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This thesis is dedicated to my friend James,
who taught me about courage.

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Summary

This study set out to compare and contrast the antimicrobial effects of freeze-dried garlic powder against the commonly occurring pathogens specifically *Escherichia coli*, and the probiotic bacterium *Lactobacillus casei*. Also to identify possible active components and sites of action of garlic, and study the reactive nature of allicin.

Freeze dried garlic proved more inhibitory to *E. coli* than *L. casei* (24 h MIC values of 1.9 mg ml^{-1} and 16.9 mg ml^{-1} respectively) This was manifested against *E. coli* as a dose-dependent extension in lag phase (no effect on specific growth rate (SGR)). Inhibitory effects of garlic powder extract against *L. casei* were manifested as a dose-dependent decrease in specific growth rate. No bacteriocidal activity was observed on treatment of *E. coli* cultures with garlic concentrations of 20 mg ml^{-1} or less over 24 hours. Bacteriocidal activity was seen against *E. coli* when using concentrations in excess of 20 mg ml^{-1} over 48 h and 72 h. Garlic powder extract proved inhibitory to MRSA strains (EMRSA 15), and displayed synergism with penicillin and methicillin.

GC-MS analysis proved garlic powder extract to be a dynamic mixture of sulphur containing compounds, including allicin and allicin reaction products. Half-life of allicin in nutrient broth (37°C) was approximately 11 h, this was reduced to 30 min on addition of *E. coli* cells.

The inhibitory effect of allicin was observed as a dose-dependent increase in lag phase (no effect on SGR). Inhibitory activity of diallyl sulphides was observed as a dose-dependent decrease in SGR and small extensions in lag phase duration. Allyl alcohol brought about a substantial decrease in SGR and culture density at 24 h (concentration independent).

Garlic powder extract caused membrane damage and abnormal morphology in *E. coli* (<2.0 mg ml^{-1}) and *L. casei* (>10 mg ml^{-1}). A reduction in glucose metabolism was observed in *E. coli* and *L. casei* on addition of garlic, also ethanol production and oxygen uptake were stimulated in *E. coli* cultures.

This investigation has highlighted differences in the nature of garlic powder extract inhibition in a susceptible and non-susceptible organism, and identified possible mechanisms of action against *E. coli* to be cell structure damage, oxidative damage, and metabolic inhibition.

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Suppliers

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Abbreviations used

AA	**Allyl alcohol**
AM	**Allyl mercaptan**
AS	**Allyl sulphide**
AMD	**Allyl methyl disulphide**
AMS	**Allyl methyl sulphide**
AMT	**Allyl methyl trisulphide**
DAD	**Diallyl disulphide**
DAP	**Diallyl pentasulphide**
DAS	**Diallyl sulphide**
DATr	**Diallyl trisulphide**
DATe	**Diallyl tetrasulphide**
DCM	**Dichloromethane**
DMD	**Dimethyl disulphide**
DMS	**Dimethyl sulphide**
DMT	**Dimethyl trisulphide**
DPD	**Dipropyl disulphide**
DPS	**Dipropyl sulphide**
DPT	**Dipropyl trisulphide**
Ery	**Erythromycin**
FIC	**Fractional Inhibitory Concentration**
FICi	**Fractional Inhibitory Concentration Index**
GC	**Gas chromatography**
GCMS	**Gas chromatography mass spectrometry**
HAI	**Hospital acquired infection**
HPLC	**High performance liquid chromatography**
IC50	**Inhibitory concentration 50%**
LAB	**Lactic acid bacteria**
Lzd	**Linezolid**

Met	**Methicillin**
MIC	**Minimum Inhibitory Concentration**
MPD	**Methyl propyl disulphide**
MPS	**Methyl propyl sulphide**
MPT	**Methyl propyl trisulphide**
MRSA	**Methicillin resistant *Staphylococcus aureus***
MSSA	**Methicillin sensitive *Staphylococcus aureus***
Ox	**Oxacillin**
Pen	**Penicillin**
Van	**Vancomycin**
2-VD	**2-vinyl dithiin**
3-VD	**3-vinyl dithiin**
VRE	**Vancomycin resistant *Enterococcus***

Chapter 1.

General introduction.

Chapter 1 General Introduction.

1.1.1 *Allium* species

The genus *Allium* contains over 600 species (Wendelbo 1971), which are widespread throughout the world. Members of the *Allium* genus are usually exclusively herbaceous, perennial, and form bulbs. Almost all *Allium* species are odorous (on flowering, or damage to the bulb). Flowers of this genus tend to be spherical umbels with small blossoms (with long pedicels), subtended by broad flat leaves. Most *Allium* species are wild and many are found growing (though naturalised rather than native) throughout the United Kingdom:

- *Allium ursinum* known as 'wild garlic' or 'ramsons', is commonly found growing in shaded forests and river banks.
- *Allium ampeloprasum* known as 'wild leek' is found growing in hedgerows.
- *Allium triquetrum* known as the 'three-cornered leek' is often seen growing near the edges of forests and woodland.
- *Allium carinatum* known as 'keeled garlic' is sometimes found growing in meadows and shaded woodland.
- *Allium roseum* or 'rose garlic' can be found in warmer parts of the United Kingdom growing on dry exposed grasslands.

Although widespread, the majority of *Allium* species are of insignificant agricultural and economic importance. Most of the commonly cultivated *Allium* species originated in Asia. Currently the *Allium* species of greatest economic importance (hence the most widely cultivated) are varieties of *Allium cepa* (onions) and *Allium sativum* (garlic).

Garlic and onions have gained overwhelming economic importance due to their culinary use. Many other *Allium* species are edible, and are used to flavour food (including chives, and spring onions). However there have been isolated reports of livestock poisoning ascribed to excessive consumption of the edible species: *Allium*

cepa – the common onion (Verhoeff 1985), and *Allium validum* – pacific or swamp onion (Van Kampen 1970).

1.1.2 *Allium sativum* – General description

Allium sativum (garlic) is a medium height (30 – 60 cm) erect, perennial herb. The below ground portion of the plant (termed the bulb) consists of usually between 4 and 20 cloves individually wrapped in a leaf skin (Figure 1.1.2), which varies in colour from white to light purple. The cloves are held together to form the bulb by a white parchment-like skin.

Each clove is capable of producing a round hollow stalk encased in tubular leaves. The flower cluster develops at the top of this stalk during the late summer. Propagation occurs by vegetative means since the flowers are usually sterile. Each flower develops into bulbils (approx 1 cm in diameter), which are dispersed by the plant. Each bulbil has the potential to develop into a complete garlic plant.

1.1.3 Historical use and cultivation of garlic

Throughout history garlic has found use as a food flavouring, a preservative, and a medicine. It is mentioned in religious writings of Islam, Judaism, Brahmanism, and Christianity

The earliest written accounts of the cultivation and use of garlic as a medicine and spice are from the Sumerians texts dated 2600 – 2100 BC (Koch *et al* 1996). Since these accounts state that garlic was already cultivated at this time, it is likely that it found use even before these dates. Garlic was also held in high regard by ancient Egyptians (2100 BC), pyramid builders were provided with garlic as part of their daily diet, Egyptians also used garlic to alleviate symptoms of gum infections and abscesses (Saynor 1995). Botany and the medicinal uses of plants was highly developed in Egypt for this time, the *codex papyrus ebers* (an ancient Egyptian document found in 1872) contains over 800 therapeutic drug formulations – 22 of these contained garlic. Garlic also found popular use amongst the Israelites residing in Egypt at this time, and is recorded in the Talmud as a food and a medicine.

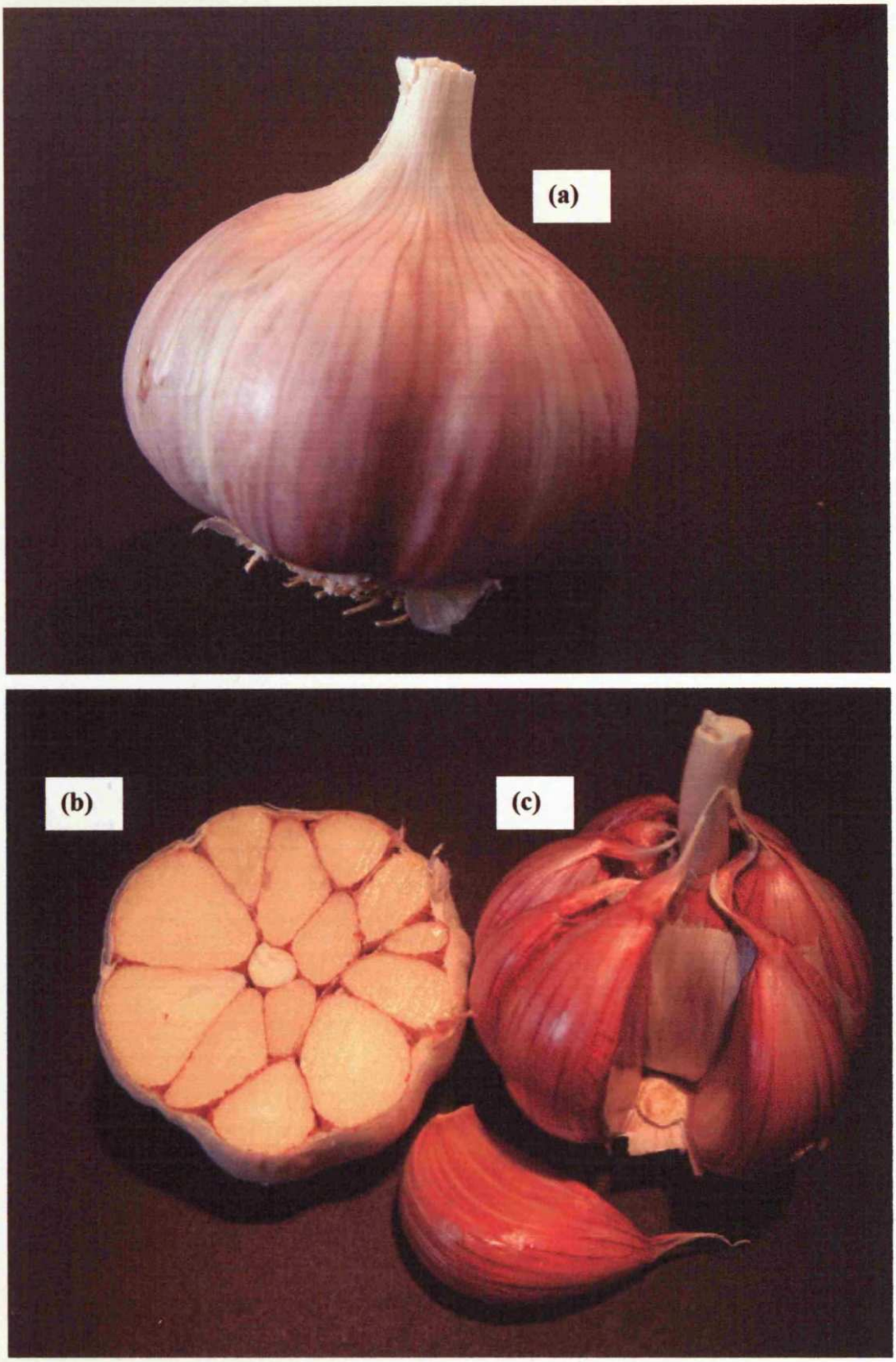


Figure 1.1.2*Allium sativum* (garlic). (a) Intact bulb with parchment-like skin, (b) longitudinal section through bulb, (c) pealed bulb with separated clove.

In ancient China and India garlic found use as a food flavouring, medicine, and preservative (Jacora 1979). It is still used in these countries today for similar reasons.

By 900 BC garlic was finding medicinal use in Ancient Greece (Baumann 1982), Homer eluded to its medicinal use in the *Odyssey* describing a garlic-like plant (given by Hermes) protecting Odysseus from transformation into a pig by Circe.
Later (approximately 400 BC) students of *Hippocrates* (father of modern medicine), described the use of garlic to treat constipation, pneumonia, and wound putrefaction.

Garlic was also finding use in ancient India and Russia, since contact between these areas with Greece and China was not thought to regularly occur, it is likely that these areas discovered the benefits of medicinal garlic independently (Barowsky *et al* 1944).

Romans learnt of the benefits of garlic from the Greeks (Fraas 1845). The naturalist Pliny the Elder believed garlic could draw away evil forces and recommended it's use to treat snake bites, dog bites, scorpion stings, gastrointestinal disorders, and madness (Harris 2001). Garlic found popular use with slaves and legionaries, as a vermifuge, and to prevent wound infection.

Use of garlic in northern European countries was not as widespread. The Vikings held it in High regard, carrying it with them on sea voyages (Bolton 1982), and garlic was brought to the Germans, Celts, and Anglo-Saxons by the Romans. But use of garlic did not become popular throughout Europe until the middle ages.

European Benedictine monks (around 768 AD) believed garlic to offer protection from the plague, and cultivated it extensively in their monasteries. The curative properties of garlic were extolled by the medical school of Salerno (a highly respected medieval Italian medical school), and St Hildegard of Bingen (1098 – 1179) advocated its use to treat jaundice (Koch 1996). The beneficial properties of garlic became strongly intertwined with the folklore of many central and eastern

European countries, often being hung over windows and doors to guard against vampires and witches.

By the 17th Century, garlic was extremely popular in Spain and France, both medicinally and as a flavouring. Spaniards believed that garlic could be used to treat: ailing horses, intestinal pain, rabies and parasitic worms (Harris, L. 1996). The conquistadores even transported garlic with them to the New World (South America).

In France garlic was used as an antiseptic for animal bites, and was also given to young infants to prevent illness. In 1721 a group of four criminals who collected bodies plague victims in Marseilles, never themselves became infected. A macerate of red wine and garlic (consumed daily by the robbers) was credited as the preventative. This famous anti-infective measure (*vinaigre des quatre voleurs*) immediately gained popularity and its use spread throughout France (Hann 1996).

Since the turn of the 20th Century, garlic appears to have gained medicinal use (especially to prevent and treat infectious disease) in civilisation worldwide. Albert Schweitzer successfully used garlic to treat amoebic colitis in Africa (Reuter *et al* 1996). In Irish hospitals (early 1900s) garlic was used to combat pulmonary infections which were rampant (Delaha *et al* 1985). During World War I garlic was used to alleviate intestinal infection and gangrenous wounds. In Asia garlic was used to combat cholera, dysentery and enteritis (Barowsky *et al* 1944).

The discovery of penicillin in 1928 by Flemming, and subsequent development of the drug by Florey, Chain, and Heatly (1935), heralded the start of the modern antibiotic era. It also brought about a decline in the usage of garlic to prevent and treat infection in many Western countries. However garlic retained much of its popularity (due to many other associated health benefits) it is still prescribed in traditional Chinese medicine, and still finds use worldwide as a food and food preservative.

1.2. The compositional chemistry of garlic

1.2.1 Background

As people became aware that garlic could be of significant medicinal benefit, interest into its compositional chemistry grew and the search for the active principals of garlic began.

Investigations into garlic chemistry are centred around its sulphur compounds (initially probably because of their odorous qualities). Garlic has an unusually high sulphur content (Table 1.2.1), accounting for approximately 0.3% of the its fresh weight (in most other vegetables, including onions, sulphur accounts for less than 0.1% fresh weight (Nielson *et al* 1991)). Many of the sulphur compounds from garlic are reactive in nature, and discovery (along with structural elucidation) of the active principles of garlic occurred in reverse chronological order of the pathways in which they occur in garlic.

1.2.2 Discovery of the diallyl sulphides

In 1844 Wertheim carried out steam distillations of crushed garlic. The essential oils he collected were pungent, and yellowish in colour. He determined these compounds contained in the oil to be organo-sulphur compounds of the formula C_6H_5S, and named the hydrocarbon grouping the 'allyl' group (after the plant from which it was extracted).

In 1892 Semmler carried out fractional distillations on garlic oil (obtained by steam distillation). He quantified each of the fractions of the garlic oil, and identified the compounds they contained: diallyl sulphide (60%), diallyl trisulphide (20%), diallyl tetrasulphide (10%), and allyl propyl sulphide (6%). He also concluded that due to the structure of the diallyl sulphides, the actual formula of the allyl group should be C_3H_5 rather than C_6H_{10}. This term is still used in organic chemistry today, and refers to the CH_2CHCH_2 hydrocarbon grouping.

Component	Amount found in garlic (% fresh weight)	Reference
Water	62 – 68	Fenwick *et al* 1985 Souci *et al* 1986 Lawson *et al* 1991
Carbohydrate	26 – 30	Darbyshire *et al* 1981
Protein	1.5 – 2.1	Lawson 1996
Lipids	0.1 – 0.2	Lawson 1996
Vitamins	0.015	Lawson 1996
Minerals	0.7	Lawson 1996
Nitrogen	0.6 – 1.3	Lawson 1996
Sulphur	0.23 – 0.37	Lawson 1996

Table 1.2.1 General compositional chemistry of fresh garlic, and references (modified from Lawson 1996).

1.2.3 Discovery of allicin

Cavallito and Bailey (1944) carried out investigations into the antibacterial action of garlic. They observed that whilst garlic was a potent inhibitor to the growth of many bacteria, the components of Semmler's fractional distillations exhibited a greatly reduced antibacterial action. However, through repeated solvent extraction at room temperatures they isolated an oily substance, with a smell characteristic of crushed garlic. This substance also displayed high antibacterial activity, they called this oily antibacterial allicin, and suggested the formula to be $C_6H_{10}S_2O$.

Allicin is a thermally unstable thiosulphinate. It rapidly forms diallyl disulphide on heating (a simultaneous loss in antibacterial efficacy is seen on heating). It is water soluble to 2.5% at 10°C, and miscible to alcohols, ether and benzene. It has and lethal dose (50%) of 60 mg kg^{-1} intravenously, and 120 mg kg^{-1} subcutaneous in mice. In 1947 Stoll and Seebeck synthesized allicin through mild oxidation of diallyl disulphide, effectively proving the structure of allicin to be $CH_2CHCH_2S(O)SCH_2CHCH_2$

Whilst the discovery of allicin, and characterisation of its properties explained the loss in antibacterial activity on steam distillations of garlic, and also accounted for the odour of crushed garlic; it did not account for the lack of odour in intact garlic cloves. Cavallito *et al* (1945) carried out a dried low-temperature acetone extraction of garlic, this did not yield diallyl sulphides or allicin. He suggested that neither the diallyl sulphides or allicin were present in garlic cloves (intact), and that allicin is produced on crushing of garlic clove by the action of an enzyme (which is unable to function in the absence of water, or in organic solvents) (Cavallito *et al* 1945).

1.2.4 Discovery of alliin and formation of allicin.

Stoll and Seebeck (1947) were the first to isolated and identify the stable precursor of allicin. Using a low temperature ethanol extraction of frozen garlic bulbs, they obtained fine, odourless crystals of (+)-S-allyl-L-cysteine sulphoxide. They named this compound alliin.

Later analyses (Fujiwara *et al* 1958, Granroth 1968) showed that garlic cloves also contained minor quantities of two other compounds similar in structure and nature to alliin: methiin ((+)-S-methyl-L-cysteine sulphoxide), and isoalliin ((+)–S-propenyl-L-cysteine sulphoxide) all of these cysteine sulphoxides are capable of producing allicin. The most abundant in garlic cloves is alliin (85% total cysteine sulphoxides), then methiin (10%), the least abundant is isoalliin (5%).

Another alliin-like compound has been identified in garlic, cycloalliin (Virtanen *et al* 1959, Ueda *et al* 1991) – a ring structure that is not thought to play a significant role in thiosulphinate production. The cysteine sulphoxides have no other function, than to act as precursors of thiosulphinates (Lawson *et al* 1994).

Alliin is odourless, and does not possess any antibacterial activity. Addition of alliin to an enzyme fraction of garlic lead to the formation of allicin. Stoll and Seebeck (1951) isolated the enzyme responsible for conversion of alliin to allicin, naming it alliin lyase or alliinase. On crushing of garlic cloves (or addition water to garlic powder) alliinase converts alliin, *via* a sulphenic acid intermediate to form allicin (Figure 1.2.4). Allicin is not the only thiosulphinate formed through the action of alliinase on cysteine sulphoxides, eight different thiosulphinates are formed from the sulphenic acid intermediates. Allicin is the dominant thiosulphinate, accounting for 70% of the total thiosulphinate content of crushed garlic (Lawson 1996).

Alliinase is a pyridoxal phosphate dependent glycoprotein (carbohydrate content approx 5%), composed of two separate subunits, and containing 448 amino acids (Van Damme *et al* 1992). Alliinase accounts for approximately 10% of the total protein content of garlic cloves.

Immuno-staining techniques (Ellmore *et al* 1994) found that alliinase was located only in a few vascular bundle sheath cells of the phloem, whilst alliin is found in the cytoplasm of most garlic cells. This spatial separation explains why formation of allicin only occurs on damage to the clove, bringing the enzyme and substrate molecules into contact.

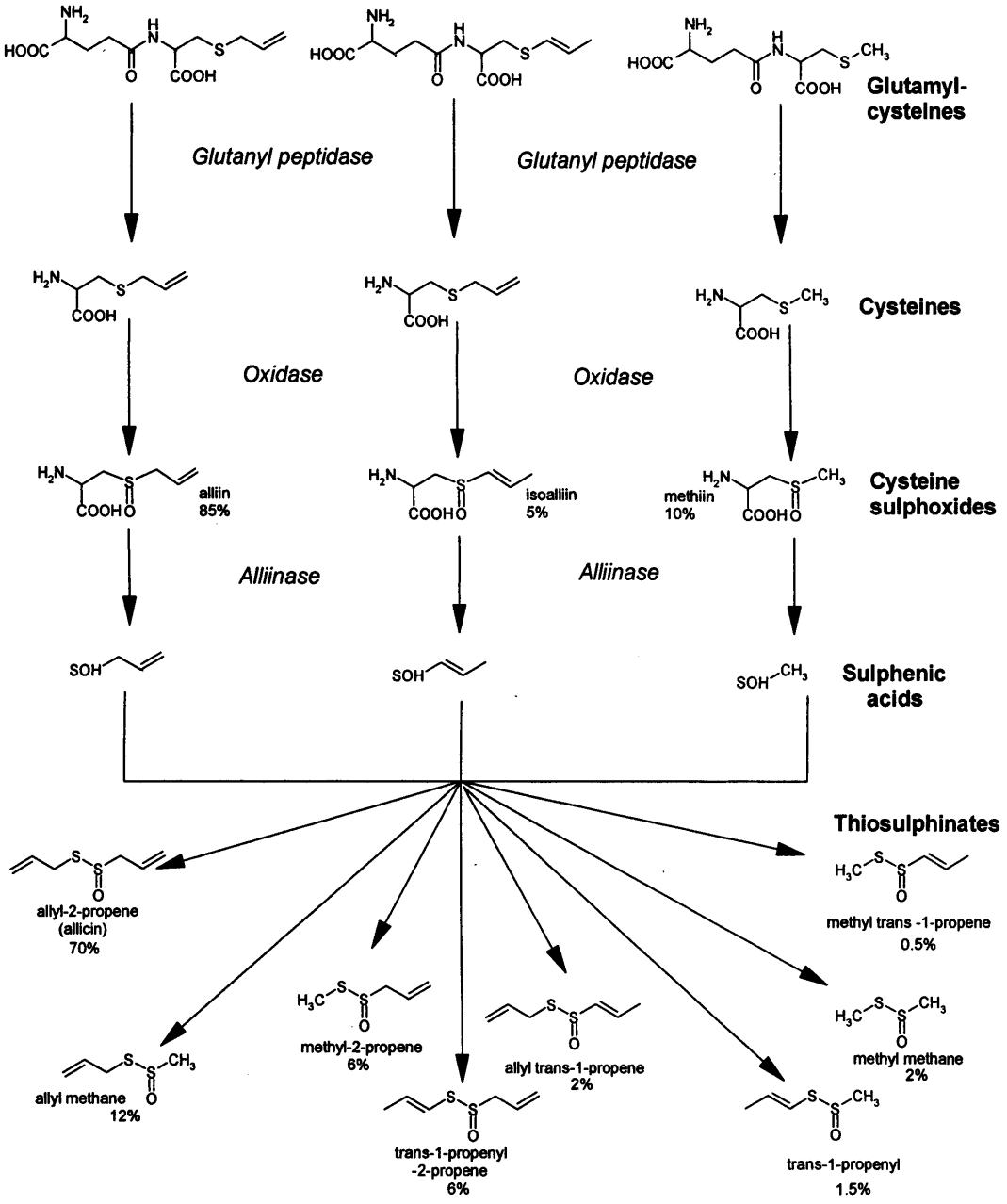


Figure 1.2.4 Formation of allicin (and other thiosulphinates), cysteine sulphoxides (alliin, isoalliin, methiin), and cysteines from γ-glutamyl cysteines. Percentages are given typical % weights found in whole cloves (cysteine sulphoxides), and crushed cloves (thiosulphinates) (Lawson 1996).

1.2.5 Discovery and formation of cysteine sulphoxides (alliin, isoalliin, methiin).

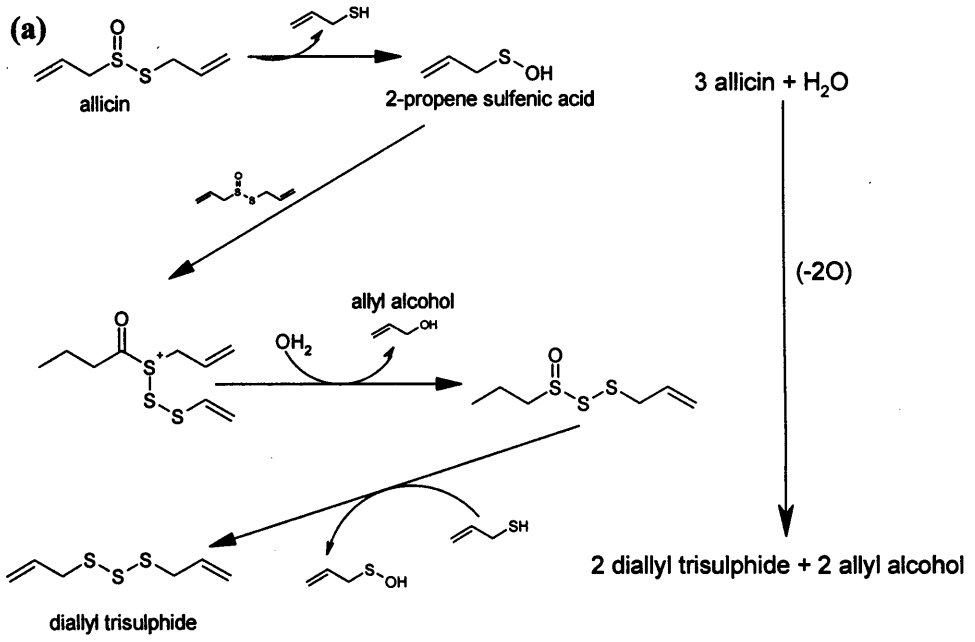
In 1961 (Virtanen *et al*, Suzuki *et al*) the parent molecules of the cysteine sulphoxides were identified as the γ-glutamylcysteines. These compounds occur abundantly in garlic cloves, and are thought to function as a cysteine sulphoxide reserve. γ-Glutamylcysteines are glutamyl peptides which contain a cysteine residue, six different γ-glutamylcysteines have been found in garlic cloves, the most abundant of which are γ-glutamyl-S-allylcystein (Virtanen *et al* 1961), and γ-glutamyl-S-trans-1-propenylcysteine (Lawson *et al* 1990).

γ-Glutamyl-S-allylcysteine, and γ-glutamyl-S-trans-1-propenylcysteine are thought to be responsible for alliin, and isoalliin production (catalysed by γ-glutamylcysteine transpeptidase). The γ-glutamylcysteine concentration in garlic bulbs decreases during sprouting (a significant increase in the concentration of cysteine sulphoxides is also seen), this may be a mechanism to ensure that young plants have enough alliin ready to repel pathogenic attacks (from micro-organisms and insects) through the antibiotic action of allicin.

1.2.6 Reactivity of allicin.

Allicin is a relatively unstable compound, readily undergoing many reactions yielding a variety of products depending on conditions. In aqueous environments allicin reacts *via* a monomolecular self-elimination reaction, or a bimolecular reaction (Block 1986) to produce diallyl trisulphide or diallyl disulphide respectively. The monomolecular self elimination reaction also produces allyl alcohol. (Figure 1.2.6.1).

Diallyl sulphide production occurs more rapidly at elevated temperatures, a greater variety of diallyl sulphide reaction products are also produced (diallyl sulphide, diallyl tetrasulphide).



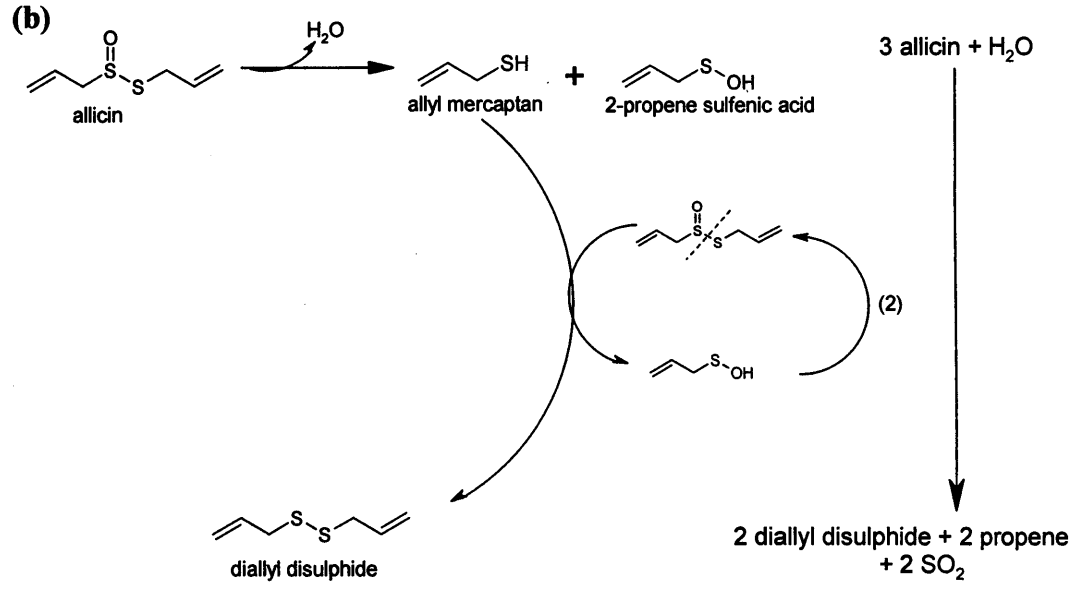


Figure 1.2.6.1 Reaction products of allicin in aqueous environments (modified from Lawson 1996) (a) monomolecular self-elimination reaction (b) bimolecular reaction.

Allicin is thought to be most stable in aqueous environments (storage is recommended in acidified water at -70°C), although estimates of allicins stability vary greatly. Brodnitz *et al* (1971) reported that pure allicin dissolved in water at 20°C completely degraded (66% diallyl disulphide, 14% diallyl sulphide, 9% diallyl trisulphide) after 24 hours. Lawson and Wang (1994) reported that pure allicin dissolved in water at 23°C has a half-life (time taken for allicin concentration to decrease by 50%) of between 30 and 40 days.

Some manufacturers now produce allicin which they claim to be stabilised. Full details of the stabilisation methods are not always given. One method involves adsorption of pure allicin onto silica gel. Analysis of this product reveals no detectable allicin (Lawson *et al* 1991, Jäger 1992). Other methods claim to have modified the allicin molecule sufficiently to make it stable.

Allicin is more unstable in organic solvents, and the reaction products vary according to the polarity of the solvent. In polar solvents (e.g. methanol) the half life of allicin is reported as 48 hours, and the predominant reaction products are *E*-ajoene (major) and *Z*-ajoene (minor). As the solvent polarity increases, the ratio of *E*-ajoene to *Z*-ajoene also increases (Lawson 1996) (Chapter 6).

In low polarity solvents (e.g. hexane) the half life of allicin is as short as 2 hours (Lawson 1996) the predominant reaction products are 2-vinyl-1,3-dithiin (major), and 3-vinyl-1,2-dithiin (minor) (Chapter 6).

The stability of allicin is also dependent on other factors: half-life of allicin in aqueous garlic solutions decreases significantly as the concentration of garlic increases (Lawson 1996) (the half-life of pure allicin in aqueous solution is relatively concentration independent). pH of the solvent also influences allicin stability; allicin degrades quickly in alkaline environments (Cavallito *et al* 1944, Block *et al* 1984, Yu *et al* 1989) producing diallyl sulphides and sulphur dioxide, allicin is more stable in acidified environments (Sovova and Sova 2001, Lawson 1996).

Allicin stability also seems to be dependant on chemical complexity of the solvent. The half-life of allicin is greatly reduced when dissolved in various biological fluids. Freeman *et al* (1995, 1997) found that when pure allicin was added to blood, it could no longer be detected after 5 minutes. Egen-Schwind *et al* (1992) found that allicin concentration decreased by 99% following a 6 minute incubation with liver homogenate. In media containing cysteine, allicin reacts quickly to form S-allylmercaptocysteine (Cavallito *et al* 1944, Lawson *et al* 1992).

1.3 Therapeutic applications of garlic

1.3.1 Effects of garlic on the cardiovascular system.

The beneficial effects of garlic and garlic components on blood pressure (Loper and Debray 1921), serum lipid (Eilat *et al* 1995) and LDL (Low Density Lipoprotein) cholesterol levels (Gebhardt and Beck 1996), blood plaques and clots (Vanderhoek *et al* 1980), and atherosclerosis have been reported for many years.

Antihypertensive effects of garlic have been proven in various animal models including: dogs (Loper and Debray 1921), cats (Malova 1951), and rats (Ogawa *et al* 1993). Garlic was found to protect against artificially induced hypertension, and generally lower blood pressure. These experimental results have also been corroborated through human clinical trials (Petkov 1966, Lutomski 1984). Allicin appears not to be responsible for the antihypertensive effects of garlic since removal of allicin by heat treatment, or inactivation of alliinase does not diminish its capacity to lower blood pressure (Rao *et al* 1981, Pantoja *et al* 1991). A chloroform soluble garlic compound has a tonic effect on the heart muscle (Torrescasana 1946). Another chloroform insoluble compound has proven to have a strong lowering effect on blood pressure – together these compounds may be responsible for the antihypertensive effect of garlic.

Low Density Lipoprotein (LDL cholesterol) is a major contributory factor for coronary heart disease and atherosclerosis. The lipid and LDL-cholesterol lowering activity of garlic appears to be due to allicin, since aged garlic extracts and steam distillates of garlic do not have the same effect (Sibler 1933, Orzechowski 1933). Garlic-induced reductions in serum lipid and cholesterol levels have been shown in rats (Ali 2000), rabbits (Karakes and Feszt 1975), and even chickens (Horton *et al* 1991). Clinical trials in humans have given conflicting results. Some trials show no significant effects on cholesterol or plasma lipid levels (Byrne *et al* 1999); other studies show a significant lowering of plasma lipids, fatty acid content, and LDL cholesterol levels (Jain 1977, Sucur 1980, Gadkari and Joshi 1991). Conflicting results may be due to difficulties in controlling variables in patients which may influence possible therapeutic effects (e.g. diet, exercise regime, previous health

problems/ medication). A recent study has shown that patient gender may also be a contributing factor (Zhang 2001), with female patients exhibiting a healthier HDL (High Density) cholesterol to total cholesterol ratio than male patients on administration of garlic oil.

Ajoene appears to be responsible for the inhibition of platelet aggregation (responsible for blood plaques and blood clots) seen in human patients on treatment with garlic (Bordia 1978, Block *et al* 1983). The activity of ajoene may be due to attachment to sulphur groups present on the surface of platelet membranes preventing interaction with fibrinogen (Jain and Apitz-Castro 1993). Diallyl trisulphide, diallyl disulphide, and diallyl sulphide have also proved to be potent inhibitors of platelet aggregation (Kung-Chi 2003). Whilst a lowering of platelet aggregation is usually seen as beneficial to health (through prevention of blood clot formation and thrombosis) it should be pointed out that this activity may lead to possible complications in patients undergoing surgery. Ingestion of excessive amounts of dietary garlic supplements is thought to have been responsible for severe haemorrhaging in a patient recently undergoing ocular surgery (Carden 2002).

1.3.2 Cancer treatment and prevention.

There are several studies which document that in geographical areas where garlic and onion consumption are high (for instance Egypt, India, China and parts of France (Reuter *et al* 1996, Wang *et al* 1989)) occurrences of cancer are lower, however the lower occurrence of cancers in these countries may also be linked to other dietary factors, or pollution levels. However further investigations into dietary factors in controlled groups indicate that increased garlic intake could play a role in prevention of cancer (Das 2003, Thomson 2003).

Recently there has been a flourish of experimental results regarding the possible use of garlic (and garlic components) to prevent or treat various forms of cancer. S-Allylcysteine (contained in garlic extract) was shown reduced development of N-nitrosodiethylamine (NEDA)-induced hepatocarcinogenesis in rats by bringing about a decrease lipid peroxidation in and an increase in antioxidant activity within liver cells (Sundaresan and Subramanian 2003). Diallyl sulphide also inhibited

onset of diethylstilbesterol (an oestrogen-like compound) induced liver cancer in rats (Green *et al* 2003). The reason for this is unclear, but is thought to be due to metabolic inhibition of the production of harmful adducts of diethylstilbesterol. Diallyl sulphide was also responsible for increasing apoptotic cell death in mouse skin tumour cells by 78% and 94% in benign and malignant tumours respectively.

Allicin reduced progress of gastric cancer (SGC-7901) cells *in vitro* by inhibiting telomere activity and also inducing cancer cell apoptosis (Sun 2003). Fractions from garlic have also proved to stimulate natural killer cell activity against breast cancer cells *in vitro* and in mice (Zuhair *et al* 2003).

Clinical trials investigating the effectiveness of garlic against forms of cancer have also yielded promising results: Ilker *et al* 2002 supplemented the diet of patients with prostate tumours (benign and malignant) with 1ml kg^{-1} body-weight aqueous garlic extract. Tumour cell mass in patients with benign prostate tumours was significantly reduced by aqueous garlic extract and symptoms were significantly alleviated in patients with prostate cancer. Tili *et al* 2003 applied ajoene topically to patients suffering from basal cell carcinoma. In 80% of patients ajoene brought about a significant reduction in tumour size (induction of apoptosis in tumour cells is thought to be responsible).

1.3.3 Antifungal and antiprotozoal activity of garlic.

Antifungal activity of garlic extracts was documented in 1936 (Schmidt and Marquardt 1936) against growth of epidermophytic organisms. In 1950 Timonin and Thexton (Timonin and Thexton 1950) reported that extremely dilute aqueous extracts of garlic inhibited growth of various soil fungi.

Since then garlic has proved effective against many fungi: *Microsporium, Trichophyton* (Amer *et al* 1980), *Malbranchea pulchella, Chrysosporium tropicum* (Shrivastava and Singh 1982), *Saccharomyces cerevisiae, Kloeckera apiculata, Candida utilis, Oospora lactis, Penicillium notatum* (Grzybowski *et al* 1988), *Botrytis cinerea, Mycosphaerella arachidicola, Physalospora piricola* (Wang and Ng 2001), *Aspergillus niger, A. flavus, A. fumigatum* (Yin and Tsao 1999).

Antiprotozoal activity of garlic is not as frequently documented, however garlic has proved inhibitory against many pathogenic organisms: *Opalina ranarum, Balantidium entozoo* (Kurnakov 1952), *Giardia intestinalis, Trichomonas foetus, T. vaginalis* (Harris 2001), *Trypanosoma spp.*, and *Entamoeba histolytica* (Lun 1994).

Allicin appears to be the primary antifungal agent in garlic, since addition of sulphydryl containing compounds brings about antagonism in the inhibitory activity. However, many other garlic components have proved inhibitory: diallyl disulphide and diallyl trisulphide have both proved inhibitory to *Candida albicans* (Avato 2000). Diallyl trisulphide has proved effective against *Cryptococcus neoformans* and displayed *in vitro* synergistic activity with amphotericin B (Shen 1996). Diallyl disulphide, diallyl sulphide and allyl alcohol all proved to be effect anti-giardial agents (Harris 2001).

1.3.4 Antiviral activity of garlic

Before vaccination programmes were established garlic was used as a preventative measure against poliomyelitis, and influenza A (Reuter *et al* 1996). Garlic has also shown inhibitory activity towards influenza B (Fenwick and Hanley 1985), cytomegalovirus (Meng *et al* 1993), rhinovirus, Human Immunodeficiency Virus (HIV) (Tsai *et al* 1985), and viral pneumonia (Harris *et al* 2001). In China preparations using diallyl trisulphide have proved active against Herpes simplex *in vitro* and *in vivo* (Reuters *et al* 1996). It appears that only allicin and its reaction products are effective antivirals (Hughes *et al* 1989, Weber *et al* 1992) alliin, and S-allyl cysteine did not display virucidal activity.

1.3.5 Antibacterial activity of garlic extract.

The first scientific investigations into the antibacterial effects of *Allium* species are attributed to Louis Pasteur, who noted the bacteriostatic activity of onion and garlic (not documented but historically believed) juices (Pasteur 1858, Reuter *et al* 1996). In the 20th Century, scientific investigations into the antibacterial efficacy of garlic began in earnest. During World War One physicians and nurses in field hospitals in the Balkans began treating cholera and dysentery patients with powdered garlic combined with phenyl salicylate (Marcovici 1995, Reuter *et al* 1996, Harris *et al* 2001).

In 1930 Lehmann found that extracts of garlic prevented growth of *Escherichia coli* and *Salmonella typhi*. Glaser and Drobnik (1939) investigated the antibacterial effects of various garlic extracts, finding high activity against Gram positive bacteria, Gram negative bacteria, and acid-fast bacteria. Cavallito and Bailey (1944) isolated allicin from garlic, which proved to be extremely inhibitory to a range of bacteria including: *Staphylococcus aureus, Streptococcus viridans, Salmonella typhi, Proteus mirabilis, Vibrio cholerae, Shigellae dysenteriae* and *Bacillus subtilis*. Its inhibitory effect was found to be considerably more bacteriostatic than bacteriocidal in nature.

In many subsequent investigations garlic has proved to be inhibitory to the growth of many bacteria. The effect of garlic is broad spectrum, inhibiting pathogenic and non pathogenic Gram positives and Gram negatives. Susceptible bacteria include: *Klebsiella, Pasteurella, Corynebacterium,* strains (Kabelik and Hejtmankova-Uhrova 1968), *Mycobacterium tuberculosum,* and *Mycobacterium leprae* (McKnight and Lindegren 1936, Jain 1993, Chaudhury *et al* 1962), *E. coli, Pseudomonas aeruginosa* (Rees *et al* 1993), *E. coli* 0157:H7 (Addler and Beuchat 2002, Sasaki *et al* 1999), *Listeria monocytogenes* (Addler and Beuchat 2002), *Enterococci,* (Jonkers *et al 1999*) and *Helicobacter pylori* (O'Gara *et al* 2000).

Jezowa *et al* (1966) reported that garlic extracts were even effective inhibitors of bacteria that had become resistant to modern antibiotics such as penicillin, and erythromycin. Jonkers *et al* (1999) found that garlic was inhibitory toward vancomycin resistant *Enterococci* (VRE), and also displayed synergistic action when combined with vancomycin.

As yet there have been no reports of bacteria developing resistance to garlic. Skyrme (1996) evaluated the antibacterial efficacy of aqueous garlic extract against a range of bacteria. Bacteria tested could be separated into two groups depending on their susceptibility to garlic inhibition (Table 1.3.5). The majority of bacteria tested were found to be susceptible to garlic inhibition, with growth being completely inhibited over the 24 hour test period at concentrations below 2.5 mg garlic powder per ml growth media. Several of the bacteria tested were found to be significantly less susceptible to growth inhibition by garlic (total growth inhibition at 24 hours only occurring at concentrations in excess of 10 mg garlic extract per ml growth media). The bacteria with reduced susceptibility to garlic were members of the Lactic Acid Bacteria group (LAB). This group of bacteria are often found inhabiting the gastrointestinal tract of many animals and are generally considered to be beneficial to the health of their host organisms (section 1.5.3).

Garlic, along with many herbal antibacterials, has been implicated as being in part responsible for the increase in bacterial resistance to conventional antibiotics (Ward *et al* 2002). Though it is impossible to say at this stage whether increased antibiotic resistance could be a result of increasing popularity of herbal treatments,

Bacterium	MIC at 24 h ($mg\ ml^{-1}$). *	Susceptibility **
Staphylococcus aureus	0.8	S
Escherichia coli	2.2	S
Bacillus cereus	1.8	S
Bacillus subtilis	1.8	S
Proteus mirabilis	1.9	S
Listeria monocytogenes	2.3	S
Salmonella dublinii	2.3	S
Salmonella enteriditis	2.4	S
Lactobacillus plantarum	12.5	NS
Lactobacillus acidophilus	16.7	NS
Lactobacillus casei	17.5	NS
Pediococcus pentosaceus	34.5	NS

Table 1.3.5 Susceptibilities of various bacteria reproduced from Skyrme 1996.

*** MIC – Minimum Inhibitory Concentration (the lowest effective concentration required to bring about total growth inhibition over 24 hours).**

**** Susceptibility- Susceptible to garlic inhibition (S), greatly reduced susceptibility (NS)**

garlic has been shown to decrease the sensitivity of *S. aureus* to ampicillin (Ward *et al* 2002.

The precise mechanisms of garlic's antibacterial action are not clear. Allicin exerts a general inhibitory effect on cell metabolism through attack of sulphydryl containing enzymes (Cavallito and Bailey 1944, Wills 1956). However allicin is not the only component of garlic that displays antibacterial action - diallyl disulphide inhibits: *H. pylori* growth *in vitro* (O'Gara 2000), antibiotic resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Tsao 2001). Diallyl sulphide, diallyl disulphide, diallyl trisulphide and diallyl tetra sulphide all proved inhibitory against *S. aureus* and Methicillin Resistant *S. aureus* (Tsao 2001).

1.4 Bacterial structure and diversity

1.4.1 The bacterial cell envelope

The term cell envelope used here refers to the components of the bacterial cell which enclose the cytoplasmic interior (cytoplasmic membrane, periplasmic space, peptidoglycan cell wall, and outer membrane (in gram negative bacteria)).

All bacteria possess a cytoplasmic membrane encased by a peptidoglycan layer (varying in thickness between different bacteria). The cytoplasmic membrane is phospholipid bilayer that surrounds the cell. It is essential to retain cell integrity, preventing intracellular cytoplasmic components from leaking out to the cell exterior. The cytoplasmic membrane acts as a highly selective permeability barrier, enabling the cell to accumulate essential nutrients and metabolites, also permitting excretion of toxic waste products.

The hydrocarbon interior of the membrane is hydrophobic in nature (the glycerol phosphate moieties of the phospholipid molecules are hydrophilic in nature, and compose the surfaces of the membrane). The most common bacterial phospholipids are diphosphatidyl glycerol, phosphatidyl glycerol, and phosphatidyl ethanolamine (Poxton 1993). Bacterial cytoplasmic membranes do not contain sterols (with the exception of methanotrophic bacteria (Madigan *et al* 2000)).

The cytoplasmic membrane contains (partially or wholly) several proteins and functions as a fluid mosaic, allowing essential interaction between membrane proteins and also other cell components. Due to the selective permeability of the cytoplasmic membrane, specific transporters are needed for many essential metabolic substrates. All transporters require energy input, either from molecules containing high energy bonds (e.g. Adenosine TriPhosphate (ATP)) or from proton motive forces. The cytoplasmic membrane also functions to maintain a proton motive force (a potential energy store).

Bacterial cytoplasmic membranes are surrounded by a cell wall composed of peptidoglycan. This is a rigid layer composed of repeating units called the glycan

tetrapeptide. This unit comprises two sugar derivatives (*N*-acetyl glucosamine, and *N*-acetyl muramic acid), with a group of amino acids (L-alanine, D-alanine, D-glutamic acid, and either diaminopimelic acid (gram negative bacteria) or lysine (gram positive bacteria) (Chapter 8, figure 8.1.1). The peptidoglycan cell wall gives the cell rigidity, its characteristic shape, and offers it protection from osmotic stress.

Bacteria are usually divided into two groups based on their ability to retain the gram stain, this is a reflection on their cell envelope structure. Gram positive bacteria retain the gram stain (an insoluble crystal violet-iodine complex formed inside the cell) on washing with alcohol. The cell envelope of these bacteria contains approximately 90% peptidoglycan (Madigan *et al* 2000), arranged in up to 25 layers (Figure 1.4.1.1). The gram positive peptidoglycan cell wall also contains acidified polysaccharides termed teichoic acids. These acids may be wholly or partially wall spanning, some are also anchored firmly to lipids of the cytoplasmic membrane (termed lipoteichoic acids). Teichoic acids give additional rigidity to the bacterial cell envelope, and contribute to the net negative charge of the cell surface.

Gram negative bacteria do not retain the gram stain on washing with alcohol. The cell envelope of these bacteria contains approximately 10% peptidoglycan (Madigan *et al* 2000), the peptidoglycan layer does not include teichoic acids, or lipoteichoic acids. Gram negative bacteria contain an additional (outer) membrane composed of lipopolysaccharides (LPS) (Figure 1.4.1.2). The inner surface of the outer (LPS) membrane is anchored to the peptidoglycan cell wall by small lipoprotein complexes. The outer surface of the outer membrane contains the LPS molecules, which are composed of three moieties: Lipid A, a core polysaccharide, and an O polysaccharide. LPS of some gram negative bacteria are toxic to host organisms (*e.g. Escherichia coli, Salmonella* and *Shigellae* strains). The lipid moiety of the LPS is responsible for its pathogenicity, whilst the polysaccharide component is needed to make the molecule water soluble (both components are needed for *in vivo* toxic activity).

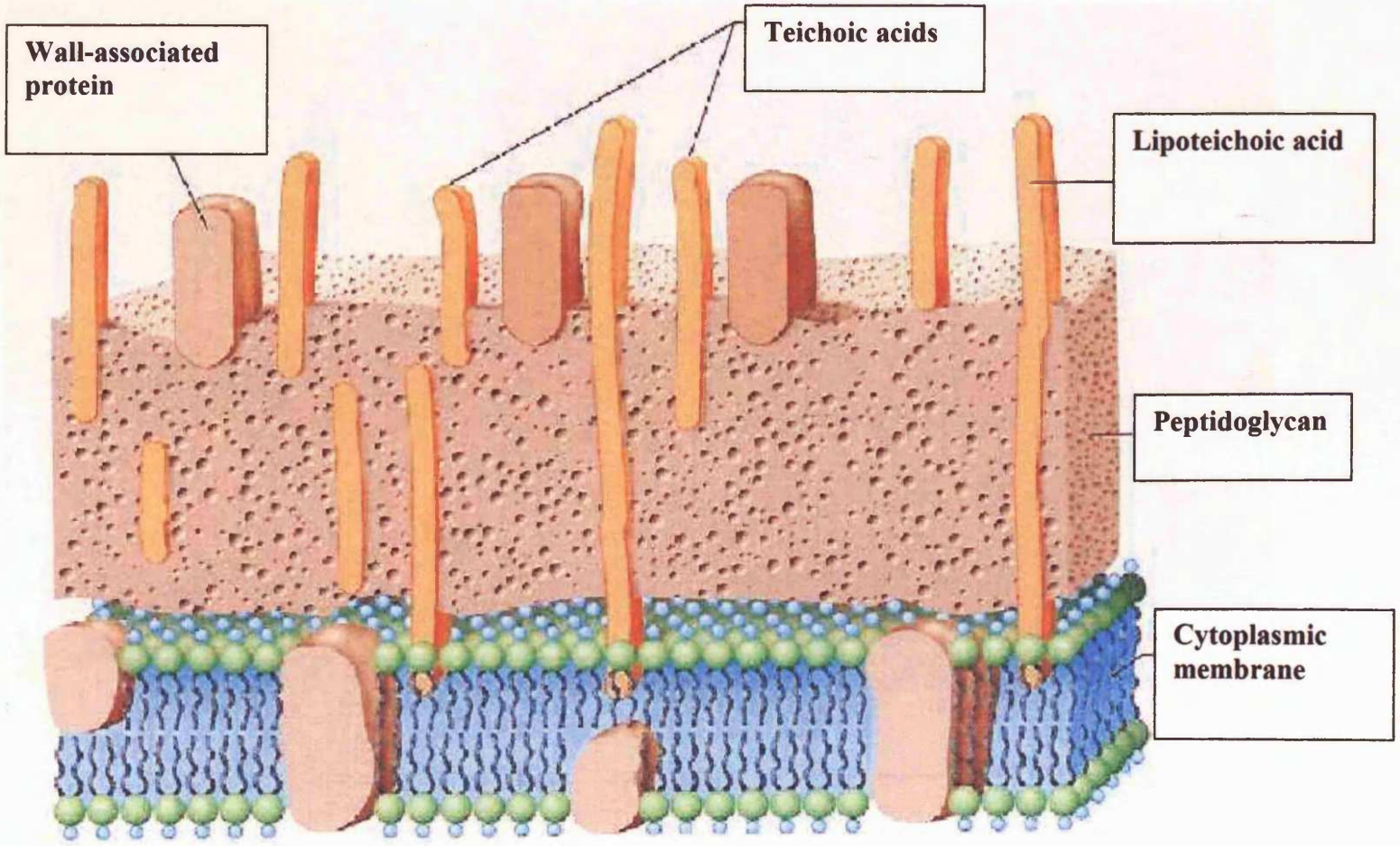


Figure 1.4.1.1 The Gram positive cell envelope (reproduced from Madigan *et al* 2000).

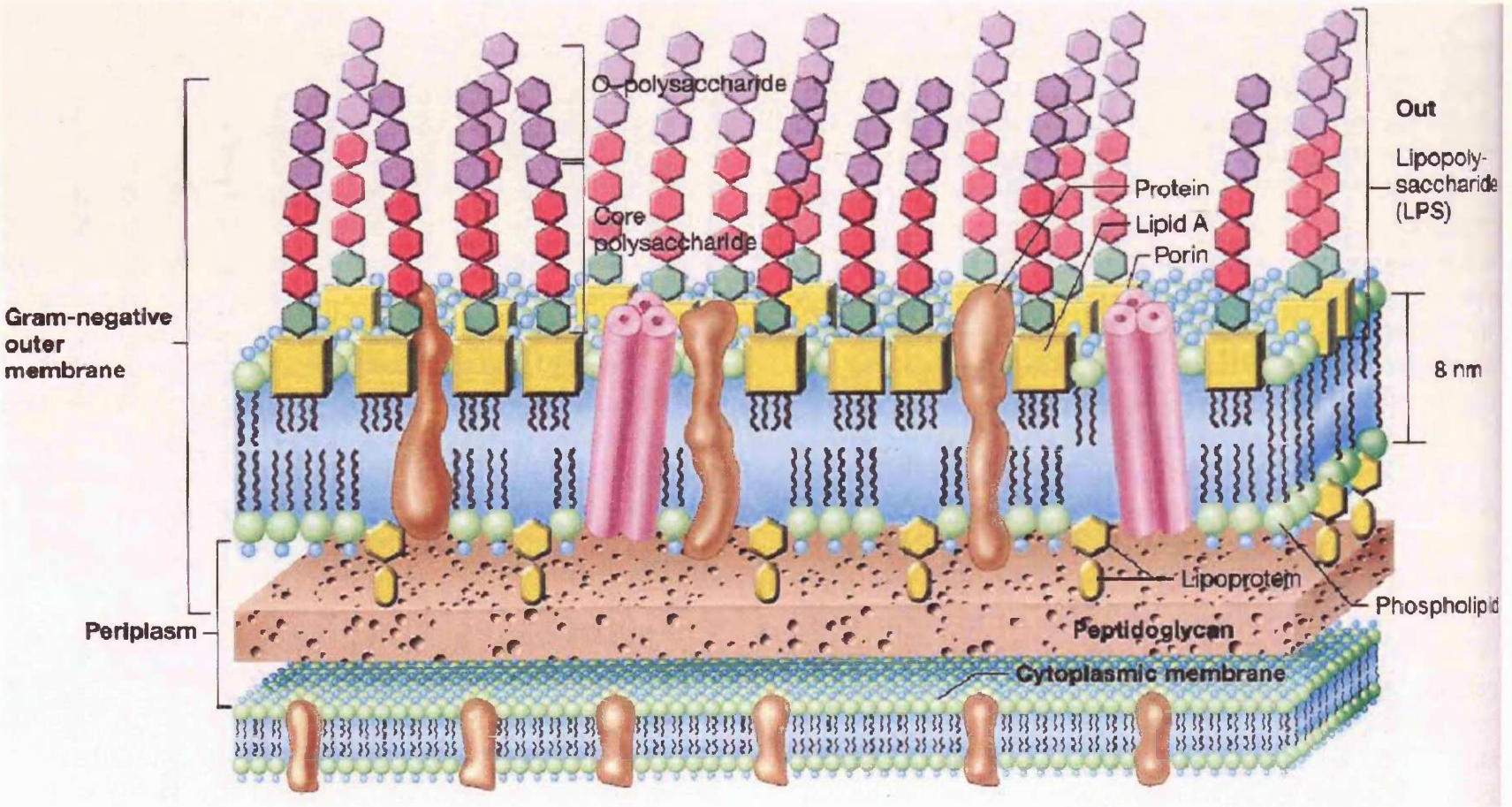


Figure 1.4.1.2 The Gram negative cell envelope (reproduced from Madigan *et al* 2000).

1.4.2 The cytoplasmic interior

Contained within the cell envelope is the cytoplasmic cell interior. This is an aqueous matrix where essential nutrients are accumulated to the levels required for biochemical reactions. Bacterial genetic material (deoxy-ribose nucleic acid (DNA), and ribose nucleic acid (RNA)) is not contained within a membrane bound nucleus, but is usually present in aggregates of closed circular molecules throughout the cytosol.

1.5. Intestinal microflora

1.5.1 Distribution of bacteria through the gastrointestinal tract

The human gastrointestinal tract can be considered in the following sections:

1. The stomach. Acidified to approximately pH 2 by secretion of hydrochloric acid from specialised cell, this acts as a chemical barrier preventing infection of the gastrointestinal tract by pathogenic organisms. The stomach usually contains few bacteria, however it can be colonised by *Helicobacter pylori* (Torres 2000), which leads to development of stomach ulcers (Noguiera 2003). Chemotherapeutic treatments which make the stomach pH more alkaline can lead to colonisation and infection of the gastrointestinal tract by opportunistic bacteria such as *S. aureus* (Yoshida 1999, Suzuki 1994).
2. The duodenum. This is the first component of the small intestine, and is moderately acidic (pH 4-5). The duodenum can be colonized by *Enterococci* and Lactic Acid Bacteria (LAB). The most frequently found LAB in the duodenum are *Lactobacilli* (Mitsuoka 1992), present in numbers of approximately 1 x 10^4 bacteria g faeces^{-1}.
3. The jejunum. Similar in pH to the duodenum, this is often colonised by *Streptococci, Bacteroides,* and *Enterobacteria* (e.g. *E. coli*) present in numbers between 1x 10^5 and 1 x 10^7 bacteria g faeces^{-1} (Mitsuoka 1992).
4. The ileum. Slightly less acidic than the jejunum, often colonised by *Enterobacteria, Bacteroides,* and *Bifidobacteria.* Bacteria here are present in higher numbers, between 1 x 10^5 and 1 x 10^7 bacteria g faeces^{-1}

5. The colon (cecum and rectum). Neutral in pH, and mainly colonised by high numbers (1 x 10^{11} bacteria g faeces^{-1}) of obligate anaerobes (e.g. *Clostridia, Ruminococcus,* and a minority of *Lactobacilli*) or anaerobic tolerant bacteria (e.g. *E. coli, S. viridans* (de San Ildefonso Pereira 2002)), other bacteria infrequently colonise the large intestine colon (*e.g. Staphylococci* (Manian 2002)).

Human intestinal microflora is a highly developed ecosystem, frequently containing in excess of 200 distinct species of bacteria (Finegold *et al* 1974). Many of these bacteria are in a symbiotic relationship with the host, and are in dynamic equilibrium with other intestinal bacteria.

1.5.2 Probiotic bacteria

A large proportion of human gastrointestinal tracts are colonized by LAB species. These bacteria are gram positive rods or cocci, which produce lactic acid as the major end product of glucose fermentation. Many LAB are termed probiotic bacteria. The term probiotic was first used in 1965 by Lilly and Stillwell (Lilly and Stillwell 1965) it refers to a 'growth promoting factor' produced by a micro-organism. In 1974 Parker extended this term to include organisms and substances which benefit the host by influencing the intestinal microflora. In 1989 Fuller proposed that the definition of a probiotic should be 'A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'. This definition restricts probiotic organisms to those supplied in food supplements, and also to bacteria of the intestinal tract. Havenaar *et al* (1992) expanded on this to give the precise definition of a probiotic:

The term probiotic applies to products which:

1. Contain live micro-organisms (e.g. freeze dried cells or liquid cultures).
2. Improve the health status of the host animal (which may include growth promotion).

3. Can have its effect in the mouth or gastrointestinal tract (whether in a food supplement or encapsulated form), the upper respiratory tract (aerosol) or in the urogenital tract (topical application).

This definition covers most uses and formulations of probiotic supplements. Recently however, investigations are being carried out into the possible use of probiotic bacteria to modify epidermal microflora, suggesting that the above definition may soon need to be modified.

1.5.3 Applications of probiotic bacteria

One major function of probiotic LAB is to enhance colonial resistance of the host organisms (i.e. maintaining indigenous microflora, reducing the risk of infection from pathogenic bacteria). Probiotic bacteria (*Lactobacilli*) have been used to combat intestinal disturbance brought about by infectious Enterobacteriaceae since 1915 (Caulk 1915, Newman 1915). Probiotic therapy has also proved effective against *Clostridium difficile* (responsible for causing diarrhoea, and colitis) (Gorbach *et al* 1987). Probiotic supplements containing *Streptococci* have been used to normalise microflora of the nasopharynx in children (Sprunt and Leidy 1988). Topical application of *Lactobacilli* to vaginal epithelial cells has proved to guard against colonisation and infection by *Candida albicans* (Reid *et al* 1988, Atanassova 2003, Jeavons 2003).

Probiotic bacteria are also responsible for stimulating hosts immune system, through transport of immuno-competent cells by the gut associated lymphatic tissue, and stimulation of helper cell production and lymphocyte differentiation (Naukkarinen and Syrjänen 1986, Berg 1983). Some pathogenic bacteria can escape the gastrointestinal tract through the mesenteric lymph nodes (translocation), leading to infection of other body tissues. *Lactobacilli* are thought to prevent translocation by occupying the lymph nodes. *Lactobacilli* are also capable of translocation from the gut to other body tissues (such as the spleen) where they may survive for several days, bringing about an increase in phagocytic activity.

1.5.4 Characteristics of an effective probiotic supplement

Different properties are required in a probiotic treatment depending on its purpose, application method, and hosts responses. In general the following characteristics are sought in a good probiotic therapy.

- The bacteria must be able to survive transit to the desired site, and should be able to withstand conditions in the desired site.

- The bacteria must be able to grow and proliferate in the required site.

- The bacteria must not produce adverse immune responses from the host organisms

- The bacteria must not be pathogenic, or potentially pathogenic. They must not bring about toxic, allergic, mutagenic or carcinogenic reactions in the host organism.

- The bacteria should be genetically stable (no plasmid transfer).

- The probiotic bacteria must be able to withstand production procedures and delivery formulations

- The bacteria should have a reasonable storage viability.

Currently amongst the most common probiotic bacteria used in commercial products are strains of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus paracasei*, and *Bifidobacterium bifidum*.

1.6 Organisms studied in this project

1.6.1 *Escherichia coli.*

E. coli is the predominant facultative anaerobe in the human intestine (Nataro and Kaper 1998). It is a motile, gram negative rod from the *Enterobacteriaceae* family.

Numerous strains of *E. coli* have been isolated, the majority of which are not pathogenic and may help in increasing the colonial resistance of the hosts gastrointestinal tract. Non pathogenic *E. coli* can lead to urinary tract infections, and travellers' diarrhoea. Many strains of *E. coli* possess enteropathogenic mechanisms resulting in food poisoning symptoms (diarrhoea, vomiting, and nausea), travellers' diarrhoea, urinary tract infections, and wound infections. A variety of pathogenicity mechanisms are available to *E. coli* strains, including: enterotoxins, adhesins, invasins, cytotoxins, and erosion of intestinal epithelial cells.

The most notorious *E. coli* strain is *E. coli* 0157. This is a cytotoxic enterohaemorrhagic (causes intestinal bleeding) strain, which can cause fatalities – particularly in young or immuno-suppressed hosts.

Cephalosporins are the drug of choice for *E. coli* infections (also nitrofurantoin, or nalidixic acid in urinary tract infections (Sleigh and Timbury 1998).

1.6.2 *Lactobacillus casei*

L. casei is an aero-tolerant (group II), non pathogenic LAB, commonly inhibiting the gastrointestinal tract of animals. *L. casei* is of growing economic importance worldwide the in dairy and fermentation industries.

L. casei has traditionally found use in yoghurt and cheese production for centuries. It is commonly found today in probiotic milk preparations (live cells suspension), live yoghurts, and encapsulated probiotic dietary supplements.

If present in high numbers in the oral cavity, LAB can cause dental caries (Hogg 1992)

1.6.3 *Staphylococcus aureus* and Methicillin Resistant *Staphylococcus aureus* (MRSA).

S. aureus is a coagulase positive, gram positive, facultative anaerobic coccus. It is a normal resident of animal epidermis and upper respiratory tract. Although usually harmless, this bacterium can cause acne, boils, wound infection, septicaemia, conjunctivitis, endocarditis, and osteomyelitis.

The majority of *S. aureus* strains now possess a β-lactamase enzyme (Chapter 4), rendering many penicillins useless against this infection. The treatments of choice against this organism are currently β-lactamase stable penicillins.

Many *S. aureus* strains also possess modified penicillin binding proteins, exhibiting a lower affinity for penicillin (including β-lactamase stable penicillins), these strains are referred to as Methicillin Resistant *S. aureus* (MRSA). At present the majority of MRSA strains are susceptible to glycopeptide therapy (e.g. vancomycin), however in recent years *S. aureus* strains with reduced susceptibility to glycopeptides/ vancomycin have been isolated (Liňares 2001), these strains are referred to as Glycopeptide Intermediate *S. aureus* (GISA), or Vancomycin Intermediate *S. aureus* (VISA).

1.7 Aims and objectives

Garlic extracts and garlic components have proved to be effective inhibitors of bacterial growth. Lactic acid bacteria (beneficial probiotic bacteria) are much less susceptible to garlic inhibition that other (including pathogenic) bacteria.

The overall objectives of this project were to assess the antimicrobial efficacy of garlic powder extract against a potential intestinal pathogen (*E. coli*), and a probiotic LAB (*L. casei*) comparing and contrasting any differences in the nature of garlic inhibition, and to investigate possible mechanisms of action for inhibitory effect of garlic against *E. coli* and *L. casei*. Also, to investigate activity of garlic powder extract against the antibiotic resistant bacteria MRSA, and assess synergistic action between garlic powder extract and conventional antibiotic treatments.

The specific aims of the work presented in this project were:

1. Assess the inhibitory capacity of garlic powder extract against *E. coli* (a potential pathogen) and *L. casei* (a probiotic LAB).

2. To compare and contrast the nature of growth inhibition exerted by garlic powder extract against selected human commensal bacteria over 24 hours. Also to carry out a detailed analysis of garlic induced growth inhibition in *E. coli* and *L. casei*, and to investigate possible bactericidal activity against *E. coli* and *L. casei*.

3. To investigate the inhibitory capacity of various garlic preparations against *E. coli* and *L. casei*.

4. To perform a detailed qualitative and quantitative analysis of the chemical composition of garlic powder extract. Also to compare the composition of garlic powder extract, to aqueous extracts of garlic preparations investigated in growth inhibition experiments (2).

5. To investigate the inhibitory capacity of garlic constituents identified in (4) against *E. coli* when used separately and in combination.

6. To assess the stability of garlic chemical constituents in conditions similar to those used in the growth inhibition experiments (2), in the presence and absence of bacteria.

7. To carry out preliminary investigations into the effect of garlic powder extract on cell morphology, and glucose metabolism in *E. coli* and *L. casei* highlighting any differences. Also to investigate the effects of garlic and garlic constituents on oxygen uptake in *E. coli*.

8. To assess antibacterial efficacy of garlic powder extract against MRSA, and investigate possible synergistic activity between garlic powder extract and the antibiotics penicillin (β-lactamase sensitive) and methicillin (β-lactamase stable).

Chapter 2

General materials and methods.

Chapter 2 – General Materials and Methods

2.1 Materials

Nutrient Broth No. 2, Nutrient agar No.2, Tryptic Soy Broth (TSB), and Tryptic Soy Agar (TSA) (used for *E. coli* growth and *S. aureus*) were purchased from Oxoid (Basingstoke UK), MacConkey agar (purity testing of *E. coli*), and MRS (deMann, Rogosa, Sharpe) broth and agar were purchased from Sigma (Poole, UK). Phosphate Buffered Saline tablets (for cell washing) was purchased from Oxoid (Basingstoke UK). Freeze dried garlic powder was supplied by Cultech Ltd (Swansea, UK).

2.2 Growth of bacteria

General growth and maintenance of all bacteria was carried out in conditions as described for *E. coli* (below) except for *L. casei* for which growth and maintenance conditions are described in 2.2.2. Purity of bacterial cultures was assayed for using Tryptic soy agar (TSA), and also MacConkey agar in the case of *E. coli*.

2.2.1 *Escherichia coli*

Overnight cultures of *E. coli* were prepared by inoculating 1 colony from a pure *E. coli* fridge stock culture plate into 10 ml autoclaved Nutrient Broth No. 2, in a 20 ml screw-capped universal bottle, bacteria were dispersed by gently vortexing. Cultures were then incubated at 37°C for the required amount of time (stationary phase occurred at approximately 12 hours). *E. coli* cultures were maintained in a fridge at 4°C on TSA plates, sub-culturing took place every 72 hours and purity of stocks was ascertained weekly using MacConkey agar.

Freezer stocks were prepared by suspending *E. coli* colonies to a final density of approximately 1 x 10^8 cfu ml^{-1} in 1 ml fresh TSB containing 10% v/v glycerol as cryoprotectant. These cultures were stored at -20°C, and used to prepare fresh fridge stocks every fourth week A back-up freezer stock was held at -70°C

2.2.2 *Lactobacillus casei*

Overnight cultures of *L. casei* were prepared by inoculating 1 colony from a pure *L. casei* fridge stock culture plate into 20 ml autoclaved MRS broth, in a 20 ml screw-

capped universal, bacteria were dispersed by gently vortexing. Cultures were then incubated at 37°C for the required amount of time (stationary phase occurred at approximately 12 hours). *L. casei* cultures were maintained in a fridge at 4°C on MRS agar plates, sub-culturing took place every 72 hours and purity of stocks was ascertained weekly. Freezer stocks were prepared by suspending *L. casei* colonies to a final density of approximately 1 x 10^8 cfu ml^{-1} in 1 ml fresh MRS containing 10% v/v glycerol as cryoprotectant. These cultures were stored at -20°C, and used to prepare fresh fridge stocks every fourth week A back-up freezer stock was held at -70°C.

2.2.3 *S. aureus* and MRSA

Overnight cultures of *S. aureus* and MRSA were prepared by inoculating 1 colony from a pure fridge stock culture plate into 10 ml autoclaved TSB, in a 20 ml screw-capped universal, bacteria were dispersed by gently vortexing. Cultures were then incubated at 37°C for the required amount of time (stationary phase occurred at approximately 6 - 8 hours in *S. aureus* cultures and approximately 8 – 10 hours in MRSA cultures.). *S. aureus* and MRSA cultures were maintained in a fridge at 4°C on TSA plates, sub-culturing took place every 72 hours and purity of stocks was ascertained weekly.

Freezer stocks were prepared by suspending colonies to a final density of approximately 1 x 10^8 cfu ml^{-1} in 1 ml fresh TSB containing 10% v/v glycerol as cryoprotectant. These cultures were stored at -20°C, and used to prepare fresh fridge stocks every fourth week A back-up freezer stock was held at -70°C.

2.3 Recovery of bacteria from freezer stocks

Bacterial stocks were removed from cold storage, and held at room temperature for 1 hour. The acclimatised freezer stock was then inoculated into the appropriate liquid growth medium, and incubated at 37°C for 18 hours. Purity of each culture was ascertained by streaking onto the appropriate agar, the culture was then used to prepare fridge stock plates.

2.4 Preparation of aqueous garlic powder extract

Stock concentrations of garlic powder extract were prepared by suspending the required amount of garlic powder in sterile distilled water (or growth media). The suspension was vortexed thoroughly for 10 min, and then allowed to stand at room temperature for 30 min. The suspension was centrifuged (3900 g) for 10 min, then sterile filtered (0.2 μm syringe filter (Supelco, Poole, UK)). Stock solutions were then used immediately to prepare required concentrations in appropriate sterile diluent.

2.5 Monitoring of bacterial growth by the Bioscreen method

A Bioscreen C analyser (Labsytems, Helsinki) was used to monitor growth of bacteria in broth cultures (Chapters 3 and 7). This method takes optical density measurements continuously over the desired test period, allowing culture growth kinetics to be assessed. The Bioscreen method of growth curve analysis has been used extensively for kinetic studies of bacterial growth and for investigating biocidal mechanisms of action (Gomez Escalada 2003, Stewart *et al* 2002, Cooper *et al* 2000, Wu *et al* 2000, Lambert *et al* 1999, Lambert *et al* 1998, Johnston 1997). The Bioscreen method has also been employed to study fungal growth kinetics (Groeneveld 2002). For growth curves presented in this thesis optical density at 540 nm of broth cultures was measured at 10 minute intervals continuously over 24 hours, unless otherwise stated. For growth curves presented in this thesis optical density at 540 nm of broth cultures was measured at 10 minute intervals continuously over 24 hours, unless otherwise stated. Inocula density was standardised to approximately 1 x 10^6 cfu ml^{-1}.

Advantages of the Bioscreen method include: the ability to screen large numbers of potential biocides, the method provides reproducible growth kinetic data (Lambert *et al* 1999, Lambert *et al* 1998, Johnston 1997), the method can also be used to study cellular uptake of components. A major disadvantage of the Bioscreen method is that there is less opportunity to tailor growth conditions to specific bacteria (e.g. anaerobic and microaerobic bacteria), also it does not permit dilution at high optical densities; thus stationary phase of growth may appear early for some microorganisms.

Chapter 3

Growth and survival of bacteria in the presence of garlic.

Chapter 3. Growth and survival of bacteria in the presence of garlic.

3.1 Introduction

Today, even with our ever improving understanding of the causes and nature of infection, diseases caused by bacteria are an increasing worldwide concern. New strains of bacteria are emerging, against which our present antibiotic treatments are ineffective. Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant *Staphylococcus aureus* (VRSA) are problematic to hospitals worldwide (Seal *et al* 2003, Campbell *et al* 2003, Quirk 2002).

Food poisoning outbreaks, often caused by pathogenic strains of *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, and Salmonella enteretidis,* are frequently reported (Agasan *et al* 2002, Guest 1996, Richards *et al* 1974, Yatsuyanagi *et al* 1996).

Other hospital acquired infections such as *Clostridium difficile* frequently cause hospital wards to close, adding additional burdens to Health Service finances and resources. Developing nations suffer from an even higher prevalence of infectious disease due to lack of adequate sanitation and hygiene resources, as well as difficulties in affording reliable preventative measures and effective treatments.

The livestock industry also suffers frequently from disease caused by microbes (Appelbee 2003, McDermott 2003), many infant animals suffer from diarrhoeal illnesses, which leads to reductions in farm productivity, increased expense, and reduced profit margins. Over-use of antibiotics within the livestock industry (Catry 2003, Roe 2003) is thought to be partly responsible for the increased levels of antibiotic resistant bacteria infecting humans.

As a result of the recently increased prevalence of bacteria with reduced susceptibility to antibiotics within the clinical setting, physicians are forced to prescribe most potent therapies currently available (*e.g.* vancomycin), or

combinations of therapies (*e.g.* β-lactams/ β-lactamase inhibitors) thereby increasing the risk of side effects to patients, and the duration of treatment. Common side effects of treatment vancomycin and penicillins include: increased urination, breathing difficulties, fever, nausea and vomiting, drowsiness and weakness. More serious side effects also observed infrequently include: abdominal cramps, convulsions, blisters, severe diarrhoea, excessive bleeding and bruising, and hearing loss.

One common major side effect of repeated use of many antibiotics is the adverse effect on natural human intestinal microflora. Exposure to repeated therapeutic doses of antimicrobials may act as a perturbant to the normal human intestinal microflora, bringing about changes in its composition and population density. Normal intestinal microflora acts as a barrier to colonisation and infection of the gastro intestinal tract by potential pathogens (Chapter 1), also aiding in the metabolism of certain nutrients and some drugs. Adverse effects on the normal intestinal microflora, can impair resistance to intestinal colonisation, leading to an increased risk of the gastrointestinal tract being colonised by enteric pathogens (such as *Escherichia coli, Salmonella* spp. and *Clostridium difficile*).

Throughout history the use of naturally occurring plant materials to prevent and combat various forms of disease and infection is well documented (Chapter 1). Many plants have traditionally found use to prevent and combat infection, now modern scientific techniques are proving that a great many traditional medicines contain active components that could play a legitimate role as a complement to, or alternative for conventional antimicrobial therapies. Garlic is amongst the oldest of the traditional medicines. Its use is documented in ancient writings such as the Dead Sea Scrolls and the Codex Ebers Papyrus. Many ancient cultures recommended garlic as a treatment for intestinal ailments, and as a preventative measure for wound infections.

Increasing numbers of researchers are turning to natural medicines to find future antimicrobial therapies. Garlic is amongst the most popular in both research and commercial value. It is known to exert significant inhibitory effect against a broad spectrum of Gram positive and Gram negative bacteria (Rees *et al* 1993, Kumar

1998). There now exists a wealth of data (Koch 1985, Adetumbi 1983) documenting the inhibitory activity of garlic against numerous bacteria. From the results of these investigations, it can be seen that one group of bacteria (the Lactic Acid Bacteria) are very much less susceptible to inhibition by garlic extract than others. Rees (1993) stated Minimum Inhibitory Concentration (MIC) values of 1.0 mg ml^{-1} for *S. aureus* and 1.4 mg ml^{-1} for *E. coli* (both potential human pathogens) when treated with aqueous garlic extract, compared to MIC values of 16.7 mg ml^{-1} for *Lactobacillus acidophilus* and 17.5 mg ml^{-1} for *Lactobacillus casei* when treated with aqueous garlic extract. From this data it is possible to conclude that garlic more is inhibitory to some pathogenic species of bacteria than it is to beneficial human intestinal microflora.

Garlic has also proved effective in *in vitro* studies against *Mycobacterium tuberculosis, Helicobacter pylori, Shigellae flexneri, Klebsiella pneumoniae and Proteus mirabilis* (Koch 1985, Skyrme 1995). *In vitro* results have indicated that garlic may prove useful in the prevention and treatment *H. pylori* (O'gara *et al* 2000), a known cause of gastric ulcers in humans. Much of the data regarding the antimicrobial properties of garlic products has been complemented through clinical trial studies. Davis *et al* (1990) concluded that although *in vitro* studies on the use of garlic against *Cryptococcal* infections proved ineffective, when injected intravenously garlic extract was seen to reduce *Cryptococcus neoformans* activity in the cerebrospinal fluid of patients with Cryptococcal meningitis. Chowdhury *et al* (1991) used a three day treatment of garlic to cure rabbits infected with *Shigellae flexneri.*

Much of the data obtained by *in vitro* analysis of the antibacterial efficacy of garlic relies on evaluation of the Minimum Inhibitory Concentration of garlic at 24 hours. MIC determination methods are extremely useful methods for screening the potential of new antibacterial preparations, and providing that methods are standardised appropriately (with respect to: inocula densities, preparation of garlic or other antibacterial agent under test, incubation conditions and measurement of culture growth) produce reliably reproducible data, even when dealing with volatile components. MIC data however provide relatively little information about the nature of inhibition, or modes of action of new antibiotics. Currently there are

few publications documenting the effects of garlic extract on growth of bacterial cultures. Clearly when considering any therapeutic antibacterial application of garlic, knowledge of inhibitory effects on the growth dynamics of target bacteria, along with adverse effects on those of normal microflora would be advantageous.

As a result of the increasing scientific and public interests in the medicinal properties of garlic, the last decade has seen a dramatic increase in the number of commercially available garlic preparations. The majority of these products contain garlic powder inside an inert capsule, usually manufactured by an independent supplier by freeze-drying fresh garlic cloves, then crushing to form a powder.

Some garlic producers use only carefully aged garlic in their preparations, claiming that volatile components of garlic (Chapter 5) such as allicin are not responsible for the majority of garlic's health benefits. The Wakunaga Corp., manufacturers of Kyolic aged garlic preparations claim that components present in this type of product are responsible for the *in vivo* lowering of blood pressure, reductions in serum levels of LDL cholesterol, and amongst others, increased antioxidant activity (Wakunaga of America Co. 2004). No claims are made regarding the antibacterial activity of aged garlic preparations by the manufacturers of Kyolic. Kyolic is prepared by soaking sliced raw garlic in aqueous ethanol for 10 months at room temperature, the extract is then filtered and concentration by reduced-pressure evaporation at low temperatures. The resulting extract is processed and sold as an odourless tablet. The most abundant constituent of Kyolic is S-allyl cysteine, the low temperature aging process ensures that concentrations of volatile oil soluble components usually associated with garlic are kept at a minimum (Wang 1998).

Recently synthetic allicin products have become available; these claim to contain allicin in a stable form while retaining *all* of its ascribed health benefits. The precise methods for stabilising allicin are trade secrets, but the manufacturer claims to have modified the enzymic production of allicin from alliin to produce a stable form. Some manufacturers (Allimax^{TM}) of 'stabilised allicin' claim that their products exceed fresh garlic in antibacterial efficacy.

The aims of the work presented in this study were firstly to evaluate the antibacterial action of aqueous garlic extract against a selection of human commensal and transient bacteria using a microtitre broth bioassay MIC method (*Escherichia coli* (chapter 1), *Lactobacillus casei* (chapter 1), *Staphylococcus aureus*, Methicillin Resistant *S. aureus*, *Enterococcus faecium*, *Salmonella enteretidis*, Vancomycin Resistant *Enterococcus*).

Enterococci are Gram positive *cocci*. They are physiologic commensal bacteria in the gastrointestinal tract and female genital tract of humans and other mammals (as well as birds) (Koch S *et ak* 2004). Whilst they usually pose no threat to the animals whose tract they inhabit, on occasion they can be responsible for serious systemic infection (Koch S *et al* 2004). Several *Enterococcal* strains have developed resistance to conventional antibiotics; the most dangerous of these strains are known as Vancomycin resistant *Enterococci* (VRE). VRE are resistant to most commonly used antibiotics including vancomycin.

Salmonella enteritidis is a Gram positive *bacillus*. It is a transient member of the intestinal microflora of humans, other mammals and birds. It is amongst the most commonly reported causes of food-poisoning associated diarrhoea in the world (Gurtler and Fehlhaber 2004).

Staphylococcus aureus is commonly found colonising the skin and nasal tracts of humans. Whilst it poses no threat to the majority of carriers, it can lead to severe infections in the immuno compromised. MRSA (Methicillin resistant *S. aureus*) is the antibiotic resistant form of *S. aureus*. Infections arising from *S. aureus* and MRSA, pathogenicity and resistance mechanisms are discussed in detail in Chapter 4.

The general effects of aqueous garlic extract on bacterial growth in aerobic conditions at 37°C was then examined by monitoring optical density at 540nm using a Bioscreen method. Growth inhibition of *E. coli* and *L. casei* was then examined in detail using the same Bioscreen method. Biocidal activity of aqueous garlic extract against *E. coli* and *L. casei* was investigated by suspension testing, using a neutralising broth and viable cell counts. This method was designed to

remove any residual inhibitory effects from the garlic extract, ensuring that counts colony forming unit give a true representation of live cell numbers at any time point. Results obtained in this manner obviate the ambiguities of some previous publications which used absence of growth on sub-culturing as an indication of biocidal activity (these quantifications make no allowance for residual biostatic inhibition from garlic extract inside and around the cell).

Growth inhibition of *E. coli* by garlic powder extract dissolved in Nutrient broth and Tryptone soya broth were compared. Finally the inhibitory actions of different commercially availably garlic preparations on aerobic growth of *E. coli* were (garlic powder extract, freshly crushed garlic clove extract, Kyolic aged garlic tablets, and Allimax stabilised allicin) compared.

Materials and Methods.

3.2.1 Minimum Inhibitory Concentration (MIC) analysis of Garlic Powder.

MIC Analysis of garlic powder was carried out using a microtitre broth dilution method. Stock concentrations of garlic were prepared as described in Chapter 2. Stock concentrations were 20 mg ml^{-1} for the *L. casei* experiments and 6 mg ml^{-1} for all other MIC experiments. Stocks were prepared in stoppered glass universal bottles. Dilutions were prepared from the stock concentration, firstly at intervals of 0.25 mg ml^{-1}, to obtain an approximate MIC value. The experiment was then repeated using concentrations of aqueous garlic extract at intervals of 0.1 mg ml^{-1} (at a range of 1.0 mg ml^{-1} around the approximate MIC value). Dilution series were prepared in sterile glass universal bottles. 100 µl aliquots of each dilution were pipetted into individual wells on a sterile flat bottomed polystyrene 96 well plate. Each well was then inoculated with 100 µl of appropriate cell suspension in Nutrient Broth (approx. $2x10^6$ CFU ml^{-1}). Negative controls contained 100 µl of garlic dilution with 100 µl of sterile nutrient broth; positive controls contained 100 µl of sterile Nutrient Broth with 100 µl of cell suspension in Nutrient Broth. Plates were then placed in a plastic container, a moist paper towel was placed on the base of the container to prevent evaporative loss from wells (NCCLS 2003). Plates were then incubated aerobically for 24 hours at 37°C. On 24 hours, optical densities were read in a multiwell plate reader with 450 nm filter. The MIC was taken to be the lowest inoculated concentration which prevented growth of bacteria. The IC50 value (concentration at which 50% of culture growth is inhibited) was taken as the concentration of garlic needed to achieve a 50% reduction in culture optical density compared to the control culture (where no is garlic present). Each optical density reading was taken in duplicate, and two experimental repeats were carried out (using independently raised inocula).

3.2.2 The effect of Garlic Powder on Bacterial growth.

Bacterial species were screened for garlic inhibition, using a Bioscreen C analyser (Labsystems, Helsinki). A general screen was carried out against *S. aureus, E. faecium, E. coli, L. casei,* Methicillin resistant *S. aureus* (MRSA) and

Vancomycin resistant *Enterococcus* using garlic powder extract serial dilutions in a broad range. Detailed analyses were carried out on *E. coli* (101418) and *L. casei.*

Stock garlic concentrations and dilutions were prepared as described previously (3.2.1). Aliquots (150 µl) were transferred to wells on a polystyrene 100 well Bioscreen plate. Wells were then inoculated with 150 µl of cell suspension in Nutrient Broth (approx 2 x 10^6 CFU ml^{-1}). Plates were then placed in the Bioscreen analyser (Incubation time 24 hours, temperature 37°C, recording intervals 10 minutes, 10 seconds of intensive shaking pre and post measurement). Growth was monitored by changes in optical density at 540 nm. The specific growth rate (µ) h^{-1} (rate of change in optical density ÷ optical density of initial culture) was calculated from logarithmic plots of the mid-exponential growth phase for each culture. Each experiment was performed in triplicate, and repeats were carried out for the *E. coli* and *L. casei* analyses. Results are tabulated as means and standard deviation of the 3 experiments.

3.2.3 Microscopy

Samples were taken from *E. coli* cultures following a 24 hour incubation (37°C) with garlic extracts at 1.0 mg ml^{-1}, 2.0 mg ml^{-1}, and 3.0 mg ml^{-1} in Nutrient Broth (prepared as described earlier). These samples were compared with a control sample taken from a control culture of *E. coli* incubated for 24 hours in Nutrient Broth (37°C), using a phase contrast (Olympus, Tokyo) microscope equipped with digital camera (Olympus, Tokyo).

3.2.4 Bactericidal effects of garlic against *E. coli* and *L. casei.*

Bactericidal effects were investigated using a suspension test method. Garlic stock was prepared in PBS buffer, subsequent dilution were prepared from this by further dilutions in PBS buffer. Bacterial cells were suspended to the appropriate density a range of garlic concentrations in PBS buffer. At timed intervals 1 ml aliquots were removed into sterile micro-centrifuge tubes, and centrifuged gently in a desktop micro-centrifuge in order to pellet. Pellets were washed with fresh PBS buffer (to remove traces of garlic extract) centrifuged, then re-suspended in fresh PBS buffer (1 ml). The samples were then added to 9 ml of Beckton and Dickinson B/E neutralising agent for 10 minutes (to remove residual bactericidal

activity from the garlic extract). Serial dilutions of each sample were prepared. 10µl of each serial dilution was dropped onto Diagnostic Sensitivity Testing (DST) agar (pre-dried for 30 minutes), and resulting colonies were counted after a 24-hour incubation period. Plates were re-checked following a further 24-hour incubation for further colony growth. Drop counts were performed in triplicate, and two repeats were carried out for each experiment.

3.2.5 The effects of commercially available garlic preparations on growth in *E. coli.*

Three commercial garlic products were tested for antibacterial activity against *E. coli* and their effects on growth curves of *E. coli* were investigated. The products tested were Kyolic™ (aged garlic tablets), Allimax™ (100% 'stabilised allicin'), and fresh garlic cloves. Stock solutions of Kyolic ™ were prepared by grinding the tablets into a fine powder using a pestle and mortar, this powder was suspended to required concentrations in Nutrient Broth. The suspension was stirred for 10 minutes, then left to stand at room temperature for 30 minutes. The suspension was then centrifuged and passed through a 0. 2 µm sterile syringe filter. This stock was immediately used to prepare required dilutions. Stock concentration of Allimax™ was prepared by removing the Allimax powder from the gelatine capsules. This powder was then used to make a suspension of the required concentration in Nutrient Broth. The suspension was then treated in the same manner as the Kyolic™ suspension. Fresh garlic cloves were removed from the bulb, peeled and crushed then pulverised using a pestle and mortar. This garlic paste was then added to Nutrient Broth to the required concentration. The fresh garlic suspension was then treated in the same manner as the two other suspensions. Prepared dilutions of the three products were then used in MIC and Bioscreen analysis (against *E. coli*) following the same protocol that was used for garlic powder (3.2.2). The specific growth rate (µ) h^{-1} (rate of change in optical density ÷ optical density of initial culture) was calculated from logarithmic plots of the mid-exponential growth phase for each culture. Each experiment was performed in triplicate and repeated.

3.3 Results

3.3.1 MIC Analysis.

MIC and IC50 values are presented in **table 3.3.1**. All bacteria tested here were subject to growth inhibition by garlic to some degree. The bacteria clearly very much less susceptible to garlic inhibition was *Lactobacillus casei* - a member of the Lactic Acid Bacteria (LAB) group. *L. casei* had an MIC value of 16.9 mg ml^{-1}. All other bacteria tested had MIC values equal to or lower than 5.5 mg ml^{-1} garlic extract. The MIC of garlic powder extract against *Enterococcus faecium* was 5.5 mg ml^{-1}, making it next least susceptible. The *Salmonella,* and *Shigellae* strains tested all had similar MIC values to garlic (3.1 mg ml^{-1} and 3.3 mg ml^{-1} respectively). Of the three strains of *E. coli* tested here, NCTC 10418 was the most susceptible (MIC 1.8 mg ml^{-1}), then *E. coli* C2 (MIC 1.9 mg ml^{-1}), ATCC was the least susceptible *E. coli* strain to garlic powder (MIC 2.7 mg ml^{-1}). The organism most susceptible to garlic inhibition was *Staphylococcus aureus* (MIC) 1.0 mg ml^{-1}).

3.3.2 Effects of garlic powder extracts on bacterial growth

Inhibitory effects of garlic powder extract against 6 different bacteria were investigated. Strains chosen for analysis were *S. aureus,* Methicillin resistant *S. aureus, E. faecium,* Vancomycin resistant *Enterococcus, E. coli, and L. casei.* A general screen was carried out to assess how garlic extract affected the growth curve of each species. A detailed analysis of the effect of garlic on the growth of *E. coli* and *L. casei* was then conducted.

3.3.2.1 Garlic and *S. aureus.*

S. aureus control cultures produced the classic bacterial growth profile (figure 3.3.2.1 and table 3.3.2.1) - consisting of an initial lag phase leading into an exponential growth phase, which is followed by a stationary phase. Decline phase was not observed in any of the *S. aureus* cultures, one reason for this may be that 24 hours was not long enough for waste product accumulation to reduce the

Bacterium	IC50 (mg ml^{-1})	MIC (mg ml^{-1})
Lactobacillus casei	14.3 ± 0.87	16.9 ± 1.13
Enterococcus faecium	4.9 ± 0.71	5.6 ± 0.77
Klebsiella pneumoniae	3.5 ± 0.08	5.5 ± 0.92
Shigellae flexneri	2.5 ± 0.59	3.3 ± 0.86
Salmonella enteritidis	1.9 ± 0.15	3.1 ± 0.97
E. coli NCTC 10418	1.4 ± 0.04	1.8 ± 0.24
E. coli C2	1.0 ± 0.02	1.9 ± 0.36
Staphylococcus aureus	0.7 ±0.01	1.0 ± 0.11
Methicillin Resistant *S. aureus 4500*	0.9 ± 0.01	2.2 ± 0.31
Methicillin Resistant *S. aureus 4467*	0.7 ± 0.01	1.0 ± 0.18

Table 3.3.1 MIC and IC50 values for selected bacteria following a 24 hour aerobic incubation at 37°C (mean values and standard deviations of three independent repeats).

optical density of the stationary phase through lytic cell death. The culture optical density continued to rise at a slower rate after the exponential growth phase. The initial lag phase seen in control cultures here is relatively short, 113 minutes (Table 3.3.2.1) The specific growth rate (μ) was 2.96 in the control culture, and optical density of the culture at the end of the 34 hour measurement period was 1.266 Absorbance units (from calibration this corresponds to a culture density of approximately 4 x 10^8 Colony Forming Units ml^{-1} see appendix for calibration). The primary effect of garlic extract on the growth curve of *S. aureus* appears to be an extension in the duration of the initial lag phase (lag phases of 327 minutes and 903 minutes in cultures incubated with 1.0 and 2.0 mg ml^{-1} garlic respectively), followed by a recovery into the exponential growth phase. Cultures incubated with garlic at concentrations of 3.0mg ml^{-1} and above did not show a recovery during the 24 hour measurement period. The Specific Growth Rate (SGR) (μ) (h^{-1}) appeared slower on addition of garlic extract, increasing the garlic concentration did not produce a further reduction in the mid exponential growth rate. The rate of increase in optical density post-exponential phase was not affected by garlic extract concentration.

3.3.2.2 Garlic and Methicillin resistant *S. aureus*.

MRSA control cultures produced growth curves similar to *S. aureus* (figure 3.3.2.2 and table 3.3.2.2), however the Specific growth rate (SGR) was not as rapid in MRSA cultures (2.96 h^{-1}), and the culture achieved a greater optical density at 24 hours (1.582 abs units). Again no decline phase was visible during the experiment, and culture optical density continued to rise after the exponential growth phase. Duration of the initial lag phase was longer in MRSA cultures (113 minutes). On addition of garlic extract again an extension in the initial lag phase was seen (lag phases of 327 minutes, and 1127 minutes on addition of 1.0 and 2. mg ml^{-1} garlic extract respectively. Garlic exerted an inhibitory effect on the SGR of the MRSA cultures tested, μ fell from 2.96 h^{-1} to 2.40 h^{-1}, on addition of 1.0mg ml^{-1} garlic extract, and to 2.3 h^{-1} on addition of 2.0mg ml^{-1} garlic extract. Optical densities of the cultures at 24 hours fell as the garlic extract concentration increased (1.582 abs, 1.521 abs, 1.152 abs, and 0.120 abs for 0 mg ml^{-1}, 1.0 mg ml^{-1}, 2.0 mg ml^{-1}, and 3.0 mg ml^{-1} respectively). The rate of increase in optical density

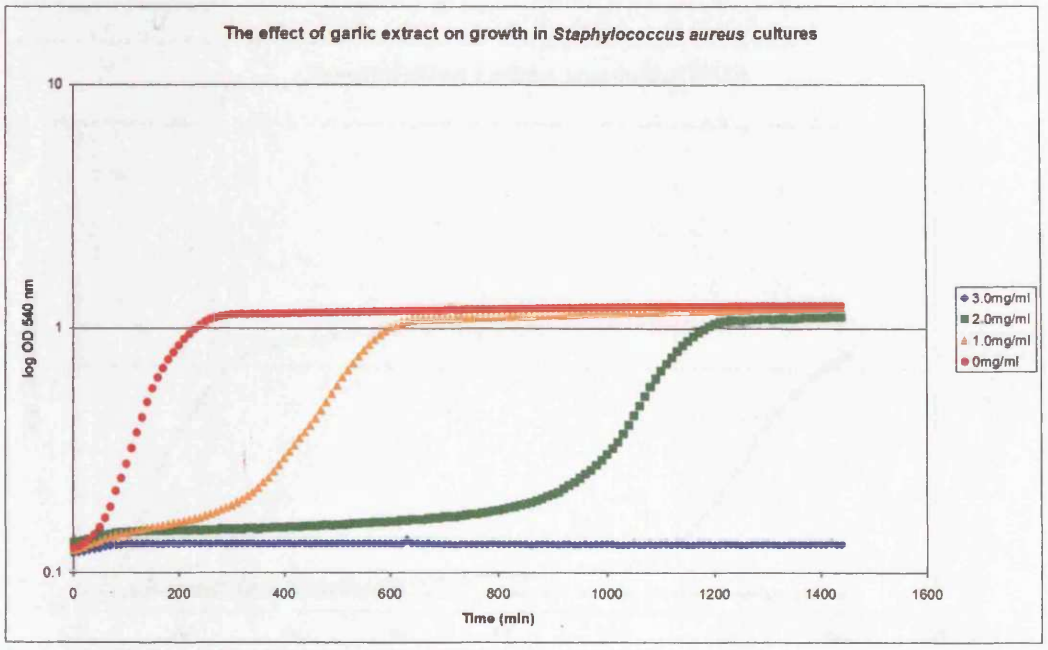


Figure 3.3.2.1 The effect of garlic powder extract on the growth curve of *S. aureus* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	1.0	2.0	3.0
Duration of initial lag phase (min)	73.33 ±11.55	345.67 ±16.92	901.00 ±10.15	>1440 -
Specific growth rate ($hour^{-1}$)	2.96 ±0.40	2.54 ±0.13	2.46 ±0.12	-
Optical density (540nm) at 24 hours	1.265 ±0.006	1.236 ±0.006	1.137 ±0.055	0.143 ±0.009

Table 3.3.2.1. The effects of garlic powder extract on growth kinetics of *S. aureus* (Figures presented are means and standard deviations of 3 independent repeats).

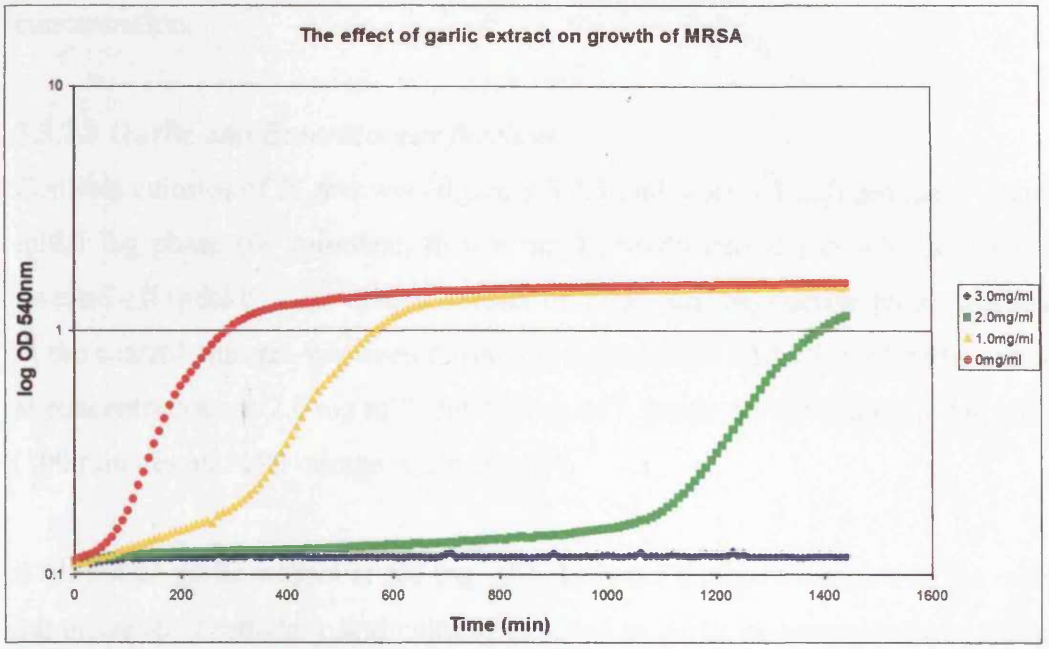


Figure 3.3.2.2 The effect of garlic powder extract on the growth curve of methicillin resistant *S. aureus* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	1.0	2.0	3.0
Duration of initial lag phase (min)	113.33 ± 5.77	326.67 ± 5.77	1126.67 ± 5.77	>1440
Specific growth rate ($hour^{-1}$)	2.96 ± 0.09	2.40 ±0.06	2.3 ± 0.03	-
Optical density (540nm) at 24 hours	1.582 ± 0.037	1.521 ± 0.101	1.152 ± 0.018	0.120 ± 0.52

Table 3.3.2.2. The effects of garlic powder extract on growth kinetics of methicillin resistant *S. aureus*. (Figures presented are means and standard deviations of 3 separate experiments).

after the exponential growth phase did not appear to be affected by garlic concentration.

3.3.2.3 Garlic and *Enterococcus faecium*

Controls cultures of *E. faecium* (figure 3.3.2.3 and table 3.3.2.3) produced a short initial lag phase (67 minutes), then a rapid growth period (μ= 4.36 h^{-1}), which levelled off quickly at an optical density of 1.070 abs. No decline phase was seen in the control cultures was seen during the experiment. Addition of garlic extract at concentrations at 2.0 mg ml^{-1} and 4.0 mg ml^{-1}, produced extensions in lag phase (100 minutes and 190 minutes respectively).

Addition of garlic extract at 6.0 mg ml^{-1} produced a large extension in the initial lag phase (640 minutes), and cultures exposed to garlic concentrations of 8.0 mg ml^{-1} an d above did not show recovery during the experiment. Garlic extract also exerted a marked effect on the SGR, with rates of 4.36 h^{-1}, 3.81 h^{-1}, 2,96 h^{-1}, and 2.53 μ in cultures containing 0, 2.0, 4.0, and 6.0 mg ml^{-1}. Along with the garlic extract induced decrease in exponential growth rates, a decrease in culture optical density on 24 hours was also seen. The action of garlic on *E. faecium* differed to the action of garlic on *S. aureus*, at concentration of 2.0 and 4.0 mg ml^{-1} garlic appeared to induce a decline phase – not visible in the control cultures.

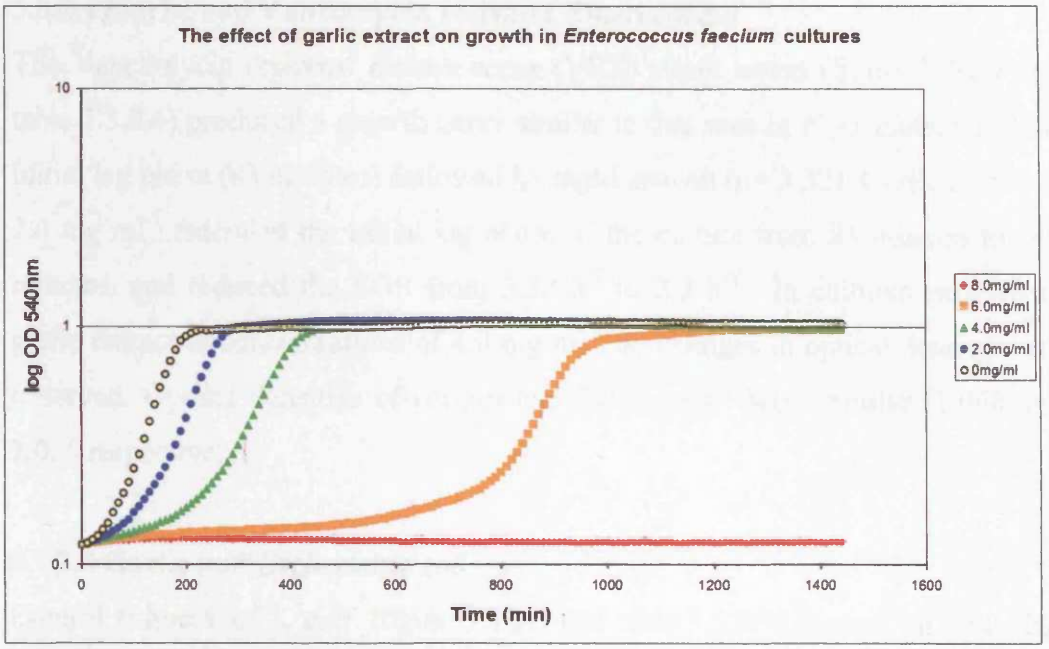


Figure 3.3.2.3 The effect of garlic powder extract on the growth curve of *E. faecium* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	2.0	4.0	6.0	8.0
Duration of initial lag phase (min)	66.67 ± 5.78	100.00 ± 10.0	190.00 ± 20.0	640 ± 20.0	>1440
Specific growth rate ($hour^{-1}$)	4.36 ± 0.11	3.81 ± 0.05	2.96 ± 0.03	2.53 ± 0.06	-
Optical density (540nm) at 24 hours	1.070 ±0.031	1.060 ±0.033	0.999 ±0.067	0.910 ±0.012	0.126 ±0.046

Table 3.3.2.3 The effects of garlic powder extract on growth kinetics of *E. faecium* (Figures presented are means and standard deviations of 3 separate experiments).

3.3.2.4 Garlic and Vancomycin resistant *Enterococcus*

The Vancomycin resistant *Enterococcus* (VRE) strain tested (figure 3.3.2.4 and table 3.3.2.4) produced a growth curve similar to that seen in *E. faecium*, i.e. short initial lag phase (83 minutes) followed by rapid growth (μ= 3.32). Garlic extract at 2.0 mg ml^{-1} extended the initial lag phase of the culture from 83 minutes to 147 minutes, and reduced the SGR from 3.32 h^{-1} to 2.2 h^{-1} . In cultures exposed to garlic extract at concentrations of 4.0 mg ml^{-1} no changes in optical density were observed. Optical densities of control and 2.0 mg ml^{-1} were similar (1.066 and 1.055 respectively).

3.3.2.5 Garlic and *Escherichia coli*

Control cultures of *E. coli* (figure 3.3.2.5 and table 3.3.2.5) showed an initial lag phase of approximately 40 minutes. After the exponential growth period culture optical density declined through the remaining test period. The most obvious effect of garlic extract on the growth curve of *E. coli*, was an extension in duration of the initial lag phase, as an approximation with every addition of 0.5 mg ml^{-1} duration of the initial lag phase doubled. (40, 90, 177, 320, 879 minutes, for cultures containing 0.5, 1.0, 1.5, and 2.0 mg ml respectively).

No significant effect of garlic extract on the SGR was apparent – growth rates for all concentrations tested were between 1.02 h^{-1} and 1.14 h^{-1}. As garlic concentration increased the culture optical density at 24 hours also increased, this may well have been a result of the extension in initial lag phase – the exponential growth phase, and onset of decline phase being delayed.

3.3.2.6 Garlic and *Lactobacillus casei*

Lactobacillus casei control cultures produced a classic sigmoid bacterial growth curve. An initial lag phase of 160 minutes was followed by a period of growth (μ 1.6), which led into a final stationary phase. On addition of garlic extract to the cultures no extension in duration of the initial lag phase was observed.

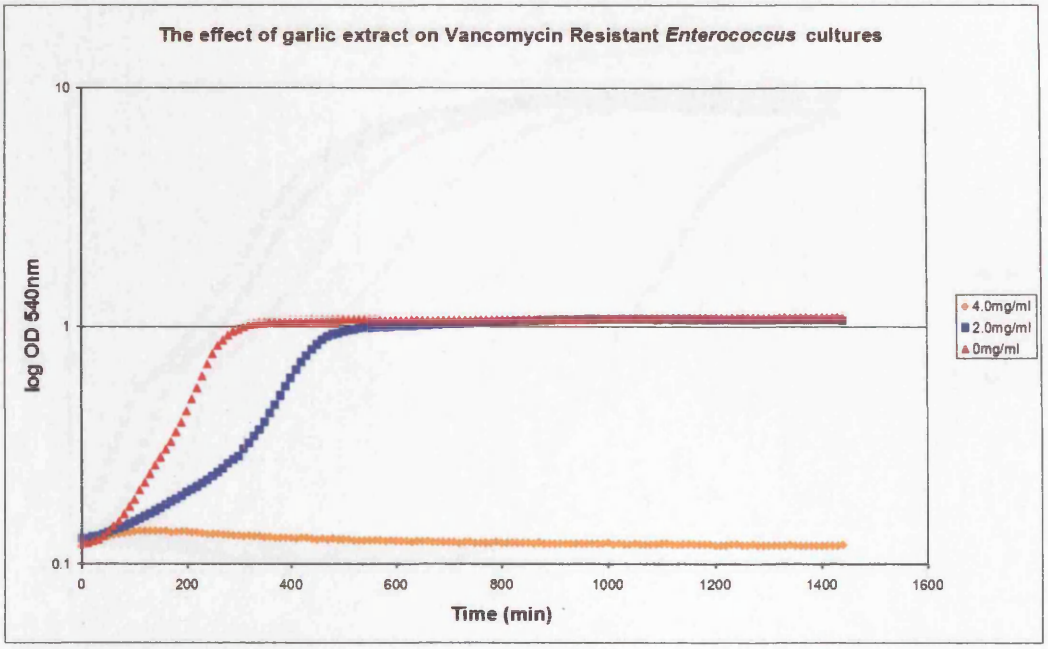


Figure 3.3.2.4 The effect of garlic powder extract on the growth curve of Vancomycin resistant *Enterococcus* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	2.0	4.0
Duration of initial lag phase (min)	83.33 ± 5.78	146.67 ± 5.78	>1440
Specific growth rate ($hour^{-1}$)	3.32 ± 0.58	2.20 ± 0.25	-
Optical density (540nm) at 24 hours	1.066 ± 0.032	1.055 ± 0.028	0.119 ± 0.043

Table 3.3.2.4 The effects of garlic powder extract on growth kinetics of Vancomycin resistant *Enterococcus* (Figures presented are means and standard deviations of 3 separate experiments).

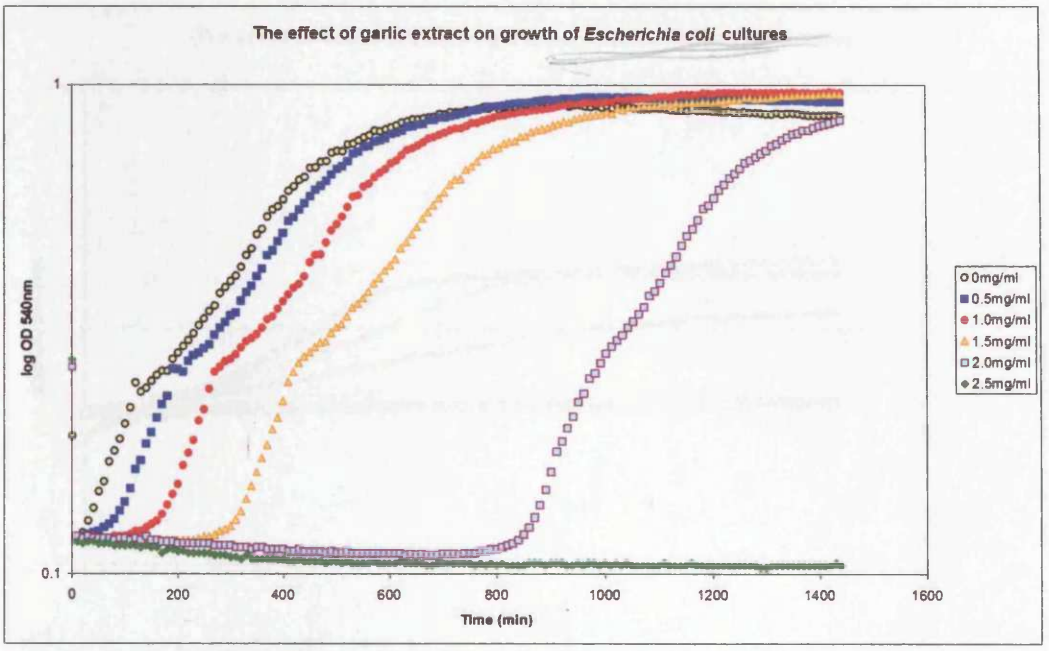


Figure 3.3.2.5 The effect of garlic powder extract on the growth curve of *Escherichia coli* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	0.5	1.0	1.5	2.0	2.5
Duration of initial lag phase (min)	40.00 ±10.00	90.00 ±10.00	176.67 ±5.78	320.00 ±10.00	880.00 ±10.00	>1440
Specific growth rate ($hour^{-1}$)	1.12 ± 0.07	1.02 ± 0.05	1.16 ± 0.03	1.08 ± 0.06	1.14 ± 0.02	-
Optical density (540nm) at 24 hours	0.850 ±0.033	0.900 ±0.029	0.970 ±0.047	0.961 ±0.022	0.909 ±0.041	0.105 ±0.036

Table 3.3.2.5 The effects of garlic powder extract on growth kinetics of *Escherichia coli* (Figures presented are means and standard deviations of 3 separate experiments).

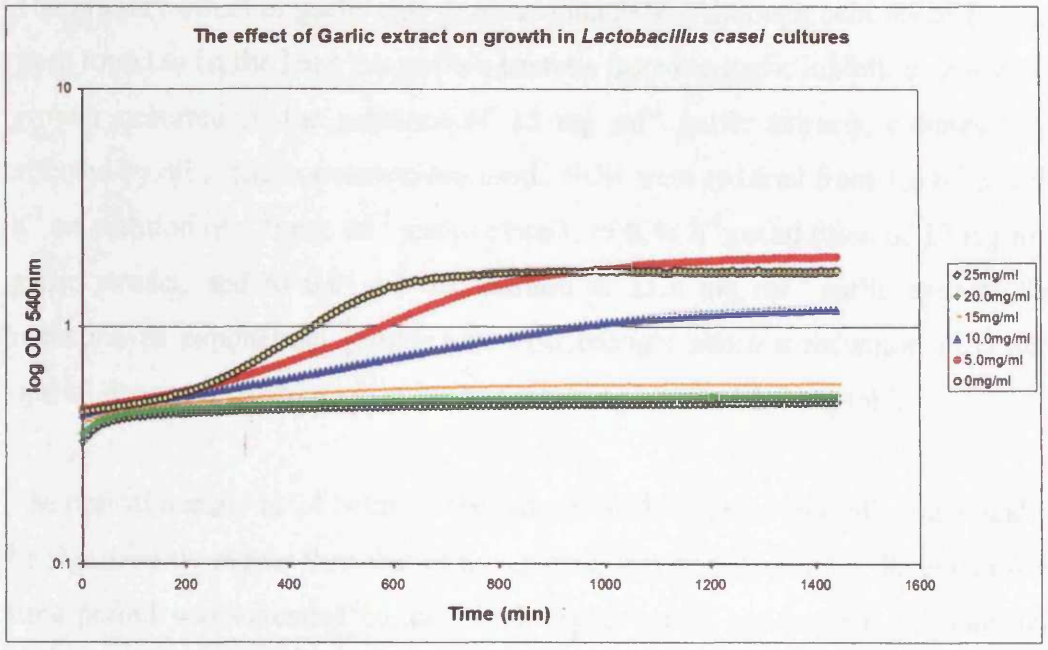


Figure 3.3.2.6 The effect of garlic powder extract on the growth curve of *Lactobacillus casei* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	5.	10.0	15.0	20.0
Duration of initial lag phase (min)	160.00 ±20.00	160.00 ±10.00	160.00 ±10.00	160.00 ± 10.00	>1440
Specific growth rate ($hour^{-1}$)	1.60 ±0.07	1.02 ±0.12	0.48 ±0.06	0.01 ±0.01	-
Optical density (540nm) at 24 hours	1.765 ±0.029	1.953 ±0.043	1.183 ±0.026	0.569 ±0.019	0.378 ±0.038

Table 3.3.2.6 The effects of garlic powder extract on growth kinetics of *Lactobacillus casei* (Figures presented are means and standard deviations of 3 separate experiments).

The primary effect of garlic was to reduce the SGR. Although cultures of *L. casei* were found to be the least susceptible bacteria tested to garlic inhibition (some cell growth occurred in the presence of 15 mg ml^{-1} garlic extract), cultures were affected by all garlic concentrations used. SGR were reduced from 1.6 h^{-1} to 1.02 h^{-1} on addition of 5.0 mg ml^{-1} garlic extract, to 0.48 h^{-1} on addition of 10 mg ml^{-1} garlic extract, and to 0.01 h^{-1} on addition of 15.0 mg ml^{-1} garlic extract. The reduction in exponential growth rate also brought about a reduction in culture optical density at 24 hours in cultures containing 10.0 to 20.0 mg ml^{-1}.

The optical density at 24 hours of the culture containing 5.0 mg ml^{-1} was found to be significantly higher than that of the control, it is possible that if the experiment time period was extended cultures with higher garlic concentrations could also achieve elevated optical densities. Out of all the bacteria tested here, *L. casei* is at least 3 times less susceptible to garlic inhibition than other species, and that the nature of the garlic inhibition is manifested in a significantly different way.

3.3.2.7 Detailed analysis of garlic effects on *E. coli* and *L. casei* cultures.

Closer analysis of the inhibitory action of garlic against *E. coli* **(Table 3.3.2.7)** proved that garlic extract extended duration of the initial lag phase in a dose-dependent manner, even at extremely low concentrations (<0.25 mg ml^{-1}). An increase in final optical density also became apparent on addition of 0.25 mg ml^{-1} to 2.0 mg ml^{-1}. Again garlic extract did not appear to effect the exponential growth rate.

Appearance of the garlic susceptible *E. coli* cultures was investigated using phase contrast microscopy following 24 hour incubations with 1 mg ml^{-1}, 2 mg ml^{-1}, 2.5 mg ml^{-1}, and 3mg ml^{-1} garlic extract. As the garlic concentration increased, appearance of the cells became less regular, individual cells became less obvious – with the majority of cells appearing to be massed together. Few cells or cell masses were seen at concentrations in excess of 2.0 mg ml^{-1}.

Conversely the major effect of garlic extract on growth in *L. casei* cultures was a dose dependant decrease in the slope of the exponential growth phase. Although cultures containing between 2.5 mg ml^{-1} to 5.0 mg ml^{-1} did not grow as rapidly as

the control cultures, final optical densities were considerably higher – indicating higher growth levels at 24 hours. Phase contrast microscopy of showed little evidence of cell aggregation, Cell numbers were seen to decrease rapidly as garlic concentration exceeded 15 mg ml^{-1}.

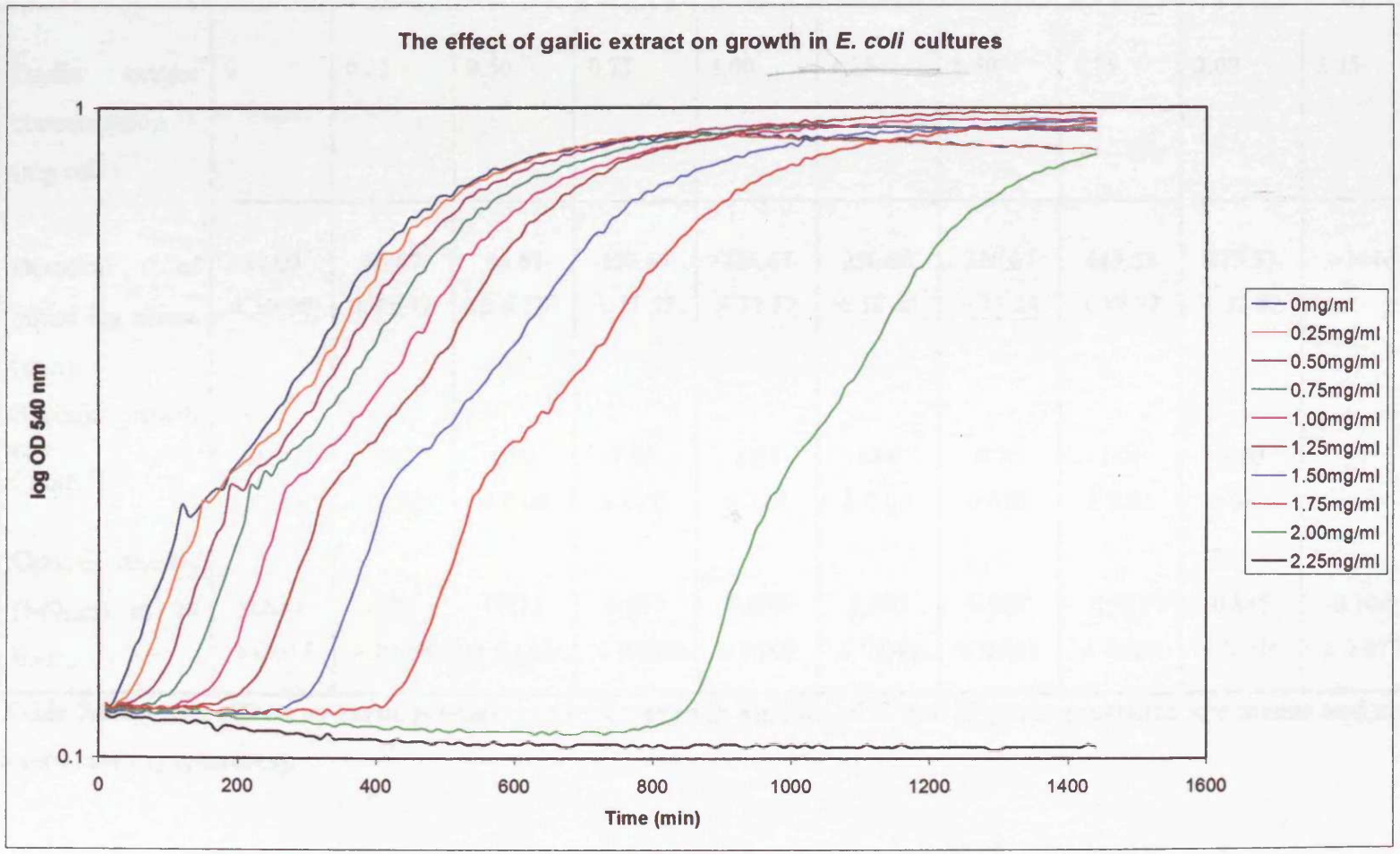


Figure 3.3.2.7 The effect of garlic powder extract on the growth curve of *E. coli* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	0.25	0.50	0.75	1.00	1.25	1.50	1.75	2.00	2.25
Duration of initial lag phase (min)	40.00 ± 10.00	66.67 ± 11.57	96.67 ± 6.12	126.67 ± 11.57	186.67 ± 11.57	230.00 ± 10.00	336.67 ± 15.28	443.33 ± 17.32	873.33 ± 21.82	>1440
Specific growth rate ($hour^{-1}$)	0.96 ± 0.03	1.02 ± 0.03	0.96 ± 0.06	0.95 ± 0.03	1.01 ± 0.03	1.04 ± 0.02	0.95 ± 0.03	1.01 ± 0.01	1.00 ± 0.04	-
Optical density (540nm) at 24 hours	0.830 ± 0.017	0.884 ± 0.028	0.913 ± 0.011	0.037 ± 0.032	0.977 ± 0.009	1.005 ± 0.066	0.987 ± 0.041	0.944 ± 0.020	0.845 ± 0.016	0.104 ± 0.077

Table 3.3.2.7 The effects of garlic powder extract on growth kinetics of *E. coli* (Figures presented are means and standard deviations of 3 separate experiments).

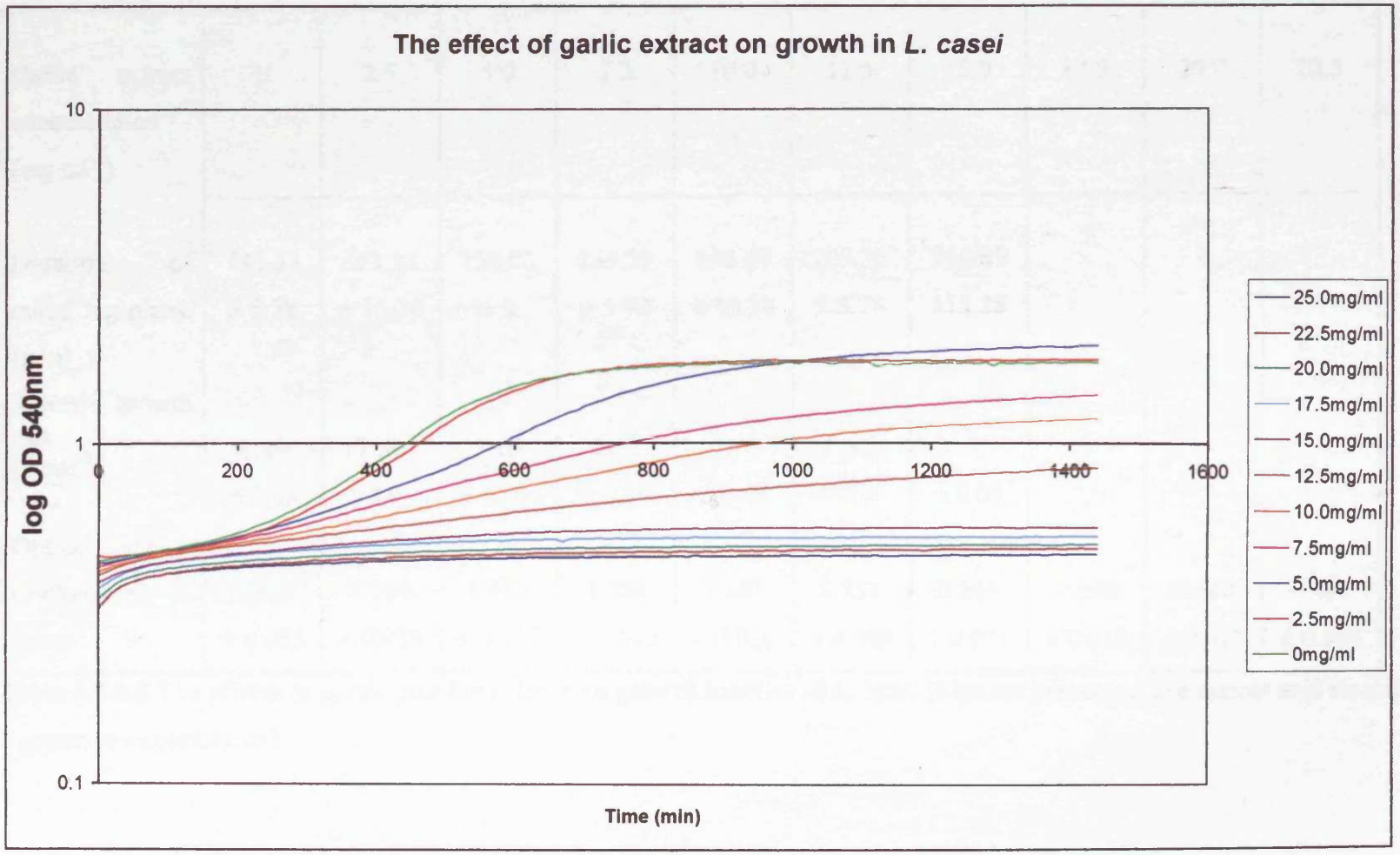


Figure 3.3.2.8 The effect of garlic powder extract on the growth curve of *Lactobacillus casei* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	22.5
Duration of initial lag phase (min)	143.33 ± 5.78	153.33 ± 15.28	150.00 ± 0	163.33 ± 5.78	176.67 ± 15.28	203.33 ± 5.78	346.67 ±15.28	-	-	-
Specific growth rate ($hour^{-1}$)	1.48 ± 0.03	1.50 ± 0.03	1.10 ± 10.06	0.52 ± 0.06	0.36 ± 0.03	0.17 ± 0.03	0.01 ± 0.03	-	-	-
Optical density (540nm) at 24 hours	1.726 ± 0.055	1.766 ± 0.028	1.953 ± 0.017	1.394 ± 0.022	1.183 ± 0.026	0.851 ± 0.063	0.569 ± 0.021	0.538 ± 0.010	0.510 ± 0.011	0.495 ± 0.034

Table 3.3.2.8 The effects of garlic powder extract on growth kinetics of *L. casei* (Figures presented are means and standard deviations of 3 separate experiments).

3.3.3 Further investigations into the effect of garlic extract on growth in *E. coli* cultures.

Growth curve analysis was carried out to determine the effects of higher doses of garlic extract against *E. coli* over a longer test period (Figure 3.3.3.0, table 3.3.3.0) During a 48 hour test period the same lag phase extension and recovery pattern was seen. Cultures exposed to garlic extract at 2.5 mg ml^{-1} showed recovery at 2300 min, exponential growth rate was not affected, optical density at 48 hours is apparently lower than seen in recovered cultures; however stationary phase had not been reach in all cultures exposed to 2.5 mg ml^{-1}.

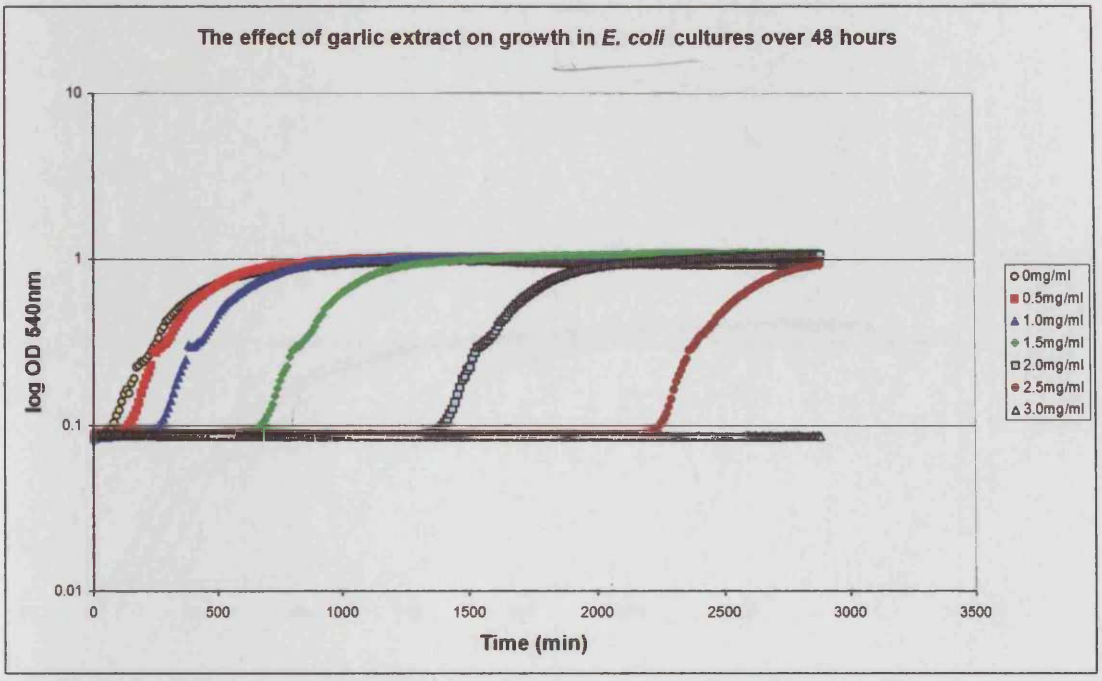
3.3.3.1 Heated garlic extract

The next experiment investigated the effect of heated garlic powder extract on the growth of *E. coli*. Garlic extract stock was prepared as previous, then heated to 96°C for 30 minutes. The active constituents of this heated extract are known to be mostly vinyl dithiins, with little allicin content (chapter 4). Heated garlic extract inhibited growth in *E. coli* at a greatly reduced capacity (table 3.3.3.1), at concentrations of 2.5 mg ml^{-1} there was no significant effect on the exponential growth rate or the duration of initial lag phase, as the concentration of garlic extract exceeded 3.0 mg ml^{-1} an extension in the initial lag phase became clear, albeit to a much lesser extent than when fresh garlic powder extract was tested, with 10 mg ml^{-1} producing an initial lag phase of 170 minutes. As concentration of garlic extract was increased, the optical density at 24 hours also increased.

3.3.3.2 The effect of commercially available garlic preparations on growth in *E. coli.*

These experiments were carried out using Tryptone soy broth (TSB) (Oxoid, Basingstoke) as growth medium, Tryptone soy broth stimulated faster growth and higher yields of *E. coli* compared to nutrient broth. Effects of garlic preparations on *E. coli* growth are compared to controls (growth media only) also carried out in TSB.

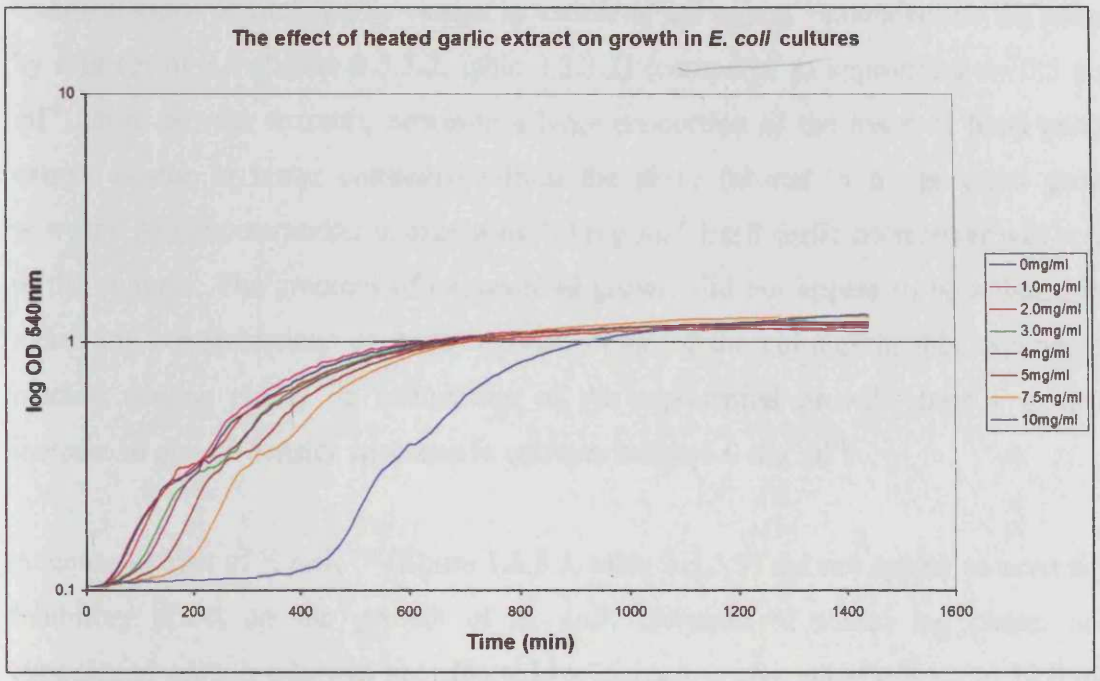
Inhibitory effects of freshly crushed garlic on growth in *E. coli* were investigated. The pattern of inhibition seen here, initial lag phase extension followed by culture recovery, was similar to the pattern seen when garlic powder was used. However extensions in initial lag phase were brought about by higher concentrations.



3.3.3.0 The effect of garlic powder extract on the growth curve of *E. coli* over 48 hours (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	0.5	1.0	1.5	2.0	2.5	3.0
Duration of initial lag phase (min)	70.00 ±10.0	103.33 ±5.78	160.00 ± 30.00	356.67 ±17.32	1036.67 ±34.64	2300.00 ±40	>2880
Specific growth rate ($hour^{-1}$)	1.20 ±0.13	1.27 ±0.13	1.13 ±0.07	1.20 ±0.07	1.20 ±0.07	1.16 ±0.01	-
Optical density (540nm) at 24 hours	0.928 ±0.010	1.053 ±0.028	0.934 ±0.030	1.056 ±0.044	1.058 ±0.041	0.859 ±0.022	0.085 ±0.019

Table 3.3.3.0 The effects of garlic powder extract on growth kinetics of *Escherichia coli* over 48 hours (Figures presented are means and standard deviations of 3 separate experiments).



3.3.3.1 The effect of heated garlic powder extract on the growth curve of *E. coli* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	1.0	2.0	3.0	4.0	5.0	7.5	10.0
Duration of initial lag phase (min)	50.00 ±10.00	40.00 ±10.00	50.00 ±20.00	60.00 ±10.00	73.33 ±5.78	90.00 ±10.00	100.00 ±20.00	170.00 ±30.00
Specific growth rate ($hour^{-1}$)	1.08 ±0.06	1.2 ±0.12	1.02 ±0.06	1.16 ±0.03	1.08 ±0.06	1.08 ±0.12	1.16 ±0.03	1.26 ±0.06
Optical density (540nm) at 24 hours	1.159 ±0.061	1.103 ±0.049	1.138 ±0.036	1.181 ±0.017	1.185 ±0.078	1.203 ±0.033	1.257 ±0.077	1.280 ±0.060

Table 3.3.3.1 The effects of heated garlic extract on growth kinetics of *Escherichia coli* (Figures presented are means and standard deviations of 3 separate experiments).

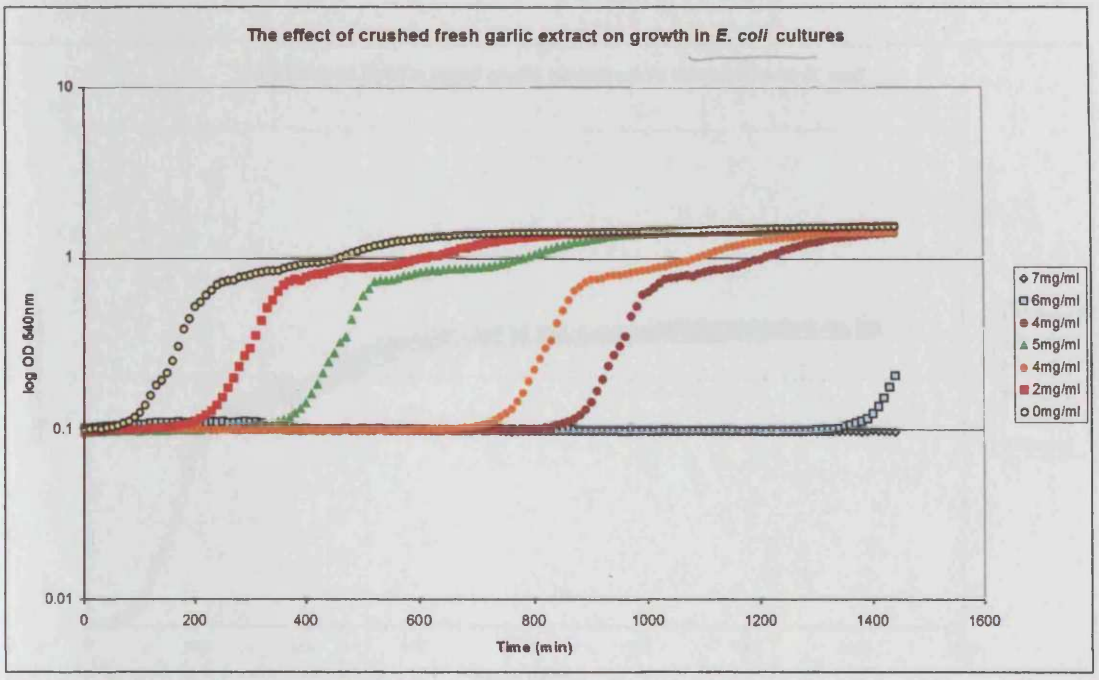
Concentrations of fresh garlic extract in excess of 2.0 mg ml^{-1} extended the lag phase by a factor of 2.2 (figure 3.3.3.2, table 3.3.3.2) (compared to approximately 0.5 mg ml^{-1} garlic powder extract), however a large proportion of the mass of fresh garlic extract is due to water contained within the clove (absent in freeze dried garlic powder). At concentrations in excess of 7.0 mg ml^{-1} fresh garlic no recover was seen in the cultures. The gradient of exponential growth did not appear to be affected by increasing concentrations of garlic extract. None of the cultures in this experiment reached decline phase, on completion of the exponential growth phase a gradual increase in optical density was seen in cultures below 6.0 mg ml^{-1}

Aqueous extract of Kyolic™ (figure 3.3.3.3, table 3.3.3.3) did not appear to exert any inhibitory effect on the growth of *E. coli*. Duration of initial lag phase, and exponential growth rate was not affected by increasing concentrations up to 10.0 mg ml^{-1}, however cultures incubated in the presence of Kyolic™ concentrations in excess of 3.0 mg ml^{-1} produced higher final optical densities at 540 nm.

Aqueous extract of Allimax™ 'stabilised allicin' did not exert significant inhibitory effect on growth of *E. coli* cultures in these tests (figure 3.3.3.4, table 3.3.3.4). A slight extension in the duration of lag phase was apparent (40 minute extension at 10.0 mg ml^{-1}), as concentration of Allimax™ was increased, specific growth rate in the cultures decreased slightly (from 3.4 h^{-1} in control cultures, to 3.18 h^{-1} in 10.0 mg ml^{-1} cultures). The predominant effect of Allimax™ was an increase in culture optical density at 24 hours (corresponding to increasing Allimax™ concentration).

3.3.4.0 Bactericidal effects of garlic powder extract

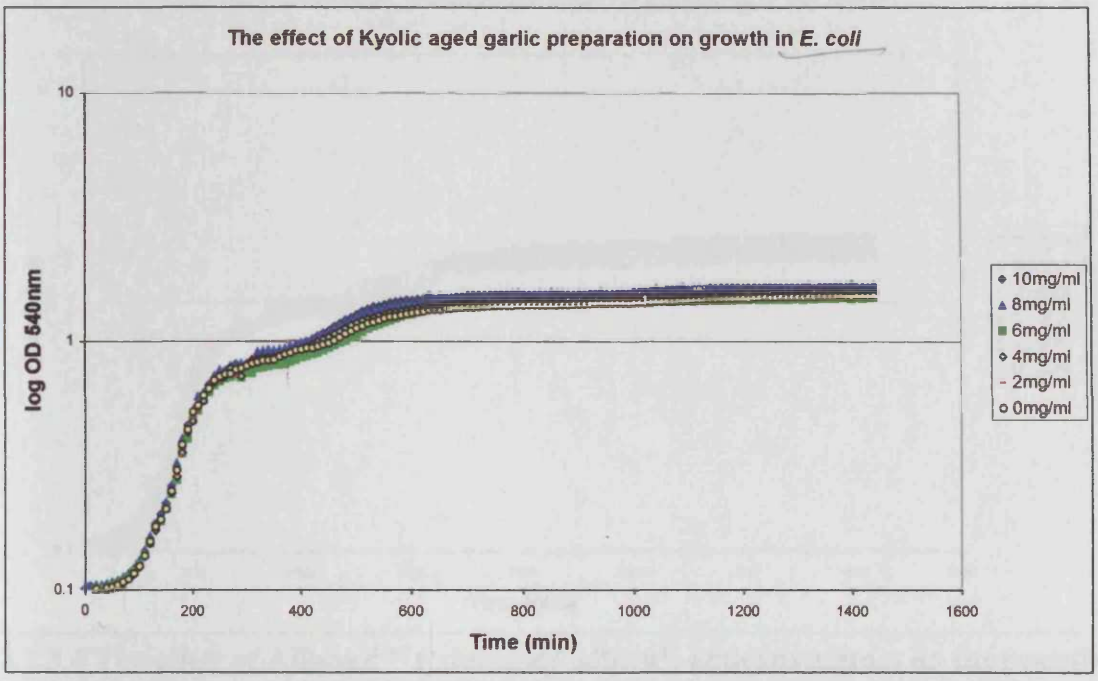
Concentrations of garlic powder extract were tested for biocidal activity against *E. coli* and *L. casei*. Concentrations ranging 1.0 mg ml^{-1} to 25 mg ml^{-1} were tested over a 5 hour time period (figure 3.3.4.0) at hourly intervals, and concentrations of 20 mg ml^{-1}, 40 mg ml^{-1}, 60 mg ml^{-1}, 80 mg ml^{-1}, and 100 mg ml^{-1} were tested over a 72 hour test period at intervals of 24 hours. Concentrations from 0 mg ml^{-1} to 25 mg ml^{-1} did not show bactericidal activity against *E. coli* or *L. casei*. Using higher concentrations



3.3.3.2 The effect of crushed fresh garlic extract on the growth curve of *E. coli* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	1.0	2.0	3.0	4.0	5.0	6.0	7.0
Duration of initial lag phase (min)	100.00 ±30.00	130.00 ±10.00	223.33 ±5.78	350.00 ±20.00	750.00 ±30.00	880.00 ±20.00	1400.0 ±60.0	>1440
Specific growth rate ($hour^{-1}$)	3.40 ±0.07	3.42 ±0.06	3.42 ±0.06	3.36 ±0.12	3.44 ±0.03	3.54 ±0.06	-	-
Optical density (540nm) at 24 hours	1.339 ±0.010	1.527 ±0.106	1.552 ±0.077	1.486 ±0.053	1.425 ±0.062	1.404 ±0.038	0.205 ±0.041	0.096 ±0.023

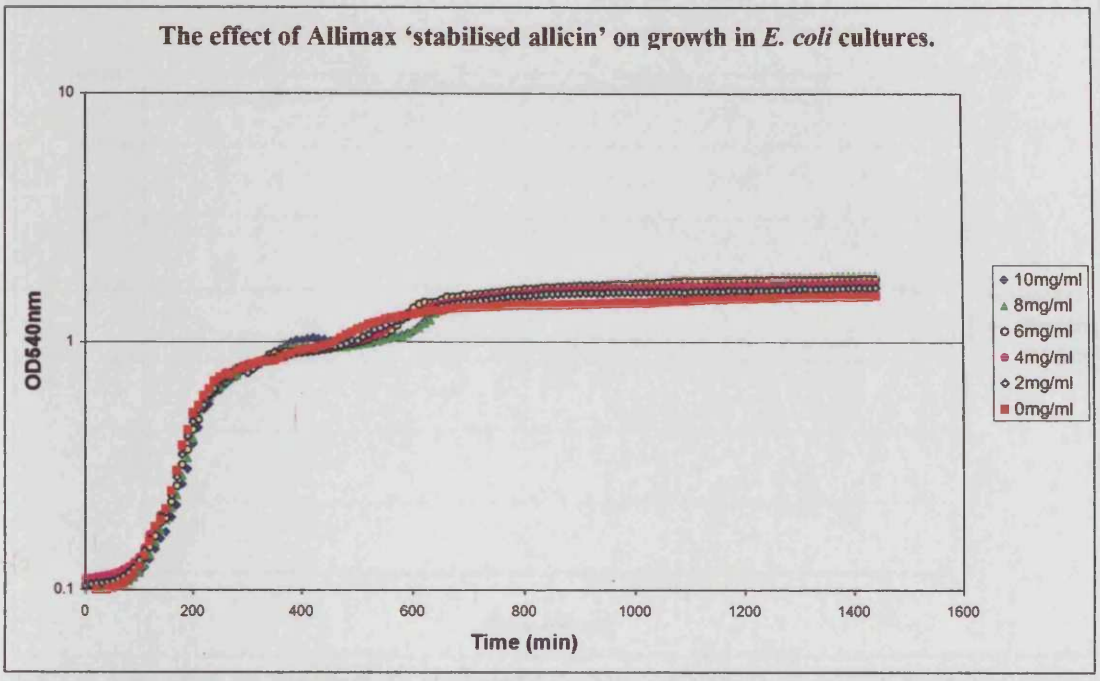
Table 3.3.3.2 The effects crushed fresh garlic extract on growth kinetics of *Escherichia coli* (Figures presented are means and standard deviations of 3 separate experiments).



3.3.3.3 The effect of Kyolic™ (aged garlic) aqueous extract on the growth curve of *E. coli* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Duration of initial lag phase (min)	100.0 ±30.0	100.0 ±10.0	100.0 ±10.0	100.0 ±10.0	100.0 ±10.0	100.0 ±10.0	100.0 ±10.0	100.0 ±10.0	100.0 ±10.0	100.0 ±10.0	100.0 ±10.0
Specific growth rate ($hour^{-1}$)	3.40 ±0.07	3.36 ±0.12	3.30 ±0.06	3.18 ±0.12	3.30 ±0.12	3.32 ±0.04	3.19 ±0.12	3.40 ±0.07	3.42 ±0.12	3.42 ±0.06	3.36 ±0.12
Optical density (540nm) at 24 hours	1.539 ± 0.051	1.539 ± 0.029	1.548 ± 0.038	1.524 ± 0.030	1.591 ± 0.027	1.622 ± 0.068	1.505 ± 0.055	1.661 ± 0.042	1.677 ± 0.035	1.687 ± 0.041	1.684 ± 0.062

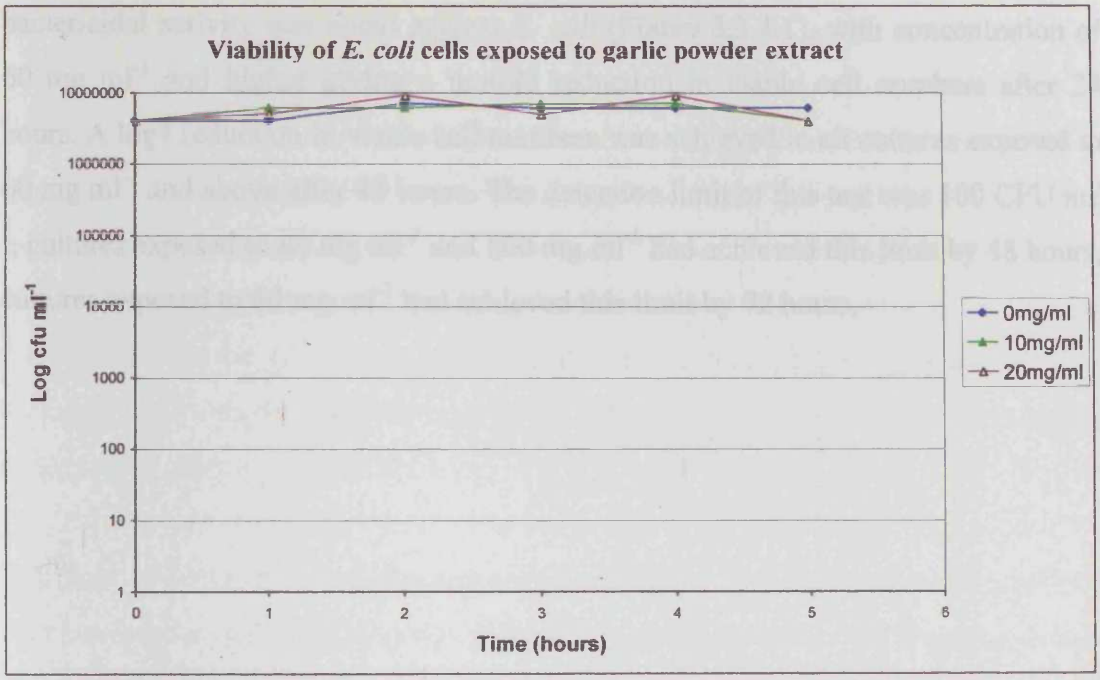
Table 3.3.3.3 The effects of Kyolic™ (aged garlic) extract on growth kinetics of *Escherichia coli* (Figures presented are means and standard deviations of 3 separate experiments).



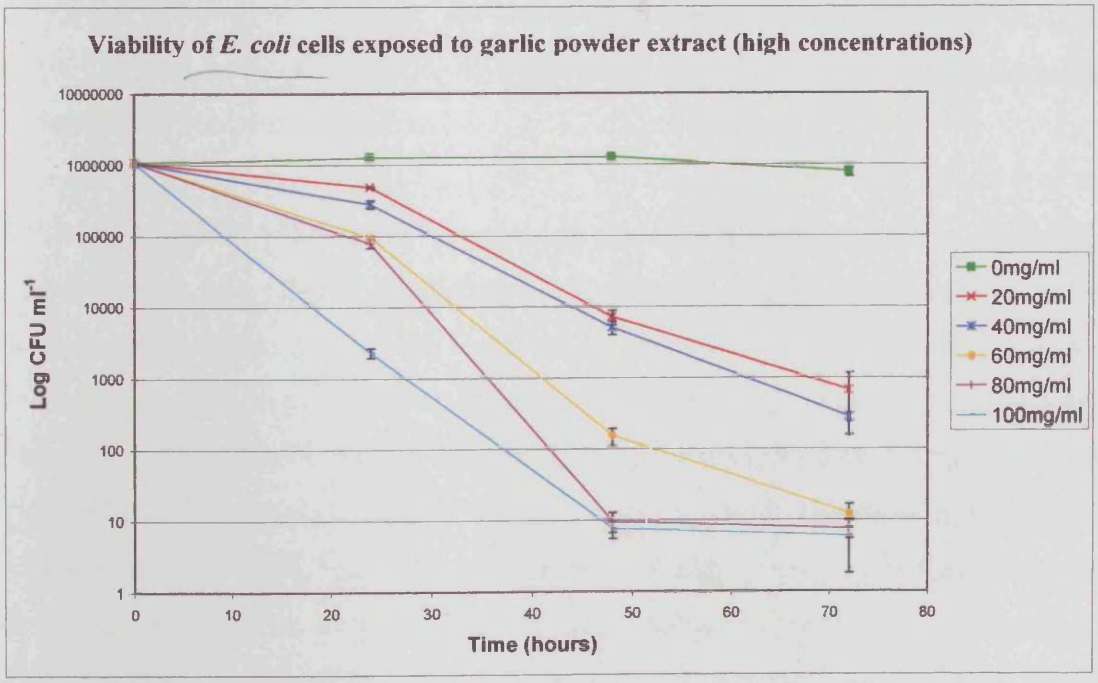
3.3.3.4 The effect of Allimax™ ('stabilised allicin') aqueous extract on the growth curve of *E. coli* (representative of 3 experiments).

Garlic extract concentration (mg ml^{-1})	0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Duration of initial lag phase (min)	70.00 ± 20.00	90.00 ± 10.00	90.00 ± 10.00	100.0 ± 10.00	70.00 ± 20.00	93.33 ± 5.78	100.0 ± 10.00	100.0 ± 10.00	116.7 ± 1.15	110.0 ± 10.00	110.0 ± 10.00
Specific growth rate ($hour^{-1}$)	3.40 ± 0.07	3.26 ± 0.04	3.12 ± 0.06	3.36 ± 0.04	3.16 ± 0.07	3.24 ± 0.12	3.16 ± 0.07	3.20 ± 0.03	3.30 ± 0.06	3.12 ± 0.05	3.18 ± 1.00
Optical density (540nm) at 24 hours	1.791 ± 0.020	1.832 ± 0.093	1.843 ± 0.034	1.817 ± 0.066	1.794 ± 0.048	1.766 ± 0.019	1.721 ± 0.023	1.666 ± 0.033	1.657 ± 0.047	1.590 ± 0.044	1.539 ± 0.028

Table 3.3.3.4 The effects of heated garlic extract on growth kinetics of *Escherichia coli* (Figures presented are means and standard deviations of 3 separate experiments).



3.3.4.0 Viability of washed *E. coli* cells in the presence of garlic powder extract (means of 3 experiments, error bars and intermediate concentrations are omitted for clarity).



3.3.4.1 Viability of washed *E. coli* cells in the presence of garlic powder extract over 72 hours (means and standard deviations of 3 experiments).

bactericidal activity was noted against *E. coli* (Figure 3.3.4.1), with concentration of 60 mg ml^{-1} and higher giving a tenfold reduction in viable cell numbers after 24 hours. A log4 reduction in viable cell numbers was achieved in all cultures exposed to 60 mg ml^{-1} and above after 48 hours. The detection limit of this test was 100 CFU ml^{-1}, cultures exposed to 80 mg ml^{-1} and 100 mg ml^{-1} had achieved this limit by 48 hours, cultures exposed to 60 mg ml^{-1} had achieved this limit by 72 hours.

3.4.1 Discussion

Some of the MIC values recorded in this series of experiments were found to be higher for some species, than previously reported data (Rees, 1993). The garlic powder extract preparation method in this study differed from previous investigations. Previously garlic extracts were prepared in distilled water, mixed for 10 minutes, allowed to stand for 30 minutes, centrifuged then sterile filtered. This garlic extract in distilled water was then diluted in a 1 to 1 ratio with double strength culture media, immediately prior to inoculation.

In this study however, a decision was taken to suspend the garlic powder directly into Nutrient broth, then mix thoroughly for 10 minutes, stand for 30 minutes, before centrifugation and sterile filtering. This decision was taken, because it was felt that it would provide a more accurate relation to the antimicrobial properties of garlic when used both in human food, and when incorporated into animal feed products. The volatile nature of garlic components mean that faster degradation of active components may take place in an environment that is increasingly chemically complex (allicin is known to attack sulphydryl groups of many compounds). This may account for the slightly lower antibacterial activity found here, although differences in garlic powder composition due to different garlic sources and manufacturing procedures must also be considered.

The general trend of MIC and IC50 values presented here agrees with data previously published. (Skyrme, 1996). The Lactic Acid Bacteria (LAB) are very much less susceptible to aqueous garlic extract inhibition, than other commonly found bacteria. The LAB group are usually held to be beneficial members of the gastrointestinal tract, whilst the group of bacteria more susceptible to garlic contains many pathogenic, and potentially pathogenic organisms. The MIC results suggest that provided garlic can be delivered in a suitable form to the intestine, it may prove to be beneficial in preventing proliferation of potentially harmful bacteria within the gastro-intestinal tract.

Data obtained from the growth curve studies here do not correlate precisely with the MIC results (table 3.3.1), however the values are relatively close. MIC results are extremely subjective, depending on methods used and experimental conditions. As far as possible conditions in the two sets of experiments were kept identical. However, since the conditions used in the Bioscreen experiments differ from those in the MIC experiments: Different well volumes and different surface areas through which loss of volatiles may occur; different multiwell plates were used; shaking regimens differed – growth curve analysis through the Bioscreen method required cultures to be shaken before each measurement, increasing possible loss of volatiles; different headspace volumes may also lead to differential rates of loss of inhibitory volatiles from the cultures. MIC values and Bioscreen inhibition data should not be compared too closely, since the purpose behind each experiment is different. MIC tests are designed to screen for inhibitory activity at a fixed time point (usually 24 hours) only, whereas the Bioscreen test uses different equipment and conditions in order to assess effects of inhibitory agents on culture growth kinetics.

A major drawback of using the Bioscreen method to analyse growth in *L. casei* cultures is that cultures are exposed to conditions that are not ideal for growth. The shaking regimen (needed to give accurate optical density readings) provides an aerobic environment throughout the experiment, whereas most LAB show maximum growth in micro-aerobic, or anaerobic environments. This may be why the *L. casei* presented in these experiments appear to be more susceptible to garlic extract than the cultures tested in MIC analyses (non-motile LAB settle to the bottom of the culture vessel; producing an anaerobic microenvironment, organic acid build up here lowers the pH to values favourable for vigorous growth of LAB).

Through overuse of popular antibacterials and misuse of many conventional antibiotics a host of bacterial species (commonly termed 'superbugs') have developed resistance to conventional treatments. These bacteria pose particular problems in hospital environments – where because of patient proximity, ineffective anti-infection/ contagion controls, and doctor-patient contact, super-bugs have become prevalent. Of these hospital acquired infections, currently the most common and problematic is Methicillin Resistant *Staphylococcus aureus* (MRSA) (PHLS 2001).

An important finding of this investigation, is that although the MRSA strains were on the whole less susceptible to garlic inhibition than the methicillin sensitive strain tested, MRSA still showed a high sensitivity to garlic powder extract - MICs for all tested strains were below 2.2 mg ml^{-1} (Inhibitory activities of active compounds from garlic against bacteria are lower still, and compare better to conventional antibiotics). These results indicate a potential role for garlic (if delivered in an effective manner) as a possible method for limiting the spread of MRSA (by inhibiting cell proliferation within the body).

From the analysis of the effects of garlic powder extract on bacterial growth curves, two distinct inhibition effects become apparent that separate the susceptible strains from the less susceptible *L. casei*. In the cases of *S. aureus,* Methicillin resistant *S. aureus, E. faecium,* and *E. coli* the predominant effect of garlic powder on the growth curve is a dose-dependent extension in the duration of initial lag phase. In the *L. casei* experiments the predominant effect exerted by garlic extract was a dose-dependent reduction in the slope of exponential growth phase.

Optical densities at 24 hours from growth curve experiments and the MIC (24 hour) experiments do not correlate exactly, it is important to remember that experimental conditions in the two tests were very different (e.g. shaking regimen, sample aerobicity, culture volume). Although trends seen in these experiments are comparable, exact MIC values (being extremely subjective to test conditions) should not be directly compared.

In *S. aureus* and MRSA screening experiments cultures exposed to 2.0 mg ml^{-1} showed recovery late in the 24 hour time period (901 minutes and 1127 minutes respectively). In both *S. aureus* and MRSA experiments, cultures exposed to garlic powder extract grew at a slower rate than control cultures (garlic powder negative). Optical densities at 24 hours in both *S. aureus* and MRSA experiments showed an increase in optical density in conjunction with increasing garlic powder extract. Because optical density continued to increase post exponential growth period at a similar rate in all test cultures, cultures showing delayed growth (exposed to higher

concentrations of garlic powder extract) could not attain the optical density of cultures showing little or no delay in entering the active growth phase,

When garlic powder extract was tested against *E. faecium* (less susceptible to garlic inhibition than *S. aureus,* and *E. coli* strains) two major inhibitory actions were observed. The same pattern of lag phase extension followed by culture growth was seen here, although higher concentrations were needed to produce similar lag phase extensions to the *S. aureus* investigations. The exponential growth rate for recovering cultures was also seen to be reduced in a dose-dependent manner. Vancomycin resistant *Enterococcus* showed a similar response to garlic powder extract as *E. faecium*, but it was found to be slightly more susceptible. Again garlic extract caused an extension in lag phase in conjunction with a slower exponential growth rate on recovery. It is important to state that the term 'recovery' when used here refers to the recovery of growth characteristics by the culture (i.e. increasing optical density), not recovery of cells from non-lethal injury – detection of the latter definition of recovery is beyond the scope of the experiment carried out here.

The inhibitory effect of garlic powder extract against *E. coli* was seen as a reproducible dose-dependent extension in initial lag phase. Between concentrations of 0 mg ml^{-1} and 1.0 mg ml^{-1} there was a relationship between lag phase extension and garlic concentration, with an extension of approximately 30 minutes for every 0.25 mg ml^{-1} garlic powder extract present. Between concentrations of 1.0 mg ml^{-1} and 2.0 mg ml^{-1} the relationship was roughly exponential, with the lag phase extension doubling for every 0.5 mg ml^{-1} added. Garlic exerted no obvious effect on the gradient of exponential growth in *E. coli*. In cultures treated with sub-MIC concentrations of garlic extract, there was found to be a concentration dependant increase in the culture optical density at 24 hours, this may suggest that *E. coli* was able to grow to higher numbers in cultures where garlic extract was present. Perhaps this is to be expected, since garlic extract is not composed solely of inhibitory compounds, but also contains a variety of carbohydrates and amino acids, which could benefit the growth of bacteria. Alternatively, garlic-induced increases in optical density could be caused by altered cell shape, or leakage of insoluble cellular material from cells (as a result of membrane damage). The effect of garlic on growth of *E. coli* in Tryptic soy broth was

comparable to the effects seen on cultures in nutrient broth. Growth of all *E. coli* cultures (including controls) in Tryptic soy broth appeared to be biphasic in nature. The reason for this is not clear, and warrants further investigation.

The Bioscreen method allows users to screen activity of antimicrobials at many different concentrations continuously over time, making it a convenient method to screen for effective antimicrobial agents. It is important to realise that the Bioscreen method used here can not be used to assess cell viability, optical density readings may not relate directly to cell numbers at any given measurement point, due to factors such as: cell size, cell lysis and accumulation of cell debris. These factors may be especially apparent during the lag phase and stationary phase of the growth curve, hence comparisons of optical density at 24 hours may not reflect differences in the number of viable cells. Although optical density measurements can provide useful data regarding approximate durations of different stages of bacterial growth and growth rates, they provide little information regarding the antimicrobial mechanisms of action.

The characteristic pattern of lag phase extension followed by culture recovery, could be due to (i) Reduction in viable cell numbers, (ii) non lethal cell injury, (iii), bacteriostatic properties of garlic. If garlic extract was affecting the growth of susceptible bacteria by bringing about a reduction in viable cell numbers, followed by a culture recovery, it would suggest that either the culture becomes tolerant to garlic extract, hence allowing cell growth; or that the substance responsible for the biocidal activity is consumed/ removed from the culture before all cells are killed.

Suspension tests were carried out in order to ascertain whether the extension in lag phase was due to cell death. Garlic concentration as high as 25.0 mg ml^{-1} did not appear to affect viability of *E. coli* cells exposed to garlic extract, over a 5 hour period. Higher concentrations of garlic were found to reduce viable cell numbers markedly over longer time periods, log-4 reductions were apparent in cultures exposed to concentrations of 80 mg ml^{-1} and higher after 48 hours, and 60 mg ml^{-1} after 72 hours. However, bearing in mind that concentrations 100 times less than the concentrations needed to bring about log 4 reduction, brought about significant

extensions in lag phase, it maybe unlikely that delay in onset of exponential phase is due to partial culture death. However this should be investigated further using more sensitive methods (*e.g.* cell viability fluorescence stains) to ascertain the numbers of cells that would need to be killed before a significant extension of lag phase becomes apparent.

Alternatively garlic could be effecting non-lethal cellular injury on the bacteria tested, bringing about a period of 'cell repair' after the inhibitory component has been degraded in the cultures, which manifests itself as a delay in the onset of the exponential growth phase.

There is also a possibility that garlic extract is not acting in a biocidal fashion against cells in experimental cultures, but is holding cell growth and division in check, allowing cell division and cultures growth only after the biostatic agents have been consumed. Pre-exposing *E. coli* inocula to garlic extract (1.0 mg ml^{-1} 2 h) before the growth curve experiment, did not produce cultures that were resistant to garlic inhibition, so it is unlikely that culture recovery is due to the tested bacteria becoming 'garlic tolerant'.

The effect of garlic powder extract against *L. casei* was remarkably different from the other bacteria tested, duration of initial lag phase was not affected (although at higher concentrations of garlic extract if was difficult to distinguish lag from the exponential growth phase, and culture growth appeared to be linear).

The pattern of lag phase extension followed by complete culture recovery is likely to be caused by either bacteriostatic properties of the garlic extract, or non lethal cell injury. In either of these cases the active components of garlic would need to be consumed or degraded for culture recovery to occur. The active components of garlic extract have long been thought to be the thiosulphinates. These compounds are thermally unstable (degrading rapidly to sulphides and vinyl dithiins at elevated temperatures). The effect of heated garlic powder extract on growth in *E. coli* was investigated in order to ascertain whether the inhibitory activity was due to thermally unstable compounds. The heated garlic extract was found to have a much diminished

inhibitory activity against *E. coli* with full recovery of cultures exposed 10.0 mg ml^{-1} showing full recovery within 17 hours. The heated garlic extract did not appear to affect the exponential growth rate to a significant degree. The results of this experiment suggest that the inhibitory actions of garlic against *E. coli* are due to compounds that are thermally unstable (such as allicin and other thiosulphinates).

Garlic extract brought about a marked dose-dependent reduction in the exponential growth rate of *L. casei* cultures with culture growth occurring 300 times less rapidly in 15.0 mg ml^{-1} cultures than in control cultures. *L. casei* still appeared much less susceptible to garlic inhibition than other bacteria tested, (indeed cultures exposed to 0.5 mg ml^{-1} to 5.0 mg ml^{-1} achieved a much higher density than the control, suggesting that although the cultures grew less rapidly on exposure to garlic extract, they were able to support higher *L. casei* numbers).

Aqueous extract of fresh crushed garlic extract brought about an effect comparable to that seen on treatment of *E. coli* with aqueous extract of garlic powder. Although the freshly crushed garlic extract appears to be less active from the growth curves, it must be remembered that a large proportion of whole garlic cloves is accounted for by water (Chapters 1 and 5), this water is absent from the garlic powder, making garlic powder seem more active when in fact the activities of the two preparations are similar (approximately 65% of the mass of garlic cloves is accounted for by water (Lawson 1996)).

Allimax and Kyolic garlic preparations had no significant effect on growth in *E. coli*. Since allicin is not found in Kyolic (chapter 1), this would appear to support the fact that allicin is necessary for the inhibitory properties of garlic against *E. coli*. It would also suggest that S-allyl cysteine (the major component of Kyolic) does act in an inhibitory manner against *E. coli*. Surprisingly, Allimax (which reports to be 100% stabilised allicin) also failed to be inhibitory to growth of *E. coli* cultures (it actually appeared to promote growth). Apparently the process by which allicin is stabilised to yield Allimax, also leads to the loss its inhibitory capacity against *E. coli*.

Chapter 4

An investigation into the inhibitory action of garlic against Methicillin resistant *Staphylococcus aureus*, when used in combination with methicillin or penicillin.

Chapter 4 -An investigation into the inhibitory action of garlic against Methicillin Resistant *Staphylococcus aureus*, when used in combination with methicillin or penicillin.

4.1. Introduction

Approximately one out of every ten patients in acute hospital care in the United Kingdom, is thought to be suffering from a Hospital Acquired Infection (HAI) (Brown, 2001). Currently the most infamous and frequently found hospital infection is caused by Methicillin Resistant *Staphylococcus aureus* (MRSA) (PHLS 2002).

Staphylococcus aureus is a gram positive coccus. It is a species of bacteria that has been notorious in causing wound, and surgical site infections throughout history (claiming the lives of many soldiers during World Wars I and II). *S. aureus* is a common cause of skin and soft tissue infections including acne, boils, ulcers, and impetigo. It has also been found to be responsible for a wide number of more serious complaints, including: post-operative bone and joint infections, post operative meningitis, prosthetic and native valve endocarditis, pneumonia, bacteraemia, septicaemia, and toxic shock syndrome – all of these complaints can prove fatal where treatment is not successful.

It was thought that with the discovery of penicillin (a naturally-occurring antibacterial agent produced by the common mould *Penicillium notatum*) by Flemming (1928), and subsequent development of the antibiotic by Florey, Chain, and their teams (1939), that an end to life-threatening *Staphylococcus* infections was in sight. However almost immediately after this first generation of antibiotics was introduced into clinical practice in 1944, the first antibiotic resistant strain of *S. aureus* was isolated and reported.

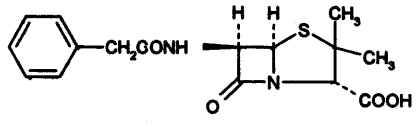
Two years later, 6% of all *s. aureus* cases reported were penicillin resistant (Livermore, 2000). Such rapid development and spread of resistance is thought to have been due to extreme over-use and ineffective use of penicillin. By 1948 above 50% (Livermore, 2000) of all reported cases were penicillin resistant. By 2000 the

percentage of clinical cases of *S. aureus* which was penicillin resistant had risen to 80% (Chen 1993, Henwood 2000).

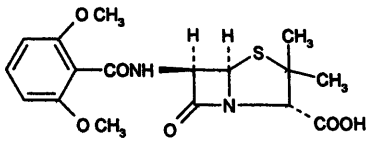
Penicillin exerts its antibacterial effect by specifically inhibiting the process which bacteria use to manufacture the cross linking components in the peptidoglycan layer of cell walls. This occurs through binding of penicillin to the active site of transpeptidase proteins located in the cell envelope (termed Penicillin Binding Proteins (PBPs) 1, 2 and 3). Penicillin (figure 4.1.0) belongs to the β-lactam class of antibiotics, so called because they contain a β-lactam ring component which is essential to their activity. *S. aureus* exerts its resistance to penicillin by use of a β-lactamase enzyme, which cleaves the β-lactam component of penicillin.

Soon after the discovery and introduction of penicillin, other natural antibiotics were developed such as tetracycline (1948), cephalosporin (1948) and erythromycin (1952). Cephalosporins are classed as β-lactams (Figure 4.1.0) and have similar binding targets, whist tetracycline and erythromycin both inhibit bacterial protein synthesis (inhibiting tRNA binding, and peptide bond formation respectively). For a time these were highly effective against *S. aureus* infections. By 1960 however, the first multi-resistant strains of *S. aureus* were found in hospitals in the United Kingdom.

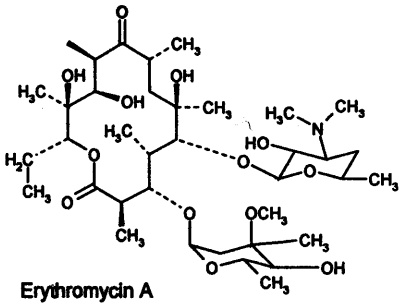
New synthetic cephalosporins (*e.g.* cephalothin) and synthetic penicillins (*e.g.* methicillin) were introduced in the 1960s. Methicillin (figure 4.1.0) is very similar in structure to penicillin, however it is protected from β-lactamase inactivation by the addition of groups (6' acyl position) which stearically hinder attack of the β-lactam component by the enzyme. *S. aureus* strains were swift in their development of resistance to these new antibiotics. In the case of methicillin, resistant strains were already identified when the drug reached the market in 1961 (Jevons, 1961), these bacteria were described as Methicillin Resistant *Staphylococcus aureus* (MRSA) – although the majority of theses strains are also resistant to many penicillins, cephalosporins, and other β-lactam antibiotics.



(a.) Penicillin G

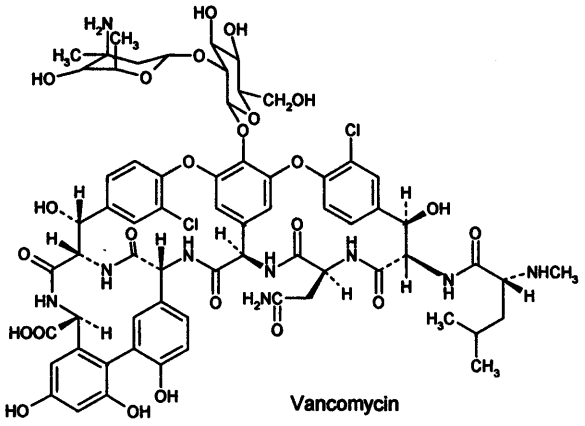


(b.) Methicillin



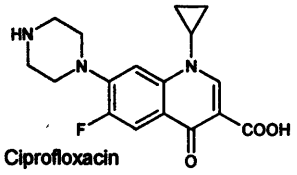
Erythromycin A

(c.)



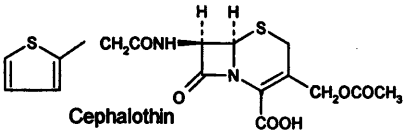
Vancomycin

(d.)



Ciprofloxacin

(e.)



Cephalothin

(f.)

Figure 4.1.0 Structures of: (**a**) penicillin G (a penicillin/ β-lactam), (**b**) methicillin (a synthetic penicillin), (**c**) erythromycin A (a macrolide), (**d**) vancomycin (a glycopeptide), (**e**) ciprofloxacin (a quinolone), (**f**) cephalothin (a synthetic cephalosporin).

Production of an additional penicillin binding protein (PBP2′) confers the methicillin resistance on *S. aureus*. PBP2′ has a much lower affinity for β-lactams than the previously detected PBPs, allowing cross linking of cell wall components even in the presence of β-lactamase stable β-lactams.

The gene for methicillin resistance (termed *MecA*) is found on large sections of chromosomally inserted DNA. No homologues for the *MecA* gene cluster have been reported in Methicillin Sensitive *S. aureus* (MSSA) suggested Methicillin resistance to be an acquired characteristic (rather than a dormant intrinsic characteristic of *S. aureus*). It is also believed that horizontal transfer of the *mecA* gene can occur, since identical systems have now been reported in Coagulase negative *Staphylococci* (Hiramatsu, 1999).This has important implications when considering possible spread of methicillin resistance throughout natural populations of *Staphylococci.*

By the 1960s increasingly more virulent *Staphylococci* were causing a problem in many hospital wards, and in communities worldwide (including the UK, Denmark and mainland Europe). In 1963 the aminoglycoside antibiotic gentamicin (interferes with DNA translation *via* attack of the bacterial ribosome) was introduced into the medical setting , and this antibiotic (amongst other measures such as infection control strategies, and strain displacement) was credited with the sharp decline of MRSA cases reported during the late 1970s. As with previous treatments, some *S. aureus* strains soon developed defence mechanisms (production of an aminoglycoside inactivating enzyme). By the 1980s gentamycin proved an ineffective treatment in many instances.

Vancomycin is currently the antibiotic of last resort for MRSA infections resistant to all other treatments. It inhibits cell wall synthesis by binding terminal ends of peptidoglycan precursors. Although this treatment is still effective in the vast majority of cases, harmful side effects of vancomycin make it unsuitable for treating many patients (including the elderly, infants, and debilitated patients suffering long-term illnesses). Reduced susceptibility of MRSA strains to vancomycin has also been reported recently (Hiramatsu 2001, Samson 1999) (substitution of terminal D-alanine residues for D-lactate is thought to confer resistance to these strains).

Today, MRSA is causing an increasing problem in hospitals the world over. At present the UK has one of the highest incidence rates in Europe. Figures show that in that a total of 250 cases of *S. aureus* infections were reported per week in the National Health Service during 2001 (PHLS, 2001). Approximately half of these cases are caused by MRSA, and the trends for both total numbers of *S. aureus* infections and resistant infections appear to be rising. *Staphylococcal* infections are increasingly common in the hospital environment; more alarmingly, the instances of multiple resistant *Staphylococcal* infections are increasing. Data suggests that, it is not merely a case of more *S. aureus* strains becoming resistant to antibiotics, but rather that the problems posed by MRSA are an additional burden on top of the generally increasing *S. aureus* infection rates.

Although there have been some recent promising developments in antibiotic therapy against *S. aureus* (Dalfopristin, linezolid etc) it is clear that at present the development of resistance seems to be outpacing the introduction of new therapies.

HAIs are currently thought to put an additional financial burden on the United Kingdom national health service of £1 billion annually. Aside from the additional financial costs incurred from treating patients with MRSA infections, infected patients can also expect significantly longer hospital stays. This in turn leads to a reduction in the number of available hospital beds, infected patient also require more intensive healthcare regimens thereby placing an additional burden on the workloads of healthcare professional. The overall results to the national health service are: increased financial costs, stressed hospital resources, lengthy hospital waiting lists, poor public image, and increased worry to the patients.

An emerging development in methods to combat bacterial resistance is the purposeful use of combinational antibiotic therapies. In the clinical setting multiple antibiotic therapies are sometimes used to combat infections, where the culprit bacteria are not yet know, or where multiple bacterial species are thought to be present. It has been found that some combinations of therapy display a synergistic inhibitory action, *i.e.* the inhibition seen is more than can be accounted for by summation of each drugs individual inhibitory capacity.

Synergism between tobramycin and piperacillin has been demonstrated against resistant *Pseudomonas aeruginosa* (Weiss *et al* 1995, Song *et al* 2002). *In vitro* synergy has also been shown between amoxicillin and ceftriaxone against *Enterococcus faecalis* (Gradelski *et al* 2000), and between vancomycin and ceftriaxone against *Streptococcus pneumoniae* (Karakoc *et al* 2002). Jonkers *et al* (1999) demonstrated that mixtures of garlic and vancomycin showed synergistic activity *in vitro* against Vancomycin resistant *Enterococci*, with the MIC to vancomycin falling from 32 $\mu g\ ml^{-1}$ to 0.5 $\mu g\ ml^{-1}$ on addition of 1.0 $mg\ ml^{-1}$ garlic extract.

Garlic exerts a broad spectrum activity against many cellular processes (Harris 2001), allicin has been shown to attack the sulphydryl groups of a variety of enzymes leading to metabolic inhibition. It is plausible that garlic may exert inhibitory actions against the processes controlling antibiotic resistance mechanisms of multiple resistant bacteria, heightening their sensitivity to the antibiotics (in addition to eliciting inhibitory responses toward compounds contained in garlic).
The antibacterial action of garlic powder extract against a clinically isolated strain of MRSA has already been demonstrated in Chapter 3.

The purpose of the work presented here was to characterise antibiotic resistance profiles of three MRSA clinical isolates, and investigate possible synergy between garlic and β-lactams (natural and synthetic) against MRSA, using a chessboard technique. The antibiotics chosen for this study were penicillin G and methicillin, being traditionally the first and last resort β-lactams for *Staphylococcal* infections.

4.2.0 Materials and methods

4.2.1 Antibiotic susceptibility testing

The sensitivity of three strains of MRSA to 12 conventionally available antibiotics was tested, using an antibiotic disc diffusion technique (NCCLS, 2002).

Mueller Hinton agar plates (9cm), containing 2% sodium chloride were prepared, and dried for 30 min in a sterile laminar flow cabinet. The plates were seeded with 1 ml of MRSA cell suspension (containing approximately 10^6 cfu ml^{-1}) using a sterile cotton swab.

The plates were left to stand for 60 min. Discs impregnated with known concentrations of antibiotics (Oxoid, Basingstoke) were then placed on the surface of the seeded plates using disc dispensing unit (Oxoid) pre-cleaned with 70% ethanol. 6 different antibiotic discs were dispensed onto each plate, and plates were prepared in triplicate. The dispensing unit was re-sterilised with 70% ethanol each time after use to prevent cross contamination.

Plates were incubated for 18 hours at 35°C. On examination at 18 hours, the diameter of each characteristic zone of growth inhibition in the bacterial lawn surrounding, antibiotic discs were measured. The measured diameters were then compared to the standard values given by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). According to the diameter of the inhibition zone, each strain could then be characterised as sensitive, intermediate or resistant to each antibiotic.

4.2.2 MIC values of methicillin and penicillin against MRSA

A Microtitre method was used; stock solutions of methicillin and penicillin G (300 $\mu g\ ml^{-1}$ and 600 $\mu g\ ml^{-1}$ respectively) were prepared by adding the required amounts of methicillin and penicillin to sterile Mueller Hinton broth and shaking for 20 minutes. Dilution series (10 $\mu g\ ml^{-1}$ intervals) were prepared and transferred to wells on a sterile 96 well plate. Wells were then inoculated with overnight cultures of the desired strain to give a final culture density of approximately 1×10^6 cfu ml^{-1}. The final culture volume in each well was 200 µl. Optical densities at 450 nm of

each Inoculated multiwell plate were recorded. The plates were covered, transferred to a plastic container (containing a small reservoir of water to prevent desiccation of plates, this is recommended for fan assisted incubators by the NCCLS 2000, volumes of liquid in each well were inspected visually after incubation), and incubated at 37° C aerobically for 24 hours without shaking.

Optical density at 450 nm was again recorded at 24 hours, the MIC value was taken as the lowest concentration of antibiotic that prevented an increase in optical density during the 24 hour test period. MIC assays were performed in triplicate using separately raised inocula, results are presented as means with standard deviations.

4.2.3 Assessment of synergy between garlic and antibiotics.

Synergistic effects were investigated using a chessboard method. Stock concentrations of penicillin, methicillin and garlic powder extract were prepared separately in Mueller Hinton broth as described previously. Dilution series were then prepared (quadruple strength to the final desired concentration after inoculation) in sterile Mueller Hinton broth. 50 µl aliquots of each test component dilution were then transferred to wells in a 96 well plate. Concentrations were arranged so that each well represented a unique combination of concentrations of component (penicillin concentrations - figure 4.2.3.1, methicillin concentrations figure 4.2.3.2). Wells were inoculated with 100 µl of MRSA cell suspension to give a final culture volume of 200 µl and a final culture density of 1×10^{6} cfu ml^{-1}. The optical density of each plate was recorded at 450 nm. 96 well plates were transferred to a plastic container (containing a small reservoir of water to prevent plate desiccation) and incubated aerobically at 37° C without shaking for 24 hours. Optical density (450 nm) was recorded at 24 hours. No observable change in the optical density of the culture after 24 hours was taken to indicate total growth inhibition.

4.2.4 Analysis of chessboard synergy test result

The chessboard synergy test was used to investigate possible synergistic action in methicillin/ garlic extract and penicillin/ garlic extract combinations. The data was analysed firstly by plotting the effect of garlic extract on the MIC of each strain to the test antibiotic.

Next the lowest concentrations of mixtures that totally inhibited growth of the test strain were used to calculate Fractional Inhibitory Concentrations (FIC) for each component.

$$FIC_{(\text{component A})} = \frac{MIC_{(A)1}}{MIC_{(A)2}}$$

Where $MIC_{(A)1}$ represents the MIC of component A when used in combination, and $MIC_{(A)2}$ represents the MIC of component A alone.

FIC values for component A (at each concentration of V) were then plotted against FIC values obtained for component B (at each concentration of A), to create an isobologram. The shape of the isobologram indicates whether the effect of the mixture is synergistic, additive, or antagonistic (see figure 4.2.4.0).

If the isobologram plot lies below the line connecting the FIC values for the single component system (*i.e.* concave) the action is said to be synergistic. If the plot of FIC values lies on this line the effect of the mixture is said to additive. If the FIC plot lies above this line (*i.e.* convex), the effect is said to be antagonistic.

The FIC index was also calculated for each experiment. The FIC index is defined as the sum of the FIC values in the most effective combination of components A and B. Although the FIC index is a commonly used measure of synergy, there is some discrepancy between authors regarding the boundaries of synergy and antagonism.

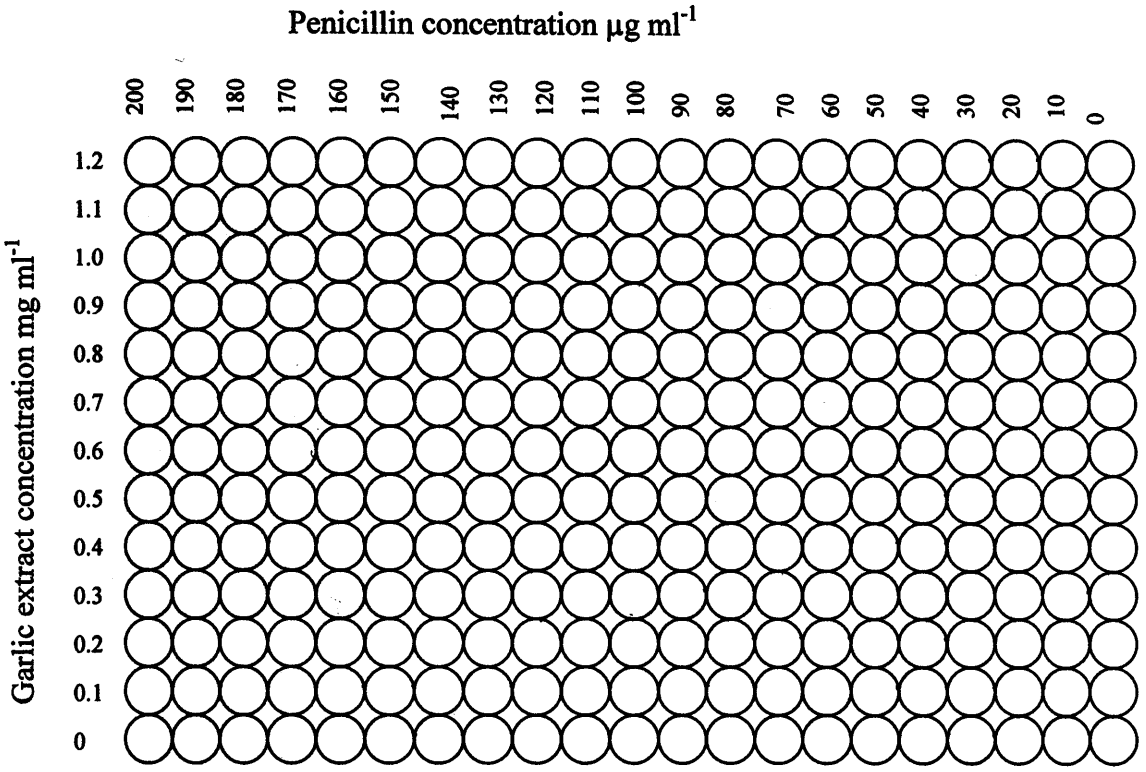


Figure 4.2.3.1 Layout of wells used in the chessboard method for investigating synergy between garlic and penicillin.

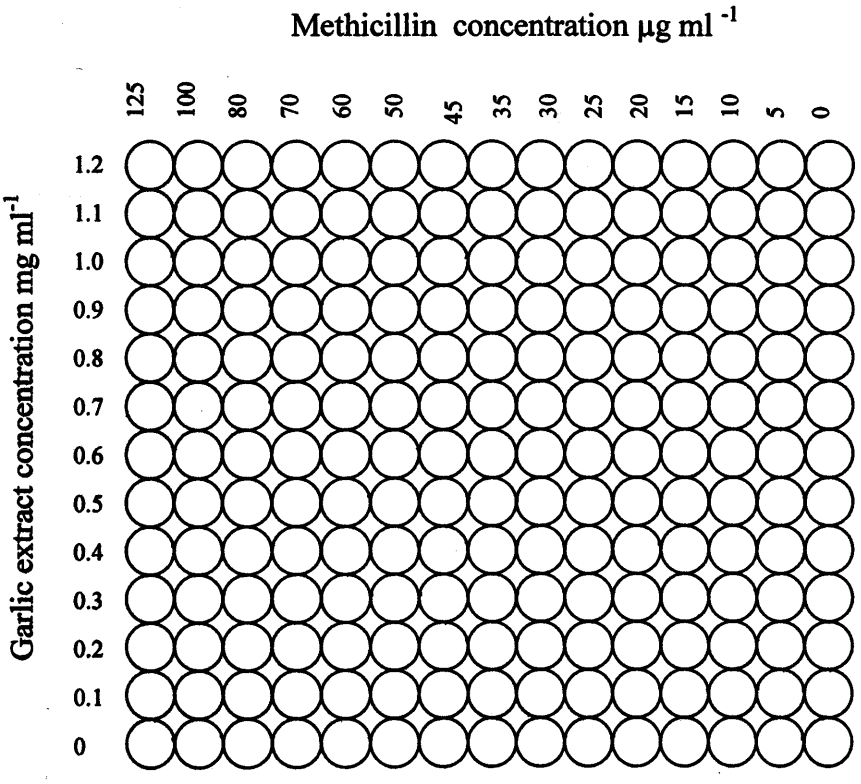


Figure 4.2.3.2 Layout of wells used in the chessboard method for investigating synergy between garlic and methicillin.

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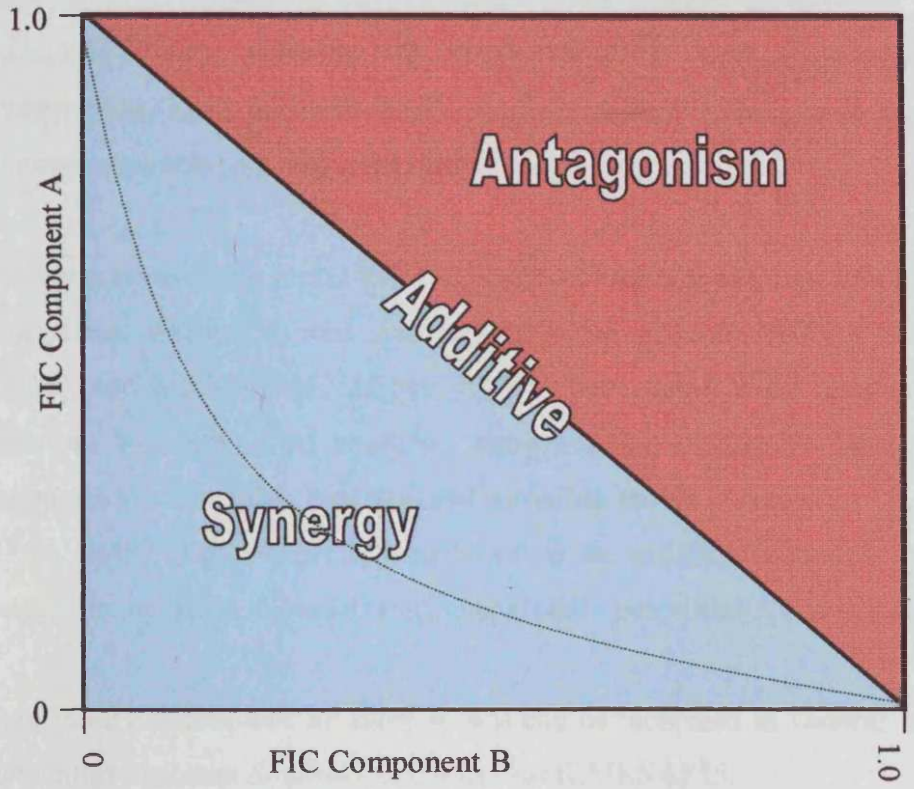


Figure 4.2.4.0 Representation of an FIC isobologram with curves showing possible synergistic, and antagonistic effects between components A and B.

Some authors state that an FIC index of 0.5 or less (Song *et al* 2003) (this represents a 4 fold reduction in the MIC of each component when used in combination) represents synergy, An FIC index between 0.5 and 2.0 represents indifference, and a value higher than 2.0 represent antagonism between the 2 components. Others maintain that an FIC index below 0.70 indicates synergy, greater than 0.70 but below 1.3 indicates indifference, and greater than 1.3 (Beale and Sutherland 1983) indicates antagonism. Others still maintain that an FIC index of less than 1 denotes synergistic action, an FIC index of 1 denotes additivity, and an FIC index of greater than 1 indicated antagonism between components. All of these limits were considered when analysing data from the chessboard tests.

4.3 Results

4.3.1.0 Antibiotic sensitivity testing

Three clinical isolates of MRSA were tested against 12 different antibiotics: methicillin (Met), penicillin G (Pen), oxacillin (Oxa), vancomycin (Van), erythromycin (Ery), linezolid (Lzd) , Also rifampicin , tetracycline, ciprofloxacin, mupirocin, gentamycin, and cephalothin.

All strains tested were found to be sensitive to Vancomycin, Linezolid, Rifampicin, tetracycline, mupirocin, and gentamycin (Table 4.3.1.0). MRSA z4500 (figure 4.3.1.0), and MRSA z4467 (figure 4.3.1.1) were found to be resistant to growth inhibition by methicillin, oxacillin, ciprofloxacin, cephalothin and penicillin G (resistance to methicillin, oxacillin and penicillin shown in figure
MRSA z4497 (figure 4.3.1.2) was found to be resistant to growth inhibition by methicillin, oxacillin, ciprofloxacin, cephalothin, penicillin G, and Erythromycin.

From these observations, all three strains can be identified as variants of Emerging Methicillin Resistant *Staphylococcus aureus* (EMRSA) 15.

4.3.2.0 Screen for MIC values of penicillin G and methicillin against MRSA

The degree of resistance to penicillin and methicillin was assayed for in MRSA z 4500, z4467, and z4497. All three strains were found to be extremely resistant to penicillin G (table 4.3.2.0), z4500 was the most susceptible strain to growth inhibition by penicillin with an MIC of 403 $\mu g\ ml^{-1}$. MRSA z4467, and 4497 showed similar susceptibilities to penicillin G, with MIC values of 451 $\mu g\ ml^{-1}$ and 447 $\mu g\ ml^{-1}$ respectively.

Antibiotic	Conc (μg)	Resistance zone diameter (mm)	Inhibition zone Diameter (mm)			Resistant (R)/ Sensitive (S)		
			MRSA Z4500	MRSA Z4467	MRSA Z4497	MRSA Z4500	MRSA Z4467	MRSA Z4497
methicillin	5	≤9	0	0	0	R	R	R
penicillin	10	≤28	0	0	0	R	R	R
erythromycin	15	≤13	21	24	0	S	S	R
oxacillin	1	≤10	0	0	0	R	R	R
linezolid	30	≤21	32	26	27	S	S	S
vancomycin	30	≤14	17	15	15	S	S	S
rifampicin	5	≤16	31	24	30	S	S	S
tetracycline	30	≤14	27	26	25	S	S	S
ciprofloxacin	5	≤15	0	0	0	R	R	R
mupirocin	5	not described	21	30	28	S	S	S
cephalothin	30	≤14	9	11	12	R	R	R
gentamycin	10	≤12	21	20	21	S	S	S

Table 4.3.1.0 Measurement of growth inhibition in MRSA strains using a disk diffusion method. Inhibition zones were compared to the standard zone diameter for resistance.

MRSA Strain	MIC to penicillin ($\mu g\ ml^{-1}$)	MIC to methicillin ($\mu g\ ml^{-1}$)	MIC to garlic ($mg\ ml^{-1}$)
Z4500	403 ± 28.2	200 ± 6.6	1.5 ± 0.1
Z4467	451 ± 20.8	198 ± 9.2	1.4 ± 0.2
Z4497	447 ± 3.5	251 ± 15.0	1.5 ± 0.1

Table 4.3.2.0 Evaluation of sensitivities of MRSA z4500, z4467. z4497 to penicillin G, methicillin and garlic (using a Microtitre broth dilution method).

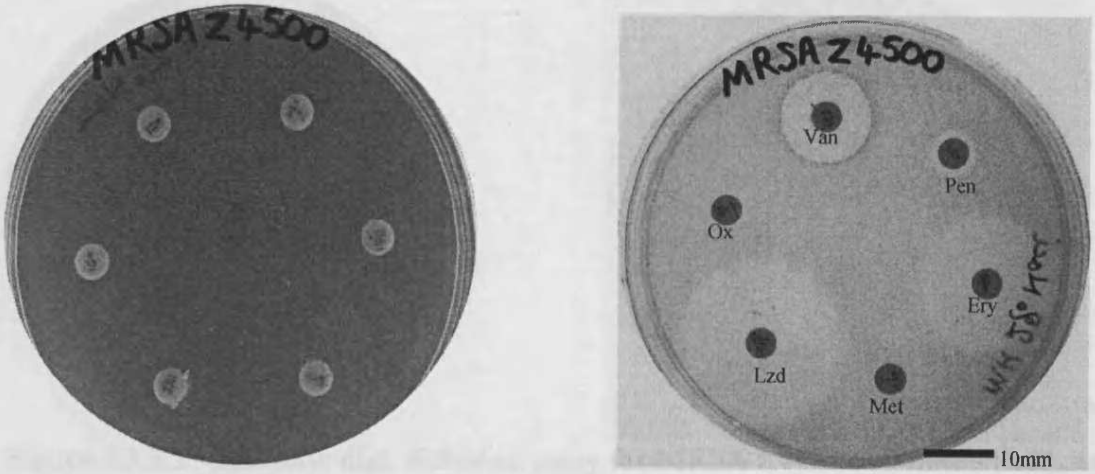


Figure 4.3.1.0 Antibiotic disk diffusion assay for MRSA z 4500 at 0 hours (left), and 18 hours (right).

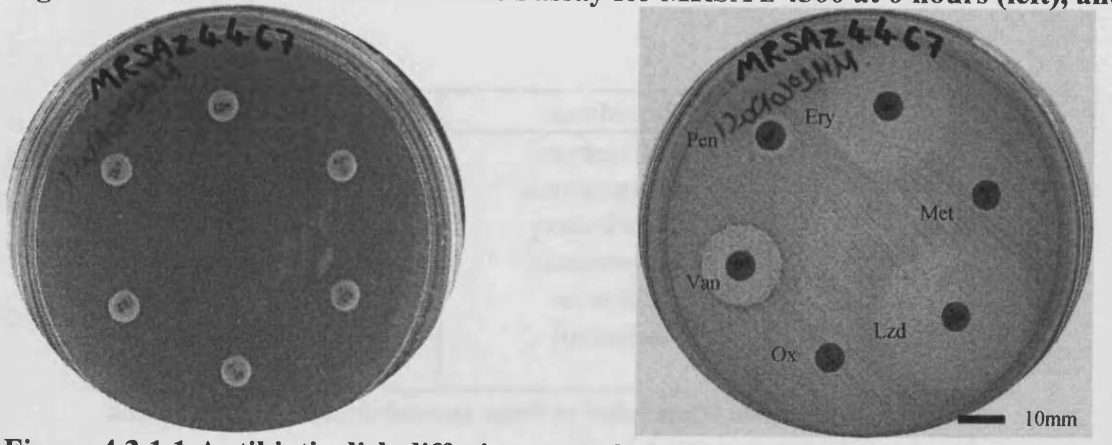


Figure 4.3.1.1 Antibiotic disk diffusion assay for MRSA z 4467 at 0 hours (left), and 18 hours (right).

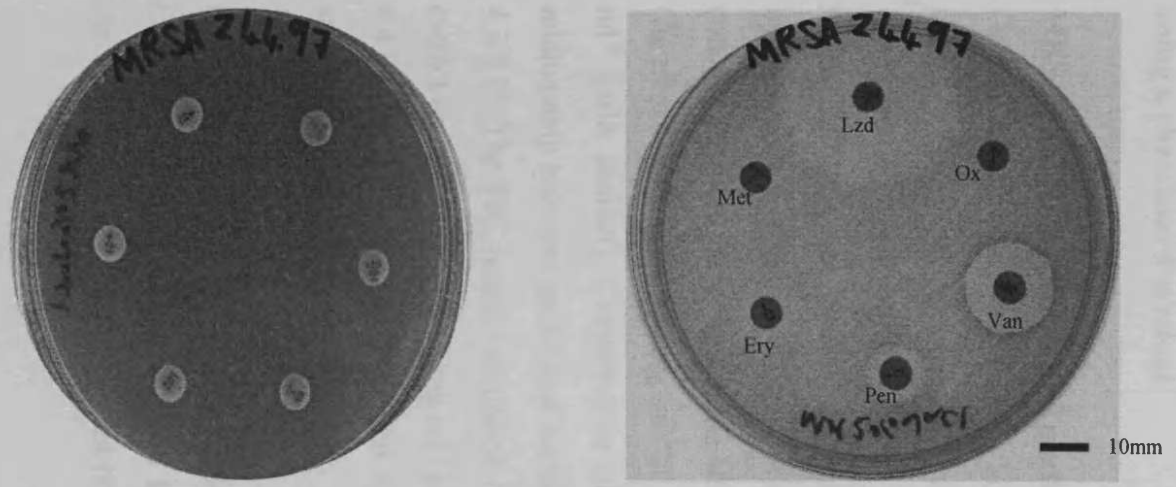


Figure 4.3.1.2 Antibiotic disk diffusion assay for MRSA z 4497 at 0 hours (left), and 18 hours (right).

Abbreviation used	Antibiotic
Met	methicillin
Ery	erythromycin
Pen	penicillin G
Van	vancomycin
Ox	oxacillin
Lzd	linezolid

Table 4.3.1.3 Abbreviations used to label antibiotic disks

MIC values to methicillin for the three strains tested were all extremely high when compared to methicillin sensitive strains (resistance breakpoint for methicillin and *Staphylococci* – 8.0 $\mu g ml^{-1}$ (NCCLS, 2002). The least susceptible strain tested was MRSA z4497 with an MIC to methicillin of 251 $\mu g\ ml^{-1}$, MRSA z4500 and z4467 showed similar MIC values to penicillin 200 $\mu g\ ml^{-1}$, and 198 $\mu g\ ml^{-1}$ respectively (resistance breakpoint for penicillin and *Staphylococci* – 0.1 $\mu g\ ml^{-1}$ (NCCLS, 2002)).

MIC values of all three strains to garlic were similar. MRSA z4500 and z4497 showed MIC values of 1.5 mg ml^{-1}, and MRSA z4467 1.4 mg ml^{-1} to garlic.

4.3.3.0 Investigations into synergistic action between methicillin and garlic using a chessboard method.

MRSA z 4500 methicillin and garlic.

Optical densities (450 nm) at 24 hours of cultures of MRSA z4500 exposed to mixtures of methicillin and garlic were lower than those of cultures exposed to corresponding concentrations of single components. Mixtures containing increasing garlic concentrations appeared to be more sensitive to growth inhibition by methicillin (figure 4.3.3.0). On addition of garlic extract at sub MIC concentrations to the cultures, the MIC of MRSA z4500 was reduced dramatically (figure 4.3.3.1), from 200 $\mu g\ ml^{-1}$ (0 mg ml^{-1} garlic extract) to 37 $\mu g\ ml^{-1}$ (1.2 mg ml^{-1} garlic extract). Construction of an FIC isobologram showed a synergistic relationship between garlic and methicillin in their action on MRSA z4500 (figure 4.3.3.2). The FIC index calculated for the most effective combination of garlic extract and methicillin (0.4 mg ml^{-1}garlic extract and 75 $\mu g\ ml^{-1}$ methicillin) (table 4.4.0) was 0.72, this confirms that the inhibitory relationship between methicillin and garlic is more than additive.

MRSA z 4467 methicillin and garlic.

Cultures of MRSA z4467 exposed to mixtures of methicillin and garlic showed the same trend as MRSA z4497. Mixtures containing increasing garlic

concentrations appeared to be more sensitive to growth inhibition by methicillin (figure 4.3.3.3).

On addition of garlic extract at sub MIC concentrations to the cultures, the MIC of MRSA z4500 was reduced (figure 4.3.3.4), from 198 μg ml^{-1} (0 mg ml^{-1} garlic extract) to 42 μg ml^{-1} (1.2 mg ml^{-1} garlic extract). Construction of an FIC isobologram showed that a synergistic relationship between garlic and methicillin existed, however when concentrations of garlic extract were high the relationship became additive then antagonistic (figure 4.3.3.5).

The FIC index calculated for the most effective combination of garlic extract and methicillin (0.5 mg ml^{-1}garlic extract and 75 μg ml^{-1} methicillin) (table 4.4.0) was 0.74, this confirms that the inhibitory relationship between methicillin and garlic is more than additive.

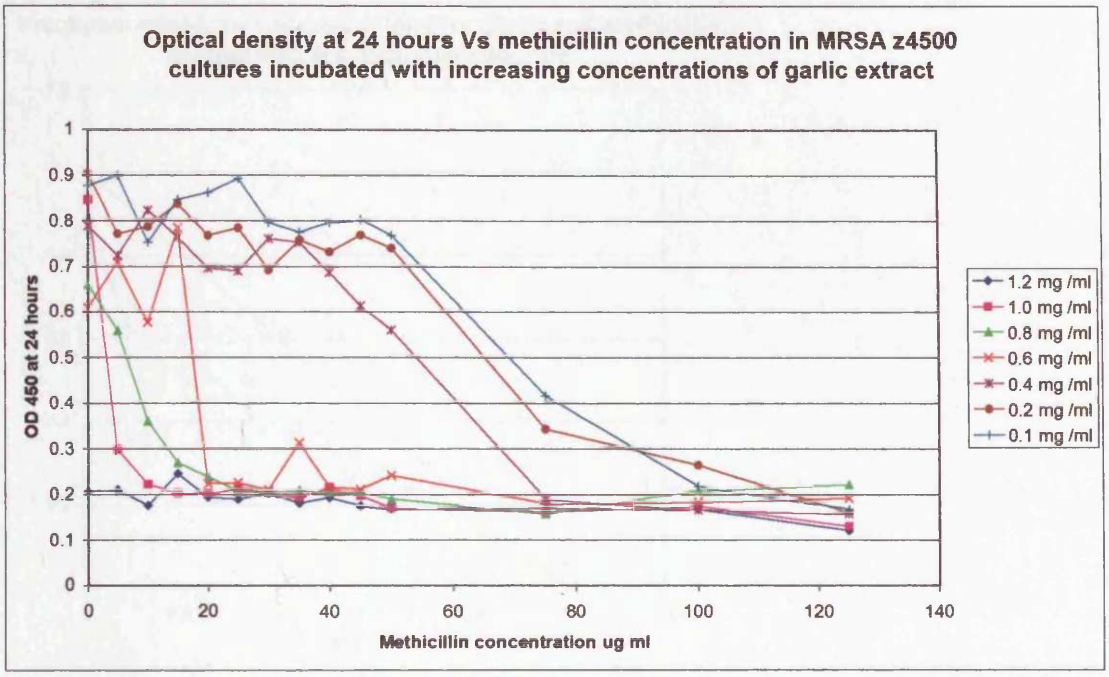


Figure 4.3.3.0 Inhibition profile of methicillin against MRSA z4500 incubated with increasing concentration of garlic extract (legend). Values plotted are the means of 3 experiments, error bars are omitted for clarity.

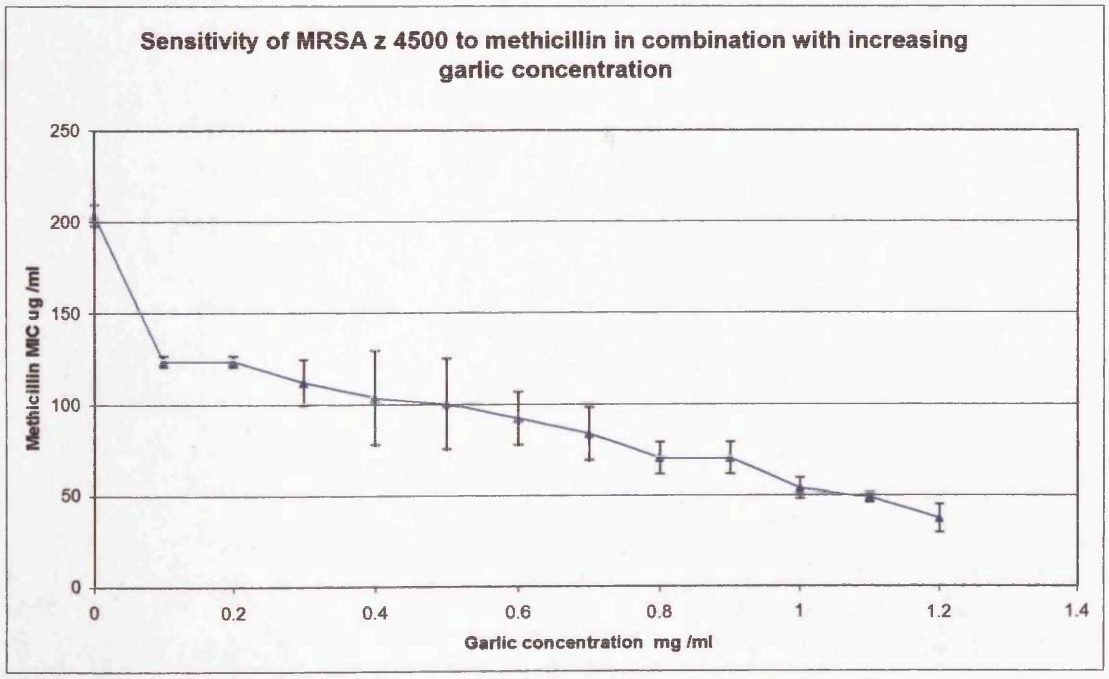


Figure 4.3.3.1 The effect of garlic extract on the MIC of MRSA z4500 to methicillin.

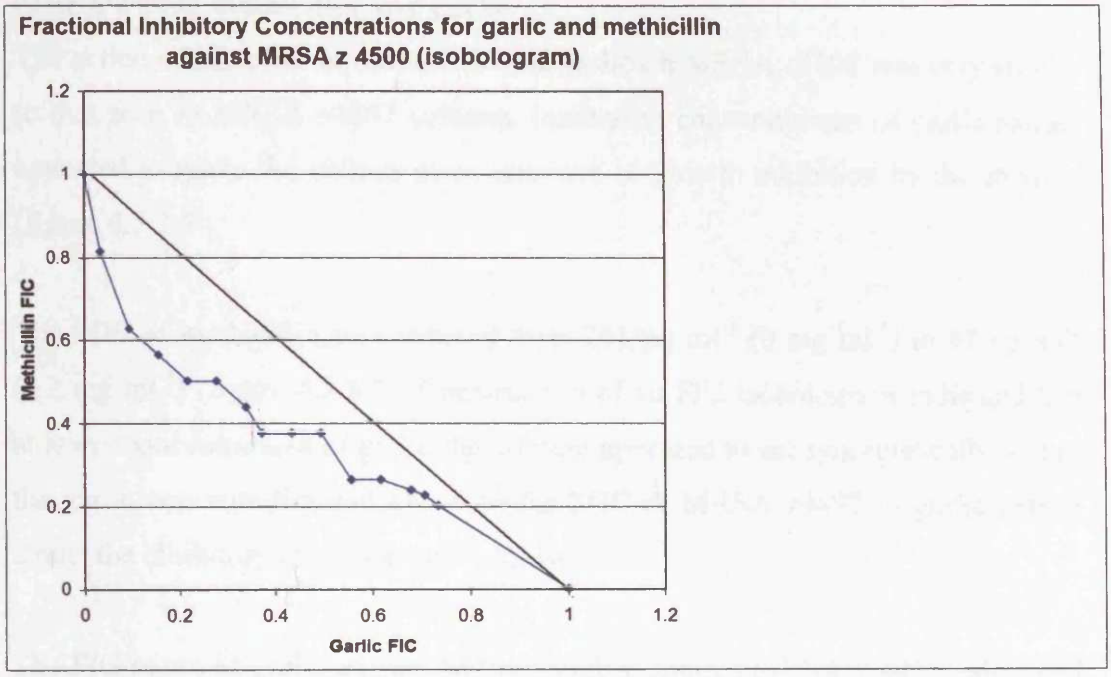


Figure 4.3.3.2 Plot of FIC to garlic extract against the FIC of methicillin when used in combination against MRSA z4500.

MRSA z 4497 methicillin and garlic.

The action of mixtures of methicillin and garlic on MRSA z4497 was very similar to that seen in MRSA z4467 cultures. Increasing concentrations of garlic extract appeared to make the culture more sensitive to growth inhibition by the mixture (figure 4.3.3.6)

The MIC of methicillin was reduced from 251 μg ml^{-1} (0 mg ml^{-1}) to 47 μg ml^{-1} (1.2 mg ml^{-1}) (figure 4.3.3.7). Construction of an FIC isobologram indicated that at lower concentrations of garlic the mixture appeared to act synergistically, but as the garlic concentration got closer to the MIC of MRSA z4497 to garlic extract alone, the inhibitory effect became additive.

The FIC index of garlic extract and methicillin against MRSA z 4497 calculated from the most effective concentration of the mixture(figure 4.3.3.8) (0.1 mg ml^{-1} garlic extract, and 150 μg ml^{-1} methicillin) (table 4.4.0), was 0.67. This is the lowest FIC index from all the tested strains, still indicating a synergistic/ indifferent relationship between garlic and MRSA z 4497 depending on which FIC standard values are to be used.

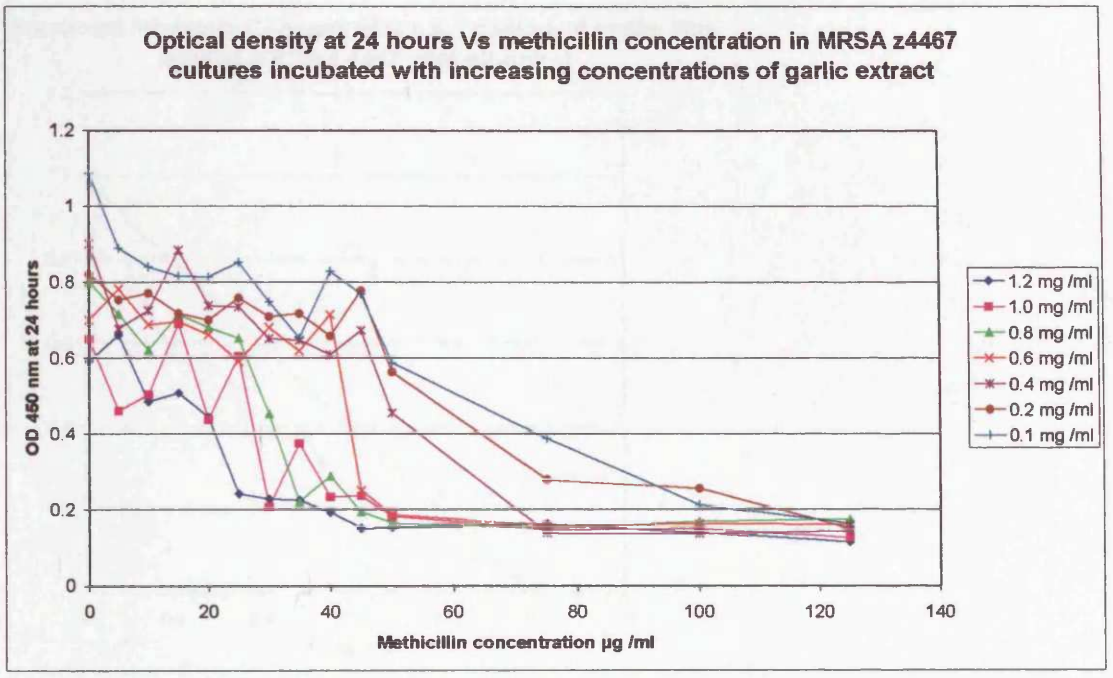


Figure 4.3.3.3 Inhibition profile of methicillin against MRSA z4467 incubated with increasing concentration of garlic extract (legend). Values plotted are the means of 3 experiments, error bars are omitted for clarity.

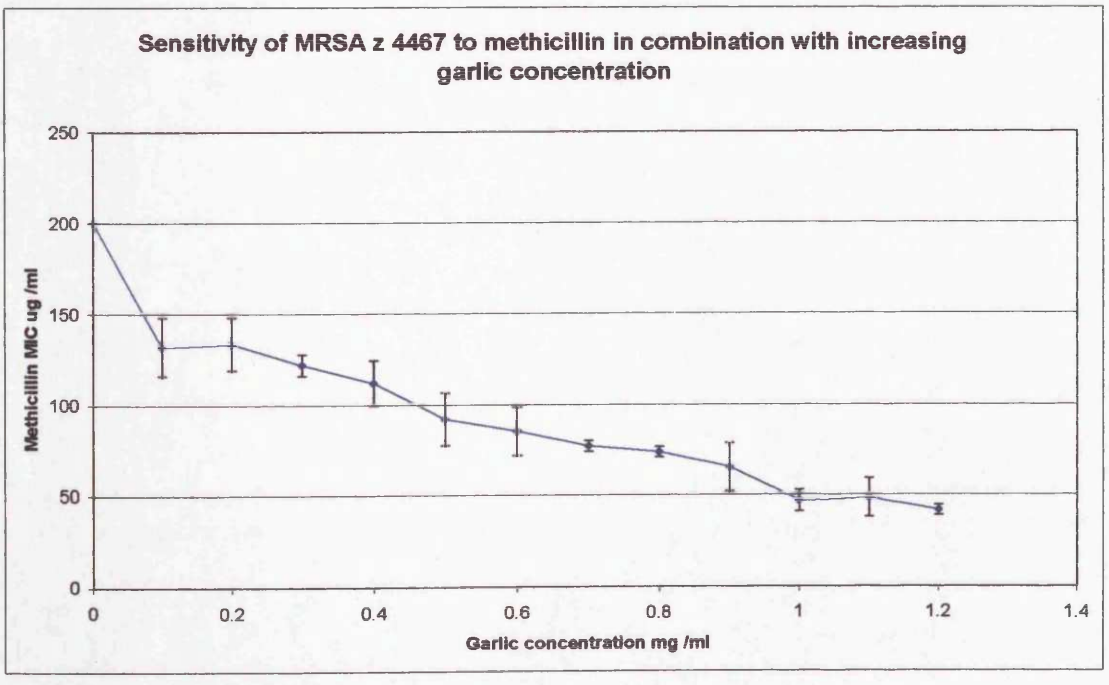


Figure 4.3.3.4 The effect of garlic extract on the MIC of MRSA z4467 to methicillin.

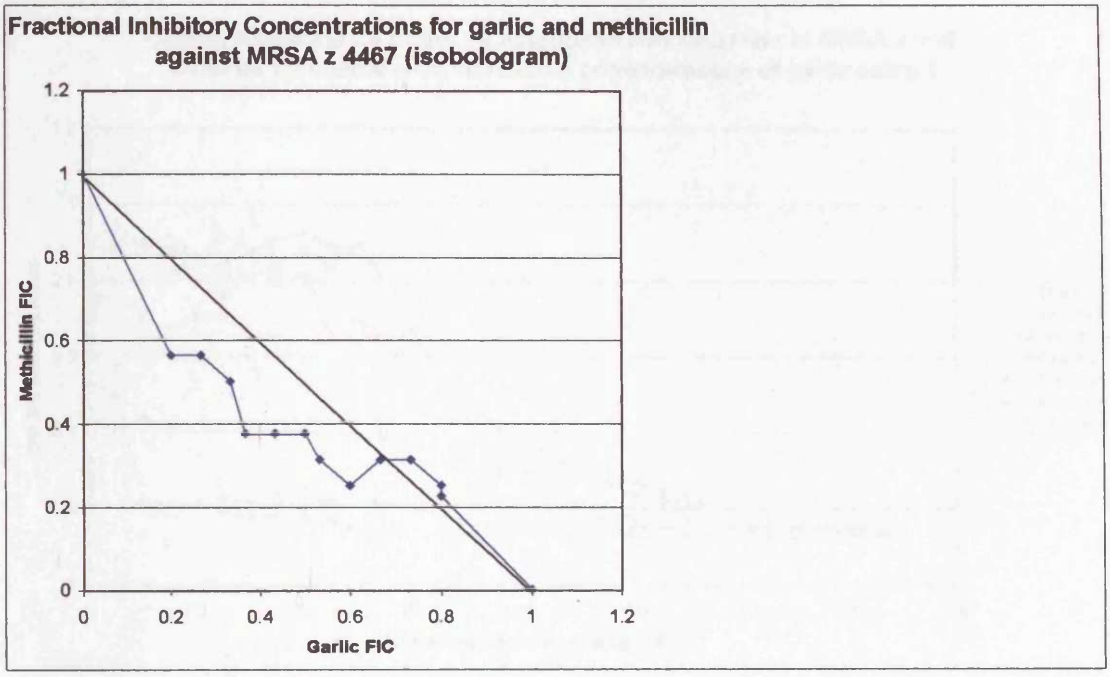


Figure 4.3.3.5 Plot of FIC to garlic extract against the FIC of methicillin when used in combination against MRSA z4467.

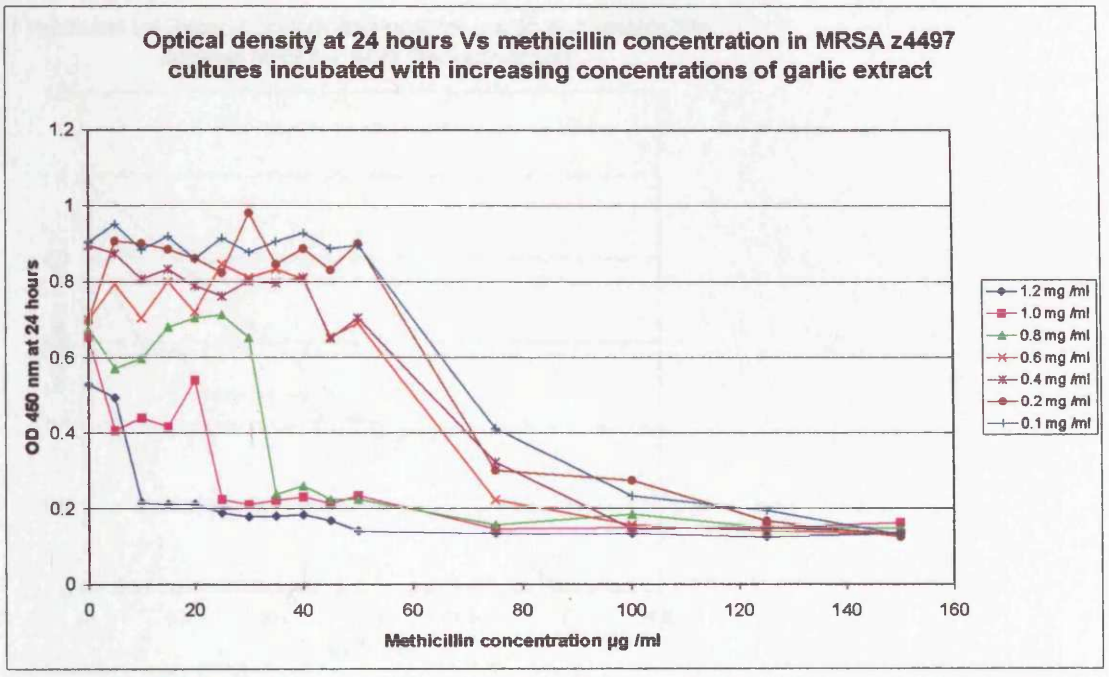


Figure 4.3.3.6 Inhibition profile of methicillin against MRSA z4497 incubated with increasing concentration of garlic extract (legend). Values plotted are the means of 3 experiments, error bars are omitted for clarity.

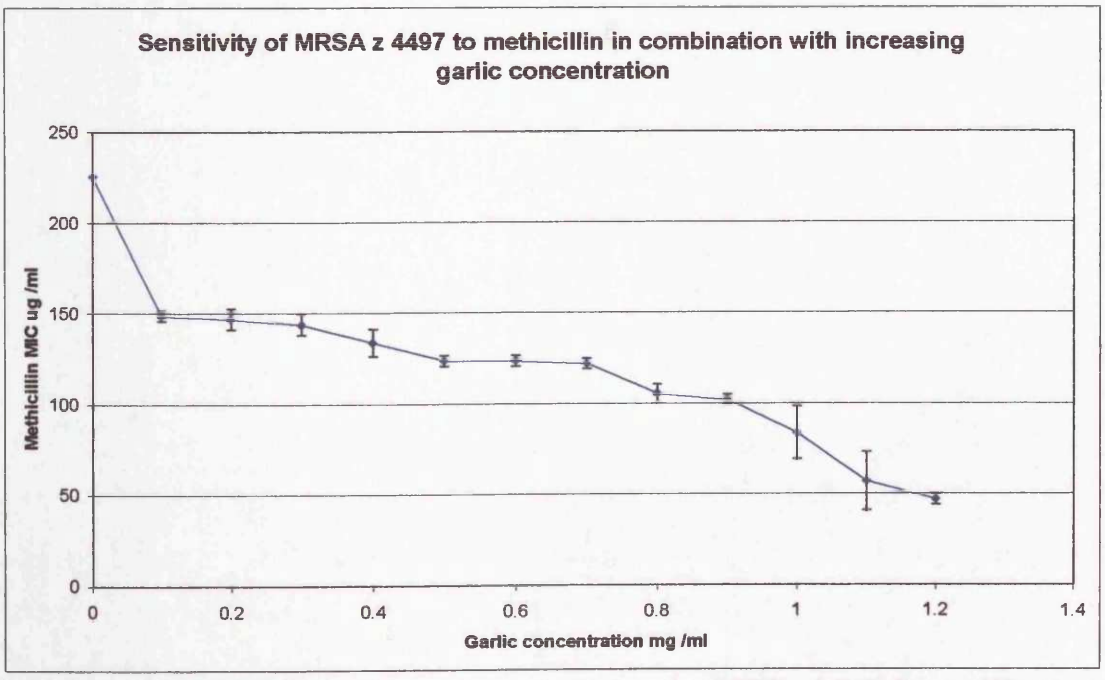


Figure 4.3.3.7 The effect of garlic extract on the MIC of MRSA z4497 to methicillin.

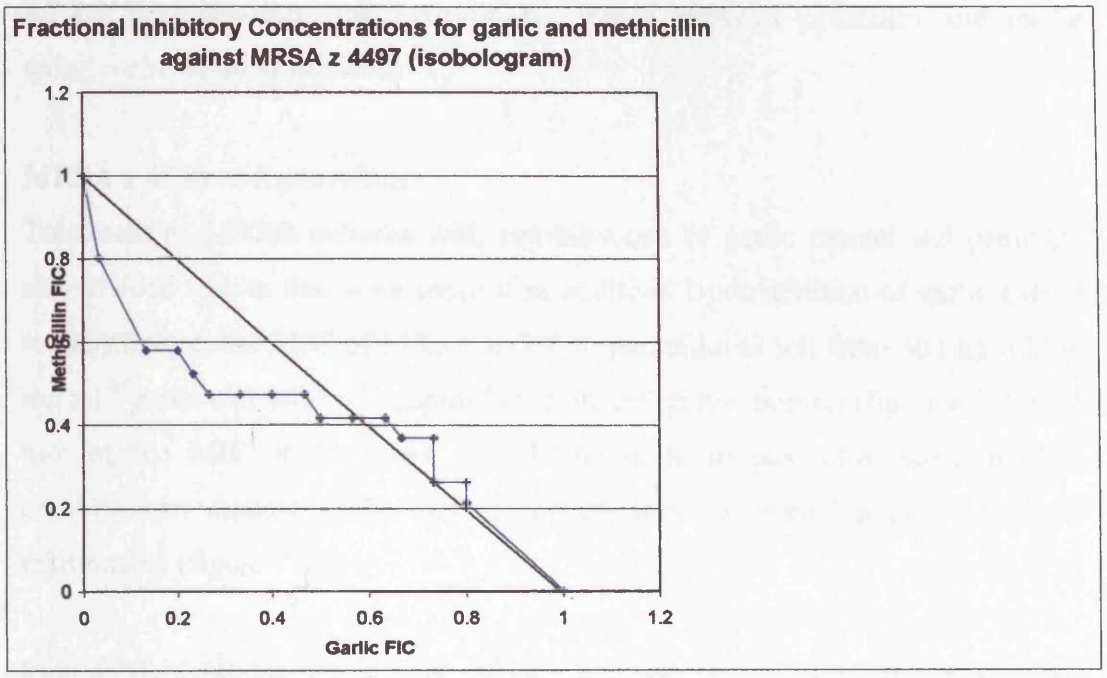


Figure 4.3.3.8 Plot of FIC to garlic extract against the FIC of methicillin when used in combination against MRSA z4497.

4.3.4.0 Investigations into synergistic action between penicillin and garlic using a chessboard method.

MRSA z 4500 and penicillin

Treatment of MRSA cultures with combinations of garlic extract and penicillin also yielded results that were more than additive. Upon addition of garlic extract to the mixture, the MIC of MRSA z4500 to penicillin G fell from 403 μg ml^{-1} (0 mg ml^{-1} garlic extract) to 53 μg ml^{-1} (1.2 mg ml^{-1}garlic extract) (figure 4.3.4.0). A plot of the MIC at 24 hours of MRSA z4500 to penicillin (when used in combination) against garlic extract concentration suggested a dose-dependent relationship (figure 4.3.4.1).

Plotting the isobologram of garlic extract FIC against penicillin FIC showed that there was a synergistic relationship between garlic extract concentration, penicillin concentration and growth inhibition in MRSA z4500 (Figure 4.3.4.2). The FIC index of garlic and penicillin against MRSA z4500 was calculated as 0.67 (most effect combination 0.6 mg ml^{-1} garlic and 110 μg ml^{-1} penicillin (table 4.4.0)).

MRSA z 4467 and penicillin

As with the other strains tested here, treatment of the cultures with garlic extract in combination with penicillin yielded an inhibitory effect that was greater than the effects seen on treatment with single components. Increasing concentrations of penicillin brought about increased growth inhibition in cultures where garlic extract was also present (figure 4.3.4.3). On addition of garlic extract the MIC of MRSA z4467 to penicillin G was reduced from 451 μg ml^{-1} (0 mg ml^{-1} garlic extract) to 80 μg ml^{-1} (1.2 mg ml^{-1} garlic extract). The MIC of MRSA z3367 to penicillin was seen to decrease in a dose dependent manner on addition of garlic extract (figure 4.3.4.4). Plotting the FIC values of each component as an isobologram revealed that the inhibitory relationship between penicillin and garlic extract was more than additive, however at higher concentrations of garlic extract, slight antagonism in the inhibitory action was seen (Figure 4.3.4.5).

The FIC index of garlic extract and penicillin against MRSA z4467 was calculated as 0.65, indicating synergy or indifference (most effective combination 0.4 mg ml^{-1} garlic extract and 170 μg ml^{-1} penicillin (table 4.4.0)).

MRSA z4497, garlic, and penicillin

Again, challenging cultures of MRSA z4497 with combinations of garlic extract and penicillin yielded inhibitory effects that were greater than those seen on treatment with single components. On addition of garlic extract, inhibitory effects were seen at sub-MIC levels of penicillin (figure 4.3.4.6). Increasing the concentration of garlic extract in the mixture from 0 mg ml^{-1} to 1.2 mg ml^{-1} decreased the MIC of MRSA z4497 to penicillin from 447 μg ml^{-1} to 60 μg ml^{-1}. Addition of garlic extract brought about a dose-dependent decrease in the MIC to penicillin (figure 4.3.4.7). The plot of FIC to garlic extract against FIC to penicillin shows that the relationship between the two components is more than additive, even at high concentrations of garlic extract (Figure 4.3.4.). The FIC index was calculated as 0.39 (most effective combination was 0.1 mg ml^{-1} garlic extract, 140 μg ml^{-1} penicillin (table 4.4.0)), making MRSA z4497 the most sensitive strain tested against garlic extract and penicillin.

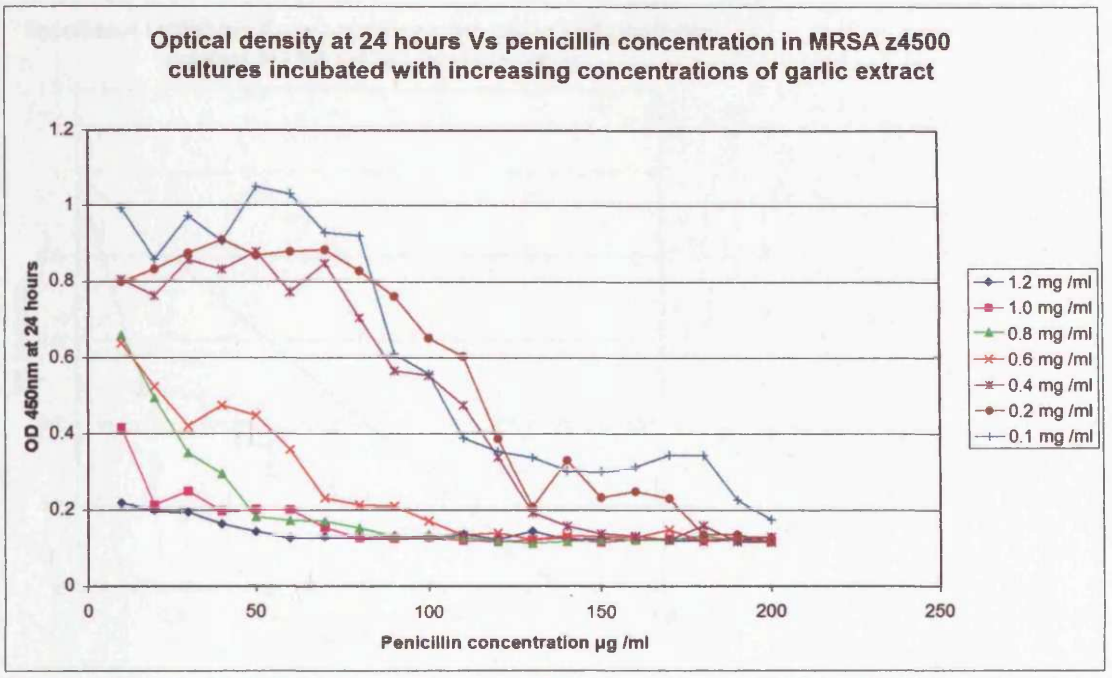


Figure 4.3.4.0 Inhibition profile of penicillin against MRSA z4500 incubated with increasing concentration of garlic extract (legend). Values plotted are the means of 3 experiments, error bars are omitted for clarity

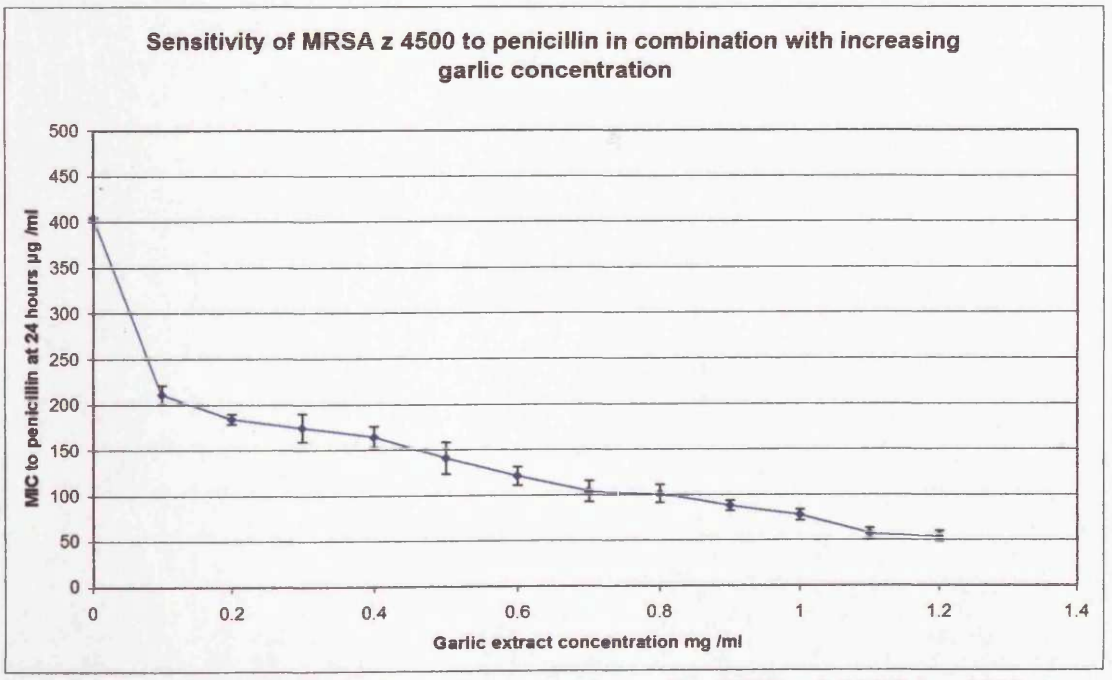


Figure 4.3.4.1 The effect of garlic extract on the MIC of MRSA z4500 to penicillin.

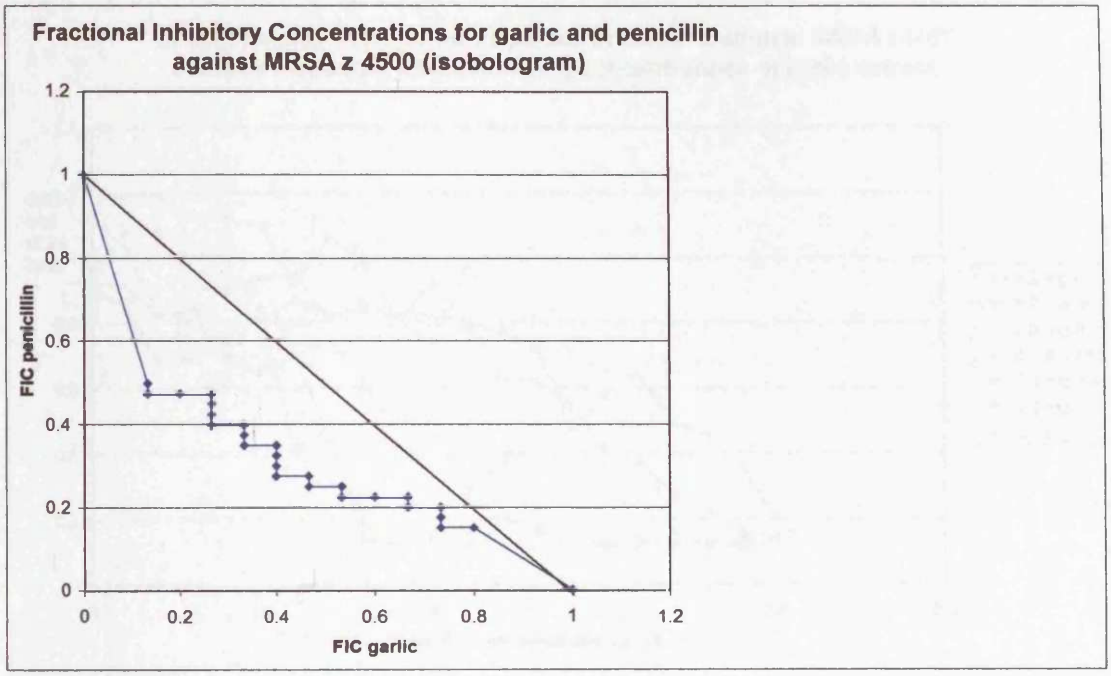


Figure 4.3.4.2 Plot of FIC to garlic extract against the FIC of penicillin when used in combination against MRSA z4500.

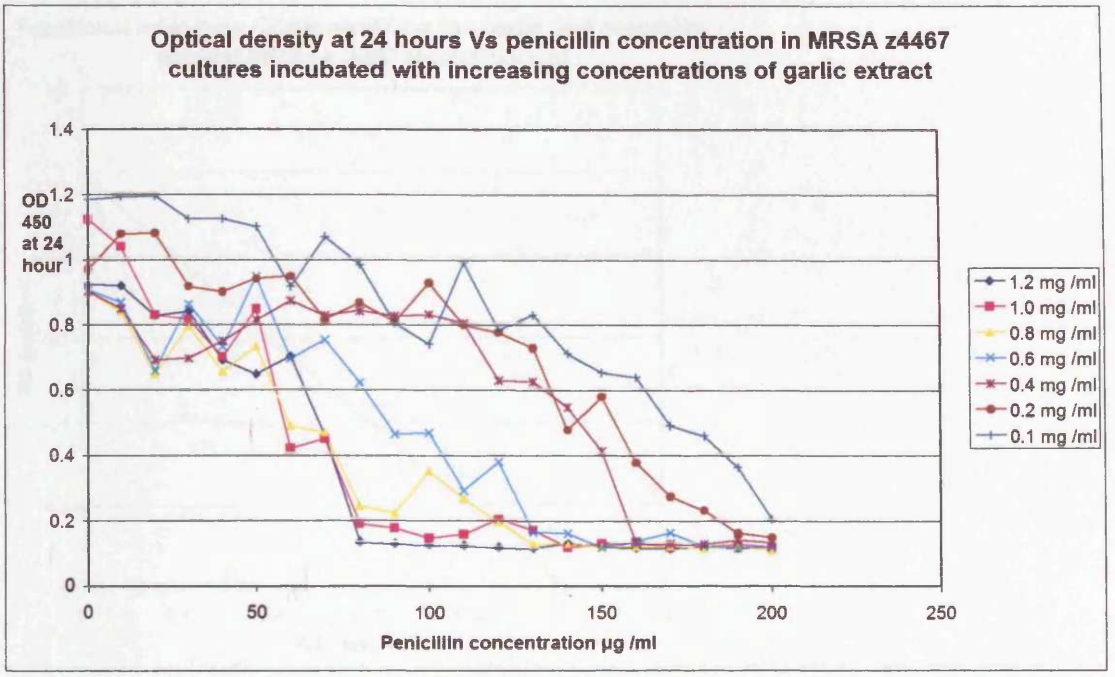


Figure 4.3.4.3 Inhibition profile of penicillin against MRSA z4467 incubated with increasing concentration of garlic extract (legend). Values plotted are the means of 3 experiments, error bars are omitted for clarity

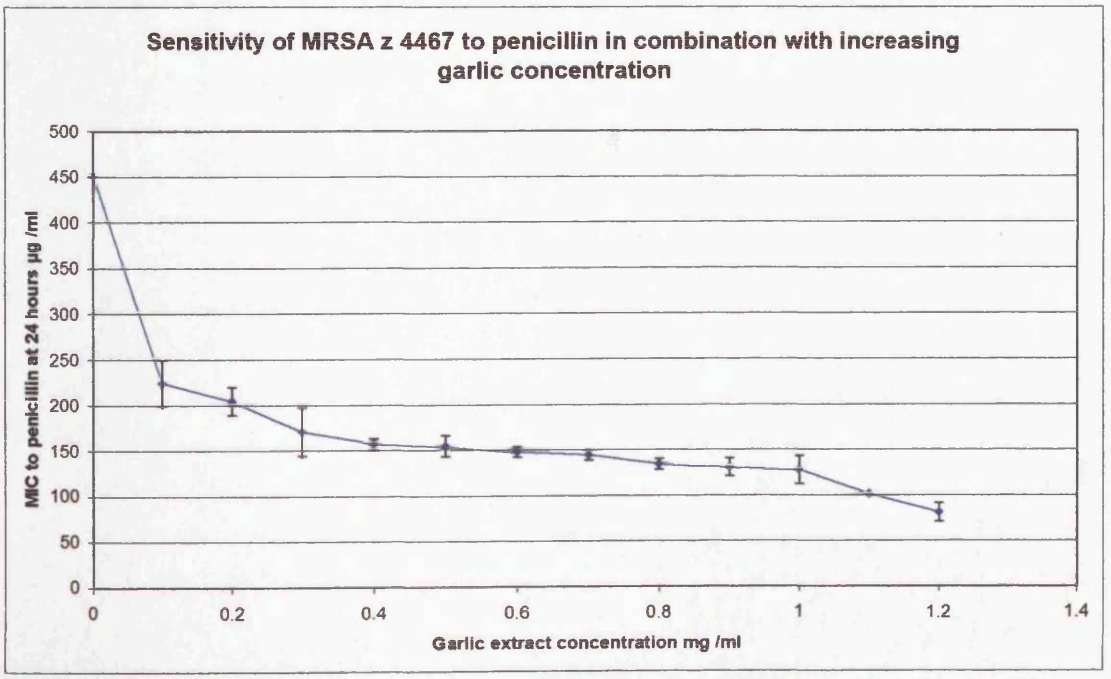


Figure 4.3.4.4 The effect of garlic extract on the MIC of MRSA z4467 to penicillin.

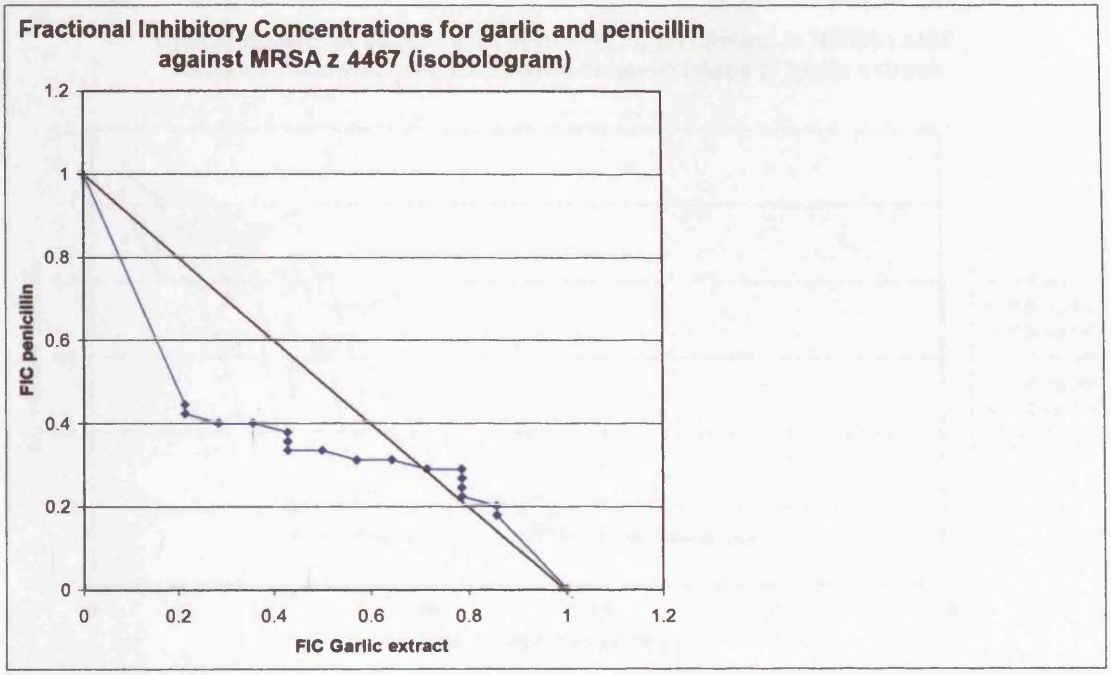


Figure 4.3.4.5. Plot of FIC to garlic extract against the FIC of penicillin when used in combination against MRSA z4467.

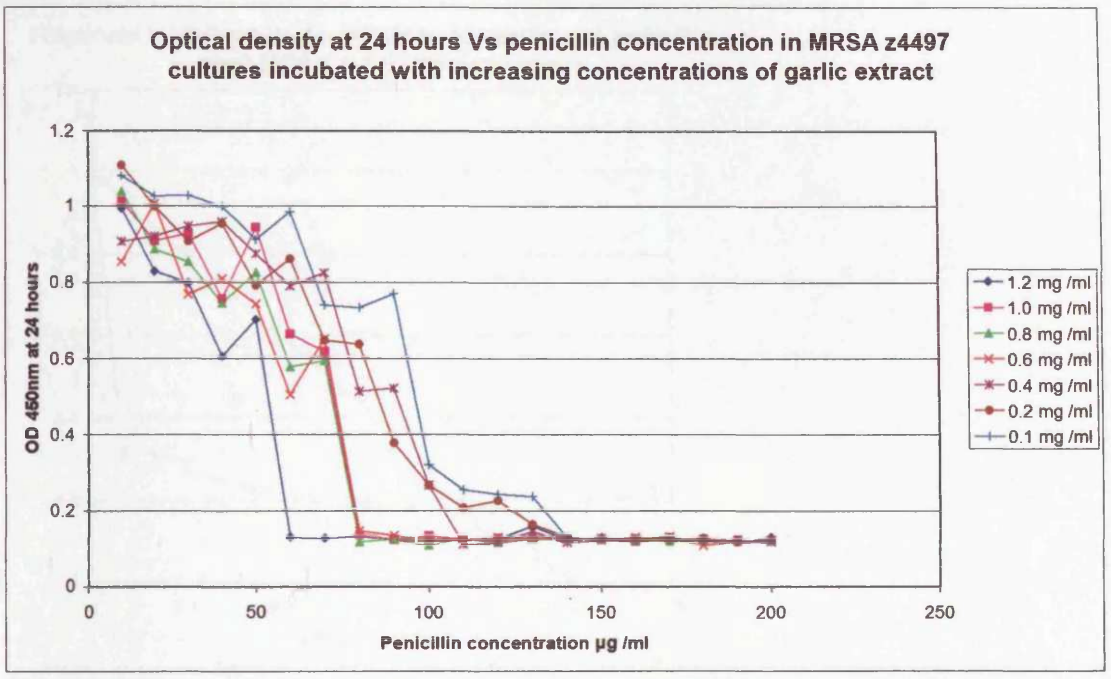


Figure 4.3.4.6. Inhibition profile of penicillin against MRSA z4497 incubated with increasing concentration of garlic extract (legend). Values plotted are the means of 3 experiments, error bars are omitted for clarity

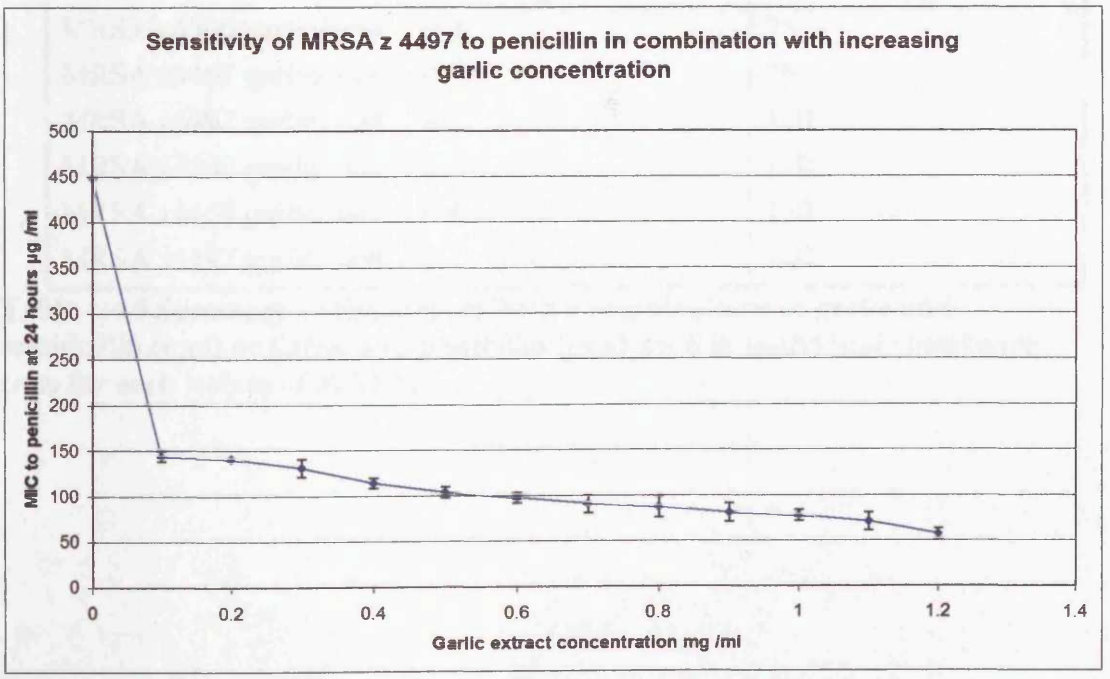


Figure 4.3.4.7. The effect of garlic extract on the MIC of MRSA z4497 to penicillin.

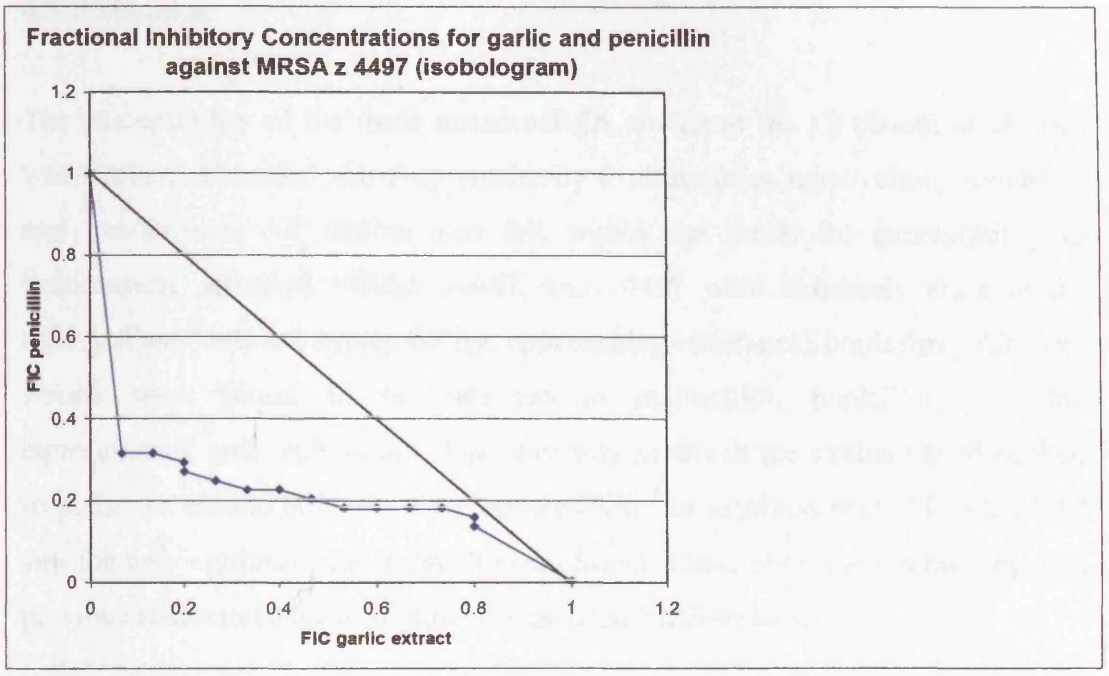


Figure 4.3.4.8. Plot of FIC to garlic extract against the FIC of penicillin when used in combination against MRSA z4497.

Experiment	Concentration of component at most effective combination used in chessboard tests	
	Garlic mg ml^{-1}	Antibiotic µg ml^{-1}
MRSA z4500 garlic/ met	0.4	75
MRSA z4467 garlic/ met	0.5	75
MRSA z4497 garlic/ met	0.1	150
MRSA z4500 garlic/ pen	0.6	110
MRSA z4469 garlic/ pen	0.4	170
MRSA z4497 garlic/ pen	0.1	140

Table 4.4.0 Summary of the most effective combinations of garlic and methicillin (met) or garlic and penicillin (pen) used in individual chessboard tests for each isolate of MRSA.

4.5 Discussion

The susceptibility of the three tested MRSA strains to the 12 chosen antibiotics was similar, all strains showing sensitivity to rifampicin, tetracycline, mupirocin and gentamycin. All strains also fell within the limits for susceptibility to vancomycin, although MRSA z4467, and z4497 were extremely close to the intermediate (reduced susceptibility, approaching resistance) borderline. All three strains were found to be resistant to methicillin, penicillin, oxacillin, ciprofloxacin, and cephalothin. The only way in which the strains varied in their response to antibiotics, was their susceptibility to erythromycin; MRSA z4497 was the only erythromycin resistant strain found. These results are in keeping with previous characterisations of clinically isolated MRSA strains.

All three MRSA strains showed high MIC values to penicillin and methicillin. The MIC values needed for *S. aureus* strains to be considered sensitive to methicillin and penicillin are 5 $\mu g\ ml^{-1}$, and 10 $\mu g\ ml^{-1}$ respectively. MIC values for the strains tested here to methicillin lay between 198 $\mu g\ ml^{-1}$ and 251 $\mu g\ ml^{-1}$, whilst MIC values to penicillin were between 404 $\mu g\ ml^{-1}$ and 451 $\mu g\ ml^{-1}$.

When the cultures were exposed to combinations of garlic and methicillin, the MIC values achieved were substantially lower than the MIC values to single components in all strains.

Exposure of MRSA z4500 to the mixture yielded an MIC to methicillin of 75 $\mu g\ ml^{-1}$ to methicillin, and an MIC to garlic of 0.4 $mg\ ml^{-1}$ (at the most effective combination), reducing the MIC to methicillin by a factor of 2.67, and to garlic by a factor of 3.75.

Treatment of MRSA z4467 with the mixture gave an MIC to methicillin of 75 $\mu g\ ml^{-1}$ and an MIC to garlic of 0.5 $mg\ ml^{-1}$. This represents a 2.64 fold reduction in the MIC to methicillin, and 2.8 fold reduction in the MIC to garlic extract.

Treatment of MRSA z4497 with the combination yielded an MIC to methicillin of 150 $\mu g\ ml^{-1}$ to methicillin, and 0.1 $mg\ ml^{-1}$ to garlic extract. This represents a

1.67 fold reduction in the MIC to methicillin, and a 15 fold reduction in the MIC to garlic extract at the lowest effective combination.

On treatment with mixtures of penicillin and garlic extract mixtures, the MIC of MRSA z4500 to garlic extract fell to 0.6 mg ml $^{-1}$ and to penicillin 110 μg ml^{-1}, at the lowest effective concentration (reduced by factors of 2.5 and respectively 3.7). On exposure of MRSA z4467 to mixtures of garlic extract and penicillin, the lowest effective concentration of the mixture yielded MIC values to garlic and penicillin of 0.3 mg ml^{-1} and 170 μg ml^{-1} respectively this represents a reduction in the MIC of MRSA z4467 to garlic extract by a factor of 4.7, and to penicillin by a factor of 2.7.

Treatment of MRSA z4497 with combinations of garlic extract and penicillin, reduced the MIC to garlic extract to 0.2 mg ml^{-1} and to penicillin to 130 μg ml^{-1}. This represents a reduction by factors of 7.5 and 3.4 in the MIC values to garlic extract and penicillin respectively.

MRSA Strain	Treatment combination	FIC index at lowest effective combination	Inference
MRSA z4500	Garlic/ methicillin	0.72	Synergy/ indifference
MRSA z4500	Garlic/ penicillin	0.67	Synergy/ indifference
MRSA z4467	Garlic/ methicillin	0.74	Synergy/ indifference
MRSA z4467	Garlic/ penicillin	0.65	Synergy/ indifference
MRSA z4497	Garlic/ methicillin	0.67	Synergy/ indifference
MRSA z4497	Garlic penicillin	0.39	Synergy/ indifference

Table 4.5.0 FIC indices and conclusions from garlic and methicillin/ garlic and penicillin synergy investigations.

Treatment of all three strains with methicillin/ garlic extract, and penicillin/ garlic extract combinations failed to produce the 4 fold reduction in the MIC values to each individual component (table 4.5.0), as suggested as the criterion for synergistic activity in many investigations. However when using the synergy FIC value limits employed by other authors (Beale and Sutherland 1983), penicillin and garlic proved to be synergistic in inhibitory nature to all three strains tested here, and methicillin and garlic displayed borderline synergy. Whichever synergy limits used, it can be said that each combination of components tested inhibited the growth of MRSA in a manner that was substantially more that additive.

It is possible that components acting synergistically do so because they have similar or related sites of action, whilst components that are additive in their inhibitory capacity act on unrelated sites. This would suggest that in the case of garlic extract/ penicillin combinations, garlic extract is inhibiting cell wall synthesis or is facilitating the inhibitory action of penicillin by attacking the synthesis or inhibitory capacity of the cells β- lactamase defence mechanism. In the case of garlic extract/ methicillin combinations, it may suggest that garlic extract could be acting to inhibit cell wall synthesis, again is facilitating the action methicillin by inhibiting the production or efficiency of the PBP2′ mechanism. This would leave methicillin free to inhibit cell wall synthesis in its usual manner (as it is unaffected by the β- lactamase mechanism). However since garlic is inhibitory to enzymes containing sulf-hydryl active site/ conformational groups (Koch 1996) a large number of growth inhibition mechanisms are possible.

In their experiments with garlic extract and vancomycin combinations against Vancomycin Resistant *Enterococci* (VRE) Jonkers *et al* 1999 suggested that garlic extract was inhibiting the resistance mechanism at a genetic level, by acting on the transposon responsible for vancomycin resistance in *Enterococci.* Garlic extract may be acting in a similar manner against MRSA, however bearing in mind that garlic extract is itself a complex mixture of volatile antimicrobials (Chapters 5, 6) many of which have multiple and different sites of action, it may be likely that garlic extract is bringing about the facilitation of antibiotic action by acting on multiple sites responsible for resistance, as well as the broad spectrum metabolic and growth inhibition previously reported in antibiotic susceptible cells.

Previously standards and limits for synergistic action were used to investigate the effects of conventional single component antibiotics, whose mechanisms of action are generally understood. Such rigid limits are possibly not as helpful when analysing combinations of natural extracts and conventional treatments, especially extracts such as garlic which represent a dynamic mixture of active components. It may be the case that if the components of garlic extract that play an active role in synergistic inhibition in combination with antibiotics are identified - and used in place of garlic extract, lower FIC indices could be obtained.

It must be stressed that the work presented here is very much a preliminary study, and needs to be expanded on before it is possible to say that garlic extract could be used to complement therapeutic antibacterials in a clinical setting. These findings suggest that further research is clearly warranted.

Chapter 5

Qualitative and quantitative analysis of components from garlic extract using Gas Chromatography – Mass Spectrometry.

Qualitative and Quantitative analysis of components from garlic extract using Gas Chromatography – Mass Spectrometry

5.1 Introduction

The compositional chemistry of garlic is complex. Nielson *et al,* (1991) showed that garlic contains an unusually high content of sulphur. Sulphur is present in all plants to some extent, contained in thiol groups of various proteins and amino acids, however the sulphur content of garlic has been recorded as in excess of three times higher (>3 mg g^{-1} fresh weight) than most other vegetables (Nielson *et al*, 1991). Most researchers are in agreement that the compounds responsible for the high sulphur content of various forms of garlic are also responsible for the plants many reported beneficial effects.

Chemical investigations into garlic composition were first documented in 1844 by the German scientist Wertheim, who distilled an essential oil rich in $C_6H_{10}S$ (diallyl sulphide). It was he who first used the term *allyl* (from *Allium*) to describe the C_3H_5 hydrocarbon structure. The investigations of Semmler (1892) identified the precise composition of Werheim's steam distillate of garlic, identifying diallyl disulphide (60%), diallyl trisulphide (20%), diallyl tetrasulphide (10%) and diallyl sulphide (6%).

Louis Pasteur is credited with the earliest scientific investigations into the antimicrobial action of garlic in 1858 (Pasteur 1858, Purvis 2001) he found that crushed garlic extract was an effective antibacterial (Delaha 1985), however it was noted that the steam distillate of garlic possessed a much reduced antibacterial activity. In their breakthrough experiments of 1944, Cavallito and Bailey suggested a compound that could be responsible for the antibacterial activity of garlic. Using a cold extraction procedure (Chapter 1) they isolated an oxygenated sulphur compound with high antibacterial activity, from crushed garlic cloves. They named this compound allicin. Investigations into garlic chemistry now preceded at pace, the first laboratory syntheses of allicin were performed in 1947 by Stoll and Seebeck, by direct oxidation of diallyl disulphide. Elucidation of allicin synthesis

within the crushed clove, and the identification of alliin and alliinase followed swiftly (Stoll and Seebeck, 1951). On crushing, the pyridoxal-phosphate dependent enzyme *alliinase* acts upon the odourless *S-allylcysteine sulphoxide* (alliin) giving rise to *allicin*, pyruvate, and ammonia (figure 5.1.0) this process is described in more detail in Chapter 1.

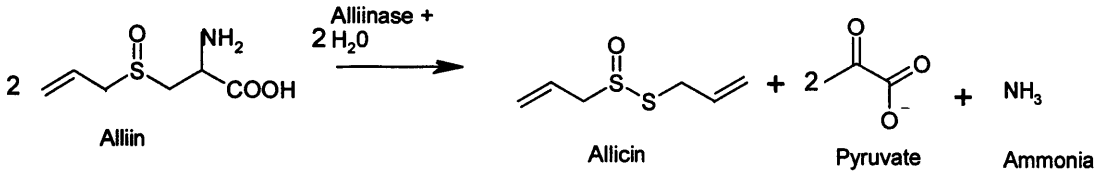


Figure 5.1.0 Enzymic production of allicin from alliin and water by alliinase.

Allicin is one of a family of thiosulphinates (Chapter 1) found in garlic extract produced by enzymic action in various cysteine sulphoxide precursors. Most chemical investigations into garlic and its extracts have concentrated on allicin as it (along with its breakdown products) is credited with many of the beneficial effects.

Allicin is a reactive compound that can form a variety of products (figure 5.1.1), depending on thermal and chemical conditions (Lawson 1996). In aqueous solution, allicin degrades quickly to produce allyl sulphides, mainly diallyl disulphide and diallyl trisulphide (Iberl *et* al 1990, Block *et al* 1992). In the case of degradation to diallyl disulphide, propene and sulphur dioxide are reaction by-products, whereas in the case of degradation to diallyl trisulphide, allyl alcohol is the reaction by-product (Block *et al* 1992). Diallyl sulphide undergoes further reaction to produce longer-chain allyl sulphides such as diallyl trisulphide, and tetrasulphide (especially at elevated temperatures) (Harris 2002), and smaller breakdown products such as allyl mercaptan (Lawson 1993), especially in environments where cysteine is present.

Breakdown of allicin in organic solvent leads to different compounds, and usually a loss in reactivity (including antibacterial activity (Block *et al* 1996)) of the breakdown products. In alcoholic environments (also when allicin is in its pure oily form) reaction products of allicin are longer-chain, more stable molecules termed *ajoenes* (Figure 5.1.1). Ajoenes are credited with garlic's ability to prevent platelet aggregation (Apitz-Castro *et al* 1993). Whilst when dissolved in low polarity solvents such as hexane, chloroform, ether or dichloromethane, the principle breakdown products are heterocyclic 6-membered ring compounds termed vinyl dithiins (Brodnitz *et al* 1971).

Clearly the highly reactive nature of the components present within garlic preparations present problems when rigorously trying to standardise garlic preparations for the commercial marketplace. A variety of analytical techniques are now used to investigate garlic compositional chemistry.

Analysis of garlic extract and its components has traditionally been carried out by High Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC); these methods still seem to be the most popular. Both techniques have various advantages and disadvantages ascribed to them.

HPLC has the major advantages that it can be performed at low temperatures, allowing direct identification and quantification of thermally unstable compounds such as allicin, and allyl sulphides. HPLC can also be used in a preparative manner, as it is usually non-destructive in its identification of chemical components. It does have drawbacks too; separated components are usually detected using ultra violet (UV) Spectra, compounds that absorb poorly in the UV region must be derivatised into compounds that will give a characteristic UV spectrum. This is not always possible, and can result in destruction of more unstable components within the mixture. Separation of chemically-similar compounds can also be problematic.

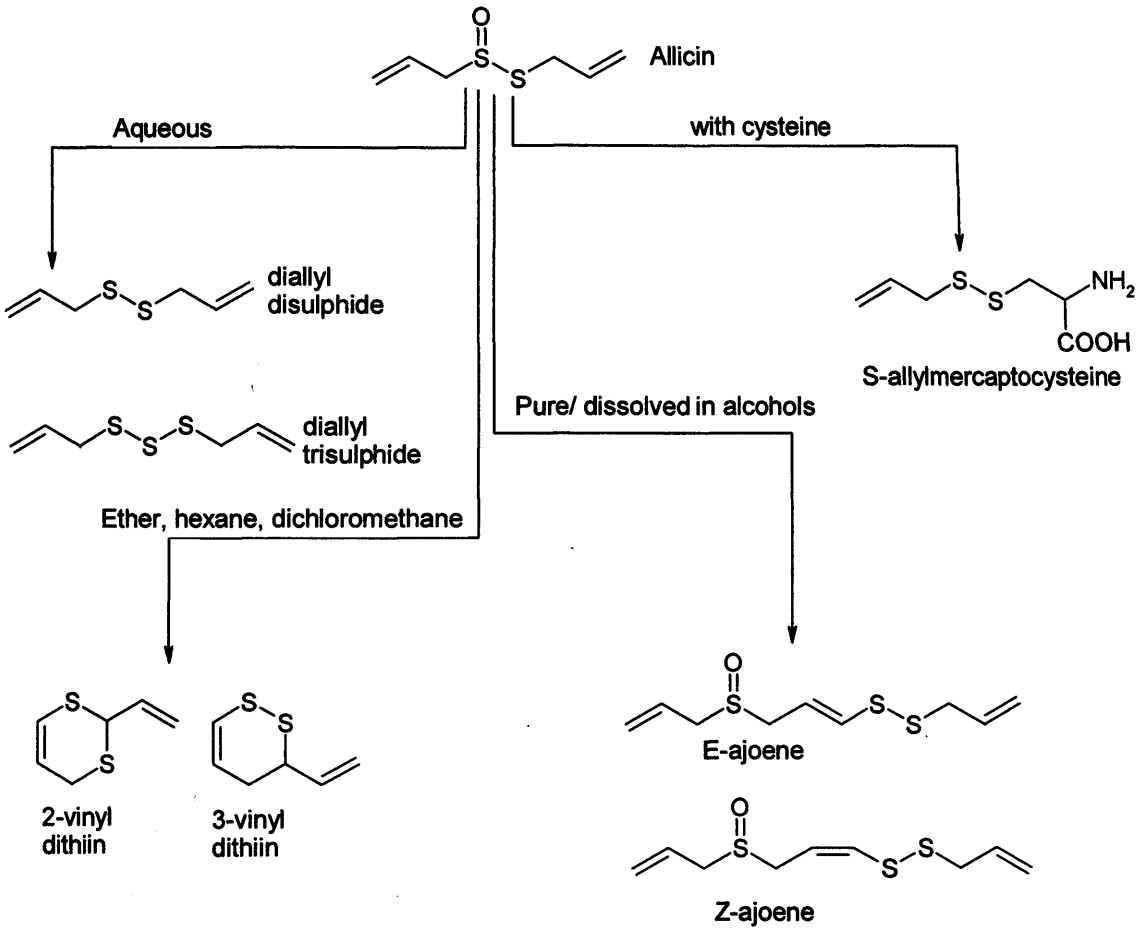


Figure 5.1.1 Breakdown of allicin under various conditions (modified from Koch and Lawson 1996).

Gas chromatography utilises the differential volatility of components within mixtures, in order to achieve separation. Small amounts of substance for analysis dissolved in a suitable solvent, are injected into the apparatus. Components present within the sample are vaporised in the injection port with the solvent. Components are then carried through a chromatography column containing a suitable packing matrix by an inert carrier gas (usually helium or nitrogen). As components are carried along the column they separate according to their volatility, molecular size, and the amount of time components spend within the packing matrix. The temperature of the column can be ramped, achieving a greater separation between more volatile and less volatile components (actually shortening analysis time, and focussing late-eluting components, theoretically providing good isothermic separation). Compounds exiting the column are then detected by a suitable detection device, and recorded as peaks on a trace known as a chromatogram. Compounds can be quantified by comparing the area contained within the peaks to external or internal standard peak areas. The use of a mass spectrometer (GCMS) as a detection device confers an advantage on the system in that it allows identification of unknown compounds through interpretation of the components' mass spectra.

The advantages of GCMS over HPLC are that GCMS can provide faster separations and results, it offers good separation and detection of components without the need for derivatisation. By modification of the column temperature program it is possible to ensure that all components are separated and identified. The disadvantages of using this system are that the analysis is destructive to the components; offering no preparative capacity, and that thermally unstable components can degrade on vaporisation, leading to detection of artefacts within the sample.

To a large extent the problems associated with thermal degradation, can be avoided by using a heated, on-column injection procedure, ensuring that thermally unstable compounds are reacted before separation. Provided that the thermal reaction products of the compounds are known; an accurate, repeatable analysis of most thermally unstable components/ mixtures can performed. To further improve accuracy and reproducibility of garlic analyses (where allicin reaction products are themselves thermally unstable), controlled heating of the aqueous garlic extract before injection can be used (Yan *et al*, 1993). This ensures that allicin degrades

completely into its identified breakdown products, which can then be quantified using internal and external standards. Extraction of volatiles from garlic extract (carried out at a temperature of 100°C in dichloromethane) ensures that all allicin is converted into vinyl dithiins (major component) or allyl sulphides. By calculating the concentrations of vinyl dithiins, and allyl sulphides (and using the reaction stoichiometry of allicin breakdown) it is possible to arrive at estimations of the original allicin and alliin content within the sample.

The experiments detailed in this chapter set out to identify and quantify components present in garlic powder and its aqueous extract, and to evaluate the allicin content of the garlic powder. Also to compare the profile of compounds achieved from heated garlic extract, commercially available 'stabilised allicin' and commercially available aged garlic extract (Chapter 3).

5.2 Materials and methods

5.2.1 GCMS parameters

Extracted garlic components were analysed on a Fisons GC 8000 instrument with attached MD 800 mass spectrometer. The column chosen for analysis was a DB-5ms (non polar), 30 m x 0.25 mm internal diameter, 0.25 μm film thickness. Helium was used as a carrier gas (40 cm s^{-1}). An on column, splitless injection procedure was used, the oven temperature program ran from 30°C to 240°C at a rate of 5°C min^{-1}, start and finish temperatures were held for 3 min. Injection port temperature was 70°C. Injection volume for all experiments presented in Chapter 5 was 1.2 μl. Components were detected after a solvent delay of 1.45 min. The mass spectrometer source temperature was 200°C (with 260°C transfer line), and scans were carried out between 35 and 215 amu (EI^+ 70eV).

5.2.2 Garlic extract preparation

Garlic extract was prepared as described previously in sterile distilled water. Concentrations used for analyses were 10 mg ml^{-1} and 50 mg ml^{-1}.

5.2.3 Solvent extraction of garlic components

Prepared aqueous garlic powder extracts (0.5ml) were inverted with dichloromethane (DCM) (analytical grade, Sigma-Aldrich, Poole UK) for 30 s in a polypropylene micro-centrifuge tube. Samples were then centrifuged gently for 2.5 min in a desktop micro-centrifuge to aid separation of solvent and aqueous layers. The solvent layer was removed, the aqueous fraction was then extracted twice again with 0.5 ml aliquots of DCM. Solvent fractions were pooled, and used immediately for injection.

5.2.4 Identification of garlic components

Components found in analysed extracts were identified by comparing retention times from the chromatogram, and mass spectra of isolated components to external standards. Where no external standard was available, compounds were identified by comparison of mass spectra to those on the GCMS software database (Masslab 1.4 with NIST library databases), or to spectra found in previously published literature.

5.2.5 Analysis of components intrinsic to freeze dried garlic powder

Garlic 0.3g powder was suspended directly into 5 ml DCM, in a stoppered glass tube, vortexed for 10 min then left to stand at room temperature for 20 min. The solvent extract was then filtered through a 0.2 μm filter and used directly for GCMS injection.

5.2.6 Quantification of garlic components through GCMS analysis

The following abbreviations have been used for garlic components:
Diallyl sulphide (DAS), diallyl disulphide (DAD), diallyl trisulphide (DAT), Diallyl tetrasulphide (DATe), allyl alcohol (AA), allyl mercaptan (AM), methyl propyl sulphide (MPS), methyl propyl disulphide (MPD), dimethyl disulphide (DMD), dipropyl disulphide (DPD), allyl methyl sulphide (AMS), allyl methyl disulphide (AMD), allyl methyl trisulphide (AMT), 2-vinyl dithiin (2VD), 3-vinyl dithiin (3VD).

Most components could be quantified using external standards, injected separately in 3 concentrations (1 ng μl^{-1}, 5 ng μl^{-1}, and 10 ng μl^{-1}) in triplicate (DAS, DAD, MPD, AMD, and AA (Sigma –Aldrich, Poole UK), DMD, DPD, MPS, AMS, and AM (Lancaster, Lancs UK). Where no external standard was readily available, concentrations were calculated by analysis of area ratio to an internal (spiked) standard of known concentration (MPS 5 ng μl^{-1}).

5.2.7 Evaluation of allicin content through analysis of total sulphides (controlled heat treated sample)

1g of garlic powder was added to 30 ml of distilled water. The suspension was vortexed thoroughly for 10 min then left to stand at room temperature for 20 min, before sterile filtering (0.2 μm filter). 5 ml of filtered aqueous extract was then placed in a screw capped glass test tube. 2 ml DCM was carefully added, the tube was then heated to 100°C in a controlled heating block. Due to the volatile nature of the DCM fraction, the tube was observed carefully throughout the heating procedure, and was removed from the heat source occasionally to prevent violent bubbling.

The tube was then allowed to slowly cool to room temperature before it was opened and the solvent layer removed. An internal standard (methyl propyl sulphide) was added (5 ngμl^{-1} final concentration) 1.2 µl was used for immediate injection.

Allicin content was then calculated (Yan *et al*, 1993) and expressed as mg per g of garlic powder (mg g^{-1}). The calculation (below) used is based on the quantification of each relevant sulphide (DAS, DAD, DATr, DATe, 2-VD, and 3-VD) from GC analysis.

$$\text{allicin content} = \sum \left[\frac{S_{\text{content}} \times 162 \times N_S}{M_S \times 2} \right]$$

Where $S_{content}$ is the pre calculated amount of sulphide (must be performed individually for each detected diallyl sulphide) found to be present in the extract, N_s is the number of sulphur atoms the sulphide contains, and M_s is the mass of the specific sulphide (g). Allicin content is calculated in g.

Using the value calculated as allicin content an approximation of the alliin content of the powder can be obtained:

$$\text{alliin content} = \frac{(A_{\text{content}} \times 177 \times N_S)}{162}$$

Where $A_{content}$ is the pre-calculated allicin content. Alliin content is calculated in g.

5.2.8 GCMS analysis of commercially available allicin and garlic preparations

'Stabilised' allicin was obtained in liquid (300 ppm) from an organisation involved in the manufacture of the commercially available allicin product used in chapter 3 (The Garlic centre, Battle UK). The aged garlic extract analysed here was manufactured by Wakunga of America Co. Ltd. (Mission Viejo USA).

200 µl Stabilised allicin were dissolved in 200 µl DCM (final concentration 150 ppm), MPS was added as an internal standard (final concentration 5 ng μl^{-1}) 1.2 µl was immediately injected and analysed in the manner described above.

Aged garlic extract tablet were crushed and powdered using a pestle and mortar, then suspended in sterile distilled water to give a final concentration of 50 mg ml^{-1}. The suspension was vortexed thoroughly for 10 min, left to stand at room temperature for 20 min, and sterile filtered through a 0.2 µm filter disc. 0.75 ml was then extracted into 0.75 ml DCM by inversion for 30 s. On separation MPS was added to the solvent fraction (final concentration 5 ng μl^{-1}) and 1.2 µl was injected and analysed in the manner described previously.

5.3 Results

5.3.1 Detection and Identification of garlic components by GCMS

Single standard components were analysed by GCMS, the retention time of each component was calculated and the characteristic molecular fragmentation patterns of each were identified (Table 5.3.1). Possible structures of molecular fragments responsible for commonly occurring m/z peaks were derived (Table 5.3.2).

Mass spectrum peaks of mass 39, and 41 were found to characteristic be of an allyl or propyl group within the component. Mass spectrum peaks of 45, 47, 72, and 73 appeared in most of the sulphide components of garlic extract and are likely to represent different hydrocarbon and sulphur groupings. A mass spectrum peak of 57 was only found to be in the 8 most abundant peaks in the spectrum of allyl alcohol, and is likely to represent the ionised form of the molecule.

5.3.2 Qualitative and quantitative analysis of garlic powder, before and after the addition of water.

Garlic powder was extracted directly into dichloromethane in order to identify components that are present in the powder before the addition of water. The profile obtained was compared (Figure 5.3.1) to the profile obtained from a freshly prepared aqueous extract (prepared in the standard way – Chapter 2).

Components were present at extremely low concentrations in the dehydrated garlic powder (<1ppm). On addition of water (standard preparation procedure – see chapter 2), concentrations of most components increased dramatically (over a 1000 fold increase in the concentrations of detected vinyl dithiins).

Garlic component Standard	Retention Time (min)	8 most prominent m/z peaks (in decreasing order of intensity)
DAS	5.836	45, 73, 41, 72, 39, 99, **114**, 71
DAD	13.195	41, **146**, 81, 39, 105, 113, 73, 45
DATr	19.998	73, 113, 41, 45, 39, 72, 71, 47
DATe	26.434	41, 39, 73, 45, 146, 105, 64, 72
AA	1.611	**57**, 41, 49, 84, 86, 51, 39, 43
AM	1.651	**74**, 41, 39, 45, 47, 73, 46, 38
DMD	2.960	**94**, 79, 45, 46, 47, 61, 96, 64
DPD	14.217	43, **150**, 108, 41, 55, 39, 73, 45
MPS	2.587	61, **90**, 48, 41, 49, 47, 43, 75
MPD	8.339	80, **122**, 43, 41, 45, 64, 47, 79
AMS	2.251	**88**, 73, 45, 41, 39, 47, 46, 61
2 – VD*	17.667	72, 71, **144**, 111, 45, 97, 39, 73
3 – VD*	16.710	111, 97, **144**, 79, 77, 103, 72, 45
AMD*	7.839	41, **120**, 30, 45, 79, 73, 47, 64
AMT*	15.426	41, 39, 45, 88, 47, 87, 48, 64

Table 5.3.1 Identification of commonly occurring garlic components using GCMS. Retention times and mass fragmentation patterns (8 most abundant mass/charge (m/z)peaks) are shown. Molecular fragment peaks are highlighted in red (molecular ion -1 in the case of allyl alcohol). Identified from fresh aqueous garlic powder extraction with dichloromethane.

*** Standard not available at time of analysis, component was identified from fresh garlic powder extract using literature reviews and computer databases.**

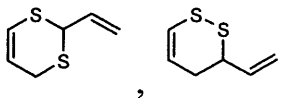
m/z peak	possible composition of fragment	m/z peak occurred in spectra of:-
39	CH_2CCH	DAS, DAD, DATr, DATe, AA, AM, DPD, AMS, 2-VD, AMT
41	CH_2CHCH_2	DAS, DAD, DATr, DATe, AA, AM, DPD, MPS, MPD, AMS, AMD, AMT
45	CHS/ CH_2S^+	DAS, DAD, DATr, DATe, AM, DMD, DPD, MPD, AMS, 2-VD, 3-VD, AMD, AMT
47	CH_3S	DATr, AM, DMD, MPS, MPD, AMS, AMD, AMT
57	$CH_2CHCH_2O^+$	AA
64	S_2	DMD, MPD, AMD, AMT
71	CH_2CC^+HS	DAS, DATe, 2-VD
72	CH_2CCHSH	DAS, DATr, DATe, 2-VD, 3-VD
73	CH_2CHCH_2S	DAS, DAD, DATr, DATe, AMS, AMD, DPD
79	CH_3SS	DMD, MPD, AMD
97	$CH_2CHSCHCCH_2^+$	2-VD,3-VD
105	CH_2CHCH_2SS	DAD, DATe
111	$CH_2CHCHSCH_2CHCH_2$	2-VD, 3-VD
113	$CH_2CHCH_2SCH_2CHCH_2$	DAD, DATr, (DAS)
144		2-VD, 3-VD
146	$CH_2CHCH_2SSCH_2CHCH_2$	DAD, DATe

Table 5.3.2 Mass spectrum m/z peaks characteristic of garlic components with suggested chemical structure for molecular fragments which produce the peaks, and mass spectra of compounds that contain these fragments.

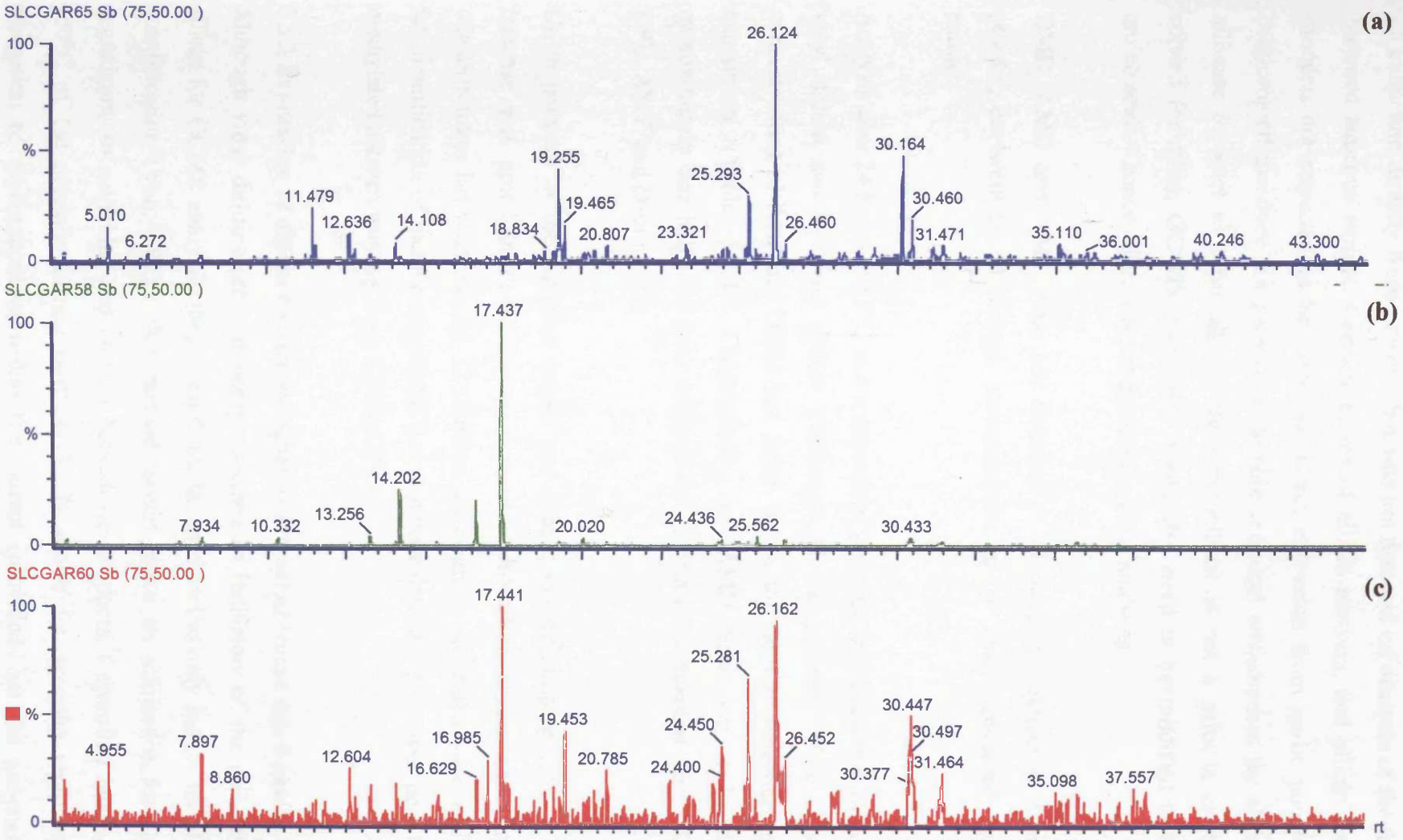


Figure 5.3.1. Analysis of extract from garlic powder before addition of water (a), on addition of water (b), and 24 hours after addition of water (25°C) (c)

The notable exception was diallyl sulphide which was present in low concentrations on extraction directly from powder, but was not detected on analysis of the freshly prepared aqueous extract. Concentrations of allicin markers, and allicin reaction products are expected to be lower on direct extraction from garlic powder, as dichloromethane does not provide a suitable chemical environment for alliin and alliinase to react to form allicin. Dichloromethane is not a suitable extraction solvent for alliin, GC-MS conditions would also need to be modified to detect amino acids – hence alliin was not detected in these analyses.

DMD, AMD and AMT were not detected at all on direct extraction from the powder, but were found in high concentrations in the freshly prepared aqueous extract.

Analysis after 24 hours at 25°C saw a substantial decrease in the concentration of 3-vinyl dithiin and 2- vinyl dithiin (indicators of the presence of allicin), the concentrations of AA, and DMD had fallen below the limit of detection in this experiment (Table 5.3.3). Concentration of AMD also was reduced (to approximately one half); 24 hour analysis saw increased concentrations of DAD, DPD, AMT, and DAT.

On preparation of the aqueous extract and subsequent standing at 25°C a large increase was noted in the concentrations of hydrocarbon chains appearing at retention times between 24 and 32 minutes, although no standards were available for identification of these compounds, the computer database proposed them to be methylated alkanes varying from C20 to C30.

5.3.3 Evaluation of allicin content in aqueous extract of freeze dried garlic

Although vinyl dithiins are commonly accepted as indicators of the presence of allicin for GCMS analysis, they should not be used as the only means for allicin quantification (Yan, 1993); this method would make no allowance for allicin degradation to sulphides and smaller breakdown products. Controlled heating to 100°C of the aqueous extract (section 5.2.7) allows for accurate reproducible estimation of allicin content within the extract (provided that the pre-analysis sample treatment is identical for each analysis).

The chromatograms obtained for heated garlic extract and freshly prepared garlic extract are compared in figure 5.3.2, chromatograms presented in this figure are not to the same scale and appear only as a graphical representation of qualitative

Compound	Concentration μg g^{-1}		
	Dehydrated	**0 hours**	**24 hours**
AA	0.16	107.5 ± 28.76	ND
AM	ND	ND	ND
MPS	-	-	-
DMD	ND	229.96±28.54	ND
DAS	0.1	ND	ND
AMD	ND	131.71±18.16	58.51 ±12.85
MPD	ND	ND	ND
DAD	0.34	180.55 ±35.45	261.1 ±21.83
DPD	0.14	50.00 ±23.01	218.28 ±15.77
AMT	ND	77.20 ±35.8	278.75 ±24.59
3-VD	0.06	871.68 ±167.11	291.64 ±11.82
2-VD	0.37	3964.07 ±199.73	1497.66 ±9.83
DAT	0.35	126.61 ±39.05	314.81 ±36.79
Methylated alkane (24.45 min)	ND	ND	1809.16 ±45.03
Methylated alkane (25.26 min ±0.30)	ND	247.71 ±22.10	992.45 ±10.38
Methylated alkane (26.157 min ±0.28)	ND	194.55 ± 43.09	3132.52 ±54.62
Methylated alkane (30.46 min ±0.13)	ND	1025.14 ±19.81	1253.33 ±73.10

Table 5.3.3 Concentrations of garlic components in garlic powder before addition of water, on addition of water, and 24 hours later (25°C). (ND – not detected).

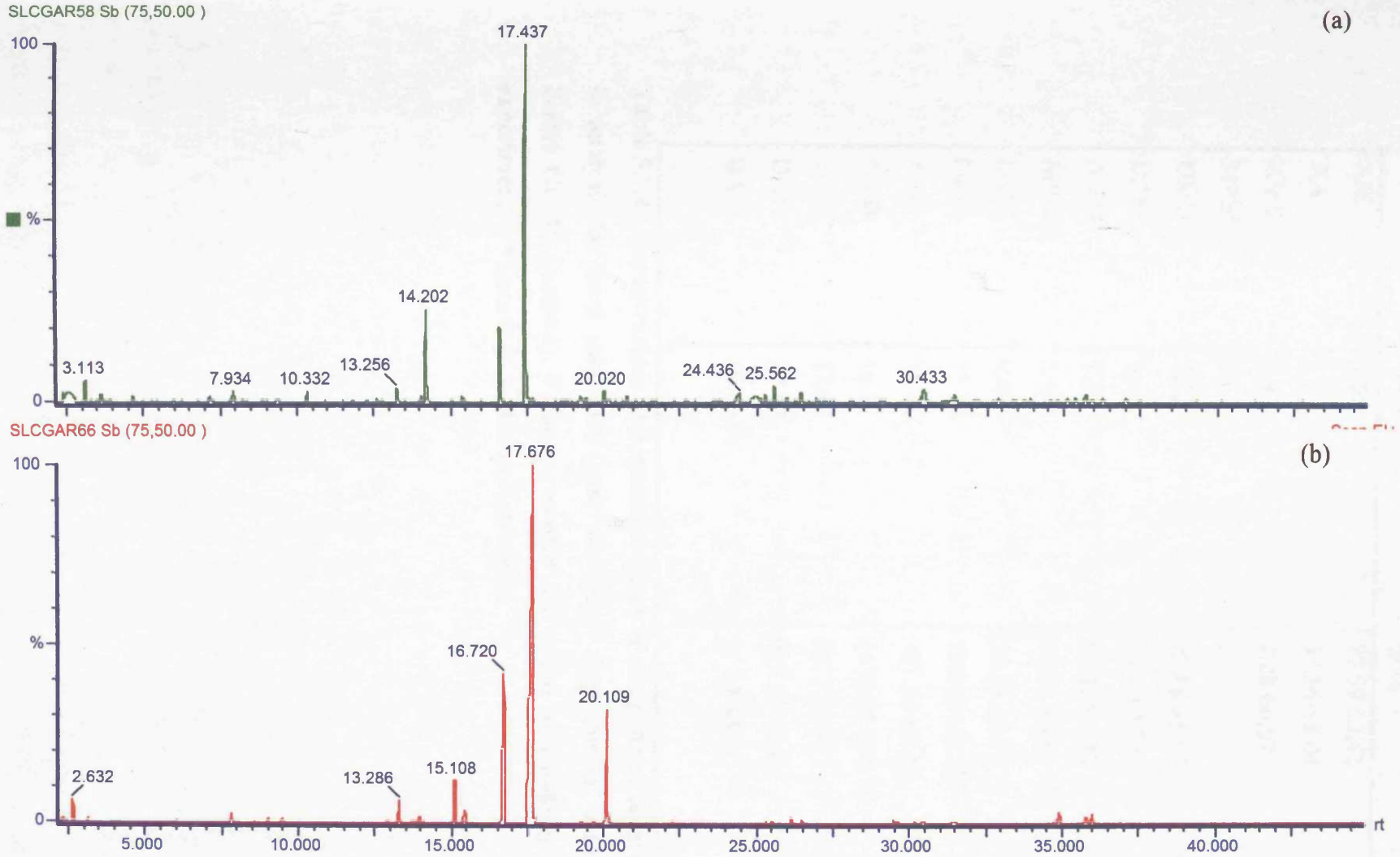


Figure 5.3.2 Chromatograms obtained from (a) freshly prepared garlic extract, (b) garlic extract following controlled heating at 100°C (30min).

Component	Mean Retention time (min)	Concentration ($\mu g g^{-1}$)
AM	1.65	79.89 ±3.43
AA	1.67	38.56 ±3.04
AMS	2.41	7.28 ±0.97
MPS*	2.63	-
DMD	3.12	22.38 ±5.93
DAS	6.03	9.04 ±0.32
AMD	7.81	67.49 ±6.43
MPD	8.56	7.011 ±0.69
DMT	9.46	29.13 ±2.17
DAD	13.28	156.95 ±5.35
AMT	15.10	301.30 ±2.42
3-VD	16.71	1435.41 ±30.99
2-VD	17.66	6621.36 ±13.57
DATr	20.10	955.11 ±7.00
DATe	26.46	31.17 ±5.51

Table 5.3.4 Concentrations and retention times of components identified in analysis for total sulphides (controlled heat degradation of allicin before GCMS analysis). Values presented are means of 3 independent experiments. * Standard added on extraction.

differences between fresh garlic extract, and heat treated garlic extract. Concentrations of components (table 5.3.4) identified in the analysis of the heat treated garlic extract differ from those identified in fresh garlic extract (table 5.3.3) as would be expected.

DAD, AA, DMD, AMD concentrations were all reduced on heating. 2-VD, 3-VD, DATr, DATe, AMT, AM, AMS, DAS, MPD, and DMT concentrations were all seen to increase. Vinyl dithiin concentrations increased dramatically from 4835.75 $\mu g\ g^{-1}$ (total) in freshly prepared extract to 8056.77 $\mu g\ g^{-1}$ in the heat treated extract. Suggesting that prior to heat treatment less allicin degraded to form vinyl dithiins during analysis, the heat treated extract represents total degradation allicin to vinyl dithiins or allyl sulphides prior to analysis.

The value obtained by calculating the allicin content of the freeze dried powder, from addition of total allyl sulphides and vinyl dithiins (Yan *et al*) is 12.107 $mg\ g^{-1}$. Using this value for the allicin content, the alliin content of the freeze dried powder is calculated as 26.456 $mg\ g^{-1}$.

5.3.4 Qualitative analysis of commercially available allicin and garlic preparations

Extraction and analysis of stabilised allicin yielded only small quantities of the components one would expect to see on analysis of allicin, or any thiosulphinate containing garlic product (table 5.3.5, figure 5.3.3). Small quantities of AMT and 2-VD were seen. Characteristic mass spectra from diallyl sulphides previously described were not detected here, neither were those of AA, and other garlic associated sulphides (e.g. DMD, and DPD).

However several compounds not recorded in the previous analyses were detected here (table 5.3.5). Unknown compounds were identified from mass spectra, and database comparisons. The largest peak identified was due to aloxipirin (a compound similar in structure to salicylic acid). This is not a compound usually associated with garlic. Two amphetamine like compounds were also identified (3-methyl amphetamine, and 2,5-dimethyl-4-(methyl sulphonyl) amphetamine), these

are also not components usually associated with allicin or other garlic constituents. These components were not quantified, as no external standards were available, and the internal standard used in previous analyses was found to mask peaks intrinsic to the stabilised allicin chromatogram.

Two garlic components were found to be present in minor quantities, allyl methyl trisulphide, and 2-vinyl dithiin. A compound (methyl-1-(methylthio)-propyl disulphide) was identified at a retention time of 23.834, this compound is structurally similar to diallyl trisulphide. AMT and 2-VD were quantified externally using previous analyses, these were present in concentrations of 0.138 $\mu g\ ml^{-1}$ and 0.124 $\mu g\ ml^{-1}$ respectively. If taken as indicators of the presence of allicin, these results correlate poorly with the reported concentration of 300 $\mu g\ g^{-1}$ claimed by the manufacturer, or when compared to the values obtained from garlic powder analyses.

On analysis of aged garlic, no allicin markers or breakdown products were detected, in addition none of the components previously detected in the studies on garlic extract and 'stabilised allicin' presented here were detected. Detection of any compound was extremely low (figure 5.3.4)., suggesting one or more of the following to be true: (a) aged garlic does not contain any of the components identified from garlic powder extract, (b) the extraction procedure used was not sufficient for detection of aged garlic components, or (c) the GC-MS analysis procedure/ GC program was not optimal for isolation and detection of aged garlic extract components.

Retention time (min)	Suggested identity (from mass spectrum)	Concentration (μgml^{-1})
2.083	Aloxipirin[+]	*
2.919	3-methyl amphetamine	*
2.604	2,2, diacetyl-3-oxo ethyl ester butanoic acid	*
5.262	aloxipirin[+]	*
14.781	4-ethyl-2-methoxy phenol (p-ethyl guaiacol)	*
15.357	AMT	0.138 ±0.022
17.431	2-VD	0.124 ±0.015
19.628	2,3-epoxy-4-methyl-pentane	*
21.233	Bis-trimethylsilyl-D-glutaraldehyde	*
23.834	methyl 1-(methylthio) propyl disulphide	*
23.944	2,5-dimethoxy-4-(methyl sulphonyl) amphetamine	*

Table 5.3.5 Compounds identified in GCMS analysis of 'stabilised allicin'. * denotes compounds not usually found in garlic extracts (not quantified due to lack of suitable standards).

[+] Possible isomeric forms of the same compound.

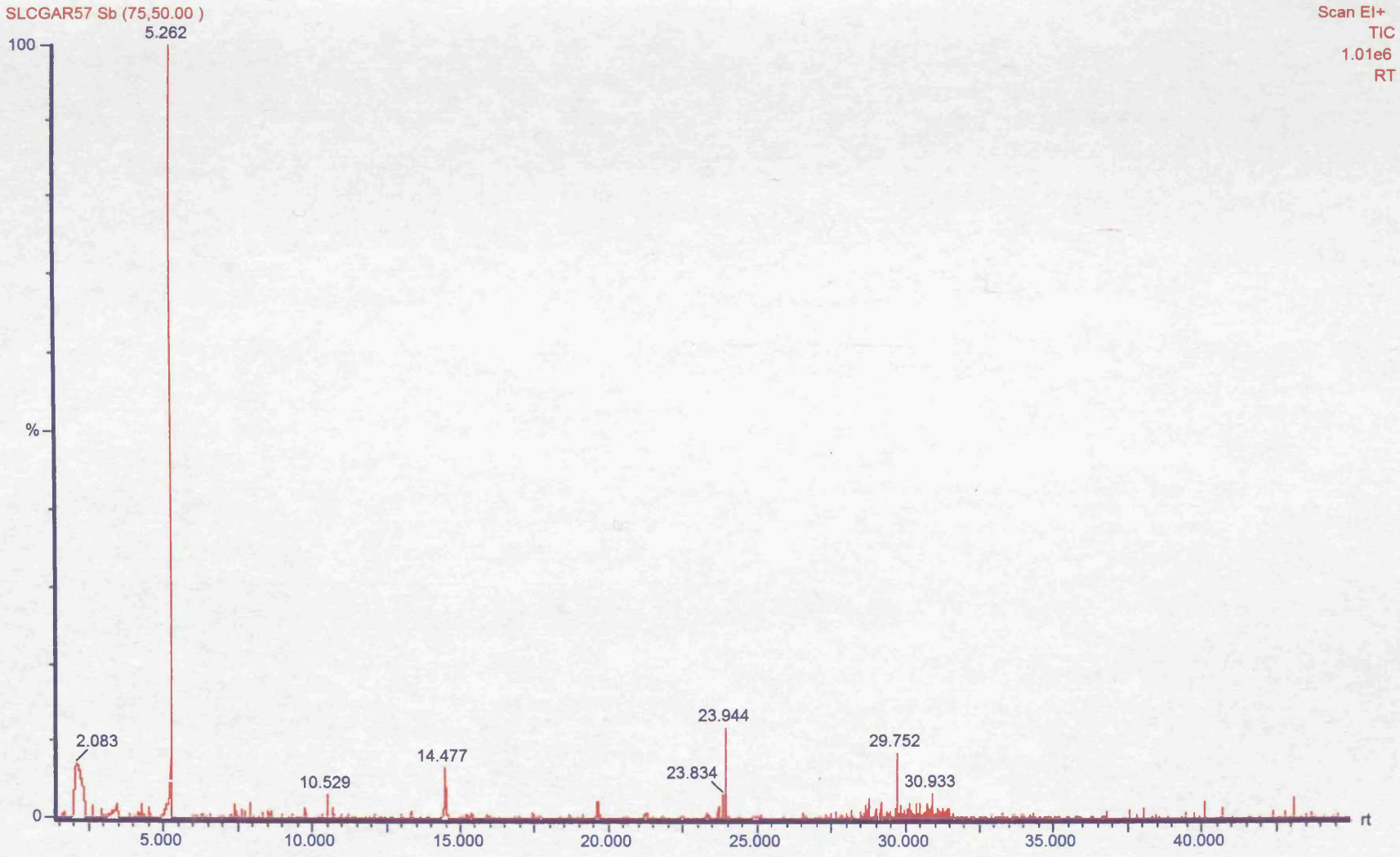


Figure 5.3.3. Analysis of stabilised allicin (150ppm final concentration).

5.4.0 Discussion

GCMS analysis of garlic extracts offers an excellent means of detection and quantification of most volatile components of garlic and components derivable from garlic exposed to high temperatures. Much available data on analysis of garlic extracts describe identifications of garlic components solely in terms of GC retention time. Providing appropriate standardisation is carried out for each instrument and experiment, this method can detect and quantify known volatile components of garlic. A drawback of this using a solely GC based method is that identification can vary considerably from instrument to instrument – depending upon: column choice, solvent choice, extraction procedure, temperature program, column age and condition, and also relies on available standards of known components. Identification of garlic components (provided adequate separation is achieved, and appropriate standards are used) by mass spectrum provides an accurate, reproducible method of detection and identification of known compounds. It is also an excellent method of detecting and estimating structures of unknown compounds which may be present in the mixture. Results here describe the characteristic mass spectrum fragmentation patterns of garlic constituents, and are presented to be an aid to further detection studies.

Extraction of volatiles directly from garlic powder, and immediate analysis showed the presence of allyl alcohol, diallyl sulphide, diallyl disulphide, dipropyl disulphide, 3-vinyl dithiin, 2-vinyl dithiin, and diallyl trisulphide. These components however were only detected at low concentrations (0.37 $\mu g\, g^{-1}$ or below). The low levels of allicin markers (e.g. vinyl dithiins), and allicin breakdown products suggests that the allicin content of the powder is also very low. Other thiosulphinate breakdown products were not detected in the dry powder solvent extract (e.g. allyl methyl disulphide, allyl methyl trisulphide, and dimethyl disulphide), indicating that concentrations of thiosulphinates other than allicin are also low.

On immediate extraction and analysis of the aqueous garlic powder preparation, substantially higher quantities of allicin markers, and thiosulphinate breakdown products were detected. Concentrations of vinyl dithiins (which are taken as the indicators of allicin presence) were in excess of ten thousand time higher – increasing from 0.43 $\mu g\ g^{-1}$ to 4835 $\mu g\ g^{-1}$.

Increased quantities of diallyl disulphide, diallyl sulphide, diallyl trisulphide, and allyl alcohol were also detected. This indicates either that some degradation of allicin had already taken place prior to analysis (time taken from addition of water to injection of extract was approximately 35 min), or that these compounds were produced by degradation of allicin during analysis – as is the case for vinyl dithiins. Also present in the extract from aqueous preparation were dimethyl disulphide, allyl methyl disulphide and allyl methyl trisulphide which had been absent from the dry powder extract, suggesting increased concentrations of other thiosulphinates on addition of water.

Several methylated long-chain hydrocarbons (identity based on analysis and interpretation of mass spectra) were also seen in the chromatogram of the aqueous extract. These compounds may be cell membrane components made accessible to solvent extraction on rehydration of the cell walls contained in the garlic powder, these compounds may not be specific to garlic cells.

Extraction and analysis of the aqueous garlic extract 24 hours after preparation, saw changes in the concentration of all detected components, indicating that garlic is a dynamic mixture of volatile components. Levels of vinyl dithiins had decreased from 4835 $\mu g\ g^{-1}$ to 1789 $\mu g\ g^{-1}$, and since these are indicators of allicin concentration, this suggests that the concentration of allicin in the aqueous extract had also decreased substantially. This is in agreement with the observation that allicin is an unstable compound and rapidly degrades in aqueous solution (Harris 2001). As would be expected, there was also a substantial increase in the levels of allicin breakdown products – most notably diallyl disulphide and diallyl trisulphide.

Levels of allyl methyl disulphide decreased, whilst levels of allyl methyl trisulphide increased, possibly suggesting that allyl methyl trisulphide is involved on the breakdown mechanism of allyl methyl disulphide.

The smaller components identified in analysis of the freshly prepared extract were not detected in the analysis of the 24 hour extract; both allyl alcohol, and dimethyl disulphide are volatile in nature and evaporative loss may have accounted for their absence.

Levels of the methylated hydrocarbons identified in freshly-prepared extract also dramatically increased, the reason for this is unclear. Since the aqueous garlic extract was filtered through a 0.2 μm filter, it is unlikely that increased decomposition of large cell wall fragments could account for this rise in the level of free hydrocarbon chains. Decomposition of cell fragments smaller than 0.2 μm could be responsible. The long chain hydrocarbons seen here may be degradation products of large molecules (e.g. lipids). Accurate detection of lipids and membrane fatty acids using GC analysis would require an additional saponification process not carried out here. Whilst being an excellent extraction solvent for sulphur components of garlic, dichloromethane is not the most suitable solvent for fatty acid and lipid analysis, it is possible that non uniform extraction and degradation of these components may also account for fluctuations in the levels of these compounds.

A major conclusion from these three analyses is that water is required for the production of allicin from freeze dried garlic powder. This has important implications for shelf life of commercial garlic powders. Water provides a suitable medium in which allicin's precursor alliin can make contact with alliinase (movement of these two molecules is severely restricted in dehydrated powder). Water also provides a suitable chemical environment for alliinase to achieve optimum activity; dichloromethane provides an unsuitable chemical environment for alliinase to action (inactivating the enzyme).

From these investigations it is also clear that aqueous extract obtained from garlic powder is a complex dynamic mixture. The reactive and unstable nature of compounds such as allicin obviously accounts for the changing bouquet of

compounds seen. The evaporative loss of some smaller more volatile components also result in changes in the concentrations of components within the system (also accounting for the pungent aroma of the 24 hour aqueous extract compared to the characteristic smell of freshly-prepared aqueous garlic extract).

Controlled heating of the aqueous garlic extract allowed for complete allicin degradation to vinyl dithiins (with other breakdown products). By calculating concentrations of all detected direct allicin breakdown products (vinyl dithiins, diallyl sulphide, diallyl disulphide, diallyl trisulphide, diallyl tetrasulphide) an approximation of the original allicin, and consequently alliin concentrations of the garlic powder can be obtained (Yan *et al,* 1992). The garlic powder used in these experiments was found to contain 12.107 mg g^{-1} allicin and 26.456 mg g^{-1} alliin, this is in agreement with previously calculated values obtained from a different batch of garlic powder from the same source (Cultech Ltd) (Harris 2001) using the Yan method.

This method of allicin quantification has a major drawback, in that it makes no allowance for allyl sulphides which may form as degradation products from other thiosulphinates present in garlic, or non-sulphide breakdown products of allicin. As such, the calculation can only provide an approximation of original allicin content. However, provided heating and analysis conditions are standardised each time the experiment is performed, this method could prove extremely useful in monitoring quality of garlic powders in terms of allicin and alliin content.

Such monitoring would prove invaluable from a quality control point of view, when optimising garlic sources, and in assessing loss of allicin (from fresh garlic cloves) during the freeze drying and testing possible improvement to the process. When used in combination with measurement of allicin (and its breakdown products) levels in the dry powder, it may also provide useful information on the stability of allicin in garlic powder during storage. The optimising recommended storage conditions requires this information.

Two commercially available garlic preparations were investigated here using the same extraction method, and analysis as used for the garlic powder (and aqueous extract) investigation. The first product investigated was a synthetic product claiming to be 100% stabilised allicin, purchased from the same manufacturers that produce the stabilised allicin investigated in chapter 3. The exact structure of stabilised allicin, and details of its production were not available from the manufacturers. GC-MS analysis yielded few of the components found in garlic extract, and expected to be found in a preparation of 100% allicin. Only small quantities of 2 vinyl dithiin, and allyl methyl trisulphide were identified. The concentration stated by the manufacturer does not compare well with the concentration of 2 vinyl dithiin found in the mixture. As no diallyl sulphides were present in the chromatograms, it is likely that the molecule claimed to be stabilised allicin, no longer has a breakdown pattern similar to allicin from natural sources. This is perhaps to be expected when investigating a version of allicin modified to have increased stability, though it must be stated that to modify a small molecule such as allicin to such an extent that it has substantially increased thermal stability would produce a molecule that is no longer allicin.

Analysis of the aged garlic preparation yielded none of the components characteristic of garlic powder (or fresh garlic extract). The process used to create the commercial garlic preparation involves aging intact cloves for extended periods. As such, allicin production is not achieved and the main sulphur compounds present are S-allyl cysteine, and mercapto-cysteines. (Wakunaga corp. USA, 2003). These compounds are beyond the scope of the analytical and extraction procedures used here.

This investigation has indicated the potential of GC-MS as a tool for identifying and quantifying compounds present in garlic powder and aqueous extract of garlic powder, however further validations in comparison to HPLC techniques (Lawson 1996) should be carried out to prove its reliability. GC analysis allows for separation of volatiles from garlic (including smaller breakdown products which are not easily identified by HPLC). Use of a mass spectrometer as the detection device gives excellent detection of separated compounds without the need for

derivatisation and also provides a means of structural elucidation of unknown components, for which reliable standards may not be available.

It is important to realise that in a system as complex as aqueous garlic solutions, whichever method of analysis chosen would not detect all thiosulphinates and thiosulphinate reaction products. The method of choice for analysis of garlic will depend on the purpose of the experiment. If the purpose of the experiment is an accurate quantitative analysis of allicin and the major allicin reaction products, then HPLC would be the most appropriate tool. If the purpose of the experiment is to identify and quantify components from garlic for which reliable reference standards are not available, then GC-MS may prove more useful.

One important criticism of the use of GC-MS for analysis of volatile components of garlic is that it is highly destructive to thermally unstable thiosulphinates, including allicin. In these investigations the use of a low temperature, on-column injection procedure, and a ramped column temperature gradient, minimised thermal degradation of volatile components prior to separation. This procedure however, does not protect components from thermal degradation post separation. For this reason allicin can not be directly detected using GC-MS techniques. At elevated temperatures, such as those experienced on a GC-MS column, allicin rapidly degrades to vinyl dithiin compounds (Brodnitz *et al* 1971). Though reference standards used in this investigation appeared to be GC stable, a detailed study of the thermal stability of allicin reaction products in garlic extract, and on column, should be carried out in order to validate determinations made from GC-MS analysis. Through careful degradation of allicin using controlled heating in a suitable solvent, and GC analysis of the degradation products it is possible to achieve an accurate estimation of allicin and alliin content (Yan *et al* 1993).

Despite its usefulness, it is clear that GC-MS is not an ideal tool for quantitative analysis of thermally unstable garlic components. The lack of reference standards for identification of thiosulphinates and thiosulphinate reaction products means that a 'gold standard' method of garlic analysis does not yet exist. HPLC-MS may prove a more useful technique than GC-MS or HPLC, minimising thermal degradation and also identifying compounds for which reliable standards are not yet available.

Chapter 6

Analysis of reactive components of garlic extract.

Chapter 6. Analysis of reactive components of garlic extract.

6.1.1 Introduction

As detailed in Chapters 1 and 5, a great deal of research has been carried out into the organosulphur chemistry of garlic and other *Allium* species (e.g. Cavallito and Bailey 1944, Block *et al* 1985, Ide 1999). The pathway that eventually leads to production of allicin and other thiosulphinates is known in detail.

A family of molecules called γ-glutamylcysteines are the precursors of the thiosulphinate parent molecules (the cysteine sulphoxides). γ-Glutamylcysteines are thought to exist as a store for cysteine sulphoxides (Block 1994), which are produced through the action of γ-glutamylcysteine transpeptidase enzymes at time of increased growth. The cysteine sulphoxide family comprises of alliin, isoalliin, methiin and cycloalliin. On damage to garlic cloves, or on addition of water to garlic powder (Chapter 1, section 1.2.4), cysteine sulphoxides are converted to thiosulphinates enzymically. Allicin production is achieved by the action of the enzyme alliinase (Chapters 1 section 1.2.4 and Chapter 5 section 5.1), on the cysteine sulphoxide alliin. Alliinase is present in excess, ensuring fast conversion of alliin to allicin (thought to occur within 30 min (Skyrme 1996). The parent molecule of alliin is though to be γ-glutamyl-S-trans-1-propenylcysteine (the most abundant γ-glutamyl cysteine found in garlic bulbs), and together with alliin accounts for approximately 72% of all sulphur content of whole garlic cloves.

Researchers are now in agreement that the majority of pharmacologically significant effects of garlic are due to the sulphur compounds it produces. Specifically, if not allicin then one of the variety of thiosulphinate reaction products produced by garlic extract under different conditions (Block and Ahmed 1984, Han 1996, Harris L. J. 1996, Koch 1996).

As the most abundant thiosulphinate in crushed garlic and the first identified highly antibacterial principle in garlic, allicin has traditionally been the focus of scientific investigation into the activity of garlic preparations. In 1978 Eric Block carried out a

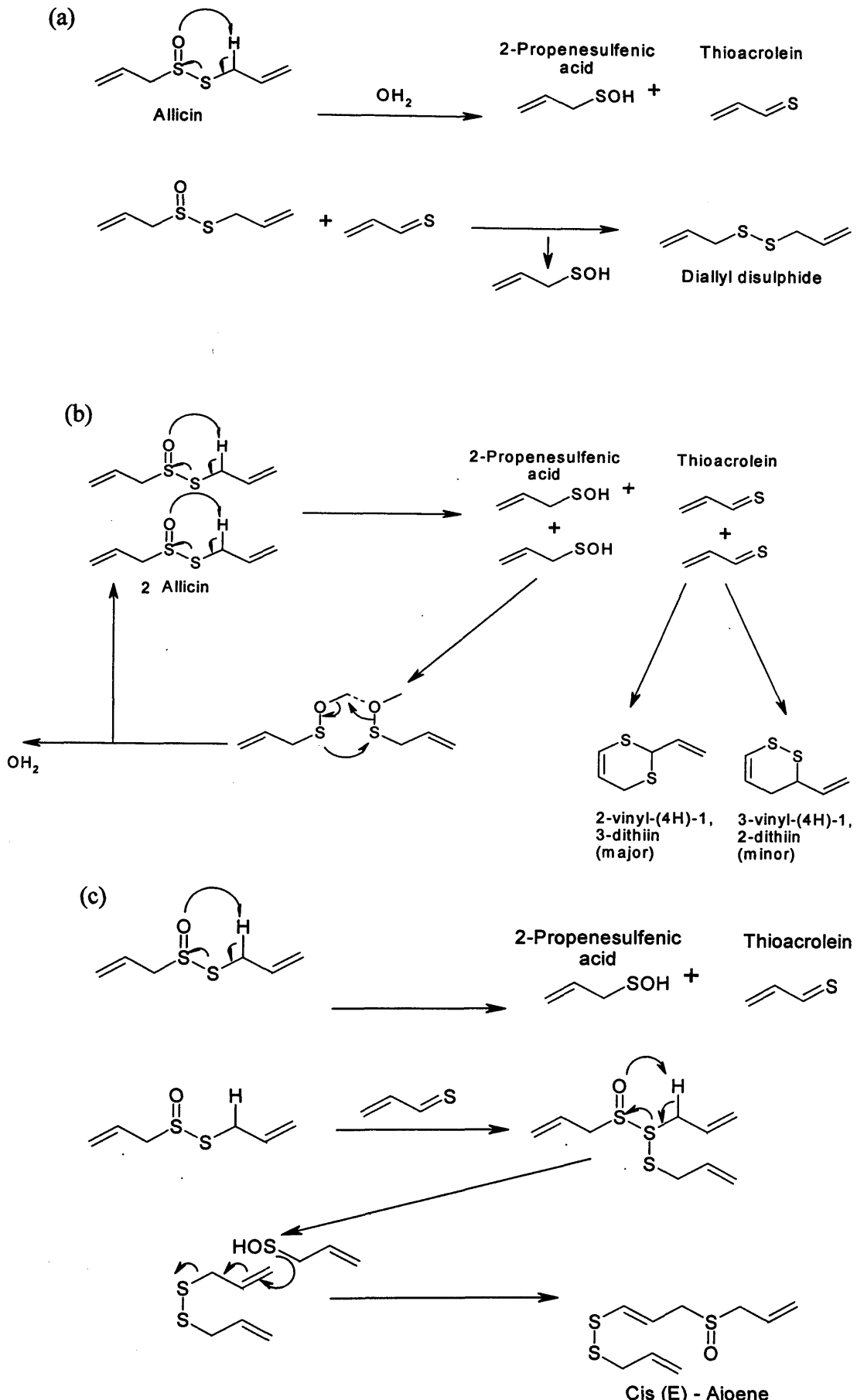


Figure 6.1.0 Reaction pathways of allicin under different conditions (a) diallyl disulphide production (b) vinyl dithiin production (c) *E* -Ajoene production and (d) diallyl trisulphide with allyl alcohol (overleaf).

(d)

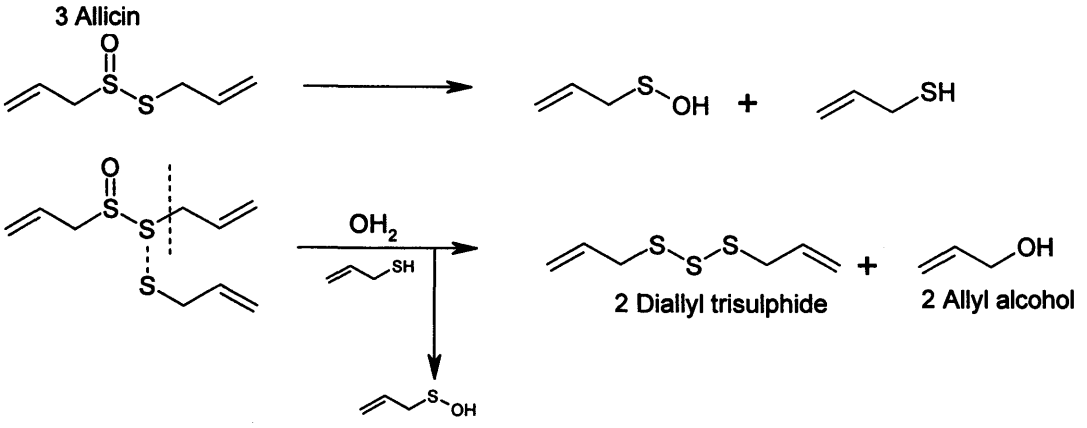


Figure 6.1.0 (ii) diallyl trisulphide and allyl alcohol production in aqueous garlic extract.

series of investigations on the smaller, more stable methane methyl thiosulphinate. Through these experiments, and later work (Block 1986, Lawson 1991, Lawson 1992, Yan 1992) a number of allicin reaction products and pathways have been identified – depending on experimental conditions (e.g. figure 6.1.0). All reaction products occur when allicin is present in aqueous garlic extract, but the proportions of each will vary according to conditions (Chapter 5). Allicin is an unstable compound, increasingly at elevated temperatures, and will breakdown spontaneously in aqueous conditions giving rise to unstable thioacrolein and 2-propenesulphenic acid intermediates, then reaction products including diallyl sulphides, vinyl dithiins, and ajoenes.

Estimates of the stability of allicin and associated products have varied considerably. The half life of allicin in water has been calculated to be 4 – 6 days (Sreenivasamurthy *et al* 1961), a matter of minutes (Koch 1988), and 15 days (Hughes and Lawson, 1991).

The huge variability in allicin stability estimates probably reflect the number techniques that have been employed. Some values of allicin half-life are calculated according to a decrease in antibiotic activity (Lawson and Hughes, 1991), but this is not necessarily a reliable method. Antibiotic activity will vary according to the strain of microbe under study, and many of the allicin reaction products posses antibiotic activity (Harris 2001) leading to generation erroneous estimates of allicin half-life. and since the breakdown pattern of allicin is dependent on the media used as the solvent, solution pH (allicin degrades rapidly in alkaline environments), and temperatue,it is likely that the breakdown pattern will also depend somewhat on the metabolism and composition, as-well as the required growth conditions of the organism under study.

Freeman *et al* (1995, 1997) investigated stability of allicin in various preparations. He found that stability of allicin depended on the dissolution media used. When using water 63% of the initial concentration was still detected after 24 h at 37°C. When allicin was added to blood, it could no longer be detected (using UV spectroscopy) after 5 minutes. However when allicin was added to only the plasma fraction of blood it was much more stable, with 35% of the initial allicin detected after 2 h. Diallyl disulphide was found to be more stable than allicin. These results have since been

questioned (Lawson, 1997). Egen-schwind *et al* (1992) reported that allicin had decreased in concentration by 99% after a 6 minute incubation with liver homogenate. The variability of allicin reactivity estimates illustrate that the reaction characteristics of allicin are influenced a great deal by its chemical and physiological environment. Hence, when studying pharmacological effects of garlic extract, and possible mechanisms of action, it is desirable to obtain a picture of how the components of garlic react within the media/ environment chosen for the investigation.

Many allicin reaction products have been ascribed different pharmacological effects, for example, ajoenes (especially (E)-ajoene) possess antithrombotic, and antihypertensive effects (Block 1984), diallyl disulphide exhibits anti-tumour activity (Knowles and Milner 2003), antibacterial activity (O'gara 2000, McNulty 2001), and vinyl dithiins are known to be mild antithrombotics (Block 1992). As a consequence the efficacy of aqueous garlic extract to treat certain conditions will vary substantially according to the age of the extract, pH and chemical environment, temperature, and extraction procedure.

When studying antimicrobial effects of garlic extract the medium of choice, is usually water in combination with a suitable growth medium for the organism under investigation. In the experiments documented in this thesis, garlic extract was prepared in Nutrient broth, or MRS broth when studying inhibitory effects against *E. coli* and *L, casei* respectively. The nutrient medium itself is a complex mixture of compounds (Table 6.1.0), which could possibly affect stability of garlic components, and the nature of reaction products.

E. coli is more susceptible to growth inhibition by garlic than *L. casei* (Chapter 1). The nature of growth inhibition is also manifested in different ways; *E. coli* shows no observable signs of growth for a period of time that is dependant of the dose of garlic administered, whereas higher concentrations of garlic extract bring about a reduction in the exponential growth rate of *L. casei.* The recovery of growth seen in *E. coli* cultures exposed to sub-lethal concentrations of garlic extract, is that due to the unstable nature of garlic components, the concentration of active inhibitory components may decrease over time until they are present at concentrations no longer inhibitory to *E. coli*, allowing colony growth.

The aim of this investigation, was to investigate the chemical composition of the garlic preparations used in Chapter 1, and detail the changing profile of garlic constituents over time when incubated in nutrient broth at 37°C over 24 hours.

Another aim of this work was to investigate whether to reactivity of allicin is increased in the presence of bacteria, with the aim of obtaining an insight into why *E. coli* cultures can recover from growth inhibition observed at low concentrations of garlic extract. Effects of *L. casei* cultures on composition of garlic constituents will also be preliminarily investigated here, highlighting differences between changes in composition of garlic extract through lag phase, and early exponential phase for *E. coli* and *L. casei*.

Nutrient Broth No. 2		**MRS Broth**	
Ingredient	Concentration (gl^{-1})	Ingredient	Concentration (gl^{-1})
'Lab-lemco' powder	10	'Lab-lemco' powder	10
Peptone	10	Peptone	8
Sodium chloride	5	Yeast extract	4
		Glucose	20
		Sorbitan mono-oleate	1(ml)
		Dipotassium Hydrogen phosphate	2
		Sodium acetate	5
		Triammonium citrate	2
		Magnesium sulphate $7H_2O$	0.2
		Magnesium sulphate $4H_2O$	0.05
pH	7.5	pH	6.2

Table 6.1.1 Compositions of Nutrient Broth and MRS Broth (used for growth of *E. coli* and *L. casei* respectively).

6.2 Materials and methods

6.2.1 Preparation of garlic extract.

Aqueous garlic extract was prepared in the manner previously described (Chapters 2, 3), in nutrient broth number 2 (Oxoid, Basingstoke UK) or MRS (Sigma, Poole UK) broth to a concentration of 5 mg ml^{-1}.

6.2.2 Solvent extraction of garlic extract.

Garlic extract was added to analytical grade dichloromethane (Fisher, Loughborough UK) (0.75 ml: 0.75 ml), in a sterile polypropylene micro-centrifuge tube. The aqueous and solvent fractions were mixed by gentle inversion for 30 s. On separation the solvent fraction was removed, and Methyl Propyl Sulphide was added to a final concentration of 10 ng μl^{-1}. 1.2 µl was used for GCMS injection

6.2.3 GCMS parameters.

The column chosen for analysis was a DB-5ms (non polar), 30 m x 0.25 mm internal diameter, 0.25 µm film thickness. Helium was used as a carrier gas (40 cm s^{-1}). An on column, splitless injection procedure was used, the oven temperature program ran from 30°C to 240°C at a rate of 5°C min^{-1}, start and finish temperatures were held for 3 min. Injection port temperature was 70°C. Injection volume for all experiments presented in Chapter 5 was 1.2 µl.

Components were detected after a solvent delay of 1.45 min. The mass spectrometer source temperature was 200°C (with 260°C transfer line), and scans were carried out between 35 and 215 amu (EI^+ 70eV).

6.2.4 Analysis of mass spectra.

Components found in analysed extracts were identified by comparing retention times from the chromatogram, and mass spectra of isolated components to external standards. Where no external standard was available, compounds were identified by comparison of mass spectra to those on the GCMS software database, or to spectra found in previously published literature.

6.2.5 Analysis of garlic extract prepared in Nutrient Broth and MRS Broth.

Garlic extract was prepared to 5 mg ml^{-1} in Nutrient broth and MRS broth. 0.75 ml was removed immediately for GCMS analysis (using the solvent extraction described above). Components were quantified using by comparison of peak area to the internal standard.

6.2.6 Analysis of sulphur components in garlic extract at 37°C over 24 hours.

Garlic extract was prepared to 5 mg ml^{-1} in Nutrient broth. 0.75 ml was removed immediately for GCMS analysis. The garlic extract (aqueous) was then placed in a regulated incubator at 37°C. At hourly intervals 0.75 ml aliquots were removed for extraction and analysis.

6.2.7 The effect of *E. coli* and *L. casei* on sulphur components in garlic extract.

An overnight culture of *E. coli* was centrifuged a 3000g for 10 min, then washed twice with sterile PBS buffer, before resuspension in 10 ml garlic extract (5 mg ml^{-1} in Nutrient Broth) to a final cell density of approximately 10^8 cfu ml^{-1}. The suspension was placed in a regulated incubator at 37°C. At hourly intervals 1 ml aliquots were removed and centrifuge in a bench-top micro-centrifuge (Eppendorf) for 1 minute at 10000g. 0.75 ml of cell free supernatant was then removed for solvent extraction and analysis.

Analysis of the effects of *L. casei* on garlic extract was carried out in a similar manner, except that MRS broth was used in place of Nutrient Broth, and cells were resuspended in 20 ml of garlic extract (5 mg ml^{-1} in MRS broth) to a final cell density of approximately 10^8 cfu ml^{-1}.

6.3 Results

6.3.1 Analysis of garlic extract prepared in Nutrient Broth and MRS Broth.

Components identified from garlic extract prepared using growth media differed from those identified in garlic extract prepared in water (figure 6.3.1, 6.3.2). Allyl alcohol, dimethyl disulphide, and allyl methyl trisulphide were not detected in either garlic-growth media preparations.

Concentrations of components found initially in the two broths were similar (table 6.3.1., figure 6.3.1.1 and figure 6.3.1.2) Vinyl dithiins were found to be present at lower levels in growth media, than in water (for the same garlic powder concentration). Components of garlic powder extract were found to be present in slightly lower concentrations in the MRS preparation (except allyl methyl sulphide, and methane methyl thiosulphinate).

Diallyl sulphide, allyl methyl disulphide, and dipropyl disulphide were detected in nutrient broth garlic extract but nor MRS broth garlic extract. Allyl methyl sulphide was detected in MRS broth garlic extract, but not Nutrient broth garlic extract.

Several compounds were detected in MRS broth at retention times 33.14 min to 43.68 min. These compounds were also found to present in the MRS-garlic extract, but are components of MRS broth rather than garlic related compounds. The mass spectra suggest long chain hydrocarbon molecules containing oxygen and nitrogen moieties.

Component	Concentration (ng μl^{-1} of 5 mg ml^{-1} aqueous garlic extract)	
	(a) Nutrient broth	(b) MRS broth
AA	nd	nd
DAS	0.454 ± 0.036	nd
AMS	nd	5.280 ± 0.103
AMD	0.184 ± 0.020	nd
MeS(O)SMe	3.644 ± 0.048	4.072 ± 0.333
DAD	3.508 ± 0.033	2.347 ± 0.122
DPD	1.727 ± 0.041	nd
3-VD	10.768 ± 0.092	5.610 ± 0.469
2-VD	49.623 ± 0.110	41.061 ± 0.071
DATr	0.715 ± 0.109	0.703 ± 0.077
DATe	0.674 ± 0.103	nd

Table 6.3.1 Comparison of components identified in garlic extract prepared using (a) Nutrient broth, and (b) MRS broth. Concentrations are given in ng μl^{-1} of aqueous garlic extract (5 mg ml^{-1}). (nd – not detected).

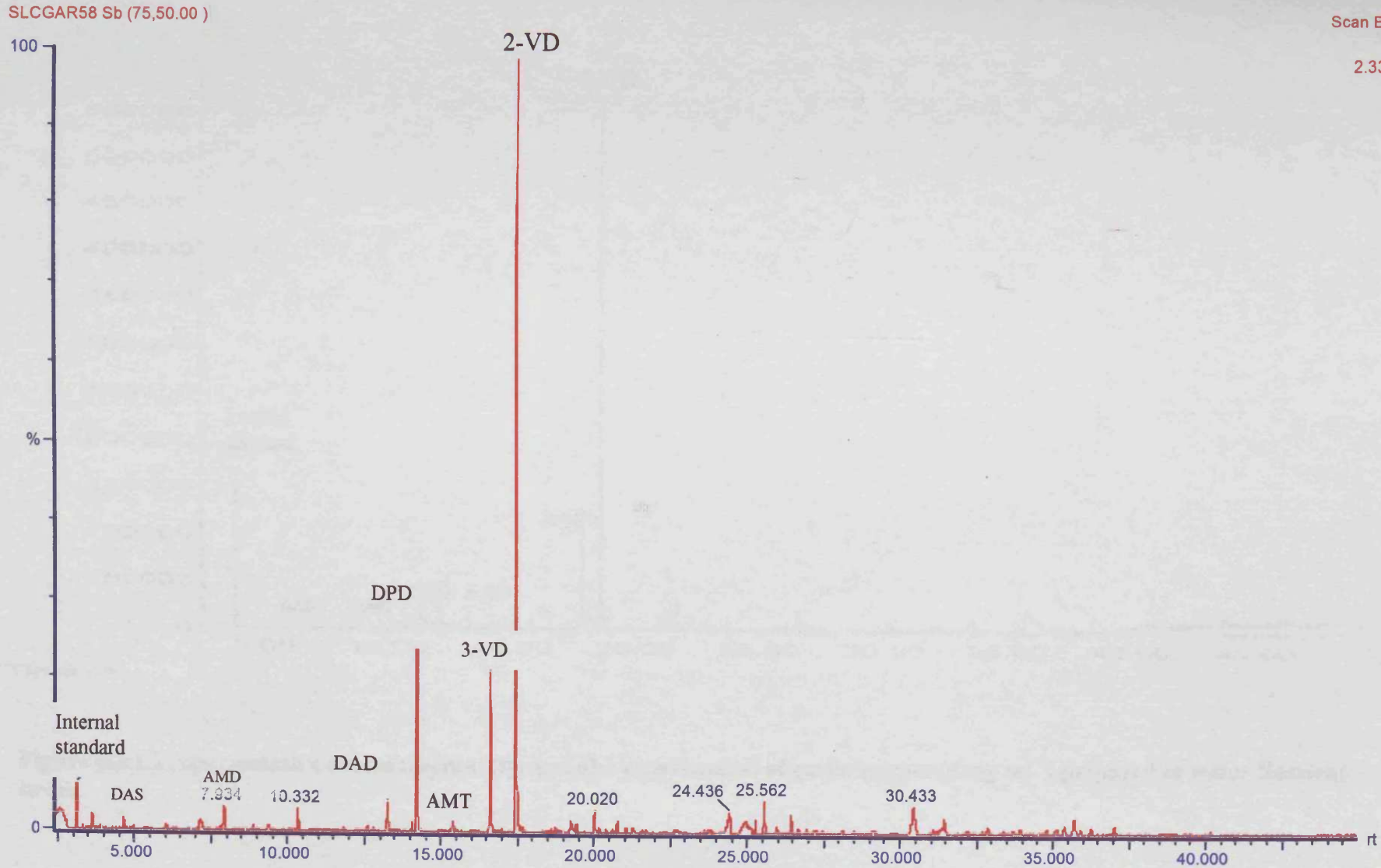


Figure 6.3.1.1 representative chromatogram (typical of 3 experiments) of garlic extract (10mg ml^{-1}) prepared in water.

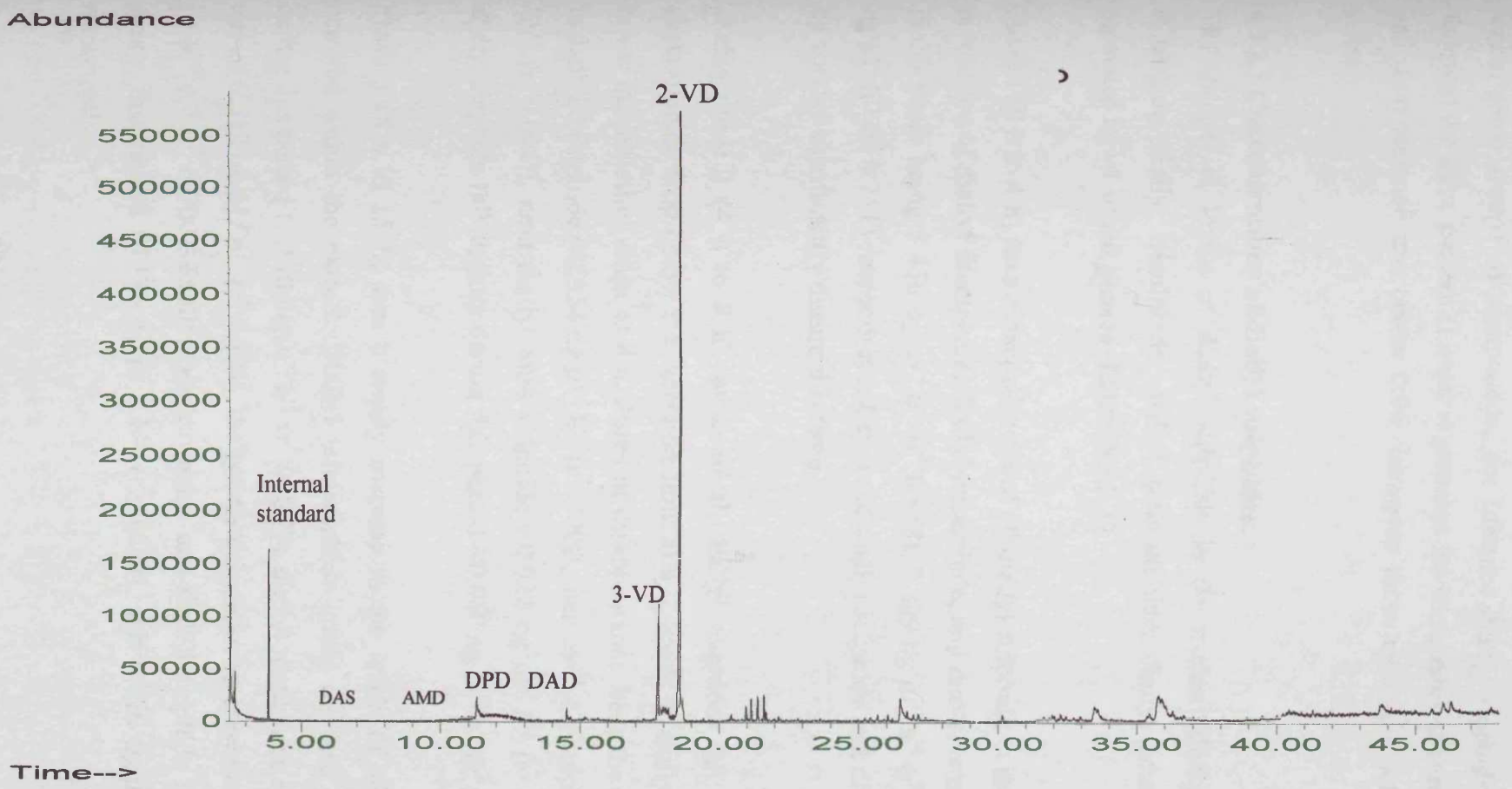


Figure 6.3.1.2 representative chromatogram (typical of 3 experiments) of garlic extract (5 mg ml^{-1}) prepared in water Nutrient broth.

6.3.2 Analysis of volatile components in garlic extract at 37°C over 24 hours.

Analyses show that concentrations of all components identified in previous experiments vary according to time (Figures 6.3.2.0 – 6.3.2.3). Patterns emerged within some groups of compounds, for instance diallyl sulphide concentrations all followed the same pattern (Linear regression for some rates of component appearance and disappearance are below 0.96, however these rates are not necessarily linear rates).

6.3.2.1 Concentrations of diallyl sulphides.

The changes in levels of diallyl sulphide in the extract (diallyl sulphide, diallyl disulphide, diallyl trisulphide, diallyl tetrasulphide, diallyl pentasulphide) can be separated into 4 broad phases (figure 6.3.2.1).

Phase 1 (0 h to 4 h) sees a rise in levels of all diallyl sulphide in the mixture. Rates of production of diallyl disulphide, diallyl trisulphide, and diallyl tetrasulphide are rapid in this phase being 5.456 ng $\mu l^{-1} h^{-1}$ (r^2 0.958), 3.829 ng $\mu l^{-1} h^{-1}$ (r^2 0.974), and 1.312 ng $\mu l^{-1} h^{-1}$ (r^2 0.751) respectively. Levels of diallyl sulphide and diallyl pentasulphide do not rise significantly during this time.

During phase 2 (4 h to 8 h) levels of all diallyl sulphides fall. Levels of Diallyl pentasulphide drop below the detection limit at 5 h, levels of diallyl tetrasulphide fall below the detection range at 8 h. Rates of disappearance from the extract are highest in diallyl disulphide (-2.954 ng $\mu l^{-1} h^{-1}$ (r^2 .790)), then diallyl trisulphide (-2.138 ng $\mu l^{-1} h^{-1}$ (r^2 0.604)), next diallyl tetra sulphide (-0.922 ng $\mu l^{-1} h^{-1}$ (r^2 0.784)). Levels of diallyl sulphide fall slightly during this period (-0.060 ng $\mu l^{-1} h^{-1}$ (r^2 0.898).

Phase 3 (8 h to 15 h) sees a steady increase in the levels of all diallyl sulphides detected within the extract. Diallyl tetrasulphide levels increase at the fastest rate during this period (1.180 ng $\mu l^{-1} h^{-1}$ (r^2 0.027)), diallyl trisulphide levels increase at a rate of 0.542 ng $\mu l^{-1} h^{-1}$ (r^2 0.824), diallyl disulphide levels increase at a rate of 0.494 ng $\mu l^{-1} h^{-1}$ (r^2 0.700). Diallyl pentasulphide and diallyl sulphide levels rise slightly during this period (0.136 ng $\mu l^{-1} h^{-1}$ (r^2 0.650), and 0.026 ng $\mu l^{-1} h^{-1}$ (r^2 0.830) respectively.

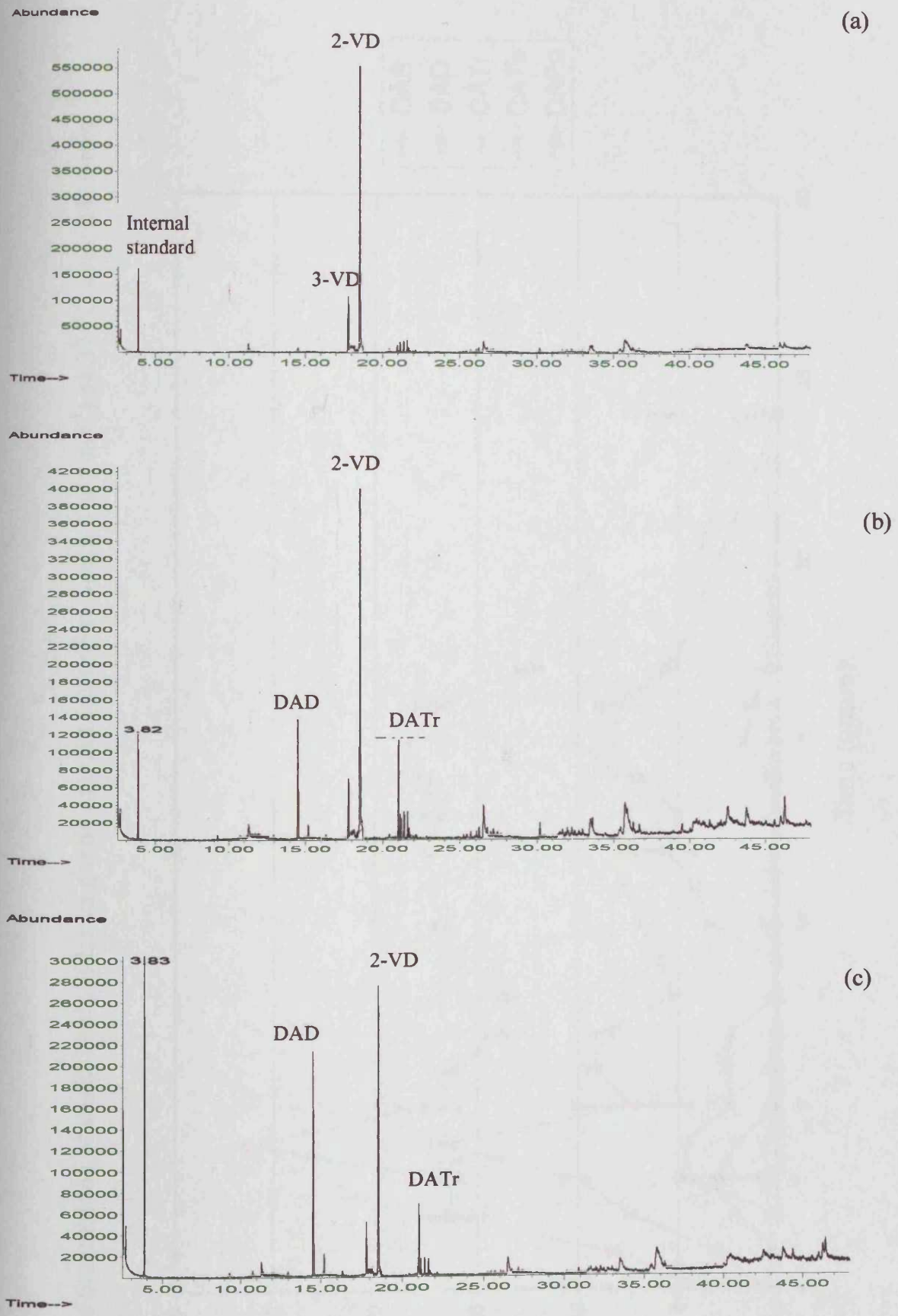


Figure 6.3.3 The changing profile of garlic extract components in Nutrient broth at 37°C (5 mg ml^{-1}). (a) 0 hours, (b) 5 hours, (c) 10 hours (most abundant components labelled).

Concentrations of diallyl sulphides in garlic extract (nutrient broth 5 mg ml^{-1}) at 37 °C over 24 hours.

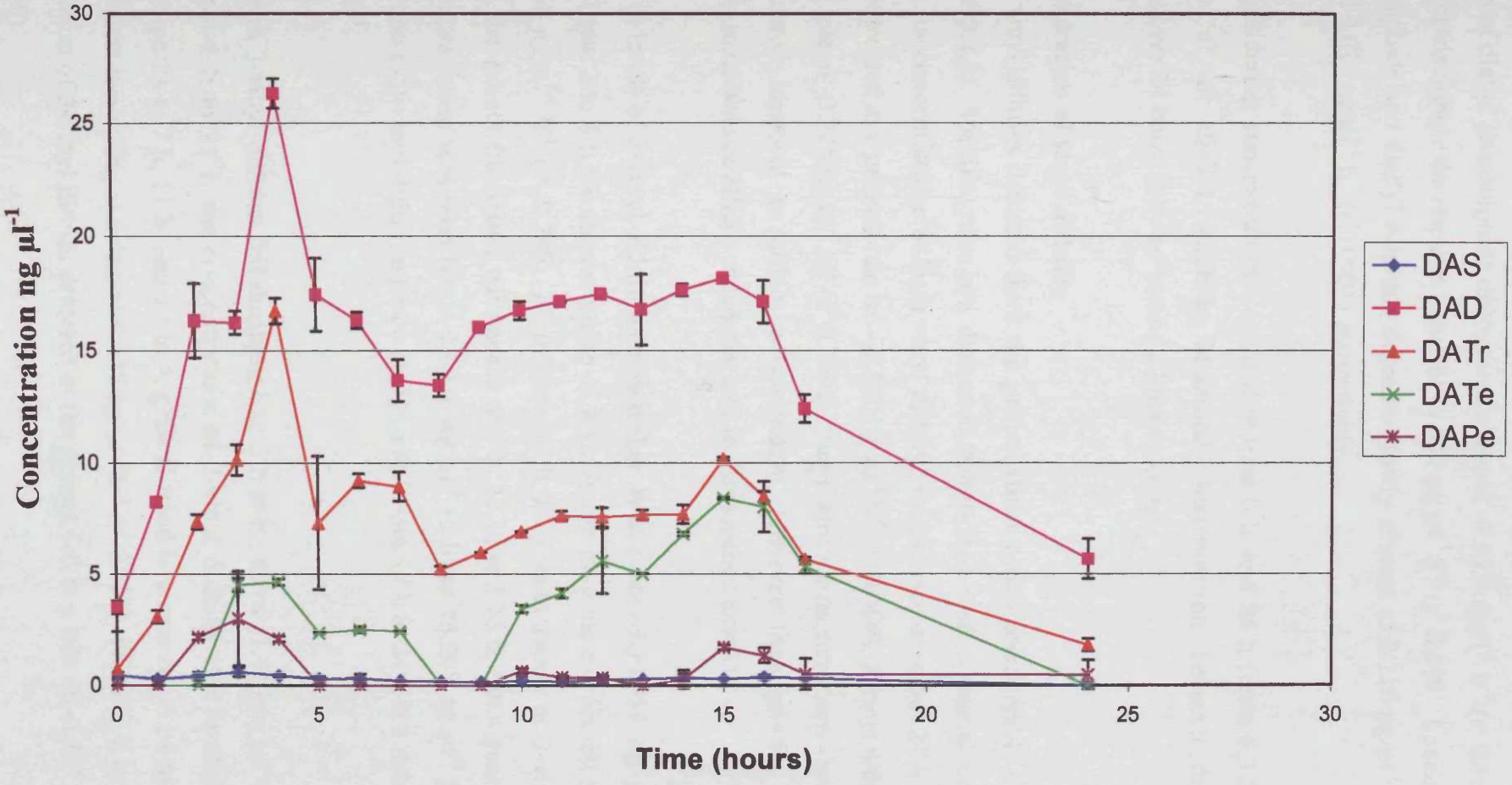


Figure 6.3.2.1 Changes in concentrations of diallyl sulphides over 24 hours in garlic extract prepared in Nutrient Broth (5 mg ml^{-1}). Values plotted are means and standard deviations from two experiments.

During phase 4 (15 h to 24 h), levels of all diallyl sulphides fall again. The rate of decrease in diallyl disulphide concentration is the most rapid (-1.339 ng μl^{-1} h^{-1} (r^2 0.926)), levels of diallyl tetrasulphide decrease at a rate of -0.933 ng μl^{-1} h^{-1} (r^2 0.963), levels of diallyl trisulphide decrease at a rate of -0.847 ng μl^{-1} h^{-1} (r^2 0.899). Levels of diallyl pentasulphide and diallyl sulphide decrease slowly at rates of 0.110 ng μl^{-1} h^{-1} (r^2 0.526), and 0.039 ng μl^{-1} h^{-1} (r^2 0.901) respectively.

When only considering concentrations recorded at time 0 h, and 24 h (table 6.3.2.1) concentrations of all diallyl sulphide increased. Juxtaposition between these concentrations over 24 hour, is by no means a linear process,

6.3.2.2 Concentration of vinyl dithiins

The levels of vinyl dithiins detected from the garlic extract varied considerably over 24 h (figure 6.3.2.2). Specific rates are discussed below, however a general trend could be seen for concentrations of both vinyl dithiins to decrease over the 24 h (2-vinyl dithiin decreased at a general rate of – 2.500 ng μl^{-1} h^{-1} (r^2 0.490), 3-vinyl dithiin decreased at a rate of -0.275 ng μl^{-1} h^{-1} (r^2 0.399)). Vinyl dithiin concentrations should not be considered identical to allicin concentration, however they provide an extremely good indication of allicin concentrations in the aqueous extract.

From 0 h to 2 h, levels of 2-vinyl dithiin increased sharply at a rate of 30.954 ng μl^{-1} h^{-1} (r^2 0.964). From 2 to 5 h the concentration of 2-vinyl dithiin in the extract fell at a rate of -27.968 ng μl^{-1} h^{-1} (r^2 0.790). From 5 to 17 h the concentration of 2-vinyl dithiin seen in the extract fluctuated with peaks at 7 h, 12 h, and 15 h, but a general decrease in concentration was seen (from 19.956 ng μl^{-1} (5 h) to 15.267 ng μl^{-1} (17 h)). Concentration of 2-vinyl dithiin seen on analysis fell from 17 h to 24 h at a rate of -1.276 ng μl^{-1} h^{-1}.

Concentrations of 3-vinyl dithiins fell during 0 h to 5 h at a rate of -1.446 ng μl^{-1} h^{-1} (r^2 0.904). From 6 h to 15 h the concentrations of 3-vinyl dithiins also oscillated somewhat, with peaks at 7 h, 11 h, and 14 h. A general trend however could be seen, with concentrations increasing at a rate of 0.188 ng μl^{-1} h^{-1} (r^2 0.707). From 15 h to 24 h the concentration of 3-vinyl dithiin detected in the extract fell at a rate of -0.347 ng μl^{-1} h^{-1} (r^2 0.958).

Concentrations of vinyl dithiins in garlic extract (nutrient broth 5 mg ml^{-1}) at 37 °C over 24 hours.

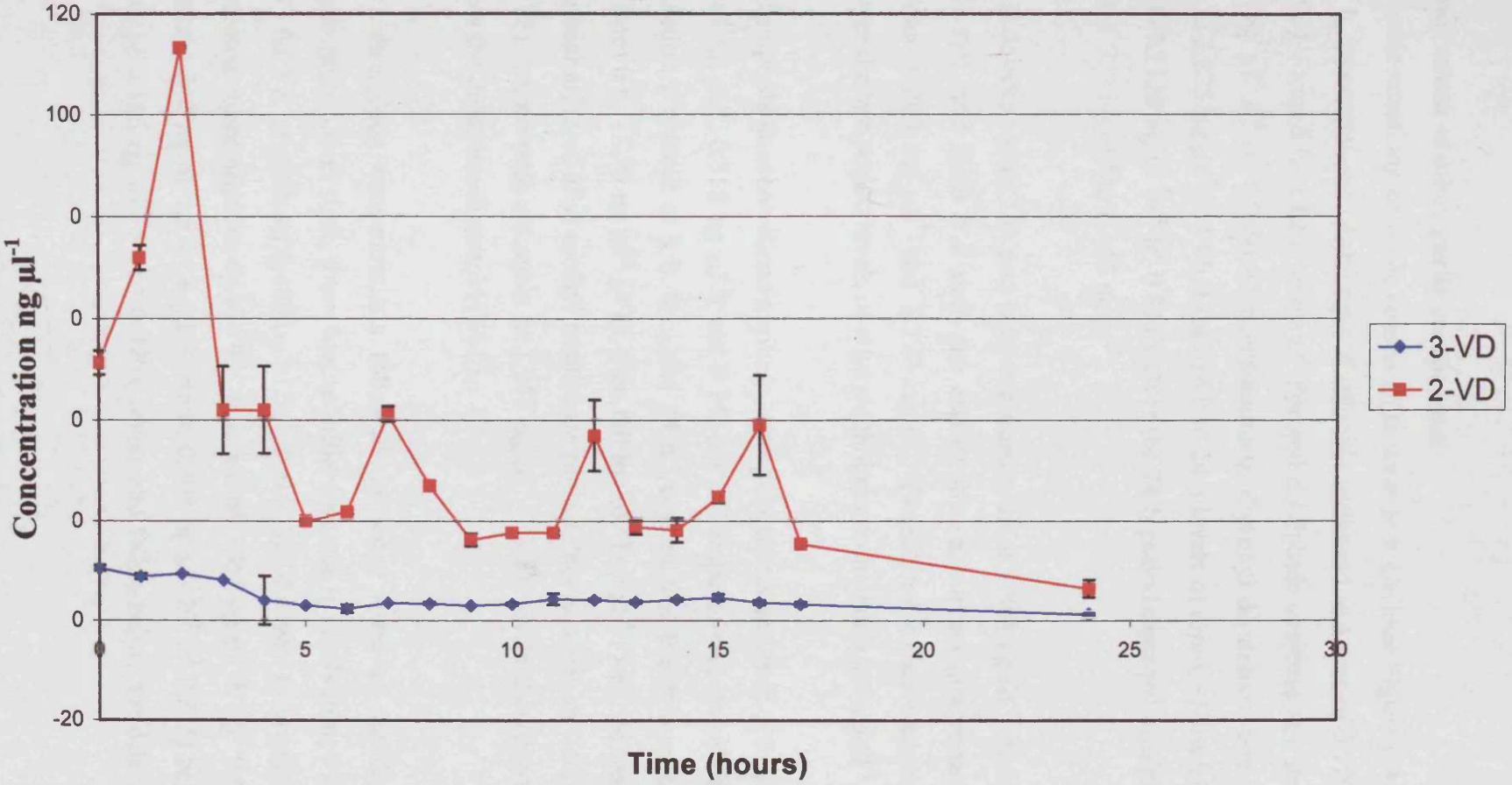


Figure 6.3.2.2 Changes in concentrations of vinyl dithiins over 24 hours in garlic extract prepared in Nutrient Broth (5 mg ml^{-1}). Values plotted are means and standard deviations from two experiments.

Over the 24 h period, levels of 2-vinyl dithiin fell from 51.289 ng μl^{-1} to 6.337 ng μl^{-1}, and concentrations of 3-vinyl dithiin fell from 10.381 ng $\mu l^{-1}$to 1.194 ng μl^{-1}.

6.3.2.3 Concentrations of other garlic components

Trends in the concentrations of other components were less obvious Figure 6.3.2.3, from 0 h to 4 h concentrations of dipropyl disulphide increased at a rate of 0.779 ng μl^{-1} h^{-1} (r^2 0.500). From 6 h to 12 h levels of dipropyl disulphide decrease at a steady rate of -0.067 ng μl^{-1} h^{-1} (r^2 0.909). Concentrations of dipropyl disulphide then rose again peaking at 2.825 ng μl^{-1} at 14 h. From 14 h to 24 h levels of dipropyl disulphide fell at a rate of -0.2328 ng $\mu l^{-1}h^{-1}$ (r^2 0.911). Over the 24 h period dipropyl disulphide levels fell from 1.727 ng μl^{-1} to 0.465 ng μl^{-1}.

Allyl alcohol is detected after 1 h, and concentrations peak at 1.868 ng μl^{-1} after 2 h. Levels quickly fall, and from 5 h until the end of the experiment concentrations fluctuate between 0.500 ng μl^{-1} and 1.227 ng μl^{-1} (found not to be statistically significant). Over the test period levels of allyl alcohol rise from 0 to 0.823 ng μl^{-1}.

Allyl methyl disulphide concentrations oscillate, with peak levels achieved at 2 h, 7 h, and 14 h (0.569 ng μl^{-1}, 0.318 ng μl^{-1}, and 0.746 ng μl^{-1} respectively). Levels drop below the detection threshold at 3 h, 8 h, and 24 h. Allyl methyl trisulphide levels climb rapidly from 0 to 1.279 ng μl^{-1} (4 h), then fall to 0.670 ng μl^{-1} (5 h). Between 5 h and 14 h concentration of allyl methyl trisulphide rise slightly at a rate of 0.025 ng μl^{-1} h^{-1} (r^2 0.475), before peaking again at 1.317 ng μl^{-1} (15 h), levels then decrease rapidly to below the detection threshold (24 h).

Methyl propyl disulphide concentrations followed the same trend as the diallyl sulphides concentrations over time, there was an initial increase in levels from 0 (1 h) to 0.977 ng μl^{-1} (at 3 h, at a rate of 0.489 ng $\mu l^{-1}h^{-1}$ (r^2 0.980)), this was followed by a decrease in detected concentrations up to 10 h, at a rate of 0.054 ng μl^{-1} h^{-1} (r^2 0.690) to a maximum of 1.348 ng μl^{-1} at 14 h (at a rate of 0.294 ng μl^{-1} h^{-1} (r^2 0.815) before falling at a rate of 0.116 ng μl^{-1} h^{-1} (r^2 0.990), levels had fallen below the detection range by 24 h.

Concentrations of components in garlic extract (nutrient broth 5 mg ml^{-1}) at 37 °C over 24 hours.

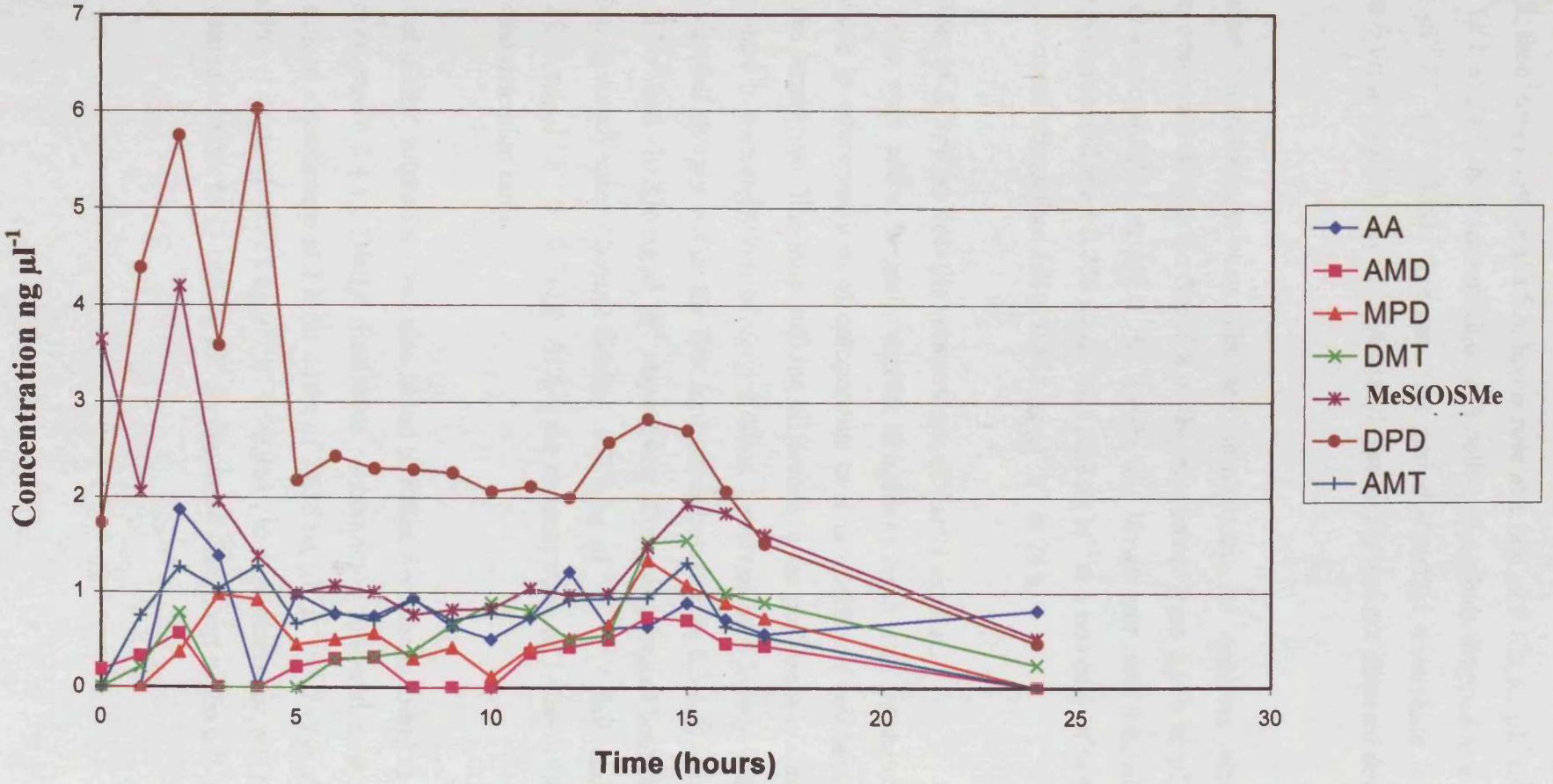


Figure 6.3.2.3 Changes in concentrations of garlic components over 24 hours in garlic extract prepared in Nutrient Broth (5 mg ml^{-1}). Values plotted are means from two experiments (error bars are omitted for clarity).

Dimethyl trisulphide was detected at 1 h (0.214 ng μl^{-1}) and 2 h (0.785 ng μl^{-1}). Levels then dropped below the detection range. Dimethyl trisulphide was not detected again until 5 h, from this point until 15 h, levels rose at a rate of 0.122 ng μl^{-1} h^{-1} (r^2 0.612). From 15 h to 24 h the concentration of dimethyl trisulphide dropped at a rate of -0.123 ng μl^{-1} h^{-1} (r^2 0.859). Over the test period dimethyl trisulphide levels increased from 0 ng μl^{-1} to 2.41 ng μl^{-1}. Dimethyl disulphide was not detected during this analysis.

Methyl methane thiosulphinate was detected throughout the analysis period. Concentrations decreased during the first 5 h of the experiment from 3.644 ng μl^{-1} to 0.988 ng μl^{-1} at a rate of 0.541 ng μl^{-1} h^{-1} (r^2 0.906), at a slower rate until 8 h, where the concentration increased from 0.768 ng μl^{-1} to 1.932 ng μl^{-1} at a rate of 0.142 ng μl^{-1} h^{-1} (r^2 0.743), concentrations then fell to 0.518 ng μl^{-1} h^{-1} at 24 h.

6.3.4.0 The effect of *E. coli* on volatile components of garlic extract.

When *E. coli* cells were added to garlic extract in nutrient broth, and incubated at 37°C, the changes in concentrations of components over time differed substantially from the cell free incubation. The most striking difference in the profiles was a much more rapid decrease in concentrations of vinyl dithiins. Levels of both 2-vinyl dithiin and 3-vinyl dithiin fell sharply within the first h of incubation (figure 6.3.4.0) at rates of -46.364 ng μl^{-1} h^{-1} and -10.336 ng μl^{-1} h^{-1} respectively. Concentrations of both vinyl dithiins then fell at steady rates (2-vinyl dithiin, -0.228 ng μl^{-1} h^{-1} (r^2 0.980) and 3-vinyl dithiin -0.013 ng μl^{-1} h^{-1} (r^2 0.711)). At 6 h the concentration of 3-vinyl dithiin dropped below the detection range.

Concentrations of diallyl sulphides were also found to differ from those found in the cell free extract (figure 6.3.4.1). Diallyl disulphide concentration followed a similar pattern, increasing to a maximum at 2 h (at a rate of 2.655 ng μl^{-1} h^{-1} (r^2 0.974), then steadily decreasing at a rate of -0.728 ng μl^{-1} h^{-1} (r^2 0.914), to a concentration at 7 h of 1.476 ng μl^{-1} (compared with to 13.666 ng μl^{-1} for the same time point in the cell free garlic extract).

Concentrations of vinyl dithiins in garlic extract (nutrient broth 5 mg ml^{-1}) at 37 °C with *E. coli*.

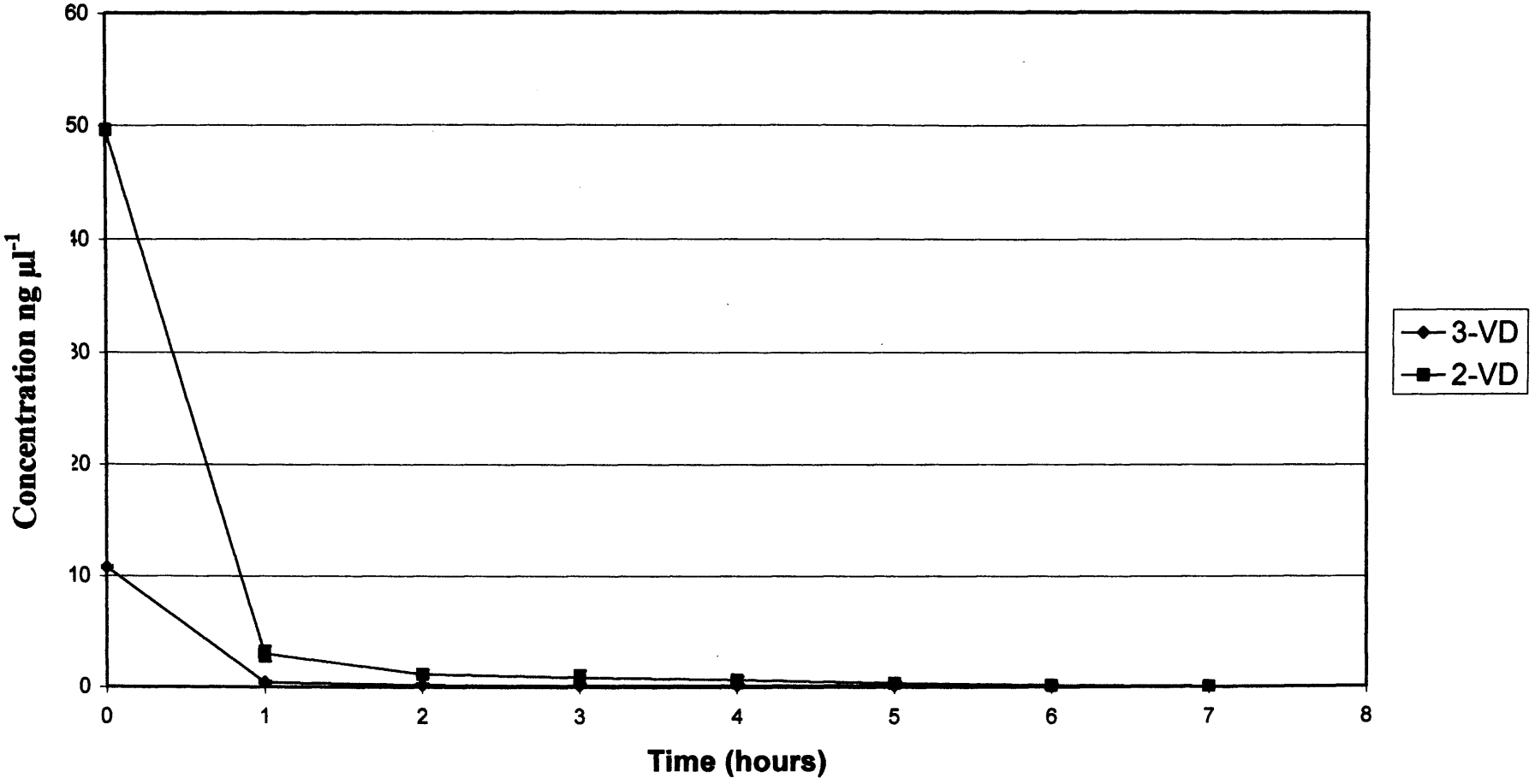


Figure 6.3.4.0 Changes in concentrations of vinyl dithiins in garlic extract prepared in Nutrient Broth (5 mg ml^{-1}) in the presence of *E. coli*. Values plotted are means from two experiments.

Concentrations of diallyl sulphides in garlic extract (nutrient broth 5 mg ml^{-1}) at 37 °C with *E. coli*.

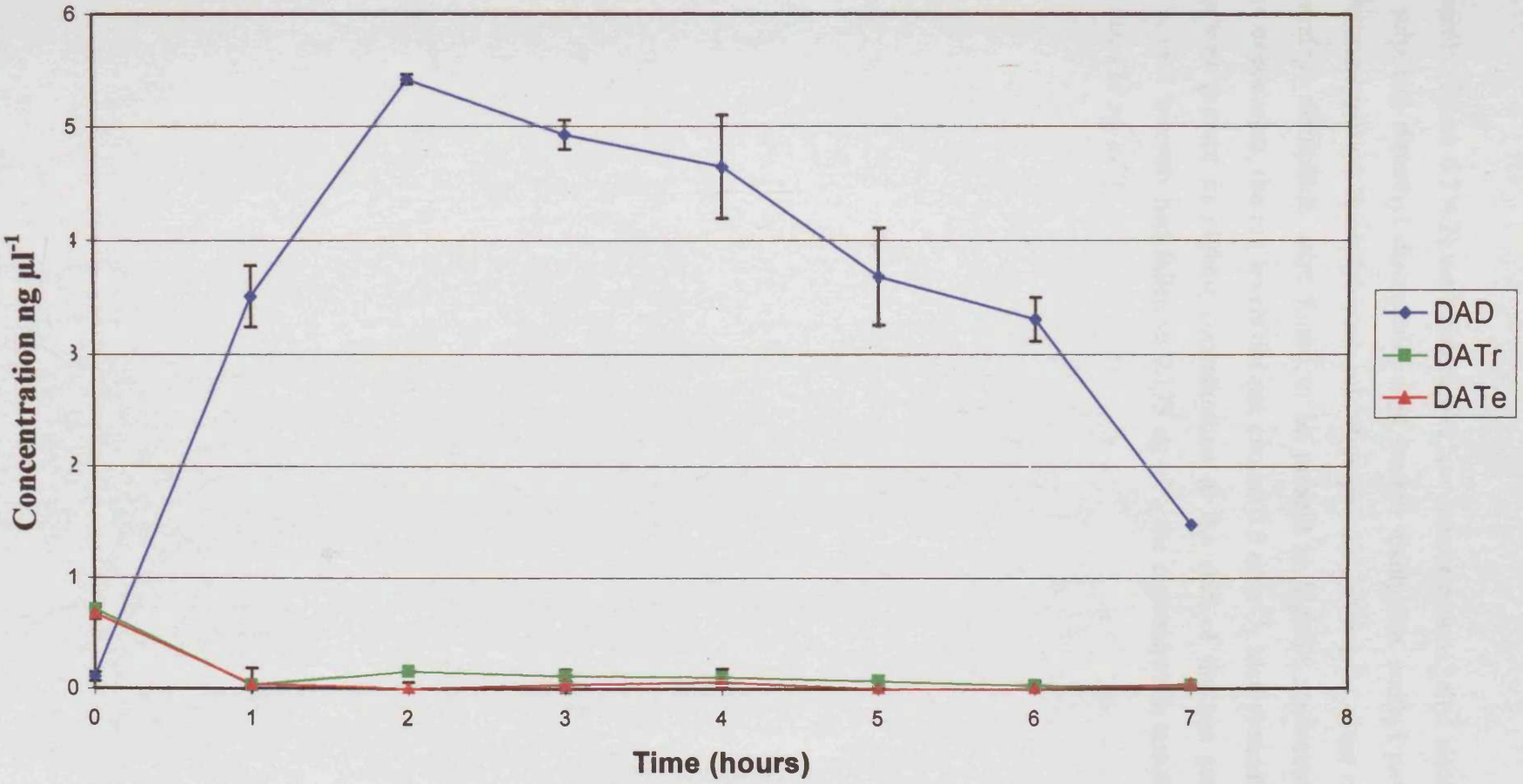


Figure 6.3.4.1 Changes in concentrations of diallyl sulphides in garlic extract prepared in Nutrient Broth (5 mg ml^{-1}) in the presence of *E. coli*. Values plotted are means from two experiments.

Diallyl trisulphide, and diallyl tetrasulphide were only detected in minor quantities throughout the test period (<0.715 ng μl^{-1}), no diallyl pentasulphide was detected

Other components (figure 6.3.4.2) were detected at low concentrations (allyl alcohol, allyl methyl sulphide, dimethyl disulphide, allyl methyl disulphide, methyl propyl disulphide, methane methyl thiosulphinate, and dipropyl disulphide), of these allyl alcohol and methyl disulphide were found to be present in highest concentration throughout the experiment, though levels did not exceed 0.5 ng μl^{-1}, Methyl methane thiosulphinate was present in highest concentration at the start of the test period (3.604 ng μl^{-1}), by 1 h levels had fallen to 0.173 ng μl^{-1}, the concentration remained constant then (±0.130 ng μl^{-1}).

Concentrations of diallyl sulphides in garlic extract (nutrient broth 5 mg ml^{-1}) at 37 °C with *E. coli*.

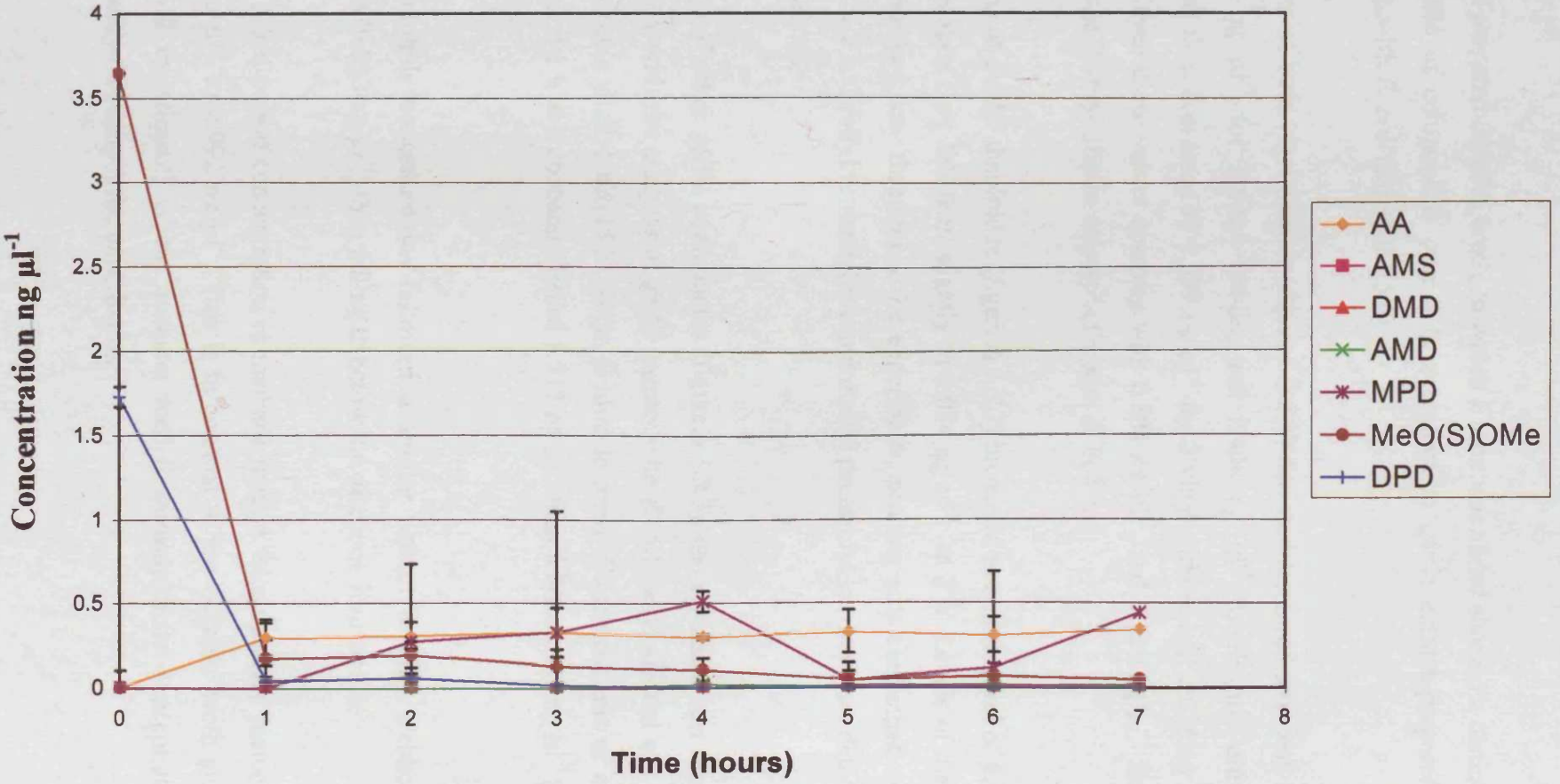


Figure 6.3.4.2 Changes in concentrations of garlic components in garlic extract prepared in Nutrient Broth (5 mg ml^{-1}) in the presence of *E. coli*. Values plotted are means and standard deviations from two experiments.

6.3.5. A preliminary investigation into the effects of *L. casei* on sulphur components of garlic extract in MRS broth.

Garlic extract prepared in MRS broth, to which *L case* was added showed a distinctly different profile of components over 3 h, compared to garlic extract prepared in Nutrient broth with *E. coli* (figure 6.3.5.0).

Concentrations of both vinyl dithiins (figure.6.3.5.1) detected increased to a peak at 3 h at 11.479 ng μl^{-1} for 2-vinyl dithiin, and 2.408 ng μl^{-1} for 3-vinyl dithiin. Concentrations then decreased to 9.539 ng μl^{-1} for 2-vinyl dithiin, and 2.028 ng μl^{-1} for 3-vinyl dithiin these values compare with 0.881 ng μl^{-1}, and 0.166 ng μl^{-1} for 2-vinyl dithiin and 3 vinyl dithiin respectively with *E. coli.*).

Concentrations of diallyl disulphide (figure.6.3.5.2) increased to a peak level of 6.247 ng μl^{-1} at 2 h, then they fell then slightly to 6.034 ng μl^{-1} at 3 h. Levels of diallyl trisulphide remained low throughout the experiment, peaking at a concentration of 0.501 ng μl^{-1} at 2 h. Diallyl tetrasulphide and diallyl pentasulphide were not detected during this analysis.

Concentrations of other garlic components (figure.6.3.5.3) also remained low during this analysis. As with the analysis of garlic extract with *E. coli*, allyl alcohol was the most abundant non diallyl sulphide/ vinyl dithiin detected. Concentrations of allyl alcohol peaked at 1 h at a concentration of 1.515 ng μl^{-1} then fell to 0.865 ng μl^{-1} at 3 h.

Dimethyl disulphide concentrations followed a similar pattern to allyl alcohol – peaking at 2 h (0.933 ng μl^{-1}) then falling to below the detection limit at 3 h.

During the 3 h test period concentration of methane methyl thiosulphinate increased from 0.405 ng μl^{-1} to 0.992 ng μl^{-1}. This is in contrast to the Nutrient broth garlic extract/ *E. coli* experiment, where methane methyl thiosulphinate concentration decreased steadily throughout the experiment.

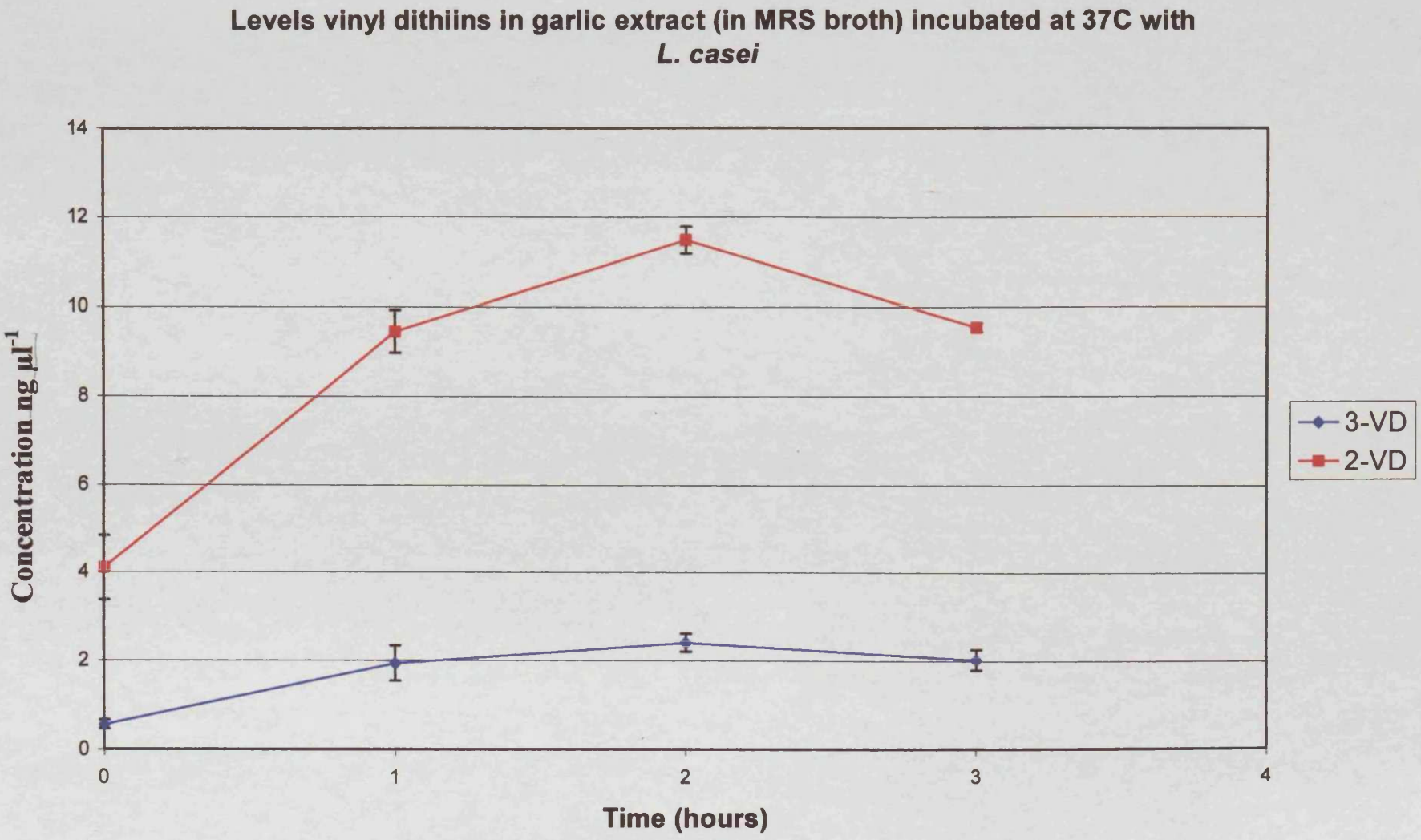


Figure 6.3.5.0 Changes in concentrations of vinyl dithiins over 24 hours in garlic extract prepared in MRS Broth (5 mg ml^{-1}) in the presence of *L. casei*. Values plotted are means from two experiments.

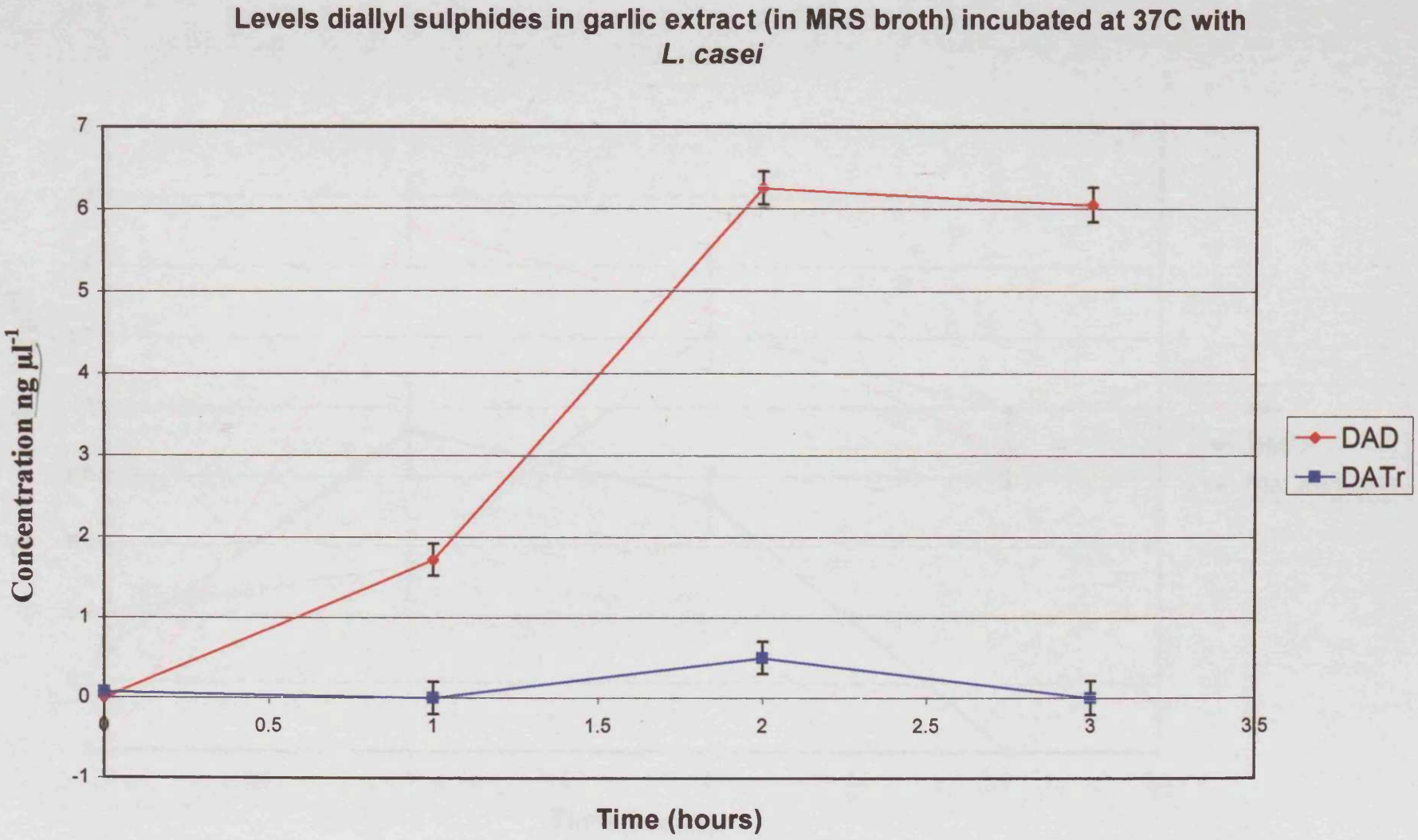


Figure 6.3.5.1 Changes in concentrations of diallyl sulphides over 24 hours in garlic extract prepared in MRS Broth (5 mg ml^{-1}) in the presence of *L. casei*. Values plotted are means and standard deviations from two experiments.

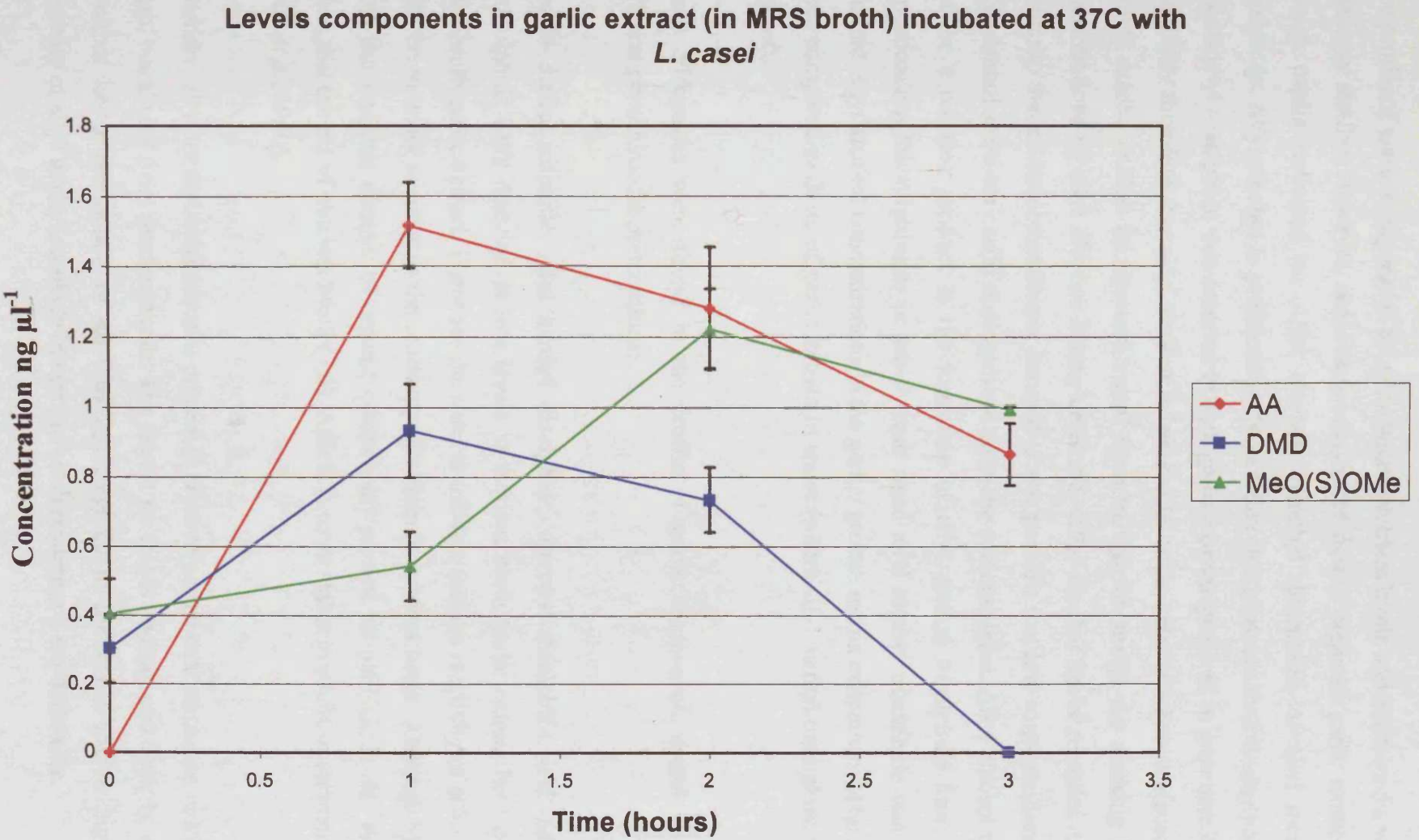


Figure 6.3.5.2 Changes in concentrations of garlic components in garlic extract prepared in MRS Broth (5 mg ml^{-1}) in the presence of *L. casei*. Values plotted are means and standard deviations from two experiments.

6.4. Discussion

The profile of garlic components found in water, Nutrient broth, and MRS media were relatively similar. However, immediate analysis of freshly prepared garlic extract in growth media indicated no Allyl alcohol, dimethyl disulphide, or allyl methyl trisulphide. Allyl alcohol is produced in the reaction that produces diallyl trisulphide, since diallyl trisulphide was detected in both garlic extracts prepared in growth media. It is likely therefore that allyl alcohol is less stable/ more volatile when produced in growth media, or that background signal from the growth media are masking low concentrations of allyl alcohol during detection. Allyl alcohol has a retention time similar to that of dichloromethane, hence it is also possible that low concentrations of allyl alcohol were not easily distinguished from the solvent signal. Allyl alcohol may also be a reaction product in the formation of allyl methyl trisulphide from it's corresponding thiosulphinate; in which case since allyl methyl trisulphide was not detected, allyl alcohol concentrations in the garlic/ growth media extract would be low when compared to those of garlic extract in water (where allyl methyl trisulphide was present).

Three differences were found in the profile of garlic components found in the different growth media preparations:

Firstly- diallyl sulphide, allyl methyl disulphide, dipropyl disulphide, and diallyl tetrasulphide were detected at low levels in nutrient broth garlic extract but not in MRS broth garlic extract. These results seem to indicate that the reactivity of allicin in MRS broth is not as great as the reactivity of allicin in nutrient broth. Although MRS broth has a greater number of organic components present, its pH (6.2) is also more acidic that the pH of nutrient broth (7.5). Allicin is more stable in acidic environments (Iberl *et al*, 1990).

Secondly, allyl methyl sulphide was present in relatively high concentrations in MRS broth, but absent from nutrient broth. The reason for this is unclear, and could be due to either lower production of allyl methyl sulphide in nutrient broth, or higher reactivity of allyl methyl sulphide in nutrient broth rendering it not detectable.

Thirdly, concentrations of vinyl dithiins were higher on analysis of nutrient broth garlic extract, than on analysis of MRS broth garlic extract. When considered individually (discounting sampling error), this could indicate that allicin is more stable when dissolved in nutrient broth than in MRS broth. However, when considered with the observation that there are higher concentrations of allicin breakdown products in nutrient broth than in MRS broth, it could suggest that less allicin was produced by conversion of alliin on addition of garlic powder to the MRS broth, than was the case for Nutrient broth. There are certainly a wider variety of compounds contained in MRS broth that could inhibit alliinase. MRS broth is also slightly acidic (pH 6.2), alliinase may function less efficiently at this pH, than at the pH of Nutrient broth (7.5). Another reason for lower concentrations of both allicin and allicin reaction products in MRS media, may be that the reaction pattern of allicin has changed, and the products may be different from those observed in Nutrient broth (leading to the production of compounds which could not be detected using this method).

Hourly extraction of components from garlic extract prepared in nutrient broth and incubated at 37°C revealed that patterns could be seen in the production and degradation of all detected components. Concentrations of all components were found to fluctuate slightly, and variation is to be expected, as the majority of components analysed here are not readily water soluble – sampling from the aqueous extract may not deliver consistent concentrations of each test component into the solvent.

General trends could be seen in the change in concentration of components (table 6.4.1). Concentrations of most components peaked twice during the test period, notable exceptions were 2-vinyl dithiin, and 3-vinyl dithiin. Concentration of 3-vinyl dithiin decreased throughout the experiment, whereas concentration of 2-vinyl dithiin increased to a maximum at 2 h, then decreased rapidly at first (then more steadily) throughout the experiment. This would suggest that the enzymic formation of allicin from alliin was not complete until the 3rd h of the experiment, this result is surprising when compared to previous observations that in water allicin reached it's maximum levels within 30 minutes (due to alliinase being present in excess). However the preparation of garlic extract in this experiment was carried out in nutrient broth no

water, and as such interference from other components of the broth may account for the continued formation of allicin.

Concentrations of all detected diallyl sulphides increased to maximum levels at 4 h, allicin levels (indicated by vinyl dithiin concentration) began to fall 2 h previous (table 6.4.1). This result supports the observations that diallyl sulphides are the primary breakdown products of allicin in aqueous solutions (Block 1985, Lawson 1996, Iberl *et al* 1990, Yan *et al*1995). On reaching a maximum at 4 h, levels of all Diallyl sulphides decrease, then increase once more reaching a second maximum at 15 h (16 h for diallyl tetrasulphide). The decrease in diallyl disulphide concentration is probably to to further reaction of the diallyl sulphide components, exactly why concentrations increase again to a second maximum is not clear. It is interesting to note that the second maxima only exceeds the first in the case of diallyl tetrasulphide, suggesting that longer chain diallyl sulphides are formed increasingly as time proceeds. Reformation of diallyl sulphides from their reaction products may account for the second maximum concentration seen. Concentrations of allyl methyl sulphides followed a similar pattern to that of the diallyl sulphides, with two maxima occurring These compounds are reaction products of the allyl methyl homologue of allicin, and it is reasonable to expect that they would have similar reaction patterns to the diallyl sulphides discussed above.

Concentrations of allyl alcohol, peak at 2 h (two h before the diallyl sulphides), then again at 12 h (again 2 h before the diallyl sulphides). Allyl alcohol production is tied in with production of allyl trisulphides, so it is expected that allyl alcohol concentration would rise as the concentration of trisulphides rise. Why the maximum levels for allyl alcohol should be achieved 2 h before diallyl trisulphide levels peak each time is not understood. Allyl alcohol is a product of the breakdown of allicin before the formation of diallyl trisulphide (which is though to occur through an oxygenated diallyl trisulphide intermediate (Koch 1988)), but it is unlikely that this would account for maximum levels of both compounds being attained 2 h apart.

Dimethyl trisulphide also showed 2 maximum concentrations, along with a general increase as the levels of methane methyl thiosulphinate (it's parent –a dimethyl homologue of allicin) compound decreased, as expected.

This analysis proved that garlic extract in nutrient broth is a complicated dynamic mixture of thiosulphinates, and their many reaction products. As general trends, diallyl sulphide levels peak 2 h after the rapid disappearance of allicin has begun. There is an increase in the proportion longer chain length diallyl, dimethyl, and allyl methyl sulphides as the experiment proceeds. Levels of allyl alcohol flux in complement to trisulphide production, and thiosulphinate levels decrease as concentration of smaller breakdown products rise.

Using the total vinyl dithiin content as a direct marker for allicin content, it is possible to calculate a tentative half- life for the component. The experiment indicated that the half-life of allicin at 37°C in garlic extract in nutrient broth is approximately 11 h. This is more rapid than the values calculated in water (4-6 day Sreenivasamurthy *et al* 1961, and 15 days – Hughes and Lawson, 1991), but substantially slower than the rates calculated by Freeman (1995) for allicin breakdown in physiological fluids. This is perhaps to be expected as the growth media here offers a chemical environment somewhere between the relative complexities seen in blood and water. Estimates of allicin half-life will always be subject to variation, as the rate of allicin breakdown depends on so many variables.

Component	Time of 1st peak (h)	Concentration achieved (ng μl^{-1})	Time of 2nd peak (h)	Concentration achieved (ng μl^{-1})
2-VD	2	113.197 ± 0.280		
3-VD	0	10.381 ± 0.548		
DAD	4	26.811 ± 0.675	15	18.158 ± 0.072
DATr	4	16.329 ± 0.553	15	10.180 ± 0.415
DATe	4	4.546 ± 0.207	15	8.417 ± 0.056
DAPe	4	2.185 ± 0.127	15	1.705 ± 0.119
AA	2	1.868 ± 0.052	12	1.223 ± 0.027
AMD	2	0.569 ± 0.03	14	0.746 ± 0.038
AMT	4	1.279 ± 0.067	15	1.317 ± 0.042
MPD	3	0.977 ± 0.245	14	1.348 ± 0.073
DPD	4	6.025 ± 0.039		
DMT	2	0.785 ± 0.092	15	1.556 ± 0.064

Table 6.4.1 Many compounds detected in analysis of garlic powder extract over 24 h appeared to peak in concentration twice. Maximum concentrations of components detected in garlic extract in nutrient broth and the times at which they occurred (concentration of some components appeared to peak twice during the analyses) are presented here.

Reactivity of allicin in garlic extract prepared in nutrient broth was substantially different when inoculated with *E. coli*. The experiment here documents the first 7 h of 37°C incubation, as a complement to the experiments carried out in Chapter 3 (beginning of *E. coli* growth curve). Production of diallyl disulphide was rapid, peaking at 2 h, although concentrations identified here were lower than those seen in cell free garlic extract. Diallyl sulphide was not detected at all, diallyl trisulphide and tetrasulphide were found to be present in only small quantities – decreasing as the experiment continued. Allyl alcohol concentrations increased until 1 h then remained stable. Concentrations of other garlic components were also found to be extremely low here, decreasing rapidly until 1 h then remaining relatively constant.

On analysis of the cell free supernatant from the cultures containing garlic extract, concentrations of total vinyl dithiin decreased rapidly. From these results a half-life of approximately 30 minutes was calculated for allicin (in nutrient broth with *E. coli* at 37°C). The results from this study would seem to indicate that components present in garlic extract are increasingly volatile (allicin half-life of 30 minutes). However it must be remembered that analyses were carried out only on cell free supernatants, and as such decreased concentrations of components detected on solvent extraction, represent a combination of component breakdown, and possible uptake of component into bacterial cell. It is known that allicin is membrane permeable, and given the hydrophobic nature of diallyl sulphides it is feasible for these molecules permeate to the interior of bacterial cell membranes.

This preliminary investigation into the effect of *L. casei* on reactive components from garlic extract, are very different from the results obtained in the *E. coli* study. Concentrations of all components detected in cell free supernatant here increase during the 1 h of incubation, then concentrations of allyl alcohol, and dimethyl disulphide decrease. Concentrations of allyl alcohol detected were in excess of 3 times higher than those detected during the *E. coli* experiment, this suggests that the reactive pathways of allicin are different in *L. casei* that in *E. coli*.

Levels of vinyl dithiins and diallyl sulphides increase until 2 h then decreased slightly. The increase in te level of vinyl dithiins would support the idea that alliinase does not perform as efficiently in nutrient media, only reaching peak allicin production at 2 h.

These results may indicate, that allicin is more stable in growth media in the presence of *L. casei*, than in the presence of *E. coli*. Possible indicating that allicin is less reactive in the presence of *L. casei* than *E. coli*, or that the reaction pathways of allicin exposed to bacteria differ significantly from the reaction pathways of allicin in cell-free environments (producing compound which could not be detected using these analyses).

These results are important in considering the differential activity of garlic towards the two bacteria. *E. coli* when incubated in the presence of garlic extract in nutrient broth brings about the rapid disappearance of allicin from the culture medium (the same can not be said for *L. casei*), whether this is through uptake, diffusion into cells, or through modified reactions of allicin, is impossible to say at this stage. But this study has highlighted a major difference in the response of *E. coli* and *L. case* towards garlic extract.

Due to time and equipment constraints a detailed 24 hour analysis of the levels of allicin and allicin reaction products was only carried out in nutrient broth. This experiment needs to be repeated using water, and MRS broth before conclusive comparisons can be made regarding differences in breakdown patterns in each medium. These 24 hour analyses should then be repeated in each medium in the presence of *E. coli* and *L. casei*. To a large extent, conclusions drawn from this chapter are tentative, providing a useful starting point for future research.

Chapter 7

The effect of garlic components on growth of *Escherichia coli.*

Chapter 7 – The Effects of Garlic components on growth in *Escherichia coli.*

7.1 Introduction

Garlic is an effective antibacterial agent (Chopra 1933, Didry *et al* 1987, Harris *et al* 2001). Traditionally the antibacterial activity of garlic has been ascribed to allicin (Cavallito 1944, Chowdhury *et al* 1991). Shashikanth *et al* (1986) demonstrated that fresh garlic has high antibacterial activity against rat intestinal microflora, whilst boiled whole garlic cloves displayed no antibacterial effect. Boiling garlic cloves denatures alliinase (responsible for allicin production on crushing of cloves), and it has been suggested from these results that allicin is responsible for the in vivo antibiotic effects of garlic (Koch 1988).

Aqueous garlic extract is a dynamic mixture containing many pharmacologically (therapeutic) significant sulphur compounds, including thiosulphinates and thiosulphinate reaction products. To say that allicin is solely responsible for the antibacterial and antibiotic effect of garlic is open to question, as it makes no allowance for antibacterial activity of other thiosulphinates and thiosulphinate breakdown products. Recently, garlic oils in which the principle ingredient is not allicin have proved to possess antibacterial activity against *S. aureus*, methicillin resistant *S. aureus* (Tsao *et al* 2001), *H. pylori* (McNulty *et al* 2001) and various enteric bacteria.

Garlic oils are produced usually by steam distillation. Due to the thermal instability of allicin it degrades during the aqueous heating procedure yielding diallyl sulphides, which then evaporate. It is the condensed volatiles of diallyl sulphides that are the principle components of garlic oil.

Diallyl sulphides in garlic oil are present in the form of: diallyl sulphide, diallyl disulphide, diallyl trisulphide, and diallyl tetrasulphide (sulphides with higher sulphur concentrations have also been detected in various heat treated garlic products).

Antimicrobial activity of diallyl sulphides has been reported against *Giardia intestinalis, Trichomonas foetus* (Harris 2001), *Pseudomonas aeruginosa* (both

antibiotic sensitive and antibiotic resistant), *Klebsiella pneumonia* (Tsao 2001), *H pylori*(O'Gara 2000), and *E. coli.*

Other allicin reaction products have also demonstrated high antimicrobial activity. Allyl alcohol inhibited growth of *G. intestinalis, T. vaginalis,* and *T. foetus* (Harris 2001). It is also known to inhibit carbohydrate metabolism in *Saccharomyces cerevisae* through competitive inhibition of alcohol dehydrogenase (Hall *et al* 1987).

Since the composition of garlic powder extract rapidly changes on addition to bacterial cell cultures (Chapter 5), and physiological fluids (Freeman *et al*, 1997). It is possible that the antimicrobial action of garlic powder extract also changes over time, depending on the antimicrobial efficacy of the combination of compounds present.

The aim of the work presented here was to Identify which components of garlic powder extract were the most effective growth inhibitors of the potentially pathogenic bacteria *E. coli* at 37°C over 24 hours (using continuous growth monitoring), and possibly indicating which components are responsible for the characteristic growth inhibition pattern observed in earlier experiments. The effects of synthesized allicin on growth in *L. casei* was also investigated, in order to identify whether a lack of allicin sensitivity could account for the differential inhibition observed between *E. coli* and *L. casei* (Chapter3).

Inhibitory effects of a mixture of thiosulphinate breakdown products (diallyl sulphide, diallyl disulphide, diallyl trisulphide, allyl alcohol, dipropyl disulphide), were also investigated, to whether a 'bouquet' of compounds produced on allicin degradation was an effective antibacterial against *E. coli*, also highlighting differences between inhibitory effects of allicin and its reaction products.

7.2 Materials and methods

7.2.1 Synthesis of Allicin.

Allicin was synthesized by direct oxidation of diallyl disulphide in acidic conditions using a modified version of the method described by Lawson and Hughes (1992).

0.5g diallyl disulphide (80% purity – Aldrich Flavours and Fragrances, Poole UK) was dissolved dropwise into 2.5 ml glacial acetic acid (Sigma-Aldrich, Poole UK) on ice, over a magnetic stirrer, in a clean glass 50 ml bulb flask(with thermometer).

0.75 ml 30% hydrogen peroxide (chilled), was added carefully (ensuring the temperature in the flask did not exceed 20°C). The oxidation of the diallyl disulphide then proceeded at 20°C for 5 hours. 7.5ml of cold distilled water was then added, and the synthetic allicin was extracted into 15 ml dichloromethane (analytical grade, Sigma, Poole UK). The dichloromethane fraction was then neutralised by washing with 15 ml aliquots of 1M sodium hydrogen carbonate solution (aqueous) until effervescence was no longer observed.

Dichloromethane was removed from the solvent fraction by rotary evaporation (30°C), yielding an oily slightly yellow residue. This residue was dissolved in 250 ml of distilled water. Un-reacted diallyl disulphide was then removed by washing the aqueous fraction twice with 25 ml n-hexane (analytical grade, Sigma, Poole UK).

The aqueous fraction was transferred to a clean glass Duran bottle, and stored until use at -70°C.

7.2.2.1 Purity of synthetic allicin

Purity of the synthetic allicin was determined by GCMS. 0.5 ml of aqueous synthetic allicin was extracted into 1 ml dichloromethane (analytical grade, Sigma, Poole UK) by gentle inversion for 30 sec. On separation, methyl propyl sulphide (Lancaster chemicals, Lancaster UK) was added to the solvent fraction as an internal standard to a concentration of 10 $ng\mu l^{-1}$. 1.2 µl were then used for GC injection. Injection port program, temperature program, column choice and mass spectrometer parameters were as described in Chapter 6.2. Using vinyl dithiin concentration as direct markers

for the presence of allicin it was determined that the aqueous allicin solution contained 61.6 μg ml^{-1} allicin.

7.2.2 The effect of Garlic components on bacterial growth.

Garlic components were screened for inhibitory activity using a Bioscreen C analyser (Labsystems Ltd, Helsinki Finland).

All components were suspended in tryptone soy broth (Oxoid, Basingstoke UK) (containing 10% Dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Poole UK) to aid dissolution). Stock solutions were prepared to 2 mg ml^{-1} in sterile glass universals, and vortexed thoroughly for 15 min. Dilution series were then made up at twice the desired final test concentration, 150 μl aliquots were transferred to wells on a 100 well Bioscreen plate.

Wells were then inoculated with 150 μl of cell suspension in appropriate broth (Nutrient Broth for *E. coli* and MRS broth for *L. casei*) (approx $2x10^6$ CFU ml^{-1}). Plates were then placed in the Bioscreen analyser (Incubation time 24 hours, temperature 37°C, recording intervals 10 minutes, 10 seconds of intensive shaking pre and post measurement). Growth was assayed using changes in optical density at 540 nm, and reported as the Specific Growth Rate (SGR) (μ) per hour. SGR was calculated from logarithmic plots of optical density graphs (mid exponential/ active growth phase), for illustrative purposes the original optical density plots are presented in this chapter (along with tabulated calculations made from logarithmic plots, presented as means and standard deviations).

7.2.3 Optical density controls

Dilutions (corresponding to those used in the bacterial growth inhibition investigations) of diallyl sulphide, diallyl disulphide, diallyl trisulphide, allyl alcohol, dipropyl disulphide, dimethyl disulphide, garlic component mixture, and allicin were prepared in growth medium with 10% (v/v) DMSO. 300 μl aliquots of each dilution were then transferred to a 100 well Bioscreen plate (Labsystems, Helsinki Finland). Plates were then placed in the Bioscreen analyser (Incubation time 24 hours, temperature 37°C, recording intervals 10 minutes, 10 seconds of intensive shaking pre

and post measurement). Optical density at 540 nm was monitored for each control, these values were then used as blanks for any components found to absorb at 540 nm.

Each experiment was repeated in duplicate performed using independently raised inocula. Growth curves presented are a typical representation of the three experiments, whilst data presented in tables are means and standard deviations of the three experiments.

Statistical significance was evaluated using one way ANOVA testing (Microsoft Excel).

7.3. Results

7.3.1 Effects of synthesized allicin on growth of *E. coli* over 24 hours at 37°C

Addition of synthetic allicin (figure 7.3.1) to *E. coli* cultures produced caused a response similar to that seen on treatment with freshly prepared garlic powder extract (Chapter 3, Figure 3.2.26). A dose dependent extension in the duration of initial lag phase was observed on addition of increasing allicin concentrations (table 7.3.1). The relationship between allicin concentration and extension in lag phase duration followed an exponential pattern; lag phase extension approximately doubling for every increase of 0.62 $\mu g\ ml^{-1}$ allicin concentration (equation of relationship $y=83.748e^{1.0724x}$ where y represents the extension in duration of initial lag phase (min) and x represents allicin concentration ($\mu g\ ml^{-1}$).

As allicin concentration increased (from 0.62 $\mu g\ ml^{-1}$ to 2.46 $\mu g\ ml^{-1}$) SGR decreased slightly from 0.6 h^{-1} to 0.53 h^{-1}. Culture optical density at 24 hours, also decreased slightly although analysis found this to be statistically insignificant ($p>0.05$).

7.3.2 Effects of synthesized allicin on growth of *L. casei* over 24 hours at 37°C

The most noticeable effect of synthesized allicin on growth of *L. casei* (figure 7.3.2) over 24 hours at 37°C, was a dramatic reduction in the rate SGR, falling from 1.18 h^{-1} (control) to 0.6 h^{-1} (6.20 $\mu g\ ml^{-1}$). This again corresponds with the inhibitory trend observed on addition of freshly prepared garlic powder extract to cultures of *L casei* (Chapter 3, figure 3.2.28). As concentrations of allicin increased, cultures did not achieve stationary phase resulting in a decrease in culture optical density at 24 hours (table 7.3.2). Duration of initial lag phase also increased slightly on addition of allicin to the cultures, however the transition from lag phase to active growth phase was less clearly defined at higher concentrations of allicin.

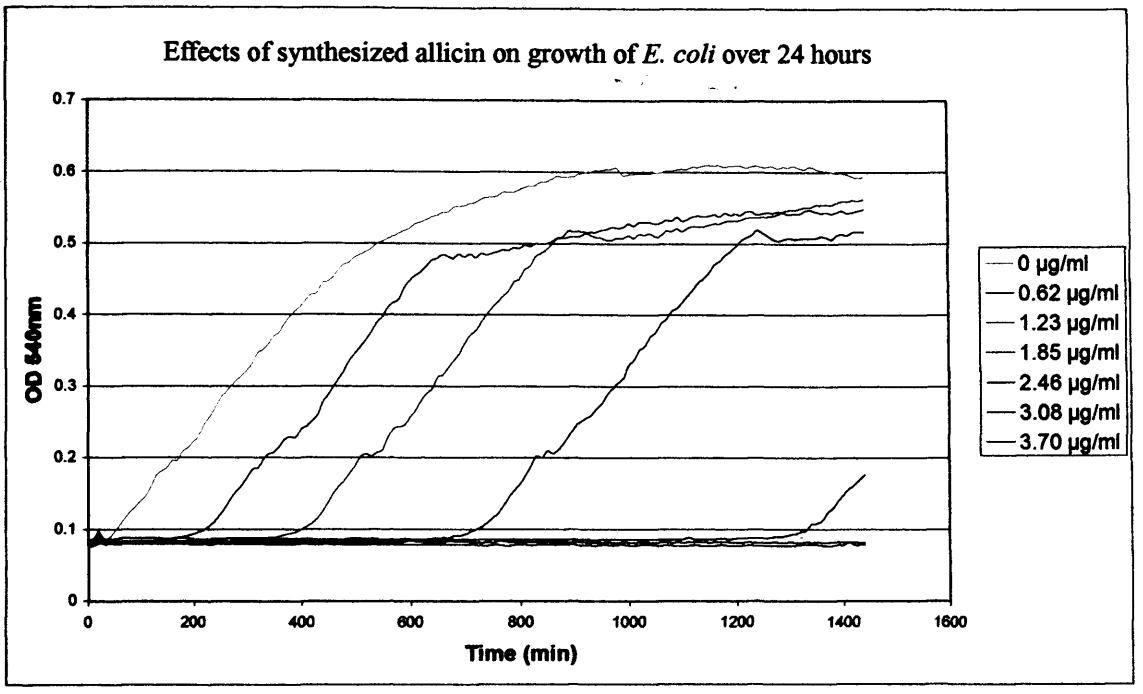


Figure 7.3.1 Growth of *E. coli* in tryptone soya broth with increasing concentrations of synthesized allicin, over 24 hours at 37°C (typical of three independent repeats).

Synthesized allicin concentration (µg ml^{-1})	0	0.62	1.23	1.85	2.46
Duration of initial lag phase (min)	66.67 ±5.77	220.00 ±10.00	400.00 ±0.00	706.67 ±23.09	1420.00 40.67
SGR (h^{-1})	0.58 ±0. 03	0.60 ±0.01	0.69 ±0.01	0.60 ±0.03	0.53 ±0.03
Optical density (540nm) at 24 hours	0.595 ±0.006	0.555 ±0.036	0.566 ±0.004	0.524 ±0.016	

Table 7.3.1 Effects of synthesized allicin on lag phase duration, SGR and culture density at 24 hours of *E. coli* (37°C). Mean values of 3 independent repeats.

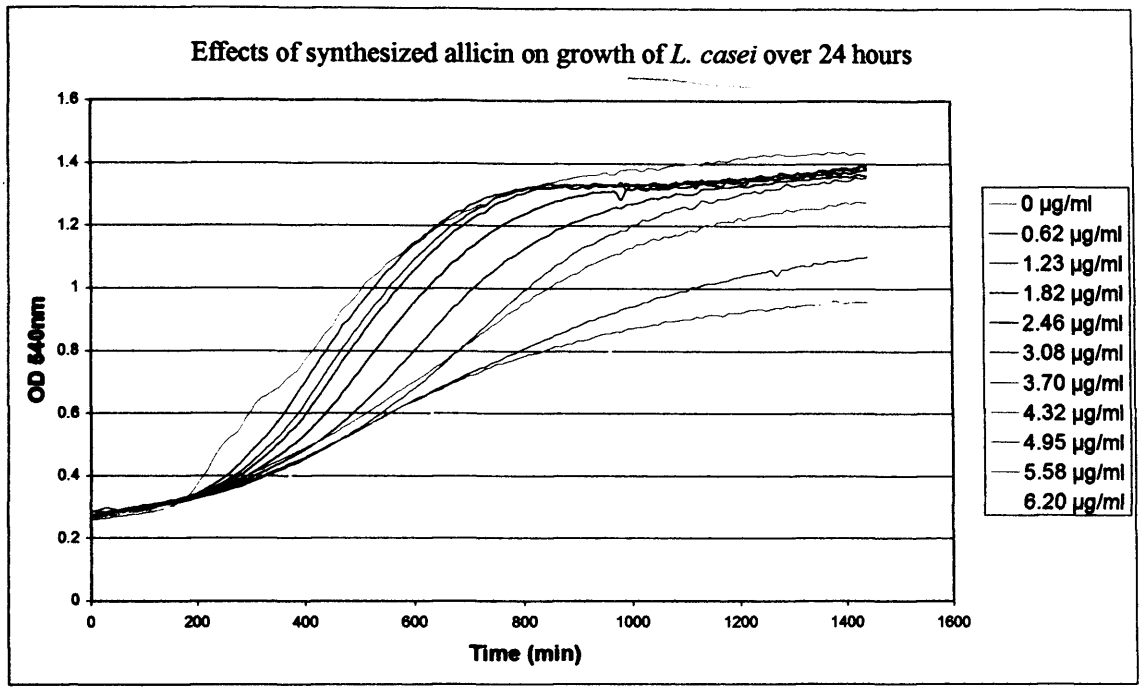


Figure 7.3.2 Growth of *L. casei* in tryptone soya broth with increasing concentrations of synthesized allicin, over 24 hours at 37°C (typical of three independent repeats).

Synthesized allicin concentration (µg ml^{-1})	0	0.62	1.82	3.08	4.32	5.58
Duration of initial lag phase (min)	200 ±10.00	260.00 ±10.00	306.67 ±15.28	306.67 ±15.28	313.33 ±5.78	306.67 ±15.28
SGR (h^{-1})	1.18 ±0.09	1.24 ±0.04	1.20 ±0.04	1.00 ±0.09	0.68 ±0.03	0.6 ±0.06
Optical density (540nm) at 24 hours	1.530 ±0.084	1.454 ±0.075	1.416 ±0.018	1.357 ±0.011	1.248 ±0.039	0.966 ±0.087

Table 7.3.2 Effects of synthesized allicin on lag phase duration, SGR, and culture density at 24 hours of *L. casei* (37°C). Mean values of 3 independent repeats.

7.3.3 Effects of diallyl sulphides on growth of *E. coli* over 24 hours at 37°C.

The effects of diallyl sulphides on aerobic growth of *E. coli* were; an increase in duration of initial lag phase, a decrease in culture optical density at 24 hours, and a reduction in exponential growth rate. These effects were found to be concentration dependent, also increasing in magnitude as sulphur content of the diallyl sulphide under test increased.

7.3.3.1 Effects of diallyl sulphide on growth of *E. coli* over 24 hours at 37°C.

Addition of diallyl sulphide to *E. coli* cultures (figure 7.3.3.1), brought about a slight decrease (0.834 to 0.791) in culture optical density, and extension (67 min to 73 min) in duration of lag phase (table 7.3.3.1), although these results proved not to be statistically significant ($p > 0.05$)). The major effect of diallyl sulphide on growth of *E. coli* at 37°C was a small (1.12 h^{-1} to 0.96 h^{-1}), but significant ($p<0.01$), decrease in the SGR (figure 7.3.1).

7.3.3.2 Effects of diallyl disulphide on growth of *E. coli* over 24 hours at 37°C.

Addition of diallyl disulphide (figure 7.3.3.2) to aerobic cultures of *E. coli* (37°C), caused a significant decrease in culture optical density at 24 hours (table 7.3.3.2) from 0.853 to 0.559 (1.0 mg ml^{-1}), an increase in the duration of initial lag phase (80 min to 240 min (1.0 mg ml^{-1})), and a larger reduction in SGR (1.07 h^{-1} (control) to 0.67 h^{-1} (1.0 mg ml^{-1})).

7.3.3.3 Effects of diallyl trisulphide on growth of *E. coli* over 24 hours at 37°C

Diallyl trisulphide caused an extension in duration of initial lag phase (table 7.3.3.3) from 70 min to 340 min (1.0 mg ml^{-1}). Culture density at 24 hours decreased from 0.835 to 0.474 as concentration of diallyl trisulphide increased to 1.0 mg ml^{-1}. The SGR was reduced (figure 7.3.3.3) from 1.14 h^{-1} to 0.48 h^{-1} on addition of 1.0 mg ml^{-1} diallyl trisulphide.

In cultures treated with diallyl disulphide slight fluctuations were seen in optical densities during the initial lag phase, these were not observed consistently in experimental repeats.

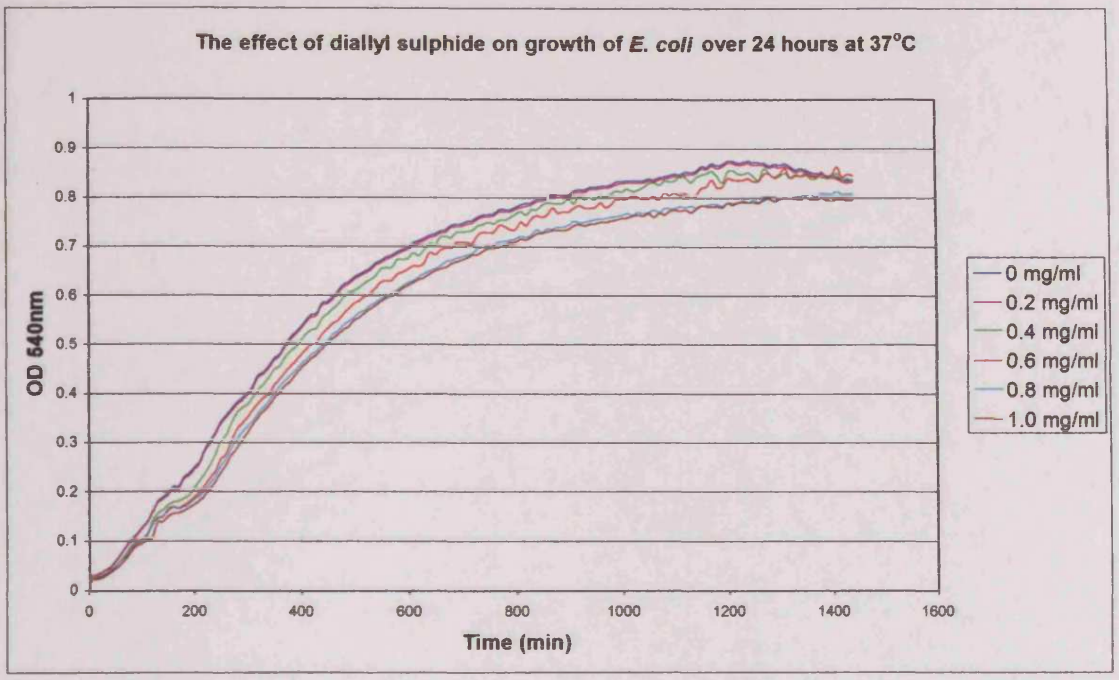


Figure 7.3.3.1 Growth of *E. coli* in Tryptone soya broth with increasing concentrations of diallyl sulphide, over 24 hours at 37°C (typical of three independent repeats).

Diallyl sulphide concentration (mg ml^{-1})	0	0.2	0.4	0.6	0.8	1.0
Duration of initial lag phase (min)	66.67 ±5.77	70.33 ±5.77	66.67 ±5.77	70.33 ±5.77	70.00 ±10.00	73.33 ±5.77
SGR (h^{-1})	1.12 ±0. 02	1.14 ±0.03	1.12 ±0.01	1.07 ±0.04	0.99 ±0.04	0.96 ±0.04
Optical density (540 nm) at 24 hours	0.834 ±0.002	0.838 ±0.003	0.836 ±0.007	0.835 ±0.017	0.805 ±0.008	0.791 ±0.005

Table 7.3.3.1 Effects of diallyl sulphide on lag phase duration, SGR, and culture density at 24 hours of *E. coli* (37°C). Mean values of 3 independent repeats.

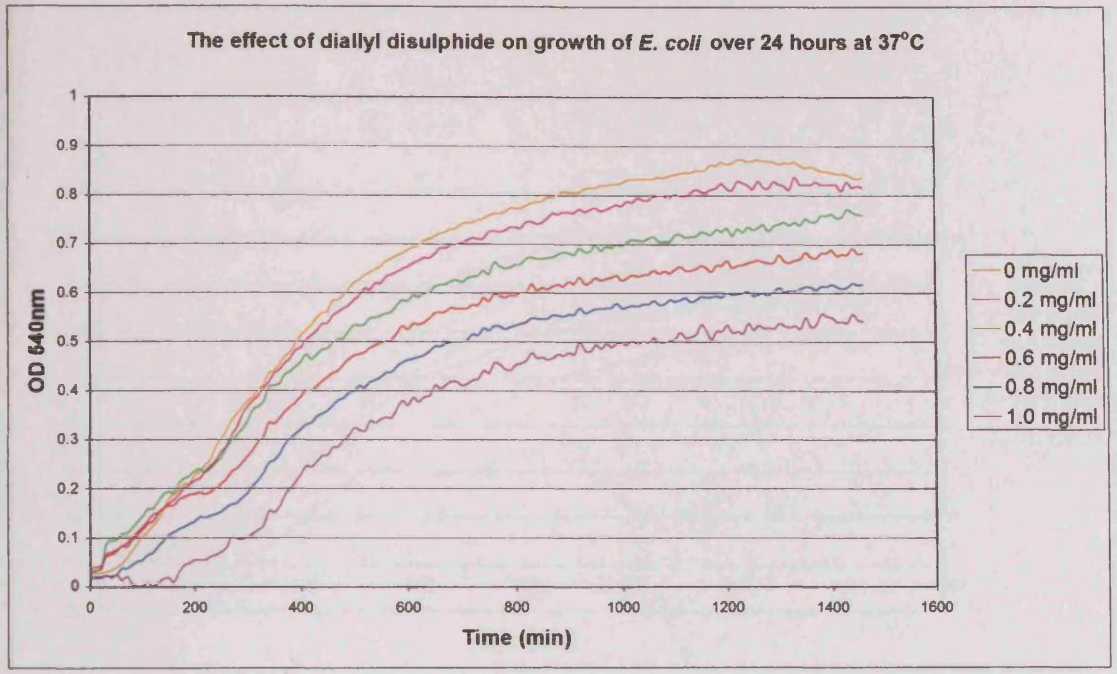


Figure 7.3.3.2 Growth of *E. coli* in Tryptone soya broth with increasing concentrations of diallyl disulphide, over 24 hours at 37°C (typical of three independent repeats).

Diallyl disulphide concentration (mg ml^{-1})	0	0.2	0.4	0.6	0.8	1.0
Duration of initial lag phase (min)	80.00 ±22.14	76.667 ±20.82	80.00 ±25.83	96.67 ±23.09	120.00 ±25.83	240.33 ±26.46
SGR (h^{-1})	1.07 ±0.05	0.98 ±0.02	0.80 ±0.14	0.77 ±0.02	0.72 ±0.14	0.67 ±0.01
Optical density (540 nm) at 24 hours	0.853 ±0.026	0.682 ±0.024	0.686 ±0.090	0.700 ±0.020	0.600 ±0.064	0.559 ±0.019

Table 7.3.3.2 Effects of diallyl disulphide on lag phase duration, SGR, and culture density at 24 hours of *E. coli* (37°C). Mean values of 3 independent repeats.

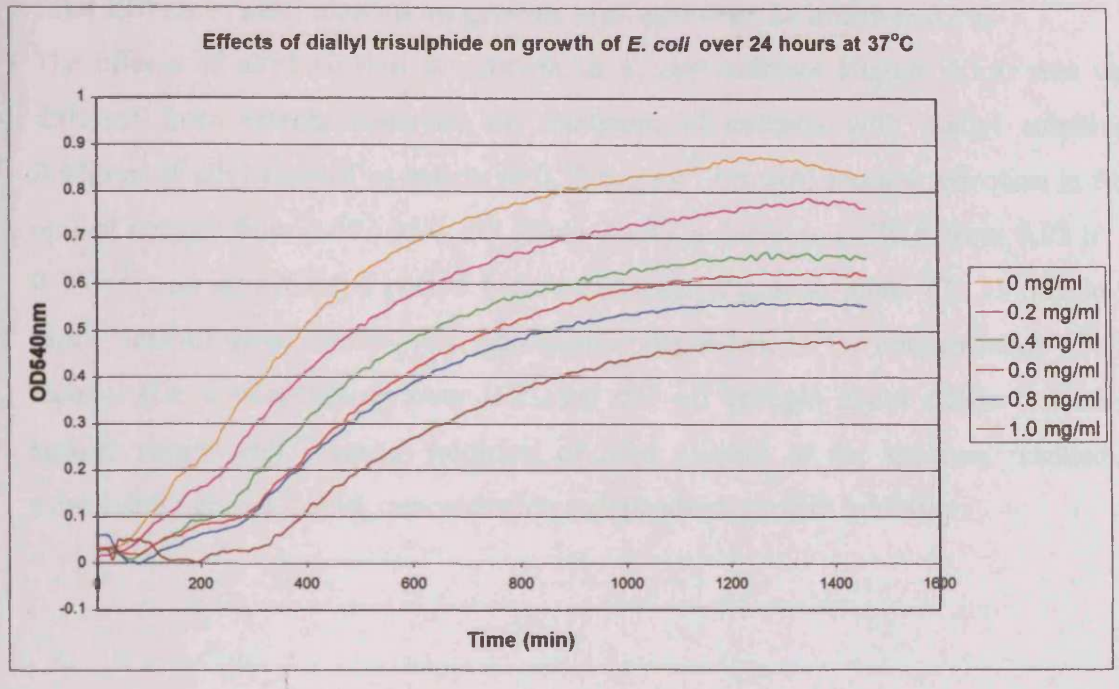


Figure 7.3.3.3 Growth of *E. coli* in Tryptone soya broth with increasing concentrations of diallyl trisulphide, over 24 hours at 37°C (typical of three independent repeats).

Diallyl trisulphide concentration (mg ml^{-1})	0	0.2	0.4	0.6	0.8	1.0
Duration of initial lag phase (min)	70.00 ±20.00	90.00 ±23.82	140.00 ±25.83	140.00 ±20.00	180.00 ±33.33	340.00 ±45.67
SGR (h^{-1})	1.14 ±0.06	0.96 ±0.13	0.88 ±0.04	0.77 ±0.01	0.72 ±0.01	0.48 ±0.07
Optical density (540 nm) at 24 hours	0.835 ±0.045	0.762 ±0.025	0.654 ±0.033	0.618 ±0.019	0.551 ±0.027	0.474 ±0.066

Table 7.3.3.3 Effects of diallyl trisulphide on lag phase duration, SGR, and culture density at 24 hours of *E. coli* (37°C). Mean values least 3 independent repeats.

7.3.4 Effects of allyl alcohol on growth of *E. coli* over 24 hours at 37°C

The effects of allyl alcohol on growth in *E. coli* cultures (figure 7.3.4) was very different from effects observed on treatment of cultures with diallyl sulphides. Addition of allyl alcohol in excess of 0.25 mg ml^{-1} brought about a reduction in final optical density from 0.533 to 0.339 (table 7.3.4), a decrease in SGR from 0.82 h^{-1} to 0.26 h^{-1}, and an extended period before exponential growth phase (73.33 min to 90 min). None of these effects were significantly dependent on the concentration of allyl alcohol (i.e. concentrations over 0.25 mg ml^{-1} all brought about similar effects of similar magnitude). Though addition of allyl alcohol to the medium resulted in substantial (almost 4-fold, concentration independent) growth inhibition.

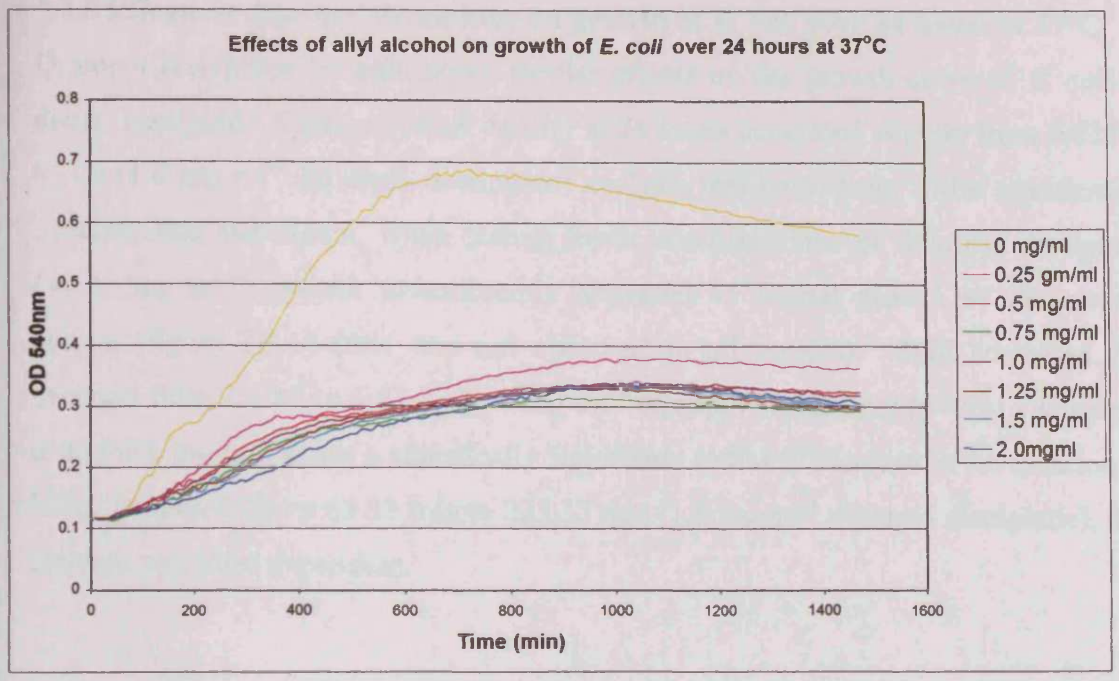


Figure 7.3.4 Growth of *E. coli* in Tryptone soya broth with increasing concentrations of allyl alcohol, over 24 hours at 37°C (typical of three independent repeats).

Allyl alcohol concentration (mg ml^{-1})	0	0.25	0.50	0.75	1.00	1.25	1.50	2.00
Duration of initial lag phase (min)	73.33 ±5.77	90.00 ±10	96.67 ±5.77	103.33 ±5.77	90.00 ±26.46	93.33 ±5.77	123.33 ±11.55	106.67 ±23.10
SGR (h^{-1})	0.82 ±0.04	0.40 ±0.01	0.34 ±0.04	0.26 ± 0.03	0.28 ±0.04	0.28 ±0.01	0.26 ±0.04	0.26 ±0.04
Optical density (540 nm) at 24 hours	0.533 ±0.056	0.339 ±0.025	0.301 ±0.018	0.295 ±0.014	0.303 ±0.012	0.298 ±0.004	0.300 ±0.002	0.310 ±0.006

Table 7.3.4 Effects of allyl alcohol on lag phase duration, SGR and culture density at 24 hours of *E. coli* (37°C). Values shown are means of 3 independent repeats.

7.3.5 Effects of dipropyl disulphide on growth of *E. coli* over 24 hours at 37°C

Dipropyl disulphide brought about similar effects on the growth curve of *E. coli* as diallyl disulphide. Culture optical density at 24 hours decreased slightly from 0.838 to 0.729 (1.0 mg ml^{-1} dipropyl disulphide) analysis this proved not to be significantly concentration dependent, when testing lower concentrations of dipropyl disulphide (<0.25 mg ml^{-1}) growth intermittently appeared to exceed growth of the control culture (figure 7.3.6) (this was not observed in all repeats). SGR increased (on average) from 1.1 h^{-1} to 1.31 h^{-1} (1.0 mg ml^{-1} dipropyl disulphide) ($p<0.01$). Dipropyl disulphide brought about a statistically significant ($p<0.01$) increase in the duration of initial lag phase (from 63.33 min to 223.33 min (1.0 mg ml^{-1} dipropyl disulphide), this increase was dose dependent.

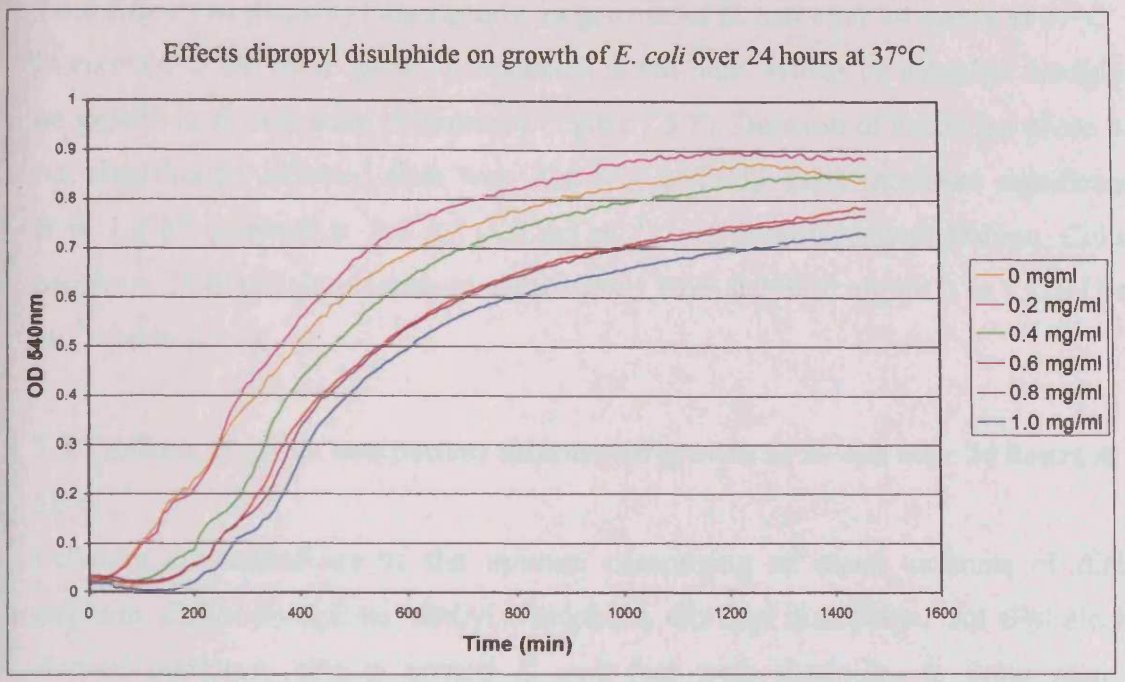


Figure 7.3.5 Growth of *E. coli* in Tryptone soya broth with increasing concentrations of dipropyl disulphide, over 24 hours at 37°C (typical of three independent repeats).

Dipropyl disulphide concentration (mg ml^{-1})	0	0.2	0.4	0.6	0.8	1.0
Duration of initial lag phase (min)	60.33 ±5.88	80.00 ±17.32	140.00 ±10.00	180 ±5.77	200 ±5.77	230 ±5.78
SGR (h^{-1})	1.10 ±0.04	1.31 ±0.04	1.41 ±0.04	1.31 ±0.08	1.37 ±0.04	1.31 ±0.05
Optical density (540 nm) at 24 hours	0.838 ±0.006	0.791 ±0.183	0.862 ±0.022	0.773 ±0.008	0.772 ±0.008	0.729 ±0.018

Table 7.3.5 Effects of dipropyl disulphide on lag phase duration, SGR, and culture density at 24 hours of *E. coli* (37°C). Values shown are means of 3 independent repeats.

7.3.6 Effects of dimethyl disulphide on growth of *E. coli* over 24 hours at 37°C

In contrast to the other garlic components tested here, effects of dimethyl disulphide on growth in *E. coli* were stimulatory (figure 7.3.7). Duration of initial lag phase was not significantly affected (one way ANOVA $p>0.05$). SGR increased significantly from 1.2 h^{-1} (control) to 1.8 h^{-1} (1.0 mg ml^{-1}) in a dose-dependent fashion. Culture density at 24 hours also increased significantly from 0.940 (0 mg ml^{-1}) to 1.220 (1 mg ml^{-1}) (table 7.3.7).

7.3.7 Effects of garlic component mixture on growth of *E. coli* over 24 hours at 37°C

Different concentrations of the mixture comprising of equal amounts of diallyl sulphide, diallyl disulphide, diallyl trisulphide, dipropyl disulphide, and allyl alcohol showed inhibitory effects toward *E. coli* that were dissimilar to those seen on treatment with single components (figure 7.3.8). There was no significant effect on the duration of initial lag phase. As seen previously in cultures treated with allyl alcohol, the SGR in control cultures were substantially faster (1.1 h^{-1}) than the exponential growth rates of cultures treated with the component mixture (0.68 h^{-1} for 0.1 mg ml^{-1}). SGR in cultures treated with different concentrations of the garlic component mixture showed no significant trend (i.e. the inhibition observed was concentration independent). Culture optical density at 24 hours decrease as concentrations of garlic component mixture increased (from 0.612 in the control culture to 0.139 in cultures treated with 1.0 mg ml^{-1}).

Cultures treated with the garlic component mixture failed to achieve 50% of the culture density seen in control cultures, in addition *E. coli* cultures treated with 1.0 mg ml^{-1} garlic component mixture entered a decline phase at approximately 620 min.

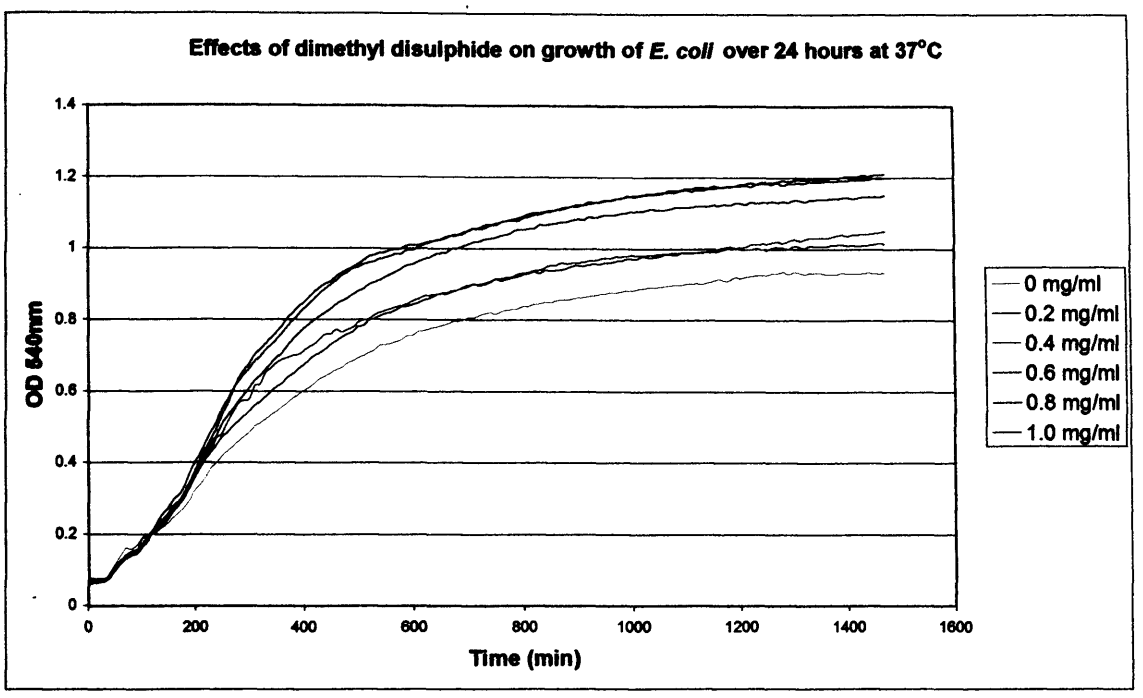


Figure 7.3.6 Growth of *E. coli* in Tryptone soya broth with increasing concentrations of dimethyl disulphide, over 24 hours at 37°C (typical of three independent repeats).

Dimethyl disulphide concentration (mg ml^{-1})	0	0.2	0.4	0.6	0.8	1.0
Duration of initial lag phase (min)	50.00 ±10.00	60.00 ±10.00	63.33 ±5.77	63.33 ±5.77	63.33 ±5.77	60 ±10.00
SGR (h^{-1})	1.20 ±0.12	1.22 ±0.13	1.23 ±0.30	1.56 ±0.04	1.65 ±0.08	1.80 ±0.07
Optical density (540 nm) at 24 hours	0.940 ±0.007	1.032 ±0.016	1.040 ±0.018	1.162 ±0.010	1.200 ±0.005	1.220 ±0.009

Table 7.3.6 Effects of dimethyl disulphide on lag phase duration, exponential growth rate, and culture density at 24 hours of *E. coli* (37°C). Values shown are means of 3 independent repeats.

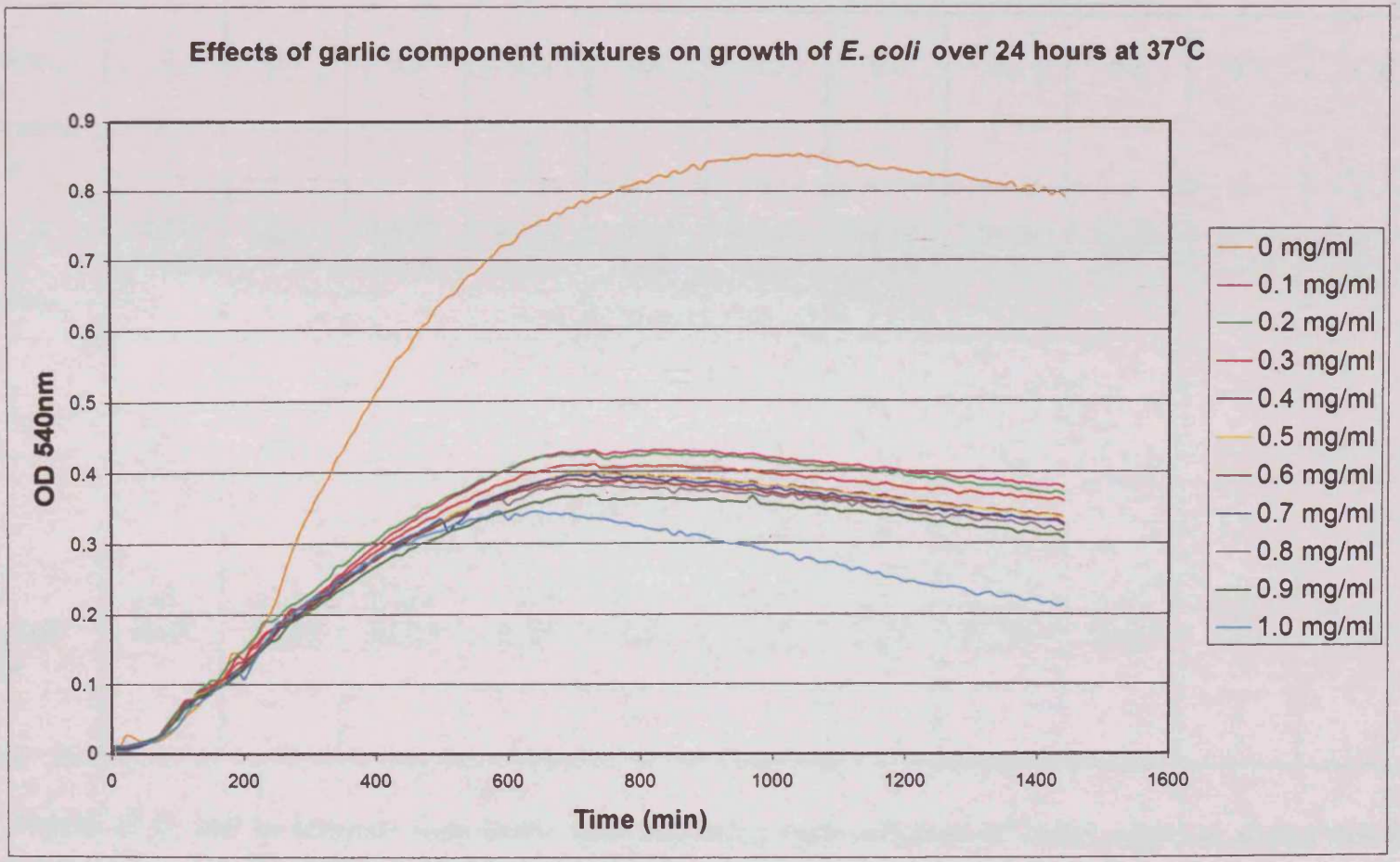


Figure 7.3.7 Growth of *E. coli* in Tryptone soya broth with increasing concentrations of diallyl sulphide, diallyl disulphide, diallyl trisulphide, dipropyl disulphide, and allyl alcohol (mixed in a 1:1:1:1:1 ratio) over 24 hours at 37°C (typical of three independent repeats).

Component mixture concentration (mg ml^{-1})	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Duration of initial lag phase (min)	70.00 ±10.00	83.33 ±5.78	86.67 ±10.00	83.33 ±5.78	86.67 ±10.00	83.33 ±17.32	80.00 ±10.00	73.33 ±5.77	80.00 ±10.00	83.33 ±17.32	80.00 ±17.32
SGR (h^{-1})	1.10 ±0.25	0.69 ±0.04	0.67 ±0.04	0.64 ±0.01	7.67 ±1.55	0.64 ±0.06	0.59 ±0.04	0.69 ±0.05	0.67 ±0.16	0.64 ±0.14	0.59 ±0.04
Optical density (540 nm) at 24 hours	0.612 ±0.007	0.347 ±0.010	0.333 ±0.011	0.320 ±0.005	0.282 ±0.007	0.269 ±0.003	0.260 ±0.011	0.248 ±0.013	0.221 ±0.003	0.207 ±0.003	0.139 ±0.011

Table 7.3.7 Growth of *E. coli* in tryptone soya broth with increasing concentrations of diallyl sulphide, diallyl disulphide, diallyl trisulphide, dipropyl disulphide, and allyl alcohol (mixed in a 1:1:1:1:1 ratio) over 24 hours at 37°C (mean values of three independent repeats).

7.4 Discussion

The effects of synthetic allicin on growth in *E. coli* cultures over 24 hours at 37°C was very similar on appearance to the effects of freshly prepared garlic powder aqueous extract (Chapter 3, Figure 3.2.26).

The relationship between extension of lag phase and concentration was not a linear relationship, fitting an exponential curve (r^2 0.9952) with the extension in duration of lag phase approximately doubling for each 0.62 μg ml^{-1} synthetic allicin.

Gradients of exponential slopes, and culture optical densities at 24 hours showed slight decreases as allicin concentrations increased. Decreases in optical density at 24 hours maybe accounted for by the reduced amount of time cultures spend in their active growth phase on addition of allicin, continuing the growth curve until all cultures reach stationary phase would determine whether this is the case.

A major difference between the effects of allicin and garlic powder extract on growth of *E. coli* cultures is that when treated with lower concentrations of garlic powder extract, cultures reached stationary phase at a higher optical density than that of the control. Cultures treated with low concentrations of synthesize allicin do not reach stationary phase, and even though the control culture has reached early decline phase by 1440 min (24 h), the optical density of cultures treated with low concentrations of allicin does not exceed that of the control. This would appear to support earlier speculation (Chapter 3 discussion) that the elevated culture optical density at 24 hours in cultures treated with concentrations of **garlic powder extract** (below 2.0 mg ml^{-1}) is caused by additional nutrients (carbohydrates, proteins, etc.) in the garlic powder extract, or by other stimulatory factors (e.g. dimethyl disulphide) – absent in the synthesized allicin.

Alternatively it could suggest that allicin is more potent when not used in garlic powder extract, results from Chapter 6 show that allicin reacts more rapidly in increasingly chemically complex dissolution media, so it is to be expected that allicin dissolved in water would be more stable than allicin dissolved in aqueous garlic

powder extract. A time course GCMS or HPLC analysis of allicin in water at 37°C would be needed to confirm this.

As with the *E. coli* cultures treated with garlic powder extract, the delay in the onset of exponential growth phase in cultures treated with synthesized allicin could be accounted for in a number of ways:

(i.) Non lethal cell injury could account for an extended lag period whilst cells repair, before entering the active growth phase.
(ii.) Low level bactericidal activity in allicin would bring about delays in the onset of exponential growth phase, with increasing concentrations of allicin killing higher numbers of cells an extended lag phase would become apparent as culture cell density recovered.
(iii.) Allicin is relatively unstable, degradation of allicin in cultures of *E. coli* at 37°C could account for the large delays in onset of exponential growth phase, as allicin content in the cultures decreases to sub inhibitory concentrations. Producing an extension in lag phase that is dependent of allicin concentration, temperature, and dissolution media (Chapter 6 discussion).

It is possible that any of the above could occur alone or in combination. When considering the results presented in Chapters 3 and 6, it is likely that the delay in onset of active growth phase is produced by allicin acting in a biostatic (or low level biocide) manner, then degrading to sub inhibitory concentrations.

Results presented here show that allicin is an effective biostatic antibacterial agent, at concentrations exceeding 3.0 $\mu g\ ml^{-1}$ no growth was observed in cultures of *E. coli*.

Addition of synthetic allicin to cultures of *L. casei* also brought about responses that were similar to those seen on addition of freshly prepared garlic powder extract (Chapter 3, figure 3.2.28). There was a slight delay in the onset of exponential growth when cultures treated with allicin were compared to control culture, however there was no significant dose dependent relationship between duration of lag phase and allicin concentration.

Growth of *L. casei* proceeded in the presence of synthesized allicin, at reduced rates. Due to the reduction in exponential growth rate however, cultures treated with higher concentrations of allicin did not reach stationary phase during the test period.
These results highlight a differential response between cultures of *L. casei* and *E. coli* on addition of synthesized allicin, as was seen with garlic extract. It is not possible to conclude why *L. casei* is so much less susceptible to the inhibitory action of allicin at this point.

Observations from Chapter 6 suggest that allicin is more stable in *L. casei*/MRS cultures than in *E. coli*/ nutrient cultures, so it is unlikely that *L. casei* is actively degrading allicin. The reduced susceptibility could be due to differences in allicins solubility in the cell envelopes of *L. casei* and *E. coli* or differences in allicins site of action within the bacteria.

Additions of diallyl sulphide, diallyl disulphide, and diallyl trisulphide produced similar effects on the growth curve of *E. coli* over 24 hours at 37°C. Duration of initial lag phase increases as concentration of sulphide increases, whilst exponential growth rate and culture optical density at 24 hours decrease with increasing sulphide concentration. The effects of diallyl sulphides on growth of *E. coli* became more pronounced as the sulphur content of the sulphide increased, this is in agreement with observations of the inhibitory action of diallyl sulphides against *Pseudomonas aeruginosa* (Tsao *et al* 2001). The mechanisms of antibacterial action of diallyl sulphides have not been elucidated, though they have proved inhibitory to a range of organisms (O'gara *et al* 2000, Harris *et al* 2001).

The effect of diallyl disulphide against *E. coli* resembled the inhibition pattern seen on incubation of *L. casei* with *E. coli*. This could suggest (provided that diallyl disulphide acts in a similar fashion against *L casei*) that allicin insensitivity could be responsible for the lack of inhibition seen in *L. casei* cultures on addition of garlic, inhibition only being seen on breakdown of allicin to diallyl sulphides.

All diallyl sulphides tested here had substantially lower antimicrobial activity than synthetic allicin or freshly prepared garlic powder extract. Given the reduced susceptibility of *E. coli* to diallyl sulphides, it seems unlikely that diallyl sulphides are

solely responsible for the inhibitory action of garlic powder extract against *E. coli*, although they would undoubtedly contribute to the antibacterial activity.

Dipropyl disulphide was very similar to diallyl disulphide in inhibitory action against *E. coli*. This is not surprising since the two compounds are very similar in structure. Dipropyl disulphide did not produce a reduction in exponential growth rate, but did cause a concentration dependent extension in duration of initial lag phase, and decrease in culture optical density at 24 hours. The inhibitory activity of dipropyl disulphide was slightly less than that of diallyl disulphide (perhaps reflecting the activity of the double bond found in the allyl grouping).

Allyl alcohol exerted inhibitory effects on growth of *E. coli* over 24 hours at 37°C, that did not resemble those seen on treatment of cultures with garlic powder extract, allicin or diallyl sulphides. *E. coli* cultures treated with concentrations of allyl alcohol in excess of 250 $\mu g\ ml^{-1}$, exhibited a much reduced exponential growth rate (approximately half), and entered stationary phase on reaching a culture optical density of approximately half of that seen in the control culture. Culture optical density at 24 hours, and exponential growth rate generally decreased as concentration of allyl alcohol increased but these trends were not found to be statistically significant. Allyl alcohol is toxic to many cells (Harris 2001), but is also extremely volatile, and rapid evaporative loss from media surrounding bacterial cells may account for the non-concentration dependent inhibition seen (allyl alcohol already diffused into cells would not be lost due to evaporation).

In contrast to the other sulphides tested, dimethyl disulphide had stimulatory effects on growth of *E. coli* cultures, exponential growth rate and culture optical density at 24 hours both increased significantly on addition of dimethyl disulphide, while there was no significant effect of duration of initial lag phase. These experiment show that longer chain-length disulphides are more inhibitory to *E. coli* than dimethyl disulphide, the different chemical structures have different solubility and different reactivity smaller compounds being more stable. Though in certain conditions (with iodine as a catalyst) dimethyl disulphide will undergo addition reactions with carbon-carbon double bonds, which may have damaging effects on cell membranes.

It is clear that dimethyl disulphide is not inhibitory to *E. coli* when used alone, but it cannot be concluded from this that it will have the same activity when used in combination with the complex mixture of components found in garlic powder extract.

Mixtures of diallyl sulphide, diallyl disulphide, diallyl trisulphide, dipropyl disulphide and allyl alcohol (figure 7.3.8) exerted inhibitory effects on *E. coli* that were different from the inhibitory effects of the individual components. All cultures treated with the component mixture grew at a very much slower rate than the control cultures (similar to allyl alcohol), the exponential growth rate was not found to be significantly concentration dependent. There was no significant concentration dependent extension in the duration of initial lag phase, this was surprising since diallyl trisulphide (and to a lesser extent diallyl disulphide and dipropyl disulphide) brought about slight extensions in lag phase at 200 $\mu g\ ml^{-1}$. Another inhibitory effect was seen when using garlic component mixture, cultures reached a maximum optical density at approximately 620 min, after which optical density decreased (more rapidly at higher concentrations).

Decreases in the optical density of an established culture, could be accounted for removal of a 540 nm absorbing compound from the media/ test component (this is unlikely as, a cell free control was used as an optical density blank). An increase in absorbance of the blank (cell free control) over time could account for decreases in the optical density of the test solution, no such increase was seen in the optical density of the cell free control was observed. Decreases in culture optical density are observed when cells enter lytic cell death phase as cells degrade and their components disperse throughout the dissolution media, optical density of the culture would diminish.

Although the Bioscreen method can be used to provide an insight into the sensitivity of bacteria to various biocides, this chapter has also highlighted some of the drawbacks. Since the Bioscreen plates are not totally sealed, they increase the risk of loss of volatile antibacterial components, this may lead to underestimation of the effects of components such as diallyl sulphides, allyl alcohol and allicin.

In conclusion results presented in this chapter suggest that allicin is the primary garlic constituent responsible for the characteristic growth inhibition pattern when *E. coli*

cultures are exposed to freshly prepared garlic powder extract. Allicin reaction products also bring about extensions in duration of lag phase when used in concentrations higher than those found naturally in garlic extract (Chapter 5). Inhibitory activity of sulphides found in garlic increases with sulphur number (in agreement with observations of the activity of diallyl sulphides against *H. pylori* (O'Gara 2000)), and molecular chain-length. Allyl alcohol (a reaction product in the formation of diallyl trisulphide from allicin) exerts growth inhibition in a different manner from garlic extract. The inhibitory pattern of garlic sulphides and allyl alcohol when used in combination is different from that seen when the components were tested individually, suggesting that the mechanism of action is also different, or that reactions occur between components giving rise to products with different sites of action.

Allicin is the most inhibitory component tested here, however since many other components tested here displayed antibacterial activity, it is likely that the inhibitory effects of garlic extract are due to the bouquet of antibacterial compounds acting in combination.

Chapter 8

The effect of garlic extract on cell morphology and carbohydrate metabolism in *Escherichia coli* and *Lactobacillus casei.*

Chapter 8. The effect of garlic extract on cell morphology and carbohydrate metabolism in *Escherichia coli* and *Lactobacillus casei.*

Introduction

8.1.1Garlic, bacterial structure and morphology

All bacterial cells are contained within a cellular envelope consisting of various different components. The major structures within the cell envelope are the cell wall, and the cell membranes.

Due to the solute content, an extremely high turgor pressure is exerted upon bacterial cells. The cell walls enable bacteria to withstand this pressure, and also provide their rigidity and characteristic cell shape. Bacteria are grouped according to their ability to retain Gram's stain (Chapter1), this is dependant on the organisation of the cell envelope components. *L. casei* is a Gram positive bacterium, *E. coli* is a Gram negative bacterium. In Gram positive organisms (able to retain Gram stain) the cytoplasmic membrane is surrounded by a thick peptidoglycan layer (approximately 90% of the cell envelope (Madigan *et al*, 2000)). Gram negative bacteria (which do not retain stain) possess a thinner peptidoglycan cell wall, sandwiched between the cytoplasmic membrane and the outer membrane (peptidoglycan accounts for approximately 10% of the Gram negative cell envelope (Madigan *et al*, 2000)).

Cell walls of all bacteria are similar in gross composition, consisting of a complex organisation of polysaccharides and amino acids – termed peptidoglycan (Figure 8.1.1). Essentially the cell wall is a thin sheet of glycan chains (glycosidically linked polysaccharides) (Poxton 1993) cross linked by peptide bonding of amino acids. The components of the glycan chains are remarkably conserved throughout bacteria, consisting of repeating sequences of glycosidically linked *N*-acetylglucosamine, and *N*-acetylmuramic acid. These components are not seen in higher organisms or archaea.

In Gram positive bacteria, cross-linking between glycan chains is usually via a peptide interbridge structure, varying in composition between bacteria. In Gram

negative bacteria cross-linking is through direct binding of a diaminopimelic acid component to the carboxyl group of D- alanine.

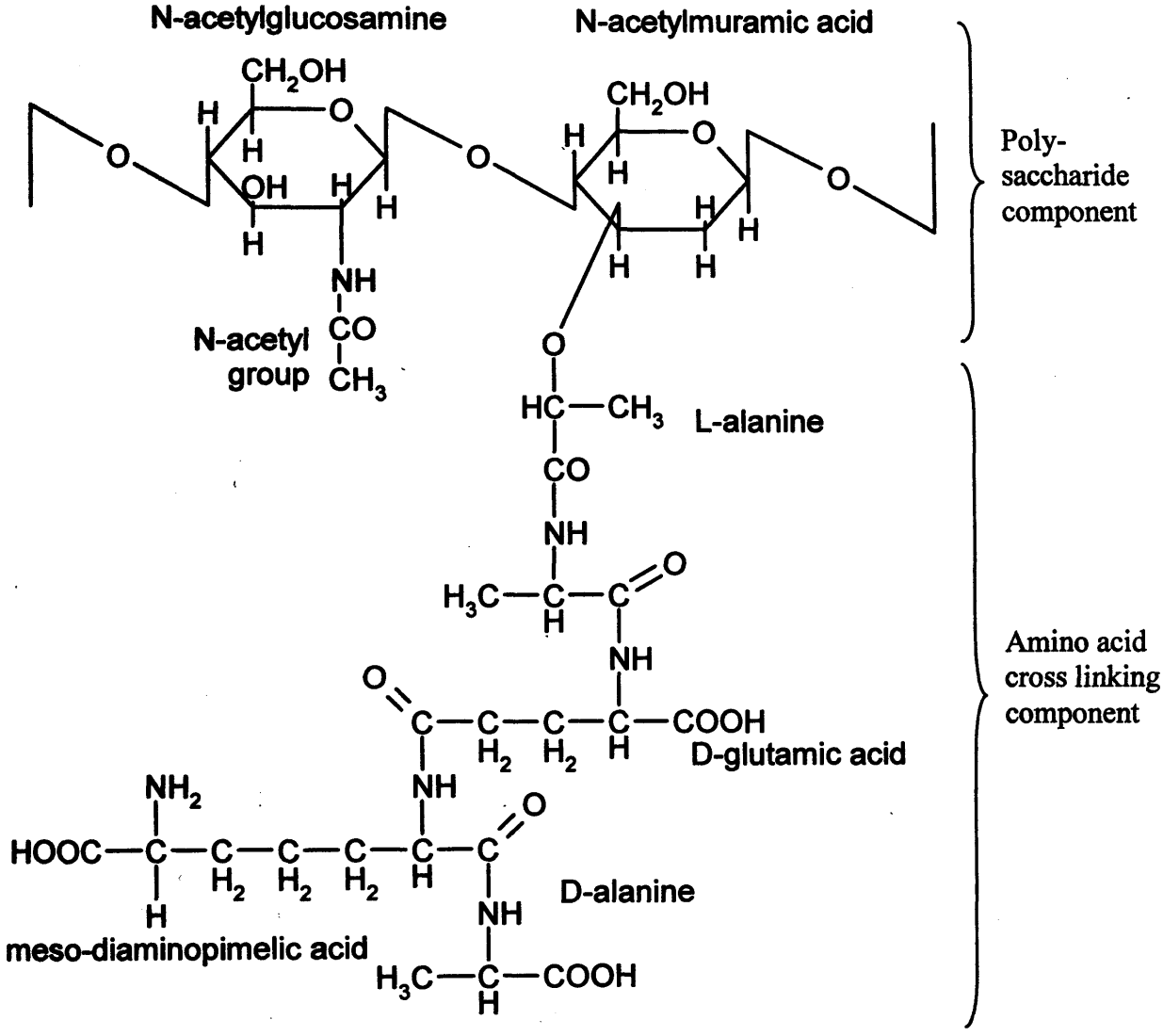


Figure 8.1.1 Peptidoglycan unit of *E. coli*. This structure is largely conserved throughout bacteria, although the amino acid composition varies.

Teichoic acids are also present in the cell wall of Gram positive bacteria, these are glycerol phosphate of ribitol phosphate residues, usually found in association with sugar molecules of alanine. Teichoic acids can be partly or wholly contained within the peptidoglycan layer, some are attached to lipids of the cytoplasmic membrane (called lipoteichoic acids) and may span the entire peptidoglycan cell wall. Teichoic acids add additional rigidity to the gram positive cell wall, and also contribute to the overall negative charge of the bacterial cell surface.

Gram negative bacteria possess an additional membrane exterior to the peptidoglycan cell wall. The outer membrane is composed of lipopolysaccharides (Chapter 1), and also contains various proteins. The outer membrane is readily permeable to hydrophilic low weight molecules due to the presence of porins – transmembrane proteins (usually composed of 3 identical subunits), which range in selectivity from non-specific water filled channels to highly selective transporters containing specific molecular binding sites. The outer membrane is not permeable to large protein molecules (e.g. enzymes), ensuring that essential enzymes and metabolic intermediates do not escape from the periplasm (the space between the cytoplasmic and outer membranes, containing many enzymes, transport and carrier proteins, and metabolic substrates) to the exterior.

Previous reports show that compounds from garlic inhibit processes responsible for production of cell envelope component. Fatty acid synthesis is inhibited through the specific action of allicin on acetyl coenzyme A sythetase (Focke, 1990), allicin and other garlic components lower serum lipid levels in rabbits (Eilat 1995), and rats (Ali 2000, Gedhardt 1996), in mice there was no significant lowering of serum lipid levels, however a reduction in fatty acid streaks responsible for artherosclerosis was seen (Abramovitz 1999). Some clinical trial into the effects of serum lipid production in humans are in conflict to the animal experiments with no significant reduction in lipid levels seen in many cases (Jain 1993, Byrne 1999, Gardner 2000). A large number of human clinical trials have reported significant reductions in serum lipids on administration of garlic (Loy 2000, Orekhov 1997, Zhang 2001), factors such as: mode of garlic delivery, patient sex, and patient diet may have contributory to the effect of garlic on lipid biosynthesis in humans and other animals.

Definitive studies into the effects of garlic components on lipids as components of bacterial membranes have not been carried out, though compounds from garlic inhibit *de novo* synthesis of membrane lipids in *Trypanosoma cruzi* (Urbina 1993) leading to a reduction in cell proliferation.

Phospholipid membranes are freely permeable to allicin (Miron, 1999). Diallyl sulphides however (direct reaction products of allicin in aqueous environments) are hydrophobic in nature and as such would be poorly soluble in the aqueous cytoplasmic interior of bacterial cells, it is possible that diallyl sulphides produced from allicin reactions within cell membranes would remain contained in the hydrophobic membrane. This could lead to compromised membrane function and structure.

8.1.2 Effects of garlic on glucose metabolism in *E. coli* and *L. casei*

A large proportion of the adenosine triphosphate (ATP) requirement for cell function is obtained through the Embden Meyerhoff pathway (or glycolytic pathway). Energy generated from the enzymatic breakdown of hexose sugar molecules (Chapter 1) is used to drive phosphorylation of ADP to ATP (the cells energy currency) which can then be used to drive non-spontaneous metabolic reactions.

E. coli is a non-fastidious heterofermentative facultative anaerobe. Under microaerobic conditions/ anaerobic conditions, *E. coli* will metabolise glucose (α – D isomer) to pyruvate (Figure 8.1.2) then either D-lactate and H^+ (*via* lactate dehydrogenase), acetate, formate H_2 and CO_2 (*via* pyruvate:formate lyase, and formate hydrogen lyase), or ethanol and CO_2 (*via* pyruvate decarboxylase, and alcohol dehydrogenase).

L. casei is a group II facultative heterofermentative lactic acid bacteria, it possess a fructose bisphosphate aldolase enzyme, and a phosphoketolase enzyme (induced on addition of pentose sugars). Under anaerobic conditions *L. casei* metabolises α-D-glucose to pyruvate, then converts pyruvate to lactate.

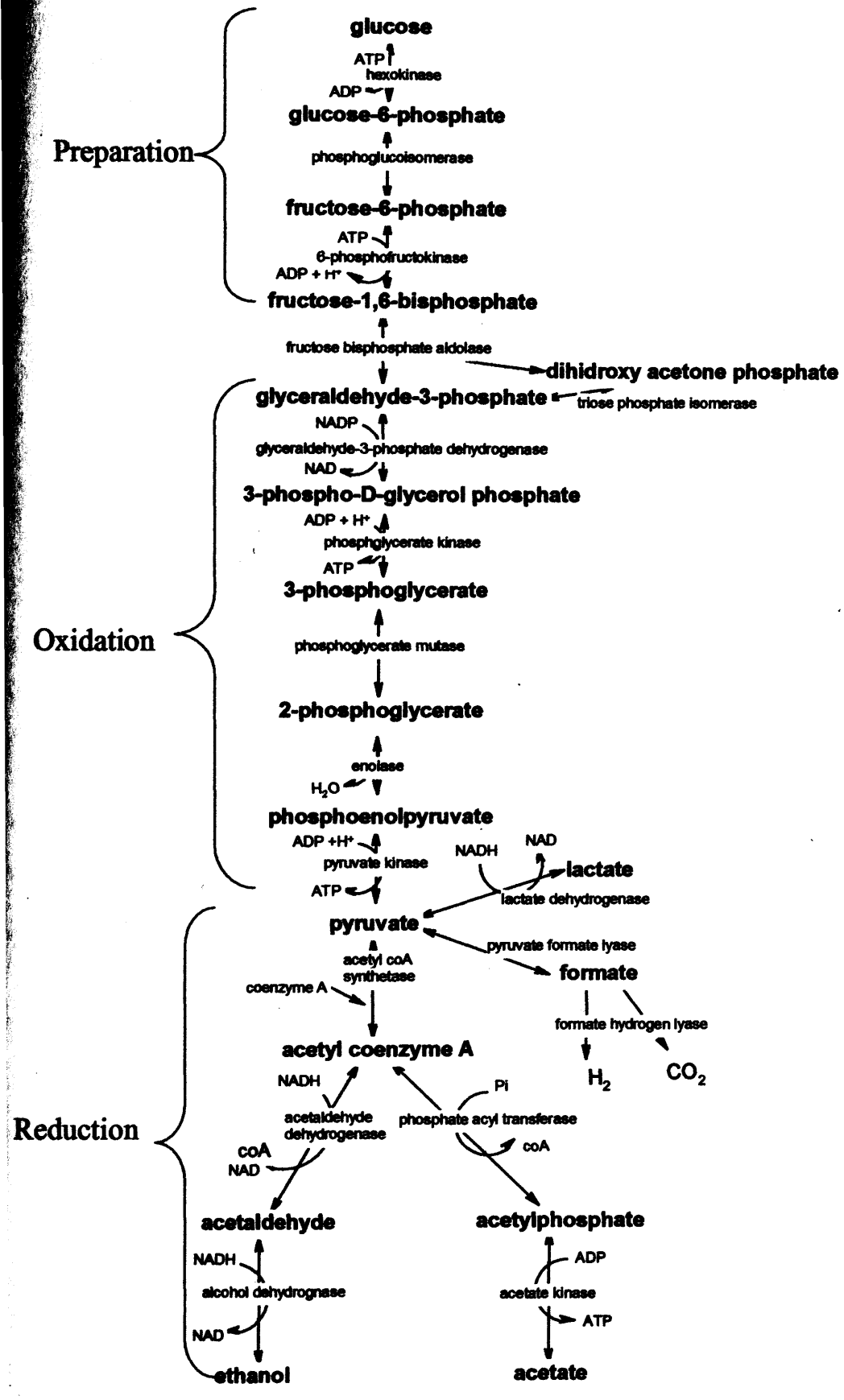


Figure 8.1.2 Glucose metabolism under anaerobic conditions (*E. coli* can metabolise to lactate, H_2 , CO_2, acetate, or ethanol. Lactate is the sole product of glucose metabolism in *L. casei*).

E. coli is a facultative anaerobe, and as such has a metabolic use for oxygen during respiration as a terminal electron acceptor. *L. casei* is an aero-tolerant anaerobe – although it will grow and proliferate in the presence of oxygen, it does not use it for respiratory metabolism (obtaining energy only from substrate level phosphorylation, not electron transport).

Previous experiments investigating effects of garlic extract on glucose metabolism and oxygen uptake in *Giardia intestinalis* (Harris 2001), have shown that aqueous garlic powder extract increases rates of glucose metabolism, and ethanol production. Garlic powder extract also exerted a stimulatory effect on oxygen consumption rates in *Trichomonas vaginalis,* and *Trichomonas foetus* (although not *Giardia intestinalis*).

Conclusive analyses of the effects of garlic powder extract on structure and metabolic function of bacteria (*E. coli* and *L. casei*) have not previously been described. The aim of work presented in this chapter was to begin investigations into the effects of garlic powder extract on cellular structure of *E. coli* and *L. casei*, highlighting possible differences in inhibitory effects. To assay for possible accumulation of hydrophobic diallyl sulphide in membranes were investigated using GC-MS. To qualitatively investigate the effects of garlic powder extract on microaerobic glucose metabolism in *E. coli* and *L. casei* using Nuclear Magnetic Resonance (NMR), and to investigate the effects of garlic powder extract on oxygen uptake in *E. coli* using a closed oxygen electrode system.

8.2 Materials and methods

8.2.1 Scanning electron microscopic analysis of the effect of garlic powder extract on *E. coli* and *L. casei* cell morphology and membrane surface appearance.

8.2.11Preparation and treatment of *E. coli* and *L. casei* with garlic powder extract.

100 µl from triplicate overnight cultures (Chapter 2) of *E. coli* and *L. casei* were inoculated (in triplicate) respectively into 10 ml of Nutrient broth (Oxoid, Basingstoke) and MRS broth (Merck, Herts) containing freshly prepared garlic powder extract (Chapter 2,) at a variety of concentrations (0 – 2.5 mg ml^{-1} in Nutrient broth, and 0 – 25 mg ml^{-1} in MRS broth). Control cultures were prepared without garlic powder extract, un-inoculated nutrient broth containing 2.0 mg ml^{-1} garlic powder extract and MRS broth containing 25 mg ml^{-1} garlic powder extract served as cell-free controls. Cultures were incubated (aerobic) at 37°C for 24 hours (without shaking).

8.2.13 Preparation of samples for scanning electron microscopy.

Before sample preparation, a containment vessel was made for cells from cultures to be analysed. This vessel consisted of a lidded hard polypropylene capsule (cylindrical in shape, with a diameter of 15 mm); the closed end of the capsule was removed carefully with a razor blade. The lid (other end) of the capsule was removed, and a circle cut into it using a razor blade. A 17 mm diameter 0.2 µm polyester filter (Nucleopore – Fisher scientific) was placed carefully between the capsule and modified lid, the lid was then fitted onto the capsule trapping the filter securely between lid and capsule.

10 ml of each culture was gently centrifuged to pellet (2000 rpm 10 min), supernatant growth media was removed and cells were washed once in PBS buffer. Pellets were then suspended in sodium cacodylate buffer containing 3% Glutaraldehyde, as a fixative for 1 hour. Cell pellets were then stained by suspending cells in 1% osmium tetroxide in cacodylate buffer (1 hour room temperature). Cells were again centrifuged to pellet, and washed twice in cacodylate buffer. 1 ml aliquots of fixed,

stained cultures were added to the vessel through the open end. Samples were then centrifuged through the vessel (2000 rpm 10 min) trapping fixed cells on the surface of the filter paper.

Samples were then dehydrated (on filter paper, in vessel) by soaking sequentially in ethanol for 10 min, at concentrations of 30%, 50%, 70%, 80%, 95%, and 100% (3 times 15 minutes each), each time ethanol was removed from the vessel by centrifugation (2000rpm, 5 min).
On drying in a critical point drier (Samdi, Maryland USA), filters were carefully removed from the vessel and mounted onto specimen carrier stubs (Agar Scientific, Stanstead). Samples were then sputter coated (EMScope, California, USA) in gold.

8.2.14 Visual analysis

Samples analysis was carried out on a XL20 scanning electron microscope (Phillips, Eindhoven Netherlands) operating at 20-30 KV accelerating voltage, equipped with Phillips control software. Images were stored digitally and presented using Photo-editor (Microsoft). Triplicate samples of each test concentration were visualised, images presented here show effects representative of 3 samples.

8.2.2 GC-MS analysis of cell membrane and cell extract fractions of *E. coli* and cultures treated with garlic powder extract.

10 ml Nutrient broth containing 1.5 mg ml^{-1} garlic powder extract (Chapter 3) was inoculated with 100 µl of *E. coli* culture (16 h) in duplicate. Cultures were incubated for 24 h at 37°C. On 24 h cultures were centrifuged to pellet (3200 rpm x 15 min), then washed twice with PBS buffer. Cells were broken using a Hughes press (Lloyd *et al* 1979) pre-cooled in dry ice to -20°C. Frozen cell extract, and cell wall fractions were collected and suspended in 1 ml analytical grade dichloromethane (Sigma, Poole). GC-MS analysis was carried out on 1.2 µl of each sample using the method previously described (Chapter 6).

8.2.3 Glucose metabolism in *E. coli* and *L, casei* cultures in the presence of garlic powder extract

Exponential phase cells from *E. coli* (in nutrient broth- Oxoid, Basingstoke UK), and *L. casei* (in MRS broth- Sigma, Poole UK), were harvested by centrifugation (3200 rpm, 15 min). As a culture control *E. coli* cells were suspended in 2 ml fresh nutrient broth to a final cell density of approx 1 x 10^8 cfu ml^{-1}. Glucose 1-^{13}C (Sigma, Poole UK) was added to the cell suspension and vortexed thoroughly for 30 sec. After filter sterilisation 0.75 ml of cell/ glucose suspension was added to a 5 mm Emperor grade NMR tube (Wilman, (Sigma, Poole UK)). Tube headspace was sparged with nitrogen for 30 s (ensuring microaerobic conditions), the tube was then placed in a temperature controlled (37°C) Bruker MSL NMR spectrometer (DPX400). One dimensional NMR analysis was then carried out. 64 scans were collated over 10 minute intervals, from 0 to 250 ppm, for a period of 60 minutes. A deuterium insert was used to provide a solvent lock. 1-C^{13} glucose was identified by comparison to a pure standard (Sigma analytical grade, all other products were identified from ppm values given in literature (Markely and Opella 1997)).

8.2.4 Measurement of oxygen uptake in *E. coli*

E. coli cells from actively growing cultures (100 µl overnight *E. coli* cultures inoculated into 10 ml Nutrient broth No.2 (Oxoid, Basingstoke UK), 6 hour incubation at 37°C) were harvested and washed twice in PBS buffer. Cells were resuspended in fresh PBS buffer to give a final density of approx 1 x 10^8 cfu ml^{-1}. 2 ml cell suspension was introduced into the reaction chamber of a Clarke-type oxygen electrode (Teflon 0_2 permeable membrane, silver anode/ platinum cathode) and magnetically stirred (200 rpm).

Oxygen content of air saturated buffer at 37°C was taken to be 258 µm (Wilhelm *et al* 1977). Glucose (final concentration 10 mM) was introduced through the capillary plug, as were biocide agents (as required). All results presented are mean values of 3 independent repeats.

Results

8.3.1 Scanning electron microscopy of *E. coli* and *L. casei* grown in the presence of aqueous garlic powder extract.

Control cultures of *E. coli* appeared as cylindrical cells, varying in length from approximately 2 μm to 6 μm, and consistently of diameter 1 μm. Characteristics of cell elongation and division could be seen in control samples (figure 8.3.1, 8.3.2) pili were not observed. Cell surfaces usually appeared as smooth or slightly wrinkled.

On addition of garlic extract at 0.5 mg ml^{-1} the cells at 24 hours took on a ridged appearance, not seen in control samples large protrusions were also visible on some cells. Surrounding the cells was a film of material resembling mucus, (figure8.3.3, 8.3.3). Some cells appeared larger, and it was harder to distinguish individual cells due to the surround material. Material leaking from/ attached to cell membranes was also observe, this phenomenon was described as 'cell blebbing' (Harris 2001). Cell blebbing is though to be caused by intracellular material leaking through the cell envelope, and could indicate membrane damage.

Cells from *E. coli* cultures exposed to garlic extract at 1.0 mg ml^{-1} (24 h, 37°C), were smaller than those previously described (few cells were longer than 4 μm) and took on an irregular/ less cylindrical appearance (figures 8.3.4, 8.3.5). The mucus material surrounding the cells seen on treatment with 0.5 mg ml^{-1} was still evident, although less visible. Cell blebbing was more extensive, completely surrounding a number of cells. Fewer cells were present (compared to control and 0.5 mg ml^{-1} treatments), and cells tended to be clustered in groups. Deposits of fibrous debris were observed; these were only found in and around the clusters of cells.

Cells treated with garlic powder extract at 1.5 mg ml^{-1} appeared swollen (figure 8.3.6) with many irregular protrusions. The morphology of many cells appeared abnormal compared to the control sample. More cells were 2 μm or less in length when compared to the previous treated, and control samples. Cell blebbing was larger, and cellular debris was more extensive. Similar to the 0.5 mg ml^{-1} and 1.0 mg ml^{-1} treatments, cells were located in clusters with cell debris.

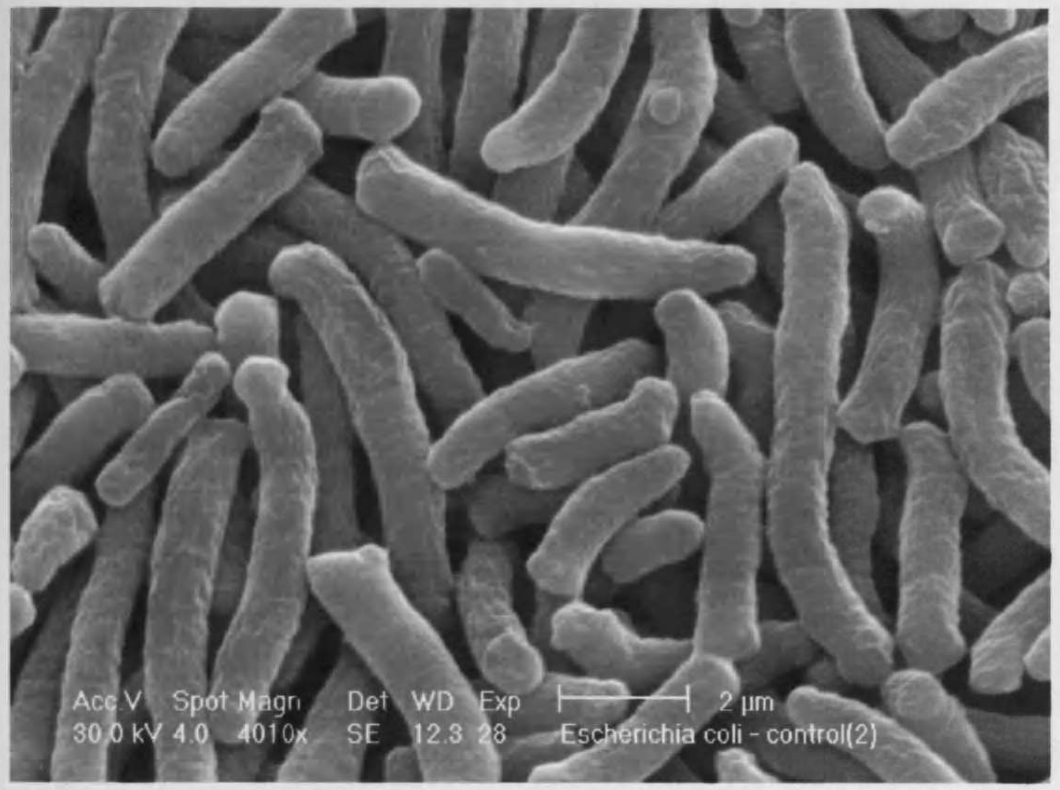


Figure 8.3.1 Scanning electron microscopy of *E. coli* cells after 24 h incubation at 37°C.

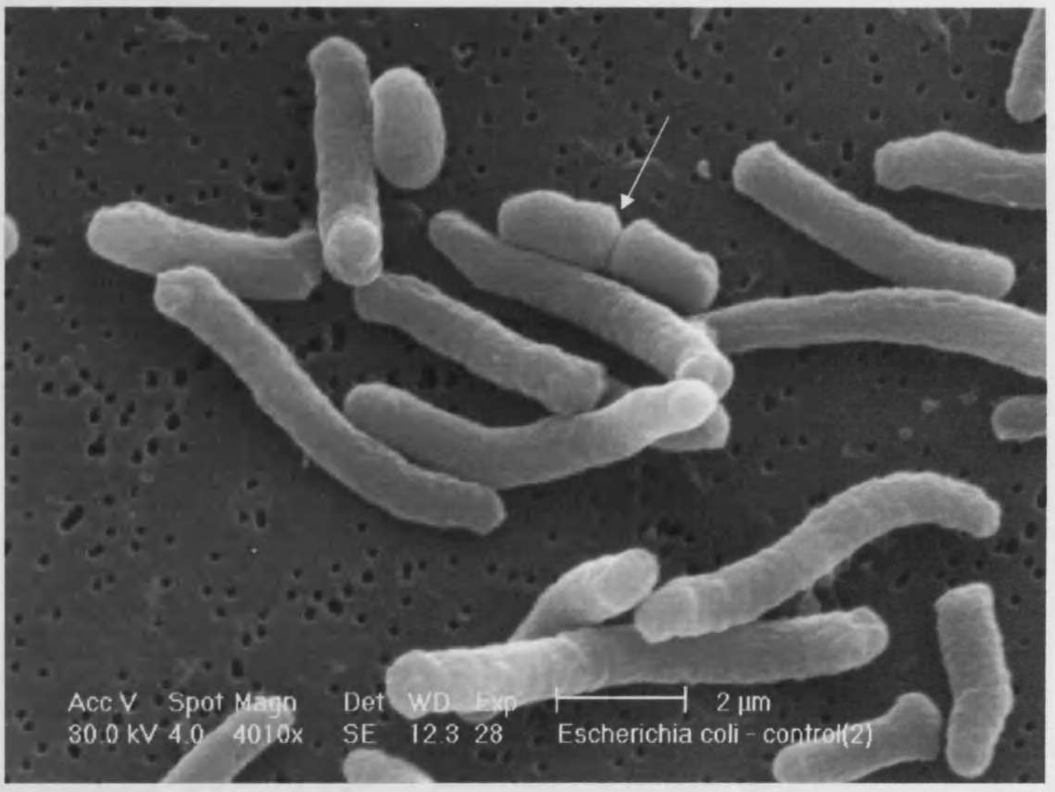


Figure 8.3.2 Scanning electron microscopy of *E. coli* cells after 24 h incubation at 37°C. Arrow points to cell division occurring.

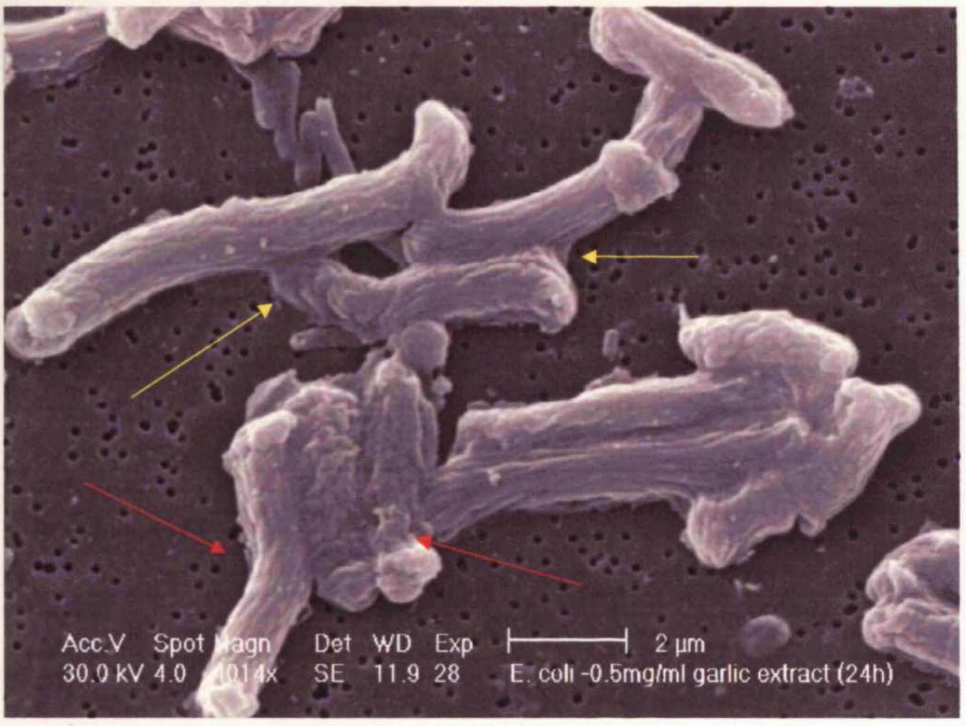


Figure 8.3.3 Scanning electron microscopy of *E. coli* cells after 24 h incubation at 37°C with 0.5 mg ml^{-1} garlic powder extract. Cells showing abnormal morphology (red) and apparent mucus (yellow).

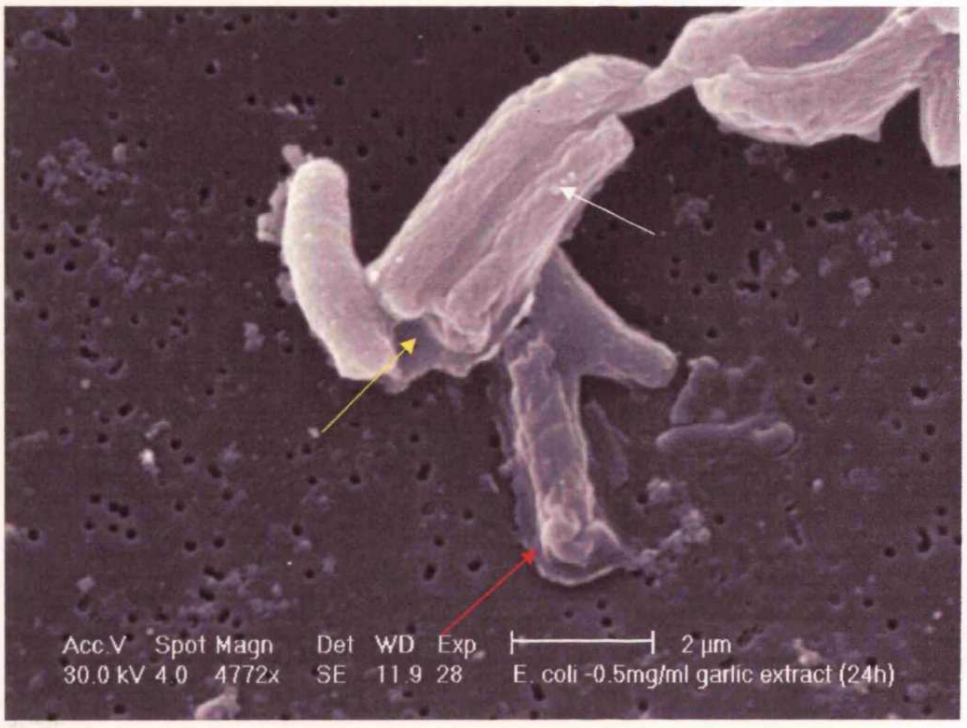


Figure 8.3.4 Scanning electron microscopy of *E. coli* cells after 24 h incubation at 37°C with 0.5 mg ml^{-1} garlic powder extract. Cells showing abnormal morphology (red) apparent mucus (yellow), and signs of 'cell blebbing' (white).

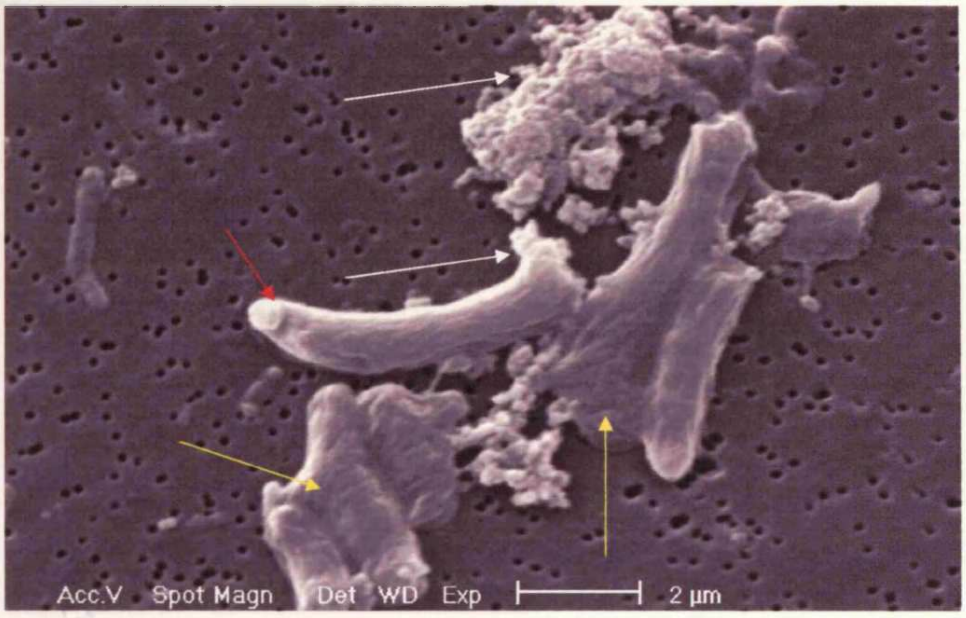


Figure 8.3.5 Scanning electron microscopy of *E. coli* cells after 24 h incubation at 37°C with 1.0 mg ml^{-1} garlic powder extract. Cells showing abnormal morphology (red) apparent mucus (yellow), and signs of 'cell blebbing' (white).

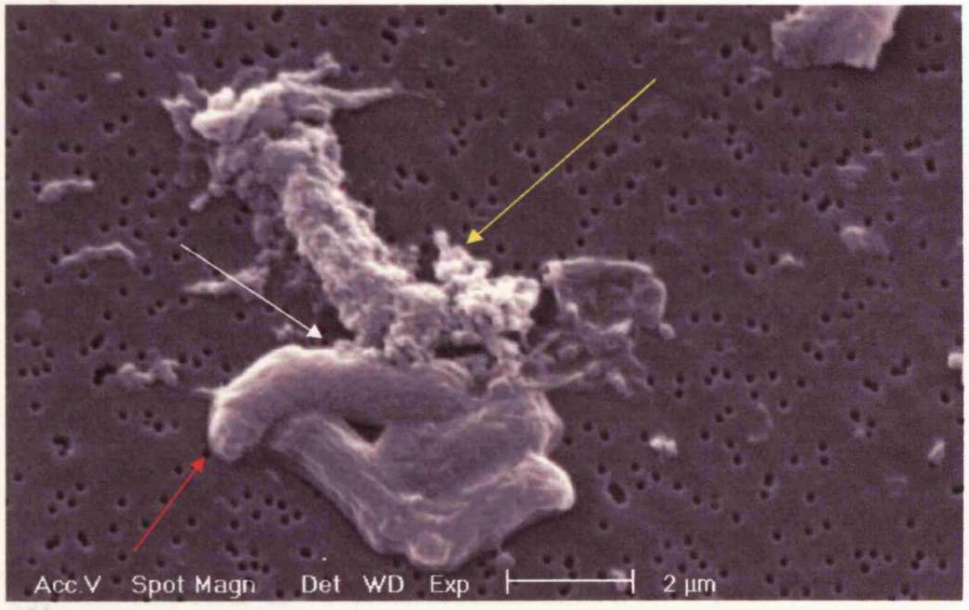


Figure 8.3.6 Scanning electron microscopy of *E. coli* cells after 24 h incubation at 37°C with 1.0 mg ml^{-1} garlic powder extract. Cells showing abnormal morphology and apparent mucus. Arrows highlight 'cell blebbing' (white), cell debris (yellow), and abnormal morphology.

Severe effects were observed in cultures treated with 2.0 mg ml^{-1} garlic powder extract. Cell numbers were greatly reduced. Virtually all cells seen on analysis exhibited abnormal morphology compared to the control sample (figure 8.3.8), many cells appeared swollen with irregular protrusions. There was a general loss of the cylindrical cell shape, many cells appeared curved, the diameter of many cells varied along there length, some cells appeared bifid (figure 8.3.9). Large masses of debris were seen surrounding cell clusters, many cells were covered in debris. Cell blebbing was difficult to distinguish because of the proximity of debris to the cells. As with the 1.5 mg ml^{-1} garlic powder extract treated cultures, there were large numbers of cells that were 2μm or less in length.

In cultures treated with garlic powder extract in excess of 2.0 mg ml^{-1} only few cells were found, these cells were shorter than those seen in the control samples. Cell blebbing appeared to be less extensive than in samples treated with 0.5 mg ml^{-1} to 2.0 mg ml^{-1}. Deposits of debris were seen (though less extensive than in previous samples tested) it may be possible that these masked any intact cells (8.3.10).

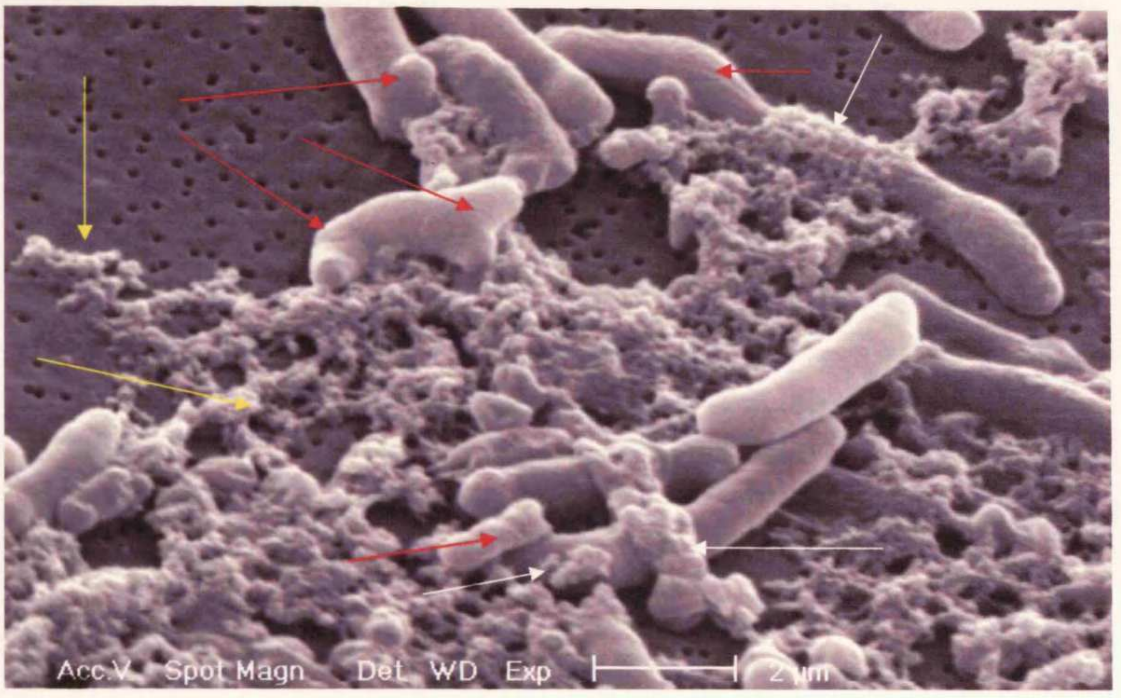


Figure 8.3.9 Scanning electron microscopy of *E. coli* cells after 24 h incubation at 37°C with 2.0 mg ml^{-1} garlic powder extract. Arrows highlight extensive cell blebbing (white), extensive cell debris (yellow), and abnormal morphology (red).

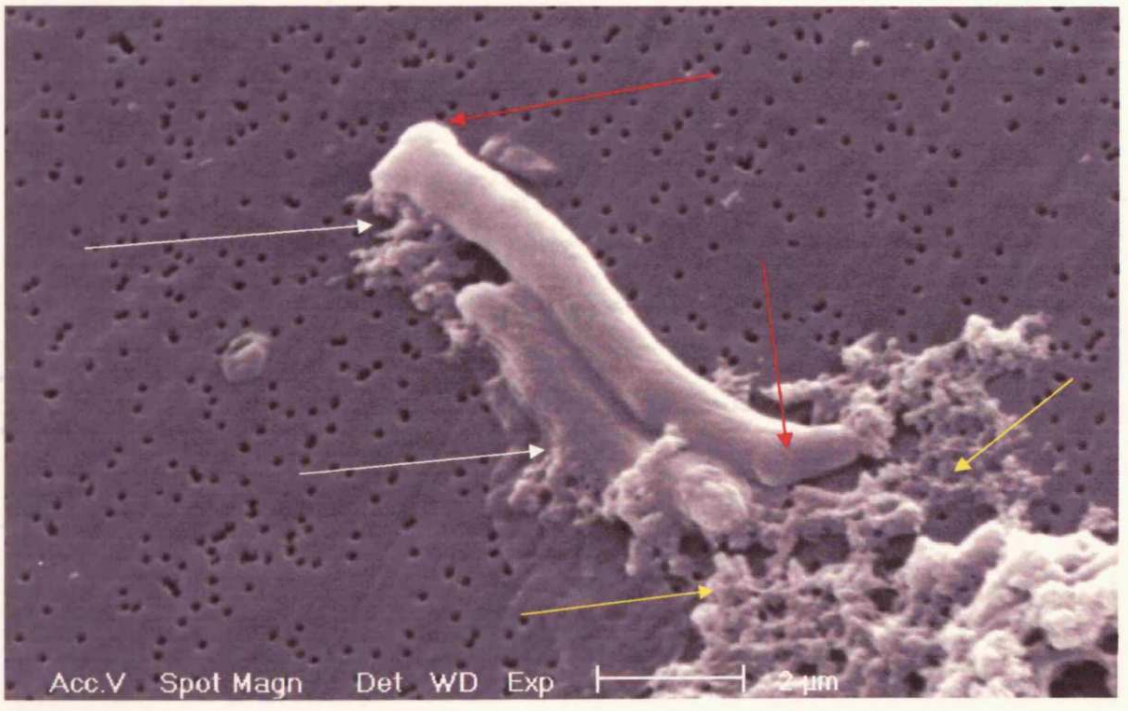


Figure 8.3.10 Scanning electron microscopy of *E. coli* cells after 24 h incubation at 37°C with 2.0 mg ml^{-1} garlic powder extract. Arrows highlight extensive cell blebbing (white), extensive cell debris (yellow), and abnormal morphology (red).

Cells of *L. casei* cultures grown at 37°C for 24 hours, were regular cylinders of 1 μm to 4 μm in length and consistently of approximately 0.75 μm diameter. Cells were seen individually or in chains of 2 to 5. Cell division could be seen occurring in cells of 2 μm in length or more (figure 8.3.11).

Cultures treated with 1.0 mg ml^{-1} garlic powder extract (24 h) did not exhibit any obvious differences from the control cultures. Small amount of cell debris were seen in isolated masses, although these masses were more frequent than observed on analysis of the control cultures, they were substantially less frequent than the masses of cell debris seen on analysis of the *E. coli* cultures treated with 1.0 mg ml^{-1} garlic powder extract.

In cultures treated with 5.0 mg ml^{-1} garlic powder extract (24 h), cells retained the regular cylindrical appearance, and cell dimensions were not substantially different from those observed in the control culture analysis. Cell blebbing was apparent here (figure 8.3.13), and cell debris was observed with increased frequency (when compared to the previous samples). There also seemed to be a higher proportion of cells grouped in chains of 3 or more.

Analysis of cultures treated with garlic powder extract at concentrations from 5.0 mg ml^{-1} to 10.0 mg ml^{-1} gave similar results. Cell dimensions did not obviously change, cells still appeared as regular cylinders. A noticeable increase in deposits of debris occurred, and cell blebbing became more frequent as garlic powder extract concentration increased (although it did not appear to increase in severity). Cells exposed to higher concentrations ($\geq$ 10.0 mg ml^{-1}) of garlic powder extract had an increased tendency to form chains (figure 8.3.14)

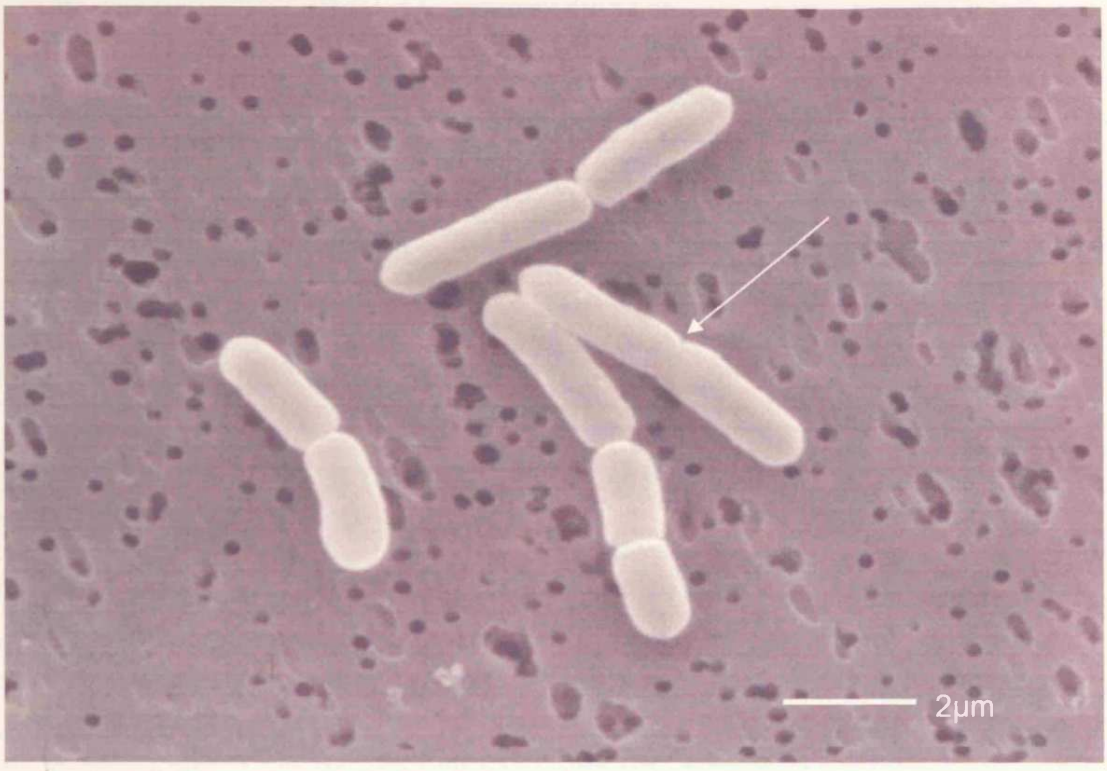


Figure 8.3.11 Scanning electron microscopy of *L, casei* cells after 24 h incubation at 37°C White arrow highlight cell division.

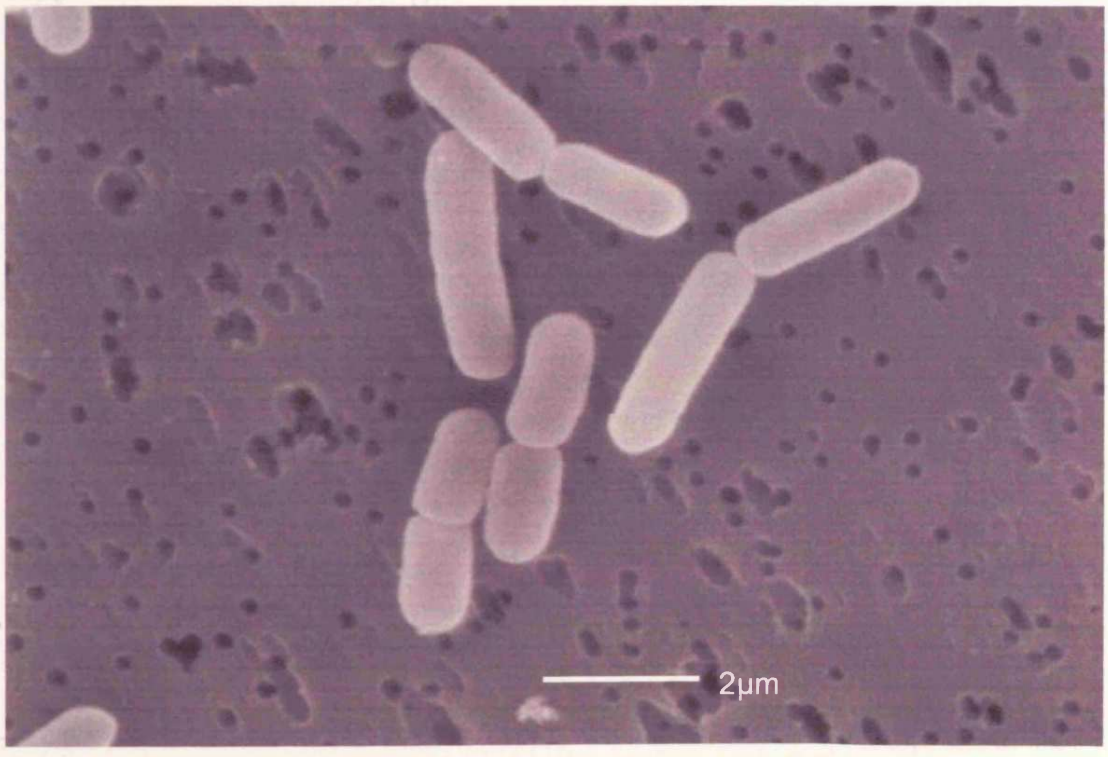


Figure 8.3.12 Scanning electron microscopy of *L, casei* cells after 24 h incubation at 37°C with 1.5 mg ml^{-1} garlic extract.

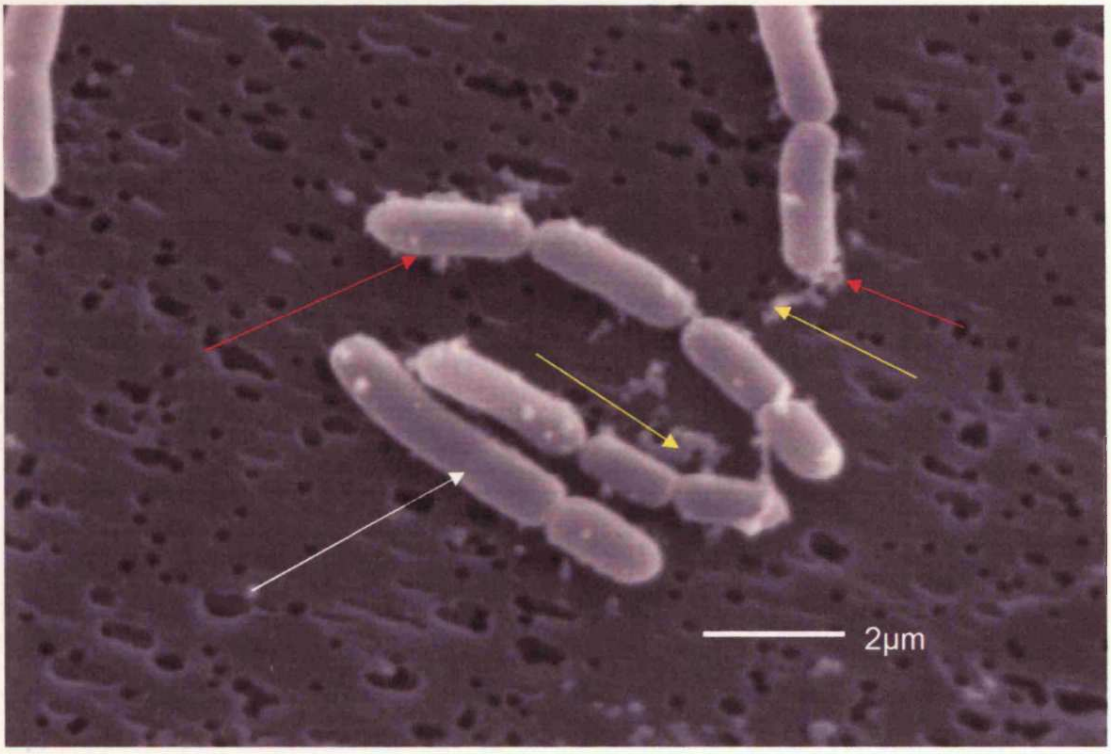


Figure 8.3.13 Scanning electron microscopy of *L, casei* cells after 24 h incubation at 37°C with 5.0 mg ml^{-1} garlic extract, highlighted: cell elongation (white), cell blebbing (red), cellular debris (yellow).

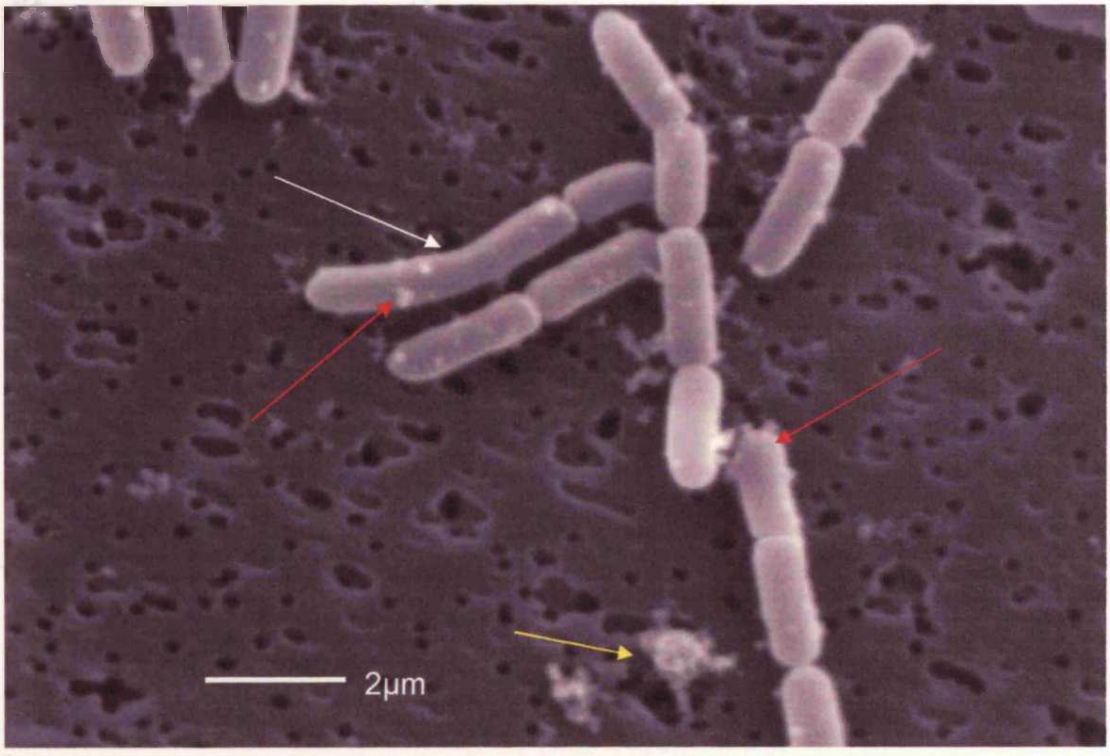


Figure 8.3.14 Scanning electron microscopy of *L, casei* cells after 24 h incubation at 37°C with 10.0 mg ml^{-1} garlic extract, highlighted: cell elongation (white), cell blebbing (red), cellular debris (yellow).

Appearance of cells grown in the presence of 15.0 mg ml^{-1} garlic powder extract, was substantially different to previously analysed cultures. Extensive cell blebbing was observed (figure 8.3.15), and large amounts of debris were seen surrounding cells. Many cells exhibited abnormal morphology compared to the control (figure 8.3.16), there was increase in the proportion of cells that were 4 μm or more in length. Many cells lost the characteristic cylindrical shape of lactobacilli; appearing curved in shape (8.3.16) some cells appeared shorter in length and more rounded than control samples.

Few cells were seen in cultures exposed to garlic powder extract at concentrations of 20.0 mg ml^{-1}. Cells were greatly reduced in size (few cells were greater than 2 μm in length). Many of the cells were rounded in appearance, some cells were curved, and some had irregular protrusions. The amount of debris seen surrounding cells was greater than the amounts seen in cultures treated with 15.0 mg ml^{-1}, however was not as great as the amounts seen when treating *E. coli* cultures with 2.0 mg ml^{-1} garlic powder extract.

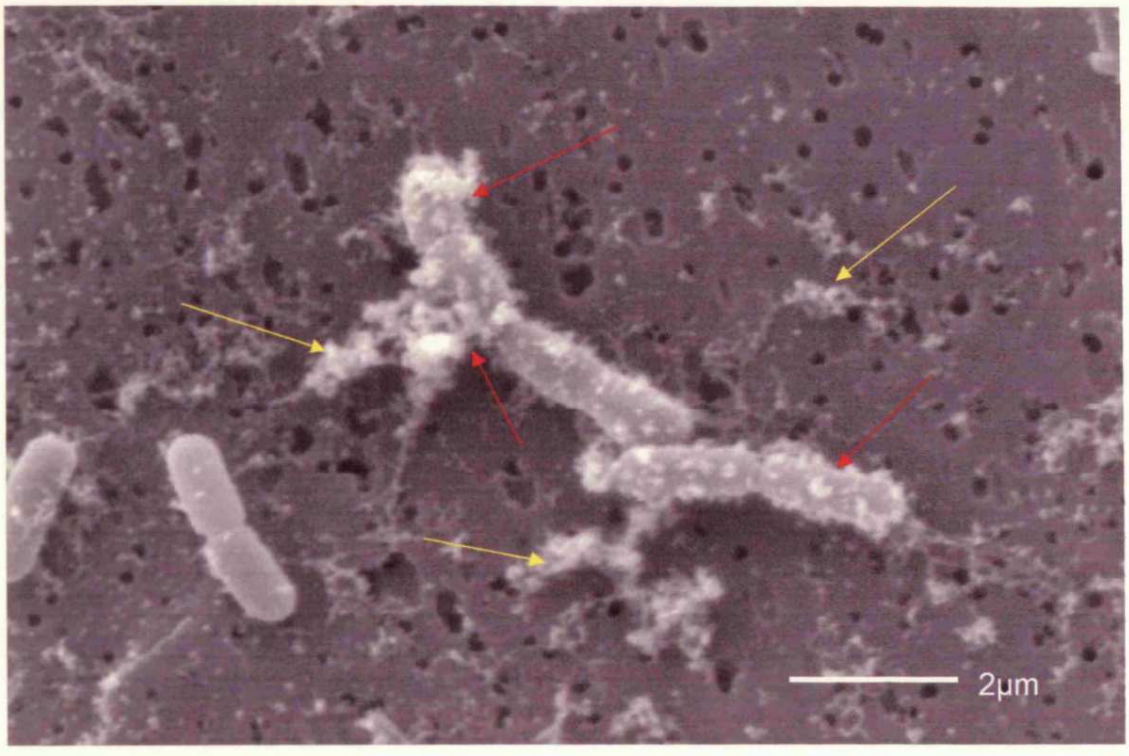


Figure 8.3.15 Scanning electron microscopy of *L, casei* cells after 24 h incubation at 37°C with 15.0 mg ml^{-1} garlic extract, highlighted: extensive cell blebbing (red), cellular debris (yellow).

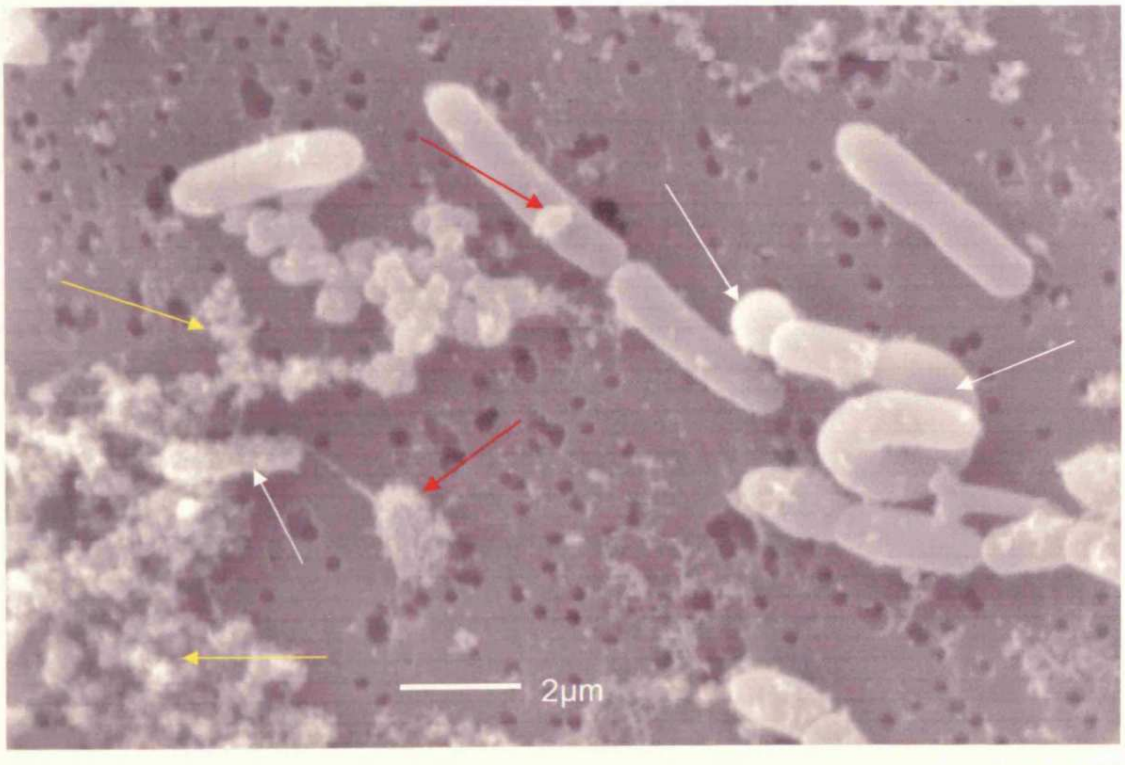


Figure 8.3.16 Scanning electron microscopy of *L, casei* cells after 24 h incubation at 37°C with 15.0 mg ml^{-1} garlic extract, extensive cell blebbing (red), cellular debris (yellow), and cells exhibiting abnormal morphology (white).

8.4.1 GCMS analysis of *E. coli* cell envelope and cell extract for garlic components.

Qualitative GCMS analysis of cell envelope fragments from control *E. coli* cells and garlic treated *E. coli* cells yielded similar results. None of the garlic components previously detected (Chapter 5) were seen in these chromatograms.

The major difference in chromatograms from control and treated cell envelope fragments were 3 peaks clustered between 3 and 4 minutes retention time. Analysis of mass spectra and comparison to standard spectra from the software database suggested these peaks were (probable identifications) due to 3-methoxy 3-pentanol, and 2 branched ketones (2-methyl 3-pentanone, and 4-methyl 3-heptanone).

Analysis of cell extract from control *E. coli* cells and garlic treated *E. coli* cells did not yield any of the garlic components detected in previous analyses (Chapter 5), there were however several differences between treated and control cells.

Chromatograms obtained from cell extracts of *E. coli* cultures treated with 1.5 mg ml^{-1} garlic powder extract, yielded three large peaks identified (probable identifications) through comparison of mass spectra to internal software database standards as short chain oxygenated hydrocarbons:

At 26.5 min a peak was seen due to bis (1,1-dimethylethyl)-phenol. At 28.1 min a peak appeared due to butanedioic acid methyl-dibutyl ester. Butanedioic acid dibutyl ester was the probable identification of the compound responsible for a peak seen at 30.5 min.

8.5.1 Glucose metabolism in *E. coli* and *L. casei* in the presence of garlic powder extract.

On incubation of 1 - ^{13}C glucose with *E. coli* (Figure 8.5.10) ^{13}C-labelled lactic acid was detected after 10 minutes, signal intensity did not alter substantially during the investigation. In the presence of 1.5 mg ml^{-1} garlic powder extract (Figure 8.5.11) ^{13}C-labelled lactic acid was detected immediately, after a slight reduction in signal intensity from 10 min to 20 min, intensity slowly rose throughout the investigation. ^{13}C-labelled ethanol was detected on incubation of ^{13}C glucose with *E. coli* in the presence of 1.5 mg ml^{-1} garlic powder extract (not detected in the garlic-free investigation). Ethanol signal intensity rose slowly throughout the investigation.

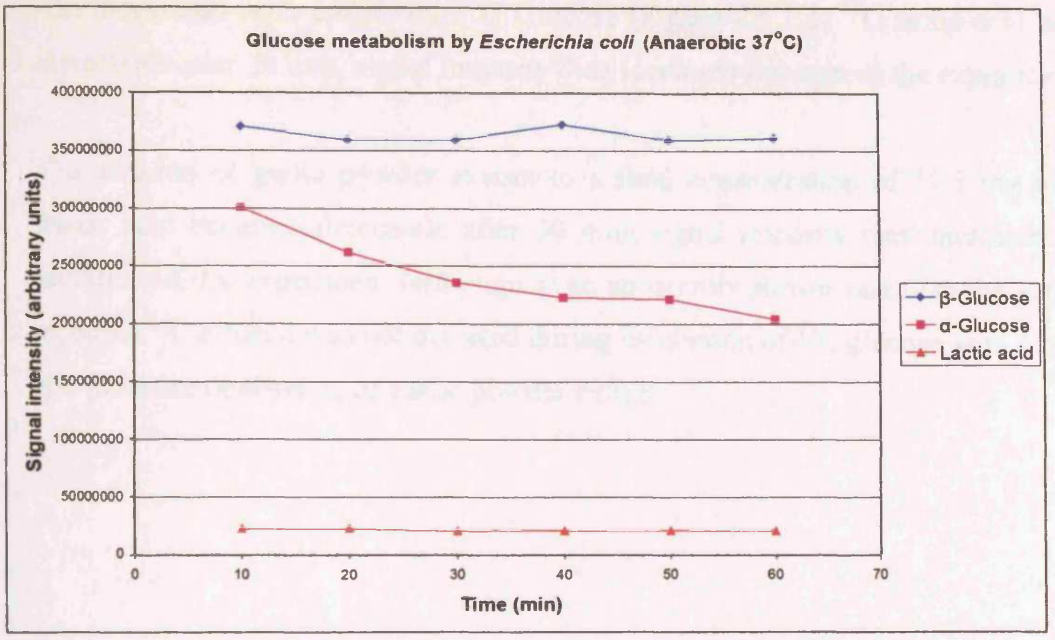


Figure 8.5.1.0 Metabolism of ^{13}C glucose by *E. coli* (anaerobic conditions at 37°C in nutrient broth). Trends shown here are typical of 2 independent repeats.

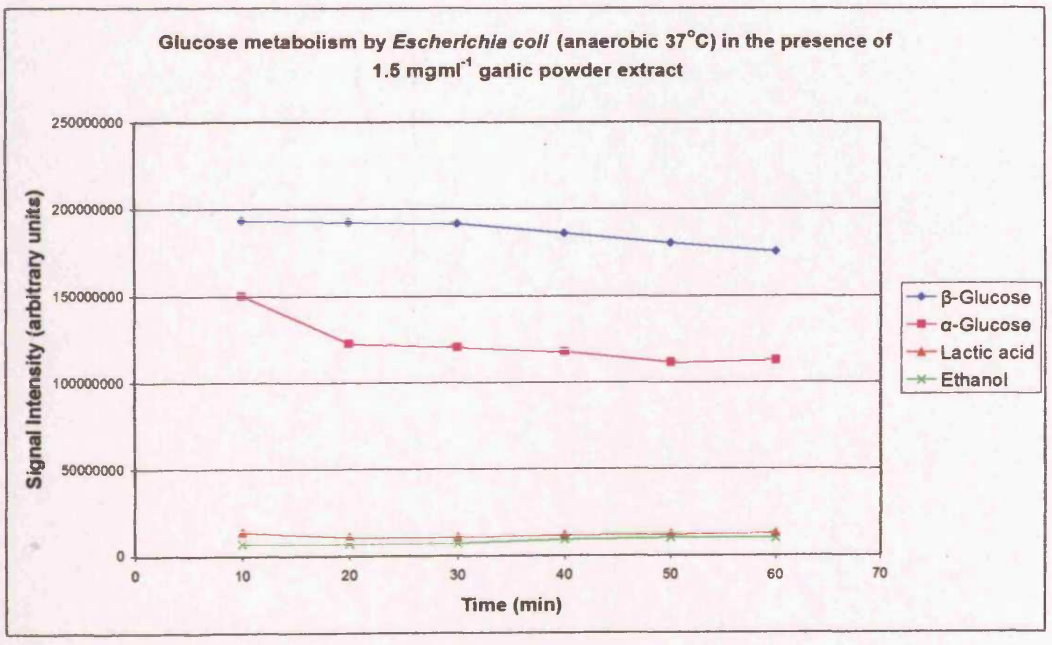


Figure 8.5.1.1 Metabolism of ^{13}C glucose by *E. coli* in the presence of 1.5 mg ml^{-1} garlic extract (anaerobic conditions at 37°C in nutrient broth). Trends shown here are typical of 2 independent repeats.

On incubation of *L. casei* with ^{13}C Glucose (Figure 8.5.1.2) ^{13}C lactic acid becomes detectable after 30 min, signal intensity then increases throughout the experiment.

On addition of garlic powder extract to a final concentration of 17.5 mg ml^{-1}, ^{13}C lactic acid becomes detectable after 30 min, signal intensity then increases for the duration of the experiment (although at an apparently slower rate than the garlic-free control).^{13}C ethanol was not detected during incubation of ^{13}C glucose with *L. casei* in the presence or absence of garlic powder extract.

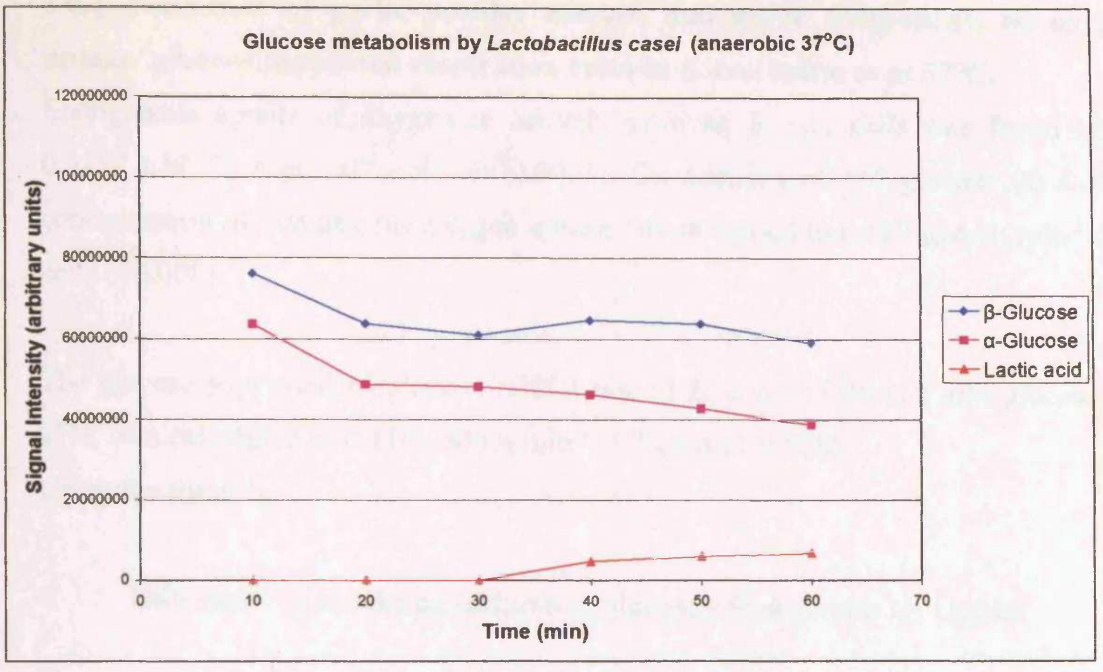


Figure 8.5.1.2 Metabolism of ^{13}C glucose by *L. casei* (anaerobic conditions at 37°C in nutrient broth).

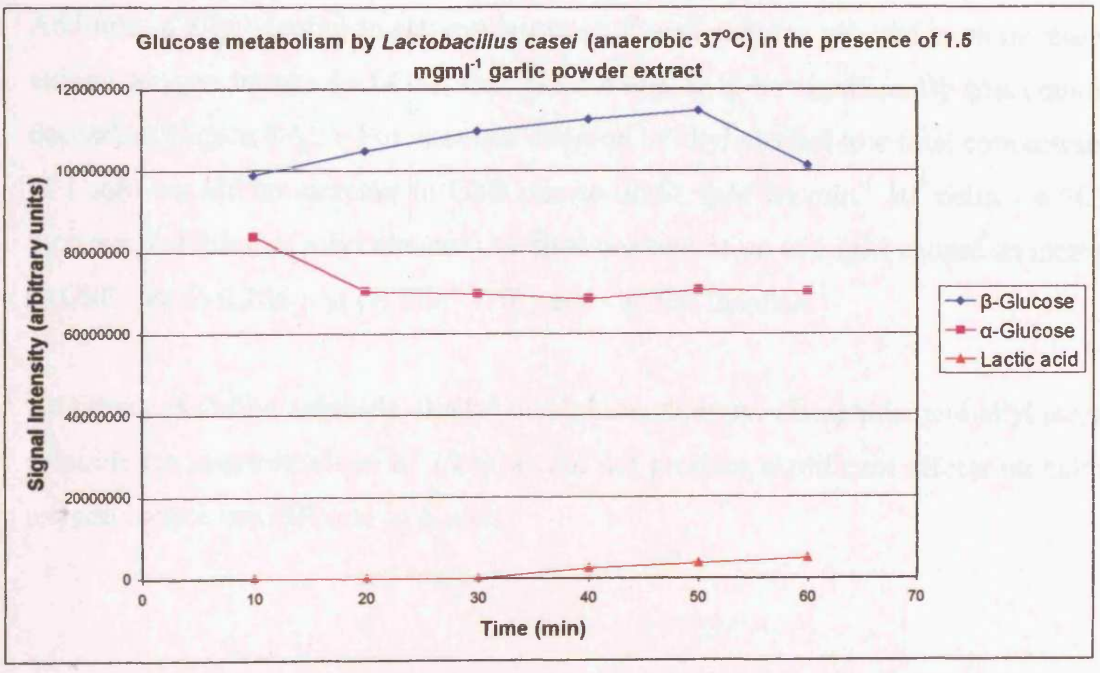


Figure 8.5.1.3 Metabolism of ^{13}C glucose by *L. casei* in the presence of 17.5 mg ml^{-1} garlic extract (anaerobic conditions at 37°C in nutrient broth).

8.6.1 The effect of garlic powder extract, and garlic components on oxygen uptake/ glucose supported respiration rates in *E. coli* cultures at 37°C.

Endogenous uptake of oxygen in actively growing *E. coli* cells was found to be 0.0152 μM O_2 min^{-1} 10^6 cells (± 0.0003). On addition of 1M glucose (to a final concentration of 10mM), the oxygen uptake rate increased to 0.129 μM O_2 min^{-1} /10^6 cells (±0.008).

The glucose supported respiration (GSR) rate of *E. coli* in PBS (10 mM glucose) at 37°C was calculated as 0.114 μM O_2 min^{-1} /10^6 cells (± 0.008).
Using the formula:

GSR rate= O_2 uptake on addition of glucose - Endogenous O_2 Uptake

Addition of garlic powder extract to the reaction culture produced a significant ($p<0.05$) dose dependent but nonlinear increase in the GSR rate (figure 8.6.1); addition of 3.0 mg ml^{-1} garlic powder extract producing a 30% increase in GSR rate.

Addition of allyl alcohol to actively growing *E. coli* cultures resulted in an increase in culture oxygen uptake and GSR that did not appear to be significantly concentration dependant (figure 8.6.2). For example addition of allyl alcohol to a final concentration of 1 mM caused an increase in GSR rate to 0.262 μM O_2 min^{-1} 10^6 cells - a 103% increase. Addition of allyl alcohol to a final concentration of 5 mM caused an increase in GSR rate to 0.256 μM O_2 min^{-1} /10^6 cells - a 99% increase.

Additions of diallyl sulphide, diallyl disulphide, dipropyl disulphide, and allyl methyl sulphide (to concentrations of 10 mM) did not produce significant effects on culture oxygen uptake or GSR rate in *E. coli.*

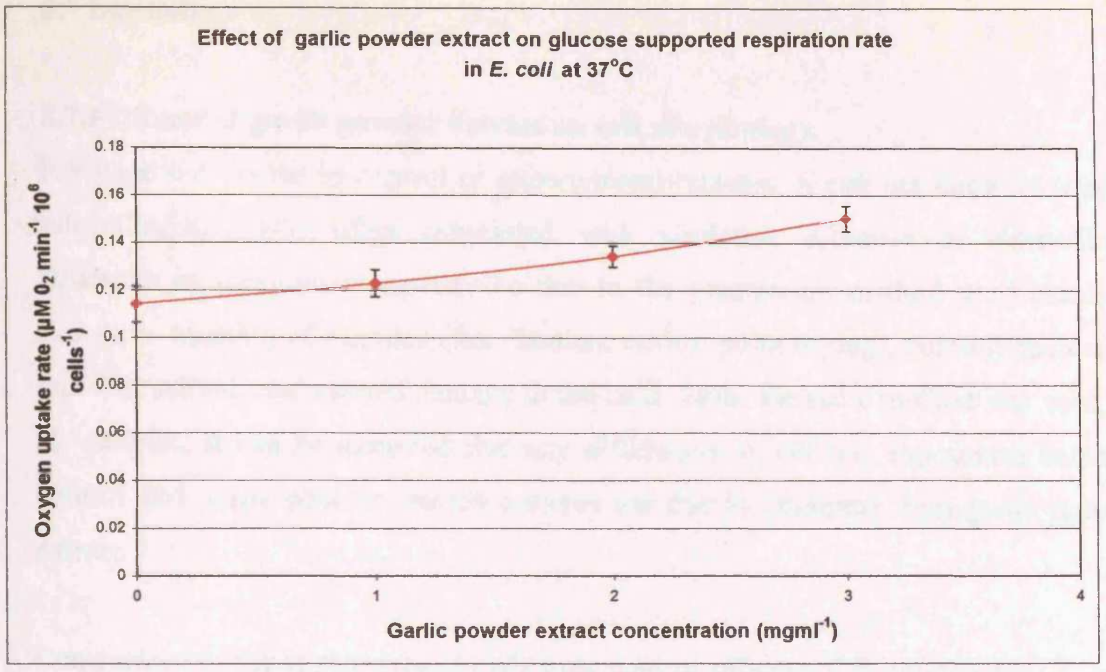


Figure 8.6.1 The effect of garlic powder extract on glucose supported respiration rate in *E. coli* cultures at 37°C, monitored in a Clarke (closed) oxygen electrode. Results presented are the mean values, and standard deviations of 3 independent repeats.

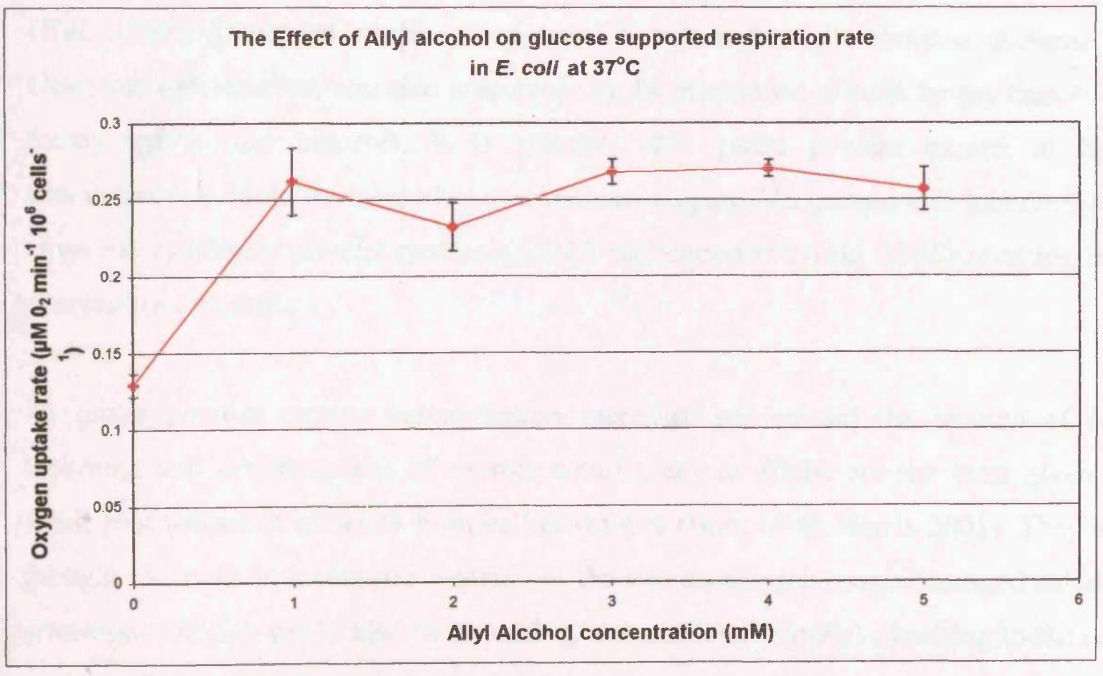


Figure 8.6.2 The effect of allyl alcohol on glucose supported respiration rate in *E. coli* cultures at 37°C, monitored in a Clarke (closed) oxygen electrode. Results presented are the mean values, and standard deviations of 3 independent repeats.

8.7 Discussion

8.7.1 Effects of garlic powder extract on cell morphology.

Pili were not visible in control or experimental cultures, *E coli* are know to possess pili (Clarke, 2003) often associated with virulence. Absence of extracellular structures in these analyses may be due to the preparation method used (fixation, excessive washing of samples after fixation, critical point drying), possibly indicating that the method used caused damage to the cells. Since the same method was used for all samples, it can be assumed that any differences in cellular appearance between control and garlic powder treated cultures are due to treatment with garlic powder extract.

Comparison of the appearance of cells from control cultures of *E. coli* to cells (*E. coli*) treated with garlic powder extract, highlights numerous differences. Treatment of cells with low concentrations of garlic powder extract had an elongating effect on some cells. Cell elongation without septation (division) has been associated with an SOS mechanism in some strains of *E. coli* induced by DNA damage within the cell (Hill, 1997). Increased garlic powder concentration brought about a decrease in elongated cell number, and also a decrease in the proportion of cells longer than 4 μm (compared to the control). It is possible that garlic powder extract at high concentrations could be inhibiting mechanisms responsible normal cell function (cell envelope synthesis, protein synthesis, DNA replication (Skyrme, 1996) resulting in a decrease in cell size.

As garlic powder extract concentration increased so too did the amount of cell blebbing, and accumulation of extract cellular debris. Blebs are the term given to small protrusions of material from cell envelopes (Itoh, 1999, Harris 2001). They are thought to be the cytoplasmic contents of the cell escaping through damaged cellular envelopes, though could also be caused by extracellular material attaching to the cell surface. The large accumulations of debris seen on treatment of bacteria of bacteria with garlic extract are assumed here to be either damaged cell envelope components, or cytoplasmic leakage induced by garlic powder extract (since the only difference between treated and control cultures was the addition of garlic powder extract, and the same debris was not seen on analysis of cell free garlic extract, or garlic free cell

controls, it is likely that debris and blebs are formed by bacterial cells on treatment with garlic powder extract). This observation is supported by the fact that as garlic concentration increases (at sub MIC concentrations) so does the frequency and severity of cell blebbing, along with the accumulation of extracellular debris. Conclusive proof of this could be achieved by assaying for leakage of cellular ions and enzymes into the supernatant medium.

Cell blebbing and accumulations of debris were less severe when *L. casei* cultures were treated with garlic powder extract (only occurring at much higher concentrations). This may be due to larger peptidoglycan layer surrounding the cell affording the bacteria structural protection, however since all Gram positive bacteria are not immune to garlic inhibition (*e.g. S. aureus* Chapter 1) any protective elements contained within the peptidoglycan layer would be Lactic acid bacteria specific (the group of bacteria that is least susceptible to garlic inhibition).

Treatment of *L. casei* with sub-MIC concentrations of garlic powder extract resulted in similar effects to those seen in treated *E. coli* cultures. Cell elongation, and irregular morphologies were seen, suggesting that production of structural components was adversely affected.

The lack of obvious cell damage at concentrations of garlic powder extract that cause damage to *E. coli* cells, suggests that garlic powder extract is less structurally damaging to components of *L. casei* cells. The lower frequency of abnormal cell morphologies also suggest that garlic powder extract if less inhibitory to the processes of cell growth and division in *L. casei*. It is not possible to conclude here whether *L. casei* possesses an internal protection process, or the *L. casei* cell envelope prevents inhibitory components contained in garlic powder extract from entering the cell.

Although scanning electron microscopy does not provide a conclusive analysis of antimicrobial action, it is a very useful tool for comparing morphology and cell surface structures in cells treated with antimicrobial cells to untreated control cultures. This provides hints to the antimicrobial mechanisms, and makes a useful starting point for further investigation.

It is not possible to pinpoint a specific site of action of garlic against *E. coli* and *L. casei* from SEM analyses. However it possible that since garlic powder extract caused membrane damage and brought about irregular abnormal cell morphologies (including cell elongation at low concentration, smaller cell size at high concentrations, loss of the regular cylindrical form) that there are multiple sites of action against *E. coli* (and *L. casei* at higher concentrations) affecting direct structural, and bringing about inhibition of cell growth and division processes.

Analysis of cell membrane fractions and intracellular fraction from *E. coli* did not identify any garlic components previously identified (Chapter 5). This suggests that either:

(1) The degradative pathway of allicin inside the bacterial cell, differs from the reaction products identified from culture supernatant analyses (i.e. the allicin reaction products formed within the cell/ cell interior on treatment of *E. coli* with garlic powder extract, are not the diallyl sulphides).

(2) A stronger extraction protocol is needed to remove diallyl sulphides from hydrophobic cell membrane interiors.

The short chain branched ketones, and alcohol found in the garlic powder extract treated cell envelope fragments could be brought about by oxidative damage of hydrocarbon membrane components, or altered metabolic intermediates. Corresponding compounds were not seen on analysis of cell free garlic powder extract, or garlic free cell controls, so it is likely that these compounds are only produced in cells on treatment with garlic powder extract.

The esters and Phenolic compounds identified on analysis of extracts from garlic powder treated cells, were not found in cell free controls or garlic free control. This suggests that they are abnormal metabolic intermediates or end products only brought about on addition of garlic powder extract.

Production of abnormal metabolic intermediates and end products with higher toxicity toward *E. coli* cells may contribute to the inhibitory capacity of garlic powder extract.

Allicin is readily permeable through phospholipid membranes (Miron *et al* 2000), assuming that the extraction method used here is sufficient, the absence of detecteble amounts of diallyl disulphide and diallyl trisulphide indicated that allicin has another degradative pathway within *E. coli* cells – the products of which were not detectable in these analyses.

8.7.2 Effects of garlic powder extract on glucose metabolism

Analysis of glucose metabolism in *E. coli* and *L. casei* using 1-^{13}C enriched D glucose revealed that α-D-glucose was preferentially metabolised rather that β-D-glucose (Figure 8.7.2.1), however glucose solutions were not equilibrated for 24 hours before use giving rise to flux between α and β isomers. As such, no conclusions can be drawn on the effect of garlic on metabolism of the separate isomers by the bacteria.

The NMR measurements here are not fully quantitative, as appropriate external and internal ^{13}C labelled standards were not available at the time of analysis, however by comparing subsequent glucose signal intensities to the glucose signal intensity at time 0; it is possible to monitor disappearance of ^{13}C labelled glucose from the system. Addition of garlic powder extract at approximately MIC concentrations resulted in a slower disappearance of glucose signal in *E. coli* and *L. casei* cultures, this is likely to be due to a decrease in the rate of glucose utilisation by the cell (NMR analysis would not detect changes in the rate of glucose uptake into cells, as even intracellular ^{13}C-labelled glucose would produce a signal).

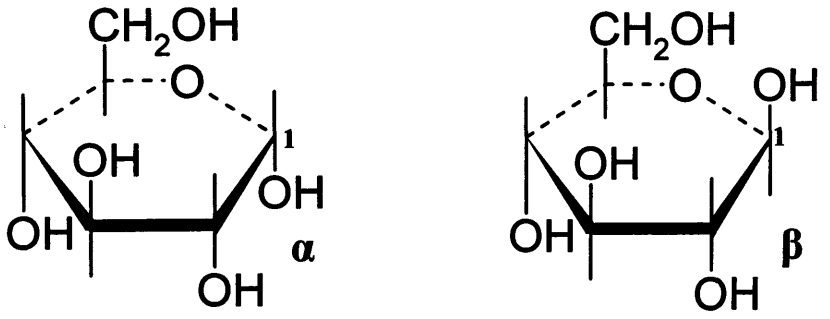


Figure 8.7.2.1 Structures of α-D-glucose and β-D-glucose. Carbon position 1 carries the ^{13}C label.

A decrease in the rate of total glucose utilisation within the cell, could be caused by a number of factors ranging from direct inhibition of glycolytic enzymes – to inhibition of enzyme production at a genetic level. Since the effect here is rapidly visible it is likely that the enzymes catabolising glucose metabolism are being inhibited by components from garlic powder extract. There are multiple sites throughout glycolysis where garlic components could be exerting inhibition, enzyme assays or radio-labelling experiments could pinpoint the inhibitory sites during operation of the glycolytic pathway.

A surprising observation from these experiments is that though garlic powder extract has an inhibitory effect on the utilisation rate of glucose, it is stimulatory for ethanol biosynthesis. Glucose metabolism to ethanol is a less efficient process than glucose metabolism to lactate (Madigan *et al* 2000) Garlic is known to increase ethanol production in protozoa (Harris 2001), and was also shown to inhibit ethanol catabolism in mice hepatic cells (Kishimoto *et al* 1999). It is important to consider that garlic extract may also contain many glycolytic enzymes (from the garlic plant), which could account for some increased production of glycolytic intermediates and end products. Analysis of ^{13}C glucose incubated with garlic extract revealed no decrease in glucose signal intensity over the 60 minute test period, suggesting that glycolytic enzymes from garlic do not significantly affect glucose utilisation and metabolism by bacteria treated with garlic extract – however, controls ^{13}C-labelled standards of each glycolytic intermediate, treated with garlic powder extract should be performed to ascertain conclusively whether enzyme interference from garlic does affect challenge study results

A decrease in the utilisation of glucose by cells of *E. coli* and *L. casei* could lead to depleted levels of ATP within the cell causing a reduced energy status within the cell. This in turn would have an inhibitory effect on all energy requiring (non spontaneous) cellular reactions (both anabolic and catabolic). Inhibition of the enzymes responsible for pyruvate reduction (*e.g.* lactate dehydrogenase) would bring about a reduction in cell NAD^+ (electron acceptor) levels, and this in turn would disturb the overall redox balance of the cell

This inhibition of carbohydrate metabolism, leading to reduced ATP synthesis could be partly responsible for garlic induced delays in the onset of the active growth phase of *E. coli* observed in chapters 1 and 5. Glucose utilisation in *L. casei* was also inhibited, though at much higher concentrations of garlic powder extract. There was no stimulation of non lactate glycolytic end products observed in *L. casei* cultures treated with garlic powder extract, this is perhaps not surprising as *L. casei* is a facultative heterofermentative that will metabolise glucose (in the absence of pentose sugars) to lactic acid. This is an important difference between the two organisms studied here, and could be (in part) responsible for the differential inhibition exerted by garlic powder extract.

Garlic powder extract and allyl alcohol were both found to increase oxygen uptake into cells. Introduction of elevated O_2 levels into *E. coli* cells would favour flux of carbohydrates into further metabolic pathways (citric acid cycle, and electron transport systems), suggesting that as glucose metabolism increased so to would energy (ATP) production. This however may not be the case, as any inhibition (garlic induced) in cytochrome function and redox balancing could increase the cellular concentrations of reactive oxygen species. Reactive oxygen species are known to be detrimental to cells – bringing about lipid peroxidation membrane damage (Urso 2003) and increasing cell membrane permeability (Lebedev 2002).

Oxidative damage to cells would support the damage to *E. coli* membranes seen on electron microscopic analysis, this would also infer that *L. casei* possesses protection mechanisms from oxidative stress sufficient to prevent damage by garlic powder extract.

Chapter 9.

General discussion.

Chapter 9 – General Discussion and Future work.

9.1 Background

The use of garlic as an antimicrobial agent is well documented throughout history (Hann 1996). It has proved to be effective against a broad range of protozoa (Harris 2001), fungi (Reuter 1996), and bacteria (Skyrme 1996). However the precise mechanisms of the antimicrobial action of garlic are not as yet elucidated

It is likely that the antimicrobial effect of garlic *in vivo* are due to the reactive sulphur compounds found in garlic. Since the pioneering investigations of Cavallito and Bailey (1944), the antibacterial actions of garlic have been ascribed to allicin. Though an excellent inhibitor of microorganism growth *in vitro*, there remains some doubt as to whether allicin is the garlic component responsible for the *in vivo* antibiotic effects. Given the reported unstable nature of allicin, especially in chemically complex environment (Egen-Schwind *et al* 1992, Freeman *et al* 1995) it is likely that reactive products of allicin also play an important role when considering the *in vivo* therapeutic effects of garlic.

Recent investigations have reported the antimicrobial efficacy of diallyl disulphide, diallyl trisulphide, and allyl alcohol (O'Gara *et al* 2000, Tsao *et al* 2001, Harris 2001). Since these three molecules are all produced during reactions of allicin in aqueous conditions, it is likely that they play a role in the *in vivo* activity of garlic extract. The picture becomes increasingly complicated when considering possible synergistic activity between allicin reaction products, and between allicin and other garlic components. In cases where high efficacy of allicin reaction products have been reported *in vivo*, the importance of allicin may be to function to deliver active components, rather that being solely responsible for garlic's therapeutic activities.

9.1.1 Garlic, growth, and metabolism in *E. coli* and *L. casei*

Garlic powder extract has proved effect against a wide range of bacteria, and there are many papers citing MIC values of garlic against various bacteria (Chapter 1). Whilst evaluation of MIC is an excellent method for quantitatively assessing the inhibitory efficacy of potential antibacterials, it provides little information on inhibitory effects on growth kinetics. Results presented here document inhibitory effects of garlic powder extract against a variety of human commensal bacteria (both pathogenic and non-pathogenic). From the results, bacteria can be divided into garlic susceptible bacteria and non-susceptible bacteria.

E. coli is a garlic susceptible bacteria, whereas *L. casei* is a non-susceptible bacteria (MIC values to garlic powder extract of 1.9 mg ml^{-1} and 16.9 mg ml^{-1} respectively). Analysis of growth kinetics seen on treatment with garlic extract revealed that in addition to the difference in MIC between the two bacteria; the nature of growth inhibition seen on treatment was also different. Garlic induced growth inhibition of *E. coli* (and other susceptible bacteria) was evident as a concentration-dependent increase in the duration of lag phase (i.e. a delay in the onset of exponential growth), whereas in *L. casei* cultures the inhibitory response was evident as a concentration-dependent reduction of the specific growth rate (which is dependent of rate of exponential growth).

During the lag phase viable cells are actively metabolising, but not reproducing (Madigan *et al* 2000), during this period the inoculum bacteria acclimatise to their surroundings, and synthesize enzymes (and other factors) needed for cell growth and proliferation. Amongst a number of factors, the duration of the lag phase is heavily dependent on:

- Inoculum size.
- Number of viable cells in the inoculum.
- Health of bacterial cells (ability to: synthesize enzymes, to synthesize cell components, accumulate nutrients, and excrete toxic waste products).

- Growth medium quality (ability of the growth medium to meet bacterial nutrient requirements).

Of these variables, inoculum size and growth medium quality were kept constant throughout the investigations. So an extension in lag phase could be due to: (i) a lower number of viable cells, brought about by garlic powder extract acting partially in a bacteriocidal capacity, or (ii) A reduction in the health status in inoculum bacteria.

Garlic did not act in a biocidal manner at concentrations which brought about substantial extensions in lag phase in *E. coli* (bacteriocidal activity was only observed at concentrations in excess of 20 mg ml^{-1}). This suggests that garlic powder brings about inhibition of exponential growth in *E. coli* cultures, by adversely affecting the health of cells in a bacteriostatic manner. There are a great many ways in which the 'health' status of bacterial cells could be affected, they will only be considered in a broad sense here.

It was observed that *L. casei* was substantially less sensitive to garlic growth inhibition than *E. coli* was. The mechanism by which *L. casei* cultures were protected against inhibition (lag phase extension) by garlic is unclear. The thick Gram positive cell wall may offer some protection to *L. casei*, however many other Gram positive (e.g. *Enterocuccus faecium, Staphylococcus aureus* (Chapter 3)) bacteria are sensitive, so the protection mechanism would have to be specific to *L casei's* (and other Lactic Acid Bacteria) cell wall.

A decrease in SGR (seen in *L. casei* cultures on addition of garlic), suggests a slower rate of cell growth and proliferation (Madigan *et al* 2000). Results obtained from chapters 3 and 7, concerning the effect of garlic extract and synthesized allicin respectively on growth indicate that the inhibitory effects of garlic against *L. casei* are due to allicin, or its breakdown products. Since the inhibitory effect does not diminish with time, if allicin is responsible for the growth inhibition it may be more stable in the presence on *L. casei* (allicin is more stable in acidic environment, such as those produced by lactic acid bacteria). Alternatively the breakdown products if allicin in aqueous solution (the diallyl sulphides and allyl alcohol) could be effecting this growth inhibition. The inhibition pattern could be brought about by inhibition of

biosynthetic pathways (such as fatty acid and lipid biosynthesis, protein biosynthesis, and nucleic acid synthesis). Inhibition of glucose metabolism by allicin could result in the cell having a lower ATP status, which would bring about an adverse effect on the specific growth rate. Since any sulphydryl containing enzyme could be a prospective target for allicin inhibition, there is abundant opportunity for allicin to act. A detailed study (possibly using NMR, or enzyme assays) of the inhibitory action of individual garlic components against metabolic pathway and enzymes would need to be conducted before conclusions could be drawn. The effects of allicin reaction products on growth kinetics of *L. casei* could be studied using a Bioscreen method similar to the method used in Chapters 3 and 7.

9.1.1.1 Adverse effects of garlic on cell structure.

Many antibiotics inhibit bacterial growth by specifically targeting the cell envelope, and cell envelope synthesis (e.g. polymixin B, penicillin, oxacillin).

Inhibition of peptidoglycan cell wall synthesis (targeted by penicillin) can lead to: a reduction in cell proliferation, loss of cell rigidity, 'abnormal' cell shape, and eventually cell lysis (due to decreased protection from osmotic pressure).

Cell membranes are also sites of antibiotic action (e.g. polymixin B). Adverse effects on cell membranes can range from subtle effects – such as decreased membrane fluidity, to extreme effects – such as loss of membrane integrity. Alterations in the fluidity of cell membranes can lead to metabolic inhibition, since many essential nutrient transporters (and other metabolic apparatus) located within cell membranes are required movement to function efficiently. Loss of membrane integrity will lead to dissipation of the proton motive force (adversely affecting the energy status of the cell), and ultimately leakage of intracellular components (ions, nutrients, enzymes, genetic material).

Garlic induced characteristics of *E. coli* cell morphology (on electron microscopic analysis) were evident as:

- Cell blebbing (leakage of cytoplasmic constituents).

- Altered cell surface appearance (mucus-like product).
- Reduction in cell proliferation (fewer actively dividing cells).
- Loss of cell rigidity.
- Abnormal cell morphology (shape).

Similar effects were seen on treatment of *L. casei* cultures with garlic powder extract at concentrations in excess of 10 mg ml^{-1} (though cells did exhibit abnormal morphology, no decreased rigidity was apparent).

This study suggests that garlic is adversely affecting *E. coli* at a structural level (further studies are needed before it is possible to conclude whether this damage brought about by direct physical attack on envelope components, or through inhibition of cell envelope synthesis). Many thiosulphinate reaction products are poorly water soluble (Chapter 5). Chapter 6 documents an increased rate of allicin disappearance from *E. coli* cultures treated with garlic extract. Given the hydrophobic nature of many allicin reaction products, it is possible that they could form aggregates within the cell, or disperse through the hydrophobic membrane interiors.

Allicin is freely permeable to biological membranes (Miron *et al* 2000), if allicin degraded within the membranes of cells, it is unlikely that the hydrophobic reaction products would exit the membrane into the aqueous cytosol. This may adversely affect membrane fluidity (and membrane integrity).

Garlic powder extract brought about an increase in oxygen uptake into *E. coli* cells. Increases in cellular oxygen levels, can disturb the redox balance of the cell, and lead to production of reactive oxygen species. Reactive oxygen species are responsible for bringing about structural damage to cell membranes through lipid peroxidation.

9.1.1.2 Adverse effects on cell metabolism

Allicin exerts an inhibitory effect on many enzyme reactions, by reacting with thiol (SH), to form S-allyl derivatives within the enzyme (Harris 2001). Pathways inhibited by allicin acting in this include lipid synthesis, DNA synthesis, RNA synthesis, and

protein synthesis (Adetumbi and Lau, 1986). RNA synthesis was inhibited through attack of a free sulphydryl contained in its α-subunit, and the effect could be reversed on addition of glutathione. Glutathione is thought to remove the S-allyl derivative from the enzyme. The presence of glutathione explains why mammalian cells are less sensitive to the effects of allicin.

The extension in lag phase seen on addition of garlic to *E. coli* cultures, and the reduced SGR observed in *L. casei* cultures on addition of garlic could be the result of inhibitory action of allicin on biosynthetic and metabolic pathways.

Carbohydrate catabolism functions to provide cells with the energy transfer molecule ATP (which is used to drive non spontaneous reactions within the cell), and produced reducing molecules (function to maintain a favourable redox balance within cells). Inhibition of glucose metabolism, will lead to a lower energy (ATP) status in the cell, and could disturb the redox balance of the cell. Lower ATP concentrations would lead to a decrease in cell growth and proliferation, as the need for energy to drive non-spontaneous biosynthetic reactions would not be met. Disturbances in cellular redox balance would adversely affect the cells ability to cope with reactive oxygen species (and inhibit many redox driven reactions in metabolic pathways).

Glucose metabolism to lactic acid in *E. coli* and *L. casei* was inhibited on addition of garlic, and ethanol production in *E. coli* was stimulated.

Inhibition of carbohydrate could account for the lag phase extension seen *E. coli* cultures treated with garlic, and (although inhibiting to a lesser extent) the lower specific growth rate observed in *L. casei* cultures on addition of garlic.

9.1.2 Inhibitory activity of garlic components on growth of *E. coli*

GC-MS analysis was used to identify the principal components of garlic extract (Chapter 5). The inhibitory effects of allicin against *E. coli* and *L. casei* were investigated. The inhibitory activity of major allicin reaction products toward *E. coli* was also investigated (Chapter 7). The effect of allicin on the growth curve of *E. coli* was very similar to the effect of garlic powder, the effect observed on addition of

allicin to *L. casei* cultures also compared well to the effect seen on addition of garlic powder extract.

E. coli cultures exposed to sub-MIC concentrations of garlic powder extract, and allicin concentration below 4.42 $\mu g\ ml^{-1}$, exhibit an extended lag phase after which, normal exponential growth is resumed. The recovery of normal growth after bacteriostatic inhibition, suggests that either cells have become immune to the antibacterial agent, or that the antibacterial agent has degraded.

Observations from Chapter 6 suggest that the half-life of allicin on addition to *E. coli* cultures is shortened from 10 hours to 30 minutes. As allicin concentration decreases below the threshold levels required for bacteriostatic activity the culture would resume growth. This theory assumes that reaction products of allicin on addition to *E. coli* cultures are of lower bacteriostatic activity than allicin.

Results from chapter 6 also suggest that allicin is more stable when incubated with *L. casei* than with *E. coli*. Insensitivity of *L. casei* to the inhibitory action of allicin could account for the differential inhibition exerted by allicin (and garlic powder extract) between *L. casei* and *E. coli*. Insensitivity could be due to a number of factors such as:

- Impermeability of *L. casei* cells to allicin.
- Lack of active sites for allicin inhibition.
- Chemical protection from inhibitory effects of allicin (e.g. glutathione un mammals).
- Enzymic inactivation of allicin.

It is not possible to suggest which of these mechanisms are responsible for offering *L. casei* protection from the inhibitory effects of allicin without further investigation.

Reaction products of allicin (diallyl trisulphide, diallyl trisulphide, diallyl sulphide, and allyl alcohol), and dipropyl disulphide, all proved much less inhibitory to growth of *E. coli* cultures than allicin. None of these components (alone or in combination)

was capable of total inhibition at concentrations below 1 mg ml^{-1}. Inhibitory activity of the diallyl sulphides increased

The Inhibition seen on addition of diallyl sulphides to *E. coli* cultures differed from that seen on addition of allicin. An concentration-dependent extension in lag phase was observed which was more pronounced as the as the molecular sulphur content increased. There was also a significant concentration-dependent decrease in SGR. Dipropyl disulphide, produced an effect similar to diallyl disulphide (the two molecules are similar in structure). Allyl alcohol exerted a concentration-independent inhibition of SGR.

Assuming that the sites of action are the same for *E. coli* and *L. casei* this could indicated that the diallyl sulphide and allyl alcohol components of garlic extract are responsible for the reduction of SGR seen on treatment of *L. casei* cultures with garlic.

9.1.3 Synergism effects of garlic penicillin, and methicillin.

Garlic powder extract in itself proved to be inhibitory to MRSA, with an MIC comparable to that of *E. coli*. Combinations of garlic with penicillin, and garlic with methicillin proved particularly effective (Chapter 4). Addition of garlic powder extract significantly reduced the MICs of all strains to penicillin and methicillin. Synergistic activity was slightly higher using combinations of penicillin and garlic, than combinations of methicillin and garlic. Penicillin resistance is conferred on *S. aureus* by the presence of a β-lactamase enzyme. Methicillin resistance is conferred by the presence of additional penicillin binding proteins, with a reduced affinity for methicillin. Facilitation of the action of penicillin could be brought about by garlic by inhibition of β-lactamase, ensuring a higher percentage of active penicillin reach their target site. Facilitation of the action of methicillin, may be brought about by inhibitory components of garlic acting on the biosynthesis of the modified penicillin binding protein.

9.2 Conclusions

Conclusions drawn from this study are:

- Garlic is significantly more inhibitory to *E. coli* than *L. casei*
- Inhibition of *E. coli* by garlic powder extract is biostatic in nature (at concentrations of 20 mg ml^{-1} or below).
- The nature of sub-MIC garlic-induced growth inhibition in *E. coli* follows a characteristic pattern of a dose-dependent increase in the duration of lag phase, followed by recovery of normal growth.
    - The recovery of culture growth may be due to the increased reactivity of allicin exposed to *E. coli* cultures (concentration decreasing below the threshold required for bacteriostatic activity)
- The nature of sub-MIC garlic-induced growth inhibition in *L. casei* follows a characteristic pattern of a dose-dependent decrease in specific growth rate.
    - The decrease in SGR may be due to allicin insensitivity, and brought about by inhibitory action of allicin reaction products.
- Commercially available stabilised allicin products (Allimax and Kyolic) do not display antibacterial activity toward *E. coli*.
- The reactivity of allicin depends strongly on dissolution media and presence of bacteria:
    - Allicin from garlic dissolved in nutrient broth has a half-life of approx 11 hours at 37°C.
    - Allicin from garlic dissolved in nutrient broth has a half-life of approx 30 minutes when incubated with *E. coli* at 37°C
- Antibacterial efficacy of allicin reaction products are dependent of the molecular sulphur content (diallyl trisulphide>diallyl disulphide> diallyl sulphide), except allyl alcohol which exerts a potent concentration-independent inhibitory effect on SGR in *E. coli*
- Mixtures of allicin breakdown products do not act in a manner similar to garlic powder extract on growth of *E. coli*.
- **Growth inhibition in *E. coli* is likely to be due to the bacteriostatic activity of allicin.**
- Garlic powder extract is structurally damaging at sub-MIC concentrations to *E. coli* and *L. casei* cells. Inducing cytoplasmic leakage from cells, abnormal morphologies, and reduced cell size.

- Garlic powder extract is inhibitory to glucose catabolism in *E. coli* and *L. casei*, and stimulatory to ethanol production in *E. coli*.
- Garlic powder extract (and allyl alcohol) increase cellular oxygen uptake in *E. coli*.
- Garlic powder extract is inhibitory to MRSA, and facilitates the action of β-lactam antibiotic (lactamase-sensitive, and lactamase-stable).

9.3 Prospects for therapeutic applications of garlic powder.

The inhibitory activity of garlic powder extract is already harnessed, to protect humans, and livestock from intestinal illness. Results presented here infer that, provided it can be delivered in a suitable format, garlic powder extract could prevent the growth of pathogenic intestinal organisms, not affecting beneficial intestinal *Lactobacilli* microflora.

Garlic powder extract could also prove a useful method of preventing and treating systemic infections antibiotic resistant bacteria such as MRSA.

9.4 Future work.

The results prevented here reveal many aspects of the antibacterial use of garlic that warrant further investigation. Some ideas for further investigations are presented below:

Clinical trials or experiments in animal models should be performed, to investigate whether garlic/ β-lactam combination therapy is effective in patients.

Synergy testing between individual garlic components and conventional antibiotics should be performed. This may provide useful information which could be used to develop effective economical treatments for MRSA infections.

Growth curve analysis should be carried out for *L. casei* with individual garlic components, to identify whether differences in inhibition seen here are due to allicin insensitivity.

The effect of garlic extract on components enzymes of glucose catabolism could be assayed for, pinpointing the active sites for garlic's activity against carbohydrate metabolism.

Cytosolic components of *E. coli* could be assayed for in cultures treated with garlic extract (e.g. potassium ions, lactate dehydrogenase), to assess membrane damage to cells.

Garlic components containing labelled sulphur could be used to identify possible accumulations, or sites of action within cells.

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