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THE POTENTIAL USE OF HEN EGG WHITE LYSOZYME AS AN ANTIMICROBIAL AGENT IN FOODS.

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ABSTRACT.

The potential use of lysozyme as an antimicrobial agent in foods was investigated in model food systems(brainheart infusion broth) using factorial designed experiments and in mussel and cottage cheese food systems. Optical density or absorbance was used as a tool to monitor the growth response of *Listeria monocytogenes* and *C. tyrobutyricum* in brain heart infusion broth under the combined influence of pH(5.5, 6.5), lysozyme (0.2mg/ml, 3mg/ml) and different chelating agents(ethylene diaminetetraacetic acid(EDTA), glycine, gluco delta lactone(GDL), citric acid, sodium phosphate dibasic(SPDB) and sodium hexametaphosphate(SHMP)(10mM, 25mM).

Using 2^3 full factorial design experiments, the yield of the organisms (expressed as the area under the curve of a plot of change in optical density at 600nm vs time) was taken as the quantitative response variable for each treatment. These yield values were then used for (a) statistical analysis to determine which of the single or interactive factors tested significantly reduced the yield, (b) formulation of a mathematical regression equation which could be used to predict microbial growth within the limits of the factors studied. Diagnostic plots were constructed to evaluate further how well the statistical model fit the observed yield values. Plots of residuals versus predicted yield values appeared to suggest that a transformation of the response would improve the fit of the models. No other serious reservations were suggested by the diagnostic plots. Goodness of fit of the models was also evaluated by the R-squared values.

Significant two-way and three-way interactions between lysozyme, pH and EDTA, GDL, citric acid and glycine were exhibited. Response surface methodology(RSM) was used to (a) characterize the response of *L. monocytogenes* to variation in treatment combinations and (b) show non-linearity of models(or interaction of factors). Generally yield was minimal in treatment where pH was low, with high lysozyme and chelator.

Based on equal molar concentrations, the antimicrobial activity of the different chelating agents was in the order EDTA > GDL > citric acid > glycine > adipic acid > SHMP > SPDB. The same ranking was true for the degree to which each chelating agent

enhanced lysozyme activity. Based on broth culture studies, the chelating agents EDTA, GDL, glycine, citric acid and adipic acid were demonstrated to have potential for use as antimicrobial agents in combination with lysozyme in food systems.

Results of a 2^5 factorial design indicated that the 5 factors, lysozyme, GDL, pH, inoculum level and temperature were important in the inhibition of *L. monocytogenes*. Results of the broth culture studies gave a good reflection of the survival of *L. monocytogenes* in the food system. The variable combinations interacted to decrease the growth of *L. monocytogenes* and extended the lag phase duration. However *C. tyrobutyricum* was more tolerant to the different treatment combinations other than EDTA.

A study of protein interference demonstrated that the antimicrobial activity of the lysozyme-GDL preservation system was not inhibited by the presence of proteins. The food system study demonstrated that the lysozyme-GDL treatment combination has potential for use as a preservative in refrigerated low pH ready-to-eat foods. The susceptibility of *L. monocytogenes* to lysozyme-GDL treatment in both broth culture and food systems increased as the temperature was reduced(25C-5C) and as the pH decreased(pH6.5-pH5.5).

Food system studies demonstrated that modified atmosphere packaging(96.58%N_2 , 2.09%O2 and 1.34% CO_2) has no influence of the growth of *L. monocytogenes*. The susceptibility of *L. monocytogenes* to lysozyme-GDL was a stable characteristic, remaining unchanged during the entire study. Attempts to select for greater lysozyme-GDL resistance by testing populations grown from lysozyme-GDL survivors isolated at the end of the food system study was unsuccessful. There was no evidence that *L. monocytogenes* was resistant to the lysozyme-GDL treatment.

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CHAPTER 1.

INTRODUCTION

The antibacterial properties of lysozyme were first reported by Flemming (1922). Lysozyme is an important natural enzyme which prevents bacterial growth. It is particularly effective in lysing cell walls of gram positive bacteria. This it does by splitting β(1-4)linkages between N-acetyl-muramic acid and N-acetyl-glucosamine, the components making up bacterial cell walls. Lysozyme is particularly attractive as a preservative since the enzyme is active between 4 and 95C, stable over a wide range of pH values, specific for bacterial cell wall and is not harmful to humans (Smolelis and Hartsell, 1952; Ryser and Marth, 1991; Johansen et al. 1994; Yang and Cunningham, 1993). Hen egg-white lysozyme is presently the most important commercially available lysozyme. Considerable interest has been stimulated in using lysozyme as a food preservative, mostly in Japan, but few scientific papers have been published (Cunningham et al. 1991).

Ethylene diaminetetraacetic acid (EDTA) is approved for use in foods primarily as a stabiliser and sequestrant (Furia, 1972; Anon, 1990). Several bacterial species have shown sensitivity to the compound or its salts. The sensitivity is caused either by sequestration of cations essential for growth or by disruption of the outer membrane, thereby affecting permeability and stability of the cell (Russel, 1971; Kranian and Shelef, 1988). Combinations of EDTA and other preservatives have been reported to enhance inhibition of several microorganisms including spores of *Clostridium botulinum* (Tompkin et al. 1979) and *Staphylococcus aureus* (Robach and Stateler, 1980).

Outbreaks of foodborne illness are often the result of one or a combination of errors that can occur during food processing, distribution, retailing and preparation. Mistakes can occur due to equipment failure, negligence, ignorance or by the inability of the food regulation and processing guidelines to keep up with rapidly evolving knowledge and technology.

Emerging foodborne pathogens have been demonstrated to cause much more serious illness. *L. monocytogenes* has been shown to cause much more serious illness in immuno-compromised individuals than in the normal population. This category includes pregnant women, unborn children, the elderly, AIDS patients, those suffering from cirrhosis of the liver, cancer patients receiving chemotherapy (especially those suffering from bowel cancer) and organ transplant recipients (Gill, 1988; Schwartz et al. 1988; Lacey, 1989). The identification of food as the vector for human listeriosis was not made until the early 1980s (Schlech et al. 1983). New concerns relate to increased evidence that foodborne microorganisms may cause diseases in organ systems other than the gastrointestinal tract. *Listeria* can migrate to the blood, cause septicaemia, a circulating blood infection and can cause meningitis. There is growing concern that the presence of non-pathogenic *Listeria spp.* in foodstuffs indicates that the conditions for contamination by and growth of *L. monocytogenes* are present.

The ability of *L. monocytogenes* to survive in various foods depends on several combined parameters in the food, these include temperature, pH, salt content, water activity, atmosphere and presence of preservatives. Emerging foodborne pathogens are capable of competitive growth at 5C in foods. *L. monocytogenes* is one of the bacteria

with this capability. The use of refrigeration (5C holding of food) can no longer be deemed sufficient to keep foods safe from bacterial hazards arising either from growth of the food pathogens or increased survival. Hazards from *L. monocytogenes* resulting from brief temperature abuse cannot be ruled out. Documented cases of temperature abuse throughout distribution in retail markets and by consumers is available (Bryan et al. 1978; Daniels, 1991; Davidson, 1987; Harris, 1989; Kalish, 1991; Van Garde and Woodburn, 1987; Wyatt and Guy, 1980). Holding of food at 5C will only delay the growth of many of the emerging foodborne pathogens. There is therefore need for extra hurdles to keep the foods safer, especially those that have extended refrigeration periods and do not receive a lethal heat treatment before consumption.

1.1 Documented characteristics of *L. monocytogenes* that are important to food processors:

1.1.1 Widespread occurrence. *Listeria monocytogenes* occurs commonly in nature, food processing environments, raw foods, in domestic and other environments. Its ubiquitous nature is well documented (Gray and Killinger, 1966; Weis and Seelinger, 1975; Fenlon, 1986; Gray, 1960; Watkins and Sleath, 1981; Al-Ghazal and Al-Azawi, 1986; Hooker, 1988).

1.1.2 pH. *L. monocytogenes* is capable of growing in a wide pH range of pH5 to pH9, but has been known to survive at much lower pH values (Conner et al. 1986; Farber et al. 1989; Parish and Higgins, 1988). Schaack and Marth (1988) demonstrated that *L. monocytogenes* can survive the fermentation and extended refrigeration storage of buttermilk and yogurt in which the final pH values were in the range 3.9-4.4 and 3.9-4.1

respectively. Growth at pH4.39 has been reported (George et al. 1988).

1.1.3 Temperature. The ability of *L. monocytogenes* to multiply at refrigeration temperature is well documented (Rosenow and Marth, 1987; Donnelly and Briggs, 1986; Gray and Killinger, 1966). *L. monocytogenes* has been isolated from frozen seafoods (Weagant et al. 1988). Other studies have reported no substantial effects on the organism in food from freezing (Kaya and Schmidt, 1989; Guyer and Jemmi, 1991; Palumbo and Williams, 1991), tolerance to repeated freezing and thawing (Bryan, 1969), growth at 0C (Gray and Killinger, 1966; Khan, et al. 1972) and extended survival at subzero temperatures (Hof et al. 1986).

1.1.4 Water activity. At relatively low NaCl concentration (6%) *L. monocytogenes* may grow even under refrigeration conditions (Hudson, 1992; Guyer and Jemmi, 1991). *L. monocytogenes* is resistant to low water activity conditions as evidenced by growth at water activity(a_w) 0.92 (Health and Welfare Canada, 1990; Petran and Zottola, 1988), survival at a_w 0.79-0.86 in fermented hard salami at 4C (Johnson et al. 1988), survival in 25.5% NaCl at 4C(a_w< 0.90) (Shahamat et al. 1980), remarkably resistant to drying (Doyle et al. 1985) and detection of the organism in alfalfa tablets (Farber et al. 1990) as well as in non-fat dry skim milk powder (Doyle et al. 1985). Thus the organism appears to withstand very dry environments.

1.1.5 Toxin production and recovery of heat damaged cells. The principal toxin of *Listeria monocytogenes* is known as listeriolysin and is synthesised maximally under cold conditions (Gray and Killinger, 1966). It is 58 kilodaltons in molecular weight and

has an optimum activity at pH5.0-5.5 (the pH of much of our food) (Kathariouv, 1988). The toxin (lysteriolysin) can be synthesised at 48C (Sokolovic and Goebel, 1988). Enhanced toxin production at low temperatures(4C) and at pH(pH5.0-5.5) has been documented (Gray and Killinger, 1966; Wood and Woodbine, 1979; Lacey, 1992). Several reports have concluded that low temperature enrichment favours the recovery of heat damaged cells of *L. monocytogenes* from heat-treated foods (Ryser and Marth, 1985; Garazaybal et al. 1987; Doyle et al. 1987; McCarthy et al. 1990) and it has been further suggested that incubation at low temperature might permit repair of heat injured organisms that are otherwise undetectable if food is examined immediately after the heat treatment (Sheeran et al. 1989; Kerr and Marth, 1992). Growth of *L. monocytogenes* can occur at temperatures as low as 0C, although as the temperature approaches zero the doubling time lengthens to about one cell division every 24hrs (Gray and Killinger, 1966). There is a theoretical possibility that just one cell division is needed to cause the maximum production of listeriolysin and this coupled with recovery of heat-damaged cells at 4C could cause problems. Under such circumstances, refrigeration which is used to restrain the growth of food poisoning agents may well be indirectly responsible for listeriosis. More recently, Mackey et al. (1994) established that cold enrichment does not allow recovery of more heat damaged cells than incubation at higher temperatures and is not optimal for the resuscitation of cells unable to grow on selective media. It was then concluded that refrigeration of heat treated foods should not therefore increase the risk that heat-injured cells will recover from the heat treatment.

1.1.6 Low infective dose. The number of *L. monocytogenes* cells required to initiate infection in an individual is not known and is difficult to establish (Lacey, 1989). There

has been considerable speculation over what comprise an infective dose of *L. monocytogenes* and whether an "acceptable level" in food can be identified. More research is needed to establish what constitutes an infective dose. Documented outbreaks rarely include data that enable precise estimation of the infective dose. A number of reports have indicated that the infective dose is "low" in susceptible populations. On the basis of listeriosis outbreaks, the best estimates were that it required 10^2 - 10^3 *L. monocytogenes* cells per gram of cheese to infect susceptible individuals (Ryser and Marth, 1987) and 10^3 - 10^5 *L. monocytogenes* cells per gram of food (Engel et al. 1990). There are reports of otherwise healthy individuals developing listeriosis and there is a suspicion that this is increasing, probably due to the ingestion of very high numbers of bacteria from, for example, refrigerated coleslaw or soft cheese (Lacey, 1989).

1.1.7 Packaging methods. *L. monocytogenes* is a microaerophilic microorganism. Growth of this pathogen is enhanced under decreased oxygen concentration and with supplementation with carbon dioxide (Seelinger, 1961; Farrag and Marth, 1992). Different packaging methods have been demonstrated to have no substantial influence on the organism's ability to grow, these include vacuum packaging (Bentley et al. 1989; Glass and Vanderlinde, 1992), controlled atmosphere packaging(CAP), modified atmosphere packaging(MAP) and plastic wrap (Berrag et al. 1989; Ingram et al. 1990; Wimpfheimer et al. 1990; Kallander et al. 1991).

1.1.8 Thermal resistance. The heat resistance of *L. monocytogenes* is well documented (Donnelly et al. 1985; Doyle, 1987; Bearns and Girard, 1958). There has been

considerable confusion and discussion about the heat resistance of *Listeria monocytogenes* particularly in regard to dairy products. Numerous conflicting reports concerning the unusual heat resistance of *L. monocytogenes* in milk can be found in early literature. An early report of the exceptional heat resistance of *L. monocytogenes* (Bearns and Girard, 1958) has not been confirmed (Donnelly et al. 1987). It was suggested that *L. monocytogenes* might survive pasteurisation if located within bovine lymphocytes (Fleming et al. 1985; Doyle et al. 1987). Bunning et al. (1986, 1988) established no evidence of thermal protection. The organism has a D value of about 1 second at 71.7C, thus it should not survive pasteurisation. It is now well established that, where the correct time/temperature relation can be assured, pasteurisation guidelines (HTST pasteurisation of 71.7C for 15sec) are adequate to destroy all *L. monocytogenes* in whole milk. The presence of *L. monocytogenes* in pasteurised dairy products denotes either underprocessing or a post pasteurisation contamination.

Listeriosis has been associated with cheese made from both pasteurised and unpasteurised milk. Cheeses are kept for long periods; such prolonged storage will also lead to high counts of *Listeria*. There are very few documented instances of infection with *Listeria monocytogenes* other than through food (Lacey, 1989). Todd (1988) estimates that the money cost per year as a result of outbreaks and recalled products in the US is $313 million per year.

1.2 Sea foods:

There is reason for concern about refrigerated seafoods destined to be eaten without exposure to heat treatment of marked microbial lethality. Contamination of seafoods

and seafood products with *Listeria monocytogenes* is a growing concern of the sea food processing industry. Consumption of shellfish or raw fish was implicated in an outbreak of listeriosis in New Zealand (Lennon et al. 1984). In seafood processing plants, crustacean exoskeletons commonly become trapped within the equipment used in cleaning and peeling, thereby contributing to the contamination problem. Processed seafoods that do not ordinarily receive additional heating before consumption (cooked, smoked, pickled or raw seafoods) are of greatest concern due to the possibility of pre- and post-processing contamination. Refrigeration of contaminated processed seafoods does not prevent further growth of *L. monocytognes* (Guyer and Jemmi, 1991; Rorvik et al. 1991).

It has been argued that the widespread distribution of *L. monocytogenes* make it impossible to control listeriosis (WHO, 1988). This may not be true since many food poisoning bacteria, *Salmonella*, *S. aureus*, *C. perfringens* and *C. botulinum* are widely distributed, yet only under certain specific conditions do they cause food poisoning. The prevention of the occurrence of these conditions have been established. Similarly, the control of listeriosis should be developed.

1.3 Modelling of microbial growth and survival in foods.

In some areas of food microbiology, namely thermal processing, it has been standard practice for many years to make use of predictive mathematical models. The commonest example of this is the "botulinum cook" that is applied to low acid foods, and is based on a well established model of the destruction of large populations of spores of *Clostridium botulinum* during heating. Models have not been extensively used

in situations where the potential for growth of contaminating microorganisms in foods, pre- or post-processing, is concerned (Gould, 1989).

Recently, several groups of workers have began to move from the traditional challenge testing and to apply modern predictive modelling technique to the growth and survival of microorganisms in foods. The models used are of various sorts. Some are probabilistic (predict the probability of hazardous events occurring, such as production of toxin, or the presence of an infective microorganism), others predict the important kinetic parameters of microbial growth, such as the lag time, microbial growth rate and generation time (Cole, 1991). The models have the universal ability to predict the response of microorganisms to the whole range of environmental influences and conditions that occur during the processing and storage of real foodstuffs within the limits examined. The models are based on collected experimental microbiological data, some of which is obtained in real food studies, but much is obtained from studies using laboratory media and automated data gathering techniques rather than actual foods and conventional counting procedures (Gould, 1989). This is because the automated techniques save so much time and can deal with so many variables with minimum expenditure of time and effort and therefore capture data very cost-effectively. The data obtained are transformed into mathematical relationships that detail the effects of the environmental changes on the microorganism that are present. Predictions about safety and keepability can be made even when the specific environmental conditions have not been tested. However, it will remain important to ensure that incorrect extrapolations are never made.

A number of models have already been constructed and applied to improve day-to-day

decision making of food safety and stability. However the techniques are still in their infancy, and development at the moment is rapid.

1.4 Combination studies.

Rarely are the effects of two or more factors tested in such a way as to allow the interactions between them to be quantified. While combinations of antimicrobials are currently utilised in the food industry, their interactions are often poorly characterised. Combined antimicrobial agents have been extensively studied in the pharmaceutical industry and methods have been developed to determine types of interactions between two antimicrobials (Barry, 1976; Krogstad and Moellering, 1986; Squires and Cleeland, 1985), however, the applicability of such procedures to combinations of food preservatives has not been extensively explored.

When two antimicrobials are used in combination, three things may occur (Barry, 1976), first there may be an "additive effect", the antimicrobial activity of an antimicrobial agent is neither enhanced nor reduced while in the presence of another agent. Secondly, a synergistic effect may occur, "the effect observed with a combination is greater than the sum of the effects observed with the two agents independently", there is enhancement of overall antimicrobial activity of a compound when in the presence of a second antimicrobial agent. Thirdly, an antagonist effect may occur. The antimicrobial activity of one compound is reduced when in the presence of a second agent. The mechanisms of antagonism are much more complex and less well studied. Examples of antagonism are combinations of bacteriostatic and bactericidal agents, use of agents with the same active site and a chemical interaction (direct or indirect)

between two agents (Krogstad and Moellering, 1986; Larson, 1984).

One way to control the growth of microorganism of concern (pathogenic or spoilage) is to manipulate the composition of foods so that growth is minimised or preferably prevented. Such manipulations could involve changing several factors, such as the intrinsic (pH, preservatives) and extrinsic factors (atmosphere, storage temperature) of the food system.

Because of the interaction of various factors, the best way to study these systems is by means of factorial designed experiments. Several variables are adjusted simultaneously in order to study their effects on some specific parameter, such as growth yield. In model food systems, predictive model equations describing the growth of organisms of concern can be produced. These models can be used to predict the growth that would occur under particular storage conditions.

1.5 OBJECTIVES OF THIS WORK.

Model food system(BHI broth).

1. To investigate the potential use of lysozyme(alone or in combination with various chelating agents) as an antimicrobial agent in foods.
2. To use full factorial designed experiments that would enable determination of interactions between various factors.
3. To investigate whether there are antimicrobial interactions of practical significance between lysozyme and various chelating agents.
4. To develop mathematical model equations that would predict the growth yield of *L. monocytogenes* and *C. tyrobutyricum* under the influence of lysozyme-chelator combinations.
5. To compare inhibition/inactivation of *L. monocytogenes* with *C. tyrobutyricum* treated in the same way.

6. To rank the ability of different chelating agents in potentiating the antimicrobial activity of lysozyme.

Food system(cottage cheese and mussels).

7. To test the efficacy of multiple barriers or hurdles(storage temperature, pH, lysozyme, chelator, atmosphere) in controlling the growth of *L. monocytogenes* in cottage cheese and mussel food systems at use and abuse temperatures.
8. To investigate whether inactivation/inhibition of microorganisms in model food systems is a good reflection of what happens in the actual food system.

CHAPTER 2.

LITERATURE REVIEW

2.1 Introduction

Microbial growth and activity are of concern for the safety and quality of most foods. It is estimated that 20% of the world's food supply is lost due to microbial spoilage (Fulton, 1981). Low temperature, chemical preservatives, reduced pH and low water activity are often used to prevent safety hazards and spoilage. Growth of some pathogenic bacteria, for instance *L. monocytogenes*, is only prevented at extreme conditions of chilling, salting or low pH. To control the safety of some foods, addition of chemical preservatives is necessary, but interest among consumers has increased for alternatives to reduce the wide use of chemical preservatives. This has resulted in the increased interest in preservation using live bacterial cultures (biopreservation) (Jeppensen and Huss, 1993), glucose oxidase (Jeong et al. 1992) and lysozyme (Chander, 1980; Teotia and Miller, 1975; Smith, 1991; Johansen et al. 1994; Buchanan and Golden, 1994). The legislative restriction on the presence of *L. monocytogenes* in foods are strict, often not allowing any *Listeria monocytogenes* to be present.

A combination of preservatives interacting synergistically to control biological deterioration can further extend the shelf-life of food systems. The bactericidal effect of egg white lysozyme is often increased by combining the enzyme with other chemicals such as EDTA (Chander, 1980), hydrogen peroxide, ascorbic acid (Miller, 1969) or amino acids like glycine (Proctor and Cunningham, 1988). Currently, research on the effectiveness of such combinations is limited.

Selecting the proper preservation system for a particular food product is not an easy process. The spoilage problem must be identified and then the possible preservation system must be evaluated in model studies in the food product in question (Branen and Davidson, 1983).

2.2 Lysozyme.

Lysozymes (1, 4-β-N acetylhexosaminodase E.C. 3.2.1.17) from various sources are well characterised enzymes. First discovered in 1922 by W. Fleming, egg white lysozyme was among the first proteins sequenced, the first for which a three dimensional structure was suggested using X-ray crystallography and the first for which a detailed mechanism of action was proposed.

The hen egg white lysozyme molecule consists of a single peptide chain of 129 amino acids. The lysozyme molecule is cross linked in four different places by disulphide bridges formed by the combination of sulphur-containing side chains between 64-80, 76-94, 6-127 and 30-115. Two of the disulfide bonds must be intact for lysozyme to maintain enzymatic activity. Lysozyme loses its activity if all the -S-S- bonds are reduced. The lysozyme molecule can be crystallised out of egg albumin. The molecular weight of lysozyme is approximately 14 300 to 14 600 and the isoelectric point is pH10.7. Lysozyme activity rate is highest from pH3.5 to 7 and the pH range of 5 to 7 is best for lysing susceptible bacterial cells. According to Vakil et al. (1969) lysozyme catalyses the lysis of certain bacteria by hydrolysing the beta linkage between N-acetylmuramic acid and N-acetylglucosamine, which are part of a long, complex sugar molecule found in the bacterial cell wall.

2.2.1 Lysozyme source and lytic activity.

Lysozyme is found abundantly in hen egg white but is also present in plants such as turnips, papaya, cauliflower and cabbage. It is also found in bacteria, tears, saliva, kidney, spleen, blood, nasal secretions, milk, human or animal fluid and tissue (Meyer et al. 1946; Vakil et al. 1969; Jolles and Jolles, 1984; Chandan and Ereife, 1981; Oishi et al. 1989). The major source for commercial extraction of lysozyme is hen egg albumin (Proctor and Cunningham, 1988). Human milk contains 39mg/100ml while bovine milk contains an average of 13µg/100ml of milk. Milk lysozyme was first isolated from bovine milk by Chandan et al. (1965) and from human milk by Parry et al. (1968). Both human and bovine lysozyme were similar to egg white lysozyme in biological activity, but differed in several chemical and biological characteristics. Egg white lysozyme, like other lysozymes, is active against Gram positive bacteria but the

activity varies depending upon the genus. Lysozyme activity is quantified by a procedure in which the reduction in turbidity of a suspension of *M. lysodeikticus* is measured in an aqueous buffer solution (Proctor and Cunningham, 1988).
According to Branwart (1979) micrococci are the most sensitive and are rapidly lysed by 1µg/ml whereas *Bacillus megaterium* and *Bacillus cereus* are barely attacked by 50µg/ml. Gram negative bacteria are generally insensitive to lysozyme alone, since the lipoprotein-lipopolysaccharide layer prevents access of lysozyme to the mucopolysaccharides of the cell wall. Susceptibility of Gram negative cells to lysozyme can be increased by treatment with EDTA. Egg white lysozyme has not been used to a great extent in food products, but Moustafa and Collins (1969) did evaluate its usefulness against *P. fragi*, a common food spoilage microorganism. A combination of 1mg/ml of EDTA and 2mg/ml of lysozyme completely prevented growth of *P. fragi* in broth, although up to 20mg/ml of lysozyme alone had no effect on growth of *P. fragi* in the broth. A combination of EDTA plus egg-white lysozyme was not effective against growth of *Pseudomonas fragi* in skim milk. The antimicrobial properties of eggs has been attributed to lysozyme and conalbumin, but other compounds have also been implicated. As with other naturally occurring antimicrobials, little or no use has been made of these materials as food additives.

In contrast to egg white lysozyme, human and bovine lysozyme inhibit Gram negative bacteria in the absence of EDTA (Vakil et al. 1969). Although the average concentration of lysozyme in milk is low, it is probable that prolonged action of low concentrations may aid the preservation of milk.

There are two commercially available lysozymes, hen egg white lysozyme and Cellosyl, a lysozyme isolated from a *Streptomyces coelicolor*. Despite slight differences (for example, the human lysozyme has 130 amino acids) the capacity for hydrolysis of acetylhexosamine polymers remains essentially the same. Because of the nature of the cell wall, Gram-positive bacteria are most susceptible to lysozyme. Lysozyme is known to kill or inhibit the growth of bacteria and fungi, and is used in Europe to control the growth of the spoilage organism *Clostridium tyrobutyricum* in cheese (Fox, 1991). It has also been proposed for use in a variety of other food preservation applications

(Proctor and Cunningham, 1988).

2.2.2 Mechanism and kinetics of cell wall lysis by lysozyme.

The mechanism of cell lysis by muramidase such as egg-white lysozyme involves the hydrolytic cleavage of the structural component of the cell wall, in the case of bacteria, the n-acetylmuramic acid and n-acetylglucosamine polymer, and in the case of fungi, the chitin (an n-acetylglucosamine polymer). By degrading the structural component of microbial cell walls, lysozyme may in the process inhibit the growth of many bacteria especially gram positive bacteria as well as certain fungi. The cells whose cell wall has been attacked are weakened, their growth inhibited and some even die. Most studies on the effect of lysozyme have been carried out with enzymes extracted from egg whites.

The kinetics of cell wall lysis by lysozyme have been studied. The kinetics in these studies have been considered as zero (Smolelis et al. 1948), first (Kerby et al. 1953) and second order (Prasad et al. 1963; Howard and Glazer 1969; McKenzie et al. 1986). Smolelis and Hartsell (1952) are of the view that the lysis is a three-step process, that is, initiation of lysis by lysozyme (adsorption and depolymerization), activity of autolytic enzymes within the cell structure and finally solution of cellular residues.

The influence of salts and ions on the activity of lysozyme has also been studied. If crystalline lysozyme is mixed with a suspension of *M. lysodeikticus* cells in triple distilled water, no visible lysis can be observed (Smolelis et al. 1952). Fleming and Allison (1922) found that 0.5% sodium chloride increased bacteriolysis and that higher concentrations caused an inhibition. Yang and Cunningham (1994) demonstrated that lysozyme activity against *M. lysodeikticus* was highly stable (maintained over 90% of the lytic activity from day 1 to day 30) in a solution of 1.0% sodium chloride. The specific effects of ions on the cell membrane may be: (a) alteration of membrane permeability which may be necessary before lysozyme can act, (b) reaction of salt with polar groups of the cell surface, (c) conditioning the solubility of cellular substances (Smolelis and Hartsell, 1952).

2.2.3 Effect of heat and chemicals on lysozyme activity.

Lysozyme is heat stable in acidic solutions, having been reported to withstand 100C with little loss of activity (Bordert, 1928; Meyer et al. 1936; Smolelis and Hartsell, 1952). Matsuoka et al. (1966) also found lysozyme to be stable in acidic solutions (pH4.5, 100C, 3min ; pH5.29 , 100C, 30min). Using loss of activity as the criterion, Beychok and Warner (1959) showed that stability in the temperature range 85-95C was maximum at about pH5.5. They reported that stability decreased rapidly on the alkaline side of pH6 but did not study lysozyme in alkaline solutions because of its low solubility. Sandow (1926) reported that lysozyme in egg white at pH8.0 was destroyed in 15min at 65C, but at pH5 and 60C no loss occurred in 60min. Cunningham and Lineweaver (1965) found lysozyme to be over 50 times more heat-stable in phosphate buffer (pH6.2) than in egg white at pH9. No inactivation of lysozyme occurred in phosphate buffer at 62.5C, even at pH9, while at 65C, inactivation occurred in 10min at pH9. Heat stability depends on the menstrum, pH and salt concentration.

Yang and Cunningham (1994) demonstrated that lysozyme activity against *M. lysodeikticus* remained relatively stable at pH7 and ionic strength < 0.1, whereas lower activity was observed at pH9 and ionic strength > 0.14. Lysozyme was highly stable (maintained over 90% of lytic activity in solutions of 1.0% sodium chloride, 100ppm sodium nitrite, 4% ethanol and 100ppm butylated hydroxytoluene). About 50% of the lysozyme activity was retained in solutions of 0.5% EDTA. Activity was lost when lysozyme was combined with 0.5% lactic acid, 4% acetic acid and 100ppm chlorine water.

2.2.4 Potential uses of lysozyme.

Because of its bactericidal properties, lysozyme has been suggested for use as a preservative in the food industry. It has the essential advantage of being found in almost all human and animal cells and does not have harmful effects (Chander and Lewis, 1980; Johansen et al. 1994). Lysozyme is specific for bacterial cell walls and has a high stability and activity under a wide range of physical conditions (Proctor and Cunningham, 1988; Yang and Cunningham, 1994; Smolelis and Hartsell, 1952; Matsuoka et al. 1966). There has been considerable interest in lysozyme as a food preservative, mostly in Japan, where the majority of the work has been performed using

lysozyme in a food system. Much of the work is locked up in patents and most research seems to involve industrial secrets.

High pressure homogenisation can be used alone or in combination with lytic enzymes or chitosan to reduce the microbial population and heat treatment damage in foods. High pressure homogenisation is used for the stabilisation of food mixtures and the recovery of cell proteins or other biochemicals from microorganisms (Herthertington et al. 1971; Kula et al. 1989). Microorganisms are disrupted by the sudden pressure drop, by tortion and shear stresses, and most probably by cavitation shock waves resulting from imploding gas bubbles (Lutz Popper et al. 1990). Lytic enzymes attack the cell walls of microorganisms and thus help to render the microorganisms more susceptible to subsequent mechanical or thermal inactivation. Lysozyme and related lytic enzymes, chitinases, glucanases and lipases may be added to the foods before homogenisation to weaken the rigid cell walls. Cell walls of bacteria vary considerably with strain, age, nutrition and on whether the bacterium is gram positive or gram negative. It is generally accepted that cell walls of gram positive bacteria are more rigid than those of gram negative bacteria.
The effect of lysozyme concentration during enzyme treatment prior to homogenisation was recently investigated by Lutz Popper et al. (1990). No synergistic effect of the combined enzyme/homogenisation treatment could be detected, but initial total counts of *Streptococcus lactis* could be reduced by increasing the enzyme concentration. The use of free and immobilised enzymes is a rapidly expanding technology (Zamorani, 1989) and lysozyme immobilised on matrices that can be easily removed from the medium and reused could be exploited in the dairy industry (Crapisi, 1993). Studies have been carried out on lysozyme covalently bounded to cellulose and polyacrylamide to evaluate its activity towards *Micrococcus lysodeikticus* (Datta, 1973).

2.3 *C. tyrobutyricum* and 'late blowing' in cheese.

C. tyrobutyricum is a rod shaped, Gram positive, strictly anaerobic spore forming microorganism. It has the following biochemical characteristics; maltose(-), mannose(+), lactose(-), starch(-), nitrate(-) and gelatin(-). The spores are subterminally positioned and it has an optimum temperature of growth in the range 25-37C.

2.3.1 The defect 'late blowing'

The defect in cheese known as 'late blowing' is typified by abnormal levels of open texture and accompanying unattractive odours and flavors may present serious problems in several important varieties of semi-hard and hard cheese including Gouda, Emmental, Provolone, Danbo and other brine salted types.

'Late blowing' in cheese is caused by the butyric acid fermentation brought about by the germination and growth of clostridial spores, especially those of *C. tyrobutyricum* present in milk. Butyric fermentation in cheese leads to considerable production of carbon dioxide and hydrogen. This results in blowing of the cheese. Organic volatile acids are formed during the fermentation and these affect the flavor and taste of the cheese.

2.3.2 The source of the causative organism.

The spores originate in the soil which contains between 500 and 8 700 spores/g of dry matter. The feeding of silage is recognised as a major source of contamination of raw milk with the pasteurisation-resistant spores of *Clostridium tyrobutyricum*. The most likely source of contamination is faecal material containing such spores. Hay contains few clostridial spores while maize silage made from the whole plant and grass silage frequently contain dangerously high numbers of spores which lead to dung being produced which if allowed to contaminate the milk is likely to lead to late blowing of cheese.

The critical numbers of clostridial spores to cause butyric acid fermentation in 12kg Gouda cheese are from 5 to 10 per litre (van den Berg et al. 1980). Galesloot and Elgersma (1975) claimed that one spore of *C. tyrobutyricum*/100 ml of milk was sufficient to cause openness in Dutch Gouda and Edam. Fryer and Hallingan (1976) recommended that the milk for Gouda production should contain less than 1 spore of *C. tyrobutyricum*/5ml. The critical number of spores to bring about butyric acid fermentation which result in cheese defects probably varies with type, since between the different brine-salted semi-hard and hard cheeses there are large differences in shape and size of the cheese, pH value, ripening time, ripening temperature and in structure including normal eye formation.

2.3.3 Control measures

The use of chemicals like nitrate is a well established effective means of preventing butyric acid fermentation in Gouda cheese. However, it is still not clear whether nitrate in cheese induces the formation of N-nitrosocompounds.

Addition of nitrate to cheese milk is a common and successful practice used to prevent the late blowing defect, which otherwise yields an unacceptable product. The active compound inhibiting germination of these spores is nitrite produced from nitrate by microbiological or enzymatic reduction or both. Nitrite, in turn may give rise to the formation of nitrosamines in foods rich in organic secondary amines. Although the incidence and amounts of nitrosamines in cheese produced with nitrate are extremely low(Elgersman et al. 1978), substitution of nitrate in cheese production may lower unwanted consumption of nitrate and nitrite (Elgersman et al. 1978).

For many years, Italian law has permitted the use of formaldehyde at approximately 25ppm, to prevent late blowing of Grana Padano and Provolone cheese. Biological control was reported by Perfilier and Gudkov (1982). A bacteriological preparation, Bioantibut consisting of a concentrate of lactic acid bacteria exerted an antagonistic action on butyric acid bacteria and when used by the cheese making industry in the USSR, resulted in the production of cheese with practically no defects caused by butyric acid fermentation.

2.4 *L. monocytogenes.*

Listeria monocytogenes is an emerging food borne pathogen which has caused great concern to both food industry and regulatory agencies since the early 1980's. The concern surrounding the presence of this organism in foods stems from the fact that: (a) it is widespread in the environment, (b) it is a psychrotrophic pathogen and thus can grow at chill temperatures in products such as the "new generation" of foods including cook-chill MAP products with extended shelf-life, (c) it is a hardy organism and appears to be more heat tolerant than many other vegetative microbes, (d) the disease it causes (listeriosis) is associated with a high mortality rate which averages 30% in food outbreak situations, (e) it is a microaerophilic microorganism, various forms of MAP and CAP have been demonstrated to have no effect on its growth but are effective on spoilage organisms, (f) the bacterium synthesises an abundance of its toxin during growth at low

temperatures, (h) heat damaged cells are able to recover at low temperatures, and (i) the infective dose is not known, although it is postulated to be low, there is little evidence to substantiate a low infective dose in susceptible individuals and the amount of toxin produced may be as equally important as the number of bacterial cells in causing listeriosis.

2.4.1 Morphology and biochemical characteristics of *L. monocytogenes.*

L. monocytogenes is a small, coccoid to rod shaped, motile, Gram-positive rod with round ends, non-spore forming, non-acid fast, 0.4-0.5µm in diameter and 0.5-2µm in length (Gray, 1960). The bacterium exhibits "tumbling motility' when grown at 20-25C (Gray and Killinger, 1966). On blood agar, colonies produce a narrow zone of β-hemolysis caused by a soluble hemotoxin (Seeliger, 1961). Initially, young cultures are without exception gram-positive, however, examination of 2 to 5 day old cultures often reveals gram-negative cells which resemble those of *Haemophilus influenzae* (Gray, 1960).

Listeria monocytogenes posses the following biochemical characteristics: catalase +, mannitol -, rhamnose +, oxidase -, urease -, and Methyl Red/Voges-Proskauer (VP) test +/+. An important reaction in defining *L. monocytogenes* is production of β-hemolysis on sheep blood agar (Christine et al. 1944).

2.4.2 Growth requirements.

Listeria monocytogenes is a nonfastidious organism that can reproduce in simple synthetic media. Excellent growth of *Listeria monocytogenes* occurs in an atmosphere with reduced oxygen, 5 to 10% carbon dioxide since the organism is microaerophilic. *L. monocytogenes* fails to grow under strict anaerobic conditions.

Minimum, optimum and maximum temperature for growth.

L. monocytogenes has a temperature growth range reported to be 2.5-44C (optimum for growth, 30-37C) (Seeliger, 1961), however growth at 0C (Khan et al. 1972) and at -0.4C (Walker et al. 1990) has been reported. However, the current edition of Berrgey's Manual of Systematic Bacteriology gives the minimum growth temperature as 1C. This

lower temperature limit for growth of *L. momocytogenes* is in agreement with the findings of Seelinger and Jones, (1986).

Minimum, optimum and maximum pH of growth.

L. monocytogenes has been reported to be unable to survive or grow poorly in low pH environments with pH5.5-5.6 being the critical cut-off point for growth (Gray and Killinger, 1966; Ryser and Marth, 1987). According to the ninth edition of Bergey's Manual of Systematic Bacteriology and based on Seelinger (1961), *L. monocytogenes* can only grow at pH from 5.6 to 9.6, with optimal growth occurring at neutral to slightly alkaline pH values, the latter was recently verified by Petran and Zottola (1989). In cottage cheese, at a pH of 5.2-5.4, *L. monocytogenes* multiplied during storage at 3C for 28 days (Ryser et al. 1985). Ryser and Marth (1987) were able to demonstrate limited growth of the organism at pH values of 5.0 to 5.1 in cheddar cheese. Recent listeriosis outbreaks linked to consumption of fermented dairy products have shown that *L. monocytogenes* can proliferate in even lower pH values, the minimum pH for growth has thus been revised downwards. More recent work has shown that *L. monocytogenes* is able to initiate growth at pH values as low as 4.39 and 5.23 at 4 and 30C, respectively (George et al. 1988). Results from independent studies by Parish et al. (1989) and Sorrells et al. (1989) confirm the ability of *L. monocytogenes* to multiply at pH4.4-4.6. Farber et al. (1989) recently observed growth of *L. monocytogenes* at a pH value as low as 4.3. While growth of *L. monocytogenes* at pH < 4.3 has not been documented, this organism appears to be fairly acid tolerant under such conditions.

2.4.3 Occurrence

L. monocytogenes is widely distributed in the environment. The widespread occurrence of *L. monocytogenes* in the general environment and how this relates to contamination of food and water has been reported (Brackett, 1988). *L. monocytogenes* has been frequently found contaminating a wide range of foods at very low and very high levels (Lund, 1990; Ryser and Marth, 1991). Surveys for *L. monocytogenes* and *Listeria spp.* have shown the ubiquity of the organism in the food production environment. *L. monocytogenes* has been found in cool damp environments, on food product contact surfaces, in standing water, in food residues, floors and drains (Slade, 1992; Walker,

1991; Venables, 1989; Hudson and Mead, 1989; Genigeorgis et al. 1990; Anon, 1988; Cox, 1989; Klauser and Donnelly, 1991). Household dish cloths and refrigerators are frequently contaminated with this organism (Cox et al. 1989; Hayes et al. 1991). Healthy intestinal carriers have been identified among cattle (Hofer, 1983; Kampelmacher et al. 1969), poultry (Schwartz, 1969), swine and sheep (Gronstol, 1979), and humans (Kampelmacher et al. 1969, 1972). The wide spread occurrence of *L. monocytogenes* is well documented (Fenlon, 1985; Ralovich, 1984; Weis and Seeliger, 1975; Gray and Killinger, 1966; Gronstol, 1979; Al-Ghazal and Al-Azawi, 1986; Watkins and Sleath, 1981).

2.4.4 The psychrotrophic nature of *L. monocytogenes*.

Although *L. monocytogenes* is generally considered to be a mesophile (optimum growth at 30 to 37C), its psychrotrophic nature has been well documented (Rosenow and Marth, 1987; Hof et al. 1986; Khan et al. 1972). The ability of *L. monocytogenes* to grow at low temperatures is a property that is used to isolate this organism from clinical and other specimens (Gray and Killinger, 1966; Donnelly and Briggs, 1986). The ability of *L. monocytogenes* to proliferate at refrigeration temperatures may make refrigerated foods especially susceptible to higher-than-usual populations of *L. monocytogenes*. Thus refrigerated storage is no guarantee of protection against growth of *L. monocytogenes* (Donnelly and Briggs, 1986). The ability of *L. monocytogenes* to initiate growth at low temperatures poses a serious threat to the food industry and particularly to manufacturers of chilled foods. Several reports (Wood and Woodbine, 1979) suggest that virulence of the bacterium is increased when it is grown at low rather than high temperatures. Thus the possibility must exist that cold storage may enhance virulence of some *L. monocytogenes* strains isolated from refrigerated foods. The findings of Hof et al. (1986) suggest that *L. monocytogenes* can remain viable as long, or longer, than most other *Listeria spp.* during extended storage at subzero temperatures.

2.4.5 Heat resistance and recovery of heat damaged cells.

An early report of the exceptional heat resistance of *L. monocytogenes* (Bearns and Girand, 1958) has not been confirmed (Donnelly et al. 1987) but the organism appears to be more heat resistant than many other nonsporing food-borne pathogens (Mackey

and Batchell, 1989). Confusion exists over the organism's heat resistance, particularly its ability to survive pasteurisation of milk (Doyle et al. 1987; Lovett et al. 1990). It was suggested that *L. monocytogenes* might survive pasteurisation if located within bovine lymphocytes (Fleming et al. 1985). Bunning et al. (1986, 1988) established no evidence of thermal protection. Findings by Sheeran et al. (1989) suggest that in food *L. monocytogenes* is more thermo-tolerant than in milk. Seafoods are usually cooked at lower temperatures than meat products. *L. monocytogenes* has been isolated from cooked and pasteurised seafoods and the effectiveness of the pasteurisation process has been questioned. More recent reports have demonstrated that *L. monocytogenes* can be easily destroyed by pasteurisation treatments employed in the seafood industry (Harrison and Huang, 1990; Budu-Amoako et al. 1992; Ben Embarek and Huss, 1993).

Several reports have concluded that low temperature enrichment favours the recovery of heat damaged *L. monocytogenes* from heat treated foods (Beuchat et al. 1986; Ryser and Marth, 1985; Garayzabal et al. 1987; McCarthy et al. 1990). Incubation at low temperature might permit repair of heat-injured organisms that are otherwise undetectable in food examined immediately after the heat treatment (Sheeran et al. 1989; Kerr and Lacey, 1992; Ryser and Marth, 1985). More recently, Mackey et al. (1994) established that cold enrichment does not allow recovery of more heat-damaged cells than incubation at higher temperatures and is not optimal for resuscitation of cells unable to grow on selective media, and concluded that refrigeration of heat-treated foods should not therefore increase the risk that cells will recover from the heat treatment.

2.4.6 Listeriosis.

Listeriosis is the disease caused by *L. monocytogenes*. The disease is generally considered a zoonosis - a disease or infection naturally transmitted between vertebrate animals and humans (Ralovich, 1984; Hyclop and Osborne, 1959; Mitscherlich and Marth, 1984). The human intestine is the primary point of entry for *L. monocytogenes* (Rosenow and Marth, 1987; Braza, 1985). The manifestation of listeriosis varies with species, sex, age, route of exposure and strain of bacterium (Gray and Killinger, 1966). People at great risk are the elderly, newborns, pregnant women and immunosuppressed individuals (Braza, 1985). However, it is not understood why a small minority, even

of vulnerable patients, acquire the disease and exactly where and how the bacterium gets into the body. The disease characteristics of *L. monocytogenes* in people at risk has been reviewed (Marth, 1988; Blume, 1987, Marth and Ryser, 1990; Ryser and Marth, 1991; EL-Gazzar and Marth, 1991).

Although the number of infections caused by *L. monocytogenes* is only a small percentage of those caused by *Salmonella* food poisoning, the number of deaths from listeriosis is about twice that from *Salmonella* (Lacey, 1989). In addition, a very substantial number of miscarriages may be due to listeriosis and have not been identified. Lacey (1989) believe that current figures underestimate the real incidences.

2.4.7 Mode of transmission.

Listeria monocytogenes is widespread in the environment, hence, humans can be exposed to the bacterium in several ways. The origin and mode of transmission of all forms of listeric infection is complex, based on some evidence provided in the literature, an attempt has been made to trace the various routes of infection using data in literature. The possible routes of transmission of *L. monocytogenes* to humans are as follows:

Humans → Humans (Ralovich, 1984).

Insect → Human (Ralovich, 1984).

Animal → Human (Seeliger, 1961).

Food → Human (Schlech et al. 1983; Fleming et al. 1985; CDC, 1985; Ho et al. 1986).

Dust(dirt) → Air → Human(Seeliger, 1961).

Feed → Animal → Human (Schlech, 1984).

Soil → Plant → Human (Schlech, 1984; Mitscherlich et al. 1984).

Animal → Soil → Plant → Human (Seeliger, 1961).

2.5 Chelating agents.

The destruction and inhibition of bacterial cells by chelating agents is well recognised. Chelating agents work to disrupt the integrity of the cell wall. They also work as potentiators of other lethal agents, either by facilitating entrance into the cell or chelating cations (magnesium, calcium and iron) essential for growth and repair of injured cells

(Borenstein, 1976; Fricke and Jensen, 1975). Chelating agents have rarely been used directly as antimicrobial agents, however several chelating agents have been shown to posses activity against some moulds and bacteria. For chelation, the sequestrant must have the proper steric and electronic configuration and be at the optimal ionic strength and pH. Citrates, pyrophosphates, ascorbic acid, glycine and GDL are by far the most popular and technically useful chelating agents.

2.5.1 Ethylene diaminetetraacetic acid (EDTA).

EDTA reacts with alkaline earth and heavy metals to form metal complexes and thereby remove reactive multivalent cations from solution. The destruction and inhibition of bacterial cells by chelating agents is well recognised. EDTA works to disrupt the integrity of the cell wall of bacteria.

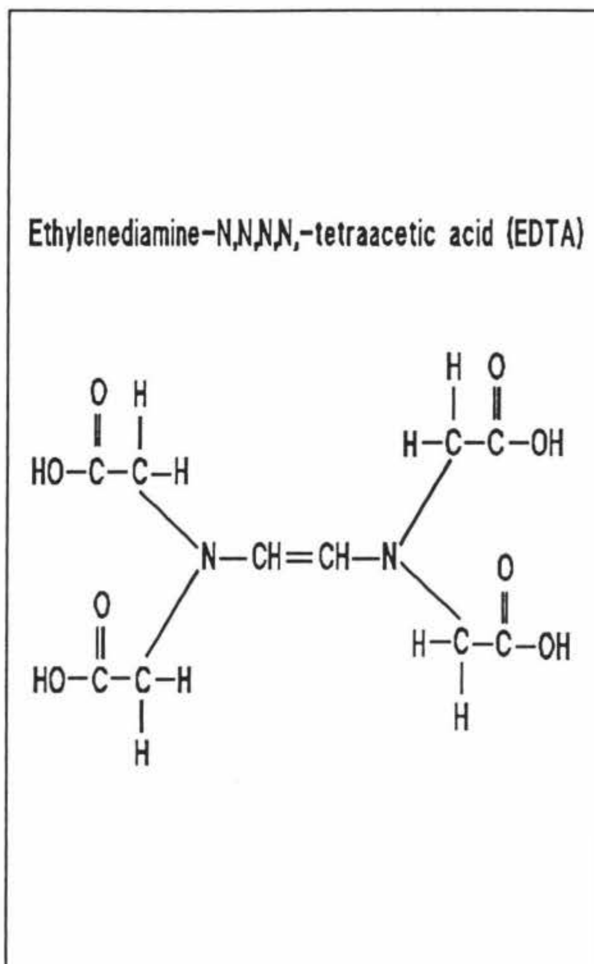
Physical and chemical properties.

EDTA becomes increasingly dissociated as pH rises and quantity of metal complexed will increase. EDTA sequesters a number of metal ions, including magnesium ions, calcium and aluminium ions. Use of EDTA in foods has been limited to the disodium salt and the disodium calcium complexes. They are available as spray-dried powders which are colorless, freely soluble in water and have only a faint saline taste at the concentrations used in foods. EDTA is approved for use in foods primarily as a stabiliser and sequestrant (Furia, 1972; Anon, 1990). Several bacterial species have shown sensitivity to the compound or its salts. The sensitivity is caused either by sequestering cations essential for growth or by disrupting the outer membrane, thereby affecting permeability and stability of the cell (Russel, 1971).

Antimicrobial activity.

EDTA may act as a direct inhibitor of several species of microorganisms or may act synergistically with other antimicrobial agents to promote bacterial destruction.
Gray and Wilkinson (1965) found that EDTA at an alkaline pH selectively solubilized a high proportion of the carbohydrate and phosphorous present in the cell walls of a number of gram-negative bacteria. EDTA was bactericidal for *P. aeruginosa* in 0.001M solution, destroying over 99.99% of the cells in suspension.

Mechanism of action.



Gray et al. (1965) theorised that EDTA was active against *P. aeruginosa* through a mechanism involving chelation. Metal cations essential for the integrity of the cell were bound on sites on the bacterium. Calcium, magnesium and other divalent cations were reported to function as a salt bridge in gram negative organisms to bind lipopolysaccharides on the surface of the cell wall. EDTA formed a complex with these cations, causing leakage of cell solutes and loss of viability. Bactericidal activity was reduced as pH was reduced supporting chelation as the mode of action. Sequestration of metal ions also appears to increase the permeability of the bacterial cell walls to compounds that can interfere with the growth of the organism.

Use in food and regulatory status of EDTA.

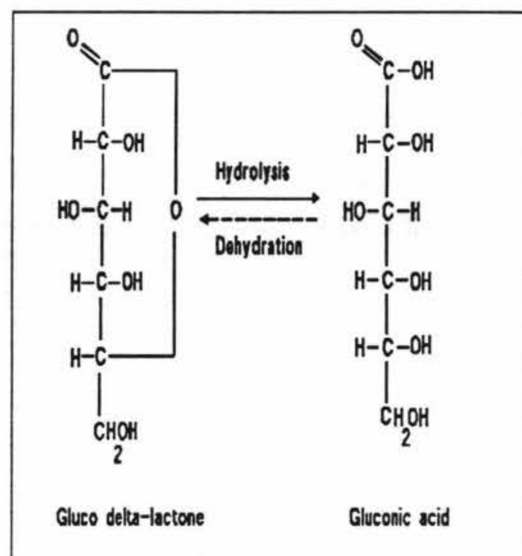
EDTA is approved as a food additive for direct addition to food in the form of calcium disodium EDTA and disodium EDTA. It is approved for use as a food preservative in a variety of foods, including salad dressing, mayonnaise, cooked canned crab meat and sausages. The maximum amount of EDTA allowed is dependent upon the type of food in which it is to be used. The FAO/WHO acceptable daily intake for EDTA is 0-2.5mg/kg body weight at 0.01% level of use.

2.5.2 Gluco delta lactone (GDL).

GDL is a fine, white almost odorless crystalline powder, soluble in water and slightly soluble in alcohol. One of the less recognised members of the acidulant group, gluco

delta lactone (GDL) is an inner ester of gluconic acid. It is produced by the fermentation of glucose, followed by crystallization of the lactone (Serpelloni, 1990). The fermentation end product consists of a blend of gluconic acid and GDL.

Upon hydrolysis of GDL, gluconic acid is formed making GDL useful in combination with chemical leavening systems. Hydrolysis occurs slowly taking three hours at room temperature to be complete, this results in a gradual but continuous decrease in pH. At the end of the hydrolysis, an equilibrium mixture exists, consisting of gluconic acid and its delta and gamma-lactones.



The rate of acid formation increases with temperature and the intensity of the acidification depends on the concentration of GDL, the temperature and the substrate. It is the slow rate of acidification and mild taste characteristics that set GDL apart from other acidulants, enabling the lactone to be used in special applications.

As GDL hydrolyses to gluconic acid, its taste characteristics change from sweet to neutral with a slight acidic aftertaste. Gluconic acid is a natural constituent of fruit juices and honey and an intermediate in glucose oxidation.

Use in food and regulatory status of GDL.

GDL is used as an acidifier, binder, curing agent, leavening agent, pH control agent, pickling agent and as a sequestrant.

It is used in communuted meat food products, sausages, dessert mixes, frankfurters and genoa salami. It is useful in any product that requires acidification but a low flavor

profile. GDL is permitted in direct-set cottage cheese and is often preferred for this application because of its slow rate of acidification which ensures development of a finer texture in the finished product, void of localised denaturation (Deane et al. 1960). GDL is readily available, nontoxic and free from objectionable properties such as insolubility of calcium salts and unpleasant taste (Moustafa et al. 1990). In the body it is a normal metabolite of glucose degradation by the pentose phosphate pathway (Serpelloni et al. 1990).

Regulatory status.

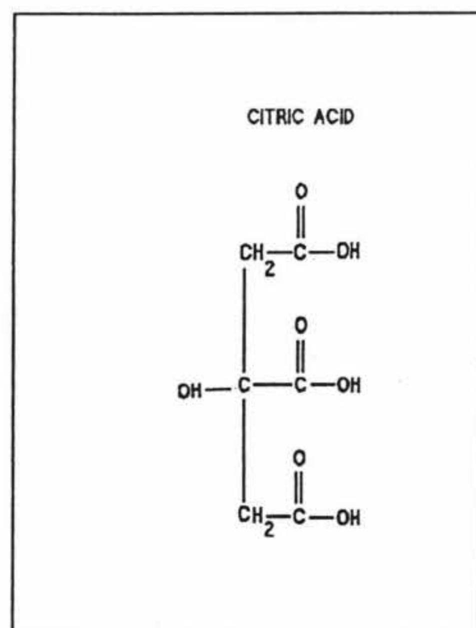
FDA - 21CFR 184.1318. GRAS when used in accordance with GMP.

2.5.3 Citric acid.

Citric acid is a colourless, translucent crystal or white granular to fine crystalline powder, odorless with a sharp acid taste. Citric acid is freely soluble in water. Citric acid is by far the most extensively used food acidulant. The acid and its salts are favorites because of its solubility, flavor character and cost. After such characteristics as food legislation, taste characteristics, physical and chemical properties and physiological properties are considered it becomes evident that many naturally occurring acids are unsuitable and only a few are widely used in foods. These food acids are mostly organic, citric acid being the most important.

Citric acid is widely distributed through nature where it not only occurs in plants but also in animals. It is found in numerous natural products. Among plant sources citric acid is most abundantly present in citrus fruits such as lemon (4.0-8%), grape fruit (1.2-2.1%), fangerines (0.9-1.2%) and oranges (0.6-1.0%) (Bouchard and Merrit, 1979). Citric acid is tribasic and is produced commercially by the fermentation of a pretreated carbohydrate (molasses, sucrose or dextrose solutions) which are inoculated with various pure strains of *Aspergillus niger* or yeasts, such as *Candida guillierondii*. Because of citric acid's role in the "Krebs cycle," it is continuously being produced and metabolised in the body of man and other creatures. It is therefore not surprising that such a physiologically important substance as citric acid is regarded by all countries as a harmless material for use in foods, drinks and pharmceutical preparations (Fricke and Jensen, 1975).

While citric acid is not used directly as an antimicrobial, it has been shown to possess activity against some moulds and bacteria. It has been demonstrated that citric acid acts as a bactericide on strains of *Staphylococcus* and other organisms (Fricke and Jensen, 1975). The dipping of animal carcases, notably poultry, in citric acid solution can be mentioned as an example of its preserving function (Fricke and Jensen, 1975). On the other hand, it is known that citrates favour microbial growth as in the manufacture of cultured flavors. The addition of 0.2-0.3% monosodium citrate to starter cultures of butter, margarine, certain cheese and cultured milk products gives a more pronounced aroma (Fricke and Jensen, 1975). Young and Foegeding (1993) reported that for certain combinations of pH and concentration, citric acid stimulated the growth of *L. monocytogenes* as compared to HCl-adjusted control cultures. They hypothesized that the effect could be due to citrate serving as a carbon source. The mechanism of inhibition by citrate has been theorised as to be related to its ability to chelate metal ions. Branen and Keenan (1970) were the first to suggest that inhibition may be due to chelation in studies with citrate against *Lactobacillus casei*. Chelation was also indicated as the reason for inhibition of *S. aureus* (Rammel, 1962) and *Arthrobacter pascens* (Imai et al. 1970) by citrate. The relative bacteristatic and bactericidal activities associated with citric, lactic and acetic acids against *L. monocytogenes* have been assessed by a number of investigators (Ahamad and Marth, 1989; Young and Foegeding, 1993; Sorrels et al. 1989).



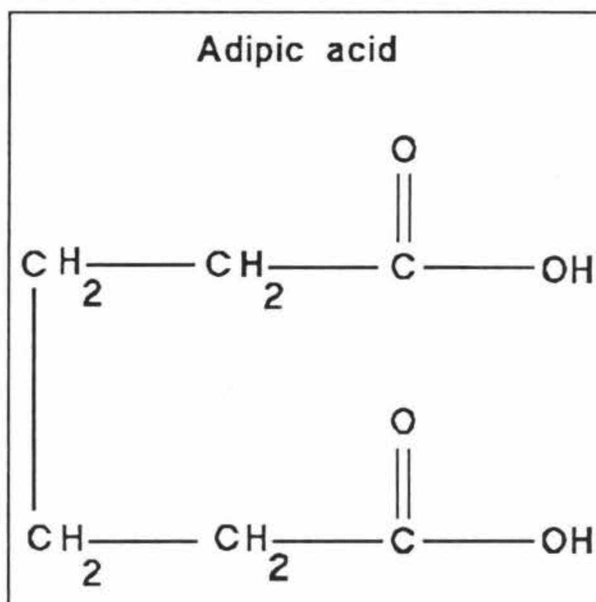
Use in food and regulatory status of citric acid.

The applications of citric acid utilise one or more of the following properties, acidity (taste, pH control), preserving properties, emulsifying/solubilising properties and as a chelating agent, due to its ability to form relatively stable complexes with metal ions. Both the acid and its salts are permitted in dairy products, various fruit juice drinks,

diluted juice beverages and nonalcoholic carbonated beverages. Citric acid and its potassium and sodium salts are classified by the FDA as GRAS for miscellaneous, general purpose or both types of uses. In the EEC citric acid is acknowledged as a food additive under No E330. The Germany Food Law does not regard citric acid as a foreign substance but as a natural flavour.

2.5.4 Adipic acid.

A white crystal or crystalline powder only slightly soluble in water, but soluble in acetone and alcohol and almost nonhygroscopic. Low hygroscopicity and lingering high tartness that complements grape - flavored products and those with delicate flavors characterise this dry, white crystalline acidulant. Characterised by a smooth, tart taste, adipic acid is used to impart a long lasting flavor note, especially to flavored dry mixes such as grape. It is made by the oxidation of cyclohexanol with nitric acid (Anon, 1974).



Aqueous solutions of adipic acid are the least acidic of all common food acidulants and show strong buffering at pH2.5 - 3.0 (McCormick et al. 1983; Gardner, 1977). The acid functions as a buffer, neutralising agent, gelling agent and sequestrant (McCormick, 1983).

Use in food and regulatory status of Adipic acid.

Applications of adipic acid include evaporated milk, instant puddings, nonstandardised jams, gelatin desserts, cheese, snack foods, powders, meat products, fat oils and flavoring extracts. Adipic acid helps extend shelf life of dry mixes and baking powders because of its extremely low rate of moisture absorption.

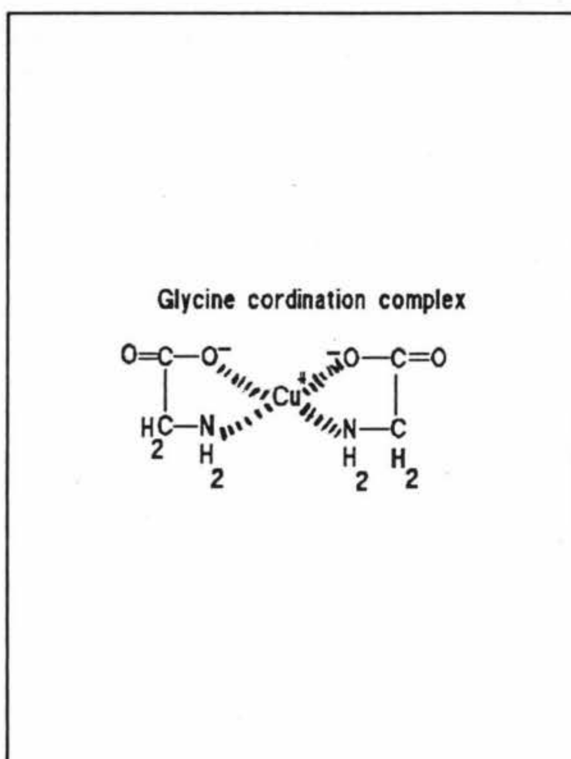
Regulatory status.

FDA - 21 CFR 172.515, 184.1009. GRAS with a limitation of 0.05% in baked goods, 0.005% in nonalcoholic beverages, 5% in condiments and relishes, 0.45% in dairy product analogs, 0.3% in fats and oils, 0.004% in frozen dairy desserts, 0.55% in gelatin and pudding, 0.1% in gravies, 0.3% in meat products, 1.3% in snack foods, 0.02% in other food categories when used in accordance with good manufacturing practice (GMP).

2.5.5 Glycine.

Glycine is the simplest of all the amino acids, {$CH_2(NH_2)COOH$}.

It appears in the UK list of additives but not in the FAO/WHO list, although it is difficult to imagine any adverse effect since it is a common constituent of proteins. Glycine form a coordination complex with copper and other metal ions. Thus it acts as a sequestrant, binding alkaline-earth metal ions in the same manner as EDTA, citrate and polyphosphates.

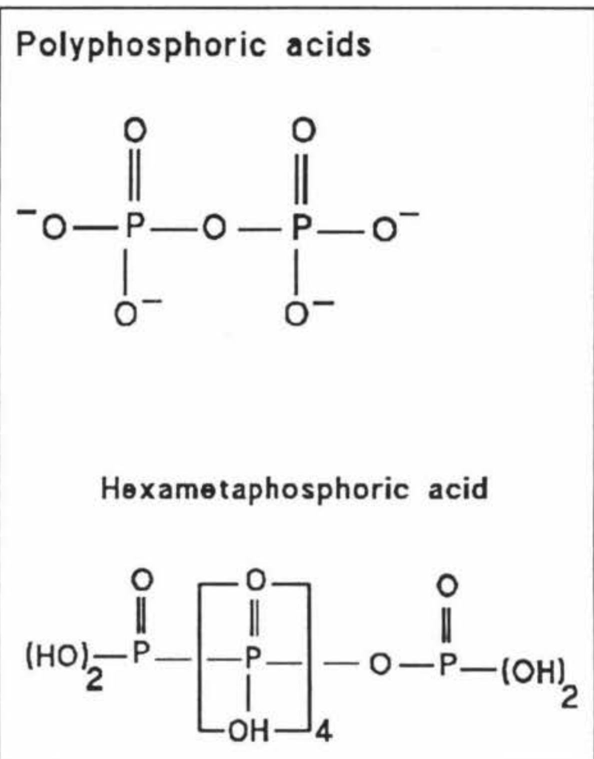


2.5.6 Phosphates.

Certain rocks, especially phosphate rock are the least expensive and the source of a diverse class of compounds commonly used as food ingredients. Emulsification, acidification, dispersion, metal chelation, buffering, water binding, leavening and imparting an acidic taste are just a few of the many functional properties phosphates play in food.

Inactivation of metal ions.

Calcium, magnesium, copper and iron cations in food systems form undesirable precipitates or participate in chemical reactions that lead to discoloration and off flavor development. Phosphates inactivate these ions by either sequestering or precipitating them. In sequestering metal cations, phosphates form a soluble complex with cations, so the metals remain in the food system, yet are rendered incapable of interfering with processing reactions. Calcium and magnesium cations and related alkaline earth metal cations are best sequestered by polyphosphates such as sodium hexametaphosphate, the complexing efficiency of these increases with increasing pH (Ellinger, 1972). Although phosphates have never been classified as antimicrobial agents (Molins, 1991), a number of investigators have reported that phosphates have indirect (Nelson et al. 1983; Sofos, 1986; Tompkin, 1983; Wagner, 1983) and direct (Kelch et al. 1958; Post et al. 1963; Lee, 1994) antimicrobial activities. That phosphates act as growth inhibitors of certain food-spoilage microorganisms has been attributed to their ability to sequester calcium, magnesium and iron, these minerals are essential for microbial growth.



Use in foods and regulatory status of phosphates.

Phosphates are used in a wide range of foods and are GRAS.

Phosphates are among the most important ingredients in dairy, bakery, meat, poultry and seafood products. They perform a variety of functions that include emulsification, buffering, metal sequestration, leaving, protection against oxidation and microbial growth (Barbut et al. 1989), control of protein coagulation and dispersion of proteins. By complexing magnesium ions, sodium hexametaphosphate and other phosphates effectively prevent struvite formation, transparent crystals of magnesium ammonium phosphate sometimes mistaken by consumers for glass (Ellinger, 1972).

Phosphates have been approved for use in meat products (U.S. Department of Agriculture, 1982). Mono-, di-, and tribasic calcium phosphate, sodium acid pyrophosphate, sodium aluminium phosphate and sodium tripolyphosphate are GRAS.

2.6 Experimental design.

The design of experiments is a plan for deliberately varying the variables in question in order to study their effects on some specific result. It enables several variables to be studied simultaneously, the greatest amount of meaningful data is obtained from the fewest number of experiments and at the lowest cost. Ultimately experimental design enables researchers to predict through model equations the effect of changes made to any of the variables found to be critical. Historically, factorial designs dates back to the 1920s when they were introduced by Sir R.A. Fisher to counter the prevalent idea that if one were to discover the effect of a factor, all other factors must be held constant and only the factor of interest could be varied. Fisher (1925) showed that all factors of interest could be varied simultaneously and the individual factor effects and their interactions could be estimated by proper mathematical treatment. The Yates algorithm and its variations are often used to obtain these estimates; least squares fitting of linear models gives essentially identical results. Factorial designs have been updated by several individuals, particularly noteworthy are the contributions of (Deming, 1987; Box, 1978, 1987; Taguchi, 1980).

2.6.1 "One-variable-at-a-time" experimentation.

This is a method which can be used for problem solving, but is neither economical nor efficient. The "one-variable-at-a-time" approach is to fix all of the variables except one

and then study the behaviour of the system at several levels of that variable. For each variable the best value is found and the process is repeated for the remaining variables until all variables have been considered. This method may be effective in some situations, but it is very inefficient. Jiang et al. (1985) and Cheng et al. (1979) optimized individual unit operations in surimi processing using the one-variable-at a time approach. Hsu (1990) demonstrated the limitations of this approach which included inability to determine the optimal conditions, inability to determine interactions and inefficiency of experimentation.

2.6.2 Factorial designs.

In two-level factorial designs, each variable is studied at only two levels, often called the (-) and (+) levels. The factorial experiment consist of conducting all possible combinations of variables and levels. For instance, to study three variables (A, B, and C) at two levels each, eight combinations of variables and levels are necessary. Such an experiment is called a two-level factorial experiment. From such a factorial experiment, the effect of each of the variables and all the possible interactions can be determined. Thus the effect of the variables A, B, and C; the two factor interactions AB, AC and BC; and the three factor interaction ABC all can be obtained. This is done by exploiting the benefits of an orthogonal design which makes it possible to identify separately the variation in the measured response attributed to each term in the fitted model equation (Cox, 1958). Full factorial designs, introduced by Fisher, are commonly used for screening but are also useful for optimization. Factorial designs can be complex and require many experimental trials if there are many factors to be investigated. The usual notation for describing factorials is m^k where k represents the number of factors and m, the number of levels of each factor. A design that involves three factors, each to be tested at two levels, is a 2^3 factorial and has 2^3 or eight factor combinations.

Fractional factorial designs.

These consist of appropriately chosen small fractions of the full factorial designs. It is clear that as the number of variables increases, the number of trials for a factorial experiment increases geometrically, creating the need for fractional factorial designs.

A large number of variables can be studied in an economical number of trials (15 variables in 16 trials). However, some of the effects will be confounded.

2.6.3 Factorial design and its advantages over 'one-variable-at-a-time' experimentation.

Factorial designs have several distinct advantages over their single-factor counterparts. Compared with traditional techniques in which only one variable is varied at a time and all other variables are held constant, designed experimentation offers several advantages. These include:

Economy

One potential advantage of a factorial experiment is a marked reduction in cost in terms of number of treatmentments, time, energy and money, for essentially the same information obtainable from corresponding single-factor experiments.

Joint manipulation of independent variables.

The essence of the factorial design is the joint manipulation of two or more independent variables. Two advantages result from this feature of factorial designs. The first advantage is that factorial designs give a better approximation of a given objective than single-factor designs.

Ability to determine interaction.

A second consequence of the joint manipulation of two independent variables is the opportunity to determine how the two combine to influence the response variable in a process. In the absence of interactions, factors would have an additive effect on the response. If there is a two factor interaction, two factors involved exert a synergistic or antagonistic effect on each other. One independent variable can change the pattern of the results observed with another independent variable when both variables are included in a factorial design. If there are interactions among the variables the "one-variable-at-a-time" method may miss the solution because it does not thoroughly explore the region of possible solutions. This inability to determine these interactions can cause very misleading conclusions.

Ability to identify true optimum settings.

The designed strategy can identify "true" optima while the traditional methods cannot. A classical example of the above is the work by Joglekar et al. (1987). Working on a bread making process, they demonstrated that the one-variable-at-a-time approach failed to determine the optimum proof time and proof temperature that would maximize the specific volume of the loaf. The approach also failed to determine interaction between time and temperature. The one-variable-at-a-time approach was also demonstrated to be inefficient. The factorial design approach could cut the cost of experimentation substantially by reducing the number of trials needed to achieve a given objective. Despite its economic benefits, experimental design is often dismissed as "taking too much time" and being "too complicated".

2.6.4 Strategy of designed experimentation.

The following is a brief review of some of the major principles of design of experiments. A more detailed explanation of the subject is however available (Box et al. 1978; Taguchi and Wu, 1980; Joglekar, 1987).

The objectives to be achieved are correctly identified.

Proper responses (dependent variables) to be measured and independent variables (factors) which are believed to have an effect on the problem and are believed to be relevant are identified.

Levels of independent variables should cover the ranges of interest and sufficient measurements collected to permit adequate analysis.

An effective experimental strategy can then be developed.

In the first stage, screening, the objective is to efficiently determine the critical factors from a collection of many potential factors (separate the "vital few" factors from the "trivial many"). Two-level factorial designs and fractional factorial designs often are best suited for this purpose. The specific screening design to be used to test the factors is selected. The experiments are conducted in a randomized order to eliminate bias. The data are then analysed using regression analysis or Yates' algorithm to 'fit the model to the data'. In performing the regression analysis, the data are coded before conducting the statistical analysis (i.e -1=low, +1=high). This allows direct comparison of the coefficients for different factors even if the factors are in different units. A model

is developed for each response evaluated, a model being an equation that will be used to predict response values for different factor levels after data have been collected and analysed. Most models will be linear, being constructed of additive terms, each of which contains only one multiplicative parameter (Deming and Morgan, 1987). A complete model for a three-factor design would be:

$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x3 + \beta_{123} x_1 x_2 x_3 + r$ where Y represents the response, β_0 stands for the y-intercept, the $x_1, x_2 .... x_1 x_2 x_3$ represent factors, the β values, called parameters, are the slopes with respect to each factor or interaction between factors, and r is the residual (or unexplained variation). User-friendly software packages can speed up construction of appropriate designs and data analysis by researchers. Some packages may be oriented toward response surface (refer section 2.6.6) but lack screening designs needed during early stages of the experimental work. Because none of the packages is comprehensive, two or three different packages may be required to meet all needs.

2.6.5 Model fitting.

Fitting the model to data involves generating an analysis of variance (ANOVA) and running a variety of diagnostic tests to assess the adequacy of the model. These tests include percentage confidence, percentage variation accounted for by the model and coefficient of variation. Analysis of residuals can also be used to check the model. Once the model is found to be adequate, it can be used to predict the effect of any combination of factor levels evaluated.

In the second stage, optimization, the objective is to find the optimum settings of the critical control factors. Multilevel response surface designs often are best suited for this purpose.

2.6.6 Response surface methodology (RSM)

Response surface designs were developed by Box and his colleagues at Imperial Chemical Industries to explore relationships such as those between the yield of a chemical process and the pertinent process variable (Box and Wilson 1951; Box, 1954; Box and Youle, 1955). In its usual form, RSM exploits simple empirical models such as low-degree polynomials to approximate relationship between a response variable and

a set of input variables over a current region of interest. Since its introduction in the early 1950's, RSM has become an accepted and widely used set of concepts and techniques.

RSM uses quantitative data to build an empirical model that describes the relationship between each factor investigated and the response. For simplicity the number of factors are usually limited to two or three. The model takes into consideration the effects of each factor, interaction between and among factors and curvature. Response surface designs have at least three levels of each variable. Graphically, the model might describe a three dimensional surface in which levels of two factors are presented on horizontally perpendicular axes and a response on the vertical axis. Response surface plots provide researchers with an understanding of how they might expect the system to behave when factor levels are changed. Equally important, plots suggest optimum levels of the factors needed for achieving specific results. One of the most popular response design is the central composite designs, which can be used to estimate parameters of a full second order polynomial model. These designs can be constructed in a sequential programme of experimentation by building on to information gathered previously from a 2^k factorial design. Joglekar and May (1987) used such a sequential method to optimize a cake formulation; they reduced the number of experimental trials from 77 done by earlier workers to 25 trials and obtained nearly identical results. A full discussion of response surface design is available (Box and Draper, 1987). Reports on the use of RSM to optimize biochemical reactions and microbial processes remain relatively scarce (Strobel et al. 1993). This situation is changing, however, with the advent of personal computers and statistical programs for the processing of experimental data.

2.6 Modified atmosphere packaging.

A general definition of a food stored in a modified atmosphere packaged (MAP) environment is one in which a food product is stored in an environment whose gas composition is different from that of air. In true modified atmosphere packaging, the particular gas mixture desired is flushed only once at the time of packaging into an evacuated or nonevacuated environment surrounding the food. MAP foods have become

increasingly more common as food manufacturers have attempted to meet consumer demands for fresh, refrigerated foods with extended shelf-life. Much information exists in the general area of MAP technology, however research on the microbiological safety of these foods is still lacking. The great vulnerability of MAP foods from a safety point of view is that with many modified atmospheres containing high levels of CO_2, the aerobic spoilage organisms which usually warn consumers of spoilage are inhibited, while growth of pathogens may be allowed or even stimulated (Farber, 1990). New safety concerns have been raised because of the emergence of psychrotrophic pathogens such as *L. monocytogenes, Aeromonas hydrophila* and *Yersinia enterocolitica*. The extended shelf-life of many MAP products may allow extra time for those pathogens to reach dangerously high levels in a food. A review of the effects of MAP on the growth and survival of foodborne pathogens is available (Farber, 1990).

2.6.1 Gases used in MAP.

Food grade oxygen, nitrogen and carbon dioxide are the three main gases used commercially. Oxygen stimulates the growth of aerobic bacteria, can inhibit the growth of strict anaerobic bacteria and in MAP meats it maintains myoglobin in its oxygenated form, oxymyoglobin, a form responsible for the bright red color which consumers associate with fresh red meat.

Nitrogen is an inert tasteless gas with little or no antimicrobial activity on its own. It has a low solubility in water, thus its presence in a MAP food prevents pack collapse that occurs when high concentrations of CO_2 are used. By displacing oxygen in a pack, oxidative rancidity is delayed and aerobic microorganisms are inhibited.

Carbon dioxide is both water- and fat-soluble and is responsible for the bacteriostatic effect seen on microorganisms grown in MAP environments. The specific effect in which CO_2 exerts its bacteriostatic effect is unknown, its overall effect on microorganisms is an extension of the lag phase of growth and a decrease in growth rate during the logarithmic phase.

There have been many theories regarding the way in which CO_2 exerts its influence on a bacterial cell (Daniels et al. 1985; Dixon et al. 1989).

These include.

Alteration of cell wall membrane function including effects on nutrient uptake

and absorption.

Direct inhibition of enzymes or decrease in the rate of enzyme reactions.

Penetration of bacterial membranes, leading to intracellular pH changes.

Direct changes of the physico-chemical properties of proteins.

The inhibitory effects of CO_2 on microorganisms in a food system are dependent on many factors. These include CO_2 partial pressure, CO_2 concentration, headspace gas volume, temperature, acidity, a_w, microorganism type, phase of microbial growth and the growth medium.

For maximum antimicrobial effects, storage temperatures of MAP product should be kept as low as possible because the solubility of CO_2 decreases dramatically with increasing temperature (Daniels et al. 1985).

2.8 Methods for testing the efficacy of food antimicrobial agents.

Methods which are used to evaluate the activity of food antimicrobials may be divided into in-vitro and application tests. The in-vitro tests may be termed 'screening methods' and might include any test in which the compound is not applied directly to the product under use conditions. Generally these tests provide preliminary information to determine potential usefulness of the test compound. The second type includes those tests in which an antimicrobial is applied directly to a food product. Screening methods may be subdivided into endpoint and descriptive test. Endpoint tests are those in which a microorganism is exposed to an antimicrobial for an arbitrary period. The results reflect the inhibitory power of a compound only for the time specified. In descriptive tests the microorganism is exposed to the antimicrobial, but periodic sampling is carried out to determine changes in viable cell numbers over time.

2.8.1 Descriptive screening methods.

While endpoint methods are excellent for screening compounds, they give little information concerning the effect of the compound on dynamic growth of microorganisms. In food products, we are interested in using the minimal antimicrobial concentration needed to cause inhibition of potential spoilage or pathogenic microorganisms. Even extension of the lag phase of a microorganism can be important under conditions of abuse. There are several possible methods for evaluating the effect

of an antimicrobial on the growth of a microorganism over time. Two of the most popular are the turbidimetric assay and the inhibition curve.

Turbidimetric Assay.

The simplest, most economical, rapid and productive growth measurement system is probably the turbidimetric assay. It simply involves following the growth of a microorganism with a spectrophotometer. Absorbance is monitored at 600nm. Two types of inhibition can be observed. First there can be suppression of final growth level compared to the control, and second, there can be a lag phase extension of the treated cells. Detecting the lag phase extension is a function of read time. Determining the lag phase extension precisely would require sampling at shorter intervals at the initiation of the assay.

Inhibition or kill curve.

In addition to the turbidimetric analysis, there is the inhibition curve, also known as the "killing" curve in clinical research (Schoenknecht et al. 1985). This test simply involves inoculation of a microorganism into a medium, addition of an antimicrobial, followed by incubation and periodic sampling to determine growth or survival. It is a more accurate analysis than the turbidimetric assay because of the wide detection range. This method is versatile but has several disadvantages.

No simple statistical method is available to detect differences.

No single statistic is produced to compare treatments such as the minimum inhibitory concentration (MIC).

It is labour intensive and expensive.

Screening methods should be used together, one endpoint and one descriptive test. The endpoint test helps to determine the approximate effective concentration and the descriptive test evaluates the effect of a compound on growth over time.

2.8.2 Application methods.

While in-vitro tests can give a good deal of information on antimicrobial performance, they cannot necessarily duplicate all the variability which might exist in a food. Therefore, once it has been determined that the antimicrobial performs well in the in-

vitro situation, it should be applied to a food system. Studies utilizing defined model systems may provide a method to investigate any interactions prior to studies involving actual food systems. For example Knox et al. (1984), used the broth dilution method to study the effect of various antimicrobials in apple juice and grape juice.

In the light of the fact that the use of antimicrobials is becoming more important in the food processing systems and at the same time more complex, there is need for a set of uniform procedures for testing the activity of these compounds. An efficient data base can be built which can be modeled.

CHAPTER 3.

MATERIALS AND METHODS

3.1 Strains and maintenance conditions.

Stock cultures of *Listeria monocytogenes* and *Clostridium tyrobutyricum* were obtained from Massey University, Department of Food Technology, Food Microbiology culture collection. The stock cultures were maintained on brain heart infusion (BHI) (Oxoid) agar slants. The slants were stored at 4C and subcultured every month to maintain viability. Culture purity and identity were confirmed at the time of each subculturing.

3.2 Chelating agents and chemical reagents.

Various chelators were examined for their ability to potentiate lysis of growing cells of *L. monocytogenes* and *C. tyrobutyricum* in the presence of lysozyme. The chelators examined were ethylene diaminetetraacetic acid(EDTA), glycine(gly), gluco-delta-lactone(GDL), sodium hexametaphosphate(SHMP), sodium phosphate (dibasic phosphate)(SPDB), citric acid monohydrate (citrate)(CA). All chelators and chemical reagents used were obtained from BDH Laboratory Supplies, Poole, BH15 ITD, England and were of analytical grade.

3.2.1 Whey protein concentrate(WPC).

WPC(Alacen 475) was obtained from New Zealand Dairy Board, Wellington.

3.3 Lysozyme

3 x Crystallized, Dialysed and Lyophilized hen egg white lysozyme(EC 3.2.1.17) with a specific activity of 48 000 units/mg solid {one unit producing a change in absorbance at 450nm of 0.0001 per minute at pH6.26 at 25C, using a suspension of *Micrococcus lysodeikticus* as substrate, in a 2.6ml reaction mixture (1 cm path)} was purchased from Sigma Chemical Co. St. Louis, USA., and was stored at 0C.

3.4 Broth culture systems: Turbidimetric assays.

The most economic, rapid and productive growth measurement system, the turbidimetric

assay, was the method of choice for monitoring microbial growth or inhibition. The method simply involves monitoring microbial growth using a spectrophotometer at 600nm.

3.4.1 Preparation of buffered media.

The basic medium for all growth experiments was BHI broth (Oxoid).

Buffered growth media pH5.5 and pH6.5.

Media

M1 :BHI containing 10mM chelator buffered to pH5.5.

M2 :BHI containing 10mM chelator buffered to pH6.5.

M3 :BHI containing 25mM chelator buffered to pH5.5.

M4 :BHI containing 25mM chelator buffered to pH6.5.

Control :BHI adjusted to pH6.5 with HCl.

Media M1-M4 were prepared in 300ml volumes. The required amount of chelator to make a 10mM or 25mM concentration in 300ml was weighed into a beaker. The required amount of BHI in 300ml of distilled water was then added. 250ml of distilled water was added and the chelator and BHI were dissolved completely. After dissolving and boiling the cooled samples were then adjusted to pH5.5 and pH6.5 using 10N NaOH, 1N NaOH, 0.1N NaOH, 1N HCl and 0.1N HCl thus obtaining buffered growth media.

The volume was then measured out in a measuring cylinder and the volume was made up to the 300ml mark using distilled water. After mixing thoroughly, the pH was then rechecked. The growth media were then dispensed in 50ml volumes into 250ml Erlenmeyer flasks. All media were sterilized by autoclaving at 121C for 15min. After cooling to room temperature the required amount of lysozyme to make a concentration of 0.2mg/ml or 3mg/ml was weighed into duplicate flasks and dissolved completely. At the end of each run, the pH was checked to establish whether buffering was

sufficient to prevent serious pH changes.

3.4.2 Inoculum preparation and inoculation procedure.

To prepare an inoculum, the test strain was transferred from the BHI slant to brain heart infusion broth and grown overnight at an incubation temperature of 37C. The culture turbidity was adjusted spectrophotometrically using BHI broth so that on inoculation of 0.5ml of culture into 50ml of each treatment the microbial load would be ca. 1×10^5 - 1×10^6 *L. monocytogenes* cfu/ml as estimated from prior determination of the relationship between viable count and absorbance at 600nm. Checks were made by absorbance and viable counts that inocula were approximately equal.

3.4.3 Growth conditions.

The media were incubated at 25C under aerobic conditions in a reciprocating shaker water bath(100 strokes/min). Flasks were equilibrated at 25C for 30 minutes before culture inoculation. All experiments were replicated twice for the full factorial experiment as well as for treatments without the lysozyme.

3.4.4 Growth measurements.

Absorbance was measured using a Hitachi Model U-2000 double beam spectrophotometer(HITACHI, Ltd. Tokyo, Japan). Measurements were made at 600nm on an aseptically sampled 2ml volume of culture.

3.4.5 Plating medium, diluent and enumeration procedure.

At various time intervals, 1ml volumes were aseptically pipetted from the different treatments and decimally diluted in 0.1% peptone water. The number of colony forming units of *L. monocytogenes* and *C. tyrobutyricum* was determined on BHI agar (Oxoid). Enumeration was done on all treatments initially, at the end of the experimental run and at appropriate intervals on treatments that showed no increase in absorbance at 600nm. Duplicate plates were inoculated with 0.1ml of each dilution which was then surface spread using a bend glass rod which was flamed with alcohol after each plate surface spreading. For *L. monocytogenes*, plates were incubated aerobically at 37C for 24-48hrs. For *C. tyrobutyricum*, plates were incubated anaerobically at 37C for 24-48hrs.

3.4.6 Confirmation of identity of microorganisms.

Test species confirmation was carried out during subculturing stages and during experimental runs.

L. monocytogenes.

An isolate was considered to be *L. monocytogenes* when the following results were obtained: Gram stain(small Gram-positive, nonspore forming rods), catalase(+), carbohydrate fermentation{xylose(-), rhamnose(+), mannitol(-)}, motility(tumbling motility at 25C in Bacto motility test medium) and unrestricted growth on Listeria Selective agar (LSA)(Oxford formulation)(Oxoid).

C. tyrobutyricum.

An isolate was considered to be *C. tyrobutyricum* when the following results were obtained: Gram stain (Gram positive spore forming rod), spore location (subterminal), catalase(-), gelatin(-), carbohydrate fermentation{maltose(-), lactose(-), starch(-) and nitrate(-).

3.5 Bacteriolytic effects of lysozyme-GDL combinations on non-growing bacteria in buffer.

The following protocol was used for all experiments:

Test species were grown to a population density of approximately 5 x 10^8 cfu/ml, centrifuged (all centrifugation was done for 20min at 5000 x g), washed twice in sterile 0.067M sodium phosphate buffer pH6.5 and resuspended in the following treatments.

(1) cell buffer

(2) Xmg/ml lysozyme and YmM of GDL in cell buffer.

The final pH of all treatment was pH6.5. Replicate treatments were incubated at 25C in a reciprocating shaker water bath (100 strokes/min). At regular intervals 2ml of culture were aseptically withdrawn and absorbance was determined at 450nm using a spectrophotometer.

3.6 Variables, experimental design and statistical analysis.

The experimental variables and levels are presented in Table 3.1(a). The actual concentration of each factor was coded to facilitate regression analysis.

Table 3.1(a).

Independent variables and their levels in 2^2, 2^3 and 2^5 full factorial designs.

Independent variable	Symbol	Level coded*	Level uncoded
Chelator	A	+1	25mM
		0	17.5mM
		-1	10mM
Lysozyme	B	+1	3mg/ml
		0	1.6mg/ml
		-1	0.2mg/ml
pH	C	+1	6.5
		0	6.0
		-1	5.5
Temperature	D	+1	25C
		0	15C
		-1	5C
Inoculum	E	+1	8E6 cfu/ml
		0	8.9E5 cfu/ml
		-1	1E5 cfu/ml

$$\text{Coded}^{*}\text{variable} = \frac{\text{uncoded value} \pm 0.5(\text{high value} + \text{low value})}{0.5(\text{high value} - \text{low value})}$$

Several full factorial designs were used at different stages. At pH7.0, a simple two factor, two level (2^2) factorial design, Table 3.1(b) with two replicates was used to investigate the effect of lysozyme and various chelators on *L. monocytogenes* and *C. tyrobutyricum* in a series of preliminary experiments. Building on the information acquired from the preliminary experiments, a full factorial, three variable, two level (2^3) design, Table 3.1(c) with two replicates was then employed to assess the effect of pH(5.5, 6.5), chelator(10mM, 25mM) and lysozyme(0.2mg/ml, 3mg/ml) on the growth response of *L. monocytogenes* and *C. tyrobutyricum*. The lysozyme-GDL system which demonstrated a potential for being applied to food systems was further investigated in a full 2^5 factorial design, Table 3.2, to give a full insight into how pH, GDL, temperature, inoculum level and lysozyme would influence microbial inhibition.

Table 3.1(b)

Variables, symbols and levels for simple 2^2 factorial design experiments:

Factor name	Symbol	Units	Low level	High level
chelator	A	mM	10	25
lysozyme	B	mg/ml	0.2	3

Response	Unit
Yield	Area under the curve of a ΔA_{600nm} versus time growth curve:

Experimental worksheet for 2^2 factorial design experiments:

Variables: 2 Response: 1 Runs: 4

Centerpoints: 0 Design: 2^2 Blocks: 0

Run	Yates code	Experimental factors: chelator(A)	lysozyme(B)
1	1	−1	−1
2	a	+1	−1
3	b	−1	+1
4	ab	+1	+1

Table 3.1(c)

Orthogonal design matrix and treatment combinations:

Experimental worksheet for 2^3 factorial experiments.

Variables: 3 Response: 1 Runs: 8

Centrepoints: 0 Design: 2^3Factorial Blocks: 0

Run	Yates code	Experimental Factors: chelator(A)	lysozyme(B)	pH(C)
1	1	−1	−1	−1
2	a	+1	−1	−1
3	b	−1	+1	−1
4	ab	+1	+1	−1
5	c	−1	−1	+1
6	ac	+1	−1	+1
7	bc	−1	+1	+1
8	abc	+1	+1	+1

Table 3.2

Experimental worksheet for a 2^5 full factorial design:

Variables:	2	Response:	1	Runs:	35
Centerpoints:	3	Design:	2^5 factorial	Blocks:	2

		Experimental factors				
Run	Yates code	A	B	C	D	E
1	1	−1	−1	−1	−1	−1
2	a	+1	−1	−1	−1	−1
3	b	−1	+1	−1	−1	−1
4	ab	+1	+1	−1	−1	−1
5	c	−1	−1	+1	−1	−1
6	ac	+1	−1	+1	−1	−1
7	bc	−1	+1	+1	−1	−1
8	abc	+1	+1	+1	−1	−1
9	d	−1	−1	−1	+1	−1
10	ad	+1	−1	−1	+1	−1
11	bd	−1	+1	−1	+1	−1
12	abd	+1	+1	−1	+1	−1
13	cd	−1	−1	+1	+1	−1
14	acd	+1	−1	+1	+1	−1
15	bcd	−1	+1	+1	+1	−1
16	abcd	+1	+1	+1	+1	−1
17	e	−1	−1	−1	−1	+1
18	ae	+1	−1	−1	−1	+1
19	be	−1	+1	−1	−1	+1
20	abe	+1	+1	−1	−1	+1
21	ce	−1	−1	+1	−1	+1
22	ace	+1	−1	+1	−1	+1
23	bce	−1	+1	+1	−1	+1
24	abce	+1	+1	+1	−1	+1
25	de	−1	−1	−1	+1	+1
26	ade	+1	−1	−1	+1	+1
27	bde	−1	+1	−1	+1	+1
28	abde	+1	+1	−1	+1	+1
29	cde	−1	−1	+1	+1	+1
30	acde	+1	−1	+1	+1	+1
31	bcde	−1	+1	+1	+1	+1
32	abcde	+1	+1	+1	+1	+1
33	zero	0	0	0	0	0
34	zero	0	0	0	0	0
35	zero	0	0	0	0	0

where A:chelator., B:lysozyme., C: pH., D: temperature., E: inoculum.

Absorbance readings of different treatment combinations were monitored to characterise microbial growth or inhibition. One dependent variable (yield) was taken as the area under the curve of a plot of absolute change in absorbance versus time. Data generated were analysed using Minitab (Minitab Release 8.2 Minitab , Inc.). Statistically significant differences ($P<0.05$) between treatments were determined using regression analysis.

3.6.1 Data processing.

The growth curves for each treatment combination were generated by taking the absolute change in absorbance and plotting it against time. The yield was then determined using a graphical software Fig.P version 6.0 (Biosoft, USA). The yield values were taken as the response variable for the statistical analysis.

3.6.2 Model equation development.

The statistical analysis and the development of linear models in terms of lysozyme, pH, chelator and their interactions were done by regression analysis using the Minitab program. Statistically significant variables and interactions identified from the statistical analysis were selected for the model. The fit of the model was evaluated using the R-squared values and analysis of residuals. For the analysis of residuals (i.e. actual - predicted values), a plot of the residual versus predicted yield was used to check that the residuals were randomly distributed about zero.

3.6.3 Response surface plots.

Response surface plots were constructed from three level designs (-1, 0, 1). The model equation developed on Minitab was used to develop the response surface plots. Surface plots were generated by a Surfer program (Golden Software Inc., Golden, Co. USA) for yield as a function of two independent variables while the other variable was held constant. Examination of the data in a graphical form such as response surfaces helps (a) to discern relationships between factors and responses and may point out regions for further investigation and (b) to show non-linearity of model (or interaction of factors).

3.7 Protein interference.

Inactivation procedure.

The bacterial strain used in this study was *L. monocytogenes*. BHI broth alone or in combination with GDL were prepared, the pH was adjusted and 50ml volumes were dispensed into 150ml conical flasks and autoclaved at 121C for 15min. 1mg/ml of whey protein concentrate (WPC) Alacen (A547) was added to each of the following four treatments.

(1) BHI broth pH5.5.

(2) 25mM GDL in BHI broth pH5.5.

(3) 3mg/ml lysozyme in BHI broth pH5.5.

(4) a combination of 25mM GDL and 3mg/ml lysozyme in BHI broth pH5.5. The treatments were equilibrated for 30min before culture inoculation and then incubated at 25C in a shaker water bath.

pH adjustment

0.1N NaOH, 1N NaOH, 0.1N HCl and 1N HCl were used for pH adjustment of the treatments 1-4.

Lysozyme.

Enough lysozyme to make the required concentration was added directly to sterile BHI broth (treatments (3) and (4) above) before culture inoculation.

Culture preparation and inoculation.

Inoculum preparation and inoculation procedure 3.4.2 was used.

Plating medium, diluent and enumeration procedure.

At appropriate intervals, 1ml of culture sample was aseptically withdrawn from each treatment, serial dilutions were performed in 9ml peptone water dilution fluid and duplicate 0.1ml volumes were spread onto modified nutrient agar containing 0.5g/l ferric ammonium citrate and 1g/l aesculin. This medium was used since it is non-selective, yet *L. monocytogenes* colonies can be readily confirmed by their surrounding black zone

caused by aesculin hydrolysis. Plates were incubated for 48hrs at 37C before counting.

Confirmation of identity of *L. monocytogenes.*

Colonies on modified nutrient agar(Oxoid) were further confirmed as *L. monocytogenes* using procedure 3.4.6

3.8 Food System.

Two food systems, cottage cheese and mussels were evaluated for the potential application of GDL and lysozyme-GDL preservative systems. Further experiments were conducted using the lysozyme-GDL treatment in MAP mussels.

3.8.1 Cottage cheese.

Raw milk was obtained from the local farm and delivered to the Food Technology Department pilot plant for processing. Uncreamed cottage cheese was produced in the pilot plant from skim milk according to the short-set method.

Preheating: use of a steam-jacketed pan.

A steam-jacketed pan was used to heat whole milk indirectly during the preheating stage. The steam-jacketed pan was half-filled with potable water and the water was then heated to boiling. Milk in 20litre plastic containers was immersed into the boiling water and was preheated to 40C to facilitate fat separation (Fig 3.1(P1)). Manual stirring was done during the preheating stage.

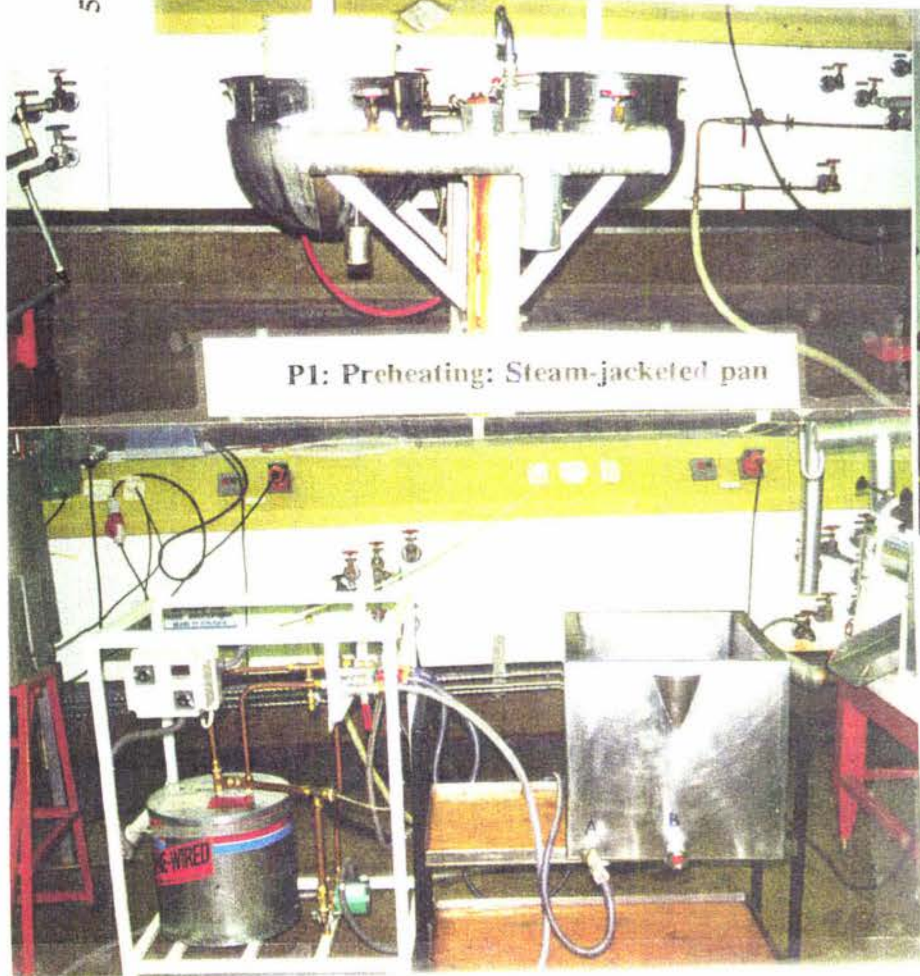
Skim milk production: use of a centrifugal separator.

A pilot plant-sized Westfalia separator model(LWA-205) was used to skim the preheated whole milk to produce the skim milk(Fig 3.1(P2)).

Skim milk pasteurization: use of an Alfa-Laval indirect UHT plant.

The equipment used is shown diagrammatically in Fig 3.1(P3). The plant was first presterilized at 120C for 15min before processing. A high temperature short time (HTST) method of pasteurization was used. During processing the skim milk was given a holding time of 16sec at 72C.

Fig 3.1 **Pictorial representation for cottage cheese production.**



P1: Preheating: Steam-jacketed pan

P4: Thermostatically-controlled water supplier connected to a cheese vat

P2: Skimming: Centrifugal separator

P3: Pasteurisation:

Alfa-Laval indirect UHT plant

Cottage cheese production

Cottage cheese was made according to the short-set procedure outlined in Table 3.3. A pilot plant-sized, water-jacketed stainless steel cheese vat was connected to a thermostatically controlled water supplier (Fig 3.1(P4)). Potable water from the mains was used to fill up the water supplier tank and the vat jacket. Once filled, the water supply from the tap was cut off and water circulated between the vat jacket and the water supplier. The temperature of the water could then be adjusted as required.
50 litres of pasteurized skim milk were placed in the cheese vat and warmed to 32C. The pH of the milk was then lowered to pH5.88 using 6N HCl. Single-strength calf rennet was then added and a holding time of 2hrs was allowed for the curd to set. Subsequent processing was as shown in Table 3.3. The thermostatically-controlled water supplier was set to increase the temperature of the curd/whey mixture. Wash water added to cool the curd was not chlorinated or acidified. A soft, white, irregular curd of cottage cheese was produced.

Cottage cheese treatment.

Preliminary experiments showed that the *L. monocytogenes* strain used in the study was representative of those strains with the ability to grow at a low temperature and low pH.

Inoculum preparation.

For all treatments *L. monocytogenes* was used. The inoculum was prepared from a culture grown in 200ml of BHI broth (Difco) for 24hrs at 37C. The culture was then centrifuged (Hermile Z320 bench centrifuge) in sterile centrifuge tubes at 5000xg for 20min and washed three times with 0.067M phosphate buffer pH6.5. The culture was then resuspended in fresh phosphate buffer pH6.5. The absorbance of the culture was then adjusted spectrophotometrically using phosphate buffer so that on inoculation of 20ml of culture the microbial load would be about 1×10^5 - 1×10^6 *L. monocytogenes* cfu/g of sample as estimated from a prior determination of the relationship between viable count and absorbance at 600nm.

Table 3.3

Processing of Raw Milk into Cottage Cheese

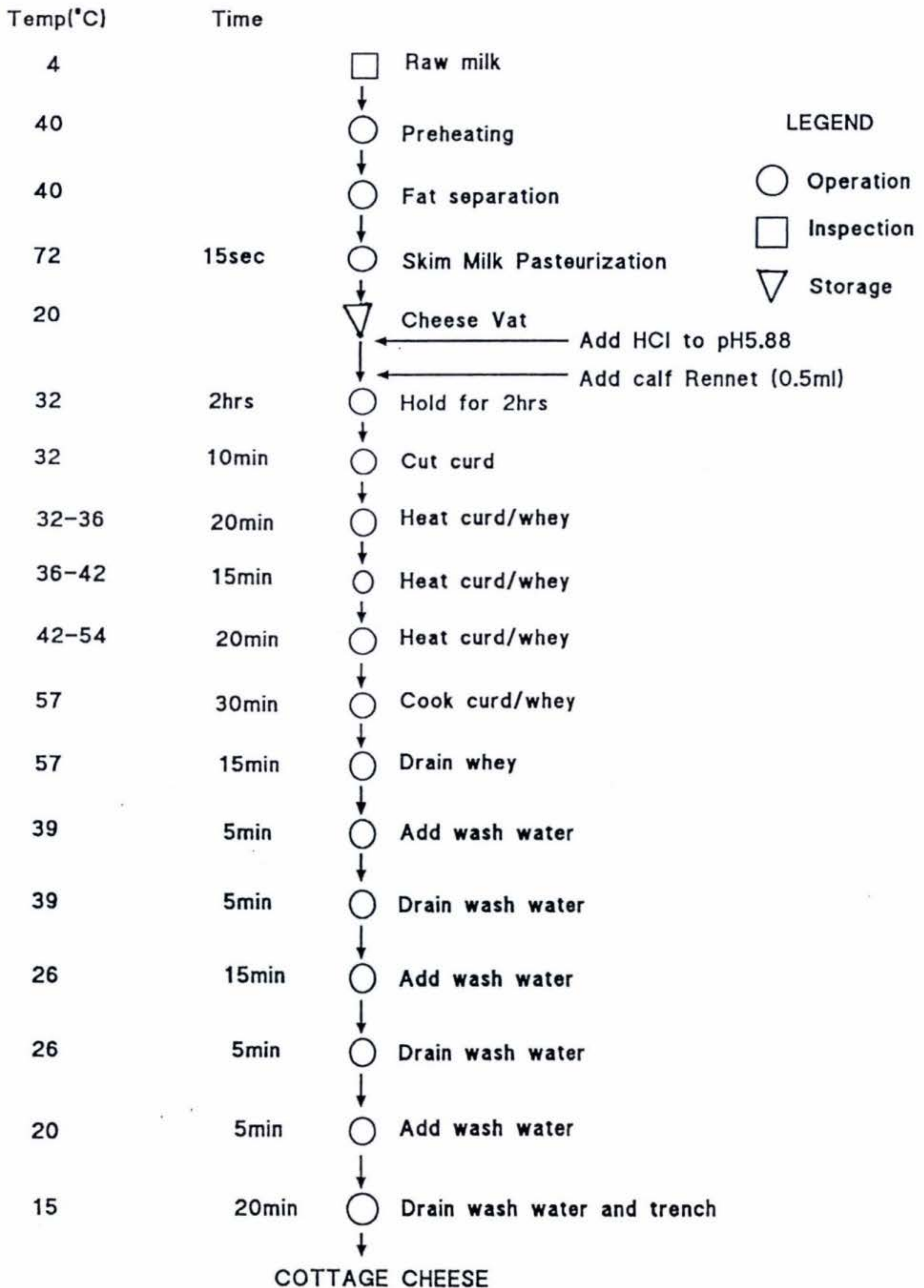
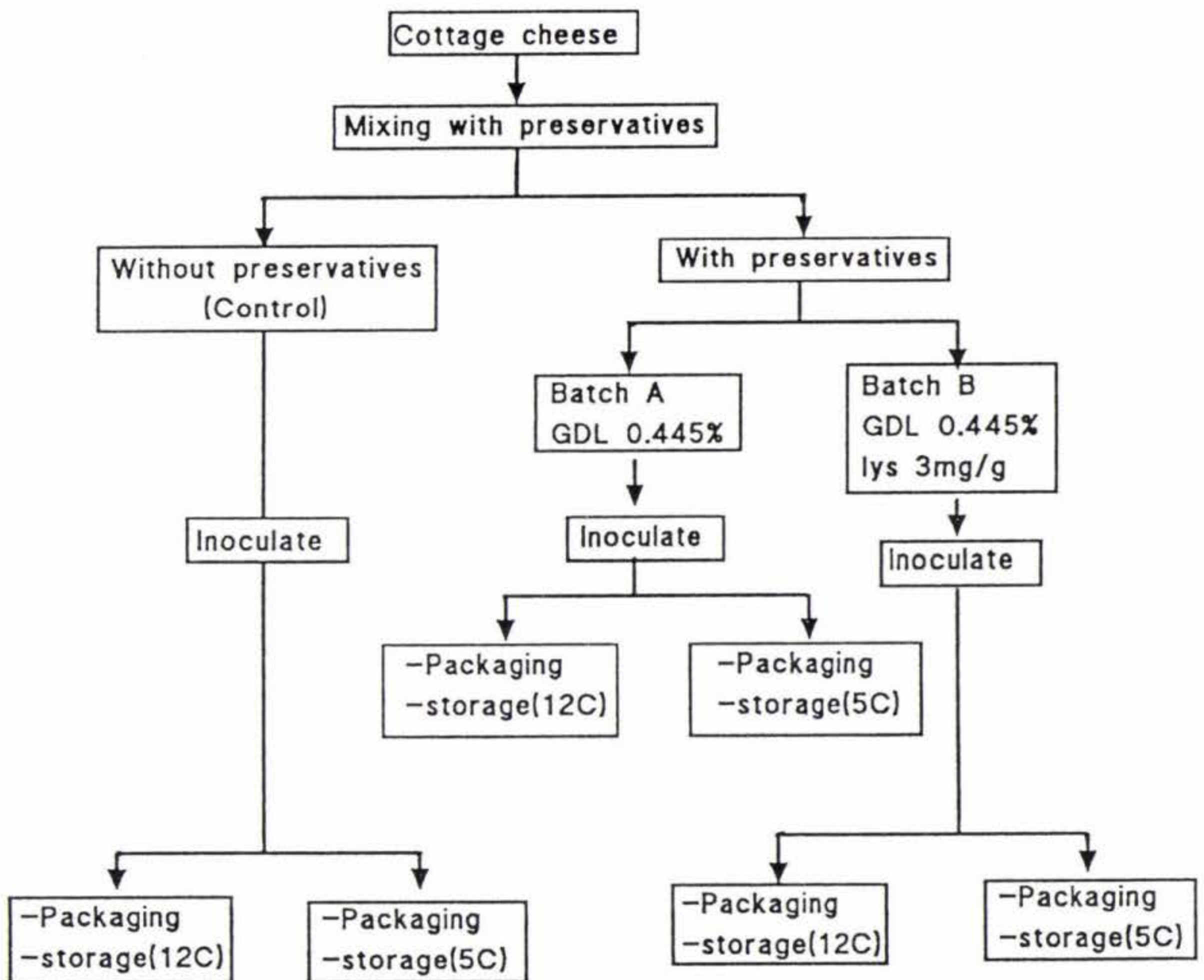


Table 3.4

Incorporation of lysozyme(lys), GDL and inoculum into the cottage cheese.



Incorporation of lysozyme, GDL and inoculum.

A blender was used to break up the cottage cheese curd batch after trenching, into irregular cottage cheese curd particles. The cheese batch was then subdivided into six 600g batches and preservatives and inoculum were then blended into the different batches as shown in Table 3.4. A food mixer (Kenwood cheff) was then used for blending all the additives. The additives were added in solution and to ensure a uniform water activity in all batches, all batches were treated to equal volumes of water, those not receiving additives were treated with the same volume water. Treatments were added in the sequence lysozyme, GDL and inoculum. A five minute blending time was allowed for each additive and inoculum to give an even distribution of lysozyme (3mg/g), GDL (0.445%) and inoculum (ca. 5 x 10^6 *L. monocytogenes* cfu/g).

Packaging and storage.

The treated batches were packed in plastic screw capped bottles in 30g portions. Samples were then incubated at 5C and 12C for 21 days.

Enumeration of viable microorganisms.

Immediately after inoculation of all batches, two 30g samples were used for enumeration of microorganisms on day 0. After incubation for 1, 3, 5, 9, 14, 17 and 21 days at 12C and 2, 4, 6, 10, 14, 17 and 21 days at 5C two samples were removed from incubation at each temperature and their microbial content enumerated. Each sample was treated in the same way. A 25g sample was removed from the stirred product, weighed into a sterile stomacher bag, diluted with 225ml 0.1% sterile peptone water and then blended in a Stomacher lab. blender 400(colworth) for 1 minute to give a homogeneous suspension. From the blended material, appropriate dilutions were made in 0.1% peptone water. *L. monocytogenes* was enumerated by plating 0.1ml of each dilution on LSA followed by incubation at 37C for 48hrs.

Aerobic plate count (APC).

0.1ml of each dilution was plated on nutrient agar and incubated at 30C for 24-48hrs.

Confirmation of identity of *L. monocytogenes*.

Isolates were confirmed as *L. monocytogenes* by using procedure 3.4.6.

pH determination.

The pH of blended samples from microbial analysis was measured using a standardised pH meter (Radiometer Copenhagen PHM82 STANDARD pH meter, Watson Victor Ltd, NZ) by immersion of the electrode into the product.

Water activity (a_w) measurement.

A water activity meter (Decagon CX-2) was used. A 5g sample was weighed into the sample pot and loaded into the a_w meter. The a_w and temperature readings were then recorded. The measurement was replicated four times and the average reading was expressed.

3.8.2 Mussels.

Raw material procurement.

Fresh green mussels(Fig 3.2(P5)) of the variety *Perna canaliculus*, harvested by A.D. & P. James Coromandel, under licence No. 373 were acquired from the local supermarket, Woolworth, Palmerston North, New Zealand.

Mussel processing.

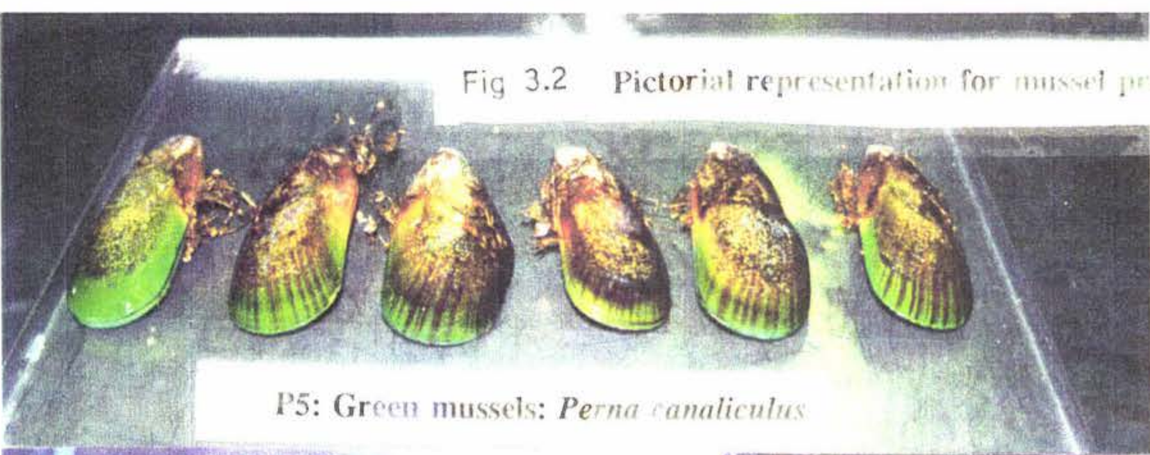
The exterior shells of the mussels were muddy and mud was often trapped inside the shells. There was no means of telling whether a shell contained mud or not, so it had to be assumed that all contained mud. In a single batch, the mussels were subjected to the following processing stages(Table 3.5).

Washing.

1. An overnight immersion in water to allow mussels to open and cleanse themselves of mud(Fig 3.2(P6)).
2. Rinsing with water to wash off exterior mud before further processing.

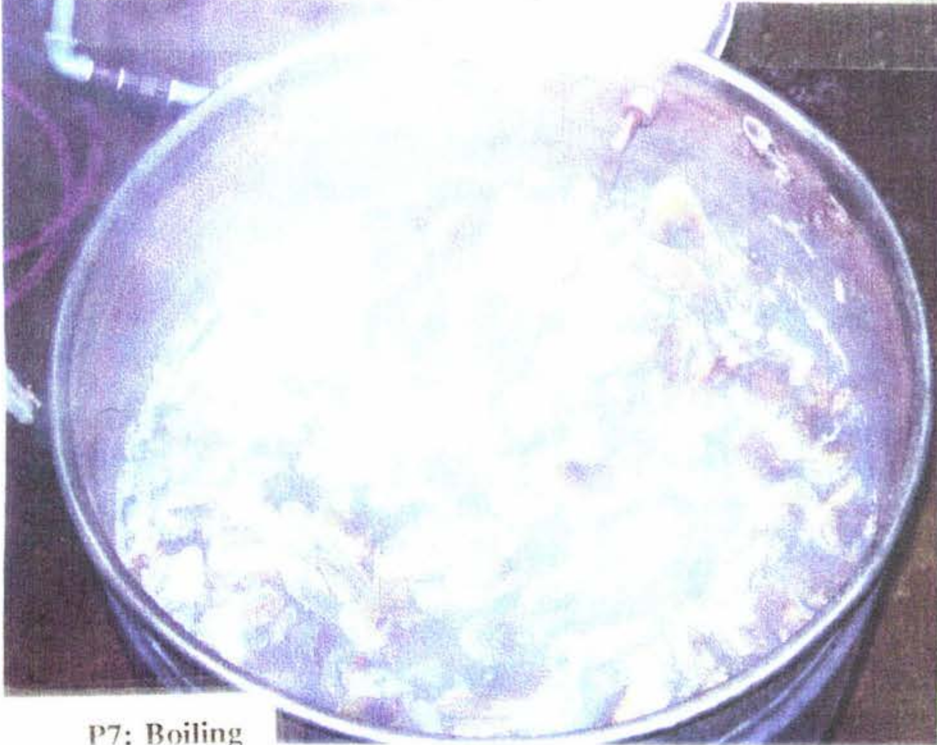
For the above operations, a stainless steel washing tank was filled with potable water

Fig 3.2 Pictorial representation for mussel processing



P5: Green mussels: *Perna canaliculus*

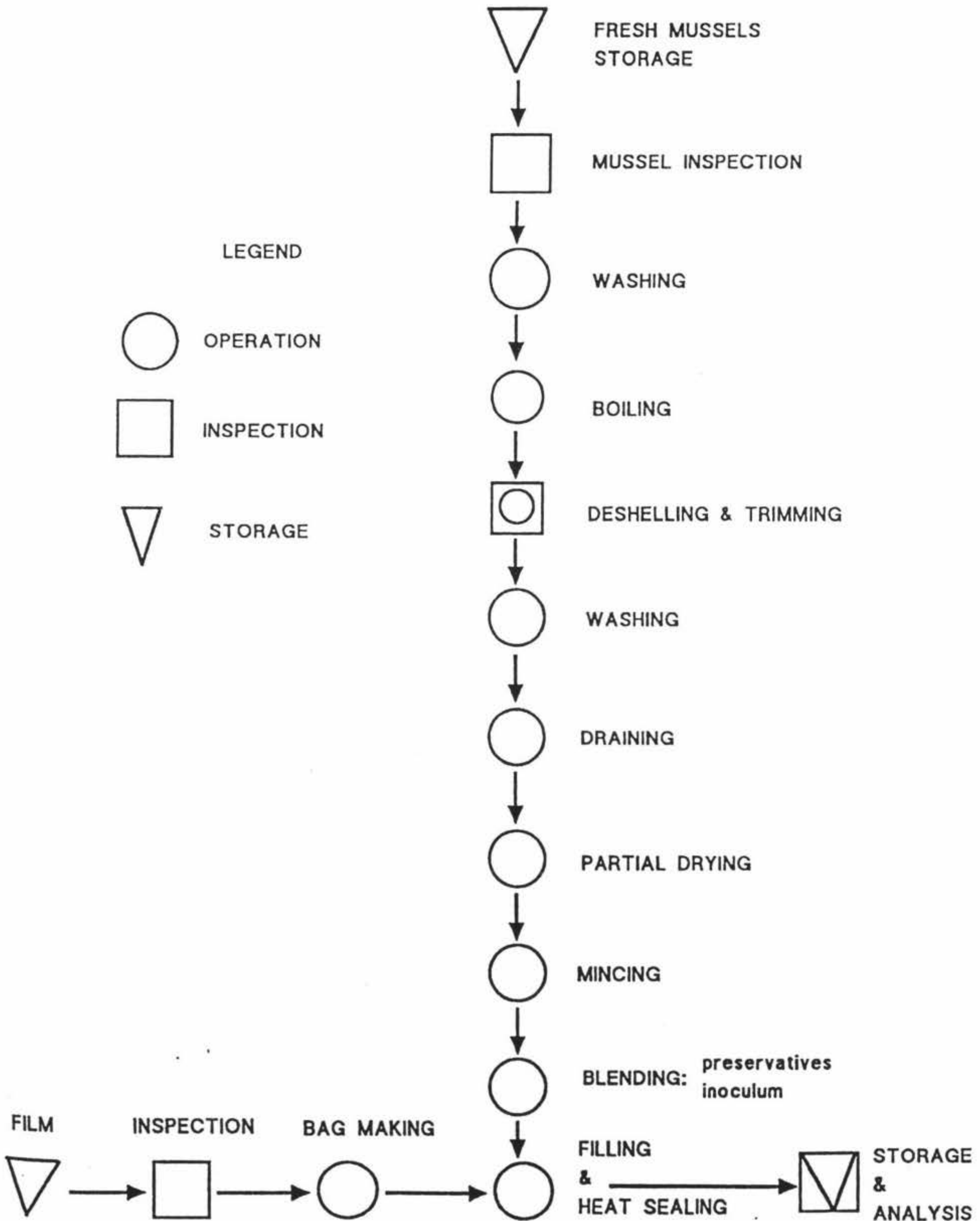
P6: Washing: Overnight immersion of mussels



P7: Boiling

P8: Shucking and trimming

Table 3.5



and 25kg of fresh mussels were loaded into the tank and left overnight. After an overnight immersion, the water was drained and excess water was further added to wash off any mud and any other extraneous matter from the shell exterior.

Boiling.

A steam jacketed vessel was filled with potable water and the steam was turned on to boil the water. The washed mussels, loaded in a wire mesh basket were then lowered into the boiling water and held for 8 minutes in boiling water(Fig 3.2(P7)). The steam was then turned off and the boiling water was then drained off. Excess cold water was then added to cool the mussels and later drained off.

Shucking and trimming.

Mussels were removed from the open shells by pulling them away from the shell leaving the adductor mussel behind(Fig 3.2(P8)). The adductor mussel (if still intact), bits of shells and other extraneous material were trimmed off the mussels and discarded.

Washing, partial drying and mincing.

The mussels were laid on a mesh tray in a monolayer and then turned 'inside-out'. They were then sprayed thoroughly with potable water ensuring that all sand, crabs, shell fragments and mud were removed. Excess water was then allowed to drain off and the mussels were then partially dried in a conventional hot air drier at 60C for 2.5hrs. The mussels were then minced using a mincer.

Treatment of minced mussels.

Inoculum preparation and incorporation of lysozyme, GDL and inoculum were done using the procedure in the cottage cheese food system (3.8.1).

Packaging and storage.

A heat sealer(model 10RS 601, Scott-Turner Industries, Ltd) was used for the construction of high barrier film bags(MET PET(12μm)/LDPE(50μm) with an oxygen transmission rate of 1cc/m^2/24hrs/atm at 75%RH 23C). The film was folded out and measured to give 20cm x 10cm size bags and it was then heat sealed from the sides at

85C. The bags were made a day before mussel processing. The treated batches were packed in the bags in 30g portions, heat sealed and the samples were then incubated at 5C and 12C.

Sampling for analysis.

Procedure 3.8.1 was used for enumeration of microorganisms and pH determination. For each parameter the average calculated values were expressed.

Lysozyme, GDL and modified atmosphere packaging.

The procedure for mussel processing (3.8.2) was used for processing and treatment of mussels for modified atmosphere packaging. Bags containing samples for modified atmosphere packaging were evacuated, backflushed with food grade nitrogen or carbon dioxide and heat sealed using a Vacuum sealer(Supervac model GK-120).

Gas composition analysis

For the analysis of the gas composition in the bags, a gas chromatograph(GC)(model SRI 8610, Alltech Associates Inc. USA) was used. The GC was equipped with a CTR 1 column and hydrogen was used as the carrier gas. The GC operated at an oven temperature of 30C and the thermal conductivity detector was run at 75C.

Calibration curves.

A calibration curve was constructed by analysis of food grade carbon dioxide and nitrogen on the GC. 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1ml volumes of each gas were analysed on the GC and the area under the curve and the peak height was noted. A plot of area under the curve/peak height versus the volume of gas was then used for the construction of the calibration curve.

Enumeration of microorganisms and pH determination.

Procedure 3.8.1 was used for microbiological analysis and pH determination.

CHAPTER 4.

PRELIMINARY EXPERIMENTS

4.1 Introduction

At the commencement of this work, published work on the antimicrobial activity of lysozyme had been done using the traditional one factor at a time technique and no information on the possible interactions with pH, temperature and other chemicals was available. Thus, this work was aimed at assessing the effects of these factors and giving insight into the various interactions that would be of benefit to increased microbial destruction. Full factorial designs were employed which had the advantages of:

1. Orthogonality: The design matrix used enabled analysis that provided mathematically independent assessment of the effects of each of the factors.
2. No confounding of main factors and interactions.
3. All main factors and all interactions can be evaluated.

The objectives of a series of preliminary experiments were to:

> Use an experimental design that identified important factors and revealed possible interactions among factors.
>
> Select an index for microbial inhibition that would be used to generate data for statistical analysis.
>
> Set a strategy and protocol for data generation for the next experimental runs in the main experiment.
>
> Estimate sampling periods and number of data points to yield data that permit construction of growth curves.
>
> Establish whether optical density is linearly correlated with viable counts for *L. monocytogenes* and *C. tyrobutyricum*.

4.1.1 Selection of the response variable.

Choice of response variable and the qualities of a response.

The response variable must be (a) quantitative, (b) precise and (c) meaningful, qualitative responses give trouble since a +++ or -- growth response and checkerboard type responses will make it impossible to perform the arithmetic operations of the

statistical analysis. To obtain sensitivity in this study, it was essential that the response variable was precise; that is, if the experiment were repeated, the same range of values would be measured with essentially the same variability. Finally, the response variable had to have a meaning related to the subject under investigation.

The experimental work was planned to be carried out in stages, each stage building on the information of the earlier stage.

4.1.2 Statistical analysis.

The output of the statistical analysis include:

The R-squared value: This provides a measure of how much of the variability in the observed response values can be explained by the experimental factors and their interactions. The R-squared value is always between zero and one. A good model explains most of the variation in the response. The R-squared value is a measure of this criterion. The closer R-squared value is to 1.00, the stronger the model is and the better it predicts the response. The R-squared value can also be expressed as a percentage (R-squared*100%). A practical rule of the thumb for evaluating R-squared value is that it should be at least 0.75 or greater.

An estimated coefficient for each term in the model.

Because data is coded before conducting the statistical analysis (i.e -1=low, +1=high), it is possible to directly compare the coefficients for the different factors among themselves.

The p-values: In this study, p-values < 0.05 were considered to be significant. Coefficients with small p-values are significant, that is they identify effects which appear to be truly important.

The standard error for each coefficient: This is a measure of the experimental error as it affects each coefficient. Because the design is balanced (each factor has the same number of high's and low's), the standard error are the same for each coefficient.

4.2 Experimental: Experimental design and procedure.

A simple 2^2 factorial design was used to study the influence of lysozyme and chelators on the growth of *L. monocytogenes* and *C. tyrobutyricum* in BHI broth.

Procedures 3.2-3.4 were employed, but this time all BHI broth culture media were

adjusted to pH7. At the same pH, runs were conducted in addition to the 2^2 factorial design treatment combinations. These included treatments of lysozyme alone(chelators excluded) and chelators alone(lysozyme excluded).

4.3 Results and Discussion.

4.3.1 Monitoring of microbial growth/inhibition.

Optical density(OD) or absorbance can be used to monitor microbial growth (McClure et al. 1993; Meynell et al. 1970; Parish et al. 1989, Hudson, 1994; Gould, 1989). Optical density has also been shown to be useful for screening the effects of combinations of antimicrobial factors on growth of microorganisms (McClure et al. 1991). OD measurements were used in this study as they were a rapid and convenient method of measuring growth. By using this method the need for diluting and spread plating samples was eliminated. However, the use of such a technique has been of concern. The initial inoculum must be sufficiently high that the instrument used to measure OD is able to detect the turbidity. Failure to meet this requirement would mean that the measured lag time becomes the time taken for the culture to reach a sufficiently high turbidity to be measured by the instrument. In this research project, all broth culture studies were carried out with an initial microbial load of 10^5-10^6 cfu/ml. Therefore the error involved in measuring the time to reach a measurable turbidity was avoided.

In this series of preliminary experiments, the reliability of the optical density method as a tool for monitoring *L. monocytogenes* and *C. tyrobutyricum* under the influence of lysozyme and various chelating agents was assessed. Bacterial growth curves were generated from the experimental data for each variable combination. An example of the growth curves obtained is shown in Fig 4.1.

The inability of the spectrophotometric method to detect microbial loads of the order less than 10^5 cfu/ml presents problems. A lack of increase in absorbance could be erroneously interpreted as a bacteriostatic effect and lethality would not be easily detected. Therefore in the work presented here, all treatments which showed no increase in absorbance were sampled and counts performed.

Most investigators support the fact that an inoculum of 10^5-10^6 cfu/ml will yield

acceptable results in a broth culture test (Lorian, 1986). This requirement limits cell concentrations which can be successfully assayed in this method (Brock, 1984). An excessively heavy inoculum tends to produce falsely high minimum inhibitory concentration values of the antimicrobial and an excessively light inoculum tends to produce a falsely low value. It is also important to use a large enough inoculum ($>10^5$ cfu/ml) so that killing can be measured accurately (Pearson, 1980). The high numbers used as inoculum in these experiments presented difficulties as comparatively few divisions were required to reach stationary phase. Measurements needed to be taken frequently to ensure sufficient points in the log phase were obtained.

4.3.2 Standardisation of inoculum.

A spectrophotometer was used for standardising the working culture inoculum. The culture turbidity was adjusted using BHI broth so that on inoculation of 0.5ml of culture into 50ml of each treatment, the microbial load would be ca. 1×10^5 - 1×10^6 cfu/ml as estimated from a relationship established between OD and microbial load.

4.3.3 'Inactivation' of antimicrobials during colony counts.

It is necessary to ascertain that carry-over of antimicrobials from the dilution tubes is not of significant magnitude to lower the resulting colony counts. Significant carryover of the antimicrobial agent(s) is not a problem when counting is performed on drops derived from the fourth or fifth dilution or beyond because the process of dilution usually reduces residual antimicrobial concentrations to negligible levels. This method of 'antimicrobial inactivation' was used in this study.

4.3.4 Recovery of microorganisms.

The recovery medium and diluents were chosen so as to prevent the influence of injury on the results. Injured cells require a nutrient rich recovery medium without selective or differential agents. Inappropriate recovery media may produce results which show the organism to have an artificially high susceptibility to the antimicrobial. In this study, BHI agar was used as the recovery medium for all broth culture studies when counts were performed initially and at the end of the experimental runs and on detection of bactericidal activity of some treatment combinations.

4.3.5 Kinetic parameters.

Full tables of kinetic data are presented in Tables 4.1 and 4.2. The maximum specific growth rate (μ_{max}) was calculated from the slope of the growth curves as illustrated in Fig 4.1. The generation time(GT) is related to the specific growth rate by the following equation.

$\mu_{max} = \ln(2)/GT$

The lag duration detection presented problems on growth curves that were generated which exhibited a decrease in the specific growth rate with little effect on lag time(Fig 4.6, treatment a). In such circumstances lag was taken as the time taken to reach a change in absorbance at 600nm of 0.08.

Table 4.1

Kinetic parameters:

Microorganism type.

	L. monocytogenes. (Fig 4.5 and 4.6)			*C. tyrobutyricum. (Fig 4.7 & 4.8)*		
Lysozyme-GDL Treatment	μ_{max} (#/hr)	GT(hrs)	Lag (hrs)	μ_{max} (#/hr)	GT (hrs)	Lag (hrs)
Control	0.193	3.59	4	0.283	2.45	3
1	0.038	18.24	4	0.135	5.13	3
a	0.023	30.14	20	0.118	5.87	3
b	0.046	15.07	32	0.129	5.37	7
ab	0.04	17.33	32	0.146	4.75	9
10mM GDL	0.066	10.50	7	0.195	3.55	3
25mM GDL	0.022	31.51	15.07	0.161	4.31	3

Table 4.2.

Kinetic parameters:

Microorganism type:

	L. monocytogenes. (Fig 4.1 and 4.2)			*C. tyrobutyricum. (Fig 4.3 and 4.4)*		
Lysozyme-EDTA Treatment	μ_{max} (#/hr)	GT(hrs)	Lag (hrs)	μ_{max} (#/hr)	GT (hrs)	Lag (hrs)
Control	0.245	2.83	3	0.230	3.01	3
1	0.137	5.06	8	0.104	6.66	15
a	0.095	7.30	10	0.110	6.30	15
b	0.103	6.73	10	0.098	7.07	6

Lysozyme-EDTA Treatment	μ_{max}(#/hr)	GT(hrs)	Lag (hrs)	μ_{max}(#/hr)	GT (hrs)	Lag (hrs)
ab	0.087	7.97	10	0.088	7.88	12
10mM EDTA	0.120	5.78	10	0.103	6.73	15
25mM EDTA	0.100	6.93	10	0.100	6.93	18

4.3.6 Expression for inhibition.

Fig 4.5 and 4.6 show some of the growth curves encountered on running the preliminary experiments. Possible forms of microbial inhibition encountered were (1) overall growth level suppression (Fig 4.5, 25mM GDL treatment), (2) a lag phase increase (Fig 4.6, treatment b) and (3) a decrease in the specific growth rate with little effect on lag time (Fig 4.6, treatment 1). In the preliminary experiments, no treatment had a significant lethal effect except in Fig 4.6, treatment (a) where there was a sudden decrease in absorbance followed by a linear increase in absorbance. A single way of expressing inhibition was sought, which would then be used for data generation for statistical analysis. Ideally such an expression should include both the kinetic parameters that are important in relation to shelf-life(spoilage) of foods and time to reach infective doses of the pathogenic microorganism.

One possible expression is:

$$Inhibition = lag \times \frac{1}{\mu_{max}} \times \frac{1}{\Delta A}$$

where lag = the lag duration and ΔA = absolute change in absorbance at the end of the run.

Data generated using the above expression gave unreliable indices of inhibition. Slight differences in the kinetic parameters (μ_{max} and ΔA) resulted in large differences in the inhibition indices for treatments which showed apparently little difference in their degree of inhibition. For instance, treatment ab and b in Fig 4.6, which are slightly different had inhibition indices of 4 125 units and 2 391 units respectively.

A second expression (Davidson et al. 1981) is:

$$PI = \frac{(OD\ control - OD\ blank) - (OD\ inhibitor - OD\ blank)100}{(OD\ control - OD\ blank)}$$

where PI = percentage inhibition., OD = optical density readings of different treatment

combinations at the end of the run.

The above expression for inhibition was in the form of an end point test. It lacked the element of lag, μ_{max} and failed to distinguish the significant difference between treatments that had long lag durations that finally had the same population density (OD) as treatments that had short lag durations (Fig 4.2, control versus treatment 1). In the work presented here, the most precise, quantitative and meaningful expression for inhibition was that of calculating the area under the curve of the different growth curves generated from the different treatment combinations and expressing the area as the 'yield'. This enabled generation of meaningful data that related to inhibition. In this particular case the goal was to minimise the yield.

4.3.7 Effect of EDTA and lysozyme on *L. monocytogenes.*

Growth/inhibition curves for the effect of EDTA and lysozyme on *L. monocytogenes* are presented in Fig 4.1 and Fig 4.2 and the kinetic data are presented in Table 4.2 for combination and single factor treatments. For treatment ab, in relation to the control, GT increased (2.83 to 7.97hrs), μ_{max} decreased (0.245 to 0.087h^{-1}), lag phase duration increased (3 to 10hrs) and the maximum population density after 10hrs decreased (2 x 10^8 to 1 x 10^5 *L. monocytogenes cfu/ml*). Starting with a level of 2.8 x 10^6 cfu/ml, counts performed on the 10th hour (Fig 4.2) were, control(2 x 10^8 cfu/ml), a(1.5 x 10^5 cfu/ml), b(2 x 10^5 cfu/ml) and 1(2 x 10^6 cfu/ml). There was a good agreement between absorbance and colony counts. Counts were performed in the middle of the run to investigate whether differences in absorbance between treatments was a good signal of differences in the actual numbers of microbes in the different treatments. A good agreement between colony counts and absorbance was also obtained for single factor treatments. The control and all treatments had no significant difference in the final microbial load.

Statistical analysis

The statistical analysis results are presented in Table 4.3. An R-squared value of 97.4%(0.974) is very close to 1.00; it can be concluded that the important effects identified in the output of the statistical analysis (Table 4.3) explain most of the variability in the assay reading. From the t-values and p-values presented in Table 4.3 for the main effects, the effect of EDTA is highly significant at ($P<0.05$) and the

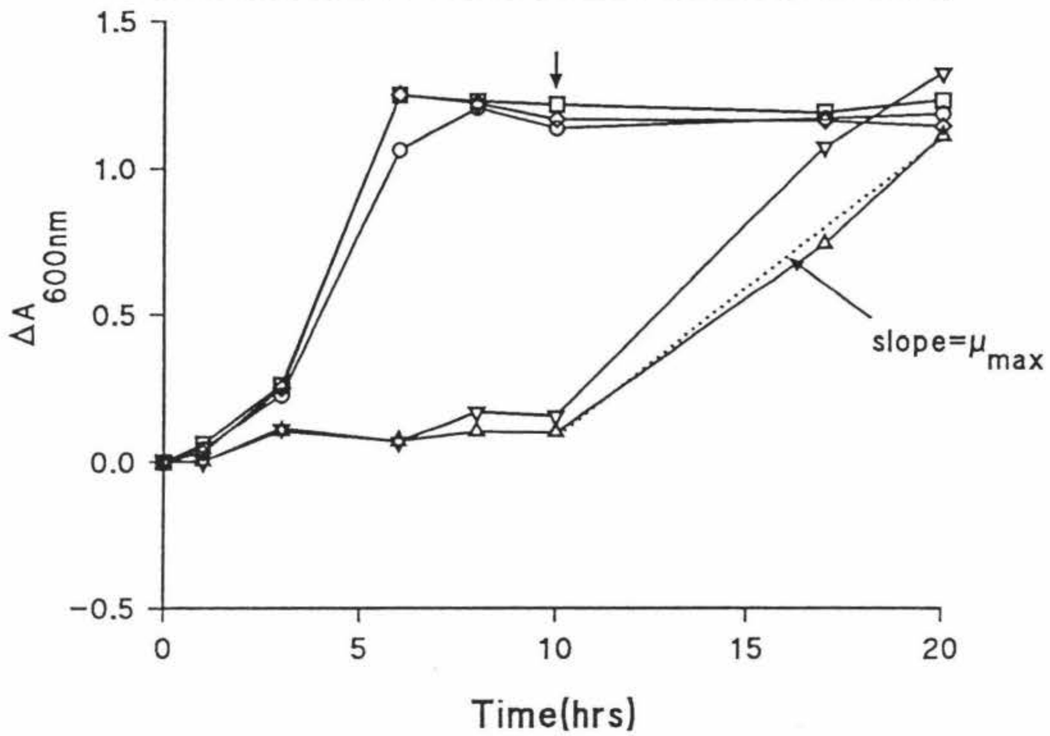


Fig 4.1

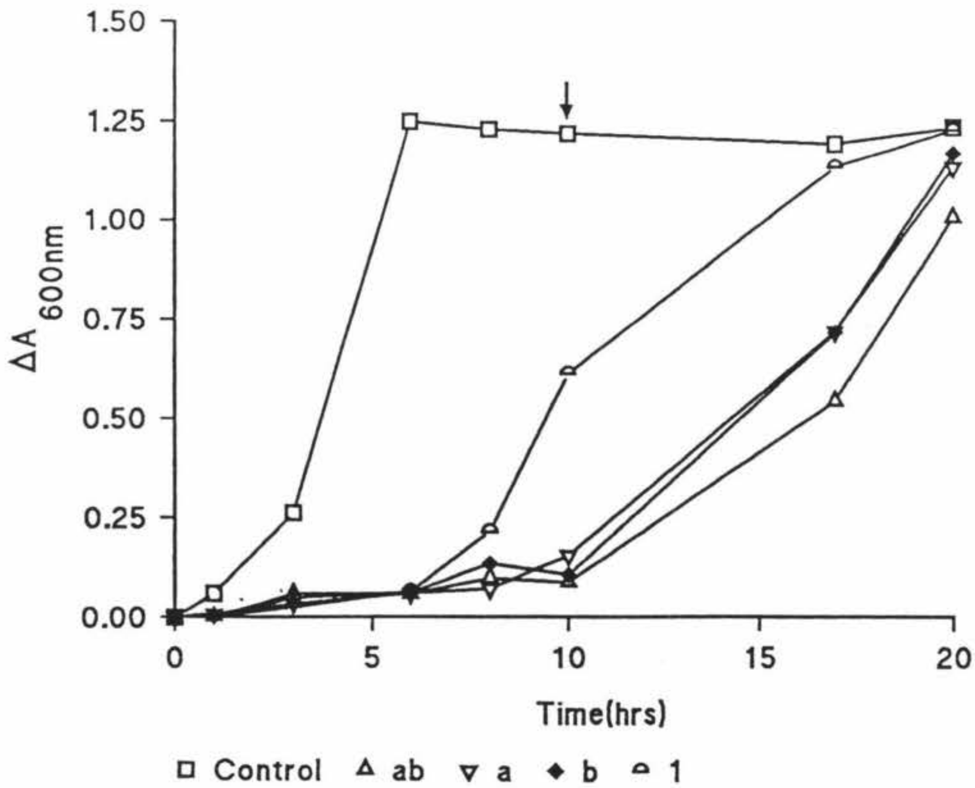


Fig 4.2

lysozyme effect is insignificant at ($P>0.05$). The two factor interaction EDTA*lysozyme is significant. Regression analysis of yield on the input variables resulted in the prediction equation:

Yield = 6.69 - 2.37EDTA - 0.524lysozyme + 1.87EDTA*lysozyme. where Yield = predicted growth yield., EDTA and lysozyme = coded levels for EDTA and lysozyme. The predicted values for yield can be calculated for each run by substituting the appropriate values (+1 or -1) of EDTA and lysozyme into the model equation.

The variable effects (coefficients) are interpreted as the average change in the response (yield) as the variable is changed from its low level to its high level. For example, the effect -2.37 on yield is due to EDTA and means that as EDTA is increased from a low (-1) to a high(+1) level, the yield on the average decreases by 2.37 units. The negative coefficients for lysozyme indicate that the yield decreases with increasing levels of lysozyme.

Table 4.3

Regression coefficients for yield of *L. monocytogenes*.

Predictor	Coef	Stdev	t-ratio	p
Constant	6.6912	0.2484	26.94	0.000
EDTA	-2.3737	0.2484	-9.56	0.001
lys	-0.5237	0.2484	-2.11	0.103
EDTA*lys	1.8662	0.2484	7.51	0.002
s = 0.706	R-sq = 97.4%		R-sq(adj) = 95.5%	

However at this stage, the regression equation was incomplete as other factors such as temperature and pH were not included.

Although lysozyme is insignificant as a main effect, its presence is still important for the interaction effect(EDTA*lysozyme).

The analysis indicate that there are two important effects to consider in trying to reduce the yield of *L. monocytogenes*. These are the main effect of EDTA and the two way interaction between EDTA and lysozyme. In order to decrease the yield of *L. monocytogenes*, the statistical analysis suggest that:

- use a treatment with a high EDTA concentration(this is because of the significance

of the main effect of EDTA.

- use a low concentration of lysozyme (because of the insignificant main effect of lysozyme and the significant EDTA*lysozyme interaction).

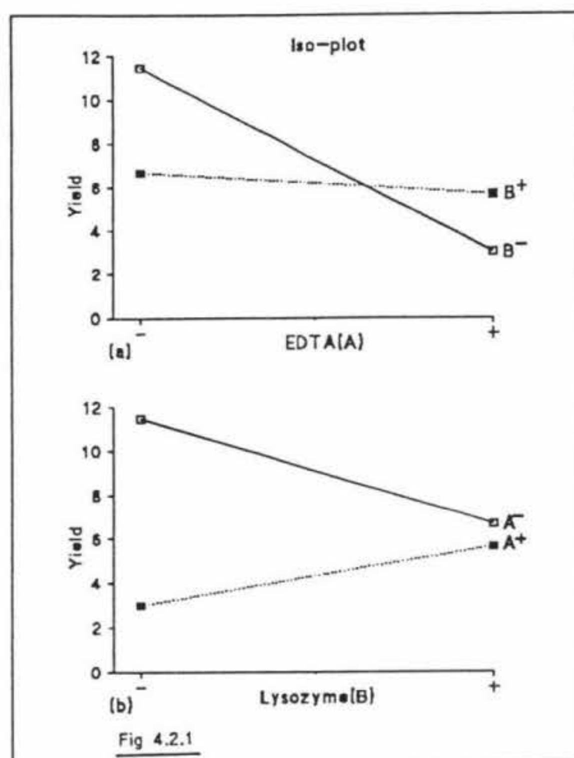


Fig 4.2.1

Interaction plots.

Interaction plots provide a means for better understanding the two factor interactions. The results are plotted in an interaction graph which is an iso-plot of the response variable for one of the factors over changes in the other factor. The interaction graph is plotted with either factor as the iso-level factor. The method of plotting iso-plots is described by Barker (1985) and Haaland (1952). The interaction plots for EDTA and lysozyme are presented in Fig 4.2.1 (a) and (b). Since the lines joining points with equal settings are not parallel, there is an interaction between the two factors. If there is no interaction, then the lines joining points with equal settings should be parallel. From Fig 4.2.1 (a) and (b) it can be deduced that increasing one factor by itself while the other stays as a low level gives a lower growth yield than if both factors are increased. This type of interaction is very important. Since treatment combinations which involve the changing of two factors at the same time do not exist in the one-variable-at-a-time design, the opportunity to make this discovery is by-passed.

To our knowledge, at the commencement of this work, little or no work had been published investigating the interactive effects of lysozyme and EDTA. Results of this combination study of lysozyme-EDTA against *L. monocytogenes* indicated that the EDTA and lysozyme had a significant interactive antimicrobial effect against *L. monocytogenes*. Similar findings were reported by Payne (1994).

4.3.8 Effect of EDTA and lysozyme on *C. tyrobutyricum*.

Growth/inhibition curves for the effect of single factor and combination treatments of EDTA and lysozyme on *C. tyrobutyricum* are presented in Fig 4.3 and Fig 4.4 and the kinetic data are presented in Table 4.2.

There was no major difference between kinetic parameters due to single factor EDTA treatment or combination treatments. The effect of lysozyme alone on *C. tyrobutyricum* at a level of 0.2mg/ml was negligible, the growth curve was the same as that of the control and was therefore not plotted. At a level of 3mg/ml, lysozyme alone had a small but insignificant effect on the growth of *C. tyrobutyricum* (Fig 4.3). Starting with a microbial load of 2.6 x 10^6 *C. tyrobutyricum* cfu/ml, counts at the end of the run were, control(8.9 x 10^9 cfu/ml), 1(3.4 x 10^8 cfu/ml), a(3.2 x 10^8 cfu/ml), b(4.2 x 10^8 cfu/ml), ab(3.4 x 10^8 cfu/ml), 10mM EDTA(3.9 x 10^9 cfu/ml) and 25mM EDTA(3 x 10^9 cfu/ml). There was a good correlation between absorbance and counts.

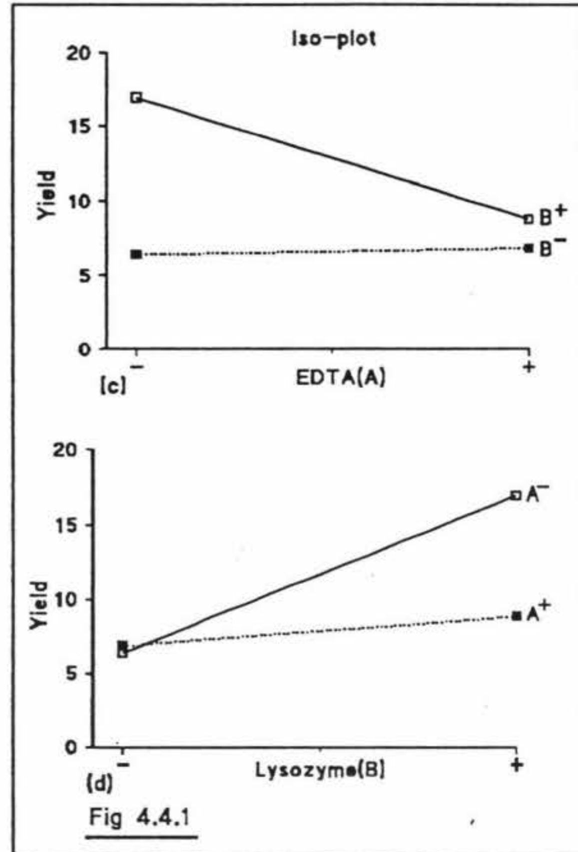


Fig 4.4.1

From the growth curves, negative absolute changes in absorbance were encountered for treatments 1, a, b and ab in Fig 4.4. The good agreement between absorbance and counts implied that such negative values in the absolute change in absorbance may have been indicative of bactericidal activity on a small proportion of the strain under test.

Statistical analysis.

Results of the statistical analysis are presented in Table 4.4.

From the t-values and p-values, the two main effects, EDTA and lysozyme were significant at ($P<0.05$). The R-squared (92.0%) and R-squared (adj) 86.1% values are high.

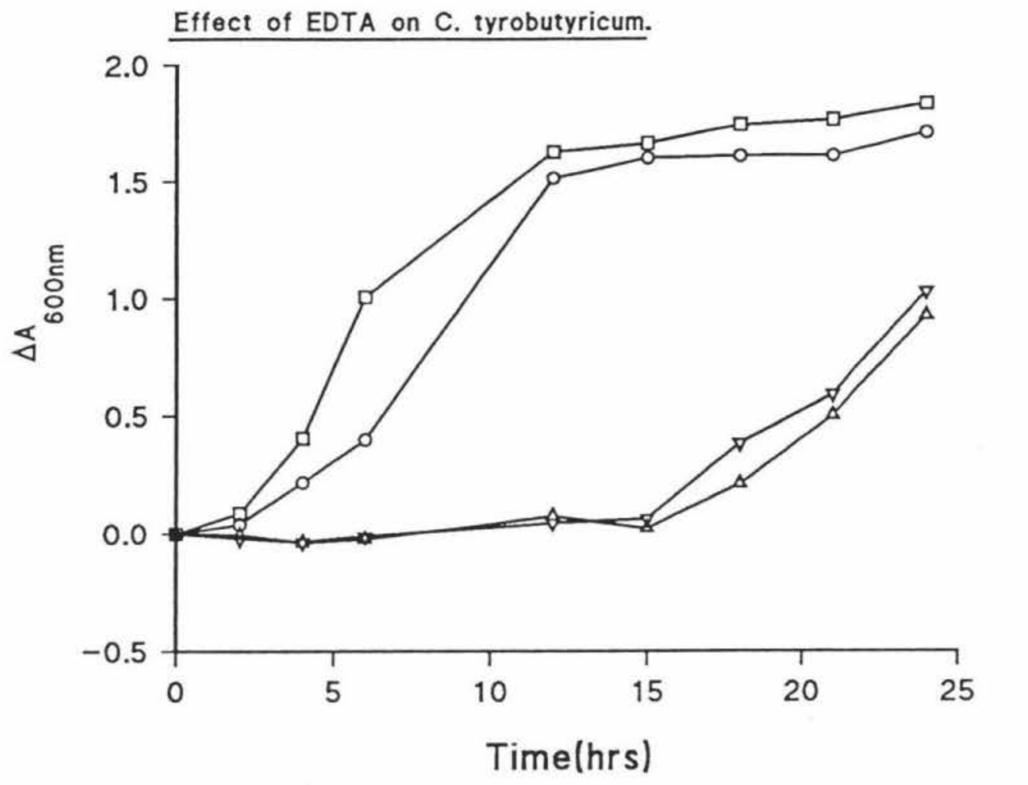


Fig 4.3

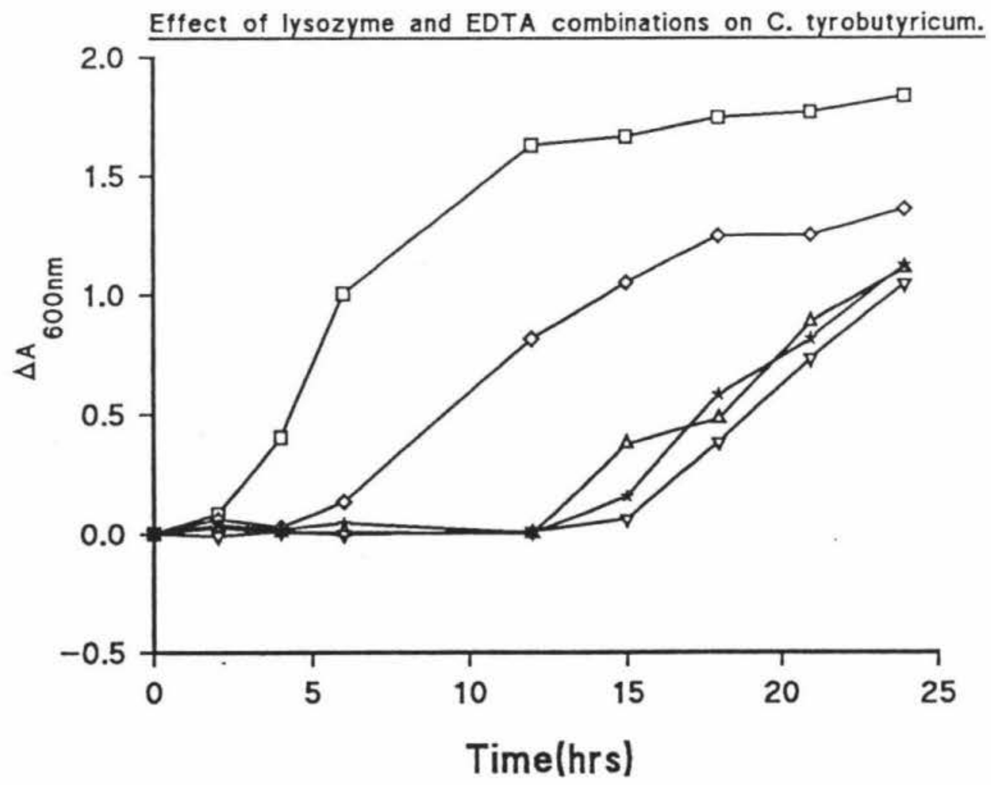


Fig 4.4

Regression analysis for yield of *C. tyrobutyricum* resulted in the equation:

YIELD = 9.75 - 1.92EDTA + 3.14lys - 2.15EDTA*lys.

Table 4.4

Regression coefficients for yield of *C. tyrobutyricum.*

Predictor	Coef	Stdev	t-ratio	p
Constant	9.7475	0.6270	15.55	0.000
EDTA	-1.9175	0.6270	-3.06	0.038
lys	3.1450	0.6270	5.02	0.007
EDTA*lys	-2.1500	0.6270	-3.43	0.027

s = 1.773   R-sq = 92.0%   R-sq(adj) = 86.1%

The analysis indicate that there are three important effects to consider in trying to reduce the yield of *C. tyrobutyricum,* namely the two main effects and the two way interactions between EDTA and lysozyme. In order to minimise the yield of *C. tyrobutyricum,* the statistical analysis suggest that:

• use a high concentration of EDTA (this is because of the significant effect of the main effect, EDTA).

• use a low lysozyme concentration.

Interaction plots

The iso-plots in Fig 4.4.1 (c) and (d) demonstrate that: (a) yield is affected by both EDTA and lysozyme, (b) the isoplots show a significant interaction between EDTA and lysozyme since the lines joining the points with equal settings in respective plots are not parallel(that is the effect is more than additive), (c) using yield as a response variable, the trend in both plots is that increasing lysozyme and EDTA together decreases the yield more than if only one of the factors is increased while the other is held at a low level.

4.3.9 Effect of GDL and lysozyme on *L. monocytogenes.*

Growth/inhibition curves for the effect of GDL and lysozyme on *L. monocytogenes* are presented in Fig 4.5 and Fig 4.6. Kinetic parameters for the different treatments are presented in Table 4.1. In comparison with the control, there was a reduction in the μ_{max}

for all treatments, an increase in the GT and an extension of the lag phase duration. Complex growth/inhibition curves were encountered (Fig 4.6) which made assessment of the lag phase difficult(treatment 1 and ab). Counts performed at the end of the experimental run confirmed the good correlation between absorbance and counts. Starting with an initial level of 2.8 x 10^6 cfu/ml, counts at the end of the run were 1.3 x 10^9 cfu/ml for the control and 9.7 x 10^8 cfu/ml for treatment 1, 3.34 x 10^6 cfu/ml for treatment ab. Lysozyme alone had no inhibitory effect against *L. monocytogenes* at 0.2mg/ml, the growth curve was superimposed on the control (growth curve not shown). The sudden drop in absorbance readings at 2hrs of treatment ab in Fig 4.6, and the absorbance readings that were below the initial readings up to the 15th hour needed further investigation. There was a possibility of the treatment ab being listericidal rather than listeristatic. This growth curve further reinforced the need to start off with a high level of inoculum when investigating the antimicrobial effects of lysozyme, thus decimal reductions in the number of the strain under test could be detected. When the antimicrobial effect is monitored by the turbidimetric assay, treatments showing no increase or a decrease in OD should be subjected to plate counts. This form of modification of the turbidimetric assay was to be employed in the main experiment when such growth curves were encountered.

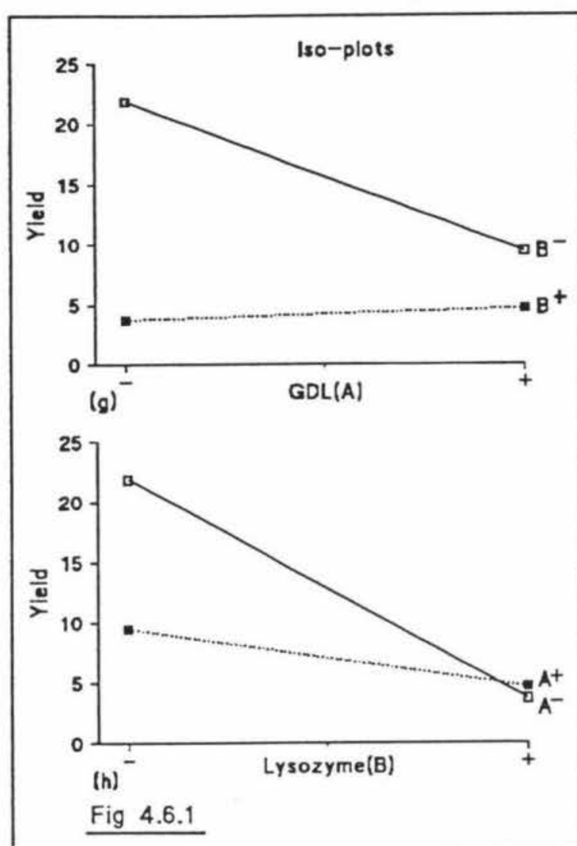


Fig 4.6.1

Statistical analysis.

Results of the statistical analysis are presented in Table 4.5. Since the model has a high R-squared value (93.8%), the factors which have an important effect on inhibition of *L. monocytogenes* have been identified.

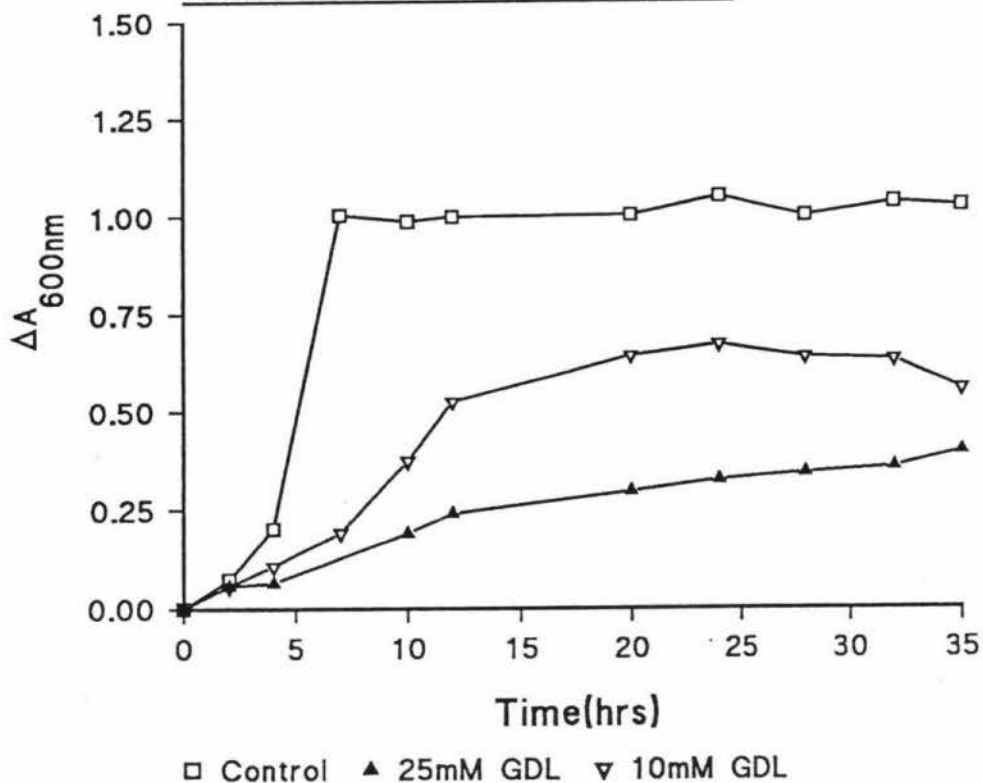


Fig 4.5

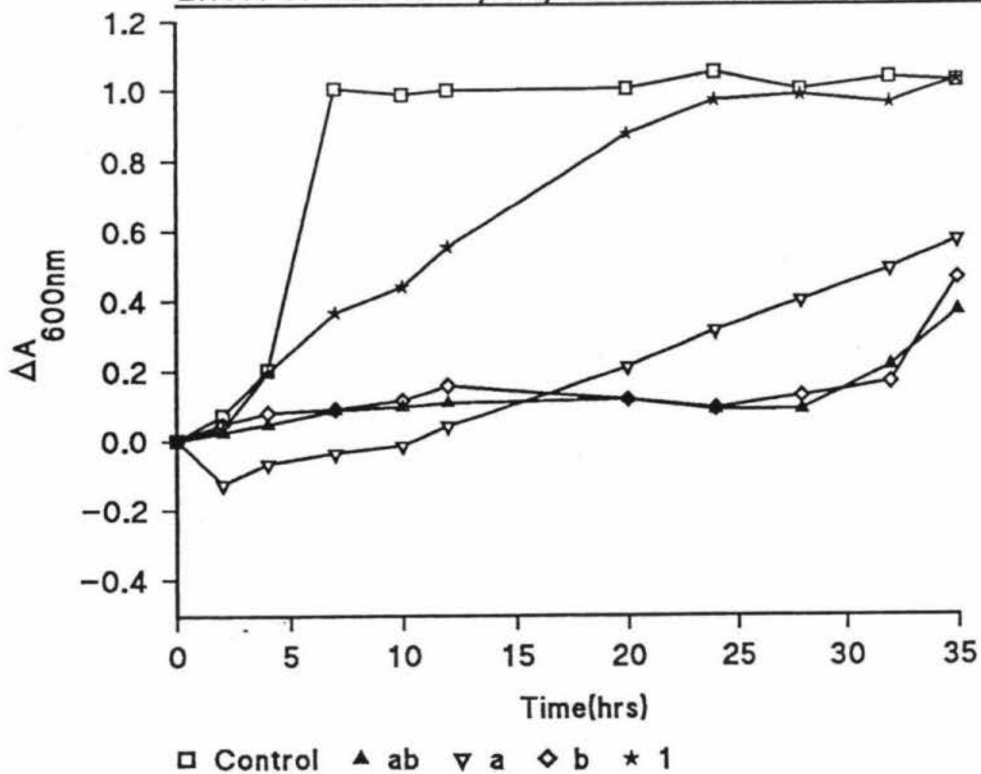


Fig 4.6

Regression analysis for yield of *L. monocytogenes* resulted in the equation:
Yield = 9.91 - 2.85GDL - 5.72lys + 3.35GDL*lys.

Table 4.5

Regression coefficients for yield of *L. monocytogenes*.

Predictor	Coef	Stdev	t-ratio	p
Constant	9.9113	0.9254	10.71	0.000
GDL	-2.8537	0.9254	-3.08	0.037
lys	-5.7238	0.9254	-6.18	0.003
GDL*lys	3.3463	0.9254	3.62	0.022

2.618 R-sq=93.8% R-sq(adj)=89.2%

From the regression coefficients, the average effect of moving from a low lysozyme level (0.2mg/ml) to a high lysozyme level (3mg/ml) is that the yield of *L. monocytogenes* decreases by 5.72 units and moving from a low GDL level(10mM) to a high GDL level(25mM) results in a decrease of *L. monocytogenes* yield by 2.85 units. The t-values and the p-values indicate that the main effects(GDL and lysozyme) and the two-way interaction(GDL*lysozyme) are all significant at ($P<0.05$). The statistical analysis suggest that in order to minimise the yield of *L. monocytogenes:*

- use a high concentration of GDL(because of the significant GDL effect).
- use a high concentration of lysozyme(because of the significant effect of lysozyme)

Interaction plots.

The isoplots in Fig 4.6.1 (g) and (h) indicate that there is an interaction between GDL and lysozyme since the lines joining point at equal settings are not parallel. Increasing one factor by itself while the other stays at a low level gives a lower growth yield than if <u>both</u> factors are <u>increased</u>.

4.3.10 Effect of GDL and lysozyme on *C. tyrobutyricum.*

Growth/inhibition curves of the effect of GDL and lysozyme on *C. tyrobutyricum* are presented in Fig 4.7 and Fig 4.8. Full kinetic parameters are presented in Table 4.1. In comparison with the control, there was no significant reduction in μ_{max} or increase in

GT for all treatments. However, treatments b and ab increased the lag phase duration. The *C. tyrobutyricum* was more resistant to such treatments when compared with *L. monocytogenes*. Negative absolute changes in absorbances were encountered for all treatments except the control. Starting with an initial level of 3 x 10^6 cfu/ml, counts at the end of the run for all treatments were not significantly different from the control.

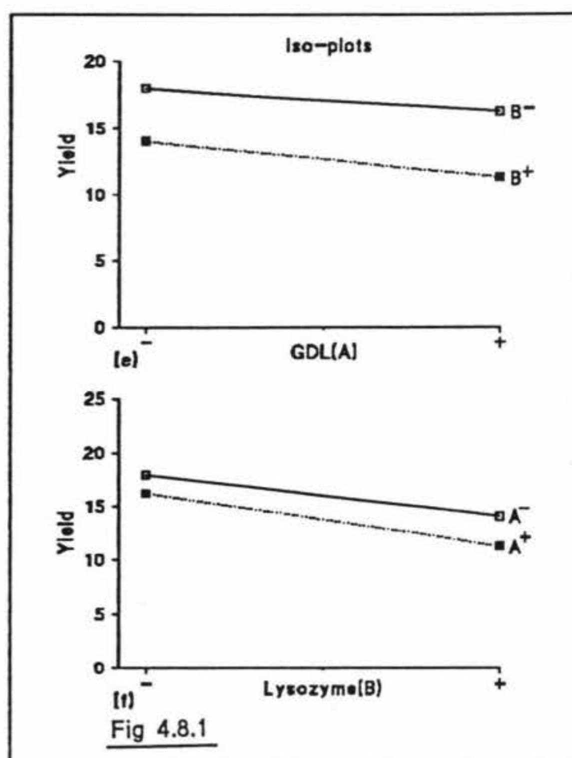


Fig 4.8.1

Statistical analysis.

Results of the statistical analysis are presented in Table 4.6. R-squared(96.8%) and R-squared-adj(94.5%) values were high. From the t-values and p-values, the main effects(GDL and lysozyme) are highly significant at ($P<0.05$) and the two-way interaction(GDL*lys) is insignificant at ($P>0.05\%$).

Regression analysis of yield of *C. tyrobutyricum* resulted in an equation:

YIELD = 14.9 - 1.13GDL + 2.24lys - 0.266GDL*lys

Table 4.6

Regression coefficients for yield of *C. tyrobutyricum*.

Predictor	Coef	Stdev	t-ratio	p
Constant	14.861	0.2276	65.31	0.000
GDL	-1.134	0.2276	-4.98	0.008
lys	-2.236	0.2276	-9.83	0.001
GDL*lys	-0.266	0.2276	-1.17	0.307
s = 0.644	R-sq = 96.8%	R-sq(adj) = 94.5%		

The statistical analysis indicate that two factors are important in minimising the yield of *C. tyrobutyricum,* namely the GDL and lysozyme. The statistical analysis suggest that in order to minimise the yield of *C. tyrobutyricum:*

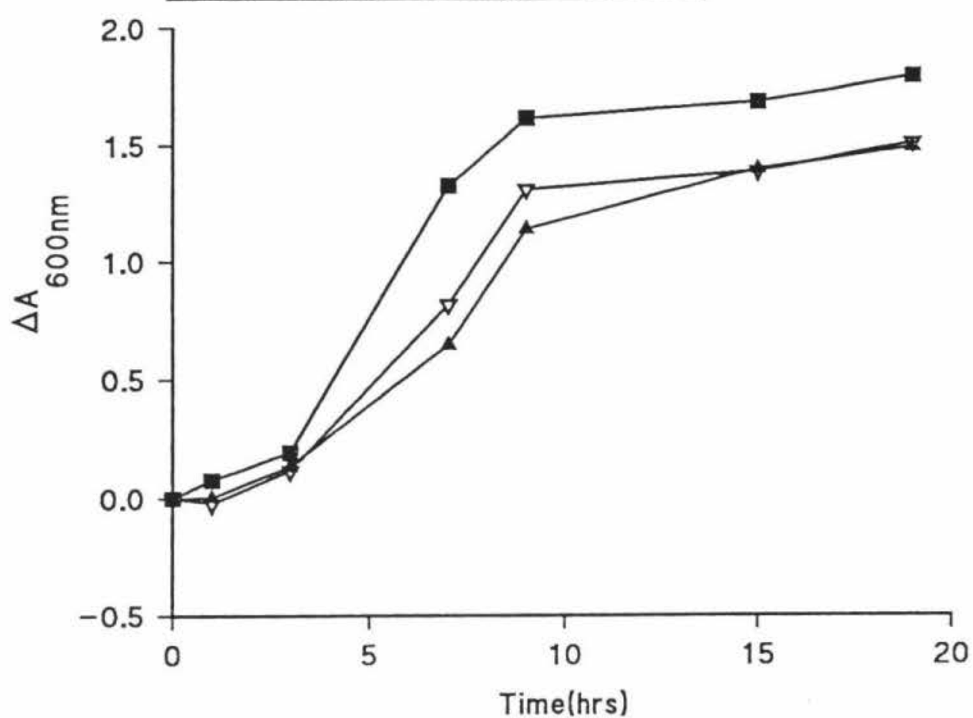


Fig 4.7

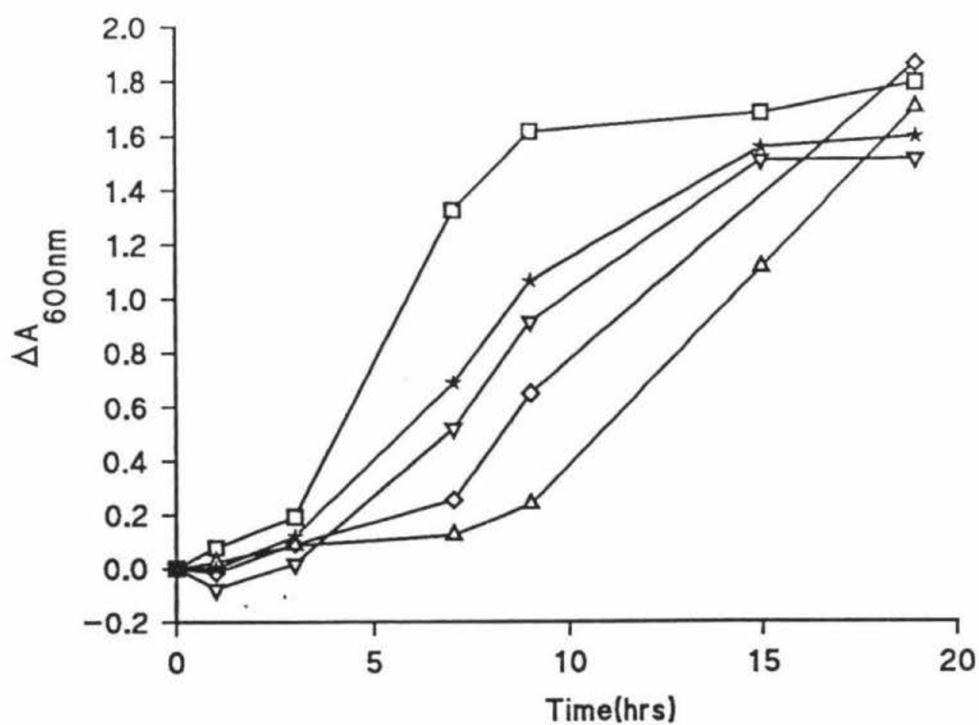


Fig 4.8

- use a high concentration of GDL(because of the significant effect of GDL).
- use a high concentration of lysozyme (because of the significant effect of lysozyme).

Interaction plots.

The iso-plot in Fig 4.8.1 (e) and (f) indicate that there is an insignificant interaction between GDL and lysozyme since the lines joining points with equal settings in the respective plots are almost parallel. The statistical analysis output confirm that the interaction is insignificant.

4.3.11 Effect of other chelators and lysozyme combinations on *C. tyrobutyricum* and *L. monocytogenes.*

Adipic acid, SPDB, SHMP, glycine and citric acid alone or in combination with lysozyme had no significant effect on *L. monocytogenes* and *C. tyrobutyricum.* Statistical analysis indicated that main effects and two-way interactions were insignificant at ($P>0.05$). Growth curves generated were either superimposed on the control curve or showed minimal inhibition of the two strains and are therefore not presented here.

4.4 Conclusions.

From the preliminary experiments, the following conclusions can be drawn:

1. Turbidimetric assay can be used to monitor the antimicrobial effects of antimicrobial agents, however where no change in absorbance or negative absolute changes in absorbance are obtained, there is need to perform colony counts to demonstrate whether the antimicrobial activity is bacteriostatic or bactericidal.
2. There is a good correlation between absorbance and colony counts.
3. Lysozyme alone at pH7.0, within the range studied, has an insignificant effect on the growth of *C. tyrobutyricum.*
4. EDTA and lysozyme interact to inhibit the yield of *L. monocytogenes* and *C. tyrobutyricum.*
5. GDL, lysozyme and their interactions have a significant effect on the growth of *C.*

tyrobutyricum and *L. monocytogenes*.

6. Complex growth curves resulting from microorganisms under extremes of environmental condition make it difficult to find an expression for microbial inhibition. However, in this study, the area under the curve has been demonstrated to be a quantitative, precise and reliable expression for generating data on microbial growth/inhibition by antimicrobial agents that can then be used for statistical analysis and development of predictive models.
7. Adipic acid, citric acid, glycine, SPDB and SHMP within the range studied, alone or in combination with lysozyme at pH7.0 have no significant effect against the growth of *L. monocytogenes* and *C. tyrobutyricum*.
8. The use of graphical displays such as interaction plots can help in understanding the effects of two-factor interactions.

4.5 General strategy for the main experiment

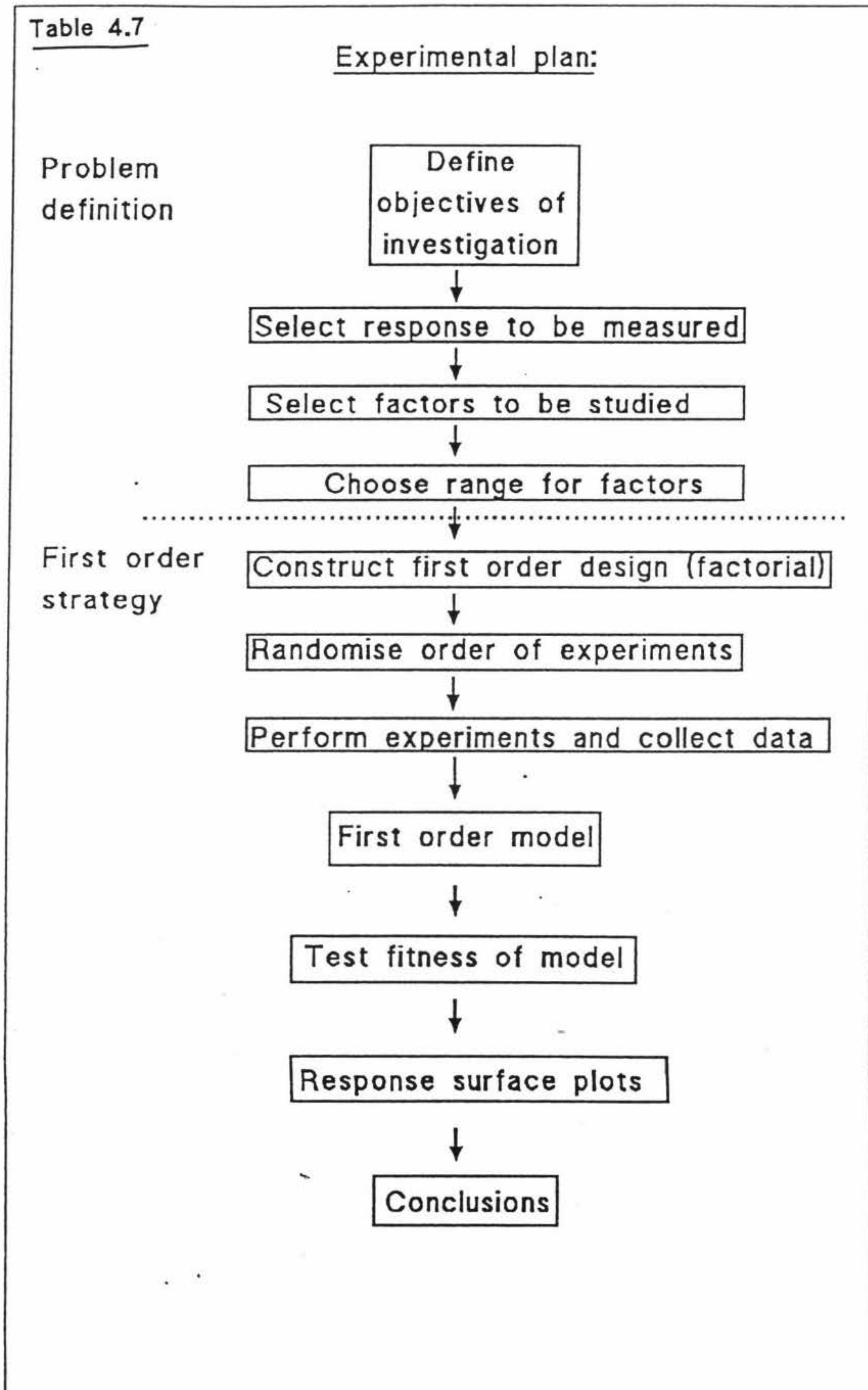
Variability.

To avoid misleading conclusions and to get the best out of the experimental runs, it was essential to design it well and to control various sources of variability. A general strategy for the main experiment was therefore laid out as in Table 4.7. Throughout the experimental runs the following aspects were standardised:

- method of maintenance of culture.
- inoculum composition(single strain).
- inoculum level.
- inoculation procedure.
- growth substrate.
- the range and levels of factors used.
- method used to adjust the pH and inoculum.
- the method of measurement and the frequency of measurement of the response variable.

Factorial designs are good for obtaining a broad understanding of preservation over a region of interest. For a particular combination of conditions, a minimum of 10 data points was adhered to (Walker, 1993). When choosing levels for design variables it was essential that they encompassed the full range over which predictions were to be made,

because extrapolation of results to any points outside would be inappropriate.



CHAPTER 5.

BROTH CULTURE SYSTEM

5.1 Introduction

Quite often, a broad spectrum of activity against microorganisms, although desired, is not easy to achieve. Very few antimicrobials have the ability to inhibit several different types of microorganisms. The antimicrobial spectrum is generally determined by following the growth of microorganisms in the presence of various concentrations of the antimicrobial. It is important to evaluate antimicrobial agents in a defined system before attempting to apply the antimicrobial agents to highly variable food systems. Methods such as the agar diffusion assay, agar dilution assay and gradient plate assay can be used for screening antimicrobial agents, each method having its own limitations. Some of these limitations can be overcome by testing the antimicrobial agent in liquid media. By following the turbidity of a culture subjected to a particular treatment, the ability of antimicrobials to prevent growth of bacteria can be established. Turbidity, however , is not always reliable and plate counts for bacteria or measurement of changes in weight may provide more accurate results. Optical density or absorbance has been used for many years to measure the concentration, expressed as mass, number or mean cell length of bacterial suspensions (Meynell et al. 1970). Recently the advent of automated turbidimetric instruments (e.g Bioscreen) has allowed microbiologists to acquire optical density measurement more efficiently. The technique has been shown to be a useful tool for screening the effects of combinations of antimicrobial factors on the growth of microorganisms (McClure et al. 1991; Davidson, 1981; Parish and Higgins, 1989; Shahamat, 1980). In this series of experiments a spectrophotometer was used to measure optical density or absorbance to assess the effects of different treatment combinations on the growth of *L. monocytogenes* and *C. tyrobutyricum.* Data obtained from different runs were used for statistical analysis. From the statistical analysis (ANOVA) significant single factor effects, two-way interactions and higher order interactions were determined. The effects(parameters) were then used to build up a predictive model equation for the system under investigation.

Predictive microbiology, where the response of microorganisms can be modeled with respect to the main controlling factors such as pH, temperature and a_w has been demonstrated to offer many advantages over traditional challenge testing (Roberts, 1989). Models are most useful as they can be used to predict changes in microbial numbers over time, even if one or more of the controlling factors affecting growth is changing. Development of such models usually requires a large number of viable count growth curves to be generated, which is time consuming and involves large quantities of laboratory media. Optical density measurements allow the generation of this information in a more cost effective way, facilitating the generation of models predicting microbial growth. Turbidimetric data have been used to generate equations which describe the growth response of different microorganisms (Cole et al. 1987; Ratkowsky et al. 1983).

In the present work, a novel method of analysing data derived from optical density measurements was used. The controlling factors in the broth culture systems were lysozyme, pH and different chelating agents. The technique involved generation of growth curves of different treatment combinations in 2^3 and 2^5 factorial designs. From optical density data, the yield (calculated as the area under the curve of the absolute change in absorbance versus time) was taken as the response variable. In this way, data generated were used to obtain predictive equations that would describe the response of the microorganism to the main controlling factors. In this way, the effectiveness of different antimicrobial systems alone or in combination was determined in a defined system.

5.2 Experimental: Experimental design and procedure.

On the basis of the knowledge acquired from a series of 2^2 factorial designs employed in the preliminary experiments, a 2^3 factorial design (Table 3.1(c)) was used to investigate in a more complete design the influence of lysozyme, different chelating agents and pH on the growth of *L. monocytogenes* and *C. tyrobutyricum*. Procedure 3.1-3.4 was used in these series of factorial designs. In a sequence of experiments, the influence of the centre point levels of pH, lysozyme and chelators on the growth of *L. monocytogenes* was investigated. Data obtained were then used to supplement the 2^3

factorial design data and at 3 levels (1, 0, -1), predictive model equations were produced and contour and response surface plots were generated.

For *C. tyrobutyricum*, no response surface plots were generated as the investigation was carried out at only two levels of each factor(1, -1).

Further investigation of the influence of GDL, lysozyme, pH, temperature and inoculum size on the growth/inhibition of *L. monocytogenes* was investigated in a 2^5 factorial design(Table 3.2). A fractional factorial design which would be desirable because of the reduced number of runs was not possible because of the significant two-way and three-way interactions that resulted from the 2^3 factorial runs. Thus to avoid confounding of the main effects, the two-way and three-way interactions, a full 2^5 factorial design was employed.

5.3 Effect of citric acid and lysozyme on *L. monocytogenes.*

5.3.1 Kinetic parameters.

Kinetic data for the influence of lysozyme, citric acid and pH on the growth of *L. monocytogenes* are presented in Table 5.1.

Table 5.1.

Kinetic parameters:

	Microorganism type:			
	L. monocytogenes		*C. tyrobutyricum*	
	Fig 5.3 and 5.4		*Fig 5.7 and 5.8*	
Lysozyme-CA Treatment	μ_{max} (#/hr)	GT(hrs)	μ_{max} (#/hr)	GT (hrs)
Control	0.263	2.64	0.132	5.25
1	0.049	14.15	0.054	12.84
a	0.043	16.12	0.068	10.19
b	0.142	4.88	0.046	15.07
ab	0.027	25.67	0.063	11.0
c	0.161	4.31	0.114	6.08
ac	0.167	4.15	0.109	6.36

Lysozyme-CA Treatment	μ_{max} (#/hr)	GT(hrs)	μ_{max} (#/hr)	GT (hrs)
bc	0.134	5.17	0.117	5.92
abc	0.134	5.17	0.113	6.13
10mM CA pH5.5	0.163	4.25	0.078	8.89
25mM CA pH5.5	0.046	15.07	0.046	15.07
10mM CA pH6.5	0.168	4.13	0.129	5.37
25mM CA pH6.5	0.073	9.50	0.124	5.59

Key

Where CA = Citric acid.

Experimental levels for citric acid, lysozyme and pH for each treatment combination(tc).

code for tc	chelator(mM)	lysozyme(mg/ml)	pH
	A	B	C
1	10	0.2	5.5
a	25	0.2	5.5
b	10	3	5.5
ab	25	3	5.5
c	10	0.2	6.5
ac	25	0.2	6.5
bc	10	3	6.5
abc	25	3	6.5

The high citric acid, low pH and high lysozyme treatment (ab) had the greatest influence on the growth of *L. monocytogenes*. In comparison with the control, the treatment resulted in a decrease in μ_{max} (0.263 to 0.027h^{-1}), an increase in the GT (2.64 to 25hrs) and a lag phase extension (3 to 72hrs)(Fig 5.1). Treatments with lysozyme were more effective in decreasing μ_{max} and increasing the generation time (Table 5.1) than treatments without lysozyme. However, when the lag phase duration is considered, treatment b and ab were the only treatments that differed in the lag duration from the treatments without lysozyme (Fig 5.1).

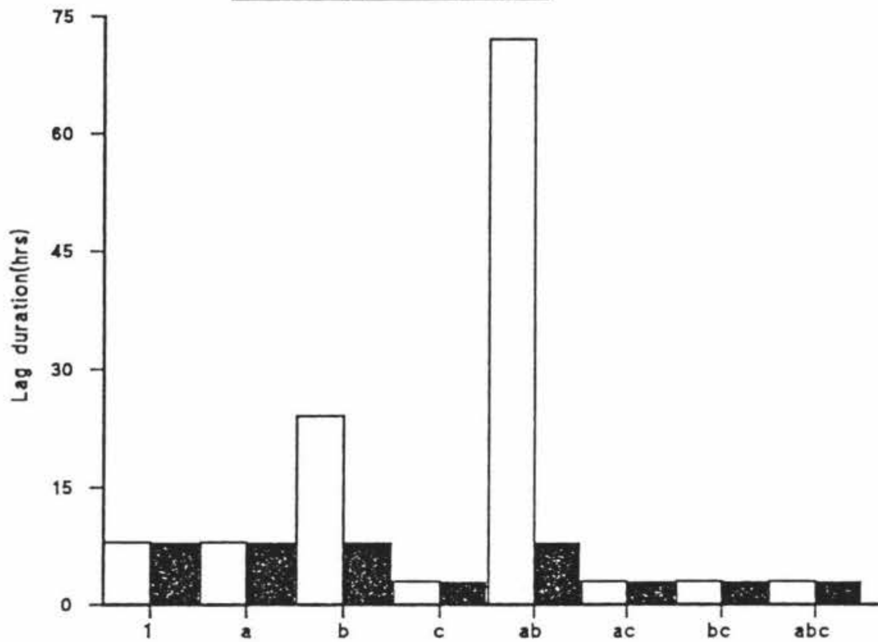


Fig 5.1

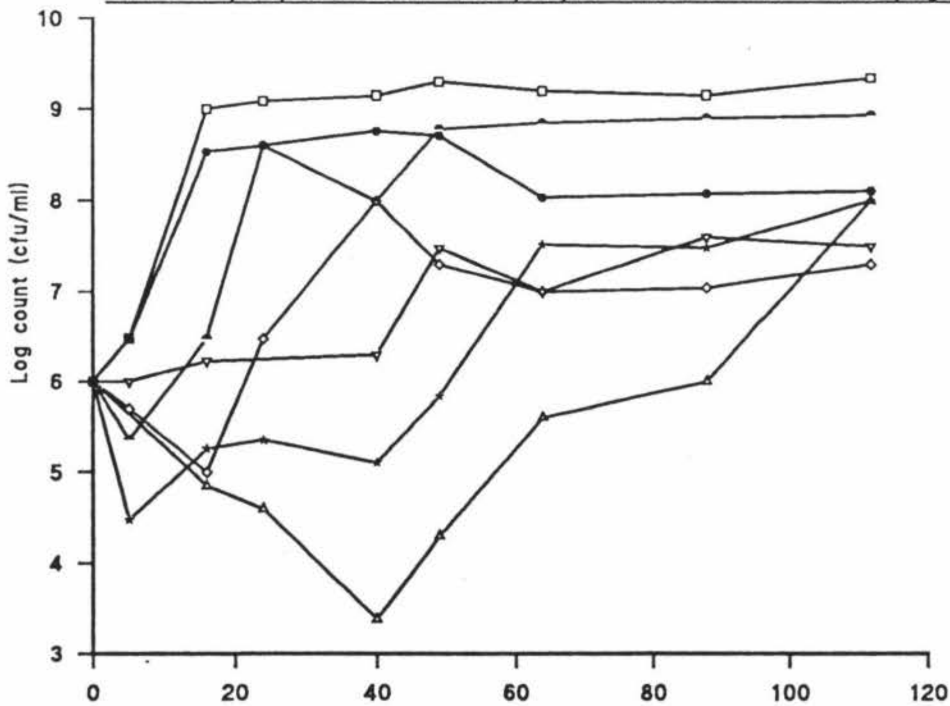


Fig 5.2

5.3.2 Growth/inhibition curves.

Fig 5.3 and Fig 5.4 represent growth curves generated from treatments with lysozyme and treatments without lysozyme respectively using the turbidimetric assay. Fig 5.2 represents the growth curves generated by the plate count method on treatment ab that demonstrated an extension of the lag phase duration in Fig 5.3. Counts were performed to investigate whether there was a listericidal or a listeristatic effect by treatment ab. Other treatments considered were lysozyme alone at different levels and pH (Fig 5.2). When identical treatments are compared in Fig 5.3 and Fig 5.2, it can be demonstrated that the turbidimetric assay generates the same information as the plate count method in terms of lag duration, μ_{max} and final population density. This then demonstrates that the turbidimetric assay can be used reliably for modelling microbial growth/inhibition. However, the turbidimetric assay has limitations in detection of the bactericidal activity of treatments as shown in Fig 5.2 and Fig 5.3 for treatment ab, that is Fig 5.3 shows a 72 hour lag, but Fig 5.2 shows that there is actually a listericidal activity.

The lysozyme activity is highly pH-dependent as demonstrated in Fig 5.2. The lysozyme activity increases as the pH decreases. This explains the ineffectiveness of lysozyme against *L. monocytogenes* at pH7 during the preliminary experiments.

The antilisteric effects of citric acid also increases as the pH decreases. Lower yields were associated with treatments at a low pH. Treatments 1 and a had short lag extensions and suppressed the maximum population density (Fig 5.3). The effect of pH on the antilisteric effect of citric acid is dramatic, only a 0.5unit change in pH (pH7.0-6.5) resulted in a marked inhibition of *L. monocytogenes*. The inhibitory effect of citric acid alone on *L. monocytogenes* has been studied (Farber, 1989; Sorrels, 1989) and on other microorganisms (Branen and Keenan, 1970; Debereve, 1987; Little, 1992). The inhibitory effect of citric acid in these studies has been theorised to be due to chelation (Branen and Keenan, 1970) and due to the undissociated form of citric acid at low pH (Debereve, 1988; 1987; Little, 1992). Our results reinforce the findings by other authors (Sorrels, 1989) that the antimicrobial effect of citric acid depends on concentration and pH. Two mechanisms may be responsible for the inhibition of microorganisms in a lysozyme-citric acid combination treatment. At pH5.5 the dissociated citric acid would, through chelation, interact with lysozyme and contribute to the inhibition of *L.*

Effect of citric acid and lysozyme combinations
on
L. monocytogenes.

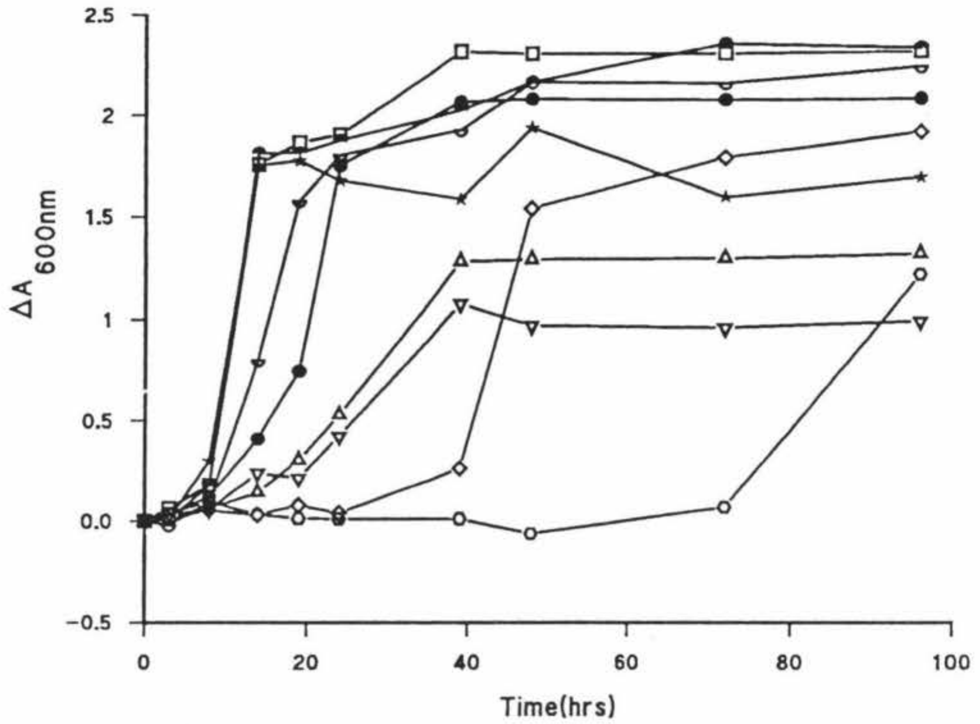


Fig 5.3

□ Control △ 1 ▽ a ◇ b ★ c ○ ab ▲ ac ● bc ▿ abc

Effect of citric acid and pH on L. monocytogenes.

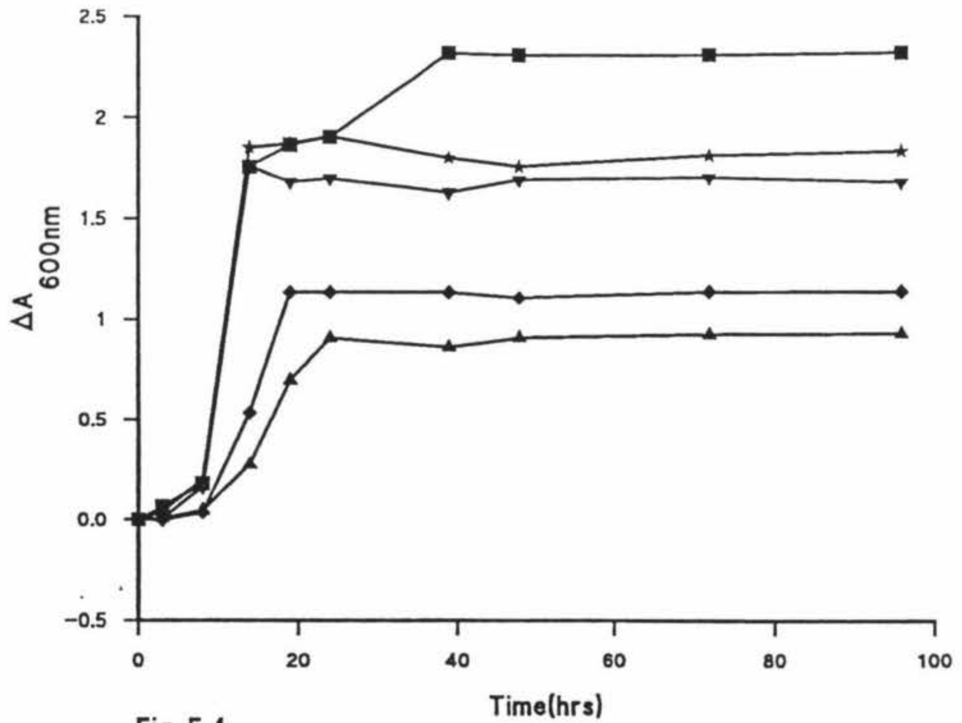


Fig 5.4

■ Control ▲ 25mM Citric acid pH5.5 ▼ 10mM Citric acid pH5.5
◆ 25mM Citric acid pH6.5 ★ 10mM citric acid pH 6.5

monocytogenes. The undissociated citric acid, would be more readily soluble in the bacterial cell membrane and therefore would be bactericidal by penetrating and acidifying the cell's interior (Little et al. 1992). However to our knowledge, the inhibitory effects of lysozyme-citric acid combination treatments and the interaction between these factors has not been published.

5.3.3 Statistical analysis.

The statistical analysis results are presented in Table 5.2. Regression analysis for yield of *L. monocytogenes* resulted in the prediction equation:

Yield = 114 - 8.96CA - 4.99lys + 46.1pH - 11.5CA*lys + 18.8CA*pH + 5.70lys*pH + 4.82CA*lys*pH.

The regression coefficients for pH and the two-way interactions(CA*lys and CA*pH) were large and significant(Table 5.2). The main effects (CA and lys), the two-way interaction (lys*pH) and the three way interaction (CA*lys*pH) were also significant at ($P<0.05$). The interactive inhibitory effect of lysozyme and low pH on the growth of *L. monocytogenes* has also been demonstrated recently (Johansen et al. 1994), and was suggested to be due to the growth retarding effect of low pH allowing enzymatic hydrolysis to exceed the rate of cell proliferation.

The statistical analysis suggest that lower yields of *L. monocytogenes* should result from the use of a high concentrations of lysozyme and citric acid and a low pH. The model equation above can be used to predict yields of *L. monocytogenes* within the experimental range studied.

Table 5.2.

Predictor	Coef	Stdev	t-ratio	p
Constant	113.832	1.881	60.5	0.000
CA	-8.960	2.050	-4.37	0.001
lys	-4.985	2.050	-2.43	0.033
pH	46.105	2.050	22.50	0.000
CA*lys	-11.545	2.050	-5.63	0.000
CA*pH	18.782	2.050	9.16	0.000
lys*pH	5.705	2.050	2.78	0.018
C8	4.820	2.050	2.35	0.038

where C8 = CA*lys*pH., CA = citric acid., lys = lysozyme.
s = 8.198 R-sq = 98.4% R-sq(adj) = 97.3%

5.3.4 Evaluating the model equation.

The R-squared value provide a measure of how much of the variability in the observed response values can be explained by the experimental factors and their interactions. The R-squared value for our example is provided in Table 5.2. Its value is 98.4%(0.984); since this value is very close to 1.0, we conclude that the important effects identified explain most of the variability in the assay reading. Further evaluation of the model was by means of the residual plot, Fig 5.5. The residual plot is the plot of differences between measured and predicted values (Fig 5.5). The residual values are small and evenly distributed above and below zero over the range of the data. However, there does appear to be more spread associated with higher predicted values, that is, as the yield increases the variance increases also. This problem is usually overcome by analysing the logarithm or the square root transformation of the response. In our case, however, this was not possible because of the negative yield values that were obtained in some treatments. Untransformed data was therefore used for statistical analysis and development of model equations.

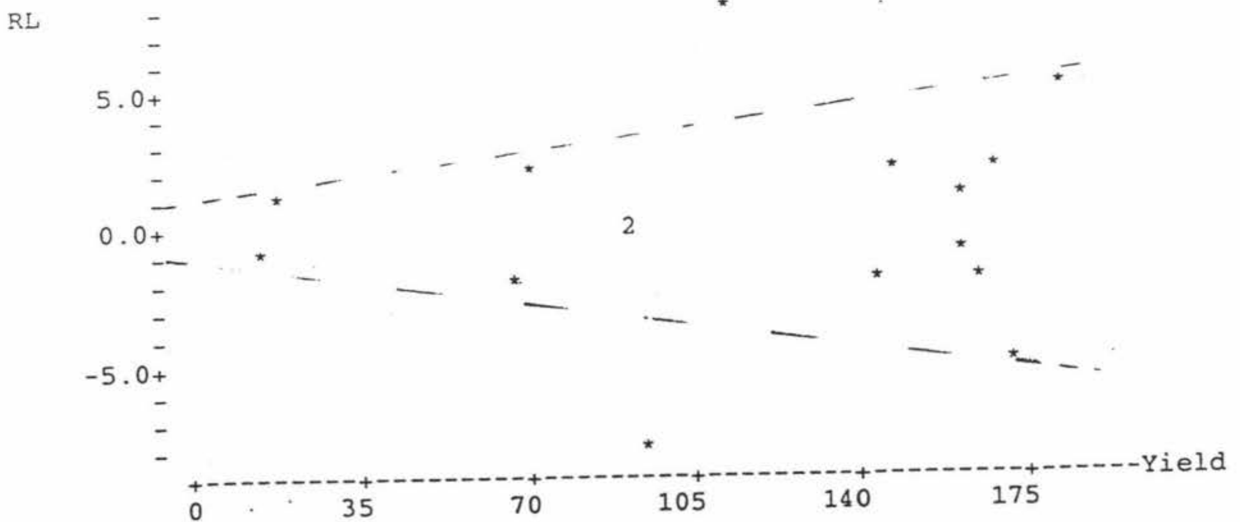


Fig 5.5. where RL = Residual values

5.3.5 Response surface plots for yield of *L. monocytogenes*.

The relationship between any two factors and yield can be quantified and displayed in the form of a contour plot(Fig 5.6(a_1)). The interaction between pH and lysozyme can be studied while holding the citric acid constant at a high level. Fig 5.6(a_2) demonstrates that by holding the citric acid concentration at a high level, the yield decreases as the pH decreases and as the lysozyme concentration increases. Fig 5.6(b_2) demonstrates that by holding the pH at a low level, the yield decreases as the lysozyme and citric acid concentrations increase.

Surface plots (Fig 5.6(a_2)) clearly demonstrate that yield values for *L. monocytogenes* depend largely on pH. This is in conformity with the large linear coefficient for pH in the model equation, Table 5.2. The model equation reveals that the two factors, lysozyme and pH affect growth(yield) of *L. monocytogenes* in both linear and interaction terms; Fig 5.6(a_2) illustrates the interaction effect of these factors; since there are no squared terms in the model equation, any curvature in the surface is due to the two factor interaction terms. The response surface is a plane. The two factor interactions in Fig 5.6(a_2) and 5.6(b_2) causes the surface to twist.

For simplicity, not all response surface plots are presented. A combination of increased citric acid and lysozyme resulted in a decrease in the yield of *L. monocytogenes*. Higher yields, analogous to short lag phase duration indicate minimal inhibition/inactivation. However an analogy between yield values and GT was not plausible. As the growth curves were complex, low GT were not always associated with high yields.

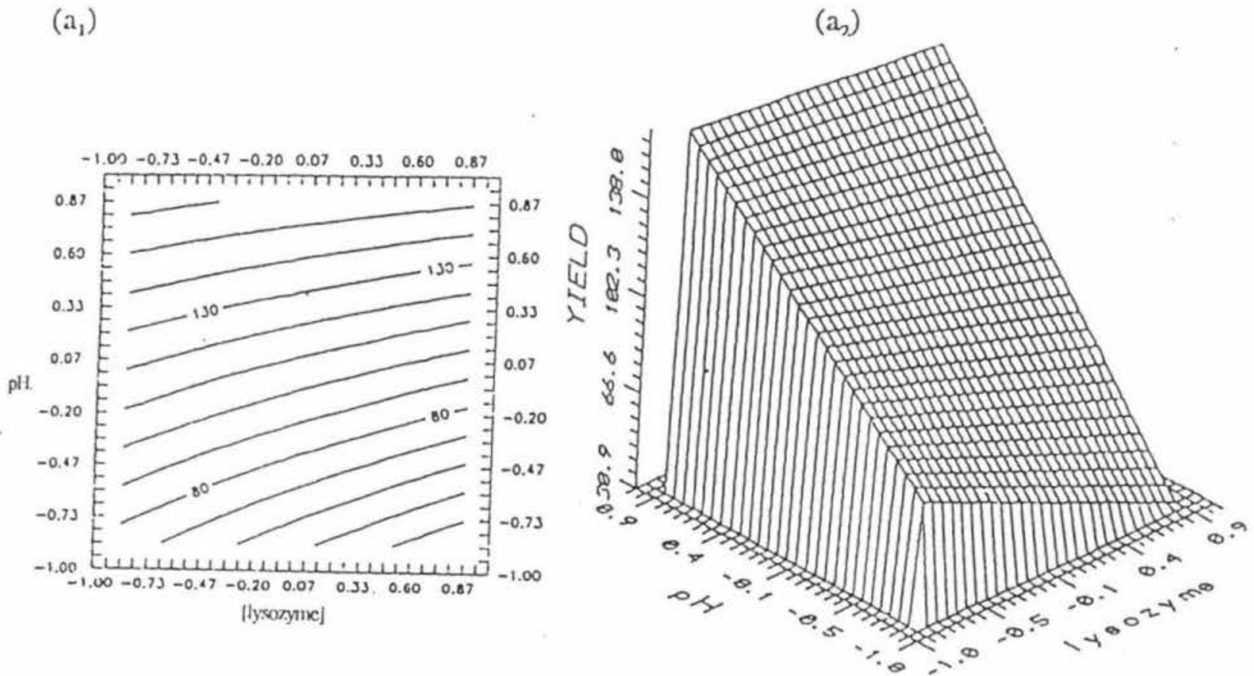
Although our data provide information on the efficacy of the lysozyme-citric acid preservative system against *L. monocytogenes*, it is inappropriate to extrapolate these findings to any food system, because there are many intrinsic factors which occur in different products that were not considered in these experiments. Examples include water activity, types of microbial flora, food components, initial microbial load and other factors. All of them might influence the antimicrobial activity of the preservative system. However, our data clearly indicate that *L. monocytogenes* is susceptible to the action of the lysozyme-citric acid system. Our findings may serve as the basis for a

Fig 5.6

Citric acid, lysozyme and pH as factors influencing growth of *L. monocytogenes.*

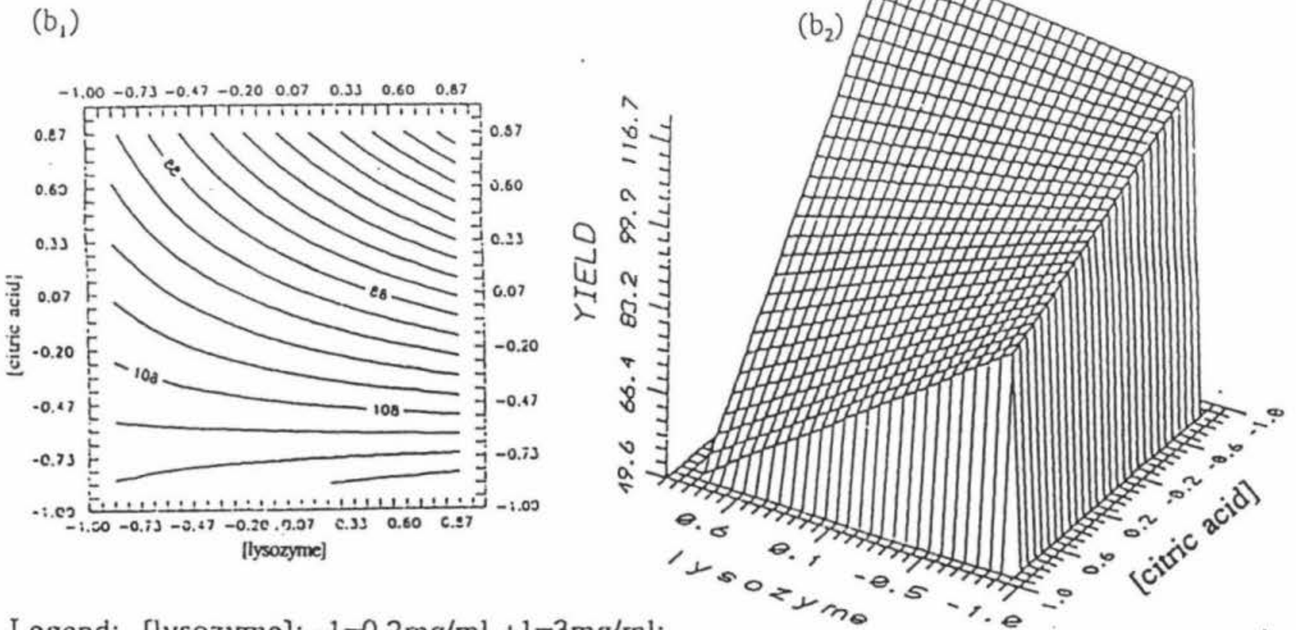
Contour(a_1) and surface(a_2) plots of yield values for *L. monocytogenes* as a function of [lysozyme] and pH. The [citric acid] was held at a high level.

(a_1) (a_2)



Contour(b_1) and surface(b_2) plots of yield values of *L. monocytogenes* as a function of [lysozyme] and [citric acid]. The pH was held at a low level.

(b_1) (b_2)



Legend: [lysozyme]: -1=0.2mg/ml +1=3mg/ml;
[citric acid]: -1=10mM +1=25mM; pH: -1=5.5 +1=6.5.

further study of this preservative system in foods.

5.4 Effect of citric acid and lysozyme on *C. tyrobutyricum*.

5.4.1 Kinetic parameters.

The kinetic parameters for *C. tyrobutyricum* under the influence of citric acid, pH and lysozyme are presented in Table 5.1. The kinetic data illustrate the resistance of *C. tyrobutyricum* to the various inhibitory factors. However, there was a reduction in μ_{max} and an increase in GT for treatments that were at a low pH. When the lag phase is considered, it can be observed from Fig 5.7 and 5.8 that there was no significant lag phase extension in any treatment. Treatments at a low pH(1, a, ab and b) in Fig 5.7 had a lower maximum population density(MPD) than the control or treatments at a higher pH.

5.4.2 Statistical analysis.

Results of the statistical analysis are presented in Table 5.3. Regression analysis for yield of *C. tyrobutyricum* resulted in the prediction equation:

Yield = 77.7 + 0.71CA - 1.0lys + 16.8pH - 0.84CA*lys - 3.37CA*pH -1.75lys*pH + 0.18CA*lys*pH.

Table 5.3

Predictor	Coef	Stdev	t-ratio	p
Constant	77.703	1.331	58.36	0.000
CA	0.707	1.331	0.53	0.610
lys	-0.998	1.331	-0.75	0.475
pH	16.844	1.331	12.65	0.000
CA*lys	-0.842	1.331	-0.65	0.545
CA*pH	-3.369	1.331	-2.53	0.035

Predictor	Coef	Stdev	t-ratio	p
lys*pH	-1.752	1.331	-1.32	0.225
C8	0.182	1.331	0.14	0.895

s = 5.326 R-sq = 95.5% R-sq(adj) = 91.5% C8 = CA*lys*pH
lys = lysozyme

In conformity with the results of the kinetic data, the statistical analysis suggest that the only important factor influencing the growth of *C. tyrobutyricum* is pH. The regression coefficient for pH is large and significant at ($P<0.05$). There is a significant interaction between citric acid and pH at ($P<0.05$). Lysozyme, citric acid, the two-way interaction(lys*pH., lys*CA) and the three-way interaction(pH*CA*lys) have insignificant effects at ($P>0.05$). The R-squared(95.5%) and the R-squared(adj)(91.5%) values are high.

Thus we draw the following tentative conclusions.

The effect of pH on the growth of *C. tyrobutyricum* is significant.

The interaction between pH and citric acid has a significant influence on the growth of *C. tyrobutyricum*.

Citric acid, lysozyme, the two-way interactions(CA*lys., lys*pH) and the three-way interaction(lys*pH*CA) have an insignificant influence on the growth of *C. tyrobutyricum*.

A reduced model equation (Haaland, 1952) for yield of *C. tyrobutyricum* under the influence of lysozyme, pH, citric acid and their interactions was of the form:

Yield = 77.70 + 16.84*pH - 3.37*CA*pH.

When identical treatments of lysozyme, citric acid and pH are considered, it is evident that *C. tyrobutyricum* is very resistant to such treatments as compared to *L. monocytogenes*.

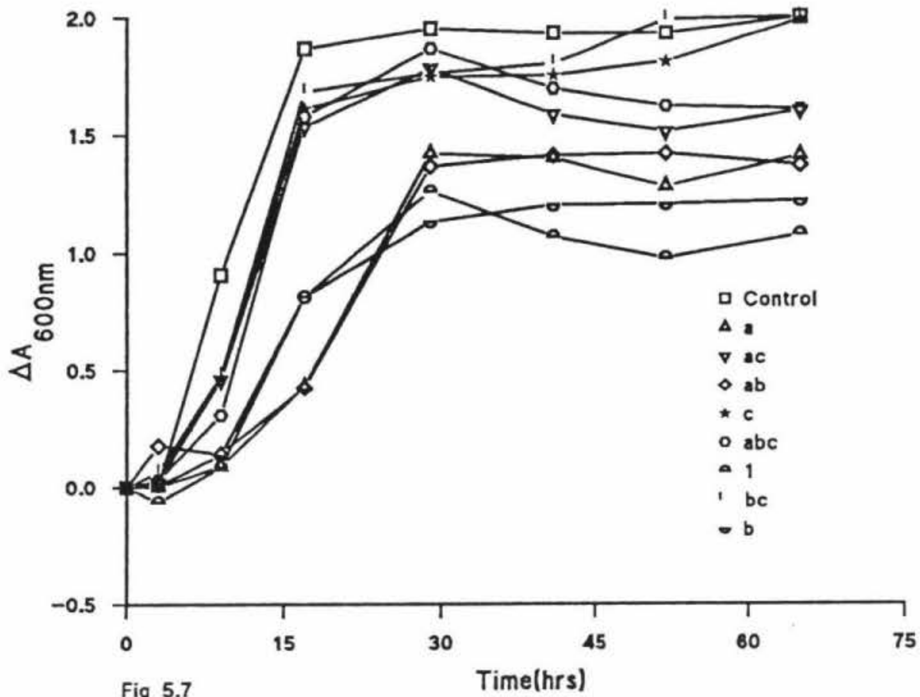


Fig 5.7

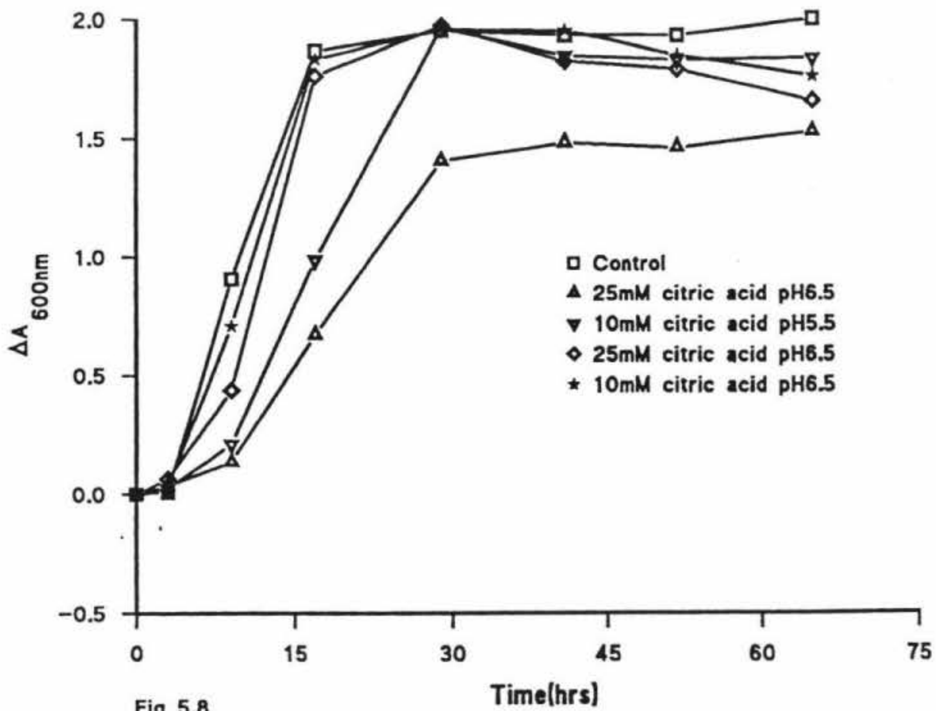


Fig 5.8

5.5 Effect of EDTA and lysozyme on *L. monocytogenes.*

5.5.1 Growth/inhibition curves.

OD curves for all EDTA and lysozyme treatments exhibited no increase in OD. Negative absolute change in absorbance values were observed. This observation implied that the various EDTA-lysozyme treatments were listeristatic or may have been listericidal. Negative yield values were obtained when the area under the curve for different treatments was calculated.

Table 5.4.

Kinetic data

	Microorganism type.			
	L. monocytogenes. *Fig 5.9 and 5.10*		*C. tyrobutyricum.* *Fig 5.12 and 5.13*	
Lysozyme-EDTA Treatment	μ_{max} (#/hr)	GT(hrs)	μ_{max} (#/hr)	GT(hrs)
Control	*	*	0.157	4.41
1	*	*	0.027	25.67
a	*	*	0.045	15.40
b	*	*	0.060	11.55
ab	*	*	0.035	19.80
c	*	*	0.034	20.39
ac	*	*	0.073	9.50
bc	*	*	0.054	12.84
abc	*	*	0.040	17.33
10mM EDTA pH5.5	*	*	0.067	10.35
25mM EDTA pH5.5	*	*	0.058	11.95
10mM EDTA pH6.5	*	*	0.036	19.25
25mM EDTA pH6.5	*	*	0.049	14.15

where * = no OD increase

Effect of EDTA, pH and lysozyme combinations on L. monocytogenes.

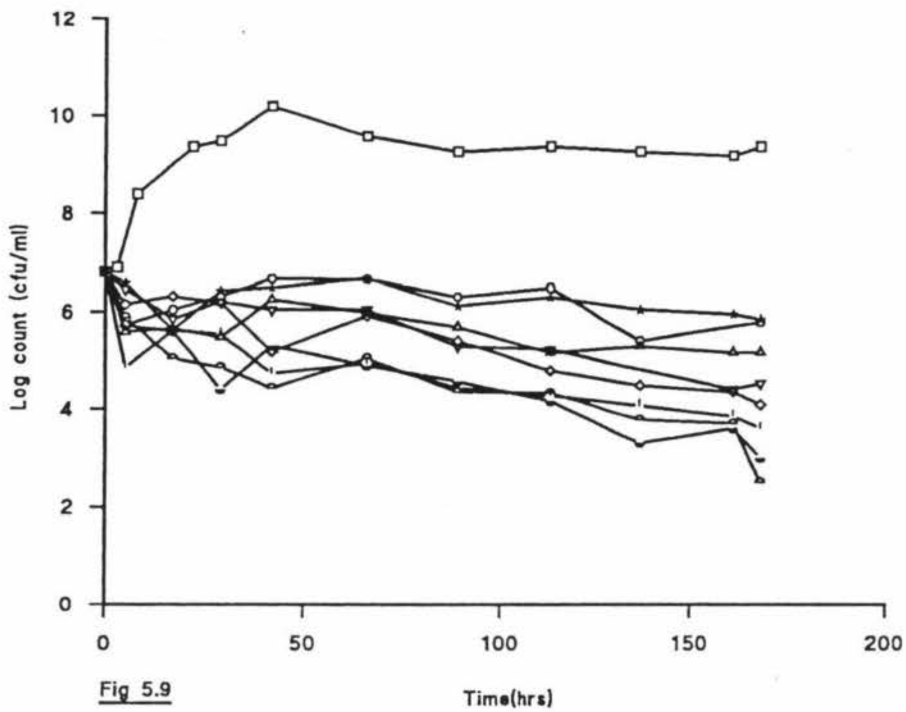


Fig 5.9

□ Control △ 1 ▽ b ◇ bc ○ ac ⌓ ab ' abc ▾ c * a

Effect of EDTA and pH on L. monocytogenes.

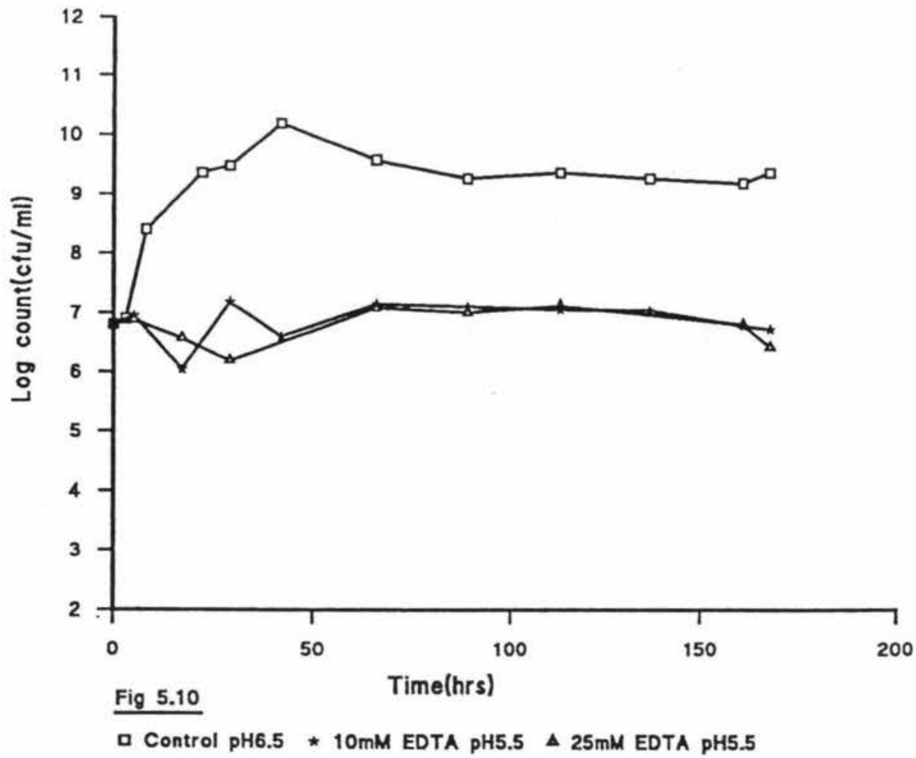


Fig 5.10

□ Control pH6.5 * 10mM EDTA pH5.5 ▲ 25mM EDTA pH5.5

For the calculation of the kinetic data, negative μ_{max} values were again obtained. For the kinetic data, all treatments were recorded as having had no growth of *L. monocytogenes*(Table 5.4).

Runs were repeated, this time the plate count method was used to monitor the growth of *L. monocytogenes* under the influence of EDTA, pH and lysozyme. From the growth curves generated by the plate count method(Fig 5.9 and Fig 5.10), it was apparent that treatments without lysozyme(Fig 5.10) were listeristatic and lysozyme-EDTA treatments were listericidal. Our results are similar to those of Chander (1980) who demonstrated that EDTA alone did not kill shrimp microflora but only inhibited their multiplication. It can also be observed that in all lysozyme-EDTA treatments, a sudden drop in numbers of *L. monocytogenes* resulted within the first 3 hours of exposure. A high antilisteric effect was exhibited by treatments with EDTA and lysozyme at pH6.5. This is probably because EDTA becomes increasingly dissociated as the pH rises and the quantity of metal ion complexed will therefore increase.

5.5.2 Statistical analysis.

This study has shown that the turbidimetric assay cannot be used for data generation from treatments that are bactericidal because the method is insensitive.

Statistical analysis of the results from the plate count data are presented in Appendix 1 Table A. All treatments were significant in reducing yield, treatment c, ab and bc were shown to be the most effective. Results of this study are in agreement with Gray and Wilkinson (1965), who demonstrated that bactericidal activity of EDTA was reduced as the pH was reduced supporting chelation as the mode of action. To our knowledge, at the commencement of this work, no study had been published investigating the interactive effects of EDTA and lysozyme against *L. monocytogenes*.

However, studies that have evaluated the effect of lysozyme against foodborne microorganisms have shown that EDTA enhances inhibition by lysozyme, producing a bacteristatic effect not observed with lysozyme alone (Bester, 1990; Chander, 1980; Smith et al. 1991; Teotia, 1975). Results of this study have demonstrated that:

When evaluated over time, EDTA-lysozyme treatment combinations are bactericidal to *L. monocytogenes*.

There is a significant antimicrobial interaction between EDTA and lysozyme against *L. monocytogenes*.

Similar findings were recently reported by Payne (1994).

In the present study, lysozyme-EDTA combinations reduced growth of *L. monocytogenes* for periods of greater than 150hrs at 25C. At lower temperatures, a slower growth rate might allow lysozyme to completely hydrolyse the cell wall, creating a more rapid bactericidal effect. Combined antimicrobial agents such as EDTA/lysozyme have potential for contributing to the microbiological safety of refrigerated food products. However, efficacy of such combinations depends upon the microorganism of concern, the food product and the storage conditions.

5.6 Effect of EDTA and lysozyme on *C. tyrobutyricum*.

5.6.1 Growth/inhibition curves.

Growth curves generated from the turbidimetric assay for the growth of *C. tyrobutricum* under the influence of lysozyme-EDTA treatment combinations and EDTA alone are presented in Fig 5.12 and Fig 5.13 respectively. Negative absolute changes in absorbance were encountered in some runs, but all treatments failed to inhibit *C. tyrobutyricum* after an extended incubation period. Fig 5.12 demonstrates that the EDTA-lysozyme treatments had different influences on the growth of *C. tyrobutyricum*. Some degree of lethality and suppression of the maximum population density was associated with treatments a and b. Other treatments had intermediate effects on the inhibition of *C. tyrobutyricum*, for instance treatment c.

5.6.2 Kinetic parameters.

Kinetic data for *C. tyrobutyricum* under the influence of EDTA and lysozyme are presented in Table 5.4. The kinetic data indicate that all treatments reduced the μ_{max} and increased the GT for growth of *C. tyrobutyricum*. Treatments for EDTA alone at a higher pH, exhibited lower μ_{max} and high GT when compared with identical treatments at a lower pH(Table 5.4). This was expected as the EDTA becomes increasingly dissociated as pH increases and the chelating ability increases. This result is in agreement with findings by Gray et al. (1965). However this trend was not so obvious

when EDTA was in combination with lysozyme. Significant interactions of lysozyme and pH; EDTA and pH may have been the reason why such a trend was not observed. When the lag phase duration is considered, it was observed that there was a general increase in the lag phase extension for most lysozyme-EDTA treatments in comparison with lag phase extensions due to treatments without lysozyme(Fig 5.11).

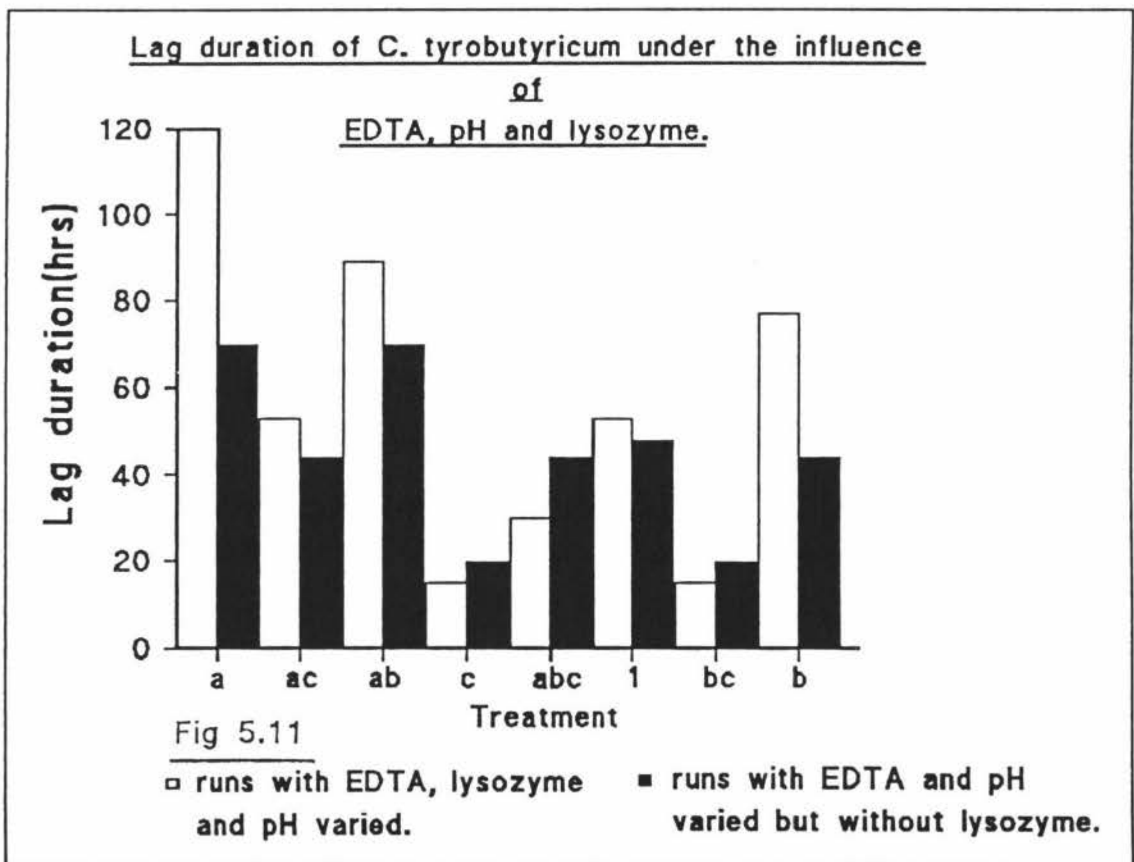


Fig 5.11

5.6.3 Statistical analysis.

Results of the statistical analysis are presented in Table 5.5.

Regression analysis for yield of *C. tyrobutyricum* resulted in the regression equation:

Yield = 117 - 24.5EDTA + 7.86lys + 46.6pH + 17.9EDTA*lys + 5.63EDTA*pH + 5.12lys*pH - 16.8lys*pH*EDTA.

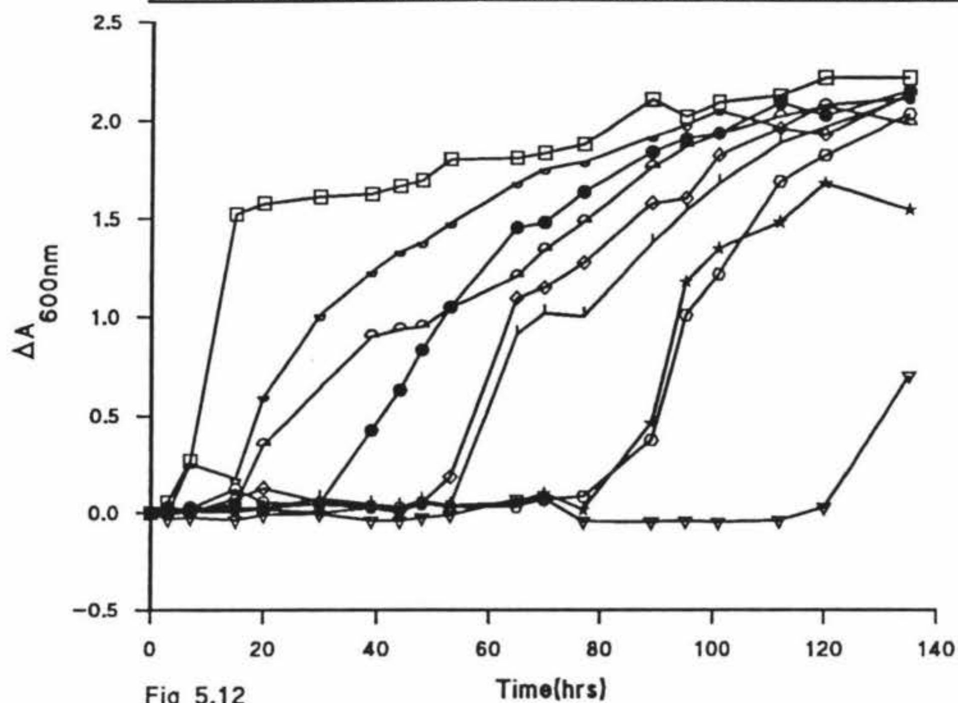


Fig 5.12

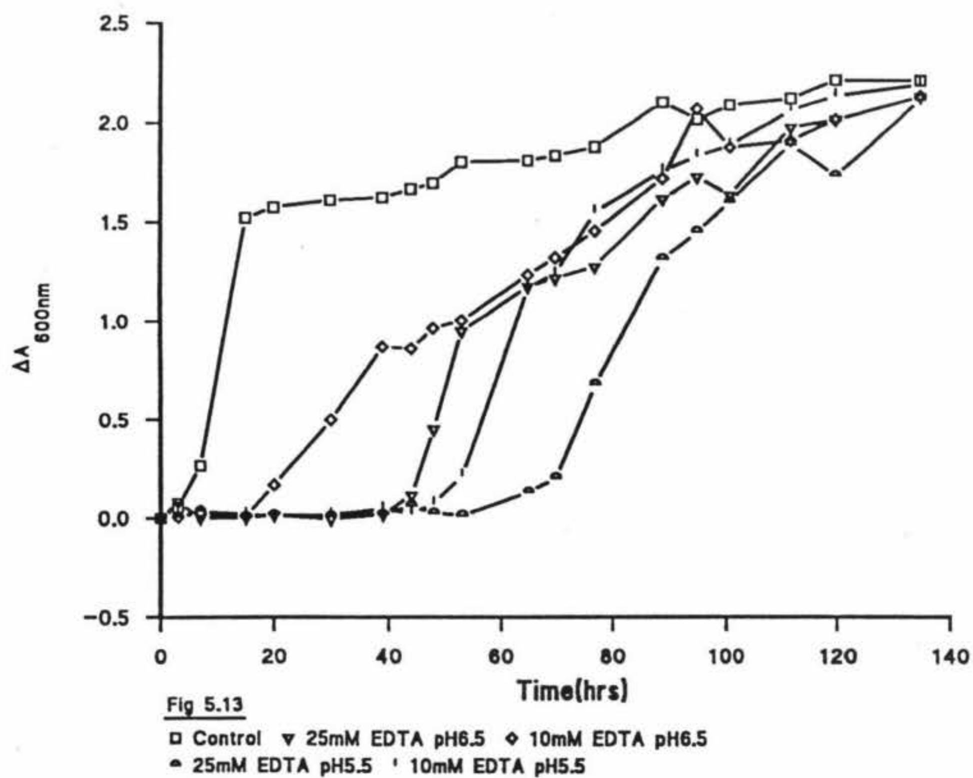


Fig 5.13

Table 5.5

Predictor	Coef	Stdev	t-ratio	p
Constant	116.544	1.256	92.79	0.000
EDTA	-24.450	1.256	-19.47	0.000
lys	7.856	1.256	6.25	0.000
pH	46.636	1.256	37.13	0.000
EDTA*lys	17.940	1.256	14.28	0.000
EDTA*pH	5.625	1.256	4.48	0.000
lys*pH	5.116	1.256	4.07	0.004
C8	-16.807	1.256	-13.38	0.000

s = 5.024 R-sq = 99.6% R-sq(adj) = 99.3% C8 = EDTA*lys*pH

All three main effects (EDTA, lysozyme and pH), the two-way interactions(EDTA*lysozyme., pH*lysozyme., pH*EDTA) and the three-way interaction(lysozyme*pH*EDTA) were significant at ($P<0.05$). The R-squared(99.6%) and the R-squared(adj)(99.3%) are high. From the regression coefficients it can be observed that there is a large pH effect, 46.64 units and a large EDTA effect -24.45 units.

Thus we draw the following tentative conclusions.

The effect of EDTA is to reduce the yield by 24.45 units.

To minimise the yield of *C. tyrobutyricum,* use a high concentration of EDTA(because of the significant effect of EDTA).

Use of a low concentration of lysozyme in combination with EDTA is ideal in minimising yield of *C. tyrobutyricum* at a low pH.

The conclusions are in conformity with the results obtained from the growth curves(Fig 5.12) where a high concentration of EDTA and a low concentration of lysozyme at a low pH had the greatest influence on the growth of *C. tyrobutyricum*.

5.7 Effect of glycine-lysozyme combinations on *L. monocytogenes.*

5.7.1 Growth/inhibition curves.

The growth/inhibition curve for *L. monocytogenes* under the influence of glycine-lysozyme combinations is presented in (Fig 5.14).

Treatments (ab) and (b) had long lag phase extensions, the lag phase extension due to treatment (a) was not different from that of the control but there was a suppression of the maximum population density(MPD). There was a general suppression of the MPD for all treatment at a low pH. Results suggest that as the pH decreases the antimicrobial activity of lysozyme-glycine combination increases. Listericidal effects were exhibited by some treatments, especially treatment ab, as negative absolute changes in absorbance were encountered during the run. However, after prolonged incubation of treatment ab, *L. monocytogenes* commenced growth at a μ_{max} similar to the other non-inhibitory treatments, for instance treatment a.

5.7.2 Kinetic parameters.

Kinetic data for *L. monocytogenes* under the influence of glycine, pH and lysozyme are presented in Table 5.6.

Table 5.6.

	Microorganism type.			
	L. monocytogenes Fig 5.14 and 5.15		*C. tyrobutyricum* Fig 5.17 and 5.18	
Lysozyme-gly Treatment	μ_{max} (#/hr)	GT(hrs)	μ_{max} (#/hr)	GT(hrs)
Control	0.217	3.19	0.117	5.92
1	0.06	11.55	0.112	6.19
a	0.04	17.33	0.098	7.07
b	0.06	11.55	0.089	7.79
ab	0.047	14.75	0.094	7.37
c	0.110	6.30	0.110	6.30
ac	0.109	6.36	0.120	5.78

Lysozyme-gly Treatment	μ_{max}(#/hr)	GT(hrs)	μ_{max}(#/hr)	GT(hrs)
bc	0.049	14.15	0.108	6.42
abc	0.036	19.25	0.100	6.93
10mM gly pH5.5	0.094	7.37	0.120	5.78
25mM gly pH5.5	0.074	9.37	0.098	7.07
10mM gly pH6.5	0.119	5.82	0.110	6.30
25mM gly pH6.5	0.115	6.03	0.094	7.37

Where gly = glycine

Generally there was a decrease in the specific growth rate and an increase in the GT for treatments that were at a low pH. However, when the lag phase duration is considered(Fig 5.15), treatments at a high lysozyme level, low pH, with either high or low levels of glycine were the only treatments that were different from the control.

5.7.3 Statistical analysis.

Results of the statistical analysis are presented in Table 5.7. Regression analysis for yield resulted in the equation:

Yield = 132 - 9.44gly - 18.0lys + 37.1pH + 7.73*gly*pH + 13.4lys*pH - 3.92gly*lys + 5.39*gly*pH*lys.

Table 5.7

Estimated Effects and Coefficients for Yield

Predictor	Coef	Stdev	t-ratio	p
Constant	131.79	1.348	97.78	0.000
gly	-9.44	1.348	-7.00	0.000
lys	-17.99	1.348	-13.34	0.000
pH	37.10	1.348	27.53	0.000
gly*lys	-3.92	1.348	-2.91	0.020
gly*pH	7.73	1.348	5.74	0.000
lys*pH	13.39	1.348	9.93	0.000
C8	5.39	1.348	4.00	0.004

s = 5.391 R-sq = 99.3% R-sq(adj) = 98.7% C8 = gly*lys*pH
gly = glycine

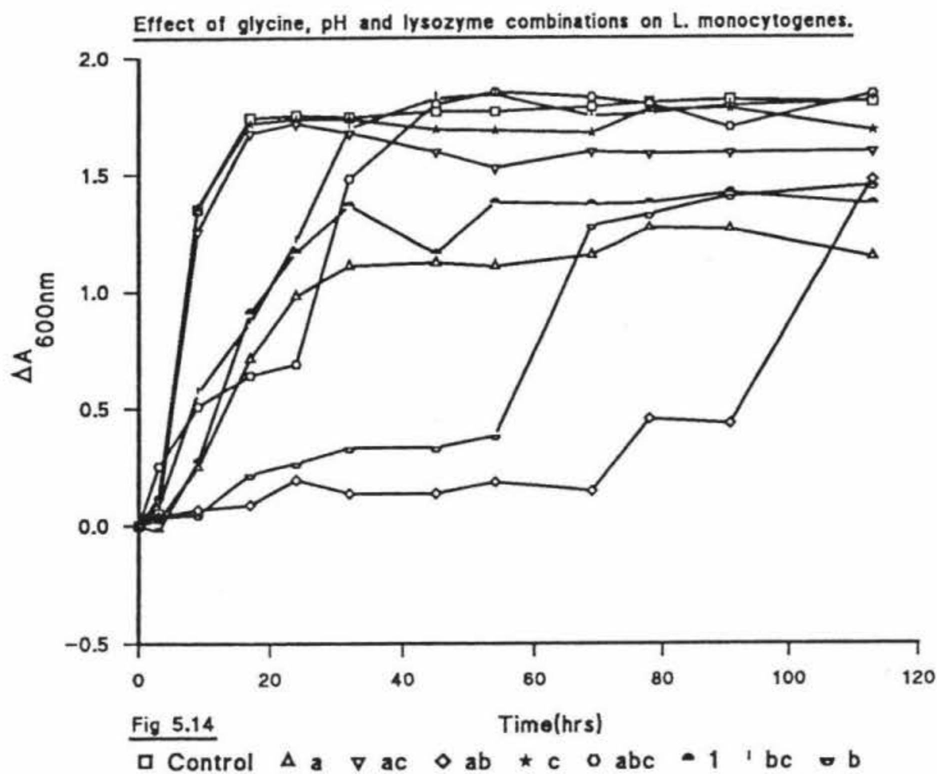


Fig 5.14

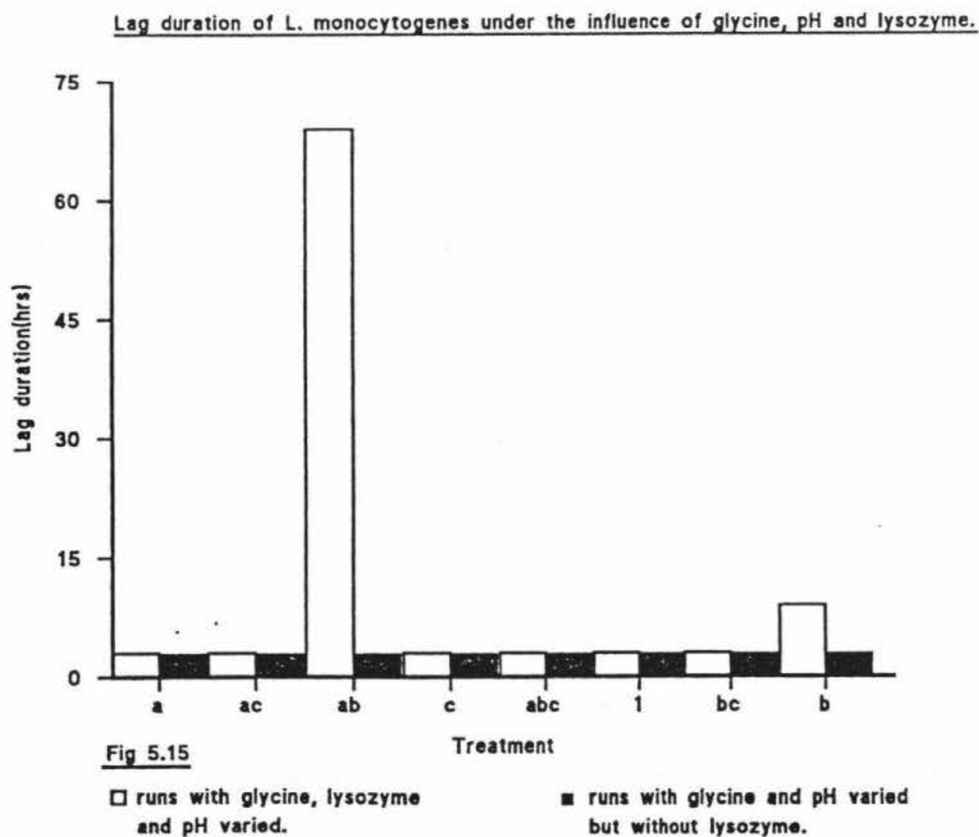


Fig 5.15

Large coefficients were associated with the main effects(pH, lysozyme and glycine) and these factors had a significant effect at ($P<0.05$). All two-way interactions(glycine*pH., glycine*lysozyme and pH*glycine) and the three-way interaction(lysozyme*pH*glycine) had a significant effect at($P<0.05$). From the statistical analysis table, we draw the following conclusions.

The effect of glycine is to reduce the yield by 9.44 units.

The effect of lysozyme is to reduce the yield by 17.99 units.

Glycine and lysozyme interact significantly at a low pH and minimise the yield of *L. monocytogenes*.

5.7.4 Evaluating the model equation.

From Table 5.7, it can be observed that R-squared (99.3%) and the R-sq(adj) (98.7%) values are very high. Further evaluation of the model by the residual plot indicated that the residual values were evenly distributed above and below zero over the range of the data. However, as the predicted yield increased, the residual values also increased.

5.7.5 Response surface plots.

The response surface plot(Fig 5.16(x_2) clearly demonstrates that the yield values of *L. monocytogenes* depend largely on pH. This is in conformity with the large linear coefficient for pH in the model equation(Table 5.7). The quantitative relationship between lysozyme, pH and yield is displayed in the form of a contour plot(Fig 5.16(x_1)). The model equation suggests that the main effects, pH, lysozyme and glycine influence the growth of *L. monocytogenes* in linear and interaction terms. Fig 5.16(x_2) and 5.16(z_2) illustrate the interaction effects of some of these factors. The 3-dimensional plot Fig 5.16(x_2) emphasises the necessity for the pH to remain low to decrease yield significantly.

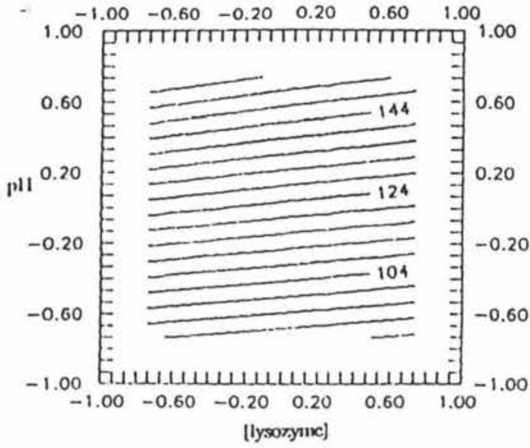
The result of this study is in agreement with the findings by Proctor (1992) who demonstrated that glycine enhanced the antilisteric effects of lysozyme. Glycine has been demonstrated to enhance the bactericidal activity of lysozyme against *Bacillus subtilis, Escherichia coli S-8* and *Staphylococcus aureus 209-P* (Yashitake, 1977). A synergistic effect of lysozyme and glycine against the three bacteria was

Fig 5.16

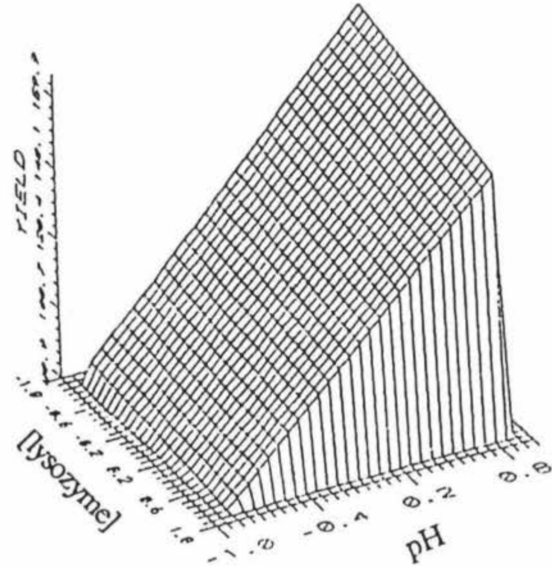
Glycine, lysozyme and pH as factors influencing growth of *L. monocytogenes*.

Contour(x_1) and surface(x_2) plots of yield values for *L. monocytogenes* as a function of [lysozyme] and pH. The [Glycine] was held at a high level.

(x_1)

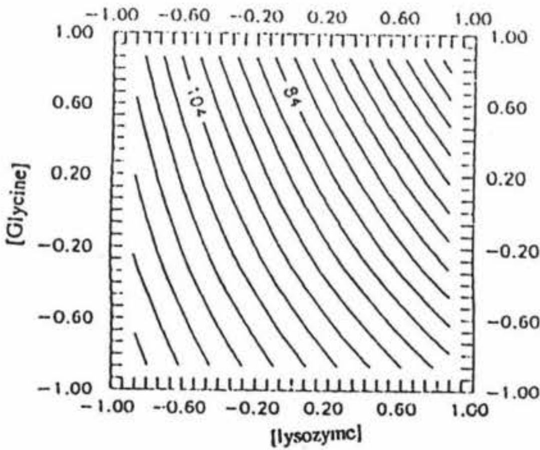


(x_2)

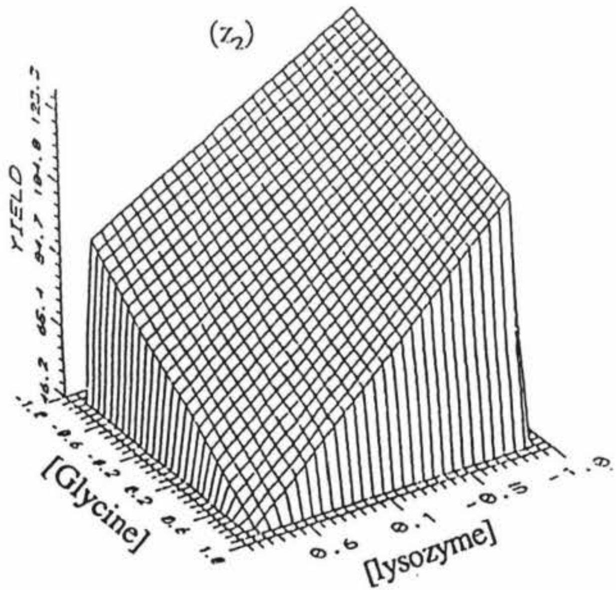


Contour(z_1) and surface(z_2) plots of yield values of *L. monocytogenes* as a function of [lysozyme] and [glycine]. The pH was held at a low level.

(z_1)



(z_2)



Legend: [lysozyme]: -1=0.2mg/ml +1=3mg/ml;
[Glycine]: -1=10mM +1=25mM; pH: -1=5.5 +1=6.5.

demonstrated.

On the basis of the results of this study, it can be concluded that lysozyme-glycine combinations may have a potential for use as a food preservative system.

5.8 Effect of glycine and lysozyme on *C. tyrobutyricum*.

5.8.1 Growth/inhibition curves.

Growth/inhibition curves for *C. tyrobutyricum* under the influence of lysozyme, pH and glycine are presented in Fig 5.17 and Fig 5.18. Results indicate that *C. tyrobutyricum* is resistant to glycine alone(Fig 5.17) and lysozyme-glycine combinations(Fig 5.18); in the latter, most treatments had minimal or no influence on the growth of *C. tyrobutyricum*.

5.8.2 Kinetic parameters.

Kinetic data for *C. tyrobutyricum* under the influence of lysozyme, pH and glycine are presented in Table 5.6. *C. tyrobutyricum* was resistant to the influence of lysozyme and glycine as there was minimal reduction in the μ_{max}, a minimal increase in the GT and a minimal lag phase extension for most of the treatments. There was also no suppression of the MPD.

5.8.3 Statistical analysis

Results of the statistical analysis are presented in Table 5.8. Regression analysis of yield of *C. tyrobutyricum* resulted in the prediction equation:

Yield = 61.5 + 0.346gly - 2.06lys + 1.34pH - 0.867gly*lys + 2.22 gly*pH + 1.14lys*pH + 0.342*gly*pH*lys.

The R-squared (94.0%) and the R-squared(adj) (92.1%) values were high. Lysozyme and the two-way interaction gly*pH are important in minimising the growth(yield) of *C. tyrobutyricum*. A reduced model equation for yield of *C. tyrobutyricum* under the influence of lysozyme, pH and glycine was of the form: Yield = 61.45 - 2.06*lys + 2.22*lys*pH. The absence of other factors from the equation means that we do not believe that the other factors have important effects on the yield.

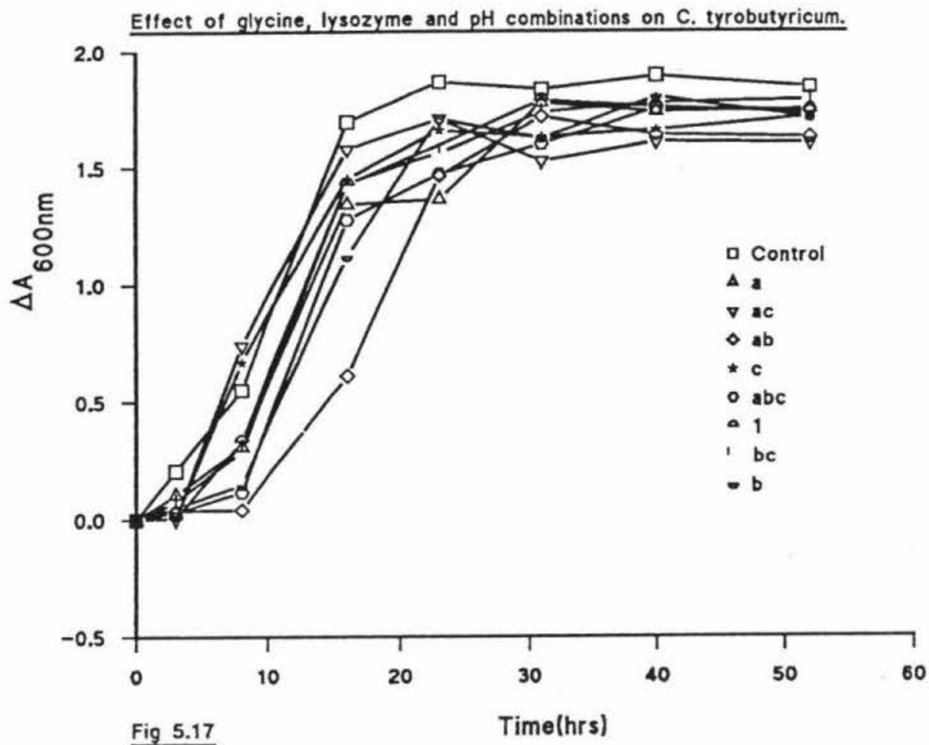


Fig 5.17

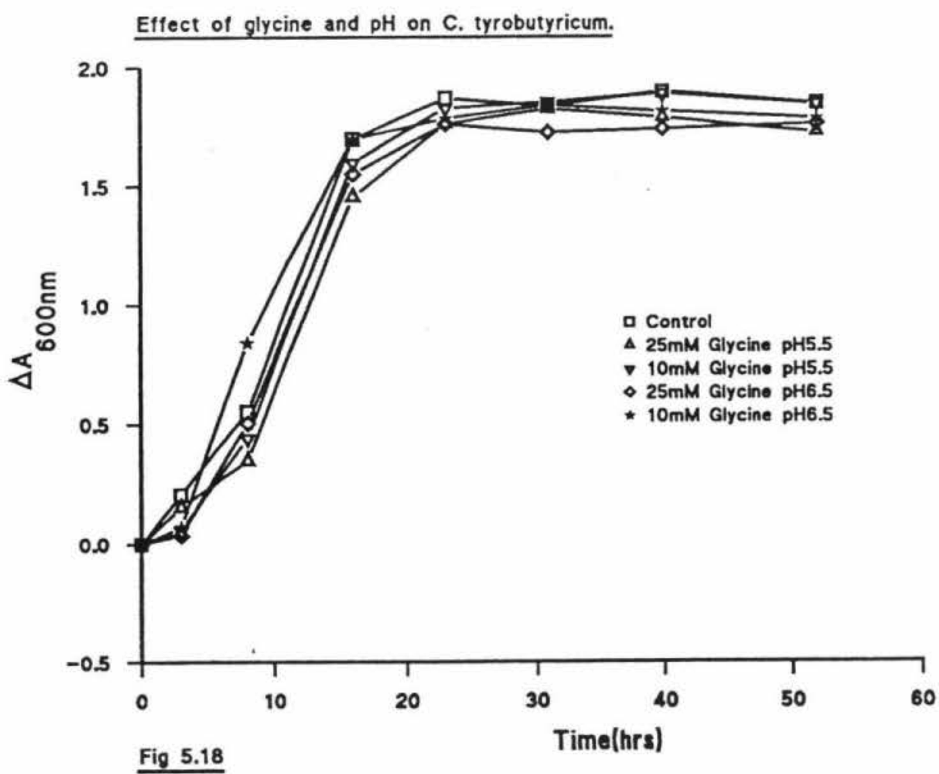


Fig 5.18

Table 5.8

Predictor	Coef	Stdev	t-ratio	p
Constant	61.4538	0.7633	80.52	0.000
gly	0.3462	0.7633	0.45	0.662
lys	-2.0600	0.7633	-2.70	0.027
pH	1.3363	0.7633	1.75	0.118
gly*lys	-0.8675	0.7633	-1.14	0.289
gly*pH	2.2188	0.7633	2.91	0.020
lys*pH	1.140	0.7633	1.49	0.174
C8	0.3425	0.7633	0.45	0.666

s = 3.053   R-sq = 94.0%   R-sq(adj) = 92.1%   C8 = gly*lys*pH

Results of this study clearly demonstrate that lysozyme-glycine treatment combinations have potential for contributing listeria-free food products produced under good manufacturing practice. However, the magnitude of inhibition of *C. tyrobutyricum* by the same antimicrobial system was much less and of little practical significance.

5.9 Effect of lysozyme and adipic acid on *L. monocytogenes*.

5.9.1 Growth/inhibition curves.

Growth curves for *L. monocytogenes* under the influence of adipic acid and lysozyme are presented in Fig 5.19 and Fig 5.20. It can be observed that complex growth curves were generated(Fig 5.19). A high lysozyme, low pH and high adipic acid treatment(ab) had the greatest influence on the growth of *L. monocytogenes,* the treatment causing a lag phase extension, a decrease in the μ_{max} and a suppression of the MPD. A high lysozyme, low pH and low adipic acid treatment(b) had no significant lag phase extension but decreased the μ_{max} and suppressed the MPD. All treatments at a low pH decreased the μ_{max} and suppressed the MPD of *L. monocytogenes*.

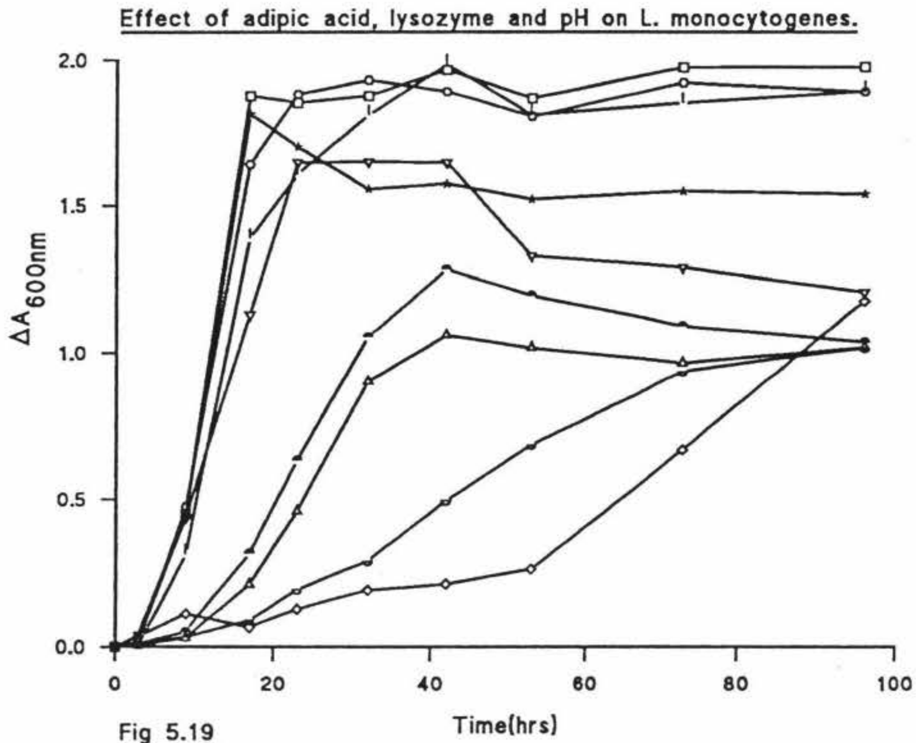


Fig 5.19

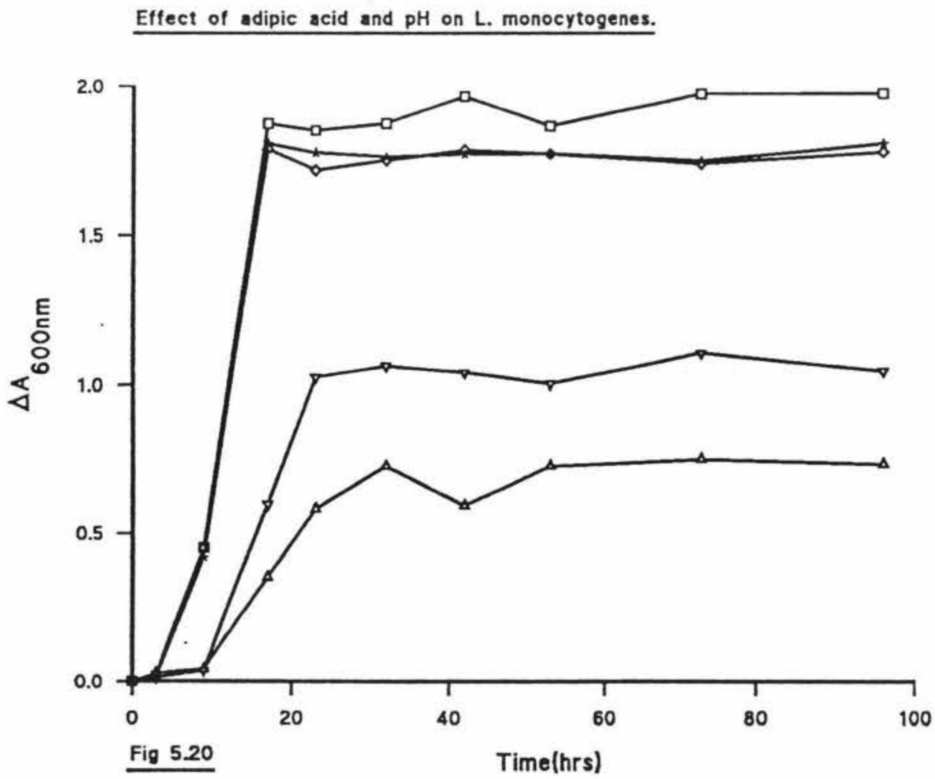


Fig 5.20

5.9.2 Kinetic parameters.

Kinetic parameters for *L. monocytogenes* under the influence of lysozyme, pH and adipic acid is presented in Table 5.9. There was a significant reduction in μ_{max} and an increase in the GT for all treatments at a low pH.

Table 5.9

Kinetic parameters.

	Microorganism type.			
	L. monocytogenes Fig 5.19 and 5.20		*C. tyrobutyricum.* Fig 5.22 and 5.23	
Lysozyme-AD Treatment	μ_{max} (#/hr)	GT(hrs)	μ_{max} (#/hr)	GT(hrs)
Control	0.180	3.98	0.181	3.83
1	0.049	14.15	0.154	4.5
a	0.031	22.36	0.118	5.87
b	0.015	46.21	0.07	9.90
ab	0.022	31.51	0.112	6.19
c	0.129	5.31	0.120	5.78
ac	0.086	8.06	0.098	7.07
bc	0.095	7.30	0.106	6.54
abc	0.118	5.87	0.085	8.15
10mM ADpH5.5	0.070	9.90	0.067	10.35
25mM AD pH5.5	0.03	23.10	0.097	7.15
10mM AD pH6.5	0.174	3.98	0.124	5.59
25mM AD pH6.5	0.168	4.13	0.121	5.73

Where AD = Adipic acid.

5.9.3 Statistical analysis.

Results of the statistical analysis are presented in Table 5.10. Regression analysis for yield of *L. monocytogenes* resulted in the prediction equation:

Yield = 104 - 3.50AD - 3.95lys + 41.7pH + 0.46AD*lys + 2.64AD*pH + 12.9lys*pH + 2.58AD*lys*pH.

Table 5.10

Predictor	Coef	Stdev	t-ratio	p
Constant	104.085	2.170	47.96	0.000
AD	-3.499	2.170	-1.48	0.167
lys	-3.946	2.170	-1.67	0.123
pH	41.656	2.170	17.61	0.000
AD*lys	0.461	2.170	0.19	0.849
AD*pH	2.639	2.170	1.12	0.288
lys*pH	12.913	2.170	5.46	0.000
C8	2.584	2.170	1.09	0.298

s = 9.459 R-sq = 96.9% R-sq(adj) = 95.0% C8 = AD*lys*pH

Results of the statistical analysis suggest that the main effects (adipic acid, lysozyme), the two-way interactions(AD*lys, AD*pH) and the three-way interaction(AD*lys*pH) have an insignificant effect at ($P>0.05$). However, the pH effect and the lys*pH interaction have a significant effect at ($P<0.05$). Therefore a reduced model equation for yield of *L. monocytogenes* under the influence of lysozyme, pH and adipic acid was of the form: Yield = 104.09 + 41.66*pH + 12.91*lys*pH.

5.9.4 Evaluating the model equation.

The R-squared(96.9%) and the R-squared(adj)(95.0%) values are high(Table 5.10). From the residuals plot, most of the residual values were evenly distributed above and below zero over the range of the data.

5.9.5 Response surface plots.

Contour and surface plots(Fig 5.21(e_1)) and 5.21(e_2)) illustrate the interactive effects of pH and lysozyme on the yield of *L. monocytogenes*. A combination of increasing levels of lysozyme and decreasing levels of pH decreased the yield of *L. monocytogenes*.

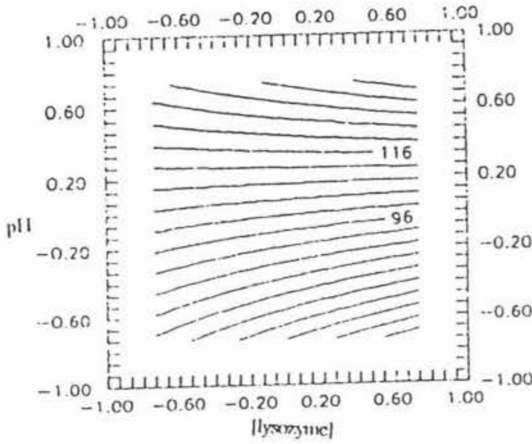
Our data show that the most significant factors affecting the growth(yield) of *L.*

Fig 5.21

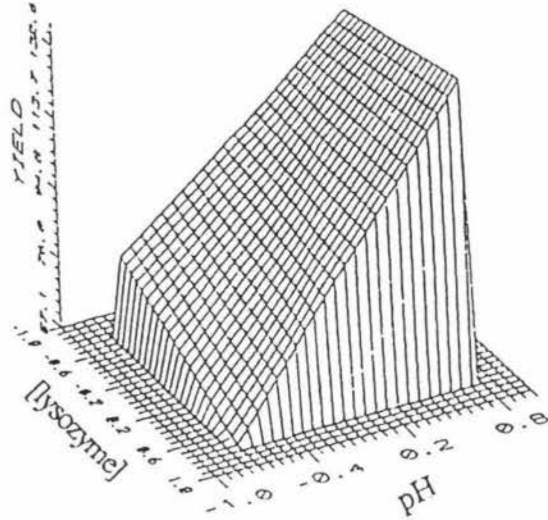
Adipic acid, lysozyme and pH as factors influencing growth of *L. monocytogenes*.

Contour(c_1) and surface(c_2) plots of yield values for *L. monocytogenes* as a function of [lysozyme] and pH. The [Adipic acid] was held at a high level.

(c_1)

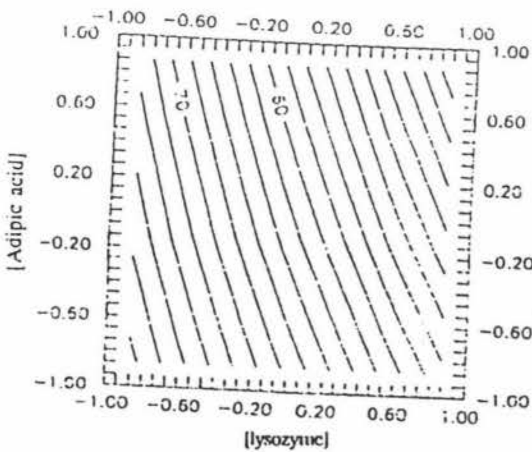


(c_2)

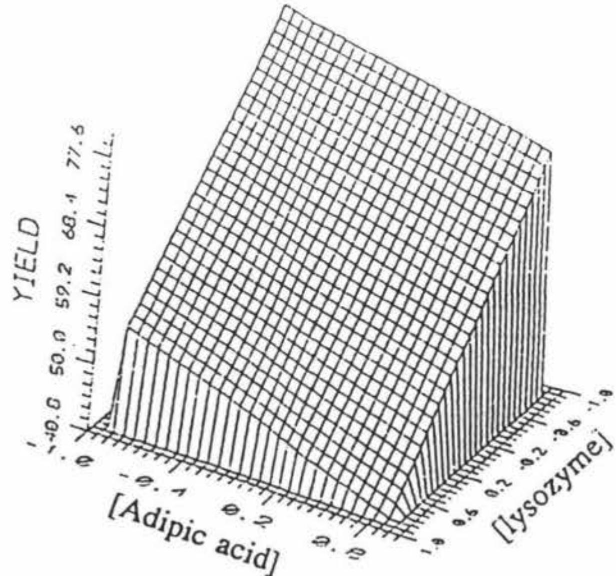


Contour(f_1) and surface(f_2) plots of yield values of *L. monocytogenes* as a function of [lysozyme] and [Adipic acid]. The pH was held at a low level.

(g_1)



(g_2)



Legend: [lysozyme]: -1=0.2mg/ml +1=3mg/ml;
[Adipic acid]: -1=10mM +1=25mM; pH: -1=5.5 +1=6.5.

monocytogenes are pH and the two-way interaction (pH*lys).

The following conclusion can be drawn for this particular system.

Although the main effect(lysozyme) is insignificant, the presence of lysozyme is still important for its interaction with pH.

Although the other insignificant factors have not been removed from the full model equation, they would be expected to contribute minimally in minimising the yield of *L. monocytogenes*.

5.10 Effect of lysozyme and adipic acid on *C. tyrobutyricum*.

5.10.1 Growth/inhibition curves.

Growth curves for *C. tyrobutyricum* under the influence of adipic acid, lysozyme and pH are presented in Fig 5.22 and Fig 5.23. The growth curves indicate that *C. tyrobutyricum* is resistant to lysozyme-adipic acid treatments. For all treatments, there was no significant lag phase extension and no significant suppression of the MPD.

5.10.2 Kinetic parameters.

The kinetic data for *C. tyrobutyricum* under the influence of adipic acid and lysozyme are presented in Table 5.9. For all treatments, there was no significant reduction in the specific growth rate and no significant increase in the GT.

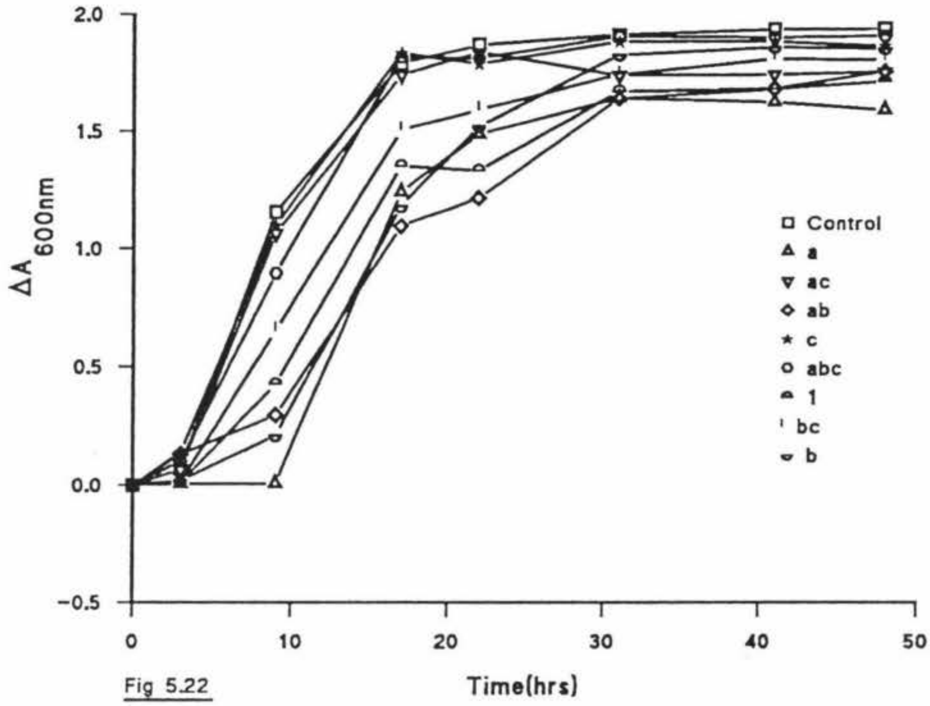


Fig 5.22

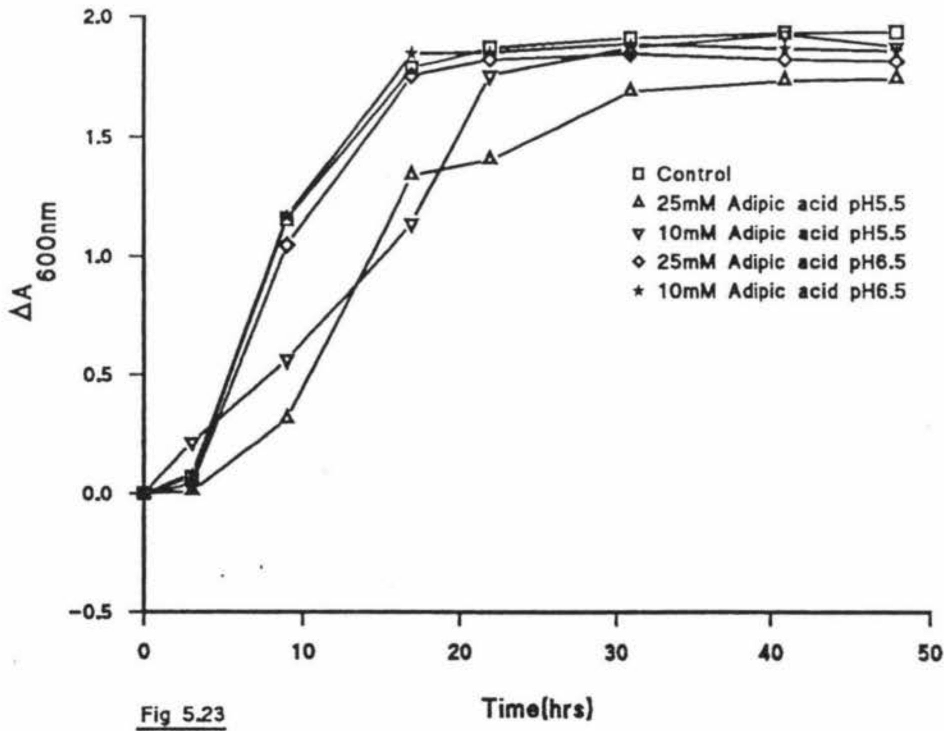


Fig 5.23

5.10.3 Statistical analysis.

Table 5.11

Predictor	Coef	Stdev	t-ratio	p
Constant	61.712	1.178	52.39	0.000
AD	0.878	1.178	0.75	0.477
lys	-2.356	1.178	-2.00	0.081
pH	4.766	1.178	4.05	0.004
AD*lys	1.658	1.178	1.41	0.197
AD*pH	2.599	1.178	2.21	0.058
lys*pH	-1.412	1.178	-1.20	0.265
C8	2.594	1.178	2.20	0.058

s = 4.711 R-sq = 81.0% R-sq(adj) = 64.3% C8 = AD*lys*pH

Results of the statistical analysis are presented in Table 5.11. No large regression coefficients were obtained. The pH was the only significant factor affecting the growth(yield) of *C. tyrobutyricum*. The main effects(adipic acid and lysozyme), the two-way interactions and the three-way interaction had an insignificant effect at ($P>0.05$). A reduced model equation for yield of *C. tyrobutyricum* under the influence of pH, lysozyme and adipic acid was of the form: Yield = 61.71 + 4.77*pH.

From the statistical analysis, it can be concluded that a lysozyme-adipic acid combination treatment is ineffective in controlling the growth of *C. tyrobutyricum* in the range studied.

5.11 Effect of sodium phosphate dibasic(SPDB), lysozyme and pH on *L. monocytogenes*.

5.11.1 Growth/inhibition curves.

Growth/inhibition curves for *L. monocytogenes* under the influence of SPDB, lysozyme and pH are presented in Fig 5.24. The growth curves indicate that lag phase extensions and decrease in the MPD was associated with treatments at a low pH.

Effect of sodium phosphate dibasic(SPDB), lysozyme and pH on L. monocytogenes.

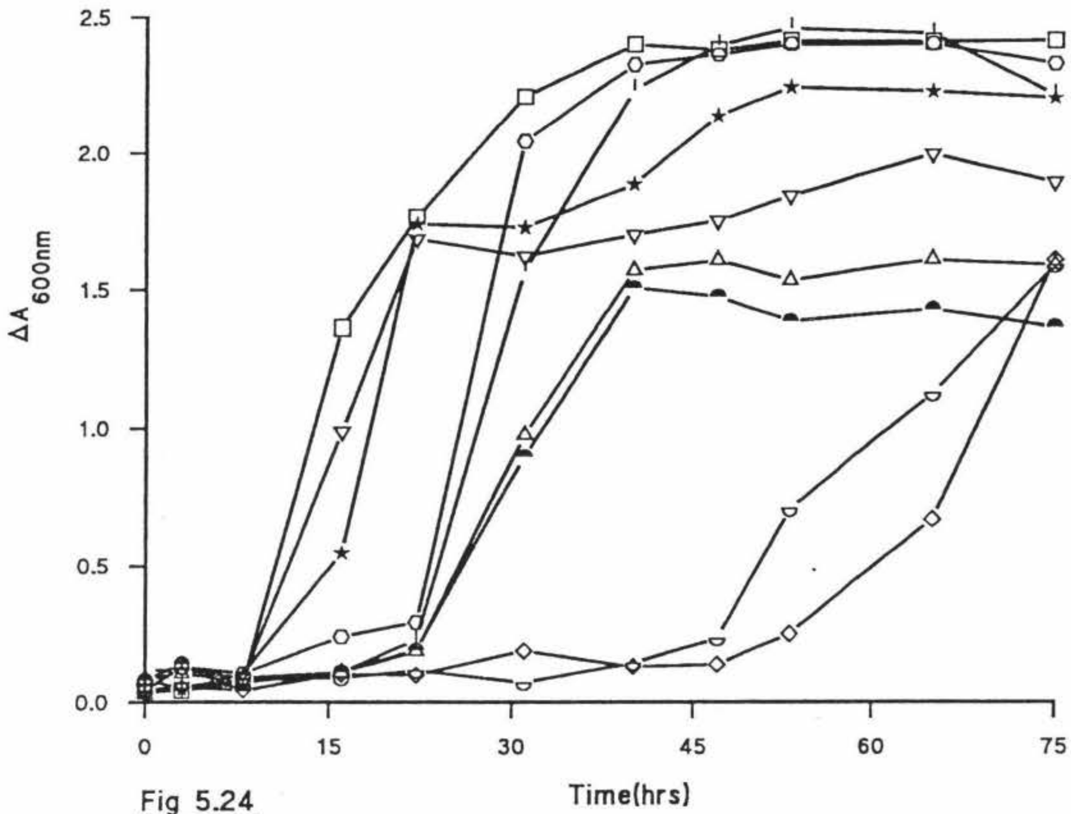


Fig 5.24

Lag duration of L. monocytogenes under the influence of SPDB, lysozyme and pH.

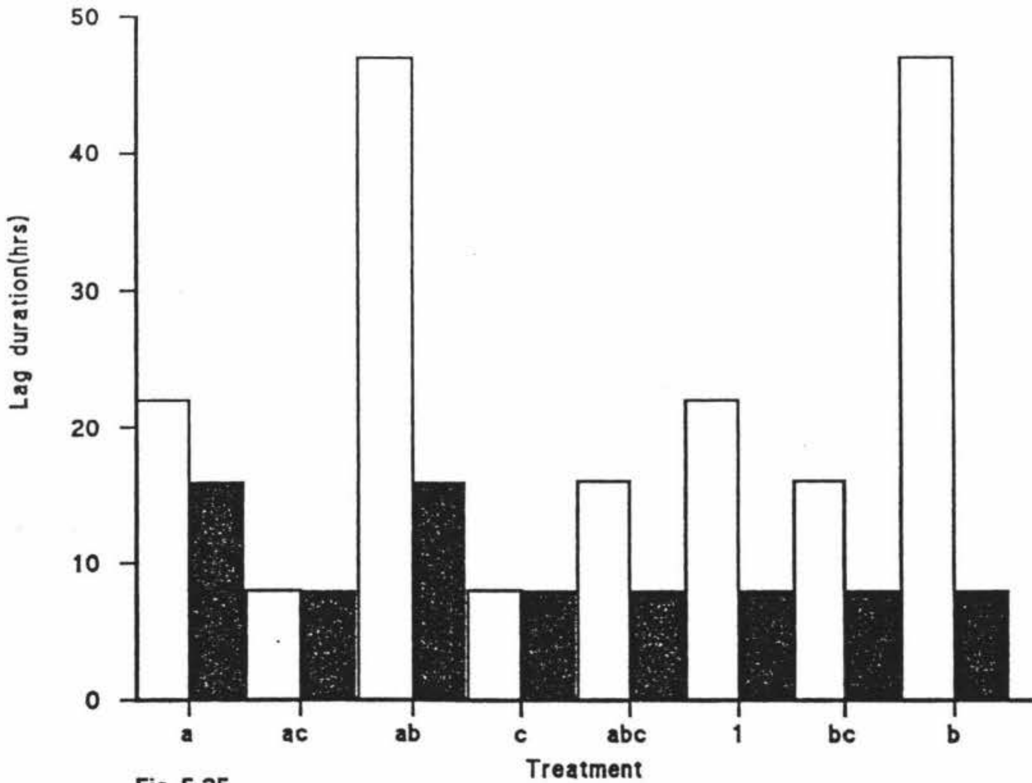


Fig 5.25

□ runs with SPDB, lysozyme and pH varied.

■ runs with SPDB and pH varied but without lysozyme.

5.11.2 Kinetic parameters.

Kinetic data for *L. monocytogenes* under the influence of lysozyme, pH and SPDB are presented in Table 5.12.

Table 5.12

Kinetic parameters.

	Microorganism type.			
	L. monocytogenes. *Fig 5.24.*		*C. tyrobutyricum.* *Fig 5.27.*	
Lysozyme-SPDB Treatment	μ_{max} (#/hr)	GT(hrs)	μ_{max} (#/hr)	GT(hrs)
Control	0.123	5.64	0.132	5.25
1	0.073	9.50	0.017	40.77
a	0.077	9.00	0.066	10.50
b	0.046	15.07	0.08	8.66
ab	0.061	11.36	0.072	9.63
c	0.113	6.13	0.115	6.03
ac	0.114	6.08	0.091	7.62
bc	0.096	7.22	0.100	6.93
abc	0.195	3.55	0.107	6.48
10mM SPDB pH5.5	0.085	8.15	0.088	7.88
25mM SPDB pH5.5	0.078	8.89	0.099	7.00
10mM SPDB pH6.5	0.122	5.68	0.089	7.79
25mM SPDB pH6.5	0.123	5.64	0.116	5.98

Where SDPB = sodium phosphate dibasic.

Treatments of SPDB at a low pH, alone or in combination with lysozyme lowered the μ_{max} and increased the GT. The same trend was also observed for lag phase extension and lowering of the MPD(Fig 5.24 and Fig 5.25). Most combination treatments had a greater lag phase extension than single factor treatments.

5.11.3 Statistical analysis.

Results of the statistical analysis are presented in Table 5.13. Regression analysis for yield resulted in the equation:

Yield = 78.2 - 1.07SPDB - 9.57lys + 31.1pH + 0.34SPDB*lys - 0.36SPDB*pH + 11.4lys*pH + 2.48SPDB*pH*lys.

Table 5.13

Predictor	Coef	Stdev	t-ratio	p
Constant	78.185	1.070	73.07	0.000
SPDB	-1.069	1.166	-0.92	0.379
lys	-9.574	1.166	-8.21	0.000
pH	31.051	1.166	26.63	0.000
SPDB*lys	0.341	1.166	0.29	0.776
SPDB*pH	-0.364	1.166	-0.31	0.760
lys*pH	11.381	1.166	9.76	0.000
C8	2.483	1.166	2.31	0.057

s = 4.664 R-sq = 98.8% R-sq(adj) = 98.0% C8 = SPDB*lys*pH

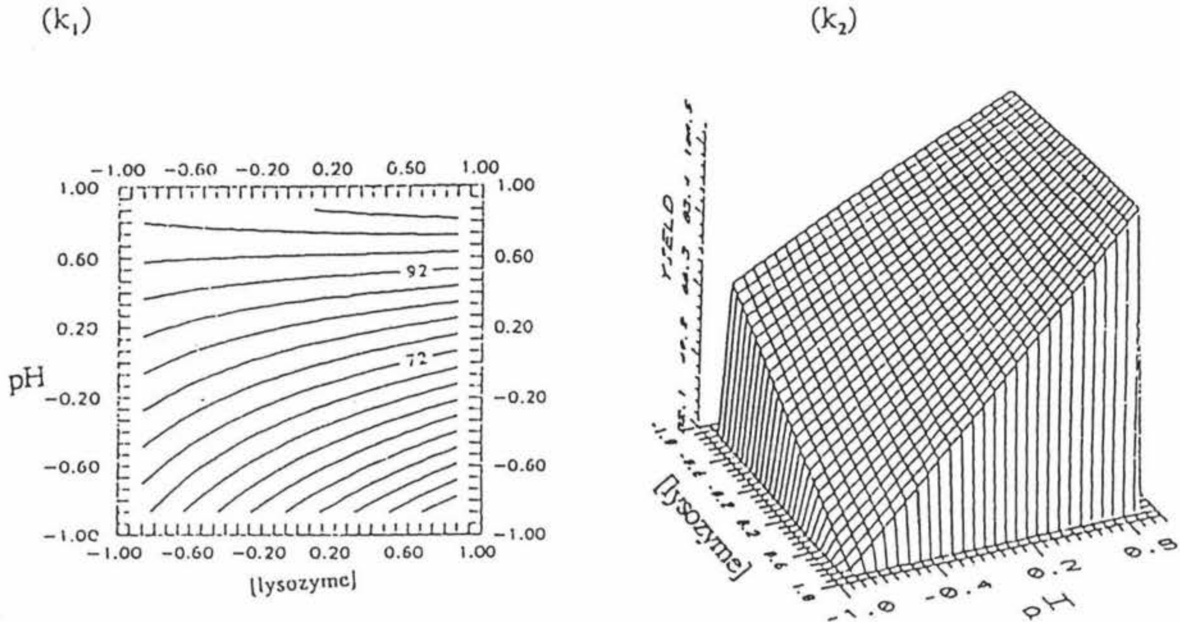
The main effects(lysozyme and pH) had a significant effect on the growth(yield) of *L. monocytogenes* and the main effect SPDB had an insignificant effect at (P>0.05). The two-way interactions (SPDB*lys and SPDB*pH) and the three-way interaction(SPDB*lys*pH) also had insignificant effects at (P>0.05). A reduced model equation for yield of *L. monocytogenes* under the influence of pH, lysozyme and SPDB was of the form: Yield = 78.19 - 9.57*lys + 31.05*pH + 11.38*lys*pH.

Fig 5.26

SPDB, lysozyme and pH as factors influencing growth of *L. monocytogenes*.

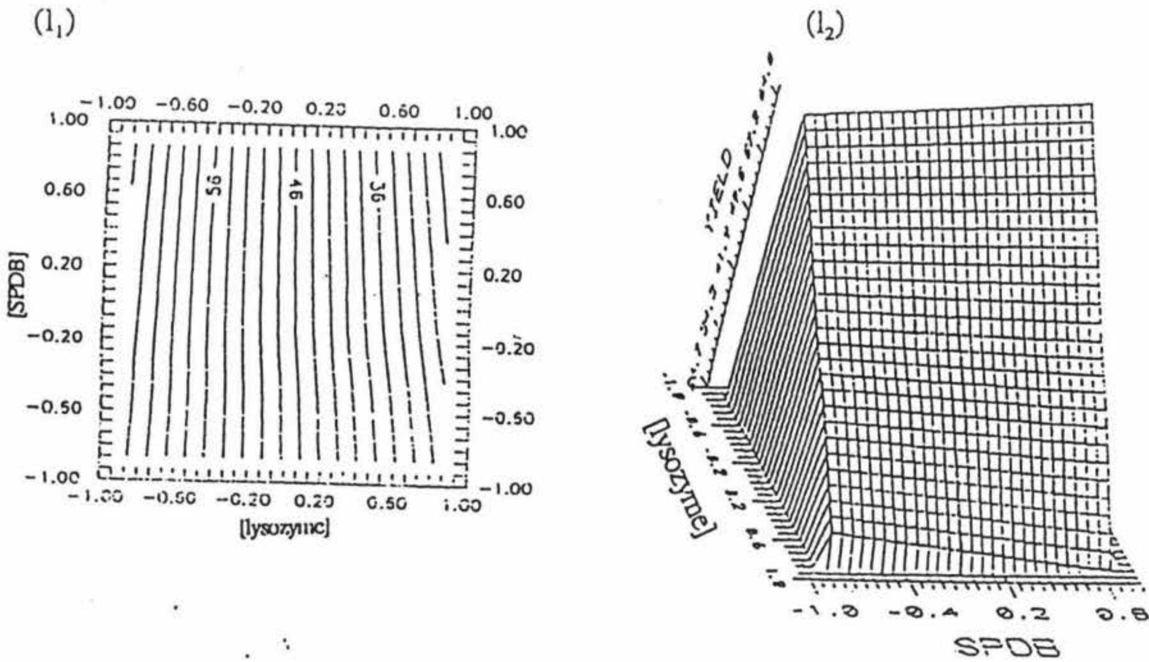
Contour(k_1) and surface(k_2) plots of yield values for *L. monocytogenes* as a function of [lysozyme] and pH. The [SPDB] was held at a high level.

(k_1) (k_2)



Contour(l_1) and surface(l_2) plots of yield values of *L. monocytogenes* as a function of [lysozyme] and [SPDB]. The pH was held at a low level.

(l_1) (l_2)



Legend: [lysozyme]: -1=0.2mg/ml +1=3mg/ml;
[SPDB]: -1=10mM +1=25mM; pH: -1=5.5 +1=6.5.

5.11.4 Evaluating the model equation.

From Table 5.13, it can be observed that the R-squared(98.8%) and the R-sq(adj)(98.0%) values are high. The residuals plot had residual values evenly distributed about the zero mark. However, the residual values increased as the predicted yield increased.

5.11.5 Response surface plots.

Response surface plot, Fig 5.26(k_2) clearly illustrate the interaction between lysozyme and pH and demonstrate that yield values for *L. monocytogenes* depend on lysozyme, pH and their interaction. In conformity with the statistical analysis, the response surface plot(Fig 5.26(l_2) demonstrate the large effect of lysozyme on the yield of *L. monocytogenes* and the insignificant effect of SPDB in decreasing the yield of *L. monocytogenes*. Although there was an interaction between lysozyme and SPDB(lys*SPDB) the interaction was insignificant at ($P>0.05$). The insignificance of SPDB and its corresponding low coefficients of these factors in the model equation imply that the use of SPDB and lysozyme as a preservative system may not be of practical significance.

5.12 Effect of SPDB, lysozyme and pH on *C. tyrobutyricum*.

5.12.1 Growth/inhibition curves.

The growth/inhibition curve for *C. tyrobutyricum* under the influence of SPDB, lysozyme and pH is presented in Fig 5.27. For all treatments there was no significant lag phase extension and treatments at a low pH exhibited a lower MPD than the control or treatments at a higher pH(Fig 5.27).

5.12.2 Kinetic parameters.

The kinetic parameters for *C. tyrobutyricum* under the influence of SPDB, lysozyme and pH are presented in Table 5.12. For all treatments, there was no significant reduction in the μ_{max} and there was no significant increase in the GT.

Effect of SPDB, lysozyme and pH on C. tyrobutyricum.

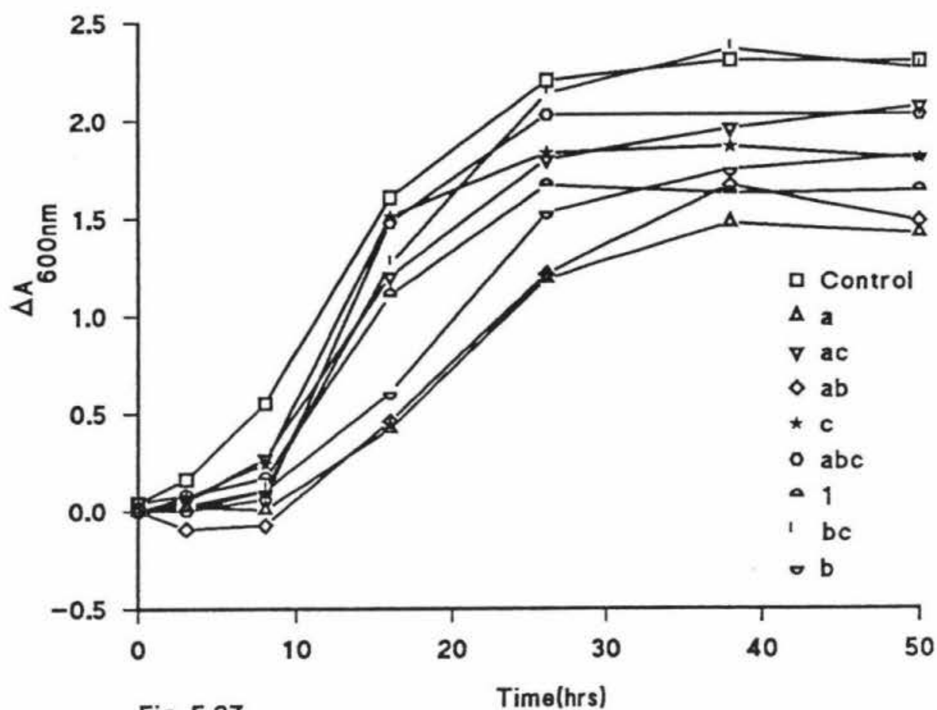


Fig 5.27

Effect of sodium hexametaphoshate, lysozyme and pH on L. monocytogenes.

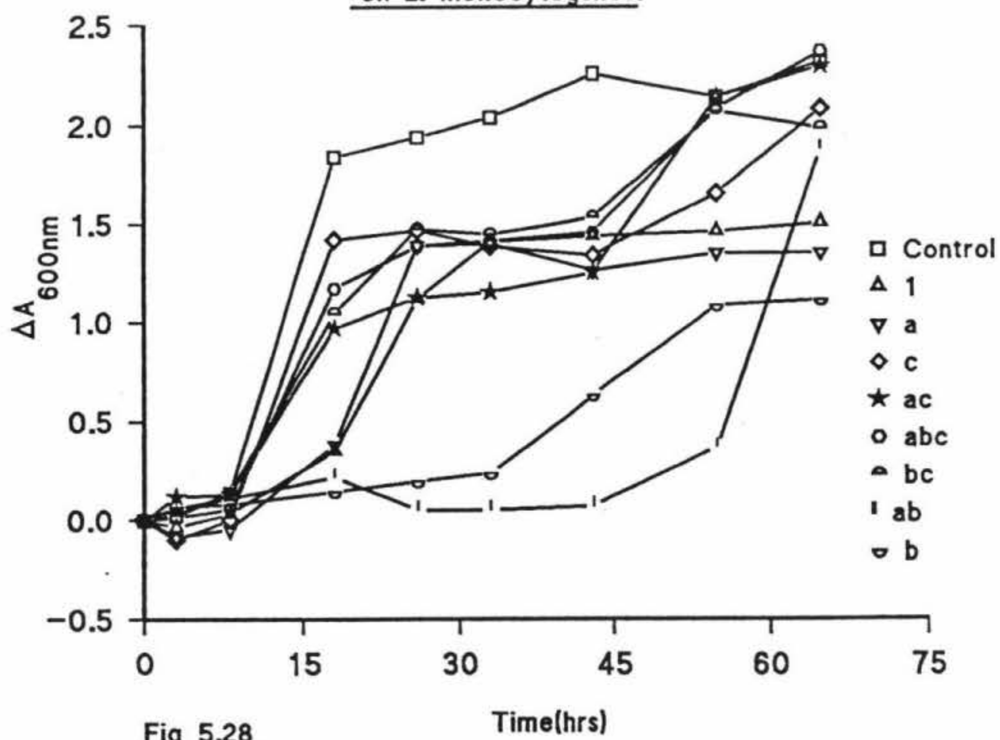


Fig 5.28

5.12.3 Statistical analysis.

Results of the statistical analysis are presented in Table 5.14.

The regression equation for yield was in the form:

YIELD = 61.7 - 1.94SPDB + 2.01lys + 10.5pH + 1.59SPDB*lys + 2.22 SPDB*pH + 0.332lys*pH + 0.458SPDB*lys*pH

Table 5.14

Predictor	Coef	Stdev	t-ratio	p
Constant	61.7256	0.9410	65.60	0.000
SPDB	-1.9381	0.9410	-2.06	0.073
lys	2.0081	0.9410	2.13	0.065
pH	10.5444	0.9410	11.21	0.000
SPDB*lys	1.5894	0.9410	1.69	0.130
SPDB*pH	2.2206	0.9410	2.36	0.046
lys*pH	0.3319	0.9410	0.35	0.733
C8	0.4581	0.9410	0.49	0.639

s = 3.764 R-sq = 94.7% R-sq(adj) = 90.1% C8 = SPDB*lys*pH

The two-way interaction (SPDB*pH) and pH were the only significant factors affecting the yield of *C. tyrobutyricum*.

The R-squared(94.7%) and the R-squared(adj)(90.1%) values are high. A reduced model equation for *C. tyrobutyricum* under the influence of pH, lysozyme and SPDB was of the form: Yield = 61.73 + 10.54*pH + 2.22*SPDB*pH.

From the statistical analysis it can be concluded that SPDB-lysozyme combination treatments have a minimal inhibitory effect on the growth of *C. tyrobutyricum* and are therefore unlikely to be worth pursuing in food systems.

5.13 Effect of lysozyme, sodium hexametaphosphate(SHMP) and pH on *L. monocytogenes*.

5.13.1 Growth/inhibition curves.

The growth/inhibition curves for *L. monocytogenes* under the influence of lysozyme, sodium hexametaphosphate and pH are presented in Fig 5.28. Treatments at a low pH had a significant degree of inhibition in either extending the lag phase duration or

suppressing the MPD. Treatment ab, for example, had a lag phase extension but the MPD was not significantly suppressed. Treatment b had the impact of extending the lag phase duration and suppressing the MPD.

5.13.2 Kinetic parameters.

The kinetic parameters for *L. monocytogenes* under the influence of lysozyme, SHMP and pH are presented in Table 5.15.

Table 5.15

Kinetic parameters.

	Microorganism type. *L. monocytogenes.* *Fig 5.28*	
Lysozyme-SHMP Treatment	μ_{max} (#/hr)	GT(hrs)
Control	0.171	4.05
1	0.059	11.75
a	0.126	5.50
b	0.038	18.24
ab	0.080	8.66
c	0.141	4.92
ac	0.056	12.38
bc	0.071	9.76
abc	0.111	6.24

Inhibition in the form of μ_{max} reduction was exhibited by a high lysozyme, low pH and low SHMP treatment(b). Although there was a lag phase extension due to treatment (ab), there was no significant suppression of the MPD, the u_{max} was $0.08h^{-1}$ and the GT was only 8.66hrs.

5.13.3 Statistical analysis.

Results of the statistical analysis are presented in Table 5.16. Regression analysis for the yield values resulted in the equation:

Yield = 58.8 - 3.79SHMP -5.43lys + 19.9pH + 0.09SHMP*lys - 0.168lys*pH - 9.17SHMP*pH + 0.18lys*pH*SHMP.

Table 5.16

Predictor	Coef	Stdev	t-ratio	p
Constant	58.787	1.552	37.88	0.000
SHMP	-3.787	1.552	-2.24	0.047
lys	-5.434	1.552	-3.21	0.008
pH	19.862	1.552	11.74	0.000
SHMP*lys	0.088	1.552	0.05	0.959
SHMP*pH	9.167	1.552	5.42	0.000
lys*pH	-0.168	1.552	-0.10	0.923
C8	0.177	1.552	0.10	0.919

s = 6.765 R-sq = 94.3% R-sq(adj) = 90.7% C8 = SHMP*lys*pH

The statistical analysis suggest that the main effects(SHMP, lys, pH), and the two-way interaction (SHMP*pH) have a significant effect at ($P<0.05$). However, the two-way interaction(lys*pH) and the three-way interaction(pH*lys*SHMP) had an insignificant effect at ($P>0.05$). Low regression coefficients were obtained for the significant factors except for the pH effect. A reduced model equation for yield of *L. monocytogenes* under the influence of lysozyme, pH and SHMP was of the form: Yield = 58.79 - 3.79*SHMP - 5.43*lys + 19.86*pH + 9.17*SHMP*pH.

5.13.4 Evaluating the model equation.

The R-squared(94.3%) and the R-squared(adj)(90.7%) in Table 5.16 are high. The residual plot had the residual values distributed evenly about zero, however there was an increase in the residual values associated with higher predicted values.

5.13.5 Response surface plots.

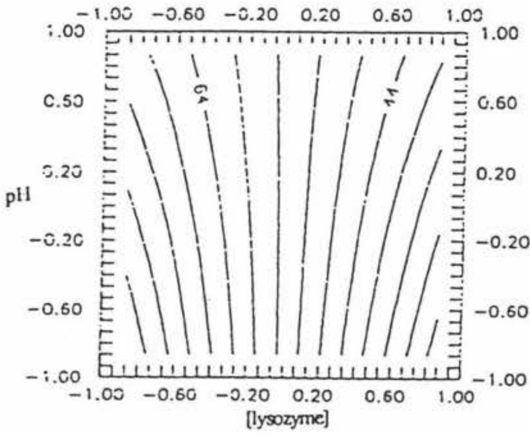
Response surface plots, Fig 5.29(h_2) and 5.29(i_2) illustrates the little interactions between lysozyme*pH and lysozyme*SHMP and how each factor markedly influence the growth(yield) of *L. monocytogenes* as it increases from its low level to its high level. Quantitative changes in yield as affected by the various factors and their interaction are presented in Fig 5.29(h_1) and 5.29(i_1).

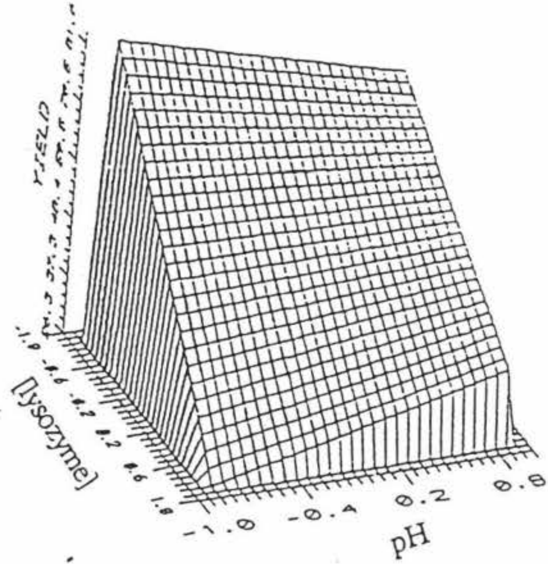
Fig 5.29

SHMP, lysozyme and pH as factors influencing growth of *L. monocytogenes*.

Contour(h_1) and surface(h_2) plots of yield values for *L. monocytogenes* as a function of [lysozyme] and pH. The [SHMP] was held at a high level.

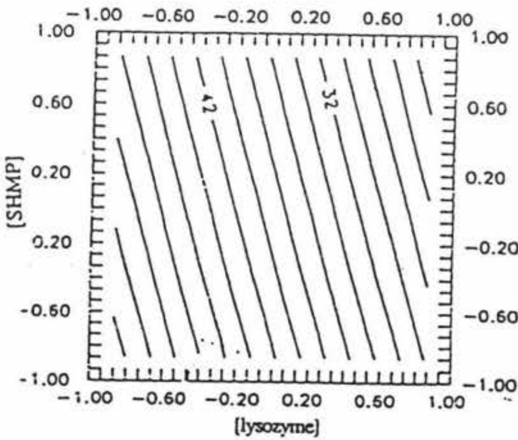
(h_1) (h_2)

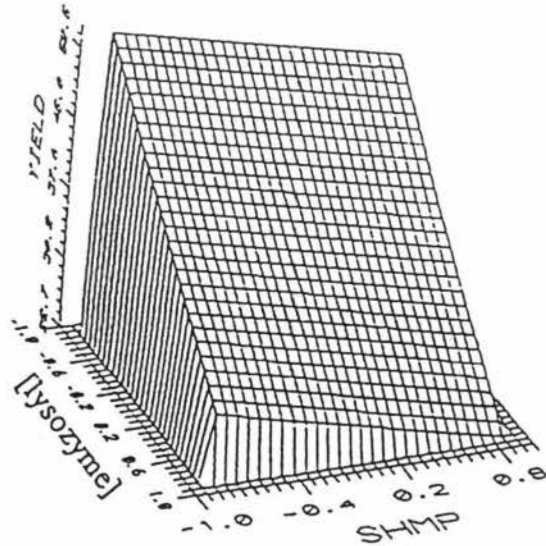




Contour(i_1) and surface(i_2) plots of yield values of *L. monocytogenes* as a function of [lysozyme] and [SHMP]. The pH was held at a low level.

(i_1) (i_2)





Legend: [lysozyme]: -1=0.2mg/ml +1=3mg/ml;
[SHMP]: -1=10mM +1=25mM; pH: -1=5.5 +1=6.5.

When the antimicrobial effects of SPDB and SHMP are compared, results of this study are in agreement with those of Lee et al. (1994) who demonstrated that the longer the chain length of phosphates, the greater the inhibition. Our results indicate that SPDB-lysozyme and SHMP-lysozyme treatment combinations are bacteriostatic to early-exponential phase cells of *L. monocytogenes*. These findings could vary when these systems are applied to the meat processing industry because phosphatases in fresh meats reduce the antibacterial effects of phosphates (Lee et al. 1994).
Inhibition of *C. tyrobutyricum* by the lysozyme-SPDB treatment combinations was so minimal to be of any practical significance.

5.15 Effect of GDL, lysozyme and pH on *L. monocytogenes*.

5.15.1 Growth curves.

Growth curves were generated from the turbidimetric assay (Fig 5.30 and Fig 5.31) and from the plate count assay (Fig 5.32 and Fig 5.34). Treatments were the same for each set of data for Fig 5.30 and 5.32 as well as for Fig 5.31 and 5.34. The growth curves are highly similar in terms of lag phase extension, suppression of the specific growth rate and suppression of the maximum population density(MPD). The only major difference is the inability of the turbidimetric assay to detect a sudden decrease in the bacterial numbers within the first 5 hours and a continued decrease in numbers for a high GDL, high lysozyme and low pH treatment(Fig 5.30 versus Fig 5.32 and Fig 5.31 versus Fig 5.34).

5.15.2 Kinetic parameters.

Kinetic parameters for *L. monocytogenes* under the influence of GDL, lysozyme and pH are presented in Table 5.17.

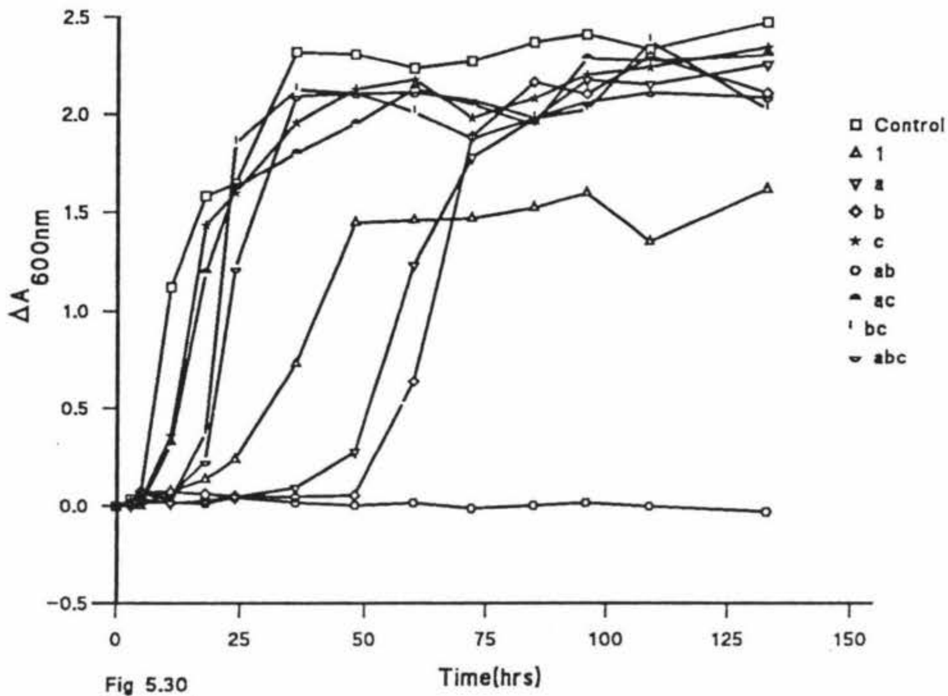


Fig 5.30

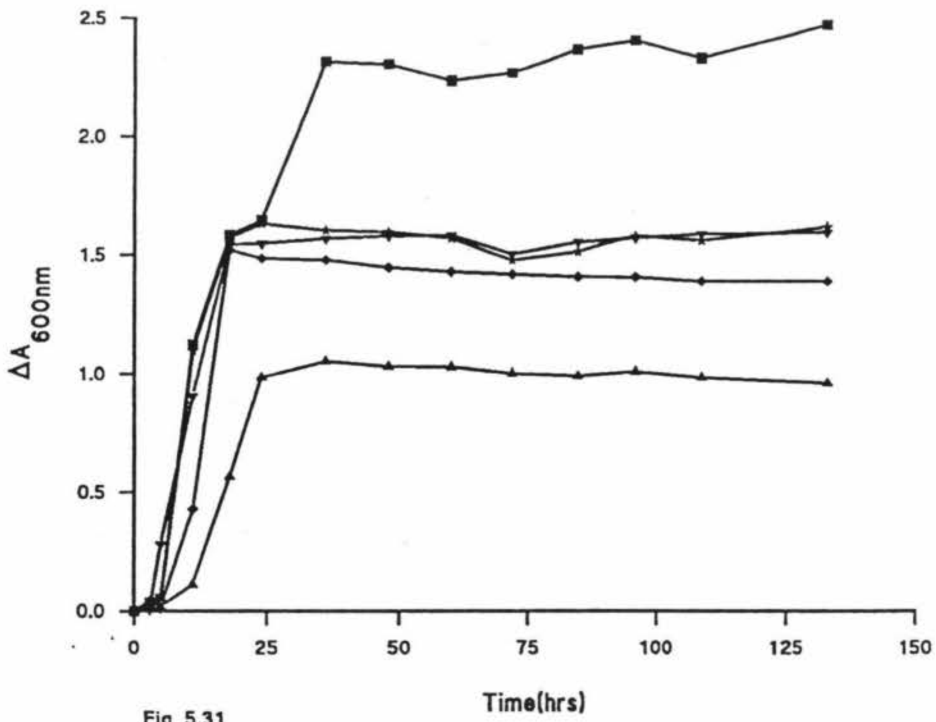


Fig 5.31

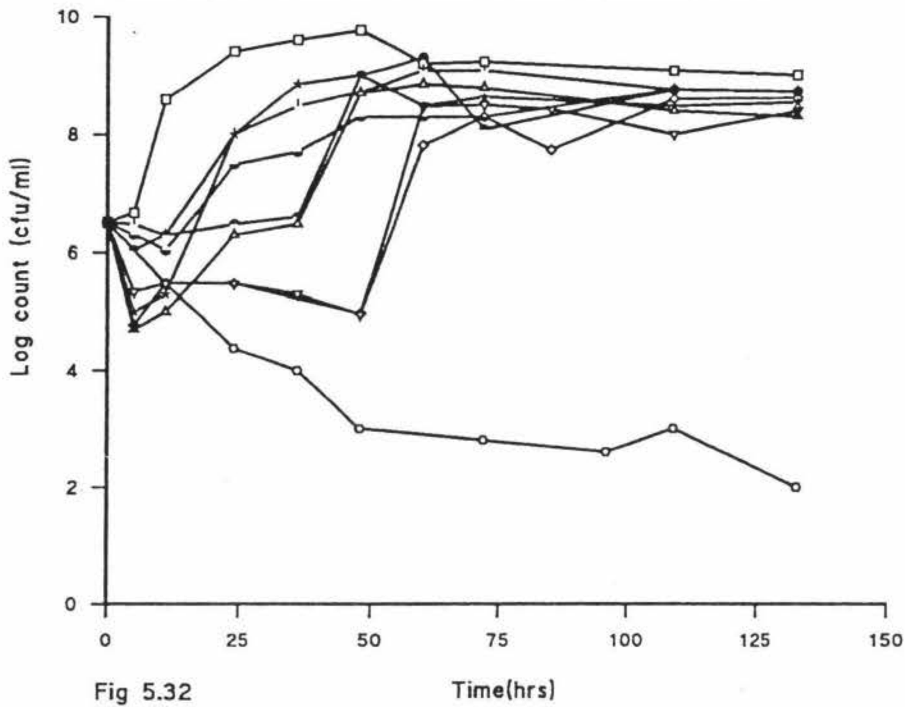


Fig 5.32

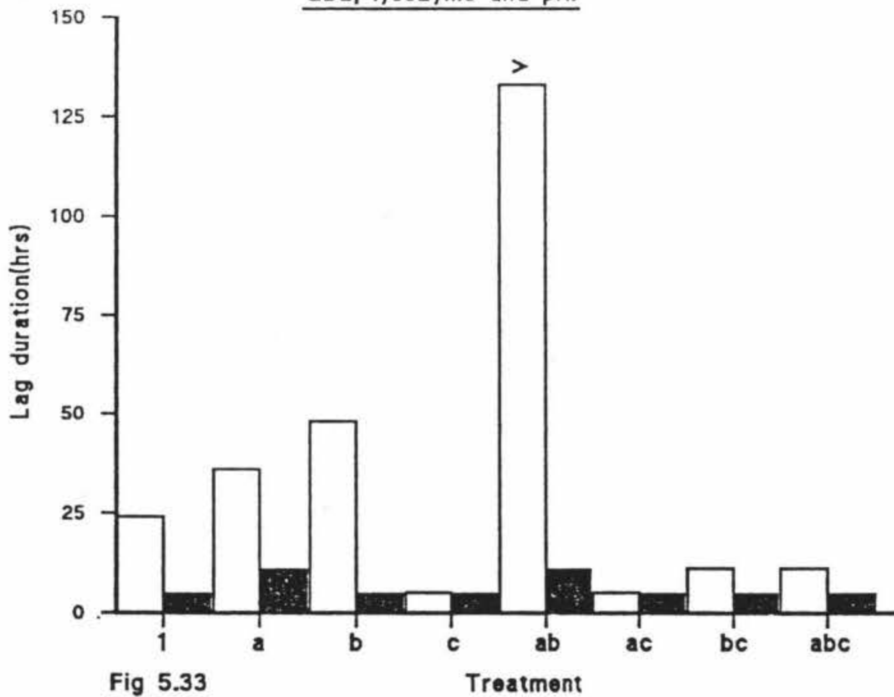


Fig 5.33

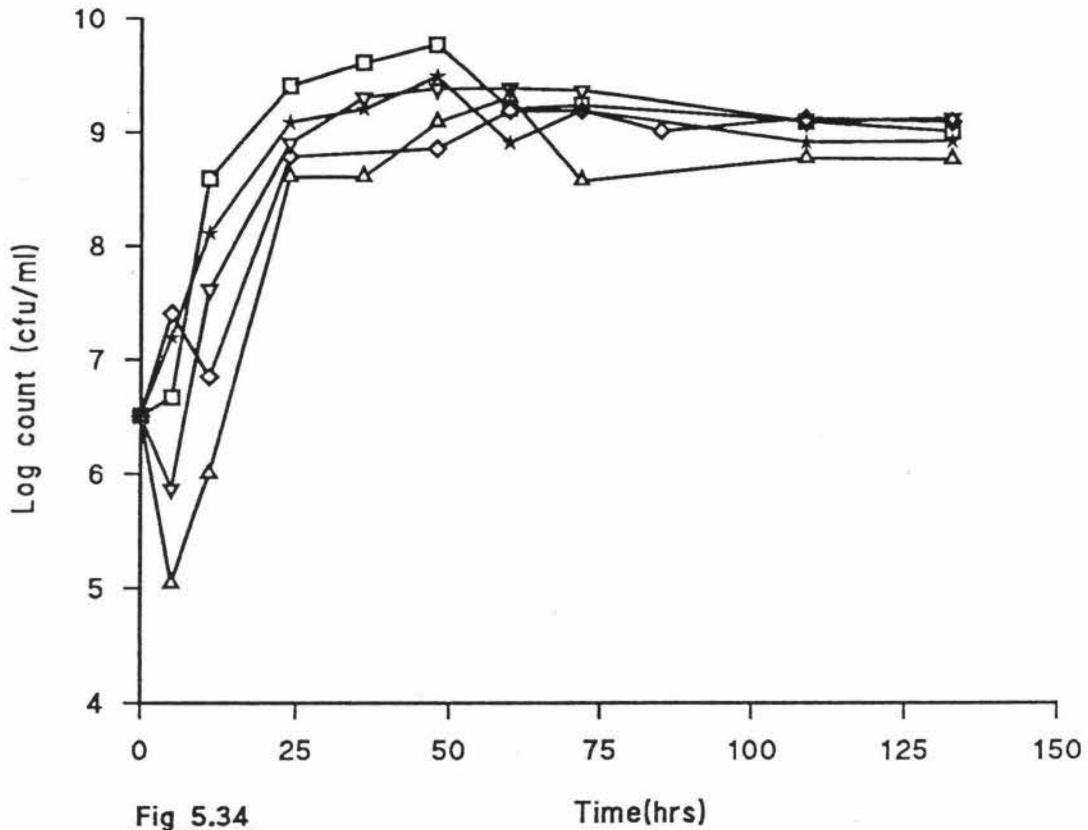


Fig 5.34

□ Control △ 25mM GDL(pH5.5) ▽ 25mM GDL(pH6.5)
◇ 10mM GDL(pH 5.5) ★ 10mM GDL(pH 6.5)

Table 5.17

	Kinetic parameters			
		Microorganism type.		
	L. monocytogenes *Fig 5.30 and 5.32*		*C. tyrobutyricum* *Fig 5.36 and 5.37*	
Lysozyme-GDL Treatment	μ_{max} (#/hr)	GT(hrs)	μ_{max} (#/hr)	GT(hrs)
Control	0.180	8.45	0.236	2.94
1	0.050	13.86	0.166	4.18
a	0.060	11.55	0.098	7.07
b	0.076	9.12	0.107	6.48
ab	*	*	0.046	15.07
c	0.066	10.50	0.220	3.15
ac	0.063	11.00	0.197	3.52
bc	0.078	8.87	0.215	3.22
abc	0.071	9.76	0.139	4.99
10mM GDL pH5.5	0.087	7.97	0.152	4.56
25mM GDL pH5.5	0.053	8.35	0.083	8.35
10mM GDL pH6.5	0.082	8.45	0.234	2.96
5mM GDL pH6.5	0.070	9.90	0.194	3.57

* = no OD increase.

There was a significant reduction in the μ_{max} of *L. monocytogenes* for all treatments (Table 5.17) as well as an increase in the GT. For treatment ab, there was no growth of *L. monocytogenes*; the treatment was listericidal and continued incubation of this treatment up to 125 hours resulted in no increase in turbidity. Fig 5.33 shows that in most treatments, the presence of lysozyme increased the lag phase duration.

5.15.3 Statistical analysis.

Results of the statistical analysis are presented in Table 5.18. Regression analysis for yield resulted in the equation:

Yield = 171 - 18.5GDL - 23.2lys + 60.8pH - 19.5GDL*lys + 16.1GDL*pH + 15.2lys*pH + 21.1GDL*lys*pH.

Table 5.18

Predictor	Coef	Stdev	t-ratio	p
Constant	170.557	4.624	36.89	0.000
GDL	-18.486	5.039	-3.67	0.004
lys	-23.186	5.039	-4.60	0.00
pH	60.780	5.039	12.06	0.000
GDL*lys	-19.530	5.039	-3.88	0.003
GDL*pH	16.104	5.039	3.20	0.009
lys*pH	15.216	5.039	3.02	0.012
C8	21.122	5.039	4.19	0.002

s = 20.15 R-sq = 95.5% R-sq(adj) = 92.6% C8 = GDL*lys*pH

All effects were significant at $P<0.05$.

According to our results, use of lysozyme, GDL and low pH(5.5) in foods offers significant potential for improving overall keeping quality and safety.

5.15.4 Evaluating the model equation.

The R-squared(95.5%) and the R-squared(adj)((92.6%) values are high. The residual plot had residual values evenly distributed about the zero mark. However, the residual values increased as the predicted yield increased. An attempt to transform the absorbance value data did not improve the R-squared value markedly no did it improve the distribution of the residuals.

5.15.5 Response surface plots.

Curvature of the response surface plots, Fig 5.35(c_2) and Fig 5.35(d_2) illustrates the interactions between lysozyme*pH and GDL*lys. Surface plots, Fig 5.35(c_2) and Fig 5.35(d_2) clearly demonstrate that yield values of *L. monocytogenes* depended largely on lysozyme, GDL, pH and their interactions.

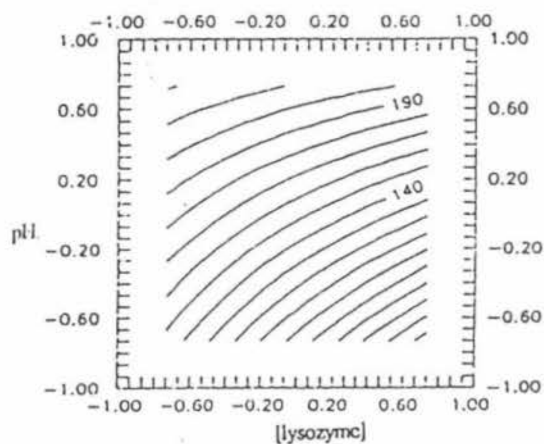
Predictions based on microbial media are useful in providing an indication of the effect that a given parameter such as pH, chelator or lysozyme may have on the behaviour of a microorganism in foods. However, other critical factors such as the physical nature

Fig 5.35

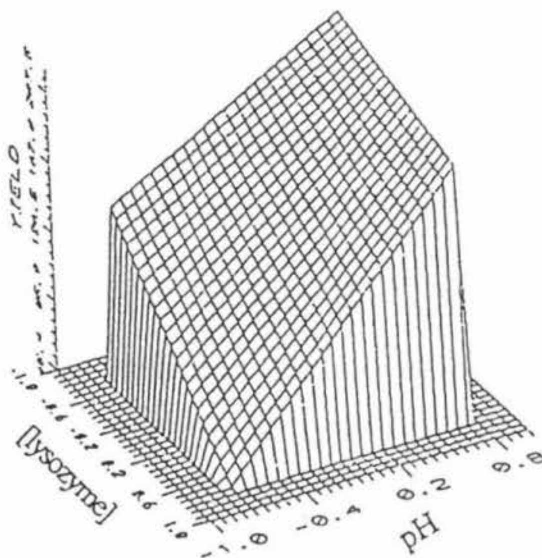
GDL, lysozyme and pH as factors influencing growth of *L. monocytogenes.*

Contour(c_1) and surface(c_2) plots of yield values for *L. monocytogenes* as a function of [lysozyme] and pH. The [GDL] was held at a high level.

(c_1)

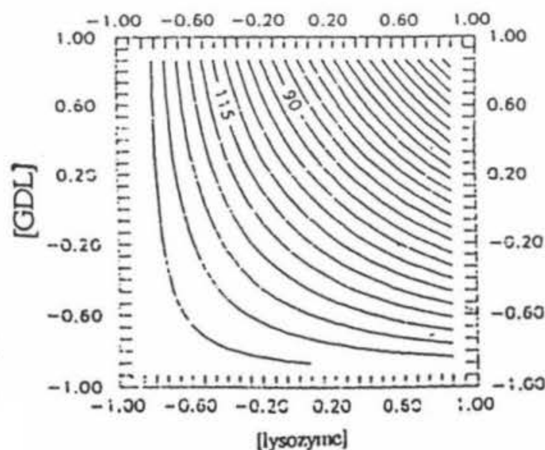


(c_2)

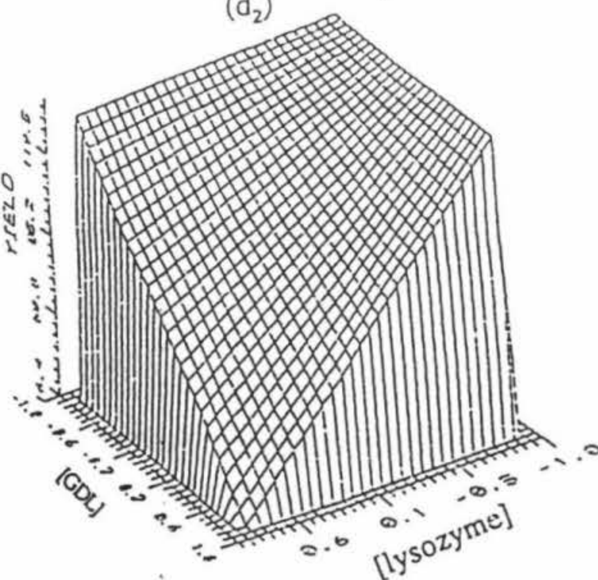


Contour(d_1) and surface(d_2) plots of yield values of *L. monocytogenes* as a function of [lysozyme] and [GDL]. The pH was held at a low level.

(d_1)



(d_2)



Legend: [lysozyme]: -1=0.2mg/ml +1=3mg/ml:
[GDL]: -1=10mM+1=25mM; pH: -1=5.5 +1=6.5.

of the food, food components, additives or processing conditions may cause deviations from these predictions. Thus, on the basis of information obtained in the present study, *L. monocytogenes* would be likely to grow in foods of pH6.5 in the presence of lysozyme and GDL, while in acidic foods of pH5.5, growth may be significantly inhibited.

The influence of GDL alone on the growth of *L. monocytogenes* has been published (El-Shenawy, 1990). Results of this study are similar to the findings by El-Shenawy (1990) who demonstrated that the growth of *L. monocytogenes* was essentially unaffected by the presence of 0.1 or 0.2% GDL(lowest pH 5.7) and that 0.3%GDL prolonged the lag phase to about 6hrs followed by growth. Differences in the kinetic parameters may have been due to differences in media, strains, temperature of incubation used and the fact that our broth culture system was buffered. However as far as the present study is concerned, no work has been published on the effect of lysozyme-GDL combinations against *L. monocytogenes*. Results of this work indicate that the antilisteric effect of GDL can be improved by taking advantage of the significant interactions between GDL, pH and lysozyme.

5.16 Effect of GDL, lysozyme and pH on *C. tyrobutyricum*.

5.16.1 Growth curves.

Growth curves for *C. tyrobutyricum* under the influence of GDL, lysozyme and pH are presented in Fig 5.36 and Fig 5.37. Most treatments caused minimal inhibition of the growth of *C. tyrobutyricum*, however treatment ab lowered the MPD. The MPD of other treatments were not significantly different from that of the control.

5.16.2 Kinetic parameters.

The kinetic parameters for the growth of *C. tyrobutyricum* under the influence of GDL, lysozyme and pH are presented in Table 5.17. There was no significant reduction in the μ_{max} and GT of *C. tyrobutyricum* under the influence of any treatment except ab. There was no significant lag phase extension caused by any treatment(Fig 5.36 and 5.37).

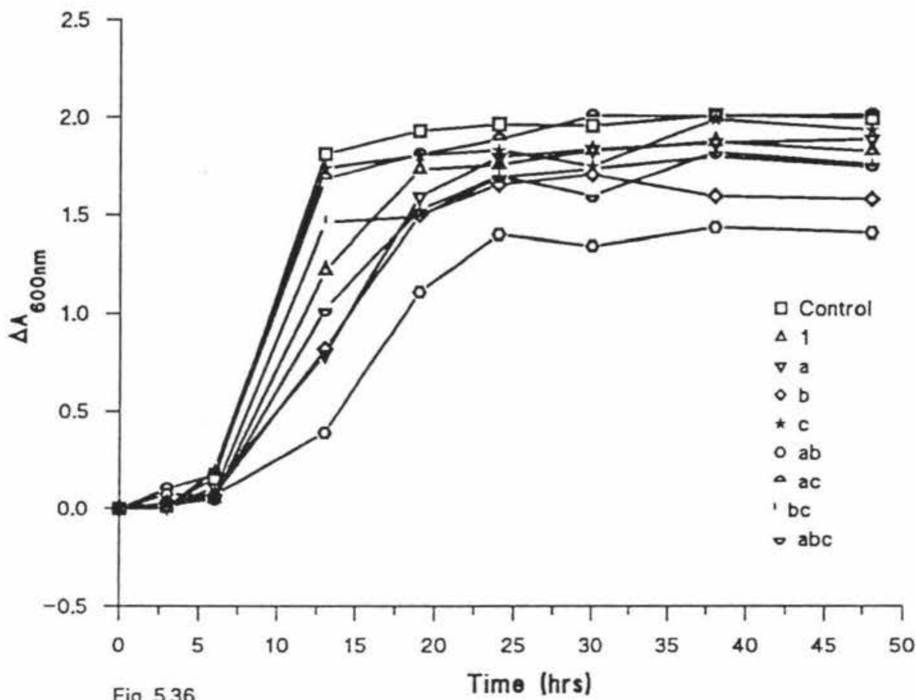


Fig 5.36

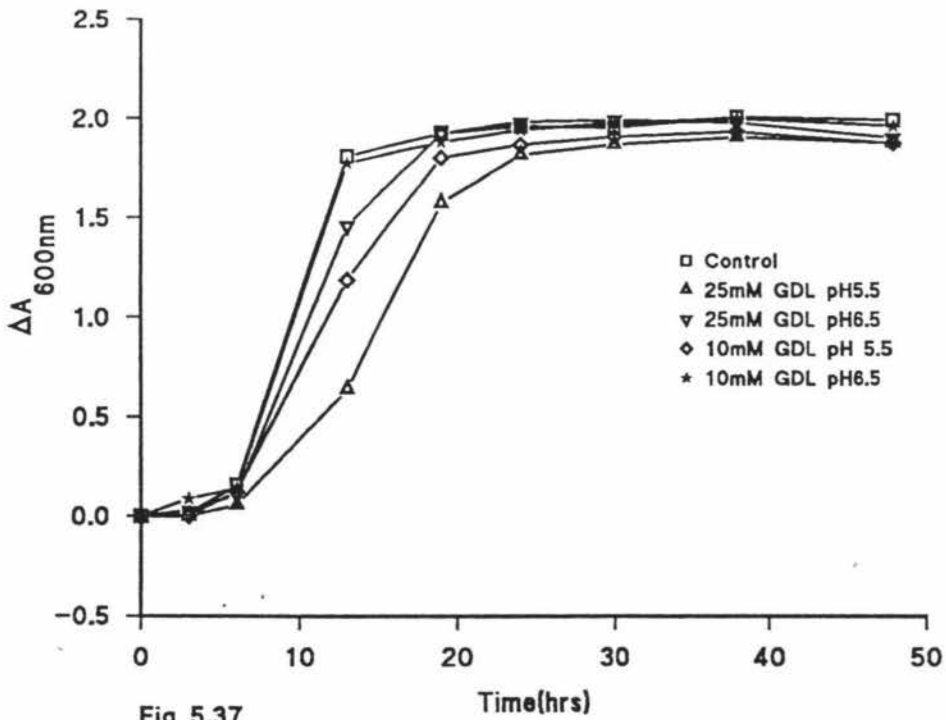


Fig 5.37

5.16.3 Statistical analysis.

Results of the statistical analysis are presented in Table 5.19. Regression analysis of the yield values resulted in the regression equation:

Yield = 65.5 + 0.524GDL - 2.68lys + 3.08pH + 0.79GDL*lys - 0.62GDL*pH - 1.77lys*pH - 2.27GDL*lys*pH.

Relatively low regression coefficients were obtained, this suggest that no large yield reduction will be effected when the main effects increase from the low to the high level. High R-squared(98.3%) and R-squared(adj)(96.9%) values were obtained.

Table 5.19

Predictor	Coef	Stdev	t-ratio	p
Constant	63.038	0.3793	166.18	0.000
GDL	-1.9469	0.3793	-5.13	0.000
lys	-5.1519	0.3793	-13.58	0.000
pH	5.5519	0.3793	14.64	0.000
GDL*lys	-1.6844	0.3793	-4.44	0.000
GDL*pH	-1.8519	0.3793	4.88	0.000
lys*pH	0.6994	0.3793	1.84	0.102
C8	0.2019	0.3793	0.53	0.609

s = 1.52 R-sq = 98.3% R-sq(adj) = 96.9% C8 = GDL*lys*pH

The t-values and p-values illustrate that the two-way interaction(lys*pH) and the three way interaction(GDL*lys*pH) have an insignificant effect at (P>0.05). The main effects, the two-way interaction GDL*lys and GDL*pH have a significant effect on the growth(yield) of *C. tyrobutyricum*.

Therefore to minimise the yield of *C. tyrobutyricum* requires:

The use of GDL at its high level.

The use of lysozyme at its high level.

The use of a low level of pH.

A reduced model equation for yield of *C. tyrobutyricum* under the influence of lysozyme, pH and GDL was of the form: Yield = 63.04 - 1.95*GDL - 5.15*lys + 5.55*pH - 1.68*GDL*lys + 1.85*GDL*pH.

5.17 2^5 Factorial design:

Yield values and statistical analysis for the different treatments in the 2^5 full factorial design are presented in Appendix 1, Table B. In this particular design, it was not possible to carry out a fractional factorial design because of the significant two-way and three-way interactions already encountered in the 2^3 full factorial design. A fractional factorial design would lead to confounding of the main effects and higher order interactions.

L. monocytogenes is a very adaptable microorganism. The temperature of incubation was important in determining the efficacy of lysozyme-GDL treatment combinations against *L. monocytogenes*. The influence of temperature and pH on the inhibitory effect of lysozyme-GDL treatment combinations is evident from the respective large coefficients in the statistical analysis output(Appendix 1, Table B). Effectiveness of treatment combinations in inhibiting the growth of *L. monocytogenes* increased as the temperature was reduced. Our results are in agreement with Smith et al. (1991) who demonstrated that *L. monocytogenes* Scott A grown at 37C were 1.8-2.5 fold more resistant to lytic action of lysozyme than cells grown at 19, 12 or 5C. At present there is no obvious answer as to why low temperature grown *L. monocytogenes* are more easily lysed by lysozyme (Smith, 1994). Similar results were obtained by El-Shenaway and Marth (1988). El-Kest and Marth (1988) also found that the smallest number of survivors of *L. monocytogenes* resulted when cells were exposed to 1ppm chlorine at 5C rather than 25C and 30C. Our experiments yielded similar results and further confirm the statement by Ingram (1976), "many pH, salt, nitrite combinations which readily permit growth under warm conditions are wholly inhibitory at lower temperatures". The interactive inhibitory effect of lysozyme and low pH against *L. monocytogenes* observed in this study has also been recently demonstrated (Johansen et al. 1994) and was suggested to be due to the growth retarding effect of low pH, allowing enzymatic hydrolysis to exceed the rate of cell proliferation. Our results suggest that lysozyme-GDL treatment combinations may be an effective preservative for controlling *L. monocytogenes* in refrigerated foods.

The statistical analysis suggests that the average effect of increasing the temperature from 5C to 25C is to increase the yield by 35.74 units. Some treatments at low

temperature were listeristatic(very low yield values) and others were listericidal(negative yield values). The yield values correlated well with the plate count at the end of the run.

The pH of the medium also contributed to the total environment which enhanced the effectiveness of the lysozyme-GDL combination treatments, for example inactivation/inhibition as reflected by yield values was greater at pH5.5 than at pH6.5. Results of the statistical analysis suggest that the main effects, pH, inoculum and temperature are significant at ($P<0.05$). Two-way interactions(GDL*temperature., GDL*pH., lysozyme*temperature and pH*temperature) were significant at ($P<0.05$). 3-way interactions(lysozyme*pH*temperature., pH*temperature*inoculum., lysozyme*pH*inoculum., GDL*lysozyme*pH., GDL*lysozyme*inoculum) were significant at ($P<0.05$), however higher order interactions were insignificant at ($P>0.05$).

5.17.1 Inoculum size effect

Palumbo et al. (1990) demonstrated that different starting counts of *Aeromonas hydrophila K144* (range over 1000 fold) did not affect lag and GT, the two parameters of greatest meaning and value to food microbiologists. Buchanan and Phillips (1990) demonstrated that the growth kinetics of *L. monocytogenes* (under aerobic conditions of 19C, pH7.5, 5g/l NaCl and 0μg/ml $NaNO_2$) were also unaffected by the size of the initial inoculum(inocula were between 2×10^1 and 2×10^4 cfu/ml). However the significance of the inoculum size has been demonstrated in other studies on parameters to control growth of *L. monocytogenes* in smoked salmon products (Guyer, 1991; Peterson, 1993; Pelroy, 1994). Cuppers and Smelt (1993) are of the opinion that only when the inoculum is subjected to extreme stress conditions might the inoculum size have an influence on the lag duration. In this particular study, the inoculum size was demonstrated to have a significant effect under conditions of stress. Our results are therefore in agreement with the assessment by Cuppers and Smelt (1993). The high levels of inocula were also chosen so that the inhibition could be evaluated under conditions of extreme contamination as might be found in some foods. As seen from our data, a low temperature(5C) enhanced the effect of lysozyme-GDL combination treatments against *L. monocytogenes*. This is important because *L. monocytogenes* is a psychrotroph (Donnelly and Briggs, 1986). In addition to its ability to grow at

refrigeration temperatures, *L. monocytogenes* becomes more virulent when grown at a low temperature (Gray and Killinger, 1966). Hence, enhancement of the antimicrobial effect of lysozyme-GDL at refrigerator temperature is desirable since this would affect the length of time a product can be refrigerated safely.

5.18 General discussion and conclusion.

The antimicrobial activity of the various chelating agents differed. Based on equal molar concentrations, the antimicrobial activity of the chelating agents against *L. monocytogenes* can be ranked in order of decreasing degree of inhibition as; EDTA > GDL > CA > Gly > Ad > SHMP >SPDB. Based on lag phase extension, the above ranking was also true when the ability of the different chelators to enhance lysozyme activity was assessed. Our results indicate that inactivation/inhibition of *L. monocytogenes* by the chelator-lysozyme combination treatments was affected by the incubation temperature, pH, concentration of chelating agent and lysozyme and the type of chelating agent.

The observation that some kinetic parameters listed in Table 5.17 for the lysozyme-GDL treatment combinations exhibited marked changes as compared to the control as the lysozyme concentration increased, pH decreased and GDL increased, has implications for these factors acting as significant growth controlling variables in foods. In the 2^5 factorial design, the observation that inactivation/inhibition of *L. monocytogenes* increased as temperature decreased is also important. Marked inhibition was achieved when the incubation temperature was 5C and when the initial microbial load was at its low level. The results are encouraging in that there is potential for the lysozyme-GDL preservative system to be able to control lower microbial loads due to inevitable contamination, or in minimally processed foods that do not receive a severe heat treatment.

We can conclude that inhibition/inactivation of *L. monocytogenes* by lysozyme-GDL combination treatment is affected by GDL concentration(as expected, GDL became more effective as the concentration was increased), lysozyme concentration, temperature(inhibition was greater at 5C than at 25C), pH of medium(inhibition was

greater at pH5.5 than at pH6.5). Although our data provide information of the efficacy of lysozyme-GDL against *L. monocytogenes,* they do not permit prediction of concentrations required commercially for preservation of all foods, because there are many environmental conditions which occur in products that were not considered in these experiments. Examples include water activity, atmosphere, type of microbial flora and certain food components. All of them might affect the antimicrobial activity of the preservative system. Thus, our observation can serve as a basis for choosing this preservative system for evaluation of the susceptibility of *L. monocytogenes* to this preservative system in refrigerated foods. GDL was the chelator of choice because it has been established as useful in the food and dairy industry. GDL is readily available, nontoxic and free from objectionable properties such as insolubility of calcium salts and unpleasant taste.

The data generated from this study suggest that pH, lysozyme and the various chelators interacted at various levels to influence the growth kinetics of *L. monocytogenes* particularly in regard to the μ_{max}, GT, MPD and lag phase durations.
Throughout all treatment combinations, the pH effect was significant. This is because cells generally react to maintain internal pH values constant in the event of, during or after a pH shift, or during growth at a suboptimal pH value. In order to do this, an organism needs to cope with an increasing leakage of protons into the cell cytoplasm as the medium pH is reduced and these must be removed if internal acidification is to be avoided. Removal of protons against what might be a steep concentration gradient of several hundreds- or thousand-fold in many acid systems is energy-demanding, so that as the external pH is reduced, more and more energy is utilised to maintain internal pH constant and less is therefore available for synthesis of cell material. The energy yielding processes situated in the cell membrane may be reversed and used to pump protons from the cell's internal space, so that enzyme reactions can proceed under favourable (neutral pH) conditions. A major consequence of this is that yield falls dramatically. Eventually, the low pH limit for growth is reached when the rate of energy generation is too low for maintenance and synthesis and when this is exceeded, substantial acidification of the interior of the cell occurs. Again, being an energy-demanding homeostatic mechanism, restriction of energy supply will improve the

effectiveness of preservation at low pH values.

The lysozyme-EDTA combination treatment was the only treatment that demonstrated significant inhibition of *C. tyrobutyricum* that would be of practical significance. *C. tyrobutyricum* was more resistant to the different chelator-lysozyme treatment combinations when compared with *L. monocytogenes*.

From this study, the following conclusions can be drawn.

1. The turbidimetric assay is a reliable method for modelling microbial growth.
2. When assessing the antimicrobial efficacy of antimicrobial agents using the turbidimetric assay, "no growth" data should not be included for statistical analysis.
3. *L. monocytogenes* becomes more susceptible to the lysozyme-GDL treatment combinations as the temperature decreases.
4. The inoculum size has a significant effect.
5. At a given temperature, differences in the antimicrobial activity between various chelating agents depend on pH, concentration and type of chelating agents.
6. Inhibition/inactivation of *L. monocytogenes* by lysozyme-chelator systems is affected by (a) temperature(inhibition was more at 5C than at 25C for lysozyme-GDL), (b) pH of medium(efficacy was greater at pH5.5 than at pH6.5 except for EDTA), (c) type of chelating agent(greater inhibition/inactivation was noted in the order EDTA > GDL > CA > Gly > AD > SHMP > SPDB).
7. Vegetative cells of *C. tyrobutyricum* were highly resistant to most lysozyme-chelator combinations studied.

CHAPTER 6.

PROTEIN INTERFERENCE ON ANTIMICROBIAL AGENTS:

6.1 Introduction.

The preservative system discussed so far were tested in a broth medium. To be useful, the system must function in foods which may contain components such as lipids, proteins and divalent cations that might influence the effectiveness of the antimicrobial compounds.

The overall antimicrobial spectrum, the mode of action and the efficacy of an antimicrobial is largely dependent on the chemical and physical properties of the antimicrobial. The polarity of an antimicrobial is probably the most important physical property. Hydrophillic properties appear to be necessary to ensure that the antimicrobial is soluble in the water phase where microbial growth occurs; highly active antimicrobial compounds which are hydrophobic tend to partition into the lipid areas of the food away from the water (Branen et al. 1980; Robach, 1980). Antimicrobials acting on the hydrophobic cell membrane appear to require some hydrophillic properties (Branen et al. 1980). Thus, as in emulsifiers, antimicrobials appear to require a specific hydrophillic-lipophilic balance for optimal activity. Lipids may cause a decrease in activity of lipophilic compounds, and since many food antimicrobials have hydrophobic character, there will invariably be some reduction (Rico-Muncoz and Davidson, 1983). Thus, lipids also contribute significantly to interference of antimicrobial activity.

Chemical reactivity of the antimicrobial with other components of the food system can also be important. Loss of antimicrobial activity can occur if the antimicrobial reacts with food components resulting in binding of the chemical to the food component or a breakdown or alteration of the chemical structure of the antimicrobial. Reactions with lipids, proteins, carbohydrates and other food additives can result in an overall decrease in the activity of the antimicrobial. Several antimicrobials can also be oxidised or hydrolysed. Binding of the antimicrobial probably results in the greatest loss of activity. Proteins can bind several compounds, and also fibre binds and inactivates some

antimicrobials. Naturally-occurring compounds other than major food components can also influence activity of the antimicrobial. Divalent cations such as Ca^{2+} may affect the activity of some antimicrobials by interacting with the antimicrobial (Rico-Munoz and Davison, 1984) or by stabilising the membrane structure (Rosen, 1982), the presence of Ca^{2+} was demonstrated to diminish butylated hydroxytoluene activity (Wanda et al. 1976).

In this study, an experiment was conducted to determine if the presence of added protein, as would be found in many food systems, interferes significantly with lysozyme activity.

6.2 Experimental procedure:

Procedure 3.7 was used to study the effects of added protein on the activity of lysozyme, GDL and lysozyme-GDL treatments against *L. monocytogenes*. In a broth culture system, the antimicrobial effectiveness of the different treatments was challenged by the addition 1mg/ml of whey protein concentrate(WPC) to the growth medium. Counts of *L. monocytogenes* were monitored on modified nutrient agar and reported plate counts were the mean of duplicate samples.

6.2.1 Statistical analysis.

Data were analysed using Fisher's pairwise comparison test (Minitab Release 8.2). The test was used to separate differences in means between treatments. Each treatment was compared with the control grown with the same concentration of protein.

6.3 Results and Discussion.

Inactivation/inhibition curves for different treatments; control, lysozyme, GDL and lysozyme-GDL treatment combination are presented in Fig 6.1. The kinetic parameters are presented in Table 6.1.

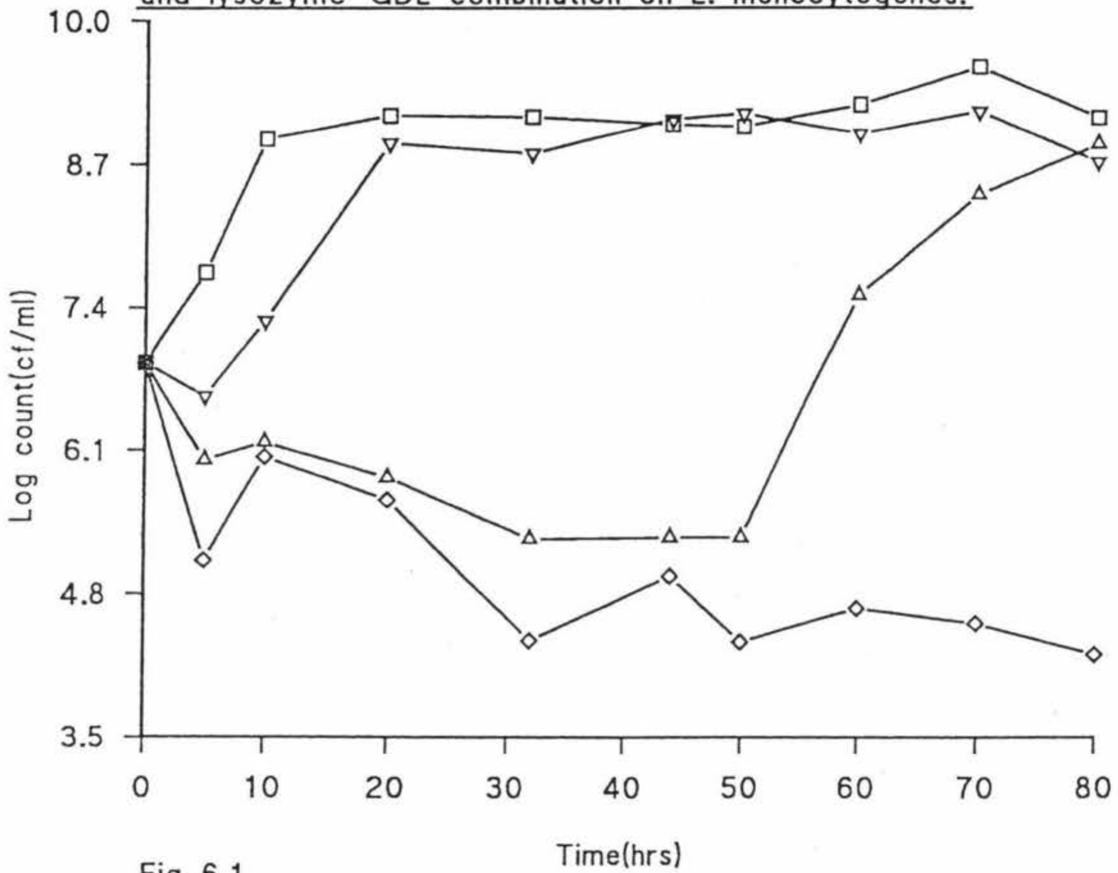


Fig 6.1

□ Control △ lysozyme(3mg/ml) ▽ GDL(25mM)
◇ lysozyme(3mg/ml)–GDL(25mM) combination

Table 6.1.

	Kinetic parameters: (Fig 6.1)			
Treatment	$\mu_{max}(h^{-1})$	GT(hrs)	lag(hrs)	MPD
Control	0.204	3.4	<1	9.12
lysozyme	0.166	6.0	50	8.88
GDL	0.155	4.47	6	8.72
lysozyme-GDL	-0.024	*	>80	4.25

All treatments, with the exception of the control were listericidal after exposure for 5hrs(Fig 6.1). The listericidal activity of the different treatments were in the order lysozyme-GDL > lysozyme > GDL. The presence of 25mM GDL at pH5.5 extended the lag duration to about 6hrs followed by growth with a GT of 4.47hrs. The treatment did not suppress the MPD. Results of this treatment were similar to the results of El-Shenawy and Marth (1990) who studied the behaviour of *L. monocytogenes* in the presence of GDL. Differences in the kinetic parameters may have been due to strain difference, media used and buffering of the media.

Enhanced listericidal activity of lysozyme alone was exhibited at pH5.5. A 0.5 log cycle reduction of *L. monocytogenes* was effected over a duration of 50hrs after which no further inhibition occurred, growth was observed with a reduced μ_{max} ($0.204-0.116h^{-1}$) and an increase in GT (3.4-6hrs) and there was no suppression of the MPD. The lysozyme-GDL combination treatment was listericidal; 2 log reductions of *L. monocytogenes* were effected. The treatment was listericidal throughout the 80hrs incubation period.

Listericidal growth curves generated by the plate count method for *L. monocytogenes* under various treatments (Fig 5.9., Fig 5.32., Fig 5.34 and Fig 6.1) all exhibited biphasic death curves (sudden death followed by a slow death or growth). The biphasic death curves suggest that a substantial percentage of the population was killed relatively fast, leaving behind a second, more resistant sub-population.

Statistical analysis of the various treatments is presented in Appendix 1, Table C. The lysozyme and lysozyme-GDL treatments were significantly different from the control as well as from the GDL treatment. However the GDL treatment was not significantly different from the control.

Results of this interference study demonstrate that there was no interference by WPC in the effectiveness of all treatments in inhibiting the growth of *L. monocytogenes*. It was also observed that lysozyme-GDL combination treatments in Fig 6.1(with WPC addition) had essentially the same listericidal activity as the same treatment in Fig 5.32 (without WPC). Equal lag phase durations(6hrs) for the GDL treatment were exhibited in Fig 6.1(with WPC addition) and in Fig 5.31(without WPC addition). This was also true for the lysozyme treatment(lag phase 50hrs) in Fig 6.1(with added WPC) and in Fig 5.2(without WPC addition).

6.4 Conclusion.

Results of this study indicate that the effectiveness of the lysozyme-GDL treatment against *L. monocytogenes* is not inhibited by the presence of 1mg/ml WPC. However, it is important not to attempt to extrapolate results of broth culture studies directly to a complex food system. The effects of many food components, especially lipids, calcium and magnesium ions on the effectiveness of lysozyme-GDL treatment combination cannot be predicted. On the basis of the results of the preliminary experiments, the broth culture system and the interference studies, the potential antilisteric use of lysozyme-GDL treatment combination was then investigated in refrigerated food systems that receive minimal or no heat treatment before consumption.

CHAPTER 7.

FOOD SYSTEM

7.1 Introduction

To demonstrate the practical use of a preservative system, its influence on the kinetic parameters (μ_{max}, GT, MPD and lag duration) of microorganisms of concern in the food itself should be known. The microbial growth/inhibition studied so far in synthetic media does not always parallel that in a food product. Thus, the final confirmatory test for the antimicrobial activity should be carried out in food. Evaluation of an antimicrobial in a food system has its own limitations. Plate count methods are often inaccurate and the number of organisms added and the overall flora may not be representative of what happens under normal circumstances.

Control of *L. monocytogenes* in refrigerated foods is imperative.
Many factors must be considered in designing an antimicrobial inactivation study for *L. monocytogenes* in a refrigerated food.
These factors include:

Type and numbers of *L. monocytogenes* cells to use as an inoculum.
Methods of production, enumeration and standardisation of inoculum.
Size of inoculum to be used.
Methods of inoculating different types of products.
Packaging of products.
Sample size and number of samples to test.
Enumeration and detection media.
Product composition.

The microorganism used should be a natural contaminant or a pathogen of interest and incubation conditions should reflect use and abuse. A satisfactory test result may be determined by increased shelf-life or prevention of problems due to abuse.
In this study, the potential use of the lysozyme-GDL preservative system as an antilisteric agent was tested in two food systems (cottage cheese and mussels), in air-packaged and MAP food samples.

7.2 Experimental procedure.

7.2.1 Survival of *L. monocytogenes* in cottage cheese or mussels, packed in air with GDL and lysozyme-GDL preservative systems.

The GDL and lysozyme-GDL preservative systems were evaluated for their potential use as antilisteric agents in two food systems. Cottage cheese and mussels were processed and treated as in procedure 3.8. Survival of *L. monocytogenes* in air-packaged samples under the influence of GDL alone(0.445%) and lysozyme(3mg/g)-GDL(0.445%) combinations was monitored for 21 days at refrigeration temperatures(5C) and at an abuse temperature(12C).

7.2.2 Survival of *L. monocytogenes* in MAP mussels with lysozyme-GDL preservative system.

In a second series of experiments, the lysozyme-GDL preservative system was incorporated in MAP mussels and was used to study the survival of *L. monocytogenes* under the same conditions as above(7.2.1). Modified atmosphere packaging of samples was effected using procedure 3.8.2. The mussels were packaged in high barrier film bags(MET PET(12μm)/LDPE(50μm) with an oxygen transmission rate of 1cc/m^2/24hr/atm at 75%RH 23C). Bags were evacuated and backflushed with food grade (1) carbon dioxide and (2) nitrogen. On performing the plate counts, sampling intervals were adjusted to observe the lag, logarithmic and stationary phases of growth in the control and treated mussels. From the inactivation/inhibition curves, kinetic parameters(μ_{max}, GT, lag duration and MPD) of practical significance were calculated and compared with the control.

7.2.3 Influence of MAP on the growth of *L. monocytogenes*.

Procedure 3.8.2 was used to study the influence of MAP on the growth of *L. monoctogenes* in minced mussels stored at 5C. No preservatives were added. One batch of the processed minced mussels was divided into two equal samples; one sample was packaged under MAP by nitrogen flushing and the other was packaged in air. Survival of *L. monocytogenes* in these samples was monitored for 20 days at 5C(using procedure 3.8.1).

7.2.4 Recovery of "resistant" *L. monocytogenes* from treated samples.

After exposure of *L. monocytogenes* to the lysozyme-GDL preservative system for 21days in mussels, survivors of this treatment were then isolated. Inactivation/inhibition of the isolate by the GDL(25mM) and lysozyme(3mg/ml)-GDL(25mM) treatment combination at pH5.5 was studied using procedure 3.4.1-3.4.3. Plate counts(procedure 3.4.5) were performed on treated samples to demonstrate whether the isolated population had become resistant to the GDL or lysozyme-GDL treatments.

7.2.5 Lysis of non-growing *L. monocytogenes* cells in phosphate buffer by lysozyme-GDL extracted from minced mussels.

Procedure 3.5 was used to investigate whether the lysozyme-GDL preservative system was still active at the end of 20 days of storage of treated mussels. At the end of the storage test(20 days), the lysozyme-GDL preservative system was extracted from minced mussels treated with lysozyme(3mg/ml)-GDL(0.445%) preservative system. Minced mussels were diluted in a ratio of 1:2 with sterile phosphate buffer in a stomacher bag and the sample was blended for two minutes. The food sample was then centrifuged at 5000 x g for 20 minutes and the supernatant fluid was double filtered through a 540µm filter paper and then through a 0.54µm filter paper. The filtrate was used to treat non-growing cells of *L. monocytogenes* in phosphate buffer(Procedure 3.5). The concentration of the lysozyme-GDL extract was calculated taking into account the dilution factor. The turbidity of the treated sample and control were then monitored at 450nm.

7.3 Results and discussion.

7.3.1 Survival of *L. monocytogenes* in air packaged cottage cheese incorporated with GDL and lysozyme-GDL preservative systems.

The growth/inhibition curves for *L. monocytogenes* in cottage cheese under the influence of GDL and lysozyme-GDL preservative systems at 5C and 12C are presented in Fig

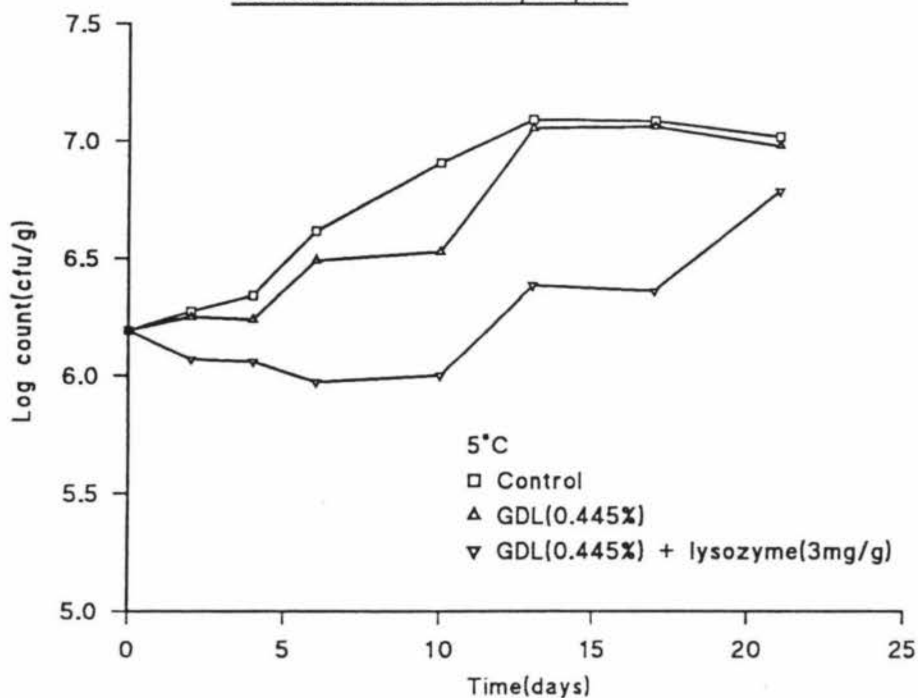


Fig 7.1

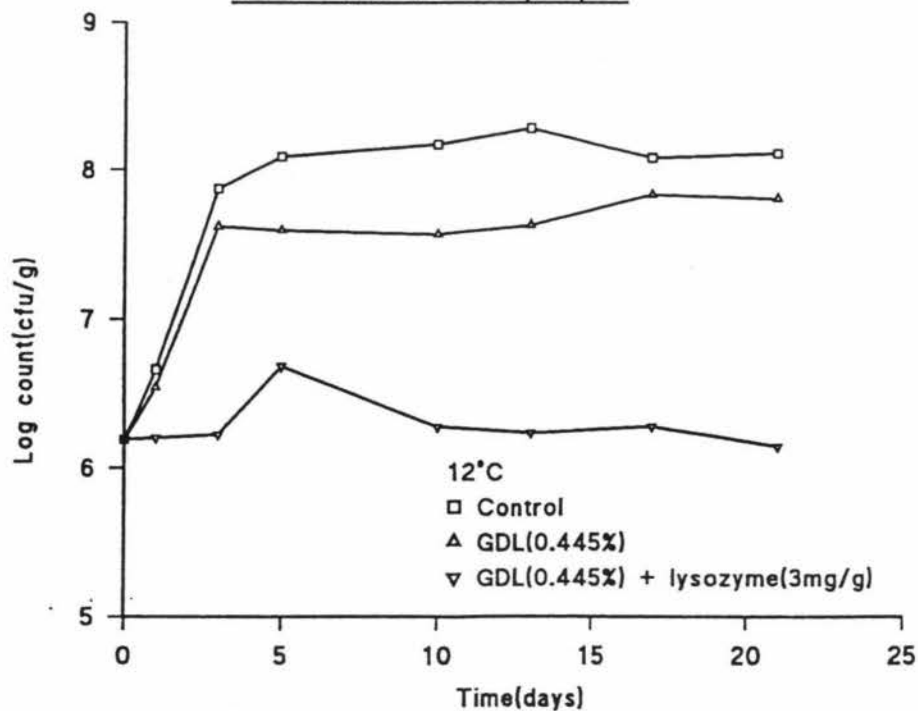


Fig 7.2

7.1 and Fig 7.2 respectively. The GDL treatment at 5C extended the lag phase duration to about 4 days (Fig 7.1) but growth of *L. monocytogenes* then occurred with no suppression of the μ_{max}(0.074-0.078 day^{-1}), no increase in the GT(9.37-8.89 days) and no suppression of the MPD.

At a typical abuse temperature(12C) the GDL treatment(Fig 7.2) effected no lag phase extension and growth occurred with no significant μ_{max} reduction(0.398-0.303 day^{-1}), no significant increase in GT(1.74-2.29 days) and there was no significant suppression of the MPD. The inhibitory effect of the GDL treatment on *L. monocytogenes* was therefore more effective at a low temperature(5C) than at a high temperature 12C.

The lysozyme-GDL treatment at 5C extended the lag phase to about 10days(Fig 7.1). The treatment was listericidal, 0.5 log reductions were effected in 10 days after which an increase in count was observed. In comparison with the control, the treatment reduced the μ_{max}(0.074-0.062 day^{-1}) and increased the GT (9.37-11.8 days). The water activity of the cottage cheese samples was 0.997 at 18.6C, thus the a_w was not a limiting factor. The pH of the cottage cheese was on average at pH6.3. Because of the strong buffering capacity of concentrated proteins, the added GDL did not significantly lower the pH with time.

To our knowledge, no work has been published on the use of a lysozyme-GDL system of preservation in foods. The increased susceptibility of *L. monocytogenes* to chemical antimicrobials has been demonstrated by El-Shenawy and Marth (1989), who found that sodium benzoate inhibited growth of *L. monocytogenes* more effectively as the temperature was lowered from 35C to 13C. Although our preservative system contained an enzyme that has a different mechanism of inhibition, our experiments yield similar results in that GDL and lysozyme-GDL systems were more effective in inhibiting the growth of *L. monocytogenes* as the temperature was reduced from 12C to 5C. The results further confirm the statement by Ingram (1976), that at a given pH, salt and nitrite combinations, warm conditions permit growth and lower temperatures are inhibitory. The effectiveness of the lysozyme-GDL combination treatment in inhibiting the growth of *L. monoctogenes* was demonstrated in a complex food system (cottage cheese) using a high level of inoculum (over 10^6 cfu/g) to illustrate the utility of the preservation system under worst case conditions.

7.3.2 Survival of *L. monocytogenes* in minced mussels packaged in air with GDL and lysozyme-GDL.

The growth/inhibition curves for the survival of *L. monocytogenes* in air packaged minced mussels under the influence of GDL and lysozyme-GDL treatments are presented in Fig 7.3(refrigeration temperature) and Fig 7.4(temperature abuse).

The GDL at 5C extended the lag phase to about 8 days. After 8 days the GDL failed to inhibit growth, which occurred with an increase in the μ_{max} as compared to the control (0.160-0.198 day^{-1}) and a corresponding reduction in the GT(4.33-3.50 days). The treatment did not effect a significant suppression of the MPD. At an abuse temperature the treatment was not listericidal(Fig 7.4), a lag phase of about 5 days was observed and *L. monocytogenes* growth occurred with a decrease in μ_{max}(0.368-0.3 day^{-1}) and a corresponding increase in GT(1.88-3.19 days) when the kinetic parameters were compared with the control treatment. The MPD was not suppressed.

The lysozyme-GDL treatment combination was listericidal at 5C and growth of *L. monocytogenes* was kept below the initial inoculum level throughout the 21 day storage period(Fig 7.3). The lysozyme-GDL preservative system remained inhibitory to the sub-population that survived on the 6th day, as this population increased by only 0.5 log cycles. This sub-population had a reduced μ_{max}(0.160-0.074 day^{-1}) and a corresponding increase in GT(4.33-9.37 days) when the treatment kinetic parameters were compared with the control. Under an abuse temperature(12C) the lysozyme-GDL treatment was listericidal for 5 days after which growth occurred, resulting in an effective lag phase extension of about 8 days(Fig 7.4). Growth of *L. monocytogenes* occurred at a decreased μ_{max}(0.368-0.30 day^{-1}) and an increased GT(1.88-2.31 days) compared with the control. The treatment effected a decrease in the MPD after 21 days(Fig 7.4).

Minced mussels in this study had a water activity of 0.968 at 19.6C; the minimum water activity for growth of *L. monocytogenes* is 0.92 (Petran and Zottola, 1989; Health and Welfare Canada, 1990). Thus the water activity was not a growth limiting factor. On average the pH of the minced mussels was at pH6.5, addition of GDL did not lower the pH because of the buffering capacity of the proteins. The trials were carried out at 10^5-10^6 *L. monocytogenes* cfu/g, the bacteria being present at higher populations than would likely be found on fresh or processed produce. This inoculum level was ideal for

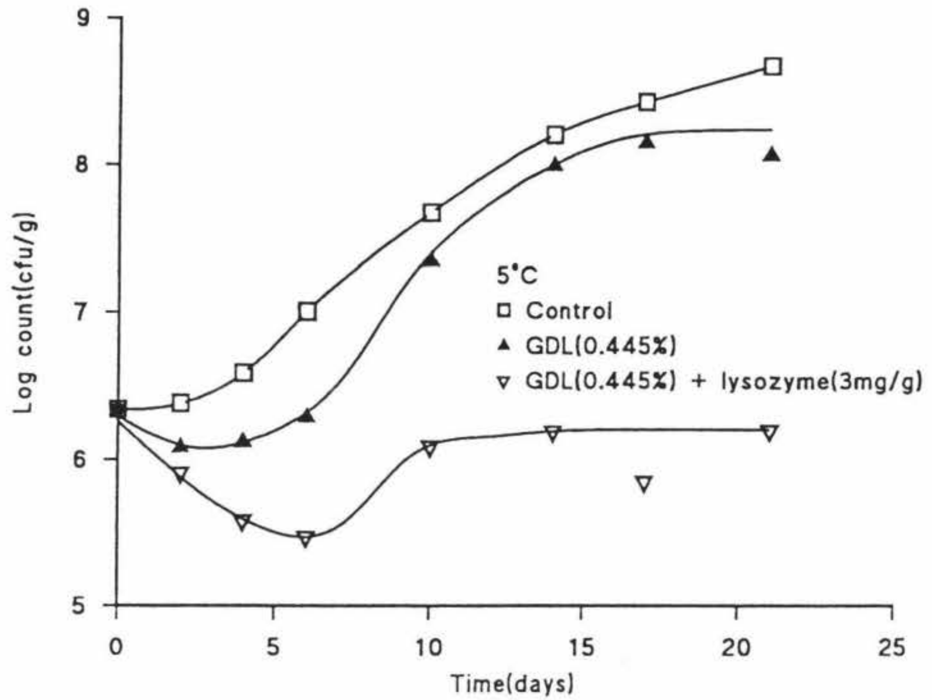


Fig 7.3

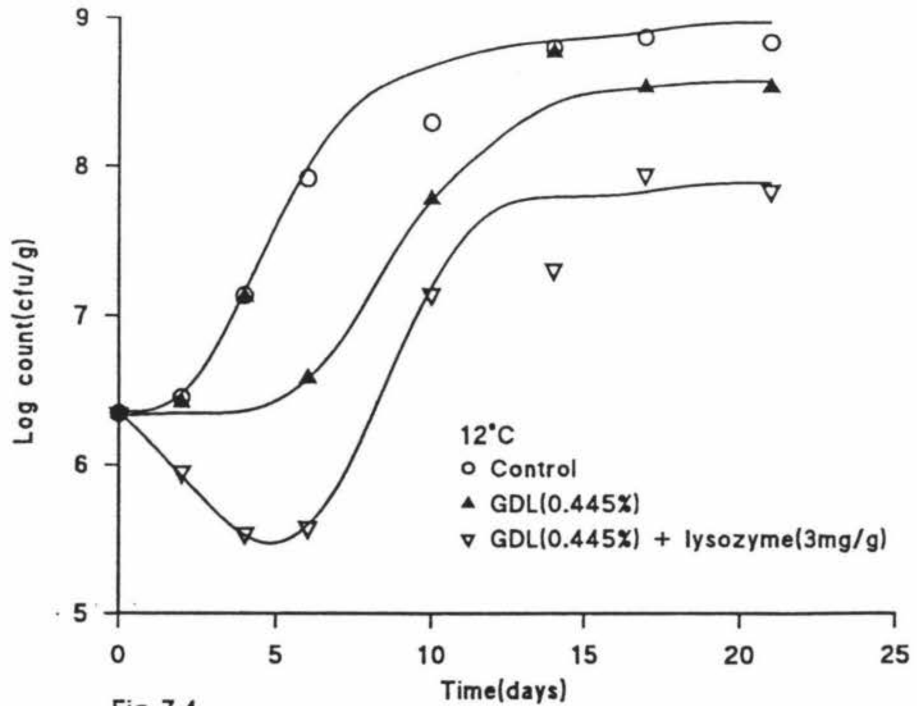


Fig 7.4

experimental purposes for it could allow 5-6 log cycle reductions to be followed and the ability of the preservative system to inhibit the microorganism of concern under the worst case scenario was tested. Results further confirm the increased susceptibility of *L. monocytogenes* to the lysozyme-GDL treatment as the temperature is reduced. Results clearly demonstrate that the lysozyme-GDL treatment combination has potential for use as an antilisteric agent in refrigerated cottage cheese and mussels and that the inhibitory effect of the preservative system even at an abuse temperature is important.

7.3.3 "Resistance" of *L. monocytogenes* to GDL and lysozyme-GDL treatment combinations.

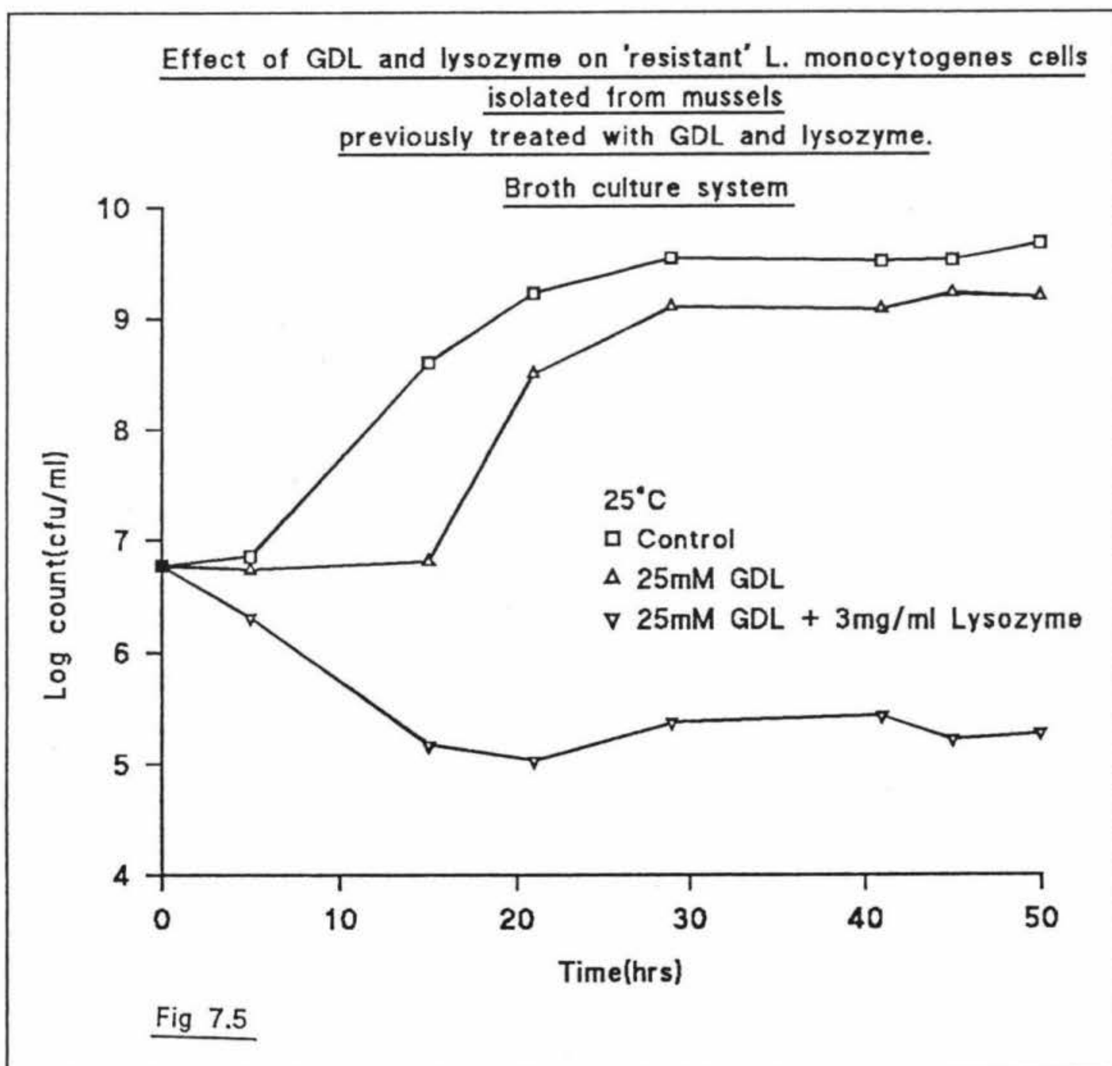


Fig 7.5

Growth/inhibition curves for "resistant" *L. monocytogenes* cells under the influence of GDL and lysozyme-GDL in BHI broth are presented in Fig 7.5. The growth curves

clearly demonstrate that the susceptibility of *L. monocytogenes* to GDL and lysozyme-GDL was a stable characteristic, remaining unchanged when an attempt to select for populations more resistant to lysozyme-GDL at the end of the storage test(21days). The growth/inhibition curves for *L. monocytogenes* isolated from the mussels(Fig 7.5) and subjected to the lysozyme-GDL treatment were essentially the same as the growth/inhibition curves observed under similar treatments throughout the study(Fig 5.32., Fig 6.1(treatment ab).
Investigators have observed unusual and complex phenomena that are incompletely resolved when the interaction of bacteria and antimicrobial agents are studied. One such complexity, referring to incomplete killing has been called the "persistance" phenomenon and relates to the small number (usually $< 0.1\%$) of the inoculum of cells which survives the lethal activity of antimicrobial agents. If persisters are retested, they appear as susceptible to the effect of the antimicrobial agent as the original isolate and no greater proportions of cells persist. Persisters have been considered to be metabolically inactive forms that were not actively growing at the time of the interaction of the antimicrobial with the inoculum and consequently were not killed by the antimicrobial (Amsterdam, 1991). However, it is still not clear how the persisters evade the action of the enzyme system as our results have demonstrated that resting cells of *L. monocytogenes* are lysed by the lysozyme-GDL preservative system.

Of the several mechanisms by which bacteria seemingly evade the killing effect of antimicrobial agents, perhaps the least understood is that of tolerance, first recognised by Tomasz et al. (1970). Reports related to the phenomenon of tolerance are available (Handwerger et al. 1985; Sherries, 1986; Tuomanen et al. 1986). Operationally, tolerance can be defined as the ability of bacteria to grow in the presence of high concentrations of antimicrobial agent so that the killing action of the antimicrobial is avoided. The tolerant strain is not lysed and loses viability at a significantly reduced rate. It is generally accepted that rapidly growing and dividing organisms are more susceptible to the inhibiting effect of cell wall-directed antimicrobials (Amsterdam, 1991).

Results of this study demonstrate that *L. monocytogenes* cells are best described as being

"persisters" in that retesting of the "persisters" (survivors) resulted in their being as susceptible to the effect of the antimicrobial agents as the original isolate and no greater proportions of cells survived.

7.3.4 Influence of lysozyme-GDL preservative system in combination with MAP(CO_2 flush) on the survival of *L. monocytogenes* in minced mussels.

Growth/inhibition curves for the survival of *L. monocytogenes* under MAP(carbon dioxide flush) in minced mussels that were packed with lysozyme-GDL treatment are presented in Fig 7.6(refrigeration storage(5C)) and Fig 7.7(temperature abused samples (12C)). The final gas composition of CO_2 flushed bags was analysed and found to be 37%CO_2, 50.4%N_2 and 12.53%O_2 (procedure 3.8.2).

The lysozyme-GDL treatment under MAP at 5C was listericidal for the first 3 days and the treatment extended the effective lag phase duration to about 7 days, after which growth occurred with a reduction in the μ_{max}(0.313-0.185day^{-1}) and a corresponding increase in the GT(2.21-3.75days)(Fig 7.6). There was however no suppression of the MPD. The mesophilic aerobic plate count indicated that the microflora was predominantly *L. monocytogenes*. At a temperature of 12C, the lysozyme-GDL system in combination with MAP extended the lag phase to about 4 days after which growth of *L. monocytogenes* occurred with no significant decrease in μ_{max}(0.386-0.375day^{-1}) and no significant increase in GT(1.80-1.85days)(Fig 7.7). There was no suppression of the MPD.

7.3.5 Influence of lysozyme-GDL preservative system in combination with MAP(nitrogen flush) on the survival of *L. monocytogenes* in minced mussels.

Growth/inhibition curves for *L. monocytogenes* under the influence of lysozyme-GDL treatment in combination with MAP(nitrogen flush) are presented in Fig 7.8(refrigerated storage(5C)) and Fig 7.9(temperature abuse(12C)). The final gas composition of the bags was 1.34%CO_2, 96.58%N_2 and 2.09%O_2,.

Nitrogen flushed samples at 5C exhibited a lag phase extension of about 5 days(Fig 7.8) after which growth occurred with a reduced μ_{max}(0.405-0.292 day^{-1}) and a corresponding increase in GT(1.71-2.37 days). There was no suppression of the MPD and the

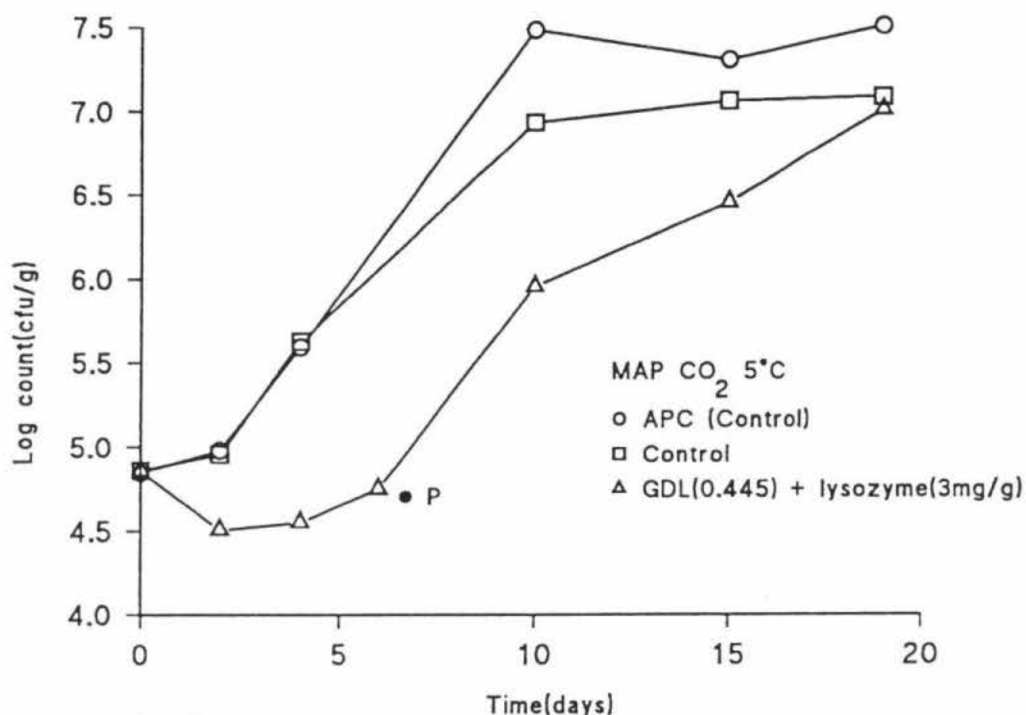


Fig 7.6

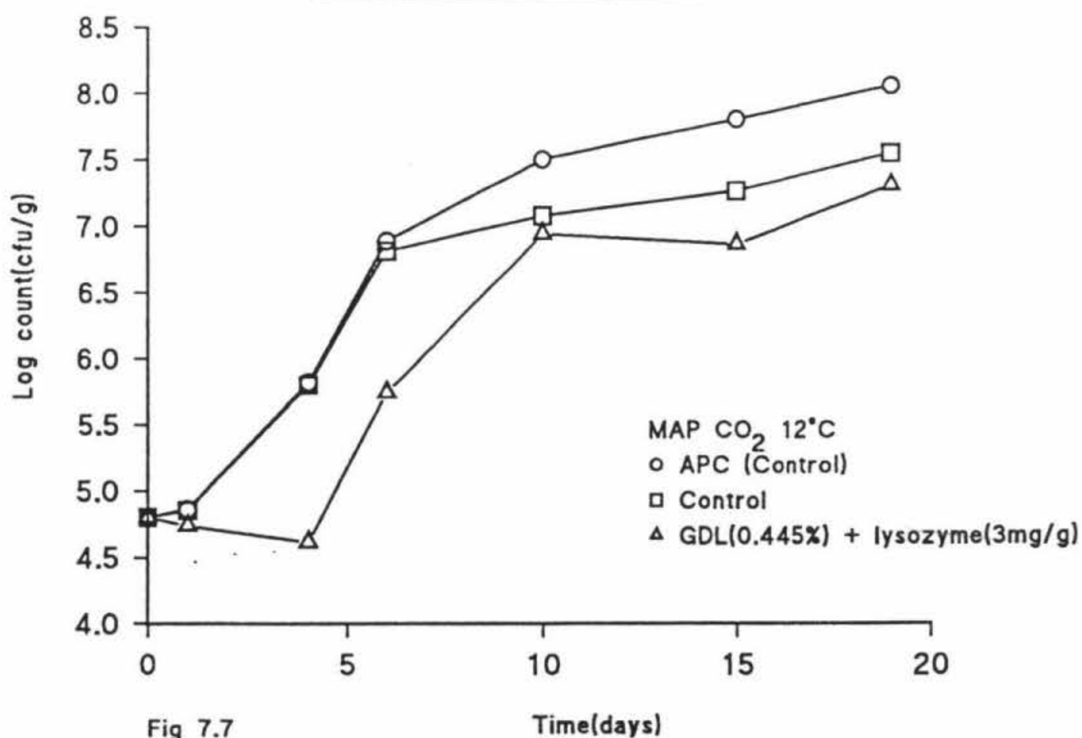


Fig 7.7

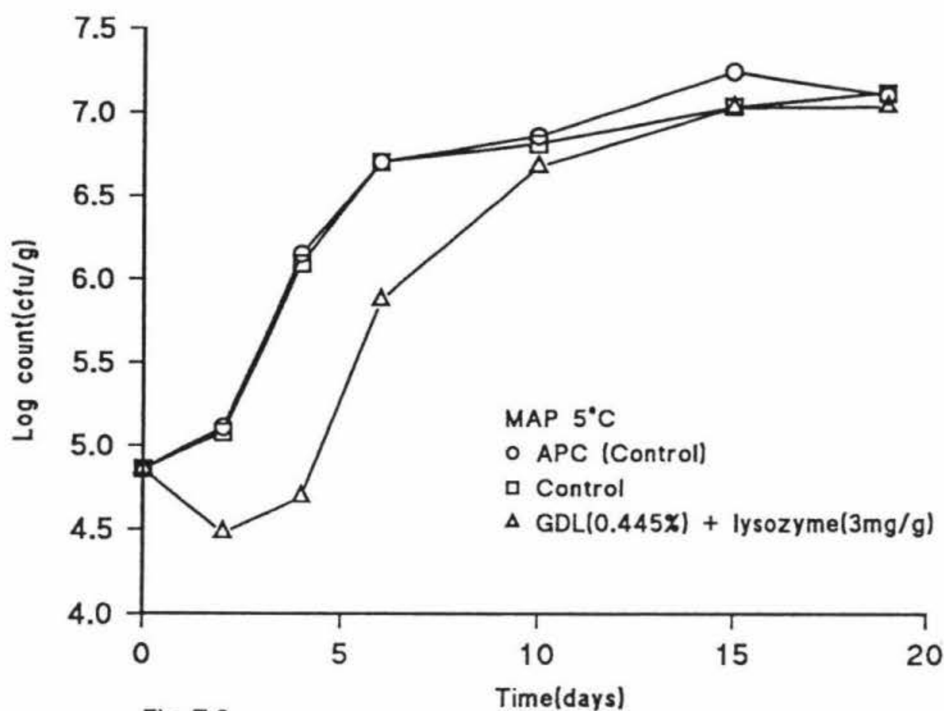


Fig 7.8

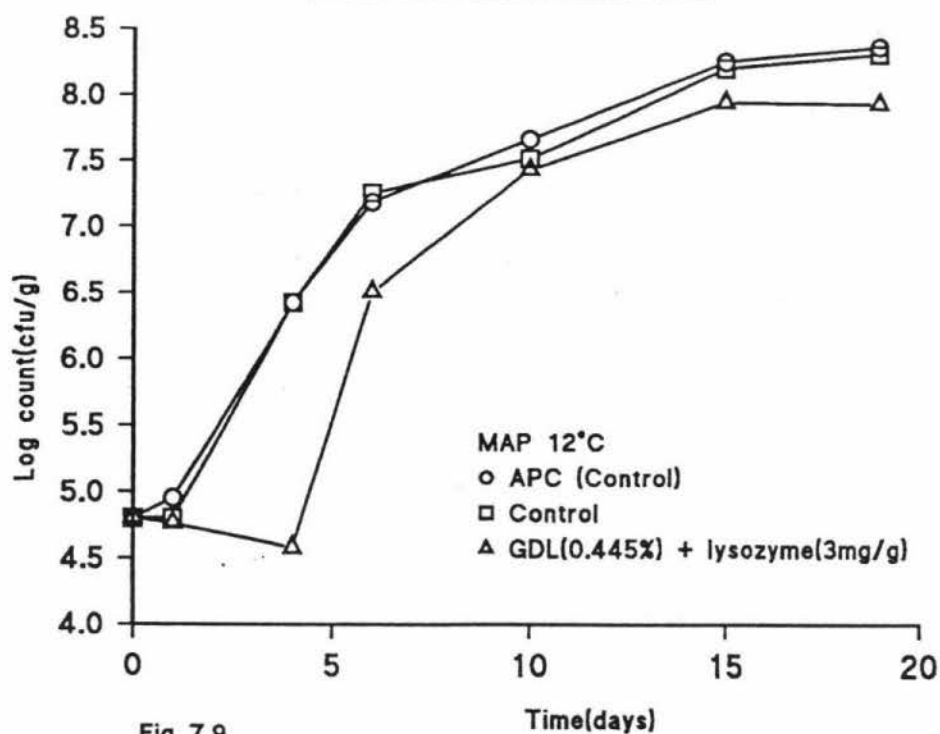


Fig 7.9

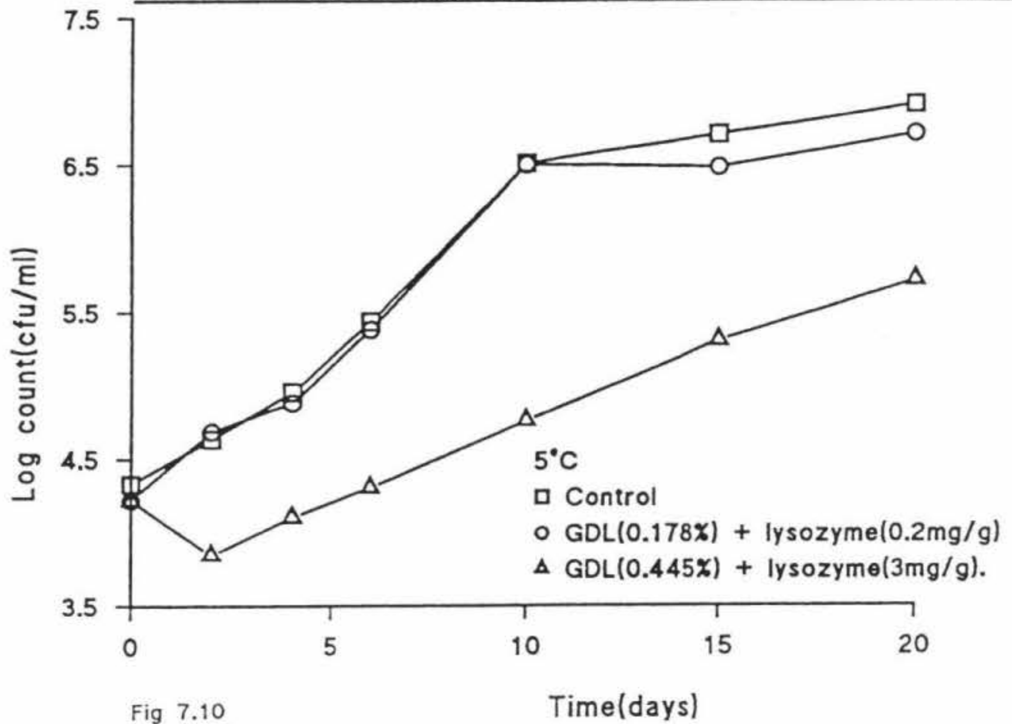


Fig 7.10

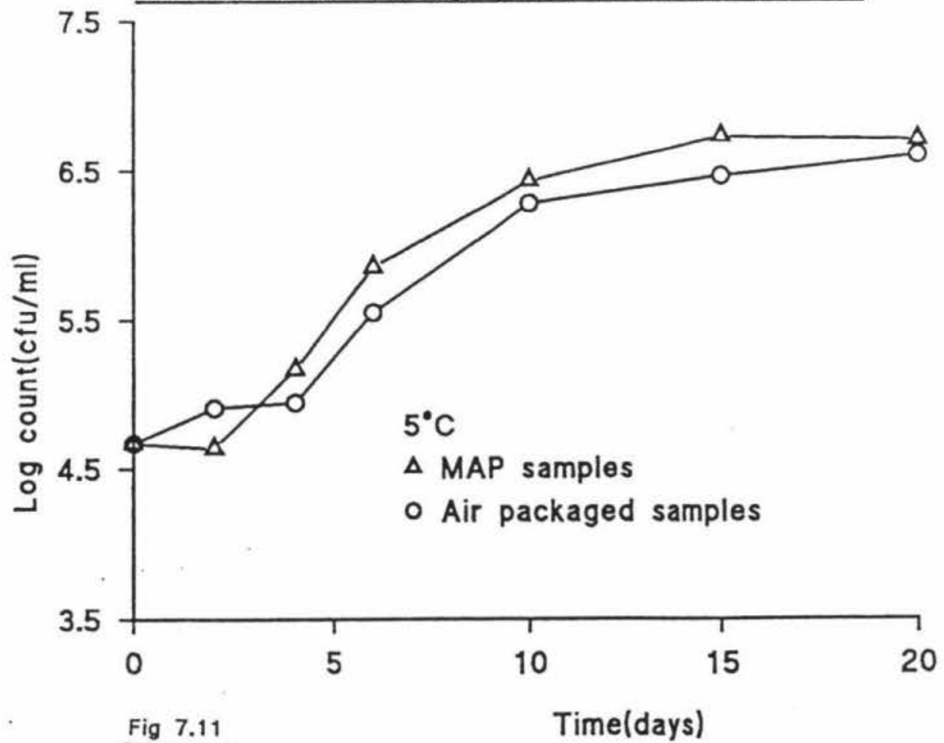


Fig 7.11

mesophilic aerobic plate count indicated that the microflora was predominantly *L. monocytogenes* (Fig 7.8). At 12C, the lysozyme-GDL treatment in combination with MAP extended the lag phase duration to about 4 days after which growth occurred with no significant reduction in the μ_{max}(0.494-0.441 day^{-1}) and with no significant increase in the GT(1.40-1.57 days). Again there was no suppression of the MPD.

When the growth/inhibition curves for *L. monocytogenes* in air packaged samples(Fig 7.3 and 7.4) were compared with the growth/inhibition curves of identical treatments under MAP(Figs 7.6, 7.7, 7.8, 7.9), it was observed that there was no enhanced inhibitory effect of MAP on the growth of *L. monocytogenes*. Kinetic parameters indicated that air packaged samples were in fact more inhibitory to *L. monocyogenes* than the MAP samples. Such an observation had a number of implications. At point **P** in Fig 7.6, one or more of the following events may have occurred.

(1) MAP may have enhanced the growth of *L. monocytogenes* by creating a microaerophilic environment conducive to the growth of *L. monocytogenes*.

(2) The competetive flora may have been inhibited, giving *L. monocytogenes* a competitive advantage. Thus the preservative system may have failed to cope with increasing numbers of *L. monocytogenes*.

(3) The preservative system may have been inhibited.

Experiments carried out to test speculation (1) indicated that MAP had no influence on the growth of *L. monocytogenes* (Fig 7.11). Although counts were slightly higher in MAP samples than air packaged samples, the difference was insignificant, thus it was concluded that MAP had no influence on the growth of *L. monocytogenes*.

Speculation (3) was disproved to some extent in that a low GDL treatment(0.178%) in combination with a low lysozyme concentration(0.2mg/g) (Fig 7.10) had no influence on the growth of *L. monocytogenes*, whereas a high GDL treatment(0.445%) in combination with a high lysozyme concentration(3mg/g) was inhibitory to the growth of *L. monocytogenes* throughout the storage period. When the kinetic parameters of *L. monocytogenes* under the influence of the above treatments were considered, the lysozyme(0.2mg/g)-GDL(0.178%) treatment had no influence in the μ_{max}(0.238-0.235 day^{-1}), no corresponding influence on the GT(2.91-2.95 days) and MPD suppression when the treatment was compared with the control. However, the lysozyme(3mg/g)-

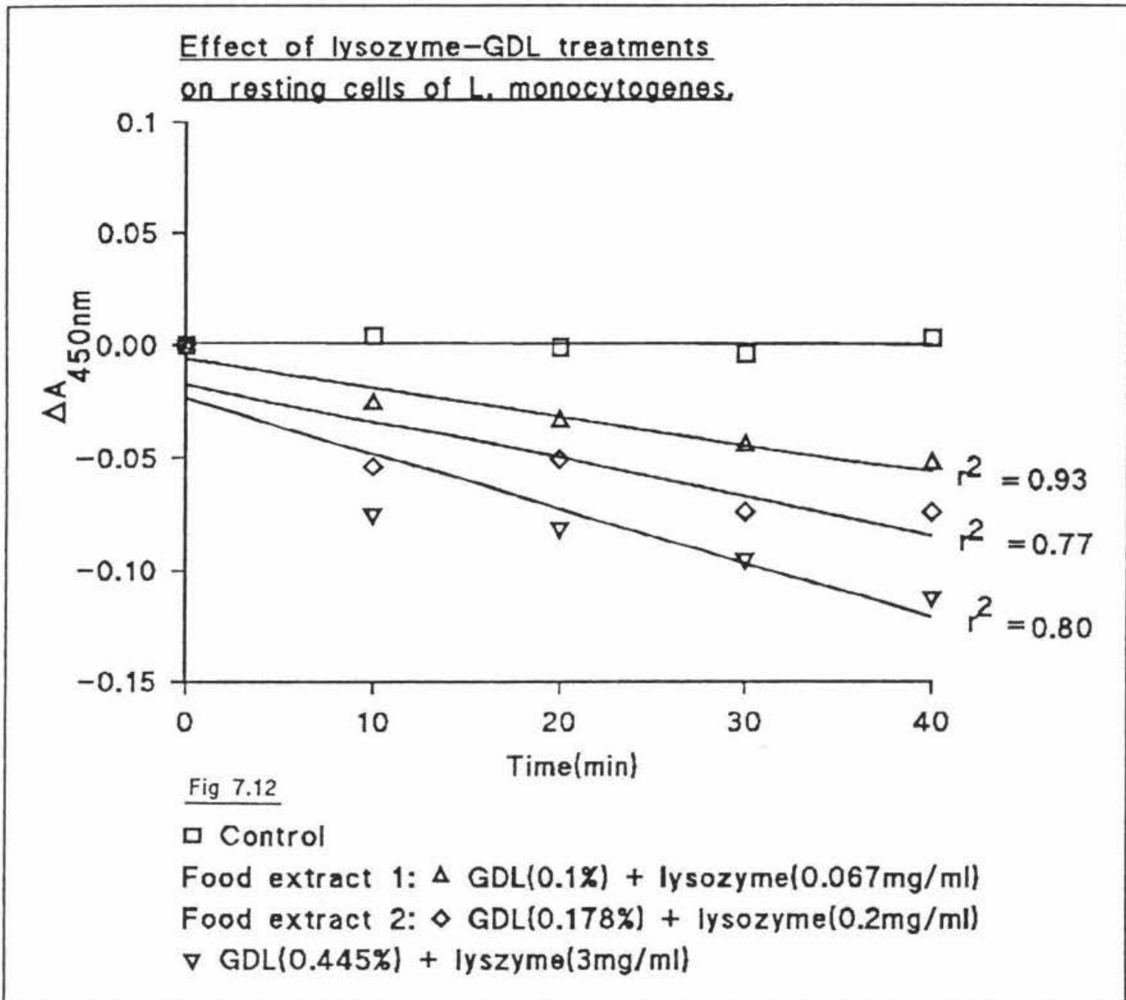


Fig 7.12

GDL(0.445%) treatment was listericidal and extended the lag phase to about 7 days, reduced the μ_{max}(0.238-0.113 day^{-1}) and increased the GT(2.91-6.13 days) and the MPD was suppressed(Fig 7.10). This observation confirms the findings in the broth culture system that the inhibitory effect of a low lysozyme(0.2mg/ml) in combination with a low GDL(10mM) was of no practical significance. Whereas the preservative system may have been partially inhibited by some other mechanism, results of this study demonstrate that the lysozyme-GDL system was active throughout the storage trial. This was further confirmed by extraction of the lysozyme-GDL preservative system from the high lysozyme-GDL treated mussels at the end of 21 days. The extracted preservative system was still active as it lysed non-growing cell of *L. monocytogenes* suspended in phosphate buffer(Fig 7.12).

Data generated in this study using nitrogen flushed mussels reinforce the conclusion of

Beauchat (1990) that MAP($3\%O_2$, $97\%N_2$) has no influence on the growth of *L. monocytogenes*. Our results are similar to the finding by Berrag et al. (1989) who demonstrated that controlled atmosphere storage of fresh vegetables does not influence the growth of *L. monocytogenes*. Various packaging methods have been demonstrated to have no influence on the growth of *L. monocytogenes*. Vacuum packaging appeared not to affect survival of *L. monocytogenes* (Bentley et al. 1989; Glass and Doyle, 1989; Harrison et al. 1991; Gau and Vanderlinde, 1992; Dillion and Patel, 1993; Hudson et al, 1994). Findings by other authors suggest that other methods of packaging such as controlled atmosphere storage, plastic wrap or MAP also have no substantial effect on the organism's ability to grow (Berrag et al. 1989; Ingram et al. 1990; Wimpfheimer et al. 1990; Kallander et al. 1991).

7.5 Conclusions.

The following conclusions can be drawn from this study:

1. The lysozyme-GDL preservative system has the potential for use as an antilisteric agent in mussels and cottage cheese stored at 5C.
2. The lysozyme-GDL preservative system was active throughout the storage trials.
3. When *L. monocytogenes* is treated with the lysozyme-GDL treatment combination, survivors of this treatment do not become resistant to the agents. They appear to exhibit "persistance".
4. MAP has no influence on the growth of *L. monocytogenes*.
5. Results obtained from the broth culture runs gave a good reflection of what happens in a food system.
6. The susceptibility of *L. monocytogenes* to lysozyme-GDL combinations increases as the temperature is reduced.

CHAPTER 8.

GENERAL DISCUSSION AND CONCLUSION

The data generated in this study suggest that pH, lysozyme and GDL interact synergistically to influence the growth kinetics of *L. monocytogenes* particularly in regard to the μ_{max}, lag phase duration, maximum population density and generation time. The results demonstrated that pH, GDL and lysozyme are effective in delaying growth initiation and total growth of *L. monocytogenes* in both broth culture and food systems. The findings are applicable for use with the hurdle concept for pathogen inhibition in foods, whereby multiple sublethal factors work synergistically to minimise microbiological safety risks. Such barriers include refrigeration, pH, preservatives and packaging techniques. There was good agreement between the numbers of microorganisms estimated from viable counts from the culture flasks and numbers estimated from OD measurements. The results of this study have demonstrated that OD can be used as a tool for screening the effects of combinations of antimicrobial factors on the growth of microorganisms. A similar finding was reported by McClure (1993).

Based on equal molar concentrations, the antimicrobial activity of the chelating agents against *L. monocytogenes* could be ranked in decreasing degree of inhibition in the order EDTA > GDL > citric acid > glycine > adipic acid > SHMP > SPDB. The same ranking was also true for the ability of the different chelators to enhance lysozyme activity. This result is probably a reflection of the relative chelating power of these chelating agents.

WPC at 1mg/ml had no influence of the antimicrobial activity of GDL, lysozyme and lysozyme-GDL treatment combinations. It would seem that if lysozyme was inactivated by higher protein concentrations, it would have to be used as a surface antimicrobial rather than attempting to incorporate it into high protein foods.

Results of this study suggest that egg white lysozyme in combination with GDL could be useful as a preservative to protect against contamination by *L. monocytogenes* in cottage cheese and mussels. There may be a potential risk if mussels are stored for a long time under MAP, even if the mussels have low numbers of *L. monocytogenes,* given the inhibitory effect of MAP on competitive flora, the ineffectiveness of MAP on *L. monocytogenes* and the low infective dose. Thus, barriers or hurdles such as the lysozyme-GDL preservative system may minimise microbiological safety risks in refrigerated, low pH, ready-to-eat foods.

The broth culture system studies emphasize the importance of preventing or reducing contamination levels(because the inhibitory effect decreased when the inoculum was at a high level). The broth and food system studies also emphasize the importance of adequate refrigeration(the greatest inhibition was achieved when the culture flasks or products were held at 5C).

The study has also demonstrated that *L. monocytogenes* is capable of growing on mussels subjected to commonly used packaging procedures used in the food industry. Several reports have measured growth of *L. monocytogenes* under MAP with various gas mixes. Zeitoun and Debereve (1991) noted a 3-log-cycle increase in numbers of *L. monocytogenes* in uncooked chicken legs incubated under 90% CO_2 , 10% O_2 and at 6C

for 13 days. Wimpfheimer et al. (1990) recorded a 6-log cycle increase in numbers of *L. monocytogenes* in raw chicken incubated at 4C under 72.5% CO_2 , 22.5% N_2 , 5% O_2 and no growth at 4C, 10C and 27C when the gas mixture was 75% CO_2, 25% N_2. Berrag et al. (1989) also demonstrated that controlled atmosphere packaging of fresh vegetables did not influence the rate of growth of *L. monocytogenes*, populations increased during storage. It is also well established that MAP inhibits the growth of aerobic spoilage organisms, while the growth of pathogens is uninhibited (Hintlian and Hotchkiss, 1986; Farber, 1991). This may be hazardous because the infective dose of *L. monocytogenes* is low(10^2-10^3) (Rosenow and Marth, 1987) and that overt spoilage may be absent when the product contains pathogens at the infective dose. Since a modified atmosphere may protect mussels against sensory quality deterioration, thus extending the shelf-life, the risk of human listeriosis resulting from consumption of infected produce may be increased.

Although the composition of the headspace gas was monitored during the study, variation of the gas composition was more pronounced in CO_2 flushed bags than on the nitrogen flushed bags. This may have been due to the increased solubility of carbon dioxide at low temperatures in the water and fat phases in the mussels, consumption of O_2 by aerobic microorganisms and higher permeability of carbon dioxide through the packaging material than O_2 into the packaging material. In addition, no testing of package integrity was done. These results imply that proper measurement of headspace gas composition, in addition to testing package integrity, is important to ensure adequate MAP performance.

L. monocytogenes was more susceptible to the effects of lysozyme-GDL in BHI broth than in any of the food systems(cottage cheese/mussels). For example in BHI broth treatment ab(Fig 5.32) continued to be listericidal during the first 125hrs(5days) compared with a similar treatments(Figs 7.1 and 7.3) in the food system. This may be explained by:

(1) differences in the ultimate pH in the two systems, BHI treatment was at pH5.5 whereas food systems where at pH5.88 for cottage cheese and pH6.5 for mussels. The buffering action of the two food systems prevented pH reductions as the lactone hydrolysed;

(2) interference with the lysozyme-GDL system by the complex food systems.

This may explain why *L. monocytogenes* was more susceptible to lysozyme-GDL in BHI than in cottage cheese or mussels. It is reasonable to conclude that inhibition of *L. monocytogenes* by the lysozyme-GDL depends on the nature of the substrate, concentration of the preservative(the higher concentrations of lysozyme-GDL caused greater inhibition), pH of the substrate(the lower the pH the more the inhibition) and incubation temperature(inactivation was enhanced at 5C compared with higher temperatures of 12C or 25C). Similar conclusions were made by El-Shenawy and Marth (1988, 1989) in a study of the susceptibility of *L. monocytogenes* to sorbic acid, benzoic and propionic acids and by Shahamat et al. (1980) who studied the susceptibility of *L. monocytogenes* to sodium nitrite.

A list of the reduced mathematical models(Haaland, 1952) for yield of *L. monocytogenes* and *C. tyrobutyricum* under the influence of lysozyme, pH and various chelating agents are presented in Table 8.1. The predicted values for yield can be calculated from each

Table 8.1
Reduced model equations for yield(Y) of *L. monocytogenes* and *C. tyrobutyricum* under the influence of lysozyme, pH and various chelating agents.

L. monocytogenes:

		r^2
GDL:		
Y =	170.56 - 18.49*GDL - 23.19*lys + 60.78*pH - 19.53*GDL*lys + 16.10*GDL*pH + 15.22*lys*pH + 21.12*GDL*pH*lys.	0.96
CA:		
Y =	113.83 - 8.96*CA - 4.99*lys + 46.1*pH - 11.5*CA*lys + 18.8*CA*pH + 5.70*lys*pH + 4.82*CA*lys*pH.	0.98
Gly:		
Y =	132 - 9.44*gly - 18*lys + 37.1*pH - 3.92*gly*pH + 13.4*lys*pH + 5.39*gly*pH*lys.	0.99
AD:		
Y =	104.09 + 41.66*pH + 12.9*lys*pH.	0.97
SHMP:		
Y =	58.79 - 3.79*SHMP - 5.43*lys + 9.17*SHMP*pH + 9.17*SHMP*pH	0.94
SPDB:		
Y =	78.19 - 9.57*lys + 31.05*pH + 11.38*lys*pH.	0.99

C. tyrobutyricum:

EDTA:		
Y =	116.54 - 24.45*EDTA + 7.87*lys + 46.64*pH + 17.94*EDTA*lys + 5.63*EDTA*pH + 5.12*lys*pH - 16.81*lys*pH*EDTA.	0.99
GDL:		
Y =	63.04 - 1.95*GDL - 5.15*lys + 5.55*pH - 1.68*GDL*lys + 1.85*GDL*pH.	0.98
CA:		
Y =	77.70 + 16.84*pH - 3.33*CA*pH.	0.96
Gly:		
Y =	61.45 - 2.06*lys + 2.22*gly*pH.	0.94
AD:		
Y =	61.71 + 4.77*pH.	0.81
SPDB:		
Y =	61.73 + 10.54*pH + 2.22*SPDB*pH.	0.95

run by substituting the appropriate values (+ or -1) of pH, lysozyme and chelator into the equation. The absence of other factors from the equation means that we do not believe that the other factors have important effects on the yield.

Model equations could be generated with experimental data when the cultural conditions allowed subsequent growth, however under extreme suboptimal conditions, this was not possible. Inclusion of the no-growth(negative yield values) data resulted in poor predictive models. It seems reasonable to recommend that no-growth responses should not be included in data for generating the predictive model equations. As suggested earlier by Cuppers and Smelt (1993), the results of this study also demonstrate that the development of a general model in terms of the kinetic parameters under sub-optimal conditions is extremely complicated.

The food system study indicated that:

L. monocytogenes did not develop resistance to the lysozyme-GDL preservative system but exhibited "persistence". The antimicrobial susceptibility of *L. monocytogenes* to lysozyme-GDL system was a stable characteristic, remaining unchanged throughout the study.

The lysozyme-GDL preservative system was not substantially inactivated throughout the storage period.

MAP(96.58%N_2., 2.09%O_2., and 1.34%CO_2) has no influence on the growth of *L. monocytogenes*.

With respect to *C. tyrobutyricum,* the EDTA-lysozyme combination treatment was the only treatment that demonstrated practical inhibition of *C. tyrobutyricum*. *C. tyrobutyricum* was more resistant to the different lysozyme-chelator treatment

combinations when compared with *L. monocytogenes.*

8.1 Conclusions.

1. The turbidimetric assay is a reliable method for modelling microbial growth.
2. The lysozyme-GDL preservative system has potential for application in food systems as an antilisteric agent.
3. When assessing the antimicrobial efficacy of antimicrobial agents, "no-growth" data should not be used for generating model equations.
4. The inoculum size effect is significant, that is the effectiveness of the lysozyme-GDL preservative system decreases with increase in size of the microbial inoculum.
5. At a given temperature, differences in antimicrobial activity between various chelating agents depend on the specific chelator, concentration of chelator and pH.
6. Inhibition/inactivation of *L. monocytogenes* by lysozyme-GDL systems was affected by: (a) temperature (inhibition was more at 5C than at 25C); (b) pH of medium(efficacy was greater at pH5.5 than at pH6.5); (c) the nature of the substrate (inhibition was more in BHI broth than in foods); and (d) concentration of preservatives.
7. The antimicrobial activity of the different chelating agents was ranked in the order EDTA > GDL > citric acid > glycine > adipic acid > SHMP > SPDB, the same ranking was true for the degree to which each chelating agent enhanced lysozyme activity.
8. There were significant two-way and three way interactions between lysozyme and the chelating agents, higher order interactions were insignificant.
9. Glycine, citric acid and adipic acid have potential for use as antimicrobial agents in combination with lysozyme in food systems.
10. *L. monocyogenes* is best described as a persister rather than a resistant population when grown under the influence of a lysozyme-GDL treatment combination.
11. MAP(96.58%N_2., 2.09%O_2 and 1.34%CO_2) has no influence on the growth of *L. monocytogenes.*
12. The lysozyme-GDL preservative system was active in the food system throughout the inoculation study.
13. Results obtained in the broth culture systems were a good reflection of what happens in the food system.

8.2 Further work.

Incorporation of the lysozyme-GDL preservative system on edible films without raising the overall concentration of the lysozyme-GDL preservative system in the food is worth investigating.

Further work is needed to determine the optimum inhibitory concentrations of the lysozyme-GDL preservative system in foods. It is also worth investigating the potential use of citric acid, glycine and adipic acid in combination with lysozyme as antimicrobial agents in food systems.

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Appendix 1

Table A.

Effect of lysozyme-EDTA combinations on L. monocytogenes.
L. monocytogenes cfu/ml.

	C1	C2	C3	C4	C5	C6	C7	C8	C9
11	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81
11	6.90	5.59	6.46	6.15	5.74	5.89	4.86	5.72	6.59
11	8.40	5.66	5.86	6.32	6.04	5.04	5.62	5.60	5.63
11	9.36	5.48	6.20	6.18	6.32	4.86	5.54	4.40	6.43
11	9.48	6.25	6.04	5.18	6.67	4.43	4.73	5.30	6.49
11	10.18	5.98	6.04	5.92	6.66	5.04	4.94	4.89	6.68
11	9.57	5.68	5.28	5.40	6.30	4.34	4.43	4.56	6.11
11	9.26	5.15	5.22	4.78	6.48	4.32	4.26	4.15	6.29
111	9.36	5.28	4.40	4.48	5.40	3.78	4.06	3.30	6.04
11	9.26	5.15	4.52	4.34	5.78	3.70	3.85	3.60	5.95
11	9.18	5.15	4.50	4.08	5.70	2.50	3.60	3.00	5.85

where c1=control c2=1 c3=b c4=bc c5=ac c6=ab c7=abc c8=c c9=a

ANALYSIS OF VARIANCE:

SOURCE	DF	SS	MS	F	p
C11	4	92.581	23.145	35.91	0.000
ERROR	50	32.229	0.645		
TOTAL	54	124.811			

```
                                       INDIVIDUAL 95% CI'S FOR MEAN
                                       BASED ON POOLED STDEV
 LEVEL      N      MEAN      STDEV   ---------+---------+---------+-------
     c1    11    8.8873     1.0863                               (---*---)
     c2    11    5.6527     0.5230     (---*---)
     c3    11    5.5755     0.8411    (---*----)
     c4    11    5.4218     0.9174   (---*---)
     c5    11    6.1727     0.4693         (---*---)
     c6    11    5.6527     0.5230     (---*---)
     c7    11    5.5755     0.8411    (---*----)
     c8    11    5.4218     0.9174   (---*---)
     c9    11    6.1727     0.4693         (---*---)
                                     ---------+---------+---------+-------
POOLED STDEV =   0.8029                      6.0       7.2       8.4
```

Fisher's pairwise comparisons
Family error rate = 0.277
Individual error rate = 0.0500
Critical value = 2.009

Treatments with overlapping asterisk(----*-----) are not significantly different.

Appendix 1

Table B:

Yield values and statistical analysis for L. monocytogenes grown under the influence of lysozyme-GDL treatment combinations in a 2 level 5 factor full factorial design:

E:\MTBWIN\DATA\FFACT05.MTW'.
Retrieving worksheet from file: E:\MTBWIN\DATA\FFACT05.MTW
Worksheet was saved on 8/23/1994
MTB > print c1-c8

ROW	GDL	lys	pH	temp	inocula	Yield
1	-1	-1	-1	-1	-1	1.59
2	-1	-1	-1	-1	-1	-0.26
3	1	-1	-1	-1	-1	3.65
4	1	-1	-1	-1	-1	0.94
5	-1	1	-1	-1	-1	35.95
6	-1	1	-1	-1	-1	40.34
7	1	1	-1	-1	-1	27.44
8	1	1	-1	-1	-1	34.83
9	-1	-1	1	-1	-1	8.14
10	-1	-1	1	-1	-1	7.04
11	1	-1	1	-1	-1	15.30
12	1	-1	1	-1	-1	13.08
13	-1	1	1	-1	-1	44.52
14	-1	1	1	-1	-1	22.25
15	1	1	1	-1	-1	27.17
16	1	1	1	-1	-1	29.22
17	-1	-1	-1	1	-1	92.92
18	-1	-1	-1	1	-1	102.22
19	1	-1	-1	1	-1	74.36
20	1	-1	-1	1	-1	80.14
21	-1	1	-1	1	-1	67.32
22	-1	1	-1	1	-1	78.22
23	1	1	-1	1	-1	15.09
24	1	1	-1	1	-1	12.44
25	-1	-1	1	1	-1	119.72
26	-1	-1	1	1	-1	114.12
27	1	-1	1	1	-1	127.29
28	1	-1	1	1	-1	114.12
29	-1	1	1	1	-1	116.27
30	-1	1	1	1	-1	95.59
31	1	1	1	1	-1	116.67
32	1	1	1	1	-1	104.98
33	-1	-1	-1	-1	1	4.31
34	-1	-1	-1	-1	1	-1.25
35	1	-1	-1	-1	1	9.46
36	1	-1	-1	-1	1	2.49
37	-1	1	-1	-1	1	34.34

ROW	GDL	lys	pH	temp	inocula	Yield
38	-1	1	-1	-1	1	49.27
39	1	1	-1	-1	1	44.43
40	1	1	-1	-1	1	54.96
41	-1	-1	1	-1	1	42.95
42	-1	-1	1	-1	1	35.23
43	1	-1	1	-1	1	26.84
44	1	-1	1	-1	1	23.80
45	-1	1	1	-1	1	18.07
46	-1	1	1	-1	1	16.72
47	1	1	1	-1	1	52.78
48	1	1	1	-1	1	59.69
49	-1	-1	-1	1	1	104.50
50	-1	-1	-1	1	1	103.58
51	1	-1	-1	1	1	82.92
52	1	-1	-1	1	1	88.57
53	-1	1	-1	1	1	102.75
54	-1	1	-1	1	1	85.95
55	1	1	-1	1	1	66.04
56	1	1	-1	1	1	56.13
57	-1	-1	1	1	1	122.91
58	-1	-1	1	1	1	122.56
59	1	-1	1	1	1	115.54
60	1	-1	1	1	1	122.73
61	-1	1	1	1	1	96.14
62	-1	1	1	1	1	106.23
63	1	1	1	1	1	115.25
64	1	1	1	1	1	93.89
65	0	0	0	0	0	85.07
66	0	0	0	0	0	83.21
67	0	0	0	0	0	77.84
68	0	0	0	0	0	90.58
69	0	0	0	0	0	90.57

```
MTB > ffactorial Yield=(GDL lys pH temp inocula)5;
SUBC> residual c9;
SUBC> EPLOT.
```

Estimated Effects and Coefficients for Yield

Term	Effect	Coef	Std Coef	t-value	P
Constant		61.30	1.377	44.52	0.000
GDL	-5.56	-2.78	1.430	-1.94	0.059
lys	1.23	0.62	1.430	0.43	0.669
pH	21.60	10.80	1.430	7.55	0.000
temp	69.75	34.87	1.430	24.39	0.000
inocula	9.91	4.95	1.430	3.47	0.001
GDL*lys	-0.62	-0.31	1.430	-0.22	0.829
GDL*pH	9.93	4.96	1.430	3.47	0.001
GDL*temp	-9.74	-4.87	1.430	-3.41	0.002
GDL*inocula	3.77	1.88	1.430	1.32	0.196
lys*pH	-2.23	-1.11	1.430	-0.78	0.441
lys*temp	-23.68	-11.84	1.430	-8.28	0.000
lys*inocula	1.61	0.81	1.430	0.56	0.576

pH*temp	15.33	7.66	1.430	5.36	0.000
pH*inocula	-3.92	-1.96	1.430	-1.37	0.179
temp*inocula	-0.27	-0.14	1.430	-0.09	0.925
GDL*lys*pH	6.74	3.37	1.430	2.36	0.024
GDL*lys*temp	-5.07	-2.54	1.430	-1.77	0.084
GDL*lys*inocula	6.63	3.31	1.430	2.32	0.026
GDL*pH*temp	7.49	3.74	1.430	2.62	0.013
GDL*pH*inocula	-1.92	-0.96	1.430	-0.67	0.506
GDL*temp*inocula	-1.41	-0.70	1.430	-0.49	0.626
lys*pH*temp	10.43	5.22	1.430	3.65	0.001
lys*pH*inocula	-7.34	-3.67	1.430	-2.57	0.014
lys*temp*inocula	3.22	1.61	1.430	1.13	0.267
pH*temp*inocula	-7.41	-3.70	1.430	-2.59	0.014
GDL*lys*pH*temp	0.98	0.49	1.430	0.34	0.733
GDL*lys*pH*inocula	2.15	1.08	1.430	0.75	0.456
GDL*lys*temp*inocula	-2.93	-1.47	1.430	-1.02	0.312
GDL*pH*temp*inocula	-2.66	-1.33	1.430	-0.93	0.358
lys*pH*temp*inocula	-1.31	-0.65	1.430	-0.46	0.650
GDL*lys*pH*temp*inocula	-4.38	-2.19	1.430	-1.53	0.134

Analysis of Variance for Yield

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	5	87387	87386.7	17477.3	133.59	0.000
2-Way Interactions	10	16432	16432.1	1643.2	12.56	0.000
3-Way Interactions	10	6477	6477.4	647.7	4.95	0.000
4-Way Interactions	5	368	368.0	73.6	0.56	0.728
5-Way Interactions	1	308	307.6	307.6	2.35	0.134
Residual Error	37	4841	4840.8	130.8		
Curvature	1	3145	3144.9	3144.9	66.76	0.000
Pure Error	36	1696	1695.9	47.1		
Total	68	115813				

Appendix 1:

Table C:
Data for counts of L. monocytogenes in the interferance study:
Counts are an average of two determinations:

MTB >

	C1	C2	C3	C4
	1	2	3	4
1	6.89	6.89	6.89	6.89
2	7.72	6.01	6.59	5.10
3	8.93	6.17	7.28	6.04
4	9.15	5.85	8.89	5.34
5	9.13	5.30	8.80	4.37
6	9.06	5.31	9.11	4.96
7	9.04	5.31	9.16	4.36
8	9.24	7.51	8.89	4.67
9	9.59	8.42	9.18	4.53
10	9.12	8.88	8.72	4.25

where 1=control 2=lysozyme 3=GDL 4=GDL-lysozyme

ANALYSIS OF VARIANCE ON C5

SOURCE	DF	SS	MS	F	p
C6	3	88.64	29.55	28.51	0.000
ERROR	36	37.30	1.04		
TOTAL	39	125.95			

```
                                     INDIVIDUAL 95% CI'S FOR MEAN
                                     BASED ON POOLED STDEV
LEVEL     N      MEAN     STDEV   -+---------+---------+---------+-----
    1    10     8.787     0.824                              (----*---)
    2    10     6.565     1.313               (----*---)
    3    10     8.351     1.012                           (----*---)
    4    10     5.051     0.847   (----*---)
                                  -+---------+---------+---------+-----
POOLED STDEV =  1.018             4.5       6.0       7.5       9.0
```

Fisher's pairwise comparisons

Family error rate = 0.197
Individual error rate = 0.0500

Critical value = 2.028