Chapter 5

Anti-microbial activity of C18 Unsaturated Fatty Acids and their Conjugated Isomers against Methicillin Resistant *Staphylococcus aureus*

Abstract

In the current study α-linolenic acid, γ-linolenic acid and stearidonic acid and their respective conjugated fatty acids CALA, CGLA and CSA were investigated for anti-microbial activity against the methicillin resistant strain *S. aureus* ATCC 43300. The study highlighted the differences in the pattern of inhibition displayed by both the unsaturated fatty acids and their conjugated fatty acids against the strain. Exposure of the strain to 0.1 mg/ml of α-linolenic acid, γ-linolenic acid, stearidonic acid resulted in 5.98 log cfu/ml, 6.19 log cfu/ml and 5.54 log cfu/ml reductions in cell viability, respectively, after 40 min. The overall activity of these unsaturated fatty acids was observed to increase with concentration and duration of exposure. Unlike their parent fatty acids the anti-microbial activity of CALA and CGLA did not increase with concentration or duration of exposure but was characterized by a 2 log cfu/ml reduction in cell viability within the first 10 min exposure. A similar trend was evident for CSA with the inhibitory activity observed within the first 10 min of exposure, however, the anti-microbial activity of this conjugated fatty acid did increase with dose. Successive exposure to either the unsaturated fatty acids or their respective conjugated derivatives did not encourage the development of resistance in the MRSA strain. The anti-microbial activity of both the unsaturated fatty acids and their respective conjugated fatty acids was observed to be strongly related to their cellular uptake but to be independent of increased cellular lipid peroxidation. Both the unsaturated fatty acids and their conjugated derivatives remained active in the presence of blood serum suggesting their potential suitability for the *in vivo* treatment of MRSA. The results suggest that unsaturated fatty acids and their conjugated derivatives may be important in the control of MRSA.

5.1 Introduction

Staphylococcus aureus (*S. aureus*) is a gram-positive bacterium that colonizes the skin of about 30% of healthy humans. Normally a harmless coloniser or commensal organism, *S. aureus* may cause a variety of human infections including abscesses, endocarditis, toxic shock syndrome, osteomyelitis, and mastitis (Fischetti, 2000; McCormick *et al.*, 2001). Despite its natural prominence as part of the natural microflora of humans, the emergence of its oxacillin-resistant form (methicillin resistant *S. aureus* (MRSA)) and of community acquired MRSA has proved a major problem, making *S. aureus* the most important cause of antibiotic-resistant nosocomial infections worldwide (Karchmer, 2000). Indeed, increases in the incidence of MRSA infection have been widely reported across Europe (EARSS, 2006).

A number of studies have reported the inhibitory properties of fatty acids on a range of gram-positive bacteria (Giamarellos-Bourboulis *et al.*, 1995; Kodicek, 1949; Lee *et al.*, 2002; Sun *et al.*, 2003). These inhibitory properties also extend to strains of *S. aureus,* which have exhibited susceptibility to a range of fatty acids including linoleic acid, α-linolenic acid and γ-linolenic acid (Butcher *et al.*, 1976; Heczko *et al.*, 1979; Kelsey *et al.*, 2006; Lacey & Lord, 1981). Indeed, there is also evidence to suggest that strains of MRSA may also be susceptible to the inhibitory properties of unsaturated fatty acids (McDonald *et al.*, 1981; Ohta *et al.*, 1995). Elucidating the mechanism through which fatty acids mediate their inhibitory properties have proved difficult, but a number of researchers have suggested an increase in the permeability of the cell membrane in the presence of unsaturated fatty acids as a potential reason (Greenway & Dyke, 1979). This increased permeability may be associated with the ability of fatty acids to act as surfactants or as a result of the incorporation of the fatty acids into the cell membrane resulting in

changes in membrane fluidity or in the case of unsaturated fatty acids increased lipid peroxidation of membrane phospholipids (Greenway & Dyke, 1979; Knapp & Melly, 1986). A recent investigation into the anti-microbial activity of linoleic acid and oleic acid against MRSA also associated fatty acids with disrupting cell energetics via membrane disruption (Kenny *et al.*, 2009). This activity was characterised by changes in the expression of genes associated with energy creation in the cell including those associated with glycolytic and fermentative pathways.

In recent years, a number of strains of bifidobacteria and propionibacteria with the ability to convert C18 unsaturated fatty acids to their respective conjugated isomers have been identified (Hennessy *et al.*, 2007; Kishino *et al.*, 2003). The ability of these strains to produce CALA, CGLA and CSA offer the prospect of the enrichment of milk and yoghurt with these fatty acids through fermentation. Indeed, there is already strong *in vivo* evidence to suggest that conjugates such as the CLA and CALA isomers can be absorbed in the intestine (Plourde *et al.*, 2006; Tsuzuki *et al.*, 2003; Tsuzuki *et al.*, 2004c). The production of conjugated fatty acids by bifidobacteria, a microbe normally abundant in the intestine presents an opportunity for the *in situ* production of bioactive conjugated fatty acids. As the substrate fatty acids from which CALA, CGLA and CSA (α-linolenic acid, γ-linolenic acid and stearidonic acid) can be found in some abundance in nature it is not unreasonable to assume their *in vivo* production is a realistic possibility (Fan & Chapkin, 1998; Li *et al.*, 2003; Whelan, 2009). Indeed further credence is given to this theory in light of the recent evidence pertaining to the *ex vivo* and *in vivo* production of CLA from dietary linoleic acid (Ewaschuk *et al.*, 2006; Wall *et al.*, 2009). Thus, it is possible that both dietary and edogenously produced conjugated fatty acids could potentially be utilised to treat MRSA infection *in vivo.*

In this study we assessed the inhibitory properties of the microbially

produced conjugated C18 fatty acids, conjugated α-linolenic acid (C18:3-*c*9, *t*11, *c*15) (CALA), conjugated γ-linolenic acid (C18:3-*c*6, *c*9, *t*11) (CGLA), and conjugated stearidonic acid (C18:4-*c*6, *c*9, *t*11, *c*15) (CSA) against MRSA. The results demonstrated that both the conjugated fatty acids and their non-conjugated parent unsaturated fatty acids displayed potent inhibitory activity against MRSA. Despite both being potent inhibitors of MRSA, the profile of inhibitory activity displayed by the conjugates and non-conjugated parent fatty acids differed substantially.

5.2 Materials and methods

5.2.1 Bacterial strains and growth conditions.

The strain *B. breve* DPC6330 was used to produce the conjugated fatty acids, CALA, CGLA and CSA. The strain was grown in cys-MRS (MRS (Difco, Detroit, MI, USA,) containing 0.5 mg/ml L-cysteine hydrochloride (Sigma Aldrich, St Louis, Mo) under anaerobic conditions (anaerobic jars with Anaerocult A gas packs, Merck Darmstedt, Germany) at 37^{o}C for 18 h. The strain of *S. aureus* used in these studies was the methicillin resistant strain *S. aureus* ATCC 43300 and was cultivated in tryptic soy broth (TSB) incubated at 37^{o}C for 24 h. Where solid medium was required 1.5% (w/v) bacteriological agar (Oxoid, Hampshire, UK) was added to the medium.

5.2.2 Microbial production of the conjugated fatty acids

All substrates were of the highest purity. α-linolenic acid (*c*9, *c*12, *c*15 octadecatrienoic acid) was obtained from Sigma Aldrich, γ-linolenic acid (*c*6, *c*9, *c*12 octadecatrienoic acid) was purchased from Nu-chek Prep (Elysian, MN), while stearidonic acid, (*c*6, *c*9, *c*12, *c*15 octadecatetraenoic acid) was obtained from Cayman Europe (Akadeemia tee, Tallinn, Estonia). A 30 mg/ml stock solution of each substrate fatty acid was prepared using 2% Tween 80 and then stored at -20^{o}C under nitrogen.

A 4% (w/v) inoculum of the strain *B. breve* DPC6330 was added to eight litres of cys-MRS containing 0.45 mg/ml of the respective unsaturated fatty acid substrate delivered in the form of a 30 mg/ml stock solution solubilised with 2% (v/v) Tween 80 (Sigma Aldrich) and incubated at 37^{o}C for 72 h. Total fatty acids were extracted from the samples using the method described by Coakley *et al.*

(2003). The hexane extract was partially concentrated using a rotary evaporator (Buchi Rotavapor R-210, Buchi Labortechnik AG, Switzerland) and the resulting concentrate washed once with 0.88% KCl and twice with water:methanol (1:1) (v/v). The remaining hexane was then evaporated by heating at 45^{o}C under a steady flow of nitrogen. The resulting lipid was resuspended at a concentration of 100 mg/ml in acetonitrile:acetate (100:0.14) (v/v) and sealed under nitrogen and stored at -20^{o}C until use.

5.2.3 Purification of the conjugated fatty acids

The conjugated fatty acids CALA, CGLA and CSA were separated from the non-conjugated fatty acids using reverse phase high performance liquid chromatography (RP-HPLC). To achieve this we employed a Luna 5u C18 (2) 100A preparative column (250 x 21.20 mm) (Phenomenex, Macclesfield, Cheshire, UK) and the Varian Prostar HPLC system (Varian Inc., CA, USA). 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 235 nm. Fractions containing the conjugated fatty acids were collected using a fraction collector and pooled. Following removal of the acetonitrile from the pooled fractions by rotary evaporation, the conjugated fatty acids were re-extracted using the method of Coakley *et al.* (2003).

5.2.4 Analysis of purified conjugated fatty acids

The fatty acid composition of the RP-HPLC fractions was confirmed by gas liquid chromatography (GLC). Fatty acid methyl esters (FAME) were created using the method 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).

5.2.5 Assessment of anti-microbial activity against MRSA

Lipids were solubilised in ethanol before addition to TSB to give final fatty acid concentrations of 0, 0.02, 0.05, 0.075 and 0.1 mg/ml. A control tube containing TSB and an equivalent amount of ethanol was also included. Culture tubes were inoculated to an O.D. 600 nm of 0.1 with the overnight culture of the *S. aureus* strain and incubated for 40 min at 37^{o}C. At 10 min intervals cell viability was determined by spot plate technique on tryptic soy agar (TSA) and the plates incubated at 37^{o}C for 18 h. All experiments were performed in triplicate.

An experiment was carried out to test for evidence of resistance to the anti-microbial effect of the unsaturated fatty acid, α-linolenic acid, and its conjugated derivative, CALA, following 40 min exposure. A fatty acid concentration of 0.05 mg/ml was chosen for testing whether resistance to α-linolenic acid or CALA can develop in the methicillin resistant strain *S. aureus* ATCC 43300. Cultures grown in the absence of α-linolenic acid or CALA were tested, in parallel, to serve as negative controls. A 13 h old culture of *S. aureus* ATCC 43300 was inoculated into three tubes containing TSB to give an O.D. 600 nm of 0.1 (approx 7 log cfu/ml). α-linolenic acid and CALA solublised in ethanol were used to give a fatty acid concentration of 0.05 mg/ml. The culture was incubated at 37^{o}C for 40 min with surviving cells spot plated onto TSA and grown for 2 days in the absence of the fatty acid (cycle 1). A single colony was removed from the plate and inoculated into 10 mls of TSB followed by incubation at 37^{o}C for 13 h (cycle 2). Three tubes of TSB were inoculated with this culture to give an O.D. 600 nm of 0.1 and again

exposed to 0.05 mg/ml of α-linolenic acid or CALA as previously described, followed by plating of the survivors (cycle 3). This process was repeated two more times. All experiments were performed in triplicate.

5.2.6 Assessing the impact of exposure to C18 conjugated or non-conjugated fatty acids on the cellular fatty acid composition of the methicillin resistant strain *S. aureus* ATCC 43300

We also determined whether the exposure of the strain *S. aureus* ATCC 43300 to sub-lethal concentrations of α-linolenic acid, γ-linolenic acid and stearidonic acid or their respective conjugated isomers, CALA, CGLA and CSA, resulted in changes in the cellular fatty acid composition following 40 min exposure. Two hundred millilitres of TSB were inoculated with 6 mls of an overnight culture of *S. aureus* ATCC 43300 containing 0.025 mg/ml of the respective fatty acids and the culture incubated at 37^{o}C for 40 min. Following incubation the culture was separated into the supernatant and pellet by centrifugation at 4000 rpm for 10 min. The pellet was washed twice in phosphate buffered saline (Sigma Aldrich) and the total fatty acids extracted using the method of Folch *et al.* (1957). Twenty millilitres of the supernatant was filter sterilised to remove any remaining cellular material and the total fatty acids extracted using the method described by Coakley *et al.* (2003). Total fatty acids from both the pellet and supernatant were methylated using the method of Park & Goins, (1994).

5.2.7 Assessing the impact of blood serum on the inhibitory activity of C18 unsaturated fatty acids and their conjugated isomers against MRSA

In our study, we investigated whether the inhibitory properties of C18 fatty acids such α-linolenic acid, γ-linolenic acid and stearidonic acid, and the conjugated fatty

acids CALA, CGLA and CSA against MRSA would be inactivated by the presence of blood serum. To achieve this, an overnight culture of the methicillin resistant strain *S. aureus* ATCC 43300 was inoculated into TSB containing 1% (v/v) fetal bovine serum (FBS) to give an O.D. 600 nm of 0.1. The fatty acids were solublised in ethanol and used to give a fatty acid concentration of 0.075 mg/ml in the medium. The culture was then incubated at 37^{o}C for 40 min with surviving cells spot plated onto TSA.

5.3 Results

5.3.1 Fatty acid composition of microbially conjugated fatty acids

Following the incubation of the strain *B. breve* DPC6330 in the presence of 0.45 mg/ml α-linolenic acid, γ-linolenic acid or stearidonic acid, and subsequent GLC analysis, the presence of substantial concentrations of CALA, CGLA and CSA were found in the respective fermentation media **(Table 5.1)**. In addition to these conjugates, the presence of residual substrate fatty acid and fatty acids such as palmitic, stearic, vaccenic and oleic acids from the medium resulted in the production of a crude oil in which the conjugated fatty acid content ranged from 29.2-73.3%. To increase their conjugate content the crude oils were subjected to RP-HPLC and the fractions containing the conjugated fatty acid collected and pooled. This process yielded oils containing ≥ 95% CALA, CGLA or CSA.

5.3.2 Effect of C18 unsaturated fatty acid and their conjugates on the survival of MRSA *in vitro*

The inhibitory activity of the C18 unsaturated fatty acids, α-linolenic acid, γ-linolenic acid and stearidonic acid, and their respective conjugated isomers, CALA, CGLA and CSA, against the methicillin resistant strain *S. aureus* ATCC 43300 were investigated in this assay. Kill curves were performed at fatty acid concentrations of 0 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.075 mg/ml and 0.1 mg/ml and cell numbers determined after 0 min, 10 min, 20 min, 30 min and 40 min incubation. These concentrations of exposure represent levels which are similar or lower than those which could be achieved as a result of the fermentation of α-linolenic acid, γ-linolenic acid, and stearidonic acid to CALA (0.3 mg/ml), CGLA (0.1 mg/ml) and CSA (0.06 mg/ml), respectively by strains of *B. breve* (Chapter 3).

Table 5.1 Fatty acid composition of conjugate rich oils produced from α-linolenic acid, γ-linolenic acid and stearidonic acid. Results represent the average of triplicate experiments ± std dev.

Fatty acids (g/100g FAME)	α-linolenic acid	γ-linolenic acid	Stearidonic acid
Palmitic acid	1.01± 0.09	1.59 ± 0.11	1.96 ± 0.19
Stearic acid	1.44 ± 0.03	1.94 ± 0.21	2.30 ± 0.22
Vaccenic acid	1.02 ± 0.03	1.38 ± 0.04	2.38 ± 0.27
Oleic acid	18.37 ± 1.01	26.61 ± 1.21	36.80 ± 1.09
Linoleic acid	0.10 ± 0.00	0.06 ± 0.00	0.11 ± 0.01
α-linolenic acid	1.06 ± 0.08	0.00± 0.00	0.00 ± 0.00
γ-linolenic acid	0.00 ± 0.00	27.14 ± 0.10	0.00 ± 0.00
Stearidonic acid	0.00 ± 0.00	0.00 ± 0.00	3.24 ± 0.31
*c*9, *t*11 CLA	0.68 ± 0.06	0.92 ± 0.05	1.18 ± 0.11
*t*9,*t*11 CLA	0.24 ± 0.02	0.20 ± 0.03	0.42 ± 0.02
*c*9, *t*11, *c*15 CALA	73.3 ± 1.22	0.0 ± 0.00	0.0 ± 0.00
*t*9, *t*11, *c*15 CALA	0.90 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
*c*6, *c*9, *t*11 CGLA	0.00 ± 0.00	29.20 ± 0.19	0.00 ± 0.00
*c*6, *t*9, *t*11 CGLA	0.00 ± 0.00	8.50 ± 0.04	0.00 ± 0.00
*c*6,*c*9,*t*11,*c*15 CSA	0.00 ± 0.00	0.00 ± 0.00	50.00 ± 0.00
*c*6,*t*9,*t*11,*c*15 CSA	0.00 ± 0.00	0.00 ± 0.00	1.20 ± 0.05

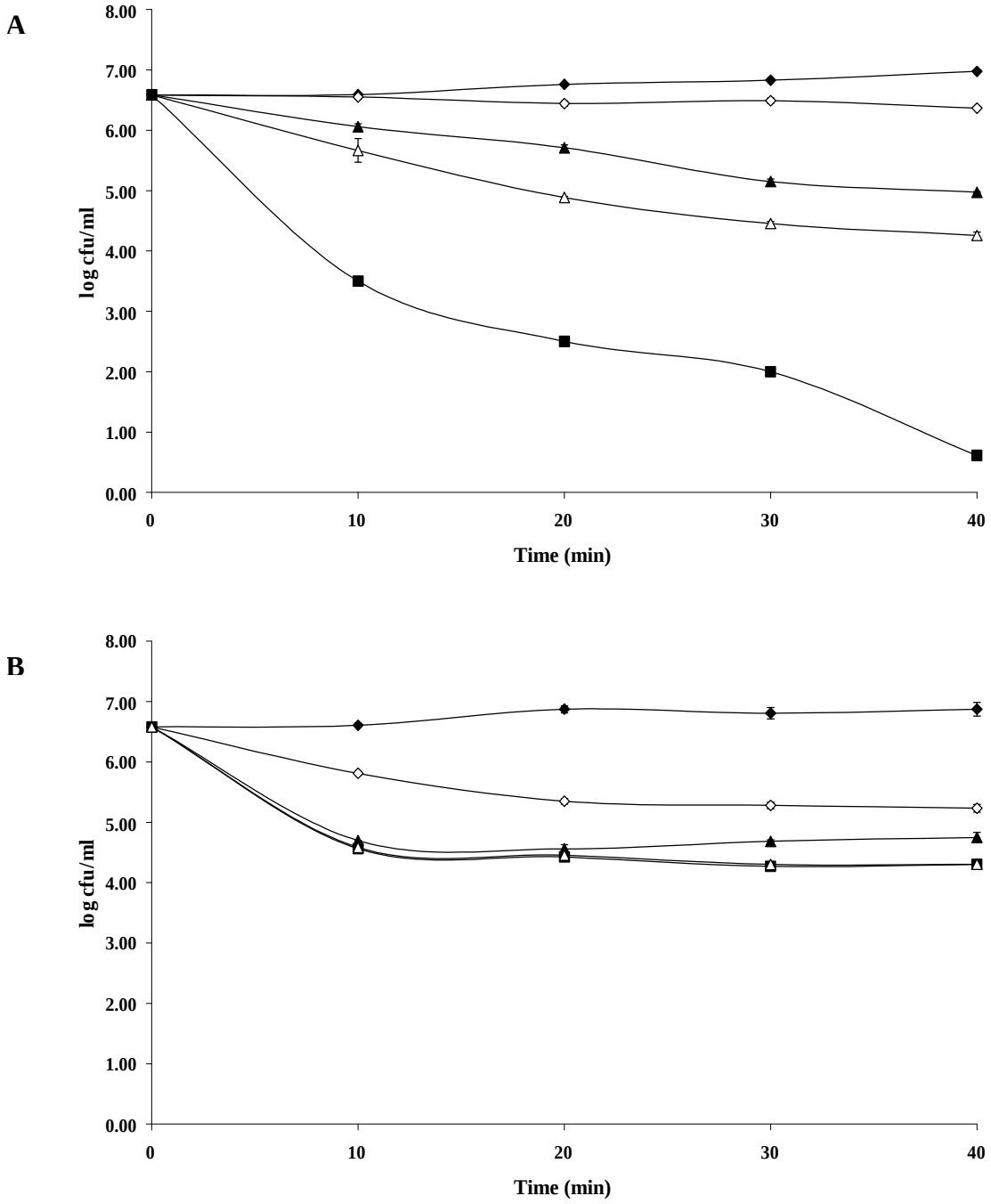

Figure 5.1 Kill curve of the methicillin resistant strain, *S. aureus* ATCC 43300, incubated at 37^{o}C in the presence of **A.** α-linolenic acid or **B.** CALA. ♦ 0 mg/ml, ◊ 0.025 mg/ml, ▲ 0.05 mg/ml, Δ 0.075 mg/ml, ■ 0.1 mg/ml. Curves represent average of cultures performed in triplicate ± std dev.

The inhibitory activity of α-linolenic acid was observed to increase with the time of exposure and in a dose dependent manner **(Figure 5.1)**. At the highest concentration assayed (0.1 mg/ml) a 5.98 log cfu/ml reduction in cell numbers was observed following 40 min exposure. The inhibitory activity of CALA differed substantially from that of α-linolenic acid. At the lowest concentrations assayed (0.025 mg/ml and 0.05 mg/ml) the inhibitory effect of CALA increased in a dose dependent manner **(Figure 5.1)**. At concentrations above this the inhibitory effect of CALA did not substantially differ from that seen at 0.05 mg/ml. At CALA concentrations of 0.05 mg/ml, 0.075 mg/ml and 0.1 mg/ml the duration of exposure did not significantly increase the inhibitory effect of the fatty acid. At concentrations of 0.025 mg/ml, 0.05 mg/ml and 0.075 mg/ml and following 10 and 20 min exposure, CALA resulted in significantly greater reductions ($P \leq 0.001$) in cell viability than were achieved with equivalent concentrations of α-linolenic acid **(Figure 5.1)**. At a concentration of 0.1 mg/ml α-linolenic acid displayed a significantly higher reduction in cell numbers than an equivalent concentration of CALA ($P \leq 0.001$). To summarize, CALA would appear to display greater inhibitory activity against the strain at lower concentrations (0.025-0.05 mg/ml) following 40 min exposure than equivalent concentrations of α-linolenic acid. In addition, the reductions observed with CALA, occur at a faster rate than those seen with α-linolenic acid at fatty acid concentrations of 0.025 mg/ml, 0.05 mg/ml, and 0.075 mg/ml.

Like α-linolenic acid the inhibitory activity of γ-linolenic acid was observed to increase with the time of exposure and in a dose dependent manner **(Figure 5.2)**. At the highest concentration assayed a 6.19 log cfu/ml reduction in cell numbers was observed following 40 min exposure. The inhibitory activity of CGLA also differs substantially from that of its parent fatty acid γ-linolenic acid, with the

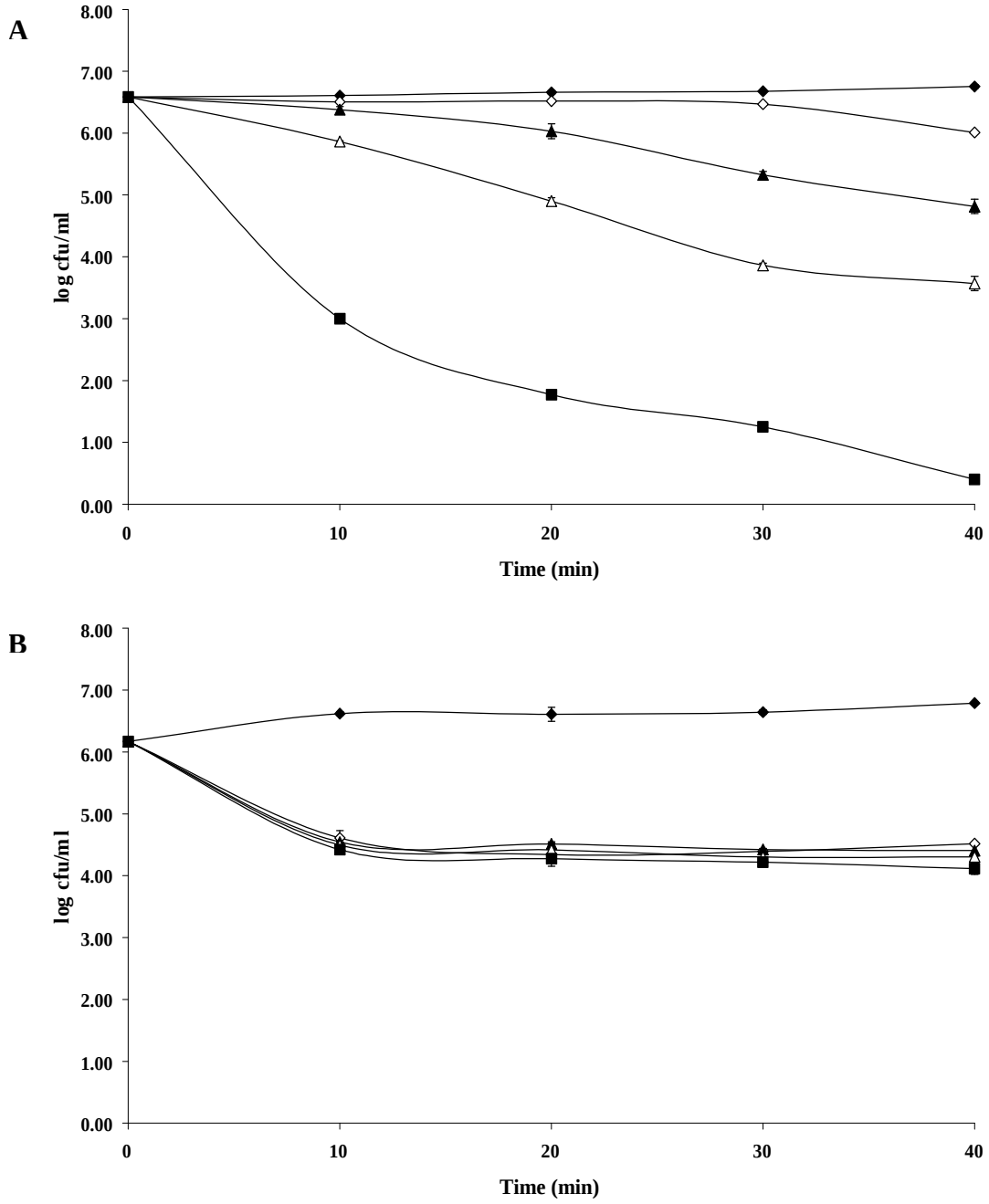

Figure 5.2 Kill curve of the methicillin resistant strain, *S. aureus* ATCC 43300, incubated at 37°C in the presence of **A.** γ-linolenic acid or **B.** CGLA. ♦ 0 mg/ml, ◊ 0.025 mg/ml, ▲ 0.05 mg/ml, Δ 0.075 mg/ml, ■ 0.1 mg/ml. Curves represent average of cultures performed in triplicate ± std dev.

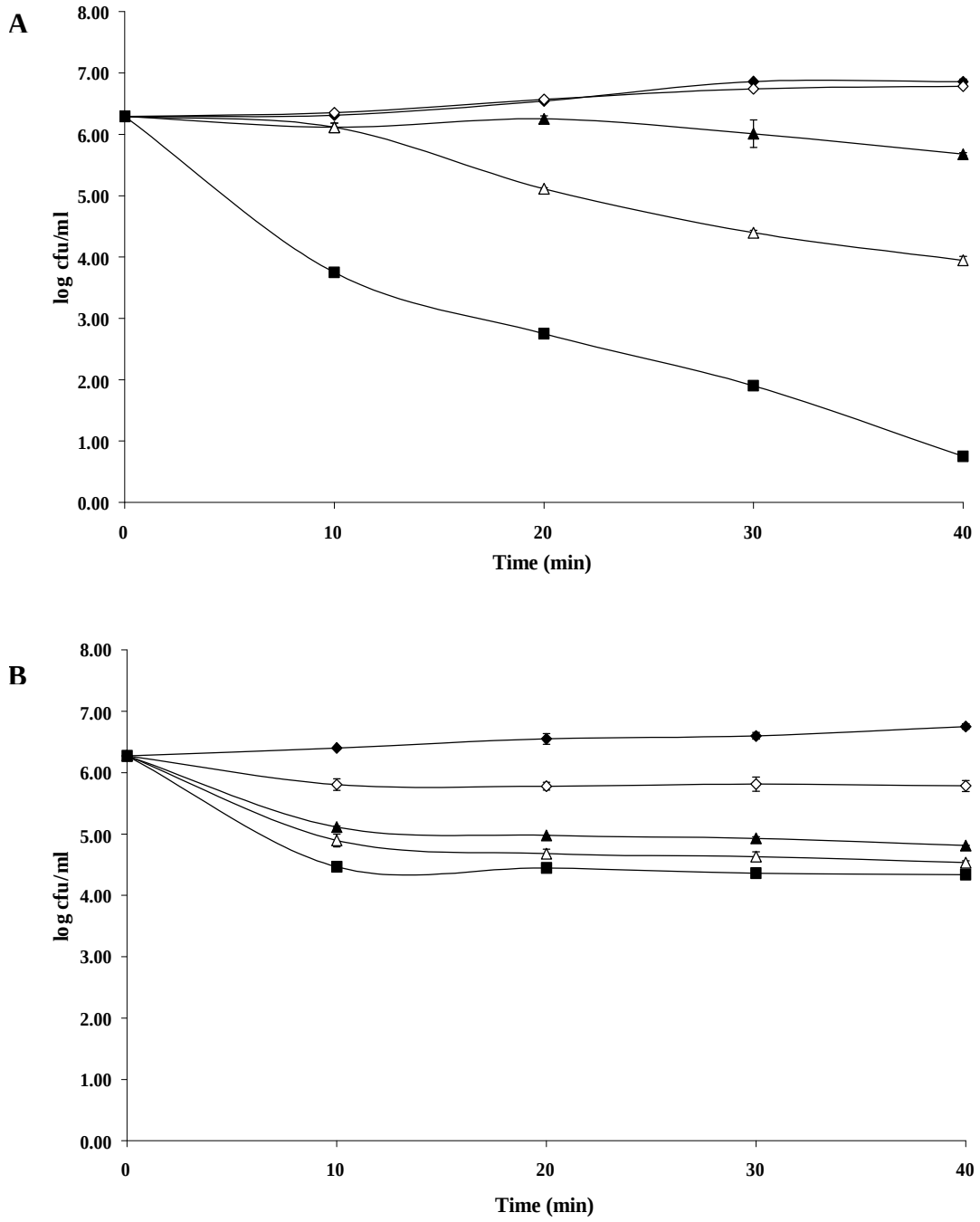

Figure 5.3 Kill curve of the methicillin resistant strain, *S. aureus* ATCC 43300, incubated at 37^{o}C in the presence of **A.** stearidonic acid or **B.** CSA. ♦ 0 mg/ml, ◊ 0.025 mg/ml, ▲ 0.05 mg/ml, Δ 0.075 mg/ml, ■ 0.1 mg/ml. Curves represent average of cultures performed in triplicate ± std dev.

reductions in cell viability occurring within the first ten minutes of exposure in a similar manner to CALA. At CGLA concentrations of between 0.025-0.075 mg/ml and following 10 min exposure, the reductions in cell viability are significantly greater than those achieved with equivalent concentrations of γ-linolenic acid ($P \leq 0.001$). Following 40 min exposure, CGLA remained significantly more active than γ-linolenic acid at fatty acid concentrations of between 0.025-0.05 mg/ml ($P \leq 0.01$). To summarize, CGLA would appear to display greater inhibitory activity against the strain at concentrations of 0.025-0.05 mg/ml following 40 min exposure than equivalent concentrations of γ-linolenic acid. In addition, the reductions observed with CGLA, occur a faster rate than those seen with γ-linolenic acid at concentrations of 0.025-0.075 mg/ml with approximately a 2 log cfu/ml reduction in cell numbers within the first 10 min.

The inhibitory activity of stearidonic acid was observed to increase with the time of exposure and in a dose dependent manner **(Figure 5.3)**. At the highest concentration assayed a 5.54 log cfu/ml reduction in cell numbers was observed following 40 min. As with the other conjugated fatty acids the inhibitory activity of CSA differed from that of stearidonic acid. The inhibitory effect of CSA occurred within the first 10 min and thereafter a significant reduction in cell numbers was not observed. Unlike the other conjugated fatty acids it was observed that the inhibitory effect of CSA increased in a dose dependent manner **(Figure 5.3)**.

Acquired resistance to anti-microbial molecules, especially to antibiotics, is a major reason why *S. aureus* infections are difficult to eradicate. To determine if resistance to α-linolenic acid or CALA could develop in the MRSA strain *S. aureus* ATCC 43300, we exposed the strain to seven cycles of exposure to both fatty acids. The incubations containing 0.05 mg/ml α-linolenic acid or CALA in each cycle always showed approximately a 2 log cfu/ml cell reduction compared to the control

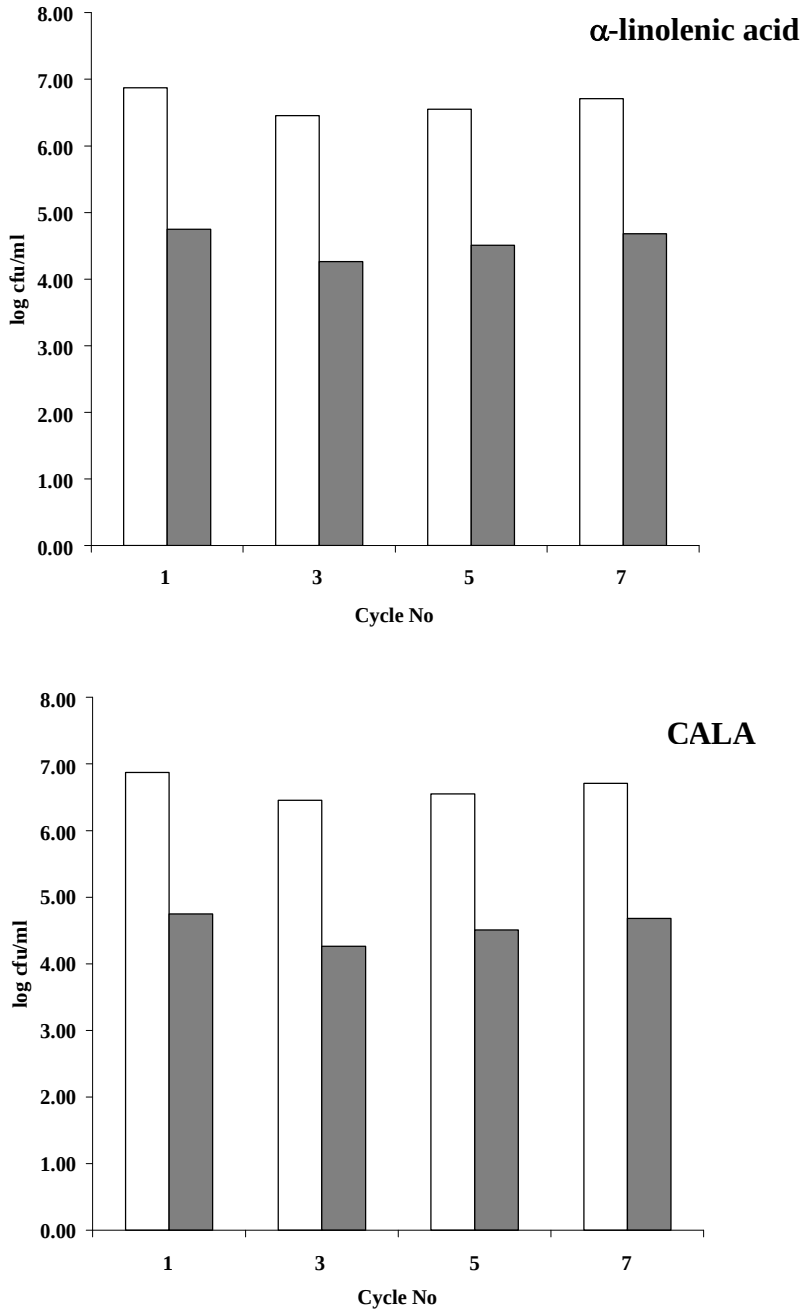

Figure 5.4 Test for acquired resistance in the methicillin resistant strain *S. aureus* ATCC 83300 to the inhibitory effect of the conjugated fatty acid α-linolenic acid or CALA after 40 min incubation at 37^{o}C for seven cycles, in the presence of 0 mg/ml (□) α-linolenic acid or CALA concentration and 0.05 mg/ml (■) α-linolenic acid or CALA**.** Results represent the average of triplicate experiments.

(Figure 5.4). Thus, there was no evidence that cells resistant to α-linolenic acid or CALA were being selected.

5.3.3 The impact of exposure to C18 conjugated and non-conjugated fatty acids on the cellular fatty acid composition of the methicillin resistant strain *S. aureus* ATCC 43300

Exposure of the cells to α-linolenic acid or CALA resulted in significant reductions in the concentration of myristoleic acid present in the cell compared to the control ($P \leq 0.001$). This reduction was significantly greater on exposure to α-linolenic acid than CALA ($P \leq 0.001$). Exposure to α-linolenic acid resulted in a significant reduction in cellular palmitic ($P \leq 0.001$) and stearic acid ($P \leq 0.01$) compared to the control and CALA supplemented samples. In contrast, CALA significantly increased the concentration of cellular oleic acid compared to α-linolenic acid or the control ($P \leq 0.001$). α-linolenic acid and CALA were found in the cellular fatty acids of both the α-linolenic acid and CALA supplemented cells, respectively. However, the uptake of α-linolenic acid was observed to be over 3-fold greater than that of CALA. In addition, supplementation with CALA caused a significant increase in cellular arachidic acid in comparison with α-linolenic acid or the control ($P \leq 0.001$).

Exposure of the cells to γ-linolenic acid or CGLA resulted in significant reductions in the concentration of myristoleic acid present in the cell relative to the control ($P \leq 0.001$) **(Table 5.2)**. Exposure to γ-linolenic acid resulted in a significant reduction in cellular palmitic ($P \leq 0.001$) and stearic acid ($P \leq 0.01$) compared to the control and CGLA supplemented samples. Like α-linolenic acid the uptake of γ-linolenic acid was observed to be over 3.5-fold greater than its

Table 5.2 Total fatty acid composition of microbial pellet following exposure to the unsaturated fatty acids α-linolenic acid, γ-linolenic acid, stearidonic acid or their conjugated isomers, CALA, CGLA, and CSA at a concentration of 0.025 mg/ml for 40 min.

α-linolenic acid & CALA									
Fatty acid (g/100g FAME)	Control			α-linolenic acid			CALA		
C6:0-C10:0	14.55	±	0.40	12.35	±	0.45	15.37	±	1.04
Lauric (C12:0)	0.82	±	0.02	0.00	±	0.00	1.21	±	0.01
Tridecanoic (C13:0)	0.93	±	0.10	1.60	±	0.10	1.77	±	0.21
Myristic (C14:0)	3.53	±	0.01	2.38	±	0.03	3.56	±	0.12
Myristeladic (C14:1 *t*)	2.24	±	0.02	0.00	±	0.00	0.00	±	0.00
Myristoleic (C14:1 *c*)	7.56	±	0.05	1.77	±	0.03	3.53	±	0.13
Pentadecanoic (C15:0)	0.62	±	0.62	0.00	±	0.00	0.00	±	0.00
Palmitic (C16:0)	33.43	±	0.50	24.56	±	0.00	32.82	±	0.61
Palmiteladic (C16:1 *t*)	0.78	±	0.03	0.00	±	0.00	0.00	±	0.00
Palmitoleic (C16:1 *c*)	1.31	±	0.09	0.00	±	0.00	0.00	±	0.00
Heptadecanoic (C17:0)	1.22	±	0.12	0.92	±	0.08	1.34	±	0.03
Stearic (C18:0)	24.22	±	1.44	17.23	±	0.06	20.60	±	0.43
Oleic (C18:1)	2.51	±	0.12	2.10	±	0.04	5.11	±	0.10
α-linolenic (C18:3)	0.00	±	0.00	36.68	±	0.00	0.00	±	0.00
CALA (C18:3)	0.00	±	0.00	0.00	±	0.18	11.27	±	0.69
Arachidic (C20:0)	0.82	±	0.01	0.00	±	0.00	2.48	±	0.12
γ-linolenic acid & CGLA									
Fatty acid (g/100g FAME)	Control			γ-linolenic acid			CGLA		
C6:0-C10:0	14.55	±	0.40	7.58	±	0.14	14.45	±	1.44
Lauric (C12:0)	0.82	±	0.02	0.00	±	0.00	1.23	±	0.02
Tridecanoic (C13:0)	0.93	±	0.10	1.90	±	1.09	1.62	±	0.17
Myristic (C14:0)	3.53	±	0.01	2.35	±	0.06	3.28	±	0.38
Myristeladic (C14:1 *t*)	2.24	±	0.02	0.39	±	0.39	0.00	±	0.00
Myristoleic (C14:1 *c*)	7.56	±	0.05	2.08	±	0.07	3.33	±	0.29
Pentadecanoic (C15:0)	0.62	±	0.62	0.00	±	0.00	0.00	±	0.00
Palmitic (C16:0)	33.43	±	0.50	21.95	±	0.46	33.86	±	1.32
Palmiteladic (C16:1 *t*)	0.78	±	0.03	0.00	±	0.00	0.00	±	0.00
Palmitoleic (C16:1 *c*)	1.31	±	0.09	0.00	±	0.00	0.00	±	0.00
Heptadecanoic (C17:0)	1.22	±	0.12	1.06	±	0.25	1.87	±	0.13
Stearic (C18:0)	24.22	±	1.44	15.14	±	1.23	20.48	±	0.58
Oleic (C18:1)	2.51	±	0.12	1.95	±	0.15	5.37	±	0.54
γ-linolenic (C18:3)	0.00	±	0.00	42.54	±	1.11	0.00	±	0.00
CGLA (C18:3)	0.00	±	0.00	0.00	±	0.00	11.96	±	0.35
Arachidic (C20:0)	0.82	±	0.01	0.56	±	0.04	1.96	±	0.27
Stearidonic acid & CSA									
Fatty acid (g/100g FAME)	Control			Stearidonic acid			CSA		
C6:0-C10:0	14.55	±	0.40	12.98	±	0.79	12.89	±	0.08
Lauric (C12:0)	0.82	±	0.02	1.00	±	0.02	0.79	±	0.04
Tridecanoic (C13:0)	0.93	±	0.10	3.85	±	0.91	1.05	±	0.02
Myristic (C14:0)	3.53	±	0.01	4.27	±	0.15	4.48	±	0.05
Myristeladic (C14:1 *t*)	2.24	±	0.02	0.00	±	0.00	1.33	±	0.05
Myristoleic (C14:1 *c*)	7.56	±	0.05	3.72	±	0.07	4.43	±	0.03
Pentadecanoic (C15:0)	0.62	±	0.62	0.00	±	0.00	0.94	±	0.20
Palmitic (C16:0)	33.43	±	0.50	36.66	±	0.81	36.09	±	0.95
Palmiteladic (C16:1 *t*)	0.78	±	0.03	0.00	±	0.00	0.34	±	0.34
Palmitoleic (C16:1 *c*)	1.31	±	0.09	0.00	±	0.00	0.94	±	0.04
Heptadecanoic (C17:0)	1.22	±	0.12	1.49	±	0.10	1.61	±	0.05
Stearic (C18:0)	24.22	±	1.44	24.12	±	0.91	23.95	±	0.24
Oleic (C18:1)	2.51	±	0.12	5.15	±	0.19	3.24	±	0.01
Stearidonic (C18:4)	0.00	±	0.00	2.11	±	0.10	0.00	±	0.00
CSA (C18:4)	0.00	±	0.00	0.00	±	0.00	0.94	±	0.15
Arachidic (C20:0)	0.82	±	0.01	0.00	±	0.00	0.85	±	0.03

conjugated derivative, CGLA. Exposure of the cells to stearidonic acid or CSA resulted in significant reductions in cellular myristoleic acid relative to the control ($P \leq 0.001$). Furthermore, exposure to stearidonic acid, and CSA, resulted in a significant increase in the concentration of cellular oleic acid in comparison to the control ($P \leq 0.01$). Interestingly, fatty acids such as palmitic acid and stearic acid which were affected by α-linolenic acid and γ-linolenic acid exposure remained relatively unaffected by exposure to stearidonic acid. The cellular uptake of stearidonic acid was found to be over 2-fold greater than that of CSA, however, uptake of stearidonic acid was found to be over 17-fold and over 20-fold lower than that of α-linolenic acid or γ-linolenic acid, respectively. This trend was also true of CSA with uptake of the fatty acid 12-fold and 13-fold lower than CALA or CGLA, respectively. Thus, it would appear that cellular uptake of the conjugated fatty acids by MRSA is lower than that of the non-conjugated parent fatty acid. The preference of the cells for the uptake of the non-conjugated parent fatty acid would appear to be in the order of γ-linolenic/α-linolenic acid > stearidonic acid, while for the conjugated fatty acids the order would appear to be CGLA/CALA > CSA.

5.3.4 Effect of α-tocopherol on the inhibitory properties of C18 fatty acids and their conjugated derivatives against the methicillin resistant strain *S. aureus* ATCC 43300

C18 unsaturated fatty acids and in particular their conjugated derivatives are particularly prone to oxidation and their incorporation into the membrane of cells has been shown to reduce cell viability (Ganzle *et al.*, 1999). This is particularly true of cancer cells where the incorporation of conjugated fatty acids into the cell membrane is associated with increased lipid peroxidation and cell death (Suzuki *et al.*, 2001; Tsuzuki *et al.*, 2004b). In these studies it has also been shown that the

application of an antioxidant such as butylated hydroxytoluene (BHT) or α-tocopherol can reduce the inhibitory activity of these fatty acids on cell viability through the prevention of lipid peroxidation.

In our study, we assessed the impact that supplementation with 0.02 mg/ml of α-tocopherol would have on the inhibitory effects of α-linolenic acid, γ-linolenic acid, stearidonic acid, CALA, CGLA and CSA at a concentration of 0.05 mg/ml over 40 min incubation at 37^{o}C. The results demonstrated that α-tocopherol did not reduce the inhibitory properties of either the C18 unsaturated fatty acids assayed or their conjugates, but on the contrary was associated with increasing the inhibitory activity of fatty acids such as γ-linolenic acid and CSA **(Figure 5.5)**. Indeed, contrary to reducing the inhibitory activity of the conjugated and non-conjugated unsaturated fatty acids, 0.02 mg/ml of α-tocopherol increased the inhibitory activity of these fatty acids against the MRSA strain *S. aureus* ATCC 43300. The results of this study demonstrate that increased cellular lipid peroxidation as a result of the uptake of C18 unsaturated fatty acids and their conjugated derivatives is not the mechanism through which these fatty acids impart their inhibitory activity.

5.3.5 Effect of serum on the inhibitory properties of C18 fatty acids and their conjugated isomers against MRSA

It has been reported that the inclusion of blood serum into the growth medium increases the resistance of *S. aureus* to unsaturated fatty acids such as α-linolenic acid (Lacey & Lord, 1981). Of the fatty acids assayed, all, with the exception of CALA, resulted in greater reductions in cell numbers when supplemented with the fatty acid and 1% (v/v) serum than when supplemented with the fatty acid alone **(Table 5.3)**. It was, however, noted that the addition of 1% (v/v) FBS had a stimulatory effect on the growth of the strain in TSB. This was reflected in the final

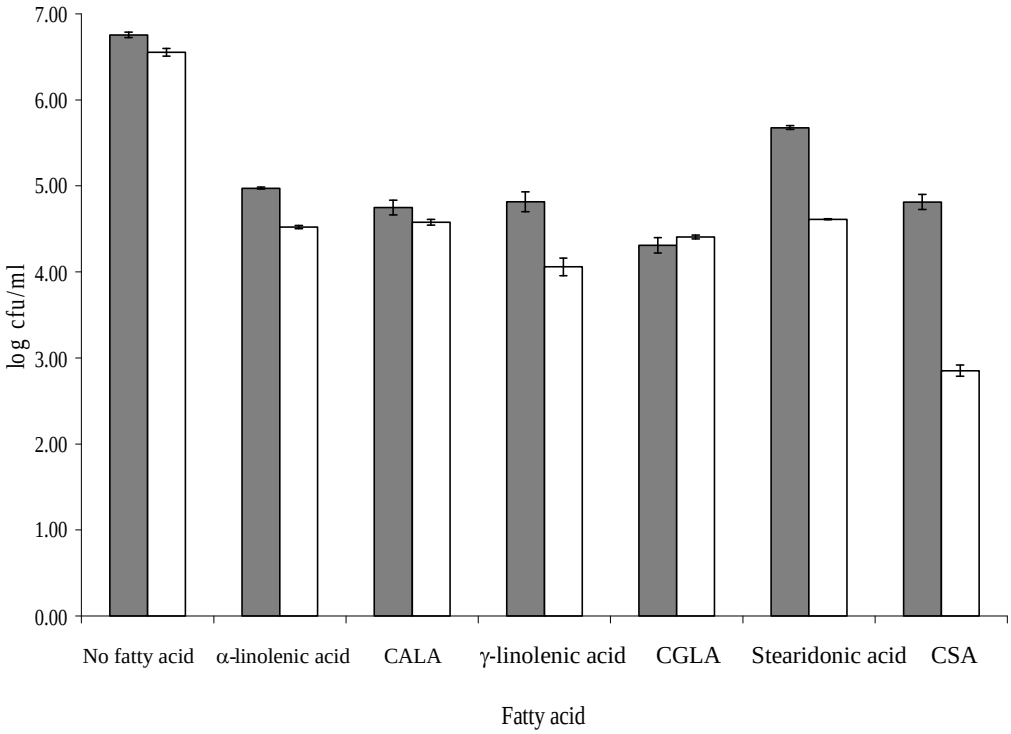

Figure 5.5 Assessing the impact of α-tocopherol on the inhibitory properties of C18 fatty acids and their conjugates against MRSA. Results represent the average of triplicate experiments. Control (■), α-tocopherol 0.02 mg/ml (□).

cell numbers which increased from 6.75 log cfu/ml in the unsupplemented control to 7.41 log cfu/ml in the medium supplemented with 1% (v/v) FBS following 40 min incubation at 37^{o}C. Thus, it would appear that the presence of FBS does not reduce the extent of the inhibitory effect of either the conjugated or the parent unsaturated fatty acid *in vitro*. However, it should be noted that FBS exerted a stimulatory effect on the growth of the strain *S. aureus* ATCC 43300 and that this may impact on the success of these fatty acids in the treatment of MRSA *in vivo*.

5.3.6 Additive inhibitory effects of γ-linolenic acid and CGLA on MRSA survival *in vitro*

At a concentration of 0.075 mg/ml the inhibitory profiles of γ-linolenic acid and CGLA against the methicillin resistant strain, *S. aureus* ATCC 43300 differ greatly. In this study, we attempted to assay if the exposure of the strain *S. aureus* ATCC 43300 to 0.075 mg/ml of a mixture of γ-linolenic acid and CGLA (50:50) (v/v) would demonstrate additive properties when compared to 0.075 mg/ml of γ-linolenic acid or CGLA individually. To achieve this, the strain was incubated for 40 min in the presence of 0.075 mg/ml of the respective fatty acids and of the mixture and incubated at 37^{o}C for 40 min with cell viability assessed by spot plate technique.

The results demonstrated that after 10 min exposure, the inhibitory activity of 0.075 mg/ml of a mixture of γ-linolenic acid and CGLA (50:50) (v/v) against MRSA was 0.7 log cfu/ml greater than that of γ-linolenic acid, but 0.7 log cfu/ml lower than that of 0.075 mg/ml of CGLA **(Figure 5.6)**. Following 40 min exposure the inhibitory effect of 0.075 mg/ml of a mixture of γ-linolenic acid and CGLA (50:50) (v/v) against MRSA was found to be 0.3 log cfu/ml lower than that of

Table 5.3 Assessing the impact of serum on the inhibitory properties of C18 fatty acids and their conjugates against MRSA. Results represent the average of triplicate experiments.

Sample	log cfu/ml (Std dev)
Control	6.75 ± 0.03
1% (v/v) foetal bovine serum	7.41 ± 0.03
0.05 mg/ml α-linolenic acid	4.97 ± 0.01
0.05 mg/ml α-linolenic acid + 1% (v/v) fetal bovine serum	5.56 ± 0.07
0.05 mg/ml CALA	4.75 ± 0.08
0.05 mg/ml CALA + 1% (v/v) fetal bovine serum	5.58 ± 0.09
0.05 mg/ml γ-linolenic acid	4.81 ± 0.12
0.05 mg/ml γ-linolenic acid + 1% (v/v) fetal bovine serum	4.80 ± 0.17
0.05 mg/ml CGLA	4.31 ± 0.09
0.05 mg/ml CGLA + 1% (v/v) fetal bovine serum	4.86 ± 0.28
0.05 mg/ml stearidonic acid	5.68 ± 0.02
0.05 mg/ml stearidonic acid + 1% (v/v) fetal bovine serum	6.07 ± 0.07
0.05 mg/ml CSA	4.81 ± 0.09
0.05 mg/ml CSA + 1% (v/v) fetal bovine serum	5.13 ± 0.38

CGLA and 1.1 log cfu/ml lower than γ-linolenic acid. Thus, it would appear that the combination of γ-linolenic acid and CGLA offers little advantage over γ-linolenic acid or CGLA individually in terms of inhibitory activity against MRSA, possessing neither the rapid action of the conjugate nor the greater inhibitory activity of γ-linolenic acid.

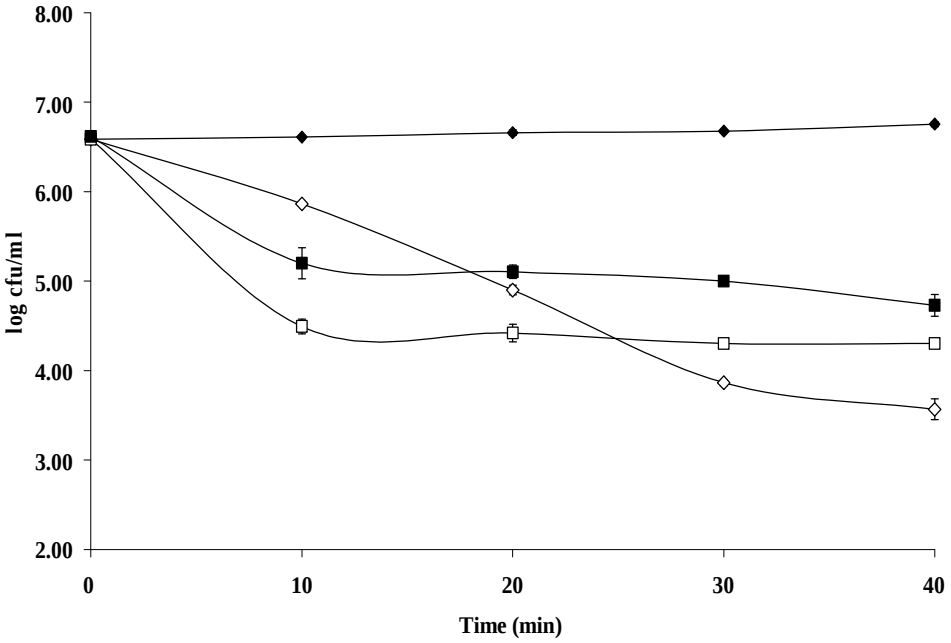

Figure 5.6 Assessment of the inhibitory activity of γ-linolenic acid and CGLA (1:1) in combination against MRSA to that of γ-linolenic acid and CGLA alone, at a fatty acid concentration of 0.075 mg/ml over 40 min incubation at 37°C. Control (♦), 0.075 mg/ml γ-linolenic acid (◊), 0.075 mg/ml CGLA (□), and 0.075 mg/ml γ-linolenic acid & CGLA (1:1) (■).Curves represent average of cultures performed in triplicate ± std dev.

5.4 Discussion

Fatty acids have long been associated with the inhibition of microbial growth. Indeed, a number of studies have associated fatty acids such as lauric acid, capric acid, linoleic acid and α-linolenic acid with the inhibition of microbes such as *Lactobacillus helveticus, Clostridium sporogenes, Bacillus subtilis,* and *Staphylococcus aureus* (Kelsey *et al.,* 2006; Laser, 1951; Nieman, 1954). The mechanisms behind the anti-microbial properties of these fatty acids are unclear, but several studies have suggested that they may be related to increases in the permeability of the cell membrane (Butcher *et al.*, 1976; Raychowdhury *et al.*, 1985) or the disruption of cell energetics (Kenny *et al*., 2009). In this study, we examined the potential of C18 unsaturated fatty acids to inhibit the growth of MRSA *in vitro.* In addition, we investigated whether the conjugated isomers of these C18 unsaturated fatty acids would possess enhanced inhibitory properties against MRSA. The production of these conjugated fatty acids from free α-linolenic acid, γ-linolenic acid and stearidonic acid has been characterized in species of bifidobacteria, propionibacteria and lactobacilli (Coakley *et al*., 2009; Ogawa *et al.*, 2005) (Chapter 3). As with the bioconversion of free linoleic acid to CLA, the conversion of α-linolenic acid, γ-linolenic acid and stearidonic acid to CALA, CGLA and CSA, respectively, most probably serves to reduce the toxicity of these fatty acids to the cultures (Coakley *et al*., 2003; Jiang *et al.*, 1998). Kelsey *et al*. (2006) recently investigated the inhibitory activity of the conjugated isomers of linoleic acid (CLA) against *S. aureus.* In the study the *c*9, *t*11 CLA isomer was found to initiate a dose dependent lag in the growth of *S. aureus* in a similar manner to linoleic acid. Given this evidence pertaining to the anti-microbial activity of conjugated fatty acids it was expected that the recently identified conjugated isomers of α-linolenic acid, γ-linolenic acid and stearidonic acid would also display

inhibitory properties against MRSA. Our results demonstrated that the conjugated isomers of α-linolenic acid, γ-linolenic acid and stearidonic acid do indeed exhibit inhibitory activity against MRSA, observing a very specific inhibitory response for both the conjugated fatty acids and their parent unsaturated parent fatty acids. Whilst the unsaturated parent fatty acids achieved inhibition through a dose dependent reduction in cell viability gradually over 40 min, the inhibitory effect of two of the conjugated isomers (CALA and CGLA) was not directly proportional to the dose level. Indeed the inhibitory activity of these conjugated fatty acids was characterized by an almost immediate (≤ 10 min) 2 log cfu/ml reduction in cell viability after which time further reductions in cell viability were not observed. The inhibitory of CSA, against MRSA was similar to that of CALA and CGLA and was characterized by a rapid reduction (≤ 10 min) in cell viability after which time further reductions in cell viability were not observed. However, the inhibitory activity of CSA was characterized by a dose dependent increase in inhibitory activity against MRSA similar to the non-conjugated parent fatty acids.

The development of anti-microbial resistance by *S. aureus* is well characterized. Indeed, the development of resistance by *S. aureus* to penicillin, methicillin, and vancomycin has been reported in detail (Fusillo *et al.*, 1953; Sievert *et al.*, 2008). In the present work the potential for the selection of *S. aureus* resistant to the anti-microbial activity of either α-linolenic acid or its conjugated derivative, CALA, was assessed by introducing a culture to seven cycles of exposure, which had been observed to reduce the viability of the strain, *S. aureus* ATCC 43300, by 2 log cfu/ml. No evidence for the development of resistance to either the conjugated fatty acid or its parent unsaturated fatty acid was found in the recovered cells. This suggests that the activity of both fatty acids was not dependent on targeting a single protein or metabolic step that could be mutated to prevent the effect.

Our research suggests that both the conjugated fatty acids and their unsaturated parent fatty acids reduce the viability of MRSA *in vitro.* However, the pattern and extent of this inhibitory activity differ substantially, which may underline major differences in the manner in which the anti-microbial activity of these two fatty acid groups is mediated. In our study, uptake of the conjugated fatty acids by the methicillin resistant strain *S. aureus* ATCC 43300 differed dramatically from that of the parent unsaturated fatty acid. We observed that after 40 minutes exposure to the fatty acids, the cellular concentrations of the conjugated fatty acids were between 2 and 2.5-fold lower than their respective parent unsaturated fatty acids. The impact of higher cellular concentrations of the unsaturated parent fatty acids on the cell could include changes in membrane fluidity and increased cellular lipid peroxidation resulting in reduced cell viability (Chamberlain *et al.*, 1991; Knapp & Melly, 1986; Tsuzuki *et al.*, 2004a), while higher concentrations of the conjugated fatty acids in the growth medium may reduce cell viability via their activity as surfactants (Pande & Mead, 1968).

Knapp & Melly (1986) related the inhibitory effect of arachidonic acid against *S. aureus* to increased cellular lipid peroxidation. Similarly, the anti-carcinogenic properties of conjugated fatty acids have also been partially attributed to increased cellular lipid peroxidation (Suzuki *et al.*, 2001; Tsuzuki *et al.*, 2004a). Despite the evidence pertaining to the role of lipid peroxidation in the inhibitory properties of conjugated fatty acids against cells we found no such evidence in our studies as the inhibitory activity of both the conjugates and their non-conjugated parent fatty acids remained unaffected by the inclusion of the natural antioxidant α-tocopherol in the growth medium.

Additive inhibitory activity between different fatty acids and lipids has previously been observed prompting us to investigate if similar additive effects

existed between our conjugated fatty acids and their parent unsaturated fatty acids (Lee *et al.*, 2002). The results of the study suggest that when supplemented at a concentration of 0.075 mg/ml the combination of conjugated fatty acids with their parent unsaturated fatty acid (50:50) neither possess the rapid inhibitory activity of the conjugated fatty acids nor the large inhibitory activity displayed by the parent unsaturated fatty acid. Indeed, the profile of inhibition displayed by the combination of γ-linolenic acid and CGLA would appear to suggest that the inhibitory activity of the combination owes more to the inhibitory activity of CGLA than γ-linolenic acid.

Strains of intestinally derived bifidobacteria and dairy propionibacteria are capable of producing conjugated fatty acids from their unsaturated parent fatty acids at concentrations in excess of those used in this study to inhibit the growth of the MRSA *in vitro* (Chapter 3). These bacteria could potentially be employed to produce CALA, CGLA and CSA enriched dairy products using fermentation based strategies similar to those being used to enrich dairy products in CLA (Hennessy *et al.*, 2009; Lin, 2003; Xu *et al.*, 2005). Indeed, given the strong evidence relating to the intestinal absorption of unsaturated and conjugated fatty acids it is likely that such a strategy could be successfully employed in the *in vivo* treatment of *S. aureus* infections of the blood and organs (Burdge & Calder, 2005; Plourde *et al.*, 2006; Tsuzuki *et al.*, 2006). Such infections can potentially lead to the development of toxic shock syndrome, necrotizing pneumonia and occasionally death. As a result of the potency of *S. aureus* and MRSA in the blood and organs it is essential that if fatty acids are to be effective against this microorganism they must remain active in the body. Previously, blood serum has been shown to inactivate the inhibitory effect of 20 mg of linolenic acid against *S. aureus* (Lacey & Lord, 1981). In our study, we found that the inclusion of 1% (v/v) FBS in the growth medium significantly increased the growth of the MRSA strain assayed (approx 0.66 log cfu/ml). The

viability of *S. aureus* when exposed to both the conjugated and unsaturated fatty acids was found to be greater in the presence of FBS, however, this increased viability was found to be related more to the stimulatory effect of FBS on the growth of the strain rather than any reduction in inhibitory effect. Indeed, with the exception of CALA, the reductions in cell viability following exposure to the fatty acids were marginally greater in the presence of FBS. Based on these results it may be concluded that the inhibitory properties of both the conjugated fatty acids and their parent unsaturated fatty acid were not compromised by the presence of serum but rather that serum itself promotes the growth of *S. aureus.*

In addition to the potential of conjugated fatty acids in the treatment of MRSA infections in the blood and organs, production of these conjugated fatty acids by strains of bifidobacteria and propionibacteria may also serve to combat the increasing incidence of intestinal *S. aureus* infection in infants and hospitalized patients (Dupeyron *et al.*, 2001; Lindberg *et al.*, 2000; Ray *et al.*, 2003). As natural residents of the human gastro-intestinal tract bifidobacteria may be capable of producing conjugated fatty acids active against *S. aureus* in the intestine from dietary α-linolenic acid, γ-linolenic acid and stearidonic acid (Chapter 3) (Fan & Chapkin, 1998; Li *et al.*, 2003; Whelan, 2009). Indeed further credence is given to this theory in light of the recent evidence pertaining to the *ex vivo* and *in vivo* production of CLA from dietary linoleic acid (Ewaschuk *et al.*, 2006; Wall *et al.*, 2009). Furthermore, Vesterlund *et al.* (2006) recently demonstrated how strains of lactobacilli and propionibacteria can be used to reduce the numbers of *S. aureus* in the intestine, suggesting a prominent role for organic acids produced by the strains in the reductions. These observations might suggest that the production of conjugated fatty acids by strains of bifidobacteria and propionibacteria could serve

not only to reduce *S. aureus* infections in the blood and organs but may also have a probiotic function in the intestine in controlling the colonisation of *S. aureus*.

5.5 Conclusions

The unsaturated fatty acid α-linolenic acid, γ-linolenic acid and stearidonic acid have been demonstrated to possess potent anti-microbial activity against MRSA *in vitro.* The anti-microbial activity of these unsaturated fatty acids can be modulated by their conjugation by certain bifidobacteria to yield CALA, CGLA and CSA, respectively. Both the parent unsaturated fatty acids and their conjugated derivatives remain active in the presence of blood serum. Previous *in vivo* studies have extensively reported the intestinal absorption of conjugated fatty acids, thus, it is likely that both the unsaturated fatty acids and their conjugated isomers may have potential in the treatment of MRSA infection. Furthermore, the production of conjugated fatty acids with inhibitory activity against *S. aureus* by intestinal bifidobacteria may serve as a probiotic trait combating the increased incidence of intestinal *S. aureus* infection.

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