Chapter 2

Optimization of a Reconstituted Skim Milk Based Medium for Enhanced CLA Production by Bifidobacteria

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Abstract

The aim of the current study was to determine the effect of a range of supplements on the bioconversion of linoleic acid to conjugated linoleic acid (CLA) by *Bifidobacterium breve* NCIMB 702258 in reconstituted skimmed milk (RSM). Seven supplements (yeast extract, casein hydrolysate, tryptone, L-cysteine hydrochloride, sodium acetate, sodium butyrate, and sodium propionate) were identified as substantially increasing the bioconversion of linoleic acid to the *c*9, *t*11 CLA isomer. Using these supplements the percentage bioconversion of linoleic acid (0.35 mg/ml) to the *c*9, *t*11 CLA isomer was elevated from 15.5 ± 1.1% in 20% (w/v) RSM to 48.1 ± 2.2% in the supplemented RSM. Through additional supplementation of this supplemented RSM with 20 mg/ml inulin and optimization of inoculum and linoleic acid concentration the percentage bioconversion to *c*9, *t*11 CLA was increased to 55.0 ± 3.2%. Thus, through supplementation the concentration of CLA produced by bifidobacteria in RSM can be increased to levels comparable to those observed in the synthetic medium cys-MRS. Overall the impact of 22 supplements on the production of the *c*9, *t*11 CLA isomer by the strain *B. breve* NCIMB 702258 in milk has been determined. The results provide a better understanding of the factors which influence CLA production by bifidobacteria in RSM.

2.1 Introduction

Conjugated linoleic acids (CLA) have been reported to exert health benefits, including prevention and regulation of a number of human medical conditions such as cancer, cardiovascular disease, diabetes, obesity, and bone disorders as well as antioxidative and growth promoting properties (Belury, 2002; Beppu *et al.*, 2006; Bhattacharya *et al.*, 2006; Roche *et al.*, 2001; Wahle *et al.*, 2004). These biogenic fatty acids exist naturally as a mixture of positional and geometric conjugated isomers of linoleic acid ($C_{18:2}$ *c*9, *c*12 octadecadienoic acid), of which the *cis*-9, *trans*-11 (*c*9, *t11*), *trans*-9, *trans*-11 (*t*9, *t11*), and *trans*-10, *cis*-12 (*t*10, *c*12) isomers have been the most extensively studied. The *c*9, *t*11 CLA isomer is most common form found in nature and is primarily associated with ruminant fats. Its presence in ruminant fat is a result of the microbial biohydrogenation of dietary linoleic and linolenic acids to stearic acid in the rumen via the action of the enzyme linoleic acid isomerase (Kepler *et al.*, 1966) or through the endogenous conversion of vaccenic acid to CLA via the action of the enzyme Δ^9-desaturase (Corl *et al.*, 2001; Griinari *et al.*, 2000). These processes lead to CLA concentrations of 0.2-3.7% in bovine milk fat (Sebedio *et al.*, 1997). However, despite the high concentration of CLA found in dairy products it is estimated that our current dietary intake is much lower than that needed for CLA to exert its health promoting activities (Ip *et al.*, 1994), and as a result strategies designed to increase dietary CLA intake have received increased attention.

In recent years, a number of dairy cultures and probiotic strains with the ability to convert linoleic acid to the *c*9, *t*11 and *t*9, *t*11 CLA isomers have been identified, including a number of bifidobacteria (Barrett *et al.*, 2007; Coakley *et al.*, 2003; Oh *et al.*, 2003; Rosberg-Cody *et al.*, 2004). Bifidobacterially produced CLA is primarily found in the form of the *c*9, *t*11 CLA isomer and to a lesser extent the

*t*9, *t*11 CLA isomer. The existence of such strains offer the potential for the development of novel CLA enriched dairy products (Lin, 2003; Xu *et al.*, 2005) or alternatively the establishment of probiotic cultures in the gastro-intestinal tract capable of producing CLA from dietary linoleic acid (Ewaschuk *et al.*, 2006).

The synthetic medium cys-MRS is most commonly used during assays designed to determine the ability of bifidobacteria to produce CLA, resulting in both an efficient and high level of bioconversion. However, given the complexity and expense of this medium, its use in large scale CLA production is not a viable option (Ventling & Mistry, 1993). A potentially more useful medium for the commercial production of CLA from linoleic acid by bifidobacteria may be milk. As a medium, milk is both abundant and cost effective, additionally offering a potential vehicle for the delivery of the microorganism and the resulting CLA to the human gastro-intestinal tract (Poch & Bezkorovainy, 1988). Propagation of bifidobacteria in milk is quite difficult due to the absence or inaccessibility of essential nutrients and growth factors (Abu-Taraboush *et al.*, 1998; Gomes *et al.*, 1998; Rasic & Kurmann, 1983; Ventling & Mistry, 1993). The relatively poor growth of bifidobacteria in milk would prove a substantial problem in CLA production, where the efficiency and extent to which linoleic acid is converted to CLA is related to the cell numbers of the converting strain (Kim *et al.*, 2000). Intensive research has been directed towards the identification of compounds capable of enhancing the growth of bifidobacteria. These studies have shown the bifidogenic properties of compounds such as prebiotics, casein hydrolysates, yeast extracts, malt extracts, reducing agents such as L-cysteine hydrochloride and short chain fatty acids (SCFA). In this study, the effect of a range of supplements on the growth and CLA production by the strain *B. breve* NCIMB 702258 in 20% (w/v) RSM was assayed. Once a range of supplements which elevate CLA production in

RSM were identified, the optimum concentration of each was determined, and the supplements blended, to produce a fermented milk where the bioconversion of linoleic acid to CLA by bifidobacteria was comparable to that found in synthetic media.

2.2 Materials and methods

2.2.1 Maintenance of bacterial strains

The bacterial strains used in this study were *B. breve* NCIMB 702258, *B. breve* NCIMB 8807, *B. breve* DPC6330, *B. breve* DPC6331, *B. longum* DPC6315 and *B. longum* DPC6320. All strains were activated in cys-MRS (MRS (Difco, Detroit, MI, USA,) containing 0.5 mg/L L-cysteine hydrochloride (cys-HCl) (98% pure, Sigma Aldrich, Poole, Dorset, U.K.)) under anaerobic conditions (anaerobic jars with Anaerocult A gas packs, Merck Darmstedt, Germany) at 37^{o}C for 48 h. Where solid media was required 1.5% (w/v) bacteriological agar (Oxoid, Hampshire, U.K.) was added to the cys-MRS media. Where it was necessary to pre-select for bifidobacteria 100 μg/ml of mupirocin was added to the media using antimicrobial susceptibility discs, as previously described (Rada, 1997). Cell counts were determined by plating serial dilutions in maximum recovery diluent (Oxoid) and pour plating with cys-MRS agar followed by anaerobic incubation at 37^{o}C.

2.2.2 Assessment of CLA production in 20% (w/v) RSM

All supplements were of the highest purity. Lactulose, yeast extract, casein hydrolysate (acid hydrolysate of casein), tryptone (enzymatic hydrolysate of casein), cys-HCl, sucrose, fructose, glucose, maltose, sodium propionate, sodium acetate, sodium butyrate, sodium propionate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol were purchased from Sigma Aldrich, Raftiline GR, Raftiline HP, Raftilose P95 and Raftilose Synergy 1 were a gift from the Orafti group, polydextrose was purchased from Danisco (Copenhagen, DEN), while transgalactooligosaccharide was a gift from Yakult Ltd (Tokyo, JPN). Lactulose, yeast extract, casein hydrolysate, tryptone, Raftiline GR, Raftiline HP,

Raftilose P95, Raftilose Synergy 1, polydextrose, transgalactooligosaccharide and cys-HCl supplemented RSM was individually prepared by the direct addition of the supplements to 20% (w/v) RSM. The supplemented RSM was then autoclaved at 90°C for 30 min. Sucrose, fructose, glucose, maltose, sodium acetate, sodium butyrate, sodium propionate, BHA, BHT and α-tocopherol supplemented RSM was prepared using sterile stock solutions. These stock solutions were filter sterilized by application to a 0.45 μm ministart filter (Sartorius AG, Goettingen, Germany) and added to 20% (w/v) RSM directly post autoclaving. Linoleic acid was delivered in the form of a 30 mg/ml stock solution containing 2% (w/v) Tween 80 (polyoxyethylene sorbitan mono-oleate, Merck-Schuchardt, Hohenbrunn, Germany) filter sterilized through a 0.45 μm Ministart filter and stored at –20°C, under nitrogen.

All strains were subcultured twice in cys-MRS and subsequently inoculated 2% (v/v) (O.D. 600nm = 0.2) in the supplemented 20% (w/v) RSM followed by anaerobic incubation at 37°C for 43 h. All experiments were performed in triplicate and the Student's *t*-test was used to determine differences between treatments.

2.2.3 Fatty acid analysis

Fatty acids were extracted from the samples following addition of 0.75 mg of the internal standard tridecanoic acid (Sigma, 99% pure) to four grams of the sample. Two milliliters of isopropanol (99% purity, Labscan, Dublin, Ireland) were then added to the sample followed by vortexing for one minute to ensure homogenization of the sample. Four milliliters of hexane were added to the mixture followed by vortexing for two min. Following this time samples were centrifuged at 2197 g for 5-6 min and the resulting clear upper layer removed to a clean glass tube. These tubes were then heated at 45°C under a steady flow of nitrogen to evaporate

the remaining hexane. The remaining lipids in the glass tube were sealed under nitrogen and stored at –20°C for gas liquid chromatographic (GLC) analysis. Extracted fatty acids were converted to fatty acid methyl esters (FAMEs) by acid catalyzed methylation (4% methanolic HCl (Supleco, Bellefonte, PA, USA) at 60°C for 20 min) as described by Stanton *et al.* (1997). The FAME were analyzed on a CP-SELECT CB column for FAME (100 m x 0.25 mm x 0.25 μm film thickness, Varian BV, Herculesweg 8, 4338 PL, Middelburgh, The Netherlands) using a Varian 3400 GLC (Varian, Walnut Creek, CA, USA) which was fitted with a flame ionization detector (FID) as previously described by Coakley *et al.* (2003). The percentage bioconversion of linoleic acid to each CLA isomer was calculated by dividing the amount of CLA present after the fermentation by the level of linoleic acid present in the sample prior to fermentation.

2.2.4 Acidity measurement

The pH of fermented milks was recorded using a digital pH recorder (Mettler Toledo,152 MP220 pH Meter).

2.3 Results

In this study, we assessed the ability of a number of *B. breve* and *B. longum* strains to bioconvert free linoleic acid (0.35 mg/ml) to the predominant *c*9, *t*11 CLA isomer in both cys-MRS and 20% (w/v) RSM. The results show that the ability of these bifidobacteria to produce the *c*9, *t*11 CLA isomer is greatly reduced when grown in a milk based medium compared with the synthetic medium, cys-MRS **(Figure 2.1)**. One such strain is *B. breve* NCIMB 702258, whose capacity to convert linoleic acid to the *c*9, *t*11 CLA isomer is markedly lower when grown in 20% (w/v) RSM (15.52 ± 1.11%) than when grown in cys-MRS (58.8 ± 2.65%) **(Figure 2.1)**. In an attempt to improve the bioconversion of linoleic acid to the *c*9, *t*11 CLA isomer by *B. breve* NCIMB 702258 in 20% (w/v) RSM, the medium was supplemented with a range of additives (n = 3). These supplements range from those used to improve the growth of bifidobacteria, to those used to improve CLA production by bacteria and include compounds of industrial significance. The impact of these additives on *c*9, *t*11 CLA production by the strain varied substantially. Compounds such as sugars (sucrose, maltose, lactose, glucose, and fructose) and antioxidants (BHA, BHT, and α-tocopherol) led to reduced *c*9, *t*11 CLA production in comparison to unsupplemented 20% (w/v) RSM **(Table 2.1)**. The impact of prebiotic compounds on *c*9, *t*11 CLA production by the strain was found to vary substantially. In the study, the prebiotics polydextrose and transgalactooligosaccharide were associated with small but significant reductions in *c*9, *t*11 production ($P \leq 0.05$) by the strain while others such as lactulose, fructooligosaccharide, and inulin were associated with small increases in *c*9, *t*11 CLA production in the range of 3-5%, these however did not reach significance **(Table 2.1)**. Supplementation with the sodium salts of the SCFA, propionate,

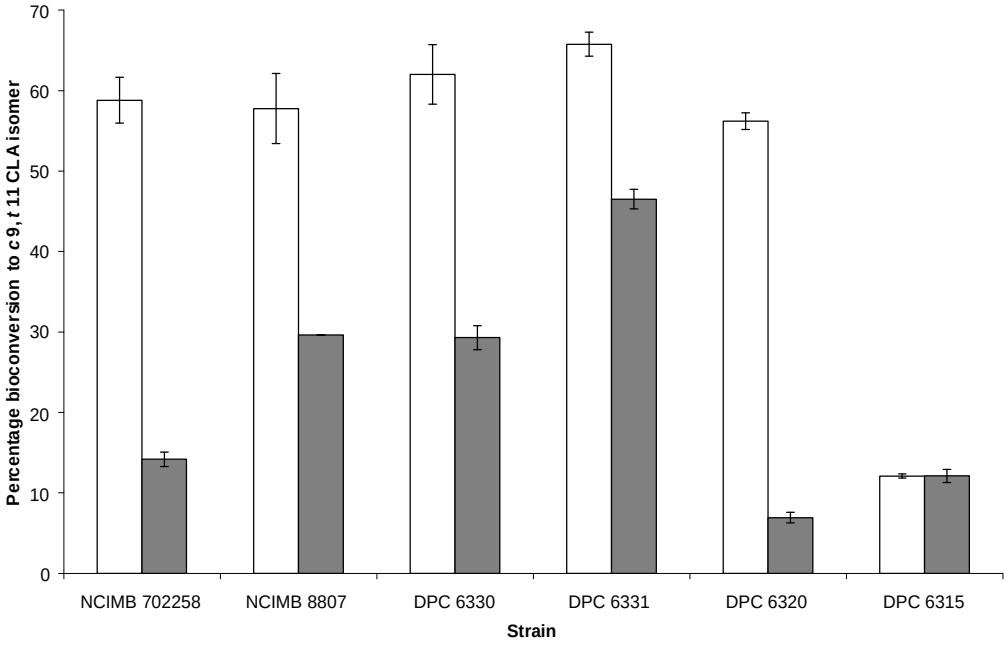

Figure 2.1 Comparison of the production of the *c*9, *t*11 CLA isomer by strains of *B. breve* (NCIMB 702258, NCIMB 8807, DPC6330, DPC6331) and *B. longum* (DPC6320, DPC6315) in cys-MRS (□) and 20% (w/v) RSM (■) in the presence of 0.35 mg/ml linoleic acid anaerobically at 37^{o}C

acetate and butyrate led to large and significant increases in the bioconversion of linoleic acid to *c*9, *t*11 CLA in comparison to the control ($P \leq 0.001$). Of the three SCFA assayed, sodium acetate supplementation (100 mM) was the most effective, increasing *c*9, *t*11 CLA production by as much as 209.02%. Supplementation with sodium propionate (100 mM) and sodium butyrate (100 mM) led to 202.32% and 127.06% increases in *c*9, *t*11 CLA production, respectively.

Supplementation with cys-HCl (250 mg/L) resulted in a significant increase (89.69%) in the bioconversion of linoleic acid to the *c*9, *t*11 CLA isomer by *B. breve* NCIMB 702258 ($P = 0.0043$) **(Table 2.1)**. The complex nutritional supplements investigated in this study included yeast extract, tryptone and casein hydrolysate. The addition of yeast extract to 20% (w/v) RSM at a concentration of 20 mg/ml resulted in a 187.11% increase in the production of the *c*9, *t*11 CLA isomer after 43 h fermentation ($P = 0.0001$) **(Table 2.1)**. Tryptone supplementation at a concentration of 20 mg/ml led to a 123.91% increase in the bioconversion of linoleic acid to the *c*9, *t*11 CLA isomer ($P = 0.0001$), while supplementation with 20 mg/ml casein hydrolysate led to a 173.20% increase in bioconversion ($P = 0.006$) compared to the control.

As a result of this study, seven supplements which substantially enhanced the bioconversion of 0.35 mg/ml free linoleic acid to the *c*9, *t*11 CLA isomer were identified **(Table 2.1)**. These additives were 100 mM sodium propionate (46.92 ± 1.32%), 100 mM sodium acetate (47.96 ± 5.46%), 100 mM sodium butyrate (35.24 ± 1.32%), 250 mg/L L-cysteine hydrochloride (29.44 ± 3.46%), 20 mg/ml yeast extract (44.56 ± 0.43%), 20 mg//ml casein hydrolysate (42.40 ± 1.27%), and 20 mg/ml tryptone (34.75 ± 2.10%).

Table 2.1 Effect of supplementation on the percentage bioconversion of linoleic acid to the *c*9, *t*11 CLA isomer by *B. breve* NCIMB 702258 in 20% (w/v) RSM containing 0.35-0.4 mg/ml linoleic acid following 43 h anaerobic incubation at 37^{o}C.

$^{+}$Also 0.025% Ascorbic acid 6-palmitate.

RSM	Supplement	Percentage bioconversion to *c*9, *t*11 CLA isomer (Std dev)	Cell count log cfu/ml (Std dev)		pH (Std dev)	
			T_0(h)	T_{43}(h)	T_0 (h)	T_{43} (h)
20%	No supplementation	15.52 ± 1.11	7.86 ± 0.01	8.84 ± 0.01	6.24 ± 0	5.11 ± 0
20%	Sucrose 60 g//L	12.67 ± 4.23	7.76 ± 0.04	9.02 ± 0.1	6.23 ± 0.01	5.02 ± 0.12
20%	Maltose 60 g/L	13.73 ± 0.34	7.76 ± 0.04	9.16 ± 0.05	6.16 ± 0.01	4.95 ± 0.11
20%	Lactose 60 g/L	6.27 ± 1.69[a]	7.76 ± 0.04	8.73 ± 0.19	6.21 ± 0.01	5.04 ± 0.05
20%	Glucose 60 g/L	10.00 ± 2.75	7.76 ± 0.04	8.93 ± 0.03	6.18 ± 0.06	5.31 ± 0.13
20%	Fructose 60 g/L	13.83 ± 0.56	7.76 ± 0.04	8.98 ± 0.05	6.28 ± 0.11	4.9 ± 0.05
20%	10 mg/ml Polydextrose	7.43 ± 5.19	7.93 ± 0.05	8.97 ± 0.04	6.31 ± 0.02	5.13 ± 0.11
20%	20 mg/ml Lactulose	18.54 ± 3.20	8.33 ± 0.08	9.04 ± 0.08	6.23 ± 0.01	4.99 ± 0.1
20%	20 mg/ml Transgalactooligosaccharides	9.55 ± 1.49	7.06 ± 0.00	ND ± ND	6.23 ± 0.05	ND ± ND
20%	20 mg/ml (Raftiline HP)	20.65 ± 1.47	7.48 ± 0.13	8.98 ± 0.03	6.27 ± 0.03	5.08 ± 0.05
20%	20 mg/ml (Raftilose P95)	18.08 ± 0.16	7.48 ± 0.14	8.95 ± 0.03	6.38 ± 0.08	5.03 ± 0.07
20%	20 mg/ml (Raftiline GR)	20.66 ± 1.81	7.48 ± 0.15	9.03 ± 0.05	6.28 ± 0.06	5.16 ± 0.03
20%	20 mg/ml (Raftilose synergy 1)	19.88 ± 5.49	7.48 ± 0.16	8.84 ± 0.07	6.26 ± 0.06	5.27 ± 0.01
20%	Sodium propionate (100 mM)	46.92 ± 1.32*	6.48 ± 0.03	8.98 ± 0.02	6.3 ± 0	5.5 ± 0.02
20%	Sodium acetate (100 mM)	47.96 ± 3.15*	6.48 ± 0.03	8.87 ± 0.01	6.3 ± 0.01	5.4 ± 0.03
20%	Sodium butyrate (100 mM)	35.24 ± 1.32*	6.48 ± 0.03	8.7 ± 0.07	6.3 ± 0	5.5 ± 0.02
20%	L-cysteine hydrochloride(250 mg/L)	29.44 ± 3.46*	7.93 ± 0.05	9.37 ± 0.03	6.23 ± 0.1	4.89 ± 0.06
20%	20 mg/ml yeast extract	44.56 ± 0.43*	7.93 ± 0.05	9.8 ± 0.01	6.26 ± 0.01	4.88 ± 0.01
20%	20 mg/ml Casein hydrolysate	42.40 ± 1.27*	6.87 ± 0.03	6.54 ± 0.08	6.38 ± 0.11	4.66 ± 0.04
20%	20 mg/ml Tryptone	34.75 ± 2.10*	6.87 ± 0.03	7.62 ± 0.04	6.28 ± 0.09	4.83 ± 0.1
20%	0.025% α-tocopherol $^{+}$	8.76 ± 0.60[a]	ND ± ND	ND ± ND	ND ± ND	ND ± ND
20%	0.005% BHT	13.03 ± 0.92	ND ± ND	ND ± ND	ND ± ND	ND ± ND
20%	0.005% BHA	13.87 ± 0.49	ND ± ND	ND ± ND	ND ± ND	ND ± ND

* = $P \leq 0.001$; significantly greater than control, [a] = $P \leq 0.05$ significantly lower than control) (N.D. = Not determined)

2.3.1 Optimisation of CLA production by bifidobacteria in RSM

2.3.1.1 Assessing the effect of supplement concentration on CLA production

From the seven supplements identified only three, sodium acetate, cys-HCl and yeast extract, were selected for further study. Of the sodium salts of the SCFA, sodium acetate displayed a significantly larger increase in *c*9, *t*11 CLA production than sodium butyrate ($P = 0.017$). In addition, during a subsequent study sodium acetate was found to result in significantly larger increase in *c*9, *t*11 CLA production than sodium propionate at concentrations greater than 100 mM ($P = 0.01$). Of the complex nutritional supplements, yeast extract displayed a significantly greater increase in *c*9, *t*11 CLA production than either casein hydrolysate ($P = 0.049$) or tryptone ($P = 0.001$). Cys-HCl was selected for further study due to its unique nature relative to the other supplements which increased *c*9, *t*11 CLA production.

The effect of sodium acetate supplementation was assayed at concentrations ranging from 50 mM to 400 mM in 20% (w/v) RSM containing 0.4 mg/ml linoleic acid (n = 3) **(Table 2.2)**. Incrementally increasing the concentration of sodium acetate from 50 mM to 200 mM resulted in a parallel increase in the production of the *c*9, *t*11 isomer **(Table 2.2)**. The highest bioconversion (47.53 ± 5.07%) was observed at a sodium acetate concentration of 200 mM. At concentrations above this (300 mM and 400 mM) a significant decrease in *c*9, *t*11 CLA production was observed ($P = 0.000$). At sodium acetate concentration of 300 mM and 400 mM a substantial decline in cell numbers and a high final pH (5.53) were observed following 43 h, suggesting poor growth by the strain **(Table 2.2)**. At all concentrations, production of the *t*9, *t*11 CLA isomer was minimal. As supplementation with 200 mM sodium acetate resulted in the production of

significantly higher concentration of the *c*9, *t*11 CLA isomer than the other concentrations assayed this concentration was selected for further study ($P \leq 0.02$).

The effect of cys-HCl supplementation was assayed at concentrations ranging from 100 mg/L to 500 mg/L in 20% (w/v) RSM containing 0.35 mg/ml linoleic acid (n = 3) **(Table 2.2)**. Increasing the concentration of cys-HCl did not significantly increase the production of the *c*9, *t*11 or *t*9, *t*11 CLA isomers ($P \geq 0.2$). Final pH and cell counts also suggest growth of the strain was also relatively unaffected by increasing the concentration of cys-HCl **(Table 2.2)**. Given this result, 100 mg/L was determined to be the optimum cys-HCl concentration and selected for further study.

The effect of yeast extract supplementation was assayed at concentrations ranging from 2.5 mg/ml to 30 mg/ml in 20% (w/v) RSM containing 0.35 mg/ml linoleic acid (n = 3) **(Table 2.2)**. Gradually increasing the concentration of yeast extract resulted in a parallel increase in the production of both the *c*9, *t*11 and *t*9, *t*11 isomers up to a concentration of 10 mg/ml **(Table 2.2)**. At concentrations of between 10 mg/ml and 30 mg/ml increases in the production of both isomers were observed, however, the concentration of *c*9, *t*11 CLA produced at a yeast extract concentration of 30 mg/ml was not significantly greater than that at 10 mg/ml ($P = 0.36$). This resulted in the selection of 10 mg/ml yeast extract for further study. Based on these results concentrations of 200 mM sodium acetate, 100 mg/L cys-HCl and 10 mg/ml yeast extract were identified as being the optimum supplement concentrations of each and selected for further study.

2.3.1.2 A comparison of the production of *c*9, *t*11 CLA isomer in the presence of sodium acetate and yeast extract

Table 2.2 The effect of supplement concentration on the growth of *B. breve* NCIMB 702258 and the bioconversion of linoleic acid to both the *c*9, *t*11 and the *t*9, *t*11 CLA isomers

	Percentage bioconversion to *c*9, *t*11 CLA isomer ± Std dev	Percentage bioconversion to *t*9, *t*11 CLA isomer ± Std dev	Percentage linoleic acid remaining ± Std dev	Cell count log cfu/ml ± Std dev T_0 (h)	Cell count log cfu/ml ± Std dev T_{43} (h)
Yeast extract					
2.5 mg/ml	29.45 ± 4.46	1.50 ± 0.18	5.15 ± 2.00	7.44 ± 0.07	5.79 ± 0.03
5 mg/ml	33.40 ± 1.05	2.82 ± 0.16	3.08 ± 0.64	7.44 ± 0.07	5.63 ± 0.10
10 mg/ml	35.32 ± 0.68	3.16 ± 0.17	5.08 ± 0.99	7.44 ± 0.07	5.55 ± 0.02
20 mg/ml	36.00 ± 6.00	3.15 ± 0.59	6.15 ± 2.67	7.44 ± 0.07	5.56 ± 0.05
30 mg/ml	37.76 ± 3.99	3.04 ± 0.50	6.19 ± 1.09	7.44 ± 0.07	5.51 ± 0.05
Sodium acetate					
50 mM	30.41 ± 1.32	0.75 ± 0.07	66.12 ± 1.79	6.83 ± 0.01	8.93 ± 0.03
100 mM	39.06 ± 1.45	0.81 ± 0.02	56.96 ± 1.12	6.83 ± 0.01	8.95 ± 0.01
200 mM	47.53 ± 5.07	0.93 ± 0.07	39.90 ± 4.65	6.83 ± 0.01	8.88 ± 0.03
300 mM	2.10 ± 0.80	0.52 ± 0.07	93.69 ± 6.26	6.83 ± 0.01	6.76 ± 0.03
400 mM	0.71 ± 0.35	0.41 ± 0.03	93.72 ± 5.03	6.83 ± 0.01	2.06 ± 0.01
Cys-HCl					
100 mg/L	27.93 ± 1.99	0.76 ± 0.10	48.76 ± 1.31	7.90 ± 0.05	9.28 ± 0.16
200 mg/L	27.92 ± 2.39	0.68 ± 0.04	29.86 ± 5.28	7.90 ± 0.05	9.33 ± 0.06
250 mg/L	26.58 ± 5.32	0.64 ± 0.15	24.07 ± 7.56	7.90 ± 0.05	9.37 ± 0.03
300 mg/L	27.68 ± 3.36	0.72 ± 0.10	20.29 ± 1.93	7.90 ± 0.05	9.38 ± 0.02
500 mg/L	31.48 ± 11.93	0.62 ± 0.17	63.24 ± 10.82	7.90 ± 0.05	9.46 ± 0.01

The growth and *c*9, *t*11 CLA production by *B. breve* NCIMB 702258 in 20% (w/v) RSM supplemented with 10 mg/ml yeast extract or 200 mM sodium acetate was compared with that of unsupplemented 20% (w/v) RSM over 40 h anaerobic incubation at 37^{o}C in the presence of 0.35 mg/ml linoleic acid (n = 3) **(Figure 2.2)**. The aim of this study was to demonstrate that while yeast extract affected an increase in *c*9, *t*11 CLA production via its well characterised bifidogenic properties, sodium acetate did not. The results showed that yeast extract did indeed increase both the growth rate and maximum cell numbers of the strain compared to the control **(Figure 2.2)**. This increased growth corresponded to an increased rate of *c*9, *t*11 CLA production, with over 13% of the available linoleic acid converted to the isomer after 8 h compared to approximately 7% in the control. After approximately 24 h incubation *c*9, *t*11 CLA production had reached its maximum (31.5% conversion), following this time there was a rapid decline in cell numbers corresponding to the pH falling below 4.3. These results suggest that it is the increased cell numbers which increase the isomerising capacity of the strain and hence the concentration of linoleic acid converted to the *c*9, *t*11 CLA isomer. The limiting factor would appear to be the rapid fall in pH to a point beyond the tolerance of the strain.

In contrast to yeast extract, sodium acetate supplementation displayed an inhibitory impact on the growth of the strain with cell numbers over one log lower than that of the unsupplemented medium after 8 h **(Figure 2.2)**. This inhibitory trend continued through 16 h, however, despite this *c*9, *t*11 CLA production in the sodium acetate supplemented RSM was significantly greater than that achieved in the unsupplemented RSM after 16 h, with 25% and 7% of the available linoleic acid converted to the *c*9, *t*11 CLA isomer, respectively. This result suggests that sodium

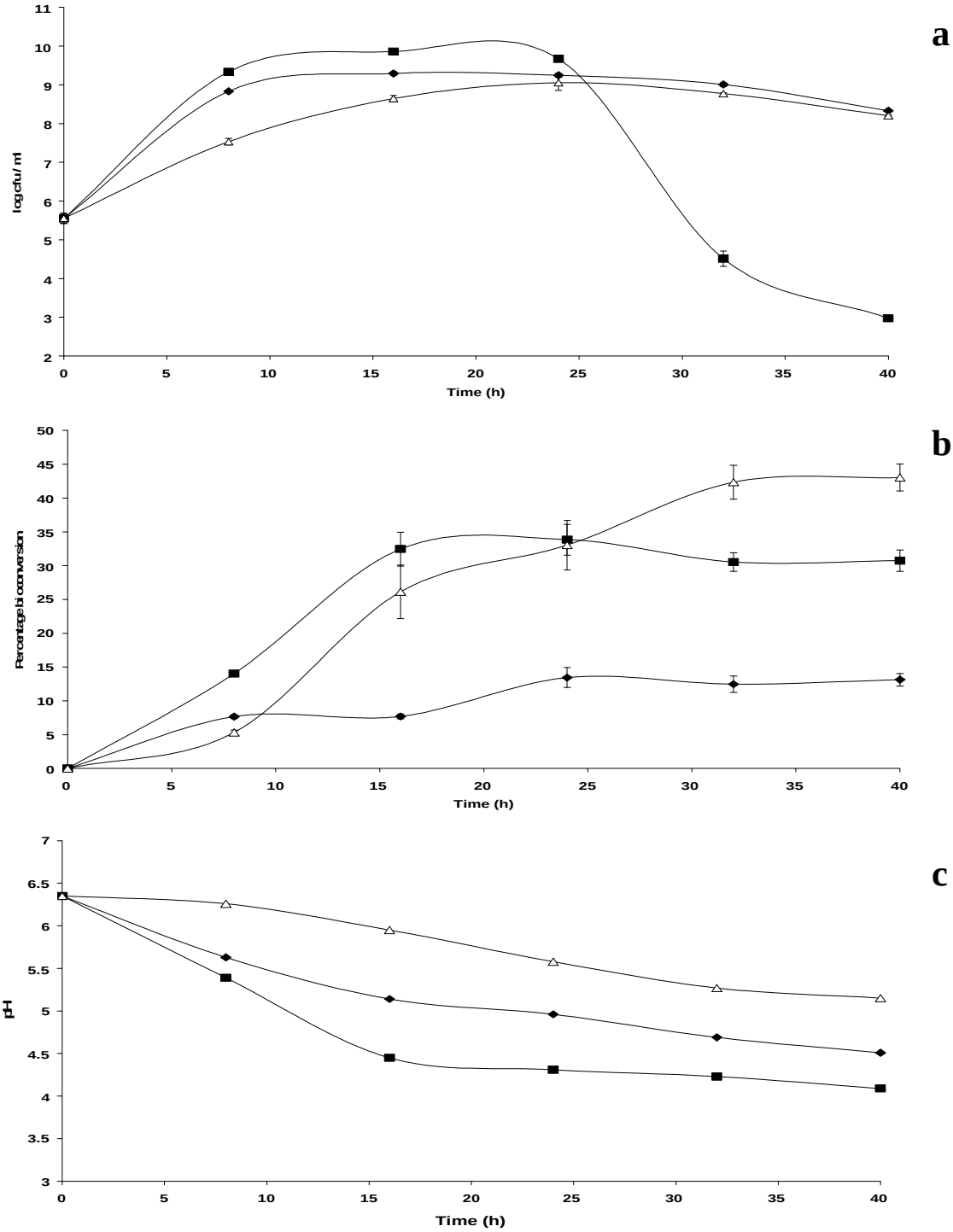

Figure 2.2 Comparison of the **a)** growth, **b)** CLA production, and **c)** pH of *B. breve* NCIMB 702258 in unsupplemented 20% (w/v) RSM (♦), 20% (w/v) RSM supplemented with 10 mg/ml yeast extract (■), or 20% (w/v) RSM supplemented with 200mM sodium acetate (Δ), in the presence of 0.35 mg/ml linoleic acid during anaerobic incubation at 37°C for 40 h.

acetate increases *c*9, *t*11 CLA production by the strain independently of growth and to levels comparable to that achieved with a growth stimulating supplement such as yeast extract. A potential reason for this increased *c*9, *t*11 CLA production may be related to the capacity of sodium acetate to act as a buffer, maintaining the pH nearer the optimum for the activity of the enzyme linoleic acid isomerase. This buffering capacity also appears to permit the production of *c*9, *t*11 CLA for a longer period than yeast extract where after 24 h the low pH had initiated the onset of late stationary phase halting the conversion of linoleic acid to *c*9, *t*11 CLA at 33%. This was in comparison to sodium acetate where the strain remains in the early phases of stationary growth with *c*9, *t*11 CLA production up to and including 40 h where over 44% of the available linoleic acid had been converted to the *c*9, *t*11 isomer.

2.3.1.3 Combining the optimum supplement concentrations

Following the identification of the optimum concentrations of the selected supplements (200 mM sodium acetate, 100 mg/L cys-HCl and 10 mg/ml yeast extract), the effect of varying combinations of each supplement on the bioconversion of linoleic acid to CLA in 20% (w/v) RSM was assayed (n = 3). Of these, it was the combination of sodium acetate and yeast extract which resulted in the significantly higher production of CLA with 48.08 ± 2.21% and 3.30 ± 0.13% of the linoleic acid bioconverted to the *c*9, *t*11 and *t*9, *t*11 CLA isomers, respectively **(Figure 2.3)**. To overcome nutrient availability becoming a limiting factor, the sodium acetate and yeast extract supplemented RSM was additionally supplemented with either 30 mg/ml casein hydrolysate or 20 mg/ml Raftiline GR (Inulin). During initial screening, both supplements had enhanced *c*9, *t*11 CLA production and it was

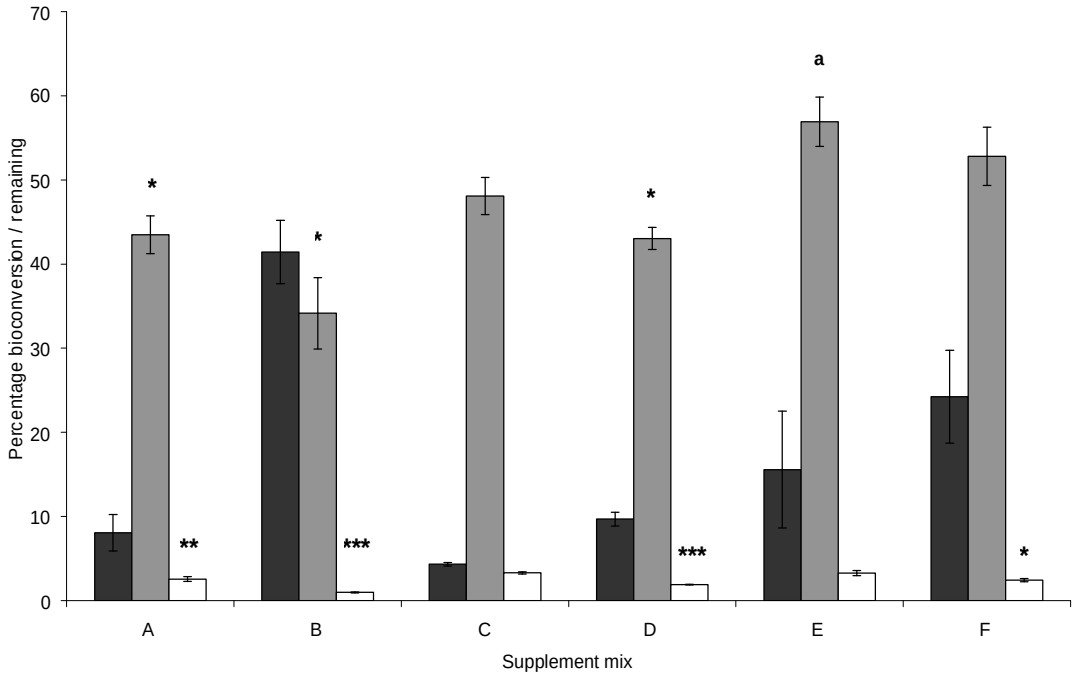

Figure 2.3 Supplementation with varying combinations of ingredients found to stimulate CLA production by *B. breve* NCIMB 702258. **A)** 200mM sodium acetate, 100 mg/L cys-HCl, & 10 mg/ml yeast extract, **B)** 200mM sodium acetate & 100 mg/L cys-HCl, **C)** 200mM sodium acetate & 10 mg/ml yeast extract, **D)** 100 mg/L cys-HCl & 10 mg/ml yeast extract, **E)** 200mM sodium acetate, 10 mg/ml yeast extract, 100 mg/L cys-HCl, & 20 mg/ml Raftiline GR. **F)** 200 mM sodium acetate, 10 mg/ml yeast extract, 100 mg/L cys-HCl, & 30 mg/ml casein hydrolysate. Percentage linoleic acid remaining (■), percentage bioconversion to the *c*9, *t*11 CLA isomer (■), and percentage bioconversion to *t*9, *t*11 CLA isomer (□). Incubated anaerobically at 37^{o}C in the presence of 0.35 mg/ml linoleic acid. (* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$ significantly lower than **C**). (a = $P \leq 0.05$ significantly greater than **C**)

anticipated their use would benefit both CLA production and bifidobacterial growth **(Figure 2.3)**.

The addition of 30 mg/ml casein hydrolysate resulted in a 4.71% increase in the production of the *c*9, *t*11 CLA isomer, however, this increase did not reach significance when compared to the RSM supplemented with sodium acetate, and yeast extract alone ($P = 0.12$). The culturing of *B. breve* NCIMB 702258 in 20% (w/v) RSM containing 20 mg/ml Raftiline GR, 200 mM sodium acetate, and 10 mg/ml yeast extract resulted in a significant increase (6.67%) in bioconversion to the *c*9, *t*11 CLA isomer, while the concentration of *t*9, *t*11 CLA remained unaffected **(Figure 2.3)**. Based on these results and the previously reported benefits of supplementation with a prebiotic, it was decided to additionally supplement 20% (w/v) RSM containing 200 mM sodium acetate, and 10 mg/ml yeast extract, with 20 mg/ml Raftiline GR (Gibson *et al.*, 2005; Veereman-Wauters, 2005; Weaver, 2005). The resulting medium was termed modified reconstituted skimmed milk (mRSM).

2.3.1.4 Inoculum and linoleic acid concentration and CLA production

In our study we aimed to determine the effect, if any, that inoculum concentration had on the bioconversion of linoleic acid to CLA in the mRSM. The medium was inoculated to give a total bifidobacterial counts of 5.48 ± 0.01 log cfu/ml, 6.48 ± 0.01 log cfu/ml or 7.07 ± 0.005 log cfu/ml and incubated at 37^{o}C anaerobically for 64 h with regular monitoring of CLA production and cell numbers (n = 3). Across the three inoculum concentrations, only marginal differences in the percentage bioconversion of linoleic acid to the *c*9, *t*11 CLA isomer were observed, ranging from 50.80 ± 3.71% to 55.00 ± 3.17% **(Figure 2.4)**. At all inoculum concentrations, the highest rate of *c*9, *t*11 CLA production paralleled the logarithmic phase of

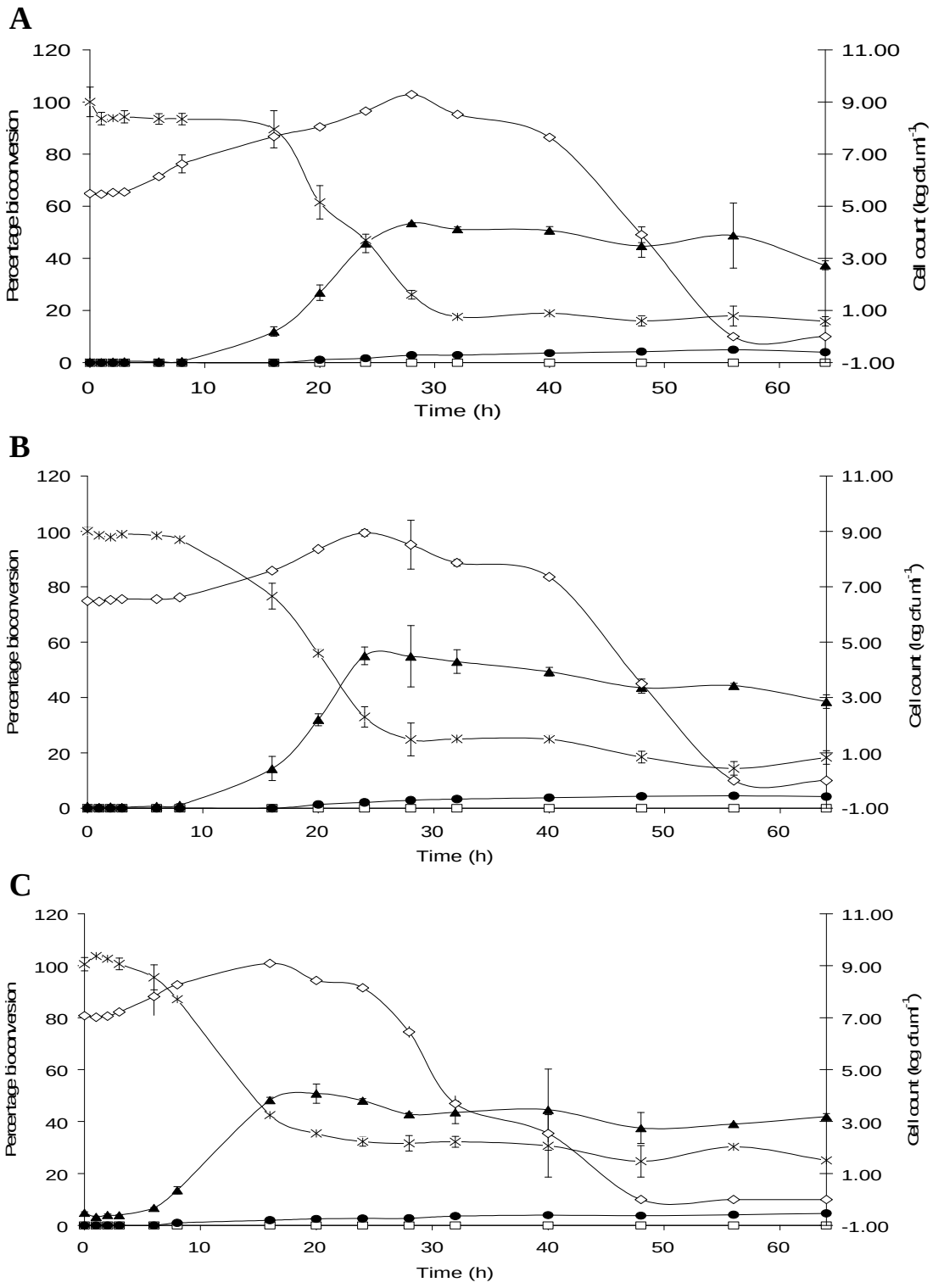

Figure 2.4 Growth and CLA production by *B. breve* NCIMB 702258 inoculated at approx. **A.** 5.5 log cfu/ml, **B**. 6.5 log cfu/ml or **C.** 7.1 log cfu/ml under anaerobic incubation at 37^{o}C in the presence of 0.37 mg/ml free linoleic acid. Percentage linoleic acid remaining (∗), percentage bioconversion to *c*9, *t*11 CLA (▲), percentage bioconversion to *t*9, *t*11 CLA (●), percentage bioconversion to *t*10, *c*12 CLA (□), and cell numbers in log cfu/ml (◇).

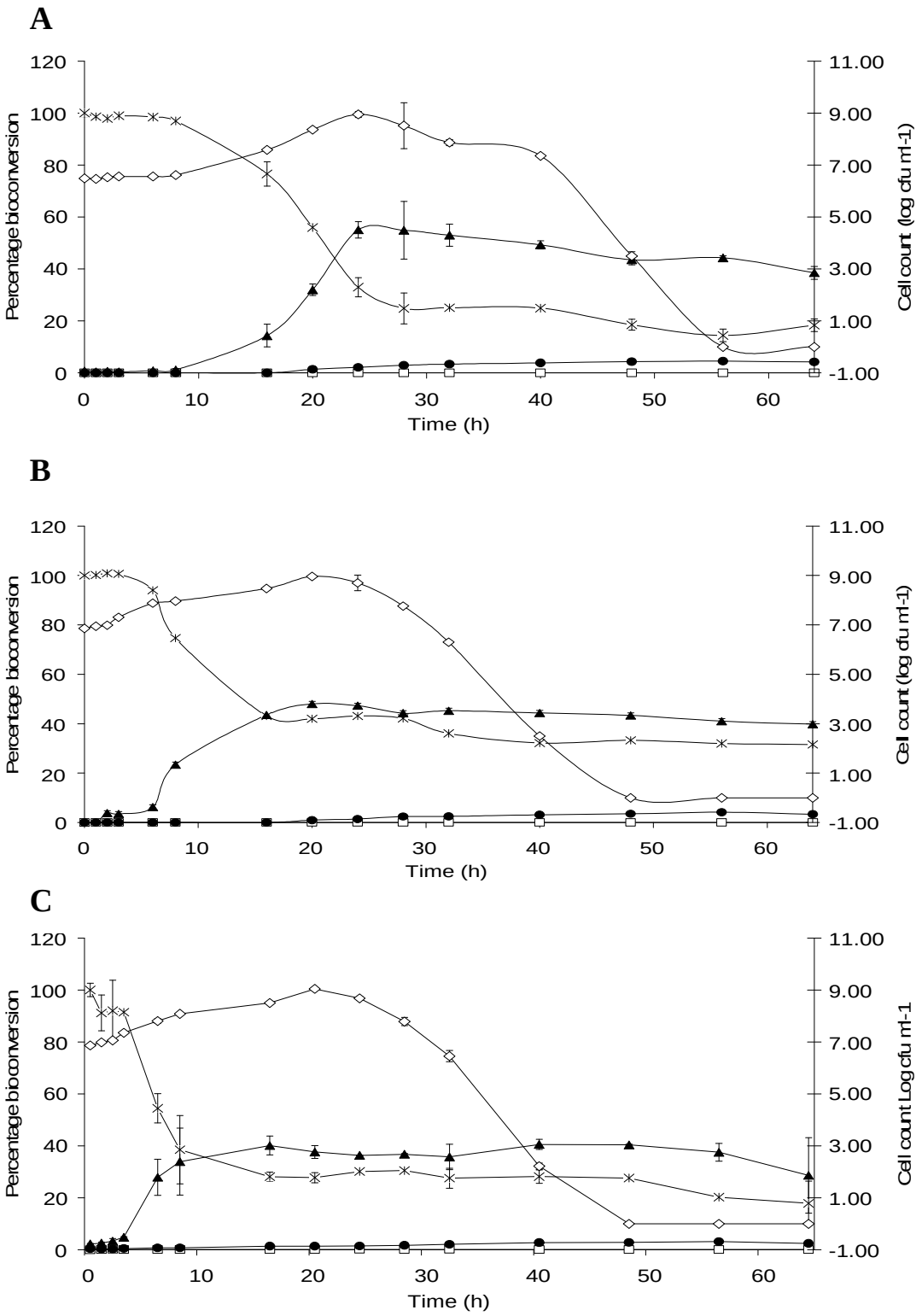

Figure 2.5 Growth and CLA production by *B. breve* NCIMB 702258 containing approx. **A.** 0.35 mg/ml, **B**. 0.45 mg/ml or **C.** 0.55 mg/ml linoleic acid under anaerobic incubation at 37^{o}C at an inoculum concentration of 5.5 log cfu/ml. Percentage linoleic acid remaining (∗), percentage bioconversion to *c*9, *t*11 CLA (▲), percentage bioconversion to *t*9, *t*11 CLA (●), percentage bioconversion to *t*10, *c*12 CLA (□), and cell numbers in log cfu/ml (◇).

cellular growth **(Figure 2.4)**. However, following this period a gradual decrease in the concentration of the *c*9, *t*11 CLA isomer and gradual increase in the *t*9, *t*11 CLA isomer were observed. This may suggest the *t*9, *t*11 CLA isomer is a product of the metabolism of the *c*9, *t*11 CLA isomer by *B. breve* NCIMB 702258.

In our study, we assayed the effect of incrementally increasing the linoleic acid substrate concentration on the growth and CLA production by the strain *B. breve* NCIMB 702258 in our mRSM. The strain was grown in mRSM in the presence of one of three linoleic acid concentrations (0.35 mg/ml, 0.45 mg/ml or 0.55 mg/ml) over 64 h with regular monitoring of cell growth and CLA production (n = 3) **(Figure 2.5)**. The results showed that increasing the linoleic acid concentration did not reduce the concentration of *c*9, *t*11 CLA produced, which ranged from 0.19 ± 0.01 mg/ml to 0.22 ± 0.01 mg/ml. However, the efficiency at which linoleic acid was converted to the *c*9, *t*11 CLA isomer was reduced with increased linoleic acid concentration, as reflected by higher residual linoleic acid concentrations and reduced percentage bioconversions **(Figure 2.5)**. Increasing the concentration of linoleic acid supplied to the strain did not significantly impact on the quantity of the *t*9, *t*11 CLA isomer produced **(Figure 2.5)**.

2.3.2 Assessment of other CLA producing bifidobacteria in mRSM

In this study we have shown how, through supplementation, the factors responsible for the poor bioconversion of linoleic acid to CLA by *B. breve* NCIMB 702258 in RSM can be offset. Furthermore, we have shown how through the use of these supplements a milk based medium (mRSM) in which bioconversion levels of linoleic acid to the *c*9, *t*11 CLA isomer of 55.01 ± 3.17% can be achieved using this strain. Following from this we assessed the production of *c*9, *t*11 CLA in this

medium by a range of CLA producing bifidobacteria including *B. breve* NCIMB 702258, *B. breve* NCIMB 8807, *B. breve* DPC6330, *B. breve* DPC6331, *B. longum* DPC6315, and *B. longum* DPC6320 over 24 h (n = 3). These results were then compared to the bioconversion achieved in 20% (w/v) RSM and cys-MRS under identical conditions **(Table 2.3)**. Five of the six strains assayed in the mRSM medium demonstrated significant increases in the bioconversion of linoleic acid to *c*9, *t*11 CLA isomer over that achieved in 20% (w/v) RSM. These increases are greatest in the four *B. breve* strains, where the percentage bioconversion is comparable to the synthetic medium cys-MRS. In addition, the mRSM medium allows for the production of a higher concentration of the *t*9, *t*11 CLA isomer and lower concentration of residual free linoleic acid than 20% (w/v) RSM or cys-MRS **(Table 2.3)**.

Table 2.3 Cell counts and percentage bioconversion of linoleic acid to *c*9, *t*11 and *t*9, *t*11 CLA isomers, following 24 h anaerobic incubation at 37°C in the presence of 0.35 mg/ml linoleic acid.

Species	Strain	Media	Percentage linoleic acid remaining ± std dev	Percentage bioconversion to *c*9, *t*11 CLA isomer ± std dev	Percentage bioconversion to *t*9, *t*11 CLA isomer ± std dev	Average cell count (log cfu/ml) ± std dev	
						T_0 (h)	T_{43}(h)
B. breve	NCIMB 702258*	cys-MRS	31.51 ± 3.04	58.80 ± 2.85	0.88 ± 0.30	7.04 ± 0.32	8.31 ± 0.04
	NCIMB 702258*	20% RSM	73.10 ± 2.50	14.30 ± 0.89	3.43 ± 0.28	7.04 ± 0.32	8.11 ± 0.17
	NCIMB 702258*	mRSM	14.14 ± 5.52	53.95 ± 1.00	8.91 ± 0.14	7.04 ± 0.32	8.29 ± 0.04
	NCIMB 8807*	cys-MRS	23.33 ± 7.33	57.78 ± 4.36	6.20 ± 0.55	7.03 ± 0.11	8.40 ± 0.06
	NCIMB 8807*	20% RSM	50.36 ± 1.41	29.90 ± 0.04	2.98 ± 0.06	7.03 ± 0.11	8.27 ± 0.10
	NCIMB 8807*	mRSM	19.42 ± 4.29	48.09 ± 3.40	8.13 ± 0.41	7.03 ± 0.11	8.37 ± 0.06
	DPC6330	cys-MRS	27.32 ± 0.28	57.85 ± 1.55	1.12 ± 0.06	7.01 ± 0.02	8.29 ± 0.02
	DPC6330	20% RSM	30.13 ± 1.75	29.31 ± 1.49	1.15 ± 0.13	7.01 ± 0.02	7.99 ± 0.02
	DPC6330	mRSM	12.00 ± 1.47	59.58 ± 4.05	2.34 ± 0.10	7.01 ± 0.02	8.27 ± 0.02
	DPC6331	cys-MRS	19.10 ± 1.62	65.77 ± 1.48	2.05 ± 0.02	7.24 ± 0.06	8.60 ± 0.05
	DPC6331	20% RSM	12.87 ± 0.21	46.52 ± 1.24	2.47 ± 0.03	7.24 ± 0.06	8.30 ± 0.05
	DPC6331	mRSM	9.21 ± 0.05	67.87 ± 2.94	2.65 ± 0.32	7.24 ± 0.06	8.57 ± 0.05
B. longum	DPC6320	cys-MRS	29.14 ± 1.03	56.20 ± 1.04	2.19 ± 0.05	6.67 ± 0.57	8.40 ± 0.33
	DPC6320	20% RSM	59.46 ± 1.53	6.93 ± 0.67	0.40 ± 0.02	6.67 ± 0.57	8.10 ± 0.33
	DPC6320	mRSM	41.33 ± 4.05	22.22 ± 3.14	2.14 ± 0.47	6.67 ± 0.57	8.38 ± 0.33
	DPC6315	cys-MRS	73.79 ± 1.32	12.10 ± 0.27	0.92 ± 0.10	6.94 ± 0.05	8.47 ± 0.05
	DPC6315	20% RSM	63.17 ± 5.18	12.11 ± 0.82	0.37 ± 0.09	6.94 ± 0.05	8.17 ± 0.05
	DPC6315	mRSM	85.33 ± 5.01	10.87 ± 0.69	0.60 ± 0.10	6.94 ± 0.05	8.45 ± 0.05

** Grown in the presence of 0.4 mg/ml linoleic acid.*

2.4 Discussion

Our study focused on screening a number of supplements which have previously been shown to stimulate bifidobacterial growth or impact on microbial CLA production with the aim of identifying those which increase the production of the *c*9, *t*11 CLA isomer by bifidobacteria. This information would then be used to develop milk based media which facilitated both high CLA production and bifidobacterial growth. Following the selection of a representative bifidobacterial strain (*B. breve* NCIMB 702258) the effect of 22 supplements (antioxidants, sugars, prebiotics, sodium salts of SCFA, reducing agents, and complex nutritional supplements) on *c*9, *t*11 CLA production was assayed with varying results.

The study showed the negative impact of antioxidants, sugars and certain prebiotic compounds on *c*9, *t*11 CLA production, with marked reductions in the percentage bioconversion of substrate to product. The antioxidant α-tocopherol has previously been shown to negatively impact on CLA production, where animals receiving vitamin-E as a supplement displayed lower milk fat CLA concentrations than animals on a control diet (Bell *et al.*, 2006). Similarly, a reduction in the bioconversion of linoleic acid to CLA by bacteria has previously been observed when the growth media was supplemented with sugars (Lin, 2000). Reduced bioconversion was attributed to an increase in the number of solute molecules reducing the CLA isomerising activity of the culture (Banwart, 1989; Lin, 2000).

The production of CLA by growing bacterial cultures is highly dependent on cell density, thus it would be expected that a bifidogenic supplement would increase *c*9, *t*11 CLA production. SCFA have previously been reported to stimulate the growth of bifidobacteria, hence it would be expected they would have an impact on *c*9, *t*11 CLA production in RSM (Kaneko, *et al.*, 1994; Roy *et al.*, 2006). Of the

supplements assayed the sodium salts of acetate, propionate, and butyrate proved to be some of the most effective compounds for increasing the production of *c*9, *t*11 CLA by the strain. Despite this the stimulatory effects of SCFA on bifidobacterial growth were less profound than other known bifidogenic compounds. Hence, it would appear that growth stimulation alone cannot account for the large increases in *c*9, *t*11 CLA seen with SCFAs. Alternative suggestions may include the ability of SCFAs to act as buffers, maintaining the pH at the optimum for the strain or possibly through some unknown effect on CLA metabolism. As SCFAs play a prominent role in both the ruminal and intestinal environment it is possible that these fatty acids may play a more important role in the microbial synthesis of CLA *in vivo* than previously known.

Prebiotic compounds have long been recognised as stimulators of probiotic growth justifying their inclusion in our study. As a whole the data set would suggest that the use of prebiotic supplements to increase *c*9, *t*11 CLA production by the strain *B. breve* NCIMB 702258 is of little benefit. However, it was noted that the addition of Raftiline GR to 20% (w/v) RSM supplemented with sodium acetate and yeast extract did cause a significant increase in the percentage bioconversion of linoleic acid to *c*9, *t*11 CLA. This increase may be a result of the provision of an energy source other than lactose, which as a result of the intense fermentation had become a limiting factor in the growth and CLA production by the strain.

Cys-HCl was one of the most effective supplements assayed in terms of increasing *c*9, *t*11 CLA production by the strain. However, investigations into the effect of increasing the concentration of the supplement demonstrated no additional benefit in terms of increased bioconversion. The ability of cys-HCl to increase *c*9, *t*11 CLA production in RSM is most likely a result of its ability to alter the redox

potential of the medium towards a reducing environment which favours bifidobacterial growth (Nebra *et al.,* 2002; Coakley *et al.,* 2003; Oh *et al.,* 2003; Rosberg-Cody *et al.,* 2004).

Bifidobacteria species possess a low proteolytic activity and as such are limited in their ability to utilize the bovine casein in milk as a source of essential amino acids and short peptides (Zbikowski & Ziajka, 1986; Klaver *et al.,* 1993; Gomes *et al.,* 1998). As a result of this low proteolytic activity complex nutritional supplements such as yeast extract, tryptone and casein hydrolysate are frequently added to improve the nutritional quality of RSM. In our study, yeast extract proved to be one of the most effective supplements in terms of increasing *c*9, *t*11 CLA production by the strain. Investigations into the reasons for this increased *c*9, *t*11 CLA production have highlighted the increased growth of the strain as the most plausible mechanism. As a supplement yeast extract is a rich source of amino acids, short peptides, B-vitamin complexes, carbon, nitrogen, minerals, and trace ingredients all of which benefit the growth of bifidobacteria (Poch & Bezkorovainy, 1988; Poch & Bezkorovainy, 1991; Klaver *et al.,* 1993; Dave & Shah, 1998; Gomes *et al.,* 1998). Similarly, the enzymatic digests of casein, tryptone and casein hydrolysates, are commonly used to stimulate the growth of bifidobacteria in milk due to the high concentration of short peptides and amino acids present (Poch & Bezkorovainy, 1988; Poch & Bezkorovainy, 1991; Klaver *et al.,* 1993; Dave & Shah, 1998; Gomes *et al.,* 1998). In our study, both tryptone and casein hydrolysate supplementation led to substantial increases in the production of *c*9, *t*11 CLA. Here the low pH values following 43 h incubation suggest a high rate of growth as the mechanism behind the increased *c*9, *t*11 CLA production.

As a result of our study a range of supplements have been screened and their

effect on bifidobacterial CLA production determined. In addition, using the data derived from this screening, a milk-based medium (mRSM) has been developed in which a percentage bioconversion of linoleic acid to the *c*9, *t*11 CLA isomer of 55.01 ± 3.17%, by the strain *B. breve* NCIMB 702258 can be achieved. This level is comparable to that seen with cys-MRS under identical fermentation conditions. Furthermore, subsequent analysis has shown that this medium is capable of increasing the percentage bioconversion of linoleic acid to *c*9, *t*11 CLA by three other CLA producing strains (*B. breve* NCIMB 8807, *B. breve* DPC6330, *B. breve* DPC6331) to levels comparable to those seen in cys-MRS. The level of bioconversion achieved using the supplemented RSM medium and *B. breve* strains compares well with other studies in which similar strains and the synthetic medium cys-MRS have been used. In our study, strains of *B. breve* have been shown to bioconvert 48-68% of the available linoleic acid (0.35 mg/ml) to the *c*9, *t*11 CLA isomer while others such as Coakley *et al.* (2003) and Oh *et al.* (2003) reported the bioconversion of 28-66% and 32% of the available linoleic acid (0.5 mg/ml) in cys-MRS, respectively. However, the medium proved less effective in stimulating CLA production by strains of *B. longum* (*B. longum* DPC6315 and *B. longum* DPC6320) and may require slight modification to suit the needs of this bifidobacterial species.

2.5 Conclusions

In conclusion, we have demonstrated both the positive and negative impact of 22 supplements on CLA production by *Bifidobacterium breve* in RSM. Of these supplements we have identified cys-HCl, the complex nutritional supplements and the sodium salts of SCFA as some of the most effective supplements in terms of stimulation of *c*9, *t*11 CLA production. Using this knowledge we subsequently developed a cost effective milk based medium where concentrations of the *c*9, *t*11 CLA isomer equivalent to synthetic media can be achieved.

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