Chapter 3

The Production of Conjugated Fatty Acids from C18 Unsaturated Fatty Acids by Strains of Bifidobacteria and Propionibacteria

Abstract

Conjugated fatty acids are regularly found in nature and as such, have had a long documented history of biogenic activity in both animals and humans. A number of these conjugated fatty acids are microbially produced and have been associated with potent anti-carcinogenic, anti-adipogenic, anti-atherosclerotic and anti-diabetogenic activities. Therefore, the identification of novel conjugated fatty acids is highly desirable. In this study, six strains of intestinally derived *Bifidobacterium* and four diary *Propionibacterium* strains, which have previously been reported to produce the *c*9, *t*11 and *t*9, *t*11 CLA isomers from free linoleic acid, were assayed for the ability to conjugate sixteen different unsaturated fatty acids. Of the fatty acids assayed, only four were converted by the strains to their conjugated isomers (linoleic acid, α-linolenic acid, γ-linolenic acid and stearidonic acid). In each case, the unsaturated fatty acid contained a methylene interrupted *c*9, *c*12 double bond conformation, which was converted to a conjugated 9, 11-double bond conformation by the isomerising activity of the strains. Of the strains assayed the bifidobacterial strain, *B. breve* DPC6330, proved to be the most effective strain assayed in terms of conjugated fatty acid production bio-converting 66% of the linoleic acid to CLA, 71% of the α-linolenic acid to CALA, 15% of the γ-linolenic acid to CGLA, and 26% of the stearidonic acid to CSA. These conjugates may potentially offer the combined health promoting of conjugated fatty acids such as CLA along those of the substrate non-conjugated fatty acids from which they are formed. Thus, these conjugated fatty acids may have potential in the treatment of diseases such as obesity, cancer, atherosclerosis and diabetes.

3.1 Introduction.

Conjugated fatty acids are positional and geometric isomers of polyunsaturated fatty acids (PUFA) and are characterised by having one or more double bonds in the *cis* (*c*) or *trans* (*t*) conformation and separated by a simple carbon-carbon linkage. These fatty acids are found naturally in ruminant milkfat and tallow (Dhiman *et al.*, 2005; Stanton *et al.*, 2003), plant seeds oils (Chisholm & Hopkins, 1967; Kohno *et al.*, 2004; Liu *et al.*, 1997; Yasui *et al.*, 2005) and marine algae (Burgess *et al.*, 1991; Lopez & Gerwick, 1987; Mikhailova *et al.*, 1995; Wise *et al.*, 1994). Furthermore, certain conjugated fatty acids may be formed synthetically via the alkaline isomerisation of PUFA (Park *et al.*, 2005; Yonezawa *et al.*, 2005). Currently, it is the *c*9, *t*11 and *t*10, *c*12 conjugated linoleic acid (CLA) isomers which have been the most extensively studied. These CLA isomers have been associated with a range of biological activities including anti-carcinogenic, anti-adipogenic, anti-obesogenic, anti-diabetogenic, anti-artherogenic, anti-thrombotic and immunostimulatory activities (Belury, 2002; Bhattacharya *et al.*, 2006; Pariza *et al.*, 2001; Wahle *et al.*, 2004).

In addition to CLA isomers, other natural and synthetically formed conjugated fatty acids have been shown to exhibit biological activities with relevance to human health. For example, conjugated α-linolenic acids (CALA) have been reported to exhibit anti-carcinogenic and anti-obese activities (Coakley *et al.*, 2009; Igarashi & Miyazawa, 2000; Igarashi & Miyazawa, 2005; Kohno *et al.*, 2004; Suzuki *et al.*, 2001). These isomers are traditionally found in the oils of plant seeds such as catalpa (42.3%), snake gourd (30-50%), bitter gourd (52.6%), pot marigold (62.6%), tung (67.7%) and pomegranate (83%) (Chapter 1.3). CALA isomers may also be produced by intestinal and ruminal bacteria via the action of the enzyme, linoleic acid isomerase, on free α-linolenic acid (Coakley *et al.*, 2009; Destaillats *et*

al., 2005; Ogawa *et al.*, 2005). Indeed, the *c*9, *t*11, *c*13 CALA and *c*9, *t*11, *c*15 CALA isomers have been found in the milk fat of ruminants at concentrations of up to 0.03% of total fatty acids (Destaillats *et al.*, 2005). Other synthetically produced conjugated isomers, such as conjugated eicosadienoic acids (C20:2) and conjugated eicosatrienoic acids (C20:3) have been associated with body fat reduction and increased lean mass (Park *et al.*, 2005). Conjugated eicosapentaenoic acids (C20:5) have been reported to exhibit anti-carcinogenic activity (Tsuzuki *et al.*, 2005b; Yonezawa *et al.*, 2005). While conjugated docosahexaenoic acids (C22:6) have been reported to exert anti-carcinogenic and anti-adipogenic activity (Danbara *et al.*, 2004; Tsujita-Kyutoku *et al.*, 2004; Tsuzuki *et al.*, 2005a). Given the extent and significance of the health promoting biological activities associated with these conjugated fatty acids, economic strategies for their production is highly desirable.

It has been reported that dairy propionibacteria and bifidobacteria of human intestinal origin have the ability to conjugate the *c*9, *c*12 double bond of linoleic acid (C18:2) yielding the *c*9, *t*11-C18:2 and *t*9, *t*11-C18:2 CLA isomers, via the action of the enzyme linoleic acid isomerase (Barrett *et al.*, 2007; Coakley *et al.*, 2003; Jiang *et al.*, 1998). The aim of this study was to assess whether strains of bifidobacteria and propionibacteria, previously shown to conjugate linoleic acid to the CLA isomers, could be utilised to conjugate a range of PUFA during fermentation and to identify and isolate any putative conjugated fatty acids formed.

3.2 Materials and Methods

3.2.1 Maintenance of bacterial strains

The strains of *Bifidobacterium* and *Propionibacterium* used in this study are detailed in **Table 3.1**, and were selected based on their ability to isomerise linoleic acid (*c*9, *c*12-C18:2) to the *c*9, *t*11 CLA isomer. The strains assayed had previously been reported to convert 0.45 mg/ml linoleic acid (*c*9, *c*12-C18:2) to both the *c*9, *t*11 and *t*9, *t*11 CLA isomers when grown in cys-MRS, confirming that the strains possessed the enzyme linoleic acid isomerase (Barrett *et al.*, 2007; Coakley *et al.*, 2003; Jiang *et al.*, 1998). All strains were grown in de Man Rogosa Sharpe (MRS) medium (Difco, Detroit, MI, USA,) containing 0.05 mg/ml L-cysteine hydrochloride (Sigma Chemical Co., St Louis, Mo) (cys-MRS) under anaerobic conditions (Anaerocult A Gas Packs, Merck Darmstedt, Germany) at 37^{o}C for 18 h. Where solid media was required, 1.5% (w/v) bacteriological agar (Oxoid, Hampshire, UK) was added to the cys-MRS medium. Cell counts were determined by plating serial dilutions in maximum recovery diluent (MRD) (Oxoid) and pour plating using cys-MRS agar.

3.2.2 Preparation of substrates for fermentation studies

All fatty acid substrates purchased for this study were of the highest purity **(Table 3.2)**. Substrates were delivered in the form of a 30 mg/ml stock solution containing 2% (w/v) Tween 80 (Merck-Schuchardt, Hohenbrunn, Germany). To ensure sterility, all substrates were filter sterilized through a 0.45 μm Ministart filter (Sartorius AG, Goettingen, Germany) followed by storage in the dark at –20^{o}C, under nitrogen.

Table 3.1 Strains of *Bifidobacterium* and *Propionibacterium*

Strain	Source	Reference
Bifidobacterium		
Bifidobacterium breve NCIMB 702258	Infant Intestine	(Coakley *et al*, 2003)
Bifidobacterium breve NCIMB 8807	Nursling Stools	(Coakley *et al*, 2003)
Bifidobacterium breve DPC6330	*C. difficile* (+) Subject	Unpublished data
Bifidobacterium breve DPC6331	*C. difficile* (+) Subject	Unpublished data
Bifidobacterium longum DPC6315	Healthy Adult	Unpublished data
Bifidobacterium longum DPC6320	*C. difficile* (+) Subject	Unpublished data
Propionibacterium		
Propionibacterium freudenreichii subsp. *shermanii* JS	Dairy Starter	(Jiang *et al*, 1998)
Propionibacterium freudenreichii subsp. *shermanii* 9093	Dairy Starter	(Jiang *et al*, 1998)
Propionibacterium freudenreichii subsp. *freudenreichii* Propioni-6	Dairy Starter	(Jiang *et al*, 1998)
Propionibacterium freudenreichii subsp. *freudenreichii* ATCC 6207	Dairy Starter	(Jiang *et al*, 1998)

Table 3.2 Fatty acid substrates

Name	Formula	Source	Purity
Linoleic acid	C18:2 (*c* 9, *c* 12)	Sigma Aldrich (Sigma Chemical Co., St Louis, MO)	>99%
α-linolenic acid	C18:3 (*c* 9, *c* 12, *c* 15)	Sigma Aldrich (Sigma Chemical Co., St Louis, MO)	>99%
γ-linolenic acid	C18:3 (*c* 6, *c* 9, *c* 12)	Nu-chek Prep (Elysian, MN, U.S.A.)	>99%
Stearidonic acid	C18:4 (*c* 6, *c* 9, *c* 12, *c* 15)	Cayman Europe (Akadeemia tee, Tallinn, Estonia)	>98%
Nonadecanoic acid	C19:2 (*c* 10, *c* 13)	Nu-chek Prep (Elysian, MN)	>99%
Eicosadienoic acid	C20:2 (*c* 11, *c* 14)	Nu-chek Prep (Elysian, MN)	>99%
Homogamma linolenic	C20:3 (*c* 8, *c* 11, *c* 14)	Nu-chek Prep (Elysian, MN)	>99%
Eicosatrienoic acid	C20:3 (*c* 11, *c* 14, *c* 17)	Nu-chek Prep (Elysian, MN)	>99%
Arachidonic acid	C20:4 (*c* 5, *c* 8, *c* 11, *c* 14)	Nu-chek Prep (Elysian, MN)	>99%
Eicosapentaenoic acid	C20:5 (*c* 5, *c* 8, *c* 11, *c* 14, *c* 17)	Nu-chek Prep (Elysian, MN)	>99%
Heneicosadienoic acid	C21:2 (*c* 12, *c* 15)	Nu-chek Prep (Elysian, MN)	>99%
Docosadienoic acid	C22:2 (*c* 13, *c* 16)	Nu-chek Prep (Elysian, MN)	>99%
Docosatrienoic acid	C22:3 (*c* 13, *c* 16, *c* 19)	Nu-chek Prep (Elysian, MN)	>99%
Docosatetraenoic acid	C22:4 (*c* 7, *c* 10, *c* 13, *c* 16)	Nu-chek Prep (Elysian, MN)	>99%
Docosapentaenoic acid	C22:5 (*c* 7, *c* 10, *c* 13, *c* 16, *c* 19)	Nu-chek Prep (Elysian, MN)	>99%
Docosahexaenoic acid	C22:6 (*c* 4, *c* 7, *c* 10, *c* 13, *c*16, *c* 19)	Nu-chek Prep (Elysian, MN)	>99%

3.2.3 Screening for microbial conjugated fatty acid production

A 4% (w/v) inoculum of the activated culture was added to cys-MRS medium containing 0.45 mg/ml of the respective PUFA substrates and incubated anaerobically at 37^{o}C (bifidobacteria) or 30^{o}C (propionibacteria) for 72 h. Fatty acids were extracted from the fermentation medium using the method described by Coakley *et al.* (2003). Extracted fatty acids were converted to fatty acid methyl esters (FAME) by acid catalyzed methylation as described by Stanton *et al* (1997). FAME were separated by gas liquid chromatography (GLC) on a CP-SELECT CB column (100 m x 0.25 mm id x 0.25 μm film thickness (Varian BV, Herculesweg 8, 4338 PL, Middelburgh, The Netherlands)) using a Varian 3400 Capillary GC (Varian, Walnut Creek, CA, USA) fitted with a flame ionization detector (FID). Helium (33.5 psi), at a flow rate of 1 ml/min at 80 psi was used as carrier gas. The initial injector temperature was held isothermally at 160^{o}C for 0.1 min and programmed to increase to 225^{o}C at a rate of 200^{o}C/min for 15 min, while the detector temperature was 250^{o}C. The column oven was held at an initial temperature of 80^{o}C for 8 min and then programmed to increase at a rate of 8.5^{o}C/min to a final temperature of 200^{o}C, and held for 72 min. Collected data were recorded and analyzed on a Minichrom PC system (VG Data Systems, Cheshire, UK). The presence of a conjugated double bond was confirmed spectrophotometrically, using a modification of the method of Barrett *et al*. (2007) using FAME as opposed to free fatty acids. Briefly, 200 μl of the methylated sample was dispensed into a UV transparent 96 well plate (Costar, Corning NY) and the absorbance of UV light at a wavelength of 234 nm measured using a 96 well plate spectrophotometer (GENios Plus, Medford, MA). All experiments were performed in triplicate.

3.2.4 Purification of microbially produced conjugated fatty acids

Total fatty acids were extracted from the samples using the method described by Coakley *et al.* (2003). The hexane extract (containing lipids) was partially concentrated using a rotary evaporator (Buchi Rotavapor R-210) and the resulting hexane concentrate washed once with 0.88% KCl (w/v) and twice with water:methanol (1:1) (v/v). The hexane was then evaporated by heating at 45^{o}C under a steady flow of nitrogen. The remaining lipid was resuspended at a concentration of 100 mg/ml in acetonitrile:acetate (100:0.14) (v/v), sealed under nitrogen and stored at -20^{o}C until use. The conjugated fatty acids were separated from the non-conjugated fatty acids using reverse phase high performance liquid chromatography (RP-HPLC). To achieve this, a Luna 5u C18 (2) 100A preparative RP-HPLC column (250 mm x 21.20 mm) (Phenomenex, Macclesfield, Cheshire, UK) and the Varian Prostar HPLC system (Varian Inc., CA) were employed. The mobile phase used to obtain optimal separation was acetonitrile:water:acetate (70:30:0.12) (v/v) at a flow rate of 10 ml/min. Conjugated fatty acids were detected using a diode array detector (DAD) at an absorbance of 234 nm. Fractions containing the conjugated fatty acids were collected using a fraction collector and subsequently pooled. Following removal of the acetonitrile from the pooled fractions by rotary evaporation, the conjugated fatty acids were re-extracted as described above. The fatty acid compositions of the pure oils were confirmed by GLC as previously described.

3.2.5 Identification of conjugated fatty acid products

FAME were prepared as described by Stanton *et al* (1997), 4, 4-dimethyloxazoline (DMOX) derivatives were prepared as described by Yurawecz *et al* (1994), 4-methyl-1, 2, 4- triazoline-3, 5-dione (MTAD) derivatives (form Diels-Alder adducts

specifically with conjugated systems) were prepared as described by Young *et al* (1990), while pyrrolidide derivatives were prepared as described by Andersson *et al* (1974). The conjugated fatty acid derivatives were identified using gas liquid chromatography mass spectrometry (GLC-MS) on an Agilent GLC-MS system (Agilent Technologies Inc., Santa-Clara, CA) fitted with a split/splitless injector (split ratio 15:1) and a HP5MS column (5% phenyl methyl siloxane, 30 m x 250 µm x 0.25 µm) (Agilent Technologies Inc) connected to a quadropole mass spectrometer at Mylnefield Lipid Analysis (Dundee, Scotland). The oven temperature was held at 160°C for 2 min, and then programmed to increase at a rate of 7°C /min to a final temperature of 325°C. Helium was used as the carrier gas at a flow rate of 1 ml/min and pressure programming was used in constant flow mode. The mass spectrometer was operated in electron impact mode at an ionization energy of 70 eV and emission current of 300 µA. The ion source and interface temperatures were at 250°C and the scan range was 35-750 atomic mass units (a.m.u.) at 0.44 scans/sec. Mass spectra were taken from the Agilent instrument software as Windows metafiles, and then annotated using Metafile Companion™ (Companion Software Inc., Moorhead, MN). Chemical formulae were drawn with ChemWindows™ (SoftShell International Ltd, Grand Junction, CO).

3.2.6 Characterization of conjugate production by the strain *B. breve* DPC6330

To determine the impact of substrate fatty acid concentration, the strain *B. breve* DPC6330 was incubated anaerobically for 72 h at 37^{o}C in the presence of linoleic acid, α-linolenic acid (Sigma Chemical Co), γ-linolenic acid (Nu-Chek Prep, Elysian, MN) and stearidonic acid (Cayman Europe, Akadeemia tee, Tallinn, Estonia) at concentrations of 0.15 mg/ml, 0.3 mg/ml and 0.45 mg/ml (n = 3). To determine the time course of CALA, conjugated γ-linolenic acid (CGLA) and

conjugated stearidonic acid (CSA) production by *B. breve* DPC6330, the strain was incubated anaerobically for 80 h at 37^{o}C at a substrate fatty acid concentration of 0.3 mg/ml. All fatty acids were delivered in the form of a 30 mg/ml stock solution containing 2% (w/v) Tween 80 (Merck-Schuchardt) as previously described. Cell counts were determined by serial dilution of the culture in MRD (Oxoid) and pour plating using cys-MRS agar. Cell numbers were expressed as colony forming units (cfu).

3.2.7 Assessing the resistance of the *B. breve* DPC6330 to simulated gastric juice (SGJ)

SGJ was prepared as previously described (Beumer *et al.*, 1992) using NaCl (2.05 g/L), KH_2PO_4 (0.60 g/L), $CaCl_2$ (0.11 g/L) and KCl (0.37 g/L), adjusted to pH 2.5 using 1M HCl and autoclaved at 121°C for 15 min. Porcine bile (0.05 g/L), lysozyme (0.1 g/L) and pepsin (0.0133 g/L) were added as stock solutions prior to use. Proteose-peptone and glucose were omitted from the SGJ as described by Beumer *et al* (1992) given their ability to affect bacterial tolerance as detailed by Corcoran *et al* (2007). Components of SGJ were obtained from Sigma Aldrich, BDH Chemicals (Poole, Dorset, UK) and Orthana (Orthana Kemisk Fabrik A/S, Kastrup, Denmark). Following overnight growth in cys-MRS, the cultures were centrifuged at 7000 x g at 4°C for 15 min, washed once in an equal volume of cold (4^{o}C) phosphate buffer solution (Sigma Aldrich) and re-centrifuged (7000 x g). Pellets were then resuspended in an equal volume of SGJ, pH 2.5, and incubated at 37°C anaerobically for 90 min with constant stirring. Samples were taken at 0, 30, 60 and 90 min, serially diluted in MRD, plated on cys-MRS and incubated at 37°C for 72 h. All experiments were performed in triplicate.

3.3 Results

3.3.1 Screening for the production of novel conjugated fatty acids by selected strains of bifidobacteria and propionibacteria

In this study, a number of strains of bifidobacteria and propionibacteria were assessed for their ability to generate conjugated fatty acids from pure PUFA substrates. The origin of the strains varied from human intestinal isolates to traditional dairy cultures **(Table 3.1)**. The performance and growth of the strains when exposed to the different PUFA (0.45 mg/ml) was similar to that observed in the unsupplemented controls, however, some exceptions were noted. Growth of Prop. freudenreichii subsp. shermanii JS, Prop. freudenreichii subsp. shermanii 9093, Prop. freudenreichii subsp. freudenreichii Propioni-6 were inhibited in the presence of 0.45 mg/ml eicosapentaenoic acid (EPA), while the growth of all strains was completely inhibited in the presence of 0.45 mg/ml of stearidonic acid. Thus, the concentration of the stearidonic acid used to screen for the production of conjugated fatty acids was reduced to 0.1 mg/ml, at which concentration, growth of the strain was determined to be unaffected **(Table 3.3)**.

3.3.2 Identification of novel conjugated fatty acids produced by strains of bifidobacteria and propionibacteria

Following GLC and UV spectrophotometric analyses, it was evident that only four of the unsaturated fatty acid substrates analysed were bioconverted to their respective conjugated isomers by the CLA producing strains. These fatty acid substrates were linoleic acid, α-linolenic acid, γ-linolenic acid and stearidonic acid, all of which contained the c9, c12 double bond **(Table 3.4)**.

Table 3.3 Production of conjugated fatty acids by growing cultures of *Bifidobacterium* and *Propionibacterium.*

Substrate		Growth	Species			
			B. breve [a]	*B. longum* [b]	*Prop. freudenreichii* subsp. *shermanii* [c]	*Prop. freudenreichii* subsp. *freudenreichii* [d]
Linoleic acid	C18:2 (*c* 9, *c* 12)	(+)	(+)	(+)	(+)	(+)
α-linolenic acid	C18:3 (*c* 9, *c* 12, *c* 15)	(+)	(+)	(+)[†]	(+)	(+)
γ-linolenic acid	C18:3 (*c* 6, *c* 9, *c* 12)	(+)	(+)	(+)	(-)	(+)*
Stearidonic acid	C18:4 (*c* 6, *c* 9, *c* 12, *c* 15)	(+)[§]	(+)	(+)	(+)	(+)
Nonadecanoic acid	C19:2 (*c* 10, *c* 13)	(+)	(-)	(-)	(-)	(-)
Eicosadienoic acid	C20:2 (*c* 11, *c* 14)	(+)	(-)	(-)	(-)	(-)
Homogamma linolenic	C20:3 (*c* 8, *c* 11, *c* 14)	(+)	(-)	(-)	(-)	(-)
Eicosatrienoic acid	C20:3 (*c* 11, *c* 14, *c* 17)	(+)	(-)	(-)	(-)	(-)
Arachidonic acid	C20:4 (*c* 5, *c* 8, *c* 11, *c* 14)	(+)	(-)	(-)	(-)	(-)
Eicosapentaenoic acid	C20:5 (*c* 5, *c* 8, *c* 11, *c* 14, *c* 17)	(+)[‡]	(-)	(-)	(-)	(-)
Heneicosadienoic acid	C21:2 (*c* 12, *c* 15)	(+)	(-)	(-)	(-)	(-)
Docosadienoic acid	C22:2 (*c* 13, *c* 16)	(+)	(-)	(-)	(-)	(-)
Docosatrienoic acid	C22:3 (*c* 13, *c* 16, *c* 19)	(+)	(-)	(-)	(-)	(-)
Docosatetraenoic acid	C22:4 (*c* 7, *c* 10, *c* 13, *c* 16)	(+)	(-)	(-)	(-)	(-)
Docosapentaenoic acid	C22:5 (*c* 7, *c* 10, *c* 13, *c* 16, *c* 19)	(+)	(-)	(-)	(-)	(-)
Docosahexaenoic acid	C22:6 (*c* 4, *c* 7, *c* 10, *c* 13, *c* 16, *c* 19)	(+)	(-)	(-)	(-)	(-)

[a] *B. breve* NCIMB 702258, NCIMB 8807, DPC6330, DPC6331

[b] *B. longum* DPC6315, DPC6320

[c] *Prop. freudenreichii* subsp. *shermanii* JS, 9093

[d] *Prop. freudenreichii* subsp. *freudenreichii* Propioni-6, ATCC 6207

* Only by *Prop. freudenreichii* subsp. *freudenreichii* Propioni-6

[†] Only by *B. longum* DPC6320

[‡] Poor growth by *Prop. freudenreichii* subsp. *shermanii* JS, *Prop. freudenreichii* subsp. *shermanii* 9093, *Prop. freudenreichii* subsp. *freudenreichii* Propioni-6

[§] Strains assayed at a fatty acid concentration of 0.1 mg/ml

Table 3.4 Bioconversion of selected PUFA to CLA, CALA, CGLA and CSA by strains of *Bifidobacterium* and *Propionibacterium*.

Strain	Concentration 0.45 mg/ml			Percentage			
	Linoleic acid	*c* 9 *t* 11 CLA	*t* 9 *t* 11 CLA	Linoleic acid	*c* 9 *t* 11 CLA	*t* 9 *t* 11 CLA	Ratio of *c*9,*t*11 to *t* 9,*t*11
Bifidobacterium							
B. breve NCIMB 702258	0.136 ± 0.013	0.254 ± 0.012	0.005 ± 0.001	31.59	58.94	1.08	54.79 : 1
B. breve NCIMB 8807	0.101 ± 0.032	0.249 ± 0.019	0.028 ± 0.002	23.42	57.92	6.39	9.06 : 1
B. breve DPC6330*	0.061 ± 0.005	0.211 ± 0.005	0.007 ± 0.000	19.10	65.77	2.05	32.06 : 1
B. breve DPC6331*	0.185 ± 0.005	0.087 ± 0.001	0.004 ± 0.000	57.85	27.32	1.12	24.29 : 1
B. longum DPC6315*	0.236 ± 0.004	0.039 ± 0.001	0.003 ± 0.000	73.79	12.10	0.92	13.20 : 1
B. longum DPC6320*	0.093 ± 0.003	0.180 ± 0.003	0.007 ± 0.000	29.14	56.20	2.19	25.70 : 1
Propionibacterium							
Prop. freudenreichii subsp. *shermanii* JS†	0.466 ± 0.011	0.044 ± 0.001	0.005 ± 0.000	90.77	8.56	1.05	8.13 : 1
Prop. freudenreichii subsp. *shermanii* 9093†	0.210 ± 0.007	0.243 ± 0.000	0.016 ± 0.000	40.97	47.37	3.17	14.94 : 1
Prop. freudenreichii subsp. *freudenreichii* Propioni-6†	0.266 ± 0.009	0.214 ± 0.007	0.016 ± 0.003	51.83	41.63	3.02	13.78 : 1
Prop. freudenreichii subsp. *freudenreichii* ATCC 6207†	0.493 ± 0.006	0.006 ± 0.000	0.002 ± 0.001	96.11	1.07	0.31	3.44 : 1

Strain	Concentration 0.45 mg/ml			Percentage			
	α-linolenic acid	CALA1	CALA2	α-linolenic acid	CALA1	CALA2	Ratio of CALA1 to CALA2
Bifidobacterium							
B. breve NCIMB 702258	0.124 ± 0.025	0.180 ± 0.013	0.019 ± 0.014	31.25	45.09	4.65	9.70 : 1
B. breve NCIMB 8807	0.051 ± 0.002	0.234 ± 0.002	0.038 ± 0.000	12.92	58.85	9.44	6.23 : 1
B. breve DPC6330	0.006 ± 0.000	0.282 ± 0.013	0.049 ± 0.002	1.41	70.79	12.18	5.81 : 1
B. breve DPC6331	0.355 ± 0.010	0.028 ± 0.000	0.003 ± 0.000	89.18	6.91	0.79	8.78 : 1
B. longum DPC6315	0.377 ± 0.004	0.000 ± 0.000	0.000 ± 0.000	94.70	0.10	0.00	0.10 : 0
B. longum DPC6320	0.375 ± 0.003	0.000 ± 0.000	0.000 ± 0.000	94.08	0.00	0.00	0.00 : 0
Propionibacterium							
Prop. freudenreichii subsp. *shermanii* JS	0.459 ± 0.012	0.018 ± 0.001	0.001 ± 0.000	89.38	3.52	0.19	18.03 : 1
Prop. freudenreichii subsp. *shermanii* 9093	0.346 ± 0.013	0.258 ± 0.017	0.017 ± 0.002	28.39	50.34	3.23	15.59 : 1
Prop. freudenreichii subsp. *freudenreichii* Propioni-6	0.422 ± 0.017	0.043 ± 0.004	0.003 ± 0.000	82.20	8.41	0.53	15.99 : 1
Prop. freudenreichii subsp. *freudenreichii* ATCC 6207	0.460 ± 0.008	0.008 ± 0.000	0.000 ± 0.000	89.62	1.65	0.00	1.65 : 0

Strain	Concentration 0.45 mg/ml			Percentage			
	γ-linolenic acid	CGLA1	CGLA2	γ-linolenic acid	CGLA1	CGLA2	Ratio of CGLA1 to CGLA2
Bifidobacterium							
B. breve NCIMB 702258	0.072 ± 0.029	0.100 ± 0.010	0.049 ± 0.012	18.11	25.13	12.34	2.04 : 1
B. breve NCIMB 8807	0.364 ± 0.013	0.004 ± 0.000	0.001 ± 0.001	91.49	1.05	0.16	6.58 : 1
B. breve DPC6330	0.196 ± 0.004	0.058 ± 0.003	0.023 ± 0.001	49.27	14.64	5.71	2.56 : 1
B. breve DPC6331	0.362 ± 0.016	0.005 ± 0.001	0.002 ± 0.000	91.02	1.24	0.51	2.41 : 1
B. longum DPC6315	0.325 ± 0.011	0.004 ± 0.005	0.004 ± 0.004	81.59	1.08	0.95	1.13 : 1
B. longum DPC6320	0.350 ± 0.019	0.002 ± 0.003	0.000 ± 0.000	87.83	0.55	0.00	0.55 : 0
Propionibacterium							
Prop. freudenreichii subsp. *shermanii* JS	0.376 ± 0.005	0.000 ± 0.000	0.000 ± 0.000	87.75	0.00	0.00	0.00 : 0
Prop. freudenreichii subsp. *shermanii* 9093	0.374 ± 0.002	0.001 ± 0.000	0.000 ± 0.000	87.16	0.13	0.00	0.13 : 0
Prop. freudenreichii subsp. *freudenreichii* Propioni-6	0.375 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	87.48	0.00	0.00	0.00 : 0
Prop. freudenreichii subsp. *freudenreichii* ATCC 6207	0.368 ± 0.005	0.000 ± 0.000	0.000 ± 0.000	85.84	0.00	0.00	0.00 : 0
Strain	Concentration 0.1 mg/ml			Percentage			
	Stearidonic acid	CSA1	CSA2	Stearidonic acid	CSA1	CSA2	Ratio of CSA1 to CSA2
Bifidobacterium							
B. breve NCIMB 702258	0.020 ± 0.001	0.018 ± 0.001	0.005 ± 0.000	22.18	19.85	5.15	3.85 : 1
B. breve NCIMB 8807	0.044 ± 0.002	0.009 ± 0.001	0.001 ± 0.000	47.56	9.49	1.46	6.48 : 1
B. breve DPC6330	0.002 ± 0.000	0.024 ± 0.001	0.001 ± 0.000	2.11	25.81	1.52	17.00 : 1
B. breve DPC6331	0.019 ± 0.000	0.014 ± 0.000	0.003 ± 0.000	21.04	14.80	3.74	3.96 : 1
B. longum DPC6315	0.052 ± 0.000	0.003 ± 0.000	0.001 ± 0.000	56.83	3.04	0.87	3.50 : 1
B. longum DPC6320	0.017 ± 0.001	0.018 ± 0.000	0.002 ± 0.000	18.00	19.25	2.11	9.10 : 1
Propionibacterium							
Prop. freudenreichii subsp. *shermanii* JS	0.058 ± 0.001	0.002 ± 0.000	0.000 ± 0.000	63.18	2.66	0.00	2.66 : 0
Prop. freudenreichii subsp. *shermanii* 9093	0.056 ± 0.002	0.003 ± 0.000	0.000 ± 0.000	60.68	3.09	0.00	3.09 : 0
Prop. freudenreichii subsp. *freudenreichii* Propioni-6	0.054 ± 0.002	0.003 ± 0.000	0.000 ± 0.000	58.35	3.58	0.00	3.58 : 0
Prop. freudenreichii subsp. *freudenreichii* ATCC 6207	0.062 ± 0.001	0.001 ± 0.000	0.000 ± 0.000	67.14	0.81	0.00	0.81 : 0

* 0.35 mg/ml linoleic acid. † 0.5 mg/ml linoleic acid.

The production of the *c*9, *t*11 and *t*9, *t*11 CLA isomers from linoleic acid by the strains was confirmed using GLC and a range of commercially available standards (Matreya LLC, PA), while the conjugated fatty acid produced from α-linolenic acid, γ-linolenic acid and stearidonic acid were identified by GLC-MS.

The predominant conjugated fatty acid isomer produced by strains of bifidobacteria and propionibacteria from free α-linolenic acid was 9, 11, 15-C18:3 (CALA1). The mass spectrum of the FAME confirmed the molecular weight of the molecule as 278 **(Figure 3.1a)**. The mass spectrum of the DMOX derivatives identified the molecule as the 9, 11, 15-C18:3 isomer **(Figure 3.1b)**. The presence of a large molecular ion at m/z = 331 is characteristic of an octadecatrienoic acid. The strong molecular ion at m/z = 262 is representative of cleavage at the centre of the bis-methylene-interrupted double bond system, and was the outstanding feature of the spectrum, confirming the presence of the 11, 15 double bond system. Gaps of 12 a.m.u. between m/z = 196 and m/z = 208 confirmed the presence of a double bond at position-9, while a similar gap between m/z = 222 and m/z = 234 confirmed the double bond in position-11. The gap to m/z = 288 also confirmed the presence of a double bond at position-15. Further confirmation of the structure of this molecule was achieved by the comparison of the spectra for the DMOX derivative of this fatty acid with that of CALA1 previously identified by both Destaillats *et al* (2005) and Winkler and Steinhart (2001) in dairy products and meat of ruminant origin.

The predominant conjugated fatty acid isomer produced by strains of bifidobacteria and propionibacteria from free γ-linolenic acid was identified as 6, 9, 11-C18:3 (CGLA1). Once again, the mass spectrum of the FAME confirmed the molecular weight of the molecule as 278 **(Figure 3.2a)**. The mass spectrum of this

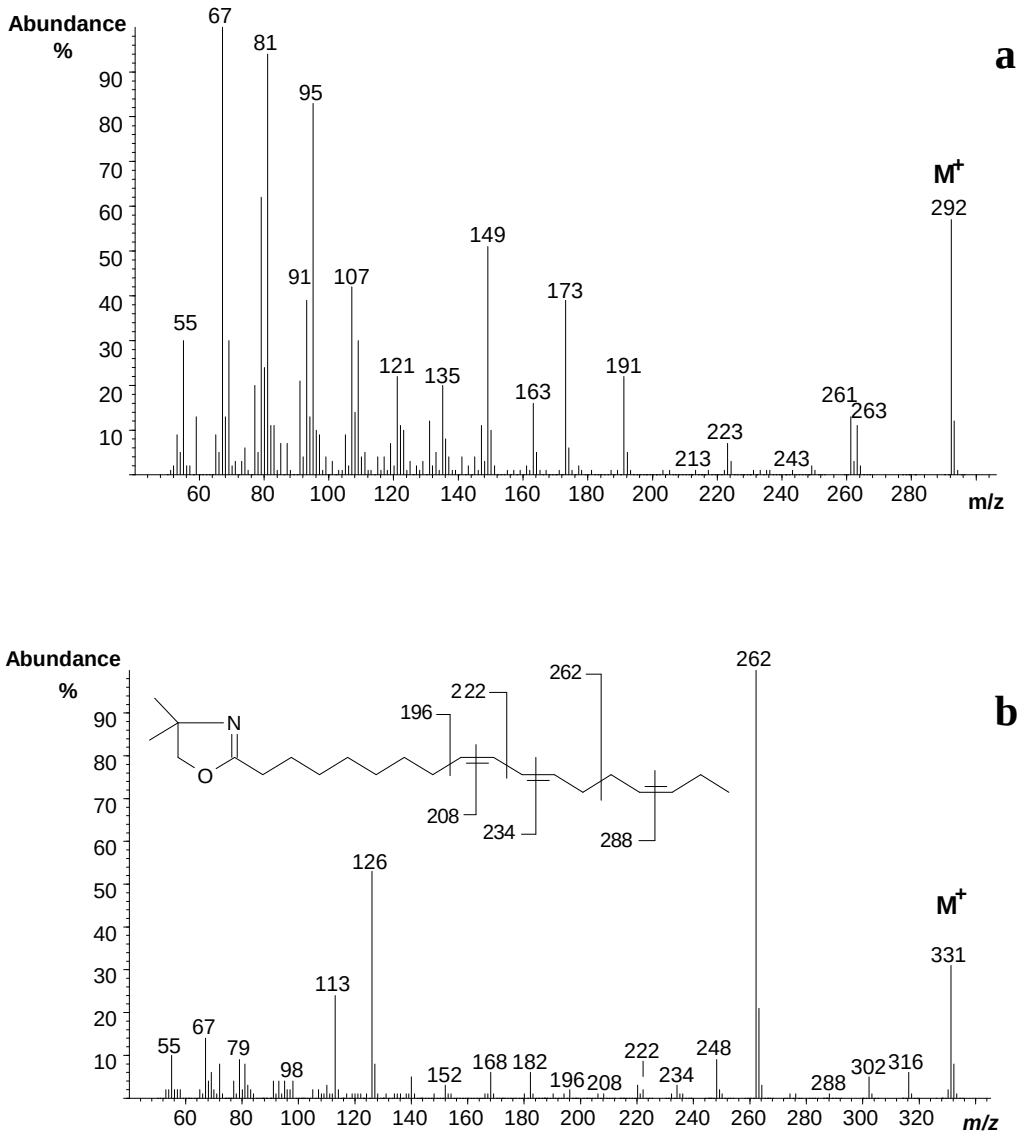


Figure 3.1 a) Mass spectrum of the FAME **b)** Mass spectrum of the DMOX derivative, of 9, 11, 15-C18:3 (CALA1).

molecule was identical to that of authentic 6, 9, 11-18:3, prepared by incubating 9, 11-C18:2 with *Spirulina platensis*, which inserts a double bond in position-6 (W.W. Christie, unpublished data). While this provided a distinct 'fingerprint' spectrum for the polyunsaturated ester, features that would assist in determining the location of the double bonds were not observed. The spectrum produced for the MTAD derivatization, with the ion at m/z = 250, confirmed that the fatty acid possessed a conjugated double bond in the 9, 11-position, with the third double bond on the carboxyl side of the conjugated double bond **(Figure 3.2b)** (Dobson, 1998). The principle behind this technique centres on the formation of a six-membered ring structure, in this instance via the formation of bonds between the carbon at position-9 and at position-11 of the carbon chain of the fatty acid with the highly reactive reagent, MTAD. This results in the loss of the double bonds which form the conjugated double bond system and the formation of a double bond at position-10 of the fatty acid molecule **(Figure 3.2b)**. During mass spectrum analysis, cleavage occurs on either side of the six-membered ring, enabling simple location of the carbons that originally constituted the conjugated double bond system. The use of DMOX derivatives in the identification of the conjugated fatty acid proved difficult as a result of the migration of the 9, 11-double bond system to the 8, 10-position during the derivatization, however, the presence of a strong molecular ion at m/z = 331 did confirm the octadecatrienoic acid structure of the molecule **(Figure 3.2c)**. Given the difficulties observed with the use of the DMOX derivatives, alternative derivatization strategies were investigated. The detection of a double bond in position-6 of the carbon chain of a fatty acid is not easily achieved, other than by a distinctive fingerprint of ions at m/z = 140, 154 and 166 which can be achieved through the formation of pyrrolidide derivatives (Andersson & Holman, 1974).

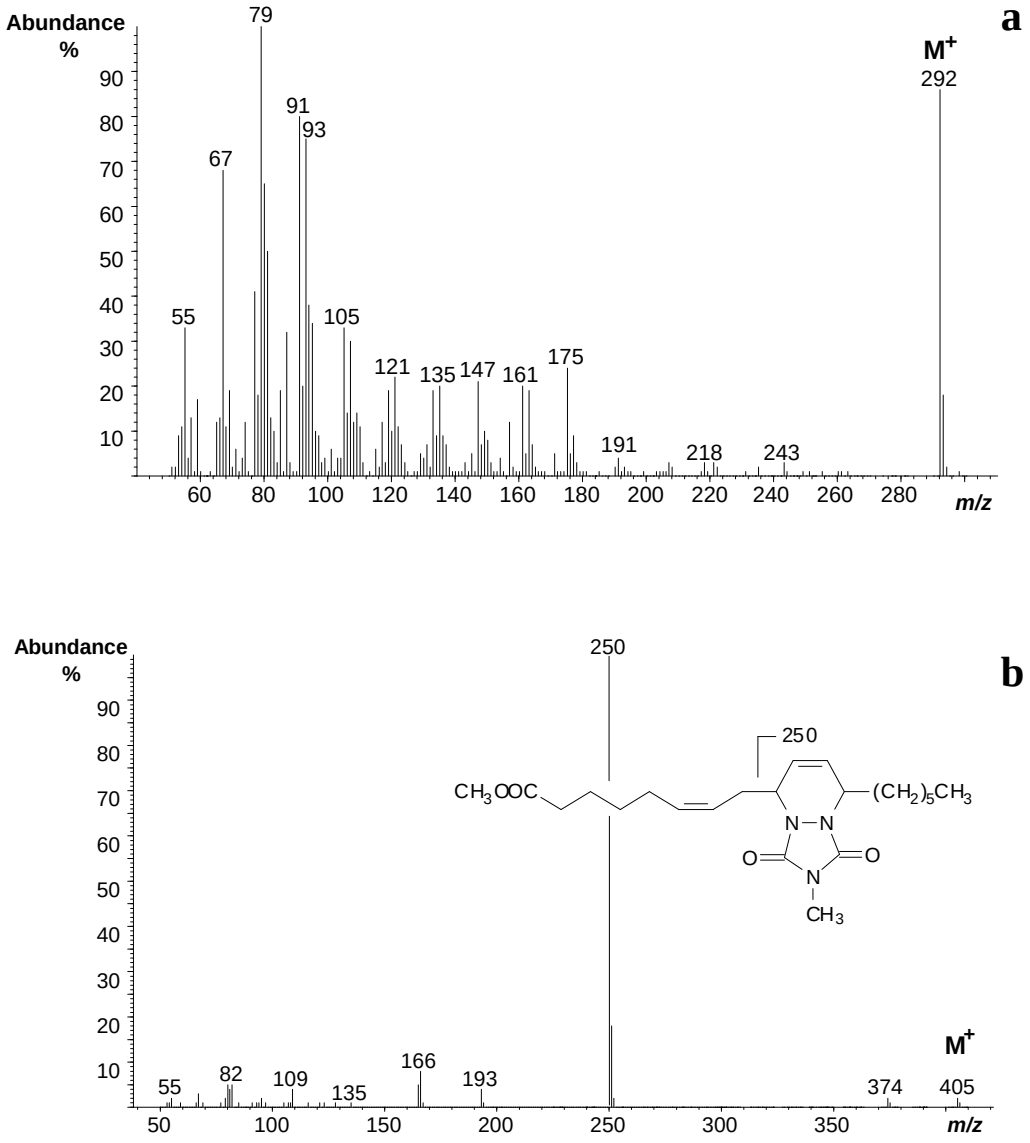


Figure 3.2 a) Mass spectrum of the FAME, **b)** Mass spectrum of the MTAD adduct, of 6, 9, 11-C18:3 (CGLA1).

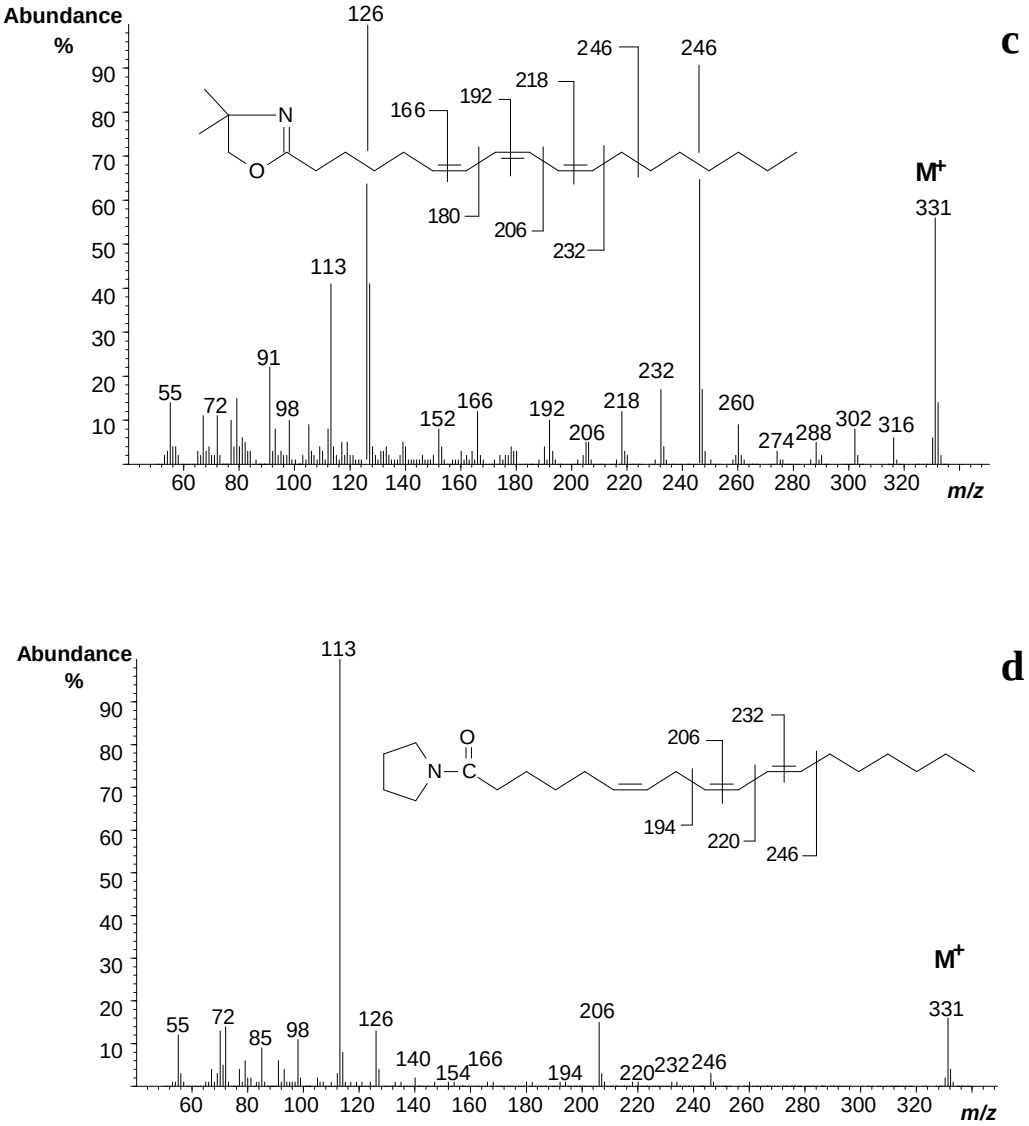


Figure 3.2 c) Mass spectrum of the DMOX derivative, **d)** Mass spectrum of the pyrrolidide derivative, of 6, 9, 11-C18:3 (CGLA1)

Such a pattern was detected in the fatty acid confirming the presence of a double bond at position-6 of the molecule **(Figure 3.2d)**. Furthermore, gaps of 12 a.m.u. between m/z = 194 and 206, and 220 and 232, confirmed the position of double bonds in the 9- and 11-positions, respectively.

The predominant conjugated fatty acid isomer produced by strains of bifidobacteria and propionibacteria from free stearidonic acid was identified as 6, 9, 11, 15-C18:4 (CSA1). Once again mass spectrum data for the FAME confirmed the molecular weight of the molecule as 276, but did not offer any detail as to the position of the double bonds **(Figure 3.3a)**. As was observed with CGLA1, DMOX derivatization of the fatty acid gave unclear results due to the migration of the 9, 11-double bond system to the 8, 10-position during the derivatization procedure, however, the large molecular ion at m/z = 329 did confirm the octadecatetraenoic fatty acid structure of the molecule **(Figure 3.3c)**. Once again pyrrolidide derivatization was used to assist in the identification of the bond position. The spectrum produced for the MTAD derivative, with the base ion now at m/z = 248, confirmed the presence of a conjugated double bond in the 9, 11-position, along with the presence of a double bond on either side of this **(Figure 3.3b)**. While the use of DMOX derivatives did not allow the identification of the position of the first three double bonds due to their migration during the derivatization process, a double bond in position-15 of the carbon backbone was confirmed by the presence of a gap of 12 a.m.u. between m/z = 274 and 286 **(Figure 3.3c)**. The mass spectrum observed with the pyrrolidide derivative of this fatty acid was very similar to that of the fatty acid identified as CGLA1 with a double bond clearly identified at position-6 along with the presence of double bonds at position-9 and position-11. However, unlike CGLA1 a further double bond was also evident as reflected by the gap to

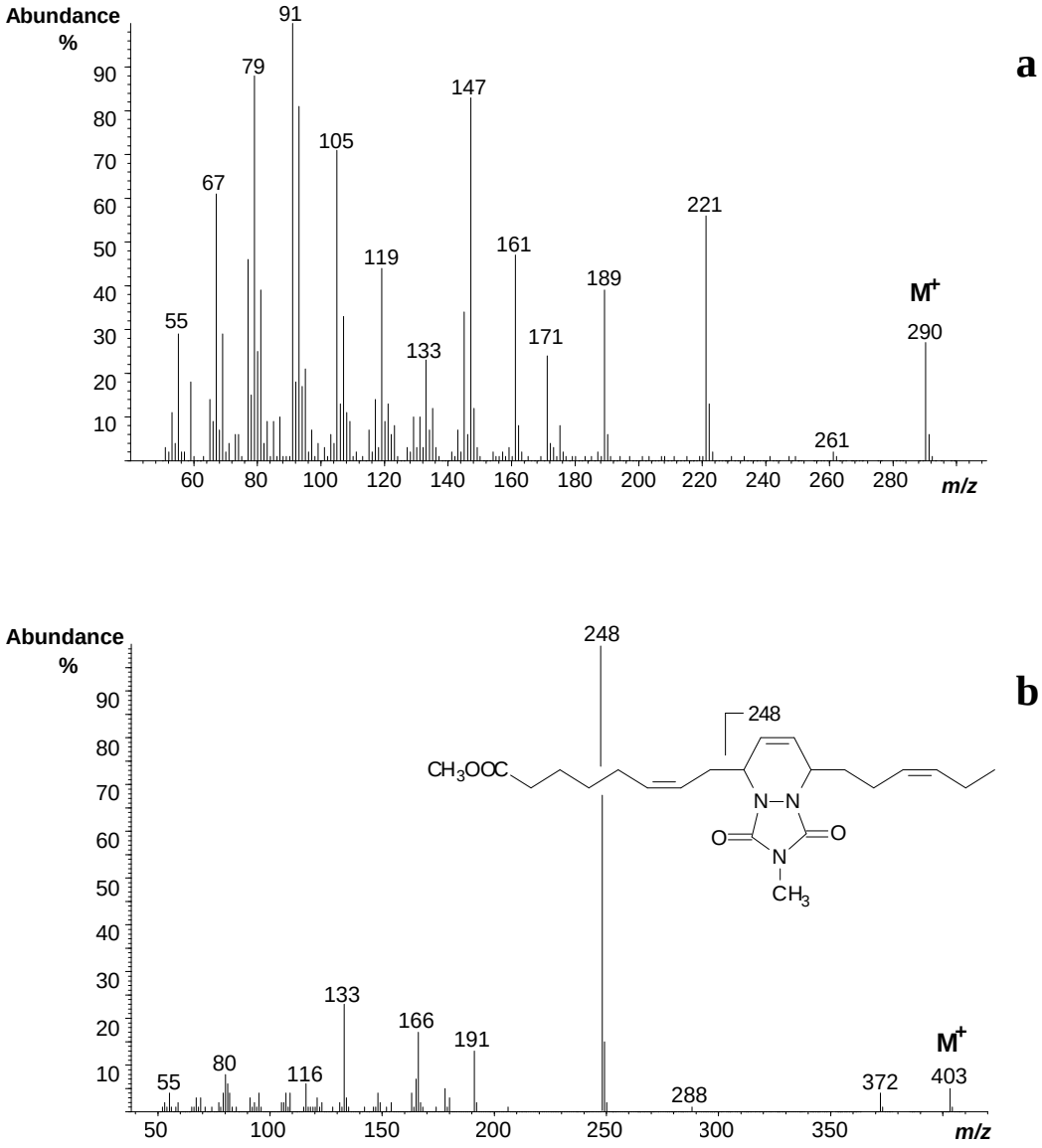


Figure 3.3 a) Mass spectrum of the FAME, **b)** Mass spectrum of the MTAD adduct, of 6, 9, 11, 15-C18:4 (CSA1).

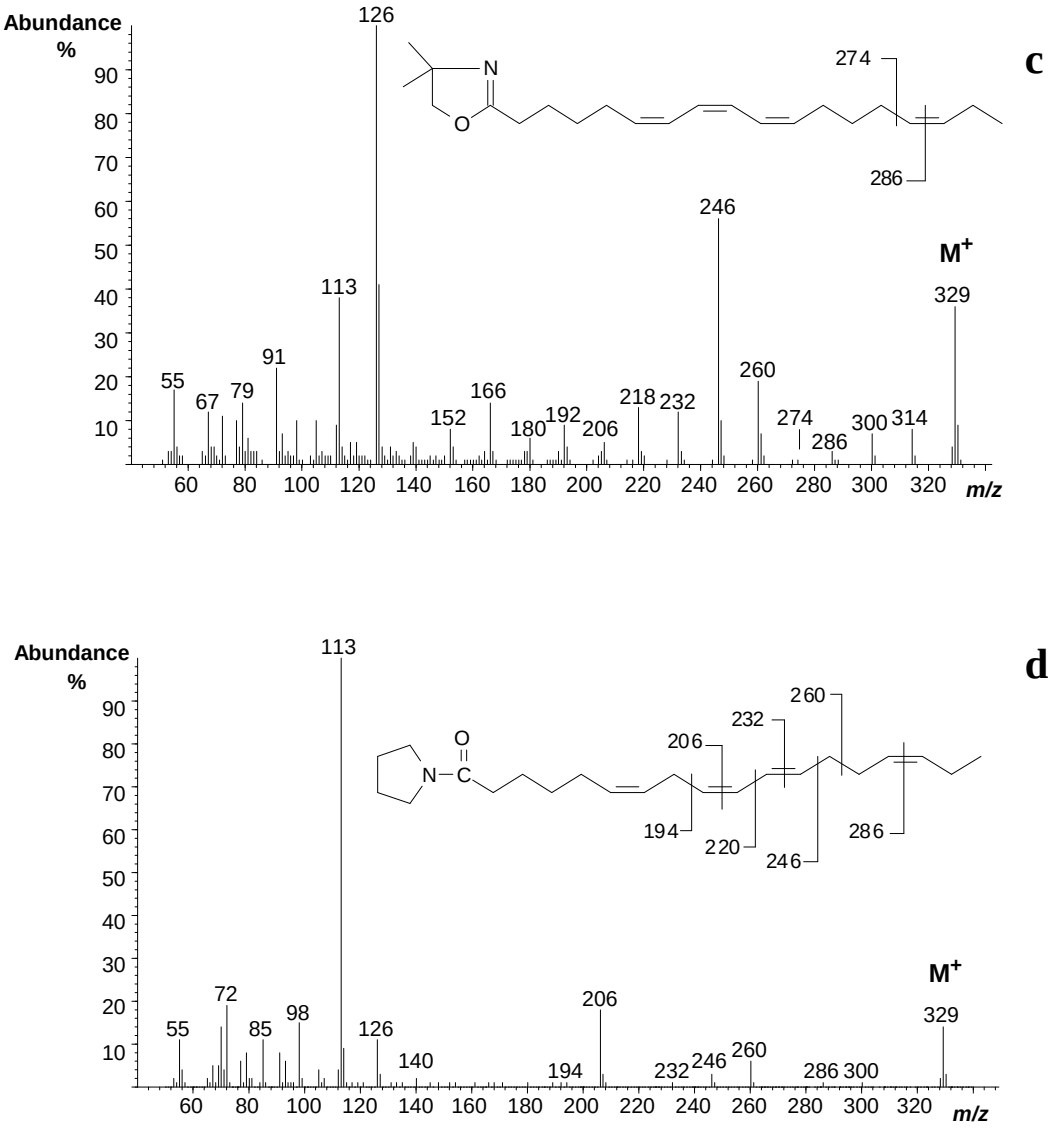
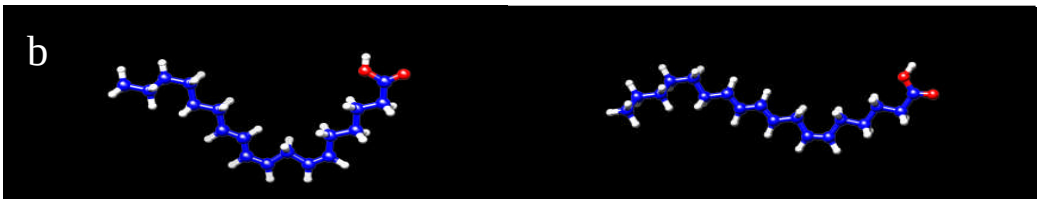


Figure 3.3 c) Mass spectrum of the DMOX derivative **d)** Mass spectrum of the pyrrolidide derivative, of 6, 9, 11, 15-C18:4 (CSA1).

m/z = 286 **(Figure 3.3d)**. Using this information and the data gathered from analysis of the DMOX derivatives; this final bond was determined to be located at position-15 of the carbon backbone.

Using GLC-MS analysis, it was not possible determine the conformation of the double bonds present in the conjugated fatty acids CALA1, CGLA1 and CSA1. The conjugation process which was responsible for the production of these fatty acids is known to be catalysed by the enzyme linoleic acid isomerase, which catalyses the conversion of the *c*9, *c*12 double bond of linoleic acid primarily to the conjugated *c*9, *t*11 bond conformation and to a lesser extent the *t*9, *t*11 bond conformation. In each of the microbially produced conjugated fatty acids found in the present study (CALA1, CGLA1 and CSA1) a conjugated double bond system was identified in the 9, 11-position. As these isomers represent the predominant conjugated isomers produced from their respective substrate fatty acids, it is likely that the conjugated bond found in the 9, 11-position of these molecules are equivalent to the predominant *c*9, *t*11 CLA isomer produced from free linoleic acid (Coakley *et al.*, 2003; Jiang *et al.*, 1998). Furthermore, as the activity of the enzyme linoleic acid isomerase has not previously been associated with conformational changes in double bonds other than at the *c*9, *c*12 position, changes in the conformation of any double bonds outside this region is unlikely. Thus, the most probable bond conformation for CALA1 is *c*9, *t*11, *c*15-C18:3, with that of CGLA1 and CSA1 being *c*6, *c*9, *t*11-C18:3 and *c*6, *c*9, *t*11, *c*15-C18:4, respectively **(Figure 3.4)**. This postulation is supported by the observations of Ogawa *et al* (2005) and Kishino *et al* (2003) who identified the production of *c*9, *t*11, *c*15-C18:3 CALA1 and *c*6, *c*9, *t*11-C18:3 CGLA1 from free α-linolenic acid and γ-linolenic acid, respectively, by strains of lactobacilli exhibiting linoleic acid isomerase activity.

CALA1 (*c*9, *t*11, *c*15-C18:3) CALA2 (*t*9, *t*11, *c*15-C18:3)



CGLA1 (*c*6, *c*9, *t*11-C18:3) CGLA2 (*c*6, *t*9, *t*11-C18:3)

CSA1 (*c*6, *c*9, *t*11, *c*15-C18:4) CSA2 (*c*6, *t*9, *t*11, *c*15-C18:4)

Figure 3.4 Structural representations of the conjugated isomers of **a)** α-linolenic acid, **b)** γ-linolenic acid, and **c)** stearidonic acid.

In addition to the production of CALA1, CGLA1 and CSA1 by strains of propionibacteria and bifidobacteria following fermentation of their respective substrate fatty acids, smaller amounts of another conjugated isomer were also observed **(Figure 3.5)**. These isomers would appear to share equivalence with the *t*9, *t*11 CLA isomer which was found in small quantities along with the predominant *c*9, *t*11 CLA isomer during conjugation of linoleic acid by strains of bifidobacteria and propionibacteria. In this study, sufficient quantities of these conjugated fatty acids could not be purified so as to facilitate their identification by GLC-MS. However, based on the results of the GLC-MS data of CALA1, CGLA1 and CSA1 and the well characterised activity of the enzyme linoleic acid isomerase, it is likely that these isomers are *t*9, *t*11, *c*15-C18:3 (CALA2) derived from α-linolenic acid, *c*6, *t*9, *t*11-C18:3 (CGLA2) derived from γ-linolenic acid and *c*6, *t*9, *t*11, *c*15-C18:4 (CSA2) derived from stearidonic acid **(Figure 3.4)**.

3.3.3 Production of conjugated fatty acids by *B. breve* DPC6330

Of the strains assayed in the present study, *B. breve* DPC6330 proved to be the most effective in terms of its ability to convert linoleic acid, α-linolenic acid, γ-linolenic acid and stearidonic acid to their respective conjugated fatty acids **(Table 3.4)**. This strain was further used to assess the impact of incrementally increasing the substrate fatty acid concentration on the production of the conjugated fatty acid isomers **(Table 3.5)**.

***Bioconversion of α-linolenic acid to CALA by B. breve* DPC6330.** Following 72 h anaerobic incubation at 37^{o}C in the presence of 0.15 mg/ml α-linolenic acid, *B. breve* DPC6330 produced 0.113 ± 0.001 mg/ml of CALA1 and 0.002 ± 0.000 mg/ml of the putative CALA2 isomer. This corresponded to 87.80% and 1.71%

(a)

(b)

(c)

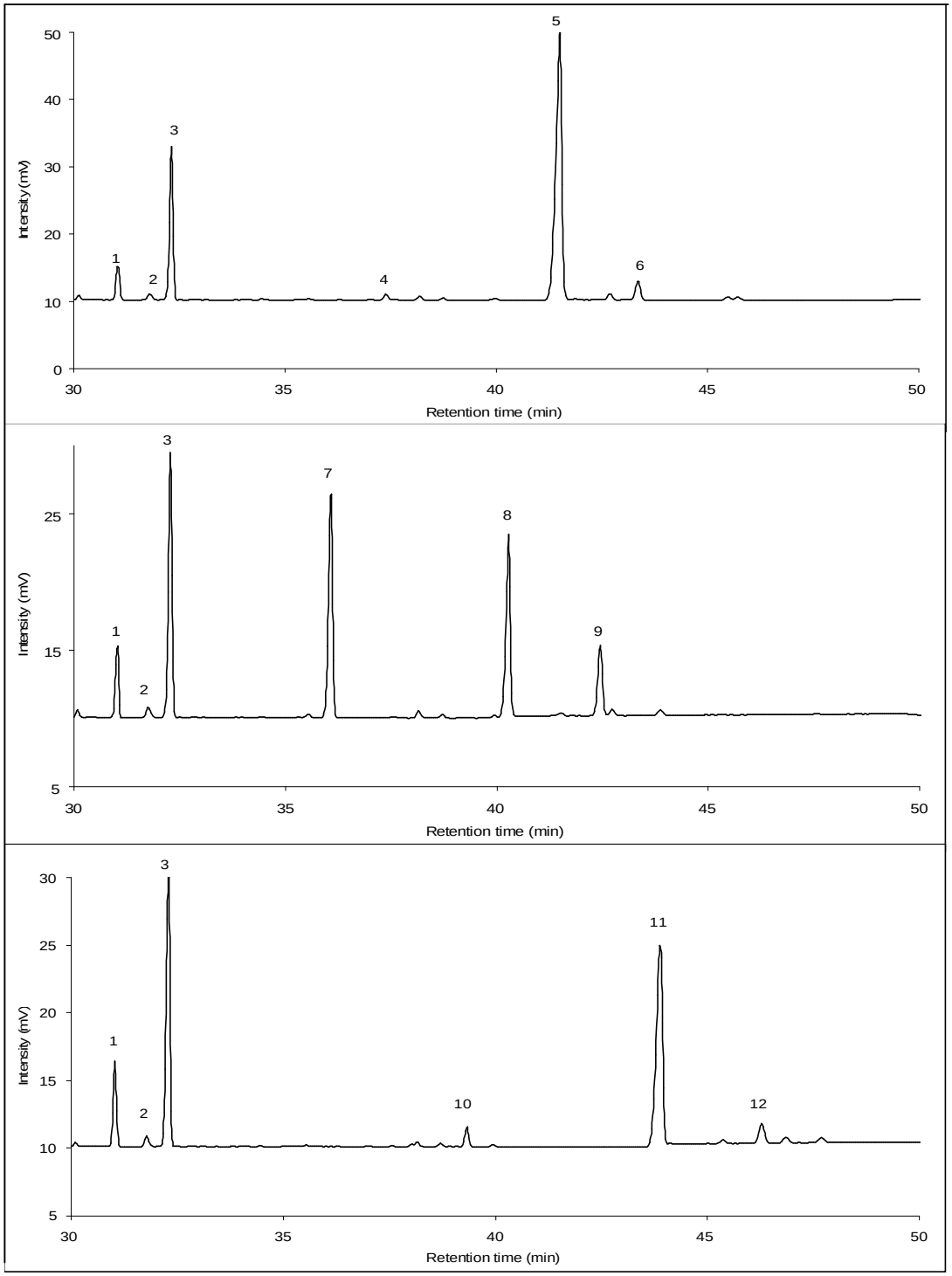


Figure 3.5 GLC profiles of *B. breve* DPC6330 grown in cys-MRS containing 0.3 mg/ml of the PUFA **a)** α-linolenic acid, **b)** γ-linolenic acid and **c)** stearidonic acid, following 80 h anaerobic incubation. 1, Stearic acid; 2, Vaccenic acid; 3, Oleic acid; 4, α-linolenic acid; 5, CALA1; 6, CALA2; 7, γ-linolenic acid; 8, CGLA1; 9, CGLA2; 10, Stearidonic acid; 11, CSA1; 12, CSA2.

bioconversion of the substrate α-linolenic acid to the CALA1 and CALA2 isomers, respectively. Production of both isomers increased 1.41-fold and 0.82-fold, respectively, upon increasing the substrate fatty acid concentration to 0.3 mg/ml. At this fatty acid concentration 89.93% and 1.34% of the substrate α-linolenic acid was converted to CALA1 and CALA2, respectively. In the presence of 0.45 mg/ml α-linolenic acid the bifidobacterial fermentation resulted in 1.04-fold and 12.25-fold increases in the concentration of CALA1 and CALA2, respectively, over those achieved in the presence of 0.3 mg/ml α-linolenic acid. These increases corresponded to percentage bioconversion levels of α-linolenic acid to CALA1 and CALA2 of 70.79% and 12.18%, respectively. The concentration of residual α-linolenic acid following 72 h fermentation also increased with increasing substrate α-linolenic acid concentrations, from a minimum of 0.001 ± 0.000 mg/ml at an α-linolenic acid concentration of 0.15 mg/ml to a maximum concentration of 0.006 ± 0.00 mg/ml at an α-linolenic acid concentration of 0.45 mg/ml **(Table 3.5)**.

In an attempt to elucidate the time-scale for the production of both CALA1 and the putative CALA2 isomer by *B. breve* DPC6330, the growth and CALA production by the strain was monitored over 80 h in cys-MRS containing 0.3 mg/ml α-linolenic acid (n = 2) **(Figure 3.6)**. From an initial inoculum of 8.0 log cfu/ml, there was a small but steady increase in cell numbers to a maximum cell count of 8.69 log cfu/ml following 20 h. This phase of logarithmic growth corresponded directly with a dramatic increase in the production of CALA1 from 0 mg/ml to 0.292 mg/ml following 16 h fermentation. Production of the putative CALA2 isomer also increased in parallel to this phase of logarithmic growth, increasing from 0 mg/ml to 0.016 mg/ml at 16 h. As the culture entered the stationary phase of growth, there was a small but gradual increase in the production of both

Table 3.5 Effect of substrate concentration on the production of conjugated fatty acids CALA, CGLA and CSA and the concentration of residual substrate following 72 h anaerobic fermentation.

Strain	PUFA concentration	Average concentration mg/ml			Average percentage remaining / bioconversion		
		α–linolenic acid	CALA1	CALA2	α-linolenic acid	CALA1	CALA2
B. breve DPC6330	0.15 mg/ml	0.001 ± 0.000	0.113 ± 0.001	0.002 ± 0.000	0.49	87.80	1.71
B. breve DPC6330	0.30 mg/ml	0.002 ± 0.000	0.272 ± 0.007	0.004 ± 0.000	0.82	89.93	1.34
B. breve DPC6330	0.45 mg/ml	0.006 ± 0.000	0.282 ± 0.013	0.049 ± 0.002	1.41	70.79	12.18
		γ-linolenic acid	CGLA1	CGLA2	γ-linolenic acid	CGLA1	CGLA2
B. breve DPC6330	0.15 mg/ml	0.040 ± 0.006	0.026 ± 0.001	0.009 ± 0.000	31.78	20.81	6.99
B. breve DPC6330	0.30 mg/ml	0.144 ± 0.024	0.047 ± 0.011	0.009 ± 0.002	51.45	16.63	3.09
B. breve DPC6330	0.45 mg/ml	0.196 ± 0.004	0.058 ± 0.003	0.023 ± 0.001	45.76	13.60	5.30
		Stearidonic acid	CSA1	CSA2	Stearidonic acid	CSA1	CSA2
B. breve DPC6330	0.15 mg/ml	0.002 ± 0.000	0.024 ± 0.001	0.001 ± 0.000	2.11	25.81	1.52
B. breve DPC6330	0.30 mg/ml	0.034 ± 0.011	0.064 ± 0.005	0.002 ± 0.000	14.87	28.45	0.97
B. breve DPC6330	0.45 mg/ml	0.418 ± 0.005	0.000 ± 0.000	0.000 ± 0.000	99.62	0.00	0.00

CALA1 and the putative CALA2 isomer, with maximum production after 64 h (0.315 mg/ml) and 56 h (0.018 mg/ml), respectively **(Figure 3.6)**. With regard to the other fatty acids found in the fermentation medium, a small decrease in the concentration of oleic acid and small increases in the concentration of vaccenic acid, stearic acid and palmitic acid were observed **(Table 3.6)**.

***Bioconversion of γ-linolenic acid to CGLA by B. breve* DPC6330.** Fermentation of *B. breve* DPC6330 in cys-MRS containing 0.15 mg/ml γ-linolenic acid at 37^{o}C for 72 h also resulted in the production of two conjugated isomers, the predominant isomer CGLA1 (0.026 ± 0.001 mg/ml) and the minor putative CGLA2 isomer (0.009 ± 0.001 mg/ml). The concentrations of CGLA1 and CGLA2 produced at this substrate fatty acid concentration corresponded to percentage bioconversion levels of 20.81% and 6.99%, respectively. Increasing the concentration of substrate γ-linolenic acid to 0.3 mg/ml resulted in a 1.81-fold increase in the concentration of CGLA1, relative to that achieved at a γ-linolenic acid concentration of 0.15 mg/ml. However, a reduction in the percentage bioconversion of γ-linolenic acid to CGLA1 (from 20.81 to 16.63%) was observed at this substrate concentration due to increased residual substrate fatty acid. While the concentration of putative CGLA2 isomer produced remained unchanged from that achieved at a substrate γ-linolenic acid concentration of 0.15 mg/ml, a small reduction in the bioconversion of the substrate fatty acid to the putative CGLA2 isomer was observed (from 6.99% to 3.09%). At a γ-linolenic acid substrate fatty acid concentration of 0.45 mg/ml, the production of both CGLA1 and the putative CGLA2 isomer increased 1.23-fold and 2.56-fold, respectively, over that achieved at a γ-linolenic acid concentration of 0.3 mg/ml. These increases corresponded to bioconversion

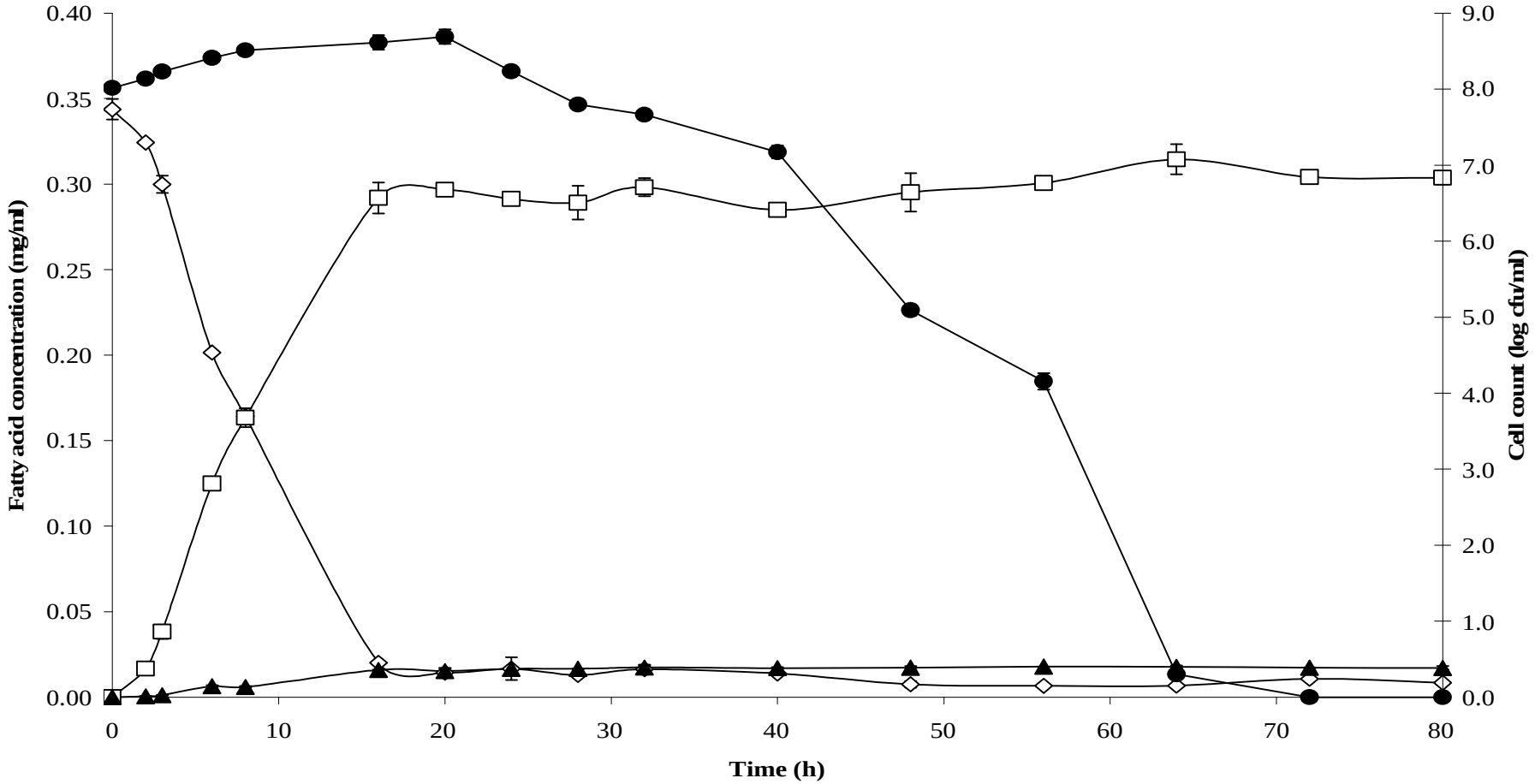


Figure 3.6 Growth and CALA production by *B. breve* DPC6330 over 80 h in the presence of 0.3 mg/ml α-linolenic acid. CALA1 (□), CALA2 (▲), α-linolenic acid (◇) and log cfu/ml (●).

α-linolenic acid				
	Concentration (mg/ml)			
Time (h)	C16:0	C18:0	C18:1-*t* 11	C18:1-*c* 11
0	0.007 ± 0.000	0.016 ± 0.001	0.003 ± 0.000	0.102 ± 0.002
2	0.006 ± 0.000	0.015 ± 0.001	0.003 ± 0.000	0.100 ± 0.000
3	0.006 ± 0.000	0.015 ± 0.000	0.003 ± 0.000	0.101 ± 0.003
6	0.006 ± 0.000	0.015 ± 0.000	0.003 ± 0.000	0.102 ± 0.004
8	0.007 ± 0.000	0.016 ± 0.000	0.003 ± 0.000	0.108 ± 0.002
16	0.007 ± 0.000	0.016 ± 0.000	0.003 ± 0.000	0.102 ± 0.000
20	0.007 ± 0.000	0.016 ± 0.000	0.003 ± 0.000	0.093 ± 0.009
24	0.007 ± 0.000	0.017 ± 0.000	0.003 ± 0.000	0.098 ± 0.003
28	0.007 ± 0.000	0.017 ± 0.001	0.003 ± 0.000	0.086 ± 0.010
32	0.008 ± 0.000	0.018 ± 0.000	0.004 ± 0.000	0.097 ± 0.006
40	0.007 ± 0.000	0.016 ± 0.000	0.004 ± 0.000	0.084 ± 0.010
48	0.008 ± 0.000	0.018 ± 0.000	0.003 ± 0.000	0.088 ± 0.009
56	0.008 ± 0.001	0.018 ± 0.001	0.004 ± 0.000	0.090 ± 0.000
64	0.008 ± 0.001	0.018 ± 0.001	0.004 ± 0.000	0.097 ± 0.003
72	0.008 ± 0.000	0.018 ± 0.001	0.004 ± 0.000	0.091 ± 0.010
80	0.008 ± 0.000	0.018 ± 0.000	0.004 ± 0.000	0.092 ± 0.002
γ-linolenic acid				
	Concentration (mg/ml)			
Time (h)	C16:0	C18:0	C18:1-*t* 11	C18:1-*c* 11
0	0.006 ± 0.000	0.014 ± 0.000	0.003 ± 0.000	0.090 ± 0.003
2	0.007 ± 0.001	0.015 ± 0.000	0.003 ± 0.000	0.098 ± 0.001
3	0.006 ± 0.000	0.014 ± 0.001	0.004 ± 0.000	0.097 ± 0.006
6	0.007 ± 0.001	0.015 ± 0.000	0.003 ± 0.000	0.091 ± 0.000
8	0.006 ± 0.000	0.014 ± 0.000	0.003 ± 0.000	0.084 ± 0.001
16	0.007 ± 0.000	0.016 ± 0.000	0.004 ± 0.000	0.100 ± 0.000
20	0.007 ± 0.000	0.016 ± 0.000	0.004 ± 0.000	0.093 ± 0.003
24	0.008 ± 0.000	0.017 ± 0.000	0.004 ± 0.000	0.089 ± 0.003
28	0.008 ± 0.000	0.018 ± 0.001	0.004 ± 0.000	0.096 ± 0.003
32	0.007 ± 0.000	0.016 ± 0.000	0.004 ± 0.000	0.091 ± 0.004
40	0.007 ± 0.000	0.016 ± 0.001	0.004 ± 0.000	0.083 ± 0.000
48	0.008 ± 0.001	0.016 ± 0.001	0.004 ± 0.000	0.079 ± 0.013
56	0.007 ± 0.000	0.015 ± 0.000	0.004 ± 0.000	0.076 ± 0.005
64	0.007 ± 0.000	0.016 ± 0.001	0.004 ± 0.000	0.081 ± 0.005
72	0.008 ± 0.000	0.016 ± 0.000	0.004 ± 0.000	0.090 ± 0.000
80	0.008 ± 0.000	0.016 ± 0.000	0.004 ± 0.000	0.087 ± 0.000
Stearidonic acid				
	Concentration (mg/ml)			
Time (h)	C16:0	C18:0	C18:1-*t* 11	C18:1-*c* 11
0	0.007 ± 0.001	0.016 ± 0.002	0.003 ± 0.000	0.098 ± 0.007
2	0.006 ± 0.000	0.014 ± 0.001	0.003 ± 0.000	0.096 ± 0.002
3	0.006 ± 0.000	0.014 ± 0.001	0.002 ± 0.000	0.091 ± 0.012
6	0.006 ± 0.000	0.014 ± 0.001	0.003 ± 0.000	0.091 ± 0.018
8	0.006 ± 0.000	0.014 ± 0.001	0.003 ± 0.000	0.097 ± 0.005
16	0.007 ± 0.001	0.015 ± 0.001	0.003 ± 0.000	0.097 ± 0.002
20	0.005 ± 0.000	0.012 ± 0.001	0.002 ± 0.000	0.085 ± 0.001
24	0.006 ± 0.000	0.013 ± 0.000	0.002 ± 0.000	0.095 ± 0.006
28	0.006 ± 0.000	0.014 ± 0.000	0.003 ± 0.000	0.095 ± 0.004
32	0.006 ± 0.000	0.014 ± 0.000	0.003 ± 0.000	0.096 ± 0.001
40	0.006 ± 0.000	0.014 ± 0.000	0.003 ± 0.000	0.099 ± 0.000
48	0.006 ± 0.000	0.016 ± 0.001	0.003 ± 0.000	0.100 ± 0.009
56	0.006 ± 0.001	0.015 ± 0.001	0.003 ± 0.001	0.091 ± 0.007
64	0.007 ± 0.000	0.015 ± 0.001	0.003 ± 0.000	0.092 ± 0.001
72	0.007 ± 0.000	0.017 ± 0.000	0.003 ± 0.000	0.107 ± 0.001
80	0.006 ± 0.000	0.015 ± 0.000	0.003 ± 0.000	0.098 ± 0.000

Table 3.6 Concentration of the other major fatty acids detected in the supernatant following the growth of *B. breve* DPC6330 in the presence of 0.3 mg/ml α-linolenic acid, γ-linolenic acid or stearidonic acid over 80 h anaerobic fermentation in cys-MRS.

levels of substrate to CGLA1 of 13.60% and CGLA2 of 5.30%. As found previously with α-linolenic acid, increasing the initial concentration of substrate γ-linolenic acid, resulted in a corresponding increase in the concentration of residual γ-linolenic acid following fermentation. This increased from a minimum γ-linolenic acid concentration of 0.040 ± 0.006 mg/ml at a substrate concentration of 0.15 mg/ml to a maximum γ-linolenic acid concentration of 0.196 ± 0.004 mg/ml at a substrate concentration 0.45 mg/ml **(Table 3.5)**.

The growth and CGLA production by *B. breve* DPC6330 was monitored over 80 h in cys-MRS containing 0.3 mg/ml γ-linolenic acid (n = 2) **(Figure 3.7)**. From an initial inoculum concentration of 8.13 log cfu/ml, growth of the strain increased at a rate of approximately 0.02 log cfu/ml per hour to a maximum cell count of 8.59 log cfu/ml after 24 h. Following this period of growth, the strain entered the late stationary phase, which coincided with a steady decline in cell numbers over the subsequent 56 h, to a final cell count of 0.15 log cfu/ml after 80 h. Production of CGLA1 was highest during the logarithmic growth phase, increasing from 0 mg/ml following 0 h to 0.035 mg/ml after 16 h, while production of the putative CGLA2 isomer increased more gradually over the duration of the fermentation **(Figure 3.7)**. The composition of the other major fatty acids in the medium remained unchanged over the course of the fermentation **(Table 3.6)**.

***Bioconversion of stearidonic acid to CSA by B. breve* DPC6330**. When incubated anaerobically at 37^{o}C for 72 h in the presence of 0.15 mg/ml stearidonic acid, *B. breve* DPC6330 produced 0.024 ± 0.001 mg/ml of CSA1 and 0.001 ± 0.000 mg/ml of the putative CSA2 isomer, corresponding to percentage bioconversions of substrate fatty acid to CSA1 of 25.81% and CSA2 of 1.52%. When the concentration of stearidonic acid in the fermentation medium was increased to 0.3

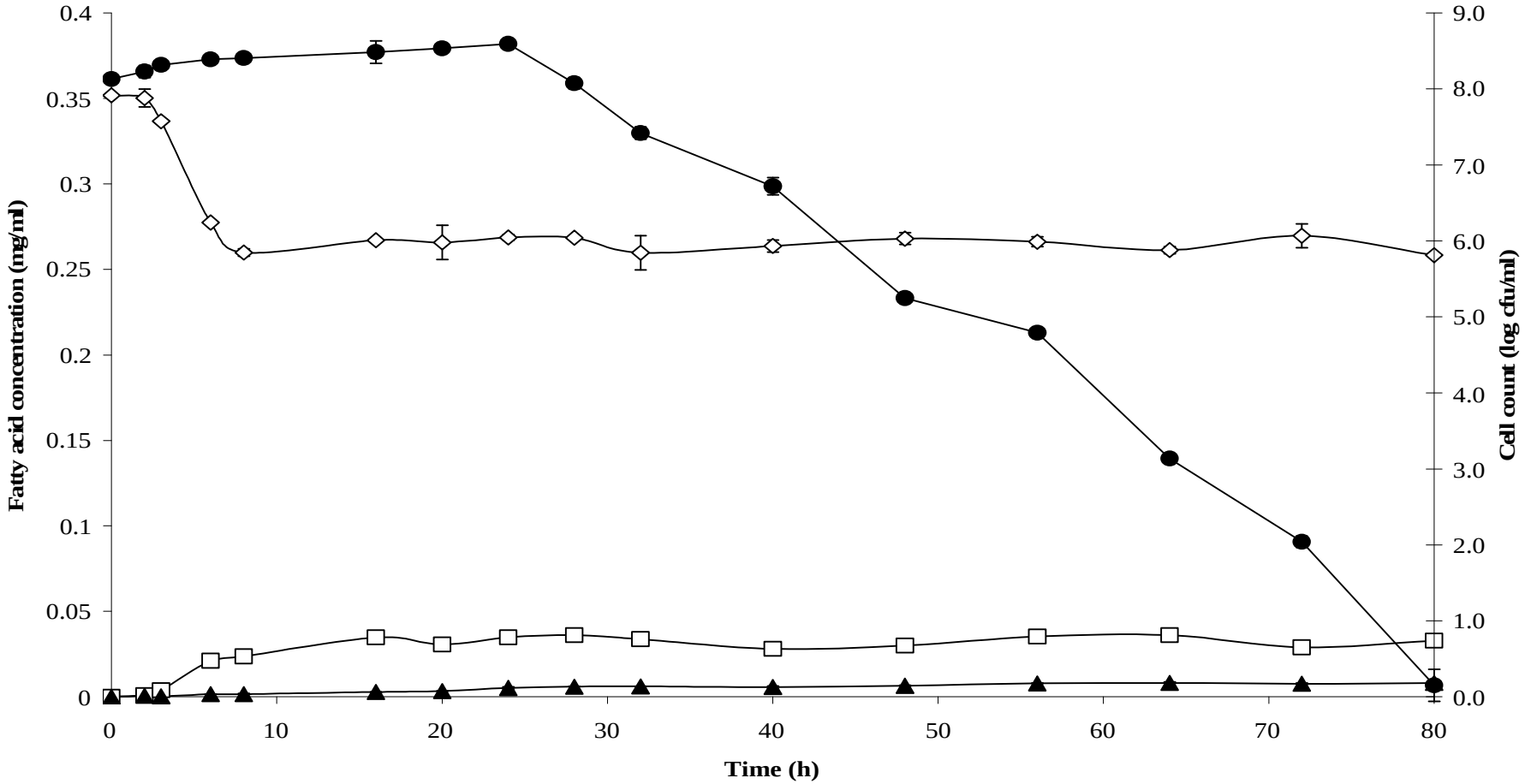


Figure 3.7 Growth and CGLA production by *B. breve* DPC6330 over 80 h in the presence of 0.3 mg/ml γ-linolenic acid. CGLA1 (□), CGLA2 (▲), γ-linolenic acid (◇) and log cfu/ml (●).

mg/ml, the production of both isomers was increased 2.67-fold and 2.00-fold, respectively. At this concentration the percentage bioconversion of stearidonic acid to CSA1 was 28.45% and to CSA2 was 0.97%. However, increasing the concentration of stearidonic acid to 0.45 mg/ml resulted in the total inhibition of growth by the strain and neither CSA isomer produced. As found previously for α-linolenic acid and γ-linolenic acid, increasing the concentration of substrate stearidonic acid supplied to the strain resulted in an increase in the concentration of residual stearidonic acid in the medium following fermentation. Following 72 h fermentation, the concentration of residual stearidonic acid increased from 2.11% at stearidonic acid substrate concentration of 0.15 mg/ml, to 14.87 % at a stearidonic acid concentration of 0.3 mg/ml and finally to 99.6% at a stearidonic acid substrate concentration of 0.45 mg/ml when growth of the strain was completely inhibited.

In an attempt to characterise the growth and CSA production by *B. breve* DPC6330 in the presence of stearidonic acid, we monitored changes in both cell numbers and fatty acid composition over 80 h in cys-MRS containing 0.3 mg/ml of stearidonic acid (n = 2) **(Figure 3.8)**. The fermentation medium was inoculated with a stationary phase culture of *B. breve* DPC6330 yielding cell counts of 8.07 log cfu/ml. Over the first three hours of the fermentation, the viability of the strain remained stable, however, this was followed by a 3.83 log cfu/ml decline in cell numbers over the subsequent 17 h. The decline in cell viability was attributed to the high toxicity of substrate stearidonic acid (0.3 mg/ml) to the strain. An increase in cell viability was observed following 20 h fermentation with cell numbers increasing from 4.30 log cfu/ml to 6.70 log cfu/ml following 28 h fermentation. This may have been a result of the development of resistance to stearidonic acid by the strain. The strain remained in the logarithmic phase of cell growth until the 56th

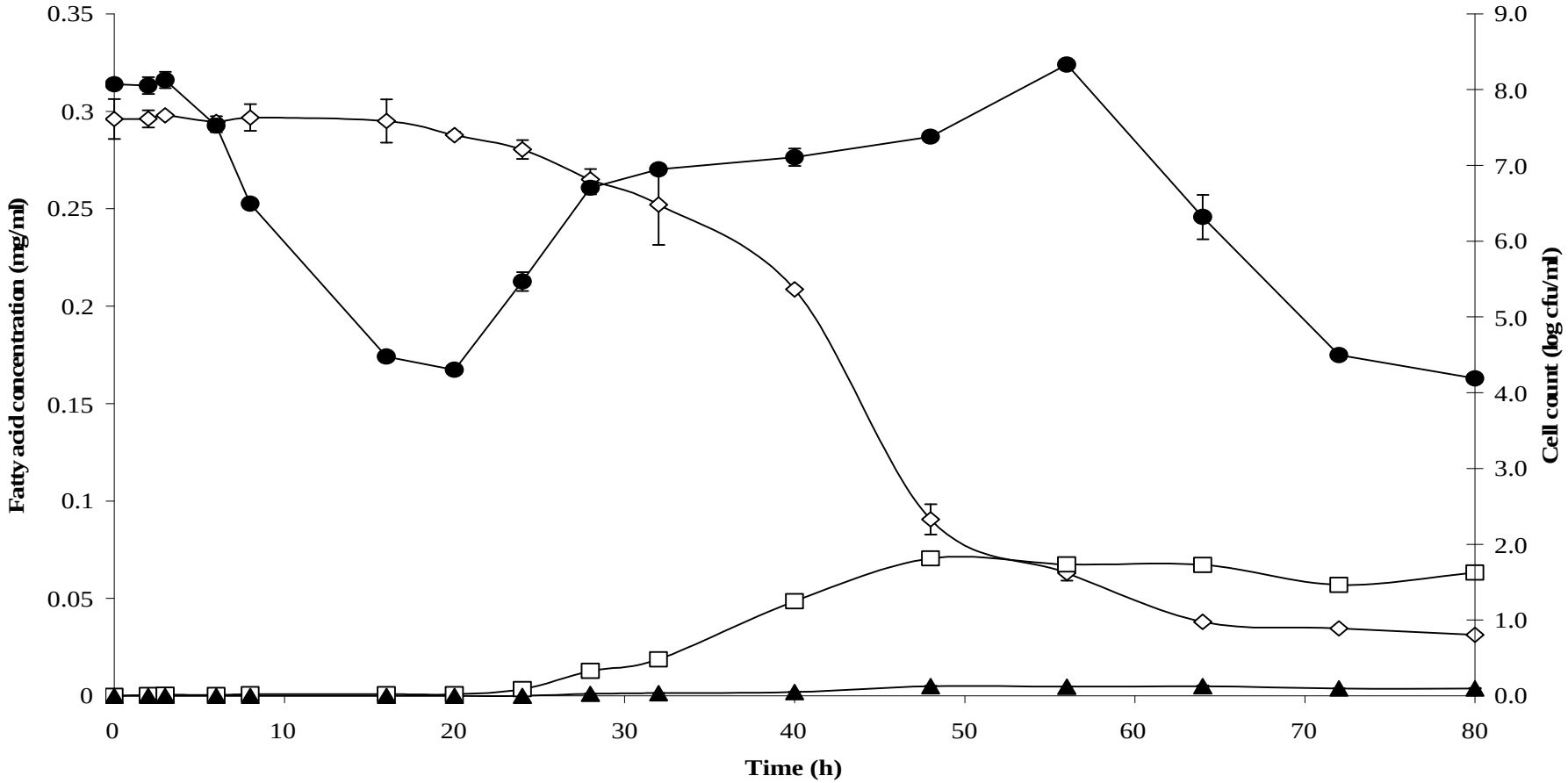


Figure 3.8 Growth and CSA production by *B. breve* DPC6330 over 80 h in the presence of 0.3 mg/ml stearidonic acid. CSA1 (□), CSA2 (▲), stearidonic acid (◇) and log cfu/ml (●).

-hour of the fermentation where a maximum cell count of 7.52 log cfu/ml was recorded **(Figure 3.8)**.

Production of CSA1 corresponded approximately with the logarithmic phase of cell growth between the 20 h and 48 h of the fermentation. During this period, the concentration of CSA1 increased from 0.001 mg/ml to 0.071 mg/ml, remaining relatively constant thereafter. Production of the putative CSA2 isomer was also at its maximum during this period, with its concentration increasing from 0 mg/ml after 20 h to 0.005 mg/ml at 48 h of the fermentation **(Figure 3.8)**. The composition of the other major fatty acids in the media remained relatively unchanged over the course of the fermentation **(Table 3.6)**.

To determine if the strain which had developed increased resistance to stearidonic acid possessed similar properties to the wild type *B. breve* DPC6330 in terms of the ability to conjugate fatty acids, the ability of both strains to produce CLA from linoleic acid was assessed. Overnight cultures of the strain displaying increased resistance to stearidonic acid and the wild type strain were inoculated (2% v/v) into cys-MRS containing from 0 mg/ml and 1.4 mg/ml linoleic acid and incubated anaerobically for 24 h at 37^{o}C. The results demonstrated that both strains displayed similar activity in terms of CLA production, exhibiting similar amounts of CLA formation at the optimum linoleic acid concentration of 0.4 mg/ml **(Figure 3.9a and 3.9b)**. However, it was observed that the capacity of the strain displaying increased resistance to stearidonic acid to convert linoleic acid to the *c*9, *t*11 CLA isomer at concentrations greater than 0.4 mg/ml was significantly higher ($p \leq 0.001$) than that of the wild type *B. breve* DPC6330 strain **(Figure 3.9a)**. Furthermore, we found that the stearidonic acid resistant strain of *B. breve* DPC6330 exhibited over 1.75 log cfu/ml greater survival than the wild type strain of *B. breve* DPC6330 in

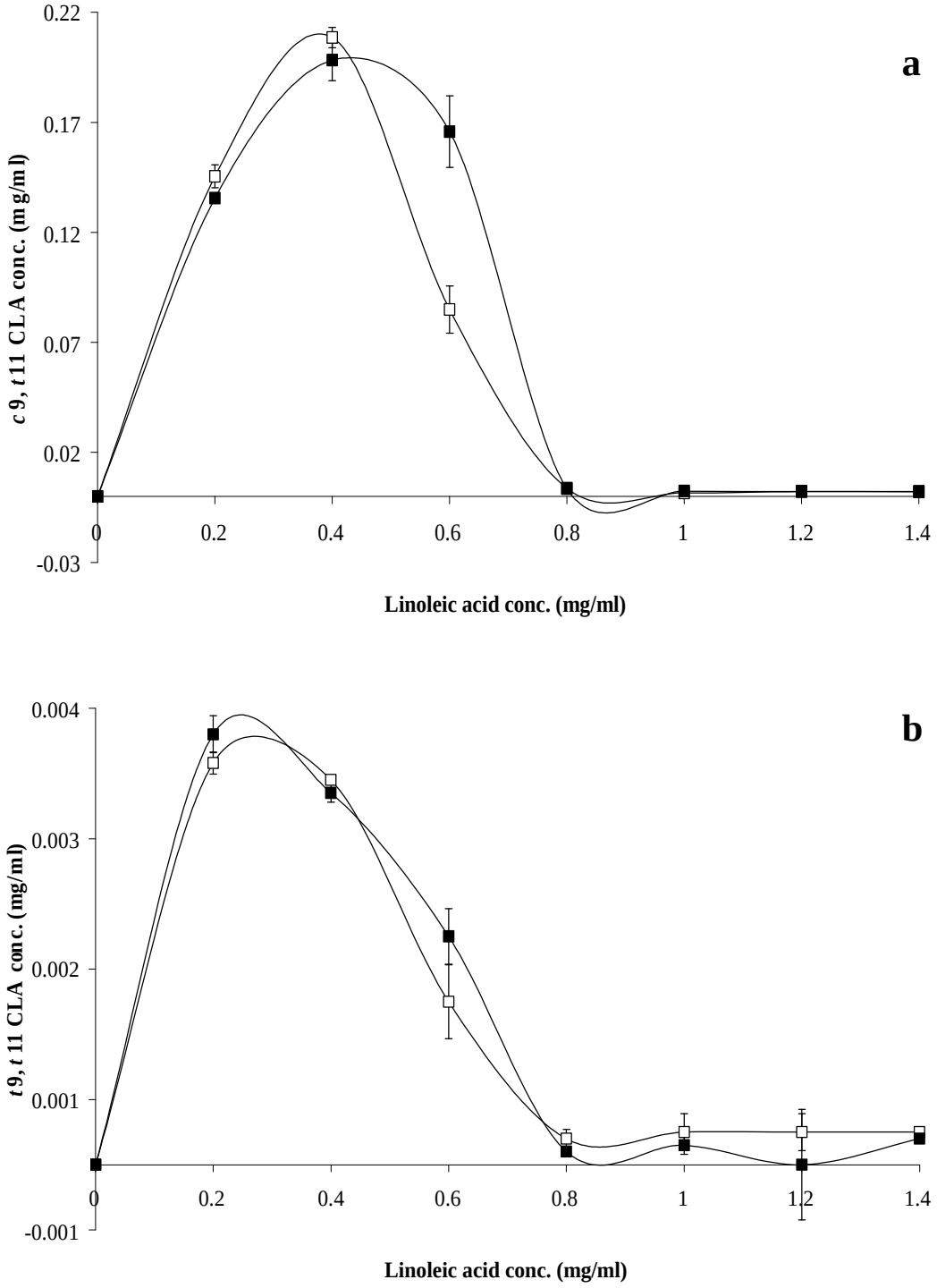


Figure 3.9 Production of **a)** the *c*9, *t*11 CLA isomer, and **b)** the *t*9, *t*11 CLA isomer by the wild type (□) and stearidonic acid resistant (■) strains of *B. breve* DPC6330.

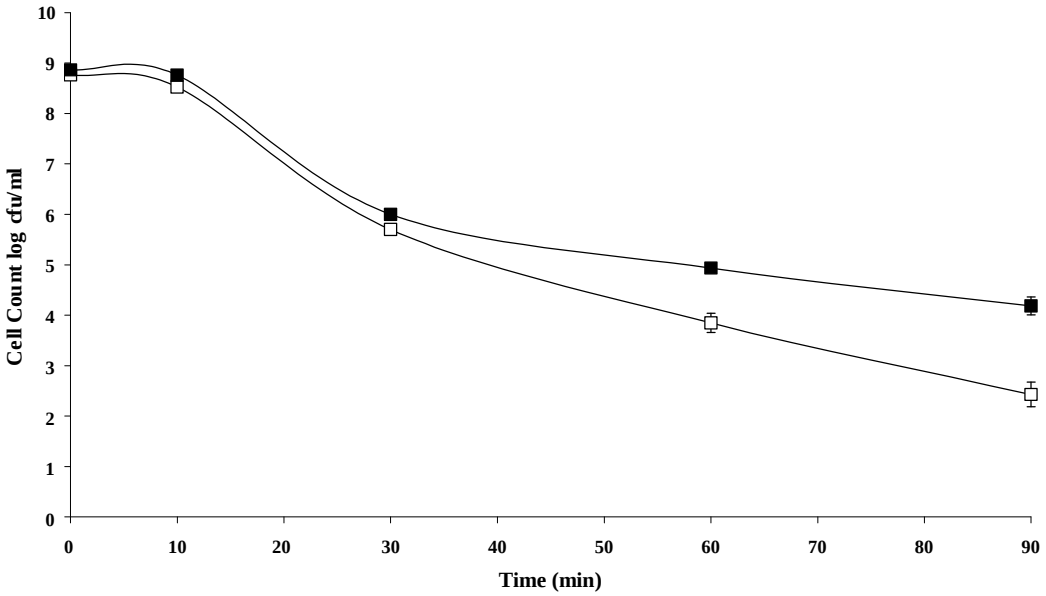


Figure 3.10 Assessing the survival of the wild type (□) and stearidonic acid resistant (■) strains of *B. breve* DPC6330 in simulated gastric juice pH 2.5.

SGJ (pH 2.5) over 90 min **(Figure 3.10)**. This observation would suggest, that rather than any specific changes in the response of the strain specifically directed against the toxic effect of unsaturated fatty acids, the mechanisms responsible for the increased tolerance of the strain to stearidonic acid and linoleic acid are more likely related to an increased overall stress tolerance.

3.3.4 Assessment of the preference of *B. breve* DPC6330 for C18 fatty acids as substrates for isomerisation

We assessed the substrate preference of *B. breve* DPC6330 to isomerise the C18 unsaturated fatty acids linoleic acid, α-linolenic acid, γ-linolenic acid and stearidonic acid (0.3 mg/ml) over an 80 h fermentation period. The activity of the strain towards these non-conjugated unsaturated fatty acids was determined by expressing the percentage bioconversion of substrate fatty acid to their respective conjugated fatty acid isomers (CALA1, CGLA1 or CSA1), relative to the ability of *B. breve* DPC6330 to convert linoleic acid to the *c*9, *t*11 CLA isomer which was alone set at 100% **(Figure 3.11)**. The results showed that *B. breve* DPC6330 has a preference for the C18 fatty acid substrates in the order α-linolenic acid (130.46%) > linoleic acid (100%) > stearidonic acid (26.58%) > γ-linolenic acid (13.74%).

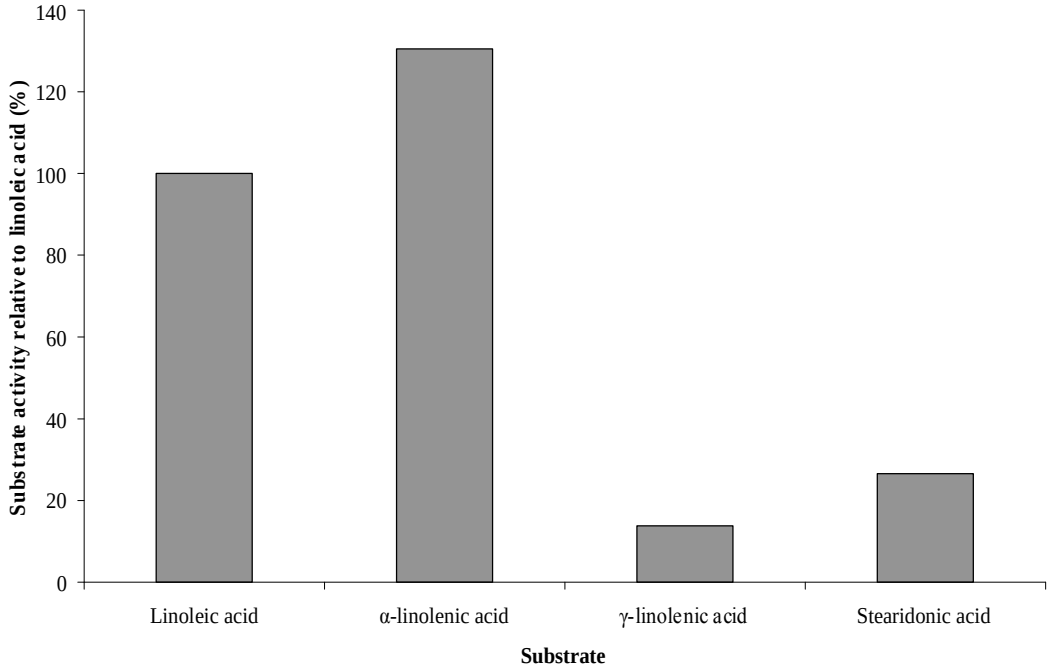


Figure 3.11 Substrate specificity of *B. breve* DPC6330, expressed relative to the strains activity when grown in the presence of linoleic acid.

3.4 Discussion

Conjugated fatty acids are commonly found in nature and many have been associated with having a beneficial effect on human health, including anti-carcinogenic, anti-adipogenic, anti-obesogenic, anti-diabetogenic, anti-artherogenic and anti-thrombotic activity as well as promoting growth, improving bone density and enhancing specific immune response (Suzuki *et al.*, 2006; Wahle *et al.*, 2004). In this study we identified a number of strains of bifidobacteria and propionibacteria with the ability to produce a range of novel conjugated fatty acids. The production of these isomers by one of these strains, *B. breve* DPC6330, was characterised and the isomers purified and identified by GLC-MS.

Of the fatty acids assayed, only four were bioconverted to their respective conjugated isomers. Linoleic acid was isomerised to the c9, *t*11 CLA and *t*9, *t*11 CLA isomers, α-linolenic acid to the CALA1 and putative CALA2 isomers, γ-linolenic acid to the CGLA1 and putative CGLA2 isomers and stearidonic acid to the CSA1 and putative CSA2 isomers, with the 9, 11-double bond common to all the conjugated fatty acids produced. While conjugation of these substrate fatty acids was found to result in the production of two conjugated isomers, it was observed that one was to be found in relative abundance while the other was present in much smaller concentrations. Similar observations have occurred during studies into the production of CLA from linoleic acid by growing cultures of CLA producing bifidobacteria (Coakley *et al.*, 2003). In these studies the primary isomer formed was the *c*9, *t*11 CLA isomer with smaller concentration of the *t*9, *t*11 CLA isomer produced. Using GLC-MS, we determined that the predominant isomer produced by both propionibacteria and bifidobacteria from α-linolenic acid possessed an 18 carbon backbone containing double bonds at positions-9, 11, and 15, with the

conjugated double bond located between positions-9 and 11. Identification of the bond conformation within the molecule was unsuccessful, however, both in this study and a number of others, the bond conformations of CALA1 was tentatively identified as being *c*9, *t*11, *c*15 (Destaillats *et al.*, 2005; Kishino *et al.*, 2003). GLC-MS was also employed to identify the predominant conjugated isomer produced by strains of bifidobacteria and propionibacteria from γ-linolenic acid. Identification of this isomer proved difficult as it was apparent that the 9, 11-double bond system had migrated to the 8, 10-position during the derivatization procedure to yield 6, 8, 10-C18:3. The difficulties in the identification of this conjugated isomer were overcome through the formation of MTAD adducts and pyrrolidide derivatives, resulting in the identification of the conjugate as 6, 9, 11-C18:3 with the conjugated double bond once again located at the 9, 11-position. Elucidation of the bond conformation of this molecule was inconclusive; however, the molecule was tentatively identified as being in the *c*6, *c*9, *t*11 bond conformation (CGLA1). A similar observation was made by Ogawa *et al* (2005) during the production of CGLA1 from γ-linolenic acid by *Lactobacillus plantarum* AKU 1009a. In that case, the bond conformation was reported as being in the *c*6, *c*9, *t*11 bond conformation. Like CGLA1, identification of the predominant conjugated isomer produced from stearidonic acid by strains of CLA producing bifidobacteria and propionibacteria proved difficult, due to the migration of the 9, 11-double bond system to the 8, 10-position during the derivatization procedure to yield 6, 8, 10, 15-C18:4. Once again the use of MTAD adducts and pyrrolidide derivatives was used to overcome these difficulties and the molecule was successfully identified as 6, 9, 11, 15-C18:4, with the conjugated double bond located in the 9, 11-position. Again, elucidation of the double bond conformations was not achieved; however, given the similarity of the

molecule to both CALA1 and CGLA1, the fatty acid was tentatively identified as being *c*6, *c*9, *t*11, *c*15-C18:4 (CSA1). The conjugation of α-linolenic acid, γ-linolenic acid and stearidonic acid resulted in the production of two conjugated isomers, respectively. While the predominant isomers were identified as being CALA1, CGLA1 and CSA1, sufficient concentrations of the less abundant isomer could not be purified to facilitate GLC-MS analysis. Based on the observations of Ogawa *et al* (2005) and the well characterised activity of the enzyme linoleic acid isomerase we putatively determined these lesser isomers as being *t*9, *t*11, *c*15-C18:3 (CALA2), *c*6, *t*9, t11-C18:3 (CGLA2) and *c*6, *t*9, t11, *c*15-C18:3 (CSA2).

The extent to which the strains were capable of converting α-linolenic acid, γ-linolenic acid and stearidonic acid to their respective conjugated fatty acid isomers differed substantially between the various strains and fatty acid substrates. Differences in the extent to which bacterial strains, even within the same species, conjugate linoleic acid to CLA have been reported with some regularity (Barrett *et al.*, 2007; Hennessy *et al.*, 2007). These differences have been attributed to variations in the toxicity of the substrate fatty acids to the strains, with the conjugation process reportedly being a detoxification mechanism (Coakley *et al.*, 2003; Jiang *et al.*, 1998). Differences in the conversion efficiency of the substrate fatty acids to their conjugated form may be more complicated and stem not only from differences in the toxicity of the substrate fatty acids but even occur as a result of the bond position of the substrate fatty acid (Kepler *et al.*, 1970). Of the fatty acids assayed in this study, stearidonic acid proved to be one of the most toxic to bifidobacteria, inhibiting cellular growth at fatty acid concentrations greater than 0.1 mg/ml. Its was observed however, that while stearidonic acid remained toxic to the strain *B. breve* DPC6330, a resistant sub-population which displayed CLA

producing abilities identical to the wild type strain could be isolated. This resistant strain was found to be capable of tolerating stearidonic acid concentrations of 0.35 mg/ml along with increased tolerance to environmental stresses such as exposure in simulated gastric juice, suggesting the resistant strain possessed a greater overall stress tolerance than the wild type strain. While the mechanism behind this increased stress response was not determined, it is likely that it may be related to changes in the expression of proteins associated with an increased overall stress response such as DnaK or GroEL or proteins related to increased resistance to bile salts or acidic pH such as proton-translocating ATPase (De Dea Lindner *et al.*, 2007; Schmidt & Zink, 2000). Alternatively, exposure of the strain to stearidonic acid may have resulted in changes in the fatty acid composition of the cell membrane which enhance the survival of the strain such as increases in the concentration of cyclopropane fatty acids (Veerkamp, 1971) and saturated fatty acids (Corcoran *et al.*, 2007; Russell *et al.*, 1995).

When the effect of substrate fatty acid concentration on the production of conjugated fatty acids was investigated, it was found that increasing the concentration of the substrate fatty acid from 0.15 mg/ml to 0.45 mg/ml resulted in a subsequent increase in conjugated fatty acid production, most likely through the increased availability of substrate fatty acid. An increase in the concentration of residual substrate fatty acid was also observed with increased substrate concentration. This increase in residual substrate is most likely a result of the substrate concentration exceeding the isomerising capacity of the growing culture or through the reported toxic effect of unsaturated fatty acids on bacteria (Jiang *et al.*, 1998; Maia *et al.*, 2006; Raychowdhury *et al.*, 1985).

Using *B. breve* DPC6330, we assessed the time scale of conjugated fatty acid production from the substrates α-linolenic acid, γ-linolenic acid and stearidonic acid by a growing culture (80 h). When grown in the presence of α-linolenic acid, *B. breve* DPC6330 rapidly converted the substrate fatty acid to the CALA1 and the putative CALA2 isomers suggesting that in this strain, the enzyme linoleic acid isomerase was highly active against α-linolenic acid. In the presence of α-linolenic acid (0.3 mg/ml) the strain displayed steady growth suggesting that the fatty acid was not inhibitory to the growth of the strain at the concentration assayed. A similar pattern of growth was also evident when γ-linolenic acid was used as the substrate fatty acid with the strain exhibiting steady stable growth in the first 24 h of the fermentation suggesting the growth of the strain was unaffected by the substrate fatty acid. Despite the stable growth of the strain, the production of CGLA1 and the putative CGLA2 isomers during this time was substantially lower than that of CALA1 or CALA2 under identical conditions, indicating that the linoleic acid isomerase enzyme of *B. breve* DPC6330 had a lower affinity for γ-linolenic acid than α-linolenic acid. When stearidonic acid (0.3 mg/ml) was employed as the substrate fatty acid, growth of the strain was substantially inhibited (at the concentration assayed). This inhibition was characterised by an almost four log cfu/ml reduction in cell numbers in the first twenty hours of the fermentation. During this time, production of CSA1 and the putative CSA2 isomer was not detected, however, in the following stage of logarithmic growth, production of both isomers increased substantially. Total conjugated fatty acid production from stearidonic acid at a concentration of 0.3 mg/ml exceeded conjugated fatty acid production from γ-linolenic acid at the same concentration but was substantially less than conjugated fatty acid production from the same concentration of α-

linolenic acid. These observations suggest that at elevated concentrations, stearidonic acid is extremely toxic to the strain relative to equivalent concentrations of γ-linolenic acid or α-linolenic acid, but as the strain appears to have a greater affinity for stearidonic acid in comparison to γ-linolenic acid, higher concentrations of its conjugated products were formed. When conjugated fatty acid production by *B. breve* DPC6330 from α-linolenic acid, γ-linolenic acid and stearidonic acid (80 h fermentation) was compared with that of linoleic acid under identical conditions, it was evident that bond position had an effect on the extent to which the fatty acids were conjugated. Indeed, it was observed that the presence of an additional double bond towards the distal end of the fatty acid molecule (e.g. α-linolenic acid) encouraged conjugation by the strain, while the addition of a double bond between the carboxyl group and the *c*9, *c*12 double bond (e.g. γ-linolenic acid) discouraged conjugation of the double bond.

Strains of both bifidobacteria and propionibacteria are important in both the production of fermented dairy products and as probiotics for the promotion of dairy and food industries. Within the human gastrointestinal tract and during dairy fermentation, bacteria are afforded regular access to the substrate fatty acids assayed in the present study. This access makes the *in situ* production of novel conjugated fatty acids with potential health promoting properties in both fermented dairy products and in the human gastro-intestinal tract a viable prospect. Indeed, the ability of CLA producing bacteria to produce CLA in a range of dairy products has been reported with some frequency (Hennessy *et al.*, 2007; Hennessy *et al.*, 2009), while more recently evidence pertaining to the *ex vivo* and *in vivo* production of CLA in the gastrointestinal tract by the intestinal microflora has emerged (Ewaschuk *et al.*, 2006; Wall *et al.*, 2009). Thus, it is likely that the strains of

propionibacteria and bifidobacteria utilised in this study could be successfully exploited to produce the conjugated fatty acids identified in both dairy products and in the human gastro-intestinal tact and that these conjugated fatty acids may subsequently contribute to improved human health.

3.5 Conclusions

In the course of this study we have investigated the ability of selected bifidobacteria and propionibacteria strains to conjugate a range of unsaturated fatty acids. Of these we have determined that only linoleic acid, α-linolenic acid, γ-linolenic acid and stearidonic acid were converted to there respective conjugated isomers. These fatty acids all contained the *c*9, *c*12 double bond system which using GLC-MS we confirmed was isomerised to the 9, 11-conjugated double bond system as a result of the activity of the strains. Of the strains assayed, *B. breve* DPC6330 proved the most effective in terms of its ability to produce novel conjugated fatty acids. These conjugated fatty acids were produced by *B. breve* DPC6330 in the logarithmic phase of cellular growth over approximately an 18 h period. The strain *B. breve* DPC6330 displayed a preference for conjugating fatty acids in the order of α-linolenic acid > linoleic acid > stearidonic acid > γ-linolenic acid. Using a Luna 5u C18 preparative RP-HPLC column we demonstrated the ease at which these conjugates could be separated from the other fatty acids found in the fermentation medium.

3.6 References.

Andersson, B. A. & Holman, R. T. (1974). Pyrrolidides for mass spectrometric determination of the position of double bonds in monounsaturated fatty acids. *Lipids* **9**, 185-190.

Barrett, E., Ross, R. P., Fitzgerald, G. F. & Stanton, C. (2007). Rapid screening method for analyzing the conjugated linoleic acid production capabilities of bacterial cultures. *Appl Environ Microbiol* **73**, 2333-2337.

Belury, M. A. (2002). Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action. *Annu Rev Nutr* **22**, 505-531.

Beumer, R. R., de Vries, J. & Rombouts, F. M. (1992). *Campylobacter jejuni* non-culturable coccoid cells. *Int J Food Microbiol* **15**, 153-163.

Bhattacharya, A., Banu, J., Rahman, M., Causey, J. & Fernandes, G. (2006). Biological effects of conjugated linoleic acids in health and disease. *J Nutr Biochem* **17**, 789-810.

Burgess, J. R., de la Rossa, R. I., Jacobs, R. S. & Butler, A. (1991). A new eicosapentaenoic acid formed from arachidonic acid in cralline red algae *Bossiella orbigniana*. *Lipids* **26**, 162-165.

Chisholm, M. J. & Hopkins, C. Y. (1967). Conjugated fatty acids in some cucurbitaceae seed oils. *Can J Biochem* **45**, 1081-1086.

Coakley, M., Ross, R. P., Nordgren, M., Fitzgerald, G., Devery, R. & Stanton, C. (2003). Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species. *J Appl Microbiol* **94**, 138-145.

Coakley, M., Banni, S., Johnson, M. C., Mills, S., Devery, R., Fitzgerald, G., Ross, R. P. & Stanton, C. (2009). Inhibitory effect of conjugated α-linolenic acid (CALA) from bifidobacteria of intestinal origin on SW480 cancer cells. *Lipids* **44**, 249-256.

Corcoran, B. M., Stanton, C., Fitzgerald, G. F. & Ross, R. P. (2007). Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice. *Microbiology* **153**, 291-299.

Danbara, N., Yuri, T., Tsujita-Kyutoku, M., Sato, M., Senzaki, H., Takada, H., Hada, T., Miyazawa, T., Okazaki, K. & Tsubura, A. (2004). Conjugated docosahexaenoic acid is a potent inducer of cell cycle arrest and apoptosis and inhibits growth of colo 201 human colon cancer cells. *Nutr Cancer* **50**, 71-79.

De Dea Lindner, J., Canchaya, C., Zhang, Z., Neviani, E., Fitzgerald, G. F., van Sinderen, D. & Ventura, M. (2007). Exploiting *Bifidobacterium* genomes: the molecular basis of stress response. *Int J Food Microbiol* **120**, 13-24.

Destaillats, F., Trottier, J. P., Galvez, J. M. & Angers, P. (2005). Analysis of alpha-linolenic acid biohydrogenation intermediates in milk fat with emphasis on conjugated linolenic acids. *J Dairy Sci* **88**, 3231-3239.

Dhiman, T. R., Nam, S. H. & Ure, A. L. (2005). Factors affecting conjugated linoleic acid content in milk and meat. *Crit Rev Food Sci Nutr* **45**, 463-482.

Dobson, G. (1998). Identification of conjugated fatty acids by gas chromatography mass spectrometry of 4-methyl-1,2,4-triazoline-3,5-dione adducts. *J Am Oil Chem Soc* **75**, 137-142

Ewaschuk, J. B., Walker, J. W., Diaz, H. & Madsen, K. L. (2006). Bioproduction of conjugated linoleic acid by probiotic bacteria occurs *in vitro* and *in vivo* in mice. *J Nutr* **136**, 1483-1487.

Hennessy, A. A., Ross, R. P., Stanton, C. & Devery, R. (2007). Development of dairy based functional foods enriched in conjugated linoleic acid with special reference to rumenic acid. In *Functional Dairy Products*, pp. 443-495. Edited by M. Saarela. Cambridge, England: Woodhead Publishing Limited.

Hennessy, A. A., Ross, R. P., Devery, R. & Stanton, C. (2009). Optimization of a reconstituted skim milk based medium for enhanced CLA production by bifidobacteria. *J Appl Microbiol* **106**, 1315-1327.

Igarashi, M. & Miyazawa, T. (2000). Newly recognized cytotoxic effect of conjugated trienoic fatty acids on cultured human tumor cells. *Cancer Lett* **148**, 173-179.

Igarashi, M. & Miyazawa, T. (2005). Preparation and fractionation of conjugated trienes from alpha-linolenic acid and their growth-inhibitory effects on human tumor cells and fibroblasts. *Lipids* **40**, 109-113.

Jiang, J., Bjorck, L. & Fonden, R. (1998). Production of conjugated linoleic acid by dairy starter cultures. *J Appl Microbiol* **85**, 95-102.

Kepler, C. R., Tucker, W. P. & Tove, S. B. (1970). Biohydrogenation of unsaturated fatty acids. IV. Substrate specificity and inhibition of linoleate delta-12-cis, delta-11-trans-isomerase from *Butyrivibrio fibrisolvens*. *J Biol Chem* **245**, 3612-3620.

Kishino, S., Ogawa, J., Ando, A. & Shimizu, S. (2003). Conjugated alpha-linolenic acid production from alpha-linolenic acid by *Lactobacillus plantarum* AKU 1009a. *Eur J Lipid Sci* **105**, 572-577.

Kohno, H., Yasui, Y., Suzuki, R., Hosokawa, M., Miyashita, K. & Tanaka, T. (2004). Dietary seed oil rich in conjugated linolenic acid from bitter melon inhibits azoxymethane-induced rat colon carcinogenesis through elevation of colonic PPARgamma expression and alteration of lipid composition. *Int J Cancer* **110**, 896-901.

Liu, L., Hammond, E. G. & Nikolau, B. J. (1997). *In Vivo* Studies of the Biosynthesis of [alpha]-Eleostearic Acid in the Seed of *Momordica charantia* L. *Plant Physiol* **113**, 1343-1349.

Lopez, A. & Gerwick, W. H. (1987). Two new icosapentaenoic acids from the temperate red seaweed *Ptilota filicina* J. Agardh. *Lipids* **22**, 190-194.

Maia, M. R., Chaudhary, L. C., Figueres, L. & Wallace, R. J. (2006). Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. *Antonie Van Leeuwenhoek* **91**, 303-314.

Mikhailova, M. V., Bemis, D. L., Wise, M. L., Gerwick, W. H., Norris, J. N. & Jacobs, R. S. (1995). Structure and biosynthesis of novel conjugated polyene fatty acids from the marine green alga *Anadyomene stellata*. *Lipids* **30**, 583-589.

Ogawa, J., Kishino, S., Ando, A., Sugimoto, S., Mihara, K. & Shimizu, S. (2005). Production of conjugated fatty acids by lactic acid bacteria. *J Biosci Bioeng* **100**, 355-364.

Pariza, M. W., Park, Y. & Cook, M. E. (2001). The biologically active isomers of conjugated linoleic acid. *Prog Lipid Res* **40**, 283-298.

Park, Y., Storkson, J. M., Albright, K. J., Liu, W. & Pariza, M. W. (2005). Biological activities of conjugated fatty acids: conjugated eicosadienoic (conj. 20:2delta(*c*11,*t*13/*t*12,*c*14)), eicosatrienoic (conj. 20:3delta(*c*8,*t*12,*c*14)), and heneicosadienoic (conj. 21:2delta(*c*12,*t*14/*c*13,*t*15)) acids and other metabolites of conjugated linoleic acid. *Biochim Biophys Acta* **1687**, 120-129.

Raychowdhury, M. K., Goswami, R. & Chakrabarti, P. (1985). Effect of unsaturated fatty acids in growth inhibition of some penicillin-resistant and sensitive bacteria. *J Appl Bacteriol* **59**, 183-188.

Russell, N. J., Evans, R. I., ter Steeg, P. F., Hellemons, J., Verheul, A. & Abee, T. (1995). Membranes as a target for stress adaptation. *Int J Food Microbiol* **28**, 255-261.

Schmidt, G. & Zink, R. (2000). Basic features of the stress response in three species of bifidobacteria: *B. longum, B. adolescentis*, and *B. breve*. *Int J Food Microbiol* **55**, 41-45.

Stanton, C., F., L., Kjellmer, G., Harrington, D., Devery, R., Connolly, J. F. & Murphy, J. (1997). Dietary influences on bovine milk *cis*-9,*trans*-11-conjugated linoleic acid content. *J Food Sci* **62**, 1083-1086.

Stanton, C., Murphy, J., Mcgrath, E. & Devery, R. (2003). Animal Feeding Strategies for Conjugated Linoleic Acid Enrichment of Milk. In *Advances in Conjugated Linoleic Acid Research, Vol 2,* pp. 123-145. Edited by J. L. Sebedio, W. W. Christie & R. Adlof. Champaign , IL: AOCS Press.

Suzuki, R., Noguchi, R., Ota, T., Abe, M., Miyashita, K. & Kawada, T. (2001). Cytotoxic effect of conjugated trienoic fatty acids on mouse tumor and human monocytic leukemia cells. *Lipids* **36**, 477-482.

Suzuki, R., Yasui, Y., Kohno, H., Miyamoto, S., Hosokawa, M., Miyashita, K. & Tanaka, T. (2006). Catalpa seed oil rich in 9*t*,11*t*,13*c*-conjugated linolenic acid suppresses the development of colonic aberrant crypt foci induced by azoxymethane in rats. *Oncol Rep* **16**, 989-996.

Tsujita-Kyutoku, M., Yuri, T., Danbara, N., Senzaki, H., Kiyozuka, Y., Uehara, N., Takada, H., Hada, T., Miyazawa, T., Ogawa, Y. & Tsubura, A. (2004). Conjugated docosahexaenoic acid suppresses KPL-1 human breast cancer cell growth *in vitro* and *in vivo*: potential mechanisms of action. *Breast Cancer Res* **6**, R291-299.

Tsuzuki, T., Kawakami, Y., Nakagawa, K. & Miyazawa, T. (2005a). Conjugated docosahexaenoic acid inhibits lipid accumulation in rats. *J Nutr Biochem* **17**, 518-524.

Tsuzuki, T., Tanaka, K., Kuwahara, S. & Miyazawa, T. (2005b). Synthesis of the conjugated trienes 5*E*,7*E*,9*E*,14*Z*,17*Z*-eicosapentaenoic acid and 5*Z*,7*E*,9*E*,14*Z*,17*Z*-eicosapentaenoic acid, and their induction of apoptosis in DLD-1 colorectal adenocarcinoma cells. *Lipids* **40**, 147-154.

Veerkamp, J. H. (1971). Fatty acid composition of *Bifidobacterium* and *Lactobacillus* strains. *J Bacteriol* **108**, 861-867.

Wahle, K. W., Heys, S. D. & Rotondo, D. (2004). Conjugated linoleic acids: are they beneficial or detrimental to health? *Prog Lipid Res* **43**, 553-587.

Wall, R., Ross, R. P., Shanahan, F., O'Mahony, L., O'Mahony, C., Coakley, M., Hart, O., Lawlor, P., Quigley, E. M., Kiely, B., Fitzgerald, G. F. & Stanton, C. (2009). The metabolic activity of the enteric microbiota influences the fatty acid composition of murine and porcine liver and adipose tissues. *Am J Clin Nutr* **89**.

Winkler, K. & Steinhart, H. (2001). Identification of conjugated isomers of linolenic acid and arachidonic acid in cheese. *Journal of Separation Science* **24**, 663 - 668.

Wise, M. L., Hamberg, M. & Gerwick, W. H. (1994). Biosynthesis of conjugated triene-containing fatty acids by a novel isomerase from the red marine alga *Ptilota filicina*. *Biochemistry* **33**, 15223-15232.

Yasui, Y., Hosokawa, M., Sahara, T., Suzuki, R., Ohgiya, S., Kohno, H., Tanaka, T. & Miyashita, K. (2005). Bitter gourd seed fatty acid rich in 9*c*,11*t*,13*t*-conjugated linolenic acid induces apoptosis and up-regulates the GADD45, p53 and PPARgamma in human colon cancer Caco-2 cells. *Prostaglandins Leukot Essent Fatty Acids* **73**, 113-119.

Yonezawa, Y., Hada, T., Uryu, K., Tsuzuki, T., Eitsuka, T., Miyazawa, T., Murakami-Nakai, C., Yoshida, H. & Mizushina, Y. (2005). Inhibitory effect of conjugated eicosapentaenoic acid on mammalian DNA polymerase and topoisomerase activities and human cancer cell proliferation. *Biochem Pharmacol* **70**, 453-460.

Young, D. C., Vouros, P. & Holick, M. F. (1990). Gas chromatography-mass spectrometry of conjugated dienes by derivatization with 4-methyl-1,2,4-triazoline-3,5-dione. *J Chromatogr* **522**, 295-302.

Yurawecz, M. P., Hood, J. K., Roach, J. A. G., Mossoba, M. M., Daniels, D. H., Ku, Y., Pariza, M. W. & Chin, S. F. (1994). Conversion of allylic hydroxy oleate to conjugated linoleic acid and methoxy oleate by acid catalyzed methylation procedures. *J Am Oil Chemists' Soc* **71**, 1149-1155.