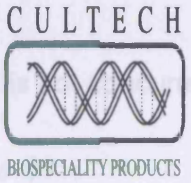

The Effects of Garlic on the Susceptibility of MRSA to β-lactam Antibiotics

By
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I would not have got this far were it not for a tremendous amount of support from a number of people.

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Publications

Wood, J., Plummer, S., Garaiova, I., Müller, C. and Lloyd, D. Review: The antibacterial effects of garlic and its sulphur containing compounds. IN PREPARATION

Wood, J., Cottrell, S.L., Plummer, S., Garaiova, I., Hann, A.C., Müller, C. and Lloyd, D. Bacteriostatic and inhibitory effects of garlic against *Escherichia coli*, *Staphylococcus aureus* and *Lactobacillus casei*. IN PREPARATION

Wood, J., Plummer, S., Garaiova, I., Müller, C. and Lloyd, D. *In vitro* reduced susceptibility of *S. aureus* to garlic through repeated sub-culture in sub-inhibitory concentrations. IN PREPARATION

Wood, J., Plummer, S., Garaiova, I., Müller, C. and Lloyd, D. The effects of garlic on the growth of MRSA and *Staphylococcus aureus*. IN PREPARATION

External poster presentations

Wood, J., Garaiova, I., Plummer, S., Müller, C., Day, M. and Lloyd, D. *The effect of garlic on the sensitivity of meticillin-resistant Staphylococcus aureus to oxacillin.* Society for General Microbiology (SGM) 162nd Meeting – Edinburgh International Conference Centre, UK, 31st March – 3rd April 2008

Norville, P., Wood, J., Garaiova, I., Plummer, S., Müller, C., Day, M. and Lloyd, D. *Different responses to combinations of green tea and oxacillin exhibited by Staphylococcus aureus small colony variants and their parents.* 13th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI) – Cairns Convention Centre, Australia, 7th – 10th September 2008

Wood, J., Garaiova, I., Plummer, S., Müller, C., Day, M. and Lloyd, D. *In vitro production of antimicrobial resistance in a methicillin-sensitive strain of Staphylococcus aureus.* 13th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI) – Cairns Convention Centre, Australia, 7th – 10th September 2008

Abstract

It is well recognised that resistance to antibiotics is a major global health problem which is increasing in significance year on year. Many authorities believe it to be the biggest healthcare issue of the 21st century. In particular, methicillin-resistant *Staphylococcus aureus* (MRSA) is a major clinical problem, particularly in the UK.

Plant-derived products, and particularly garlic, have been recognised as having antimicrobial activity for centuries. This traditional use has now been substantiated by credible science. Recent studies have begun to investigate whether there is any synergism between clinically-employed antimicrobials and plant-derived products, which could, in theory, make use of our currently redundant antibiotics

In this study sub-MIC concentrations of garlic (MICs 1-2 mg mL^{-1}) were shown to potentiate oxacillin susceptibility (MIC $\leq$ 4 $\mu g\ mL^{-1}$) against MRSA, and increase the sensitivity of MRSA to penicillin. Results suggest that increasing antibiotic susceptibility was as a result of the cumulative effects of the garlic and antibiotic rather than interaction between the two. It therefore appears that garlic does not exhibit the same mechanism of action as oxacillin or penicillin. Low concentrations of garlic (<0.5 mg mL^{-1}), however, decreased the susceptibilities of MRSA and *S. aureus* to oxacillin or penicillin. Garlic caused thickening of the primary Staphylococcal cell wall, but did not damage the cell wall integrity.

This is the first study to show *in vitro* decreasing sensitivity to garlic through repeated sub-culture in sub-inhibitory concentrations (0.5x MIC). Such isolates exhibited morphological changes but did not differ from control cultures in terms of growth dynamics. Acquired resistance to garlic has not previously been reported, suggesting these data are worthy of further investigation.

The present study lends further support to the continued investigation into potentiation of antibiotic susceptibility in antibiotic-resistant organisms by phytochemicals. The results presented here do however suggest that these interactions may be more complex than anticipated and that low concentrations of phytochemicals could potentially exacerbate the problem of antibiotic-resistance.

Abbreviation	Term in full / Definition
AA	Allyl alcohol
BSAC	British Society for Antimicrobial Chemotherapy
C. albicans	*Candida albicans*
CFU	Colony forming units
DADS	Diallyl disulphide
DATS	Diallyl trisulphide
E. coli	*Escherichia coli*
FGP	Freeze-dried garlic powder
FIC	Fractional Inhibitory Concentration
FICi	Fractional Inhibitory Concentration Index
Garlic (except in **1 – Introduction**)	Aqueous suspensions made from FGP
LAB	Lactic Acid Bacteria
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth (2% w/v NaCl)
MHSC	Mueller-Hinton agar (2% w/v NaCl)
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NA	Nutrient agar
NaCl	Sodium chloride
NB	Nutrient broth
OD	Optical density
PBS	Phosphate buffered saline
Ps. aeruginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus*

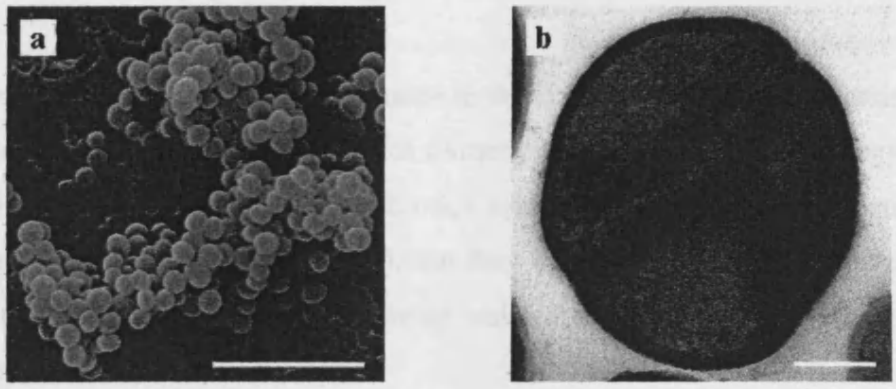
1 – General Introduction

1.1 – *Staphylococcus aureus* and MRSA

1.1.1 – Morphology

Staphylococcus aureus (*S. aureus*) is a Gram-positive, spherical bacterium approximately 0.6 – 0.8 μm in diameter that grows as irregular aggregates of cells (**Fig. 1.1**). *S. aureus* is a human commensal and asymptomatically inhabits the skin and mucosal surfaces of many warm-blooded vertebrates. It is estimated that *S. aureus* transiently, or persistently, colonises the nostrils of 18-38% of adults (LaPlante, 2007). *S. aureus* are opportunistic pathogens that cause diseases of varying severity; from minor skin and wound infections (impetigo, cellulitis, boils) through to life-threatening diseases such as toxic shock syndrome, meningitis, septicaemia and bacteraemia. The majority of *S. aureus* strains are β-haemolytic and coagulase-positive, although coagulase-negative *S. aureus* is an increasing clinical problem and can lead to false identification in routine MRSA screening in clinical laboratories (Olver *et al.*, 2005).

Fig. 1.1 – The morphology of *Staphylococcus aureus*



Scanning and transmission electron micrographs (**a** and **b**, respectively) of *Staphylococcus aureus* exhibiting typical morphologies; organisms grow in irregular 'grape-like' clusters (**a**) and are spherical with a well defined cell wall (**b**). Note the presence of two daughter cells which have not yet split apart (**b**). Scale bars and magnification: 5 μm, x 20,000 (**a**) and 200 nm, x 63,000 (**b**). Micrographs by J Wood, 2009

The species name, aureus, is derived from the Latin for gold, and refers to the orange / yellow pigment associated with many strains. Over 90% of clinical isolates are pigmented (Chamberlain *et al.*, 1991), although long-term storage can give rise to colonies with sectored pigmentation, or pigmentation can be lost completely (Servin-Masseiu, 1961).

1.1.2 – Methicillin-resistant *Staphylococcus aureus* (MRSA)

Methicillin-resistant *S. aureus* (MRSA) refers to strains that have acquired resistance to methicillin, a semi-synthetic β-lactam antibiotic. Typically the acquired β-lactam resistance mechanisms of MRSA strains mediate cross-resistance to almost all β-lactam antibiotics.

Clinicians consider MRSA a lethal organism, capable of killing a patient in one to two days (Schentag, 2001). It is important to highlight however that MRSA and *S. aureus* do not differ in terms of virulence, or the infections and diseases they cause (Frees *et al.*, 2005). Under normal circumstances, a healthy immune system is sufficient to halt the progression of a Staphylococcal infection. In the elderly, young, or immuno-compromised however, antibiotics would also be required. If the infection is caused by an MRSA strain, the majority of available antibiotics are ineffective and therefore the infection becomes life-threatening.

Today, more than 90% of *S. aureus* strains in the U.S.A express acquired resistance to penicillin and other β-lactam antibiotics (American Society for Microbiology, 1995). There are currently few effective antibiotics available to treat MRSA infections (for example vancomycin and teicoplanin), but they are expensive and have undesirable side effects and clinical strains expressing resistance are increasingly being isolated (Smith *et al.*, 1999).

1.2 – Antibiotic resistance

1.2.1 – Antibiotic resistance mechanisms and β-lactam resistance in MRSA

Antibiotic resistance refers to either an innate or acquired ability of an organism to survive or grow with concentrations of antibiotic that are inhibitory or lethal to the rest of a population (Russell, 2003). Bacterial antibiotic resistance mechanisms can be grouped into four categories: inactivation of the antibiotic, modification of the antibiotic-target site, alteration of the metabolic pathway, or reduced accumulation of the antibiotic (usually by active efflux). Resistance to β-lactam antibiotics is mediated by both inactivation of the antibiotic and alteration of the target-site.

β-lactam antibiotics (penicillins, cephalosporins and carbopenems) target bacterial cell wall synthesis. Specifically, β-lactams target the enzymes (the so-called penicillin-binding proteins; PBPs) which catalyse cross-linking between peptidoglycan strands in the bacterial cell wall. The β-lactam ring, the active site common to those groups of antibiotics, is homologous to the binding-site of the PBPs. The antibiotic therefore binds to the PBPs, altering the active sites and thus reduces the extent of cross-linking. Cell death is as a result of poorly formed cell walls (although bacteria continue to grow and begin division; Labischinski, 1992). As previously mentioned, there are two β-lactam resistance mechanisms; activation or induction of β-lactamase enzymes and expression of PBP2a, which confers resistance to β-lactamase stable antibiotics. Penicillin inactivating enzymes, β-lactamases, were first recorded in 1940 (Abraham *et al.*, 1941), before penicillin had become commercially available (**Fig. 1.2**). β-lactamases cleave the β-lactam ring, rendering the antibiotic ineffective and PBPs uninhibited. As a consequence, β-lactamase stable antibiotics (i.e. antibiotics in which the β-lactam ring was protected from cleavage), for example methicillin and oxacillin, were developed and produced commercially (Enright *et al.*, 2000). Resistance to those antibiotics is mediated by expression of an altered PBP (PBP2a) which has a much lower affinity for the antibiotics, thus efficiently catalysing peptidoglycan cross-linking even at high antibiotic concentrations (Labischinski, 1992). MRSA is a generic term, referring to those strains carrying *mecA*, the gene encoding PBP2a (Hiramatsu, 1998) although most strains also express β-lactamase (Hackbarth and Chambers, 1993). The *mecA* gene is carried by a large mobile genetic element, the so called Staphylococcal cassette

chromosome *mec* (SCC*mec*; Oliveira *et al.*, 2002) of which four different types have been identified. In addition to *mecA* the SCC*mec* element carries genes (the presence of which varies between SCC*mec* types) which are integration sites for other antibiotic resistance determinants, therefore allowing the acquisition of multiple antibiotic-resistances (Ender *et al.*, 2004). Throughout the present study the term "methicillin resistance" implies resistance to β-lactamase stable β-lactam antibiotics conferred by expression of PBP2a.

1.2.2 – Heterogeneous methicillin resistance in MRSA

In many Staphylococcal populations, methicillin resistance is not homogeneous. There exists, in each population, not only sensitive organisms with normal growth characteristics, but also slower growing methicillin-resistant organisms that prefer lower temperatures and higher NaCl concentrations (Sutherland and Rolinson, 1964). This heterogeneity has caused problems in clinical laboratories; the resistant population is not evident on solid agar after the standard 24 h growth period. This results in false negative results (McDougal and Thornsberry, 1984). An incubation period of 48 h can overcome the problem of heterogeneous methicillin resistance, but this extended period for growth is not required if a hypertonic medium is used (Drew, 1972).

Theoretically, increasing the agar NaCl concentration favours either the expression of methicillin resistance through increased synthesis of PBP2a, or provides protection against lysis (Chambers and Hackbarth, 1987; Montanari, 1990). There is, however, some disagreement regarding the required NaCl concentration. Whilst Barber (1964) and Hewitt *et al.* (1969) reported better growth at 5% NaCl than in media without NaCl, Jones (1997) cautioned against using NaCl concentrations >2.5% because of lag phase extensions with some strains. For the purposes of this study, 2% NaCl (w/v) was added to all media for MRSA and *S. aureus* unless otherwise stated, as specified by the Clinical Laboratory Standards Institute (CLSI, 2007).

1.2.3 – Antibiotic resistance: a global heath problem

Antibiotic resistance mechanisms predate the antibiotic-era (Koch, 2007). The excessive and imprudent use of antibiotics has, however, facilitated the lateral transfer of those resistance mechanisms from non-pathogenic bacteria into those of clinical

and agricultural importance (Alanis, 2005; Koch, 2007). The acquisition of resistance mechanisms by clinical pathogens, particularly MRSA, has been rapid (**Fig. 1.2**). Primarily, resistance acquisition is facilitated by rapid bacterial generation times (hence mutation rates are high) and the ease of genetic exchange between bacteria. There are, however, many other factors which have accelerated the process, including the use of antibiotics as food supplements in agriculture, longer human-life expectancy with increased antibiotic use in the elderly, and a lack of effective preventive infection control measures (i.e. hand-washing; Alanis, 2005).

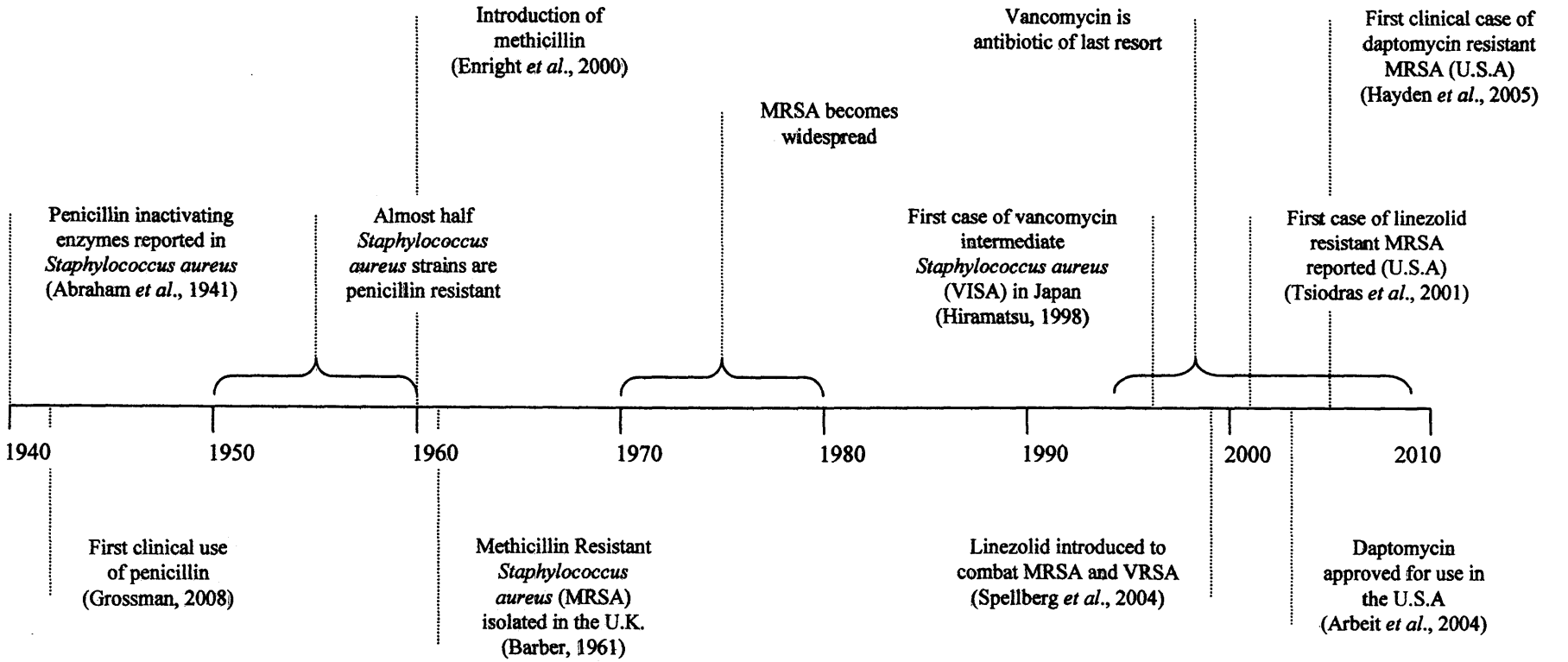
In the U.S.A alone it is estimated that approx. 14,000 people die from antibiotic-resistant, hospital-acquired infections per annum (Chea, 2005). At least one mechanism of resistance has developed to every class of commercially employed antibiotics to date. In many cases, bacteria have acquired resistance to multiple drug classes, exacerbating the treatment and raising the financial costs (Sefton, 2002; Levy and Marshall, 2004). The net results are higher mortality, morbidity, costs and, perhaps more worryingly, an ever decreasing number of effective antimicrobial agents (Alanis, 2005).

The additional financial burden resulting from antibiotic resistance is difficult to calculate. It is perhaps easier to calculate the extra costs for an individual case. Treatment of a tuberculosis infection resistant to several drugs could cost 100-fold more than traditional treatment of a susceptible infection, from U.S $20 – U.S $2000 (World Health Organisation, 2001). The extra costs relate to the prolonged hospital stay, in particular the longer duration of Intensive Care Unit admission, and the intensity of therapy (Neiderman, 2001).

In May 2005, the 58th World Health Assembly passed a resolution stating that antimicrobial resistance required urgent action, especially in view of the decreasing development of novel antimicrobials (Levy and O'Brien, 2005). The outbreak of Severe Acute Respiratory Syndrome (SARS) in 2003 caused global panic and illustrated how easily and rapidly an infectious disease, with limited therapeutic options, could spread across national borders (Zhang *et al.*, 2006). Multidrug resistant pathogens are no longer only spread locally, due to increased international travel and increased foreign trade. Worryingly, especially in light of the increase in foreign

trade, the rapid acquisition of antimicrobial resistance is more severe in the poor and developing countries due to easy access to these inappropriate antibiotics without prescription or the benefits of expert clinical advice (Alanis, 2005).

Fig. 1.2 – The global progression of acquired antibiotic resistance in *S. aureus* in relation to the development and release of antibiotics



NB – not all dates of publication correlate directly with dates of occurrence

1.2.4 – Combating antibiotic resistance

Two independent reports published by the American Society of Microbiology (ASM; 1995) and the World Health Organisation (WHO; 2001) proposed a number of strategies to try and combat, or limit, the problems associated with antibiotic resistance. The WHO report listed suggestions under the following headings:

- Increase awareness of the antibiotic resistance problem
- Improve surveillance of antibiotic resistance
- Improve antibiotic resistance in people
- Regulate antibiotic use in animals
- Encourage new product development
- Increase resources to curb antibiotic resistance in the developing world
- Increase funding for surveillance, research and education

These were largely the same recommendations as made in the ASM report seven years previously. Both reports concluded that a global surveillance programme was required and that the curricula of healthcare professions should be strengthened in the subjects of antibiotic resistance, disinfection and sterilisation in order to reduce the spread of infectious agents and should encourage a more prudent use of antibiotics (American Society for Microbiology, 1995). Despite these recommendations, there is still no global surveillance program. Surveillance programs have been created in certain countries; the UK Department of Health, for example, introduced the Mandatory Bacteraemia Surveillance Scheme in October 2005 with a target to reduce MRSA by 50% by 2008 (Finch, 2006). However, in isolation, surveillance programmes are only a means of assessing the extent of the resistance crisis. The only substantive mechanism employed to reduce the progression of resistance acquisition was the EU ban on feeding antibiotics and related drugs to livestock for growth-promotion purposes with effect from January 1st 2006 (Blondeau, 2006). This ban has not yet been applied in the U.S., where it is estimated that 70% of antibiotics (and related drugs) are actually used in animals (Blondeau, 2006).

Although it has often been suggested that a decreased use of antibiotics overall could help to slow the acquisition of antibiotic resistance (Hughs and Tenover, 1994; American Society for Microbiology, 1995; Goldman *et al.*, 1996) the best strategy appears to be strict limitation of unnecessary antibiotics and shortening of the overall course of treatment (Fagon *et al.*, 2000; Singh *et al.*, 2000). However, several studies have shown that whilst rotation of antibiotic regimes and restriction of overused antibiotics can lead to a reversal in the incidence of infections due to highly antibiotic-resistant pathogens (Kollef *et al.*, 1997; Seppala *et al.*, 1997; Gruson *et al.*, 2000), this is at the expense of increased resistance to the substituted antibiotic(s) (Meyer *et al.*, 1993; Go *et al.*, 1994; Rahal *et al.*, 1998; Landman *et al.*, 1999).

1.3 – Garlic

1.3.1 – Botany and classification

Garlic (*Allium sativum*, **Fig. 1.3**) is a herbaceous, perennial plant native to central Asia. Above-ground the plant grows resembling 'green spears' from where the name garlic possibly arose (from the Old Anglo-Saxon words "ger", meaning spear and "leek", a general term for any *Allium* plant; Hahn, 1996a). The "characteristic" part of garlic, the bulb, grows underground. The edible bulb consists of 4-20 garlic cloves tightly bound with a parchment-like skin, which is generally white to pale purple in colour (**Fig. 1.3**). Garlic not only plays an important part in the cuisine of many cultures, but has also been widely used as a preservative and medicine. Due to its popularity in cooking and medicine, garlic has high economic value.

Fig. 1.3 – A garlic bulb, garlic clove and cross-section showing the clustering of cloves within a bulb

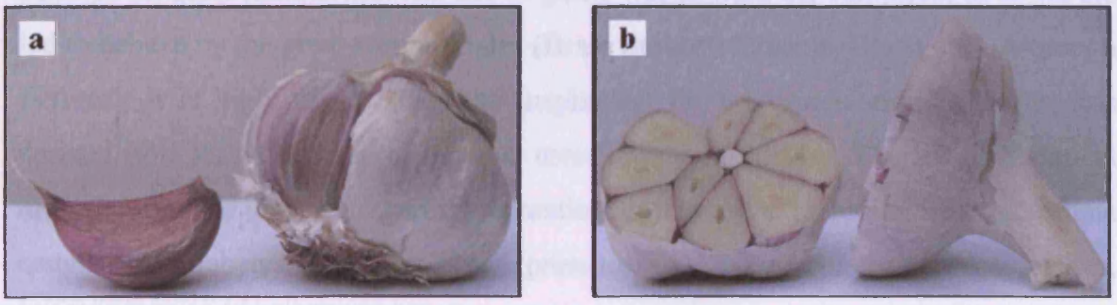


Image of a garlic bulb and an individual clove (**a**) and a cross-section of the same garlic bulb showing tightly packed bulbs (**b**). Note the parchment-like tissue enclosing the cloves and the pale purple skin which encloses each clove. Images by Wood, J., 2009

Garlic is of the genus *Allium*, as are onion (*Allium cepa*), leek (*Allium ampeloprasum*), shallot (*Allium ascalonium*) and wild garlic (*Allium ursinum*; Hahn, 1996b). *Allium* species typically form bulbs, and many species are used as spices and vegetables. *Allium* species produce cysteine sulfoxides (thought to be exclusively produced by the Alliacae family) from which they get their characteristic odours (Hahn, 1996b). Numerous species of the genus *Allium* have many reported health benefits and antimicrobial activities, and contain many of the active compounds found in garlic, although to a lesser extent.

Although "garlic" refers to the plant as a whole, for the purposes of this study (and indeed the majority of the literature citing the effects of garlic) the term refers to the bulb or the mixture of the compounds derived from it in fresh extracts unless otherwise specified.

1.3.2 – The historical uses of garlic

The earliest written records of garlic (2600 – 2100 BC) are Sumerian, and refer to garlic as a cultured plant, suggesting that its uses as a spice and medicine predate this (Hahn, 1996a). The use of garlic as both condiment and cure is documented throughout the written history of many civilisations, including the Greek and Roman (Hahn, 1996a). The remains of garlic bulbs were even found in the tomb of Tutankhamen (buried 1352 BC), which suggests garlic was held in high regard by the Ancient Egyptians (Hahn, 1996a).

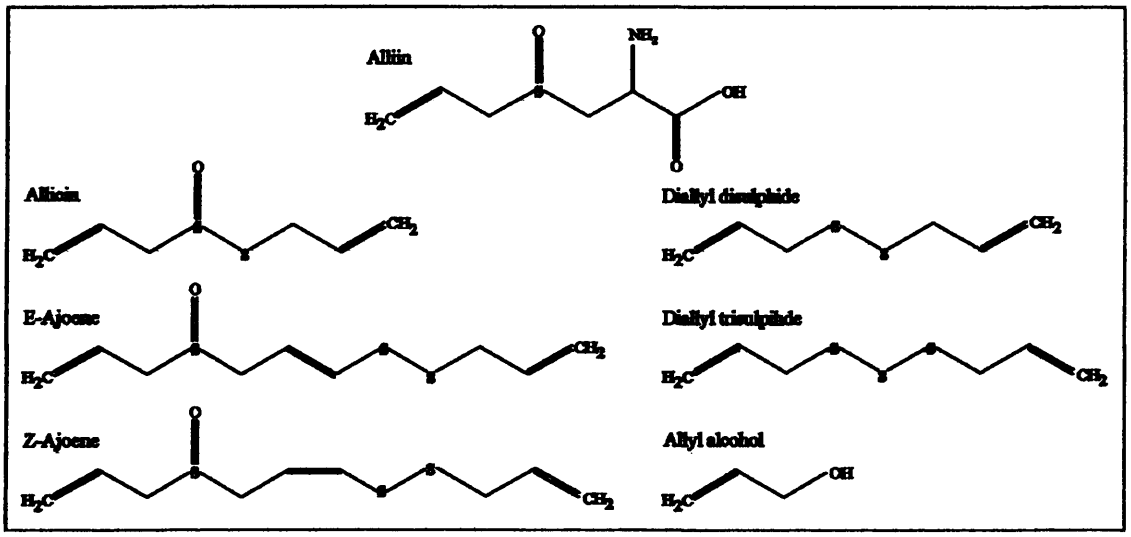
Perhaps the most famous medical use of garlic was to ward off vampires, or so we are led to believe by the great vampire tales (Bram Stoker's Dracula, Blade etc). Although fictional, it is highly likely that the inspiration for this application of garlic was derived from its wide range of medical uses throughout history. The list of historical medicinal uses of garlic and garlic preparations is extensive, including treating asthma coughs and diabetes, lowering blood pressure and cholesterol, impotence, cancer, small-pox, leprosy, epilepsy, dropsy, rheumatism, and even curing baldness as an ingredient of a topically applied lotion (Hahn, 1996a). In ancient Greece Hippocrates treated infections, wounds and intestinal disorders with garlic preparations and it is thought that the garlic was used by the first Olympic athletes as a stimulant (Whitmore and Naidu, 2000). Additionally, there are at least two reports of garlic protecting against the bubonic plague (circa 1340). It is said that the Jewish population of Basle, who regularly ate garlic, had a much lower fatality rate to the plague (Hahn, 1996a). In Marseille, gangs of men robbed the sick and dying plague victims, but did not become infected themselves. This was attributed to the regular consumption of a mixture of vinegar, wine and garlic, now known as 'Four Thieves Vinegar' (*vinaigre des quatres voleurs*; Hahn, 1996a).

1.3.4 – The sulphur chemistry of garlic

The antimicrobial properties of garlic are attributed to its complex sulphur chemistry; elimination of the volatile thiosulfinates results in a substantial loss of antibacterial and antifungal activity (Lawson, 1996). The numbers and proportions of sulphur compounds vary depending on whether the bulb is crushed, heated or intact, and on the strain and growing conditions. Garlic has very high sulphur content compared with other *Allium* species and other plants in general. Sulphur contributes 0.35% of the fresh weight of garlic (approximately 1% of the dry weight); in most other vegetables this proportion is much less (roughly 0.1%; Nielson *et al.*, 1991).

The identities of the reactive sulphur compounds of garlic were determined in investigations carried out throughout the 19th century by steam distillations. Work by Semmler (1892) on the quantification of garlic oil components led to the elucidation of the allyl group. The isolation and identification of allicin (**Fig. 1.4**) was performed by Cavallito and Bailey (1944). The structures of the major thiosulfinates in garlic are shown in **Fig. 1.4**, although the chemicals obtained, and their proportions, depend on the method of extraction, and on the factors previously mentioned.

Fig. 1.4 – Structures of some thiosulfinates from garlic with antimicrobial properties



Allicin is the major antibacterial thiosulphinate formed from alliin when garlic cloves are damaged. Ajoene (*E*- is the dominant conformation), allyl alcohol and diallyl sulphides are formed upon the decomposition of allicin under different conditions

Allicin has been identified as the major antimicrobial compound of garlic (Cavallito and Bailey, 1944) but is not present in intact bulbs. The parent compound, alliin, was identified later (Stoll and Seebeck, 1947) but has no antimicrobial properties itself. Although alliin is present at approximately 5-14 mg g^{-1} fresh weight in intact bulbs it is not detectable in crushed garlic (Lawson, 1996). Alliin is converted to allicin (and other thiosulfinates) by the enzyme alliinase, the conversion taking less than 10 s at room temperature (which explains the lack of alliin in crushed garlic; Lawson, 1996). Approximately 40% of alliin is converted to allicin, although allicin accounts for 70% of the thiosulfinate products of the reaction (pyruvic acid and ammonia are also formed; Lawson, 1996). Alliinase contributes roughly 10% of total clove protein; approximately equal to the alliin content. This perhaps explains the rapid conversion to allicin (Lawson, 1996). Enzymes are seldom present in such large amounts; therefore these reactions must be of great importance to the plant. The conversion of alliin to allicin does not occur in intact bulbs because alliinase and its substrate are physically separated, and only come into contact on damage to the garlic tissues (Lawson, 1996). This separation explains why intact garlic cloves have no distinctive

smell. Interestingly, there are reports of some bacteria (*Escherichia coli E. coli* and *Bacillus subtilis*) converting alliin to allicin *in vitro*, implying the expression of alliinase (Murkami, 1960a; 1960b). This is potentially important because both cooking and stomach acid inactivate alliinase, therefore alliin, rather than allicin, would enter the digestive tract (assuming whole cloves are consumed). These data are questionable, however, because *E. coli* (in particular), is very susceptible to allicin and garlic (see **Section 1.3.10**). It is therefore unlikely that this bacterium would catalyse a reaction producing such a harmful product.

1.3.4.1 – Allicin

Allicin (**Fig. 1.4**) exhibits excellent broad spectrum antimicrobial activity, but it is highly unstable and is only produced when a garlic clove is cut or crushed (Lawson, 1996). Allicin has a relatively short half life (2-10 h), although this is dependent on temperature, pH and the solvent in which it is kept (Moore and Atkins, 1976; Barone and Tansey, 1977; Yamada and Azuma, 1977; Caporaso *et al.*, 1983 Naganawa, 1996; San-Blas, 1997). For example, the half-life of the anti-Candidal activity of garlic extract is 15 d at room temperature (Lawson, 1996). Although allicin is very unstable, it can reportedly be stabilised by incorporation into a gel (Cutler *et al.*, 2009).

Allicin slowly reacts with itself at room temperature and is converted to diallyl disulphide (DADS; **Fig. 1.4**) and diallyl trisulphide (DATS; **Fig. 1.4**), both potent antimicrobials. During this reaction allyl alcohol (AA; **Fig. 1.4**), another antimicrobial compound, is produced as a by-product (Lawson, 1996). This conversion is accelerated at increased temperatures, such as during steam distillation (Lawson, 1996). Hence, the major antimicrobial compounds of garlic oil (produced by steam distillation) are diallyl sulphides (Lawson, 1996). Alliin, allicin and also the enzyme alliinase are all degraded by stomach acid, so there is almost no conversion to allicin in the stomach, and only a small amount of active compounds may pass through the stomach. Even in the small intestine it is unlikely that any allicin will remain active, as allicin readily reacts with cysteine (Lawson, 1996). As allicin breaks down, a cascade of sulphur containing compounds is produced some of which are potent antimicrobials (Lawson, 1996).

Decomposition of allicin can occur in two ways, resulting in the production of different antimicrobial compounds; allicin molecules either combine to form ajoene (**Fig. 1.4**), or degrade into 2-propenensulfenic acid and thioacrolein, which then decompose further to form 2- and 3-vinyldithiins.

1.3.5 – Aqueous preparations of freeze-dried garlic powder (FGP)

Despite the numerous potential antimicrobials in garlic, the present study investigated the anti-Staphylococcal effects of aqueous preparations made from freeze-dried garlic powder (FGP) as a whole. Garlic powders represent the true composition of garlic cloves better than any other preparation because they are simply peeled, dehydrated and pulverised cloves (Lawson, 1996). Even so the preparation used throughout the present study has been shown to have a lower allicin content than fresh garlic extracts (Lemar *et al.*, 2002). The major reason such preparations were used in the present study is, however, one of resistance. To date there have been no reported instances of acquired resistance to garlic, and it is unlikely that resistance could develop due to the number of antimicrobial compounds and therefore the theoretical multitude of targets. If each component was isolated and purified for medicinal use, resistance to even one of these could potentially develop rapidly. Differential kinetics of release of antimicrobial breakdown products of allicin produce a succession of inhibitory effects that overcome potential resistance mechanisms. There may also be synergistic interactions between the individual compounds in the aqueous preparation which would not occur if single compounds were employed separately. For example, the activity of garlic oil is reportedly four-times greater than expected from the activities of its constituent compounds employed separately, indicating interactions that result in enhanced activity (Reuter *et al.*, 1996). Similarly, the overall biological activity of oregano oil is as a result of its chemical complexity, and possible synergistic interactions between components acting on different cellular targets (Bouhdid *et al.*, 2009). Whilst it is known that consumption of garlic is non-toxic to humans, there is some toxicity in other mammals; dogs and cats, for example, are extremely sensitive to allicin and ajoene (Cope, 2005). Purified compounds may however cause toxicity to humans due to their use at much higher concentrations relative to those normally present in experiments using whole garlic or its derived preparations.

The use of whole garlic in antimicrobial research is however not without problems. Due to the complex chemistry potential targets are numerous and difficult to identify. The other major drawback of using whole garlic extract is differences in potency due to the variability of the raw material. Although the FGP used in the present study is produced to a minimum potency (14,000 ppm alliin), and all batches are compared with a standard, there is invariably some variation, which could give erroneous results. To try and minimise the effect of sample variability the same production batch of garlic powder was stored at low temperature (4°C) and used throughout the present study.

1.3.6 – Reported health promoting benefits of garlic

Garlic preparations are widely taken for their reported multifunctional health benefits, including the lowering of blood pressure (Loeper and Debray, 1921) and of LDL (low density lipoprotein) cholesterol levels (Gebhardt *et al.*, 1994), as well as raising fibrinolytic activity and inhibiting thrombocyte aggregation (Whitmore and Naidu, 2000). Studies have also investigated the impact of garlic or garlic compounds on cancer (Sun and Wang, 2003).

1.3.7 – Antiprotozoal effects of garlic

Garlic and garlic-derived compounds exert antimicrobial activity against a wide range of protozoal pathogens (Table 1.1). A study into the effect of garlic on *Giardia*, *Trichomonas vaginalis* and *Tritrichomonas foetus* showed that AA and aqueous preparations of FGP actively collapsed the trans-membrane electrochemical potential of the plasma membranes of those organisms (Harris, 2001). A separate study reported extensive internal and external morphological changes in *Giardia* due to the action of the same compounds (Harris *et al.*, 2000). Protozoa that have been exposed to garlic exhibited an increase in membrane permeability and fragility (Bogin, 1973). These effects which were not seen by exposure to allicin, suggesting that allicin has a different mode of action (Miron *et al.*, 2000). Ankri *et al.* (1997) determined that allicin inhibited the ability of *Entameoba histolytica* trophozoites to destroy monolayers of baby hamster kidney cells. It was also found that cysteine proteinases (amoebic virulence factors) as well as alcohol dehydrogenase were strongly inhibited by allicin.

Table 1.1 – The antiprotozoal effects of garlic derived compounds

Organism	Compound	Reference
Giardia spp.	Garlic, allyl alcohol, diallyl trisulphide	Soffar and Mokhar, 1991 Lun *et al.*, 1994 Harris *et al.*, 2000; 2002 Harris, 2001
Trichomonas vaginalis	Garlic, allyl alcohol	Harris, 2001
Tritrichomonas foetus	Garlic, allyl alcohol	Harris, 2001
Opalina ranarum	Garlic	Reuter *et al.*, 1996
Opalina dimidicita	Garlic	Reuter *et al.*, 1996
Balantidium entozoon	Garlic	Reuter *et al.*, 1996
Entamoeba histolytica	Garlic	Mirelman *et al.*, 1987 Reuter *et al.*, 1996
Trypanosomes	Garlic	Urbina *et al.*, 1993 Reuter *et al.*, 1996
Leishmania	Garlic	Reuter *et al.*, 1996
Leptomonas	Garlic	Reuter *et al.*, 1996
Crithidia	Garlic	Reuter *et al.*, 1996

NB – Garlic refers to aqueous preparations made from freeze-dried, homogenised (ground) garlic cloves (Freeze-dried garlic powder)

1.3.8 – Antifungal effects of garlic

There are studies on the effect of garlic compounds against numerous fungi. The fungistatic effects of garlic and onion juice were first reported in 1925, before allicin was determined as the major antimicrobial compound of garlic (Walker *et al.*, 1925). Ledzema *et al.* (1996) reported that ajoene could be successfully used as a short-term therapy for athlete's foot (tinea pedis). Ajoene was also inhibitory to *Aspergillus* spp. (Yoshida *et al.*, 1987) whilst DATS was effective against cryptococcal meningitis (Cai, 1991). Other fungi susceptible to garlic compounds include *Torulopsis, Trichophyton, Cryptococcus* (Fromtling and Bulmer, 1978), *Trichosporon and Rhodoturala* (Tansey and Appleton, 1975). However, the anti-fungal effects of garlic have most commonly been reported against *Candida albicans C. albicans.*

Aqueous preparations of FGP and AA affected both the growth and respiration of *C. albicans,* and both these compounds also induced oxidative stress (Lemar *et al.*, 2005; Lemar, 2005). Aqueous preparations of FGP and fresh garlic extracts both also prolonged the lag phase, and reduced growth rates during the exponential growth phase. Fresh garlic extract was found to be twice as effective as aqueous preparations of FGP, probably due to a higher allicin content (Lemar *et al.*, 2002). Two different garlic compounds, AA and DADS caused contrary cell death mechanisms in *C. albicans*. Low concentrations of AA triggered necrosis, whereas at higher concentrations cell death was apoptotic. The converse was caused by DADS (Lemar, 2005; Lemar *et al.*, 2007). Depletion of glutathione pools by DADS and consequent oxidative stress induces apoptotic and necrotic cell death in *C. albicans* (Lemar *et al.*, 2003; 2007). Depletion of mitochondrial inner membrane potential, elevation of intracellular ROS (reactive oxygen species) levels and lowered respiration, were accompanied by F_1F_0 ATPase activity. Thus the primary effects were on mitochondrial function in this highly dangerous opportunistic pathogen (Lemar *et al.*, 2003; Lemar, 2005). Aqueous preparations of FGP inhibited Candidal attachment to buccal epithelial cells and caused structural damage at low concentrations, whereas cellular integrity was severely disrupted at higher concentrations (Garaiova *et al.*, 2005). Diallyl sulphides were also reported as rigidifying Candidal membranes (Tsuchiya and Nagayama, 2008). A synergistic interaction was reported between garlic and *Lactobacillus acidophilus* against Candidal buccal epithelial attachment (Garaiova *et al.*, 2005).

1.3.9 – Antiviral effects of garlic

Although studies into the antiviral activity of garlic are not as numerous as on other organisms, garlic has proven activity against some major human viruses:

- Influenza A and B (Fenwick and Hanley, 1985)
- Cytomegalovirus (Guo *et al.*, 1993; Meng *et al.*, 1993)
- Rhinovirus (Tsai *et al.*, 1985)
- AA and DADS against HIV infected cells *in vitro* (Shoji *et al.*, 1993)
- Herpes simplex virus type 1 (Tsai *et al.*, 1985)
- Rotavirus (Rees *et al.*, 1993)

Weber *et al.* (1992) also reported *in vitro* virucidal effects of fresh garlic extract, and some compounds derived from garlic against herpes simplex virus type 1, herpes simplex virus type 2, parainfluenza virus type 3, vaccinia virus, vesicular stromatitis virus, and human rhinovirus type 2. The order of virucidal activity was ajoene > allicin > allyl methyl thiosulfinate > methyl allyl thiosulfinate (Weber *et al.*, 1992).

1.3.10 – Antibacterial effects of garlic

A huge number of pathogenic and food-spoilage bacteria are inhibited by garlic (**Fig. 1.5**). In fact, the only group of bacteria reported to be insensitive are the lactic acid bacteria (LAB), Skyrme, 1996), which form a separate clade to garlic 'sensitive' bacteria (**Fig. 1.5**). Sensitivity to garlic appears to be indicated by an MIC $\leq$5 mg mL^{-1} (e.g. *S. aureus*, *E. coli*, *Helicobacter pylori*, *Salmonella* spp.; Skyrme, 1996), whilst insensitive bacteria exhibit MICs ranging from 10 mg mL^{-1} – 40 mg mL^{-1} (e.g. *Lactobaciilus* spp., *Pediococcus pentosaceus*; Skyrme, 1996). Considering that the major antimicrobial component of garlic is allicin, it is unsurprising that the majority of studies report the inhibitory effects of aqueous preparations of FGP or allicin. There are, however, reports regarding the antibacterial effects of some other garlic compounds.

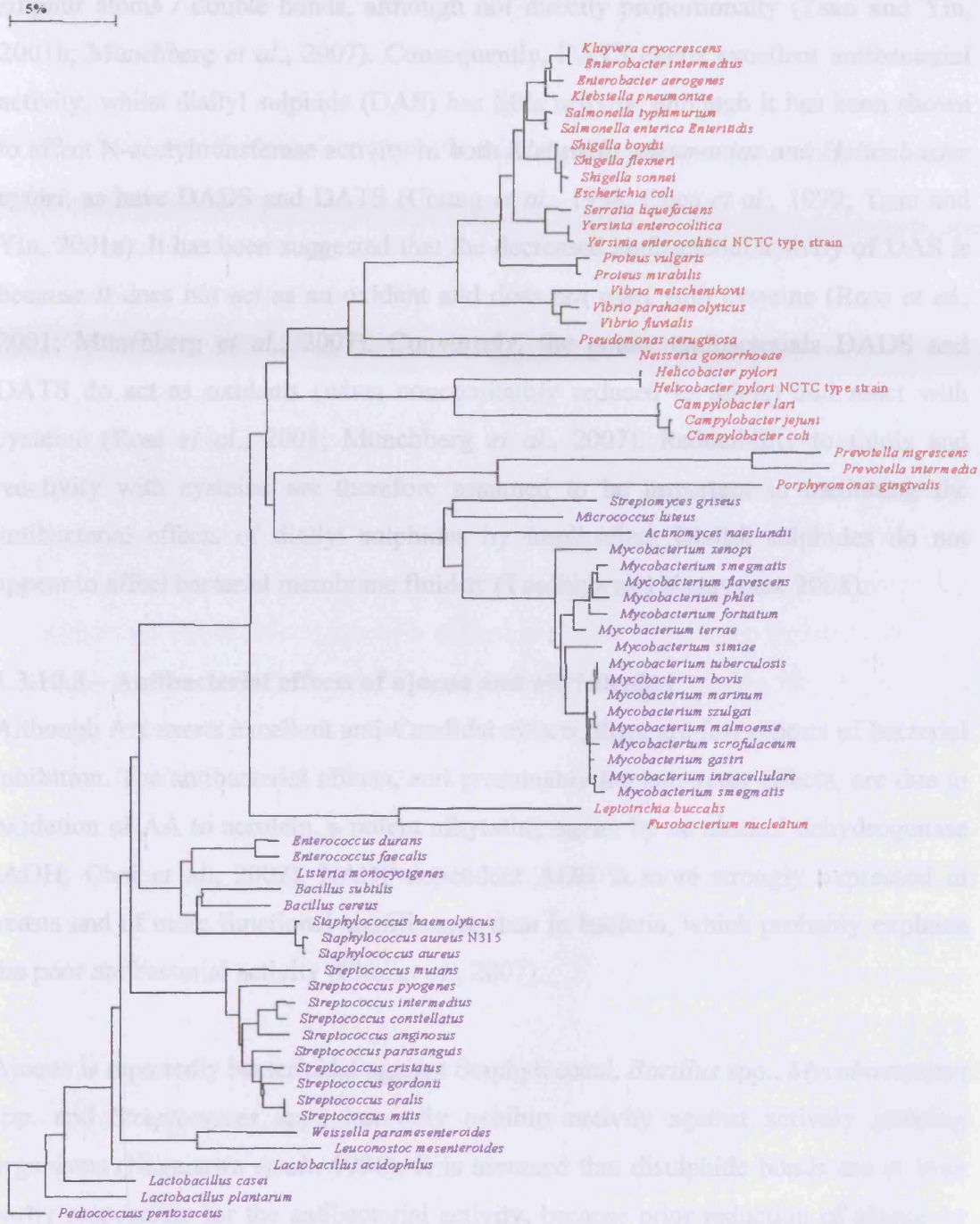
1.3.10.1 – Antibacterial effects of allicin (or garlic)

It is well documented that the major antibacterial effects of allicin are due to a rather non-specific interaction with thiol (R-SH) containing enzymes (Focke *et al.*, 1990;

Rabinkov *et al.*, 1998; Ankri and Mirelman, 1999; Curtis *et al.*, 2004; Choi *et al.*, 2007). There are numerous thiol-containing enzymes inhibited by allicin, including xanthine oxidase, urease, cysteine proteases and alcohol dehydrogenases (Curtis *et al.*, 2004; Choi *et al.*, 2007). Furthermore, there is an apparent correspondence between the concentrations of allicin at which these enzymes were inhibited and the range of concentrations at which allicin exerts its bactericidal effects (Wills, 1956). Binding to thiol containing enzymes appears to be non-covalent and reversible (Feldberg *et al.*, 1988; Focke *et al.*, 1990; Ankri and Mirelman, 1999). The interaction with thiol groups has been attributed to the sufinyl group of allicin (-S(O)-S-), and was not shown for –S-, -S-S- or –SO- groups (Choi *et al.*, 2007). This inhibition of thiol groups also correlates with an inhibition of RNA synthesis by garlic or allicin (Feldberg *et al.*, 1988; Ankri and Mirelman, 1999; Choi *et al.*, 2007). In cultures of *Salmonella typhimurium* treated with allicin there was a 15 min lag (independent of allicin concentration or culture density) between addition of allicin and inhibition of growth – this time may correlate with that between the completion of chromosome replication and cell division. These results suggested that the division of organisms which had completed DNA synthesis was not affected, whereas those undergoing replication were inhibited (Feldberg *et al.*, 1988). Furthermore, DNA and protein synthesis were partially inhibited by allicin, but recovered to control levels within 40 min. Conversely, RNA synthesis was inhibited immediately after allicin addition and after 40 min was still inhibited to 90% of control values (Feldberg *et al.*, 1988). Considering that bacterial RNA polymerase contains a thiol group, it is reasonable to assume this is a target of allicin (Feldberg *et al.*, 1988; Ankri and Mirelman, 1999).

Allicin has also been proposed as targeting the bacterial cell wall (Cottrell, 2003). It would therefore be reasonable to assume Gram-negative bacteria would be more susceptible to garlic than Gram-positives due to their thinner, and hence more osmotically fragile, cell walls. However, Skyrme (1996) reported that *S. aureus* was more sensitive to garlic than *E. coli*. This is possibly because *E. coli* has a ten-fold higher cell wall lipid content than *S. aureus*. It is therefore suggested that allicin may become trapped in the lipids, or interact with them, resulting in reduced allicin uptake, and therefore decreased inhibition (Fujisawa *et al.*, 2008). Other effects of allicin include the inhibition of ^{14}C acetate incorporation into fatty acids (Ankri and Mirelman, 1999).

Fig. 1.5 – Phylogenetic tree of bacteria which have been tested for susceptibility to garlic (Wood, J., unpublished)



16S phylogenetic tree showing sequence diversion between bacteria for which the MIC of garlic has been assessed. TREECON was used to construct this tree (Van de Peer and De Wachter, 1994). Organisms highlighted in red indicate Gram-negative bacteria; organisms highlighted in purple indicate Gram-positive bacteria. This tree is rooted with *Pediococcus pentosaceus* (a lactic acid bacterium). Scale bar represents percentage homology

1.3.10.2 – Antibacterial effects of diallyl sulphides

The antibacterial activity of diallyl sulphides increases with an increasing number of sulphur atoms / double bonds, although not directly proportionally (Tsao and Yin, 2001b; Münchberg *et al.*, 2007). Consequently, DATS exerts excellent antibacterial activity, whilst diallyl sulphide (DAS) has little activity, although it has been shown to affect N-acetyltransferase activity in both *Klebsiella pneumoniae* and *Helicobacter pylori*, as have DADS and DATS (Chung *et al.*, 1998; Chen *et al.*, 1999; Tsao and Yin, 2001a). It has been suggested that the decreased antibacterial activity of DAS is because it does not act as an oxidant and does not react with cysteine (Ross *et al.*, 2001; Münchberg *et al.*, 2007). Conversely, the potent antibacterials DADS and DATS do act as oxidants (when concomitantly reduced to thiols) and react with cysteine (Ross *et al.*, 2001; Münchberg *et al.*, 2007). Reducibility to thiols and reactivity with cysteine are therefore assumed to be important in mediating the antibacterial effects of diallyl sulphides by implication. Diallyl sulphides do not appear to affect bacterial membrane fluidity (Tsuchiya and Nagayama, 2008).

1.3.10.3 – Antibacterial effects of ajoene and allyl alcohol

Although AA exerts excellent anti-Candidal effects, there are few reports of bacterial inhibition. The antibacterial effects, and presumably the anti-yeast effects, are due to oxidation of AA to acrolein, a potent alkylating agent, by an alcohol dehydrogenase (ADH; Choi *et al.*, 2007). NADP-dependent ADH is more strongly expressed in yeasts and of more functional significance than in bacteria, which probably explains the poor antibacterial activity (Choi *et al.*, 2007).

Ajoene is reportedly bactericidal against Staphylococci, *Bacillus* spp., *Mycobacterium* spp. and *Streptomyces* spp., but only exhibits activity against actively growing organisms (Naganawa *et al.*, 1996). It is assumed that disulphide bonds are at least partly responsible for the antibacterial activity, because prior reduction of ajoene by cysteine abolishes the antibacterial activity (Naganawa *et al.*, 1996). However, *E*-ajoene (**Fig. 1.4**) has inferior activity to *Z*-ajoene (a structural isomer), which suggests that the configuration about the double bond is also important for activity (Yoshida *et al.*, 1999). The reaction of allicin with cysteine was partly attributed to the sufinyl group (-S(O)-S) as discussed previously. Ajoene, like allicin, possesses a sufinyl

group, which suggests that this may also be important for antibacterial activity (Naganawa *et al.*, 1996).

1.4 – Increases in turbidity as a measure of growth of organisms in liquid cultures

In the present study, estimations of bacterial growth were often determined from absorbance measurements. Indirect measurement of growth using turbidity is rapid, non-destructive and relatively inexpensive (Augustin *et al.*, 1999; Dalgaard and Koutsoumanis, 2001; Lindqvist, 2006). Traditional estimations of growth parameters using viable count determinations require large numbers of replicates, and are both time-consuming and very labour intensive (Augustin *et al.*, 1999; Francois *et al.*, 2005). It is generally assumed that there is a proportional relationship between optical density (OD) and viable counts (Augustin *et al.*, 1999). However, turbidity-based methods have numerous limitations. Primarily, there is concern regarding the relationship between absorbance and bacterial numbers, particularly when assessing the effects of stress-inducing agents (Francois *et al.*, 2005; Lindqvist 2006). For example, changes in OD may be detected as a result of increases in cell size, cell shape or cellular debris rather than cell division. Furthermore, OD is not a valid measure of viability because both live and dead cells absorb and scatter light. Many studies have attempted to define the relationship between OD values and viable counts using a series of calibration curves (Augustin *et al.*, 1999; Lindqvist, 2006). In the present study, however, such an approach would negate the purpose of using absorbance as a measure of growth. Considering that the present study investigated the combined effects of two inhibitory compounds on the growth of *S. aureus*, and that both compounds theoretically affect cellular morphology or result in progressive accumulation of cellular debris as a consequence of damage and release of contents, it would be necessary to perform calibrations for every combination tested.

In an attempt to minimise the limitations of using an indirect method to measure bacterial growth, the following steps were taken:

1) Calibration curves were obtained by assessing the viable count of suspensions of known OD using an adapted Miles and Misra plate count technique (Miles and Misra, 1938). This confirmed a general correlation between increasing OD and increasing viable counts (although only for control cultures). These are provided in **Appendix**.
2) Viable counts were also routinely performed on Bioscreen "wells" at random after 24 h to correlate increases in OD with increases in viable count from starting populations.
3) Estimations of bacterial growth parameters from absorbance data were only compared within the present study, and not with published data. Furthermore, "growth rates" were presented as the rate of change of OD per h when the culture was growing most rapidly rather than specific growth rates.
4) Negative (i.e. uninoculated) controls were performed to minimise the effects of any increase in the OD of test media over time.

2 – General Materials and Methods

Specific materials and methods are discussed in the relevant Chapters. Methods were designed using adapted CLSI guidelines: "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically" (CLSI, 2007). General materials and methods were as follows.

2.1 – Strains and isolates

Strains and isolates of *S. aureus* were donated as indicated (Table 2.1). All strains and isolates were maintained at –80°C on Cryobeads™ (Pro-Lab, Cheshire, UK) and were re-isolated when required onto Mueller-Hinton (2% NaCl) agar plates. *E. coli* NCTC 10418 was from Cultech Ltd and was maintained and re-isolated as above, except that nutrient agar and nutrient broth were used.

Table 2.1 – Description of thesis strains / isolates

Thesis isolate	Description of isolate	Antibiotic resistance	Reference
EMRSA-15[a]	MRSA reference strain	Aml, Amc, Amp, Ctx, Cro, Cxm, Ox, P	Richardson and Reith, 1993
EMRSA-16[a]	MRSA reference strain	Aml, Amc, Amp, Cip, Clr, E, Ox, P	Cox *et al.*, 1995
MRSA N315[b]	MRSA reference strain	Aml, Amc, Amp, Ctx, Cro, Cxm, Clr, E, Ox, P	Kuroda *et al.*, 2001
MRSA JW4[a]	Clinical isolate	Aml, Amc, Amp, Ctx, Cro, Cxm, Cip, Clr, E, Ox, P	N/A
MRSA JW6[a]	Clinical isolate	Aml, Amc, Amp, Ctx, Cro, Cxm, Ox, P	N/A
MRSA 4500[c]	Clinical isolate	Aml, Amc, Amp, Ctx, Cro, Cxm, Ox, P	N/A
NCTC 6571[a]	Antibiotic susceptibility testing reference strain	None	Heatley, 1944
RN450[c]	*S. aureus* reference strain	None	Novick, 1967

Strains / isolates kindly donated by Prof. Rose Cooper, UWIC, UK[a]; Dr. Paul Seaman, Cardiff University, UK[b]; Obsidian Research Ltd, Port Talbot, UK[c]. Antibiotics tested: Amoxicillin (Aml), amoxicillin / clavulanic acid (Amc), Ampicillin (Amp), Ceftoxamine (Ctx), Ceftriaxone (Cro), Cefuroxime (Cxm), Ciprofloxacin (Cip), Clarithromycin (Clr), Erythromycin (E), Gentamicin (Gen), Mupirocin (Mup), Oxacillin (Ox), Penicillin (P), Rifampicin (Rif), Tetracycline (Tet), Vancomycin (Va). N/A indicates that no reference is available for this isolate

2.2 – Media

Mueller-Hinton agar (MHA; Oxoid, Hampshire, UK) and Mueller-Hinton broth (MHB; Oxoid, Hampshire, UK) were made according to manufacturer's instructions with the addition of 2% w/v NaCl according to CLSI guidelines (CLSI, 2007), unless otherwise stated. Phosphate buffered saline (PBS; Oxoid, Hampshire, UK) was made according to manufacturer's instructions. Throughout all experiments MHSC refers to MHA with 2% (w/v) NaCl. MHB was always made with 2% (w/v) NaCl. Nutrient agar (NA; Oxoid, Hampshire, UK) and nutrient broth (NB; Oxoid, Hampshire, UK) were made according to manufacturer's instructions. Incubation, unless otherwise stated, was at 37°C, without agitation, for 24 h.

2.3 – Preparation of aqueous garlic solutions

Freeze-dried garlic powder (FGP; Batches 8711 A and C) was kindly provided by Cultech Ltd and stored at 4°C. Aqueous preparations made from FGP are henceforth known as garlic. Stock solutions of garlic were prepared from FGP when required as follows. FGP was suspended in sterile MHB to required concentrations and vortexed for 10 min, and then left for 10 min at room temperature. This solution was centrifuged at 3900 x *g* for 20 min, and the resulting supernatant passed through 0.2 μm sterile filters and diluted to required concentrations with sterile MHB.

Stock solutions of garlic were frozen and maintained at -80°C, and were thoroughly defrosted prior to use. Freezer stocks were replaced every four weeks.

2.4 – Preparation of oxacillin and penicillin solutions

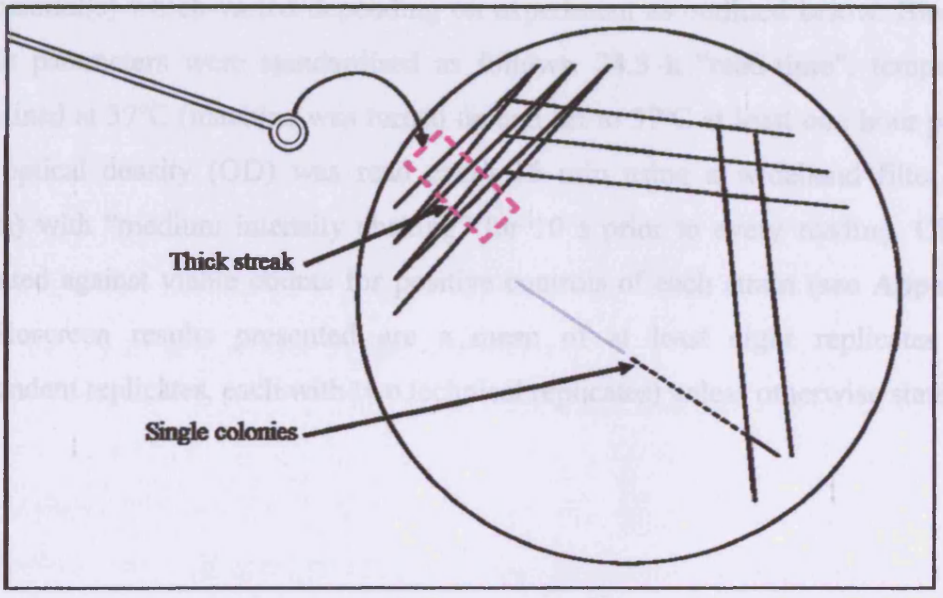
Oxacillin (Sigma-Aldrich, Poole, UK) and penicillin G (Sigma-Aldrich, Poole, UK) were stored at 4°C and made into suspensions when required as follows: antibiotic powders were suspended in MHB to required concentrations and vortexed for 10 min, and then left for 10 min at room temperature. The solutions were passed though 0.2 μm sterile filters and diluted to required concentrations with sterile MHB.

Stock solutions of oxacillin and penicillin were frozen and maintained at -80°C, and were thoroughly defrosted prior to use. Freezer stocks were replaced every four weeks.

2.5 – Inocula (based upon CLSI guidelines; CLSI, 2007)

Unless otherwise stated, inocula were standardised as follows. Prior to preparation of inocula (24 h), one cryobead was streaked out onto a fresh MHSC plate and incubated. A loopful of culture (from the "thick streak", rather than single colonies – **Fig. 2.1**) from the 24 h MHSC plate was transferred into 10 mL MHB and incubated for 2-6 h. The absorbance (630nm; Dynamax; ThermoFisher, Leicestershire, UK) was adjusted to 0.5 McFarland using sterile MHB ("optically adjusted inocula"). This equated to a cell density of approx. 1 x 10^8 CFU mL^{-1}. Viable cell counts (**Section 2.6**) were performed on all inocula to confirm initial cell densities.

Fig. 2.1 – Area of streak plates used for preparation of inocula



Inoculation was made from the "thick streak" (red dotted area) rather than from single colonies to reduce the possibility of selecting mutants. Single colony morphology and characteristics were routinely examined for purity of cultures

2.6 – Determination of viable counts (plate counts)

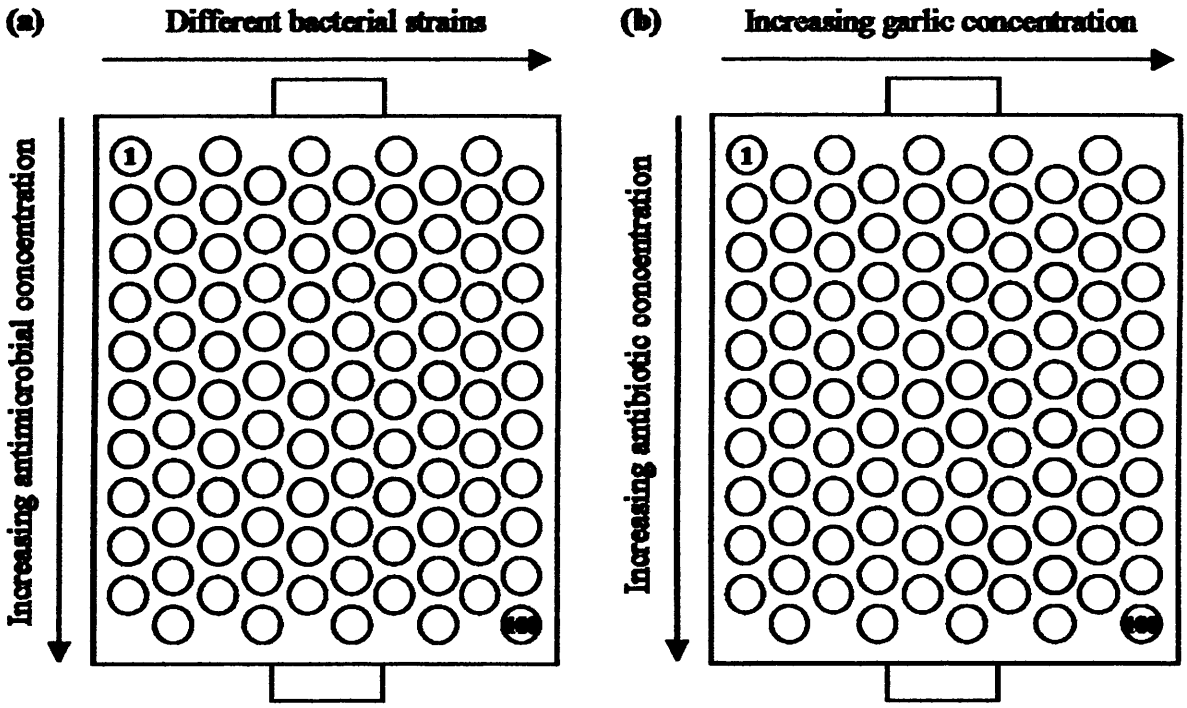
Viable cell counts were performed using an adapted Miles and Misra plate count technique (Miles and Misra, 1938). Six serial ten-fold dilutions were made in sterile PBS (Oxoid, Hampshire, UK). Each dilution was dispensed in triplicate (10 µL drops) onto MHSC plates divided into six sections representing the six dilutions. Drops were allowed to dry and then plates inverted and incubated (37°C, 24 h). Viable counts are reported as colony forming units (CFU) mL^{-1}.

2.7 – Bioscreen C – Determinations of MICs and chequerboard assays

2.7.1 – Bioscreen C growth analyser parameters and set-up

Determinations of minimum inhibitory concentrations (MICs) and chequerboard assays were conducted using a Bioscreen C growth analyser (ThermoFisher, Leicestershire, UK) unless otherwise stated. The Bioscreen allowed large numbers of test conditions to be assessed simultaneously, under identical conditions, and also provided data on the effects of various compounds on bacterial growth dynamics. The final well volumes of Bioscreen "honeycomb well-plates" (**Fig. 2.2**) were always 200 μL. For all experiments 100 μL of this total volume consisted of optically adjusted inoculum (diluted to 1 x 10^6 CFU mL^{-1}) and the other 100 μL consisted of test material(s) which varied depending on experiment as outlined below. Bioscreen growth parameters were standardised as follows: 24.5 h "read-time", temperature maintained at 37°C (machine was turned on and set to 37°C at least one hour prior to use), optical density (OD) was read every 15 min using a wideband filter (480-560nm) with "medium intensity shaking" for 10 s prior to every reading. OD was calibrated against viable counts for positive controls of each strain (see **Appendix**). All Bioscreen results presented are a mean of at least eight replicates (four independent replicates, each with two technical replicates) unless otherwise stated.

Fig. 2.2 – The layout of Bioscreen "honeycomb well-plates" for determinations of MICs (a) and chequerboard assays (b)



2.7.2 – Determination of MICs using a Bioscreen

The MICs of oxacillin, penicillin and garlic were often determined by measurement of OD, with the lowest concentration which inhibited an increase in OD taken as the MIC. Bioscreen "honeycomb well-plates" were set up as in **Fig. 2.2a**, with increasing antimicrobial concentrations in each row, and each column containing a different strain. Each well contained, as previously stated, 100 μL of inoculum, and 100 μL of antimicrobial at twice the required final concentration, resulting in total well volumes of 200 μL and approximate initial densities of 5×10^5 CFU mL^{-1}.

2.7.3 – Chequerboard assays using a Bioscreen

Chequerboard assays allowed the combined effects of two compounds on bacterial growth to be assessed simultaneously over a wide range of concentrations. The capacity of the Bioscreen was 200 wells, comprised of two separate 100 well plates, allowing the effects of 100 different combinations to be assessed in duplicate. Bioscreen "honeycomb well-plates" were set-up as depicted in **Fig. 2.2b**; increasing concentrations of the antibiotic (50 μL) were dispensed into each row and increasing

concentrations of garlic (50 μL) dispensed into each column. Both antibiotic and garlic were dispensed at 4x the required final concentrations. Well 1 (**Fig. 2.2b**) was therefore a positive control, and well 100 (**Fig. 2.2b**) contained the highest concentrations of both compounds. Negative control assays, containing test materials and 100 μL sterile broth (in place of inoculum) were also performed – there were no changes in the OD values of the contents of those wells after 24 h. Optically adjusted inocula (1 x 10^6 CFU mL^{-1}), as stated above, were dispensed in 100 μL volumes into each well, resulting in total well volumes of 200 μL, 5 x 10^5 CFU mL^{-1} approximate initial densities.

2.7.4 – Determination of growth dynamics from Bioscreen growth curves

The data were transformed from ASCII into Microsoft Excel format, plotted as semi-logarithmic plots and analysed. Lag times were only determined from growth curves in which there was an obvious exponential growth phase, and was taken as the period of time (h) from $T_{=0}$ until the beginning of the exponential growth phase. "Growth rates" were calculated as the maximum change in optical density per hour for reasons highlighted in **Chapter 1**.

2.8 – Visualisation of cellular morphologies by electron microscopy

The following fixing, staining and dehydration steps were repeated for both scanning and transmission electron microscopy. Further specific preparation steps are described following the general sample preparation.

Bacterial suspensions (standard growth conditions, treatment dependent on experiment) were sedimented by centrifugation at 8,000 x *g* for 10 min. Supernatants were discarded and pellets fixed with 2% glutaraldehyde (prepared in PBS) for 1 h, washed in PBS and then stained with 1% osmium tetroxide for 1 h. After fixing and staining, pellets were washed twice; firstly in PBS and subsequently in double distilled water. Pellets were then sequentially dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 90% and three passages in 100%), each dehydration step lasting 15 min.

2.8.1 – Further sample preparation for scanning electron microscopy (SEM)

Samples were disaggregated onto 0.2 μm cellulose acetate filters (Millipore, Billerica, MA, USA) soaked in 100% ethanol and were dried at the critical point (SamiDri, Maryland, USA) in 100% ethanol. Filters were then mounted onto 10 mm aluminium stubs (Agar Scientific, Stanstead, UK) and splutter coated with gold (EMScope, UK). Scanning electron micrographs were obtained using a Philips XL20 scanning electron microscope (Philips, Surrey, UK) at a 15 kV accelerating voltage.

2.8.2 – Further sample preparation for transmission electron microscopy (TEM)

The ethanol was discarded and the pellets were immersed in 1,2-Epoxyethane for 10 min. The 1,2-Epoxyethane was replaced with a fresh 1:1 mixture of 1,2-Epoxyethane and araldite (see **Appendix**) and samples were left unsealed overnight to allow solvent evaporation and araldite infiltration of the cells. Pellets were then transferred into sectioning tubes and set in fresh araldite for 48 h. Sections of the polymerised resin embedded pellets (60-90 nm thick) were cut using an ultramicrotome (Reichert-Jung, NY, USA) and were floated onto copper grids. Sections were stained with 2% uranyl acetate for 10 min and Reynolds lead citrate for 5 min (Reynolds, 1963) and allowed to dry (grids were washed twice in double distilled water after each staining step). Transmission electron micrographs were obtained using a Philips EM208 transmission electron microscope (Philips, Surrey, UK) at an accelerating voltage of 80 kV.

3 – The Effects of Garlic on the Growth of MRSA and *S. aureus*

3.1 – Introduction

3.1.1 – The problem of antibiotic resistance

Methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile* and vancomycin-resistant Enterococci (VRE) are currently the major causative agents of hospital acquired antibiotic-resistant infections (Nicolle *et al.*, 2009). Such infections have become so prevalent over the past decade in particular that the terms "hospital superbug" and "MRSA" are now commonplace in the media. Each year in U.S.A alone it is estimated that approx. 14,000 people die from antibiotic-resistant hospital acquired infections (HAIs; Chea, 2005). Importantly, these antibiotic-resistant bacteria are, in most cases, no more virulent than their antibiotic-susceptible counterparts (Frees *et al.*, 2005). Under normal circumstances a healthy immune system is sufficient to halt the progression of most hospital acquired infections regardless of whether the causative agent is antibiotic-resistant or not. The elderly, young, or immuno-compromised (i.e. those groups regularly admitted to hospitals), however, would require antibiotics to fight off such an infection and since the majority of antibiotics are ineffective against these resistant pathogens, infections become life-threatening.

For the patient, antibiotic-resistant infections can result in longer hospital stays, the possibility of recurrent infections, increasingly toxic treatments (for example, vancomycin) with highly unpleasant side-effects and most importantly, the possibility of death. MRSA, for example, is considered a lethal organism, capable of killing a patient in one to two days (Schentag, 2001). Antibiotic-resistant infections also result in increasing financial costs which relate to, for example, longer lengths of stay (particularly in Intensive Care Units) and more expensive antibiotics over a longer period of time. The additional financial burden of antibiotic resistance is difficult to calculate, but it was estimated in the U.S.A to be $4-$5 billion in 2001 (World Health Organisation, 2001).

3.1.2 – The rapid progression of antibiotic resistance

The acquisition and evolution of antibiotic resistance in human pathogens has been very rapid. At least one mechanism of resistance has developed to all 17 different classes of antibiotics produced to date (Alanis, 2005). In many cases, bacteria have acquired resistance to multiple drug classes, exacerbating the treatment and raising the financial costs (Sefton, 2002; Levy and Marshall, 2004). A comparison between the first clinical applications of antibiotics and the first reports of antibiotic resistance to the same drugs show that in almost every case antibiotic-resistant pathogens were isolated within two years of the antibiotics become commercially available. For example, β-lactamase enzymes (the resistance mechanism against penicillin) were first isolated in 1940 (Abraham and Chain, 1940), whereas the first clinical treatment with penicillin was in 1942 (Grossman, 2008). Similarly, methicillin was commercially produced by Beecham (as Celbenin) in 1959 (Enright *et al.*, 2000), and methicillin-resistant Staphylococci were first noted in 1961 (Barber, 1961). There is a well-documented proportional relationship between increasing antimicrobial use and increasing antimicrobial resistance both in hospitals and the community. The selective pressure of widespread antimicrobial use is one of the major factors in the development of antibiotic resistance (American Society for Microbiology, 1995).

Whilst the media portray antibiotic resistance as a new phenomenon, this is not the case. Most antibiotic resistance mechanisms, including drug modification and efflux pumps, existed long before the antibiotic era (Koch, 2007). It is important to note that most antibiotics are derived from naturally produced secondary metabolites which confer combative advantages to the organisms that produce them (in terms of acquisition and utilisation of resources). Considering the rapid rate of bacterial growth and division, and the corresponding high mutation rates, it is perhaps of little surprise that bacterial and fungal "countermeasures" (i.e. resistance mechanisms) also existed. The transfer of antibiotic resistance mechanisms into micro-organisms of clinical or agricultural importance was only as a result of the inappropriate usage of antibiotics by humans (Koch, 2007).

The outlook for the development of significant new classes of antibiotics is bleak. Over the past two decades there has been a steady decrease in the approval of new antibiotics (Spellberg *et al.*, 2004). Only nine antibacterial compounds were approved

in the U.S.A from 1998-2003, and of those only daptomycin and linezolid had truly novel mechanisms. The cost of research and development of new antibiotics is considerable. It has been estimated that each new drug from discovery to pre-approval cost $802 million in 2000 (DiMasi *et al.*, 2003), a figure four times greater than 10 years previously (DiMasi *et al.*, 1991). Considering these costs and the rapid development of antibiotic resistance it is no surprise that pharmaceutical companies are investing less in antibiotic discovery. Increasingly, funding is directed towards development of new treatments for many common, previously untreated conditions (such as osteoporosis) or diseases that, whilst not life threatening, affect the "lifestyle" of those suffering them (e.g. erectile dysfunction; Alanis, 2005).

3.1.3 – Alternative therapies to traditional antibiotics

Currently the only drugs available to treat MRSA infections (e.g. vancomycin and teicoplanin) are expensive, have undesirable side effects and are becoming increasingly redundant due to bacterial resistance (Smith *et al.*, 1999). Although there have been some recent developments in anti-Staphylococcal treatment, for example linezolid and the streptogramin / dalfopristine mixture Synercid® (Tsiodras *et al.*, 2001), efficacy is already reduced due to resistance. Varying 'traditional' antibacterial structures has nearly been exhausted and it is extremely unlikely that slight structural changes will have any impact on the problem of antibiotic resistance (Alanis, 2005). There are examples, however, of non-antibiotic therapies being used in clinical settings, particularly in wound management, which are effective against antibiotic-resistant bacteria:

Silver was used as an antimicrobial before the discovery of penicillin. With the advent of antibiotics the therapeutic use of silver diminished, but recently there has been an increase in the clinical use of wound dressings incorporating varying levels of silver in response to antibiotic-resistant bacteria (Chopra, 2007). There are, however, numerous reports of silver resistance in a variety of pathogenic bacteria, although resistance mechanisms remain unclear (Chopra, 2007). Therefore, although some silver based dressings currently provide an alternative to antibiotics in wound infection management, dressings with low silver concentrations (especially those at sub-minimum inhibitory concentrations) are likely to select for resistance.

Honey, and in particular, manuka honey (*Leptospermum scoparium*), also appears to be an effective wound antiseptic (Cooper *et al.*, 2002; Lay-flurrie, 2008). Clinical trials with honey incorporated wound dressings have shown promising results in terms of clearing of wound infections, and promotion of wound healing, particularly in wounds caused by antibiotic-resistant bacteria (Lay-flurrie, 2008). The antibacterial mode of action of honey is not fully elucidated, but H_2O_2 and osmolarity are apparently important (Cooper *et al.*, 2002). In addition to antibacterial properties, honey also promotes wound clearing and is also thought to stimulate the growth of epithelial tissue, promoting wound healing (Cooper *et al.*, 2002; Lay-flurrie, 2008).

3.1.4 – Antimicrobials derived from plants

Considering that almost all clinically employed antibiotics are derived from naturally produced bacterial and fungal metabolites, it is perhaps reasonable to assume that nature will also hold some "answers" to antibiotic resistance. A vast number of plants and plant compounds with wide ranges of antimicrobial properties have been identified. Throughout history, and across the world, plants have been used to treat a wide range of ailments and infections, and now some of these traditional applications have been substantiated by science. Some of the plants with notable antibacterial properties include:

- Oregano (*Origanum vulgare*; Lambert *et al.*, 2001))
- Cinnamon (*Cinnamomum zeylanicum*; Singh *et al.*, 2007)
- Tea tree (*Melaleuca alternifolia*; Loughlin *et al.*, 2007)
- Aleppo Oak (*Quercus infectoria*; Chusri and Voravuthikunchai, 2009)
- Green tea (*Camellia sinensis*; Cho *et al.*, 2008)
- Myrrh (primarily from *Commiphora myrrha*; Gibbons, 2004)

Garlic (*Allium sativum*) is another plant with a broad spectrum of antimicrobial and health promoting activities. As well as notable antiviral, antifungal and anti-protozoal activities (Harris *et al.*, 2001), garlic has excellent antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria. The antimicrobial properties of garlic are attributed to the bouquet of sulphur containing chemicals which also provides the characteristic smell (Lawson, 1996). The major antibacterial sulphur

containing compound of garlic is allicin (Cavallito and Bailey, 1944), although numerous other antimicrobial compounds are also present in garlic preparations (Lawson, 1996). The exact compounds and their proportions vary depending on the strain of garlic and the extraction procedure (Lawson, 1996; Pentz and Siegers, 1996). Garlic is relatively non-toxic to human cells, as would be expected considering its wide use in the cuisine of many cultures. Reportedly there is, however, a toxic effect on the human liver, although garlic must be administered at extremely high doses; 100 mg / kg, the equivalent of approx. 500 raw cloves per person per day (Koch, 1996)!

The antibacterial mechanism(s) of action of garlic and allicin are unknown, but the major antibacterial effect of allicin has been proposed as inhibition of SH- containing enzymes (Rabinkov *et al.*, 1998; Ankri and Mirelman, 1999). There are, however, likely to be numerous targets and mechanisms of action for each individual compound: several cellular targets for allyl alcohol, a compound produced by self-condensation of allicin (Lawson, 1996), were identified in *Candida albicans* (Lemar *et al.*, 2005). Furthermore, numerous targets of garlic compounds have been proposed (in a range of pathogenic bacteria) including: RNA polymerase (Ankri and Mirelman, 1999), N-acetyltransferase (Chung *et al.*, 1998, Tsao and Yim, 2001a) and lipid biosynthesis (Choi *et al.*, 2007). The wide range of antimicrobial activity is attributed to the ease by which garlic compounds pass through cell membranes (Miron *et al.*, 2000). Despite reports of garlic constituents (diallyl sulphides) altering the lipid fluidity of *Candida* spp., no such effect was reported on bacterial membranes (Tsuchiya and Nagayama, 2008).

One of the most promising aspects of garlic as an antibacterial, particularly against antibiotic-resistant bacteria, is the lack of resistance. There have been no reports of bacteria acquiring resistance to garlic, but considering the complex nature of the chemistry and presumed numerous targets this is perhaps unsurprising. It is feasible, however, that resistance to individual garlic compounds could occur, although it has been suggested that development of allicin resistance would be 1000x more difficult than developing resistance to β-lactam antibiotics (Ankri and Mirelman, 1999). Interestingly, the only bacteria (in general) that exhibit decreased sensitivity to garlic are the non-pathogenic lactic acid bacteria (LAB; Rees *et al.*, 1993), whilst most pathogens appear to be susceptible. Typically MICs of garlic against LAB are ≥ ten-

fold greater than those of susceptible bacteria (including *E. coli* and *S. aureus*; Rees *et al.*, 1993).

3.1.5 – The present study

It has been widely reported that both MRSA and *S. aureus* are susceptible to garlic (for example: Reuter *et al.*, 1996; Harris *et al.*, 2001; Cottrell, 2003). It was also previously reported, in a limited study, that garlic affected the lag phase duration, but not the specific growth rates of the two garlic susceptible pathogens *E. coli* and *S. aureus* (Cottrell, 2003). Furthermore, the garlic insensitive lactic acid bacterium *Lactobacillus casei* responded very differently; garlic caused dose-dependent decreases in the specific growth rate but had no effect on lag phase (Cottrell, 2003). It was hypothesised that garlic inhibited cell wall formation in *E. coli* and *S. aureus*, resulting in the lag phase extension (Cottrell, 2003). The present study tested the hypothesis of the cell wall as a target by investigating the effect of garlic on the integrity of the Staphylococcal cell wall and also on cellular morphology (by electron microscopy). Furthermore, the effect of garlic on the growth dynamics of a number of strains of MRSA and *S. aureus* was investigated.

3.1.6 – Aims

- Confirm that these strains of MRSA and *S. aureus* are susceptible to garlic
- Investigate the effect of garlic on the growth dynamics of MRSA and *S. aureus*
- Investigate the suggestion that garlic targets the cell wall of MRSA and *S. aureus*
- Identify any morphological abnormalities induced by garlic using electron microscopy

3.1.7 – Hypotheses

1. The growth of MRSA and *S. aureus* will be inhibited by garlic
2. MRSA will be no less susceptible to garlic than *S. aureus*
3. Garlic will affect the lag phases but not growth rates of MRSA and *S. aureus*
4. The osmotic tolerance of MRSA and *S. aureus* will be decreased due to garlic induced cell wall damage
5. Garlic will induce obvious damage to the structure of MRSA and *S. aureus*, accounting for the inhibition of growth

3.2 – Materials and Methods

3.2.1 – Effect of garlic on the growth of MRSA and *S. aureus*

Garlic stock solutions were prepared as previously described (**Chapter 2.3**) and diluted in MHB to desired concentrations in final volumes of 10 mL. Optically adjusted inocula were prepared, as previously described (**Chapter 2.5**), and garlic solutions were inoculated to achieve an initial cell density of approx. 5 x 10^5 CFU mL^{-1}. Viable cell counts were enumerated (**Chapter 2.6**) on MHSC after 24 h incubation at 37°C. MICs were determined after 24 h as the lowest garlic concentrations which inhibited increases in cell numbers from initial populations.

3.2.2 – Effect of garlic on the growth dynamics of MRSA, *S. aureus* and *E. coli*

Growth dynamics of MRSA and *S. aureus* were determined from Bioscreen growth curves obtained as previously described (**Chapter 2.7.4**). Growth dynamics of *E. coli* were determined using the same method, but with NB in place of MHB.

3.2.3 – Effect of garlic on the osmotic tolerance of MRSA and *E. coli*

Garlic stock solutions were prepared as previously described (**Chapter 2.3**) and diluted in MHB / NB (for MRSA and *E. coli* respectively) to desired concentrations in final volumes of 10 mL. Suspensions of organisms were optically adjusted, as previously described, and garlic solutions were inoculated to achieve initial cell densities of approx. 5 x 10^5 CFU mL^{-1}. MHA was prepared containing 0, 2, 5 and 10% (w/v) NaCl (for MRSA), and NA prepared containing 0, 2, 4 and 5% (w/v) NaCl (for *E. coli*), on which viable cell counts were enumerated (**Chapter 2.6**) after 24 h incubation at 37°C. Plates were examined for growth after 24 h and 48 h incubation.

3.2.4 – Effect of garlic on the cellular morphology of MRSA and *S. aureus* determined using scanning electron microscopy

Garlic stock solutions were prepared as previously described (**Chapter 2.3**) and diluted in MHB to desired concentrations (0, 0.25 and 0.6 mg mL^{-1}) in final volumes of 10 mL. Optically adjusted inocula were prepared, as previously described (**Chapter 2.5**), and garlic solutions were inoculated to achieve initial cell densities of approx. 5 x 10^5 CFU mL^{-1}. Samples were prepared for scanning electron microscopy, and imaged, as previously described (**Chapter 2.8** and **2.8.1**) after 24 h incubation.

3.3 – Results

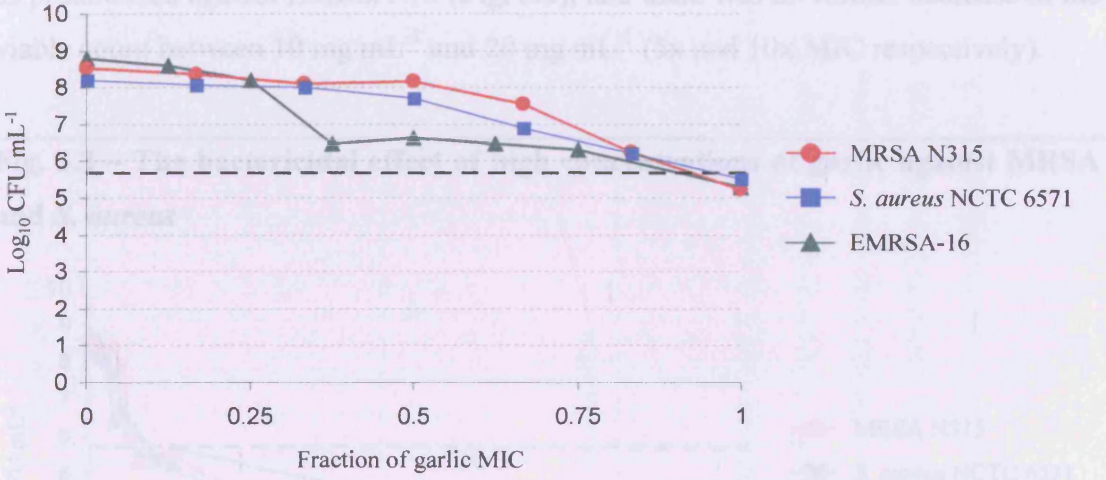
Garlic inhibited the growth of all strains of MRSA and *S. aureus*, except for EMRSA-16, at ≤1.5 mg mL^{-1} (determined by viable count; Table 3.1). MICs of garlic were lower when determined by optical density than by viable count (Table 3.1). The MIC of garlic against EMRSA-16 was the same as all other strains when measured by optical density (Table 3.1). The MIC value of EMRSA-16 by viable count (2 mg mL^{-1}; Table 3.1) was not considerably higher than those of other strains. The difference between the MIC of EMRSA-16 and other strains is only highlighted because in numerous cases the response of EMRSA-16 to antimicrobials was atypical compared with other strains.

Table 3.1 – The MICs of garlic against MRSA and *S. aureus* determined by two different methods

Strain	MIC determined by viable count on MHSC (mg mL^{-1})	MIC determined by optical density (Bioscreen) in MHB (mg mL^{-1})
MRSA N315	1.5	0.8
S. aureus NCTC 6571	1.5	0.8
EMRSA-16	2	0.8

At garlic concentrations <0.5x MIC there was no effect on the extent of growth of MRSA N315 and NCTC 6571 after 24 h (**Fig. 3.1**). At garlic concentrations between 0.5x MIC and the MIC there were dose-dependent decreases in the extent of growth of both strains after 24 h (**Fig. 3.1**). There was decreased growth of EMRSA-16 at 0.25x MIC, but increasing concentrations of garlic (up to 0.75x MIC) had no further effect on the extent of growth after 24 h (**Fig. 3.1**).

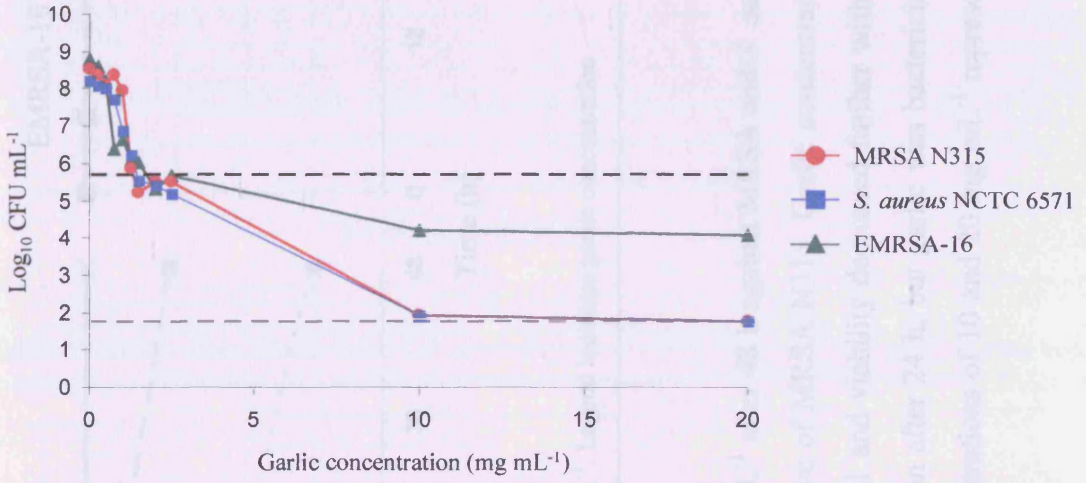
Fig. 3.1 – The yield of *S. aureus* and two strains of MRSA after 24 h exposure to garlic



Legend indicates strain. Black dashed line indicates initial population (5×10^5 CFU mL^{-1})

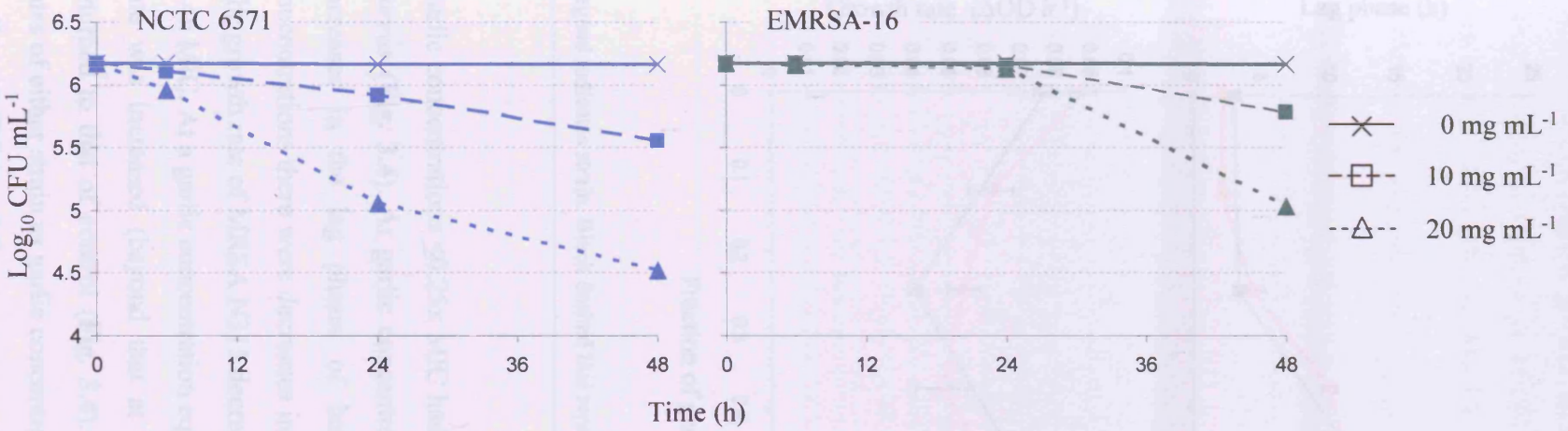
Garlic was bacteriostatic (cells were inhibited from growth but were not killed) against MRSA and *S. aureus* at concentrations up to 2x MIC (**Fig. 3.2**). At garlic concentrations of 10 mg mL^{-1} and 20 mg mL^{-1} (representing 6.5x and 13x MIC; 5x and 10x for EMRSA-16) there was a bactericidal effect, i.e. a decrease in the number of viable cells from the initial populations (**Fig. 3.2**). For MRSA N315 and NCTC 6571 cell numbers decreased from 5 x 10^5 CFU mL^{-1} with garlic concentrations representing 2x MIC to 6.5x MIC (**Fig. 3.2**). It is unclear whether cell numbers continued to decrease as garlic concentration increased from 10 mg mL^{-1} – 20 mg mL^{-1} because the limit of detection was reached. The bactericidal effect of garlic was not as pronounced against EMRSA-16 (**Fig. 3.2**), and there was no further decrease in the viable count between 10 mg mL^{-1} and 20 mg mL^{-1} (5x and 10x MIC respectively).

Fig. 3.2 – The bactericidal effect of high concentrations of garlic against MRSA and *S. aureus*



Legend indicates strain. Black dashed line represents initial population (approx. 5 x 10^5 CFU mL^{-1}; grey dashed line indicates limit of detection

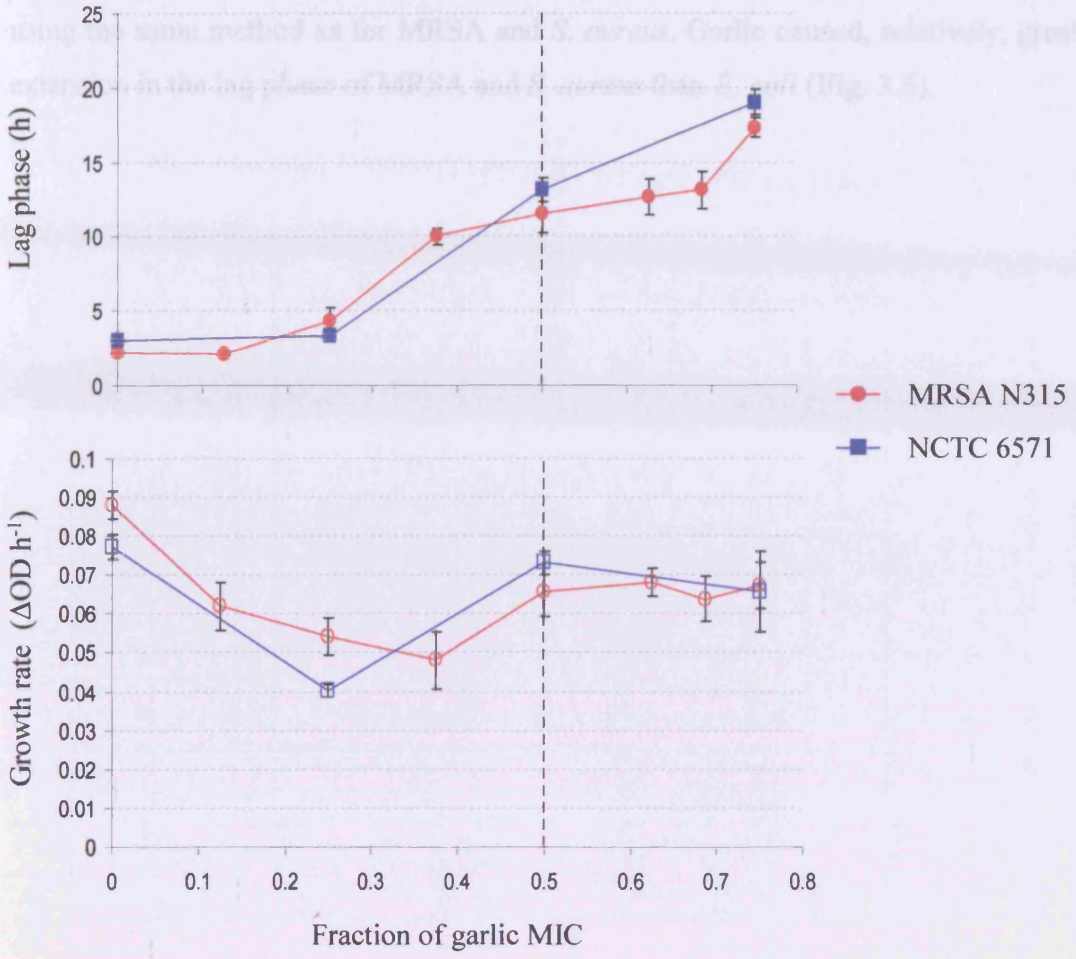
Fig. 3.3 – The effect of garlic on the viability of MRSA and *S. aureus* in PBS over 48 h



Initial populations were approx. 1 x 10^6 CFU mL^{-1}. Legend indicates garlic concentration

Garlic was also bactericidal at 20 mg mL^{-1} after 48 h against MRSA and *S. aureus* (**Fig. 3.3**) maintained in PBS at room temperature. *S. aureus* NCTC 6571 is also representative of MRSA N315. Garlic concentrations of 10 mg mL^{-1} and 20 mg mL^{-1} caused proportional decreases in the viability of NCTC 6571 and viability decreased further with time. Conversely, there was no effect on the viability of EMRSA-16 at either garlic concentration after 24 h, but garlic was bactericidal at both concentrations after 48 h (although to a lesser extent than NCTC 6571). Garlic concentrations of 10 and 20 mg mL^{-1} represented approx. 5x / 6.5x and 10x / 13x MIC against NCTC 6571 and EMRSA-16 respectively.

Fig. 3.4 – The effect of garlic on the growth dynamics of MRSA and *S. aureus*

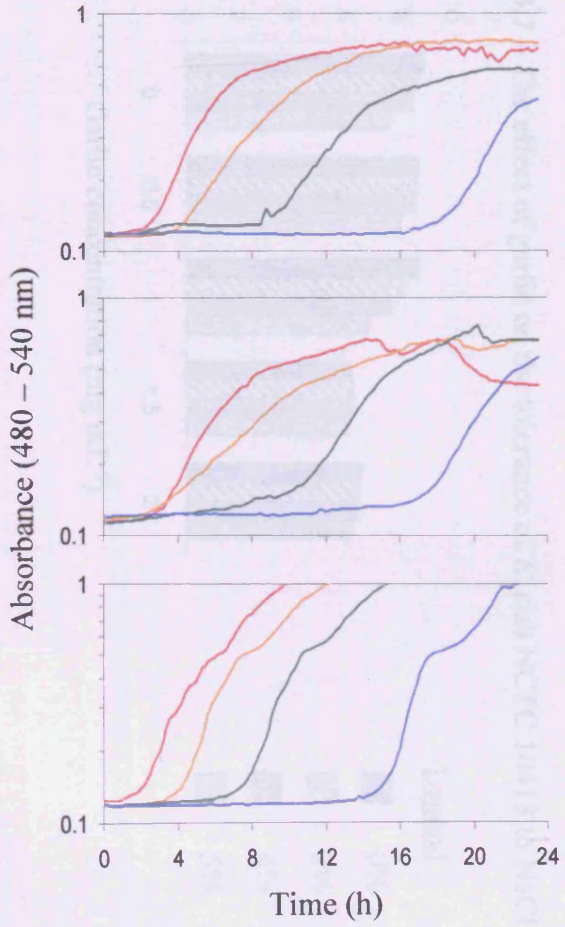


Legend indicates strain. Black dashed line represents a garlic concentration of 50% of the relative MIC

Garlic concentrations ≤0.25x MIC had little effect on the lag phases of MRSA and *S. aureus* (**Fig. 3.4**). At garlic concentrations >0.25x MIC there were dose-dependent increases in the lag phases of both strains (**Fig. 3.4**). At the lowest garlic concentrations there were decreases in the growth rates of both MRSA and *S. aureus*. The growth rate of MRSA N315 decreased further as garlic concentration increased to 0.4x MIC. At a garlic concentration equivalent to 0.5x MIC of both strains, the growth rate was increased (beyond that at lower garlic concentrations) and was almost returned to that of control (**Fig. 3.4**). There were no further changes in the growth rates of either strain as garlic concentrations increased above 0.5x MIC. The effects of garlic on MRSA and *S. aureus* were obviously different to those on *E. coli* (**Fig. 3.5**).

Cottrell (2003) reported that garlic caused extensions in the lag phase of *E. coli*, but had no effect on specific growth rates. These data have been replicated here (**Fig. 3.5**) using the same method as for MRSA and *S. aureus*. Garlic caused, relatively, greater extension in the lag phase of MRSA and *S. aureus* than *E. coli* (**Fig. 3.5**).

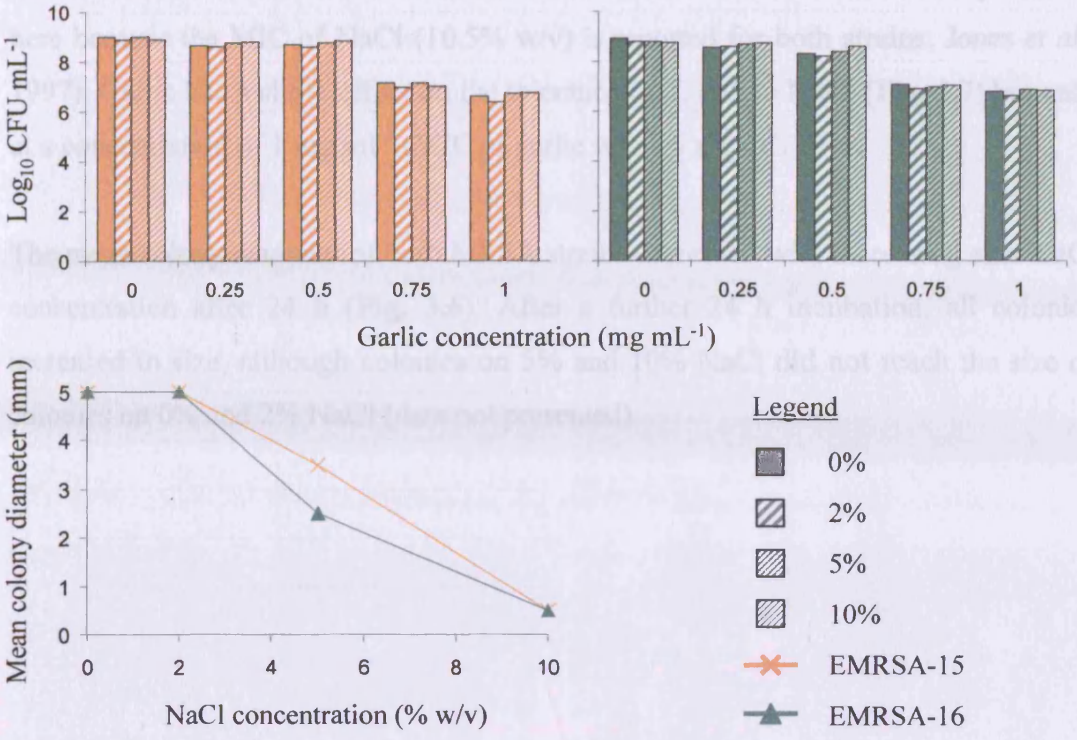
Fig. 3.5 – Representative growth profiles of MRSA, *S. aureus* and *E. coli* with garlic



Strain	Fraction of garlic MIC	Lag phase duration (h)	Fraction of control growth rate
MRSA N315	0	2.2	1
	0.25	4.3	0.66
	0.5	12.6	0.75
	0.75	16	0.77
S. aureus NCTC 6571	0	3	1
	0.25	3.3	0.52
	0.5	13	0.95
	0.75	19	0.97
E. coli NCTC 10418	0	2.7	1
	0.25	4.5	0.95
	0.5	8	0.96
	0.75	14.6	0.98

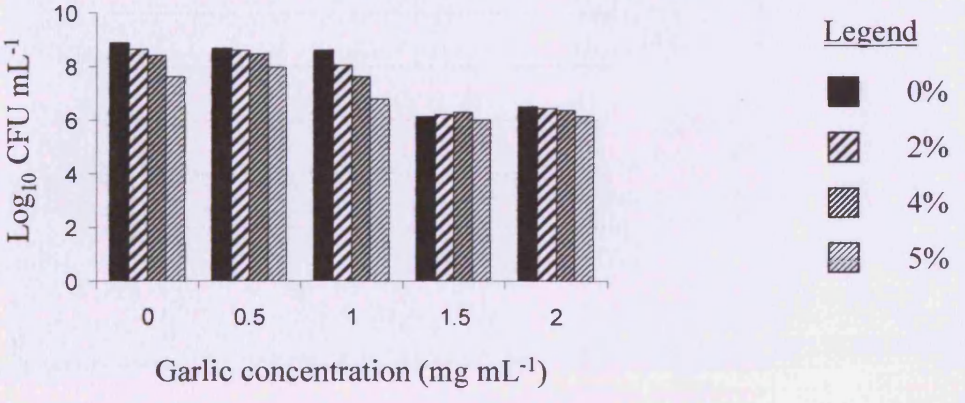
Semi-logarithmic growth profiles were obtained using a Bioscreen (ThermoFisher, Leicestershire, UK). Growth dynamics are presented as means, and were not measured directly from the profiles but rather shown here merely as examples. Growth profiles of *E. coli* are limited to an OD of 1 to allow direct comparison with *S. aureus*.

Fig. 3.6 – The effect of garlic on the tolerance of two MRSA strains to NaCl



Legend indicates NaCl concentration (% w/v) and strain. Colony diameters were measured from control isolates on NaCl containing agar after 24 h incubation

Fig. 3.7 – The effect of garlic on the tolerance of *E. coli* NCTC 10418 to NaCl

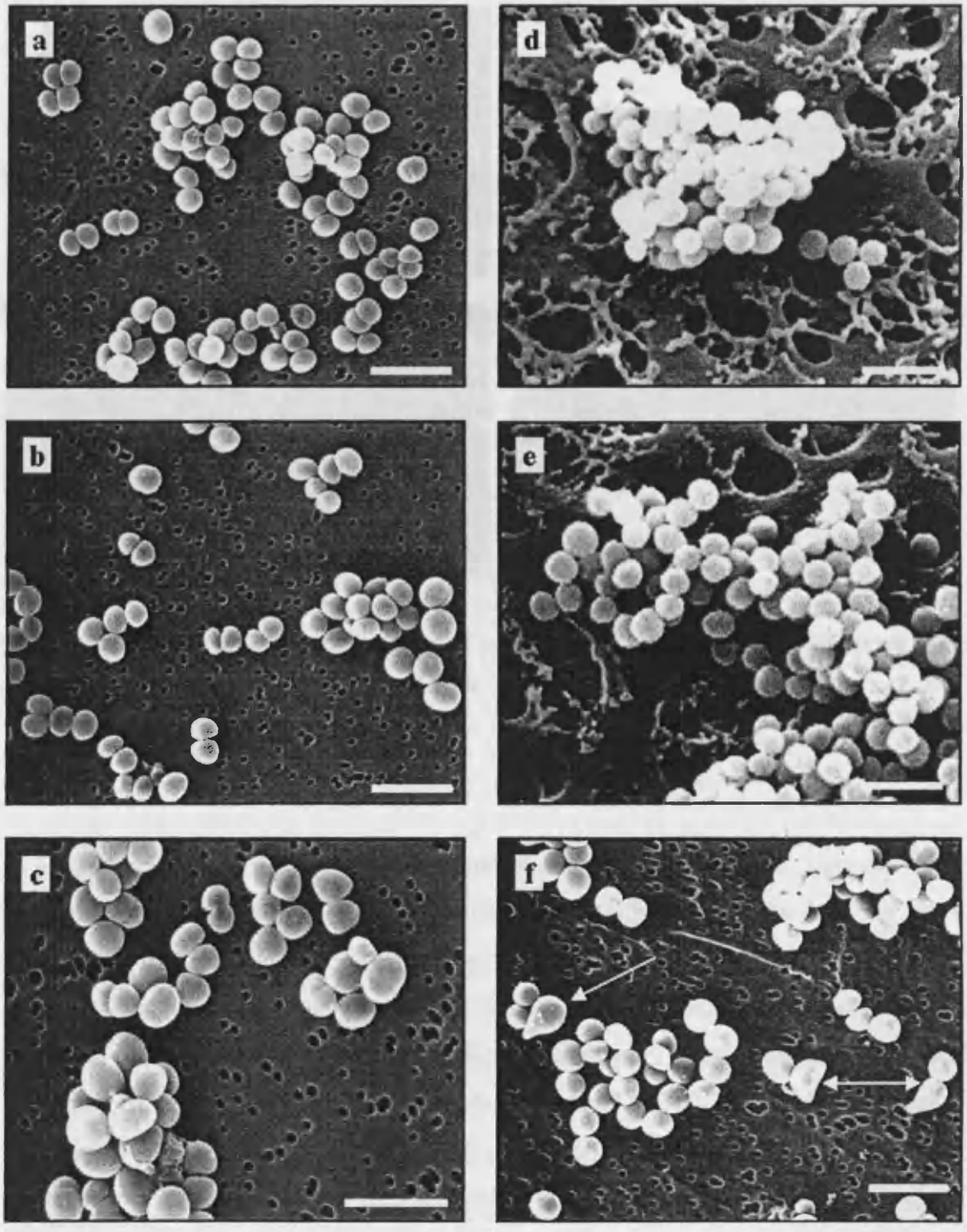


Legend indicates agar NaCl concentration (% w/v)

Garlic had no effect on the tolerance of MRSA to NaCl (**Fig. 3.6**) at NaCl concentrations close to the MIC (NB – EMRSA-15 and EMRSA-16 are presented here because the MIC of NaCl (10.5% w/v) is reported for both strains; Jones *et al.*, 1997). Garlic had a slight effect on the tolerance of *E. coli* to NaCl (**Fig. 3.7**) but only at a concentration of 1 mg ml^{-1} (MIC of garlic was 1.5 mg mL^{-1}).

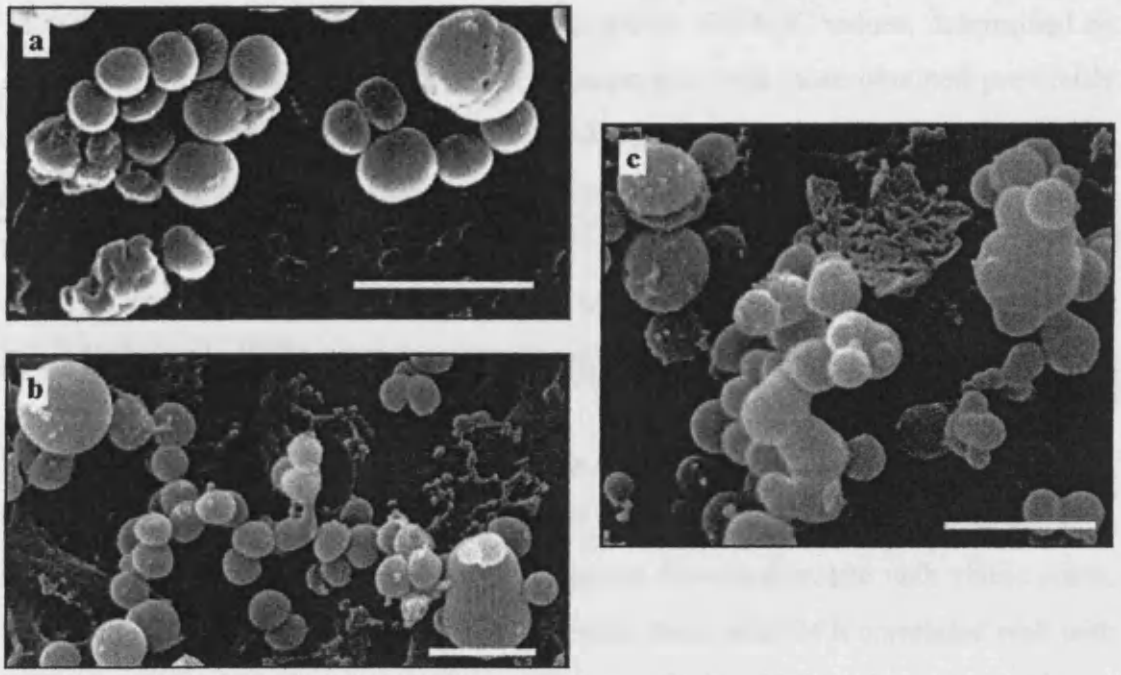
The mean colony diameter of both MRSA strains decreased with increasing agar NaCl concentration after 24 h (**Fig. 3.6**). After a further 24 h incubation, all colonies increased in size, although colonies on 5% and 10% NaCl did not reach the size of colonies on 0% and 2% NaCl (data not presented).

Fig. 3.8 – Electron micrographs of MRSA N315 (a-c) and *S. aureus* NCTC 6571 (d-f) treated with sub-MIC concentrations of garlic for 24 h



MRSA N315 (**a** – **c**) and NCTC 6571 (**d** – **f**) treated with 0 mg mL^{-1} garlic (**a**, **d**), 0.25 mg mL^{-1} garlic (**b**, **e**) and 0.6 mg mL^{-1} garlic (**c**, **f**). White arrows indicate cells with abnormal morphologies. Note that **c** is not to the same scale as other images. Scale bars indicate 2 µm. Magnification, x15,000 (except **c** x20,000)

Fig. 3.9 – Electron micrographs of abnormal *S. aureus* cellular morphologies induced by garlic at sub-MIC concentrations



Cells of NCTC 6571 (**a**) and EMRSA-16 (**b**, **c**) with abnormal morphologies induced by sub-MIC concentrations of garlic (**a** – 0.25 mg mL^{-1}; **b**, **c** – 0.6 mg mL^{-1}). Scale bars indicate 2 μm; magnification x15,000

In general, garlic had little effect on the morphology of MRSA and *S. aureus*, when viewed by scanning electron microscopy (**Fig. 3.8**). Cells treated with 0.25 mg mL^{-1} and 0.6 mg mL^{-1} garlic resembled (in terms of appearance and size) control cells grown without the presence of garlic. There was no evidence of "blebbing" or structural damage. With increasing garlic concentration there was an increased frequency of cells with abnormal morphologies (**Fig. 3.8, 3.9**), numerous cells being 1.5 - 2 μm in diameter (control size ~ 0.8 μm), but these were exceptions (n < 1%). There was a higher frequency of cells with abnormal morphologies in cultures of EMRSA-16, and at a lower concentration of garlic than for other strains (0.25 mg mL^{-1}; **Fig. 3.9**).

3.4 – Discussion

3.4.1 – The susceptibility of MRSA and *S. aureus* to garlic

MRSA and *S. aureus* were both sensitive to garlic. The MIC values, determined by either viable count or optical density, are comparable with those obtained previously for other "sensitive" organisms (e.g. 0.8 – 3.3 mg mL^{-1}; Rees *et al.*, 1993; 1 – 5.5 mg mL^{-1}; Cottrell, 2003). The MIC values were also at least 10x lower than those previously determined for "less susceptible" organisms such as *Lactobacillus casei* (MIC 16.9 mg mL^{-1}; Cottrell, 2003) or *Pediococcus pentosaceus* (MIC 34.5 – 39.7 mg mL^{-1}; Rees *et al.*, 1993).

The MIC values of garlic against MRSA and *S. aureus* determined by viable count were approximately two times higher than those determined by absorbance. It is important to reiterate that optical density does not directly correlate with viable count. However, viable counts obtained from Bioscreen plates after 24 h correlated well with MICs determined by absorbance (data not presented). In addition, the culture volumes of both methods were very different (although the proportions of organisms and garlic were the same), and the cultures in which MIC was determined by absorbance were agitated before each reading, possibly impacting on oxygen transfer. Despite the differences between MICs, it is important to highlight that using either method the organisms were susceptible to garlic. The MIC values determined using either method were much lower than those for less-susceptible organisms (i.e. LAB).

Compared with those for commonly-used antibiotics, the MIC values of garlic were very high. For example, the Clinical Laboratory Standards Institute (CLSI, 2007) states that oxacillin-resistant organisms can grow at oxacillin concentrations >4 μg mL^{-1}, so the MICs of garlic presented here (in the region 800 – 1500 μg mL^{-1}) were very high. Some relatively inert compounds may be inhibitory at such high concentrations (Gibbons, 2004), but it is important to highlight that the high vegetable content of garlic powder is probably responsible for its low potency (O'Gara *et al.*, 2000). As previously stated, the major antibacterial compound in garlic is allicin (Reuter *et al.*, 1996), which is formed from alliin by the enzyme alliinase (Lawson, 1996). The alliin content of the garlic powder used in these investigations was approx. 10,000 ppm (1% w/w), and approx. 40% of alliin is converted to allicin by allinase

(Lawson, 1996). Taking these values into consideration, a very crude MIC of allicin against MRSA and *S. aureus* of 3 - 6 μg mL^{-1} can be obtained; a comparable value to many clinically employed antibiotics. It must be highlighted that this value is only a crude comparison, and is only provided to demonstrate that the low efficacy of garlic is due to the high vegetable content. There are in the research literature many examples of MICs for commercially-available garlic preparations quoted as "MIC of allicin", which have been converted in the manner above. Considering the complex chemistry of garlic compounds, and the likely interactions between them (which could increase or decrease efficacy), it is unrealistic to state such MICs as if they may be attributed to a single compound.

MRSA N315 was resistant to β-lactam antibiotics as well as two macrolide antibiotics. EMRSA-16 and MRSA JW4 were additionally resistant to ciprofloxacin (a quinolone; see **Appendix** for antibiotic susceptibility profiles). *S. aureus* NCTC 6571 had no antibiotic resistance, yet was as susceptible to garlic as strains with multiple antibiotic resistances. These data therefore suggest that the mechanism(s) of garlic appear to be different from those of the antibiotics to which these strains have resistance. Any antibiotic resistance mechanisms of these MRSA strains seemingly have no impact on susceptibility to garlic. Conversely, it is also possible that due to the hypothesised multiple targets / mechanisms of action of garlic, the effect of reduced susceptibility caused by antibiotic resistance mechanisms at one or more of the targets are limited.

3.4.2 – Effect of garlic on Staphylococcal growth dynamics

Garlic caused both lag phase extension and decreased growth rates of MRSA and *S. aureus*. Previously, Cottrell (2003) reported that garlic caused extended lag phases in *E. coli* but had no effect on specific growth rates (Data reproduced here; **Fig. 3.5**) and conversely, caused decreased growth rates in *Lactobacillus casei* (*L. casei*) but had no effect on the lag phase. Furthermore, garlic reduced the maximum specific growth rate of the LAB *Lactobacillus curvatus* (Verluyten *et al.*, 2004) and *Salmonella typhimurium* treated with allicin grew at a reduced rate compared with untreated controls (Feldberg *et al.*, 1988). These data therefore suggest that the major targets of garlic in *E. coli* and *S. aureus* are different from those of *L. casei* and that either there are different major targets in *E. coli* and *S. aureus* or that there are numerous major targets in *S. aureus*. The effects of garlic on Staphylococcal growth dynamics shown

here were complex. Although low concentrations of garlic (< 25% of the MIC) had little effect on the lag phase of MRSA and *S. aureus*, there were sharp decreases in the growth rates. This suggests that either:

1. extended lag phases and decreased growth rates are caused by different mechanisms
2. higher concentrations of garlic are required to halt the progression into the exponential phase than to affect the growth rate
3. extended lag phases and decreased growth rates are caused by different compounds

It is interesting that the growth rates increased, in some cases almost to those of control, at garlic concentrations >0.5x MIC. This pattern was seen in all strains, although the extent to which the growth rates changed did vary with strain (see **Appendix** for full details). Without further work, or elucidation of the mechanism of action / targets of garlic, the reasons for these effects on growth rates remain unclear.

3.4.3 – Effect of garlic on the osmotic tolerance of MRSA

The results presented here suggest that the Staphylococcal cell wall is unlikely to be a major target of garlic against *S. aureus*. There was no cell blebbing, or visible cell wall damage in cells treated with 0.6 mg mL^{-1} garlic (representing 0.4x MIC) viewed by electron microscopy (although an intact cell structure does not necessarily indicate a perfectly formed cell wall). Garlic at the MICs also exerted a bacteriostatic effect on MRSA and *S. aureus*; if the cell wall were damaged cells would most likely die as a result of an osmotic imbalance. Finally, increasing garlic concentrations had no effect on the NaCl tolerance of MRSA. *S. aureus* is extremely halotolerant, with strains reportedly capable of growing in 3.5 M NaCl (approx. 20.3% NaCl; Scott, 1953) and the growth of some epidemic strains (including EMRSA-15 and EMRSA-16) inhibited by 1.8 M (approx. 10.5% NaCl; Jones *et al.*, 1997). Most bacteria have an internal osmotic pressure ranging from 5 to 20 atm (Zhao *et al.*, 2001), and are capable of withstanding low external osmotic pressures due to their high-tensile-strength cell walls. The Gram-positive cell wall consists of up to 50 sheets of peptidoglycan external to the cell membrane (Labischinski and Maidhof, 1994). Any damage to the cell wall would decrease NaCl tolerance. It is therefore unlikely that

garlic, at these concentrations, causes sufficient damage to peptidoglycan for the cell to lose its osmotic tolerance. The bactericidal effect and loss of viability at higher concentrations of garlic does however suggest damage to the Staphylococcal cell wall. This is, perhaps, further supported because the loss of viability was greater in MHB (after 24 h) than in PBS. The relative NaCl concentrations in MHB and PBS are 2% and 0.8% respectively. The increased loss of viability in MHB, compared with PBS, could be due to the increased NaCl concentration, and the associated loss of osmotic tolerance caused by garlic induced damage of the cell wall. The difference in the bactericidal effects of garlic in these two media could be caused by the difference in temperatures (MHB at 37°C, PBS at 20°C). This is however unlikely because garlic is a more effective antibacterial at lower temperatures (Marques *et al.*, 2008) and the degradation of allicin is more rapid as temperature increases (Lawson, 1996).

The Gram-negative cell wall consists of a much thinner peptidoglycan layer than the Gram-positive wall; typically one or two sheets. Garlic may damage the peptidoglycan layer of *E. coli* sufficiently to create the blebbing previously reported (Cottrell, 2003). The data presented here support a hypothesis of the cell wall as a target of garlic against *E. coli*; increasing concentrations decreased the tolerance to NaCl. However, it appears that the cell wall of *E. coli* is not the major target of garlic. There was only a slight decrease in NaCl tolerance at a high garlic concentration, and the data presented previously (Cottrell, 2003) would suggest a decrease in osmotic tolerance at lower garlic concentrations if the cell wall were the major target.

Although there were, in general, no effects of garlic on the morphology of MRSA and *S. aureus* at sub-MIC concentrations, there were increased numbers of abnormal cells. The most frequent abnormal morphology was a much larger cell. These larger cells could be so called "pseudomulticellular Staphylococci" (Giesbrecht *et al.*, 1998). Asymmetric pseudomulticelluar Staphylococci (Giesbrecht *et al.*, 1998) are formed when autolytic enzymes are inhibited, preventing the separation of daughter cells after successful cell division. Inhibition of autolytic enzymes has been previously shown at low concentrations (0.1 $\mu g\ mL^{-1}$) of penicillin (Giesbrecht *et al.*, 1998). Additionally, cinnamaldehyde (derived from cinnamon, *Cinnamomum zeylanicum*) was shown to strongly inhibit cell separation in *Bacillus cereus* (Valero and Francés, 2006), although the mechanism was not reported. In some of the large cells it was possible to

see protrusions which resemble none separated Staphylococcal cells (see **Fig. 3.9c** in particular) which lend support to this suggestion.

3.4.4 – Effect of garlic on EMRSA-16

In many cases, EMRSA-16 exhibited markedly different results to those seen for the other strains. EMRSA-16 was slightly less sensitive to garlic (when determined by viable count), although growth was inhibited to an extent by proportionally lower concentrations (i.e. those relative to the respective MIC values) of garlic than other strains. As previously mentioned, although the difference between the MICs obtained through different methods is minimal, other data support the atypical responses of this strain. The viability of EMRSA-16 was also less affected than other strains in PBS after 24 h, although after 48 h viability was decreased. Cells of EMRSA-16 with abnormal morphologies were also more frequent than of other strains and were obvious at 0.25 mg mL^{-1} (0.125x MIC), a much lower concentration than required for other strains (0.6 mg mL^{1-}; 0.4x MIC). In terms of the effect of garlic on growth dynamics, EMRSA-16 showed the same responses as all other strains. It is unclear why EMRSA-16 showed such different responses to garlic. In some respects it appeared to be more susceptible to the effects, and yet was able to survive and grow at higher concentrations of garlic. Studies of the antibacterial effects of garlic on a much wider range of strains might result in other strains exhibiting similar responses, from which it might be possible to determine reasons for these atypical responses. EMRSA-16 is one of the most prevalent strains in hospitals (Moore and Lindsay, 2002), perhaps suggesting an innate ability to persist in unfavourable conditions.

3.5 – Conclusions

- MRSA and *S. aureus* are both susceptible to garlic – **Hypothesis 1 ACCEPTED**
- The antibiotic resistance mechanisms of MRSA had no impact on garlic sensitivity; MRSA and *S. aureus* were similarly susceptible to garlic – **Hypothesis 2 ACCEPTED**
- The effects of garlic on MRSA and *S. aureus* are bacteriostatic at concentrations $\leq$2x MIC and bactericidal at concentrations $\geq$5x MIC
- Garlic affects both the lag phase and growth rates of MRSA and *S. aureus* – **Hypothesis 3 REJECTED**
- Garlic had no effect on the tolerance of MRSA to NaCl – **Hypothesis 4 REJECTED**
- Sub-MIC concentrations of garlic have little effect on the morphology of *S. aureus* – **Hypothesis 5 REJECTED**
- The Staphylococcal cell wall is not a major target of garlic

4 – The Effects of β-lactam Antibiotics on the Growth of MRSA and *S. aureus*

4.1 – Introduction

4.1.1 – The bacterial cell wall

Whilst Gram-negative and Gram-positive bacteria differ in the structures of their cell walls (a basis upon which the differential Gram stain works), they share the same essential cell wall polymer; peptidoglycan (Labischinski and Maidhof, 1994). Gram-positive bacteria have a thick cell wall consisting of 30-40 sheets of peptidoglycan, whereas the Gram-negative cell wall is much thinner, containing just 1 or 2 sheets (Labischinski and Maidhof, 1994). The formation of pentaglycine links between peptidoglycan polymers, so called "cell wall cross-linking", is catalysed by transpeptidases referred to as penicillin binding proteins (PBPS) in both Gram-positive and –negative bacteria (Giesbrecht *et al.*, 1998). In Gram-positive cell walls there is a much higher degree of cross-linking (up to 70%) compared with Gram-negative cell walls (25-30%; Labischinski, 1992). Staphylococci, however, have an extremely high degree of cross-linking (up to 90%) due to a slightly different cell wall arrangement (Labischinski, 1992).

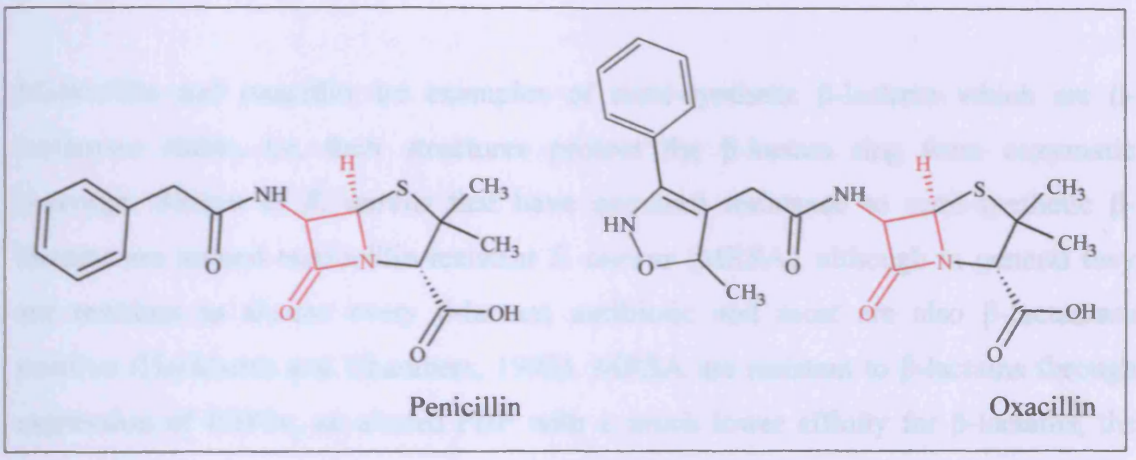
4.1.2 – β–lactam antibiotics

Penicillin and oxacillin (used in the present study; **Fig. 4.1**) are examples of β-lactams; broad spectrum, cell wall active, bactericidal antibiotics. Penicillins (with the suffix –cillin e.g. penicillin, oxacillin, methicillin), cephalosporins (with the prefix ceph- or cef- e.g. cefuroxime, ceftriaxone) and carbopenems (with the suffix –penem e.g. imipenem, meropenem) are all grouped as β-lactam antibiotics due to their universal active site, the β-lactam ring (**Fig. 4.1**). The discovery of the first β-lactam, penicillin, was attributed to Sir Alexander Fleming in 1929 (Fleming, 1929). It is reported that Fleming found a zone of clearing around a fungal contaminant on a plate of *Staphylococcus* left on his bench. The fungal contaminant is now known as *Penicillium notatum* and the zone of clearing produced by the antibiotic penicillin F.

β-lactam antibiotics specifically target peptidoglycan synthesis, and therefore most are more active against Gram-positive bacteria than Gram-negatives. The β-lactam ring (**Fig. 4.1**) is an analogue of the final amino acid residues on peptidoglycan between

which cross-linking occurs. The β-lactam ring binds and irreversibly acylates the active site of the PBPs, preventing the final transpeptidation reaction and thus inhibiting cross-linkage between peptidoglycan molecules (Labischinski and Maidhof, 1994). Penicillin, for example, reduces cross-linking in the Staphylococcal cell wall from approx. 85% to 60% (Giesbrecht *et al.*, 1998). Interestingly, there is no correlation between the degree of cross-linking inhibition and harm to the cell (Labischinski, 1992).

Fig. 4.1 – The chemical structures of penicillin and oxacillin



The β-lactam ring common to all β-lactam antibiotics is highlighted in red

Initially, the inhibition of cross-wall linkage was thought to result in immediate cell death, because the poorly formed walls could not withstand the high internal cellular pressure (up to 2 MPa; Labischinski, 1992) causing the cells to burst. However, this appears to be an oversimplification. Penicillin only affects growing and dividing cells. Furthermore it only affects the part of the cell that is actually enlarging i.e. (with the exception of rod shaped cells) the nascent cross-walls during cell-division (Giesbrecht *et al.*, 1998). Treatment with low, but still bactericidal concentrations of penicillin (at which PBPs are not inhibited; Labischinski, 1992) does not inhibit cell wall formation, but the new walls are deformed, made of loose fibrillar material and are often incomplete (Giesbrecht *et al.*, 1998). Cell death does not occur until the second cell division after treatment (Labischinski, 1992), and is a consequence of a fully functioning cell separation system, but poorly formed cell walls, or wall material deposited in the wrong areas. When the daughter cells begin to separate, the overall

cellular assembly is not structurally sufficient to protect from the internal pressure, and the cells burst (Labischinski, 1992).

4.1.3 – Mechanisms of β-lactam resistance

The first clinical application of penicillin occurred in 1942 (Grossman, 2008), and yet penicillin degrading enzymes in Staphylococci were reported in 1940 (Kirby, 1944). Penicillin-resistant bacteria express β-lactamases; enzymes which cleave the β-lactam ring, thus inhibiting the action of the antibiotics. β-lactamase expression can be either inducible (by the antibiotic) or constitutive in strains which have lost the repressor gene (Koch, 2007).

Methicillin and oxacillin are examples of semi-synthetic β-lactams which are β-lactamase stable, i.e. their structures protect the β-lactam ring from enzymatic cleavage. Strains of *S. aureus* that have acquired resistance to semi-synthetic β-lactams are termed methicillin-resistant *S. aureus* (MRSA), although in general they are resistant to almost every β-lactam antibiotic and most are also β-lactamase positive (Hackbarth and Chambers, 1993). MRSA are resistant to β-lactams through expression of PBP2a, an altered PBP with a much lower affinity for β-lactams, the expression of which is induced by the antibiotic. Interestingly, there is no correlation between the level of PBP2a production and the degree of antibiotic resistance (Labischinski, 1992).

Although *S. aureus* have four PBPs, MRSA strains produce functional cell walls with just one (PBP2a; Labischinski, 1992). It has been noted, however, that organisms expressing PBP2a in high concentrations of oxacillin (enough to inhibit all other PBPs) exhibit extreme changes in morphology, such as impaired cell separation and hypo-cross-linking of peptidoglycan. Despite the morphological defects of PBP2a derived cell walls, the current major healthcare problem of MRSA (Harbarth *et al.*, 2006) suggests that the growth, replication and virulence of the bacteria are unaffected.

The genes for both β-lactamase and PBP2a expression are located on plasmids (Hackbarth and Chambers, 1993). Furthermore, the transcription of both resistance mechanisms is regulated by the same system (Hackbarth and Chambers, 1993). There

are no equivalent genes to those for β-lactamase and PBP2a in β-lactam-susceptible strains of *S. aureus*, and the genes are also non-chromosomally encoded. This indicates that neither resistance mechanism evolved in *S. aureus* but rather they were transferred by lateral gene transfer (Koch, 2007). The origin species of both of these resistance mechanisms are unknown, but *Enterococcus hirae* has been proposed for PBP2a due to its intrinsic β-lactam resistance mediated by a very similar PBP (PBP5) to PBP2a (Labischinski, 1992).

4.1.4 – The use of oxacillin and penicillin in the present study

The overall subject of this thesis is the effect of garlic on the β-lactam susceptibility of MRSA and *S. aureus*. Although the modes of action of oxacillin and penicillin are well known, it is important to define how these two antibiotics affect the growth and viability of the thesis strains so that the effect of garlic on β-lactam susceptibility in terms of these parameters can be determined.

4.1.5 – Aims

- Determine the effects of oxacillin and penicillin on the growth dynamics of MRSA and *S. aureus*

4.2 – Materials and Methods

4.2.1 – Detection of β-lactamase expression

β-lactamase production and expression was assessed using β-lactamase (Nitrocefin) sticks (Oxoid, Hampshire, UK) according to manufacturer's instructions, briefly; colonies were assessed after 24 h growth on MHSC (constitutive expression) or MHSC containing 6 μg mL^{-1} oxacillin (inducible expression). A small sample of organisms from a single colony was applied to each stick, moistened with distilled water and examined for colour change (positive = red, negative = no change) after 1 min, 5 min and 60 min. Results are expressed as either positive constitutive, positive inducible or negative (see **Appendix** for full results).

The sensitivities of MRSA and *S. aureus* to oxacillin were determined using three different methods (sensitivity to penicillin was assessed using two methods).

4.2.2 – Disc diffusion

The disc diffusion method was conducted according to BSAC guidelines (BSAC, 2008). Inocula were prepared as previously described (**Chapter 2.5**). Inocula were diluted 1 in 10 with sterile PBS to give initial cell densities of approx. 1 x 10^7 CFU mL^{-1}. Adjusted inocula were swabbed in three directions using sterile cotton swabs (Eurolabs, Manchester, UK) onto dried MHSC plates (to create semi-confluent lawns after 24 h incubation). Antibiotic discs (Oxacillin 1 μg, Penicillin 10U; Oxoid, Hampshire, UK) were placed centrally onto each plate, and incubated (24 h, 37°C). Zone diameters were measured digitally (using IMAGE J, NIH) from images of each plate taken after 24 h incubation. Antibiotic resistance / sensitivity was interpreted according to BSAC guidelines (BSAC, 2008).

4.2.3 – Viability on oxacillin containing agar

Viability in the presence of oxacillin was determined by viable cell counts on oxacillin-containing agars (with varying oxacillin concentrations) using a modified Miles and Misra (Miles and Misra, 1938) plate count technique. MHB (10 mL) was inoculated with inocula, prepared as previously described (**Chapter 2.5**), to give initial densities of approx. 5 x 10^5 CFU mL^{-1} and incubated (24 h, 37°C). Viable cell counts were performed on MHSC plates containing a range of oxacillin

concentrations (two-fold increments), prepared from stock solutions of oxacillin in PBS. MICs were determined as the lowest concentration of oxacillin to limit growth to initial cell densities.

4.2.4 – Effect of oxacillin and penicillin on MRSA and *S. aureus* growth dynamics

Growth dynamics were determined from growth curves obtained using a Bioscreen C growth analyser (ThermoFisher, Leicestershire, UK) as previously described (**Chapter 2.7.4**). Lag phase duration and growth rates were calculated from each plot; results presented are means of at least four independent replicates. MICs of penicillin and oxacillin against MRSA and *S. aureus* measured by absorbance were determined from these growth curves.

Unless otherwise stated, results for MRSA N315 and *S. aureus* NCTC 6571 are representative of all other strains tested, and are representative of at least four independent replicates.

4.3 – Results

MRSA N315 expressed an inducible β-lactamase, and was resistant to both oxacillin and penicillin using all three methods (Table 4.1; Table 4.2). *S. aureus* NCTC 6571 was confirmed as being sensitive to both oxacillin and penicillin using all three methods, and did not express a β-lactamase (Table 4.1; Table 4.2).

Table 4.1 – The susceptibilities of a strain of MRSA and a strain of *S. aureus* to oxacillin and penicillin by disc diffusion

	MRSA N315		*S. aureus* NCTC 6571	
β-lactamase expression	Inducible		Negative	
4.2.2 – Sensitivity by disc diffusion (mm ± SEM; n ≥ 4)				
Oxacillin (1 μg)	6 ± 0	R	26.85 ± 0.38	S
Penicillin (10U)	9.24 ± 0.37	R	40.78 ± 0.67	S

Inhibition zones were determined using BSAC standard methodology (BSAC, 2008). Breakpoints are as follows: Oxacillin (1 μg disc) Resistant (R) < 14 mm, Sensitive (S) > 15 mm; Penicillin (10U disc) R < 24 mm, S > 25 mm

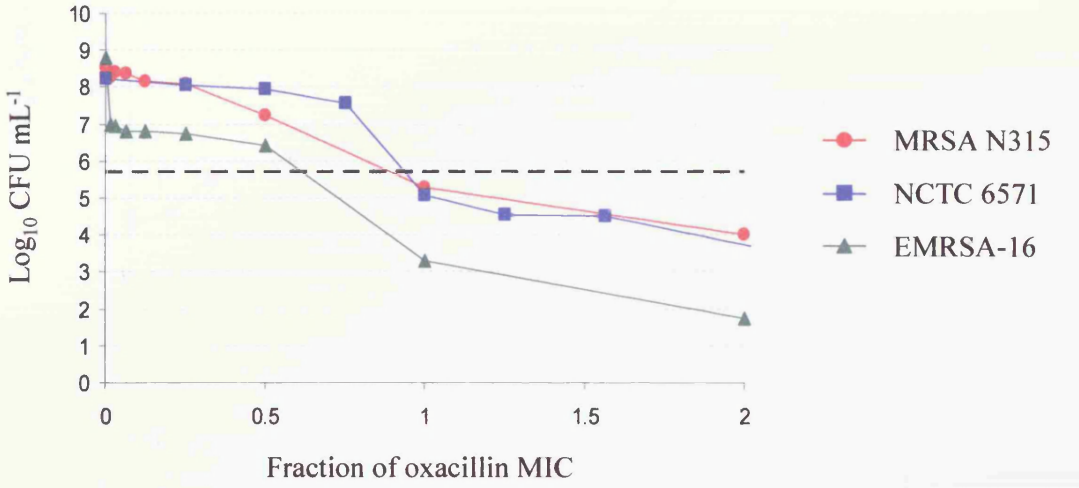
Table 4.2 – The MICs of oxacillin and penicillin against a strain of MRSA and a strain of *S. aureus*

Method of MIC measurement	MRSA N315		*S. aureus* NCTC 6571	
4.2.3 – MIC of oxacillin determined by viable count performed on antibiotic containing MHSC (μg mL^{-1})	128	R	0.08	S
4.2.4 – MIC of oxacillin determined in MHB (μg mL^{-1})	64	R	0.125	S
4.2.4 – MIC of penicillin determined in MHB (μg mL^{-1})	64	R	0.05	S

MICs determined on MHSC and in MHB using CLSI breakpoints for oxacillin (CLSI, 2007) as follows: Resistant (R) = MIC > 4 μg mL^{-1}, Sensitive (S) = MIC < 2 μg mL^{-1}

Oxacillin caused dose-dependent inhibition of the growth of MRSA N315 (**Fig. 4.2**), whereas there was little decrease in the growth of *S. aureus* NCTC 6571 at sub-MIC concentrations. The general pattern of growth inhibition of EMRSA-16 was very similar to that of NCTC 6571, except there was a ten-fold reduction in the growth of EMRSA-16 at the lowest oxacillin concentration (compared with control). At concentrations between MIC and 2x MIC there was a slight bactericidal effect against all strains.

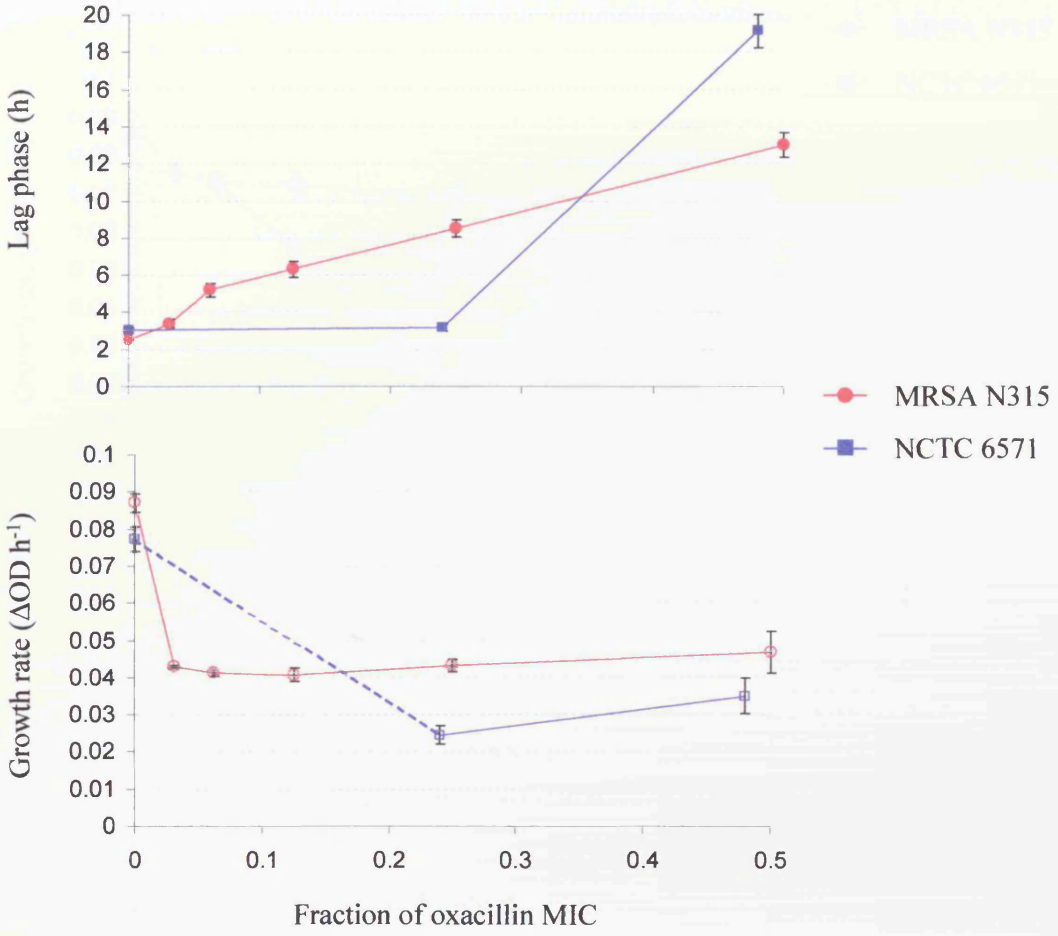
Fig. 4.2 – The effect of oxacillin on viable counts of MRSA N315 and *S. aureus* NCTC 6571



Oxacillin concentration (X-axis) is presented as a fraction of the relative MIC (MRSA N315, NCTC 6571 and EMRSA-16; 128 μg mL^{-1} and 0.08 μg mL^{-1} and 128 μg mL^{-1} respectively). Black dashed line represents initial cell densities. Legend indicates strain

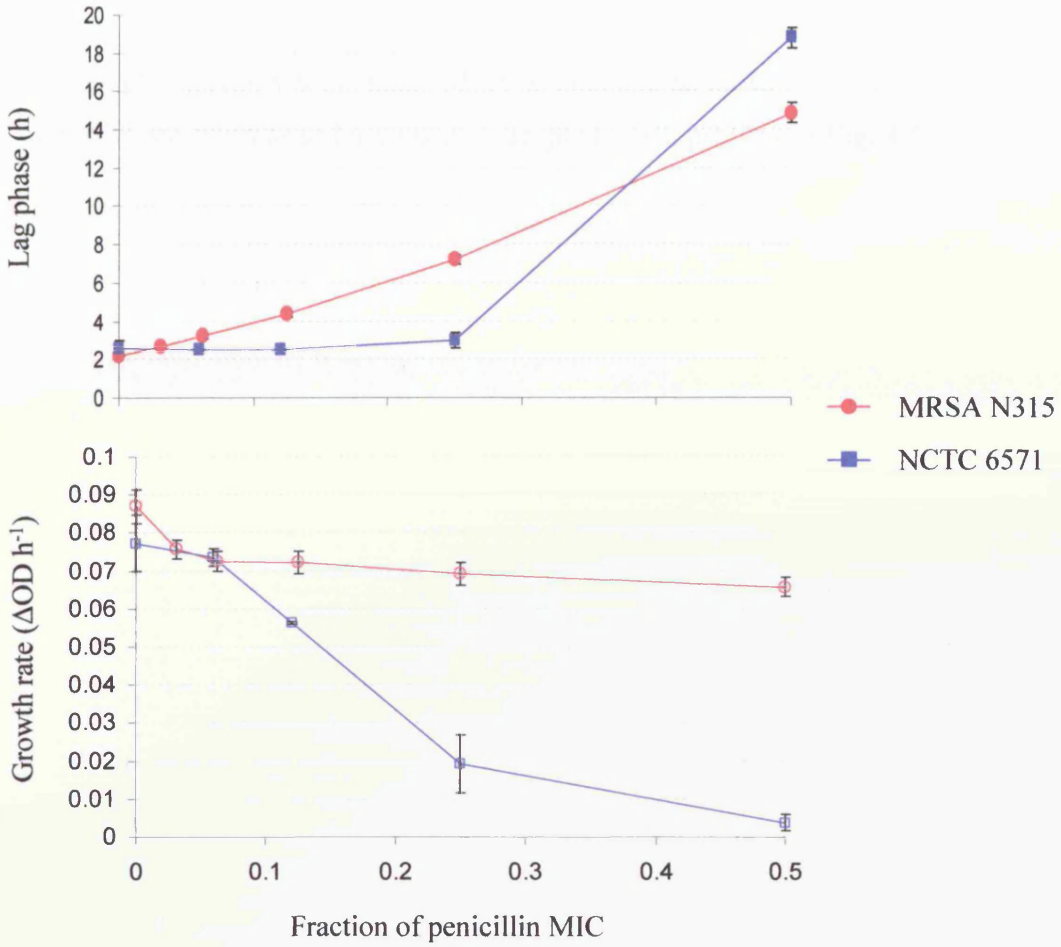
Oxacillin caused dose-dependent increases in the lag phase of MRSA N315 (**Fig. 4.3**). The lag phase of NCTC 6571 was not affected by oxacillin concentrations between 0.25 – 0.5x MIC. Conversely, the lowest oxacillin concentrations caused (approximately) 50% decreases in the growth rates of both MRSA N315 and NCTC 6571. The growth rates of both strains increased slightly with increasing oxacillin concentrations, although these increases were negligible (**Fig. 4.3**).

Fig. 4.3 – The effect of oxacillin on the growth dynamics of MRSA and *S. aureus*



Growth dynamics were determined for MRSA and *S. aureus* from growth curves obtained using a Bioscreen C growth analyser (ThermoFisher, Leicestershire, UK). Broken line between 0 and 0.25x MIC of NCTC 6571 (growth rate) indicates that no concentrations between these values were tested, and this line is therefore theoretical. Oxacillin concentration (X-axis) is presented as a fraction of the relative MIC (64 μg mL^{-1} and 0.125 μg mL^{-1} respectively). Bars indicate standard error of the mean (n ≥4). Legend indicates strain

Fig. 4.4 – The effect of penicillin on the growth dynamics of MRSA and *S. aureus*



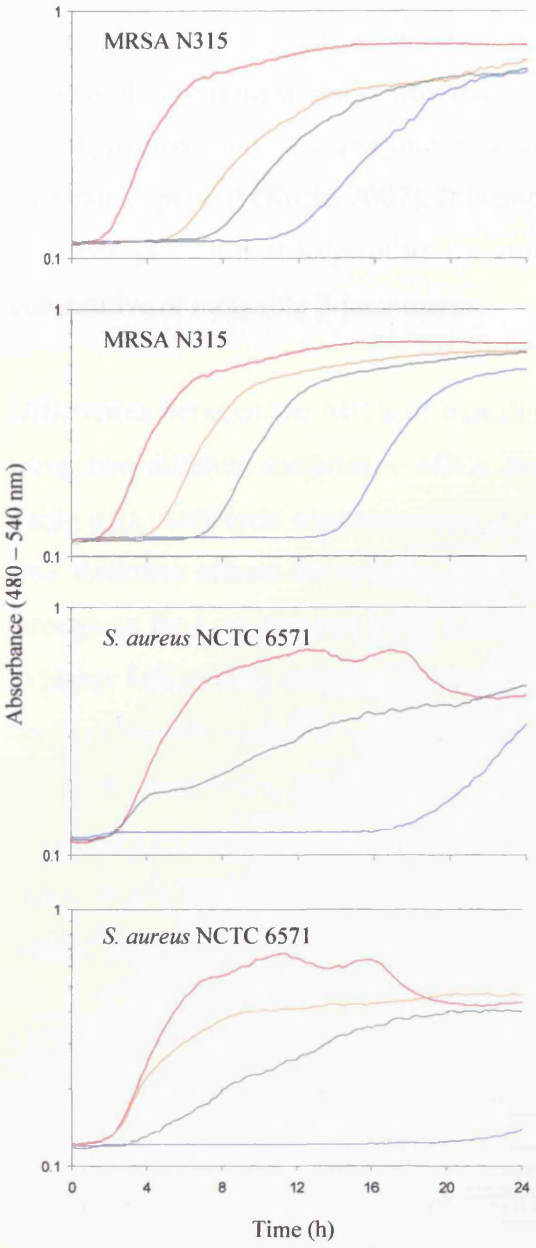
Growth dynamics were determined for MRSA and *S. aureus* from growth curves obtained using a Bioscreen C growth analyser (ThermoFisher, Leicestershire, UK). Penicillin concentration (X-axis) is presented as a fraction of the relative MIC (MRSA N315 and NCTC 6571; 64 μg mL^{-1} and 0.005 μg mL^{-1} respectively). Bars indicate standard error of the mean (n ≥4). Legend indicates strain

Penicillin affected the lag phase duration of both MRSA N315 and NCTC 6571 in a similar manner to oxacillin (**Fig. 4.4**). There were dose-dependent increases in the lag phase of MRSA N315, and the lag phase of NCTC 6571 was unaffected by penicillin concentrations <0.25x MIC. The effect of penicillin on the growth rates of the two strains was however different from that of oxacillin (**Fig. 4.4**). Penicillin caused a slight decrease in the growth rate of MRSA N315, although even at a penicillin concentration of 0.5x MIC there was only a 25% decrease in growth rate compared with control. Increasing penicillin concentrations caused sharp dose-dependent

decreases in the growth rate of NCTC 6571. At 0.25x MIC, the growth rate was approx. 25% of control.

The lag phases, growth rates and MICs of both strains in the presence of oxacillin and penicillin are summarised alongside example growth profiles in **Fig. 4.5**.

Fig. 4.5 – Representative growth profiles of MRSA and *S. aureus* with oxacillin or penicillin



Oxacillin				
Ox conc.	0%	12.5%	25%	50%
Lag phase	2.5	6.3	8.5	13
ΔOD h^{-1}	0.087	0.041	0.043	0.047
MIC	64 μg mL^{-1}			

Penicillin				
Pen conc.	0%	12.5%	25%	50%
Lag phase	2.2	4.4	7.2	14.9
ΔOD h^{-1}	0.087	0.072	0.069	0.066
MIC	64 μg mL^{-1}			

Oxacillin				
Ox conc.	0%		25%	50%
Lag phase	3		3.2	19
ΔOD h^{-1}	0.077		0.025	0.035
MIC	0.125 μg mL^{-1}			

Penicillin				
Pen conc.	0%	12.5%	25%	50%
Lag phase	2.6	2.5	3	18.8
ΔOD h^{-1}	0.077	0.056	0.019	0.004
MIC	0.05 μg mL^{-1}			

Growth profiles, presented on semi-logarithmic graphs, were obtained using a Bioscreen (ThermoFisher, Leicestershire, UK). Lag phases (h), growth rates (ΔOD h^{-1}) and MICs are presented as means (see **Fig. 4.3, 4.4**), and were not measured directly from the profiles but rather shown here merely as examples. Antibiotic concentrations are presented as a percentage of the respective MIC.

4.4 – Discussion

MRSA N315 has an inducible β-lactamase (Table 4.1). Inducible β-lactamases are only expressed when the substrate, a β-lactam ring (**Fig. 4.1**), is present (Hackbarth and Chambers, 1993). The antibiotic acts as an inducer for expression of the enzyme. Several of the strains tested constitutively expressed β-lactamases (data not presented; see **Appendix**), i.e. the enzyme is always expressed, regardless of whether the substrate is present (Koch, 2007). It is important to note that no differences were seen in either penicillin susceptibility or growth dynamics of those strains expressing constitutive or inducible β-lactamases.

Differences between the MICs of oxacillin against MRSA and *S. aureus* were noted using two different methods – MICs determined on agar and in broth (Table 4.1; Table 4.2). Antibiotic concentrations in agar are not as uniform as in broth; firstly, agar thickness affects the relative concentration and secondly antibiotic distribution throughout the agar may not be as homogenous as in broth. These factors could result in higher MICs being obtained using agar-based methods. Conversely, increasing OD does not directly correlate with bacterial growth, and does not differentiate between live and dead cells (see **Chapter 1**). Despite the differences between MICs determined by these two methods, it was very clear that MRSA N315 was resistant to oxacillin and penicillin, and NCTC 6571 was sensitive to both antibiotics. Neither method provided ambiguous results.

The differences between the effects of these two β-lactam antibiotics on the growth of MRSA and *S. aureus* were probably caused by a combination of the following factors:

1) Oxacillin resistance is not homogenous (Sutherland and Rolinson, 1964):

   Many populations of MRSA consist of both β-lactam-resistant and -sensitive organisms, the antibiotic-resistant portion being slower growing (Sutherland and Rolinson, 1964). This, in part, explains why the growth rate of MRSA decreased in the presence of oxacillin compared with control. It is likely that the presence of oxacillin inhibited the antibiotic-susceptible population. Expression of PBP2a is at an energy cost to the organism and although *S. aureus* can grow using just one PBP (for example PBP2a, as is the case for MRSA), growth is slower and not to the same extent as that of control

organisms expressing the four typical PBPs (Labischinski, 1992). Therefore, the portion of the population capable of surviving and growing in oxacillin do so at a slower rate than control.

2) Although the exact functions of each individual PBP is unknown, it is likely that they are inhibited to different extents by β-lactam antibiotics, hence as antibiotic concentration increases, the activity of each PBP decreases (Giesbrecht *et al.*, 1998):

   The exact function of each PBP in *S. aureus* is unknown (Giesbrecht *et al.*, 1998). However, although growth can occur with just one PBP, all four would not be expressed typically unless their expression facilitated optimal growth. Furthermore, because the PBPs have different functions, it is likely that they will be inhibited to different extents by β-lactam antibiotics. Therefore, the decline in the growth rate of *S. aureus* in the presence of oxacillin and penicillin is likely to relate to increasing inhibition of PBP action. This suggestion is supported because the lag phase duration of *S. aureus* did not increase with increasing concentrations of either antibiotic <0.5x MIC. This indicates that the antibiotics were not at a sufficient concentration to inhibit all four PBPs but did partially inhibit some PBP action. Therefore although growth was not inhibited, the rate of growth was decreased.

3) Penicillin is a more effective antibiotic against *S. aureus* than oxacillin

   Although *S. aureus* was sensitive to both penicillin and oxacillin, results showed that *S. aureus* was much more sensitive to penicillin than oxacillin (Table 4.2). It is possible that although the side chain of oxacillin protects from cleavage by β-lactamase, it also reduces the efficacy of the antibiotic. Increasing concentrations of penicillin caused proportional decreases in the growth rate if *S. aureus*, but this was not the case for oxacillin, lending support to the suggestion of reduced efficacy.

4.5 – Conclusions

- MRSA N315 is resistant to penicillin and oxacillin and expresses an inducible β-lactamase
- NCTC 6571 is susceptible to penicillin and oxacillin
- Penicillin and oxacillin caused proportional increases in the lag phase duration of MRSA, and caused decreased growth rates (compared with control) that did not change with increasing antibiotic concentration
- Penicillin and oxacillin at concentrations <0.5x MIC did not affect the lag phase of *S. aureus*, but did cause decreased growth rates
- The effects of penicillin and oxacillin on the growth dynamics of MRSA and *S. aureus* can be explained due to heterogeneous oxacillin resistance and differential inhibition of PBP activity

5 – The Effects of Garlic on the β-lactam Susceptibility of MRSA and *S. aureus*

5.1 - Introduction

5.1.1 – Activity of antimicrobials derived from plants compared with traditional antibiotics

Although numerous plant-derived compounds exert antimicrobial effects (see **Chapter 3.1**), their efficacies are typically weaker than those of traditionally employed antibiotics. The MICs of some plant antimicrobials are >1000 μg mL^{-1} (for example, those reported of garlic against MRSA and *S. aureus*; **Chapter 3**), concentrations at which some relatively inert compounds would also become inhibitory (Gibbons, 2004). The high MICs of plant compounds are often due to the high vegetable content (O'Gara *et al.*, 2000) as a result of freeze-drying and milling whole plants or plant tissues rather than direct extraction of antimicrobial compounds. Purification of plant antimicrobial compounds is, in some cases, a way to produce MICs comparable with those of traditionally antibiotics. For example, if allicin is taken as the major antibacterial compound in garlic (Cavallito and Bailey, 1944), then the allicin equivalence of the MICs of garlic against MRSA and *S. aureus* reported in **Chapter 3** are 3-6 μg mL^{-1} (garlic powder alliin content was 1% of which approx. 40% is converted to allicin (Lawson, 1996); MICs of garlic were 1-2 mg mL^{-1}). This of course is not a realistic MIC because it attributes all the antimicrobial activity to allicin and does not account for enhancement or antagonism of the activity of allicin by other compounds in garlic, some of which may also exert an additional antimicrobial effect. The comparison does however highlight the increase in MICs that the high vegetable content of plant preparations can account for.

Purification of antimicrobial compounds from plants also removes any interactions between components which may enhance activity or reduce the risk of developing bacterial resistance. Hence, if purified compounds are used the likelihood of bacteria developing / acquiring resistance mechanisms is increased. For these and other reasons there have recently been large increases in the area of research of interactions between whole plant extracts / preparations (as well as purified compounds) and traditional antibiotics. It appears that many plant-derived compounds enhance the activity of traditional antibiotics, of numerous classes, at sub-inhibitory

concentrations. Furthermore, potentiation of antibiotic susceptibility in antibiotic-resistant pathogens has been demonstrated with many plant compounds.

5.1.2 – Potentiation of antibiotic susceptibility in MRSA

Several plant compounds have been shown to potentiate antibiotic susceptibility against MRSA (i.e. induce antibiotic sensitivity) in addition to having moderate antibacterial activities. Sophoraflavanone G (a flavanone isolated from *Sophora* (Kowhai spp.) was synergistic / additive with both vancomycin and fosfomycin (fractional inhibitory concentration (FIC) indices (FICi) of 0.16 – 0.73; Sakagami *et al.*, 1998). Galangin, a compound with moderate antibacterial activity (MIC 62.5 – 125 μg mL^{-1}) found in *Alpinia officinarum* (Lesser galangal) was highly synergistic (FICi 0.19 – 0.25) with gentamicin (Lee *et al.*, 2008). Bidwillon B (an isoflavanone isolated from *Erythrina variegate* (Tiger's Claw)) acted synergistically with mupirocin (Sato *et al.*, 2004). *Camellia sinensis* (Tea), *Lawsonia inermis* (Henna), *Punica granatum* (Pomegranate), *Terminalia chebula* (Black myrobalan) and *Terminalia bellirica* (Bastard myrobalan) were all synergistic with tetracycline (Aqil *et al.*, 2005).

The majority of plant-antibiotic interactions research appears to be directed towards β-lactam antibiotics. Numerous plant compounds have shown potentiation of, or have synergistic relationships with, β-lactam antibiotics against MRSA (Table 5.1). Whether this prevalence of research into plant compounds and β-lactam activity enhancement is because β-lactams are the most clinically important class of antibiotics (Labischinski, 1992) and are therefore the subject of a greater research interest, or whether there is a mechanistic reason for the potentiation of β-lactam susceptibility compared with other antibiotic classes is unclear.

Table 5.1 – Plant-derived compounds that potentiate β-lactam susceptibility against MRSA

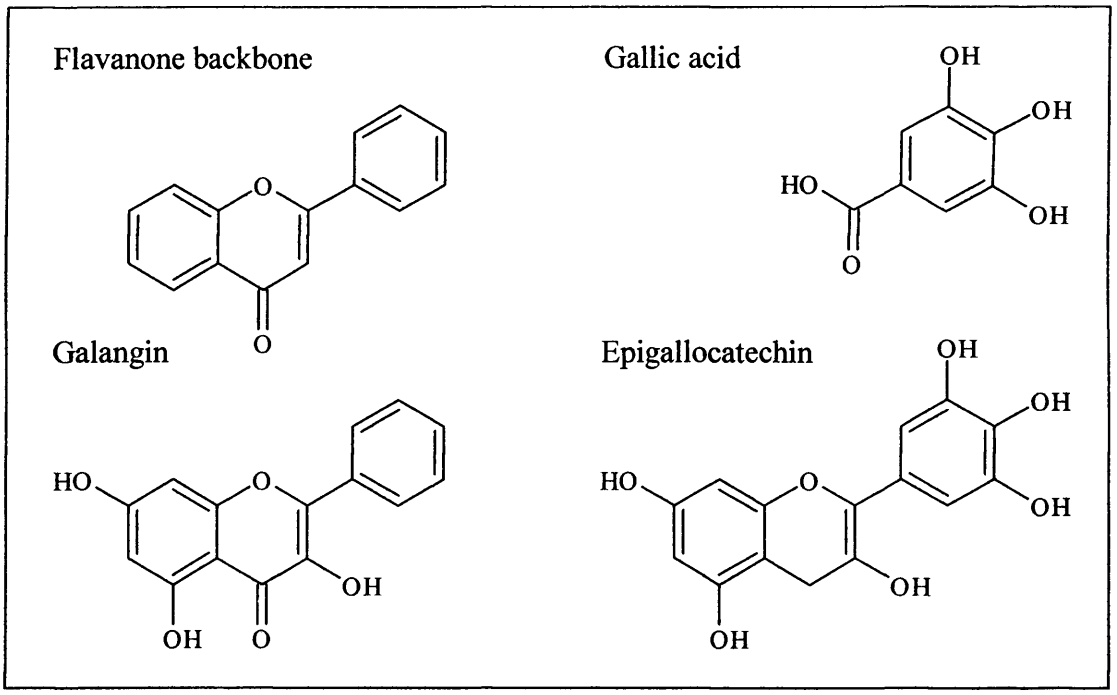
Compound	Plant derived from: (Latin binomial / common name)	FICi	References
Farnesol	Numerous including: Citronella, Lemon grass	0.012 – 0.055	Lee *et al.*, 2008
Quinic acid gallates	*Caesalpinia spinosa* / Tara	N/A	Kondo *et al.*, 2006
Ethyl gallates			Shibata *et al.*, 2005
Alkyl gallates			Shibata *et al.*, 2005
Corilagin	*Arctostaphlos uva-ursi* / Bearberry	N/A	Shimizu *et al.*, 2001
Baicalin	*Scutellaria amoena* / Baikal Skullcap	0.282 – 0.314	Liu *et al.*, 2000
Epicatechin gallate	*Camellia sinensis* / Green tea	0.199 – 0.393	Stapleton *et al.*, 2004b
Epigallocatechin gallate		0.422 – 0.891	Stapleton *et al.*, 2004b

An FIC index of ≤0.5 indicates synergism, 0.5 – 2 indicates additivity (White *et al.*, 1996). N/A indicates that no FICi was reported or calculable from the available data

A high proportion of plant compounds which potentiate antibiotic susceptibility are flavanones or gallates (e.g. galangin or epigallocatechin respectively; **Fig. 5.1**). In green tea compounds the gallate moiety appears to be critical for potentiation of β-lactam susceptibility; related compounds without the gallate moiety do not have the same effect (Stapleton *et al.*, 2004a; 2004b; Shibata *et al.*, 2005). Interestingly, compounds with gallate moieties are more often associated with potentiation of β-lactam susceptibility (Shimizu *et al.*, 2001; Stapleton *et al.*, 2004b; Shibata *et al.*, 2005; Kondo *et al.*, 2006), whereas flavanones are associated with potentiation of the activities of other antibiotic classes (Sakagami *et al.*, 1998; Lee *et al.*, 2008). This is interesting considering the structural similarity between flavanone and gallate

compounds, but, again, it is unclear whether this finding is as a result of the available published literature; for example baicalin (a flavanone derived from *Scutellaria amoena*, the Baikal Skullcap) was shown to potentiate β-lactam susceptibility in MRSA (Table 5.1; Liu *et al.*, 2000).

Fig. 5.1 – Structures of flavanones and gallates, potentiators of antibiotic-susceptibility against MRSA



Backbone structures of flavanones and gallates, potentiators of antibiotic susceptibility in MRSA, and examples of a flavanone, galangin (Lee *et al.*, 2008) and a gallate compound, epigallocatechin (Stapleton *et al.*, 2004a). Note the structural similarity between galangin and epigallocatechin

The potentiation of β-lactam susceptibility by green tea and green tea compounds (catechins) are perhaps the most studied interactions between plant compounds and antibiotics. In isolation green tea has a moderate antibacterial effect (MICs typically >100 μg mL^{-1}), but several of the compounds in green tea potentiate oxacillin (or penicillin) susceptibility in MRSA at sub-MIC concentrations. For example, oxacillin susceptibility has been induced by green tea (Cho *et al.*, 2008), epicatechin gallate (Stapleton *et al.*, 2004b) and epigallocatechin gallate (Stapleton *et al.*, 2004b) against MRSA but not by catechin (Stapleton *et al.*, 2004b), which does not contain a gallate

moiety. Potentiation of oxacillin susceptibility is thought to be exerted from a locus in, or on, the bacterial cell wall, and inhibition of PBP2a (along with other PBPs) has been proposed as the basis for this potentiation (Zhao *et al.*, 2001). Epigallocatechin gallate also modulates tetracycline sensitivity in Staphylococci by inhibiting tetracycline efflux pumps (Sudano-Roccaro *et al.*, 2004), but there was no enhancement of vancomycin activity by epicatechin gallate against MRSA (Hamilton and Shah, 1999). These data therefore suggest that potentiation of antibiotic susceptibility by green tea compounds is mediated by interaction with, or facilitation of, specific antibiotic resistance mechanisms.

5.1.3 – Interactions between garlic compounds and antibiotics

There has also been a lot of research on interactions between garlic compounds and antibiotics against a range of pathogenic bacteria. Many garlic compounds have been shown to enhance antibiotic efficacy (Table 5.2; Table 5.3). For example, diallyl sulphides enhanced the activity of a range of β-lactams (meropenem, ceftazidime, imipenem) and gentamicin (aminoglycoside) against *E. coli*, *Enterococcus faecalis*, *Enterobacter cloacae* and *Citrobacter freundii* (Yin *et al.*, 2002; Maldonado *et al.*, 2005). The enhancement of activity increased with the increasing number of sulphur atoms (or disulphide bonds) of the diallyl sulphides. It has also been noted that the antibacterial activity of these compounds increases similarly; i.e. diallyl sulphide is a less effective antibacterial and enhancer of antibiotic activity than diallyl trisulphide. It is interesting to note that the majority of other plant compounds shown to potentiate antibiotic susceptibility contain cyclic hydrocarbons (for example, **Fig. 5.1**), whereas there are no garlic compounds with such structures (**Chapter 1**).

Conversely, garlic and allicin have also been reported as having no effect on antibiotic efficacy in several interactions (Table 5.2; Table 5.3). The data available on enhancement of antibiotic activity vary depending on garlic compound, antibiotic and also detection method, which might suggest that garlic (or garlic compounds) specifically interact with some antibiotics, or facilitate their action as a result of similar targets. For example, it is assumed garlic and omeprazole are synergistic against *Helicobacter pylori* because both compounds reportedly target enzymes with –SH groups (Jonkers *et al.*, 1999b). Synergism between garlic and nisin against *Listeria monocytogenes* (*L. monocytogenes*), however, is thought to be due to

dissipation of membrane potential by nisin, which presumably facilitates the action of allicin (Singh *et al.*, 2001). Although allicin freely permeates bacterial membranes (Miron *et al.*, 2000), there was no effect on bacterial membrane fluidity (Tsuchiya and Nagayama, 2008), making it unlikely that both allicin and nisin have the same target.

Table 5.2 – Interactions between garlic and antibiotics against a range of pathogenic bacteria

Antibiotic	Antibiotic Class	Organism	Interaction	Reference
Tobramycin	Aminoglycoside	*Ps. aeruginosa* (biofilm associated)	Synergistic	Rasmussen *et al.*, 2005
Vancomycin	Glycopeptide	*Enterococcus* spp.	Synergistic	Jonkers *et al.*, 1999a
Omeprazole	N/A	*Helicobacter pylori*	Synergistic	Ceylan and Fung, 2004
Nisin	N/A	*Listeria monocytogenes*	Synergistic	Singh *et al.*, 2001
Amoxicillin	β-lactam	*Helicobacter pylori*	None	Jonkers *et al.*, 1999b
Clarithromycin	Macrolide	*Helicobacter pylori*	None	Jonkers *et al.*, 1999b
Metronidazole	Nitroimidazole	*Helicobacter pylori*	None	Jonkers *et al.*, 1999b
Anti-tuberculosis drugs	N/A	*Mycobacterium* spp.	None	Abbruzzese *et al.*, 1987

Omeprazole is a proton-pump inhibitor. Nisin is a polycyclic peptide antimicrobial. "Synergistic" indicates that FICi values of ≤ 0.5 were quoted. "None" indicates that there was no enhancement of antibiotic activity by garlic

Table 5.3 – Interactions between allicin and antibiotics against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Antibiotic	Class	Organism	Interaction	Reference
Cefazolin	β-lactam	*S. aureus*	Synergistic	Cai *et al.*, 2007
		Ps. aeruginosa	Enhancement	Cai *et al.*, 2008
Oxacillin	β-lactam	*S. aureus*	Synergistic	Cai *et al.*, 2007
		Ps. aeruginosa	Enhancement	Cai *et al.*, 2008
		S. aureus	None	Betoni *et al.*, 2006
Cefoperazone	β-lactam	*Ps. aeruginosa*	Synergistic	Cai *et al.*, 2007
		Ps. aeruginosa	Enhancement	Cai *et al.*, 2008
Ampicillin	β-lactam	*S. aureus*	None	Betoni *et al.*, 2006
Cefoxitin		*S. aureus*	None	Betoni *et al.*, 2006
Penicillin		*S. aureus*	None	Betoni *et al.*, 2006
Tetracycline	Tetracycline	*S. aureus*	Enhancement	Betoni *et al.*, 2006
Cotrimaxazole	Sulfonamide	*S. aureus*	Enhancement	Betoni *et al.*, 2006
Gentamicin	Aminoglycoside	*S. aureus*	Enhancement	Betoni *et al.*, 2006
Tobramycin		*Ps. aeruginosa*	None	Cai *et al.*, 2008
Netilmicin		*S. aureus*	None	Betoni *et al.*, 2006
Ciprofloxacin	Fluoroquniolone	*Ps. aeruginosa*	None	Cai *et al.*, 2008
Ofloxacin		*S. aureus*	None	Betoni *et al.*, 2006
Chloramphenicol	Chloramphenicol	*S. aureus*	None	Betoni *et al.*, 2006
Erythromycin	Macrolide	*S. aureus*	None	Betoni *et al.*, 2006
Vancomycin	Glycopeptide	*S. aureus*	None	Betoni *et al.*, 2006

S. aureus = *Staphylococcus aureus*; *Ps. aeruginosa* = *Pseudomonas aeruginosa*. "Synergistic" indicates that FICi values of ≤ 0.5 were quoted. "Enhancement" indicates that antibiotic activity was increased but not quantified. "None" indicates that there was no enhancement of antibiotic activity by allicin

The variability between the available data perhaps also highlights that some of the methods employed to determine interactions may not have been ideal. Enhancement of antibacterial activity by garlic (or garlic compounds) has been determined using both broth-based and agar based methods. Broth-based methods (for example; Jonkers *et al.*, 1999a; Tsao and Yin, 2001a; Singh *et al.*, 2001; Yin *et al.*, 2002; Maldonado *et al.*, 2005; Cai *et al.*, 2008) were either chequerboard experiments, or larger volumes of liquid media containing both garlic compounds and antibiotics in combination. In general chequerboard experiments provided a more comprehensive investigation into these interactions, whilst other broth methods investigated a limited range of garlic concentrations, typically 0.25x or 0.5x MIC. Agar based methods (Jonkers *et al.*, 1999b; Betoni *et al.*, 2006) either utilised agars containing the garlic-compound alone or the garlic compound and antibiotic together. Where "garlic agars" were used, antibiotic containing discs were applied to the agar surface (Betoni *et al.*, 2006). In both investigations using agar-based methods, enhancement of activity was only shown with some antibiotics. It is important to note that preparation of garlic-compound containing agars may not be an ideal method to quantify these types of interactions. As previously discussed, allicin and other garlic compounds are volatile, and degradation of these compounds is more rapid at higher temperatures (e.g. when incubated; Lawson, 1996). Therefore, garlic containing agars are likely to lose activity more rapidly than broth due to the larger surface area. It has, for example, been reported that the anti-Candidal activity of "garlic-agar" decreased with time (Barone and Tansey, 1977). Whilst it is accepted that these investigations may report a lack of antibiotic enhancement by garlic compounds due to different cellular targets, or even inhibition of the activity of one compound by the other, they could also have reported false negative results as a result of lower than expected agar garlic concentrations. For example, a synergistic relationship was reported between allicin and oxacillin against *S. aureus* using a broth based method (Cai *et al.*, 2008) whilst using an allicin-containing agar method there was no enhancement of oxacillin activity (Betoni *et al.*, 2006).

5.1.4 – Quantification of interactions between two antimicrobial compounds

The combinatorial effects of inhibitory agents are of great research interest because of their clinical potential, particularly against antibiotic-resistant pathogens (Greco *et al.*, 1995). At the very least, combinations that display some degree of synergy are deserving of further in-depth study (Greco *et al.*, 1995). In antimicrobial chemotherapy, minimum inhibitory concentrations (MICs) are the most frequently used measure of the efficacy of an agent (Andrews, 2001). MICs are a quantification of the lowest concentration of a compound that inhibits growth above a starting point over a set period of time (traditionally 24 h and measured using absorbance or viable cell counts; Andrews, 2001). The combinatorial effects of two compounds can be quantified similarly using fractional inhibitory concentrations (FICs). FICs are, as the name implies, an indication of the fraction of the MIC of each compound required to inhibit growth over a set period of time in combination. To define, or describe an interaction, FICs for each compound are summed to create an FIC index (FICi) which is compared with set-breakpoints for synergism, antagonism or additivity. In the present study a FIC index of ≤ 0.5 was considered to represent synergism, a value of between >0.5 and ≤ 2 to represent additive and >2 to represent antagonism (White *et al.*, 1996). A synergistic interaction is one where growth is inhibited at lower concentrations of both compounds than would be expected considering their efficacy when employed separately. Equally, an antagonistic interaction would require higher concentrations of both compounds to inhibit growth than expected. FICs and FIC indices (FICi) are calculated from MICs using the following equation:

$$FICi = FIC(Antimicrobial) + FIC(Oxacillin)$$

Where:

$$FIC(Antimicrobial) = \frac{MIC\ of\ Antimicrobial\ in\ Combination}{MIC\ of\ Antimicrobial\ Alone}$$

And:

$$FIC(Oxacillin) = \frac{MIC\ of\ Oxacillin\ in\ Combination}{MIC\ of\ Oxacillin\ Alone}$$

5.1.5 – The present study

There are reports of synergism between allicin and oxacillin against both *S. aureus* and *Pseudomonas aeruginosa* (Cai *et al.*, 2007; 2008). Furthermore, Cottrell (2003) presented preliminary data which suggested there was a synergistic relationship between aqueous preparations of garlic and oxacillin against MRSA and *S. aureus*. It was suggested that the proposed action of garlic on the Staphylococcal cell wall facilitated the activity of oxacillin against MRSA and *S. aureus* (Cottrell, 2003). However, previous results in the present study suggest that garlic does not damage the integrity of the Staphylococcal cell wall (**Chapter 3**). These investigations therefore expanded on those of Cottrell (2003), investigating the interaction between garlic and oxacillin against MRSA and *S. aureus* using two different approaches:

1) The effect of treating MRSA and *S. aureus* with garlic on oxacillin susceptibility determined by viable count
2) The effect of combinations of garlic and oxacillin (or penicillin) on the growth of MRSA and *S. aureus* determined in broth cultures by chequerboard assays

These experiments are presented separately to create a clear distinction between the two approaches. The aims, methods and rationales behind each experimental approach are also presented separately.

5.1.6 – Aims

Specific aims and hypotheses are dealt with in the relevant sections, but the overall aim was:

- Determine the effects of garlic on the susceptibility of MRSA and *S. aureus* to β-lactam antibiotics

5.2 – The effect of pre-treatment with garlic on the oxacillin susceptibility of MRSA and *S. aureus*

5.2.1 – Background

Preliminary work by Simon Cottrell (unpublished) suggested that treatment of both MRSA and *S. aureus* with garlic increased oxacillin sensitivity in a synergistic manner. It was also suggested that garlic affected the cell wall formation or cell wall integrity of MRSA and *S. aureus* resulting in lag phase prolongation but no effect on specific growth rates (Cottrell, 2003). The proposed target of the cell wall in part explained the increased sensitivity to oxacillin (a cell wall active antibiotic; **Chapter 4.1**) of garlic treated organisms.

Previously in this study, however, it was shown that garlic did not affect the tolerance of MRSA and *S. aureus* to NaCl, therefore, in part, implying that the Staphylococcal cell wall was not a major target of garlic (in addition to other results which implied the same; **Chapter 3**). The sensitivity of garlic treated MRSA and *S. aureus* to oxacillin was therefore reassessed using the same method as that of Cottrell (unpublished) with a number of strains.

5.2.2 – Aims

- Determine the effect of treatment with garlic on the susceptibility of MRSA and *S. aureus* to oxacillin using viable count as an indicator of growth

5.2.3 – Hypotheses

1. Treatment with garlic will increase the sensitivity of MRSA and *S. aureus* to oxacillin
2. Oxacillin sensitivity (i.e. an MIC <4 μg mL^{-1}) will be induced in MRSA at sub-MIC concentrations of garlic)

Although it was previously shown in this study that garlic did not affect the integrity of the Staphylococcal cell wall (**Chapter 3**), it was also shown that garlic inhibited the growth of both MRSA and *S. aureus* (**Chapter 3**). It is therefore reasonable to suggest that the inhibitory effect of garlic on MRSA and *S. aureus* will increase sensitivity to another inhibitory compound.

5.2.4 – Materials and methods

MHA plates were prepared according to manufacturer's instructions (with 2% w/v NaCl), but with only 450 ml water per 500 ml agar required, allowing for the addition of 50 ml oxacillin solution. Stock solutions of oxacillin were prepared in PBS, and diluted with sterile PBS to required concentrations (Doubling dilutions; 50 mL volumes). Oxacillin additions were made into molten, sterile MHSC, and mixed thoroughly before plates were poured. Oxacillin agar plates were kept for a maximum of 2 weeks at 4°C before use, and were dried in a laminar flow cabinet when required.

Stock solutions of garlic were prepared as previously described (**Chapter 2.3**) and diluted in MHB to desired concentrations in final volumes of 10 mL. Optically adjusted inocula were prepared as previously described (**Chapter 2.5**) and garlic solutions were inoculated to achieve initial cell densities of approx. 5 x 10^5 CFU mL^{-1}. After 24 h incubation (37°C) the susceptibility of garlic exposed cultures to oxacillin was assessed by viable cell counts on agar plates containing a range of concentrations of oxacillin using a modified Miles and Misra plate count technique as previously described (**Chapter 2.6**). Colonies were counted after 24 h incubation at 37°C.

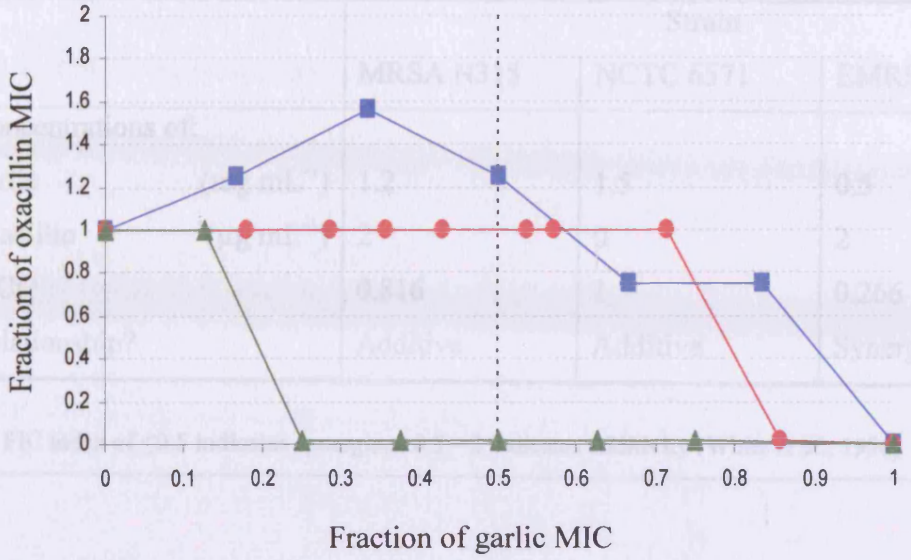
Results are presented as the mean of at least four independent replicates, and unless otherwise stated are representative of all other MRSA and *S. aureus* strains tested.

5.2.5 – Results

With the exception of EMRSA-16, garlic concentrations <0.5x MIC had either no effect on the oxacillin sensitivity of MRSA and *S. aureus*, or actually decreased oxacillin sensitivity (i.e. increased the MIC; **Fig. 5.2**). The decrease in the oxacillin sensitivity of NCTC 6571 at garlic concentrations <0.5x MIC is considered later (**Section 5.4**). Treatment with garlic concentrations 0.67x – 0.8x respective MIC induced oxacillin sensitivity (i.e. reduced the MIC of oxacillin to ≤4 μg mL^{-1}; CLSI, 2007) in 4/6 MRSA strains with the exception of EMRSA-16 (oxacillin sensitivity induced at 0.25x MIC of garlic; see **Appendix** for further information). Treatment with garlic at concentrations >0.5x MIC also slightly increased the sensitivity of *S. aureus* to oxacillin (**Fig. 5.2**).

EMRSA-16 treated with low concentrations of garlic (0.5 mg mL^{-1}; 0.25x MIC) did not grow on agar containing ≥2 μg mL^{-1} oxacillin and was therefore oxacillin-sensitive (CLSI, 2007). Potentiation of oxacillin susceptibility with garlic concentrations <0.5x relative MIC was not seen for any other strain.

Fig. 5.2 – The effect of pre-treatment of MRSA and *S. aureus* with garlic on the MICs of oxacillin



MIC and threshold values for induction of oxacillin sensitivity by garlic	MRSA N315	*S. aureus* NCTC 6571	EMRSA-16
MIC of garlic (mg mL^{-1})	1.5	1.5	2
MIC of oxacillin (μg mL^{-1})	128	0.08	128
Induction of oxacillin sensitivity (mg mL^{-1} garlic)	1.2	N/A	0.5

Garlic and oxacillin concentrations are presented as fractions of the MICs. Black dotted line indicates 0.5x MIC of garlic Table indicates MIC values for garlic and oxacillin for three strains, and also threshold values for induction of antibiotic sensitivity (CLSI, 2007) by garlic

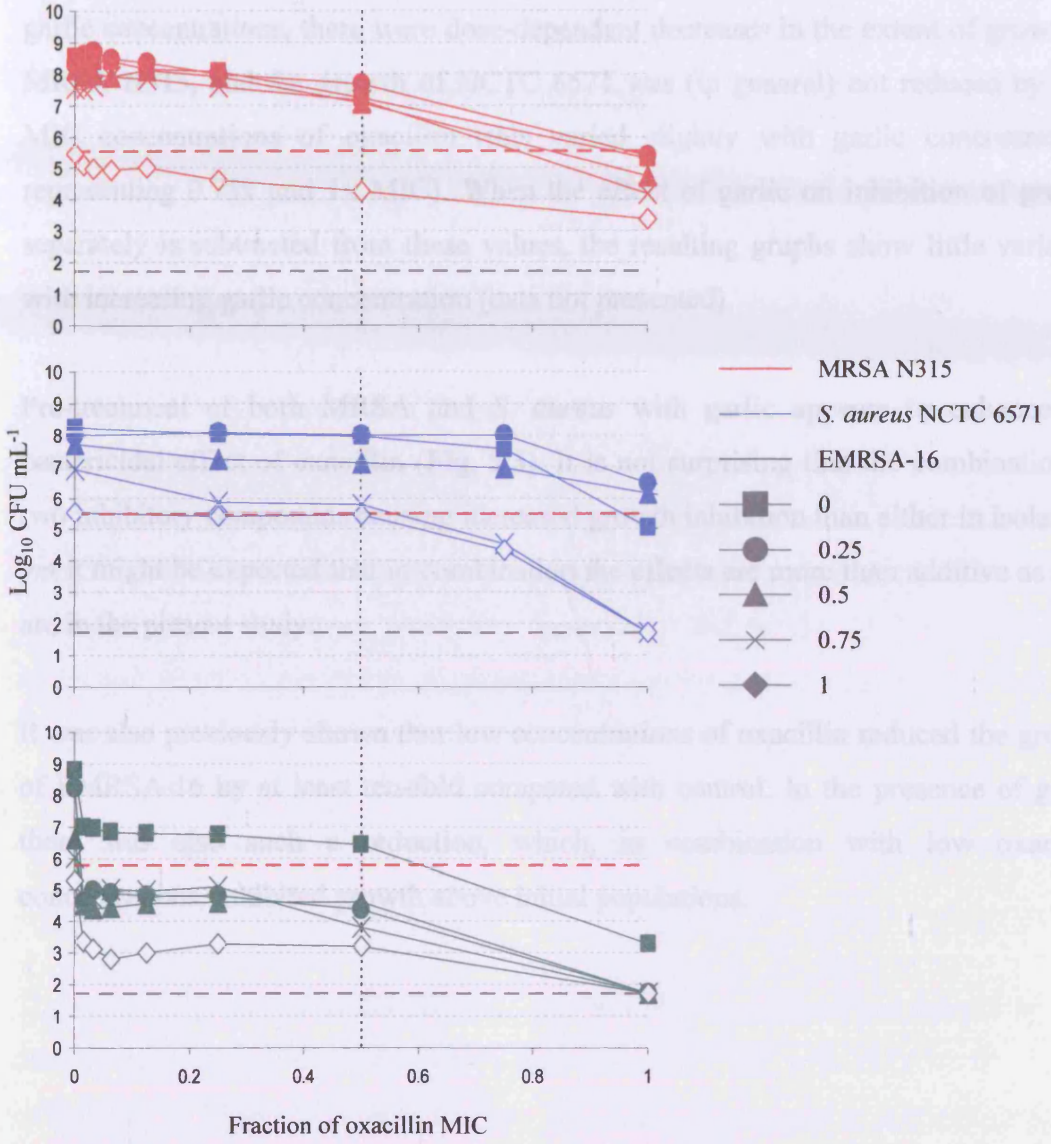
Table 5.4 – The most effective combinations of garlic and oxacillin against MRSA and *S. aureus*

	Strain		
	MRSA N315	NCTC 6571	EMRSA-16
Concentrations of:			
garlic (mg mL^{-1})	1.2	1.5	0.5
oxacillin (μg mL^{-1})	2	0	2
FICi	0.816	1	0.266
Relationship?	Additive	Additive	Synergistic

An FIC index of ≤0.5 indicates synergism, 0.5 – 2 indicates additivity (White *et al.*, 1996)

The most effective combinations of garlic and oxacillin were, in general, additive (i.e. with FIC values <2 but >0.5; Table 5.4). For some strains, garlic had no effect on oxacillin susceptibility (in terms of MIC), and hence the best FIC index was 1 (i.e. no combination was more effective than either garlic or oxacillin employed separately). Only one synergistic combination was noted; treatment of EMRSA-16 with 0.5 mg mL^{-1} garlic on MHSC containing 2 μg mL^{-1} oxacillin (Table 5.4).

Fig. 5.3 – The effect of garlic on the growth of MRSA and *S. aureus* in the presence of oxacillin



Legend indicates strain and garlic concentration (as fractions of the MICs of garlic). Oxacillin concentration (X axes) presented as fractions of the relative MICs. Red dashed lines indicate initial populations (approx. 5 x 10^5 CFU mL^{-1}). Grey dashed lines indicate limit of detection. Black dotted lines (vertical) indicate 0.5x MIC of oxacillin.

It was previously shown that increasing concentrations of oxacillin reduced the growth of MRSA and *S. aureus* after 24 h (**Chapter 4**). The presence of garlic had no impact (relatively) on the pattern of this reduction in growth, (**Fig. 5.3**) i.e. with all garlic concentrations, there were dose-dependent decreases in the extent of growth of MRSA N315, and the growth of NCTC 6571 was (in general) not reduced by sub-MIC concentrations of oxacillin (this varied slightly with garlic concentrations representing 0.75x and 1x MIC). When the effect of garlic on inhibition of growth separately is subtracted from these values, the resulting graphs show little variation with increasing garlic concentration (data not presented).

Pre-treatment of both MRSA and *S. aureus* with garlic appears to enhance the bactericidal effect of oxacillin (**Fig. 5.3**). It is not surprising that the combination of two inhibitory compounds causing increased growth inhibition than either in isolation, yet it might be expected that in combination the effects are more than additive as they are in the present study.

It was also previously shown that low concentrations of oxacillin reduced the growth of EMRSA-16 by at least ten-fold compared with control. In the presence of garlic there was also such a reduction, which, in combination with low oxacillin concentrations, inhibited growth above initial populations.

5.2.6 – Discussion

Although the best combinations of garlic and oxacillin were not synergistic (except for EMRSA-16), treatment of MRSA and *S. aureus* with garlic enhanced oxacillin susceptibility, but only at garlic concentrations >0.5x MIC. These data are therefore in disagreement with those of Cottrell (unpublished), which showed dose-dependent decreases in the sensitivity to oxacillin with increasing garlic concentration. At low concentrations of garlic (<0.5x MIC), the MIC of oxacillin against NCTC 6571 actually increased. This is termed hormesis, where a low concentration of an inhibitory compound is actually beneficial to the organism (Mattson, 2008). Hormesis, and decreasing oxacillin sensitivity caused by low concentrations of garlic is dealt with in more detail later (**Section 5.4**)

It was shown previously in this study that garlic (up to 2x MIC) was bacteriostatic against MRSA, therefore suggesting that organisms are growth inhibited at these concentrations, rather than physically damaged (**Chapter 3**). That garlic had no additional effect on the extent of growth inhibition by oxacillin lends support to this suggestion of bacteriostatic action. These data also strongly suggest that garlic and oxacillin have different mechanisms of action against MRSA and *S. aureus* and that the increasing sensitivity to oxacillin is due to the cumulative inhibitory effects of both compounds.

The effect of garlic on the limitation of growth in the presence of oxacillin (**Fig. 5.3**) provides an explanation for the synergistic relationship seen with EMRSA-16. Viable cell counts of EMRSA-16 were ten-fold less in the presence of oxacillin than in the control cultures. Garlic also limited the growth of EMRSA-16; hence garlic treated cells were additionally growth limited in the presence of oxacillin, and this combined effect inhibited growth to fewer than initial populations. The patterns of growth inhibition of both MRSA N315 and EMRSA-16 by oxacillin in increasing concentrations of garlic (**Fig. 5.3**) are very similar, with the exception of the initial ten-fold reduction seen in EMRSA-16. It therefore appears that although EMRSA-16 is slightly more tolerant to garlic than other strains (**Chapter 3**), it is atypically affected by oxacillin (**Chapter 4**), and is therefore highly susceptible to the combination of these two compounds.

5.2.7 – Conclusions

- Pre-treatment of MRSA and *S. aureus* with garlic concentrations >0.5x MIC caused increased sensitivity to oxacillin – **Hypothesis 1 ACCEPTED**
- Pre-treatment of MRSA with garlic concentrations >0.67x MIC induced oxacillin sensitivity (i.e. MIC <4 μg mL^{-1}; CLSI, 2007) in most strains – **Hypothesis 2 ACCEPTED**
- The synergistic relationship between garlic and oxacillin against EMRSA-16 is likely to be a result of an atypical response to oxacillin of this strain rather than increased tolerance to garlic

5.3 – The effect of combinations of garlic and oxacillin against MRSA and *S. aureus*

5.3.1 – Background

Treatment with garlic reduced the susceptibility of MRSA and *S. aureus* to oxacillin. It is, however, likely that should such a combination be used clinically, both antimicrobials would be administered simultaneously. It is therefore necessary to determine whether garlic and oxacillin in combination are more effective than when employed separately, and whether garlic enhances oxacillin sensitivity when both compounds are present in combination. The effect of garlic on penicillin susceptibility will also be investigated to determine whether enhancement of β-lactam activity is dependent on antibiotic resistance mechanisms.

5.3.2 – Aims

- Determine the combined effects of garlic and oxacillin against MRSA and *S. aureus* using chequerboard assays
- Determine the combined effects of garlic and penicillin against MRSA and *S. aureus* using chequerboard assays
- Compare the growth dynamics of MRSA and *S. aureus* treated with β-lactam antibiotics with those of the same strains treated with both garlic and β-lactam antibiotics.

5.3.3 – Hypotheses

3. Combinations of garlic and oxacillin will be more effective in terms of inhibition of MRSA and *S. aureus* than either compound employed separately
4. Combinations of garlic and penicillin will be more effective in terms of inhibition of MRSA and *S. aureus* than either compound employed separately
5. Oxacillin or penicillin sensitivity (i.e. an MIC <4 μg mL^{-1}; CLSI, 2007) will be induced in MRSA at sub-MIC concentration of garlic

5.3.4 – Materials and methods

The combined effects of garlic and oxacillin (or penicillin) were determined by the chequerboard method using a Bioscreen C growth analyser (ThermoFisher, Leicestershire, UK) as previously described (**Chapter 2.7.3**). Inocula were prepared as previously described (**Chapter 2.5**) and diluted 1:100 in sterile MHB to create suspensions of approx. 1 x 10^6 CFU mL^{-1}. Inocula were used within 15 min of preparation. Sterile suspensions of garlic (prepared as stock solutions in sterile MHB as previously described; **Chapter 2.3**) were diluted to four times required concentrations (garlic was only 50 µL in a total volume of 200 µL, therefore concentrations were higher than required to allow for dilution by oxacillin and the inoculum). Oxacillin and penicillin stock solutions were prepared as previously described (**Chapter 2.4**) and diluted to four times required concentrations in MHB. MICs were determined as the lowest concentrations of garlic / antibiotic to inhibit any increase in optical density over 24 h. Duration of lag phases and growth rates were calculated from each plot as previously described (**Chapter 2.7.4**); results presented are means of at least four independent replicates, all with two technical replicates. Although the chequerboard experiments investigated the combined effects of garlic and oxacillin (or penicillin) on the growth of MRSA or *S. aureus*, the results presented refer to the effect of garlic on the oxacillin (or penicillin) susceptibility of MRSA and *S. aureus* i.e. the effect of garlic on oxacillin (or penicillin) tolerance.

5.3.5 - Results

In general, increasing concentrations of garlic caused increased sensitivity of MRSA and *S. aureus* to both oxacillin and penicillin (**Fig. 5.4**). Concentrations of garlic <0.5x MIC increased penicillin sensitivity by 50% (with the exception of EMRSA-16), but the same concentrations of garlic had no effect on, or decreased oxacillin sensitivity (i.e. the MIC increased; **Fig. 5.4**). There were dose-dependent increases in the susceptibility of all strains (MRSA and *S. aureus*) to both antibiotics with garlic concentrations >0.5x MIC.

Garlic induced oxacillin sensitivity (i.e. reduced the MIC of oxacillin to ≤ 4 μg mL^{-1}; CLSI, 2007) in most MRSA strains (5/6) at concentrations of 0.75x – 0.94x respective MIC of garlic. Conversely, garlic only induced penicillin sensitivity in one MRSA strain (1/3) at 0.75x MIC (see **Appendix** for further information).

The pattern of decreasing antibiotic MIC with increasing garlic concentration differed between oxacillin and penicillin. There was a linear relationship between increasing garlic concentration and decreasing MIC of penicillin, but a sigmoidal relationship between increasing garlic concentration and decreasing MIC of oxacillin (**Fig. 5.4**).

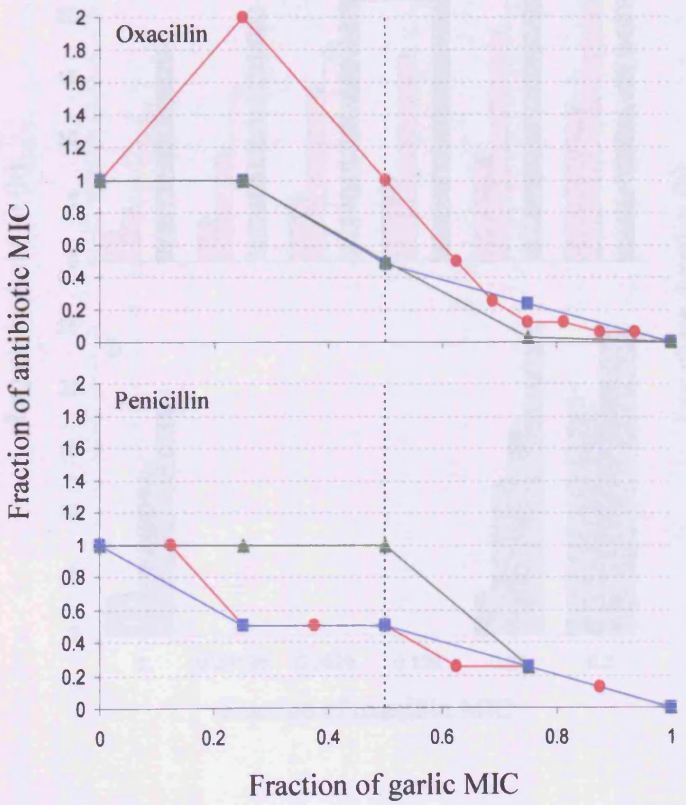
The best combinations of garlic and oxacillin or garlic and penicillin were additive for all strains (Table 5.5). Interestingly, the best combinations of garlic and penicillin comprised low concentrations of garlic (<0.5x MIC; except for EMRSA-16), conversely the best combinations of garlic and oxacillin comprised high concentrations of garlic (>0.5x MIC).

Table 5.5 – The most effective combinations of garlic and β-lactam antibiotics against MRSA and *S. aureus*

Antibiotic		Strain		
		MRSA N315	NCTC 6571	EMRSA-16
Oxacillin	Concentrations of:			
	garlic (mg mL^{-1})	0.55	0.4	0.6
	oxacillin (μg mL^{-1})	16	0.06	2
	FICi	0.875	0.98	0.7813
	Relationship?	Additive	Additive	Additive
Penicillin	Concentrations of:			
	garlic (mg mL^{-1})	0.2	0.2	0.8
	penicillin (μg mL^{-1})	32	0.025	0
	FICi	0.75	0.75	1
	Relationship?	Additive	Additive	Additive

An FIC index of ≤0.5 indicates synergism, 0.5 – 2 indicates additivity (White *et al.*, 1996)

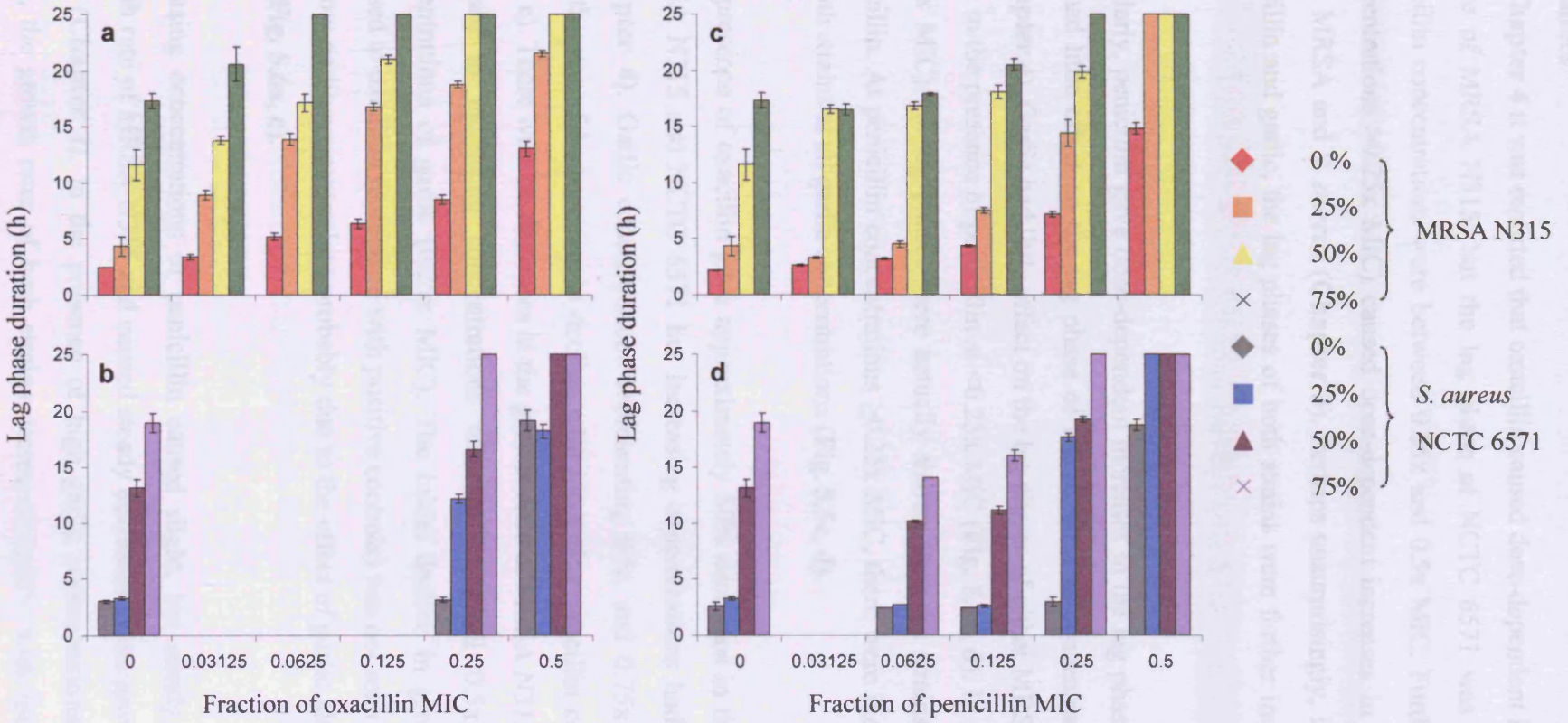
Fig. 5.4 – The effect of garlic on the MICs of oxacillin or penicillin against MRSA and *S. aureus*



MIC and threshold values for induction of antibiotic sensitivities by garlic	MRSA N315	*S. aureus* NCTC 6571	EMRSA-16
MIC of garlic (mg mL^{-1})	0.8	0.8	0.8
MIC of oxacillin (μg mL^{-1})	64	0.125	64
Induction of oxacillin sensitivity (mg mL^{-1} garlic)	0.7	N/A	0.6
MIC of garlic (mg mL^{-1})	0.8	0.8	0.8
MIC of penicillin (μg mL^{-1})	64	0.05	64
Induction of penicillin sensitivity (mg mL^{-1} garlic)	0.8	N/A	0.8

Garlic and oxacillin (or penicillin) antibiotic concentrations presented as fractions of the relative MICs. Black dotted line indicates 0.5x MIC of garlic. Table indicates MIC values for garlic, oxacillin or penicillin for three strains, and also threshold values for indication of antibiotic sensitivity by garlic

Fig. 5.5 – The effect of garlic on the lag phase duration of MRSA (a, c) and *S. aureus* (b, d) in the presence of oxacillin (or penicillin)



Effect of garlic on the lag phase duration of MRSA (**a, c**) and *S. aureus* (**b, d**) in the presence of oxacillin (**a, b**) and penicillin (**c, d**) Legend indicates strain and concentration of garlic (as a percentage of the relative MICs). Oxacillin or penicillin concentrations (X axes) presented as fractions of the relative MICs. Bars indicate standard error of the mean ($n \geq 4$)

5.3.5.1 – The effect of garlic and β-lactam antibiotics on Staphylococcal growth dynamics

In **Chapter 4** it was reported that oxacillin caused dose-dependent increases in the lag phase of MRSA N315, but the lag phase of NCTC 6571 was not affected until oxacillin concentrations were between 0.25x and 0.5x MIC. Furthermore, garlic (at concentrations >0.25x MIC) caused dose-dependent increases in the lag phases of both MRSA and *S. aureus* (**Chapter 3**). Perhaps unsurprisingly, in combinations of oxacillin and garlic, the lag phases of both strains were further increased (**Fig. 5.5a, c**).

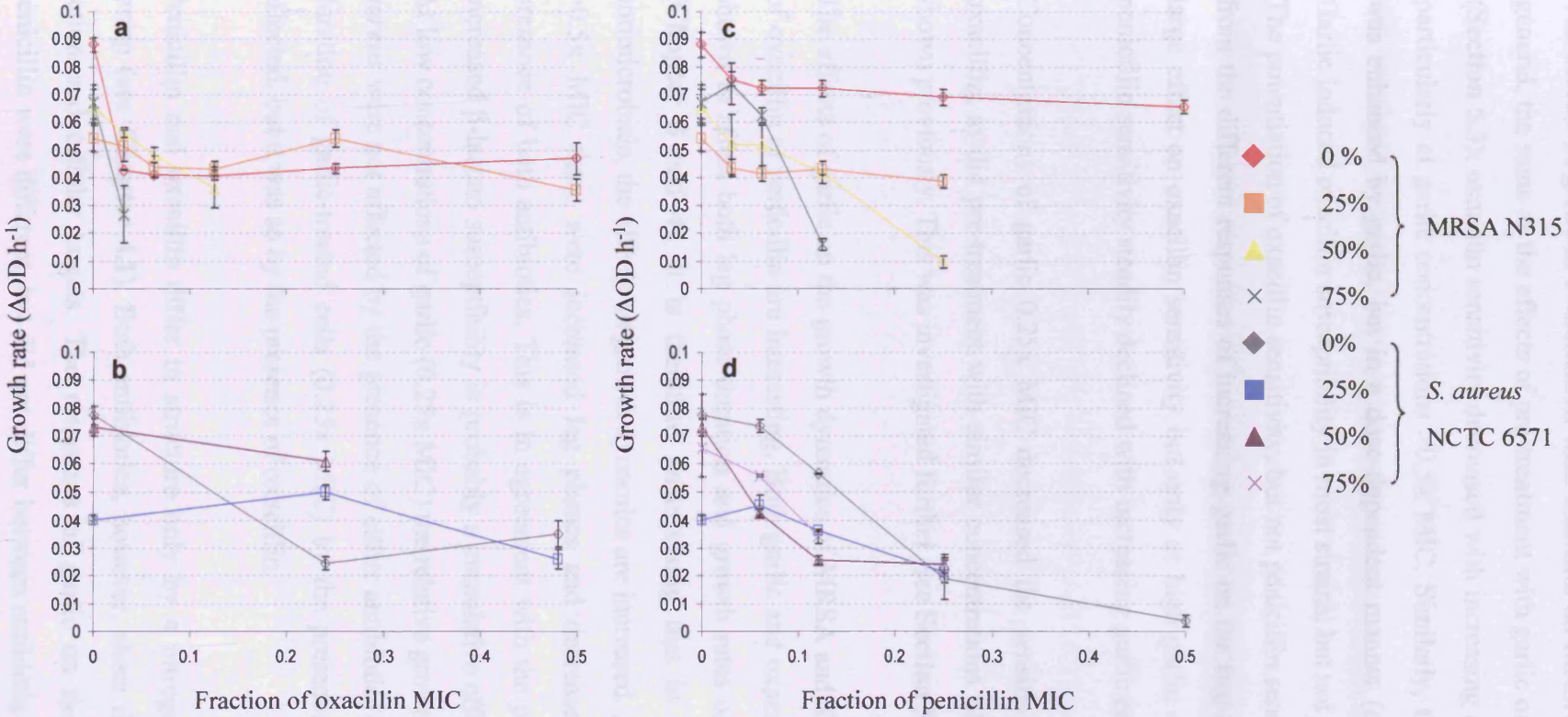
Similarly, penicillin gave dose-dependent increases in the lag phase of MRSA N315 but had little effect on the lag phase of NCTC 6571 at concentrations <0.25x MIC (**Chapter 4**). Garlic had little effect on the lag phases of either MRSA N315 or NCTC 6571 in the presence of penicillin at <0.25x MIC (**Fig. 5.5c, d**). In some cases (garlic >0.5x MIC) the lag phases were actually shorter than the strains just treated with penicillin. At penicillin concentrations ≥0.25x MIC, there were increased lag phases of both strains at all garlic concentrations (**Fig. 5.5c, d**).

The presence of oxacillin gave approximately 50% decreases in the growth rates of MRSA N315 and NCTC 6571 but increasing concentrations had no further effect (**Chapter 4**). Garlic concentrations representing 0.5x and 0.75x MIC caused the growth rates of both strains to decline with increasing oxacillin concentration (**Fig. 5.6a, c**). There were no decreases in the growth rates of MRSA N315 and NCTC 6571 exposed to increasing concentrations of oxacillin (until 0.5x MIC) and low concentrations of garlic (0.25x MIC). The initial decline in growth rate of cells exposed to oxacillin (compared with positive controls) was not seen in the presence of this low garlic concentration; probably due to the effect of garlic alone on the growth rate (**Fig. 5.6a, c**).

Increasing concentrations of penicillin caused slight, but steady decreases in the growth rate of MRSA N315 and caused steady decreases in the growth rate of NCTC 6571 (**Chapter 4**). In the presence of high garlic concentrations (0.5x and 0.75x MIC), the growth rates of both strains decreased rapidly with increasing penicillin concentration (**Fig. 5.6b, d**). At low garlic concentration (0.25x MIC), the effect of

garlic on the growth rates of cells in increasing penicillin concentrations was similar to those in oxacillin: there was little change in the growth rate (until penicillin concentration was 0.5x MIC).

Fig. 5.6 – The effect of garlic on growth rates of MRSA (a, c) and *S. aureus* (b, d) in the presence of oxacillin (or penicillin)



Effect of garlic on the growth rates of MRSA (**a, c**) and *S. aureus* (**b, d**) in the presence of oxacillin (**a, b**) and penicillin (**c, d**) Legend indicates strain and concentration of garlic (as a percentage of the relative MICs). Oxacillin or penicillin concentrations (X axes) presented as fractions of the relative MICs. Bars indicate standard error of the mean ($n \geq 4$)

5.3.6 – Discussion

The effects of garlic in combination with oxacillin on MRSA and *S. aureus* were, in general, the same as the effects of pre-treatment with garlic on oxacillin susceptibility (**Section 5.3**): oxacillin sensitivity decreased with increasing concentrations of garlic, particularly at garlic concentrations >0.5x MIC. Similarly, the activity of penicillin was enhanced by garlic, but in a dose-dependent manner (except for EMRSA-16). Garlic induced oxacillin susceptibility in most strains, but not penicillin susceptibility. The potentiation of oxacillin sensitivity, but not penicillin sensitivity is likely to result from the different responses of increasing garlic on the two antibiotics: garlic had a large effect on oxacillin sensitivity but only at high garlic concentrations, whereas penicillin sensitivity steadily declined with increasing garlic concentration.

Concentrations of garlic 0.25x MIC decreased the sensitivity of MRSA N315 to oxacillin, as did pre-treatment with similar concentrations of garlic on NCTC 6571 shown previously. This was investigated further (see **Section 5.4**).

The effects of garlic on the growth dynamics of MRSA and *S. aureus* in the presence of oxacillin or penicillin are interesting. Both garlic and oxacillin (or penicillin) were shown to affect both lag phase duration and growth rates of MRSA and *S. aureus* (**Chapter 3** and **4**). It is therefore unsurprising that in combinations of these antimicrobials, the effects on growth dynamics are increased. At garlic concentrations >0.5x MIC there were increased lag phases and decreased growth rates in the presence of both antibiotics. This is in agreement with the previous suggestion that increased β-lactam susceptibility is probably a cumulative effect of both compounds. At low concentrations of garlic (0.25x MIC) the relative growth rates of MRSA and *S. aureus* were not affected by the presence of either antibiotic. Similarly, the lag phase duration of garlic-treated cells (0.25x MIC) by the presence of penicillin was not affected, but it was so by the presence of oxacillin.

Penicillin and oxacillin differ in structure only by a nitrogen containing cyclic R group (see **Chapter 4.1**). Both antibiotics, however, share the same mechanism of action and cellular targets. The responses of garlic on the MIC of oxacillin or penicillin were different, but did not differ between antibiotic-resistant and -sensitive strains. This suggests that the difference in response was not affected by antibiotic

resistance mechanisms. It is therefore unclear what mediated the different responses of increasing garlic concentration on oxacillin and penicillin sensitivity.

5.3.7 – Conclusions

- Combinations of garlic and oxacillin or garlic and penicillin were more effective, in terms of growth inhibition, than the antimicrobials employed separately – **Hypotheses 3 and 4 ACCEPTED**
- Oxacillin sensitivity was induced in MRSA at sub-MIC concentrations of garlic, but penicillin sensitivity was not. This is likely to result from different responses of garlic on the sensitivity of MRSA to these antibiotics – **Hypothesis 5 PARTIALLY ACCEPTED**
- Combinations of garlic and oxacillin or garlic and penicillin had, in general, greater effects on the growth dynamics of MRSA and *S. aureus* than the antimicrobials employed separately

5.4 – The effects of low concentrations of garlic on the susceptibility of MRSA and *S. aureus* to oxacillin

5.4.1 – Background

The sensitivity of MRSA N315 and NCTC 6571 to oxacillin decreased in the presence of 0.25x MIC of garlic (**Fig. 5.4** and **5.2** respectively). These data were contrary to the effects of higher concentrations of garlic (>0.5x MIC) on oxacillin susceptibility and implied that low concentrations of garlic were beneficial to the organisms in terms of tolerance to oxacillin. This was investigated further by reassessing the effect of combinations of garlic and oxacillin on MRSA and *S. aureus* but with smaller oxacillin concentration increments (MRSA N315 = 8.5 μg mL^{-1} increments and *S. aureus* NCTC 6571 = 0.01 μg mL^{-1} increments). The effect of low concentrations of garlic on the morphology of *S. aureus* was also investigated to try and elucidate whether structural changes could have caused the decreased sensitivity to garlic.

5.4.2 – Aims

- Confirm that low concentrations of garlic (0.25x MIC) increase the MIC of oxacillin against both MRSA and *S. aureus*
- Determine whether morphological or structural changes to *S. aureus* can account for the increased MICs

5.4.3 – Materials and methods

5.4.3.1 – Effect of combinations of garlic and oxacillin (at small increments) on MRSA and *S. aureus*

MICs of oxacillin against N315 and NCTC 6571 were determined using small increment changes in oxacillin concentration. Oxacillin MICs were determined in combination with 0, 0.2 and 0.4 mg mL^{-1} garlic, representing 0x, 0.25x and 0.5x the MIC of garlic of both strains. MICs were determined as previously described (**Chapter 2.7.2**) but using sterile 96 well Microtitre plates (Sterilin, London, UK) in place of "Honeycomb well-plates". The optical density of each plate was measured at $T_{=0}$, at 630nm (Dynamax; ThermoFisher, Leicestershire, UK). Microtitre plates were incubated for 24 h at 37°C with lids on and sealed in plastic bags with a piece of moistened laboratory paper to maintain humidity. OD was measured at $T_{=24}$, from which the $T_{=0}$ values subtracted. MICs were determined as the lowest concentrations of garlic and oxacillin to inhibit any increase in optical density.

5.4.3.2 – Transmission electron microscopy of *S. aureus* NCTC 6571 treated with 0.375 mg mL^{-1} garlic

Garlic stock solutions were prepared as previously described (**Chapter 2.3**) and diluted in MHB to desired concentrations (0 and 0.375 mg mL^{-1}) in final volumes of 10 mL. 0.375 mg mL^{-1} garlic represented 0.25x MIC of garlic in 10 mL volume (**Chapter 3**). Optically adjusted inocula were prepared as previously described (**Chapter 2.5**), and garlic solutions were inoculated to achieve initial cell densities of approx. 5 x 10^5 CFU mL^{-1}. Treated suspensions of organisms were prepared for microscopy and imaged as previously described (**Chapters 2.8** and **2.8.2**), after 24 h incubation. The effects of treatment with garlic on cell wall thickness were determined by digital analysis (using IMAGEJ, NIH) of transmission electron micrographs. Cell wall thickness was measured from ten treated (0.375 mg mL^{-1} garlic) and ten non-treated (0 mg mL^{-1} garlic) cells (five random measurements from each cell). A two-way T-test (Minitab) was applied to mean cell wall thickness data (n = 50) to determine whether variation in thickness was significant. Data were confirmed as being normally distributed and with equal variances by the Anderson-Darling test (Minitab).

5.4.4 – Results

The MIC of oxacillin against MRSA N315 increased from 59.5 μg mL^{-1} (control) to 85 μg mL^{-1} with 0.2 mg mL^{-1} garlic (0.25x MIC); an increase of 42% (Table 5.6). Similarly the MIC of oxacillin against *S. aureus* NCTC 6571 increased by 37.5%, from 0.08 to 0.11 μg mL^{-1} with 0.2 mg mL^{-1} garlic (0.25x MIC; Table 5.6). At 0.5x MIC of garlic, the MIC of oxacillin of both strains was that of control cells (Table 5.6).

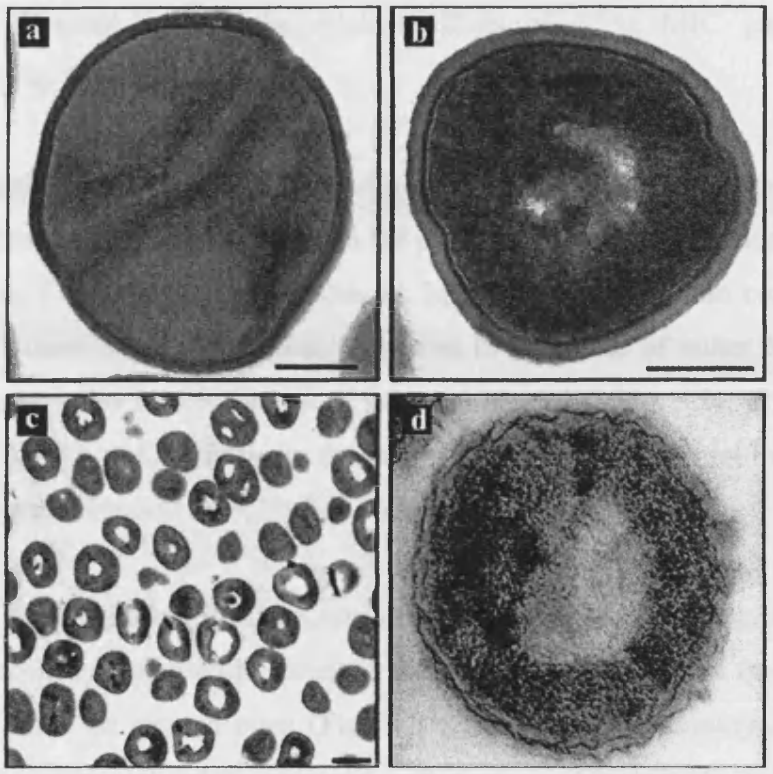
Table 5.6 – A comparison between cell wall thickness, growth rate and MIC of oxacillin of *S. aureus* NCTC 6571 treated with low concentrations of garlic

		Garlic concentration (x MIC)		
Measurement		0	0.25	0.50
Cell Wall	Mean (nm)	28.60	34.46	*
Thickness	SE	± 0.52	± 0.95	*
	% of control	100%	120%	*
MIC of	Mean (μg mL^{-1})	0.08	0.11	0.08
oxacillin	% of control	100%	137.5%	100%
Growth Rate	Mean (h^{-1})	0.077	0.040	0.073
	SE	± 0.003	± 0.002	± 0.003
	% of control	100%	52.3%	94.8%

SE indicates standard error of the mean. Cell wall thickness was not determined at a garlic concentration 0.5x MIC, because experiments yielded insufficient material for sectioning. * indicates that data were not determined

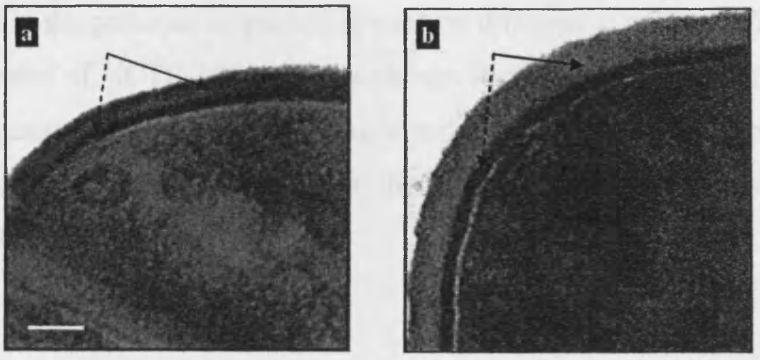
Transmission electron micrographs of both control organisms and organisms treated with 0.375 mg mL^{-1} garlic were obtained to elucidate any structural changes which could have resulted in the decreased sensitivity to oxacillin. The mean cell wall thickness of untreated NCTC 6571 cells was 28.60 ± 3.68 nm (**Fig. 5.7a**). In the presence of 0.375 mg mL^{-1} garlic (0.25x MIC) the cell wall was significantly thicker (**Fig. 5.7b**); 34.46 ± 6.73 nm ($T = 5.394$, $df = 98$, $P < 0.0005$). The secondary cell walls of both treated and untreated cells were of a similar cross-section size, hence only the primary cell wall was thickened by treatment with garlic (**Fig. 5.8**). Additionally, treated cells contained electron sparse areas of cytoplasm in most cells (**Fig. 5.7**). Magnesium limited *Pseudomonas putida* (Sykes and Tempest, 1965) also contained similar areas and images are presented for comparison (**Fig. 5.7d**). A summary of cell wall thickness, oxacillin sensitivity and growth rates of *S. aureus* NCTC 6571 (taken from **Chapter 3**) in the presence of low concentrations of garlic is provided in Table 5.6.

Fig. 5.7 – The effect of low concentrations of garlic on the morphology of *S. aureus* NCTC 6571 by transmission electron microscopy



S. aureus cells treated with 0 mg mL^{-1} (**a**) and 0.375 mg mL^{-1} garlic (**b**, **c**). Micrographs of magnesium limited *P. putida* (**d**; Sykes and Tempest, 1965) provided as a comparison with **c**. Scale bars represent 200 nm, magnification x63,000 (**a**, **b**) and x20,000 (**c**). Magnification and scale not provided for **d**

Fig. 5.8 – Thickening of the primary cell wall of *S. aureus* NCTC 6571 caused by 0.25x MIC of garlic



The micrographs show a thickening of the primary cell wall (solid arrow) in 0.25x MIC treated cells (**b**) compared with control (**a**). The thickness of the secondary cell wall (dotted arrow) remained similar in both. Scale bar represents 40 nm

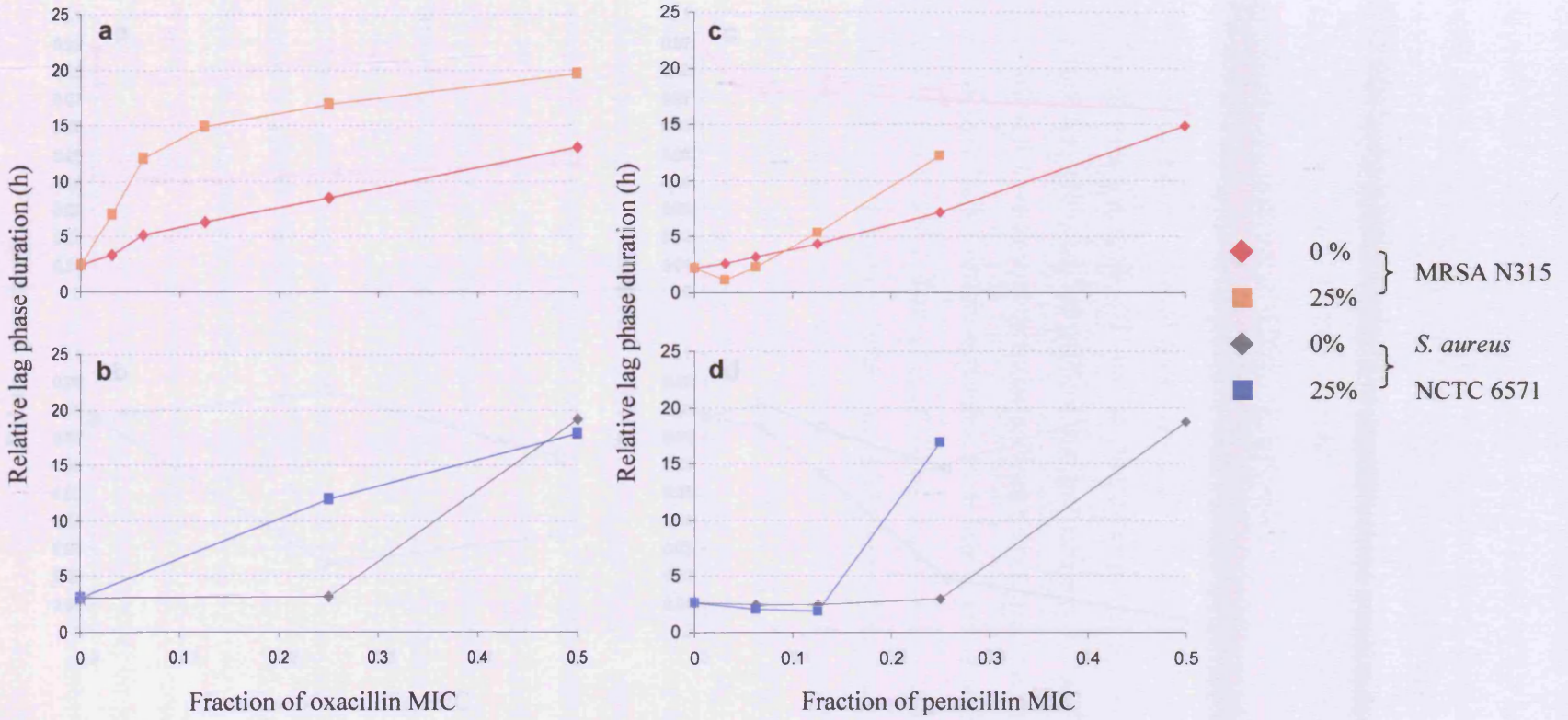
The effect of 0.2 mg mL^{-1} garlic (0.25x MIC) on the growth dynamics of MRSA and *S. aureus* were reassessed, and are presented here with the effect of garlic employed separately subtracted so that the relative effects of 0.25x MIC garlic can be determined (**Fig. 5.9** and **Fig. 5.10**).

Low concentrations of garlic (0.25x MIC) caused large increases in the lag phase duration of both MRSA and *S. aureus* in the presence of oxacillin, particularly at low concentrations (<0.125x MIC; **Fig. 5.9a, b**). In contrast, at penicillin concentrations <0.25x MIC, there were no additional increases in lag phase of either MRSA or *S. aureus* caused by low concentrations of garlic (0.25x MIC; **Fig. 5.9**). The lag phase durations of MRSA and *S. aureus* in the presence of 0.25x MIC penicillin (**Fig. 5.9c, d**) were however increased by 0.25x MIC of garlic.

The relative effects of low concentrations of garlic (0.25x MIC) on the growth rates of MRSA and *S. aureus* exposed to oxacillin appear to be very different from the effect of oxacillin alone on growth rates (**Fig. 5.10a, b**). Oxacillin, employed separately caused decreased growth rates compared with controls (**Chapter 4**), but increasing concentrations had little effect. Low concentrations of garlic (0.25x MIC) had no relative effect on the growth rates of MRSA and *S. aureus* in oxacillin concentrations <0.5x MIC. Comparison of **Fig. 5.10a, b** with **Fig. 5.6a, b** shows that this can probably be accounted for because of the effects of garlic employed separately on the growth rates of MRSA and *S. aureus*.

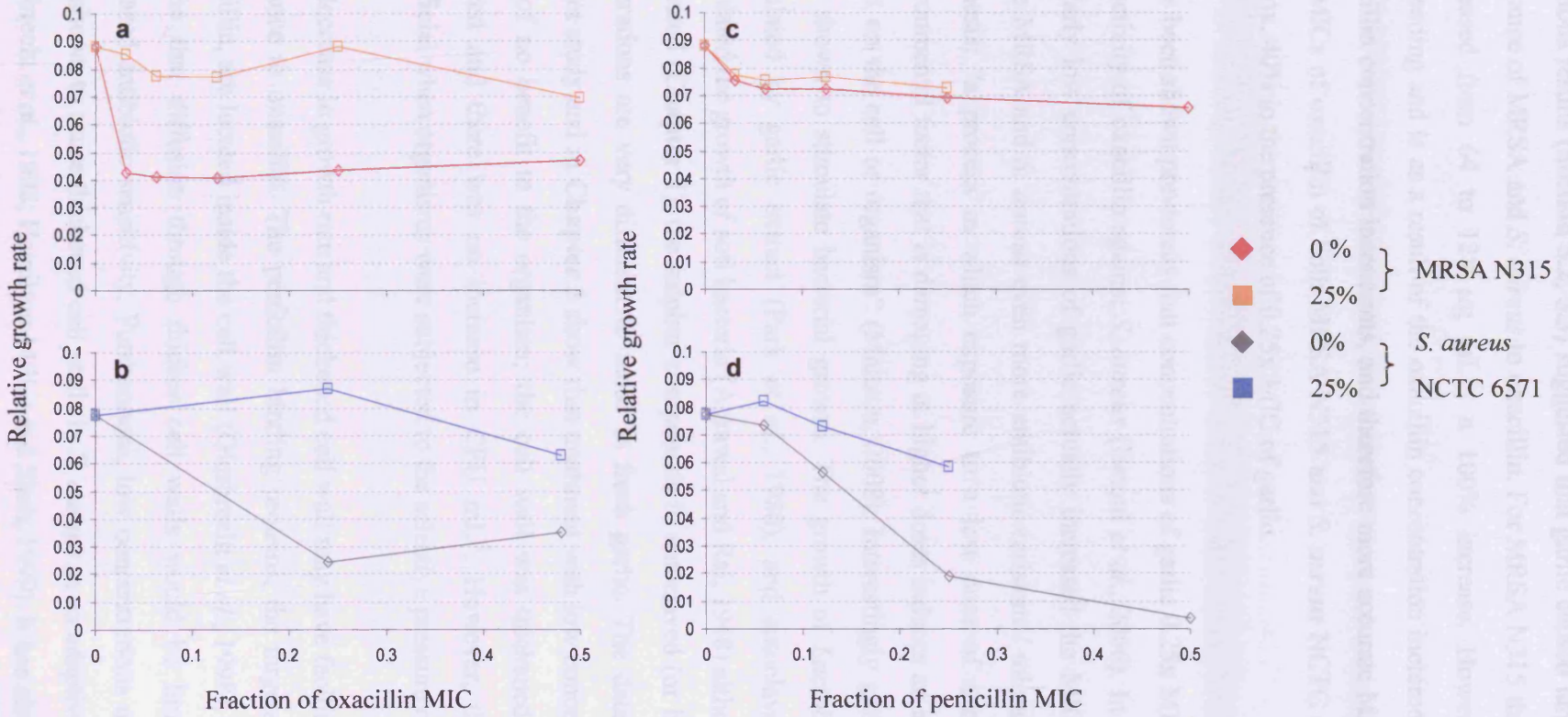
The relative effects of low garlic concentrations (0.25x MIC) on the growth rates of MRSA N315 in the presence of penicillin were no different to control (**Fig. 5.10c, d**). The growth rates of NCTC 6571 did not change in penicillin concentrations 0.125x MIC in the presence of low concentrations of garlic. With 0.25x MIC of penicillin, the growth rate decreased slightly, but not to the same extent as cultures without garlic (**Fig. 5.10c, d**).

Fig. 5.9 – The relative effects of low concentrations of garlic on the lag phase duration of MRSA (a, c) and *S. aureus* (b, d) in the presence of β-lactam antibiotics



Data re-scaled to allow direct comparison between the effects of garlic on lag phase duration of MRSA (**a, c**) and *S. aureus* (**b, d**) in the presence of oxacillin (**a, b**) and penicillin (**c, d**) Legend indicates strain and concentration of garlic (as a percentage of the relative MICs). β-lactam concentrations (X axes) presented as fractions of the relative MICs.

Fig. 5.10 – The relative effects of low concentrations of garlic on the growth rates of MRSA (a, c) and *S. aureus* (b, d) in the presence of β-lactam antibiotics



Data re-scaled to allow direct comparison between the effects of garlic on growth rates of MRSA (**a, c**) and *S. aureus* (**b, d**) in the presence of oxacillin (**a, b**) and penicillin (**c, d**) Legend indicates strain and concentration of garlic (as a percentage of the relative MICs). β-lactam concentrations (X axes) presented as fractions of the relative MICs.

5.4.5 – Discussion

Previous results (**Section 5.2, 5.3**) suggested that garlic caused large increases in the tolerance of MRSA and *S. aureus* to oxacillin. For MRSA N315 the MIC of oxacillin increased from 64 to 128 μg mL^{-1}, a 100% increase. However, this value is misleading and is as a result of the oxacillin concentration increments. Using smaller oxacillin concentration increments, and therefore more accurate MIC determinations, the MICs of oxacillin of both MRSA N315 and *S. aureus* NCTC 6571 increased by approx. 40% in the presence of 0.25x MIC of garlic.

It has been shown previously that concentrations of garlic 0.25x MIC did not enhance the activity of oxacillin against *S. aureus* (Betoni *et al.*, 2006). In the present study, similarly low concentrations of garlic actually increased the MIC of oxacillin, i.e. made MRSA and *S. aureus* even more antibiotic-resistant / tolerant. This is termed hormesis; "a process in which exposure to a low dose of a chemical agent or environmental factor that is damaging at higher doses induces an adaptive beneficial effect on the cell or organism" (Mattson, 2008). Interestingly garlic has previously been shown to stimulate bacterial growth. The growth of *Lactobacillus casei* was stimulated by garlic extract (Park *et al.*, 1980), and autoclaved garlic extracts stimulated the growth of soil bacteria (Agrawal and Rai, 1948) although as previously discussed (**Chapter 1**), the sulphur compounds in autoclaved (or heat treated garlic) preparations are very different to those in fresh garlic. The data presented in the current study and in **Chapter 3** show that treatment with low concentrations of garlic was of no benefit to the organism; the cell wall was thickened, the growth rate reduced and there was no increase in CFU mL^{-1}. However, these effects were beneficial when organisms were subjected to the selective pressure of oxacillin.

The decrease in growth rate and thickened cell wall may have facilitated the increased tolerance to oxacillin. The penicillin binding proteins, the targets of oxacillin and penicillin, are located inside the cell wall (Giesbrecht *et al.*, 1998). It is reasonable to assume that diffusion through thicker cell walls would be limited, resulting in decreased antibiotic sensitivity. Furthermore, low concentrations of antibiotics have been shown to cause thickened cell walls in *S. aureus* as an adaptive / stress response (Giesbrecht *et al.*, 1998; Hamilton-Miller and Shah, 1999). It has also been shown that oxacillin (and other β-lactam antibiotics) are only effective against actively growing

organisms (Giesbrecht *et al.*, 1998), and consequently organisms with decreased growth rates (e.g. Staphylococcal small colony variants) are less susceptible (McNamara and Proctor, 2000). The decreased growth rates of garlic treated *S. aureus* relative to those of control organisms was possibly accompanied by decreased numbers of ribosomes per organism (Sykes and Tempest, 1965). This would be one explanation for the electron-sparse cytoplasm observed in 0.25 mg mL^{-1} garlic treated *S. aureus* (**Fig. 5.7c**). RNA polymerase and RNA synthesis have been proposed as targets of garlic (Feldberg *et al.*, 1988; Ankri and Mirelman, 1999; Choi *et al.*, 2007) due to targeting of the RNA-polymerase sulphydryl group by allicin (Ankri and Mirelman, 1999). This does not, however, explain why at higher garlic concentrations, the growth rates increased towards those of control organisms (see **Chapter 3**). It seems unlikely that garlic at low concentrations whereas not at higher concentrations would affect ribosome occupancy. Electron-sparse areas of cytoplasm have also been noted after growth of *S. aureus* in elevated NaCl concentrations (Hajmeer *et al.*, 2006). These areas were either cytoplasm-sparse or nuclear regions which were exposed due to removal of cytoplasmic material. Similar electron sparse areas in terpinen-4-ol treated *Pseudomonas aeruginosa* (Longbottom *et al.*, 2004) have been described, although they appeared to be an accumulation of tepinen-4-ol inside the cell.

In the present study the effect of low garlic concentrations on oxacillin susceptibility was only assessed for two strains. It is unlikely that garlic specifically interacts with the antibiotic resistance mechanisms of MRSA because the tolerance of both MRSA and *S. aureus* (methicillin susceptible) to oxacillin was increased. It is therefore likely that *S. aureus* species in general would exhibit the same response. The effect on the MICs of penicillin using smaller increment changes was not assessed. As penicillin and oxacillin have the same mechanism of action against *S. aureus*, and the increased tolerance to oxacillin was not related to antibiotic resistance mechanisms, it is reasonable to assume tolerance to penicillin would also be increased by low concentrations of garlic. These results have potentially important consequences; for example, does consumption of garlic affect the efficacy of antibiotic treatment regimes? The effect of low concentrations of garlic on *S. aureus* and MRSA susceptibility to penicillin and other antibiotics (of various classes) in addition to biocides should be determined.

5.5 – Overall discussion

Susceptibility of MRSA and *S. aureus* to oxacillin was increased when organisms were pre-treated with garlic, or when garlic and oxacillin were present in combination. Oxacillin, but not penicillin, sensitivity (i.e. an MIC <4 μg mL^{-1}; CLSI, 2007) was induced in MRSA at sub-MIC concentrations of garlic. It is noted, however, that antibiotic sensitivity is a pre-determined value, and although this breakpoint was not attained, susceptibility to both antibiotics was increased with the presence of garlic. The most efficient combinations of garlic and oxacillin were additive regardless of whether organisms were pre-treated with garlic, or if garlic and oxacillin were present in combination. Garlic and oxacillin were previously shown to affect lag phase and growth rates of MRSA and *S. aureus* when employed separately, and in combination the effects on Staphylococcal growth dynamics were increased. Synergism between allicin and oxacillin has been reported against both *S. aureus* and *Pseudomonas aeruginosa* (Cai *et al.*, 2007; 2008 respectively). These results therefore imply that allicin is the major compound responsible for potentiation of β-lactam susceptibility in MRSA and *S. aureus*, although considering it is the major antimicrobial compound both in efficacy and abundance this is perhaps not surprising.

Interestingly, the susceptibility of both MRSA and *S. aureus* to oxacillin was decreased (i.e. the MIC was increased) by concentrations of garlic 0.25x MIC. Decreased oxacillin sensitivity was accompanied by thickened cell walls, slower growth rates and electron sparse areas of cytoplasm. It is assumed that these factors, at least in part, were responsible for the decreased oxacillin susceptibility. It is reasonable to assume that decreased susceptibility was not mediated by interference or enhancement of antibiotic resistance mechanisms because both MRSA and *S. aureus* were more tolerant of oxacillin in the presence of these garlic concentrations. Although it was shown previously that garlic did not damage the integrity of the Staphylococcal cell wall (**Chapter 3**), the cell wall is obviously targeted in some way, hence the thickening of the primary wall shown here. Although the primary wall appears thickened, and is obviously no less osmotolerant, there are no data to suggest whether it is perfectly or imperfectly formed. For instance, MRSA grown in high penicillin concentrations develop functional cell walls, but also exhibit gross morphological abnormalities due to a dependence on PBP2a for cell wall formation (Labischinski, 1992).

That pre-exposure of MRSA and *S. aureus* to garlic increased oxacillin susceptibility as well as garlic and oxacillin in combination supports the suggestion of independent mechanisms of action / targets of garlic and oxacillin. It also supports the suggestion that increased susceptibility to oxacillin is as a result of the cumulative effects of both inhibitory compounds. These results therefore possibly suggest that garlic impairs the activity of PBPs, which would account for increasing susceptibility of both MRSA and *S. aureus* to .oxacillin and penicillin. The roles of the PBPs in Staphylococci are currently unknown (Giesbrecht *et al.*, 1998), so this hypothesis would not be easily tested. It is clear, however, that elucidation of the target sites and modes of action of garlic and garlic compounds on *S. aureus* would greatly increase our understanding and be of great benefit in identifying compounds with which synergy might occur.

The relationships between garlic and oxacillin (or penicillin) presented here were generally quantified as "additive", and are therefore contrary to those of Cottrell (2003 and unpublished). The conventional breakpoints used in this study (White *et al.*, 1996) defined an additive relationship as a FICi between 0.51 and 1.99. Additivity can be described as a relationship in which the sum total of the inhibitory effects is as expected considering the effects of each compound in isolation. Hence a FICi of 1 could be derived by a combination of two compounds, both at concentrations of 0.5x MIC and therefore both contributing half their efficacy. The sum total effect would therefore be the same as one of the compounds at 1x MIC. However, FICi values of between 1 and 1.99 are termed additive, and yet a value greater than 1 signifies that the combined effects of the two compounds are less than either compound in isolation. Many of the combinations of garlic and oxacillin (or penicillin) resulted in FICi > 1. This raises the question: should such a combination, which is less effective than one of the components alone still be termed additive? A better classification perhaps is FICi < 0.5 = synergistic, 0.51 – 1 = additive and 1.01 – 1.99 = indifferent. In fact, the Journal of Antimicrobial Chemotherapy state that any literature submission containing FICi data must be restricted to interpretations of 'synergy' (FICi $\leq$0.5), 'antagonism' (FICi >4.0), and 'no interaction' (FICi >0.5-4.0) to encourage conservative interpretation of results (Odds, 2003).

Classification of antimicrobial combinations using FICi provides a number by which different combinations of compounds can be compared. By doing this, it appears that

the combination of garlic and oxacillin / penicillin against MRSA was not particularly effective. Epicatechin gallate (from green tea) and oxacillin are synergistic against MRSA, as are farnesol and numerous β-lactams, with FICi of 0.199 – 0.393 (Stapleton *et al.*, 2004a) and 0.012 – 0.055 (Kuroda *et al.*, 2007) respectively. However, MRSA is a major hospital problem (see **Chapter 1**), and current therapies include toxic antibiotics; these have highly undesirable side effects and in many cases are unsuccessful. That garlic and several other plant antimicrobials (see **Section 5.1**) potentiate the activity of currently redundant antibiotics with few serious side effects may prove so significant so as to warrant re-evaluation of interactions between growth-inhibiting agents. The classification of a combination as "synergistic" or "additive" is therefore perhaps not so relevant to the assessment of therapeutic efficacy.

5.6 – Conclusions

- Garlic, at concentrations >0.5x MIC, caused increased oxacillin and penicillin sensitivity against MRSA and *S. aureus*
- At concentrations >0.67x MIC, garlic induced oxacillin, but not penicillin, sensitivity (CLSI, 2007) in MRSA
- At garlic concentrations 0.25x MIC, oxacillin susceptibility of MRSA and *S. aureus* was decreased
- Increased sensitivity of garlic treated MRSA and *S. aureus* to oxacillin and penicillin appears to be a culmination of the inhibitory effects of both compounds rather than a direct interaction between the antimicrobials
- Combinations of garlic and oxacillin (or penicillin) increased the lag phase duration and decreased growth rates of both MRSA and *S. aureus*

6 – Induction of Antimicrobial Resistance in Oxacillin-Susceptible *S. aureus*

6.1 – Introduction

6.1.1 – Bacterial resistance to garlic

Although garlic has excellent antibacterial properties against a wide range of pathogens, some bacteria, in particular the lactic acid bacteria (LAB) are relatively insensitive. MICs of garlic against LAB are typically 5-10x higher than for susceptible pathogens such as *E. coli* and *S. aureus* (Rees *et al.*, 1993). In some cases, for example *Pediococcus pentosaceus*, the MIC can be almost 40 mg mL^{-1}; approx. 40x higher than that of *S. aureus* (Skyrme, 1996). Populations of garlic tolerant bacteria have also been cultured from garlic cloves; for example *Leuconostoc mesenteroides* (LAB; Kyung *et al.*, 2006) and non-pathogenic *Pseudomonas* spp. (it is important to note that although allicin-insensitive *Pseudomonas* spp. are reported (Slusarenko *et al.*, 2008) the susceptibility to garlic was not quantified). *Bacillus* spp. and *Erwinia* spp. have also been isolated from garlic powder at very low levels (around 10^2 CFU g^{-1}; Brown and Jiang, 2008) but the garlic susceptibility of these strains was not assessed. Allicin is not present in garlic powder and such low populations suggest survival in the powder rather than growth. Interestingly, although numerous fungal species rot garlic cloves, only one bacterial species is noted as doing so; *Bacillus cepivorans* (Hahn, 1996b). Any damage to garlic cloves will result in the production of allicin. It therefore seems strange that no studies into the garlic susceptibility of this bacterium have been conducted because presumably it is also tolerant of allicin.

One of the most promising aspects of garlic as an antibacterial is the lack of reported induced resistance in any bacterial species. Despite there being bacteria with inherently low susceptibility to garlic, there are no examples of sensitive bacteria becoming less sensitive to garlic either *in vivo* or *in vitro*. It has been estimated that development of resistance to β-lactam antibiotics is 1000x easier than to allicin (Ankri and Mirelman, 1999), although it is unclear what this estimation is based on. It is noted, however, that only one study appears to have attempted to reduce the sensitivity of a bacterial species to garlic, and that was without success (Skyrme, 1996).

6.1.2 – Mechanisms of antibiotic resistance

Antibiotic resistance has recently been brought to the public's attention (particularly over the past decade) but it is hardly a new phenomenon. In fact most resistance mechanisms pre-date the antibiotic era (1930s onwards; Alanis, 2005), and it is only the use of antibiotics by humans that has lead to lateral gene transfer of resistance mechanisms into pathogens of clinical or agricultural importance (Koch, 2007). Antibiotic resistance can be intrinsic, but in pathogenic bacteria mechanisms of resistance are more commonly acquired through transfer of genetic material. There are numerous factors which have facilitated the evolution and / or acquisition of antibiotic resistance mechanisms by pathogens; primarily, excessive and irrational use of clinical and agricultural antibiotics (Alanis, 2005).

Antibiotic resistance mechanisms exist for every different class of commercially employed antibiotics. Resistance mechanisms can develop over months or years (Davies, 1996), and can be specific to one antibiotic, or more frequently, provide protection against numerous antibiotics (as in the case of MRSA). Antibiotic resistance has lead to decreasing numbers of effective antimicrobials and increasing treatment times, hospital costs and mortality (Sefton, 2002; Levy and Marshall, 2004; Alanis, 2005). Antibiotic resistance mechanisms vary between species, strains and antibiotics, but in general they can be grouped into one of four classes:

1 – Inactivation / modification of the antibiotic (e.g. penicillin resistance in *S. aureus*)

The first penicillin-resistant *S. aureus* strains were isolated and characterised in the early 1940s (Kirby, 1944) before penicillin became commercially available. Penicillin resistance is mediated by the enzyme β-lactamase which cleaves the active site of penicillin, the β-lactam ring, thus rendering the antibiotic ineffective. It remains unclear in which species β-lactamase enzymes originated, but it is fairly certain that they were acquired from another species (Hawkey, 1998). It has been suggested that the first organisms to produce β-lactamases also produced β-lactams; the enzymes providing protection to toxic concentrations of the antibiotics (Koch, 2007).

2 – Alteration of the target site (e.g. methicillin resistance in *S. aureus*)

Methicillin, a semi-synthetic penicillin, was designed to be β-lactamase stable i.e. the β-lactam ring could not be cleaved. All penicillin and cephalosporin antibiotics exert a bactericidal action by acylating the active site of the penicillin binding proteins (PBPs), which facilitate cross-linking in the bacterial cell wall. The modified enzymes cannot catalyse the final step of peptidoglycan synthesis; hence cell walls are poorly formed. The cells continue to grow and undergo division, but the weakened cell walls cannot withstand the internal cell pressure, causing the cell to "burst" (Giesbrecht *et al.*, 1998). Resistance to methicillin (and other β-lactamase stable penicillin antibiotics) is mediated by an altered PBP (PBP2a), which has a lowered affinity for β-lactams and can therefore produce functional cell walls in the presence of high concentrations of antibiotics. The genes for PBP2a are located on non-chromosomal DNA and have no equivalent in methicillin-susceptible strains (Labischinski, 1992). It is therefore likely that PBP2a evolved a long time ago in a different organism (Koch, 2007), possibly *Enterococcus hirae*, which has intrinsic methicillin resistance due to a very similar PBP (Labischinski, 1992).

3 – Alteration of the metabolic pathway (e.g. sulphonamide resistance in *E. coli*)

Sulphonamide antibiotics exert a bacteriostatic effect by interrupting the synthesis of folate, required to synthesise nucleic acids, and thereby inhibiting bacterial growth. Sulphonamides are structural analogues of *p*-aminobenzoic acid, the substrate for a catalytic enzyme (dihydropterate synthase, DHPS) in the folic acid biosynthesis

pathway (Petteren and Boerlin, 2003). Sulphonamide resistance is typically mediated by an alternative DHPS gene, the product of which has a lower affinity for sulphonamides (Petteren and Boerlin, 2003). These alternative genes are present on a resistance-plasmid (R-plasmid), and the resistant enzyme is therefore expressed alongside the antibiotic-sensitive enzyme (Wise and Abou-Doria, 1975).

4 – Reduced drug accumulation / increased active efflux (e.g. Tetracycline resistance)

Tetracyclines are bacteriostatic enzymes which inhibit bacterial protein synthesis (McMurry *et al.*, 1980; Guardabassi *et al.*, 2000). Tetracycline resistance is mediated by actively transporting the antibiotic out of the cell via the Tet protein (Levy, 1992), and is therefore an energy requiring process (McMurry *et al.*, 1980). The Tet protein is induced by tetracycline, and is only found in tetracycline-resistant organisms (Levy, 1992). Most tetracycline resistance genes are located on R-plasmids or transposons (Guardabassi *et al.*, 2000) and it is therefore assumed that tetracycline resistance is mainly obtained by gene transfer.

6.1.3 – Induction of non-β-lactamase mediated resistance to penicillin

Although there are four classical methods of antibiotic resistance, decreased antibiotic sensitivity can by caused by morphological adaptations (Paul *et al.*, 1995) or changes in growth dynamics. For example, Staphylococcal small colony variants exhibit an increased tolerance to cell wall active antibiotics due to slower growth rates (McNamara and Proctor, 2000). Furthermore, during the first studies into the action of penicillin, non-β-lactamase mediated resistance was induced in a strain of *S. aureus* (Abraham *et al.*, 1941). *S. aureus* was repeatedly sub-cultured in the presence of penicillin. After a few daily sub-cultures, there was evidence of decreased penicillin sensitivity and after eight weeks treated bacteria were able to grow at concentrations of penicillin 1000x greater than the MIC of the parent strain. The main effects of the adaptation to penicillin were reduced growth rates and enzyme activities when the treated cultures were grown in normal growth media. There was also no evidence of β-lactamase activity, indicating that resistance was mediated by the creation of a population with reduced innate penicillin susceptibility.

The present study attempted to reduce the garlic sensitivity of one strain of *S. aureus* using a method adapted from that of Abraham *et al.* (1941). Initially it was hoped that a chemostat-based approach could be used, but as it is inadvisable to culture such large volumes of an opportunistic pathogen, a continuous sub-culture method was devised. In addition this study also attempted to induce oxacillin resistance in a strain of *S. aureus* using the same method. This was in part to replicate the work of Abraham *et al.* (1941) using a semi-synthetic β-lactam, oxacillin (and thus induce non-PBP2a mediated resistance), and also to demonstrate that the method would result in the creation of a population with reduced antimicrobial susceptibility.

It is important to highlight that this study was designed to create an antimicrobial "resistant" population. Antimicrobial concentrations were therefore increased as the microbial populations became less sensitive to the antimicrobial.

6.1.4 – Aims

- Induce oxacillin resistance in an oxacillin-sensitive strain of *S. aureus* (NCTC 6571) by repeated sub-culture in the presence of sub-MIC concentrations of oxacillin
- Reduce the susceptibility of the same *S. aureus* strain to garlic by the same process

The specific treatment conditions were as follows:

1) Control – sub-culture in MHB
2) Induce oxacillin resistance (Ox-RI) – Sub-culture in 0.5x MIC of oxacillin
3) Reduce garlic susceptibility (G-RI) – Sub-culture in 0.5x MIC of garlic

The MICs of both oxacillin and garlic were measured every 7 d, and the concentrations of oxacillin or garlic were subsequently adjusted to achieve 0.5x new MIC. The experiments were terminated when oxacillin resistance was induced; i.e. when oxacillin MIC was >4 μg mL^{-1} (CLSI, 2007). All three treatments were set-up from a single inoculum. Experiments were repeated using independent inocula but the results presented represent a single experiment.

6.1.5 – Hypotheses

The hypotheses (and the rationales behind them) were as follows:

1. Oxacillin resistance (MIC >4 μg mL^{-1}) will be induced in *S. aureus* through repeated sub-culture in sub-MIC levels of oxacillin

   Using a similar method to the present study, Abraham *et al.* (1941) induced non-β-lactamase penicillin resistance in *S. aureus*. Furthermore, Staphylococci have been shown to respond to sub-lethal concentrations of β-lactam antibiotics by producing a thickened cell wall (Paul *et al.*, 1995). Considering that the target of β-lactam antibiotics is the cell wall, it is reasonable to assume that cell wall thickening could, in part, lead to reduced β-lactam susceptibility. Cell wall thickening has been recognised as a factor in reducing the susceptibility of *S. aureus* to vancomycin (Longzhu *et al.*, 2006)

2. Garlic resistance (MIC >8 mg mL^{-1}) will not be induced in *S. aureus* through repeated sub-culture in sub-MIC levels of garlic

   There have been no examples of *in vitro* or *in vivo* reduced susceptibility to garlic in *S. aureus* as previously mentioned. It has been suggested that this is due to both the number of antimicrobial compounds in garlic and the possible interactions between them. Furthermore, several of the constituent antimicrobial compounds exhibit multiple target sites (Lemar *et al.*, 2003). For the purposes of this study garlic "resistance" was assigned a value of (MIC) >8 mg mL^{-1}. This concentration represents 10x MIC of *S. aureus*, and a garlic tolerance similar to some LAB (MIC ranges of aqueous garlic preparation: *Lactobacillus plantarum* 10 to 15 mg mL^{-1}, *Lactobacillus acidophilus* 10 to 25 mg mL^{-1}; Skyrme, 1996)

3. Repeated sub-culture in the presence of oxacillin will have no effect on garlic susceptibility (and visa versa)

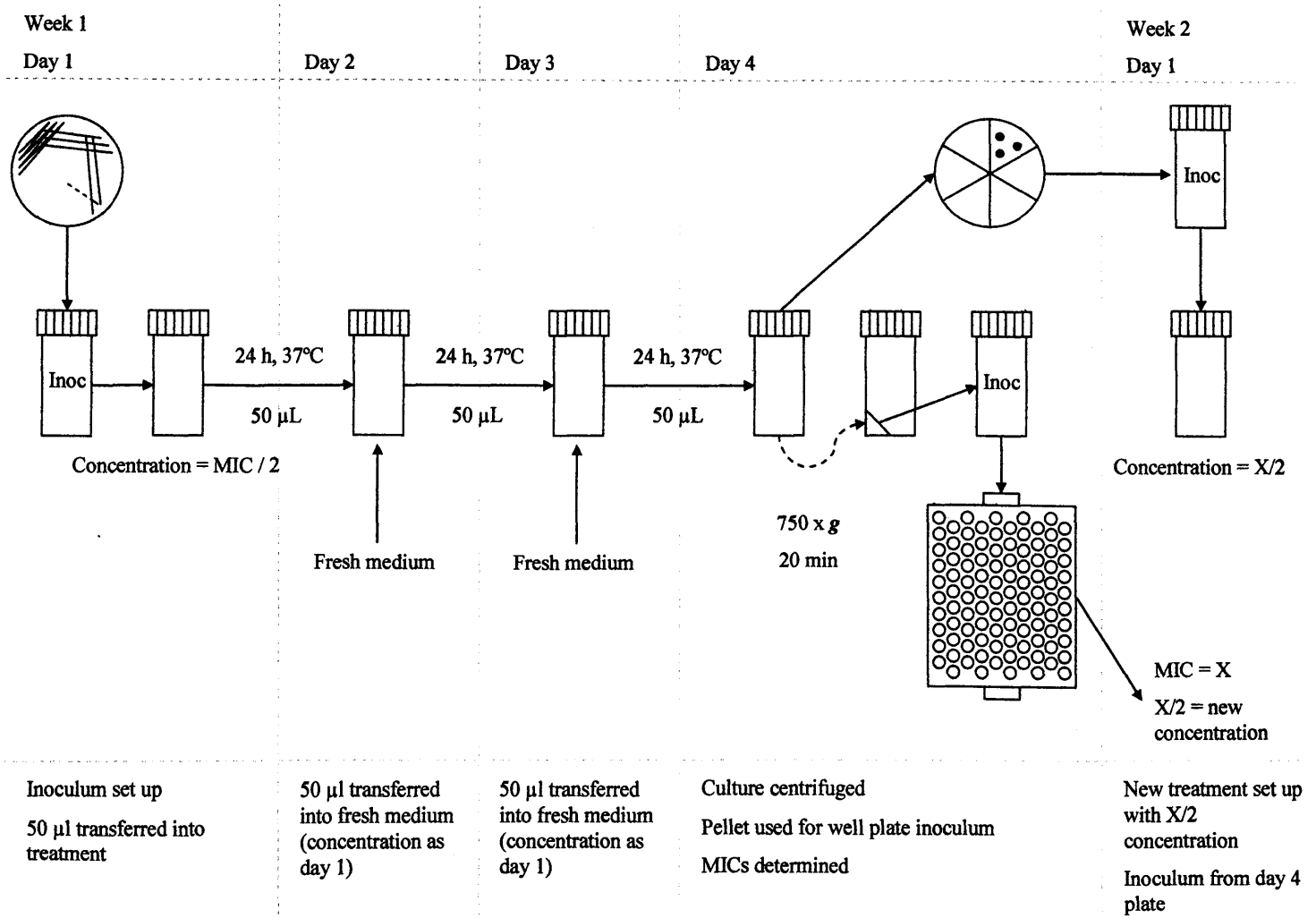
   The results presented in **Chapter 3** and **5** suggested that although the target of garlic against *S. aureus* is unknown, it is unlikely to be solely the cell wall or peptidoglycan (the targets of β-lactam antibiotics). Furthermore, the presence of low concentrations of garlic decreased the sensitivity of MRSA to oxacillin, which lends support to the suggestion that oxacillin and garlic have different targets and mechanisms against MRSA and *S. aureus*. It is therefore reasonable to assume that decreasing susceptibility to garlic would have no effect on oxacillin susceptibility

6.2 – Materials and methods

6.2.1 – General method

The following method is additionally described as a flow-chart (**Fig. 6.1**). An optically adjusted inoculum of *S. aureus* NCTC 6571 was prepared as previously described (**Chapter 2.5**). Stock solutions of garlic and oxacillin (prepared as previously described; **Chapter 2.3**; **2.4**) were diluted to required concentrations (see Table 6.1) in MHB. Each tube was inoculated with 50 µL inoculum to achieve an initial cell density of approx. 5 x 10^{-5} CFU mL^{-1}. Every 24 h tubes were thoroughly mixed and 50 µL transferred into fresh media containing the same antimicrobial concentrations. After three passages (i.e. 72 h), viability was determined by viable cell counts on MHSC and the MICs of garlic and oxacillin of each isolate were determined. Inocula used to determine the MICs of garlic or oxacillin were prepared by centrifuging the suspensions for 20 min at 750 x *g*. The supernatant was discarded and the pellet twice washed in PBS. A small amount of pellet was transferred into fresh MHB and incubated for 2 – 3 h; inocula were subsequently treated as all other inocula (see **Chapter 2.5**). Minimum inhibitory concentrations of garlic and oxacillin were determined optically following the method described in **Chapter 2.7.2**. The resulting MICs of oxacillin / garlic were used to adjust the antimicrobial concentrations of each treatment for the following week. Inocula on day 1 of each subsequent week were raised from the growth off the viable cell count plates obtained on day 4 of the previous week following the procedure for inoculation as described in **Chapter 2.5**.

Fig. 6.1 – The passage of "resistance induction" isolates through one week



Experimental procedure is described in more detail in **Section 6.2.1**

Table 6.1 – The antimicrobial concentrations at $T_{=0}$ of "resistance induction" experiments

Treatment	Concentration of garlic (mg mL^{-1})	Concentration of oxacillin (μg mL^{-1})
1 – Control	0	0
2 – Oxacillin (Ox-RI)	0	0.05
3 – Garlic (G-RI)	0.4	0

6.2.2 – Effect of treatment on growth dynamics

Lag phase duration and growth rates were determined from growth curves obtained using a Bioscreen C growth analyser as previously described (**Chapter 2.7.4**). Growth curves were obtained from treated isolates grown in the absence of the inducing agent (in sterile MHB), to determine whether changes in the MICs of oxacillin or garlic over time corresponded with altered growth dynamics.

6.2.3 – Visualisation of abnormal cellular morphologies by scanning and transmission electron microscopy

The effects of treatment on cellular morphology after 35 d were assessed visually by both scanning and transmission electron microscopy. Samples were prepared as described in **Chapter 2.8**. The effects of the treatments on cell size, cell wall thickness and cell volume were estimated by digital analysis (using IMAGEJ, NIH) of both scanning and transmission electron micrographs. Cellular diameter and cell volumes were estimated from scanning electron micrographs using the mean diameter of randomly measured cells (n = 50); cell wall thickness was estimated from six cells from each treatment (five random measurements from each cell) using transmission electron micrographs.

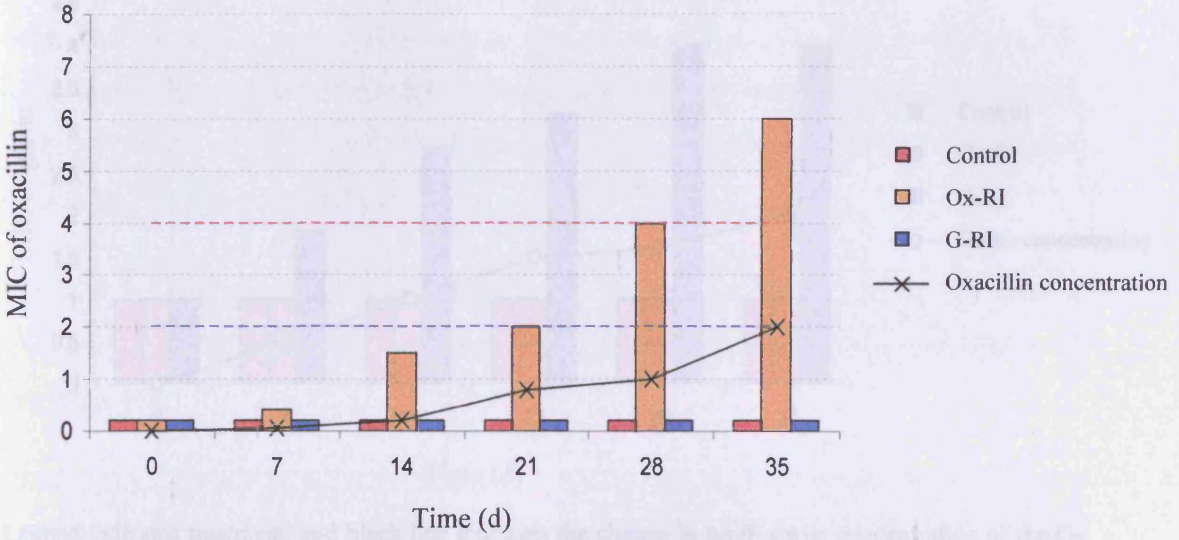
6.2.4 – Statistical analysis

Two-way T-tests (Minitab) were applied to mean cell diameter data to determine whether variation in cell size was significant. Data were confirmed as being normally distributed and with equal variances by the Anderson-Darling test (Minitab).

6.3 – Results

The MIC of oxacillin of Ox-RI increased over time (**Fig. 6.2**). The isolate lost oxacillin sensitivity (MIC <2 μg mL^{-1}; CLSI, 2007) after 21 d and became oxacillin-resistant (MIC >4 μg mL^{-1}; CLSI, 2007) after 35 d. The MIC of oxacillin of control and G-RI did not change. Ox-RI isolates were β-lactamase negative, determined by β-lactamase (Nitrocefin) sticks (Oxoid, Hampshire, UK).

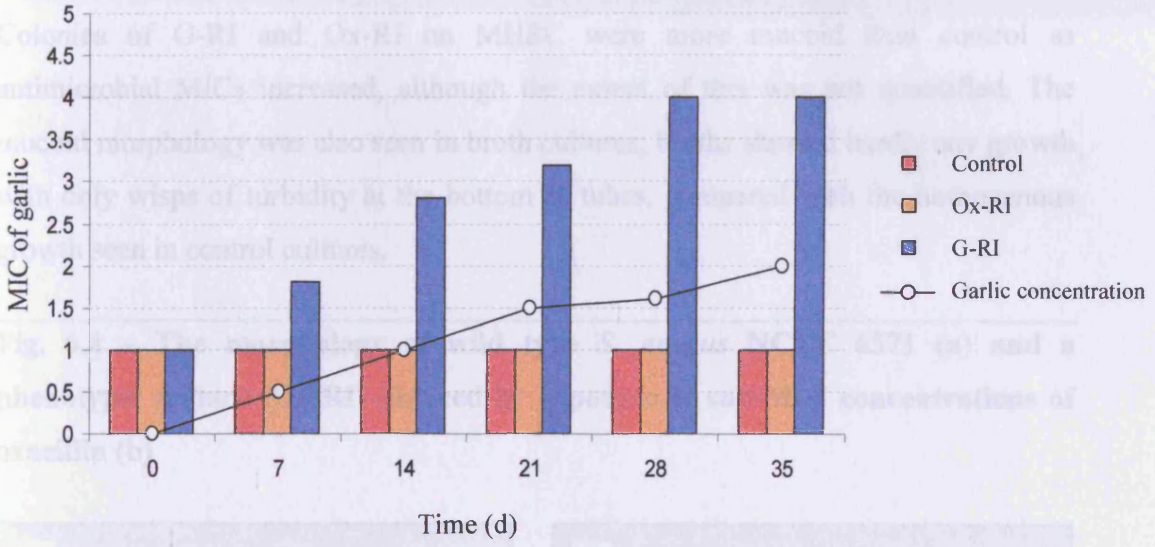
Fig. 6.2 – The change in the MIC of oxacillin against three isolates of NCTC 6571 treated with oxacillin, garlic or not treated, over a 35 d period



Legend indicates treatment, and black line indicates the change in broth oxacillin concentration of Ox-RI (in response to the increasing MIC of oxacillin). The red dotted line and blue dotted line indicate oxacillin resistance and sensitivity respectively (CLSI, 2007)

G-RI became progressively less sensitive to garlic due to incubation in increasing sub-MIC concentrations of garlic over 35 d (**Fig. 6.3**). The MIC of garlic increased 5x (from 0.8 mg mL^{-1} to 4 mg mL^{-1}) but G-RI did not become "resistant" to garlic according to the cut-off point proposed in this study (MIC >8 mg mL^{-1}). The MICs of garlic against both Ox-RI and control did not change over the same period.

Fig. 6.3 – The change in the MIC of garlic against three isolates of NCTC 6571 treated with garlic, oxacillin or not treated, over a 35 d period



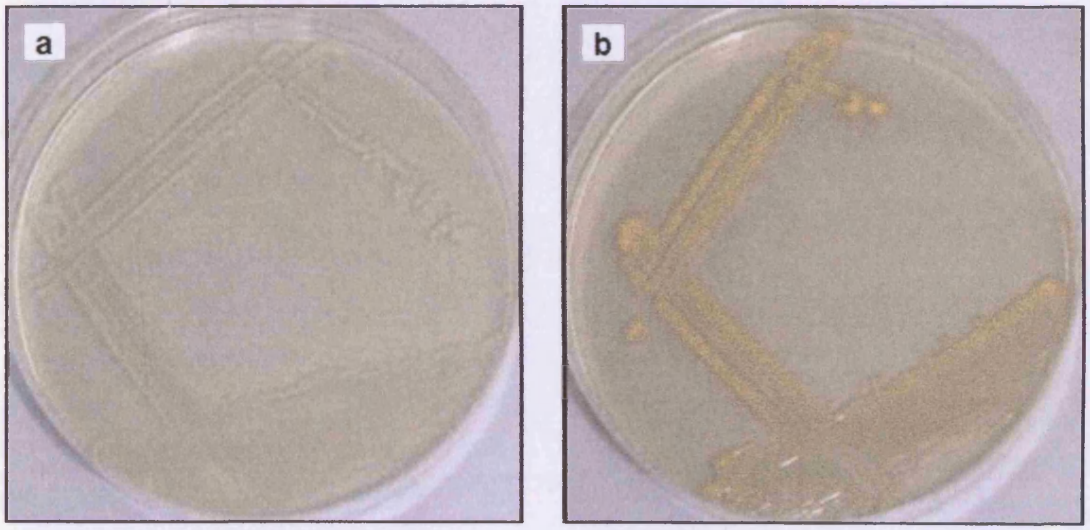
Legend indicates treatment, and black line indicates the change in broth garlic concentration of the G-RI (in response to the increasing MIC of garlic)

6.3.1 – Colonial morphological changes

Ox-RI developed orange pigmentation (**Fig. 6.4**) after 7 d, compared with the non-pigmented parent (*S. aureus* NCTC 6571). All subsequent colonies of Ox-RI were pigmented and slightly smaller (after 24 h incubation) than control colonies. There was no loss of this pigmentation throughout the experiment. Pigmented colonies were compared with wild type by randomly amplified polymorphic DNA (RAPD) profiling to ensure they were the same strain (see **Chapter 7**), and were identified as *S. aureus* by API strip (BioMerieux, France).

Colonies of G-RI and Ox-RI on MHSC were more mucoid than control as antimicrobial MICs increased, although the extent of this was not quantified. The mucoid morphology was also seen in broth cultures; broths showed hardly any growth with only wisps of turbidity at the bottom of tubes, compared with the homogenous growth seen in control cultures.

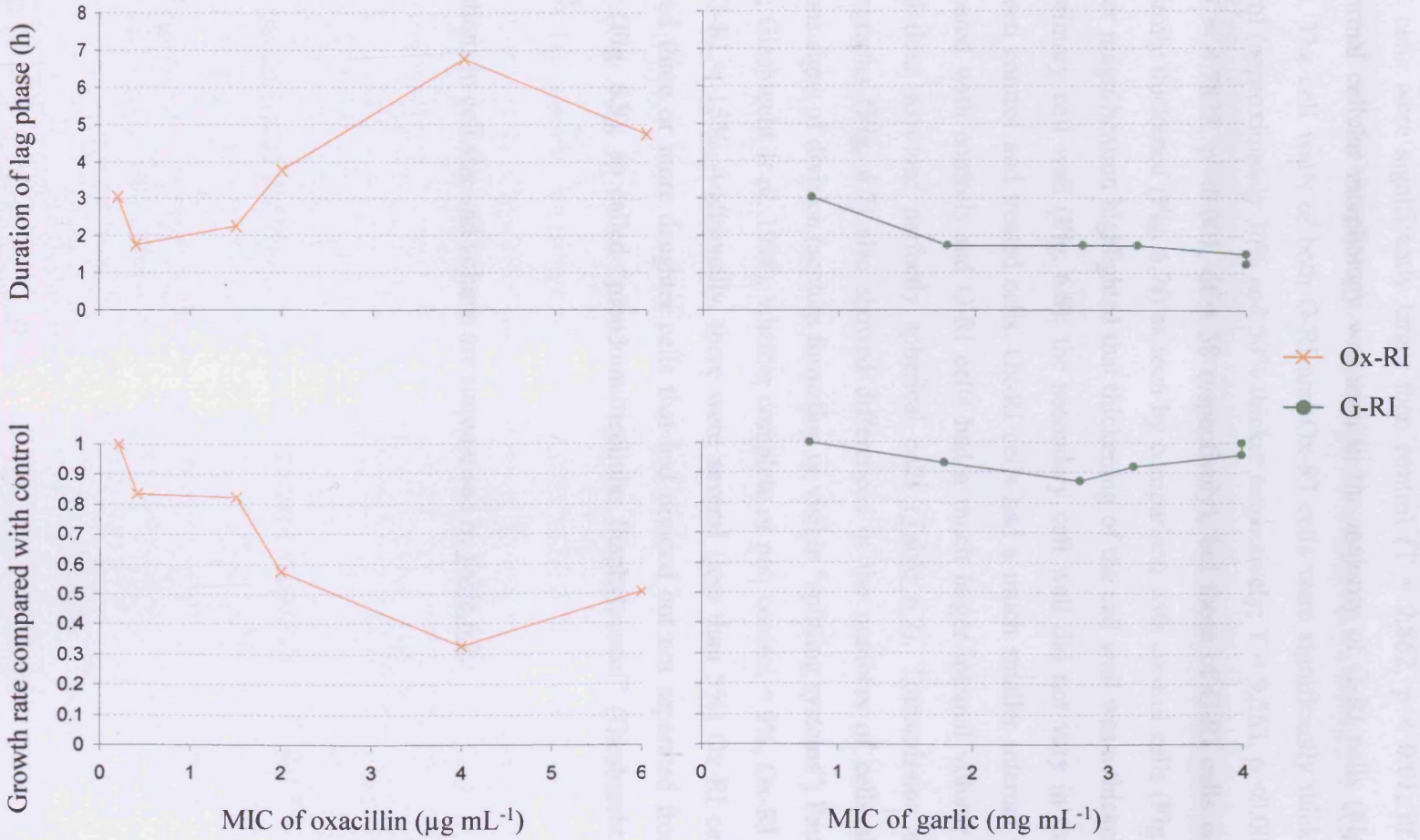
Fig. 6.4 – The morphology of wild type *S. aureus* NCTC 6571 (a) and a phenotypic mutant (Ox-RI) induced by exposure to sub-MIC concentrations of oxacillin (b)



6.3.2 – The effect of treatment on growth dynamics

There were correlations between the increasing MIC of oxacillin of Ox-RI and both decreasing growth rates and increasing lag phases (**Fig. 6.5**) of the same isolates grown in MHB. There was only a slight correlation between increasing MIC of garlic and decreasing lag phase of G-RI, but there was no effect on the growth rates (**Fig. 6.5**).

Fig. 6.5 – Changes in the growth dynamics of "resistance induction" isolates in relation to increasing time and MICs of antimicrobials



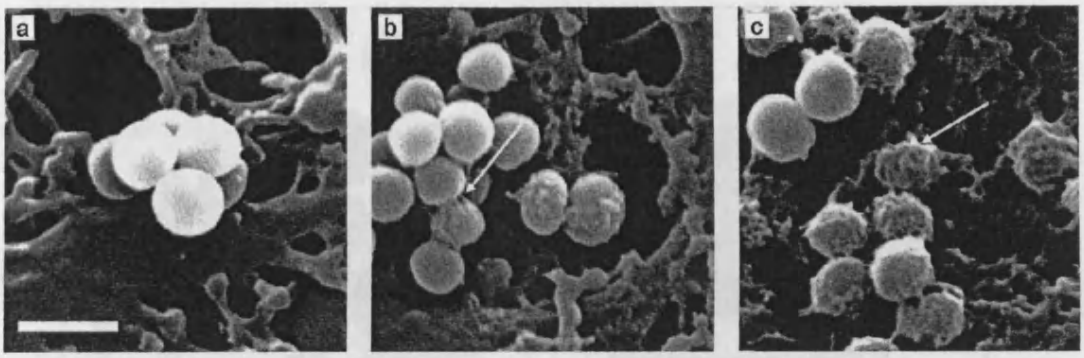
Legend indicates the treatment, and each point indicates time (d)

6.3.3 – Cellular morphological changes

The resistance induction procedures induced changes in cell size and shape of both Ox-RI and G-RI (**Fig. 6.6** and **Fig. 6.7**; summarised in Table 6.2). Ox-RI cells were significantly smaller than control cells ($T = 4.398$, $p < 0.001$, $df = 98$) and conversely, G-RI cells were significantly larger than control ($T = 2.862$, $p < 0.01$, $df = 98$). Abnormal cellular morphology was seen in the majority of G-RI cells (**Fig. 6.6c**, **6.7c**). The cell walls of both G-RI and Ox-RI cells were significantly thicker than control (approximately 30% and 50% thicker respectively; $T = 9.563$, $p < 0.001$, $df = 58$; $T = 4.7823$, $p < 0.001$, $df = 58$ respectively), but those of G-RI cells were not uniformly thickened (**Fig. 6.7f**) as seen by comparison with control cells (**Fig. 6.7d**). Higher magnification highlighted that thickening of the cell wall was a thickening of the primary cell wall (**Fig. 6.8**); the secondary cell wall did not vary in thickness between control and treated cells. Ox-RI cells had a much smaller internal volume compared with controls and G-RI cells had a much larger internal volume. These calculations assumed perfectly spherical cells (Table 6.2). Transmission electron micrographs (**Fig. 6.7**) also showed differences in the numbers of cells showing obvious signs of division (septum formation or visible "splitting systems"; Paul *et al.*, 1995; Giesbrecht *et al.* 1998), whether complete or not; control = 9%, Ox-RI = 34% and G-RI = 15%. Additionally, there were several (less than 5%) Ox-RI cells that showed three or more daughter cells that had divided but not separated from each other (**Fig. 6.9**), so called "pseudomulticellular Staphylococci" (Giesbrecht *et al.*, 1998).

Variations in cell size and volume are summarised in Table 6.2.

Fig. 6.6 – Scanning electron micrographs showing changes in the cellular morphology of *S. aureus* NCTC 6571 resulting from the "resistance induction" procedure after 35 d

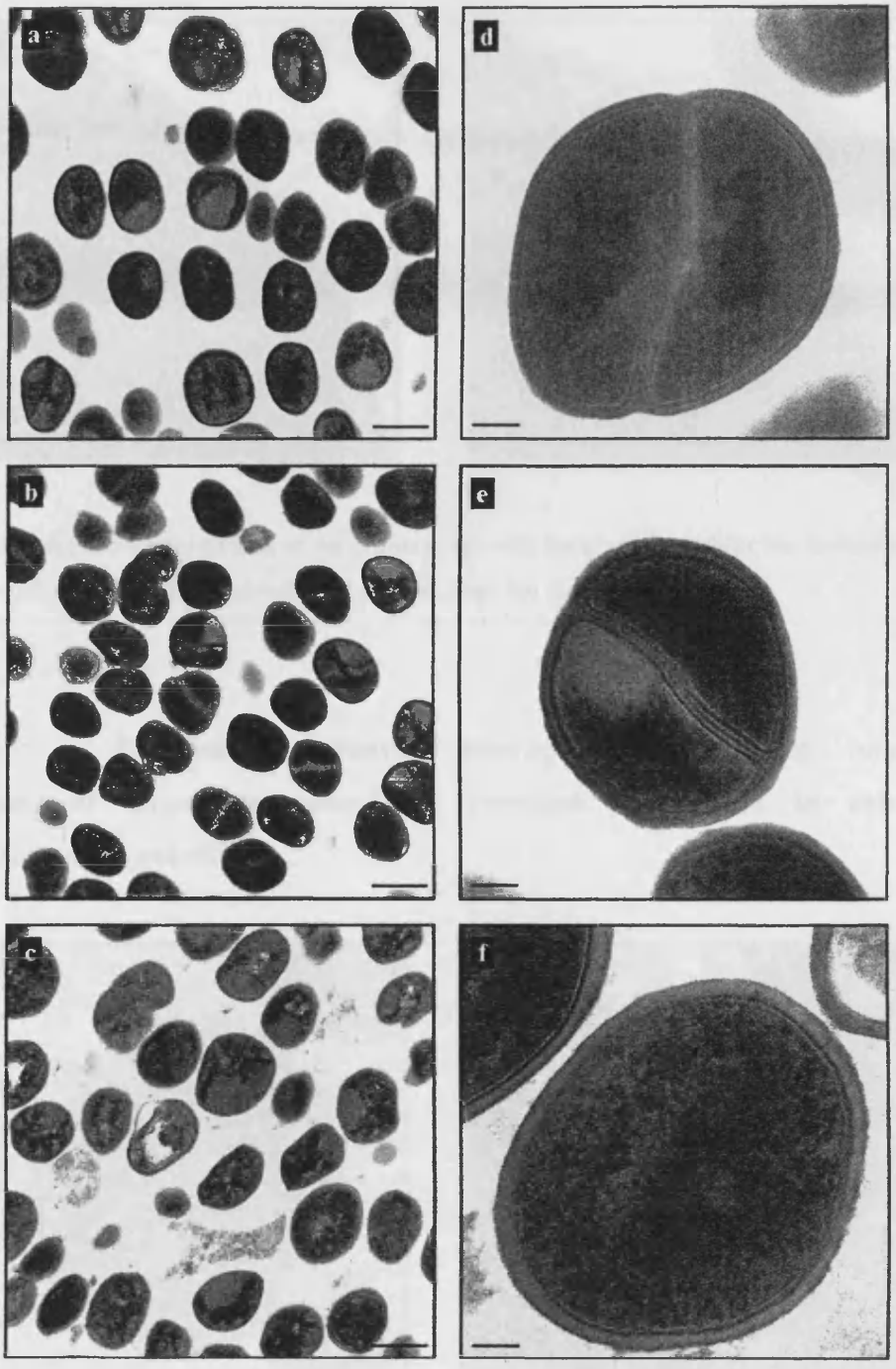


a) Control **b**) Ox-RI **c**) G-RI. White arrows denote the presence of extracellular polysaccharide (**b**) and abnormal cellular morphology (**c**). Scale bar represents 1 μm, all images to scale (15,000x magnification)

6.3.4 – Maintenance of resistance

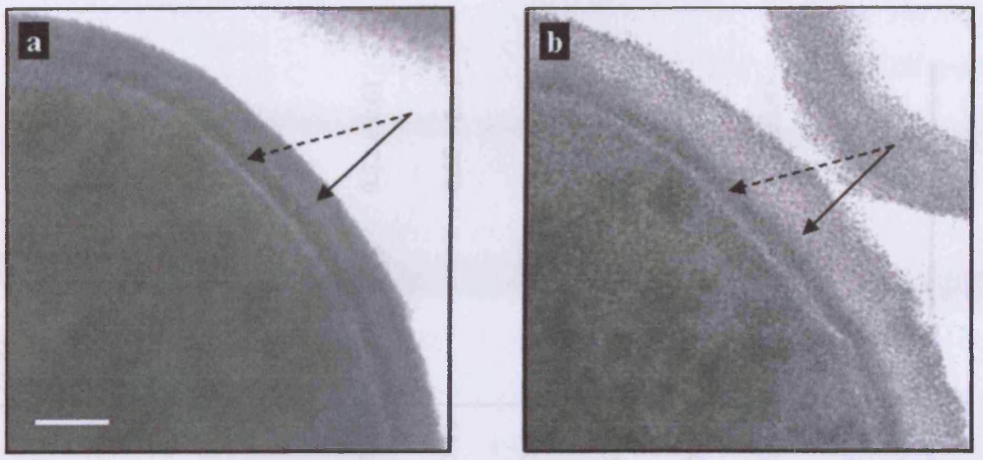
Ox-RI and G-RI isolates were sub-cultured on MHSC every week for eight weeks after termination of the present study and were also maintained on Cryobeads™ (Pro-Lab, Cheshire, UK) at -80°C. MICs of oxacillin and garlic (respectively) were measured every week. The MICs of oxacillin and garlic (respectively) did not decrease over an eight week period. Furthermore, MRSA N315 (a strain of MRSA expressing oxacillin resistance via PBP2a) was sub-cultured on MHSC alongside these isolates. After eight weeks the MIC of oxacillin had decreased from 64 μg mL^{-1} to <1 μg mL^{-1}, indicating a loss of antibiotic resistance.

Fig. 6.7 – Transmission electron micrographs illustrating changes in cell size and cell wall thickness induced by sub-culture in sub-MIC levels of oxacillin or garlic



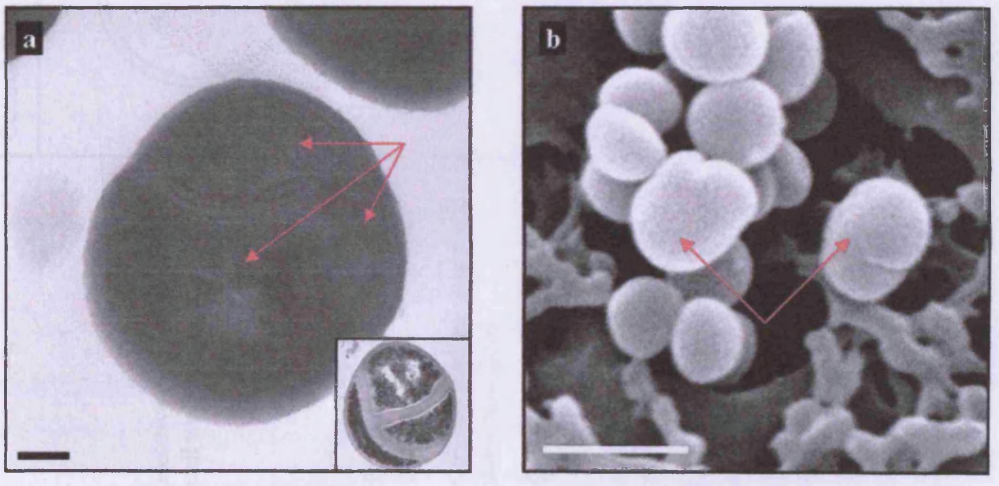
Control cells (**a**, **d**) had a regular morphology and cell wall of uniform thickness (29.47 nm). Ox-RI cells (**b, e**) were smaller than controls, and had thicker cell walls (43.62 nm). G-RI cells (**c, f**) were larger than controls, with thicker cell walls (of irregular thickness: 22.3 – 55.74 nm) and were irregularly shaped. Magnification x20,000 (**a – c**) and x80,000 (**d – f**). Scale bars represent 500 nm (**a – c**) and 100 nm (**d – f**)

Fig. 6.8 – A comparison between the cell wall thickness of control (a) and G-RI (b)



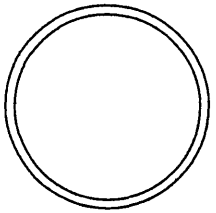
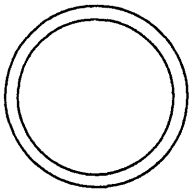
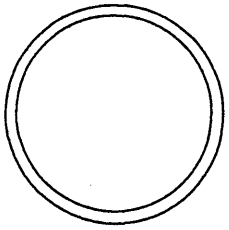
The micrographs show a thickening of the primary cell wall (solid arrow), whilst the thickness of the secondary cell wall (dotted arrow) remained similar. Scale bar represents 30 nm

Fig. 6.9 – Transmission electron micrographs illustrating impaired Staphylococcal separation caused by repeated sub-culture in sub-MIC concentrations of oxacillin



TEMs (a) and SEMs (b) showed some evidence of cells which had divided but not separated (red arrows), so called "pseudomulticellular Staphylococci" (Giesbrecht *et al.*, 1998 – see inset). Scale bars represent (a) 100 nm, (b) 2 µm. Magnification (a) x80,000, (b) x15,000

Table 6.2 – Changes in the cellular dimensions of *S. aureus* NCTC 6571 caused by the "resistance induction" procedure

	Control	Ox-RI	G-RI
Relative mean cell size and cell wall thickness			
Estimated mean cell diameter (μm)	0.67 ± 0.01	0.59 ± 0.01	0.72 ± 0.01
Cell diameter minimum and maximum (μm)	0.52 – 0.89	0.46 – 0.86	0.58 – 0.91
Cell diameter range (μm)	0.38	0.40	0.33
Estimated mean cell wall thickness (nm)	29.47 ± 0.64	43.62 ± 1.33	37.74 ± 1.38
Cell wall thickness maximum and minimum (nm)	23.58 – 39.08	30.09 –56.11	22.3 –55.74
Cell wall thickness range (nm)	15.5	26.01	33.45
Estimated percentage total volume / percentage internal volume (% of control volume)	100 / 100	72 / 59	124 / 118

Cell volumes were estimated from mean cell diameters (n = 50) and assume regular, spherical cells. Mean cell diameters and cell wall thicknesses (n = 30; 6 cells, 5 random measurements) are presented with standard error of the mean

6.4 – Discussion

6.4.1 – Induction of oxacillin resistance

Abraham *et al.* (1941) reported induction of non-β-lactamase mediated resistance to penicillin through repeated sub-culture of *S. aureus* in the presence of sub-MIC concentrations of the antibiotic. In the present study, non-PBP2a mediated oxacillin resistance was induced in *S. aureus* using a similar methodology. Oxacillin resistance was accompanied by slower growth rates and increased lag phases (**Fig. 6.5**), and a thickening of the primary cell wall (**Fig. 6.8**). Abraham *et al.* (1941) also reported slower growth rates and increased lag phases in isolates with non-β-lactamase mediated penicillin resistance. Typically, oxacillin resistance is mediated by expression of a lower-affinity penicillin-binding protein (PBP2a), and penicillin resistance is as a result of β-lactamase expression. These results suggest that thickening of the cell wall and altered growth dynamics can also contribute to reduced β-lactam susceptibility. It is important to note that increasing cell wall thickness was not the primary cause of the increased tolerance to oxacillin; the MIC of oxacillin against G-RI did not change (**Fig. 6.2**) despite thickened cell walls. It is unclear whether the decreased cell size of Ox-RI had any effect on oxacillin tolerance. It has previously been shown that approximately half the population of Staphylococcal SCVs, which exhibited reduced susceptibility to penicillin, were larger in size than wild type (Seaman *et al.*, 2007).

Classical oxacillin resistance (mediated by PBP2a) is induced by the antibiotic, and is therefore only expressed when it confers a selective advantage to the cell. Staphylococcal PBP2a was almost certainly derived from another species (Koch, 2007) and consequently does not function as well as the four constitutive PBPs (Labischinski, 1992), resulting in poorly formed cell walls. The expression of PBP2a is also an energy cost to the cell, hence the inducible expression only when it provides an advantage. That oxacillin resistance was not lost through repeated sub-culture without the inducing agent suggests that the causes of resistance were constitutive, intrinsic characteristics. The Ox-RI population was therefore likely to comprise mutants with increased oxacillin tolerance as a consequence of different growth characteristics or gene expression. Furthermore, a strain of MRSA expressing classical PBP2a mediated oxacillin resistance, carried on plasmid DNA, became

susceptible following repeated sub-culture in the absence of oxacillin, indicating that the plasmid was lost because it was not required. This has previously been shown with other PBP2a expressing strains (Sutherland and Rolinson, 1964) and lends support to the suggestion that the induced oxacillin resistance of Ox-RI was as a result of an intrinsic growth characteristic.

The presence of some asymmetric "pseudomulticelluar Staphylococci" in the Ox-RI population suggested an effect on the expression or action of autolytic enzymes, preventing the separation of daughter cells after division. Inhibition of autolytic enzymes has been previously shown at low concentrations (0.1 $\mu g\ mL^{-1}$) of penicillin (Giesbrecht *et al.*, 1998), although this was typically reversed upon removal of the inducing agent. The pseudomulticellular morphologies seen in the present study were induced by low concentrations of oxacillin, but were maintained upon removal of oxacillin, suggesting that the expression or action of autolytic enzymes was inhibited. It is important to note that only few pseudomulticellular Staphylococci were seen (n <5 %): however, none were seen in the other treatments, and a higher proportion of non-dividing cells with intact splitting systems were seen in Ox-RI compared with other treatments (n = 34%). These observations lend support to the proposal of an inhibition or downregulation of autolytic enzyme expression / activity.

Ox-RI isolates became more mucoid during the study than control, possibly due to increased production of extra-cellular polysaccharide. Bacterial biofilms typically have elevated MICs of antibiotics and the third stage of biofilm formation, maturation, is associated with increased polysaccharide production (Blanco *et al.*, 2005). The increased antibiotic MICs of biofilms are, in part, attributed to the additional diffusion barrier the polysaccharide matrix creates (Donlan and Costerton, 2002). It is therefore feasible that the mucoid phenotype of Ox-RI resulted in a correspondingly lower uptake of the antibiotic, hence the elevated MICs. Interestingly, some antibiotics at sub-inhibitory concentrations have been shown to stimulate exo-polysaccharide production; for example erythromycin and tetracycline in *Staphylococcus epidermidis* (Anderson and O'Toole, 2008).

The induction of pigmentation in a non-pigmented strain of *S. aureus* through exposure to sub-MIC concentrations of an antibiotic is very interesting. Over 90% of

clinical *S. aureus* isolates are pigmented (Chamberlain *et al.*, 1991), but the predominance of *in vivo* pigmentation does not appear to be coincidental. A number of traits which would enhance *in vivo* success have been identified in pigmented, but not non-pigmented strains including; impaired antimicrobial attack by neutrophils (Liu *et al.*, 2002), stabilisation of bacterial membranes (Chamberlain *et al.*, 1991) and production of alternative σ factors (which co-ordinate the expression of stress response genes; Giachino *et al.*, 2001). Pigmentation is also lost during long term storage (Servin-Masseiu, 1961), which supports a hypothesis that pigment production is upregulated as part of the stress response in *S. aureus* (Giachino *et al.*, 2001). Investigation into the effects of sub-MIC concentrations of antibiotics on pigment production in non-pigmented strains of *S. aureus* would test this suggestion, or show whether the strain presented here was atypical.

6.4.2 – Decreased garlic susceptibility in *S. aureus* NCTC 6571

It is difficult to define "resistance" to garlic. As previously mentioned, LAB typically have MICs approximately five to ten times higher than *S. aureus* or *E. coli*. For the purposes of these experiments an increase in MIC from 0.8 mg mL^{-1} to 8 mg mL^{-1} would have been considered substantial. Whilst such an MIC was not acquired (MIC was 4 mg mL^{-1} after 35 d), sensitivity to garlic of G-RI decreased throughout the experiment (**Fig. 6.3**). There was no increase in the MIC of garlic after 28 d, a finding that was consistent with other replicates of the same procedure, which perhaps suggests that further increases in MIC using this method are not possible – repeating the procedure over a longer time period may lend support to this suggestion. A similar degree of reduced garlic susceptibility was induced in a strain of MRSA (N315; data not presented) using the same method, which lends further support to the suggestion that garlic and oxacillin have different targets in *S. aureus*.

Garlic causes dose-dependent decreases in the specific growth rate of LAB, but does not affect the specific growth rate of *E. coli* (Cottrell, 2003). It was previously shown that garlic affects the lag phase of *S. aureus* but only affects the growth rate at low concentrators (**Chapter 3**). It has been suggested, therefore, that the increased tolerance of LAB to garlic is associated with its effect on growth rates (Cottrell,

2003). In the present study, however, despite increasing garlic tolerance, there was no change in the growth rate of G-RI compared with control.

As previously mentioned, not only does garlic contain numerous antimicrobial compounds, but some of the breakdown products of these compounds also exert antibacterial effects. It is feasible that through the 35 d period G-RI developed a tolerance to one (or more) of the antimicrobial compounds, and was comparatively less sensitive to the other antimicrobials, allowing growth in increasing concentrations of garlic. After 28 d it seems that either the adaptation conferring decreased sensitivity failed to provide further protection, or that the isolates became inhibited by another garlic compound. This hypothesis could be tested by measuring the MICs of individual garlic compounds against control and treated isolates, although this would not take into account any interactions between the antimicrobial components in garlic.

The altered cellular morphology of Ox-RI was fairly regular, and, in part, helped to explain the elevated MICs. Treatment with garlic however appeared to induce a number of morphological changes – most noticeably, cells became enlarged and were irregular in shape and texture (**Fig. 6.6**). Additionally, there appeared to be large amounts of extra-cellular polysaccharide. As previously discussed, this could account for increased tolerance to garlic due to decreased uptake through the polysaccharide matrix (Donlan and Costerton, 2002). The isolates also lost some structural integrity, because the sample preparation procedure for transmission electron microscopy resulted in numerous "empty" cells and a large amount of cellular debris – neither of which were seen in the other treatments. This suggests that although thicker, the cell walls were not properly formed in G-RI. An initial hypothesis could be that the thickening of the cell wall facilitated the decreased sensitivity to garlic however this is unlikely for several reasons:

1. It was previously shown that *S. aureus* treated with low garlic concentrations exhibited thicker cell walls after 24 h incubation, but had longer lag phases than control (**Chapter 5**)
2. As previously discussed (**Chapter 3**), garlic does not damage the integrity of the Staphylococcal cell wall after 24 h exposure, and peptidoglycan is unlikely to be a major target of garlic

3. Cell walls were not thicker in all cells, and even in individual cells the wall was not uniformly thick (note the range of cell wall thickness Table 6.2)
4. There was no change in the MIC of garlic against Ox-RI cells, despite thicker cell walls

All of the above do not imply a decreased tolerance to garlic as a result of thickened cell walls. It is unclear why these isolates developed decreased sensitivity to garlic; however as the mode of action of garlic against *S. aureus* is complex and remains to be investigated in detail, this is perhaps unsurprising. This is, however, the first study to report decreased sensitivity to garlic of any bacterial species through continued exposure.

6.5 –Conclusions

- Oxacillin resistance (MIC >4 μg mL^{-1}) was induced in *S. aureus* using a method similar to that of Abraham *et al.* (1941) – **Hypothesis 1 ACCEPTED**
- Oxacillin resistance was accompanied by thickened primary cell walls, smaller cells, decreased growth rates, increased lag phases, induction of pigmentation and an increasingly mucoid phenotype
- Sensitivity of *S. aureus* to garlic was decreased reproducibly using a method based on that of Abraham *et al.* (1941), although garlic resistance (using breakpoints defined in the present study; MIC >8 mg mL^{-1}) was not induced – **Hypothesis 2 REJECTED**
- Decreased garlic sensitivity was accompanied by abnormal cellular morphologies, non-uniform thickening of the primary cell wall and an increasingly mucoid phenotype. There was little / no change in growth dynamics
- Repeated sub-culture in the presence of oxacillin, and the corresponding decrease in oxacillin sensitivity, had no effect on the susceptibility of *S. aureus* to garlic (and visa versa) – **Hypothesis 3 ACCEPTED**

7 – *S. aureus* Ox-RI is a Phenotypic Mutant of *S. aureus* NCTC 6571: Confirmation by RAPD Fingerprinting

7.1 – Introduction

Molecular genotypic typing methods have been widely used both to identify bacterial isolates and to discriminate between strains of the same species, a level of discrimination which is much more difficult to achieve using traditional phenotypic techniques (Lipuma, 1998). There are numerous methods used to genotype bacteria including DNA hybridisation techniques, pulsed field gel electrophoresis (PFGE) and PCR based techniques (Lipuma, 1998). The ability to discriminate between strains of MRSA has allowed the evolutionary history of MRSA to be studied, and has identified five major pandemic MRSA clonal lineages (Oliveira *et al.*, 2002). MRSA typing has been achieved using a combination of molecular techniques including digestion and separation of chromosomal DNA by PFGE, detection of polymorphisms in the *mecA* gene and, more recently, multi-locus sequence typing (MLST) and *spa* typing (Oliveira *et al.*, 2002).

Randomly amplified polymorphic DNA (RAPD; Welsh and McClelland, 1990; Williams *et al.*, 1990) fingerprinting is a polymerase chain reaction (PCR) based technique, which helps to differentiate between genetically diverse isolates. The technique produces a strain-specific unique fingerprint which can be used to elucidate whether two isolates are the same strain.

PCR amplifies part of a genome using a pair of 'primers' – short pieces of DNA with complementary sequences to part of the nucleic acid. The primers are added to a solution of nucleic acid to which they bind under the appropriate conditions. The nucleic acid acts as a template and by adding Taq polymerase (a thermostable DNA polymerase derived from *Thermus aquaticus*; Chien *et al.*, 1976), then cyclically heating and cooling the reaction, a specific length of nucleic acid is amplified. RAPD fingerprinting works in precisely the same fashion except only one short (10 nucleotides) primer is used. This may randomly bind to any genome in several places on either strand of DNA. The random attachment of the primer to the nucleic acid is one of the advantages of RAPD fingerprinting since it requires no prior genetic knowledge of the genome (Welsh and McClelland, 1990). It is unknown where the

primer will bind and therefore what pattern of banding will occur; however when the same DNA template is used with the same primer the resulting fingerprint will always be the same. If a different DNA template is used a different pattern will result. This therefore allows bacterial strains to be uniquely identified. The resolving power of RAPD fingerprinting is increased if several primers are used separately.

7.1.1 – Use of RAPD fingerprinting in the present study

RAPD fingerprinting was used in the present study to confirm whether the phenotypic mutant isolated through continuous sub-culture in sub-MIC levels of oxacillin (see **Chapter 6**) was the same strain as the parent (*S. aureus* NCTC 6571) from which it was derived.

The RAPD method outlined below was carried out essentially as described by Mahenthiralingam *et al.*, (1996). The primers used were chosen specifically to discriminate between different strains of *Ps. aeruginosa*, and hence might not have been optimal to discriminate between strains of *S. aureus*. Preliminary investigations, however, showed that several bands could be consistently obtained from *S. aureus* with each of the three primers, thereby providing enough information to suggest whether two isolates were the same strain.

RAPD products from the phenotypic mutant (Ox-RI), from three different primers, were obtained in duplicate (on two separate occasions), and were run alongside the RAPD products of wild type colonies in duplicate (also obtained on two separate occasions). In addition RAPD profiles were obtained for MRSA N315 (in triplicate) to show the degree of homology between the RAPD profiles of two different *S. aureus* strains.

7.1.2 – Aims

- Confirm that the phenotypic mutant obtained in **Chapter 6** was *S. aureus* by Gram-morphology and substrate utilisation
- Determine whether the phenotypic mutant (Ox-RI) obtained in **Chapter 6** was the same strain as the parent strain from which it was derived by comparison between RAPD profiles

7.1.3 – Hypothesis

1. The phenotypic mutant (Ox-RI) is *S. aureus* NCTC 6571 in which pigmentation has been induced through continued sub-culture in sub-MIC concentrations of oxacillin

7.2 – Materials and methods

7.2.1 – Identification by Gram-morphology and substrate utilisation

The phenotypic mutant (Ox-RI) was compared with the parent strain by Gram-stain morphology and the presence or absence of catalase. Substrate utilisation of both parent and phenotypic mutant was assessed using API ID 32 Staph (BioMérieux, France) identification strips.

7.2.2 – Chelex® 100 DNA extraction method

DNA was prepared directly from single colonies of 24 h pure cultures on MHSC. Colonies were picked and transferred in 50 µL aliquots of sterile 5% (w/v) Chelex® 100 resin solution (Sigma-Aldrich, Poole, UK) in duplicate. DNA extraction tubes were stored at –20°C until required.

DNA extraction tubes were pricked to prevent a build up of pressure and were boiled for 5 min and immediately placed on ice for 5 min; this heating and cooling process was repeated. Samples were briefly centrifuged (3,000x *g* for 5 min) to remove the resin, yielding a sheared DNA sample suitable for use as a PCR template.

7.2.3 – RAPD method

Primers 272, 277 and 287 (Table 7.1; MWG Biotech, Covent Garden, London, UK) were prepared from desiccated reagents as 100 pmol μL^{-1} stock solutions.

Table 7.1 – DNA sequences of the three RAPD primers used in the present study

Primer	Sequence (5' to 3')
272	AGCGGGCCAA
277	AGGAAGGTGC
287	CGAACGGCGG

All other reagents (Qiagen Ltd, Crawley, UK) were thawed and mixed in a 25 μL reaction mixture containing: 10.3 μL sterile polished deionised water, 2.5 μL PCR buffer, 5 μL Q-solution, 1.5 μL 25 mM $MgCl_2$ (1.5 mM final concentration), 4 μL of RAPD primer (10 pmol stock), 0.5 μL 10 mM dNTPs (200 μM final concentration), 0.2 μL Taq polymerase (5 units μL^{-1} stock) and 2 μL template DNA (approx. 40 ng). The PCR thermal cycles were carried out on a Flexigene Thermal Cycler (Techne Ltd., Newcastle, UK) as follows: (i) 4 cycles of: 94°C for 5 min, 36°C for 5 min and 72°C for 5 min; (ii) 30 cycles of: 94°C (1 min; ramp time = 66 sec) 36°C (1 min; ramp time = 22 sec) and 72°C (1 min; ramp time = 17 sec); (iii) a final extension of 72°C (10 min). The reaction was then held at 4°C until required.

7.2.4 – Gel electrophoresis

RAPD profiles were separated by standard agarose gel electrophoresis (Mahenthiralingam *et al.*, 1996) using 1.5% agarose (Sigma-Aldrich, Poole, UK) gels and were visualised with ethidium bromide (0.667 $\mu g\ mL^{-1}$ final in Tris Borate EDTA (TBE) buffer) for 30 min. Molecular size standards were included alongside RAPD products on all gels (1 Kb Plus DNA Ladder; Invitrogen, Paisley, UK).

7.2.5 – Imaging

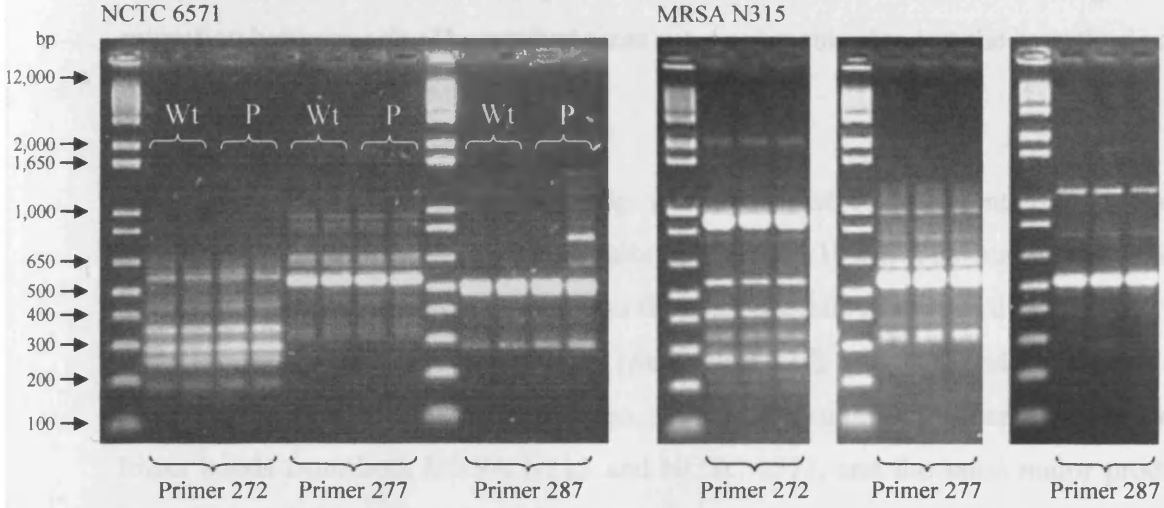
Imaging was performed using the gel Doc-it TS imaging system (Jencons, Leicestershire, UK).

7.3 – Results

The phenotypic mutant was confirmed as *S. aureus* by both Gram-stain morphology and substrate utilisation (API ID 32 Staph, BioMerieux, France).

The RAPD fingerprints obtained with all three primers were the same for both wild type NCTC 6571 and the phenotypic mutant (**Fig. 7.1**). The same numbers of bands were produced from both duplicated isolates with each primer. The RAPD profiles from MRSA N315 (**Fig. 7.1**) were clearly different to the profiles of both NCTC 6571 and the phenotypic mutant regardless of the primer they were derived from.

Fig. 7.1 – RAPD profiles of *S. aureus* NCTC 6571, MRSA N315 and a phenotypic mutant of NCTC 6571 (Ox-RI) obtained through repeated sub-culture in sub-MIC concentrations of oxacillin



Wt indicates the parent strain (*S. aureus* NCTC 6571); P indicates the phenotypic mutant (Ox-RI). Ladders (1 Kb Plus; Invitrogen, Paisley, UK) were used to compare product movement due to molecular size, but were not required to determine the actual size of the products. RAPD profiles of MRSA N315 using the same primers are also presented to highlight the very different profiles obtained from different strains

7.4 – Discussion

The homology between the profiles of the phenotypic mutant and wild type *S. aureus* NCTC 6571, and the lack of homology between those profiles and those from MRSA N315, indicated that the phenotypic variant was *S. aureus* NCTC 6571. **Hypothesis 1** is therefore accepted: pigmentation was induced by the continued presence of sub-MIC concentrations of oxacillin, possibly as a stress response. Pigmentation was not seen in the wild type of this strain, at any point, and the pigmented morphology was maintained throughout the experiment, which lends support to the hypothesis of methodological indication of pigmentation rather than selection of a pigmented mutant.

Marking ladders (of known molecular sizes) are typically used to determine the molecular size of PCR products. In the present study the ladders were only used to compare between the banding patterns, and to correct for different degrees of migration between gels. The product sizes were not required to test the hypothesis and were therefore not determined.

The RAPD method used in this study was optimised for differentiation between strains of *Ps. aeruginosa* (Mahenthiralingam *et al.*, 1996). The number of bands amplified from two different strains, and the reproducibility between duplicates of the same strain suggest that these primers (particularly 272 and 277) and conditions are also suitable for distinguishing between strains of *S. aureus*. Primer 287 produced fewer bands from both MRSA N315 and NCTC 6571, and the same major product from both MRSA strains. It is therefore suggested that primer 287 is not ideal for differentiating between strains of *S. aureus*, and another primer with a higher resolving power should be used where such discrimination is required.

8 – The Susceptibility of MRSA and *S. aureus* to Plant Antimicrobials Other than Garlic

8.1 – Introduction

Data presented in **Chapter 3** and **5** confirmed that garlic was an effective anti-Staphylococcal agent and that at sub-MIC concentrations of garlic β-lactam susceptibility could be restored against β-lactam-resistant strains of *S. aureus*. There are many compounds derived from plants other than garlic with proven antibacterial and antimicrobial activity, including essential oils of oregano and cinnamon, and green tea powder. Many of these plant compounds also potentiate antibiotic sensitivity in antibiotic-resistant bacteria (see also **Chapter 3.1**). It is thought that plant-derived antimicrobials could be used alongside clinically employed antibiotics, particularly in wound management, as one method to counteract the problems associated with antibiotic resistance.

8.1.1 – Oregano – *Origanum vulgare*

Oregano essential oil (OEO) contains two principle antimicrobial components, the phenols thymol and carvacrol (Si *et al.*, 2008), and their precursor molecules, γ-terpinene and ρ-cimene (respectively). In addition there are at least 30 other compounds (some with limited antimicrobial activity), although these are relatively low in abundance. The proportions of these compounds vary hugely depending on the plant variety, environmental and climate conditions, and extraction procedures (Faleiro *et al.*, 2005). Carvacrol concentrations, for example, have been reported ranging from 14% (Nostro *et al.*, 2004) to 75.71% (Peñalver *et al.*, 2005), and similarly, thymol concentrations from a complete absence (Peñalver *et al.*, 2005) to 33% (Faleiro *et al.*, 2005). Consequently, literature citing the antimicrobial activities of OEO should contain data expressing the relative abundances of thymol and carvacrol at the very least.

OEO exerts a bacteriostatic or bactericidal (depending on species) effect against numerous pathogens including *S. aureus* / MRSA, *E. coli*, *Ps. aeruginosa*, *Salmonella typhimurium* and also the probiotic bacteria *Lactobacillus carvatus* and *Lactobacillus sake* (Lambert *et al.*, 2001; Chorianopoulos *et al.*, 2004; Nostro *et al.*, 2004; Faleiro *et al.*, 2005; Peñalver *et al.*, 2005; Preuss *et al.*, 2005; Gill and Holley, 2006). MICs of

OEO against Staphylococci were found to be typically higher than those of thymol and carvacrol alone at relative concentrations (Nostro *et al.*, 2004), however, as previously mentioned, OEO does not consist solely of these two compounds, and hence this result is perhaps unsurprising. It has been reported, however, that thymol and cavracrol had neither an antagonistic nor synergistic effect on each other (Lambert *et al.*, 2001).

OEO appears to be a cytoplasmic membrane active biocide and causes proton, phosphate and potassium ion leakage (Lambert *et al.*, 2001). Thymol and carvacrol also disrupt bacterial cell membranes (Lambert *et al.*, 2001), which probably explains the membrane disrupting activity of OEO. In addition, carvacrol inhibited the flagella motors of both *E. coli* and *L. monocyotgenes*, although it was unclear whether these were direct effects on the flagellum motor or indirect due to structural damage, and also had ATPase activity (Gill and Holley, 2006). OEO interacted with some antibiotics (including Doxycycline, Amoxicillin, Ceftriaxone and Levofloxacin) in either a synergistic or additive manner against *E. coli* (Si *et al.*, 2008).

8.1.2 – Cinnamon – *Cinnamomum zeylanicum* (Synonym: *Cinnamomum verum*)

Cinnamon essential oil (CEO) is extracted from the bark of *Cinnamomum zeylanicum* (*C. zeylanicum*), the major antimicrobial compound of which is cinnamaldehyde (or cinnamic aldehyde). There are various other species also known as "cinnamon", including *Cinnamomum osmophloeum* and *Cinnamomum cassia* both of which contain different antimicrobial compounds to *C. zeylanicum*. The following information has been obtained only from studies on *C. zeylanicum*.

As previously mentioned, the major antibacterial compound of CEO is *trans*-cinnamaldehyde, which constitutes approx. 60% of the essential oil (Inouye *et al.*, 2001; Shahverdi *et al.*, 2007). As with many essential oils, however, the chemical composition varies with climate and growing conditions, and extraction procedures, hence reports of cinnamaldehyde content vary from 52.42% (Prabuseenivasan *et al.*, 2006) to 75% (Singh *et al.*, 2007). In addition to cinnamaldehyde, eugeneol, another antimicrobial compound, is often reported in CEO, at concentrations of up to 8% (Valero and Francés, 2006; Senhaji *et al.*, 2007).

CEO, cinnamaldehyde and eugeneol exert bacteriostatic or bactericidal (dependent on species) effects against a wide range of bacteria, including: *S. aureus* / MRSA, *E. coli*, *Helicobacter pylori, Bacillus subtilis, Klebsiella pneumoniae, L. monocytogenes, Ps. aeruginosa, Lactobacillus sake, Vibrio harveyii* and *Salmonella typhimurium* (Niu and Gilbert, 2004; Gill and Holley, 2006; Niu *et al*, 2006; Prabuseenivasan *et al.*, 2006; Singh *et al.*, 2007).

In contrast to OEO, CEO and cinnamaldehyde do not appear to cause damage to cytoplasmic membranes (Valero and Francés, 2006; Senhaji *et al.*, 2007), although eugeneol did disrupt the cellular membranes of *E. coli, L. monocytogenes* and *Lactobacillus sake* (Gill and Holley, 2006). It is likely that at MIC concentrations of CEO eugeneol is not present in sufficient quantities to disrupt cellular membranes; hence CEO does not act in this manner. Cinnamaldehyde has been shown to inhibit cell separation in both *Bacillus cereus* (Valero and Francés, 2006) and *E. coli* (Niu and Gilbert, 2004) but not in *S. aureus* (Valero and Francés, 2006); this effect was probably caused by interference with, or inhibition of, the autolytic enzymes which facilitate cell division following separation (Giesbrecht *et al*, 1998). Furthermore cinnamaldehyde and eugeneol affected quorum sensing at sub-MIC concentrations in *Vibrio harveyii* (Niu *et al*, 2006; Brackman *et al.*, 2008). The two compounds also have targets which are independent of the other compound: cinnamaldehyde decreased biofilm formation in *E. coli* and several *Pseudomonas* spp. (Niu and Gilbert, 2004; Niu *et al*, 2006), whereas eugeneol possessed ATPase inhibiting activity (Gill and Holley, 2006).

8.1.3 – Green tea (*Camilla sinensis*)

Green tea contains a number of polyphenols (collectively termed 'catechins') with a wide range of antimicrobial and health promoting effects. The major antibacterial compounds, and their relative abundances, are summarised in Table 8.1.

Table 8.1 – The main antibacterial compounds in green tea

Compound	Abbreviation	Abundance (%)
Epicatechin	EC	2 - 7
Epicatechin gallate	ECg	1-20
Epigallocatechin	EGC	10-24
Epigallocatechin gallate	EGCg	18-60

Abundances were summarised from papers citing the antimicrobial effects of green tea (Sankanaka *et al.*, 2000; Hisano *et al.*, 2003; Gradiar *et al.*, 2006; Cho *et al.*, 2008)

Green tea contains approximately 10-40% catechins (Hisano *et al.*, 2003; Navarro-Martínez *et al.*, 2005), corresponding to >200 mg catechins per 240 mL cup of green tea (Gradiar *et al.*, 2006, Navarro-Martínez *et al.*, 2005). Numerous bacteria are sensitive to green tea and the catechins it contains, including: *S. aureus* / MRSA (planktonic and biofilm associated), *Helicobacter pylori*, *Yersinia enterocolitica*, *Streptococcus pyogenes*, *Neisseria* spp., *Klebsiella pneumoniae*, *Ps. aeruginosa*, *Stenotrophomonas maltophila*, *Haemophilus influenzae* and *E. coli* (Tombola *et al.*, 2003, Yam *et al.*, 1997). Gram-positive pathogens are generally more sensitive to green tea catechins than Gram-negative bacteria. This is possibly due to repulsion of the negatively charged catechins by the negatively charged surfaces of Gram-negatives (Taguri *et al.*, 2004) or attraction of the catechins to the positively charged lipids in the Gram-positive membrane (Hisano *et al.*, 2003). In general, the antibacterial effect increases with the size of the molecule or the presence of a gallate moiety (Taguri *et al.*, 2004) and follows the order: EGCg > ECg > EGC > EC (Sakanaka *et al.*, 2000). The bactericidal action of EGCg is probably due to H_2O_2

production (by reaction with dissolved oxygen in aqueous solution) and is inhibited by catalase (Arakawa *et al.*, 2004).

Although green tea catechins do not posses comparable activity with most clinically employed antibiotics (MICs typically > 100 μg mL^{-1}; oxacillin resistance in *S. aureus* is defined as an MIC > 4 μg mL^{-1}; CLSI, 2007), several of the compounds have been shown to potentiate antibiotic susceptibility in *S. aureus* at sub-MIC concentrations. Green tea (Cho *et al.*, 2008), ECg (Stapleton *et al.*, 2004b) and EGCg (Stapleton *et al.*, 2004b) acted synergistically with oxacillin against MRSA but catechin did not (Stapleton *et al.*, 2004b). It therefore appears that the gallate moiety is essential for modulation of oxacillin sensitivity (Stapleton *et al.*, 2004a; 2004b). Potentiation of oxacillin resistance is thought to be exerted from a locus in or on the bacterial cell wall, and inhibition of PBP2a (along with other PBPs) has been proposed as the basis for this potentiation (Yam *et al.*, 1998; Zhao *et al.*, 2001; Stapleton *et al.*, 2004a). EGCg also modulates tetracycline sensitivity in Staphylococci by inhibiting tetracycline efflux pumps (Sudano-Roccaro *et al.*, 2004), but ECg does not act synergistically with vancomycin (Hamilton-Miller and Shah, 1999).

8.1.4 – The present study

The anti-Staphylococcal effects of OEO, CEO and green tea powder were confirmed. Additionally, the effects of CEO and OEO on the oxacillin susceptibility of one strain of MRSA were determined and compared with the reported synergistic relationship between green tea and oxacillin, and the previously shown additive relationship between garlic and oxacillin (**Chapter 5**). These compounds in particular were chosen because they are used in some product formulations by Cultech Ltd.

8.1.5 – Aims

- Confirm the anti-Staphylococcal activities of OEO and CEO and green tea powder compared with garlic against MRSA and *S. aureus* using a disk diffusion method
- Determine whether CEO and OEO potentiate oxacillin susceptibility in MRSA N315
- Replicate the reported synergistic relationship between green tea and oxacillin against MRSA
- Compare the interactions between OEO, CEO or green tea and oxacillin with the effect of garlic on the oxacillin susceptibility of MRSA

8.1.6 – Hypotheses

1. OEO and CEO will exhibit antibacterial activity against MRSA and *S. aureus*
2. MRSA will be no less susceptible than *S. aureus* to these plant-derived oils / aqueous preparations – the antibiotic resistance mechanisms of MRSA will have no effect on sensitivity
3. Treatment of MRSA with OEO and CEO will result in an increase in sensitivity to oxacillin

8.2 – Essential oils and antimicrobial powders

All essential oils and powders were from Cultech Ltd. The compositions of the essential oils or powders used, in terms of the major antibacterial compounds (see **Section 8.1**) were as follows:

Oregano oil	- 74.3% (v/v) Carvacrol - 0.35% (v/v) Thymol - 11.74% (v/v) ρ-Cymene - 3.38% (v/v) γ-Terpinene
Oregano powder	- 25% (w/v) oregano oil emulsified with tapioca starch and maltodextrin
Cinnamon bark oil	- 70% (v/v) Cinnamaldehyde - 4.5% (v/v) Eugeneol
Cinnamon powder	- 33% (w/v) cinnamon bark oil emulsified with tapioca starch and maltodextrin
Green tea powder	- 52.53% (w/v) polyphenols (unspecified)
Garlic powder	- 10,000 ppm (w/v) alliin (1% alliin)

8.3 – The anti-Staphylococcal effects of oregano and cinnamon oils and green tea powder

Experiments were repeated with four MRSA strains and one *S. aureus* strain, but in general only results for MRSA N315 and *S. aureus* NCTC 6571 are presented. Unless otherwise stated the presented results are also representative of the other strains.

8.3.1 – Materials and methods

The following method was adapted from the BSAC Methods for Antimicrobial Susceptibility Testing (BSAC, 2008). Inocula of MRSA N315 and NCTC 6571 were prepared as previously described (**Chapter 2.5**) and diluted 1:10 in sterile distilled water to create suspensions of approx. 1 x 10^7 CFU mL^{-1}. MHSC plates were swabbed (sterile cotton swabs; Eurolabs, Manchester, UK) with inoculum suspensions in three directions (to create semi-confluent bacterial lawns after 24 h incubation at 37°C). Plates were left to dry for 15 min.

Stock solutions of non-sterile aqueous green tea and garlic were prepared by suspending the corresponding powders in sterile distilled water and mixing thoroughly for 20 min. Stock solutions were diluted to required concentrations in sterile distilled water.

Sterile filter paper discs (5 mm) were applied to the centre of each plate, onto which volumes (0 – 6 µL) of antimicrobial oils or non-sterile garlic and green tea suspensions (5 µL volumes, 0 – 200 mg mL^{-1}) were dispensed. Inhibition zones were measured (using IMAGEJ, NIH) using digital images taken after 24 h incubation at 37°C. Results are means of at least four independent replicates. Results are presented with standard error of the mean.

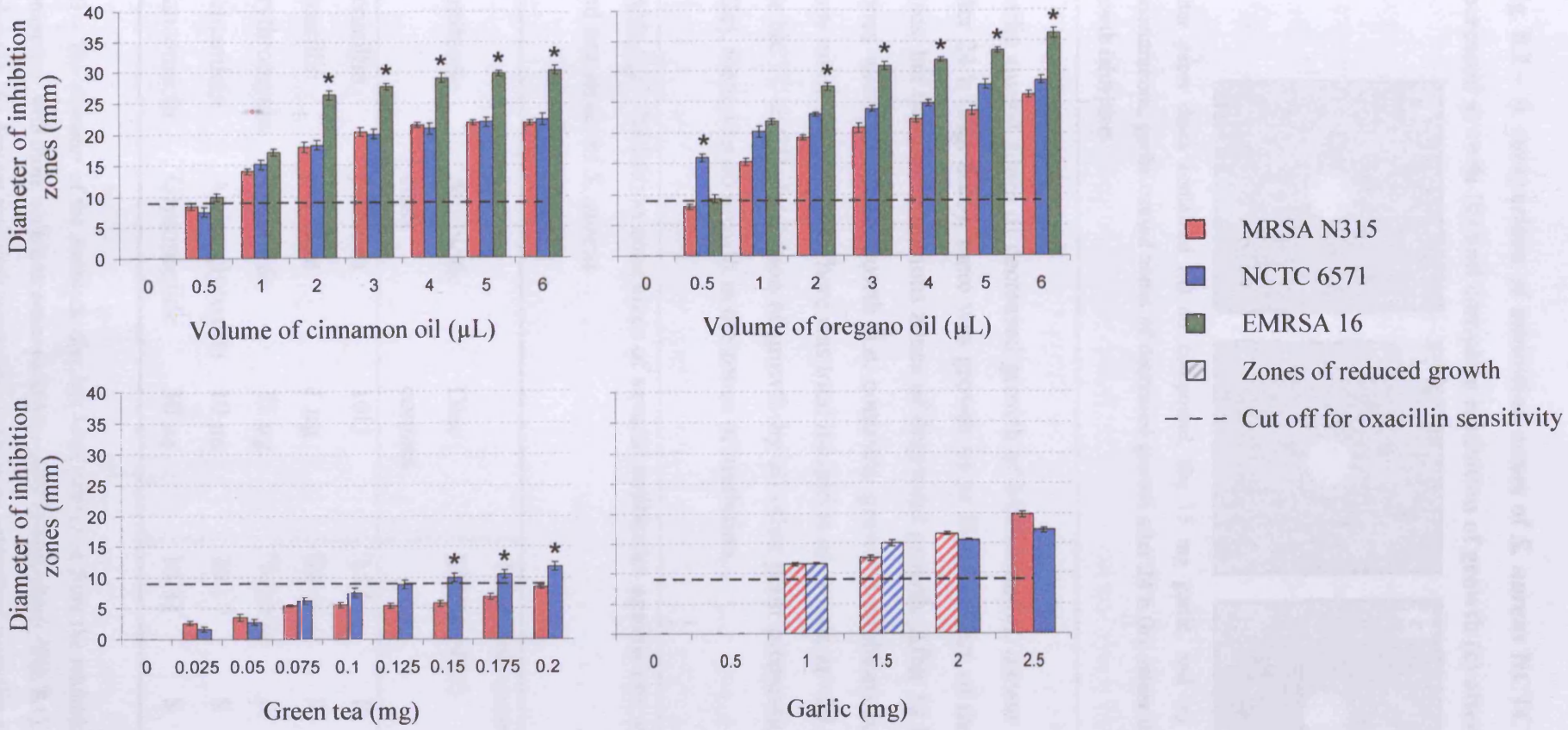
Two-way T-tests (Minitab) were applied to mean inhibition zone diameters to confirm significant differences between inter-strain susceptibilities to essential oils / aqueous preparations. Data were confirmed as being normally distributed and with equal variances by the Anderson-Darling test (Minitab).

8.3.2 – Results

All four antimicrobial preparations inhibited the growth of both MRSA and *S. aureus* (**Fig. 8.1**). There was no significant difference between the susceptibilities of MRSA N315 and NCTC 6571 to CEO or OEO, but EMRSA-16 was significantly more susceptible than both strains to discs containing >2 μL of either oil ($p<0.005$; see **Appendix** for full details). NCTC 6571 was significantly more susceptible to green tea (discs containing ≥0.15 mg) than MRSA N315 ($p<0.005$; see **Appendix** for full details).

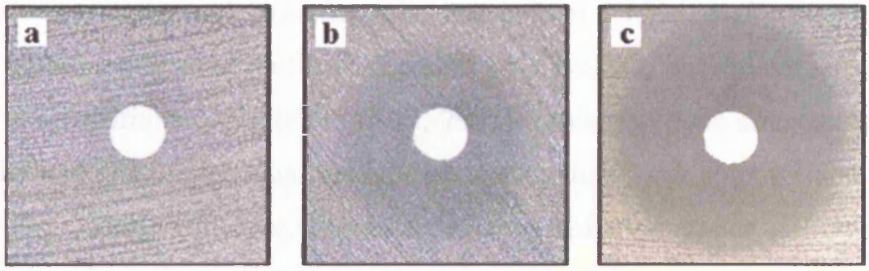
Based on the size of the inhibition zones alone, the activity of two antimicrobial oils against MRSA and *S. aureus* followed the order OEO > CEO. Green tea was inhibitory against MRSA and *S. aureus* at lower concentrations than garlic. The sizes of the inhibition zones were comparable with the zones created by some antibiotics (Table 8.2). Zone sizes of >9 mm are taken to indicate sensitivity to oxacillin (1 μg; BSAC, 2008; **Fig. 8.1**).

Fig. 8.1 – The antibacterial effects of four plant-derived antimicrobial oils / aqueous suspensions against MRSA and *S. aureus*



Results are the mean of at least four independent replicates and are presented with standard error of the mean. Legend indicates strain. Bars with block colour indicate complete inhibition of growth; broken colour indicates zone of reduced growth. Black dashed line indicates oxacillin susceptibility (disc containing 1 µg; BSAC, 2008). Significant differences between strains indicated by * ($p<0.005$; see **Appendix** for details). Diameters of filter discs have been subtracted from inhibition zone measurements

Fig. 8.2 – A comparison of inhibition zones of *S. aureus* NCTC 6571 illustrating decreased growth (b) and complete inhibition of growth (c) after 24 h



Filter paper discs contained (**a**) no compound, (**b**) 15 mg garlic, and (**c**) 1 µL CEO. At low concentrations, garlic caused zones of decreased growth after 24 h (**b**), rather than zones of complete growth inhibition

Garlic caused zones of decreased growth of MRSA and *S. aureus* growth at <15 mg after 24 h (**Fig. 8.2b**); there was growth up to the periphery of the garlic containing discs, but there were obvious zones of decreased growth. After 12 h incubation, these zones were devoid of growth (i.e. complete growth inhibition) but after 48 h there were no obvious zones. There was total inhibition of growth at >20 mg garlic (>15 mg for NCTC 6571). Inhibition of growth by all other plant preparations was total (**Fig. 8.2c**), there was no growth in the zones of inhibition.

Table 8.2 – Inhibition zone sizes of several antibiotics against one strain of MRSA and one strain of *S. aureus*

Antibiotic	Antibiotic class	Disc content	Inhibition zones (mm) for strains MRSA N315		NCTC 6571	
Penicillin	β-lactam	10U	3.24	R	34.78	S
Oxacillin	β-lactam	1 µg	No zone	R	20.85	S
Erythromycin	Macrolide	15 µg	No zone	R	22.87	S
Gentamicin	Aminoglycoside	10 µg	10.17	S	13.58	S
Vancomycin	Glycopeptide	30 µg	10.81	S	11.97	S

NB – The diameter of the antibiotic disc has been subtracted from the inhibition zone sizes to allow comparison with those inhibition zones caused by plant antimicrobials (**Fig. 8.1**). (R) denotes antibiotic resistance; (S) denotes antibiotic sensitivity. Zones were determined according to BSAC methodology (BSAC, 2008)

8.3.3 – Discussion

Disc diffusion is a rapid way of determining the antimicrobial properties of plant-derived suspensions and essential oils, but it is not ideal. Early studies into the antibacterial properties of garlic oil reported little activity, but did not account for oil volatility or hydrophobicity (Ross *et al.*, 2001). Inhibition zone sizes are affected by solubility and rate of diffusion through the agar medium, and volatilisation of the oils (Benkeblia, 2004). However, the sizes of the inhibition zones (CEO and OEO) reported in this study, and the increasing size of inhibition zones with increasing oil disc content, do not suggest a huge loss of activity through volatility or that the antibacterial compounds were of a hydrophobic nature. These data therefore suggest that OEO and CEO are very potent against MRSA and *S. aureus*, with EMRSA-16 being significantly more susceptible to both compounds. The CEO and OEO used in the present study contained 74.65% and 74.5% active compounds respectively (see **Section 8.2**). Due to these comparable proportions it is reasonable to suggest that OEO is more inhibitory to MRSA and *S. aureus* than CEO. Diffusion rates of water based suspensions (derived from powders) through agar are likely to differ from those of oils. Furthermore, the garlic and green tea preparations were derived from powders containing 52.53% polyphenols and 0.4% allicin (see **Chapter 3**) respectively. Therefore the anti-Staphylococcal activities of CEO and OEO cannot be compared with those of garlic and green tea powders. Additionally, although small amounts of CEO and OEO caused inhibition zones of similar sizes to some clinically employed antibiotics, it is not possible to draw any conclusions about relative activities for the reasons highlighted above.

Green tea has been shown to cause thickened cell walls and impaired cell separation in MRSA, but not *S. aureus* (Stapleton and Taylor, 2002). Strains of MRSA and *S. aureus* were also shown to have similar MICs of catechin, ECg and 3-*O*-octonoyl-(-)-epicatechin (Compounds from green tea; Stapleton *et al.*, 2004b). It is therefore surprising that in the present study *S. aureus* was significantly more susceptible to green tea than MRSA at high concentrations. Susceptibility testing of more *S. aureus* strains to green tea might highlight whether this result was specific to the strain used in the present study, or whether the antibiotic resistance mechanisms of MRSA facilitate reduced susceptibility to green tea.

The zones of partial inhibition of both MRSA and *S. aureus* caused by garlic were probably due to the bacteriostatic action of garlic. Inhibition zones were examined after 12 h, 24 h and 48 h incubation (data not presented). After 12 h incubation, there were zones of total growth inhibition at all garlic concentrations $\geq$ 1 mg. As shown in **Fig. 8.1**, after 24 h there were clear zones of decreased growth at some of the garlic concentrations. After further incubation (an additional 24 h) there was growth across the inhibition zones at all garlic concentrations. The zones of inhibition caused by CEO and OEO remained clear at all concentrations after 48 h incubation, suggesting a bactericidal action against MRSA and *S. aureus*. There was growth across some of the inhibition zones caused by green tea, suggesting a bacteriostatic action.

8.3.4 – Conclusions

- CEO and OEO exhibit antibacterial activity against MRSA and *S. aureus* – **Hypothesis 1 ACCEPTED**
- MRSA and *S. aureus* were both inhibited by all four antimicrobial compounds but *S. aureus* was significantly more susceptible to green tea than MRSA – **Hypothesis 2 PARTIALLY ACCEPTED**
- Green tea and garlic exerted bacteriostatic effects on MRSA and *S. aureus*, whilst CEO and OEO exerted bactericidal effects

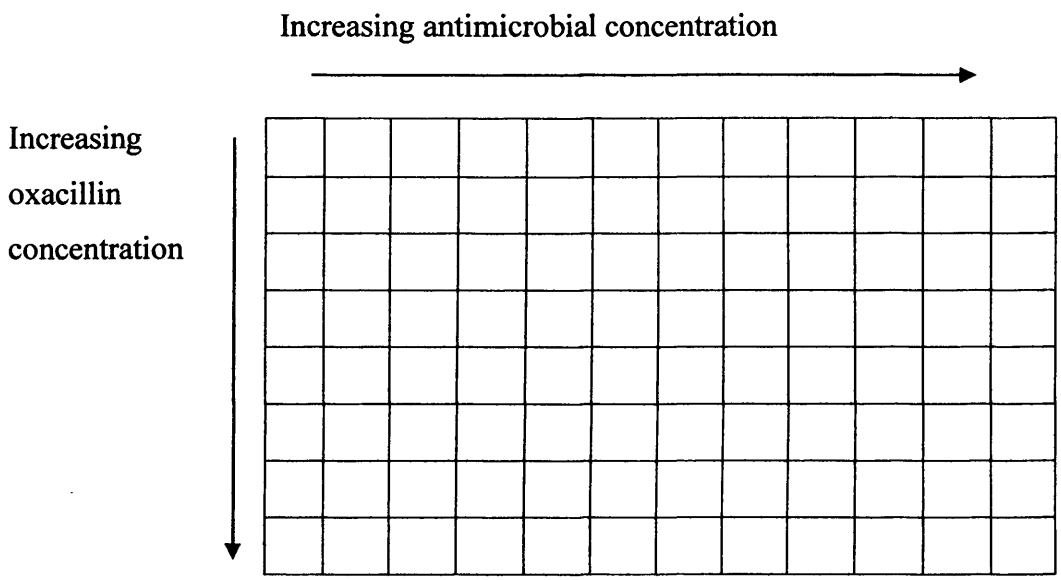
8.4 – The effects of aqueous preparations of cinnamon, oregano, green tea and garlic on the oxacillin susceptibility of MRSA N315

8.4.1 – Materials and methods

Aqueous preparations of garlic, green tea, cinnamon and oregano were prepared from the corresponding powders as stock solutions in sterile MHB and mixed thoroughly for 20 min. Suspensions were centrifuged for 30 min at 8200 x *g* and passed through Whatman no. 3 filters (Whatman, Kent, UK) and then 0.2 μm sterile filters (Millipore, Billerica, MA, USA).

Inocula were prepared as previously described (**Chapter 2.5**) and diluted 1:100 in sterile MHB to create suspensions of approx. 1 x 10^6 CFU mL^{-1}. Inocula were used within 15 min of preparation. Sterile suspensions of cinnamon, oregano, green tea and garlic were diluted to four times required concentrations (plant suspensions were only 50 μL in a final volume of 200 μL, therefore concentrations were higher than required to allow for dilution by oxacillin and the inoculum). Oxacillin stock solutions were prepared as previously described (**Chapter 2.4**) and diluted to four times required concentrations in MHB. The effect of each antimicrobial on oxacillin sensitivity was determined by the chequerboard method (based on **Chapter 2.7.3**) using 96 well Microtitre plates (Sterilin, London, UK). Increasing concentrations of the antimicrobial solutions (50 μL volumes of each) were dispensed into each column (increasing in concentration from left to right; **Fig. 8.3**) and oxacillin solutions (50 μL) into each row (increasing in concentration from top to bottom; **Fig. 8.3**). Optically adjusted inocula were dispensed into each well (100 μL) to create a final well volume of 200 μL, and OD measured at 630nm (Dynamax; ThermoFisher, Leicestershire, UK) at $T_{=0}$. Microtitre plates were incubated for 24 h at 37°C with lids on and sealed in plastic bags with a piece of moistened laboratory paper to maintain humidity. OD was measured at $T_{=24}$, from which the $T_{=0}$ values subtracted. MICs were determined as the lowest concentrations of antimicrobials / oxacillin to inhibit any increase in OD.

Fig. 8.3 – The layout of antimicrobial solutions in chequerboard plates



8.4.2 – Assessment of synergism, additivity or antagonism by determining fractional inhibitory concentrations

FICs and FIC indices (FICi) were calculated from MICs using the following equation:

$$FICi = FIC(Antimicrobial) + FIC(Oxacillin)$$

Where:

$$FIC(Antimicrobial) = \frac{MIC\ of\ Antimicrobial\ in\ Combination}{MIC\ of\ Antimicrobial\ Alone}$$

And:

$$FIC(Oxacillin) = \frac{MIC\ of\ Oxacillin\ in\ Combination}{MIC\ of\ Oxacillin\ Alone}$$

A FIC index of ≤ 0.5 was considered to represent synergism, a value of between >0.5 and ≤ 2 to represent additivity and >2 to represent antagonism (White *et al.*, 1996). See also **Chapter 5.1.4.**

8.4.3 – Results

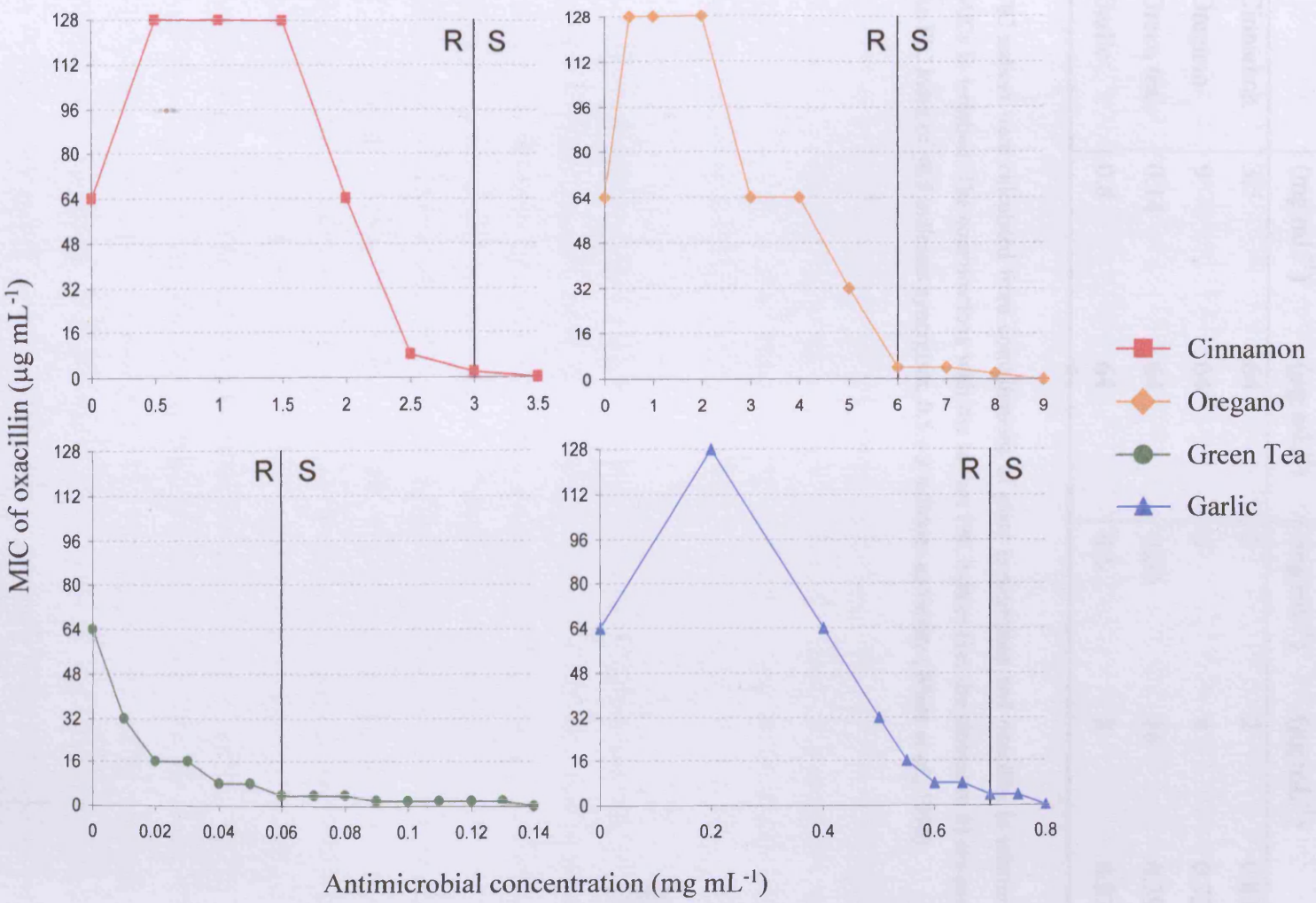
The general effects of aqueous preparations of oregano or cinnamon on the oxacillin sensitivity of MRSA N315 were similar to those of garlic – there was no increase in oxacillin sensitivity until (plant preparation) concentration was greater than 0.5x MIC (**Fig. 8.4**). As the concentration of plant preparation increased above 0.5x MIC, there were dose-dependent increases in oxacillin sensitivity. Interestingly, the lowest concentrations of either cinnamon or oregano caused an increase in the MIC of oxacillin (or a decrease in oxacillin sensitivity), as was also seen with garlic (see also **Chapter 5**). This effect (i.e. an inhibitory compound being beneficial at low concentrations) is termed hormesis (Mattson, 2008; see also **Chapter 5**).

Green tea had an effect completely different from that of garlic, cinnamon or oregano on the oxacillin sensitivity of MRSA N315 (**Fig. 8.4**). Even at the lowest green tea concentration, there was an increase in oxacillin sensitivity, and further increases in sensitivity occurred with increasing green tea concentrations.

All four aqueous plant preparations reduced the MIC values for oxacillin to <4 μg mL^{-1}, i.e. they all induced oxacillin sensitivity (CLSI, 2007) at sub-inhibitory concentrations (**Fig. 8.4**). The concentration of the aqueous suspension required to induce oxacillin sensitivity differed between preparations. Green tea induced oxacillin sensitivity at 0.42x MIC, the other plant antimicrobials induced oxacillin sensitivity at concentrations >0.65x MIC: cinnamon = 0.86x, oregano = 0.67x, garlic = 0.875x.

In general, the relationships between oregano, cinnamon and garlic and oxacillin were additive, whereas the relationship between green tea and oxacillin was synergistic. The lowest (i.e. those closest to 0) FIC indices (and the combinations from which they were derived: Table 8.3) show that only green tea had a synergistic relationship with oxacillin against MRSA N315.

Fig. 8.4 – The effect of four aqueous plant preparations on the oxacillin susceptibility of MRSA N315



Legend indicates aqueous plant preparation. Black line indicates a MIC of oxacillin <4 µg mL^{-1}: at plant concentrations to the right of the black line strain is sensitive (S) to oxacillin; at plant concentrations to the left of the black line strain is resistant (R) to oxacillin

Table 8.3 – The most effective combinations of four aqueous plant preparations and oxacillin against MRSA N315

	MICs		Best combination		
	Antimicrobial ($mg\ mL^{-1}$)	Oxacillin ($\mu g\ mL^{-1}$)	Antimicrobial ($mg\ mL^{-1}$)	Oxacillin ($\mu g\ mL^{-1}$)	FICi
Cinnamon	3.5	64	3	2	0.839
Oregano	9	64	6	4	0.729
Green tea	0.14	64	0.02	16	0.393
Garlic	0.8	64	0.6	8	0.875

FIC indices were calculated from combinations of plant preparations and oxacillin in relation to the MICs in isolation. The combinations with the lowest FIC indices (i.e. the closest to 0) are presented. An FIC index of ≤ 0.5 indicates synergism, 0.5 – 2 indicates additivity (White *et al.*, 1996)

8.4.4 – Discussion

The inhibition zones shown previously (**Fig. 8.1**) suggested that these cinnamon and oregano oils were very potent against MRSA. The percentages of active compounds in the oils were 74.65% (cinnamon oil) and 74.5% (oregano oil). As previously stated (**Section 8.2**), cinnamon powder contained 33% cinnamon oil, and oregano powder contained 25% oregano powder, the oils (in both cases) being emulsified with starch. The relative percentages of active compounds in the powders were therefore 24.6% (cinnamon powder) and 18.63% (oregano powder). Whilst the antimicrobial activities of garlic, green tea, oregano and cinnamon powders cannot be directly compared (due to interactions between compounds, and different powder formulations), the MICs of cinnamon and oregano (3.5 and 9 mg mL^{-1} respectively) seem very low. It is possible that the preparation of aqueous suspensions from these powders resulted in a loss of activity. Despite a possible loss of activity, aqueous suspensions of these two powders still exerted antibacterial effects against MRSA N315. It is likely that even without a loss of activity, the pattern of increasing sensitivity to oxacillin with increasing cinnamon / oregano concentrations would remain the same (all concentrations were made from the same stock, hence any loss of activity was uniform throughout all tested concentrations).

The effect of green tea on the oxacillin susceptibility of MRSA was very different to the effects of the other three plant-derived compounds. There was a synergistic relationship between green tea and oxacillin against MRSA, whereas the relationships between garlic, cinnamon or oregano and oxacillin were additive. Green tea targets peptidoglycan (Zhao *et al.*, 2001) thereby indirectly facilitating the action of oxacillin. It is therefore reasonable to assume that any plant antimicrobial which targets peptidoglycan or cell wall synthesis will directly or indirectly facilitate the action of β-lactam antibiotics. This provides further support for the suggestion that garlic does not damage the integrity of the Staphylococcal cell wall (see **Chapter 3** and **5**). These data also therefore suggest that cinnamon and oregano also do not target the Staphylococcal cell wall. As with garlic, the increase in the oxacillin susceptibility of MRSA with increasing concentrations of oregano or cinnamon, and subsequent induction of oxacillin sensitivity (at sub-MIC concentrations of plant antimicrobial) is probably due to the cumulative inhibitory effects of both compounds. Interestingly, the major antimicrobial compounds in oregano (carvacrol and thymol) and cinnamon

(cinnamaldehyde and eugenol) all contain cyclic six-carbon rings, as do the β-lactam potentiating gallate compounds in green tea; these structures are not found in any garlic compounds.

Low concentrations of cinnamon and oregano powders caused decreased oxacillin sensitivity (i.e. an increased MIC) in MRSA, as garlic was also previously shown to do (**Chapter 5**); so termed 'hormesis' (Mattson, 2008). Aqueous preparations of both cinnamon and oregano contain high concentrations of maltodextrin and starch (emulsifiers of the oils in the powder preparations; see **Section 8.2**). It is feasible that at low concentrations of these preparations (i.e. those at which oxacillin sensitivity is decreased; ≤0.43x MIC of cinnamon and ≤0.22x MIC of oregano) the starch is utilised as an additional energy source, whilst the oil is not at a sufficient concentration to inhibit the organism (conversely, as concentrations of these preparations increase, the starch:oil ratio changes). This would therefore increase the available starch in the growth medium (MHB contains approx. 1.5% (w/v) starch; Oxoid product information). It has been shown that Staphylococci grown in the presence of glucose utilise different metabolic pathways to those grown without glucose; the tri-carboxylic acid (TCA) cycle is virtually absent, and there is an increase in glucose oxidation via the Embden-Meyerhof pathway (Blumenthal, 1972). It is therefore possible that utilisation of the additional starch facilitated the increased tolerance to oxacillin, perhaps through variations in metabolism. This could be determined by assessing the oxacillin sensitivity of MRSA in MHB containing different concentrations of starch or glucose.

8.4.5 – Conclusions

- Cinnamon or oregano, at concentrations >0.5x MIC, decreased the sensitivity of MRSA to oxacillin
- Cinnamon or oregano potentiated oxacillin susceptibility at sub-MIC concentrations – **Hypothesis 3 ACCEPTED**
- Green tea was confirmed as having a synergistic relationship with oxacillin against MRSA, and subsequently the relationships between cinnamon, oregano or garlic and oxacillin were confirmed as additive rather than synergistic
- Low concentrations of cinnamon or oregano caused decreased oxacillin sensitivity as was previously shown with garlic

8.5 – Effect of filter sterilisation on the efficacy of cinnamon, oregano, green tea and garlic powders

8.5.1 – Background

The efficacies of oregano and cinnamon powders using the chequerboard method (**Section 8.4**) was much less than expected considering the inhibition of growth by low concentrations of the oils using the disc diffusion method (**Section 8.3**). The powders were created by emulsification of the corresponding oils with starch (see **Section 8.2**). One suggestion was that the centrifugation and subsequent filter sterilisation used to prepare sterile aqueous suspensions from the powders had resulted in a loss of some antimicrobial activity. This suggestion was tested by comparing the efficacies of filter sterilised and non-sterile aqueous preparations (from powders) using a well diffusion method. Sterile and non-sterile preparations of garlic and green tea were also compared in the same manner; the efficacy of these preparations in **Section 8.4** was as expected considering the results obtained in **Section 8.3** (garlic and green tea powders were made by freeze-drying and milling whole plants or parts of plants).

8.5.2 – Materials and Methods

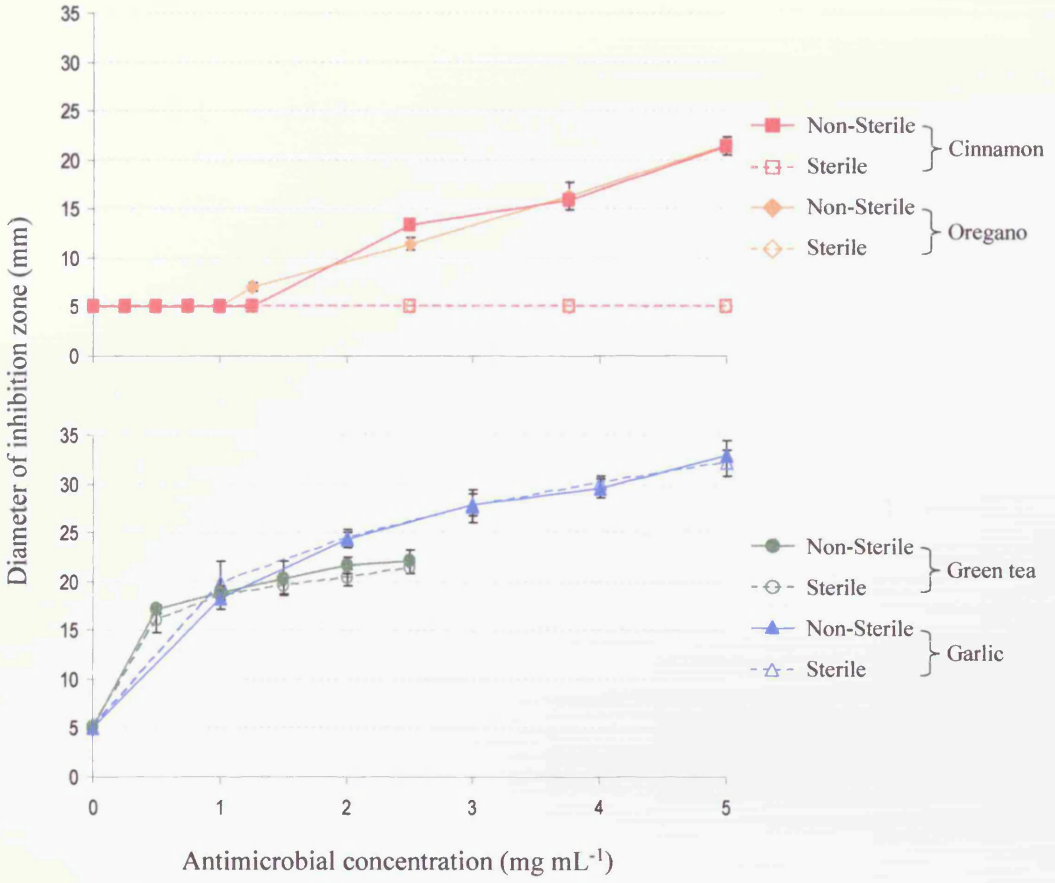
Stock solutions of non-sterile aqueous plant preparations were created by suspending the corresponding powders in sterile distilled water and mixing thoroughly for 20 min. To create sterile preparations, half of each solution was centrifuged (8,200 x *g*, 20 min) and the resulting supernatant passed through Whatman (Whatman, Kent, UK) no. 3 filters and then 0.2 μm sterile filters (Millipore, Billerica, MA, USA).

MHSC plates were swabbed (sterile cotton swabs; Eurolabs, Manchester, UK) with inocula (prepared as previously described; **Chapter 2.5**) of MRSA N315 (to create semi-confluent bacterial lawns after 24 h incubation at 37°C) as described above (**Section 8.3.1**). Wells (5 mm diameter) were removed from the centre of each plate using a flame sterilised cork-borer. Aqueous antimicrobial preparations, both sterile and non-sterile, were diluted to required concentrations with sterile distilled water and 50 μl volumes dispensed into each well. Inhibition zones were measured from digital images (using IMAGEJ, NIH) taken after 24 h incubation at 37°C.

8.5.3 – Results

Filter sterilisation had no effect on the efficacy of aqueous preparations of either garlic or green tea (**Fig. 8.5**), but it resulted in a substantial loss of the antibacterial activities of both cinnamon and oregano.

Fig. 8.5 – The effect of centrifugation and filter sterilisation (0.2 µm pore size) on the efficacy of four aqueous preparations from plant-derived antimicrobial containing powders



Data are presented as the means of at least four independent replicates. Bars indicate standard error of the mean. Legend indicates powder and preparation method

Interestingly, the inhibition zones caused by garlic using two different methods, but the same amount of antimicrobial, resulted in slightly different inhibition zone sizes. There was a partial inhibition zone (11.64 mm) with a disc content of 1 mg (**Fig. 8.1**), but the same content of garlic using a well diffusion method resulted in a zone of complete growth inhibition (18.32 mm; **Fig. 8.5**). This discrepancy is possibly due to the increased errors in preparing a garlic solution of such high concentration for the disc method (a large amount of powder was re-suspended in a relatively small volume of liquid) and / or the filter paper (used in **Section 8.3**) to some extent acted as a barrier against diffusion through the agar.

8.5.4 – Discussion

The data presented confirm the suggestion that centrifugation and filter sterilisation of aqueous preparations of cinnamon and oregano oil resulted in a large loss of activity. These data also confirm that the antimicrobial efficacies of cinnamon and oregano exhibited in **Section 8.4** were less than expected considering the efficacy of the oils in **Section 8.3**. Filter sterilisation of green tea and garlic extracts resulted in no loss of activity, suggesting the antimicrobial constituents of these compounds were completely water soluble.

It is assumed that the majority of the antimicrobial activity of cinnamon and oregano powders was retained in the pellet after centrifugation, possibly bound by the starch with which it was emulsified. Therefore, powders prepared by emulsification of essential oils with starch and subsequent freeze-drying should not be re-hydrated for determination of antimicrobial activity using the aqueous preparation method outlined previously (**Section 8.4**).

Non-sterile, optically dense, aqueous preparations are adequate for a well diffusion technique, but are not suitable for a broth method using turbidity as a measure of growth. Chequerboard experiments are ideal for determining the effects of plant preparations on antibiotic sensitivity because of the high number of concentrations that can be tested simultaneously. Chequerboard assays, however, require sterile, transparent solutions to allow changes in OD to be used as an indicator of growth. Chequerboard assays could be conducted using opaque, but sterile solutions, with viable cell counts taken from each well to determine MICs. This would, however, be very labour intensive, thus negating the benefits of the high throughput capacity of chequerboard assays. Alternatively, a pre-chequerboard screening assay could be used, with fewer concentrations, to narrow down the range of concentrations to be tested. Such a screening assay could be conducted using a "sloppy-agar" method; liquid growth medium containing a very low concentration of agar, which holds the oil in suspension but does not restrict the growth of microorganisms. To measure MICs in "sloppy-agar" assays the redox indicator rezazurin is added to each well after incubation, and the resulting colour change from deep purple (oxidised form) to light pink (reduced form) indicates respiration and therefore growth. Again, this technique is labour intensive and difficult to set up; the "sloppy-agar" must be kept at >37°C to

prevent the agar from solidifying. Tea tree (*Melaleuca alternifolia*) oil can be solubilised in Tween 80 (Mondello *et al.*, 2009) at a concentration of 0.25% v/v (Brady *et al.*, 2006). It is therefore possible that cinnamon or oregano oils could be solubilised in such a way, allowing the efficacies of the oils to be tested directly using an adapted chequerboard method as outlined previously (**Chapter 2.7.3**).

These results also suggest that well diffusion assays are more suitable for determination of the antimicrobial activity of garlic than disc diffusion assays. The required garlic concentrations are much lower, and there are no additional barriers against diffusion of the antimicrobials through the agar.

8.6 – Overall conclusions

- MRSA and *S. aureus* are susceptible to preparations of cinnamon, oregano, green tea or garlic
- S. *aureus* is more susceptible to green tea than MRSA, suggesting that the two β-lactam resistance mechanisms also provide protection against the antimicrobial compounds in these plant preparations
- Green tea is confirmed as having a synergistic relationship with oxacillin against MRSA. The relationships between increasing cinnamon, oregano or garlic concentration and oxacillin sensitivity of MRSA are additive
- Cinnamon, oregano, garlic or green tea potentiate oxacillin susceptibility in MRSA at sub-MIC concentrations
- Centrifugation and filter sterilisation of powders comprised of essential oils emulsified with starch results in a substantial loss of activity

9 – General Discussion and Further Work

9.1 – MRSA and *S. aureus* are susceptible to garlic

In this Thesis it was confirmed that garlic, administered as a standardised freeze-dried preparation had antibacterial properties against both MRSA and *S. aureus*. The resistance mechanisms of MRSA (β-lactamase enzymes and PBP2a) had no impact on garlic susceptibility. Similarly, MRSA strains expressing resistance to macrolides and quinolones were no less susceptible to garlic than strains sensitive to those antibiotics. This implies that either garlic has different mechanisms of action to β-lactams, macrolides and quinolones, or that resistance mechanisms to those antibiotics do not inhibit the action of garlic.

9.2 – Garlic does not damage the integrity of the Staphylococcal cell wall

In general, garlic had little effect on the growth dynamics of MRSA and *S. aureus* (except at low concentrations, which is considered later), but caused proportional prolongation of their lag phases. This suggests, as previously, that one of the targets of garlic is the Staphylococcal cell wall. Cells treated with low concentrations of garlic (0.25x MIC), or those treated with garlic over a long time period, showed thickening of the primary cell wall, lending support to the suggestion of the cell wall as a target. However, there are several findings which in combination suggest that garlic does not affect the integrity of the Staphylococcal cell wall:

1. Treatment of MRSA with garlic up to MIC concentrations had no effect on NaCl tolerance
2. β-lactam antibiotics specifically target peptidoglycan. It would therefore be reasonable to assume that β-lactam resistance mechanisms would result in increased tolerance to garlic
3. Green tea specifically targets the Staphylococcal cell wall and is synergistic with oxacillin against MRSA. The interaction between garlic and oxacillin was very different from that of green tea and oxacillin
4. *S. aureus* repeatedly sub-cultured in sub-MIC concentrations of oxacillin exhibited a thickened cell wall, but the sensitivity to garlic did not change

Taking all these findings into consideration it appears likely that the integrity of the Staphylococcal cell wall is not damaged by garlic. However, this does not rule out the cell wall as a target of garlic. For example, MRSA in high concentrations of β-lactam antibiotics grows using just one PBP (PBP2a) and as a result exhibits atypical, but functional and osmotically tolerant cell walls.

9.3 – Does garlic affect Staphylococcal RNA synthesis?

S. aureus treated with low concentrations of garlic exhibited electron sparse areas of cytoplasm, which resembled those induced by magnesium limitation (Sykes and Tempest, 1965). In the magnesium-limited cells, there was decreased ribosomal occupancy, which accounted for the electron sparse regions of cytoplasm. Garlic-dependent inhibition of RNA synthesis and specifically inhibition of RNA polymerase have been reported (Feldberg *et al.*, 1988; Ankri and Mirelman, 1999; Choi *et al.*, 2007). The inhibition of *E. coli* RNA polymerase was thought to be as a result of the interaction between allicin and thiol groups (Ankri and Mirelman, 1999). A decrease in ribosome occupancy is certainly one suggestion for the electron sparse areas observed here (Sykes and Tempest, 1965), and is supported by the associated decrease in growth rates. However, there are very few data presented here, and much more detailed structural and functional investigations, particularly targeted towards the effects of garlic on Staphylococcal ribosome activity, would be required to make this suggestion more than just circumstantial. Feldberg *et al.*, (1988) reported an immediate inhibition of RNA synthesis in *Salmonella typhimurium* by allicin using pulse-labelling experiments. It is suggested that a similar method could be employed to determine whether garlic has any effect on protein, DNA or RNA synthesis in *S. aureus*.

9.4 – Garlic enhances the activity of oxacillin and penicillin against MRSA and *S. aureus*

Garlic, at concentrations >0.5x MIC caused large increases in the oxacillin or penicillin susceptibility of MRSA and *S. aureus*. Although the FICi values were greater than for some plant-derived constituent-antibiotic interactions (see **Chapter 5.1.2**), and were quantified as additive rather than synergistic, these results suggest that possible interactions between garlic compounds and antibiotics warrant further investigation. Although purification of garlic compounds could result in an increased

likelihood of resistance development (discussed later), and removes any possible enhancement of activity by interaction between compounds, it is certainly an area of research that deserves consideration.

9.5 – EMRSA-16 exhibited different responses to garlic and oxacillin compared with other strains

In general both MRSA and *S. aureus* exhibited the same responses to garlic. As previously mentioned, antibiotic resistance mechanisms appeared to have no effect on garlic sensitivity. EMRSA-16, however, exhibited different responses to both garlic and oxacillin. Whilst the slightly increased tolerance of EMRSA-16 to garlic compared with other strains was negligible, and the effect of garlic on growth dynamics was no different to other strains, different overall responses were reported on several occasions. It appears that the enhanced activity of oxacillin on growth limitation of EMRSA-16 (**Fig. 4.2**) is the primary cause of the synergistic relationship reported between garlic and oxacillin against this strain. However, EMRSA-16 was less susceptible to the bactericidal effects of garlic than other strains, both in MHB at 37°C and in PBS at 20°C. There are no similar reports in the research literature of atypical responses of this strain; in fact, EMRSA-16 is one of the most frequently isolated MRSA strains in UK hospitals (Moore and Lindsay, 2002). It is reported that EMRSA-16 is adapted to success in the hospital environment by an unknown basis (Moore and Lindsay, 2002); this might account for some of the different responses reported here.

9.6 – Low concentrations of garlic decrease the oxacillin susceptibility of MRSA and *S. aureus*

This is the first study to report hormesis in garlic. It appears that whilst low concentrations of garlic are not inhibitory to the organism, they are certainly not beneficial (decreased growth rates, thickening of the primary cell wall). However, under the selective pressure of oxacillin treatment with low concentrations of garlic, perhaps the associated morphological and physiological alterations reported here provide an advantage to those organisms. Indeed the available data suggest that decreased growth rates, and thickened primary cell walls facilitated these increases in oxacillin tolerance, but other factors are also likely. These results therefore suggest that the effects of garlic on MRSA and *S. aureus* are more complex than previously

suggested (Cottrell, 2003). Further investigation into the effects of low concentrations of garlic on decreasing MICs of other antibiotics is warranted. Furthermore, determination of the mechanism(s) of action of garlic is imperative in investigating this response further. These findings also have potential ramifications for other plant-derived constituents-antibiotic interaction studies. Currently, many investigations determine the MIC of the phytochemical and then assess antibiotic susceptibility only at one or two sub-inhibitory concentrations of the plant compounds. The data presented here suggest that further investigation should be directed towards to the effects of low concentrations of plant compounds on antibiotic sensitivity. Preliminary screens using smaller antibiotic increments at low phytochemical concentrations are advisable.

9.7 – Sensitivity of *S. aureus* to garlic can be decreased *in vitro*

This is also the first study to report decreasing sensitivity of a bacterial species to garlic through repeated exposure. Whilst the MIC of treated organisms remained much lower than those "less susceptible" bacteria, i.e. LAB, there were approx. five-fold increases in the MIC. These data warrant further investigation into the effect of repeated exposure of bacteria to sub-inhibitory concentrations of garlic. Primarily, a repeat of the presented experiment, over a longer time period is advised, to determine whether the MICs reported here represent a "threshold" of inducible decreased sensitivity. Furthermore, investigation into the resulting "G-RI" strains is advised, particularly regarding any variation in antibiotic sensitivity, sensitivity to specific garlic compounds, growth dynamics, and biochemistry; perhaps the use of techniques such as global transcriptome expression using DNA micro-arrays would provide clues as to the cause of the decreased sensitivity, and may help with elucidation of the mechanisms of action of garlic. Investigation into decreasing susceptibility to individual garlic compounds is also warranted.

9.8 – Cinnamon and oregano oils potentiate oxacillin susceptibility in MRSA

Cinnamon oil and oregano oil were shown to potentiate oxacillin susceptibility in MRSA. These results provide further evidence that research into the effects of plant antimicrobials on the enhancement of traditional antibiotic therapy is justified. These results also highlighted problems in preparing aqueous solutions from powders comprising starch emulsified essential oils. It is advisable to investigate the solubility of these essential oils in Tween 80 (Brady *et al.*, 2006; Mondello *et al.*, 2009) or DMSO, and the efficacy of the resulting solutions.

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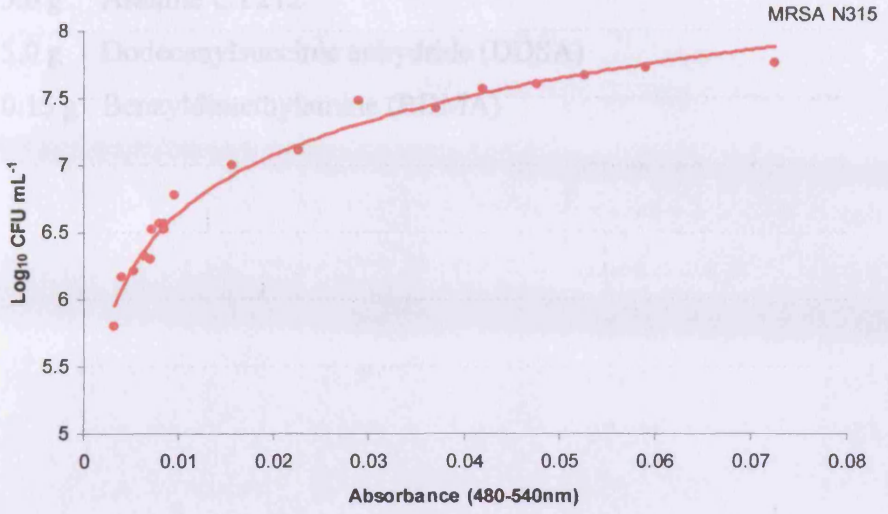
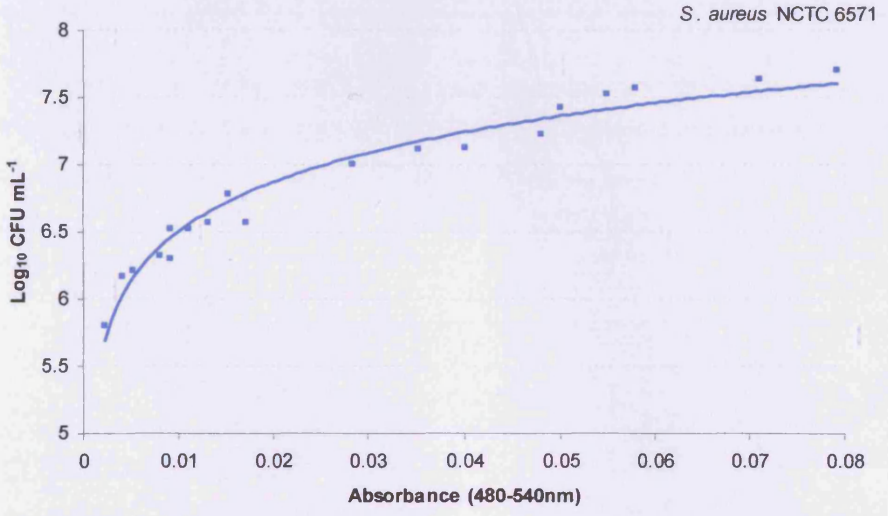
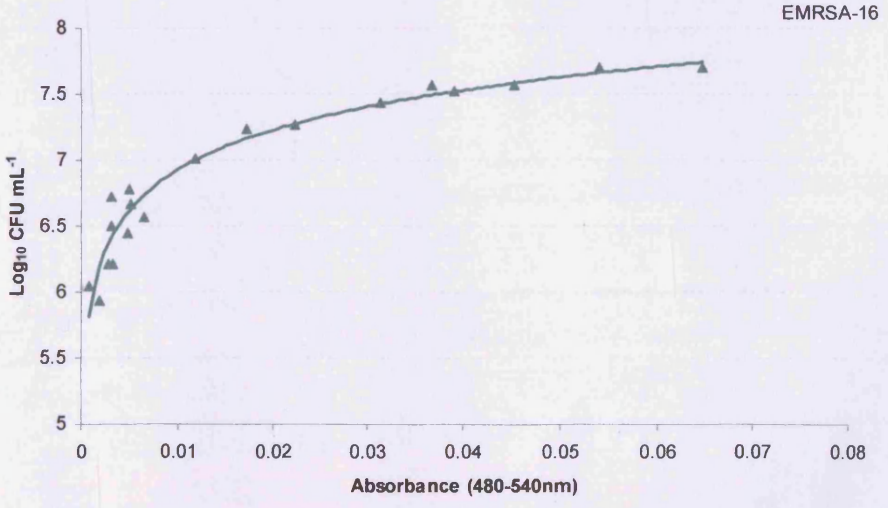
Chapter 1 – Calibration curves relating changes in optical density with viable counts of three *S. aureus* strains grown in MHB







Chapter 2 – Preparation of araldite for transmission electron microscopy

5.0 g Araldite CY212

5.0 g Dodecenylsuccinic anhydride (DDSA)

0.15 g Benzyldimethylamine (BDMA)

Chapter 3 – Summary of the effects of low garlic concentrations on the "growth rates" of MRSA and *S. aureus*

	Garlic concentration (% of relative MIC)		
Strain	0%	25%	50%
MRSA N315	0.095	0.054	0.066
	(100%)	(57.2%)	(69.3%)
Five MRSA strains	0.077 – 0.099	0.048 – 0.058	0.054 – 0.075
	(100%)	(54.3% - 66.1%)	(59.3% - 81.9%)
NCTC 6571	0.077	0.040	0.073
	(100%)	(52.3%)	(94.6%)
Two *S. aureus* strains	0.077 – 0.098	0.04 – 0.05	0.073 – 0.093
	(100%)	(51.1% - 52.3%)	(94.6% - 94.9%)

"Growth rates" expressed as $\Delta OD\ h^{-1}$. Values in parentheses represent % of control "growth rates". Data for MRSA N315 and *S. aureus* NCTC 6571 are presented in **Chapter 3**. Data presented are the maximum and minimum values for MRSA and *S. aureus*, not mean values.

Chapter 4 – Expression of constitutive (C) or inducible (I) β-lactamase expression

Strain	MHSC agar		MHSC agar with 6 μg mL^{-1} oxacillin		Expression?
	5 min	60 min	5 min	60 min	
EMRSA-15			✓	✓	I
EMRSA-16	✓	✓	✓	✓	C
MRSA N315			✓	✓	I
MRSA JW4	✓	✓	✓	✓	C
MRSA JW6			✓	✓	I
MRSA 4500			✓	✓	I
NCTC 6571					None
RN450					None

Enzyme expression detected using Nitrocefin sticks (Oxoid, Hampshire, UK). Colour change determined after 5 and 60 min (as per manufacturer's instructions). Constitutive expression (C) determined from growth on agar MHSC plates, inducible expression (I) determined from growth on MHSC agar plates containing 6 μg mL^{-1} oxacillin

Chapter 5 – FICi of garlic and oxacillin (or penicillin) against of several strains of MRSA and *S. aureus*

FICi of garlic and oxacillin – Pre-incubation with garlic on oxacillin susceptibility

Strain	Garlic conc. (mg mL^{-1})	Oxacillin conc. (μg mL^{-1})	FICi
EMRSA-15	1.25	0	1
EMRSA-16	0.5	2	0.256
MRSA N315	1.2	2	0.817
MRSA JW4	1	2	0.671
MRSA JW6	0.75	2	0.766
MRSA 4500	1.5	0	1
NCTC 6571	1.5	0	1
RN450	1.5	0	1

FICi of garlic and oxacillin – Chequerboard method

Strain	Garlic conc. (mg mL^{-1})	Oxacillin conc. (μg mL^{-1})	FICi
EMRSA-15	0.8	0	1
EMRSA-16	0.6	2	0.781
MRSA N315	0.6	8	0.875
MRSA JW4	0.7	16	0.938
MRSA JW6	0.6	2	0.781
MRSA 4500	0.3	8	0.85
NCTC 6571	0.4	0.06	0.98
RN450	0.4	0.06	0.98

FICi of garlic and penicillin – Chequerboard method

Strain	Garlic conc. (mg mL^{-1})	Penicillin conc. (μg mL^{-1})	FICi
EMRSA-15	0.8	0	1
EMRSA-16	0.8	0	1
MRSA N315	0.2	32	0.75
NCTC 6571	0.2	0.025	0.75

Chapter 5 – Concentrations of garlic required to induce sensitivity to oxacillin (or penicillin) in several strains of MRSA and *S. aureus*

Induction of oxacillin sensitivity by treatment with garlic – Pre-incubation with garlic on oxacillin susceptibility

Strain	MIC of garlic (mg mL^{-1})	Garlic concentration to induce oxacillin sensitivity	% of garlic MIC to induce oxacillin sensitivity
EMRSA-15	1.25	1.25	100
EMRSA-16	2	0.5	25
MRSA N315	1.5	1.2	80
MRSA JW4	1.5	1	66.67
MRSA JW6	1	0.75	75
MRSA 4500	1.5	1.5	100

Induction of oxacillin sensitivity by treatment with garlic – Chequerboard method

Strain	MIC of garlic (mg mL^{-1})	Garlic concentration to induce oxacillin sensitivity	% of garlic MIC to induce oxacillin sensitivity
EMRSA-15	0.8	0.8	100
EMRSA-16	0.8	0.6	75
MRSA N315	0.8	0.7	87.5
MRSA JW4	0.8	0.75	93.75
MRSA JW6	0.8	0.6	75
MRSA 4500	0.8	0.6	75

Induction of penicillin sensitivity by treatment with garlic – Chequerboard method

Strain	MIC of garlic (mg mL^{-1})	Garlic concentration to induce penicillin sensitivity	% of garlic MIC to induce penicillin sensitivity
EMRSA-15	0.8	0.6	75
EMRSA-16	0.8	0.8	100
MRSA N315	0.8	0.8	100

Chapter 8 – Statistical analysis

Significant differences between the susceptibility of MRSA and *S. aureus* to cinnamon essential oil ($p<0.005$)

	Disc content (μL)				
E16: NCTC 6571	2	3	4	5	6
Calculated T value	6.5984	8.85474	6.1743	8.28282	5.15359
Critical T value	3.196574	3.196574	3.196574	3.196574	3.196574
P value	6.09×10^{-5}	4.79×10^{-5}	0.000105	8.64×10^{-6}	0.000429

Significant differences between the susceptibility of MRSA and *S. aureus* to oregano essential oil ($p<0.005$)

	Disc content (μL)
NCTC 6571:E16	0.5
Calculated T value	4.18315
Critical T value	3.196574
P value	0.00092

	Disc content (μL)				
E16: NCTC 6571	2	3	4	5	6
Calculated T value	7.65102	9.35437	14.5899	7.2465	7.4257
Critical T value	3.196574	3.196574	3.196574	3.196574	3.196574
P value	1.74×10^{-5}	2.92×10^{-6}	4.56×10^{-8}	2.77×10^{-5}	2.25×10^{-5}

Significant differences between the susceptibility of MRSA and *S. aureus* to green tea powder ($p<0.005$)

	Disc content (mg)		
N315: NCTC 6571	0.15	0.175	0.2
Calculated T value	4.194965	4.915674	4.100501
Critical T value	3.196574	3.196574	3.196574
P value	0.001843	0.000609	0.002143