereas a diet high in corn oil increased PFTase activity It was demonstrated that corn oil increased ras expression and localization in the rat colon without affecting farnesylation of ras (Davidson, *et al*, 1999) In another study the elevated ras membrane cytosol ratio in

malignant transformed young adult mouse colon (YAMC)-Ras cells, overexpressing v-H-Ras, supplemented with corn oil was not due to increased farnesyl protein transferase activity or prenylate state as nearly all detected ras was in the prenylated form This study demonstrated that the ras membrane cytosol ratio was reduced in docosahexaenoic acid (DHA) treated cells compared to LA treated cells DHA partially blocked events downstream of ras such as reduction of the effector MAP kinase (Collett, *et al*, 2001) A decrease in cellular AA was also observed, a known antagoniser of GAPs (Tsai, *et al*, 1991) Reduction in AA would be expected to lead to reduced antagonism of GAPs and in turn a reduction in GTP bound ras Inhibition of ras association with membranes could also be due to an interference with palmitoylation, a process necessary for localisation of ras at the inner leaf of the plasma membrane (Hancock, *et al*, 1989, James and Olson, 1989) Overall it is likely that dietary fat affects ras expression, membrane localization and/or signaling pathways known to play a role in tumour development

Hydroxymethylglutaryl CoA (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, catalyses the formation of mevalonate, a precursor to farnesol Goldfarb and Pitot, (1971) observed that dietary fat can interfere with the activity of this enzyme El-Sohemy and Archer (1997) demonstrated a reduction in HMG-CoA reductase due to dietary fish oil which would lead to a decrease in levels of farnesol available for the farnesylation reaction These studies strongly suggest that individual components of fat, particularly n-3 PUFAs, may inhibit cancer development by interfering with post-translational modifications and membrane localisation of ras (Singh, *et al*, 1997a, Singh, *et al*, 1998)

Previously, it was found that the *c*9, *t*11 CLA isomer significantly reduced PFTase activity in a colon cancer cell line (SW480), a cell line in which the K-ras mutation is overexpressed However, the *t*10, *c*12 CLA isomer enhanced PFTase activity This suggests that the anticarcinogenic activity of the *c*9, *t*11 CLA isomer may be due to an inhibition of membrane ras (O'Shea, 2000) One of the objectives of the present study was to determine if a CLA mixture of isomers or specific CLA isomers have an inhibitory effect on ras expression Of the individual CLA isomers, *c*9, *t*11 has been implicated as the most biologically active because it is the predominant isomer incorporated into the phospholipids of cell membranes (Ip, *et al* , 1991, Hayek, *et al* , 1999) However, more recent studies have shown the *t*10, *c*12 CLA isomer to be even more potent In colon cancer cell lines the potent anticarcinogenic effects of CLA have been attributed either to mixtures of CLA isomers that contain primarily the *c*9, *t*11 and *t*10, *c*12 forms in approximately equal proportions, with other CLA isomers at considerably lower levels (Ha, *et al* , 1990, Schut, *et al* , 1997, Pariza and Hargraves, 1985) or to the pure *c*9, *t*11 isomer or the pure *t*10, *c*12 CLA isomer (Ip, *et al* , 1999) A dose- and time-dependent growth inhibitory effect, induction of apoptosis and an induction of antioxidant enzymes in CLA-treated mammary and colon cancer cells as an adaptive response to induction of lipid peroxidation by synthetic CLA (O'Shea, *et al* , 1999, Miller, 2003, Miller, *et al* , 2003) and CLA-enriched milk fat (O'Shea, 2000, Miller, 2003) has previously been reported

2 1 9 Objective of Experiment

The aim of this study was to determine if a mixture of CLA isomers shown to be anticarcinogenic (Hayes, *et al*, 1997, O'Shea, *et al*, 1999, O'Shea, *et al*, 2000, Schønberg and Krokan, 1995, Shultz, *et al*, 1992) or if CLA in the pure form (*c*9, *t*11 and *t*10, *c*12 CLA), inhibited cell growth of the human colon cancer line, SW480, via modulation of ras expression

2 2 Materials and Methods

2 2 1 Materials

Human colon (SW480) cancer cell lines were obtained from the American Type Culture Collection, (Manassas, VA) Culture media and supplements were purchased from GIBCOBRL (Paisley, Scotland) The SW480 cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 5% (v/v) fetal bovine serum, 0 2 mM L-glutamine, 1 mM HEPES, and 1 unit/ml penicillin and streptomycin Triton-X 100, linoleic acid (LA) and *trans*-vaccenic (TVA) were purchased from Sigma-Aldrich Ireland Ltd , (Dublin) The mixture of CLA isomers were obtained from Nu Chek Prep Inc (Elysian, MN), and pure *c*9, *t*11 and *t*10, *c*12 CLA isomers were obtained from Matreya (Pleasant Gap, PA) Primary antibody, pan-ras monoclonal antibody, Ab2 and p21 H-*ras* standard were obtained from Oncogene (Oncogene Science, Manhasset, NY) Secondary antibody, anti-mouse horse radish peroxidase (HRP) antibody was purchased from Amersham (Amersham, Little Chadford, Buckinghamshire, UK) Microcon centrifugal filter devices (YM-10, with molecular cut off of 10,000 kDa) were obtained from Millipore (Millipore Corporation, Bedford, MA) Hybond enhanced chemiluminescence (ECL) membrane was purchased from Amersham, Little Chadfort, Buckinghamshire, UK The Trans-blot electrophoretic transfer cell was purchased from Bio-Rad, Hemel Hempstead, Hertfordshire, UK

2 2 2 Cell Viability

SW480 cells were seeded at 1 x 10^6 cells in 75cm^2 flasks in DMEM (O'Shea, *et al* , 1999) The pH of the media was maintained at 7 2-7 4 by a required flow of 95% air and 5%

CO_2 Cells were cultured for 24 h to allow the cells to attach to the substratum The medium was then replaced with medium alone containing ethanol to a final concentration of 0 1% (v/v) (control) or medium supplemented with 20 μg/ml CLA, LA, *c*9, *t*11 and *t*10, *c*12 CLA dissolved in ethanol Following 4 days of incubation, cells were harvested using trypsin-EDTA and counted using trypan blue exclusion

2 2 3 Cell Culture for Western Blot Analysis

SW480 cells were cultured and maintained as described in **Section 2 2 2** SW480 cells were seeded at 5 x 10^6 cells/150cm^2 flask in DMEM (O'Shea, *et al* , 1999) The media was then replaced with media containing either (i) a CLA mixture of isomers (ii) *c*9, *t*11-CLA (iii) *t*10, *c*12-CLA and (iv) linoleic acid (LA) (v) C18 1 *t*11 (vaccenic acid) (all at a concentration of 20 μg/ml and dissolved in ethanol) An equivalent volume of ethanol was added to a control flask Quercetin was used as a negative control at a concentration of 10 μM Quercetin is a plant flavanoid which has been shown to inhibit ras expression in human colon cancer cell lines and the growth of human colon carcinoma cell lines and primary colorectal tumours (Hosokawa, *et* al , 1990, Ranelletti, *et al* , 1992, 2000)

2 2 4 Preparation of Cell Extracts

Cells were harvested after 24 h and 4 days incubation using phosphate buffered saline (PBS) containing 0 25% (w/v) trypsin Cells were spun 1,000 x g for 5 min The resulting pellets were washed twice in ice-cold PBS For total ras determination cell pellets were sonicated on ice for 10 min in 500 μL lysis buffer (containing 10 mM sodium phosphate buffer (pH 7 2), 100 mM NaCl, 10 mM sodium deoxycholate, 1 mM PMSF, 1% Triton-X

100, 0 1 mM leupeptin and 0 2 µg/ml aprotinin) The lysates were concentrated using Microcon YM-10 filters The lysates were centrifuged at 10,000 x g at 4°C until 100 µL remained in the upper chamber of the tube This chamber was then inverted and centrifuged at 1,000 x g for 3 min to collect the concentrated lysate The protein content of the lysates was determined using the Bradford assay (Bradford, 1976) For membrane ras determination, cell pellets were sonicated on ice in 500 µL PBS/PMSF buffer (containing 10 mM sodium phosphate buffer (pH 7 2), 100 mM NaCl, 0 2 mM PMSF, 0 1 mM leupeptin and 0 2 µg/ml aprotinin) for 10 min The suspensions were centrifuged at 100,000 x g in a Beckman Ultracentrifuge for 1 h at 4°C The supernatant (cytosolic fraction) was removed and the pellet resuspended in lysis buffer as for total ras The suspension was incubated on ice for 20 min followed by centrifugation at 15,000 x g for 15 mm The resulting supernatant (membrane fraction) was removed, concentrated and analysed for protein content as for total ras

2 2 5 Gel Electrophoresis and Western Blot Analysis

Cell lysates were electrophoresed using 12% polyacrylamide gel, adding 70 µg of protein in loading buffer (16 µL in total) to each well The loading buffer consisted of 62 5 mM Tris-HCl (pH 6 8), 2% w/v sodium dodecyl sulphate (SDS), 10% glycerol, 50 mM dithiothreitol (DTT) and 0 01% bromophenol blue The gel was run at a current 0 06 mA for 1 hr The separated proteins were transferred onto a Hybond ECL membrane in a Trans-blot electrophoretic transfer cell at 80V for 1 5 hr The membrane was then covered with Ponceau S stain (0 1% Ponceau S in 5% acetic acid) to ensure transfer of proteins To account for degradation of samples and differences in sample loading, the intensity of the

stain was measured using densitometry (NIH Image, Version 1 61) and the value obtained was used to quantify ras, as described below Blocking buffer consisted of 5% non-fat dried milk in PBST The membrane was incubated with the primary antibody (1/5,000 dilution in phosphate buffered saline containing 0 1% Tween-20 (PBST) and 0 5% non-fat dry milk overnight at 4°C followed by rinsing with PBST The membrane was incubated with the HRP-linked secondary antibody 1/10,000 dilution in PBST containing 0 5% non-fat dry milk followed by rinsing with PBST Presence of ras was analysed using ECL and autoradiography (Amersham Pharmacia Biotech Hyperprocesser, Model AM4) Ras was quantified using densitometry, with an accurate measurement being achieved by multiplying the intensity of a band by its area (Singh, *et al*, 1997a, Cerda, *et al*, 1999) This value was then multiplied by the intensity of the corresponding Ponceau S stain

2 2.6 Statistical Analysis

Three independent experiments were performed in triplicate The Student's *t* test was used to determine significance between treatments

2 3 Results

A dose dependent growth inhibitory effect of the CLA mixture of isomers in both the MCF-7 and SW480 cancer cell lines has been previously demonstrated (O'Shea, *et al* , 2000) High fat fish oil diets are known to inhibit tumour growth, inhibit ras expression (Singh, *et al* , 1997, Singh, *et al* , 1998, Bartsch, *et al* , 1999) and farnesylation (Schønberg and Krokan, 1995) in animal models of breast and colon carcinogenesis The effect of various CLA isomer preparations and linoleic acid on growth and ras expression in the SW480 colon cancer cell line was investigated in this study

2 3 1 Growth of SW480 Cells

The Nu Chek Prep mixture of CLA isomers and the pure *c*9, *t*11 and *t*10, *c*12 CLA (Matreya) isomers significantly inhibited growth in SW480 cells following 4 days incubation at 20 µg/ml compared with control cells (**Figure 2 6**) The individual *t*10, *c*12 CLA isomer (51% growth inhibition, P<0 01) was slightly more effective at reducing cell numbers than the *c*9, *t*11 CLA isomer (42% growth inhibition, P<0 01) and the CLA mixture of isomers (38% growth inhibition, P<0 01) LA (20 µg/ml) had a stimulatory effect on growth of SW480 cells following 4 days, increasing cell growth by almost 20% (P<0 05) compared with ethanol control

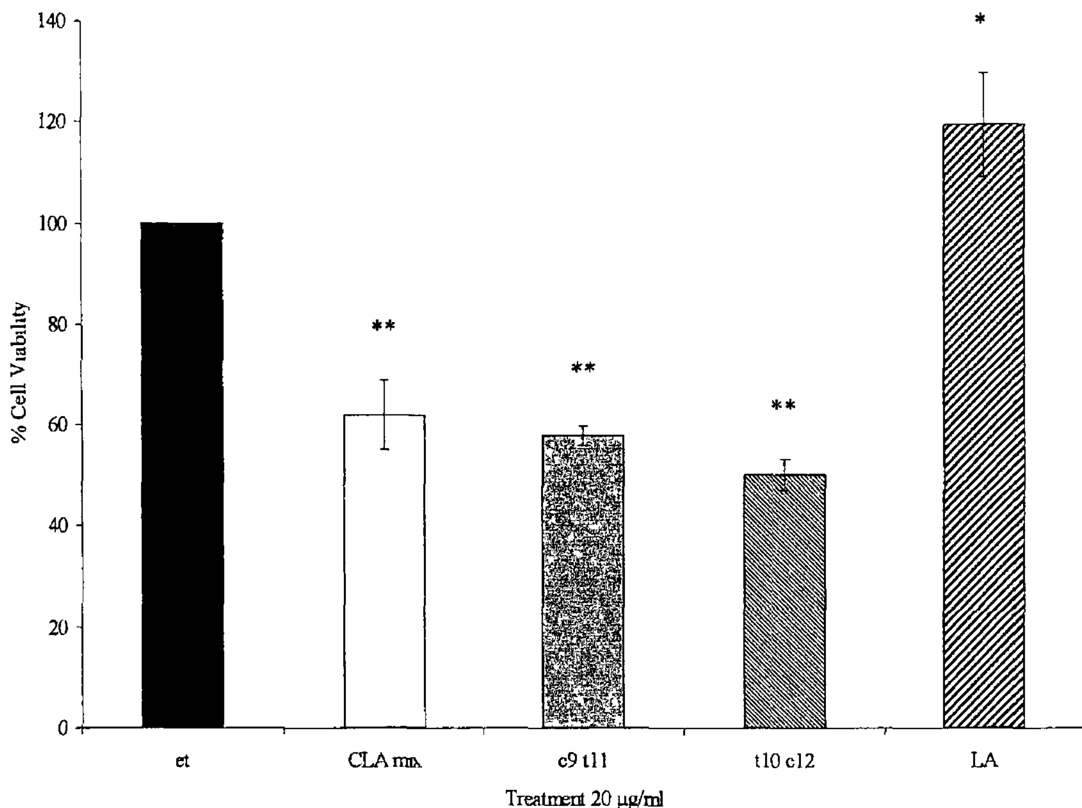

Figure 2 6 Cell viability for SW480 cells incubated with fatty acids for 4 days (20 µg/ml Nu-Chek Prep CLA, *c*9, *t*11 and *t*10, *c*12 pure CLA isomers (Matreya) and LA * denotes values that are significantly different ($P<0\ 05$) compared with et (ethanol control) ** denotes values that are significantly different ($P<0\ 01$) compared with et (ethanol control)

2 3 2 Western Blot Analysis of Ras

Figures 2 7 and **2 8** show representative examples of Western blot analysis of total ras p21 expression in cells treated with CLA isomers, LA, and vaccenic acid (VA) all at a concentration of 20 µg/ml and 10 µM quercetin for 24 h and 4 days respectively VA is a precursor to CLA in ruminal biohydrogenation A recent study carried out in the laboratory showed that VA (20 µg/ml) was converted to *c*9, *t*11 CLA in SW480 cells and reduced ($P<0\ 05$) cell growth by approximately 30% (Miller, *et al*, 2003) compared to ethanol

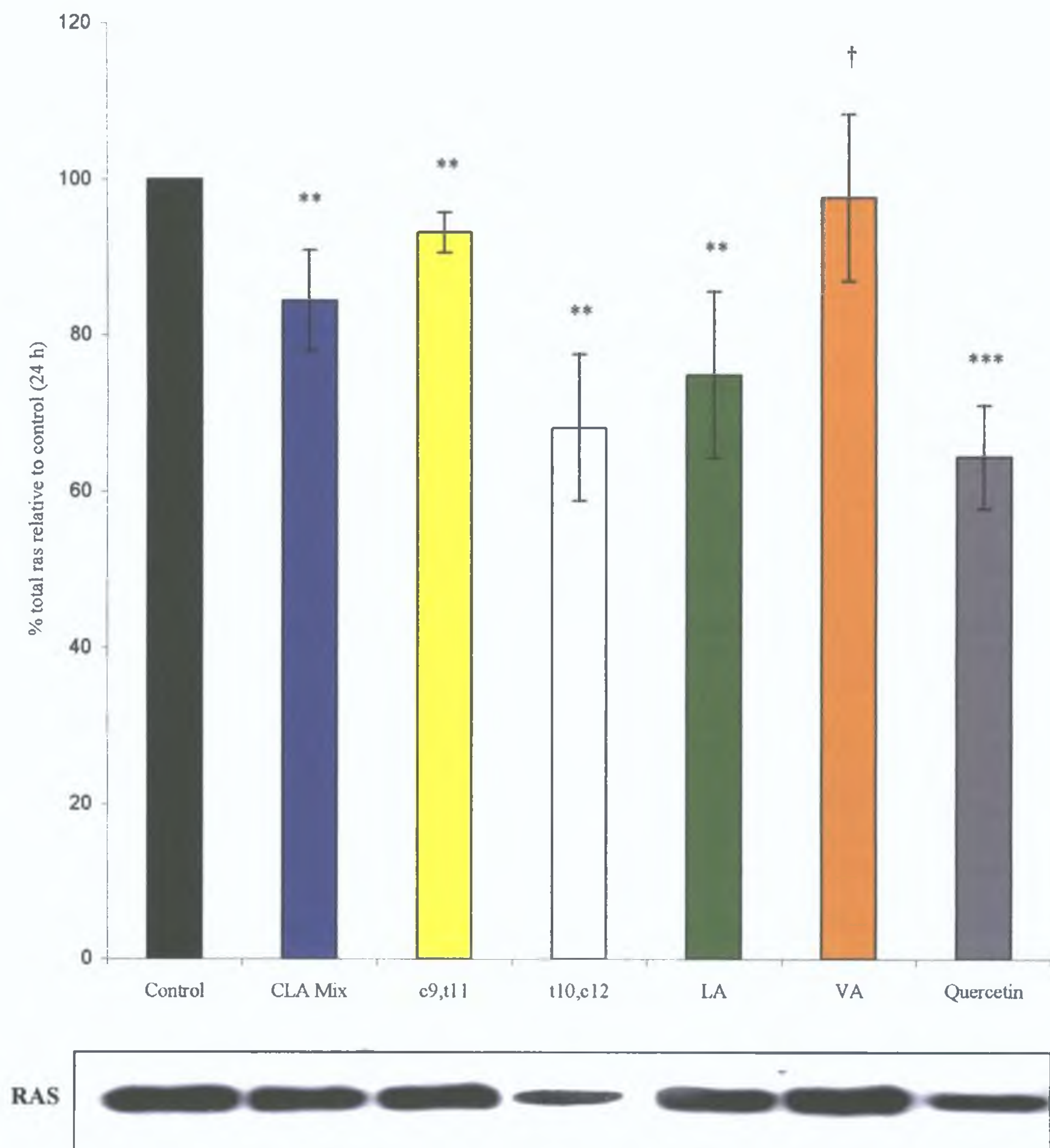

Figure 2.7 Effect of fatty acids (20 μg/ml Nu-Chek Prep CLA, *c*9, *t*11 and *t*10, *c*12 pure CLA isomers (Matreya), VA, LA) and 10 μM quercetin on total ras after 24 h incubation

**P<0.01; *** P<0.001 significantly different compared with ethanol control
† significantly different compared to quercetin, P<0.05

Total ras is visible as a dense band or doublet due to the presence of both membrane and non-membrane bound ras, resolution of the doublet of total ras was more apparent after 4 days incubation compared with 24 h incubation

It is apparent from **Figure 2 7** that quercetin inhibited total ras expression by 35% Of the fatty acids investigated, *t*10, *c*12 CLA and LA were the most potent, inhibiting total ras expression by 32% and 25% respectively The *c*9, *t*11 CLA isomer was less potent (inhibiting ras expression by 7%) compared with the CLA mix, which inhibited total ras expression by 16% Vaccenic acid did not have a significant (P<0 05) effect on total ras after 24 h incubation

Extending the incubation period to 4 days resulted in a greater inhibition of total ras by all of the treatments, with the exception of LA (**Figure 2 8**) Quercetin was the most potent inhibitor, inhibiting total ras by 57% compared with the control The *c*9, *t*11 and *t*10, *c*12 CLA isomers, the CLA mixture of isomers and VA reduced total ras expression by 22%, 47%, 35% and 45% respectively By contrast, the inhibition of total ras by LA after 4 days incubation (24%) was similar to that observed after 24 h incubation The *c*9, *t*11 CLA isomer and LA were significantly (P<0 05) less potent as total ras inhibitors compared with the quercetin control

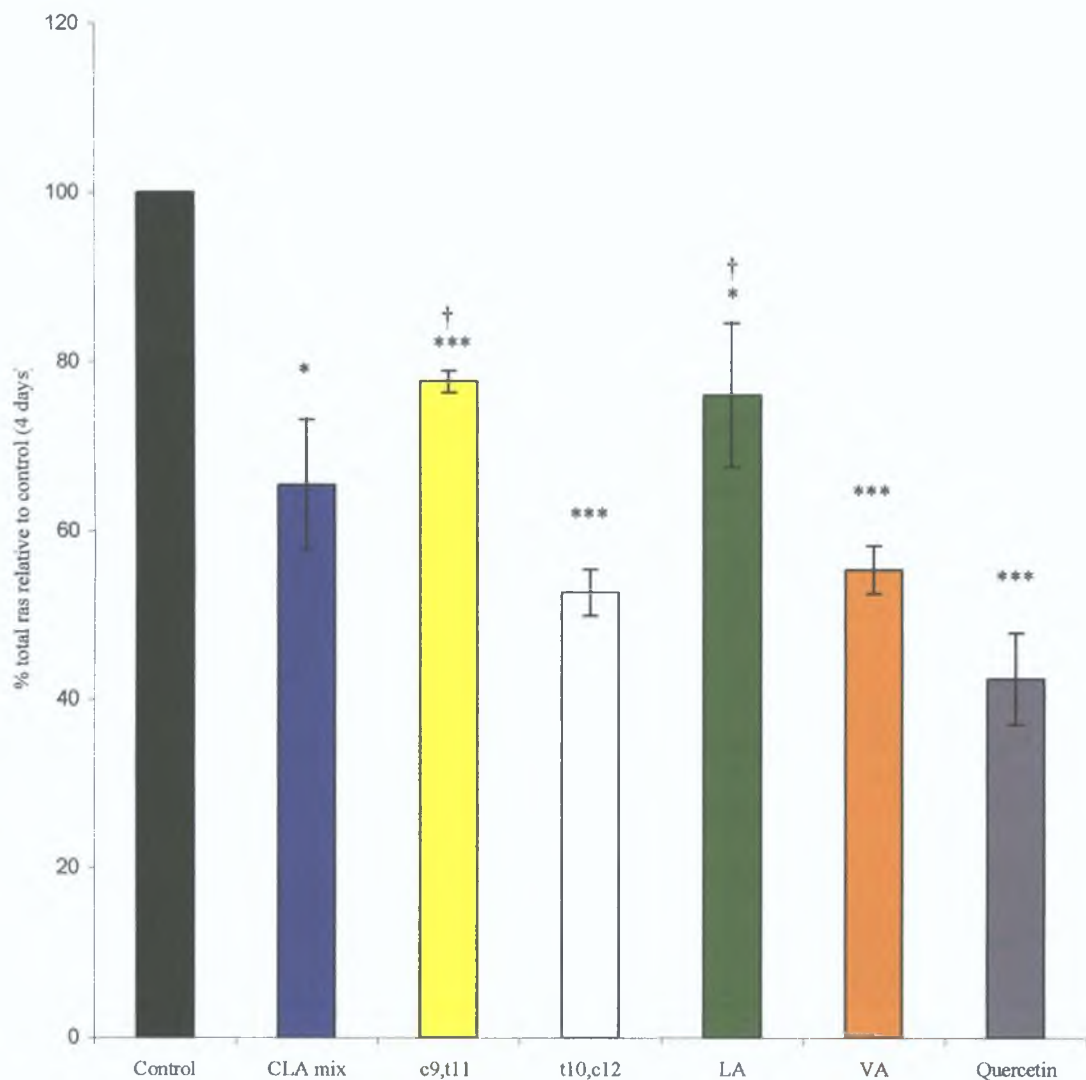

Figure 2.8 Effect of fatty acids (20 μg/ml Nu-Chek Prep CLA, *c*9, *t*11 and *t*10, *c*12 pure CLA isomers (Matreya), VA, LA) and 10 μM quercetin on total ras after 4 days incubation

* P<0.05; *** P<0.001 significantly different compared with ethanol control
† significantly different compared to quercetin, P<0.05

Figures 2 9 and **2 10** represent examples of Western blot analysis of membrane ras p21 in cells treated with CLA isomers, LA, VA and quercetin for 24 h and 4 days respectively The ras protein was expressed as a duplet comprising mainly farnesylated ras at 23 kDa and unfarnesylated ras at 21 kDA Farnesylated ras is membrane bound and this band was quantified using densitometry **Figure 2 9** shows the reduction of membrane ras after 24 h treatment with the fatty acids compared to the control, which was taken as 100% The *c*9, *t*11 CLA isomer and LA significantly (P<0 05) reduced membrane ras by 16% and 30% respectively The CLA mixture of isomers did not significantly (P<0 05) reduce membrane ras The *t*10, *c*12 CLA isomer and quercetin significantly (P<0 01) reduced membrane bound ras by 47%, and 22% respectively Vaccenic acid significantly (P<0 001) reduced membrane ras by 47%

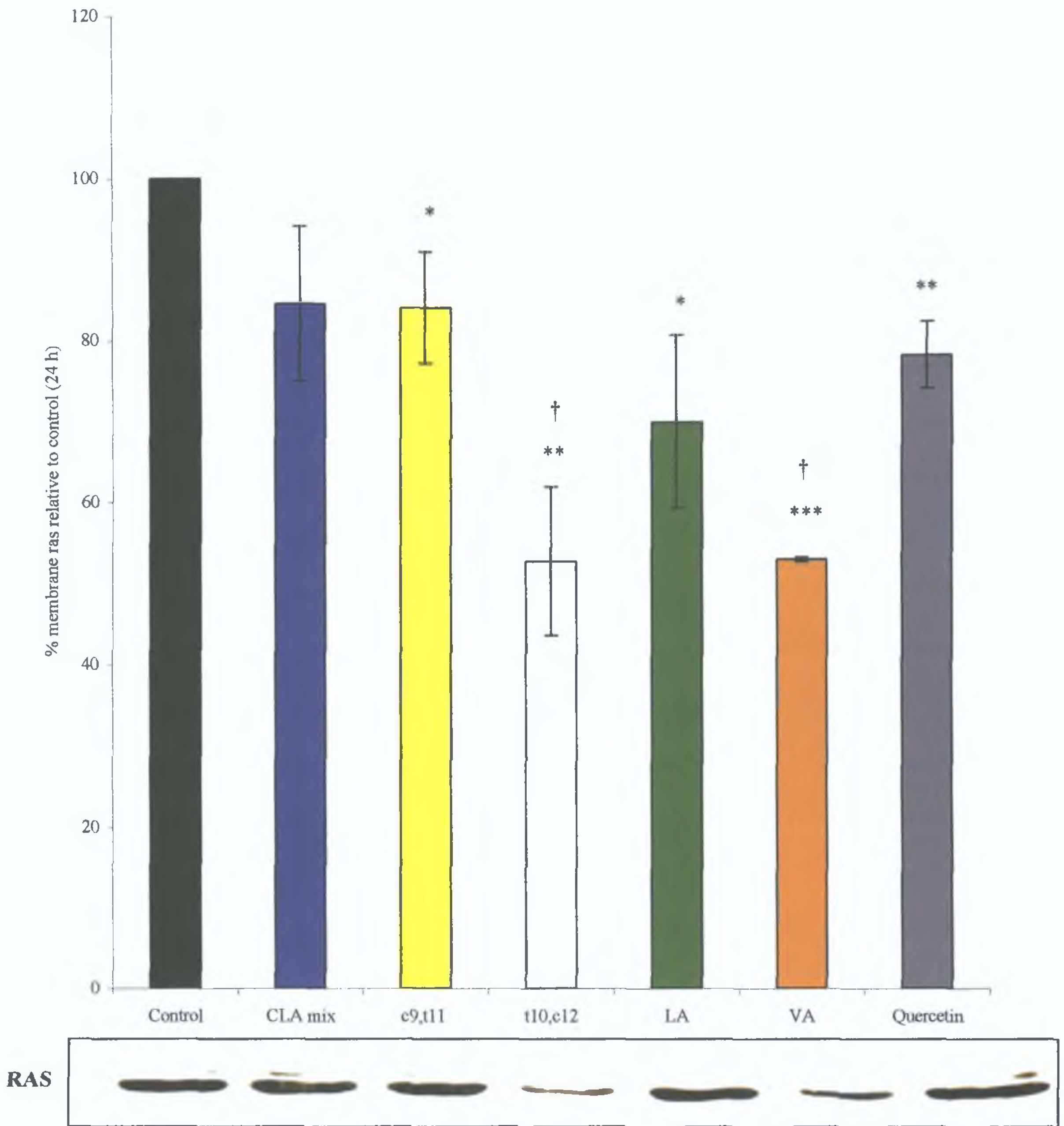

Figure 2.9 Effect of fatty acids (20 μg/ml Nu-Chek Prep CLA, *c*9, *t*11 and *t*10, *c*12 pure CLA isomers (Matreya), VA, LA) and 10 μM quercetin on membrane ras after 24 h incubation

* P<0.05; **P<0.01; *** P<0.001 compared with control
† significantly different compared to quercetin, P<0.05

Figure 2.10 Effect of fatty acids (20 μg/ml Nu-Chek Prep CLA, *c*9, *t*11 and *t*10, *c*12 pure CLA isomers (Matreya), VA, LA) and 10 μM quercetin on membrane ras after 4 days incubation

**P<0.01; *** P<0.001 compared with control

† significantly different compared to quercetin, P<0.05

Figure 2 10 presents the effect of the fatty acid treatments on membrane ras after 4 days incubation with SW480 cells The *c*9, *t*11, *t*10, *c*12 CLA isomers, LA and quercetin significantly (P<0 01) reduced membrane ras after 4 days incubation by 26%, 27%, 29% and 52% respectively Vaccenic acid did not significantly (P<0 05) reduce membrane ras compared with the ethanol control Quercetin was significantly (P<0 05) more potent at reducing membrane ras after 4 days incubation

2 4 Discussion

Many mechanisms of action have been suggested for the anticarcinogenic effect of CLA, including a prooxidant effect (O'Shea, *et al* , 1999, Schønberg and Krokan, 1995), inhibition of mutagen activation (Liew, *et al* , 1995) and modulation of eicosanoid production (Belury, *et al* , 1996, Li and Watkins, 1998) In addition, it has been proposed that growth regulatory genes such as *c-myc* and *c-jun*, may be influenced by binding of CLA to PPARs (Moya-Camarena, *et al* , 1999b) Incubation of cancer cells with CLA has been shown to be associated with incorporation of CLA into membrane phospholipids and neutral lipids (Miller, 2003) As a result of incorporation into membrane phospholipids CLA may modulate subsequent signal transduction pathways which depend on the proper membrane localisation of oncogene encoded proteins, for example ras It has been proposed that modulation of ras activation by a high fat fish oil diet containing 31% n-3 fatty acids may partly explain why dietary fish oil protects against colon cancer development (Singh, *et al* , 1997a, 1998) Whether CLA and LA differentially modulate oncogenic ras expression has not been studied This study examined the effects of equal amounts (20 μg/ml) of various CLA preparations, VA and LA on total ras and membrane bound ras expression in the SW480 cell line in which mutated K-ras is overexpressed

2 4 1 Effect of *c*9, *t*11 CLA on Ras

Data from this study suggest that a reduction in membrane ras expression may underly the growth inhibitory effect of the c9, t11 CLA isomer The reduction in membrane bound ras by 16% and 26% after 24 h and 4 days respectively suggests that *c*9, *t*11 CLA decreased ras membrane localisation This together with findings of a previous study showing that

*c*9, *t*11 CLA inhibited PFTase activity suggests that diminished farnesylation and/or membrane localisation may result in ras remaining in its biologically inactive form thus preventing growth signals from reading proliferation genes in the nucleus This isomer also reduced total ras expression, this may suggest interference in ras transcription and/or degradation of ras transcripts A previous study (Ip, *et al*, 1997) demonstrated that the anticancer effect of dietary CLA did not depend on the presence or absence of mutated ras in mammary tissue However, the data from this study has been generated using a colon cancer cell line in which the K-ras mutation is overexpressed, hence different anticancer mechanisms can be expected in different cell lines and tissues The specificity of certain exchange factors such as Smg GDS which is specific for K-ras or Ras GRF which is specific for H-Ras activation (Orita, *et al*, 1993, Jones and Jackson, 1998) may account for different ras responses to CLA treatment Another possibility for expected differences is that K-ras is localised to the plasma membrane differently to other Ras isoforms Membrane polar lipid head groups which form a high affinity bond with a stretch of lysine residues on K-ras4B are responsible for maintaining membrane localisation of this ras isoform All other ras isoforms are held deep in the plasma membrane by their palmitate group(s) (Roy, *et al*, 1999, Sternberg and Schmid, 1999)

2 4 2 Effect of LA on Ras

The proliferative effect of LA observed in this study supports previous studies showing LA can stimulate cancer cell growth (Shultz, *et al*, 1992, Cunningham, *et al*, 1997) However, LA significantly reduced membrane and total ras in the SW480 cell line This suggests that LA interferes with the cell proliferation cascade downstream of ras (Collett,

et al, 2001) Therefore, despite reduced levels of ras, LA may interfere with effectors downstream of ras such as MAPK, leading to increased cellular proliferation (Brandy and Saker, 2001)

2 4.3 Effect of *t*10, *c*12 CLA on Ras

Incubation with the *t*10, *c*12 isomer showed a reduction in total and membrane bound ras in addition to an antiproliferative effect in the cell line However, this isomer caused a 66% increase in the activity of PFTase towards exogenous H-ras (O'Shea, 2000) This suggests that this isomer inhibits SW480 cell growth by targeting *ras* gene expression rather than farnesylation of ras One theory may be that although PFTase activity is stimulated, farnesylation is reduced due to inhibition of *ras* gene expression A likely consequence of a reduction in the amount of ras protein in the cytosol that is available for farnesylation is a reduction in membrane bound ras and in turn cell proliferation Therefore increased activity of PFTase is redundant in the absence of adequate concentrations of its substrate The *t*10, *c*12 CLA isomer may also target events downstream of ras farnesylation and thereby inhibit proliferation Such downstream effectors include NF-κB which has an anti-apoptotic function (Irani, *et al*, 1997, Romashkova and Makarov, 1999, Ozes, *et al*, 1999), or the AKT/PKB pathway which promotes cell survival (Khwaja, 1999)

2 4 4 Effect of Mixture of CLA Isomers on Ras

The two predominant CLA isomers inhibited both membrane and total ras after 24 h and 4 days incubation when present in culture media at a concentration of 20 μg/ml in pure

form However, the CLA mixture of isomers containing 6 μg/ml *c*9, *t*11 and 6 μg/ml *t*10, *c*12 CLA did not inhibit membrane bound ras, whereas this treatment inhibited PFTase activity (O'Shea, 2000) and was found to have a significant effect on total ras after 24 h and 4 days in the SW480 cell line This indicates a possible effect at gene level, where the mixture of CLA isomers may be inhibiting *ras* gene expression The K-*ras* mutation is overexpressed in the SW480 cell line K-ras can become membrane bound by either farnesylation or geranylgeranylation and both processes must be inhibited in order to inhibit membrane localization of K-ras (Whyte, *et al*, 1997) Although a previous study showed that the CLA mixture of isomers containing 6 μg/ml *c*9, *t*11 and 6μg/ml *t*10, *c*12 CLA inhibited exogenous H-ras farnesylation, no studies have been conducted on the affect of specific CLA isomers on K-ras farnesylation or on the geranylgeranylation process to determine which isomers, if any, might have a dominant effect and to determine the critical concentration at which an effect is observed

2 4 5 Effect of Vaccenic Acid on Ras

Vaccenic acid, a precursor to CLA, has been found to inhibit growth of SW480 cells after 4 days incubation (Miller, *et al*, 2003) In this study it was found that 24 h incubation with VA was found to inhibit membrane ras by 47%, however, increasing the incubation period to 4 days resulted in VA not effecting membrane ras Miller, *et al* (2003) found that SW480 cells are capable of converting VA to *c*9, *t*11 CLA – 29 4% of 20μg/ml VA was converted to *c*9, *t*11 CLA after 4 days incubation with SW480 cells The reduced inhibitory effectiveness of VA after 4 days compared to 24 h may be due to unavailability of VA due to its involvement in conversion to *c*9, *t*11 CLA It is likely that the extent of

conversion to *c*9, *t*11 CLA at a concentration of approximately 6 µg/ml was inadequate to inhibit membrane ras Total ras was not affected by incubation of SW480 cells with VA for 24 h However, VA significantly reduced ($P<0\ 001$) total ras when the incubation period was extended to 4 days This inhibitory affect may be due to the combined affect of VA and the *c*9, *t*11 CLA produced after 4 days incubation

2 4 6 Conclusions

Ras is involved in a number of complex signalling pathways affecting growth Interference with several steps in these pathways can affect the end result This study suggests that CLA isomers may modulate the activity of ras by affecting production and/or localisation The fact that both N- and K-ras can undergo farnesylation and geranylgeranylation further complicates the issue It is important that the effects of CLA on downstream targets of the ras pathway be studied It has also been shown that activated ras can up-regulate *bcl-2*, a suppressor of apoptosis (Kinoshita, *et al* , 1995) Therefore, it may be possible that a reduction of ras due to CLA treatment may underly the down regulation of *bcl-2* by CLA and hence induction of apoptosis, reported by Miller, *et al* (2002)

2 4 7 Summary

Ras oncoproteins have been implicated in a phosphorylation cascade which results in the activation of proliferation genes and, in turn, tumour development The objective of this study was to determine if the anticarcinogenic fatty acid CLA inhibited ras in the SW480 cancer cell line A mixture of CLA isomers (*c*9, *t*11-, *t*10, *c*12-, *c*11, *t*13- and minor

amounts of other isomers), *c*9, *t*11 CLA, *t*10, *c*12 CLA (all at 20 μg/ml) significantly (P<0 01) suppressed growth of SW480 cells by 38%, 42% and 51% respectively following 4 days incubation compared with untreated controls Incubation with LA (20 μg/ml) had a significant (P<0 05) stimulatory effect (~20% increase) on cell growth after 4 days Of the CLA isomers analysed the *t*10, *c*12 isomer appeared to be the most potent ras inhibitor This isomer reduced total ras by 32% (P<0 01) and 47% (P<0 001) after 24 h and 4 days incubation respectively, while *c*9, *t*11 CLA reduced total ras by 7% (P<0 01) and 22% (P<0 001) after 24 h and 4 days respectively The *t*10, *c*12 CLA isomer reduced membrane ras to a similar extent as the *c*9, *t*11 isomer after 4 days incubation, but at 24 h, was more potent than *c*9, *t*11 CLA reducing membrane ras by 47% (P<0 01) after 24 h compared with 16% (P<0 05) by *c*9, *t*11 CLA Although LA was found to be growth stimulatory after 4 days incubation, this fatty acid reduced both total and membrane ras after 24 h and 4 days, however this effect did not appear to be time dependant with total ras reduced by ~25% after 24 h and 4 days, and membrane ras reduced by ~30% after 24 h and 4 days The CLA mixture of isomers, which contained primarily *c*9, *t*11 and *t*10, *c*12 CLA, reduced total ras in a time dependant manner, however only a small reduction in membrane ras was observed after 24 h and no significant effect after 4 days Vaccenic acid, a CLA precursor, only inhibited total ras after 4 days incubation and membrane ras after 24 h incubation The results suggest that measurement of ras expression alone cannot account for growth effects produced by these fatty acids in the SW480 cell line

CHAPTER 3

ANTICARCINOGENIC ACTIVITY OF CLA PRODUCED BY BIFIDOBACTERIA

3.1 Introduction

3.1.1 Ruminal Biohydrogenation and Microbial CLA Production

In milk, CLA is produced directly in the rumen as a by-product of microbial biohydrogenation of LA (Harfoot and Hazlewood, 1997) and indirectly from VA. Vaccenic acid is a product of further biohydrogenation of *c*9, *t*11 CLA and is converted back to *c*9, *t*11 CLA by the action of Δ9-desaturase enzymes in the mammary gland of the lactating ruminant (Griinari, *et al.*, 2000). Consequently CLA content of milk is markedly different from the CLA content of the diet of ruminant animals and is strongly influenced by diet (Chilliard, *et al.*, 2000; Stanton, *et al.*, 2003).

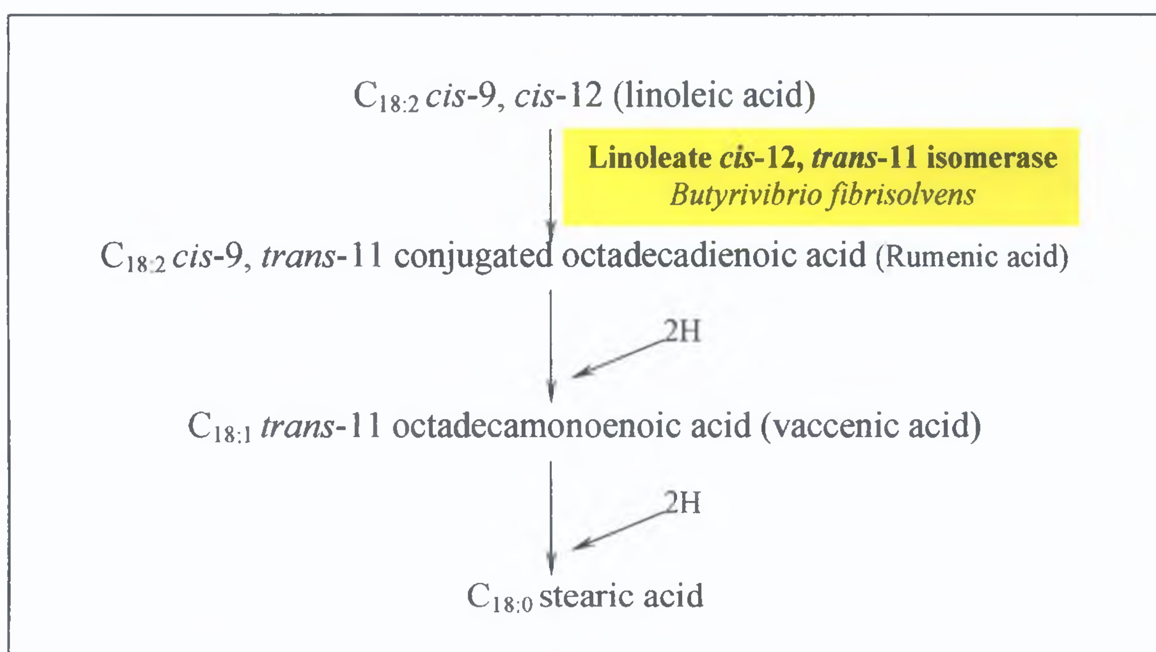

Figure 3.1 Ruminal biohydrogenation of linoleic acid

The first step in the ruminal biohydrogenation of LA involves its isomerisation to preferentially form the *c*9, *t*11 CLA isomer (Kepler, *et al.*, 1966) (**Figure 3.1**). This isomer has been given the name rumenic acid (Kramer, *et al.* 1998). The reaction is

catalysed by LA isomerase produced by ruminal bacteria such as *Butyrivibrio fibrisolvens* (reviewed in Harfoot and Hazlewood, 1988) Once formed, the *c*9, *t*11 CLA isomer may be absorbed or may undergo additional biohydrogenation in the rumen to form VA which is further biohydrogenated to produce C18 0 (stearic acid) This reaction is much slower than the previous one and so leads to an accumulation of VA in the rumen (Keeney, 1970) It has been suggested that biohydrogenation is a detoxification process used by bacteria to protect against PUFAs (Dawson and Kemp, 1970), which are known to be highly toxic (Kepler and Tove, 1967)

3 1 2 Microbial CLA Production

Chin, *et al* (1994) demonstrated that microflora isolated from the intestine of rats fed a diet containing LA had the ability to form the *c*9, *t*11 CLA isomer Some strains of dairy starter cultures have also been shown to possess the ability to produce CLA The *c*9, *t*11 isomer was the predominant CLA isomer found, accounting for as much as 70% of total CLA (Jiang, *et al*, 1998) The pathway utilised by *Propionibacteria freudenreichii* subsp *freudenreichii* and *Propionibacteria freudenreichii* subsp *shermanii* differed from that of *Butyrivibrio fibrisolvens* in that higher concentrations of C18 1 *c*9 (oleic acid) were produced during LA biohydrogenation by the dairy starter cultures compared to high levels of VA during LA biohydrogenation by the ruminant bacteria This study also demonstrated that the presence of components such as 0 1% Tween-80 and 3% milk protein influenced the microbial production of CLA Such components counteract the antimicrobial effect of fatty acids such as LA (Dubos, 1947, Ledesma, *et al*, 1977, Baker, *et al*, 1983, Cummins and Johnson, 1986, Boyaval, *et al*, 1995) It has also been

proposed that milk proteins may act as hydrogen donors for the production of CLA in cheese (Gurr, 1987) Other strains such as *Lactobacillus acidophilus* (Lin, *et al* , 1998, Kishino, *et al* , 2002) and Bifidobacteria strains *B breve* and *B dentium* (Nordgren, 1999, Coakley, *et al* , 2003) have been shown to be capable of producing CLA from LA After selecting CLA producing strains from a range of intestinal and probiotic strains it was demonstrated that of the strains studied, *Bifidobacterium* in particular *B breve* and *B dentium* produced the highest concentration of CLA with *B breve* NCFB 2258 converting 65% of LA to *c*9, *t*11 CLA (Coakley, *et al* , 2003) A pathway for the production of CLA by *L acidophilus* was suggested by Ogawa, *et al* (2001) It was found that incubation of LA with *L acidophilus* under microaerobic conditions resulted in the formation of hydroxy fatty acids and in turn *c*9, *t*11, *t*9, *c*11 and *t*9, *t*11 CLA isomers at concentrations as high as 5 mg/ml The concentration of substrate and incubation period are critical in optimising production of CLA using such cultures (Lin, *et al* , 1999a)

3 1 3 Probiotics and Cancer

Probiotics are bacteria which impart beneficial health effects on the host when ingested and have been defined as viable microorganisms that (when ingested) have a beneficial effect in the prevention and treatment of specific pathologic conditions (Havenaar and Huis in't Veld, 1992) It was Metchnikoff (1907) who suggested that these effects are due to the positive influence of probiotics on the intestinal microorganisms Many health benefits have been attributed to probiotics some of which have also been attributed to CLA e g stimulation of the immune system (Sekine, *et al* , 1995, reviewed in Belury, 2002) and inhibition of carcinogenesis (reviewed in Hirayama and Rafter, 2000, Holzapfel

and Schillinger, 2002, reviewed in Belury, 2002) Singh, *et al* (1997b) and Abdelali, *et al* (1995) both demonstrated the inhibitory effect of Bifidobacterium strains against colon cancer

In relation to carcinogenesis, a number of mechanisms have been suggested Probiotics may inhibit cancer development by (1) inhibiting bacteria that produce carcinogens after certain reactions, (2) direct inhibition of tumour formation, (3) inactivation of carcinogens (reviewed in Rolfe, 2000) – these possible mechanisms are discussed below Cellular proliferation in colonic crypts of patients with colon adenocarcinomas was reduced due to treatment with *L acidophilus* and *B bifidus* (Biasco, *et al* , 1991) *B longum* has been associated with inhibition of azoxymethane induced colon adenocarcinomas and aberrant crypt foci (ACF) in male F344 rats (Kulkarni and Reddy, 1994, Singh, *et al*, 1997b) In addition, *B longum* inhibited 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) induced mammary tumours and to a greater extent colon tumours in F344 rats (Reddy and Rivenson, 1993) A number of studies have demonstrated that ingestion of *L acidophilus* or *L casei* resulted in reduced levels of enzymes which convert pre-carcinogens to carcinogens in faecal specimens (reviewed in Rolfe, 2000) These enzymes include glycosidase, β-glucuronidase, azoreductase and nitroreductase Thus inhibition of such enzymes can lead to the prevention or reduction of carcinogen formation Ornithine decarboxylase (ODC) is an enzyme which plays an important rate limiting role in the synthesis of polyamines – essential for cellular proliferation and differentiation CLA has been shown previously to inhibit proliferation of human cancer cell lines (O'Shea, 2000) It has been demonstrated that *B longum* can lead to reduced levels of ODC in colon

tumours ın conjunctıon wıth a decrease ın colonıc mucosal cell prolıferatıon (Reddy, 1999) *B longum* has also been associated wıth a reductıon m the expressıon of total and mutated ras ın colonıc mucosa, correlatıng wıth a reductıon ın occurrence of colon tumours (Reddy, 1999) As demonstrated m **Chapter 2**, CLA has an ınhıbıtory effect on ras expressıon ın the SW480 human colon cancer cell lıne *Bıfidobacterıum* has been shown to produce CLA from LA (Rosberg, 2002, Coakley, *et al*, 2003) It may be possıble that the antıtumour effect of some *Bıfidobacterıum* specıes ıs partly due to an abılıty to produce CLA whıch ın turn may act on ras expressıon

3 1 4 Objectıve of Experıment

It has been demonstrated that certaın human derıved Bıfidobacterıa can produce CLA from LA (Rosberg, *et al*, 2002, Coakley, *et al*, 2003) In partıcular, the straın Bıfidobacterıum (*B breve* NCFB 2258) could convert up to 65% LA to CLA wıth smaller amounts of *t*9, *t*11 CLA also formed (Coakley, *et al*, 2003) The objectıve of thıs study was to examıne the antıcarcınogenıc actıvıty of fatty acıds produced by selected *Bıfidobacterıa*, m partıcular *c*9, *t*11 and *t*9, *t*11 CLA, agaınst the SW480 colon cancer cell lıne The fatty acıds produced ın the medıa due to fermentatıon of LA enrıched medıa by *B breve* NCFB 2258 were compared wıth that of another *Bıfidobacterıum* straın (*B lactıs* Bb12) straın as well as unfermented medıa, rıch ın LA

3 2 Materials and Methods

3 2 1 Materials

Human colon (SW480) cancer cell line was obtained from the American Type Culture Collection, (Manassas, VA) Culture media and supplements were purchased from GIBCOBRL (Paisley, Scotland) The SW480 cells were maintained in DMEM supplemented with 5% (v/v) fetal bovine serum, 0 2 mM L-glutamine, 1 mM HEPES, and 1 unit/ml of penicillin and streptomycin CLA was obtained from Nu Chek Prep Inc (Elysian, MN, USA), this material consisted of a mixture of isomers – 21% *c*11, *t*13, 29% *t*10, *c*12, 29 5% *c*9, *t*11, and 12 3% *c*8, *t*10 CLA and pure *t*9, *t*11 CLA was purchased from Matreya and pure *c*9, *t*11 was kindly donated by Natural ASA, Norway Stearic acid was purchased from Sigma Chemical Co St Louis, MO, USA *Trans*-vaccenic acid was purchased from Sigma-Aldrich Ireland Ltd, (Dublin) Isopropanol (99% purity) was obtained from Alkem Chemicals Ltd Cork, Ireland Hexane (99% purity) was obtained from LabScan Ltd Dublin, Ireland GC column Chrompack CP Sil 88 column was purchased from Chrompack, Middleburg, The Netherlands *B breve* NCFB 2258 and *B lactis* Bb12 (previously obtained from the National Collection of Food Bacteria and Chr Hansen respectively) were used for bioconversion of LA to CLA, as previously described (Coakley, *et al*, 2003)

3 2 2 Preparation and Extraction of Fermented Oils

B breve NCFB 2258 and *B lactis* Bb12 cultures were cultured in MRS broth containing 0 05% (w/v) cysteine and 0 5 mg/ml LA The cultures were then grown for 48 h at 37°C prior to lipid extraction from the supernatant The unfermented oil was prepared by the

incubation with MRS broth containing 0 05 % cysteine and 0 5 mg/ml LA without the addition of a bacterial strain for 48 h at 37°C followed by the extraction of the oil To extract the oil, 10 ml of the fermented media were centrifuged at 2197 x g for 20 min at room temperature (20°C), using a Sanyo Mistral 2000 R centrifuge To the 4 ml of the supernatant was added 0 75 mg C 13 0 as an internal standard The lipid content of the broth was extracted by adding 2 ml isopropanol and 1 5 ml hexane to the supernatant and mixed by vortexing This was followed by the addition of 3 ml of hexane, mixing using a vortex and centrifugation at 2197 x g for 5 min The upper layer (hexane layer containing fatty acids) was removed and transferred to a screw capped glass tube and flushed with N_2 gas

Extracted lipids were methylated using acid-catalysed methylation by resuspending in 12 ml, 4% methanolic HCl (v/v) in methanol and mixed using a vortex for 10 s This was followed by incubation at 60°C for 1 h, mixing every 10 min using a vortex After incubation 2 ml of water-saturated hexane and 5 ml of hexane were added and the mixture was allowed to stand for 30 min The FAME containing top layer was transferred to a tube and 2 ml of water-saturated hexane were added and the solution was mixed for 30 s using a vortex and allowed to stand for 30 min Following this, the top layer was transferred to a new tube and the methylation reaction terminated by addition of 0 5 g anhydrous sodium sulphate and mixed for 5 s using a vortex This was allowed to stand for 1 h, and then the top layer was removed and stored at -20°C prior to GC analysis

The free fatty acids (FFAs) were analysed using a gas liquid chromatograph (GLC-Varian 3400, Varian, Harbor City, CA, USA) fitted with a flame ionization detector (FID) and a Septum Programmable Injector (SPI) Separation of fatty acids was performed on a Chrompack CP Sil 88 column (100 m x 0 25 mm i d , 0 20 μm film thickness), using He as carrier gas at a pressure of 33 psi The injector temperature was held isothermally at 225°C for 10 min and the detector temperature was 250°C The column oven was held at an initial temperature of 140°C for 8 min, and then programmed at an increase of 8 5°C/min to a final temperature of 200°C, which was held for 41 min Data was analyzed on a Minichrom PC system (VG Data System, Manchester, UK) The *c*9, *t*11 CLA isomer was identified by retention time with reference to CLA standards, VA and stearic acid were identified by retention time with reference to their standard fatty acids

3 2 3 Cell Culture

Cells were grown in 6 well plates and maintained at 37°C in a humidified atmosphere The pH of the media was maintained at 7 2-7 4 by a required flow of 95% air and 5% CO_2 Initially, 5 x 10^4 cells were seeded in wells and cultured for 24 h at 37°C allowing the cells to attach to the substratum prior to treatment with the fatty acids, as described below The medium was replaced with medium supplemented with varying concentrations of fatty acids extracted from media following incubation with the 2 strains of *Bifidobacterium* Each oil was dissolved in ethanol and added to the cells to yield a final concentration of 84 μg of oil/ml The concentration of the oils was reduced to yield a concentration of 20 μg LA/ml in the unfermented oil, which equaled an oil concentration of 33 μg/ml Control flasks were supplemented with ethanol to a final concentration of

0 1% (v/v) as in experimental wells Following incubation, cells were harvested by treatment with 500 µL of trypsin (0 25% w/v) in PBS at 37°C for 5 min Viable cells were counted after 24 h, 48 h and 4 days incubation Cell viability was determined using the Trypan Blue exclusion (0 1% w/v) method Harvested cells were resuspended in 1 ml of fresh media followed by addition of 200 µL of Trypan Blue Viable cells were counted using an inverted microscope and a haemocytometer Non-viable cells were visible as blue cells and were not included in the final count

3 2 4 Effect of Fatty Acid Components of Fermented Oils on SW480 Cells

As the oils may also have contained components other than the fatty acids identified by GC analysis it was necessary to determine if the fatty acids present had similar growth inhibitory effects as the fermented oils In addition, it was important to identify the fatty acid components responsible for the growth inhibitory effect of the oils SW480 cells were treated by combining synthetic isomers of the primary fatty acids present in the oils, as determined by GC analysis These combinations were prepared to represent the fatty acid composition of the three oils and hence analyse the effect of the oils on SW480 cell growth with respect to each individual fatty acid and different combinations of these fatty acids It was important to determine the growth inhibitory effect of different combinations of fatty acids as the inhibitory effect of an individual fatty acid may be altered in the presence of others (Noble, *et al* , 1974, Hudson, *et al* , 1996) Standard curves of the effect of each of the four fatty acids, LA, oleic acid, *c*9, *t*11 and *t*9, *t*11 CLA on SW480 cell growth were also set up in the ranges 0-55, 0-40, 0-20 and 0-10 µg/ml respectively for periods of 24 h, 48 h and 4 days The concentration ranges used were representative of the

ranges present in the oils The three time points were chosen as it has previously been demonstrated that CLA significantly inhibits SW480 cell growth after 4 days incubation whereas LA significantly increased cell growth after 4 days incubation (O'Shea, 2000) The other two time points of 24 h and 48 h were chosen in order to determine if growth inhibition increased with time As the effect of the fermented oils on the growth of SW480 cells had not been previously studied it was important to determine the time required to observe a significant effect on SW480 cell growth and to determine if such growth effects were time dependant Control flasks were supplemented with ethanol to a final concentration of 0 1% (v/v) as in experimental wells Following incubation for 24 h, 48 h and 4 days, cells were harvested and viable cells counted as in **Section 3 2 3**

3 2 5 Statistical Analysis

Three independent experiments were performed in triplicate The student's *t* test was used to determine significant differences between treatments

3 3 Results

After GC analysis of the fermented oils it was found that the oils consisted primarily of LA, oleic acid, *c*9, *t*11 and *t*9, *t*11 CLA as presented in **Table 3 1** This chapter initially describes the effects of these single fatty acids on SW480 cell growth

Table 3 1 Fatty acid composition (mg/g fat) of the unfermented oil, the *B lactis* Bb12 fermented oil and the *B breve* NCFB 2258 fermented oil

	Unfermented oil (mg/g fat)	*B lactis* Bb12 fermented oil (mg/g fat)	*B breve* NCFB 2258 fermented oil (mg/g fat)
Linoleic acid	610	397	251
Oleic acid	264	400	353
*c*9, *t*11 CLA	8 0	16	239
*t*9, *t*11 CLA	4 9	11	19

The effects of an unfermented oil extract and two microbially fermented oil extracts on SW480 cell growth were then analysed using the oils at a concentration of 84 μg oil/ml and 33 μg oil/ml as presented in **Tables 3 2** and **3 3** respectively

Table 3 2 Fatty acid composition of bacterially fermented oil treatments when at a concentration of 84 μg oil/ml in ethanol

Fatty Acid	Unfermented Oil μg/ml	*B lactis* Bb12 Oil μg/ml	*B breve* NCFB 2258 Oil μg/ml
Linoleic acid	51 2	33 3	21 1
Oleic acid	22 2	33 6	29 6
*c*9, *t*11 CLA	0 7	1 4	20
*t*9, *t*11 CLA	0 4	0 9	1 6

Table 3.3 Fatty acid composition of bacterially fermented oil treatments when at a concentration of 33 μg oil/ml in ethanol

Fatty Acid	Unfermented Oil μg/ml	*B lactis* Bb12 Oil μg/ml	*B breve* NCFB 2258 Oil μg/ml
Linoleic acid	20	13	8 2
Oleic acid	8 7	13 1	11 6
*c*9, *t*11 CLA	0 3	0 5	7 8
*t*9, *t*11 CLA	0 2	0 4	0 6

Finally, the effects of various combinations of the four fatty acid constituents of the oils (LA, oleic acid, *c*9, *t*11 CLA and *t*9, *t*11 CLA) were examined

3 3 1 Effect of C18 Fatty Acids on SW480 Cell Growth

Figure 3 2 represents % cell viability of SW480 cells following incubation with either LA (0-55 µg/ml), oleic acid (0-40 µg/ml), *c*9, *t*11 CLA (0-20 µg/ml) and t9, t11 CLA (0-10 µg/ml) relative to control cells (100% viability) treated only with 0 1% (v/v) ethanol Incubation of SW480 cells with LA (25 µg/ml) had negligible effects after 24 h but increased viable cell numbers by 10% and 27% (P<0 05) after 48 h and 4 days respectively (**Figure 3 2A**) However, at higher concentrations, LA (40 and 55 µg/ml) significantly reduced (P<0 001) cell growth by 61% and 95% respectively after 24 h incubation, 82% and 92% respectively after 48 h incubation and 89% and 99% respectively after 4 days incubation

Incubation with oleic acid at concentrations of 30 and 40 µg/ml significantly (P<0 01) reduced SW480 cell growth by 30% and 32% respectively after 24 h and by 57% and 67% after 48 h (P<0 001) In addition, oleic acid at a concentration of 20 µg/ml significantly (P<0 05) reduced SW480 cell growth by 25% after 48 h incubation (**Figure 3 2B**) Decreased potency of oleic acid was observed after 4 days, with cell growth being reduced by 32% (P<0 05) and 41% (P<0 01) following incubation with 30 and 40 µg/ml respectively These data suggest that there is an optimum concentration (40 µg/ml) and incubation period (48 h) for observing inhibition of cell growth by oleic acid

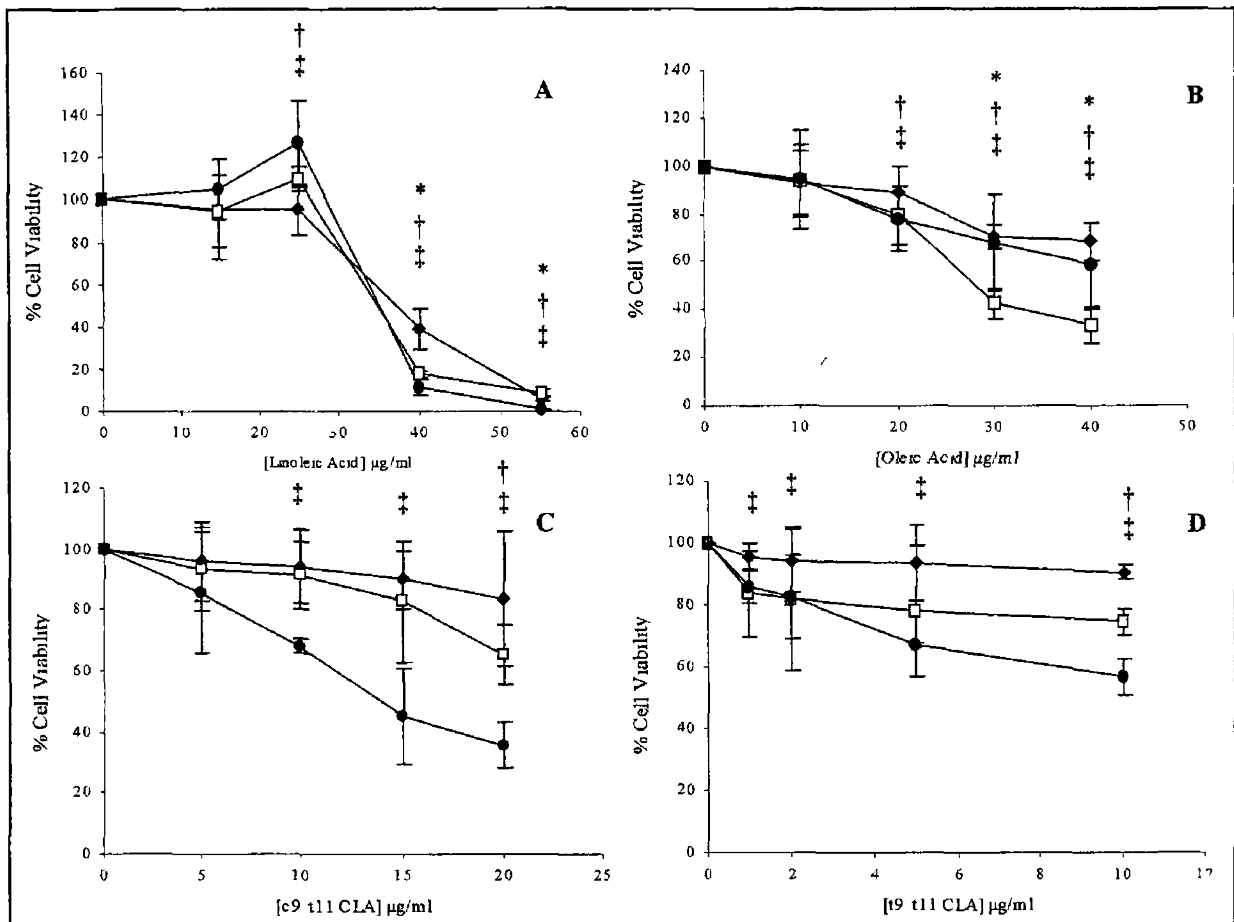

Figure 3 2 % viability of SW480 cells following incubation with (A) linoleic acid, (B) oleic acid, (C) *c*9, *t*11 CLA, (D) *t*9, *t*11 CLA for 24 h (♦), 48 h (□), and 4 days (●) compared with ethanol control Analysis was carried out three times in triplicate, data represent the mean of the three individual experiments
* denotes values that are significantly different compared with ethanol control after 24 h incubation † denotes values that are significantly different compared with ethanol control after 48 h incubation ‡ denotes values that are significantly different compared with ethanol control after 4 days incubation For clarity, P values are inserted in text

SW480 cell growth was not significantly affected by *c*9, *t*11 CLA (5-20 µg/ml) after 24 h incubation compared with ethanol control However, after 48 h *c*9, *t*11 CLA (20 µg/ml) significantly ($P<0\ 05$) reduced cell growth by 35% while after 4 days *c*9, *t*11 CLA at 10, 15 and 20 µg/ml all reduced growth ($P<0\ 001$) by 32%, 55% and 65% respectively (**Figure 3 2C**) These data indicate the increase in potency of this CLA isomer with time

The *t*9, *t*11 CLA isomer (1-10 µg/ml) did not significantly influence SW480 cell growth after 24 h incubation However, this CLA isomer at a concentration of 10 µg/ml significantly reduced growth by 26% (P<0 001) after 48 h incubation and by 43% (P<0 001) after 4 days incubation In addition 1, 2 and 5 µg/ml of *t*9, *t*11 CLA reduced SW480 cell growth by 14% (P<0 05), 21% (P<0 05) and 33% (P<0 001) respectively after 4 days (**Figure 3 2D**) As observed with the *c*9, *t*11 CLA isomer, the potency of the *t*9, *t*11 CLA isomer was much greater after 4 days incubation compared with 24 h and 48 h

3 3 2 Effect of Oils on SW480 Cell Growth

SW480 cells were incubated with the unfermented oil, the *B lactis* Bb12 fermented oil and the *B breve* NCFB 2258 fermented oil all at a concentration of 84 µg/ml for periods of 24 h, 48 h and 4 days This concentration was chosen as at this concentration the *B lactis* NCFB 2258 fermented oil contained 20 µg/ml of the *c*9, *t*11 CLA isomer, a concentration which has been shown previously to inhibit SW480 cell growth (O'Shea, 2000) Following incubation viability of the cells was determined by Trypan Blue exclusion The composition of each oil at this concentration is presented in **Table 3 2** As with the individual fatty acids (**Section 3 3 1**) all treatment of SW480 cells was compared to control cells treated with 0 1% (v/v) ethanol, the viability of which was taken as 100%

All three oils significantly (P<0 05) reduced SW480 cell growth relative to the ethanol control in a time dependant manner after 24 h, 48 h and 4 days (**Figure 3 3**) The unfermented oil reduced growth by 47% after 24 h (P<0 01), 83% after 48 h (P<0 001) and 98% after 4 days (P<0 001) Both the *B lactis* Bb12 and *B breve* NCFB 2258

fermented oils were significantly (P<0.05) more potent than the unfermented oil after 24 h (**Figure 3.3**) reducing growth by 62% and 57% respectively after 24 h. No significant difference in growth inhibition was observed between the oils after 48 h and 4 days incubation.

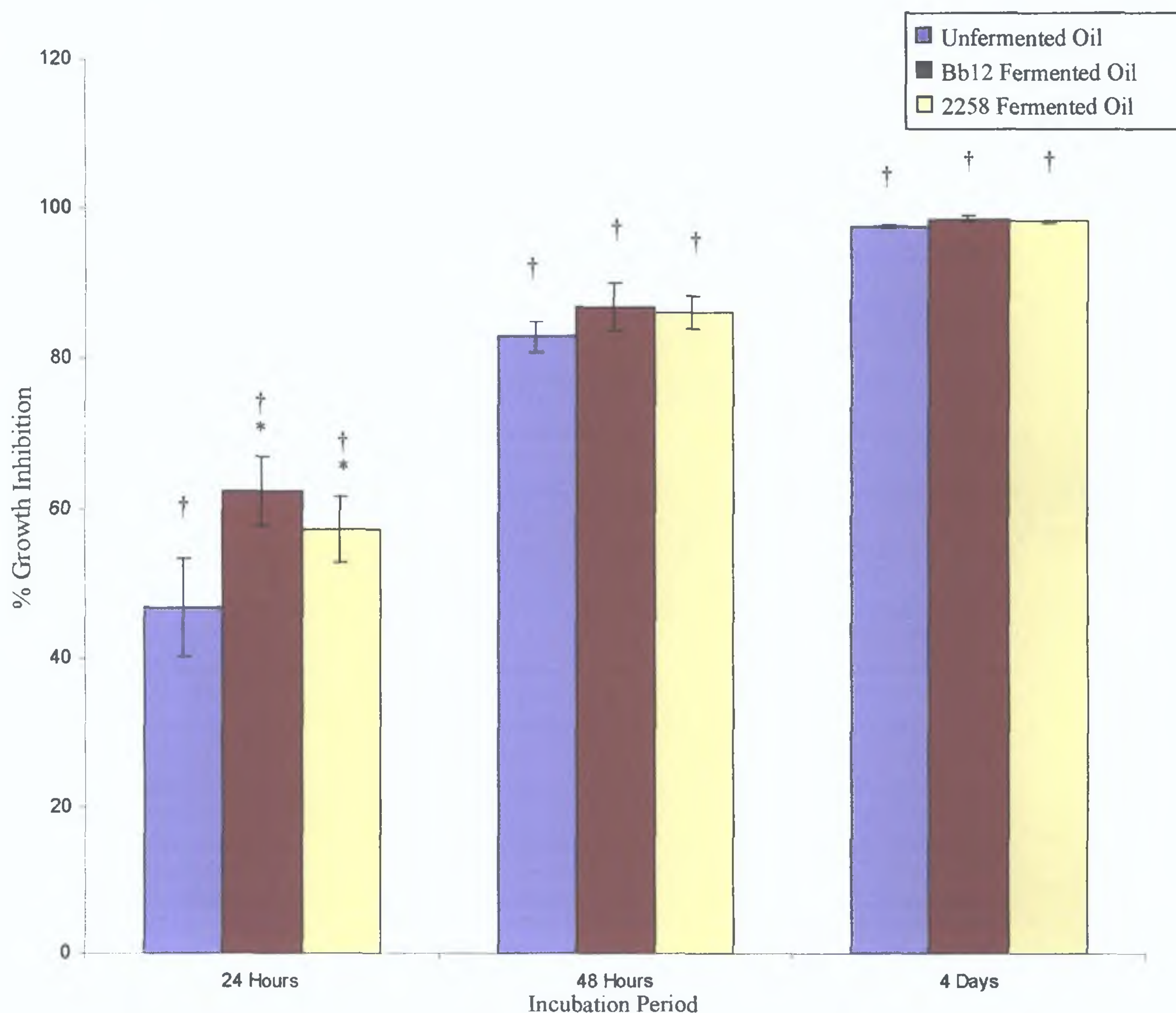

Figure 3.3 Effect of *B. lactis* Bb12 and *B. breve* NCFB 2258 fermented oils on SW480 cell growth compared with unfermented oil at a concentration of 84 µg/ml after 24 h, 48 h, and 4 days incubation. * denotes values that are significantly different (P<0.05) compared with unfermented oil. † denotes values that are significantly different (P<0.05) compared with ethanol control (0% inhibition).

There was no significant difference between the growth inhibitory effect of the *B lactis* Bb12 fermented oil and that of the *B breve* NCFB 2258 fermented oil at each of the three time points

3 3 3 Effect of Fermented Oils on SW480 Cell Growth at 40% Reduced Concentration

At a concentration of 84 μg/ml oil, the *B breve* NCFB 2258 fermented oil contained 20 μg/ml of the *c*9, *t*11 CLA isomer – a concentration which has previously been demonstrated to be cytotoxic to the SW480 cell line (O'Shea, 2000) However at this concentration the unfermented oil contained a very cytotoxic concentration of LA (51 μg/ml) as seen in **Figure 3 2A** LA has been shown to stimulate SW480 cell growth at a concentration of 20 μg/ml (O'Shea, 2000) To exclude the inhibitory effect of LA when the oils are present at 84 μg/ml, the concentration of each oil was reduced to 33 μg/ml before incubation with SW480 cells for 24 h, 48 h and 4 days **Table 3 3** presents the fatty acid composition of the three oils when at a concentration of 33 μg/ml At a concentration of 84 μg/ml the *B breve* NCFB 2258 fermented oil contained 20 μg/ml *c*9, *t*11 CLA, whereas 33 μg/ml of the unfermented oil contained 20 μg/ml LA

All three oils were significantly cytotoxic to cell growth when presented to cells at 33 μg/ml after 24 h, 48 h and 4 days (**Figure 3 4**) The unfermented oil reduced growth by 23% after 24 h, 62% after 48 h and 96% after 4 days The *B lactis* Bb12 fermented oil was more potent ($P<0.05$) than the unfermented oil after 24 h, reducing growth by 62% However after 48 h and 4 days, no significant difference was observed between the unfermented oil and either of the two fermented oils

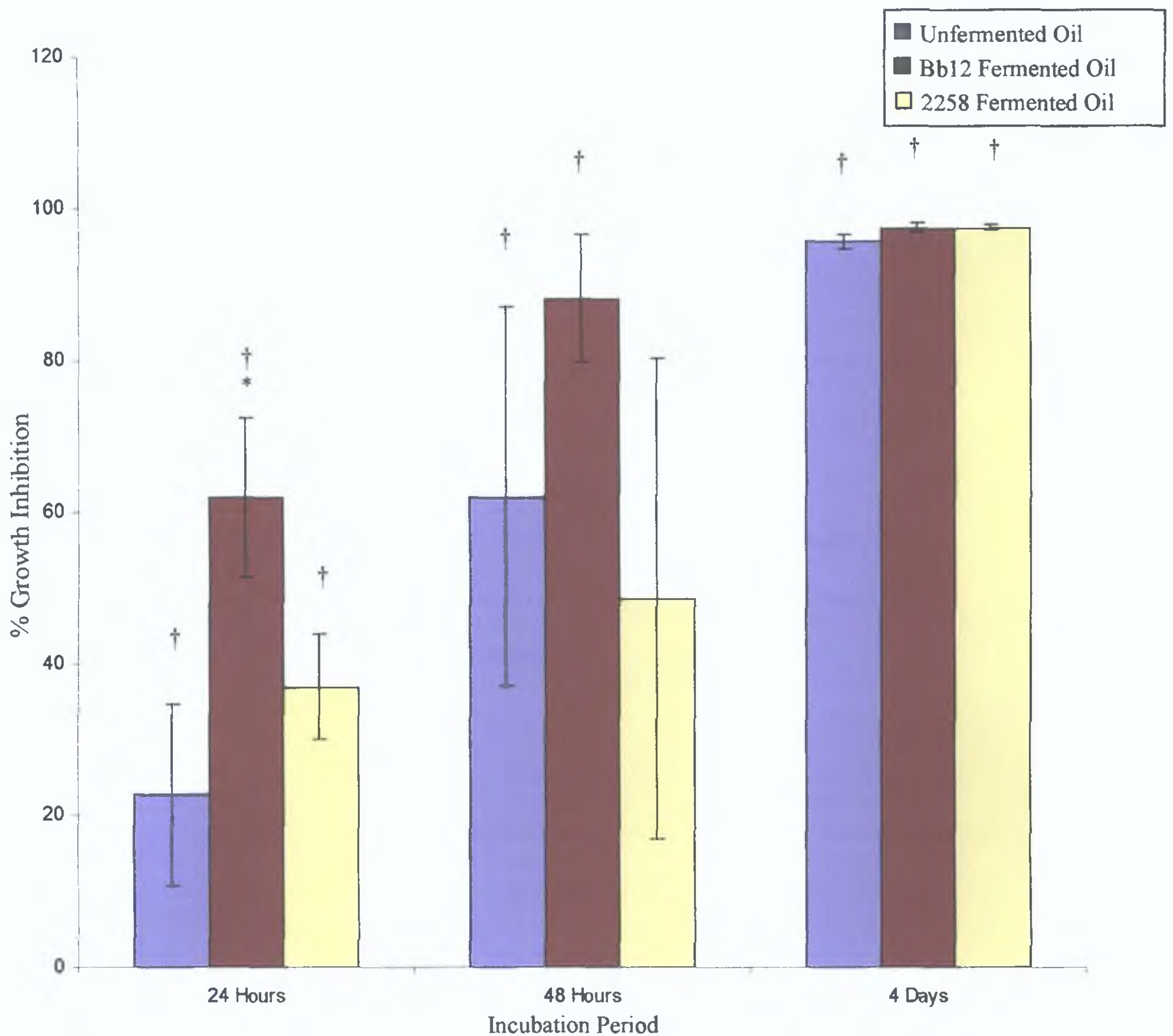

Figure 3.4 Effect of *B. lactis* Bb12 and *B. breve* NCFB 2258 fermented oils on SW480 cell growth compared with unfermented oil at a concentration of 33 µg/ml after 24 h, 48 h, and 4 days incubation. * denotes values that are significantly different ($P<0.05$) compared with unfermented oil. † denotes values that are significantly different ($P<0.05$) compared with ethanol control (0% inhibition).

Figure 3.5 compares the growth inhibitory effects of the three oils at concentrations of 84 µg/ml oil and 33 µg/ml at the various time points. It is apparent that reducing the concentration of the oils by 40% did not significantly reduce the inhibitory effect of the unfermented oil at any time point.

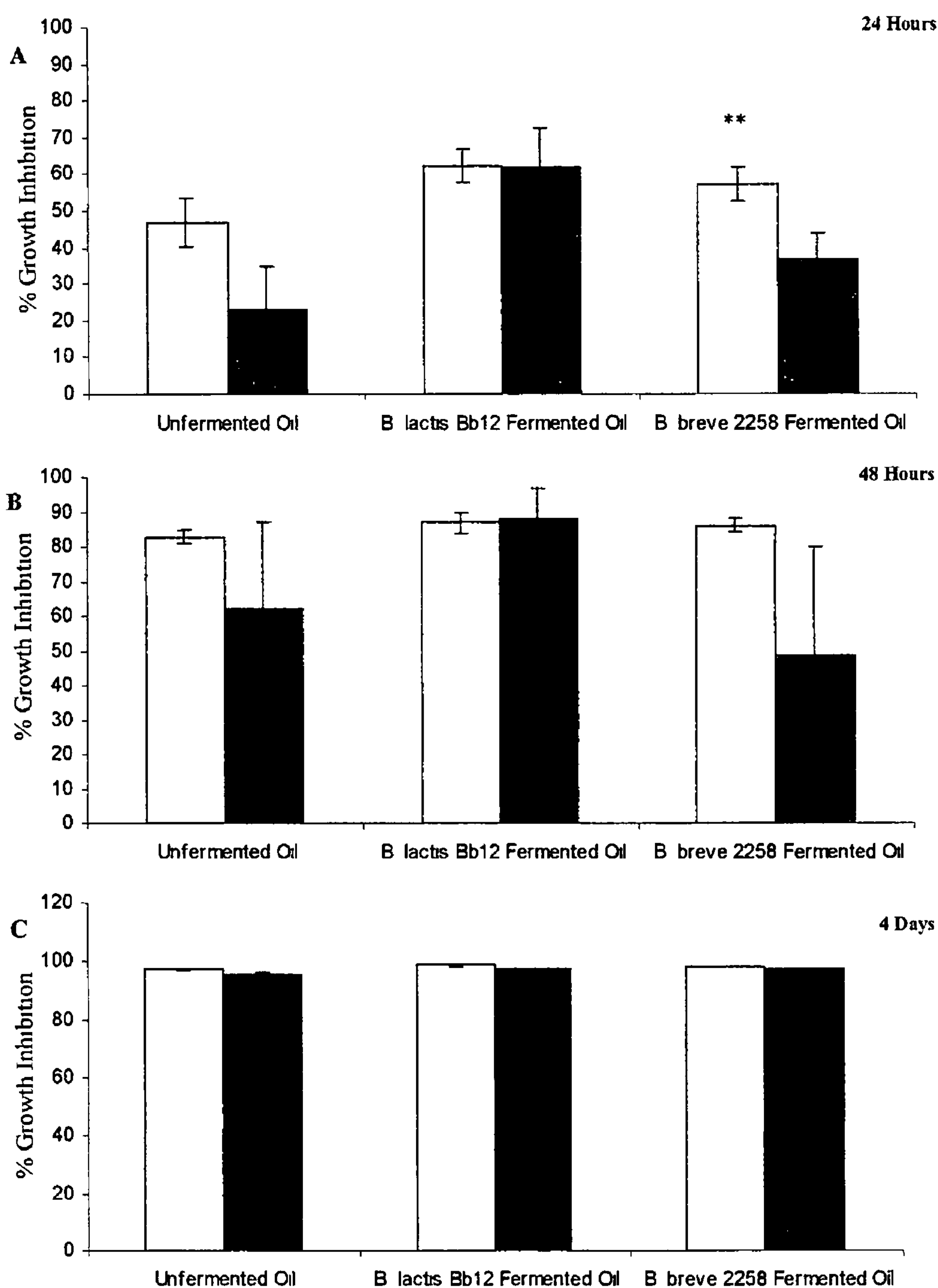

Figure 3 5 Comparison between the growth inhibitory effect of 84 μg/ml (□) and 33 μg/ml (■) of each of the three oils after (A) 24 h, (B) 48 h and (C) 4 days incubation All data values are relative to ethanol control (0% growth inhibition) ** denotes values which significantly different (P<0 01) compared with 33 μg/ml of oil

Only incubation of cells with *B breve* NCFB 2258 (33 µg/ml) for 24 h was significantly (P<0 01) less inhibitory than the corresponding treatment at 84 µg/ml

3 3 4 Elucidation of Inhibitory Fatty Acids in Unfermented Oil

The unfermented oil was prepared by the addition of LA to media without fermentation, incubated under the same conditions as the fermented oils and extracted as described in **Section 3 2** The extracted oil was diluted m ethanol When added to SW480 cells at a concentration of 84 µg/ml the unfermented oil treatment consisted of 51 µg/ml LA, 22 µg/ml oleic acid, 0 7 µg/ml *c*9, *t*11 CLA and 0 4 µg/ml *t*9, *t*11 CLA in ethanol

As was seen in **Figure 3 3**, incubation with the unfermented oil significantly (P<0 05) reduced cell numbers after 24 h, 48 h and 4 days incubation, with growth inhibition increasing with time

To obtain a clearer picture of the effect of the individual fatty acids present in the oils on SW480 cell growth, synthetic isomers of these fatty acids were analysed individually at concentrations representative of those present in each of the three oils analysed In addition, to account for any interactions between these fatty acids in the oils, a number of combinations of the fatty acids were prepared and incubated with SW480 cells These preparations were called synthetic representations as they consisted of synthetically produced isomers of the fatty acids The LA synthetic representation of the unfermented oil consisted of 51 µg/ml of pure LA dissolved m ethanol

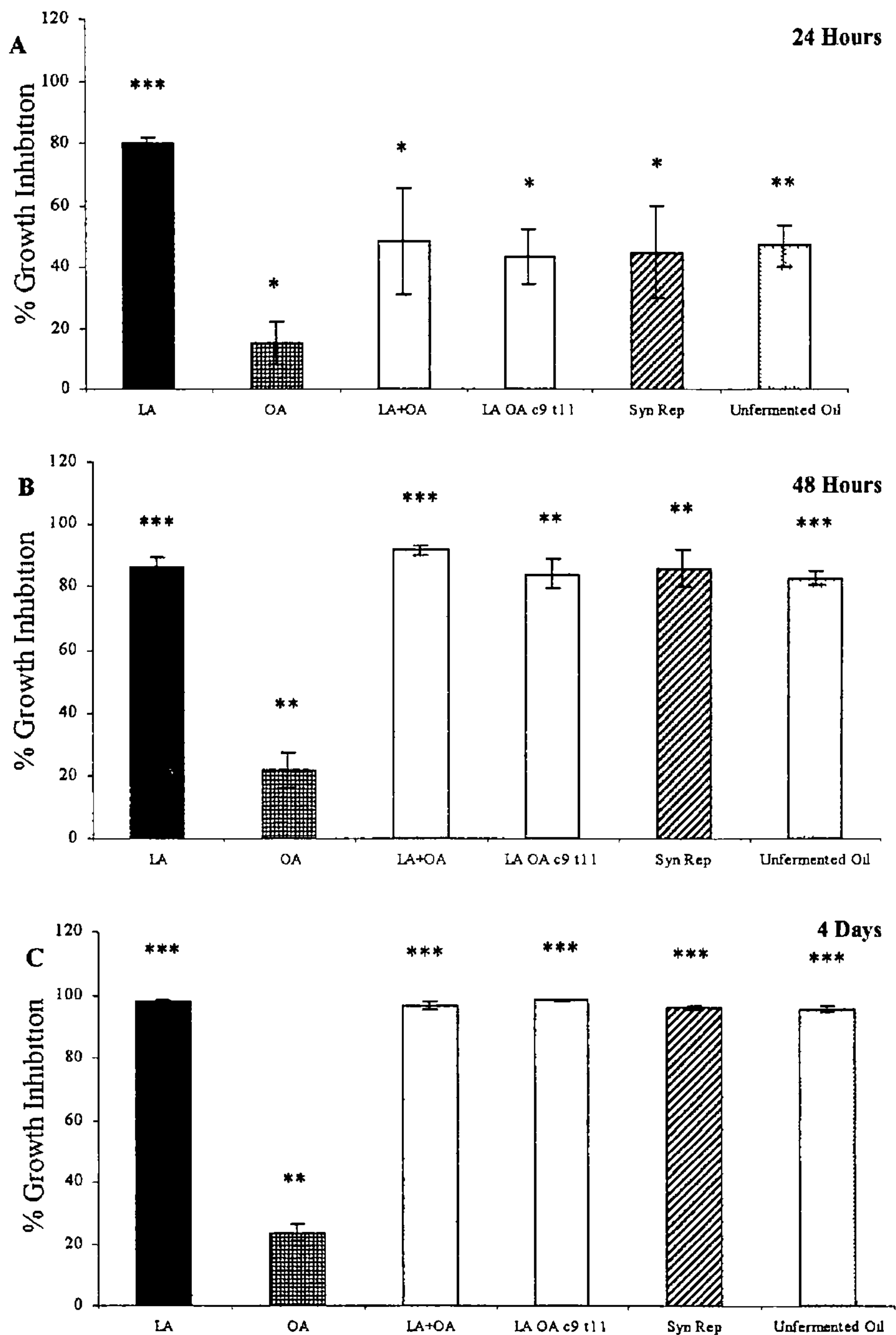

Figure 3 6 Effect of 84 µg/ml Unfermented Oil and the individual fatty acids present in the oil on growth after (A) 24 h, (B) 48 h and (C) 4 days expressed as % growth inhibition The composition of the Unfermented Oil and hence the Synthetic Representation (Syn Rep) was LA (51 µg/ml), oleic acid (OA) (22 µg/ml), *c*9, *t*11 CLA (0 7 µg/ml) and *t*9, *t*11 CLA (0 4 µg/ml) * denotes values that are significantly different (P<0 05) compared with ethanol control (0% inhibition), ** denotes values that are significantly different (P<0 01) compared with ethanol control, *** denotes values that are significantly different (P<0 001) compared with ethanol control

After 24 h incubation, the LA synthetic representation reduced SW480 cell numbers by 80% (P<0 001) compared with the ethanol control (**Figure 3 6A**) Pure oleic acid alone at a concentration of 22 µg/ml significantly reduced (P<0 05) SW480 cell growth by 15% after 24 h

Combining LA and oleic acid at a concentration of 51 µg/ml and 22 µg/ml respectively and incubating with SW480 cells for 24 h significantly (P<0 05) reduced growth by 48% compared with the ethanol control suggesting the possibility of a protective effect of oleic acid against the growth inhibitory effect of linoleic acid (**Figure 3.6A**)

A combination of LA, oleic acid and *c*9, *t*11 CLA at the concentrations present in the unfermented oil treatment also significantly reduced (P<0 05) cell growth compared with the ethanol control, as did a combination of all four fatty acids – LA, oleic acid, *c*9, *t*11 CLA and *t*9, *t*11 CLA (a complete representation of the unfermented oil) However, neither of these two combinations produced effects that were different to the combination of LA and oleic acid It is apparent that the inhibition (45%) caused by the complete synthetic representation of the unfermented oil was similar to that produced by the unfermented oil (47%)

Of the combinations of synthetic fatty acids used to represent the fatty acid composition of the unfermented oils, LA alone at a concentration of 51 µg/ml had the most significant inhibitory effect on SW480 cell growth after incubation for 24 h

After 48 h LA (51 µg/ml) further reduced cell growth by 86% (P<0 001) compared with control cells (**Figure 3 6B**) Oleic acid alone (22 µg/ml) significantly reduced (P<0 01) cell growth by 22% after 48 h Incubation of SW480 cells with a combination of LA (51 µg/ml) and oleic acid (22 µg/ml) for 48 h significantly reduced (P<0 001) cell growth by 92% A combination of LA, oleic acid and *c*9, *t*11 CLA (0 7 µg/ml) also reduced growth (84%) but was not significantly different to the effect produced by the combination of LA and oleic acid

The synthetic representation of the unfermented oil containing the four fatty acids significantly reduced (P<0 01) cell growth after 48 h by (86%) but was not significantly different to the effect produced by the combination of LA, oleic acid and *c*9, *t*11 CLA As observed after 24 h incubation, it was apparent that the inhibition caused by the complete synthetic representation of the unfermented oil was similar to the produced by the unfermented oil (83%)

After 4 days incubation with SW480 cells the treatment consisting of 51 µg/ml of synthetic LA significantly reduced (P<0 001) cell growth by 98% (**Figure 3 6C**) Oleic acid alone at a concentration of 22 µg/ml significantly reduced (P<0 01) cell growth by 24% The combination of LA and oleic acid significantly reduced (P<0 001) cell growth by 97% while the combination of LA, oleic acid and *c*9, *t*11 CLA significantly reduced (P<0 001) SW480 growth by 98% The synthetic mixture of all four fatty acids representative of the unfermented oil significantly reduced (P<0 001) cell growth by 96% and was similar to the effect produced by the unfermented oil (96% inhibition) Together

these data suggest that it was the fatty acids present in the unfermented oil, in particular the high content of LA, which led to the growth inhibitory effect of 84 µg/ml of the unfermented oil

When comparing the inhibitory effect of the treatments presented in **Figure 3 6**, it was found that the inhibitory effect of LA (51 µg/ml) significantly increased (P<0 05) with time (from 80-98%) Though the inhibitory effect of oleic acid (22 µg/ml) changed only slightly with time (from 15-24%) the effect of the combination of LA and oleic acid changed from 48-97% over the 4 days Similar time dependant effects were observed for the combination of LA, oleic acid and *c*9, *t*11 CLA (43-98%), the combination of LA, oleic acid, *c*9, *t*11 CLA and *t*9, *t*11 CLA (45-96%) as well as the unfermented oil (47-96%) Thus, an incubation period of 4 days yielded optimal inhibition of SW480 cell growth It has previously been demonstrated that 4 days incubation with CLA are required to observe substantial growth inhibition of SW480 cells (O'Shea, *et al* , 2000)

When added to SW480 cells at a concentration of 33 µg/ml, the unfermented oil consisted of 20 µg/ml LA, 8 7 µg/ml oleic acid, 0 3 µg/ml *c*9, *t*11 CLA and 0 2 µg/ml *t*9, *t*11 CLA As was seen in **Figure 3 4**, maximum inhibition was obtained following incubation with the unfermented oil for 4 days **Figure 3 7** presents the effects of various combinations of these four fatty acids over 24 h, 48 h and 4 days incubation periods

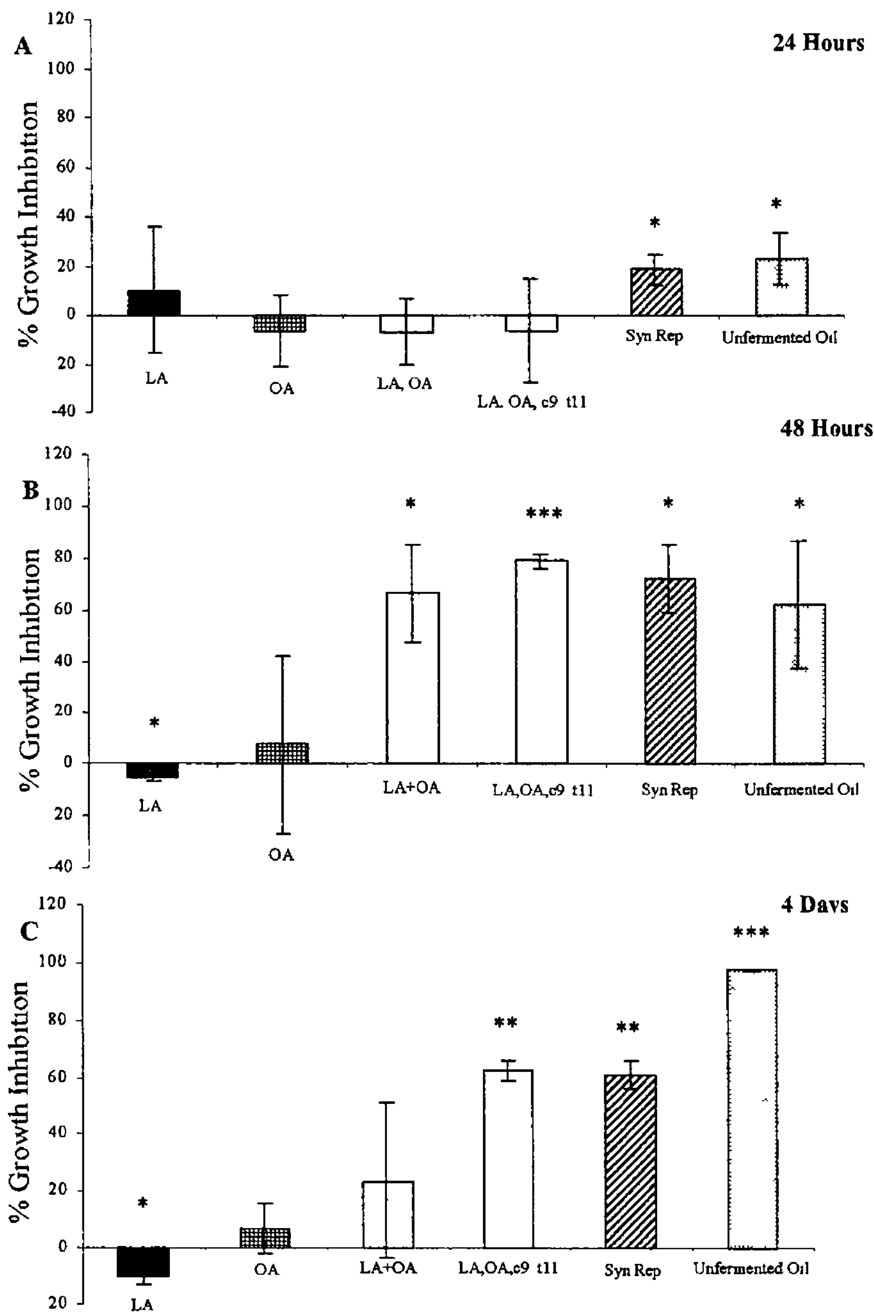

Figure 3 7 Effect of 33 µg/ml Unfermented Oil and the individual fatty acids present in the oil on growth after (A) 24 h, (B) 48 h and (C) 4 days expressed as % growth inhibition The composition of the Unfermented Oil and hence the Synthetic Representation (Syn Rep) was LA (20 µg/ml), oleic acid (OA) (8 7 µg/ml), *c*9, *t*11 CLA (0 3 µg/ml) and *t*9, *t*11 CLA (0 2 µg/ml) * denotes values that are significantly different (P<0 05) compared with ethanol control (0% inhibition), ** denotes values that are significantly different (P<0 01) compared with ethanol control, *** denotes values that are significantly different (P<0 001) compared with ethanol control

It is apparent that neither LA alone, oleic acid alone nor the combination of LA and oleic acid had any effect on cell growth after 24 h The combination of LA, oleic acid and *c*9, *t*11 CLA had a negligible effect after 24 h but the combination of all four fatty acids – LA, oleic acid, *c*9, *t*11 CLA and *t*9, *t*11 CLA did decrease growth by approximately 18% after this incubation period, similar to that produced by the unfermented oil (23%) (**Figure 3 7A**)

After 48 h incubation LA alone significantly (P<0 05) increased cell growth by 5%, while the combination of synthetic isomers of LA and oleic acid, the combination of LA, oleic acid and *c*9, *t*11 CLA and the combination of synthetic isomers of all four fatty acids present in the unfermented oil all significantly reduced cell growth by 66% (P<0 05), 79% (P<0 001) and 72% (P<0 05) respectively (**Figure 3 7B**) As demonstrated in **Figure 3 4** the unfermented oil reduced cell growth by 62%

Following 4 days incubation of fatty acids with SW480 cells, only the synthetic combinations containing *c*9, *t*11 CLA and *t*9, *t*11 CLA were inhibitory by 61-62% LA alone (20 μg/ml) significantly increased (P<0 05) cell growth by 10% (**Figure 3 7C**) In **Figure 3 2A** it was demonstrated that 25 μg/ml LA stimulated SW480 cell growth after 4 days incubation The unfermented oil significantly reduced (P<0 001) cell growth by 98% It is clear that LA did not influence the inhibitory effect of the oil at this concentration as the concentration of LA present in 33 μg/ml of the unfermented oil had a stimulatory effect on SW480 cells after 4 days incubation Interestingly, the unfermented oil was significantly more potent (P<0 01) at inhibiting SW480 cell growth compared with the

synthetic mixture of all four fatty acids of the oil (61% growth inhibition) or the mixture of LA, oleic acid and *c*9, *t*11 CLA (62% growth inhibition) This suggests that another inhibitory factor, more potent than *c*9, *t*11 CLA or *t*9, *t*11 CLA, may be present in the unfermented oil at the concentration tested Although the potency of the synthetic mixture of all four fatty acids and the combination of LA, oleic acid and *c*9, *t*11 CLA seemed to decrease between 48 h and 4 days the potency of the unfermented oil increased over this time period These data further suggest the presence of another inhibitory factor in the oil which is responsible for the growth inhibitory effect of the oil at a concentration of 33 µg/ml The diminished inhibitory effect of the synthetic fatty acids is upheld by **Figure 3 2** in which such concentrations of LA enhanced cell growth, whereas such concentrations of oleic acid, *c*9, *t*11 CLA and *t*9, *t*11 CLA had negligible effects on cell growth A difference in how fatty acids are presented to cells (e g bound or 'free') may also account for the difference in potency observed between the oil and the synthetic combination

3 3 5 Elucidation of Inhibitory Fatty Acids in *B lactis* Bb12 Fermented Oil

B lactis Bb12 was incubated in the presence of LA (0 5 mg/ml) for 48 h and the fatty acid composition of the spent media was analysed The fatty acids were extracted and dissolved in ethanol prior to anticancer activity analysis At a concentration of 84 µg/ml the *B lactis* Bb12 fermented oil consisted of LA (33 µg/ml) in addition to the other three fatty acids After 24 h incubation LA (33 µg/ml) significantly reduced ($P<0.05$) cell growth by 30% (**Figure 3 8A**) Similarly, oleic acid (34 µg/ml), the concentration of oleic acid present in the *B lactis* Bb12 fermented oil, reduced cell growth by 27% ($P<0.05$)

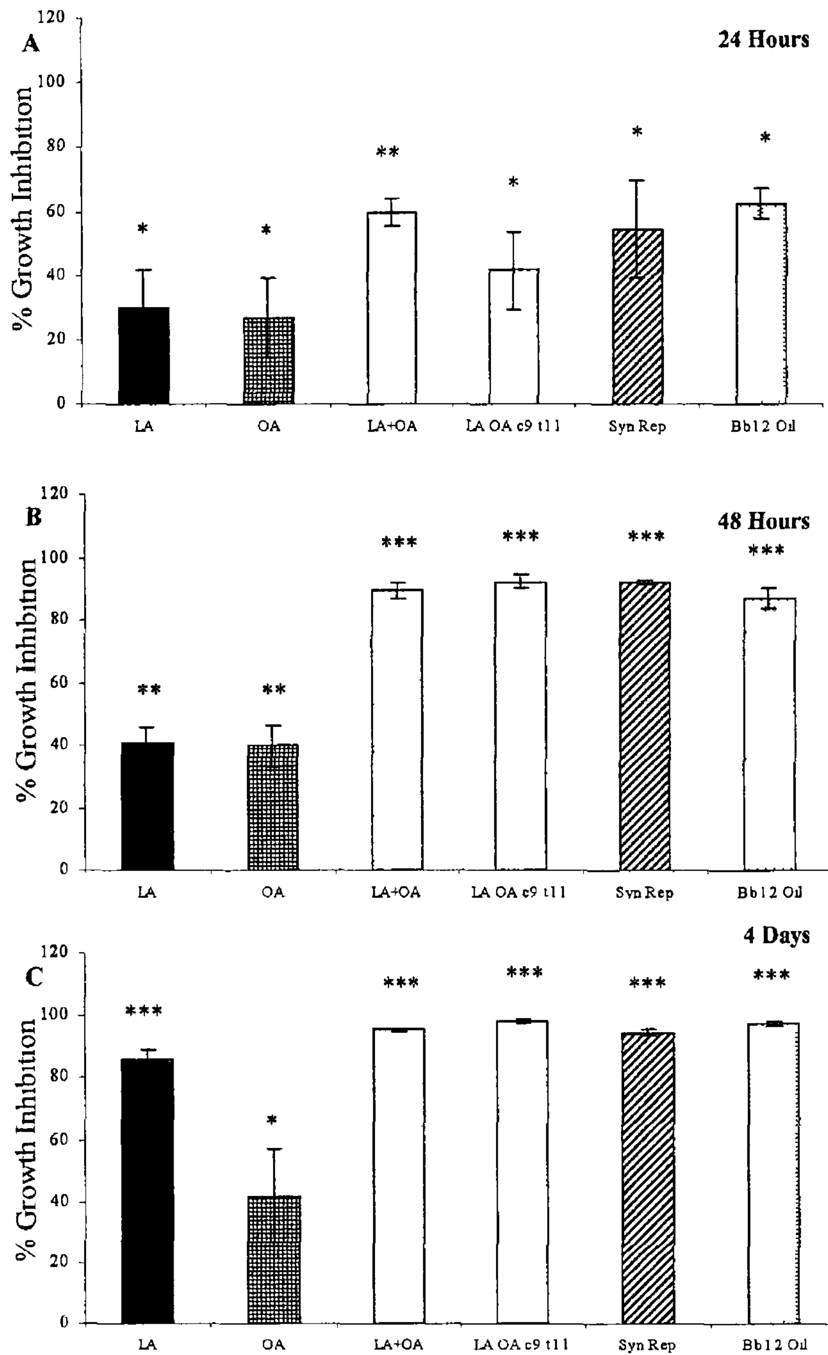

Figure 3 8 Effect of 84 μg/ml *B lactis* Bb12 fermented oil and the individual fatty acids present in 84 μg/ml of the oil on growth after (A) 24 h, (B) 48 h and (C) 4 days expressed as % growth inhibition compared with ethanol control (0% inhibition) The composition of the *B lactis* Bb12 fermented oil and hence the Synthetic Representation (Syn Rep) was LA (33 μg/ml), oleic acid (OA) (33 μg/ml), *c*9, *t*11 CLA (1 4 μg/ml) and *t*9, *t*11 CLA (0 9 μg/ml) * denotes values that are significantly different (P<0 05) compared with ethanol control (0% inhibition), ** denotes values that are significantly different (P<0 01) compared with ethanol control, *** denotes values that are significantly different (P<0 001) compared with ethanol control

Both of these observations are consistent with the data shown in **Figure 3 2** showing inhibitory effects of LA at concentrations >25 μg/ml and of oleic acid at concentrations >30 μg/ml Combining LA and oleic acid at the concentrations present in the *B lactis* Bb12 fermented oil led to greater reductions in growth (60% growth inhibition) compared to these fatty acids used separately

A combination of LA (33 μg/ml), oleic acid (34 μg/ml) and *c*9, *t*11 CLA (1 4 μg/ml) also reduced cell growth by 41% (P<0 05) while the mixture of synthetic isomers of the four fatty acids present in the *B lactis* Bb12 fermented oil reduced SW480 cell growth by 54% (P<0 05) The *B lactis* Bb12 fermented oil significantly (P<0 01) reduced cell growth by 62% after 24 h incubation with SW480 cells This inhibitory effect was not significantly different compared to that of the synthetic fatty acid representation of the *B lactis* Bb12 fermented oil after 24 h incubation

After 48 h incubation, both LA and oleic acid significantly reduced growth by 40% (P<0 01) (**Figure 3 8B**) Combining these two fatty acids in the concentrations present in the *B lactis* Bb12 fermented oil significantly reduced growth (P<0 001) by 90% and therefore represented a much greater inhibition than either fatty acid alone Similar reductions in cell growth (92%, P<0 001) were observed following incubation with a combination of LA, oleic and *c*9, *t*11 CLA and with the mixture of synthetic isomers of the four fatty acids (92%, P<0 001) It is apparent that the inhibition (92%) caused by the complete synthetic representation of the *B lactis* Bb12 fermented oil was similar to that produced by the fermented oil itself (87%)

After 4 days incubation all synthetic combinations containing LA reduced growth by 86-98% and were not significantly different The *B lactis* Bb12 fermented oil significantly reduced (P<0 001) SW480 cell growth by 97% compared with the ethanol control (**Figure 3 8C**) There was no significant difference between the growth inhibitory effect of the *B lactis* Bb12 fermented oil and the synthetic representation of this oil which reduced cell growth by 95% (P<0 001) Oleic acid alone was less potent than when present in combination with LA, reducing growth by 42% relative to ethanol control The data suggest that cytotoxic concentrations of fatty acids such as LA and oleic acid are contributory factors to growth inhibition caused by the *B lactis* Bb12 fermented oil

When added to SW480 cells at a concentration of 33 μg/ml the *B lactis* Bb12 fermented oil consisted of 13 μg/ml LA, 13 μg/ml oleic acid, 0 5 μg/ml *c*9, *t*11 CLA and 0 4 μg/ml *t*9, *t*11 CLA As was seen in **Figure 3 4**, incubation with the fermented oil significantly (P<0 05) reduced cell numbers after 24 h (62%), 48 h (88%) and 4 days (99%) incubation, with growth inhibition increasing with time **Figure 3 9** presents the effects of the various combinations of these fatty acids on cell growth after 24 h, 48 h and 4 days incubation

As expected neither LA nor oleic acid influenced cell growth after 24 h, 48 h or 4 days incubation Interestingly the combination of LA and oleic acid did inhibit (P<0 05) growth by 15%, 38% and 16% after 24 h, 48 h and 4 days respectively

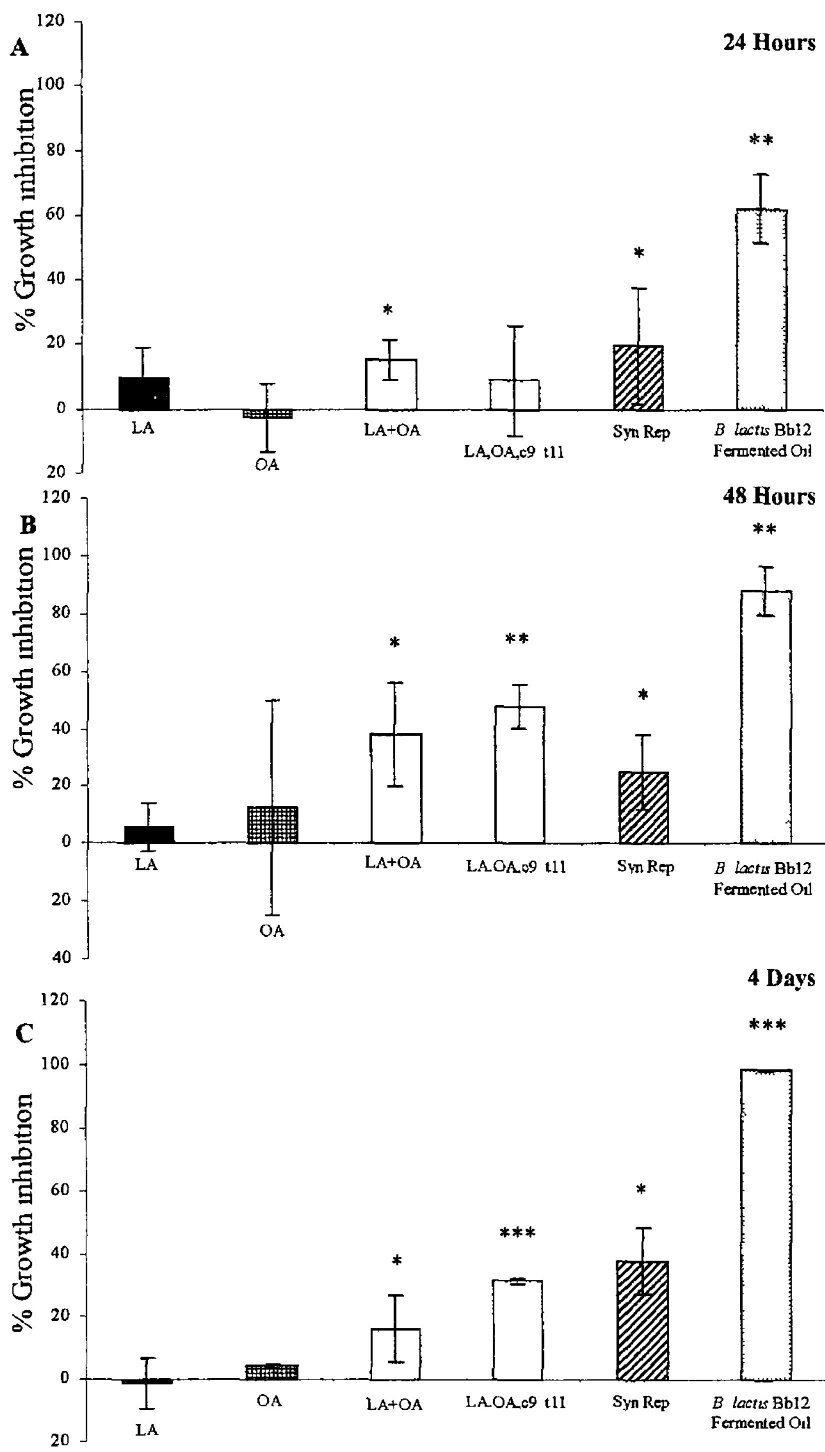

Figure 3 9 Effect of 33 µg/ml of the *B lactis* Bb12 fermented oil and the individual fatty acids present in the oil on growth after (A) 24 h, (B) 48 h and (C) 4 days expressed as % growth inhibition The composition of the Unfermented Oil and hence the Synthetic Representation (Syn Rep) was LA (13 µg/ml), oleic acid (OA) (13 µg/ml), *c*9, *t*11 CLA (0 5 µg/ml) and *t*9, *t*11 CLA (0 4 µg/ml) * denotes values that are significantly different (P<0 05) compared with ethanol control (0% inhibition), ** denotes values that are significantly different (P<0 01) compared with ethanol control *** denotes values that are significantly different (P<0 001) compared with ethanol control

The synthetic combination containing LA, oleic acid and *c*9, *t*11 CLA had a negligible effect after 24 h, but decreased growth by 48% (P<0 01) and 32% (P<0 001) after 48 h and 4 days respectively

Interestingly, the synthetic combination of all four fatty acids significantly inhibited growth by 20% after only 24 h, with growth being reduced further following 48 h (25%) and 4 days (38%) However, this synthetic combination was less potent than the *B lactis* Bb12 fermented oil after 48 h and 4 days suggesting that other inhibitory components may be present in the oil

3 3 6 Elucidation of Inhibitory Fatty Acids in *B breve* NFCB 2258 Fermented Oil

B breve NFCB 2258 was incubated in the presence of LA (0 5 mg/ml) for 48 h and the fatty acid composition of the spent media was analysed (**Table 3 1**) The fatty acids were extracted and dissolved in ethanol At a concentration of 84 µg/ml of the *B breve* NCFB 2258 fermented oil consisted of 21 µg/ml LA, 30 µg/ml oleic acid, 20 µg/ml *c*9, *t*11 CLA and 1 6 µg/ml *t*9, *t*11 CLA

After 24 h incubation, LA alone had a negligible effect but was stimulatory towards cell growth after 48 h and 4 days incubation Oleic acid alone reduced cell growth (P<0 05) by 27-37% over 4 days The combination of LA and oleic acid was inhibitory, reducing growth by 33-75% over the same period

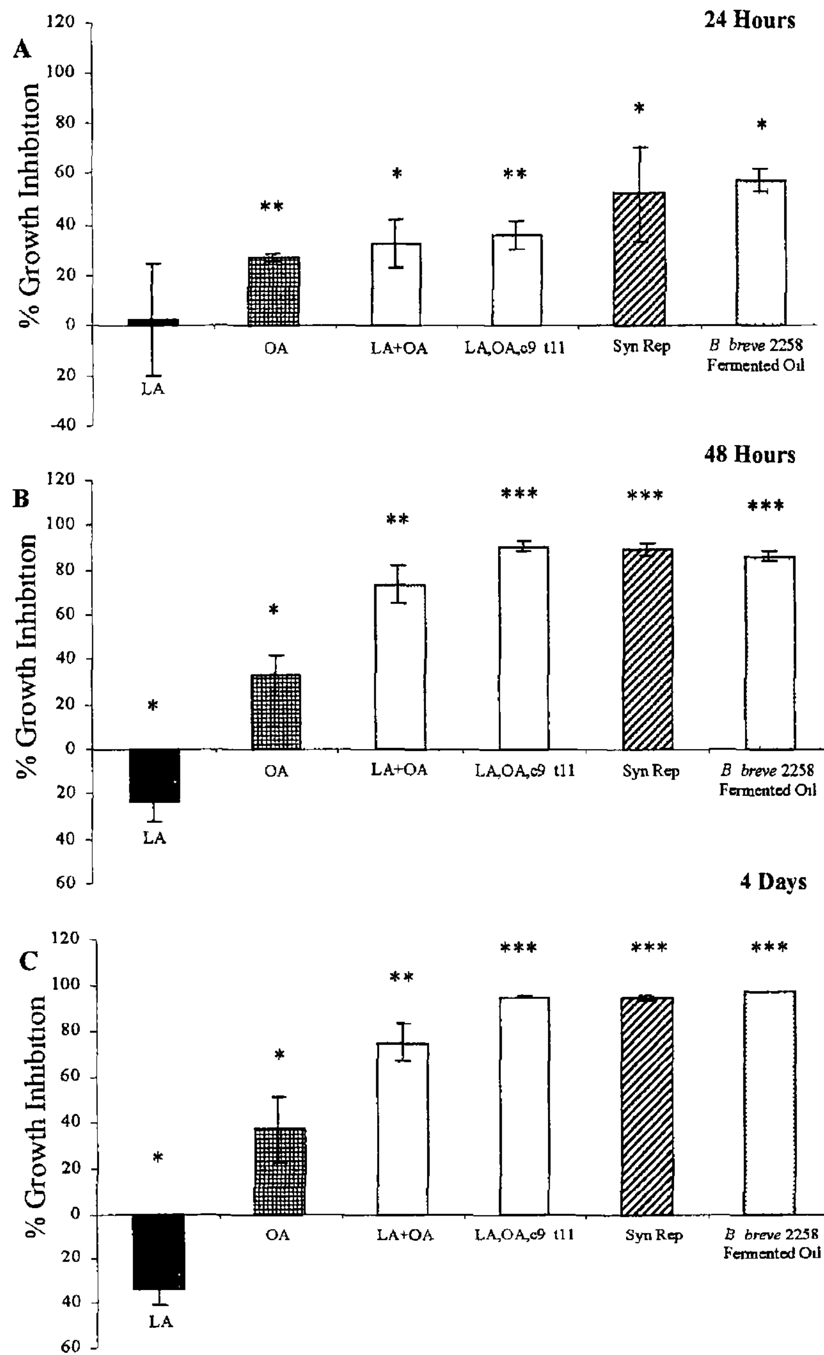

Figure 3 10 Effect 84 μg/ml of the *B breve* NCFB 2258 fermented oil and the individual fatty acids present in the oil on growth after (A) 24 h, (B) 48 h and (C) 4 days expressed as % growth inhibition compared with ethanol control (0% inhibition) The composition of the *B breve* NCFB 2258 fermented oil and hence the Synthetic Representation (Syn Rep) was LA (21 μg/ml), oleic acid (OA) (30 μg/ml), *c*9, *t*11 CLA (20 μg/ml) and *t*9, *t*11 CLA (1 6 μg/ml) * denotes values that are significantly different (P<0 05) compared with ethanol control, ** denotes values that are significantly different (P<0 01) compared with ethanol control *** denotes values that are significantly different (P<0 001) compared with ethanol control

The synthetic combinations containing *c*9, *t*11 CLA and/or *t*9, *t*11 CLA further reduced growth with the greatest inhibition (95%) observed after 4days incubation of SW480 cells in the presence of the combination of LA, oleic acid and *c*9, *t*11 CLA At each time point, the synthetic representation of the *B breve* NCFB 2258 fermented oil produced an effect similar to the fermented oil itself suggesting that the specific combination and concentration of oleic acid and CLA isomers present in the fermented oil account for the inhibition observed

The standard curve for LA (**Figure 3 2A**) demonstrated that 25 µg/ml LA did not significantly increase SW480 cell growth after 24 h incubation, but significantly increased cell growth after 48 h and 4 days The standard curve also demonstrated that as the concentration of LA is increased it becomes a growth inhibitor It is demonstrated in **Figure 3 10** that 21 µg/ml LA significantly (P<0 05) increased cell growth after 48 h and 4 days incubation Therefore it appears as though there is an optimum concentration at which LA stimulates SW480 cell growth Combining the data from **Figure 3 2A, Figure 3 7** and **Figure 3 10** suggests that this optimum concentration is in the range of 20-25 µg/ml LA

After 4 days incubation the growth inhibitory effect of the combination of LA, oleic acid and *c*9, *t*11 CLA (95% growth inhibition) was significantly (P<0 05) greater than that of the combination of LA and oleic acid (75% growth inhibition) This suggests that the *c*9, *t*11 CLA isomer had a significant influence on the growth inhibitory effect of the *B breve* NCFB 2258 fermented oil

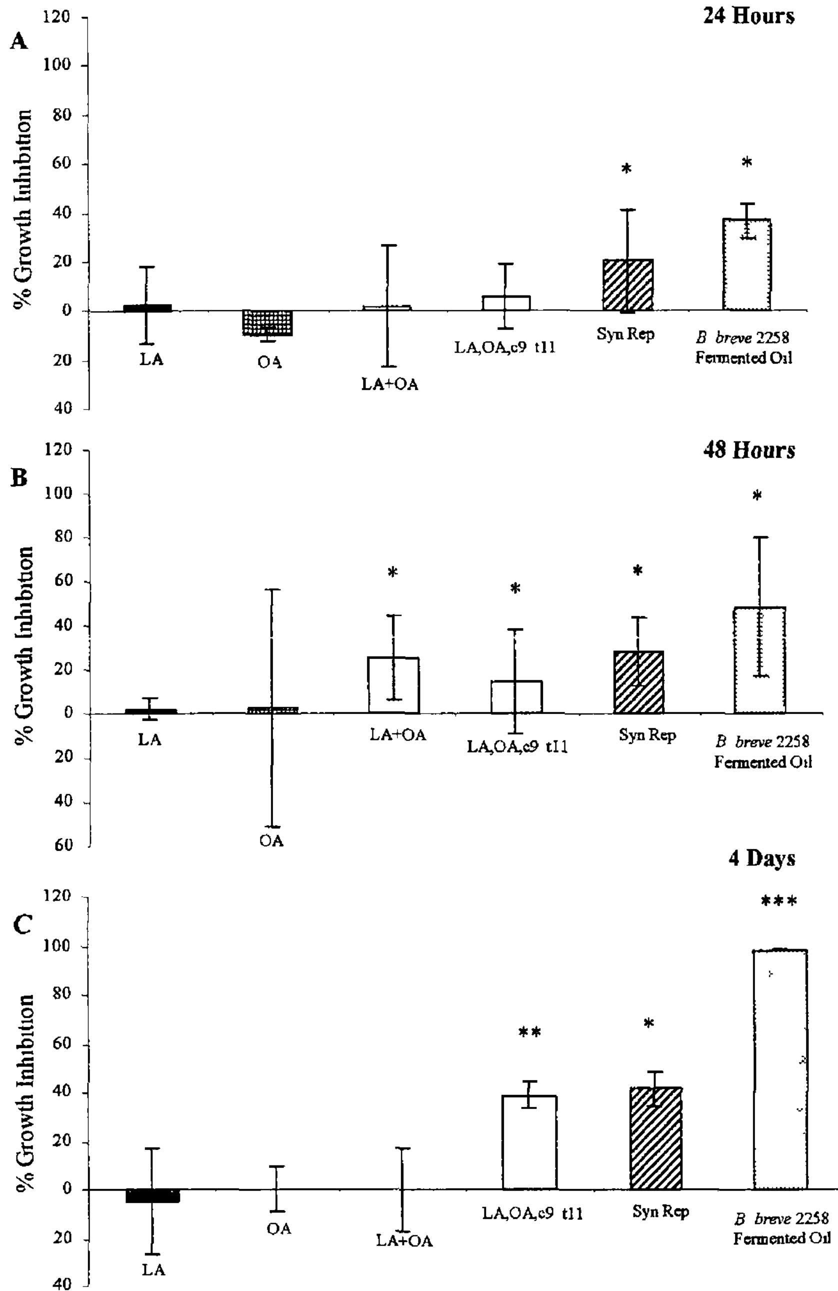

Figure 3 11 Effect of 33μg/ml of the *B breve* NCFB 2258 fermented oil and the individual fatty acids present in the oil on growth after (A) 24 h, (B) 48 h and (C) 4 days expressed as % growth inhibition compared with ethanol control (0% inhibition) The composition of the *B breve* NCFB 2258 fermented oil and hence the Synthetic Representation (Syn Rep) was LA (8 2 μg/ml), oleic acid (OA) (12 μg/ml), *c*9, *t*11 CLA (7 8 μg/ml) and *t*9, *t*11 CLA (0 6 μg/ml) * denotes values that are significantly different (P<0 05) compared with ethanol control, ** denotes values that are significantly different (P<0 01) compared with ethanol control *** denotes values that are significantly different (P<0 001) compared with ethanol control

When added to cells at a concentration of 33 µg/ml of oil, the *B breve* NCFB 2258 fermented oil consisted of 8 2 µg/ml LA, 12 µg/ml oleic acid, 7 8 µg/ml *c*9, *t*11 CLA and 0 6 µg/ml *t*9, *t*11 CLA As was seen in **Figure 3 4**, incubation with the fermented oil significantly (P<0 05) reduced cell numbers after 24 h, 48 h and 4 days incubation, with growth inhibition increasing with time **Figure 3 11** presents the effects of various combinations of these four fatty acids on cell growth Neither LA nor oleic acid alone influenced cell growth The combination of LA and oleic acid was negligible after 24 h and 4 days, though this treatment reduced growth by approximately 25% (P<0 05) after 48 h

The combinations containing *c*9, *t*11 CLA and *t*9, *t*11 CLA reduced growth by approximately 20-40% Though the synthetic combination of the *B breve* NCFB 2258 fermented oil was as potent as the oil up to 48 h, the oil was more potent than the synthetic representation of the oil after 4 days suggesting that other inhibitory components in the oil may have become active during the longer incubation period

3.4 Discussion

Many beneficial health effects have been associated with ingestion of probiotic bacteria (reviewed in Zubillaga, *et al* , 2001), including anticancer activity, although mechanisms of action have not been clearly established Some microbial strains used in food fermentations have been shown to have an ability to produce CLA, including propionibacteria (Jiang, *et al* , 1998) and Bifidobacteria (Coakley, *et al* , 2003) The aim of this study was to investigate whether the fatty acids produced from LA by two probiotic strains of *Bifidobacterium, B breve* 2258 and *B lactis* Bb12, were inhibitory towards the human colon cancer cell line, SW480

Each of the three oils – an unfermented oil, and two oils produced by the fermentation of linoleic acid by *B lactis* Bb12 and *B breve* NCFB 2258 – was used to treat SW480 human colon cancer cells at two concentrations of oil (84 and 33 µg/ml) These two concentrations were chosen in order to observe the antiproliferative activity of the fatty acid components present in the oils at different concentrations A synthetic representation of the four major fatty acids present in the oils were also used to treat the cells at concentrations representative of the concentrations present in the bacterially transformed fatty acids The four primary fatty acids present were LA, oleic acid, *c*9, *t*11 CLA and *t*9, *t*11 CLA The effects of these fatty acids formed microbially were investigated individually In addition, various combinations of fatty acids were used to determine effects of combining the fatty acids on SW480 cell growth Combining the four fatty acids present in the corresponding concentrations, formed models of the oils consisting of synthetically produced fatty acids

The unfermented oil was not exposed to any bacteria and was extracted from media enriched in LA Linoleic acid was present at a very high concentration in the unfermented oil due to the fact that it was used as the substrate for production of CLA However, small quantities of other fatty acids were also found to be present after incubation in the absence of any microbial fermentation The reason for this is not known but may relate to some non-enzymatic hydrogenation of LA occurring under incubation conditions At a concentration of 84 µg/ml the unfermented oil and the mixture of synthetic isomers of the four fatty acids present in the oil reduced cell growth to a similar extent throughout the experiment From the standard curves produced in this study it was expected that both LA and oleic acid would inhibit growth of the SW480 cell line at concentrations of 51 and 22 µg/ml respectively It is therefore very likely that such high concentrations of LA and oleic acid in the unfermented oil greatly influenced the growth inhibitory effect of this oil At the reduced concentration of 33 µg/ml the unfermented oil was significantly more potent compared with the synthetic mixture of fatty acids At this concentration of oil, LA was present at a concentration (20 µg/ml) which was seen to enhance cell growth and so clearly LA would not be expected to have the same inhibitory influence as was observed at the higher oil concentration Similarly, oleic acid (8 7 µg/ml) would not have been expected to affect growth Fatty acid analysis determined total fatty acid content and did not distinguish between free and bound fatty acids Therefore, it may be possible that bound fatty acids present in the oil influenced SW480 cell growth differentially compared with the FFAs used to produce the synthetic representation of the oil

It was noted that a small amount of both CLA isomers were produced in the unfermented oil suggesting that incubation conditions can result in the formation of CLA from LA However higher concentrations of these CLA isomers were present in the fermented oils, in particular the *B breve* NCFB 2258 fermented oil At a concentration of 84 µg/ml, there was no significant difference between the growth inhibitory effect of the oils produced by the two Bifidobacteria strains However, both *B lactis* Bb12 and *B breve* NCFB 2258 fermented oils reduced growth compared with the unfermented oil after 24 h This led to an investigation as to which fatty acid components were responsible for the growth inhibitory effect of each individual oil

In the *B lactis* Bb12 fermented oil, LA and oleic acid were present at similar concentrations and the data suggest that it was the combination of these two fatty acids that reduced cell growth when cells were treated with a concentration of 84 µg/ml of *B lactis* Bb12 fermented oil for 24 h and 48 h Addition of the *c*9, *t*11 CLA isomer did not result in further cell growth inhibition However, this concentration of *c*9, *t*11 CLA is very low (1 4 µg/ml) and has not been previously reported to inhibit SW480 cell growth It was apparent that the synthetic *t*9, *t*11 CLA isomer at a concentration of 1 µg/ml significantly reduced SW480 cell growth after 4 days incubation However addition of *t*9, *t*11 CLA (0 9 µg/ml) to a mixture containing 33 µg/ml LA, 34 µg/ml oleic acid and 1 4 µg/ml *c*9, *t*11 CLA had a negligible additional effect thus demonstrating the relatively greater potencies of LA and oleic acid compared to CLA isomers

At a concentration of 84 µg/ml, the growth inhibitory effect of the *B lactis* Bb12 fermented oil appeared to be due to the fatty acids present, however this was not the case at a concentration of 33 µg/ml When the concentration of the *B lactis* Bb12 fermented oil was reduced to 33 µg/ml of oil, it was significantly more potent (P<0 05) compared with fatty acid treatments which were representative of the *B lactis* Bb12 fermented oil (i e 13 µg/ml LA, 13 µg/ml oleic acid, 0 5 µg/ml *c*9, *t*11 and 0 4 µg/ml *t*9, *t*11 CLA) (**Figure 3 8**) This suggests that the fatty acid composition of the oil at this concentration was not solely responsible for the growth inhibitory effect observed on SW480 cells and suggests that there may be another component(s) present in this oil which was responsible for growth inhibition The effect of this component may be masked by the fatty acids present when used at a higher concentration, as the inhibitory effect of the *B lactis* Bb12 fermented oil at 84 µg/ml appeared to be due to the fatty acids present

After 24 h, 48 h and 4 days incubation there was no significant difference between the *B breve* NCFB 2258 fermented oil (84 µg/ml) and the synthetic mixture of all four fatty acids This suggests that the combination of all four fatty acids was responsible for the growth inhibitory effect of the *B breve* NCFB 2258 fermented oil The data show that although LA was at a concentration which increases cell growth of the SW480 cell line, combining it with oleic acid resulted in enhancing the growth inhibitory effect of oleic acid to 75% inhibition Similar results were observed by Takeshita, *et al* (1997) when in the presence of a high LA diet, oleic acid maintained its anticancer activity Addition of the *c*9, *t*11 CLA isomer, which was present at a concentration of 20 µg/ml, a concentration

which has been shown to be cytotoxic in this cell line after 48 h and 4 days (**Figure 3 2C**), appeared to have a strong influence reducing cell growth to 95%

However, when a lower concentration (33 µg/ml) of the *B breve* NCFB 2258 fermented oil was used to treat the cells for 4 days, there was no significant difference between this concentration and the higher concentration of 84 µg/ml Therefore, reducing the fatty acid content of the oils by over half did not significantly reduce the inhibitory effect of the oil The combination of the synthetic fatty acids representative of the *B breve* NCFB 2258 fermented oil at the lower concentration (33 µg/ml) did not reduce cell growth to the same extent as the oil itself suggesting that when used at the lower concentration of 33 µg/ml the fatty acids present may not have been solely responsible for the growth inhibitory effect observed As the bacterial strains used in this study are probiotic bacteria it is likely that, like the *B lactis* Bb12 fermented oil, another component could be contributing to the growth inhibitory effect

As already mentioned differences between the inhibitory effects of the oils and the synthetic mixture of fatty acids representing the oils may either be due to bound fatty acids or to the presence of other fermented products, in the case of *B lactis* Bb12 and *B breve* NCFB 2258 fermented oils Such components may be vitamins such as vitamin K, a fat-soluble vitamin (reviewed by Cummings and Macfarlane, 1997) or butyric acid, a short chain fatty acid which can be formed during bacterial fermentation Butyric acid has been shown to inhibit colon carcinogenesis via increasing apoptosis in the colon (Hague, *et al* , 1993)

At the higher concentration (84 µg/ml) of the oils used, the effect of the fatty acids appeared to cancel out the effect of the other component, however when the concentration of the oils was reduced to concentrations which do not have a strong inhibitory effect on cell growth, the growth inhibitory effect observed may have been due to the other component having a stronger influence on cell growth inhibition This component would also be reduced as the concentration of the oil is reduced, however it may be possible that with the removal of the growth inhibitory effect of the fatty acids present its growth inhibitory effect is more obvious Another possibility is that it has an optimum concentration at which it displays its anticarcinogenic activity in a manner similar to that of the growth stimulatory effect of LA as observed in this study

It is clear that the oils produced by fermentation with the two *Bifidobacterium* strains inhibited growth of the SW480 cell line The *B breve* NCFB 2258 strain produced significant amounts of CLA – concentrations that have been shown previously and in this study to significantly inhibit SW480 cell growth When used at a lower concentration of 33 µg/ml of the oils, there was still a similar potent inhibitory effect on SW480 cell growth This suggests that the presence of another component may be partially responsible for the growth inhibitory effect observed, as the synthetic fatty acid representations of the oils did not mimic the inhibitory effect of the bacterially transformed oils, unlike when the oils were used at a higher concentration of 84 µg/ml Further study is required to identify this component It may be beneficial to isolate the CLA produced by this bacterial strain and analyse its growth inhibitory effect independently It may also be beneficial to grow these probiotic strains with the cancer cells as attenuated Bifidobacterium are known to

selectively multiply in tumours and inhibit growth (reviewed in Bermudes, *et al* , 2002) Such a study would be difficult due to the differences in incubation requirements of the cancer cells and the Bifidobacteria, but would be very useful in further solving the relationship between the ability of probiotics to produce CLA and the anticarcinogenic activity of probiotic strains

3 4 1 Conclusion

As far back as 1907, it has been suggested that certain microorganisms can be beneficial to human health when ingested (Metchnikoff, 1907) However, the mechanisms used by many probiotics to benefit the health of the host are not fully understood, although some theories have been suggested in relation to anticarcinogenic activity (discussed in **Section 3 1 3**) including inhibition of carcinogen producing bacteria, inhibition of tumour formation and inactivation of carcinogens The data from the study by Coakley, *et al* (2003) shows that *B breve* NCFB 2258 can produce high concentrations of CLA isomers that are shown in this study to inhibit cancer cell growth These data suggest that ingestion of *B breve* NCFB 2258 may exert anticarcinogenic effects However, further studies are needed to confirm any potential anticarcinogenic activity associated with ingestion of CLA-producing Bifidobacteria in animal models of carcinogenesis

3 4 2 Summary

The objective of this study was to analyse the anticancer activity of microbially produced conjugated linoleic acid (CLA) Linoleic acid was fermented in the presence of two probiotic strains of *B lactis* Bb12 and *B breve* NCFB 2258 The resulting oils contained

1 6% and 23 9% *c*9, *t*11 CLA respectively and 1 1% and 1 9% *t*9, *t*11 CLA respectively At a concentration of 84 μg/ml , *B lactis* Bb12 and *B breve* NCFB 2258 fermented oils significantly reduced growth of SW480, a human colon cancer cell line, in a time dependant manner compared with an ethanol control An unfermented oil extract containing 0 8% *c*9, *t*11 CLA and 0 5% *t*9, *t*11 CLA, 61% LA and 26% oleic acid also reduced cell growth Synthetic models of the oils containing their four main constituent fatty acids, LA, oleic acid, *c*9, *t*11 CLA and *t*9, *t*11 CLA at a concentration of 84 μg/ml reduced growth to a similar extent as the oils suggesting that the fatty acid composition of the oils had a major inhibitory influence on growth The *c*9, *t*11 CLA isomer was at a concentration of 20 μg/ml in the *B breve* NCFB 2258 fermented oil (84 μg/ml) When added to a combination of LA and oleic acid, the resulting growth inhibition relative to control was 95%, compared with 75% inhibition when cells were incubated with a LA-oleic acid mixture The *t*9, *t*11 CLA was at a concentration of 1 6 μg/ml in the *B breve* NCFB 2258 fermented oil (84 μg/ml) Though inhibitory effects of this isomer were not apparent when co-incubated with inhibitory concentrations of oleic acid and *c*9, *t*11 CLA, this study demonstrated for the first time that *t*9, *t*11 CLA alone was more potent than *c*9, *t*11 CLA, reducing cell growth by 14% (P<0 01) at a concentration of 1 μg/ml and as much as 43% (P<0 001) at a concentration of 10 μg/ml Upon reducing the concentration of the oils to 33 μg/ml the synthetic fatty acids did not inhibit cell growth to the same extent as the oils, suggesting that another growth inhibitory factor may be present in the oils

CHAPTER 4

AN INVESTIGATIVE STUDY TO ENRICH MILK FAT CLA IN HIGH CLA-PRODUCING COWS

4.1 Introduction

4 1 1 Milk Lipids

Triglycerides are the major class of lipid found in milk fat making up approximately 98% of the total fat (**Table 4.1**) The primary fatty acids found in milk are long chain fatty acids – $C_{14\ 0}$ (myristic), $C_{16\ 0}$ (palmitic), $C_{18\ 0}$ (stearic), $C_{18\ 1}$ (oleic) and the minor fatty acids are short chain $C_{4\ 0}$ (butyric), $C_{6\ 0}$ (caproic), $C_{8\ 0}$ (caprylic), $C_{10\ 0}$ (capric) (reviewed in Christie and Clapperton, 1982, Parodi, 1983, Timmen and Patton, 1988, Jensen, *et al*, 1991, Jensen, 2002)

Table 4 1 Milk fat composition (reviewed in Jensen, 2002)

Lipid Class	% Total Milk Fat
Triglycerides	98
Diacylglycerides	0 25-0 48
Monoacylglycerides	0 02-0 04
Phospholipids	0 6-1 0
Cholesterol	0 2-0 4
Glycolipids	0 006
Free fatty acids	0 1-0 4

Milk fatty acids can be synthesised by either *de novo* synthesis or by ruminal fermentation and carried to various parts of the body via the circulatory system (**Figure 4 1**) (reviewed in Barber, *et al*, 1997) In the rumen, glycolipids, phospholipids and triglycerides consumed in the diet undergo lipolysis resulting in the formation of FFAs, which are isomerised and hydrogenated by the activity of ruminal microorganisms (reviewed in Chilliard, *et al*, 2000) In the case of LA (C18 2), hydrogenation by an isomerase produced by *Butyrivibrio fibrisolvens* leads to the formation of fatty acids such as VA (C18 1 *t*11) and following further hydrogenation, stearic acid (C18 0) The fatty acids formed and the pathways occurring depend on the microbial ecosystem in the rumen (Keeney, 1970)

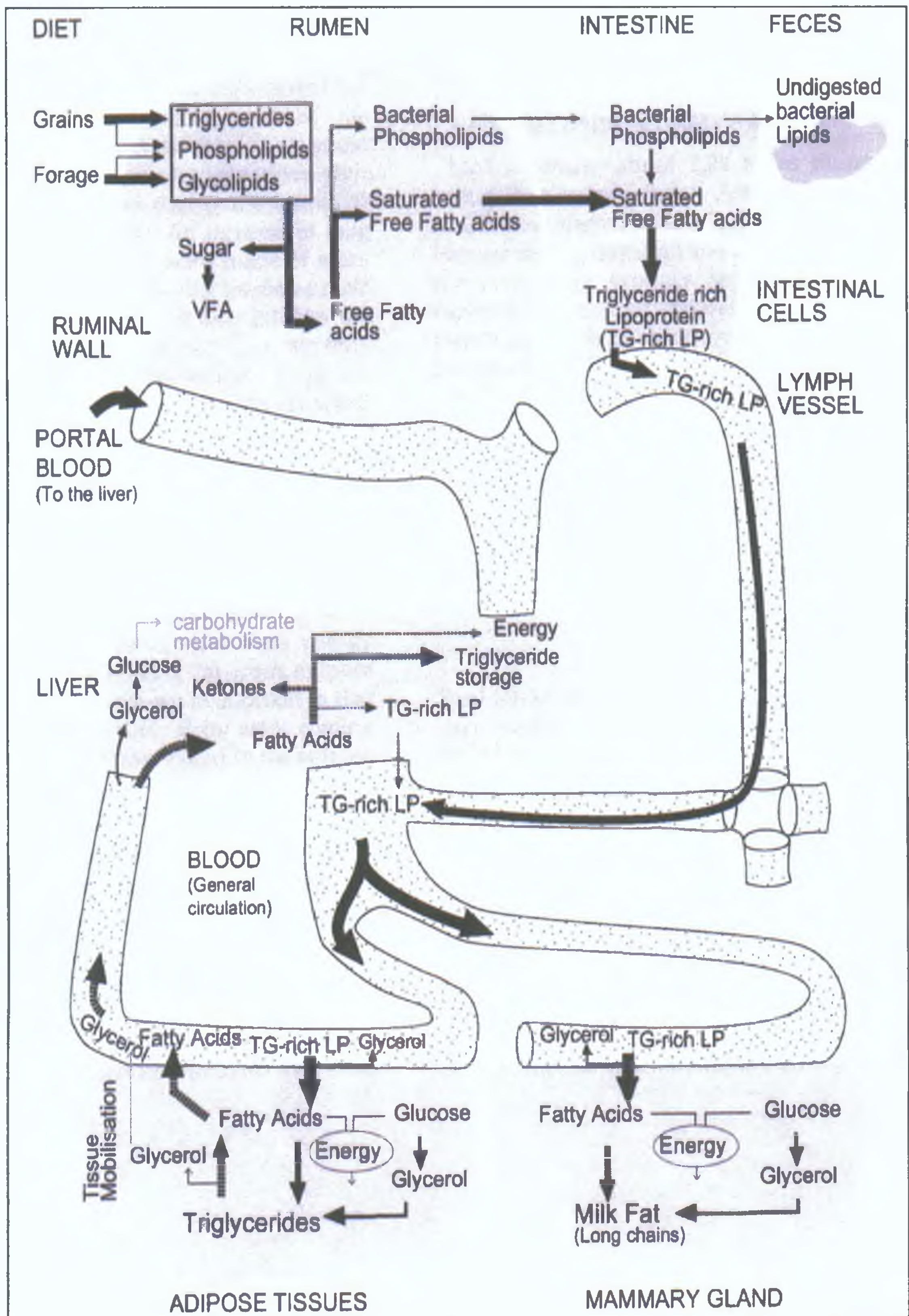

Figure 4.1 Summary of bovine lipid metabolism (Wattiaux and Grummer, 1997)

Lipids leaving the rumen consist of 85-90% saturated FFA and 10-15% phospholipids Palmitic acid and stearic acid make up the majority of the saturated FFAs and are bound to particles such as microbial or feed particles FFAs leaving the rumen are available for absorption into the circulatory system and incorporation into milk fat

De novo synthesis takes place in the cytoplasm of the mammary epithelial cell and leads to the formation of short and medium chain fatty acids In ruminant animals, cellulose and hemicellulose are fermented by ruminal microorganisms to produce volatile acids consisting mainly of acetic, propionic and butyric acid, acetate and β-hydroxybutyrate (BHB) being the carbon sources utilised in fatty acid synthesis Two enzymes are involved in this process – acetyl-CoA carboxylase (a rate limiting enzyme) and fatty acid synthetase (required for elongation of fatty acid chains) (reviewed in Barber, *et al*, 1997) High concentrations of propionate (achievable by feeding a high concentrate diet) can lead to reduced milk fat (reviewed in Doreau, *et al*, 1999) Very low density lipoproteins (VLDL) present in the blood, are produced in the liver and intestine and consist of 90-95% of lipids (55-60% triglycerides), and the remainder consisting of a protein outer surface Lipoprotein lipase (LPL) hydrolyses VLDL triglycerides in the mammary capillaries The resulting FFA, monoacylgylcerides, diacylglycerides and glycerol can be absorbed by the mammary epithelial cells and are available for synthesis of triglycerides In the mammary gland, triglycerides are synthesised at the cytoplasmic surface of the smooth endoplasmic reticulum The FFA are then esterified to the hydroxyl group of glycerol units resulting in triglycerides Of the fat present in milk, approximately 50% comes from fatty acids produced in the mammary gland (reviewed in Chilhard, *et al*, 2000) As they are produced, the triglycerides coalesce to form droplets of lipids which are released into

the cytoplasm These may come together to form larger droplets, they may be released from the cells as small milk fat droplets or may combine with cytoplasmic droplets to form cytoplasmic lipid droplets Eventually large milk fat droplets are released at the apical surface of the cell (reviewed in Mather and Keenan, 1998)

4.1.2 Ruminal Production of Milk Fat CLA

The *c*9, *t*11 CLA isomer is an intermediate in the biohydrogenation of LA to stearic acid (Kepler, *et al*, 1966) During microbial hydrogenation LA is first isomerised to *c*9, *t*11 CLA by LA isomerase of rumen bacteria such as *Butyrivibrio fibrisolvens* (reviewed in Harfoot and Hazlewood, 1988) The *c*9, *t*11 CLA is quickly hydrogenated to VA by reductase enzymes and this is then further hydrogenated to form stearic acid While some of the *c*9, *t*11 CLA is believed to be absorbed directly, the major source of milk fat CLA is due to the activity of mammary Δ9-desaturase activity which converts VA to *c*9, *t*11 CLA in the mammary gland (Bickerstaffe and Annison, 1970, Bickerstaffe and Johnson, 1972, Kinsella, 1972, Ward, *et al*, 1998) The Δ9-desaturase results in a second point of desaturation at carbon 9 (C9) of VA (C18 1 *t*11) in the *cis* configuration, producing *c*9, *t*11 CLA The mammary glands appear to be the major site of endogenous synthesis of CLA in ruminants (Bickerstaffe and Annison, 1970, Kinsella, 1972) A number of factors indicate this In the first instance, *c*9, *t*11 CLA appears to be a transient intermediate in the rumen, unlike VA which accumulates There appears to be a linear relationship between VA and *c*9, *t*11 CLA which remains constant across a range of diets suggesting a precursor/end-product relationship (reviewed in Griinari and Bauman, 1999) Abomassal infusion of VA, the substrate for Δ9 desaturase, resulted in a 40% increase in milk fat CLA, while sterculic acid, a Δ9 desaturase inhibitor, dramatically reduced milk fat *c*9, *t*11 CLA content

(Corl, *et al* 1998) Endogenous synthesis of CLA is estimated to account for 78-80% of the total *c*9, *t*11 CLA in milk fat (Corl, *et al*, 2001, Lock and Garnsworthy, 2002) The fact that a 100% reduction in CLA concentrations due to sterculic acid was not observed by Corl, *et al* (1998) indicates that endogenous synthesis is not the sole source of CLA

Although *c*9, *t*11 is the predominant CLA isomer produced during microbial biohydrogenation of LA, other ruminal micro-organisms appear to have an ability to produce the *t*10, *c*12 CLA isomer, although these micro-organisms have not been identified (Fellner, *et al*, 1999) It appears that this isomer forms as a result of biohydrogenation of *trans*-10-octadecenoic acid due to ruminal bacterial metabolism (Griinari and Bauman, 1999) It is unclear if humans possess the Δ12 desaturase enzyme A study by Adlof, *et al* (2000) did not detect it within a detection limit of <2 ng FAME/ml plasma, however it was suggested that there is a possibility that humans may possess such an enzyme Therefore ruminal biohydrogenation is the most likely source of the *t*10, *c*12 CLA isomer

Kim, *et al* (2000) showed that *B fibrisolvens* A38 produced high concentrations of CLA in the presence of high concentrations of LA (>350 μM) High concentrations of LA resulted in incomplete hydrogenation However, when this occurred the cells were no longer viable This suggests that CLA is not a normal end product of these cultures and the CLA in milk fat is the result of another process i e endogenous synthesis

4.1.3 Manipulation of the Diet to Enhance Milk Fat CLA

Dairy products are the main source of CLA in the human diet (discussed in **Chapter 1**) Due to the many health benefits associated with CLA, such as activity against carcinogenesis, atherosclerosis and diabetes (reviewed in Belury, 2002), it is of interest to increase CLA concentrations in bovine milk It has been known since 1935 that dairy products contain fatty acids with conjugated double bonds (Booth, *et al* , 1935) when butter fat exhibited spectrophotometric absorption at 230nm Parodi (1977) demonstrated that *c*9, *t*11 CLA is the predominant CLA isomer in milk fat

There are many factors which influence the fatty acid composition of milk and there tends to be a wide variation in the *c*9, *t*11 CLA content of milk A number of factors have been implicated in this such as, dietary supplementation (reviewed in Chilliard, *et al* , 2001), animal age (Lal and Narayanan, 1984, Stanton, *et al* , 1997), stage of lactation (Lal and Narayanan, 1984, Stanton, *et al* , 1997) and animal breed (Lawless, *et al* , 1999, White, *et al* , 2001) Because production of CLA involves a microbial biohydrogenation pathway and mammary Δ9-desaturase activity, there are two possible approaches used to enrich milk fat CLA CLA concentrations are high in the milk of cows on pasture (reviewed in Bauman, *et al* , 2000a) compared with cows on conserved forages Linolenic acid is the principal fatty acid in fresh pasture and biohydrogenation of LA in the rumen does not result in CLA as an intermediate However, VA is produced during this process and this fatty acid can be converted to *c*9, *t*11 CLA via Δ9 desaturase activity (Grinari *et al* , 2000, Santora *et al* , 2000) Further enhancing the concentration of substrates for the biohydrogenation process is one possible approach to enhancing milk fat CLA The second approach is to modify the activity of ruminal microorganisms involved in the biohydrogenation process via

optimisation of their environmental conditions (Griinari and Bauman, 1999) Animal fat (tallow), plant oils (soybeans, linseed, rapeseed and sunflower), fish oil in addition to pasture feeding have all been used successfully to alter the fatty acid profile of bovine milk and enrich milk fat CLA

Table 4.2 Summary of effect of dietary variations on CLA content of milk fat (taken from Jensen, 2002)

Dietary Factor	Content of CLA in milk fat
a *Lipid substrate*	
Unsaturated vs saturated fat	Increased by addition of unsaturated fat [(Griinari *et al* 1998a)]
Plant oils	
Type of plant oil	Increased with oils high in unsaturated fatty acids [(Tesfa *et al* 1991 Dhiman, *et al* 2000 Chouinard *et al* 1998a Kelly *et al* 1998a)]
Level of plant oil	Dose-dependent increase [(Tesfa *et al* 1991 McGuire *et al* 1996 Dhiman, *et al* 2000)]
Ca salts of plant oils	Increased [(Chouinard, *et al* (1998a)]
High-oil corn plant seeds	
Raw seeds	No effect [(Dhiman, *et al* 2000 Chouinard, *et al* 1998a)]
Processed seeds	Increased [(Stanton, *et al* 1997 Chouinard *et al* 1998a Lawless *et al* 1998, Dhiman, *et al* 1999)]
High-oil corn grain and silage	Minimal effect [(Dhiman, *et al* 1996, Chouinard *et al* 1998a)]
Animal fat by-products	Minimal effect [(Chouinard *et al* 1998a)]
b *Modifiers of rumen environment*	
Forage Concentration ratio	Variable effect [(Jiang *et al* 1996 Chouinard, *et al* 1998b Griinari *et al* 1998a)]
Nonstructural carbohydrate level	Minor effect [(Chouinard, *et al* 1998b Solomon, *et al* 2000)]
Restricted feeding	Variable effect [(Timmen and Patton, 1988 Jiang *et al* 1996 Stanton *et al* 1997)]
Fish oil/fish meal	Increased [(Dhiman, *et al* 1996 Chouinard, *et al* 1998a Chilliard, *et al* 1999)]
Marine algae	Increased [(Franklin, *et al* 1999)]
Ionophores	Variable effect [(Dhiman, *et al* 1996 Chouinard, *et al* 1998b Sauer *et al* 1998)]
Dietary buffers	Little effect with sufficient fibre [(Chouinard *et al* 1998b)]
c *Combination of a and b*	
Pasture	Higher than on conserved forages [(Timmen and Patton, 1988 Dhiman *et al* 1996 Zegarska *et al* 1996 Jahreis *et al* 1997 Precht and Molkentin 1997, Kelly *et al* 1998b)]
Growth stage of forage	Increased with less mature forage [(Chouinard *et al* 1998b)]
CLA supplement	Dose-dependent increase [(Loor and Herbein, 1998 Chouinard *et al* 1999a Chouinard, *et al* 1999b Giesy *et al* 1999)]

It is possible to further enhance the effect of lipid supplements by processing the oilseed to make the oil more readily available for conversion to CLA (reviewed in Chilliard, *et al.*, 2000).

Extensive study has been carried out on the effects of dietary supplementation on the fatty acid profile and the CLA content of milk fat. The results of these studies are summarised in **Table 4.2** and will be discussed in detail below.

4.1.4 Dietary Supplementation with Plant Oils to Elevate Milk Fat CLA

Plant oils rich in LA such as cottonseed, soyabean, sunflower and safflower when fed to lactating cows have been shown to enhance the CLA content of milk fat. In addition, plant oils rich in linolenic acid, e.g. linseed oil (Kelly, *et al.*, 1998a), and rapeseed oil rich in oleic and linoleic acid (Stanton, *et al.*, 1997) have also been shown to enhance CLA concentrations when fed to lactating cows.

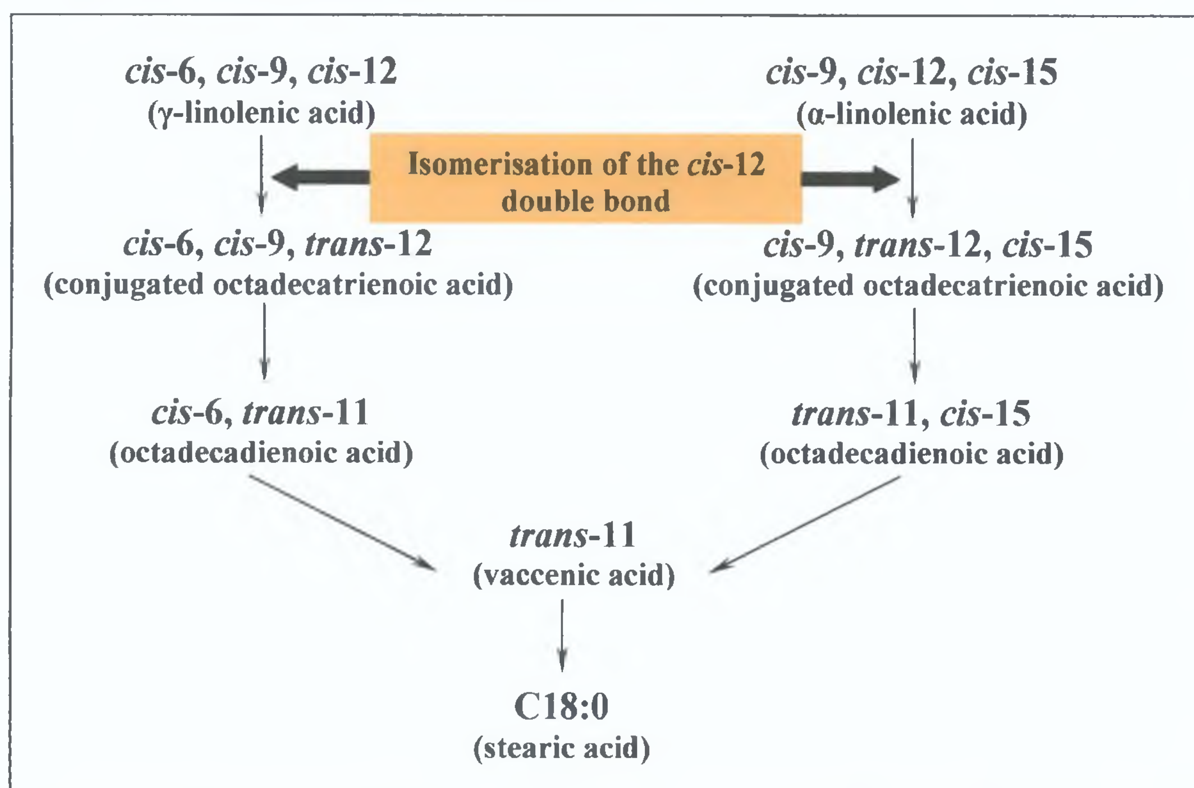

Figure 4.2 Biohydrogenation of γ- and α-linolenic acid

Linolenic acid, when biohydrogenated, leads to the formation of VA (C18 1 *t*11), although *c*9, *t*11 CLA is not formed during this biohydrogenation pathway (**Figure 4.2**)

Following absorption of VA, this fatty acid is converted to CLA by the action of Δ9-desaturase in the mammary gland (Harfoot and Hazlewood, 1988, Griinari, *et al*, 1998a) Kelly, *et al* (1998a) demonstrated that sunflower oil (rich in LA) was more effective at increasing CLA concentrations in milk fat compared to peanut and linseed oil Feeding sunflower oil to cows housed indoors increased CLA concentrations to 24 4 mg/g milk fat compared to 13 3 mg/g milk fat and 16 7 mg/g milk fat for peanut and linseed oil, respectively This study suggested that dietary supplementation of the CLA precursor LA resulted in the largest increases in milk fat CLA Sunflower oil had much higher LA content (69%) compared with linseed oil (15 4%) Lock and Garnsworthy (2002) demonstrated that when linoleic and/or linolenic acid were present in the diet at high concentrations, milk fat *c*9, *t*11 CLA was highest Olive oil, linseed oil, rapeseed oil, soya oil and sunflower oil were combined at different concentrations resulting in four diets varying in linoleic and linolenic acid concentrations which were fed to lactating cows It was demonstrated in this study that 80% of the *c*9, *t*11 CLA was produced via conversion of VA by stearoyl CoA desaturase in the mammary gland (Lock and Garnsworthy, 2002)

Feeding cows a diet rich in full fat soybean and ground full fat rapeseed (FFR) significantly increased CLA in the milk produced, to concentrations of 19 1 and 23 7 mg/g fat respectively (Lawless, *et al* 1998) It may have been that the soybean yielded lower CLA concentrations, because it was not as available to ruminal microrganisms

as was the ground rapeseed due to heat treatment Not only does the oil source and quantity affect the content of CLA in the milk fat but also the processing of the oil supplemented (Dhiman, *et al* , 1999, Murphy, *et al* , 1990, Lawless, *et al* , 1998) and the level of oil present Dhiman, *et al* (2000) fed 180 g/kg raw cracked soybean, 180 g/kg roasted cracked soybean, 22 g/kg linseed oil and 44 g/kg linseed oil to dairy cows and found that feeding raw soybeans had no effect on the CLA content of the milk fat, while feeding roasted soybeans resulted in a doubling of CLA concentrations However as Lawless, *et al* (1998) demonstrated roasting of the oil source may not be as effective as grinding in providing a substrate for ruminal biohydrogenation It may also be due to the fact that rapeseed has been found to provide a more suitable substrate for ruminal biohydrogenation (Lawless, *et al* , 1998) Extrusion involves forcing feed through metal holes to break it up, releasing its components, including oil The advantage of this process with regard to elevating CLA concentrations in milk fat is that the oil becomes more readily available for microbial biohydrogenation in the rumen Feeding extruded soybeans (ground full fat soybeans were heated to 138-149°C for 1-3 min) significantly increased CLA concentrations m milk fat compared to a diet without extruded soybeans (Solomon, *et al* 2000), a demonstration of the beneficial effect of processing soybeans Heat-processing soybeans produced similar increases in milk fat CLA as extrusion of soybeans (Chouinard, *et al* , 1998a) A soybean oil content of feed, equivalent to 5 and 10g/kg dry matter (DM) produced a CLA content of 7 5 and 7 6 g/kg total fatty acids respectively, however on increasing the soybean oil concentration to 20 and 40 g/kg DM the resulting CLA contents were 14 5 and 20 8 g/kg total fatty acids respectively (Dhiman, *et al* 2000) A supplemental grain diet containing mechanically extracted soybean meal as opposed to solvent extracted soybean meal, increased concentrations of *c*9, *t*11 CLA in milk fat (Loor, *et al* 2002)

again demonstrating the differential effects of different oil processing systems on CLA concentrations in milk fat The effect of processing on CLA enrichment of milk fat was again demonstrated when different processing systems were used to process the soybeans increased CLA concentrations in milk fat by 2 to 3-fold compared to a control diet which consisted of raw ground soybeans The processes used were extrusion, micronizing (a short time, high temperature process using humidity, temperature (infra-red energy) and mechanical pressure) and roasting (cooking with dry heat) of the soybeans (Chouinard, *et al* , 2001)

4 1 5 Marine Oils

Marine oils, including both oil from fish and marine plants, have been shown to have an ability to elevate CLA concentrations in milk fat when included in the diet (reviewed in Chilliard, *et al* , 2000 and 2001) Fish oils are already known for their beneficial health effects due to the presence of long chain PUFAs namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) The mechanism by which CLA is enriched is unknown, but increases in ruminal and milk VA concentrations has been observed (reviewed in Chilliard, *et al* , 2000) One theory is that reduction of VA is prevented by inhibition of the growth of ruminal bacteria involved in this process due to the presence of long chain PUFAs in the marine oil (Griinari and Bauman, 1999) It has been observed that supplementing the diet with fish oil reduces the fat content of milk (Doreau, *et al* , 1999, Abu-Ghazaleh, *et al* , 2001)

There have been a number of studies on the effect of marine oils on CLA content of milk fat (reviewed in Chilliard, *et al* , 2001) One such study was undertaken by Jones,

et al (2000) in which an increase in milk fat CLA and VA was demonstrated after dietary supplementation with 3% tallow fish oil mixture, however there was no effect on milk yield or composition The optimum concentration of dietary fish oil resulting in maximal increases in milk fat CLA was demonstrated to be 20 g/kg of DM with no additional increase m milk fat CLA observed with a further increase in fish oil concentrations (Donovan, *et al* , 2000, Baer, *et al* , 2001, Chouinard, *et al* , (2001) The concentrations of fish oil can be decreased by partial substitution with dietary plant oils Incorporation of menhaden fish oil at a concentration of 20 g/kg into the diet of cows increased milk fat CLA concentrations to 23 g/100g fatty acids compared to 2 17 g/100g fatty acids for a mixture of 10 g/kg menhaden fish oil and 10 g/kg fat from extruded soybeans (Ramaswamy, *et al* , 2001a) Abu-Ghazeleh, *et al* (2001) demonstrated that dietary supplementation with fish oil can be more effective than plant oils in elevating milk fat CLA, when an increase from 0 39 g CLA/100g fatty acids to 0 72 g CLA/100g fatty acids was observed due to fish oil supplementation Other studies have demonstrated higher concentrations *c*9, *t*11 CLA and VA in milk fat after fish oil supplementation compared with soybean oil supplementation (Whitlock, *et al* , 2002)

It is also possible to enhance milk fat CLA using algae as a supplement (Franklin, *et al* , 1999) A species of marine algae, *Schizochytrium* sp , is a rich source of long chain n-3 fatty acids The diet was supplemented with 910 g of these algae either protected by coating with xylose or unprotected Both protected and unprotected algae led to similar increases of 6 to 7-fold in milk fat CLA compared with the control

It is also important that the use of fish oil as a dietary supplement does not adversely affect the sensory qualities of milk or dairy products produced using the milk No differences in the sensory qualities of milk and butter were observed after a three-month storage period (Ramaswamy, *et al* , 2001a) There was no difference between control milk and milk from the 20 g/kg fish oil diet according to consumer evaluation (Ramaswamy, *et al* , 2001b)

4 1 6 Animal Fats

Another supplement used to enrich milk fat is animal fat (also known as tallow) (Palmquist, 1988) Animal fat is rich in long chain saturated fatty acids such as palmitic (23-27%) and stearic acid (14-29%), being low in LA at 1-5% (reviewed in Chilliard, *et al* , 2001) Enhancing milk fat CLA using animal fat as a supplement is not as successful as the other dietary supplements discussed above This is due to the fatty acid profile of animal fat, which is low in PUFAs Only low increases in CLA (increased from 0 2 to 0 5%) and VA (from 0 9 to 1 6%) were obtained, after dietary supplementation with 40-50 g/kg tallow (Pantoja, *et al* , 1996, Chouinard, *et al* , 1998a) Tallow supplemented at 0, 20 and 40g/kg of the diet DM did little to increase VA concentrations in milk (Onetti, *et al* , 2001) It has been demonstrated that tallow can reduce saturated fatty acids from C6-C16, while increasing C18 0 and C18 1 in milk fat (reviewed in Chilliard, *et al* , 2001) There still remains the question of safety regarding the use of animal fat in foodstuffs Due to the risk of BSE associated with animal products it is important that all traces of protein be removed from the fat before it is used as a dietary supplement This is possible although there is the risk of contaminants such as hair fragments remaining (Padley, *et al* , 1994, Palmquist, 1988)

4.1 7 Pasture

The fatty acid content of pasture around the world varies greatly but is typically 1-3% fat Tropical climates produce grass containing 150-400 g/kg α-linolenic acid, while in temperate climates α-linolenic acid accounts for 550-650 g/kg of the fatty acids, the grass containing 10-30 g/kg fat Fatty acid content of pasture varies throughout the year reaching its highest in the spring and autumn months (Kudzal-Savoie, 1965, Bauchart, *et al*, 1984) When cows are fed on pasture the concentrations of oleic acid increase and of palmitic acid decrease (reviewed in Chilliard, *et al*, 2001) A number of studies have demonstrated that pasture feeding can greatly increase milk fat CLA (Timmen and Patton, 1988, Stanton, *et al*, 1997, Kelly, *et al*, 1998b, Agenas *et al*, 1999, Dhiman, *et al*, 1999) The LA in grass is extensively hydrogenated in the rumen leading to the formation of VA and in turn *c*9, *t*11 CLA The effect of pasture on *c*9, *t*11 CLA synthesis is also attributed to its influence on ruminal microflora and in turn biohydrogenation (Griinari and Bauman, 1999) A sharp increase in conjugated dienes was observed when cows were turned out to pasture after winter-feeding on grass silage, hay and beets, while no further increase was observed after 5 days (Kudzal-Savoie and Kudzal, 1961, Kudzal-Savoie, 1965) Both VA and CLA were increased when cows were put on pasture (Lawless, *et al*, 1999) Although CLA concentrations increase with grass allowance (Stanton, *et al*, 1997) they are not affected by the maturity of the grass (Griinari, *et al*, 1998b) Stanton, *et al* (1997) found that 20 kg grass/cow per day resulted in higher milk fat CLA concentrations than 16 kg grass/cow per day in a 19 week feeding trial Kelly, *et al* (1998b) and Dhiman, *et al* (1999) both demonstrated that pasture alone resulted in higher CLA concentrations in milk than when combined with other dietary components such as cottonseed, legume hay, roasted soybeans or alfalfa hay Likewise, milk from pasture (crabgrass – *Digitaria*

sanguinalis) fed cows contained proportionally 0 83 more *c*9, *t*11 CLA than cows fed a mixed diet which included corn, silage, alfalfa silage, ground corn, soybean meal and whole cottonseed (White, *et al* , 2001) Similar results regarding CLA content of intra-muscular fat of steers were observed by French, *et al* (2000) It is also clear that the variety of grass used to supplement the diet is another factor which influences the CLA content of milk fat Loyola, *et al* (2002) demonstrated the effect of grass using four varieties of ryegrass, these were Spelga and Portstewart (both diploid), and Napoleon and Millennium (both tetraploid) with different heading dates Although the four varieties had similar linoleic and linolenic acid contents, milk fat *c*9, *t*11 CLA content varied when a diet of Spelga or Napoleon was administered Millennium and Portstewart resulted in 1 72 g/100g FAME and 1 71 g/100g FAME in the milk respectively Intermediate concentrations (1 54 g/100g FAME) were achieved by feeding with Spelga, whereas Napolean produced the lowest milk fat CLA concentrations of 1 35 g/100g FAME

Another factor affecting the CLA content of milk fat is the forage to concentrate ratio CLA and VA concentrations in milk fat can be increased with a diet which has a low forage to concentrate ratio compared to a diet with a high forage to concentrate ratio (Chouinard, *et al* , 1998b) This low forage to concentrate ratio diet led to reduced ruminal pH Similar results were observed by Kalscheur, *et al* (1997) in a study where buffer was added to the low fibre diet Addition of the buffer to the diet led to increased pH and a reduction in production of *trans*-octadecenoic acids Griinari, *et al* (1998a) found that a diet with a low forage to concentrate ratio led to enrichment of milk fat with the *t*10, *c*12 CLA isomer

4.1.8 Protected Oils

Fats and oils can be protected against biohydrogenation by rumen microorganisms Physical protection involves heat-treating oilseeds The high temperatures alter the protein matrix around the fat droplet, the result being protection against biohydrogenation (reviewed in Kennelly, 1996) Similarly, whole oilseeds are protected against biohydrogenation due to the presence of an intact hull The level of protection provided depends on the size, physical and chemical properties of the hull Chemical protection can be achieved by emulsification or encapsulation (Kim, *et al* , 2000) in a protein matrix, one example being the treatment of a protein with formaldehyde *In vitro* studies have demonstrated that this technology can provide as much as 85% protection, whereas a maximum of 65% protection has been shown *in vivo* (Ashes, *et al* 1979, 1992, Storry, *et al* 1980) This reduced effectiveness is due to physical breakdown of the protective protein matrix by mastication and digestive processes within the rumen Another form of chemical protection is the formation of calcium (Ca) salts An increase in milk fat CLA of 3 to 5-fold after supplementing the diet with Ca salts of canola, soybean, and linseed oil has been achieved (Chouinard, *et al* , 2001) This was most likely due to breakdown of the protection mechanism within the rumen

Although one of the aims of supplementing the diet with plant oils is to enhance milk fat CLA, protection of the substrate against ruminal digestion seems contradictory – even though Chouinard, *et al* (2001) showed otherwise It would, however, appear more advantageous to protect CLA supplements against such activity In an *in vitro* study, 80-90% of unprotected CLA was hydrogenated under anaerobic conditions at 38°C for 24 h Encapsulating the CLA in a matrix of protein resulted in only 30%

hydrogenation of CLA isomers under the same hydrogenation conditions, with 70% protection achieved (Gulati, *et al*, 2000)

4 1.9 Effect of Processing on the CLA Content of Milk

Not only is it of interest to enrich milk with CLA but also it is important that this beneficial component is not negatively altered during processing of the milk fat It has been demonstrated that the means by which dairy products are processed can lead to wide variation in CLA content (Ha, *et al*, 1989, Chin, *et al*, 1992, Parodi, 1994, Lin, *et al*, 1995) Microbial fermentation during cheese ripening has led to increases in CLA content of the final product (Aneja and Murthi, 1990, Lin, *et al*, 1999b) However, such changes have not been observed m yoghurt production and it has been suggested that it is the dietary supplement used to produce the CLA enriched milk that will determine the CLA content of yoghurt rather the processing of the product (Shantha, *et al*, 1995, Boylston and Beitz, 2002) CLA enriched milk was produced following dietary supplementation of a 13 5% soybean meal diet with 12% full fat extruded soybeans or full fat extruded cottonseed with a 109% and 77% increase respectively in milk fat CLA compared with milk fat from the control diet Production of Mozzarella cheese from this CLA enriched milk did not alter CLA concentrations (Dhiman, *et al*, 1999) Bauman, *et al* (2000b) produced CLA enriched butter after feeding a low forage diet supplemented with sunflower oil The result was the production of milk with increased concentrations of CLA It was necessary to collect the milk for the study over the first few days from the cows that produced the highest concentrations of CLA as the increased concentrations of CLA were transient and declined over a three-week period The resulting butter had CLA concentrations 7-fold

greater than that of the control butter with the *c*9, *t*11 isomer being the most abundant isomer at 91%

4 1.10 Objective of Experiment

Previously it has been demonstrated that after 18 days feeding of ground FFR *c*9, *t*11 CLA concentrations in milk increased to 31 8 mg/g fat compared with 13 4 mg/g fat in the milk of control cows on the unsupplemented diet (Lawless, *et al* , 1998) Stanton, *et al* (1997) also demonstrated that a diet supplemented with high concentrations (1650 g/day per cow) of FFR resulted in higher milk fat CLA than diets consisting of pasture alone or containing lower concentrations (825 g/day per cow) of FFR

There is great variation in milk fat CLA concentrations between individual cows, with concentrations as low as 2 mg/g fat and as high as 30 mg/g fat being observed (Lawless, *et al* , 1998, Jiang, *et al* , 1996) This variation may be due to differences in the expression of Δ9-desaturase between cows, although this has yet to be proven It would therefore be beneficial to select cows with a natural ability to produce high concentrations of CLA in combination with the manipulation of the diet by supplementation with plant oil with a view to further elevate milk fat CLA

In this study, cows were selected as low or high CLA producing animals These were then supplemented with FFR to determine (a) the response of cows with different CLA-producing abilities on a control diet of pasture only and a diet supplemented with rapeseed and (b) the period of time required to achieve a maximum concentration of CLA due to dietary supplementation

4 2 Materials and Methods

4 2 1 Materials

The supplemental feed (Pura 42™) was purchased from J W Green and Co Ltd , Cork, Ireland Pura 42™ is a blend of whole rapeseed and pulses which is processed using "fluidised bed" cooking technology The blend temperature is raised to 125°C and then steam cooked for approximately 20 min at over 100°C The cooked product is then ground and pelleted Linoleic acid (LA) and Tridecanoic acid (C13 0) (99% pure) were purchased from Sigma-Aldrich Ireland Ltd , (Dublin) Pure *c*9, *t*11 and *t*10, *c*12 CLA isomers were obtained from Matreya (Pleasant Gap, PA) Methanolic HCl (12% v/v) was obtained from Supelco Inc (Bellefonte, PA) Hexane, methanol and chloroform were purchased from LabScan Analytical Services Ltd , Unit T26, Stillorgan Industrial Park, Co Dublin, Ireland

4.2 2 Experimental Design

The experiment was a 2 (high and low CLA producing cows) x 2 (unsupplemented and supplemented) factorial design Milk from 59 individual cows was analysed for CLA content and subsequently 40 cows (16 autumn-calvers per treatment and 4 spring-calvers per treatment) were selected and divided into two groups of high and low CLA producers These two groups were further divided into cows fed a control diet and cows fed a diet supplemented with 3 3 kg/day Pura 42™ Supplementation (8 autumn-calvers and 2 spring-calvers per diet) continued for 8 weeks

4 2 3 Collection of Milk Samples

Samples were taken at the evening milking for the first ten days of the trial beginning the day prior to commencing dietary supplementation (day 0) These samples were

composited according to yield into 1 sample per treatment group for analysis A sub-sample was removed from the day 8 sample of each cow and analysed individually It was anticipated that differences in *c*9, *t*11 CLA concentrations would be evident by day 8 of supplementation based on previous observations (Lawless, *et al* , 1998)

4.2 4 Fatty Acid Analysis of Milk Fat

Milk fat samples were obtained by collecting the cream by centrifugation at 1,000 x g for 20 min at 4°C Following overnight storage at -18°C the cream was heated to 60°C for 10 min and centrifuged at 1,600 x g for 7 min The top layer (milk fat) was removed and stored at -20°C prior to methylation (Murphy, *et al* , 1990) Milk fat was methylated using acid-catalysed methylation as described by Stanton, *et al* (1997) with reference to the internal standard $C_{13\,0}$ Milk fat (30 mg) and C13 0 (1 5 mg/g fat) were resuspended in 12 ml of 12% HCl (v/v) in methanol and incubated at 60°C for 20 min, vortexing every 10 min The sample was then washed with 2 ml water saturated hexane and 5 ml hexane, vortexed for 30 sec followed by centrifugation at 1,000 x g for 5 min The top layer containing the FAMEs was removed and washed with 2 ml water saturated hexane, vortexed and centrifuged at 1,000 x g for 5 min The top layer was removed and 0 5 g anhydrous sodium sulphate was added, vortexed for 5 sec and allowed to stand for 1 hr The top layer was then removed and diluted for GC analysis (150 µL sample and 850 µL hexane) Separation of the FAME was performed on a Chromopack CP Sil 88 column (100 m x 0 25 mm i d , 0 20µm film thickness, Chromopack, Middleburg, Netherlands) using a Varian 3400 GLC (Varian, Walnut Creek, CA, USA) fitted with a FID Helium was used as the carrier gas at a pressure of 32 7 psi The injector was held at an initial temperature of 160°C for 0 1 min and programmed to increase at a rate of 200°C/min to a final temperature of 225°C, which

was held for 15 mın The detector temperature was 250°C The column oven was held at an ınıtıal temperature of 80°C for 8 mm and then programmed to ıncrease at a rate of 8 5°C/mın to a final temperature of 200°C, whıch was held for 77 mın Data were recorded and analysed usıng a Mınıchrom PC system (VG Data Systems, Cheshıre, UK) CLA ısomers were ıdentıfied by retentıon tıme wıth reference to CLA standards

4.2.5 Fatty Acıd Analysıs of Feed

Oıl was extracted from Pura 42™ and pasture usıng the Folch wash method (Chrıstıe, 1989) A volume of 10 ml of methanol were added to 4 g Pura 42™ and vortexed for 1 mın To thıs mıxture was added 20 ml chloroform followed by vortexıng for 2 mm The mıxture was fıltered, followed by resuspensıon of the solıd resıdue ın 30 ml chloroform-methanol (2 1 v/v) and vortexmg for 3 mın The mıxture was agaın filtered and the resıdue washed wıth 20 ml chloroform, vortexed for 1 mm, filtered and washed wıth 10 ml methanol, and agaın vortexed for 1 mın and filtered The filtrates from each step were combıned A volume equal to 0 25 of the total volume of the filtrates was added to the filtrate ın the form of 0 1 M potassıum chlorıde ın water The solutıon was shaken vıgorously and centrıfuged at 1,000 x g for 5 mın The upper layer was removed A volume equal to 0 25 of the volume of the lower layer was added ın the form of water-methanol (1 1 v/v) The solutıon was centrıfuged at 1,000 x g for 5 mın The bottom layer, contaınıng purıfied lıpıds, was carefully removed and concentrated usıng rotary evaporatıon The fat was resuspended ın hexane followed by dryıng wıth nıtrogen gas and stored for methylatıon Methylatıon and GC analysıs were performed as for mılk fat samples usıng acıd-catalysed methylatıon

4.2.6 Blood Analysis

Blood samples were taken from coccygeal vessels into 7 ml heparinised vacutainers once after the morning milking in the 8th week of the experiment Plasma was prepared by centrifugation at 800 x g for 10 min at 4°C and analysed for the following β-hydroxybutyrate (BHB), glucose, protein, urea, cholesterol, trigylceridedes and non-esterified fatty acids (NEFA) using Cobas Mira biochemical Analyzer (Roche Diagnostics, Basel, Switzerland)

4.2 7 Statistical Analysis

Statistical procedures were carried out using SAS (SAS Institute, 1991), however correlations were carried out using MS Excel, 1997 The mean values for the experimental period for milk, fat, protein and lactose yields and milk fat, protein and lactose concentrations were analysed as a randomised block design The model used for these analyses had terms for block (CLA group and supplementation), pre-experimental data where appropriate and the interactions between CLA group and supplementation treatment The fatty acid content (at selection, day 0 and day 8) of the milk and the blood metabolite data were analysed as a randomised block and the model used had terms for block, CLA group (high or low) and supplementation treatment (none vs supplementation) Differences between treatments were tested for significance using student's *t* test

4.3 Results

4.3.1 Feed Analysis

Compositional analysis of pasture demonstrated that DM was 908 g/kg DM compared with 935 g/kg (DM) for the Pura 42™ supplement (**Table 4.3**) Fatty acid analysis demonstrated that the rapeseed supplement, Pura 42™ had a typical fatty acid profile for rapeseed (Lawless, *et al*, 1998) Oleic acid was the predominant fatty acid at a concentration of 56% FAME, followed by LA at a concentration of 23% FAME The other fatty acids, palmitic acid, stearic acid, arachidonic acid and linolenic acid were present at lower concentrations (**Table 4.3**)

Table 4.3 Chemical composition of pasture and rapeseed supplement

Component	Pasture	Pura 42™
Dry matter (DM), g/kg	908	935
Ash, g/kg DM	84	51
Crude protein, g/kg DM	168	218
Organic matter digestibility (OMD), g/kg DM	800	-
Fatty acids (% FAME)		
C14 0 myristic acid	0 8	-
C16 0 palmitic acid	12 4	6 7
C18 0 stearic acid	1 9	1 9
C18 1 *c*9 oleic acid	2 6	56 3
C18 2 linoleic acid	10 9	22 9
C18 3 linolenic acid	63 8	8 9
C20 4 arachadonic acid	-	0 5

Analysis of pasture produced a typical fatty acid profile for pasture (**Table 4 3**) (Dhiman, *et al*, 1995), being rich in linolenic acid (64% FAME) with smaller quantities of linoleic (11% FAME) and palmitic acid (12% FAME) present Also present were myristic acid, stearic acid and oleic acid at lower concentrations

4.3.2 Effect of Supplementation on Milk Composition

Production and composition data are presented in **Table 4 4** On average, milk yield was significantly (P<0 001) increased from 17 1 kg to 20 5 kg due to rapeseed supplementation Supplementation also led to significant increases in milk fat yield (12% increase), protein yield (19% increase) and lactose yield (21% increase) (**Table 4.4**) However, the fat content was significantly reduced with rapeseed supplementation from 41 2 to 38 8 g/kg Supplementation had no significant effect on protein or lactose content

Table 4 4 Average effect of CLA content and supplementation on milk composition

Blood Component	CLA Content				Supplementation			
	Low	High	SEM	p-value	None	+3 5kg	SEM	P-value
Milk Yield	18 8	18 7	0 58	NS	17 1	20 5	0 55	***
Fat Yield	0 74	0 74	0 02	NS	0 70	0 78	0 02	*
Protein Yield	0 66	0 67	0 02	NS	0 61	0 73	0 02	***
Lactose Yield	0 85	0 87	0 03	NS	0 78	0 94	0 03	***
Fat (g/kg)	39 8	40 2	0 69	NS	41 2	38 8	0 66	*
Protein (g/kg)	36 0	36 5	0 32	NS	36 3	36 3	0 31	NS
Lactose (g/kg)	45 3	46 3	0 29	*	45 6	46 0	0 26	NS

NS = not statistically significant
* P<0 05
*** P<0 001

4.3 3 Relationship Between CLA Content and Milk Composition

Comparison of the high and low CLA producing cows showed that lactose content was significantly (P<0 05) higher in high CLA producing cows (46 3 g/kg) compared with that of low CLA producing cows (45 3 g/kg) There were no significant differences for the other parameters measured due to CLA content of milk fat (**Table 4 4**)

4 3 4 Effect of Supplementation on Blood Components

It was found that the rapeseed supplementation significantly (P<0 01) reduced NEFA by 34% Supplementation significantly (P<0 05) increased blood protein by 3 5% and blood cholesterol (P<0 001) by 44% The supplement had no significant effect on blood glucose, BHB, urea, or triglycerides (**Table 4 5**)

Table 4 5 Effect of supplementation on blood components

Blood Component	CLA Content			Supplementation		
	Low	High	P-value	None	+3 5 kg	p-value
Glucose, mmol/L	4 18	4 47	***	4 27	4 38	NS
NEFA, mmol/L	0 205	0 141	*	0 209	0 137	**
BHB, mmol/L	0 389	0 382	NS	0 347	0 424	NS
Protein, g/L	85 13	84 66	NS	83 45	86 34	*
Urea, mmol/L	5 88	5 89	NS	5 85	5 92	NS
Triglycerides, mmol/L	0 216	0 246	NS	0 223	0 239	NS
Cholesterol	5 01	4 87	NS	4 06	5 83	***

NS = not statistically significant
* P<0 05
** P<0 01
*** P<0 001

4.3 5 Blood Components in Cows Selected for High and Low CLA Groups

Of the blood components analysed, glucose and NEFA differed significantly between the high and low CLA producing cows (**Table 4.5**) Glucose was higher (P<0 001) in the blood of the high CLA group (4 47 mmol/L) compared with that of the low CLA group (4 18 mmol/L), whereas NEFA were lower (P<0 05) in the high CLA group (0 141) compared with the low group (0 205) The other blood components (BHB, protein, urea, triglycerides and cholesterol) analysed were not significantly different in low CLA-producing cows compared with high CLA-producing cows

4.3.6 Effect of Supplementation on Fatty Acid Composition of Milk Fat

When comparing milk of supplemented cows with that of control cows (**Figure 4 3**) there was a notable difference in the concentration of a number of the milk fatty acids

after 8 days of supplementation In general, the Pura 42™ supplemented diet led to a decrease in the saturated fatty acids and increases in unsaturated fatty acids in the milk fat Palmitic acid (C16 0) was significantly reduced (P<0 001) by 29% due to rapeseed supplementation, whereas oleic acid (C18 1 *c*9) was significantly increased (P<0 01) by 24% due to the supplemented diet LA (C18 2) was also significantly higher (P<0 001) (by 39%) in supplemented cows Linolenic acid (C18 3) was significantly lower (P<0 05) in the milk of supplemented cows (3 7 mg/g fat) compared with control milk (4 4 mg/g fat) Surprisingly, rapeseed supplementation had no significant effect on *c*9, *t*11 CLA, the concentration being 8 6 mg/g fat in the control group compared with 9 2 mg/g fat in the supplemented group On the other hand, VA was significantly increased (P<0 01) by 22% after rapeseed supplementation from 20 to 25 mg/g fat However *t*10, *c*12 CLA was significantly reduced (P<0 001) from 0 51 mg/g fat to 0 33 mg/g fat due to rapeseed supplementation Elaidic acid (C18 1 *t*9) was significantly higher (P<0 001) in the milk fat of supplemented cows (5 47 mg/g fat) compared with control cows (1 9 mg/g fat), an increase of 188% A significant (P<0 05) 10% increase in the concentration of C18 0 (stearic acid) was observed in the milk fat of cows on the supplemented treatment Although concentrations of C4 0 and C6 0 were not significantly affected by the rapeseed supplement, concentrations of other short to medium chain fatty acids (C8 0 to C16 1) were reduced due to rapeseed supplementation, whereas concentrations of long chain fatty acids in milk fat were increased with the exception of *t*10, *c*12 CLA, *c*9, *t*11 CLA and linolenic acid (C18 3)

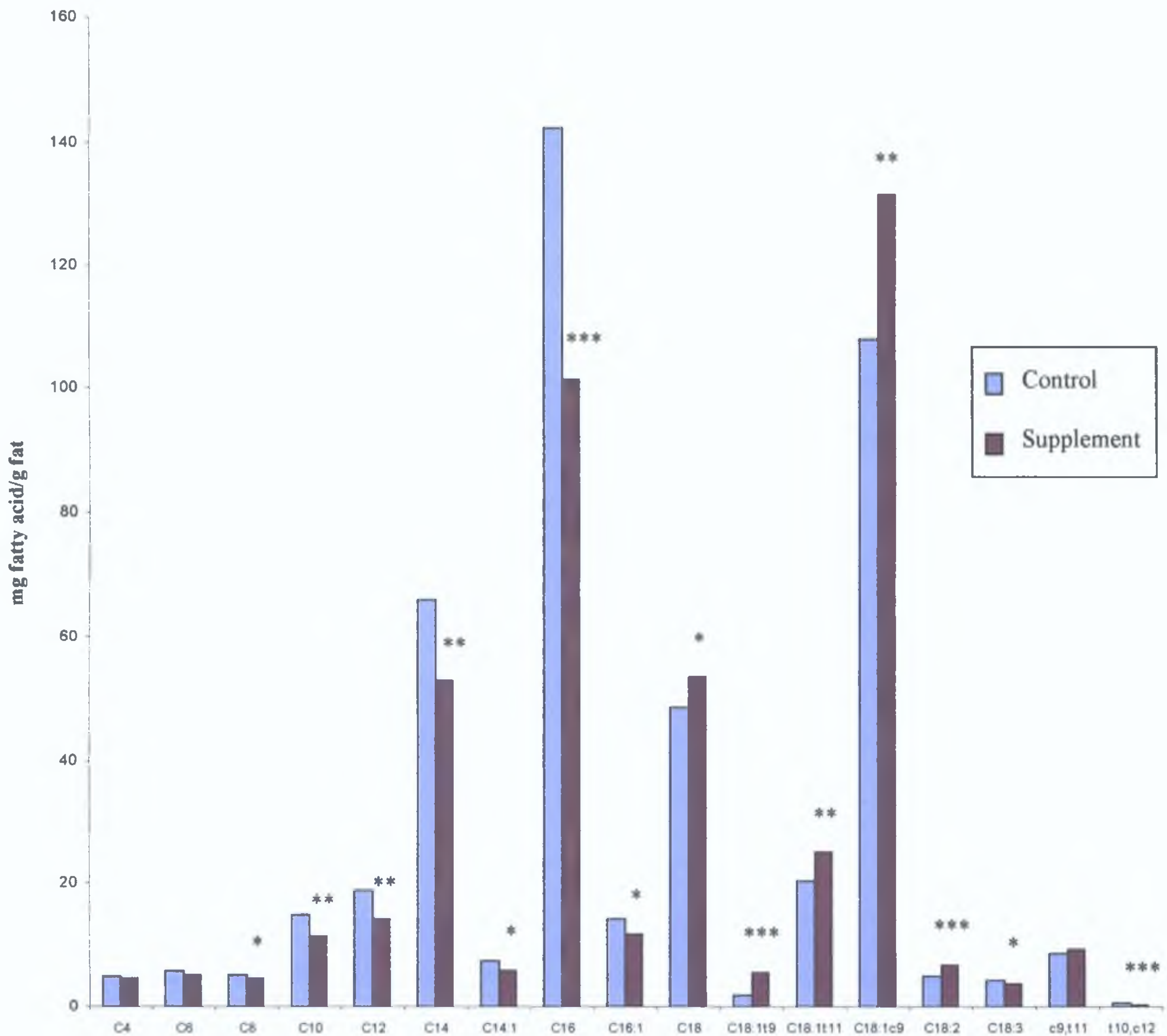

Figure 4.3 Effect of 8 days rapeseed supplementation on fatty acid profile of milk fat. Values shown are mean values of individual cows

* $P<0.05$
** $P<0.01$
*** $P<0.001$

4.3.7 Relationship Between CLA Content and Fatty Acid Composition of Milk Fat

There were a number of significant differences between the fatty acid profiles of the high and low CLA treatment groups after 8 days supplementation (**Figure 4.4**).

Most of the fatty acid concentrations were higher in the milk of the low CLA producing cows (**Figure 4 4**) The concentrations of C4 0 C6 0, C8 0, C10 0, C12 0, C14 0, C14 1, C16 0, C16 1, C18 0, C18 1 *c*9, C18 2, and C18 3 were significantly higher in the milk fat of low CLA producing cows There was no significant difference in the concentrations of C14 1, C18 1 *t*9, C18 1 *t*11, *c*9, *t*11 CLA and *t*10, *c*12 CLA in the milk fat obtained from the two groups

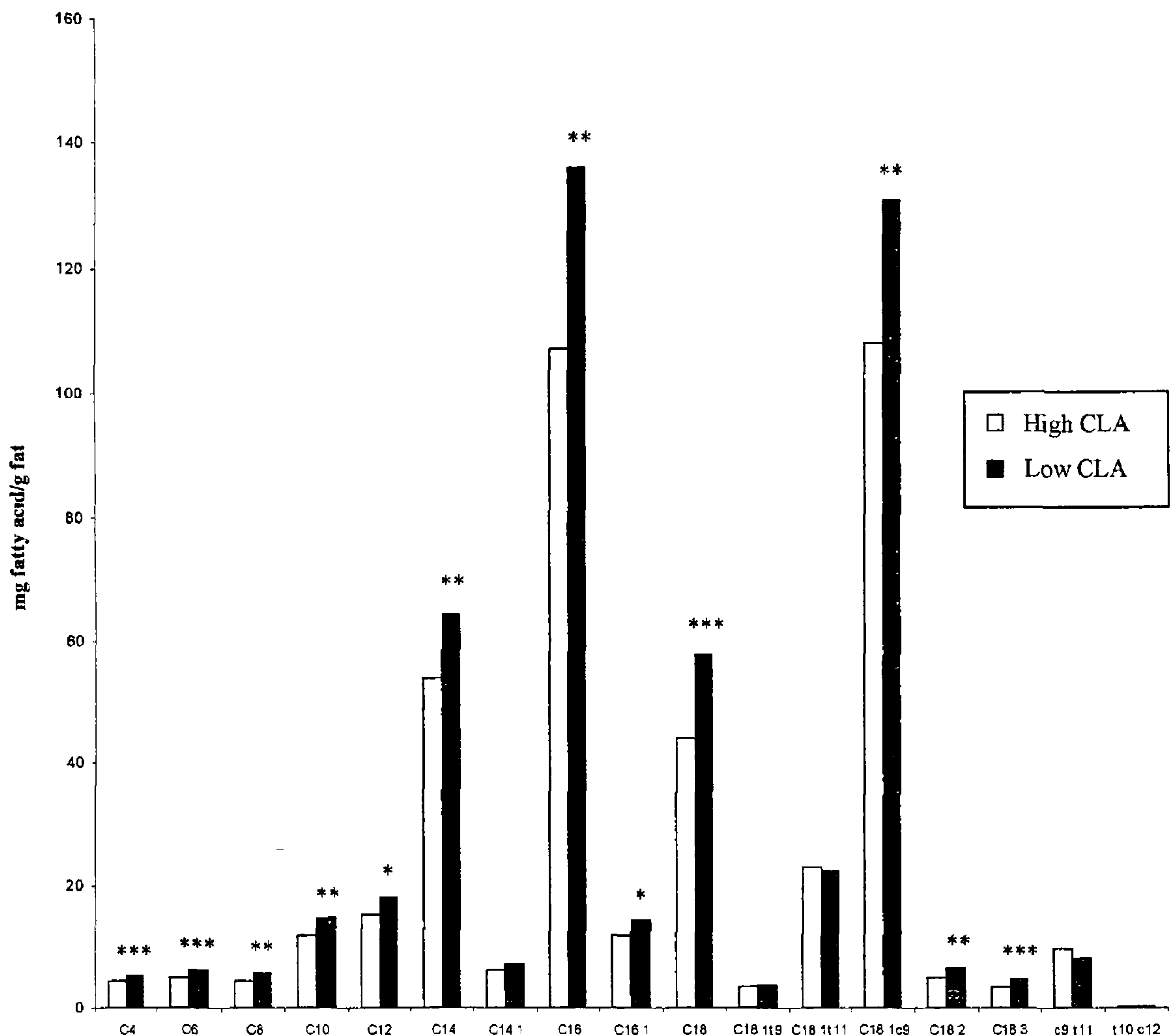

Figure 4 4 Fatty acid profile of milk fat from cows selected for high and low CLA groups regardless of diet after 8 days supplementation

* P<0 05
** P<0 01
*** P<0 001

4 3.8 Relationship between Dietary Supplementation and Milk Fat CLA over the First Ten Days of the Trial

Figure 4 5 presents the *c*9, *t*11 CLA content of the composites of each of the four treatment groups collected over the first ten days of the trial Surprisingly, there was no significant difference due to rapeseed supplementation in *c*9, *t*11 CLA content of the milk fat from the low CLA unsupplemented group and the low CLA supplement group (**Figure 4.5**) This was also observed in the two high CLA groups Milk fat CLA concentrations did not increase in a consistent way on any of the treatment groups and therefore, the time required to maximally enrich milk fat with CLA could not be determined

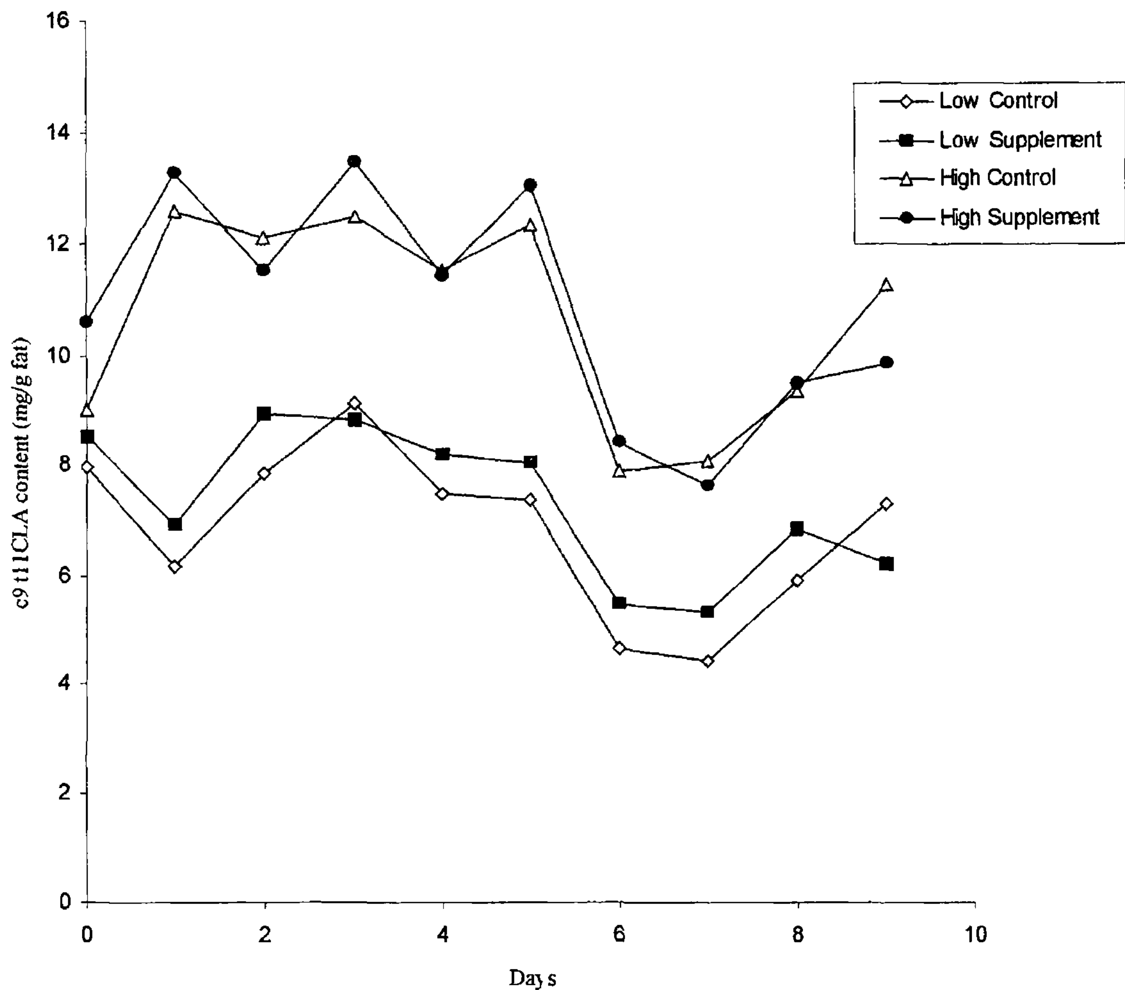

Figure 4 5 *c*9, *t*11 CLA content of milk fat in composite samples over 10 days

When comparing the differences between the *c*9, *t*11 CLA content of the high and low CLA producing cows on the unsupplemented diet it was found that the *c*9, *t*11 CLA content in the high CLA control group composite was significantly higher (P<0 01) than that of the low CLA control group by 13% at day 0 The *c*9, *t*11 CLA content of milk fat from the high CLA unsupplemented group was again significantly higher at day 2 (P<0 001) by 54%, day 5 (P<0 05) by 68%, and day 6 (P<0 05) by 70% compared with that of the low CLA unsupplemented group Similar observations were made when comparing the *c*9, *t*11 CLA content of the two supplemented groups *c*9, *t*11 CLA was significantly higher in milk from the high CLA supplemented group at day 0 (P<0 05) by 24%, day 1 (P<0 05) by 92%, day 5 (P<0 01) by 62%, day 6 (P<0 05) by 54% and day 9 (P<0 05) by 55% compared with that of the low CLA supplemented group However, the four composite groups appear to follow the same pattern of increases and decreases in *c*9, *t*11 CLA after 1 day of supplementary treatment It was noted that there was no significant difference in the *c*9, *t*11 CLA content of composites from control groups compared with supplemented groups at 8 days, the day on which individual samples were taken and in which no significant changes in *c*9, *t*11 CLA content were observed

4 3 9 Changes in CLA Content from Selection to Day 0 of Rapeseed Supplementation

The correlation (r = 0 0435) between the CLA concentrations in samples taken during the selection process and that of samples taken at day 0 was very weak (**Figure 4.6**) This indicates that there were large changes in the CLA content of the milk of individual cows over the time period between collection of these two sets of milk samples (20 days)

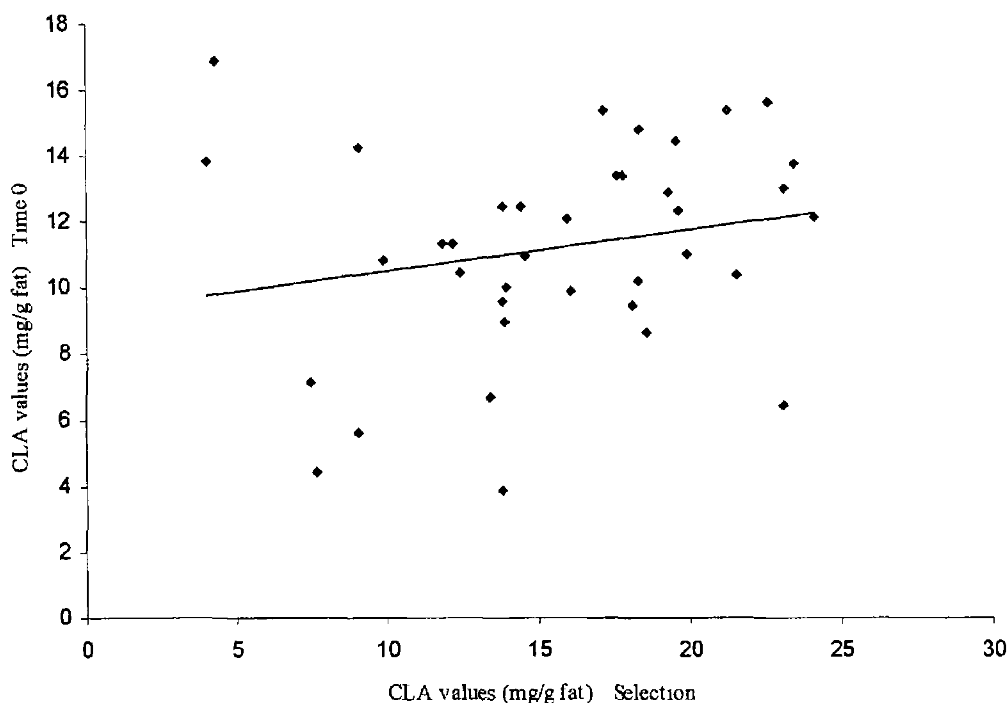

Figure 4 6 Correlation between CLA concentrations in milk fat used for selection process and concentrations in milk fat at day 0 of the feeding trial (y = 0 1238x + 9 2589) (MS Excel 1997)

4 3.10 Relationship Between Vaccenic Acid and *c*9, *t*11 CLA

Figure 4 7 presents the correlation between *c*9, *t*11 CLA and VA from the time of selection to 8 days supplementation (**Figure 4 7A**), the day on which rapeseed supplementation began – day 0 (**Figure 4.7B**) and after 8 days of rapeseed supplementation (**Figure 4.7C**) **Figure 4.7B** shows that a strong correlation (r = 0 8685) existed between the concentration of *c*9, *t*11 CLA and VA The relationship between *c*9, *t*11 CLA isomer and VA in the milk fat was still present at day 0 – the day on which rapeseed supplementation commenced, but prior to supplementation However, after 8 days supplementation the correlation (r = 0 0281) between *c*9, *t*11 CLA and VA was very weak (**Figure 4.7C**)

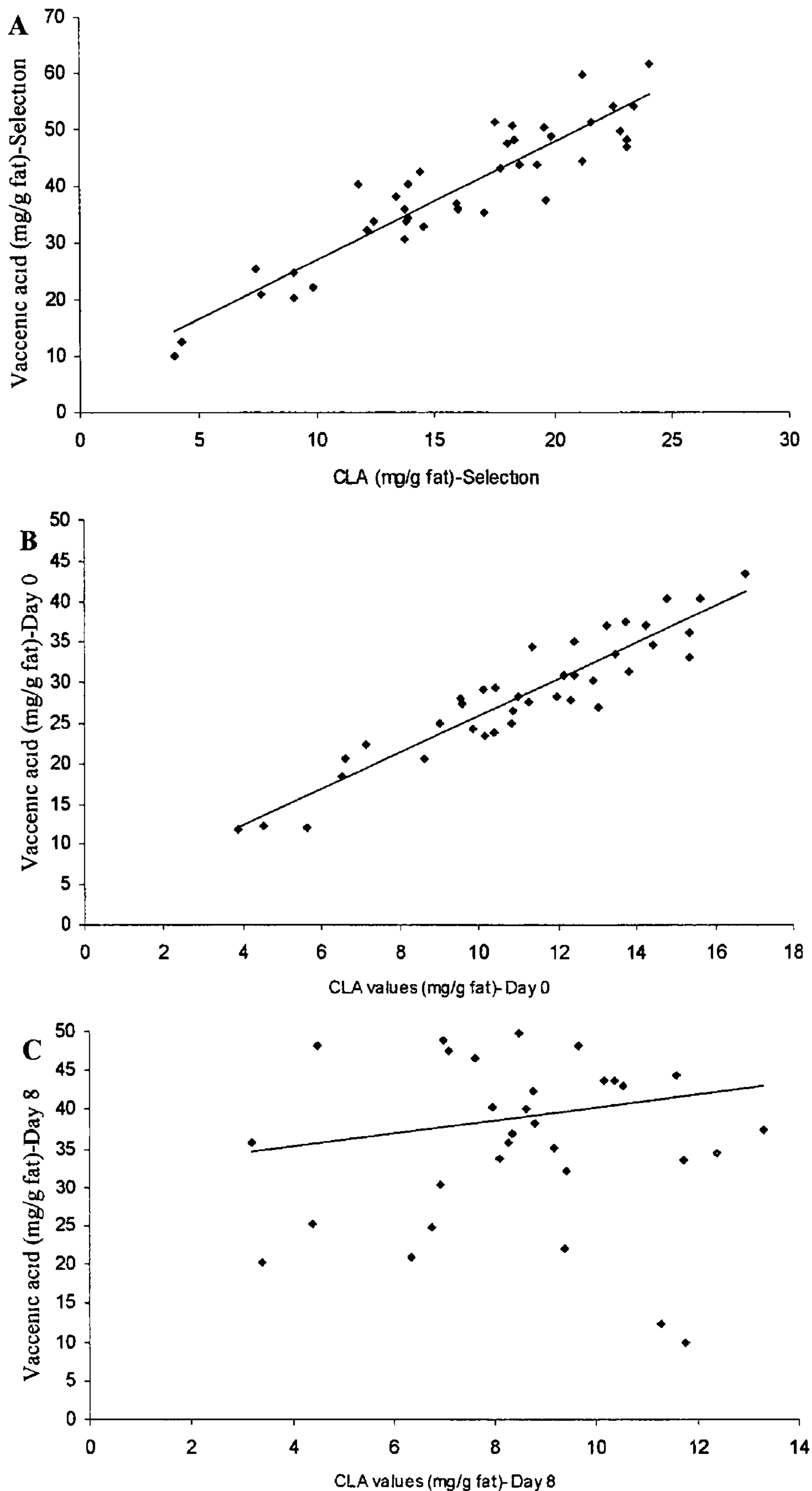

Figure 4 7 Correlation between *c*9, *t*11 CLA and VA at different time points (A) Correlation between *c*9, *t*11 CLA and VA values in milk fat at selection (y = 2 0829x + 6 0943, r=0 8685) (B) Correlation between *c*9, *t*11 CLA and VA values in milk fat at 0 days supplementation (y = 2 2673x + 3 2046, r=0 8481) (C) Correlation between *c*9, *t*11 CLA and VA values in milk fat after 8 days supplementation (y = 0 8309x + 32 056, r=0 0281) (MS Excel 1997)

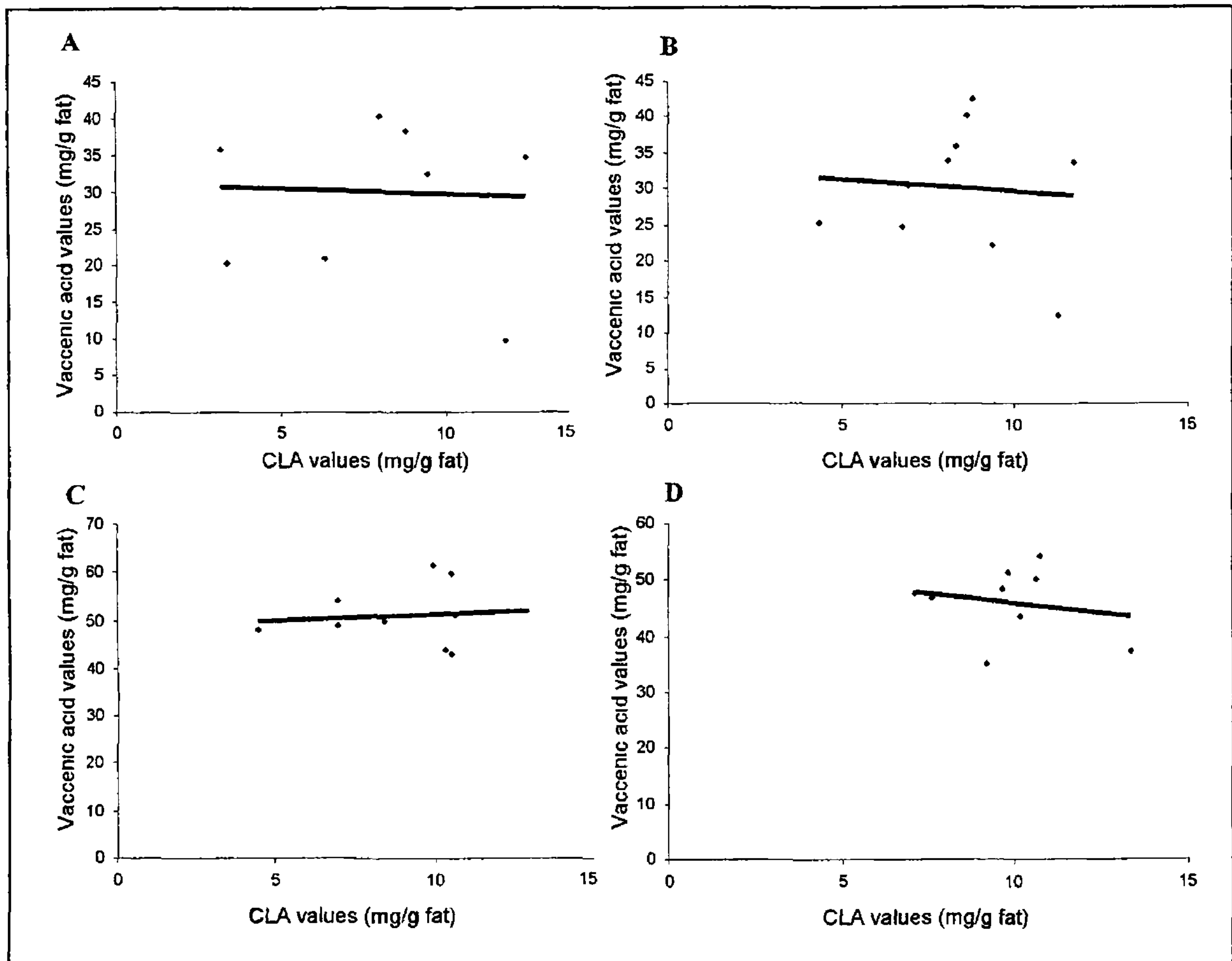

Figure 4 8 Correlations between CLA and vaccenic acid in each of the 4 treatment groups after 8 days supplementation (A) Low CLA Control Group, (B) Low CLA Supplement Group, (C) High CLA Control Group, (D) High CLA Supplement Group

Figure 4.8 shows the correlation between CLA and VA in milk fat obtained from each of the treatment groups after 8 days of the trial There was a very weak correlation between VA and *c*9, *t*11 CLA in the milk of both the low CLA producing control (r = 0 0025) and supplemented group (r = 0 0067) (**Figure 4.8A**) Similarly there was a weak correlation between VA and *c*9, *t*11 CLA in milk fat of the high CLA producing control (r = 0 0081) and supplemented (r = 0 0517) groups This suggests that VA was not converted to *c*9, *t*11 CLA to the same extent observed at the time of selection Therefore it appears that the relationship between VA and *c*9, *t*11 CLA observed at the time of selection no longer existed 8 days after the trial began regardless of diet

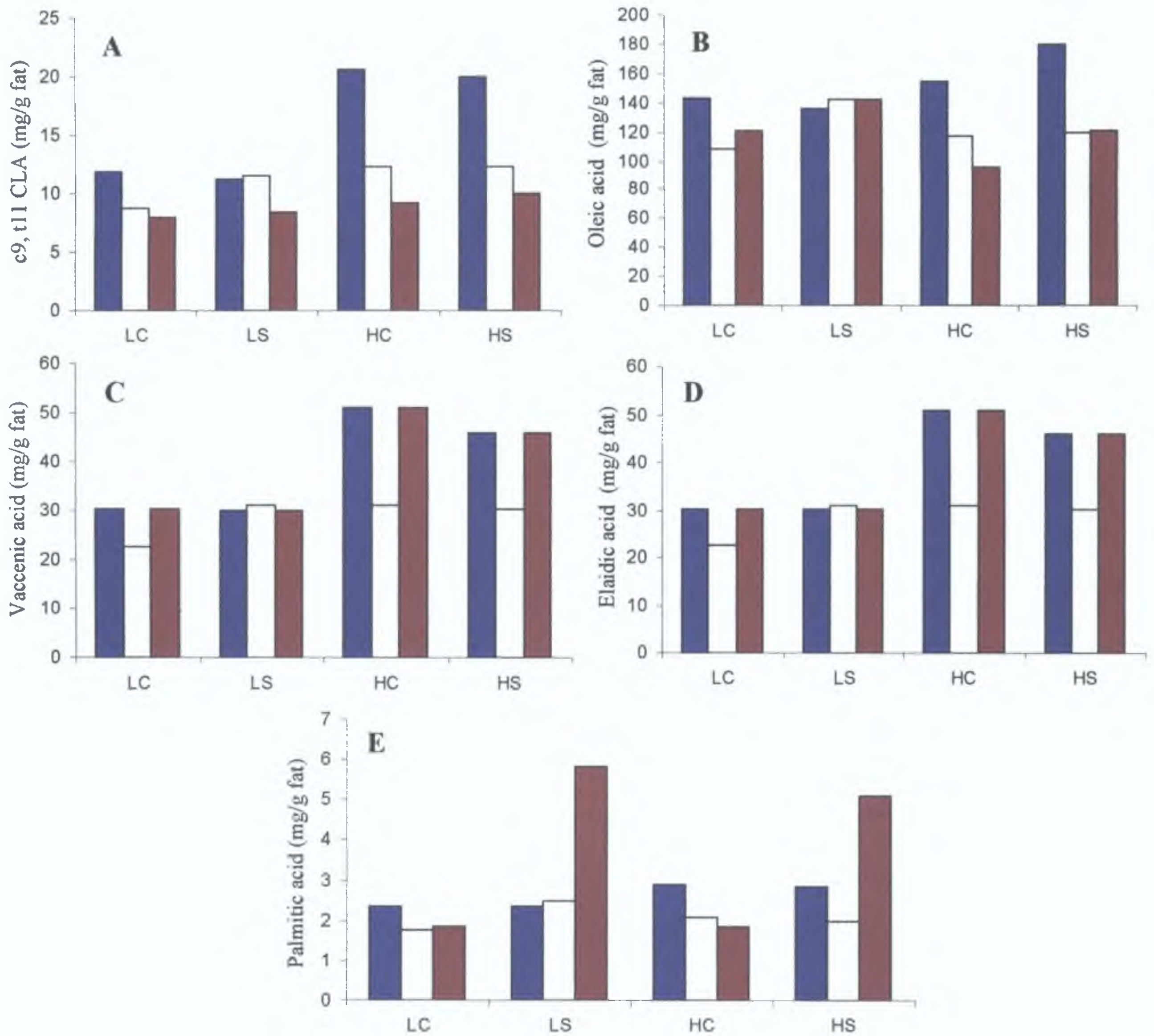

Figure 4.9 Changes in fatty acid content of milk of low control (LC, low supplement (LS), high control (HC) and high supplement (HS) cows with time. ■ Selection, □ Day 0, ■ 8 Days. A = *c*9, *t*11 CLA, B = oleic acid, C = vaccenic acid, D = elaidic acid and E = palmitic acid

Five fatty acids were chosen to represent the changes in fatty acid content from time of selection up to 8 days supplementation. These fatty acids were *c*9, *t*11 CLA, oleic acid, VA, elaidic acid and palmitic acid. Fatty acid content at the time of selection (20 days prior to commencement of dietary supplementation) and at day 0 should remain quite similar, as there was no change in diet. However this was not so, with individual fatty acid content being lower at day 0 compared with samples taken at the time of selection

– with the exception of the low supplement cows where the concentration of the fatty acids present remained similar between these two time points (**Figure 4.9**) After 8 days supplementation, milk fat *c*9, *t*11 CLA was at its lowest of the three time points despite the supplementation of the diet with rapeseed

Oleic acid content was lower in milk taken from cows in the high control group after 8 days compared to samples taken at selection and day 0 Concentrations of this fatty acid were similar in day 0 samples and after 8 days supplementation in the other three treatment groups

Vaccenic acid increased in the low control, high control and high supplement groups after 8 days supplementation Elaidic acid remained similar in control groups although it was slightly higher at selection However, in the supplemented groups elaidic acid increased greatly after 8 days Palmitic acid decreased at each time point for each of the treatment groups except the low control group which increased slightly after 8 days

4.4 Discussion

Dietary supplementation with the aim of enhancing milk fat CLA has been well studied, therefore it is of interest to explore the manipulation of other factors which may further enhance CLA concentrations in milk fat One of the aims of this study was to determine if purposefully selecting cows with a natural ability to produce high concentrations of milk fat CLA together with supplementation of the diet with rapeseed would lead to further elevated concentrations of milk fat CLA

4 4 1 Effect of CLA Producing Ability on Fatty Acid Profile of Milk Fat

When comparing the effect of CLA producing ability of the cows, C4 0 C6 0, C8 0, C10 0, C12 0, C14 0, C14 1, C16 0, C16 1, C18 0, C18 1 *c*9, C18 2, and C18 3 fatty acids were higher in the low CLA producing cows whereas there was no significant difference in concentrations of C14 1, C18 1 *t*9, C18 1 *t*11, *c*9, *t*11 CLA and *t*10, *c*12 CLA between the two groups Despite little change in CLA concentrations of individual milk samples due to supplementation, analysis of composite samples of the four treatment groups collected over the first 10 days of the trial showed some significant differences in CLA concentrations between high and low CLA groups Composites of milk from the high CLA control group contained 13%, 54%, 68%, and 70% more *c*9, *t*11 CLA than composites of milk from the low CLA control group at days 0, 2, 5 and 6 respectively Similarly there were differences in the *c*9, *t*11 CLA content of milk from the high CLA supplemented group and the low CLA supplemented group Composites of milk from the high CLA supplemented group collected on days 0, 1, 5, 6 and 9 were 24%, 92%, 62%, 54% and 55% (respectively) higher than composites of milk from cows in the low CLA supplemented group collected on the same days

Due to the fact that CLA concentrations m the milk fat were not enhanced by rapeseed supplementation as expected, it is difficult to compare the influence which CLA producing ability has on the other fatty acids present in milk fat Some of the composites showed differences in CLA content between low CLA groups and likewise in high CLA groups, whereas no such differences were observed in individual cows However, there were no significant differences in CLA between composite groups on day 8 – the time at which the individual samples were collected therefore, these two sets of data are not contradictory

Analysis of the fatty acid profile of milk fat suggests that an ability to produce high concentrations of CLA led to lower concentrations of other fatty acids However there were differences in the blood components of cows from the two groups Blood glucose levels were higher in the high CLA producing cows whereas NEFA were lower in this group compared to low CLA producing cows This suggests that the high CLA cows were in a better energy status than the low CLA cows

Why cows differ in their ability to produce CLA is unclear However, as endogenous synthesis is the primary source of milk fat CLA (reviewed in Grinari and Bauman, 1999) it is likely that variations in CLA producing ability is due to genetic variations regarding Δ9-desaturase Another possibility is differences in ruminal environment between individual cows, with the rumen of some cows supporting optimal CLA production compared with that of other cows (reviewed in Grinari and Bauman, 1999) However, in this study the CLA producing ability of the cows changed in the 2 week period between time of selection and day 0 The reason for such a change is unclear It may be possible that Δ9-desaturase activity may have been inhibited or it is

more likely that the unusual weather conditions experienced throughout the trial may have interfered with the content and quality of the pasture eaten by the cows, interfering with CLA production This possibility is discussed below

4 4 2 Effect of Rapeseed Supplementation on CLA Content of Milk Fat

Despite the abundance of evidence regarding the positive relationship between plant oil supplementation and increase in milk fat CLA, no such relationship was observed in this study Five fatty acids that are known to change after rapeseed supplementation or were observed to change in this study were plotted against time to determine the presence of any pattern over time In general, these fatty acids decreased between selection and day 0 (prior to commencing supplementation) VA was seen to increase, however an increase was observed in milk from the low and high control groups which did not receive supplementation, suggesting changes in pasture as this was the only form of feed available to these cows An increase in elaidic acid was observed after supplementation of the diet with rapeseed, rather than an increase in *c*9, *t*11 CLA Unlike VA, increases in elaidic acid were only observed in the milk of supplemented cows Elaidic acid concentrations in milk fat have not been reported, although a review by Jensen (2002) states that other C18 1 *trans* isomers constitute 3% of reported oleic acid concentrations Increases in milk fat elaidic acid due to rapeseed (rich in oleic acid) supplementation have not been reported previously with studies reporting increase in C18 1 and not differentiating between the various isomers of this fatty acid (Murphy, *et al*, 1995, Lawless, *et al*, 1998) Mosley, *et al* (2002) demonstrated that a culture of mixed gut microbes from the stomach of cattle had an ability to produce a variety of trans isomers, including elaidic acid, from oleic acid However, it was difficult to determine the percentage of this isomer formed from oleic acid as the oleic

acid substrate was contaminated with elaidic acid Elaidic acid is also produced from LA biohydrogenation in the rumen in the same manner as VA (Kepler, *et al* , 1966) In the present study it may be possible that elaidic acid was the dominant by-product of ruminal biohydrogenation as opposed to VA, although the conditions required for this to occur are unclear As the majority of *c*9, *t*11 CLA is produced endogenously from VA (Griinari, *et al* , 2000), a lack of VA would in turn lead to reduced production of *c*9, *t*11 CLA Van Soest, (1994) demonstrated that reducing ruminal pH led to changes in bacterial populations and in turn, alterations in the pattern of fermentation end products Griinari, *et al* (1998a) demonstrated that conditions of decreased ruminal pH due to feeding of a high-concentrate, low fibre diet led to the formation of *t*10, *c*12 CLA and in turn C18 1 *t*10 Griinari and Bauman (1999) have proposed that the activity of a *c*9, *t*10 isomerase can increase, resulting in the formation of *t*10, *c*12 CLA This theory is supported by a study by Griinari, *et al* (1999) in which a low fibre diet led to increased concentrations of the *t*10, *c*12 CLA isomer in milk fat Therefore, it may also be possible that an isomerase, leading to the formation of elaidic acid, can increase in activity It has been demonstrated that the presence of fatty acids such as elaidic acid are not responsive to diet, however in this study we observed an increase in elaidic acid only in the milk of cows on the supplemented diet Kalscheur, *et al* , (1997) demonstrated that low ruminal pH due to a high concentrate diet resulted in increased flow of total 18 1-*trans* isomers to the duodenum

Fatty acids consisting of up to 16 carbons were significantly lower in supplemented cows as previously demonstrated by Murphy, *et al* (1990) after 3 weeks supplementation However, LA content decreased in the milk of supplemented cows in the study by Murphy, *et al* (1990) whereas in the present study LA increased due to

rapeseed supplementation, although the data in the present study were collected after a shorter supplementation period

The supplement used in the present study consisted primarily of rapeseed, a plant oil which has previously been demonstrated to enhance CLA concentrations in milk fat (Stanton, *et al*, 1997, Lawless, *et al*, (1998) Both the supplement and pasture showed typical fatty acid profiles indicating that the fatty acid content of the diet was not a reason for the unusual results Murphy, *et al*, (1990) demonstrated that milk yield and lactose yields were increased by supplementation with ground FFR in agreement with the results of the present study

CLA content of milk fat is known to vary with seasons (reviewed in Jensen, 2002) The period over which this feeding trial was undertaken had an unusual weather pattern for the time of year – there was excessive rainfall over the summer months It may be possible that this would have affected the quality of the pasture and consequently the outcome of the experiment At the time of selection and day 0 of the trial, the VA and *c*9, *t*11 CLA content m milk fat were closely correlated (r = 0 85) However, after 8 days supplementation the strong correlation disappeared – correlations of these two fatty acids within each treatment group showed similar patterns after 8 days supplementation This suggests changes in the biohydrogenation process taking place in the rumen However, despite the close correlation between VA and *c*9, *t*11 CLA at day 0 (r = 0 8481), unexpected reductions m *c*9, *t*11 CLA were observed at this time compared with that at time of selection It was observed that at day 0 milk fat VA was at its lowest concentration of the 3 time points, hence the correlation with the reduced concentration of *c*9, *t*11 CLA observed at this time point

It may be possible that at day 0 the rate of conversion of VA to *c*9, *t*11 CLA by the action of Δ9-desaturase in the mammary gland was inhibited It may also be possible that at day 0 VA concentrations were reduced in turn leading to reduced concentrations of *c*9, *t*11 CLA produced both via ruminal biohydrogenation and endogenous synthesis At 8 days VA concentrations had increased, but normal biosynthesis of *c*9, *t*11 CLA had not had sufficient time to reach its optimum

There were no effects on blood glucose and urea as a result of dietary supplementation in the present study Previously, Lawless, *et al* (1998) demonstrated that glucose was reduced and urea increased in the blood of cows supplemented with rapeseed Alterations in blood urea concentrations are an indication of the protein content of the feed Although urea levels were not altered in this study, blood protein levels increased in cows on the supplemented diet Changes in blood glucose levels may occur when metabolism is altered, while no such changes due to rapeseed supplementation were observed in this study Of the other blood components analysed, cholesterol increased significantly, while NEFA decreased in cows fed rapeseed Changes in both of these components were expected as the dietary supplementation was primarily of a lipid nature

Many of the changes expected due to rapeseed supplementation were observed in this study, a decrease in short-chain fatty acids and an increase in long-chain fatty acids in particular oleic acid and stearic acid As expected VA increased with rapeseed supplementation but not to the extent expected A 22% increase in VA was observed after supplementation compared to a 42% increase in a study by Lawless, *et al* (1998) However, CLA concentrations were not altered

4 4 3 Conclusion

There are many factors which influence CLA content of milk fat Therefore, it is difficult to determine the reason for the unexpected lack of elevation of *c*9, *t*11 CLA in milk fat due to rapeseed supplementation As the fatty acid composition of the supplement used is typical for rapeseed it may be possible that another component of the rapeseed supplement interfered with ruminal fermentation, altering the level of CLA produced The unexpected elevation of elaidic acid also suggests an interference with the fermentation process Although CLA concentrations were not increased due to rapeseed supplementation expected increases and decrease in the other major fatty acids present were observed, however not to the extent expected This suggests that ruminal biohydrogenation may have been slowed One possible reason for this may be the unusual weather conditions which prevailed during the period of the feeding trial – May and June (2002) The rainfall for the month of May 2002 (126 mm) was over twice the average rainfall (59 mm) in May for the previous 11 years (Nash, J , personal communication) The rainfall in June was also higher than the average rainfall for that month (78 mm compared with 56 mm) The rainfall in July and August was lower than the average Such weather conditions could have led to changes in the pasture, suggested in the elevated concentrations of VA in control groups after 8 days of the trial despite no change in feeding regime

4 4.4 Summary

Cows were selected for their ability to produce high concentrations of milk fat CLA High CLA-producing groups were put either on a control diet (pasture alone), or pasture supplemented with 3 5 kg rapeseed/day Low CLA-producing groups were put either on a control diet (pasture alone) or pasture supplemented with 3 5 kg

rapeseed/day Milk fat from these groups was then analysed to determine differences in CLA content between high and low CLA-producing groups over a 10 day period There was no significant difference between high CLA-producing cows and low-CLA producing cows in the *c*9, *t*11 CLA concentration of milk fat from individual cows after 8 days supplementation However when analysing the fatty acid content of composite samples taken over the first 10 days of the trial, it was found that milk fat of the high CLA control group contained 13% (day 0), 54% (day 2), 68% (day 5) and 70% (day 6) more *c*9, *t*11 CLA compared with composites of the low CLA control group Similarly, the milk fat of composites of the high CLA supplemented group contained 24% (day 0), 92% (day 1), 62% (day 5), 54% (day 6) and 55% (day 9) more *c*9, *t*11 CLA than composites of the low CLA supplemented group This study yielded a number of unexpected findings There was no significant effect on *c*9, *t*11 CLA due to rapeseed supplementation over 8 days, although elaidic acid significantly increased (P<0 001) by 188% due to dietary rapeseed with a concentration of 5 47 mg/g fat in milk fat from the high CLA supplemented group compared with 1 9 mg/g fat in milk fat from the low CLA supplemented group A correlation between VA and *c*9, *t*11 CLA which existed at the time of selection no longer existed 8 days into the trial These data suggest that alterations in the quality of the pasture and/ or rate of biohydrogenation in the rumen during supplementation period may have interfered with CLA production

CHAPTER 5

DISCUSSION

5 Discussion

In the modern world there is much emphasis on the importance of a healthy diet This is due to the fact that a number of human diseases such as cancer, coronary heart disease (CHD) and osteoporosis, have been attributed to an unbalanced diet It was once believed that milk and dairy products were undesirable in the human diet due to its fat content However, there is an increasing amount of evidence which suggests that milk contains components which impart beneficial health effects when consumed Such components include, calcium, vitamin A and D, β-carotene, unsaturated fatty acids, n-3 fatty acids, phospholipids, sphingolipids, butyric acid, essential fatty acids and conjugated linoleic acid (CLA) (Boland, *et al* 2001)

Studies investigating the mechanisms by which CLA inhibits carcinogenesis are still ongoing One of the objectives of this study was to determine if CLA inhibited the activity of ras, an oncogene which is known to be involved in a number of human cancers such as prostate and colon cancer Although data regarding the effect of CLA on this mechanism is limited, previous studies have demonstrated conflicting results Ip, *et al* (1997) suggested that CLA inhibition of mammary cancer was independent of the presence or absence of mutated ras O'Shea (2000) found that CLA isomers inhibited PFTase (an enzyme necessary for activation of ras) activity in colon and mammary cancer cell lines Experiment 1 in this study suggested that the *c*9, *t*11 CLA isomer inhibited growth of SW480 cell growth by inhibition of the activation of ras and also by inhibition of expression of the *ras* gene As suggested previously, CLA isomers differentially modulate carcinogenesis (Palombo, *et al*, 2002) and this was demonstrated m the present study The *t*10, *c*12 CLA isomer inhibited membrane and total ras, however O'Shea (2000) demonstrated that this CLA isomer stimulated

PFTase activity by 66% compared with a control Therefore, unlike the *c*9, *t*11 CLA isomer, the data suggest that the *t*10, *c*12 CLA isomer inhibited *ras* gene expression, resulting in reduced levels of ras available for activation despite the increased production of PFTase These data also suggest that growth of SW480 cells is only partially controlled by farnesylation It is of interest that the much less studied VA – a CLA precursor – also demonstrated an ability to inhibit ras The SW480 cell line has been shown to convert VA to *c*9, *t*11 CLA (Miller, *et al* 2003) It is clear that not only are there numerous mechanisms by which CLA inhibits cancer development (reviewed in Belury, 2002), but also each isomer of CLA is capable of acting against cancer in both similar and different ways

In the second experiment of this study, the anticarcinogenic activity of oils produced by the fermentation of LA with 2 Bifidobacterium strains (*B breve* NCFB 2258 and *B lactis* Bb12) was investigated Both strains have been shown previously to possess an ability to produce CLA (Coakley *et al*, 2003) The study was inconclusive in that when SW480 cells were incubated with 84 μg/ml of each oil the growth inhibitory effect observed appeared to be due to the concentrations of the primary fatty acids present in the oils However when the concentration of the treatment was reduced to 33 μg/ml the growth inhibitory effect of the oils did not mimic that of the fatty acids present As suggested in **Chapter 3**, another component may be present in the oils which is responsible for the growth inhibition observed at the reduced concentration The identity of this component requires further investigation The presence of an unidentified component in the oils fermented by *B breve* NCFB 2258 and *B lactis* Bb12 could be explained by the possibility of the production of a fermentation product such as butyric acid (Hague, *et al*, 1993) or vitamin K (reviewed by Cummings and

Macfarlane, 1997) However the fact that the control oil (which was not fermented by any bacterial strain) also produced similar results is not so easy to explain One possibility is the differential anticancer activity between esterified fatty acids and FFAs There is the potential to use these *Bifidobacterium* probiotic strains with the added value of proven anticancer activity due to an ability to produce CLA Another interesting finding from this study was the potent anticarcinogenic activity of the *t*9, *t*11 CLA isomer The *c*9, *t*11 CLA isomer inhibited growth of the SW480 cells at a concentration of 10-20 µg/ml after 4 days incubation, whereas the *t*9, *t*11 CLA isomer inhibited growth of SW480 cells at a concentration of 1-10 µg/ml after 4 days incubation The anticarcinogenic activity of this CLA isomer has not been reported previously

With the large amount of interest associated with the potential use of CLA in the development of functional foods it is of great significance to enrich CLA concentrations in milk Of the many methods previously studied to enrich milk fat CLA, the previously proven CLA enhancing dietary supplement, rapeseed, was chosen for Experiment 3 The aim of this experiment was to determine if rapeseed supplementation of the diet of cows with an ability to produce high concentrations of CLA on pasture would further enhance milk fat CLA concentrations However, unexpectedly, *c*9, *t*11 CLA was not enhanced by rapeseed supplementation, contrary to what has previously been observed by Lawless, *et al* (1998) and Stanton, *et al* (1997) Therefore it was not possible to determine if purposely selecting high CLA producing cows further enhanced milk fat CLA CLA concentrations had dropped prior to rapeseed supplementation suggesting that the lack of CLA enrichment was due to a factor other than the supplement One possibility is the unusual weather conditions

experienced at the time of the feeding trial Excessive rainfall may have interfered with the composition of the pasture – rainfall for the month of May was over twice the average rainfall for that time of year Pasture has been shown to affect the fatty acid composition of milk by its fat content but also by its influence on the microbial environment in the rumen (Griinari and Bauman, 1999) Therefore, it is possible that an imbalance in the rumen microbial environment led to inadequate production of CLA Analysis of milk samples collected later in the feeding trial is required to obtain a fuller picture of the fatty acid profile of supplemented milk over time

Further study of CLA is necessary in many areas In terms of enrichment of CLA, numerous supplements have been investigated for their ability to enhance milk fat CLA However, as already mentioned the balance of ruminal bacteria also strongly influences CLA synthesis in the ruminant Manipulation of these microorganisms has been suggested by Boland, *et al* (2001) in that reducing bacteria responsible for the saturation of *c*9, *t*11 CLA and VA in the biohydrogenation of LA would lead to enhanced levels of the two fatty acids available for absorption Increased concentrations of absorbed *c*9, *t*11 CLA can be incorporated into milk fat whereas, increased concentrations of absorbed VA will lead to increased concentrations available for the activity of Δ9-desaturase and in turn synthesis of *c*9, *t*11 CLA The aim of Experiment 3 was to select cows with an ability to produce high concentrations of milk fat CLA This characteristic is presumably due to genetic expression of high quantities of Δ9-desaturase – the activity of which is responsible for the synthesis of the majority of *c*9, *t*11 CLA found in milk fat (reviewed in Griinari and Bauman, 1999) In Experiment 3, at day 0 there was very little variation in CLA concentration in milk fat compared with milk collected at selection compared to the variation at the

time of selection Therefore, repeating this experiment when CLA in milk fat has returned to concentrations with normal variation may lead to more useful data In addition, investigation of the expression of Δ9-desaturase in relation to CLA synthesis would further develop the possibilities of enriching milk fat with CLA

The potential for CLA is immense Fish oil, which has also been implicated with beneficial health effects in humans, must be administered at a dietary concentration of 10% (reviewed in Ip, *et al*, 1994b) However, CLA has been shown to inhibit mammary tumours in rats at a concentration as low as 0 1% It is important to keep in mind the different effects which synthetically produced CLA may have on human diseases compared with naturally produced CLA It is clear that each CLA isomer has different effects and when combined with one or more of the other CLA isomers may have different effects than those observed when administered as a single isomer When used as a chemotherapeutic it is more achievable to administer CLA in the quantities and combinations required to obtain the necessary results However, when used in functional foods, the composition of CLA will be primarily *c*9, *t*11 CLA with smaller quantities of other isomers present and may be in both esterified and free form Therefore, it is important to develop the potentials for synthetically produced CLA and naturally occurring CLA separately In terms of functional foods, it may be more beneficial to investigate the effect of milk and dairy products high in CLA on humans In addition, foods rich in CLA are generally also high in fat Therefore, foods rich in CLA may not be attractive to those who wish to keep their fat intake to a minimum As fat tends to have negative health effects it is important that the food as a whole be investigated for its beneficial health effects Already, dairy products containing

probiotic bacteria are popular Use of strains which can produce CLA in products already enriched in CLA would further enhance the marketability of such products

It is important that investigation of the anticancer mechanisms of CLA continues in order to determine which isomers are most effective and to determine if combining specific isomers of CLA is more effective than single isomers alone

With all the interest and potential of CLA it is possible that animal fats may become more desirable in the human diet, paving the way for the development of a much wider variety of dairy products Not only will these products appeal to the health conscious, but such products can be developed to enhance the beneficial effects, for example, further enrichment with CLA using methods discussed throughout this thesis In addition, negative aspects can be manipulated and in turn reduced or removed completely in order to render the products more attractive Such development of dairy products will result in products with beneficial health effects which outweigh any negative health effects

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