Use of *Carnobacterium piscicola* to limit the growth of *Listeria monocytogenes* in mussel products

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Scientific theory is a contrived foothold
in the chaos of living phenomena.

Wilhelm Reich

Austrian psychologist 1897-1957

Abstract

Bacteria were screened in order to find an organism antagonistic to *Listeria monocytogenes* which could be applied to mussel products and enhance their safety, especially when temperature-abused.

A *Listeria monocytogenes* isolate from the seafood industry was selected as the target organism.

Strains of *Lactobacillus reuteri* and *Enterococcus fecium* were screened on plates incubated at 35°C and 10°C for anti-listerial compounds, but none were found.

A non-bacteriocinogenic strain of *Carnobacterium piscicola, A9b-* was selected as the antagonist for detailed examination of growth in broth, agar and mussel systems at 10°C. This temperature was chosen to represent temperature abuse of refrigerated products.

To distinguish between the growth of the *Carnobacterium piscicola* strain and wild-type *Listeria monocytogenes* a "semi-selective" agar was developed using phenol-red indicator, and mannitol as the sole carbohydrate source.

Growth rates of *Carnobacterium piscicola* and *Listeria monocytogenes* were compared when grown alone and as a co-culture in agar and broth. Growth rates of *Listeria monocytogenes* when grown alone, and in the presence of *Carnobacterium piscicola,* were determined on mussels.

Regression analyses were done for the inhibition of *Listeria monocytogenes* by *Carnobacterium piscicola*. In all cases *Carnobacterium piscicola* significantly inhibited the growth of *Listeria monocytogenes* (P_{broth}= 0.018, P_{agar} <0.001, $P_{mussels}$< 0.001).

Growth of both organisms was faster in broth, than on mussels or agar. The greatest inhibition of *Listeria monocytogenes* was observed in broth reaching $\log_{10}$ 4.8 at 41 hours of incubation, prior to decreasing after this time. In agar and mussels the inhibition lasted longer and had not decreased at the end of the trial. The $\log_{10}$ reduction in growth of *Listeria monocytogenes* in agar was measured at 3.4 and in mussels measured at 1.6. These results were statistically significant ($P<0.001$ for all).

Inhibition of wild type *Listeria monocytogenes* was also shown in broth when a much lower concentration of *Carnobacterium piscicola* was used.

These results should be considered as preliminary and further confirmatory work should be done. However, *Carnobacterium piscicola* A9b- shows promise as an antagonistic organism to assist in the control of *Listeria monocytogenes* in mussel products along with industry-accepted good hygienic practices.

Acknowledgements

The outcomes of this small piece of research are vast. Only a few of the outcomes are described here, in the text of this document. Like many research projects, most of the outcomes had more to do with my personal learning than the research topic itself.

I learned some important lessons about science and microbiology in general and I have gained a new respect for the dedication of all those who attempt to gather information about the biological mysteries of life. I have been awe-struck by the complexity and variability of microbes and all biological materials. Our reliance on the use of very blunt instruments for intricate and detailed measurements has been an eye-opener to me and I have learned how little we really know about "simple" single celled organisms.

There are many people who helped me in my learning, and for all of their inputs I am very grateful. I would particularly like to thank the following:

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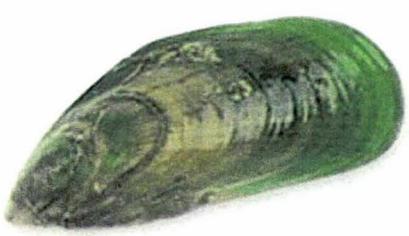
Contents

List of tables

List of figures

Chapter 1 Introduction



1 Introduction

1.1 The concept

Several years ago, I was working with a seafood company. Like all seafood companies making ready-to-eat product, they were grappling with *Listeria* control.

The idea came to me that competitive micro-flora could be used as a means of control rather than the current methods of non-specific destruction of all organisms.

There were two major influences on my thinking at the time, namely:

- Studies providing evidence that simply reducing the numbers of micro-organisms may not be effective in controlling pathogens (Jay, 1995).
- Guidance to processors from US Food and Drug Administration not to heat cold-smoked sea-food products too harshly as naturally occurring lactic acid bacteria are more heat-sensitive than pathogens they suppress, such as *Listeria* and *Clostridium* species. (US FDA, 2001) Hence the use of too much heat treatment could make the product less safe.

Some years later the time was right to test this idea. The concept was being intensively studied, with most focus on characterising bacteriocins. Most work had been done in the dairy industry, less in meat and less again in seafood. A few control methods using the products of competitive micro-flora had reached the marketplace.

Mussels were selected as the seafood product for study.

1.2 Objectives and outcomes

Current methods of processing mussels use heat treatment. This serves two purposes:

- To enable the shell to be removed (shucking) and
- To destroy organisms on the surface.

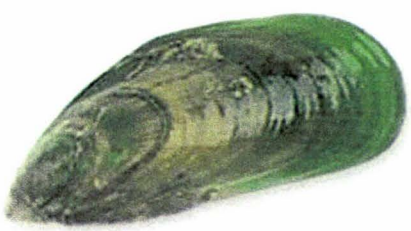
Ready-to-eat products are required by law to undergo sufficient treatment to destroy *Listeria monocytogenes* cells, unless a further treatment such as acidification is used. In New Zealand the levels of *Listeria* are considered to be extremely low on the raw shellfish entering the shucking process (Gosnell, Personal Communication 1998). Therefore mussels are frequently over-processed in an effort to destroy organisms that may not even be present.

This project was aimed at investigating an alternative to current methods of processing that would allow a less severe heat treatment, but increased protection later in the product life, when it is required. The feasibility of using antagonistic bacteria to inhibit pathogens that could be present in the final mussel product was investigated. The use of probiotic antagonists was the preferred approach as these organisms are likely to have more consumer acceptance and may provide additional health benefits to consumers.

Specifically the project was designed to determine the extent of inhibition by selected bacteria to pathogens that could be present in mussel products, such as *L. monocytogenes* and to serve as a proof of concept for this alternative control method.

The knowledge obtained in this project could assist seafood-processing companies to more effectively control the growth of food pathogens and spoilage organisms using antagonistic bacteria. If successful this approach would result in an increase in product safety and quality. Also it would provide opportunities to decrease costs associated with over-processing, rejected product and limited shelf-life.

Chapter 2 Literature Review



2. Literature Review

2.1 Background

Consumers demand a safe and consistent food supply. Food products have changed markedly over time and include a higher proportion of refrigerated, ready-to-eat, long shelf life, pre-cooked and/or minimally processed foods. Yet these products often provide ideal conditions for pathogen growth, especially as they may be temperature-abused once they reach the marketplace.

With these products there has been an increase in the numbers of cold-tolerant pathogens implicated in food-borne illness (FSANZ, 2004b). As a response industry has moved to taking more stringent sanitary measures and regulators around the world are imposing increasingly higher standards.

Surveillance and monitoring by a number of countries indicates the incidence of food-borne illness has increased substantially during the 1980s and 1990s (FSANZ, 2004b). Reliable data for food-borne illness incidence are not available due to a number of factors, including under-reporting of cases. It is estimated from surveys that less than one percent of cases are captured in Australia and this rate appears to be similar here. The estimated cost to the Australian community is $2.6 billion every year (FSANZ, 2004b). On a population basis, the situation in New Zealand is likely to be similar.

Most current methods of pathogen control result in a non-selective destruction of bacteria, yet it is well known that many pathogens are inhibited by the growth of competitive microflora. In our efforts to destroy all micro-organisms we destroy beneficial microbes that would normally suppress the growth of pathogens. Often these beneficial microflora are more sensitive to the bactericidal treatments used and are destroyed before the pathogens we are trying to control (Jay, 1995).

Some countries have recently reported a downturn in some illnesses due to special efforts at targeting the problem. The USA reported substantial decreases in the incidence of infections from some pathogens in 2003 consistent with

government initiatives (CDC, 2004). The USA FDA Seafood HACCP[1] rule issued in December 1995 added to pressure on seafood manufacturers worldwide to follow HACCP principles. This has not decreased the incidence of listeriosis (CDC, 2005).

2.2 Purpose of this review

This project is aimed at investigating the use of active cultures of competing microflora for control of bacterial pathogens in mussel products. It is assumed that this would be additional to current good manufacturing practices and hygiene controls

The use of probiotic bacteria is preferred as this will prove more acceptable to the consumer. Although the use has potential to enhance the health of the consumer, this is unlikely unless very large quantities are consumed frequently.

In order to determine strategies to assist the industry it is important to understand the following.

- Properties concerning the organisms that limit shelf-life and cause food safety problems. For the purposes of this study *Listeria* control is the main area of concern (see section 2.4).
- Characteristics of the organisms that may be used as controls, including how they grow, conditions under which they demonstrate anti-microbial activity and how they are affected by the product including its composition, processing and existing microflora.
- What is currently done in the seafood industry or in similar industries.
- Experimental methods that may be used in this application.

[1] Hazard Analysis Critical Control Point is the accepted food safety assurance methodology and is defined by Codex Alimentarius in Recommended International Code of Practice General Principles of Food Hygiene *CAC/RCP 1-1969, Rev.4- 2003.*

This review will also be concerned with the methods of antagonism and how these may be utilised.

2.3 Concerns with New Zealand Seafood Products

In New Zealand mussel exports have risen dramatically from NZ$24m in 1988 and 5,794,105 kg to NZ$185m in 2002 and 28,809,245 kg. (MIC, 2004)

Improved seafood storage and handling techniques and developments in value-added products have improved export returns. Improving storage techniques for live and fresh shellfish is particularly important because of the large distances to New Zealand's markets (SeaFIC, 2004). The focus on value-added products emphasises the need to provide high value, high quality and microbiologically sound products whether they be fresh or further processed.

Mussel products are processed and sold in a variety of forms including ready-to-eat, frozen, marinated, smoked and further processed. Half-shell mussels have been heat-treated to open the shell, a shell is removed and the product snap frozen after applying a glaze (usually water). This study is applicable to these types of products.

In New Zealand seafood products species of *Vibrio, Aeromonas, Listeria* and *Clostridium* have been identified as possible pathogens (Fletcher, 1996).

Aeromonas species are emerging as organisms of concern. These Gram-negative organisms are widespread, will grow well under refrigeration at 4-5°C and have been found in aerobic and vacuum-packed fish products (ICMSF, 1996a). In a retail survey of New Zealand shellfish motile Aeromonas were found in 66% of samples. The risk of illness is considered to be low but more investigation is needed as some species are pathogenic (Fletcher, 1996).

Strains of non-01 *Vibrio cholera* are believed to be endemic in the New Zealand environment and our seafood cannot be ruled out as a potential source of intoxication (Fletcher, 1996). However, low numbers of cholera infections are

reported in New Zealand (17 between 1980 and 2001) and frequently the source has been traced to infection overseas (Sneyd et al., 2002).

There is a possibility of *Clostridium botulinum* type E being found in seafood as it is known to be present in the marine environment overseas. Fortunately cases have been very rare in New Zealand (Fletcher, 1996). There is almost no information about prevalence and location of this organism in New Zealand.

In New Zealand *L. monocytogenes* contamination has been identified as the main food safety concern for our seafood industry especially in ready-to-eat products (Fletcher et al., 1998).

The principal spoilage organisms of New Zealand Greenshell mussels have not been reported. Overseas data indicate that for shucked molluscs, bacterial populations normally increase to 10^7 or more when spoiled. Gram-negative proteolytic bacteria, usually *Pseudomonas* and *Vibrio*, are prominent as well as Gram-positive saccharolytic species of *Lactobacillus* spp (ICMSF, 1998).

2.4 The problems with *Listeria*

2.4.1 It causes illness

Twenty-six cases of listeriosis were reported by the New Zealand Health authorities in 2004. Three were perinatal, resulting in 2 fatalities and of the remaining 23 non-perinatal cases, 3 resulted in death (ESR, 2005). This is typical of the incidence in New Zealand and follows patterns in other countries. These notifications represent the more severe cases and actual incidence is likely to be much higher. Although the incidence of listeriosis is relatively low, the fatality rate is high and is approximately 20% in the US (CDC, 2005).

Listeriosis usually occurs in certain high-risk groups of people including pregnant women, newborn babies and immunocompromised individuals, and occasionally in persons without known underlying conditions. In non-pregnant adults *Listeria* primarily causes meningitis, septicaemia and meningocephalitis with a mortality rate of 20-25%. In neonates sepsis, meningitis and pneumonia are seen, while

pregnant women may experience only mild flu-like symptoms, but the foetus may abort (Swaminathan, 2001).

2.4.2 It is resilient

L. monocytogenes is a Gram-positive, microaerophilic, non-spore forming rod, measuring 0.4-0.5μm in diameter and 0.5-2μm in length. In 3-5 day old cultures, long peritrichous flagella are found, giving *Listeria* species its characteristic tumbling motility. The organism is not fastidious and grows well in most common nutrient media. Survival of *L. monocytogenes* in foods below 0°C has been reported (Miliotis and Bier, 2003) and it will survive several weeks at -18°C. The organism can grow in a temperature range of -0.4 to 45°C. It grows in a pH range of 4.4 to 9.4 and minimum water activity of 0.92 (ICMSF, 1996b).

L. monocytogenes is found in a wide variety of habitats, including water, soil, raw materials, people and food processing environments. Foods appear to be a major vector of human listeriosis infection (ICMSF, 1996b) and mussels have been identified as a cause of illness in New Zealand as well as overseas (Baker and Wilson, 1993; FAO, 1999). However there have been no incidences of *Listeria* detected in mussels entering processing facilities in New Zealand, and in industry, it is widely accepted that mussels become contaminated while in the processing environment (Gosnell, Personal Communication, 1998).

Listeria can readily enter the food-processing environment and once there rapidly establish a biofilm. When attached to a surface the biofilm bacteria produce extra-cellular material that provides further protection to an organism already capable of withstanding relatively harsh conditions. It grows easily in a cold environment and its survival in certain adverse conditions, such as higher salt concentrations, is enhanced at low temperatures (Swaminathan, 2001).

Cleaning regimes have to be thorough and high mechanical activity provides the most effective biofilm removal. Sanitizer alone is not sufficient (Gibson et al., 1999). Some areas such as conveyor belts used in the seafood industry are particularly difficult to clean. Work at Crop & Food Research Ltd (Seafood Research Unit, Nelson) indicates that extracellular polysaccharide material produced by attached bacterial species, including *L. monocytogenes* and

Flavobacterium spp, and growth within the weave of the belt, further protect cells from the effects of the sanitizer (Boase, 2002). *Pseudomonas* and *Flavobacterium* spp are commonly found in seafood and the seafood processing environment (ICMSF, 1998). These organisms act together as primary colonisers enhancing the formation of biofilms of *Listeria* (Jeong and Frank, 1994) and survival of *Listeria* in a biofilm on a stainless steel surface is significantly enhanced in the presence of *Flavobacterium* spp. (Bremer et al., 2001).

2.4.3 There are differing standards for *Listeria* levels

The infective dose of *L. monocytogenes* is not known and microbiological limits for this organism in foods differ around the world. Most data show that at least 100 cfu/g are required for disease to develop (Swaminathan, 2001). Some countries such as France, Germany, Denmark and Canada allow up to 100/g at point of sale for some foods. Others such as New Zealand and the USA operate essentially a zero tolerance while England and Wales tolerate low levels in ready-to-eat foods (Lake et al., 2002). The Food Standards Code adopted by Australia and New Zealand does not allow any *L. monocytogenes* to be detected in 25 g for ready-to-eat mussel products but allows detection of up to 100/g in one sample of 5 for ready-to-eat processed finfish products (FSANZ, 2004a). To further complicate matters the sampling criteria and methods of testing for *L. monocytogenes* differ around the world (FAO, 1999).

FAO propose a simple decision tree for the establishment of *L. monocytogenes* criteria in foods. The decision is HACCP based. It is concerned primarily with whether there could be multiplication to >100 cfu/g within the stated shelf life and recommended storage conditions, assuming that in addition, there is no listeriocidal treatment prior to consumption. (FAO, 1999)

Preliminary studies provide some evidence of trade impact due to differing standards around the world (FAO, 1999). New Zealand manufacturers, for example, have to meet internal standards of zero tolerance as well as meeting the requirements for overseas market access. This may make our product more expensive entering a "*Listeria* tolerant" market compared to that from an equally "tolerant" market.

2.5 Use of microbial control mechanisms

To be effective, efforts to reduce the level of pathogens and spoilage organisms in our foods must use a multi-hurdle approach, particularly in ready-to-eat or minimally treated foods. The use of microbial control mechanisms should be considered as another hurdle.

Microbial growth has the potential to affect the growth of other organisms by changing the environment and can be expedited in a number of different ways. These ways could include:

- Antagonism, such as by change in pH, depletion of essential nutrients required for growth of a particular strain, or production of substances which act directly on target organisms.
- The production of bacterial signals, allowing expression of phenotypic traits that could influence the growth of other organisms.
- Supply of nutrients from other micro-organisms which could allow the growth of target species (metabiosis) (Gram et al., 2002).

For control of pathogen growth in mussels we are interested in activities from this list that suppress the growth of target organisms and will focus on the first point, as this has been the most well studied.

An antagonistic micro-organism is one that has the ability to suppress the growth of another organism. This includes probiotic organisms. An increasing number of organisms are being identified as probiotic and this is likely to continue as more organisms are characterised. Many organisms useful for control are found within the lactic group, therefore these will be considered in more detail in a later section.

2.5 1 Use of bacteriocins (static approach)

Many micro-organisms produce bacteriocins, which can be used to inhibit other bacteria.

There have been a number of efforts to isolate bacteriocins and to use these on food products to control bacterial growth. Nisin was the first bacteriocin to be isolated and approved for use in foods and has GRAS (Generally Regarded As Safe) status in the USA (FDA, 2002). It is a relatively broad spectrum lantibiotic-type bacteriocin produced by *Lactococcus lactis* (McMullen and Stiles, 1996). This has been used in foods such as cheese spreads to prevent the outgrowth of *C. botulinum* spores (Riley and Wertz, 2002).

Difficulties may arise in the requirement to obtain GRAS status for isolated bacteriocins and the need to declare these as additives.

Many studies have considered the use of isolated bacteriocins as a means of bacterial control. A drawback from this static approach is that there may be no control over increasing numbers of pathogens if the product is temperature-abused. Product temperature-abuse is a very common factor in reported cases of food-borne illness (Taoukis and Labuza, 2004).

2.5.2 Use of live cultures (active approach)

Cultures may be more acceptable, especially if these have a safe history of use by food industries prior to the 1958 Food Additives amendment in the USA (Muriana, 1996). Active cultures are more likely to provide assurance of protection in cases of temperature-abuse where cultures can grow at enhanced rates in a similar fashion to pathogens and spoilage organisms.

The use of cultures for bio-control is becoming increasingly accepted, not only in food products but also in other applications. In June 2000 the company BLIS Technologies was launched in New Zealand. This company uses lactic acid bacteria as protective agents for throat and mouth conditions and these are now sold over the counter in New Zealand pharmacies and in other countries.

Known probiotic organisms are preferred as active cultures because they may confer benefits to the host. At least, they are not seen as harmful, while suppressing growth of pathogens in food products

Numerous bacteriocin-producing lactic acid bacteria inhibitory to *L. monocytogenes* and other pathogens have been isolated from fermented and other food products (Muriana, 1996) and many have been tested directly in foods as an active control system.

Considerable work has been done in the dairy industry and a large number of organisms including nisin-producing *Lactococcus lactis* strains have been successfully used in dairy applications. However many such as *L. lactis* are mesophilic and have not been used successfully in meat systems where products are refrigerated (McMullen and Stiles, 1996).

Applications have been patented using the expertise gained from the dairy industry in starter cultures. For example, the company Danisco claims probiotic cultures to inhibit pathogenic and other undesired bacteria in a variety of dairy and non-dairy products without influencing sensory properties (Skoymose, 2002). These include a protective culture using *Lactobacillus plantarum* for use in meat and seafood products (Danisco, 2004).

There has been less work published related to seafood applications and much has been studied in liquid systems such as the cold-salmon juice extracts described by Nilsson (Nilsson et al., 1999). More work has been done in the meat industry, which can provide useful background information for mussel products.

2.5 3 Use of a combined approach

Logically, one would expect, the most effective control may be brought about by the use of bacteriocin to provide immediate inhibition along with an active culture. The active culture would maintain longer term control when numbers of the target organism increase beyond the point of bacteriocin control.

2.6 Mechanisms of antagonism

The spoilage organisms for many products, including mussels, have been identified as belonging to the lactic acid bacteria (LAB) group, as have most identified probiotic bacteria. Many LAB demonstrate antibacterial activity preventing the growth of other organisms including pathogens of concern in seafood products, such as *L. monocytogenes* (Carr et al., 2002).

Antagonism can occur by a variety of mechanisms including those outlined below. Although these mechanisms have been principally studied in the LAB group it is logical to expect that the mechanisms also apply to other groups of bacteria.

2.6.1 Lowering pH and production of organic acids

Lowering the pH is a common method of antagonism and is often found associated with the production of organic acids. Because of their weak dissociation constant organic acids can easily pass through the cell membrane where they will dissociate in the higher pH environment of the cytoplasm. This disrupts cellular metabolism by effects of the acid anion and by the movement of hydrogen ions out of the cell causing loss of energy. In numerous studies of production of acidic conditions by LAB, relatively large numbers of the antagonist need to be present to effectively inhibit a particular pathogen (Adams and Nicolaides, 1997).

This effect is non-specific and will inhibit a wide range of organisms.

2.6.2 Production of metabolites such as ethanol, hydrogen peroxide, diacetyl and CO_2

A large number of compounds produced during cellular metabolism can have antagonistic effects. These compounds generally have non-specific activity against a number of organisms, both Gram-positive and Gram-negative (Ouwehand, 1998). A few examples are described below.

Fermentation of sugars by heterofermentative lactic acid bacteria produces CO_2 which can be lethal to some organisms. Gram-negative bacteria are more susceptible than Gram-positive so spoilage organisms such as *Pseudomonas* may be more inhibited in vacuum-packed products (Adams and Nicolaides, 1997). Production of CO_2 may however be undesirable in vacuum-packed products.

Lactic acid bacteria may produce hydrogen peroxide (H_2O_2) in the presence of oxygen but lack catalase required to break it down. H_2O_2 inhibits a range of organisms including *Staphylococcus aureus* and *Pseudomonas* spp, whereas LAB can be more resistant. The amount of H_2O_2 accumulated by LAB cultures is quite variable and depends on the degree to which the medium is oxygenated and the temperature. Low temperatures favour H_2O_2 production as the solubility of oxygen is lower (Adams and Nicolaides, 1997). Therefore in an oxygenated chilled food product this could offer protection. This would generally be restricted to the surface of foods where oxygen tension is adequate but where contamination is most significant.

Ethanol and diacetyl have established anti-microbial activity and are produced by various lactic acid bacteria. The antibacterial activity of diacetyl has been described for a number of organisms including *A. hydrophila, E. coli, Pseudomonas* spp., and *Salmonella* spp., but the levels required to produce appreciable inhibition are generally considered too high to be palatable (Adams and Nicolaides, 1997). Lower concentrations may be satisfactory at lower temperatures (Archer et al., 1996).

The spoilage reactions of certain Gram-negative bacteria may produce ammonia and trimethylamine, which are toxic to a number of other organisms and sometimes to the producer itself.

2.6.3 Nutrient depletion and crowding

It is possible that the anti-microbial activity of some organisms is simply due to outgrowing competitors and by using up the most readily assimilable or growth-limiting nutrients (Adams and Nicolaides, 1997).

One area of nutrient depletion that has been looked at in detail is the competition for iron. When iron levels are low siderophore production (iron chelating systems) is induced in groups of organisms such as *Pseudomonas* (Gram and Melchiorsen, 1996). This may be significant in some fish products where iron is limiting, but less likely in NZ Greenshell™ mussels with a higher iron content of 7mg/100g (Sanford, 2003).

Work carried out by Buchanan and Bagi (1997) has shown that *L. monocytogenes* Scott A growth was suppressed by *Carnobacterium piscicola*[2] strains. Although two of the *C. piscicola* strains used produced bacteriocin, the depression of growth was only slightly greater than the suppression with the non-bacteriocin producing strains. The effects were shown to be independent of pH depression, peroxide production or oxygen depletion for at least one strain and it was thought that suppression may be due to nutrient depletion since the effect was dose-related.

A non-bacteriocin producing strain of *C. piscicola*, A9b-, was observed to suppress the growth of *L. monocytogenes* under experimental conditions. The effect is likely to be due to nutrient depletion by the non-bacteriocin producing strain. The degree of depression of maximum cell number was due to the initial cell density of the inhibitory organism, but did not require cell-to-cell contact as was demonstrated by growing the organisms apart in a diffusion chamber. When glucose was introduced back to the media, the depression was abolished. However the lag phase was longer and evidence suggests that acetate production is also involved in this effect (Nilsson et al., 2005).

2.6.4 Production of low molecular weight substances that appear to have a broad spectrum of activity

The most well known, reuterin (β-hydroxypropionaldehyde), is produced by *Lactobacillus reuteri* and has anti-microbial activity toward a range of food borne

[2] Organisms previously classified as *Carnobacterium piscicola* and *Lactobacillus maltaromaticum* have been reclassified as *Carnobacterium maltaromaticum* (Mora et al, 2003). The term used by the supplier of the organisms and by authors has been used in this report for easy reference.

pathogens and spoilage organisms including both Gram-positive and Gram-negative bacteria (Ouwehand, 1998).

Pyroglutamic acid or 2-pyrorolidone–5-carboxylic acid (PCA), produced by some strains of *Lactobacillus casei,* is inhibitory against *Bacillus subtilis, Enterobacter cloacae* and *Pseudomonas putida* (Ouwehand, 1998).

2.6.5 Production of a range of higher molecular weight substances

The term bacteriocins has defied concise definition for many years and therefore is used in different ways by different workers. A common definition is that bacteriocins are proteins that are generally narrow acting against similar species (Adams et al.,1997, Ouwehand, 1998, Tagg et al., 1976). It is now evident that bacteriocins take many forms and elicit bactericidal activity beyond species that are closely related or confined within a particular niche (Klaenhammer, 1993). For example, some from the *Pediococcus* genus have very broad spectrum of action including inhibition of *Pediococcus, Lactobacillus, Leuconsotoc, Enterococcus, Micrococcus, Listeria, Staphylococcus, Bacillus* and *Clostridium* (Nieto-Lozano et al., 2002).

Because of the difficulty in classifying bacteriocins many workers favour the use of the term Bacteriocin-Like Inhibitory Substance (BLIS) introduced by Tagg. Many have been discovered that are smaller than true proteins and their activity may not be as restricted as first thought (Tagg et al., 1976).

For simplicity, the term bacteriocins is used in this report to include all bacteriocin-like inhibitory substances, i.e. anti-microbial substances produced by bacteria that are genetically coded as opposed to being produced as a catabolic product. Hence specificity and size are not considered important.

Use of these bacteriocins as a control mechanism for pathogens in foods has been the focus of much research in recent years. These will be discussed in more detail later.

2.7 Bacteriocins

Much work recently has involved the use of bacteriocins as a pathogen control method in foods. This obviously has huge potential for this application whether it is used as a static, or as an active control mechanism. Therefore bacteriocins will be considered in more depth.

For use in a food system it is important to understand some characteristics of these compounds and their production by lactic bacteria.

2.7.1 Production of bacteriocins

Some organisms can produce more than one bacteriocin and they may be plasmid- or chromosomally-coded. *Carnobacterium piscicola* LV17, for example, produces two plasmid-encoded bacteriocins at different stages of the growth cycle whereas chromosomal determinants for the production of bacteriocin are found in *C. piscicola* UAL (Klaenhammer, 1993).

Features of bacteriocin production important to their use in food applications are considered here.

Stage of life cycle

Bacteriocins appear to be produced during different stages of the life cycle of the bacteria, depending on the organism and conditions. Reported to be primarily produced in the growth phase (Nes et al., 2002), some are produced early in the growth cycle while some are produced during later stages including late exponential phase (McMullen and Stiles, 1996). Differences within strains of the same species have been noted. For example, one strain of *C. piscicola* isolated from fish produced bacteriocin during the mid-exponential phase of growth (Stoffels et al., 1992), whereas maximum yield of the bacteriocin produced by *C.piscicola* CS526 was reached at the end of the exponential phase (Yamazaki et al., 2003).

Other organisms produce bacteriocins during late exponential phase or stationary phase, some producing them in bursts while others release them continuously (Tagg et al., 1976).

To minimise unrestricted pathogen growth in food products, production of the bacteriocin should occur early in the growth cycle.

Culture conditions

Many bacteriocins are produced in anaerobic conditions and many of the producers are strict anaerobes or facultative anaerobes. Bacteriocins from strains of *Lactococcus lactis, Pediococcus pentosaceus, Lactocobacillus rhamnosus* and *Enterococcus faecium* have been used to protect against Clostridium spore outgrowth in anaerobic conditions. Inhibition was greater at 10°C than 15°C or 25°C (Rodgers et al., 2003). By contrast, aeration of cultures has been found to greatly increase the yield of staphylococcal bacteriocins (Tagg et al., 1976).

Bacteriocin production in some strains of *C. piscicola* isolated from fresh fish was shown between 15 and 34°C but was completely abolished at 15°C and below (Stoffels et al., 1992). Conversely, increased production of bacteriocins has been shown at low temperatures (Buchanan and Klawitter, 1992).

In many organisms, much of the bacteriocin adsorbs to the producer cell, especially close to pH 6.0. Adsorption is lowest at pH 1.5-2.0 (Ouwehand, 1998). This is likely to be due to charges on the protein groups at different pH values.

The particular chemical composition of the culture medium is important. Various key nutrients such as amino acids and metal ions have been shown to be required in vitro for the production of different bacteriocins (Tagg et al., 1976).

In some instances the production of bacteriocins has been demonstrated only on solid media (Tagg et al., 1976), whereas many other applications have used liquid media, such as the starter cultures for yoghurts and many other fermented foods (Ray, 1992). The production of bacteriocin from *C. piscicola* A9b strains has been observed in cold-smoked salmon juice systems but has not been detected in cold smoked salmon pieces (Nilsson et al., 1999).

De Vuyst (1996) notes that bacteriocin production may be increased when the producer cells are stressed. Therefore any adverse environmental or culture conditions may increase bacteriocin production. This may help explain some of the variability in bacteriocin production under different growth condition such as temperature and level of oxygen.

Inducibility of bacteriocins

The production of some bacteriocins of Gram-positive bacteria has been shown to be inducible in a manner analogous to prophage induction (Tagg et al., 1976).

The production of bacteriocins by strains of *C. piscicola* has been reported as being regulated by the bacteriocins themselves (Saucier et al., 1995), and this may account for the enhanced bacteriocin production observed in solid media as opposed to liquid media. This is however contrary to the observation by Nilsson et al. (1999) in salmon juice compared to salmon pieces (see previous section) where no bacteriocin production was seen in the solid product. This may be due to characteristics of different strains of *C. piscicola* or to some other difference between the experimental systems. Later work by Nilsson et al. (2004), states induction of bacteriocin may also be triggered by an extracellular secreted peptide, the bacteriocin itself, and acetate in laboratory media and in cold-smoked salmon juice.

Ideally, in a solid food system, bacteriocins produced would be inducible or triggered by an extra cellular compound to maximise production.

2.7.2 Actions of bacteriocins and development of resistance

In vitro studies have suggested that the cytoplasmic membrane of sensitive cells is the target of many bacteriocins, with Gram-positive cells being more sensitive due to the structure of the lipopolysaccharide layer (Abee et al., 1995). However some Gram-positive organisms such as *Lactobacillus acidophilus, Bacillus cereus* and some *Streptococci* have been shown to inhibit Gram-negative organisms. The mechanism of action in Gram-negative cells appears also to affect the cell wall (Tagg et al., 1976).

Recent studies show that for some bacteriocins the peptide concentration required to cause membrane changes does not necessarily cause cell death. The effects on the cell membrane may serve as a mechanism for allowing the bacteriocin to enter the cell where it acts (Cleveland et al., 2001).

The development of bacteriocin resistance has been frequently observed (Klaenhammer, 1993) and is very variable. In some cases bacteriocin resistance is a stable trait as observed in pediocin-resistant mutants of *Listeria monocytogenes* and less stable in nisin-resistant mutants of *Listeria monocytogenes* (Gravesen et al., 2002). The fitness, or resistance to stress, of the organisms also varies. Pediocin-resistant mutants were shown to be less capable than nisin-resistant mutants of withstanding environmental stressors such as salt concentration, reduced pH and reduced temperature *in vitro*. However in a saveloy-type meat model at 5°C there were no observed differences in fitness between resistant and wild type strains. Nisin resistance at 10°C was enhanced in one strain of *Listeria* when the salt concentration was increased, but this effect was not noted at higher temperatures (Gravesen et al., 2002). Considering that there are major structural differences between these bacteriocins, it is reasonable to expect different responses.

Unlike antibiotic resistance it appears that bacteriocin resistance may not be genetically determined (Cleveland et al., 2001), but more work is needed to substantiate this. However bacteriocin producers themselves have developed a protection system against their own bacteriocin that is genetically defined (Nes et al., 1996)

2.8 Factors affecting bacteriocin activity in food systems

The use of cultures or bacteriocins as control agents in food systems provides many challenges.

In food matrices bacteriocin activity may be affected by a number of factors (Ganzle et al., 1999). While these effects have been studied mostly for bacteriocins many are applicable to other mechanisms of antagonism.

2.8.1 Changes in solubility and charge

Any change to the growth media will affect the activity of substances within that matrix. Changes in pH will have a profound effect on the extent of dissociation of organic acids, their charge and their actions *in situ*.

Their protein nature may make bacteriocins particularly vulnerable to biochemical reactions involving amino acid side chains or hydrophobic interactions that may interfere with their intended interaction with target cells (Muriana, 1996). Factors such as pH and temperature have also been reported to have effects; this may be due to the solubility of the particular bacteriocin at different pH values (Ray, 1992) and will differ according to the different chemical structure of a bacteriocin.

2.8.2 Interaction (including binding) with food components

Antagonistic molecules such as bacteriocins may interact with food molecules and this changes their activity. Gänzle et al. (1999) reported the activities of the bacteriocins nisin, sakacin P, and curvacin A against *Lactobacillus curvatus* and *Listeria innocua*, were reduced in the presence of lecithin and this effect was related to the bacteriocin rather than to the target organism. Casein and the divalent cations magnesium, manganese, and calcium also reduced bacteriocin activity, but glycerin monooleate increased the activity of sakacin P and nisin.

Nisin has been shown to have higher activity in sausages with lower fat content (Davies et al., 1999), and lower activity on raw meats due to the presence of glutathione (Cleveland et al., 2001).

2.8.3 Inactivation by enzymes

Nisin has been considered as a bacteriocin for use on meats but it has not proven effective (McMullen and Stiles, 1996). The reasons for this have not been fully established and may vary according to each specific application. Possible factors could include degradation by muscle or bacterial proteases.

2.8.4 Large molecules may hinder diffusion

Bacteriocins considered for food application (commonly 4000-8000 Da) are large compared to organic acids and other anti-microbial chemicals used in food preservation (<100 Da). Lucke (2000) comments that it is commonly observed that bacteriocins are less effective in solid foods than in liquid media. The size of these molecules may impede diffusion, posing a physical constraint in delivery of sufficient quantities to be effective. However one would expect the production of auto-induced bacteriocins to be greater in solid systems than in liquid as the inducers are likely to remain in close proximity at relatively higher local concentrations in solid systems compared to liquid.

As bacteriocin molecules tend to be specific in their interactions with target organisms, less may be required than for a non-specific anti-microbial substance provided it can reach the target organisms.

2.8.5 Changes to the expression of bacteriocin genes

Bacteriocin production by lactic acid bacteria is often inconsistent and low in food products, possibly because of repression of bacteriocin synthesis. The reasons for this are not known at this time but could be due to environmental factors such as pH, presence of ethanol or sodium chloride. (Nilsson et al., 2004).

2.8.6 Particular strain(s) and state of pathogen present

It is likely that for any strain of target organism, the physiological state it is in at the time will also affect lethality of any antagonistic mechanism.

Bacteriocins originating from *Lactococcus* UW and *Lactobacillus sake* 148 did not express any inhibitory effects on any *Listeria* serotypes tested, while those arising from *Lactobacillus sake* 265 and 706, and *Pediococcus* 347, had a listeriocidal effect towards almost every, but not all, serotypes tested (Mirjana et al., 2004).

Environmental factors inducing changes in the cell envelope will affect the response to the bacteriocin (Ganzle et al., 1999). Changes in pH may play a role here, as this is likely to affect charges on protein groups.

Stressors placed on bacteria have profound effects. These are well documented in many texts and in many cases stressed bacteria appear more resistant to adverse effects than those that are non-stressed (Sanders et al., 1999).

The stage of the life cycle of a bacterium can also have an effect on its physiological state and it may be that organisms are more or less susceptible to antagonists at various stages of the life cycle. Gram-positive cells, for example, can grow very rapidly in the exponential phase of growth in a rich medium and produce a Gram-negative response to the Gram stain. This is because the cell wall becomes thinner during rapid growing periods and they lyse during staining (Beveridge, 2001). Although data are not available for such effects it is logical to expect that the physiological state of the organism has a large bearing on effects of antagonists.

2.8.7 Physical state of substrate

Only a few studies have considered the effectiveness of bacterial cultures or bacteriocins in solid food media. Various trials have been done in minced (Ganzle et al., 1999; Nieto-Lozano et al., 2002) or liquid systems (Duffes et al., 1999; Himelbloom et al., 2001; Nilsson et al., 2004). These studies may have limited relevance to applications in solid systems.

As discussed earlier (section 2.7.1.2), bacteriocins produced by *C. piscicola* A9b in salmon juice were not detected when the organism was grown on cold smoked salmon slices stored at 5°C, although the growth of *L. monocytogenes* was suppressed (Nilsson et al., 1999). High cell numbers of numbers of *C. piscicola* were used. It is possible that the effect was due to depletion of an essential nutrient but this has not been investigated in solid foods.

2.8.8 Bacterial Resistance

The build up of bacterial resistance could limit the use of bacteriocins as protective agents in food systems. This may be mediated by the use of active antagonistic cultures, which act in a number of ways, in addition to producing bacteriocin, and/or by using a multi-hurdle approach. It may be that unless resistant organisms re-enter the factory environment this will not be significant.

2.9 Confusing aspects

The overwhelming impression gained from the literature is the extremely large variation not only between the growth conditions for producer and target organism but also variations between different strains of the same species.

It is likely that each specific antagonist and antagonistic mechanism will also have specific reactions with each food system including the packaging, other organisms and their metabolites. Storage conditions such as temperature may also influence the activity. Biological variation in a foodstuff composition is likely to be significant. No information appears to be available to indicate how differences due to growing condition, season, type of feed, or composition of growing water may impact on the mussel conditions for growth of organisms.

An example of the complexity that needs to be understood for any particular application can be shown by consideration of the interactions between the organisms used in this study, *C. piscicola* and *L. monocytogenes*.

C. piscicola A9b- is a non bacteriocin-producing strain of the bacteriocin producing strain C. *piscicola* A9b+. The inhibitory effect of *C. piscicola* A9b- was

shown to be partly due to glucose depletion when organisms were grown at 30°C in broths. (Nilsson et al., 2005). In these studies, the organism also produced acetate. Growth of *L. monocytogenes* was inhibited.

Although not tested, it is likely that acetate also plays an inhibitory role on the growth of pathogens such as *Listeria.* Acetate also has an inhibitory effect on *C. piscicola* strains (Nilsson et al., 2002).

In broth systems, acetate stimulated bacteriocin production in *C. piscicola* A9b+ (Nilsson et al., 2002) however, bacteriocin production was not seen in cold smoked salmon pieces (Nilsson et al., 1999).

Bacteriocin production is often increased as a response to conditions of stress (De Vuyst, 1996). Whether the acetate acts in its own right as an inducing agent or is causing cells to become stressed and therefore produce bacteriocins has not been investigated. However, in a mixed culture including bacteriocin-producers, acetate production may confer an additional advantage.

C. piscicola A9b+ has the capability of producing a bacteriocin but its production has not been detected in solid food systems (Himelbloom et al., 2001; Nilsson et al., 1999). Given this, it may be that the inhibitory effects of the strain A9b+ in solid foods are due to the same mechanisms operating as for the non-bacteriocinogenic strain, rather than the production of bacteriocin. This is yet to be investigated. If this is so concerns regarding bacteriocin resistance amongst target strains are not important.

In addition to identifying whether or not a strain has activity in a particular food system, it is important to determine details on how the effect occurs over time. The changes may not be linear and many differences in the food system could impact on the protection. There may in fact be periods when the bio-effect is not acting sufficiently to protect against unacceptable levels of pathogens.

2.10 Use in industry

Most applications have produced reductions of 1 to 3 log cycles in *L. monocytogenes* in foods (Muriana, 1996), and this is likely to be sufficient for many applications, including mussel products.

Most applications are found in the dairy industry where the use of bacteriocins and protective cultures has been widely studied. These have been reviewed by many authors (Ray, 1992).

Some work has been done in the meat industry and less in the seafood industry.

Meat industry examples are more relevant than dairy examples to the seafood industry. *Pediococcus acidilactici*, *Lactobacillus curvatus, Lactobacillus pentosus* and *Lactobacillus plantarum*, when used as commercial starter cultures, showed inhibitory action against a wide range of Gram-positive bacteria. *P acidilactici* showed the most activity against *L. monocytogenes, L. innocua, C. perfringens, B. cereus, B. lichenoformis,* and *B. subtilis* (Nieto-Lozano et al., 2002). Many other strains have been or are being investigated, including *Enterococcus spp, Leuconostoc spp* and *Lactobacillus spp* (De Martinis and Freitas, 2003)

Species of *C. piscicola* are found in the endogenous microflora of many seafood and meat products and their use has been investigated (Nilsson et al., 1999; Paludan-Muller et al., 1998; Schobitz et al., 2003; Yamazaki et al., 2003). The organism has been considered as suitable for use in cold smoked salmon (Nilsson et al., 1999; Nilsson et al., 2004; Yamazaki et al., 2003) and refrigerated poultry products (Barakat et al., 2000).

For preservation of meats Lucke (2000) suggests a three step approach:

- Selection of psychotropic LAB with bacteriocins active against *L. monocytogenes* and other undesirable Gram-positive organisms.
- Selection of psychotropic bacteria that produce enough lactic acid to inhibit the growth of other psychotropic bacteria but not form compounds with adverse flavour.

- Addition of mesophilic LAB that become active rapidly if product is temperature abused.

These principles equally apply to mussels.

2.10.1 Considerations for mussel products

Ideally, for an antagonist application to be successful for any selected mussel product it will fulfil the following conditions:

- Growth under refrigerated conditions (allowing for temperature abuse, temperatures could be up to 10°C).
- Anti-microbial activity at neutral to acidic pH ranges.
- Freeze-thaw stability as mussels may be frozen directly after processing.
- Facultative metabolism with the ability to tolerate oxygen, as most mussels are not vacuum-packed.
- Ability to outgrow or out-compete *L. monocytogenes*.
- Activity against a wide variety of strains of *L. monocytogenes* and preferably other organisms.
- Activity by more than one mechanism to minimise the chance of build up of resistant organisms.
- No off-flavours, odours, colours or textural changes such as slime production.
- No harm to consumers. Probiotics are likely to be harmless but this needs to be confirmed. Different strains, different levels and a different way of ingesting may cause harm. Other antagonistic organisms may need to be assessed even more thoroughly.
- No harm to people during the application process, including no allergies from breathing or from skin contact.
- No gas produced or packaging and aesthetics may be affected.
- Easily applied to product. Many products could be dipped or sprayed with the antagonist.

Use of a combination of organisms possibly augmented by use of a chemical such as an organic acid or bacteriocin may in fact be a better way to achieve effective control than use of cultures only.

2.11 Where to from here?

The vast amount of literature on this topic demonstrates an overwhelming complexity in the anti-microbial activities and substances produced in a wide range of environments by a possibly even wider range of organisms. Hence, for any given application it is important to test the actions empirically.

Each strain of a given species can be expected to react differently to the various conditions existing in a foodstuff, including its chemical composition, physical state, pH, temperature, gaseous environment and the presence of other micro-organisms. Changes in the environment may alter the expression of the bacteriocin genetic code, thereby altering the production of bacteriocin. However effects observed are not always due to the production of bacteriocin.

Studies demonstrating *in vitro* activity or activity in one system cannot necessarily be applied to another system.

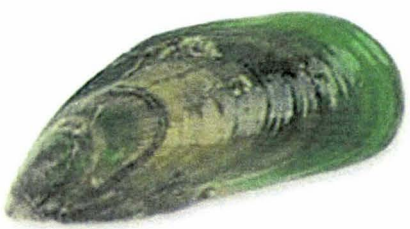
Anti-microbial effects as have been described earlier, need also to be considered in relation to the time sequence of events. There is no reason to expect the anti-microbial activity to occur in a time dependent fashion and most studies have not demonstrated the rate of activity in a system.

The biological variability in a specific mussel product could also be extremely large and be affected by the location of the growing beds, feed, season, water temperature and composition, and this would affect subsequent growth of micro-organisms.

In this work it was seen to be important to replicate as far as possible the conditions that would be found in the final product, but it was also recognised that fundamental data obtained from laboratory systems can be helpful to our understanding of the industrial system.

Chapter 3

Overview of methods and materials



3 Overview of methods and materials

3.1 Rationale for methodology

3.1.1 Selection of target organism

As *Listeria monocytogenes* has been identified as the main organism of concern for the New Zealand shellfish industry this organism was chosen as the target organism. A strain was sourced that had been found as a contaminant by Sealord Processing from their environmental monitoring at the mussel processing site in Nelson. Throughout this text this is referred to as the seafood variant. The organism was confirmed to be *L. monocytogenes* by standard biochemical and Christie, Atkins, Munch-Peterson (CAMP) testing (Jones, 1991), but as yet is an unidentified strain.

Strains from other sources were also used; initially before the seafood variant was obtained, and later to determine strain specificity of antagonism.

3.1.2 Selection of antagonist organism

From the literature search it appeared that *Carnobacterium piscicola* strains were potential candidates as antagonist organisms. Initially a stock culture was sourced from the Communicable Disease Centre (Porirua) for preliminary trials. This was the only known supply of the organism in New Zealand.

Two strains of *C. piscicola* (A9b+ and A9b-) were kindly donated by Dr Lone Gram of the Danish Fisheries Institute. Both these strains have demonstrated anti-listerial activity in their laboratory (Nilsson et al., 1999, 2002).

Also tested were a range of probiotic cultures obtained from Crop & Food Research Ltd. These organisms were of human origin.

A culture of *Lactobacillus plantarum,* marketed under the Holdbac™ brand, was kindly donated by Dansico Ltd. This had been tested in food systems and showed antagonism to *L monocytogenes* but had not been tested in seafood.

3.1.3 Screening for activity against *Listeria monocytogenes*

Preliminary experiments were conducted on this range of 17 strains to determine activity against *L. monocytogenes*.

For detection of anti-listerial activity, using active cultures, several common techniques were used. These are detailed in further in section 4.2.

A combination of actively growing organisms and extracted bacteriocins was also seen as a possible method of control. Crop & Food Research Ltd provided 13 strains of *Lactobacillus reuteri* and one strain of *Enterococcus fecium* which were tested for production of anti-listerial compounds, or bacteriocins, against the seafood variant of *L. monocytogenes*. These had showed the production of anti-microbial substances in broths at 30°C, under anaerobic conditions, by well-diffusion assays. The extracts had demonstrated activity against a number of pathogens including *L monocytogenes*, *Esherichia coli* and *Staphylococcus aureus*. (Lu, Personal Communication, 2004). Crop & Food Research Ltd also provided a sensitive strain of *L. monocytogenes*, which was used as a control in the screening of the strains of LAB they had supplied.

For detection of anti-listerial activity of the extracts the "spot on the lawn" technique was chosen whereby the anti-microbial agent (in this case 20μL of supernatant) was placed as spots on a lawn of test organism (in this case *L. monocytogenes*). Schillinger (1990) found spot tests showed more positive reactions than well tests.

The "spot of the lawn" technique approximates a hypothetical industrial situation where extracts are likely to be placed directly on the surface of a product enabling it to come into contact with contaminants. The spot technique gives direct contact between anti-microbial extract and the test organism.

Alternative methods are available, the most common is the well diffusion method where target organisms are added to molten agar, and extracts applied in wells drilled into the agar after solidification. This method requires diffusion of molecules through agar in order to make contact with the target organism. There is a chance larger molecular weight compounds will not diffuse through the agar

and therefore not react with the test organism, so this method was not selected. Although the reuterin produced by most of the strains tested is a small molecule, larger bacteriocins may also be produced. In addition, heat-sensitive organisms may be destroyed in molten agar methods.

3.1.4 Practical considerations

Growth at low temperatures

To be useful in practice the antagonistic organisms selected need to be able to grow appreciably in cold conditions where *Listeria* growth occurs.

Most references suggest that *Lactobacillus reuteri* strains do not grow under 15°C (Hammes et al., 1991). However, Muthukumarasamy et al. (2003) applied an *L. reuteri* strain to meat products to protect against *E. coli* growth. This was done at 4°C but required 10 or 20 days for the full protective effect depending on the initial concentration of *L. reuteri* used. This length of time is likely to be unacceptable for the control of *L. monocytogenes* which grows significantly faster than *E. coli* at 4°C. Mussel products are likely to be spoiled long before a protective effect is obtained. Since there could be significant differences between strains within a species, it was decided to determine if the strains supplied by Crop & Food Research Ltd grew at low temperatures.

Less information was available concerning *Enterococcus fecium* so this was also included in the growth temperature study. Growth at as low as 0.1 C was reported in bologna-type sausage but this was very slow (Zanoni et al., 1993).

Reports from Danisco have indicated that their strain of *L. plantarum* is capable of growth at low temperatures and that it is active against *L. monocytogenes* (Schwarz et al. 2004). Because of commercial sensitivities, information about this organism is not readily available.

These organisms were initially screened for their ability to grow in cold conditions. Literature reviews showed that *C. piscicola* was capable of strong growth at low temperatures, so this organism was also selected for study.

Strains of potential antagonists showing ability to grow in cold conditions were tested against the *L. monocytogenes* strain obtained from the seafood industry. Preliminary tests were carried out to determine if there was an effect in laboratory systems and in mussels before extensive trials were commenced.

Freeze-thaw stability

Since many mussel products are frozen it was decided to carry out an additional check to determine if the chosen antagonist strain would survive freezing.

Choice of growth media

To carry out *in vitro* laboratory trials, it was necessary to identify media which would allow the growth of both organisms and allow the antagonist to grow at a rate sufficient to compete effectively with *L. monocytogenes*. A number of different broth and agar systems were trialled, and the final antagonist was selected for detailed study.

Growth in liquid media has been studied more frequently than in solid media and the methodology is better established. It was therefore decided to use a liquid system for this work. Growth in solid systems differs from liquid systems in a number of ways (Stringer et al., 1995), so it was decided to also grow organisms in solid agar systems. There was less information available as to how to enumerate from an agar surface, so it was decided to simply stomach[1] the agar prior to dilution and plating to determine levels of organisms. As the growth of organisms varies with the substrate, growth on mussels also was critical. It must be noted that mussels are a more variable substrate than laboratory media and this makes interpretation of results more difficult.

[1] Solid media was placed in heavy duty plastic bags to which diluent was added, and a Stomacher Lab-Blender was used to emulsify this by the action of paddles.

Antagonist chosen

Carnobacterium piscicola A9b- was chosen for detailed studies. The reasons for selection are given in the discussion of experimental results (section 4.3.3).

C. piscicola A9b- does not produce a bacteriocin, unlike the closely related strain *C. piscicola A9b+* (Nilsson et al., 2005; Nilsson et al., 2002). This was seen to be an advantage as there should be no risk of build-up of immunity in prolonged use.

3.1.5 Preparation for determination of antagonistic effects and growth rates

Before the final experiments were completed a number of preparatory steps were needed.

Selection of a selective agar for *Carnobacterium piscicola*

A medium that would support the growth of *C. piscicola* A9b- and allow it to be distinguished from the wild-type *L. monocytogenes* was required to allow more accurate enumeration of *Carnobacterium* growth in the presence of *L. monocytogenes.*

Determination of *C. piscicola* by difference (subtraction of *L. monocytogenes* counts as recorded on Oxford agar from total counts recorded on Brain Heart Infusion Agar) was considered as an option if a suitable medium was not found. However, if the level of *L. monocytogenes* was considerably higher than *C. piscicola* a non-specific media would not allow the *C. piscicola* to be enumerated by a difference method.

Initially, "selective" media used by other workers were trialled. However the target *Listeria* strain grew well on these media so a search was conducted to find a more selective growth medium. A number of different agars were made and tested.

Determination of the pH of mussels

This was to enable the preparation of laboratory media at a similar pH to mussels for the laboratory trials.

Selection of a rapid method to determine concentrations of organisms in broths to enable a consistent level to be used in each part of the final experiments

To ensure a that consistent concentration of organisms was used in the final experiments a method was needed to asses the cell concentration in case the growth conditions changed slightly and altered the final viable numbers in the active broths.

The measurement of optical density (OD) was selected (Begot et al., 1996) as a quick, simple and non-destructive method. However, as the method does not distinguish between live and dead cells it was not considered acceptable for use as the means of measuring viable numbers in the final experiments.

Confirmation of purity of strains

Checks were made to confirm the purity of the organisms. Testing techniques were not available to type the *Carnobacteria.* This would require genetic detection methods and was outside the scope of this work.

Determination of Listeriocidal action of *Carnobacterium piscicola* A9b- in a broth culture

A preliminary trial was done before planning the extensive testing for the final experiments.

3.1.6 Determination of antagonistic effects and growth rate in mussels

The final experiments were designed to investigate growth rates and interactions between the antagonist and the target organisms. Initially broth was used, then

agar and finally mussels. The objective was to provide a proof of concept that could be further investigated to provide practical application to the seafood industry.

Knowledge of growth rates gives information of effects over time. In mussels this is useful in order to determine extent of protection during the shelf life of the product. For the laboratory studies it was useful to provide more information about the interaction of these organisms and to provide a basis for further studies to characterise them.

3.1.7 Determination of concentration effect

It was thought that the effects of the antagonist could be due to the large difference in cell concentration between the antagonist and target organisms, rather than intrinsic characteristics of the antagonist. Hence an additional trial was carried out using a lower concentration of *Carnobacterium* in broth.

3.2 Cultures used

These are shown in Table 3.1 below.

Table 3.1 Cultures used

Organism	Source
Carnobacterium maltaromaticum (DSM 20730 ATCC 35586)	NZ Communicable Disease Centre Institute of Environmental Science and research Ltd Communicable Disease Group ESR Kenepuru Science Centre PO Box 50 342 Porirua 6215 New Zealand.
Carnobacterium piscicola strains A9b+ and A9b-[2]	Danish Fisheries Institute Dept of Seafood Research Søltofs Plads Technical University of Denmark DK-2800Kgs. Lyngby Denmark.
Enterococcus fecium	Crop & Food Research Ltd Private Bag 92169 Auckland New Zealand.
Lactobacillus plantarum	Danisco Holdbac™ Ltd Danisco New Zealand Ltd Private Bag 76911 Manukau City Auckland New Zealand.
Lactobacillus reuteri (13 strains)	Crop & Food Research Ltd Private Bag 92169 Auckland New Zealand.

[2] *C. piscicola* A9b+ is treated by acriflavin, to construct a strain *C. piscicola* A9b- which does not produce a bacteriocin (Nilsson, et al. 2004).

Organism	Source
Listeria monocytogenes (Massey var.)	Institute of Food Nutrition and Human Health Massey University Private Bag 11222 New Zealand.
Listeria monocytogenes (Crop & Food var.)	Crop & Food Research Ltd Private Bag 92169 Auckland New Zealand.
Listeria monocytogenes (Seafood var.)	Sealord Group Ltd PO Box 11 Nelson New Zealand.

3.3 Media

The following were used:

- Brain Heart Infusion (BHI) broth (Merck)

- Buffered Brain Heart Infusion (B-BHI) broth (Merck) was used for the growth of *Listeria* and *Carnobacterium*. This was buffered in a phosphate buffer using equimolar amounts of 0.1M K_2HPO_4 and 0.05M KH_2PO_4 and 0.1% Tween 80 was added (Nilsson et al., 1997). HCl was added as necessary to adjust the pH to between pH 6.6 and 6.8.

- BHI agar (Merck) was used for growth of *Listeria* and *Carnobacteria*. For the final experiments in an agar system, BHI agar was pH adjusted to between pH 6.6 and 6.8 before autoclaving.

- Blood agar pre-poured plates (Difco) were used to confirm the β-haemolysis reaction of *L. monocytogenes.*

- Infant formula agar was prepared by adding one packet of a commercial brand of Toddlers Infant Formula to cooled molten agar (15g agar-agar to 1L water) before pouring plates.

- De Man, Rogosa and Sharpe (MRS) broth or agar (Merck) was used for the growth of all *Lactobacillus* strains, including Danisco *Lactobacillus plantarum*, and also for *Enterococcus fecium.*

- Modified MRS (D-MRS) from Hammes et al. (1991). Acetate was removed and pH adjusted to 8.5.

To minimise the risk of caramelisation during heat treatment of the agar, dehydrated MRS broth media was mixed into a boiled 1.5% agar solution prior to autoclaving.

- Nitrite-Polymixin (N-P) agar from Nilsson (2004) based on Davidson and Cronin (1973).

- Modified N-P agar (M-N-P). The glucose was removed and replaced by mannitol at the same concentration of 7.5 g/L.

- Modified N-P-BCP (Modified Nitrite-Polymixin Bromocresol-Purple) agar. Bromocresol purple indicator was added at concentration of 0.01g/L to modified NP agar.

- Oxford agar (Fort Richard) was used for selective detection and enumeration of *Listeria* throughout (Paranjpye et al., 1992).

- Peptone water for dilutions (Merck) was made at 5g/L.

- Phenol-Red Mannitol agar (PRMA). Made from phenol-red base (Merck) according to instructions on bottle, 1% mannitol and 0.15% agar-agar (Merck) were added prior to autoclaving.

- Water used was de-ionised.

- Mussels were kindly supplied by Sealord Group Ltd. Products were supplied directly from the processing operation in Nelson and were from the same batch, reasonably close in size, from the same harvest area, harvest date, and season. Half shell mussels and mussel "meat" were supplied. Half shell have had one shell removed, mussel meat has both shells removed.

3.4 Methods

Cell concentrations of *C. piscicola* were chosen for the final experiments based on literature values (Nilsson et al., 1999) and information received with the cultures from Dr L. Gram. For *L. monocytogenes* the concentration needed to be sufficient to enable detection and enumeration, but as low as possible. It is known in industry that the level of contamination by *L. monocytogenes* occurring during processing is very low and in most cases, initial levels would be below the level of detection.

Details of specific methods can be found in the relevant sections (sections 4&5). Throughout the experimental work certain standard procedures were adopted. These are described here to avoid duplication in later sections.

3.4.1 Storage of organisms

Stock cultures were received as agar slants, plates or freeze dried. These were grown to active cultures as described below. Where cultures were going to be needed for a significant period of time (some months) active cultures were stored in glycerol on plastic beads from Prolab Microbank at –80 °C. For temporary storage active cultures were kept at 4°C on agar slopes, stabs or plates. For all of the final experiments active cultures were freshly prepared from beads at -80°C immediately prior to use.

3.4.2 Preparation of active cultures

Organisms from stock cultures were used to inoculate broths, either by picking off a colony from agar or dropping a bead into broth. These were routinely incubated at 35°C overnight (except where stated otherwise). A second inoculation was made into a fresh broth and again incubated overnight at 35°C.

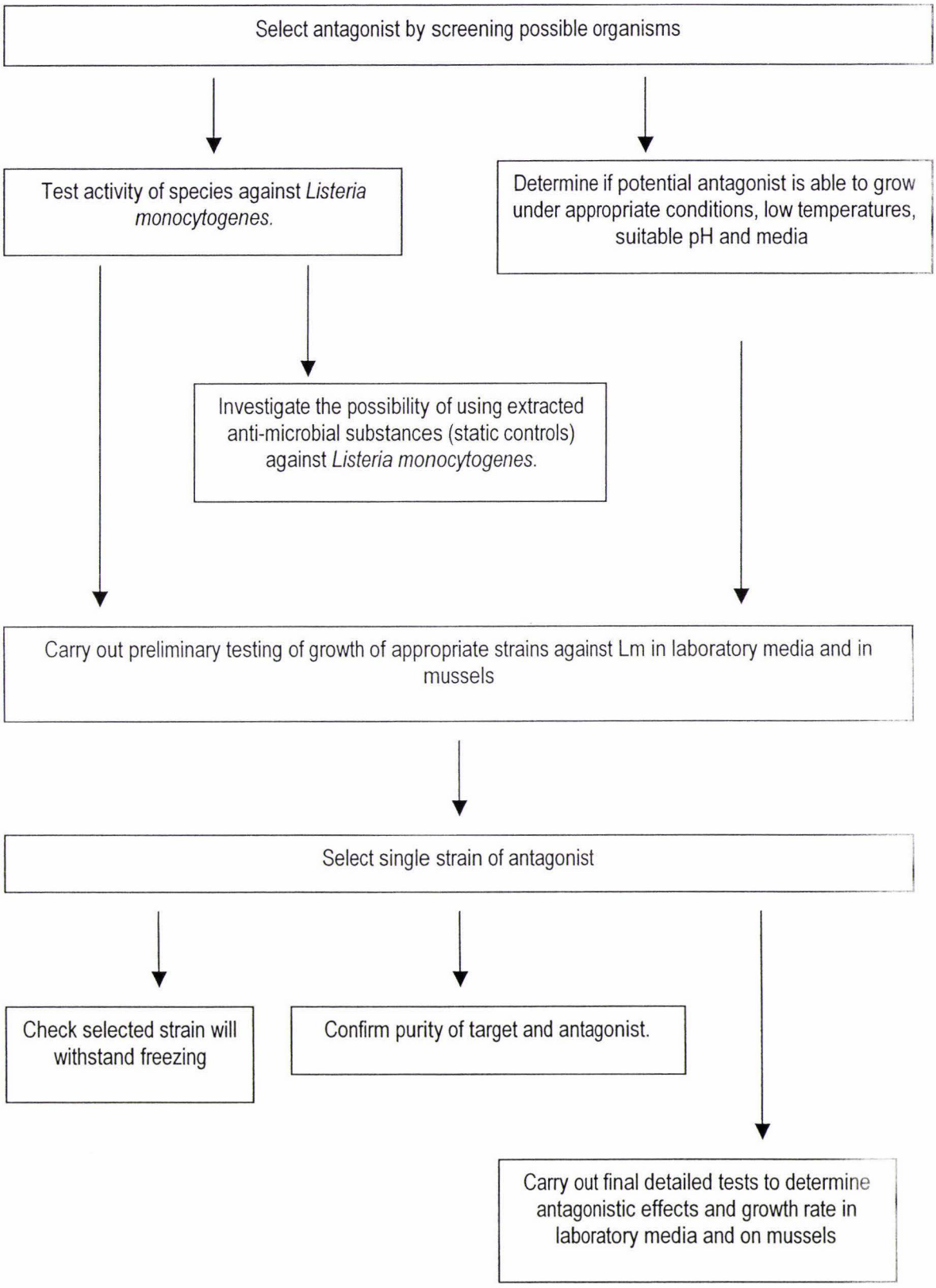
3.4.3 Plate counts

Plate counts were carried out by the spread plate method. This method was chosen to minimise the heat applied to organisms. Except where stated elsewhere spread plates were prepared by applying 0.1mL of the required dilution to the plate and spreading with a sterile glass spreader. Wherever possible counts were made of plates with between 30 and 300 colony forming units.

3.4.4 Good laboratory practice

Normal laboratory procedures were followed in terms of media controls, sterile techniques, and calibration of equipment including temperature checks of incubators.

3.5 Summary of experiments carried out



Chapter 4 Preliminary experiments

4 Preliminary experiments

4.1 Selection of target organism

As discussed previously, *L. monocytogenes* was chosen as the target organism and a "wild-type" was obtained from a mussel processor. Strains from other sources were also used; initially before the seafood strain was obtained, and later to determine strain specificity of antagonism.

4.2 Selection of antagonist

4.2.1 Determination of growth conditions

Measurement of pH of mussels

Method

The pH of mussel flesh was determined using a Testo model 205 probe manufactured by Testo Ag, Lenzkirch, Germany. Three measurements were made on 3 mussels at random points.

Results

All readings were between pH 6.6 and 6.8.

Cold tolerance of *Lactobacillus reuteri*, *Enterococcus fecium* and *Lactobacillus plantarum* strains

Method

Crop & Food Research Ltd provided anaerobic culture plates of 13 strains of *L. reuteri* and 1 of *E. fecium*. These had demonstrated activity against *L. monocytogenes* (Lu, Personal Communication). Organisms were picked off plates and placed onto MRS agar slants by the stab and streak method and

incubated at 35°C for 2 days. The streak provided aerobic growth conditions whereas the stab gave largely anaerobic conditions.

Broth cultures of *L. plantarum* were prepared in MRS broth by growing from the freeze-dried sample overnight at 35°C, then sub-culturing into fresh MRS broth and incubating for a further 24 hours. MRS agar slants were inoculated by stab and streak methods as described previously.

All slants were incubated at 35°C or 10°C for up to two weeks. Aerobic growth was more pronounced than anaerobic. After growth had appeared the slopes were refrigerated before applying cultures to MRS agar plates.

Samples from the surface of each slope were taken and applied by swab to MRS agar plates. Fifty-six plates were set up. Each plate was divided in half and each strain streaked onto a plate so that half the plate was streaked from the 10°C slope and the other half from the 35°C as shown in Figure 4.1. The aerobic portion of the slope was used. The plates were incubated at 10, 15, 20 and 35°C for 5 days.

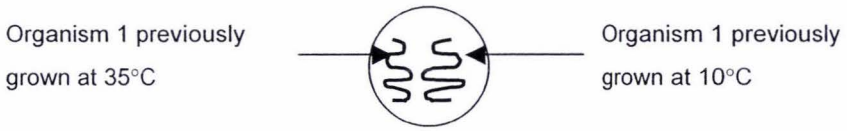


Figure 4.1 Method for inoculation of plates.

Samples from the 10°C slopes were made despite the fact that there was very little, if any, growth observed on the chance that a small population of cold resistant organisms might have been present.

Results

Moderate growth of *L. plantarum* was seen at 20°C after 1 day, slight growth at 15°C after 2 days, and significant growth at all temperatures after 5 days.

Moderate growth of *L. reuteri* and *E. fecium* was seen at 35°C when swabs from the slant grown at 35°C were plated, less growth was seen at 35°C when swabs

from the 10°C slants were plated. This is expected, as the size of the inoculum at 10°C is likely to be significantly less.

There was no evidence of cold-adapted organisms growing within this time period.

L. plantarum appears to be the only strain tested to grow sufficiently well to be useful against *L. monocytogenes* in cold conditions. Strains of *C. piscicola* were not available at this stage for testing.

Identification of a suitable culture medium for growing both *Listeria monocytogenes* and *Lactobacillus plantarum* spp in mixed culture

Method

Broth cultures of *L. monocytogenes* (Crop & Food var.) and *L. monocytogenes* (seafood var.) were prepared in BHI broth and incubated overnight at 35°C. *L. plantarum* was prepared in MRS broth and incubated for 2 days at 35°C.

Each organism was streaked onto BHI agar, MRS agar and Oxford agar. A mixture of equal volumes of *L. plantarum* broth and each of the *L. monocytogenes* strains was also streaked onto each type of plate.

A range of media with various modifications was trialled and plates were incubated at 10°C. These are detailed in the Section 3.3 and listed in the results below.

A final trial was carried out of an agar made by adding infant formula to agar after autoclaving as it was considered likely that both organisms would grow in this medium.

Results

After 2 days incubation at 35°C no growth of *L. plantarum* was seen on either BHI or on Oxford agar. Both strains of *L. monocytogenes* grew well on BHI and Oxford agar but showed no growth on normal (non pH-adjusted) MRS agar.

The range of plates incubated at 10°C was examined after 7 and 13 days. In all cases where growth was observed the colony characteristics were very similar between the organisms. Colonies were small, off-white, round, smooth shiny and slightly convex.

Growth of cultures is summarised in Table 4.1.

Table 4.1 Growth of *L. plantarum* and *L. monocytogenes* strains in different agar at 10°C

Agar	Mixed *L. plantarum* and *L. monocytogenes*	*L. plantarum*	*L. monocytogenes* (Seafood var.)	*L. monocytogenes* (Crop & Food var.)
TSB	Appreciable growth seen	Very slight growth observed at 7 and 13 days	Appreciable growth at 7 and 13 days	Appreciable growth at 7 and 13 days
MRS	Very slight growth observed at 7 days, appreciable growth at 13 days	Very slight growth observed at 7 days, appreciable growth at 13 days	No growth	No growth
MRS @ pH 6.50	Very slight growth observed at 7 days, appreciable growth at 13 days	Very slight growth observed at 7 days, appreciable growth at 13 days	Very slight growth observed at 7 days, appreciable growth at 13 days	Very slight growth observed at 7 days, appreciable growth at 13 days
LSB @ pH 6.58	Very slight growth observed at 7 and 13 days	Very slight growth observed at 7 and 13 days	No growth	No growth
BHI	Appreciable growth at 7 and 13 days	No growth	Appreciable growth at 7 and 13 days	Appreciable growth at 7 and 13 days
PCA	Appreciable growth at 7 and 13 days	Very slight growth observed at 7 and 13 days	Appreciable growth at 7 and 13 days	Appreciable growth at 7 and 13 days
Infant formula agar	Many contaminants grew on all plates	Many contaminants grew on all plates	Many contaminants grew on all plates	Many contaminants grew on all plates

Growth of *L. plantarum* in the media tested was generally too slow compared with *L. monocytogenes*. MRS agar (pH adjusted) was the best compromise but a 13-

day incubation makes its use difficult in practice. Merck were unable to suggest a suitable media.

Confirmation of freeze-thaw stability of *Carnobacterium piscicola* A9b-

Method

Whole frozen mussels (both shells removed) were thawed overnight under refrigeration before being packed into stomacher bags. These were autoclaved at 121°C for 15 mins, before dipping into B-BHI broth containing *C. piscicola* Ab- at 10^7 cfu/mL for 15 mins, draining and repacking into 4 stomacher bags.

Mussels from 2 bags were stomached in peptone, diluted and tested for levels of *C. piscicola* by plating onto PRMA.

2 bags were placed in a laboratory freezer at -18°C for 6 weeks. After 6 weeks the bags were removed and thawed overnight under refrigeration and levels of *C. piscicola* were measured as before.

Results

Levels of organisms were not reduced by the freezing process, but increased significantly. $P<0.001$.

Before freezing levels were:
6.34 $\log_{10}$ cfu/g (average of 6 readings) S.D 5.42

After freezing levels were:
6.50 $\log_{10}$ cfu/g (average of 6 readings) S.D 5.55.

4.2.2 Determination of activity against *Listeria* strains

Activity of *Carnobacterium maltaromaticum* against *Listeria monocytogenes*

Method

Active cultures were prepared in BHI broth and incubated at 37°C, *C. maltaromaticum* from a freeze-dried ampoule, and *L. monocytogenes* (Massey var.) from stock frozen on beads. Gram stains of the first generation cultures were made.

Dilutions of *L. monocytogenes* and *C. maltaromaticum* were prepared in peptone water. Spread plates of *L. monocytogenes* were prepared and left for 30 minutes until all liquid had been absorbed. Dilutions of *C. maltaromaticum* were applied to sterile filter paper discs and placed onto these *L. monocytogenes* plates. The levels of organisms used were determined by plate counts.

Results

Gram stains of both organisms showed Gram-positive rods; *L. monocytogenes* was slightly shorter than *C. maltaromaticum* which measured 1-2µm in length.

C. maltaromaticum was applied at levels of $2.8X10^7$, $2.8X10^4$, and $2.8X10^2$ to the filter papers.

Growth of *L. monocytogenes* was observed on plates incubated at 35°C after 1 day and further growth was seen after 2 days. At 10°C growth of *L. monocytogenes* was noted after 1 week.

No inhibition of *Listeria* was noted on any plates.

Activity of *Lactobacillus plantarum* against *Listeria monocytogenes* strains in laboratory media

Because *L. plantarum* showed appreciable growth at 10°C, further tests were made to determine activity against *L. monocytogenes.*

Method

Two strains of *L. monocytogenes* (Seafood and Massey var.) and *L.plantarum* were grown overnight in BHI broth at 35°C. Both *L. monocytogenes* samples showed vigorous growth. As *L. plantarum* showed poor growth, culture was added directly from the agar plate until the broth looked cloudy. Samples were then cross-streaked onto BHI agar plates and incubated at 35°C and 10°C.

Results

At 35°C both strains of *L. monocytogenes* showed strong growth after one day. Some growth of *L. plantarum* streaks was observed after 3 days but no inhibition was noted. Good growth of *L. plantarum* and both *L. monocytogenes* strains was seen after 6 days with no inhibition.

At 10°C both strains of *L. monocytogenes* showed strong growth after 5 days, but no growth of *L. plantarum* was observed. After 10 days there was still no observed growth of *L. plantarum.*

Activity of cell free extracts from *Lactobacillus reuteri* and *Enterococcus fecium* strains against *Listeria monocytogenes*

Method

Cultures of *L. reuteri* and *E. fecium* were grown anaerobically (2 days at 35°C) in MRS broth, with glycerol (250mM) added to allow *L. reuteri* strains to produce reuterin. The cultures were centrifuged at 4000rpm for 20 minutes and the supernatant was collected for use. Both the seafood *L. monocytogenes* strain and the same strain of *L. monocytogenes* that *Crop* & Food Research Ltd had previously used in research with these organisms were grown overnight at 35°C in BHI broth.

Spread plates using *L. monocytogenes* (0.1mL of 10^{-2} dilution) were prepared and incubated overnight at 37°C until a lawn was just visible. The extracted supernatant was applied as spots directly onto the surface of these plates prior to incubation at 10°C and 35°C.

Results

The pH of extracts was tested and results were between 4 and 5 (most were close to pH 4.5).

No bacteriocidal activity was noted at 35 or 10°C (plates were left for 16 days).

Hence, the extracts did not inhibit either strain of *L. monocytogenes* in the systems used here.

Activity of *Lactobacillus plantarum* against *Listeria monocytogenes* on mussels

Method

Bagged individual quick-frozen (IQF) half-shell mussels were thawed at ambient temperatures immediately before use. The meat surface of each mussel was inoculated by flooding with 2 mL of *L. monocytogenes* (seafood var.) culture of approx 10^4 cfu/mL, diluted with BHI broth, and/or *L. plantarum* culture undiluted. The mussels were left for half an hour to adsorb the inoculum.

A piece of flesh was excised from each of three mussels (surface layer only) of each treatment group, placed into pre-weighed bottles of peptone with a glass bead and vortexed for 1 min. Duplicate plate counts were made to determine the initial level of *L. monocytogenes* (seafood var.) and *L. plantarum* on these samples, using Oxford plates for *L. monocytogenes* and MRS for all other organisms. It was assumed that the majority of other organisms present were *L. plantarum*. The remainder of the mussels were placed into sterile (stomacher) bags and left at 4°C for one week. Samples were taken from the flesh and treated as before.

Because contamination with *Listeria* is associated with the surface, it was decided to remove the surface flesh from the mussels rather than use the more usual technique of stomaching whole mussels. This avoided the dilution of *Listeria* levels by interior surfaces, which are unlikely to be contaminated. The technique was difficult and time consuming and was not used in the subsequent, final experiments.

Results

Levels of *L. monocytogenes* observed after 5 days growth were on average lower in the presence of *L. plantarum* than in its absence (Fig 4.2). However as this was the first trial in a mussel system there were difficulties in applying the culture and some of the *L. monocytogenes* (seafood var.) may have been washed off with the subsequent application of *L. plantarum*. Counts also were outside acceptable counting range in the initial samples.

Therefore it is not appropriate to perform a statistical analysis on these results. They should be viewed as indicative only and contributing to the development of methodology.

Results from the MRS plates are not reported due to the large number of other organisms which grew on these plates, making it impossible to accurately count the numbers of *L. plantarum*.

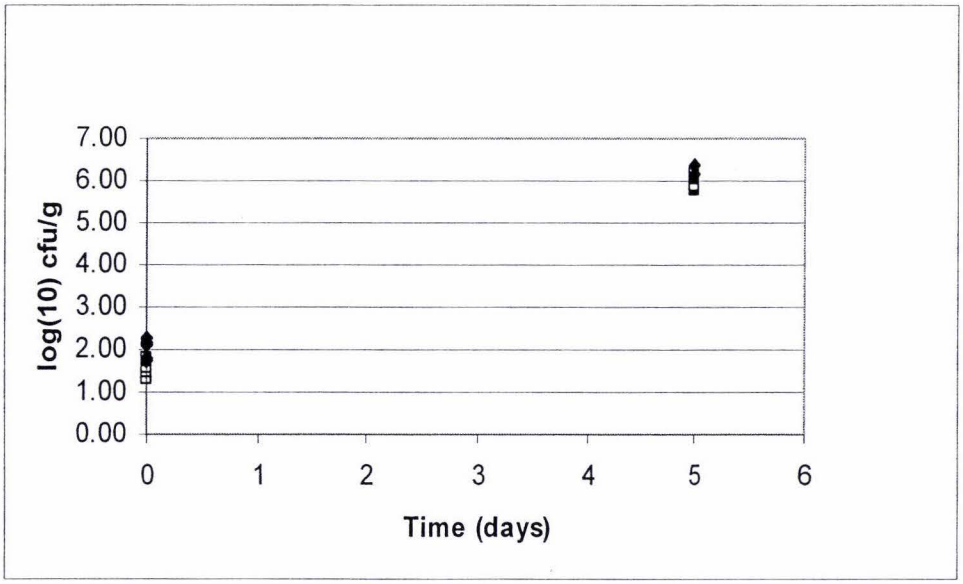


Figure 4.2
Growth of *L. monocytogenes* in mussels in presence and absence of *L. plantarum*

Key: ◆ *L. monocytogenes* grown alone, □ *L. monocytogenes* grown with *L. plantarum.*

Activity of *Carnobacterium piscicola* strains against *Listeria monocytogenes* in a laboratory (agar) system

Method

Active cultures of *L. monocytogenes* (seafood var.) and both strains of *C. piscicola* were prepared in BHI broth and diluted so that *L. monocytogenes* was approximately 10^2 cfu/mL and *C. piscicola* 10^6 cfu/mL . *L. monocytogenes* (seafood var.) alone and *L. monocytogenes* with *C. piscicola* A9b+, and with *C. piscicola* A9b- were plated in duplicate on BHI plates and incubated at 10°C.

After 6 days the agar was transferred from the petri plates into stomacher bags. Peptone was added and the agar was stomached for two minutes. Serial dilutions were made and plated onto Oxford or BHI agar to determine levels of *L. monocytogenes* and *C. piscicola* respectively. Duplicate plates of each sample were prepared.

Results

After six days at 10°C the levels of *L. monocytogenes* were lower when either strain of *C. piscicola* was present. (Figure 4.3 and Table 4.2).

Both strains of *C. piscicola* reached 10^9 cfu/g in six days when grown alone and when grown with *L. monocytogenes*.

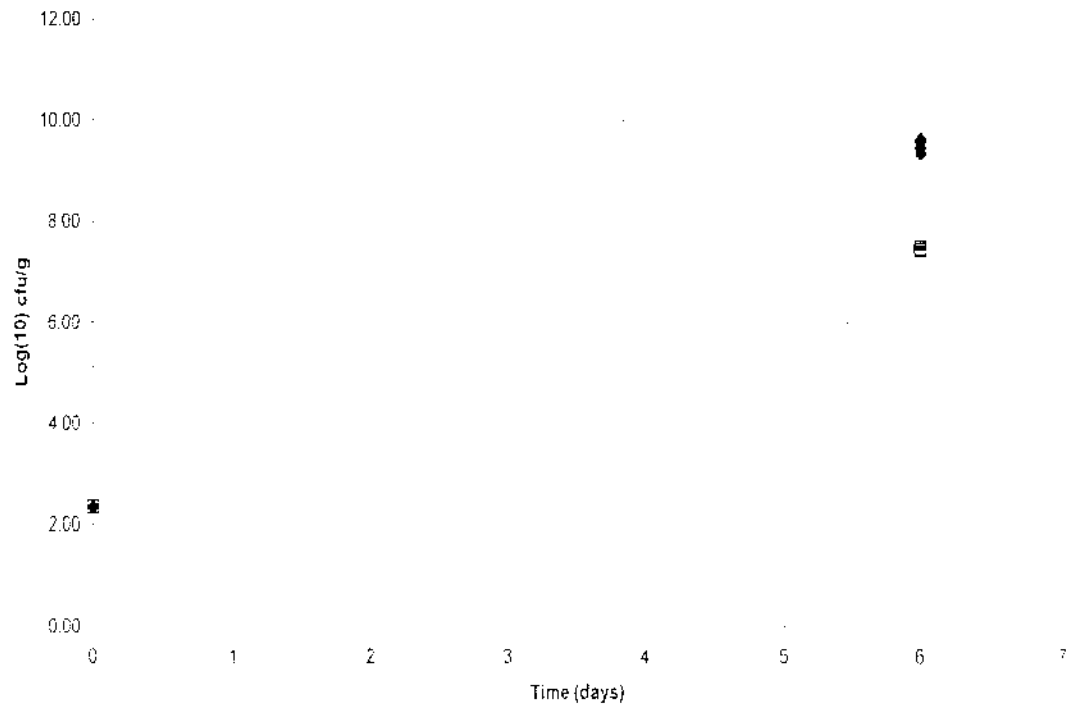


Figure 4.3 Growth of *L. monocytogenes* in BHI agar with and without *C. piscicola* A9b+ and A9b-
Key: ◆ *L. monocytogenes* grown alone, □ *L. monocytogenes* grown with C. piscicola A9b+, – *L. monocytogenes* grown with *C. piscicola* A9b-.

Table 4.2
Extent of inhibition of *L. monocytogenes* by *C. piscicola* A9b- and A9b+ after 6 days in BHI agar at 10°C

	Log reduction in *L. monocytogenes*[a]	P [b]
C. piscicola A9b+	**2.04**	**<0.0001**
C. piscicola A9b-	**2.01**	**N.S[c]**

[a]Log reduction = Log $_{10}$(av of L. monocytogenes grown alone) - Log $_{10}$(av of L. monocytogenes when grown with C. piscicola).

[b]P values determined by student t-test 4 observations, one tailed, unequal variances.

[c] N.S = Not significant

These results indicate that *C. piscicola* Ab+ has inhibited *L. monocytogenes* in the laboratory system. *C. piscicola* A9b- may have inhibited *L. monocytogenes.*

Activity of *Carnobacterium piscicola* against *Listeria monocytogenes* on mussels

Method

L. monocytogenes (seafood var.) and both *C. piscicola* strains A9b+ and A9b- were grown in BHI broth overnight at 37°C.

Cultures of approx 10^5 cfu/mL *L. monocytogenes* and 10^7 cfu/mL of each *C. piscicola* strain were prepared in BHI broth. Mussels were thawed and inoculated as before (Page 46) and left for 6 days at 10°C.

A piece of flesh was excised from each of 3 mussels at each treatment and vortexed as previously described. Cultures were diluted and *L. monocytogenes* plated to Oxford plates. Three replicates of each plate at each dilution were prepared.

Results

Technical problems prevented initial counts being made.

After 6 days at 10°C *L. monocytogenes* counts were lower when grown as a co-culture with either *C. piscicola* strain than when grown alone (Table 4.3).

Table 4.3 Extent of inhibition of *L. monocytogenes* by *C. piscicola* A9b- and Ab+ in mussels at 10°C

Organism	Time (days)	Log reduction in *L. monocytogenes*[a]	P [b]
C. piscicola Ab+	6	**0.46**	**0.0001**[b]
C. piscicola Ab-	6	**0.47**	**ND**[c]

[a] Log reduction =
Log $_{10}$(av of L. monocytogenes grown alone) - Log $_{10}$ (av of L. monocytogenes when grown with C. piscicola).

[b] P values determined by student t-test 4 observations, one tailed, unequal variances.

[c] Insufficient data points for determination of statistical significance.

These results indicate that *C. piscicola A9b+* inhibited *Listeria* growth in mussels while the A9b- strain may also have done so.

Activity of *Carnobacterium piscicola* A9b- against *Listeria monocytogenes* in a mussel system

It appeared that both *Carnobacterium* strains were capable of inhibiting *L. monocytogenes* in mussels. As there were only a small number of results for *C. piscicola* A9b-, it was decided to do further preliminary tests on this strain before the final experiments were conducted with this organism.

Method

Active cultures of *C. piscicola* A9b- and *L. monocytogenes* (seafood var.) were prepared as described previously.

Mussels were thawed as before and drained of any free liquid before inoculation by flooding as before, with *L. monocytogenes* or *L. monocytogenes* mixed with *C. piscicola*. These were packed into sterile stomacher bags and incubated at 10°C for 5 days.

Flesh was excised from mussels and treated as before. Five mussels at each treatment were sampled. *L. monocytogenes* levels were determined by plating onto Oxford agar in triplicate.

Results

After 5 days the growth of *L. monocytogenes* was significantly reduced by the presence of *C. piscicola*. (See Tables 4.4 and Figure 4.4)

The initial count of *L. monocytogenes* was approximately 10^4 cfu/g mussel flesh. The level of *C. piscicola* was not be measured.

Table 4.4 Extent of inhibition of *L. monocytogenes* by *C. piscicola* A9b- in mussels at 10°C

Time (days)	Log reduction in *L. monocytogenes*[a]	P [b]
5	1.21	<0.0001

[a]Log reduction = Log $_{10}$(av of L. monocytogenes grown alone) - Log $_{10}$ (av of L. monocytogenes when grown with C. piscicola).

[b] P values determined by student t-test 10 observations, one tailed, unequal variances.

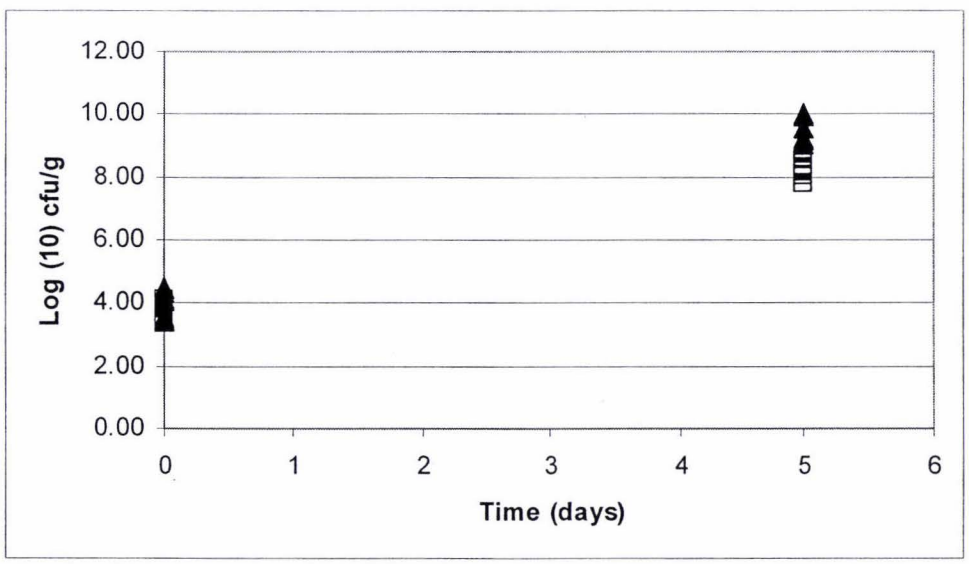


Figure 4.4 Growth of *L. monocytogenes* on mussels in the presence and absence of *C. piscicola* A9b-
Key: ▲ *L. monocytogenes* grown alone, □ *L. monocytogenes* grown with *C. piscicola* A9b-.

4.3 Discussion

4.3.1 Selection of target organism

The seafood strain of *L. monocytogenes* seemed to be more robust than other strains as it grew well in the "semi-selective" medium used by Nilsson et al. (1999). Organisms that colonise seafood premises are likely to well adapted to harsh conditions and tolerant to the chemicals used in sanitizers and well adapted to low temperature growth. Hence they may be more robust than lab strains as they are able to resist environmental stressors (Begot et al., 1997).

4.3.2 Selection of antagonist

A large number of strains (17) were obtained trialled but few were suitable for this application.

Carnobacterium maltaromaticum

This organism was chosen for testing because the literature indicated it may be suitable. The strain tested did not show any activity against the *L. monocytogenes* strain used as the target organism.

Schillinger (1990) reported that this strain of *C. maltaromaticum* showed no activity against tested strains of *L. monocytogenes*, so it was decided not to use this organism in the present work. This paper was not found until after the organism was tested.

Lactobacillus plantarum

The Danisco Holdbac™ product appeared to show effective inhibition of the *L. monocytogenes* strains tested in mussel products.

It was not possible to determine if this strain of *L.plantarum* shows activity against *L. monocytogenes* in laboratory media as no suitable medium was found that would allow co-culture of both organisms.

Lactobacillus reuteri and *Enterococcus fecium* strains

Although these organisms have shown promise in other laboratories (*Crop &* Food Research Ltd) their use was limited, as they did not grow well in the cold under the conditions used here. These organisms were therefore discounted.

It is possible that cold adaptation may be a mechanism for obtaining the higher growth rates needed to provide control against *L. monocytogenes*. Studies of *E. fecium* pre-incubated in cold conditions have shown enhanced growth in these conditions and greater freeze-thaw stability than organisms grown in warmer conditions. Increasing the length of time of cold-incubation gave a greater degree of adaptation (Thammavongs et al., 1996). However for these strains of *L. reuteri* and for *E. fecium*, there was no evidence of growth at 10°C after pre-incubation at this temperature. Further work would need to be done to determine if cold adaptation was possible.

The pH of the extracts had dropped during the growth of the strains but it was not expected to exert an effect, as there would be a large dilution when applied to the agar.

The *L. reuteri* strains had been shown to produce compounds with anti-listerial activity in *Crop &* Food Research Ltd laboratories, but this was not demonstrated here. Strain variability may account for the lack of response seen with the seafood strain of *L. monocytogenes*, but the *Crop &* Food var. also was not inhibited.

The methods used for this experiment differed from those used by *Crop &* Food Research Ltd (Lu, Personal Communication) where *L. monocytogenes* was added to molten agar (48°C), 4mm wells were drilled, 40μL extract was added and the plates were left at room temperature (approximately 25°C) for 12 hours. The type of agar used was not specified.

The different results may be simply due to the level of anti-listerial compounds being too low to be effective under the experimental conditions used. The type of

agar may make a difference to the growth characteristics and susceptibility of *L. monocytogenes.* Studies indicate lag times of *L. monocytogenes* varied from 4 hours to 4 days under different growth conditions (Begot et al. 1997).

Carnobacterium piscicola strains Ab+ and Ab-

These strains were known to grow in cold conditions from the literature; hence they were not specifically tested but demonstrated the ability to grow well in the cold conditions used in these preliminary experiments. As both of these strains also showed activity against *L. monocytogenes* in the preliminary experiments, they were used for further investigations.

C. piscicola A9b- demonstrated the ability to withstand freezing and thawing and the numbers had in fact increased after freezing. This could have occurred during the cooling process before freezing occurred and also in the thawing process.

Both strains of *C. piscicola* demonstrated the ability to inhibit *L. monocytogenes* in solid media, although strain *C. piscicola* A9b+ was not studied in detail.

Carnobacterium piscicola A9b- was selected for the detailed studies planned in the final experiments for a number of reasons including the following:

- It is capable of strong growth in cold conditions.
- It was possible to co-culture with *Listeria* in easily obtainable laboratory media.
- It had demonstrated potential to suppress the growth of wild type *Listeria* obtained from the seafood industry in mussels and laboratory media in preliminary trials.
- It does not produce a bacteriocin, therefore resistance of target organisms is less likely to be an issue.

Chapter 5

Determination of antagonistic effects and growth rate

5.1 Preparation for determination of antagonistic effects and growth rate

5.2 Determination of antagonistic effects

5.3 Discussion of results

5 Determination of antagonistic effects and growth rate

5.1 Preparation for determination of antagonistic effects and growth rate

5.1.1 Confirmation of purity of strains

Method

Active cultures of *C. piscicola* A9b- and *L. monocytogenes* (seafood var.) were grown and plated to BHI agar. Single colonies were isolated and plates were streaked for single colony isolation. This process was repeated 2 further times then these colonies used for confirmation of purity (i.e. 3rd generation colonies were used).

To check the overall physical characteristics of the organisms, gram stains were made of both cultures directly off plates and from broth cultures before freezing.

Confirmation of *Listeria* to genus level was carried out using Merck Single Path Listeria Gold Labelled Immnunosorbent Assay (GLISA) rapid test kit (MSD, Newmarket, Auckland).

Confirmation of *L. monocytogenes* to species level was done by selected biochemical reactions; fermentation of mannitol and xylose using carbohydrate fermentation broth tubes (Collins, 1984) and β-haemolysis of blood agar.

Full confirmation of the strain of *C. piscicola* was not possible, as this would require genetic determination methods which were not available and were outside the scope of this work. However the following tests were carried out to confirm characteristics as far as possible:

- Fermentation of mannitol using sugar-broths
- Growth in acetate agar at pH 5.4 (MRS was used)
- Growth at pH 9 (BHI was made and pH was adjusted using NaOH to pH 9).

Results

Results are summarised in Table 5.1. These results indicate that the organisms were true to type. The anomalies in the Gram-stain reaction are not uncommon and are considered in more detail later (Section 5.7).

Table 5.1. Confirmatory tests for *L. monocytogenes* and *C. piscicola*

	***L. monocytogenes* (seafood var.)**	***C. piscicola* A9b-**
Gram-stain	G +ve and G-ve very short rods <1.0μm on agar and in broth	Some G +ve and predominantly G-ve short rods 1- 2 μm on agar G+ve rods 2-3 μm in pH 9 broth
GLISA *Listeria* test	+ve	-ve
Mannitol fermentation	-ve	+ve
Xylose fermentation	-ve	Not tested
β-haemolysis	+ve	Not tested
Growth in acetate agar	Not tested	No growth
Growth at pH 9	Not tested	Growth

5.1.2 Trial of OD method to determine initial levels of organisms in broths

Method

Buffered BHI was used to grow active cultures of *L. monocytogenes* (seafood var.) and *C. piscicola* A9b-. OD measurements at 600nm were taken at various concentrations diluted in B-BHI broth. These were compared with standard plate counts on BHI agar to enable a standard curve to be plotted. Two runs were completed to determine consistency. Plates were incubated at 35°C for 2 days.

Dilutions were also plated to Oxford and N-P agar. N-P plates were incubated at 35°C and at 25°C for 2 days. 25°C plates were also examined after 3 days.

Results

There was no growth of *C. piscicola* A9b- on Oxford agar. Both organisms grew well on N-P agar at both temperatures and at similar rates. Colonies were visually very similar, off-white, convex and of similar size.

Viable counts of organisms grown on BHI agar were used for the calculation of standard curves (see Figures 5.1 and 5.2). Results of both trials are combined.

Some differences were noted between runs but these were not seen as sufficient to affect the full trial, as these were less than one log value where measurements were made.

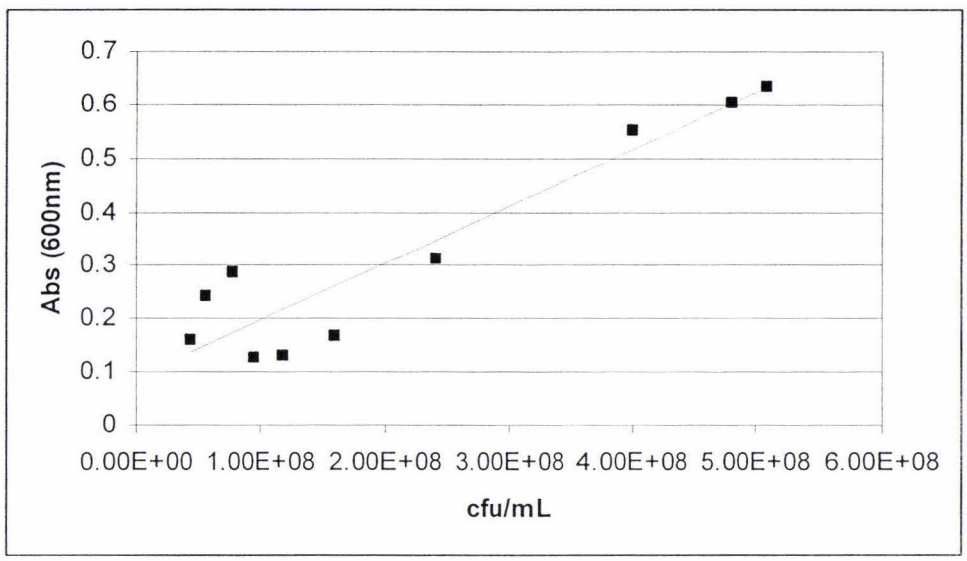


Figure 5.1. Standard curve of OD vs cfu/mL for *C. piscicola* A9b-

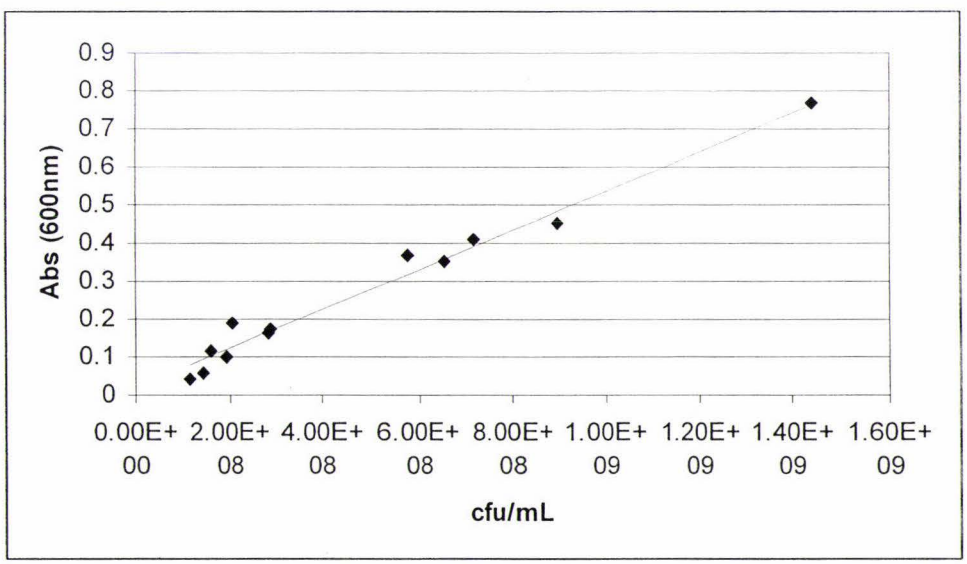


Figure 5.2. Standard curve of OD vs cfu/mL for *L. monocytogenes*

5.1.3 Selection of a selective agar for *C. piscicola*

A selective medium was necessary to facilitate the enumeration of *C. piscicola* in the presence of *L. monocytogenes*.

Method

Initially the NP agar used by other workers was trialled. However, this was not sufficiently selective, so other media were prepared and evaluated.

Table 5.2. Solid media tested to identify a selective medium for *C. piscicola* in the presence of *L. monocytogenes*.

Agar type	Reason for selection
N-P agar	Original agar selected based on literature (Nilsson et al., 2004).
Modified N-P agar	Mannitol was used instead of glucose as *C. piscicola* ferments mannitol.
Modified N-P-BCP agar	Bromocresol purple indicator was added in order for pH changes due to growth to be seen more clearly.
MRS	Tested as a common method for growing LAB (Hammes et al., 1991).
D-MRS	Use of higher pH to select for *Carnobacteria* spp (Hammes et al., 1991).
PRMA	Agar was based on reagents used for carbohydrate fermentation tests and solidified. The added indicator enabled changes to be seen more easily.

The pH was measured before plating for agar types as shown in Table 5.3 below.

Results

Results are summarised in Table 5.3.

Table 5.3.
Growth of *C. piscicola* and *L. monocytogenes* on different agars after 2 days @ 35°C or 25°C

	MRS pH 5.4	D-MRS pH 9.0	NP 25°C pH 6.9	NP 35°C pH 6.9	M-NP	MNPBCP	PRMA pH 7.3
C. piscicola A9b-	-	-	++	++	++	++	++
L. monocytogenes	-	++	++	++	++	++	+

Key: - no growth + slight growth ++ moderate growth

L. monocytogenes did not grow in unmodified MRS as demonstrated previously so it was not expected here.

Growth characteristics in D-MRS were unexpected this medium had been used by workers (Hammes et al., 1991), who suggested this media for the growth of *Carnobacterium* species. However this medium is not commonly seen in references pertaining specifically to the growth of *C. piscicola* so species and strain differences may exist.

L. monocytogenes (seafood var.) also showed significant growth on N-P agar and on both types of modified N-P agar. It was impossible to visually distinguish from *C. piscicola* colonies.

Gram stains were done on these colonies. Both showed Gram-variable characteristics. The cells from *C. piscicola* were slightly larger, at 1-2 µm than *L. monocytogenes* at up to 1 µm.

Strong growth of *C. piscicola* was observed on PRMA plates, while there was only slight growth of *L.monocytogenes*.

C. piscicola colonies were slightly convex, smooth, yellow about 1mm in diameter. Colonies of *L.monocytogenes* were smaller, pale and did not show yellow colouration.

PRMA plates were used for subsequent experiments (see figure 5.3 and 5.4). Mannitol was readily fermented by *Carnobacteria* but not by *Listeria spp.* The phenol red indicator enabled this fermentation to be very easily seen and gave a distinct yellow colouration.

Colony characteristics can be seen in Figure 5.4 below.

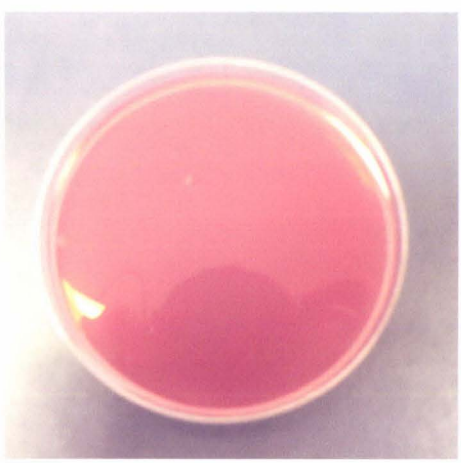


Figure 5.3. Uninoculated PRMA plate showing red-coloration.

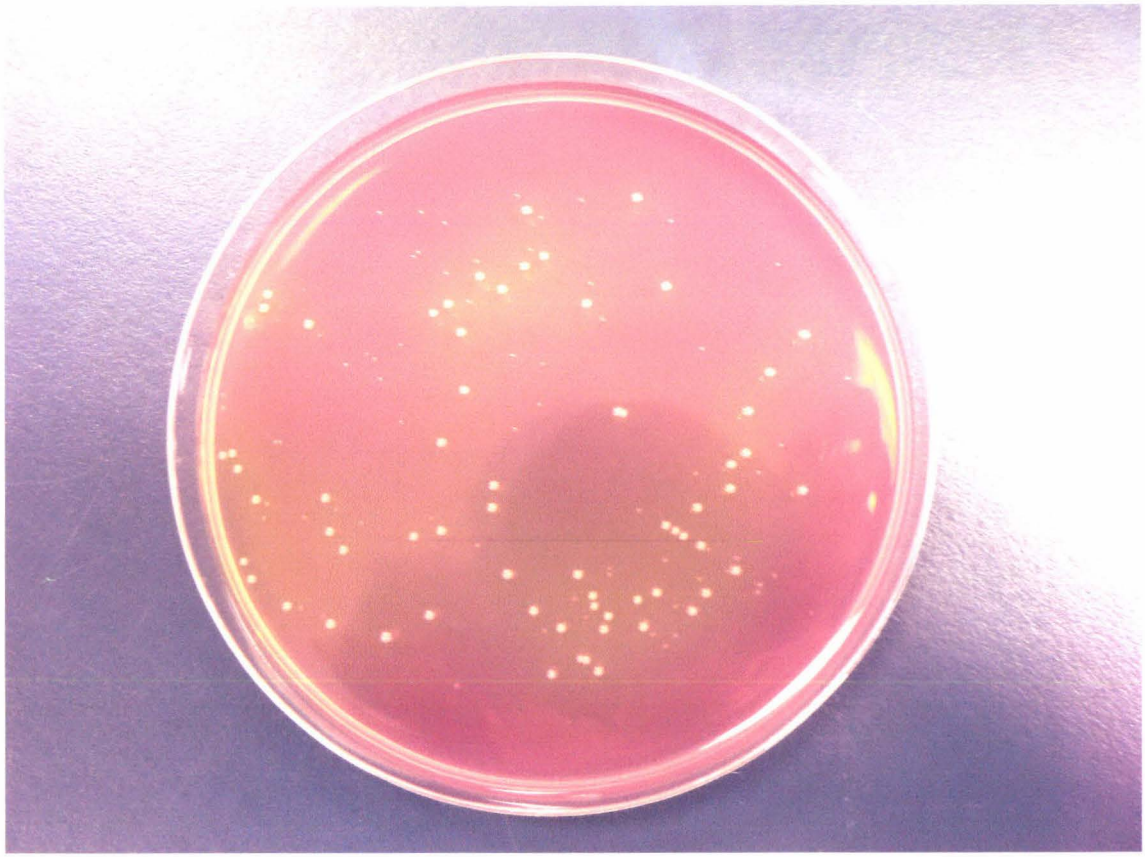


Figure. 5.4
C. piscicola colonies are seen as larger colonies on yellow background, while *L. monocytogenes* are seen as very faint small colonies generally with no change in background colouration except where growing adjacent to *C. piscicola* colonies.

5.1.4 Confirmation of inhibition of *Listeria monocytogenes* by *Carnobacterium piscicola* A9b- in a broth culture.

Method

Buffered BHI was used to grow active cultures of *L. monocytogenes* (seafood var.) and *C. piscicola* A9b-. OD measurements were made and organisms were diluted in B-BHI broths so that the final concentration of *L. monocytogenes* was approximately 10^2 cfu/mL and *C. piscicola* A9b- approximately 10^7 cfu/mL. Broths of *L. monocytogenes* alone, *C. piscicola* alone, and a mixed broth were placed on an orbital shaker and incubated at 10°C. Samples were incubated for 0, 1, 2 and 4 days before being plated in duplicate to Oxford agar and PRMA to determine levels of *L. monocytogenes* and *C. piscicola* A9b- respectively.

Table 5.4 Growth of *L. monocytogenes* and *C. piscicola* A9b- in broth at 10°C

Time (days)	Mean cfu/mL *C. piscicola*	Mean cfu/mL *L. monocytogenes*	Mean cfu/mL *C. piscicola* + *L. monocytogenes* together	
			C. piscicola	*L. monocytogenes*
0	1.15×10^7	1.85×10^4	1.15×10^7	1.85×10^4
1	1.15×10^9	$>4.12 \times 10^4$	8.35×10^8	3.27×10^4
2	1.10×10^9	Not able to determine*	2.70×10^9	2.58×10^3
4	6.30×10^8	2.55×10^8	$<5.10 \times 10^8$	Not able to determine*

* No plates at a suitable dilution to determine numbers.

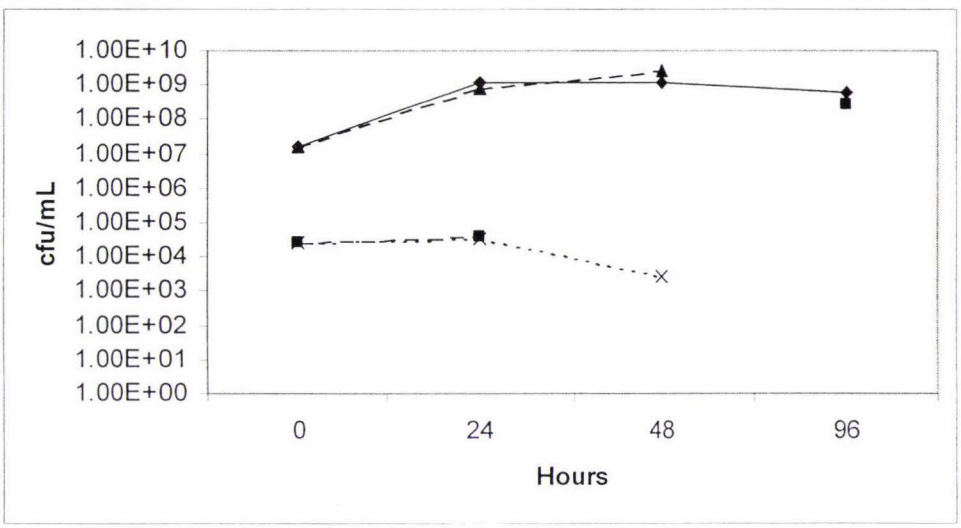


Figure 5.5. Growth of *C.piscicola* and *L.monocytogenes* with time when grown separately and together in broth at 10°C

Shown is *C. piscicola* only (cfu/mL) ◆, *L. monocytogenes* only (cfu/mL) ■, *L. monocytogenes* (cfu/mL) grown with *C. piscicola* ▲ , *C. piscicola* (cfu/mL) grown with *L. monocytogenes* x.

5.2 Determination of antagonistic effects

5.2.1 Growth rate of *Listeria monocytogenes* and *Carnobacterium piscicola* in broth

These final experiments were performed so that sufficient data would be collected to enable the quantification of the antagonistic effects of *C. piscicola* on *L. monocytogenes*. The experiments were performed in broth, on agar, and on mussels.

Method

Active cultures of *L. monocytogenes* (seafood var.) and *C. piscicola* A9b- were grown in B-BHI broth. Broths were made of *L. monocytogenes* alone, *L. monocytogenes* with *C. piscicola* A9b-, and *C. piscicola* A9b- alone. The broths were diluted so that the final concentration of *L. monocytogenes* was approximately 10^2cfu/mL and *C. piscicola* A9b- was approximately 10^6 cfu/mL. One mL of each type of broth was placed into a series of disposable tubes and incubated on an orbital shaker at 10°C. At appropriate time intervals, samples were removed from each treatment and concentrations of *L. monocytogenes* and *C. piscicola* A9b- were measured by conducting total viable counts, using Oxford agar and PRMA respectively. Peptone water was used as diluent. Times of sampling *C. piscicola* were based on interpolation of data from Nilsson et al. (2004), at different temperatures, and *L. monocytogenes* from the Pathogen Modelling Program (USA, ARS, NAA, ERRC, MFSRU PMP 7.0). Plates were incubated for 2 days at 35°C. Three samples were taken at each time period and counts were performed in triplicate.

Results

The *C. piscicola* concentration was slightly higher than intended due to the inaccuracy of the O.D. measurements. The concentration of *C. piscicola* increased from 10^7 cfu/mL, to a maximum of 10^9 cfu/mL within 15 hours, when grown alone and also when grown with *L. monocytogenes*. The *L. monocytogenes* concentration increased to a maximum of over 10^8 cfu/mL within 41 hours when grown alone. When grown with *C. piscicola*, this organism did not reach its maximum concentration of over 10^8 cfu/mL until after 140 hours (Figure 5.6).

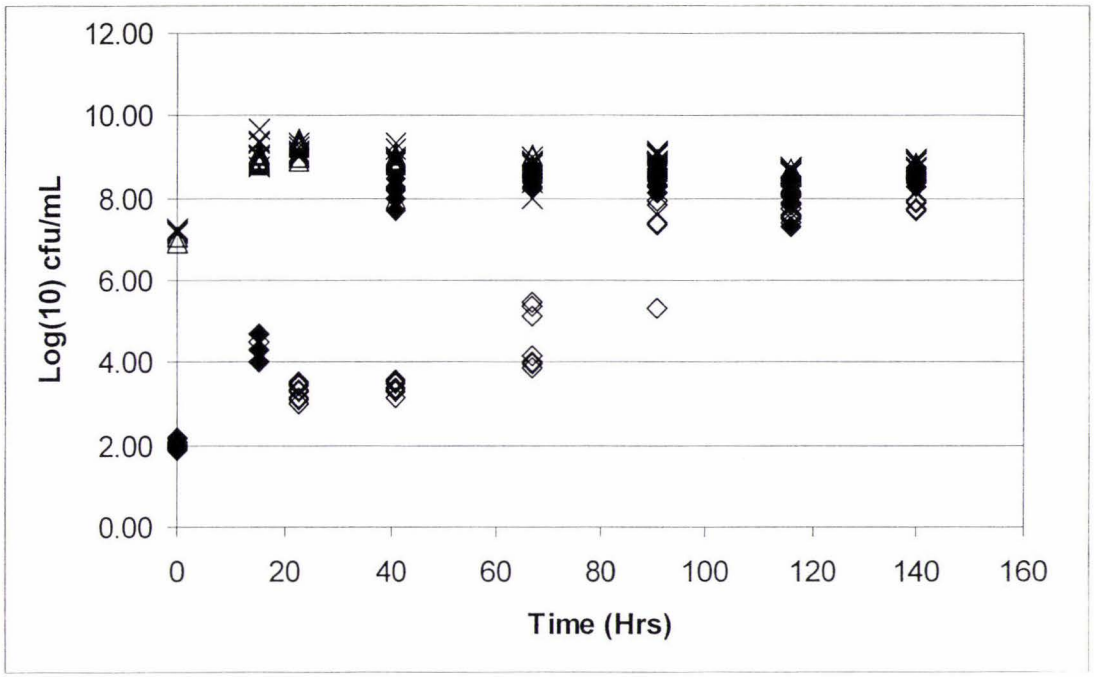


Figure 5.6. Counts of *C. piscicola* and *L. monocytogenes* when grown separately and together in B-BHI broth at 10°C

Shown is: ◆ Log $_{10}$ *L. monocytogenes* (cfu/mL), × Log $_{10}$ C. *piscicola* (cfu/mL), ◇ Log $_{10}$ L. *monocytogenes* grown in co-culture with *C. piscicola* (cfu/mL), and △ Log $_{10}$ *C. piscicola* grown in this co-culture with *L. monocytogenes* (cfu/mL).

There seemed to be no adverse effect of *L. monocytogenes* on the growth of *C. piscicola* under these experimental conditions.

The growth rates of *L. monocytogenes* grown alone and *L. monocytogenes* grown in co-culture with *C. piscicola* were compared and a linear regression analysis was conducted using Minitab 14. Although a curved line would have been more accurate, a linear fit was used for simplicity and calculations were made omitting the values at either end where the data did not show a straight line relationship.

The growth rate of *L. monocytogenes* (seafood) was significantly reduced by *C. piscicola.* (P=0.018).

Numbers measured at various time intervals were compared. *C. piscicola* significantly suppressed the numbers of *L. monocytogenes* at different time intervals as shown in table 5.5.

Table 5.5. Extent of inhibition of *L. monocytogenes* by *C. piscicola* A9b- in B-BHI broth at 10°C

Time (hrs)	Log reduction in *L. monocytogenes* [a]	P[b]
41	4.78	<0.0001
67	4.03	<0.0001
91	1.67	0.0027
116	0.40	0.0048
141	0.68	<0.0001

[a] Log reduction = Log 10(av of *L. monocytogenes* grown alone) - Log 10 (av of *L. monocytogenes* when grown with *C. piscicola*).

[b] P values determined by student t-test, one-tailed, unequal variances.

During the course of the trial the broth temperature varied between 10°C and 14°C and the pH of the broth fluctuated within 0.2 pH units as shown below (Figure 5.7).

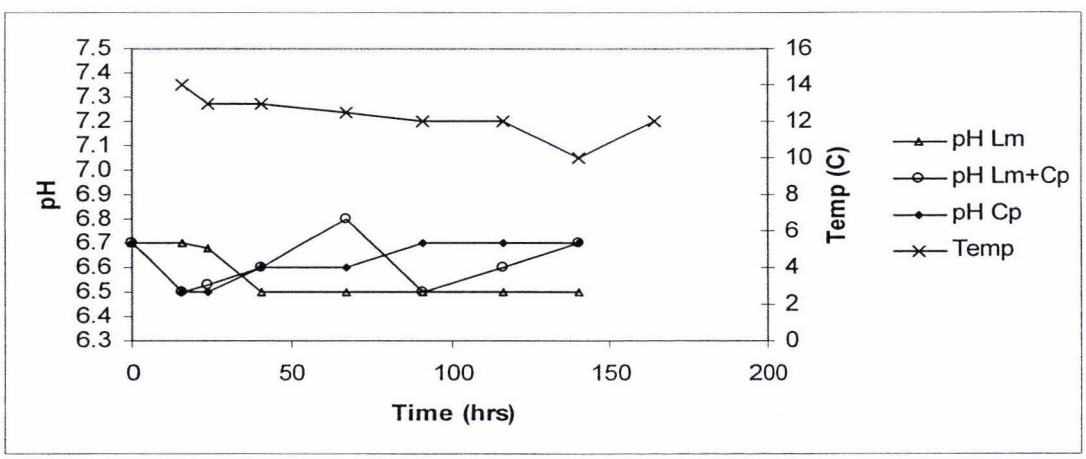


Figure 5.7. Graph of pH and temperature variation during broth trial

5.2.2 Growth rate of *Listeria monocytogenes* and *Carnobacterium piscicola* in agar

Method

Active cultures were grown and diluted to give final concentrations of approximately 10^2 cfu/mL for *L. monocytogenes* and 10^6 cfu/mL for *C. piscicola* based on OD measurements as before, then plated to 3 series of BHI spread-plates (*L. monocytogenes* alone, *L. monocytogenes* with *C. piscicola* A9b- and *C. piscicola* A9b- alone). These plates were incubated at 10°C. At specified times three plates were removed from each treatment for determination of viable counts of *L. monocytogenes* and *C. piscicola* in triplicate. The agar from the plates was aseptically transferred to stomacher bags and peptone water was added prior to stomaching for 1.5 min. Samples were then diluted as before and plated in triplicate to PRMA or Oxford agar for the determination of *C. piscicola* or *L. monocytogenes*.

Results

As for the broth trial, there seemed to be no adverse effect of *L. monocytogenes* on the growth *of C. piscicola*. The count of *L. monocytogenes* when grown alone rose to a maximum by 80 hours of incubation, compared to half this time in broth. When present in the mixture, the count of *L. monocytogenes* rose more slowly and did not reach the same level as when grown alone, even after 160 hours of incubation (Fig 5.8).

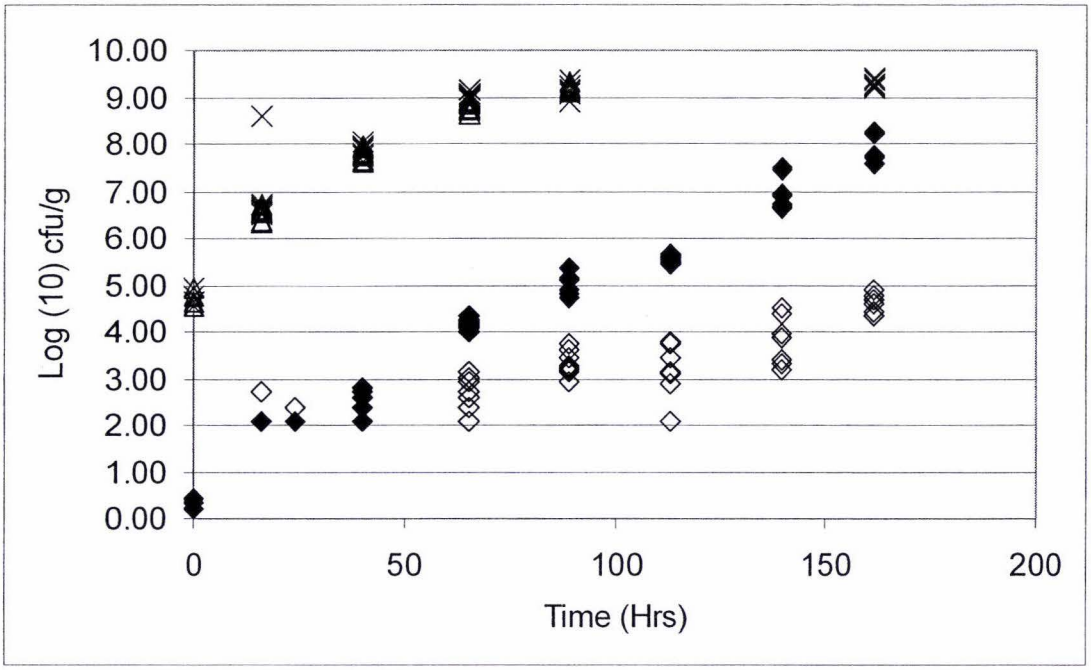


Figure 5.8. Change of concentrations of *C. piscicola* and *L. monocytogenes* with time when grown separately and together in BHI agar at 10°C

Shown is: ◆ Log $_{10}$ *L. monocytogenes* (cfu/g), × Log $_{10}$ C. *piscicola* (cfu/g), ◇ Log $_{10}$ L. *monocytogenes* grown in co-culture with *C. piscicola* (cfu/g), and △ Log $_{10}$ *C. piscicola* grown in this co-culture with *L. monocytogenes* (cfu/g).

A regression analysis on growth rates of *L. monocytogenes* grown alone and grown with *C. piscicola* was done as previously described.

The growth rate of *L. monocytogenes* (seafood) was significantly reduced by *C. piscicola.* (P=<0.001).

Numbers measured at various time intervals were compared. *C. piscicola* demonstrated significant reduction in the numbers of *L. monocytogenes* when measured at 65 hours and beyond (Table 5.6).

Table 5.6. Extent of inhibition of *L. monocytogenes* by *C. piscicola* A9b- in BHI agar at 10°C

Time (hrs)	Log $_{10}$ reduction in *L. monocytogenes*[a]	P[b]
40	0.04	0.33
65	1.54	<0.0001
89	1.73	<0.0001
113	2.30	<0.0001
139	3.13	<0.0001
161.5	3.35	<0.0001

[a] Log reduction = Log $_{10}$(av of L. *monocytogenes* grown alone) - Log $_{10}$ (av of *L. monocytogenes* when grown with *C. piscicola*).

[b] P values determined by student t-test, one-tailed, unequal variances.

5.2.3 Growth of *Listeria monocytogenes* and *Carnobacterium piscicola* in mussels

Method

Actively growing cultures of *L. monocytogenes* (seafood var.) and *C. piscicola* A9b-were prepared in B-BHI and approximate numbers determined by OD measurement as before. Dilutions in B-BHI broth were made to give final concentrations of 10^3 cfu/mL (*L. monocytogenes*) and 10^7 (*C. piscicola*) in B-BHI broth. Two broths were made up, one of equal volumes of *L. monocytogenes* (seafood var.) culture and uninoculated B-BHI, and one of equal volumes of *L. monocytogenes* and *C. piscicola* cultures. Frozen, shucked mussels with shell completely removed were thawed overnight at 4°C. These were then fully immersed in the broths for 15 minutes before draining on plastic mesh for 2 minutes on each face. Mussels were then aseptically transferred to stomacher bags and incubated at 10°C.

Three stomacher bags from each treatment were removed and stomached for 2 minutes after the addition of peptone.

Counts of *L. monocytogenes* were measured in triplicate as described in sections 5.1 & 5.2.

Results

C. piscicola could not be enumerated due to the numbers of other organisms which also grew on the PRMA plates. Growth of colonies with similar characteristics to *C. piscicola* was seen on PRMA. An example can be seen in Figure 5.9. No *C. piscicola* had been added to this sample.

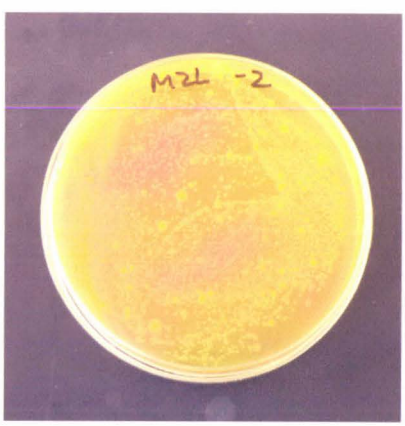


Figure 5.9
Growth of colonies from mussels on PRMA. Note presence of mixed flora including many colonies with similar appearance to *C. piscicola*.

The growth of *L. monocytogenes* was significantly reduced in the presence of *C. piscicola* (Figure 5.10).

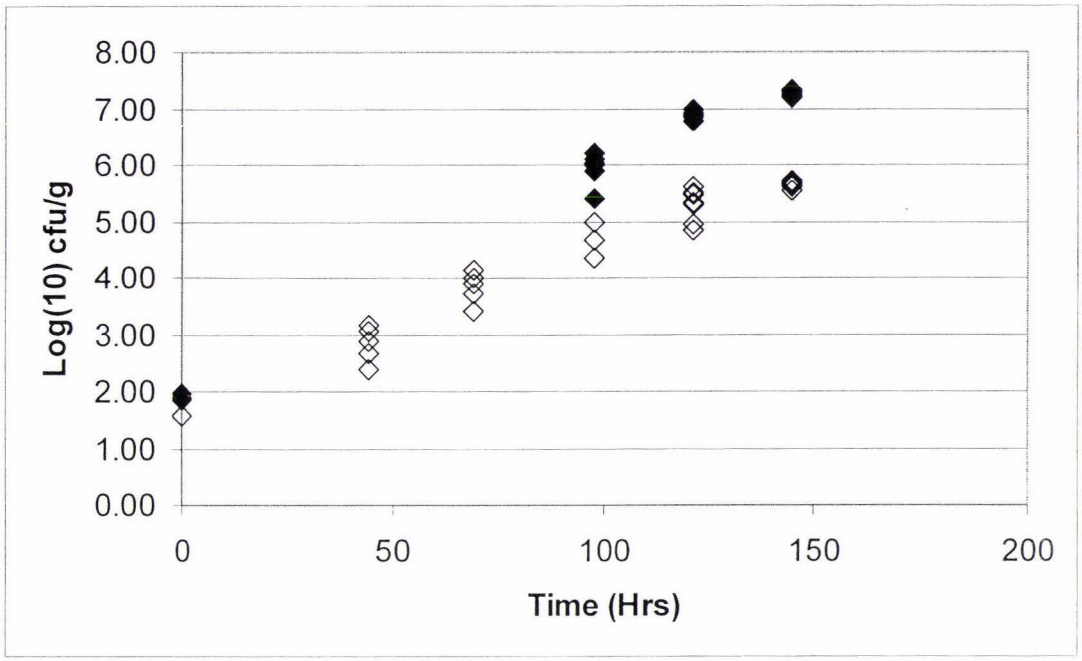


Figure 5.10 Change of concentration of *L. monocytogenes* with time when grown on mussels in the presence and absence of *C. piscicola*

Shown is: ◆ Log $_{10}$ *L. monocytogenes* (cfu/g), ◇ Log $_{10}$ L. *monocytogenes* grown in co-culture with *C. piscicola* (cfu/g).

As the growth of *L. monocytogenes* alone was slower than expected the dilutions 44.5 and 67.5 hours were too high for measurements to be made.

A regression analysis on growth rates of *L. monocytogenes* grown alone and grown with *C. piscicola* was done as previously described.

The growth rate of *L. monocytogenes* (seafood) was significantly reduced by *C. piscicola.* (P=<0.001).

Again, numbers measured at various time intervals were compared. *C. piscicola* demonstrated significant reduction in the numbers of *L. monocytogenes* when measured at 97.5 hours and beyond (Table 5.7). There were insufficient measurements of *L. monocytogenes* only to determine reduction in *L. monocytogenes* at earlier times.

Table 5.7 Extent of inhibition of *L. monocytogenes* by *C. piscicola* A9b- on mussels at 10°C

Time	Log $_{10}$ reduction in *L. monocytogenes*[a]	P[b]
97.5	1.32	0.0001
121	1.57	<0.0001
145	1.59	<0.0001

[a] Log reduction = Log 10(av of L. *monocytogenes* grown alone) - Log 10 (av of *L. monocytogenes* when grown with *C. piscicola*).

[b] P values determined by student t-test, one-tailed, unequal variances.

5.2.4 Comparison of agar, broth and mussel systems

The results for the three different systems can be seen in Figure 5.11. Initial loadings of *C. piscicola* and of *L. monocytogenes* are shown in Table 5.8. These values are considered to be approximate as the extraction rate of organisms from the solid systems is not known.

For comparison of systems slopes of graphs should be considered rather than actual values. This is discussed in section 5.3.

Figure 5.11 Reduction in *L. monocytogenes* (shown as Log$_{10}$ values) when grown as a co-culture with *C. piscicola* A9b- for broth, agar and mussel systems

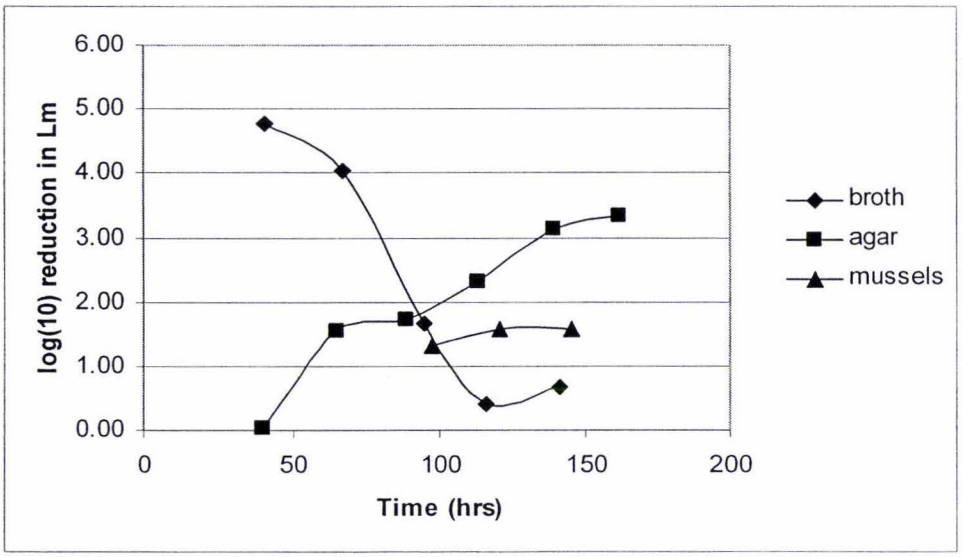


Table 5.8 Approximate initial concentrations of organisms used in the systems tested

System	*L. monocytogenes*	*C. piscicola*
Broth	1.0 $X10^2$ cfu/mL	1.0 X 10^7
Agar	2.4 cfu/g	1.9 $X10^6$
Mussel	0.8 $X10^2$ cfu/g*	2.2 $X10^{6*}$

*Values are determined from actual counts extracted from mussels after stomaching in peptone. Levels of *C. piscicola* have been taken from the freeze-thaw stability trial where organisms have been applied to autoclaved mussels.

The broth system showed the largest degree of inhibition at 41 hours but the effect diminished more rapidly than for the agar and mussel systems. Inhibition in the mussel system was less than the agar system.

5.2.5 Determination of concentration effect

The concentrations of organisms used previously were based on other published studies and personal communication from Dr Lone Gram. As mentioned previously (section 3) any observed inhibitory effects may have been due only to the very large difference in cell concentrations rather than to intrinsic characteristics of the *C. piscicola* strain. For this reason, an experiment was performed in broth using lower initial counts of *C. piscicola*.

Method

Broths of *L. monocytogenes* (seafood var.) and *C. piscicola* A9b- were set up as described in section 5.2.1, but the concentration of *C. piscicola* used was reduced to approximately 10^4 cfu/mL, and treatments were kept in bulk instead of being placed in individual tubes. Three measurements only were carried out for each treatment at each time interval.

Results

Although the initial growth of *L. monocytogenes*, for the first 40 hours, was not inhibited by *C. piscicola*, the growth rate was considerably slowed after this period when the concentration of *C. piscicola* had reached high levels (>10^8 cfu/mL).

A linear regression analysis was conducted showing, the growth rate of *L. monocytogenes* (seafood) was significantly reduced by *C. piscicola.* (P=0.015)

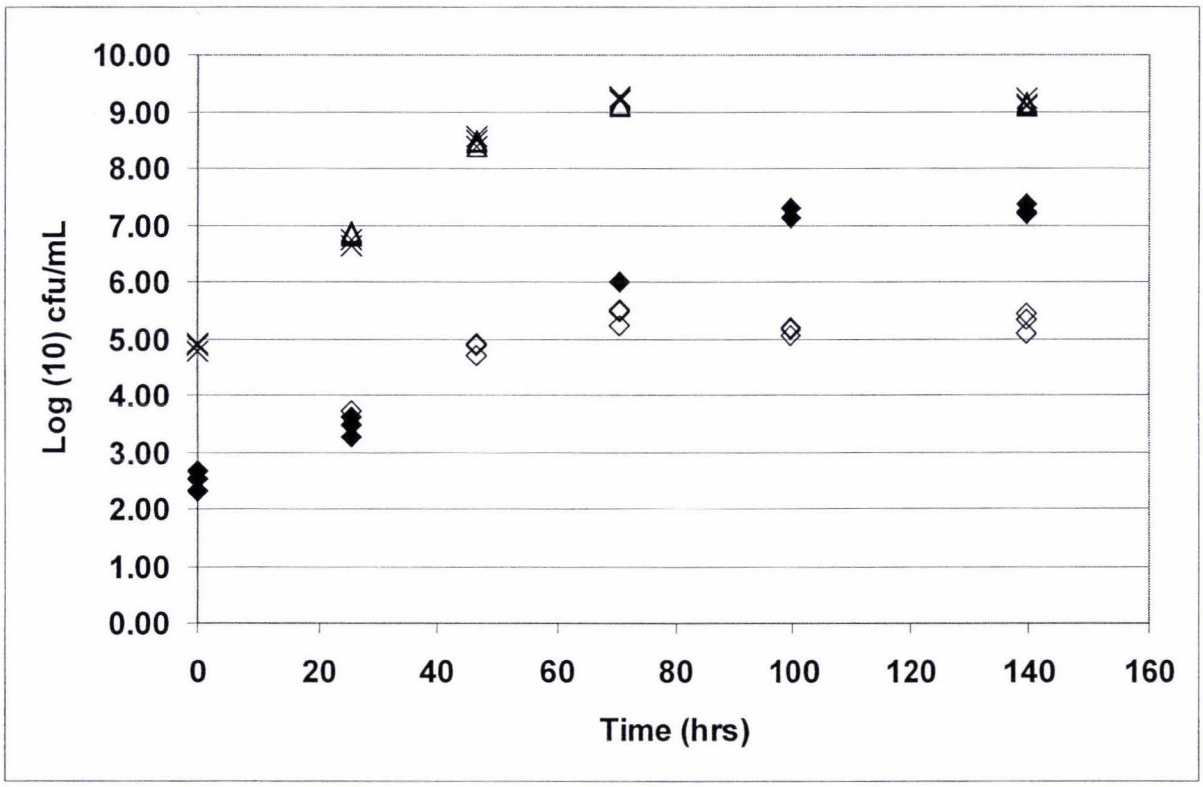


Figure 5.12 Cell counts of *C. piscicola* and *L. monocytogenes* with time when grown separately and together in broth at 10°C

Shown is: ◆ Log $_{10}$ *L. monocytogenes* (cfu/mL), × Log $_{10}$ C. *piscicola* (cfu/mL), ◇ Log $_{10}$ *L. monocytogenes* grown in co-culture with *C. piscicola* (cfu/mL), and △ Log $_{10}$ *C. piscicola* grown in this co-culture with *L. monocytogenes* (cfu/mL).

5.3 Discussion of results

5.3.1 Preparation for determination of antagonistic effects and growth rate

Confirmation of purity of strains

The results from these tests indicated that the strains were true to type. Resources were not available to carry out a full identification of *C. piscicola* A9b-.

It is clear from these results, however, that interpretation of the Gram stain needs to be done with caution. Both of these organisms are reportedly Gram-positive (Collins et al., 1987; Jones, 1991) but commonly displayed Gram-negative characteristics. Reasons for Gram-variability involve breakdown in the integrity of the wall of Gram-positive bacteria (Beveridge, 2001). This would suggest significant changes to the physiology and metabolism of the cell if basic processes of cellular transport were changed. It is interesting that the only occasion where consistent Gram-positive results were obtained for *C. piscicola* A9b- was when the organism had been grown in pH 9 broth. The cells may be growing more slowly at this high pH and therefore the cell walls are less likely to show thinning. *L. monocytogenes* showed a more variable result with both Gram-positive and Gram-negative organisms seen.

The implications from this change in cell wall structure may be highly significant in terms of how the cells will perform at any given time in any given conditions. In a number of cases exposure to a single type of stress provides resistance to other adverse conditions (Sanders et al., 1999). If the cell wall structure is thicker due to cells growing more slowly, this may be another important factor to consider when accounting for discrepancies observed between the sensitivity of bacteria to bacteriocins or other inhibitory substances.

Selection of agar

The N-P agar was selected as previous work had used this agar in experiments with these organisms (Nilsson et al., 2004). Strong growth of *L. monocytogenes* therefore was not expected. However the *L. monocytogenes* strain used here grew well so an alternative "semi-selective" agar was needed to distinguish *C. piscicola* A9b-. The base medium contains significant levels of

glucose, which supports growth of *Listeria* spp and other ingredients do not seem likely to fully suppress *L. monocytogenes.* This result is likely to be due to a strain difference. Considerable strain variations of *L. monocytogenes* have been reported (Begot et al., 1997; Buncic et al., 2001). In contrast, the strains of *L. monocytogenes* used by other workers grew only slowly on N-P agar (Nilsson et al., 2004).

L. monocytogenes (seafood var.) also showed significant growth on both types of modified N-P agar. It was impossible to visually distinguish from *C. piscicola* colonies.

L. monocytogenes did not grow in MRS agar at pH 5.4 but did grow in D-MRS agar at pH 8.5.

Carnobacteria may be inhibited by acetate in standard MRS media and some strains do not grow at the pH of this agar (Begot et al., 1997; Buncic et al., 2001; Hammes et al., 1991). *C. piscicola* was expected to grow in D-MRS as this has been suggested for the growth of *Carnobacterium* species in general (Hammes, 1991). However the lack of growth could be due to strain differences and other workers have not cited this medium as a growth medium for this strain.

The agar chosen, PRMA, was developed for these experiments. Its degree of accuracy in detecting levels of organisms was not determined, although some parallel comparisons with BHI agar were made and results were comparable (not reported). The results are determined by difference therefore absolute values are not required. Consistency between replicates was noted.

5.3.2 Determination of antagonistic effects

Comparison between broth, agar and mussels

These experiments have demonstrated that *L. monocytogenes* (seafood var.) is inhibited by *C. piscicola* A9b- in all of the systems trialled. The effect was also observed with a high and with a low initial starting concentration of *C. piscicola* in the broth system.

In broth, it was seen that the effect was not due to a pH change. In agar the pH changed from the initial pH of 7.3 to a pH of 6.5 or below as shown by the change of phenol-red from red to yellow in the PRMA. It is possible that the observed effect is partly due to a pH change in this system. Measurements of changes in pH of mussels were not made but it is expected that mussel tissue would provide buffering.

From the slopes of the graphs in Fig 5.11, it is tempting to speculate on the extent of inhibition of *L. monocytogenes* in the 3 systems. There is disparity between agar and broth, while there appears to be similarity between agar and mussels, but there are insufficient data for accurate comparisons, particularly in the mussel system.

These experiments were conducted at different times over the study period with different cultures of the same strains of the organism. Also, the methods of detecting *L. monocytogenes* counts differed between the solid and liquid substrates. It is not known what the extraction rate for organisms is from agar and mussels through the stomaching process. More accurate data would be needed at the early stages of the trials to be conclusive as for some of these early measurements the levels of *L. monocytogenes* observed for plate counts were below the recommended levels of 30-300 cfu/plate needed for accurate determination. Therefore, it is not appropriate to compare absolute values but comparison of trends is valid and further work would need to be done to enable specific comparisons between solid and liquid systems.

L. monocytogenes numbers were reduced by *C. piscicola* to a greater extent in broth than in agar or mussels. This would be expected as the effect is likely to

be dependent on diffusion of molecules (such as acetate and glucose), and diffusion would be more rapid in a liquid than in a solid system.

Comparison between high and low concentrations of *C. piscicola*

In broth, *L. monocytogenes* was inhibited by *C. piscicola* at both high and low initial concentrations. Simple nutrient depletion does not appear to be the sole cause for inhibition. The total concentration of organisms that was supported by the broth was larger in the first experiment where high numbers of *C. piscicola* were used than in the second where lower numbers were used. At 140 hours the total concentration of organisms grown in co-culture with high levels of *C. piscicola* was 10^{16}-10^{18}, compared to approximately 10^{14} with lower levels of *C. piscicola*. The experiments were done at different times and conditions could not be considered to be exactly the same. Therefore it is not possible to make direct comparisons between the two sets of data and further investigation may be warranted. The use of a lower concentration of *C. piscicola* may be a viable option and it would be worthwhile comparing inhibitory effects with different cell concentrations of the antagonist organism.

5.3.3 Methodology and interpretation of results

Conclusions from these results are based on the assumption that the methods used are a true measure of what is happening under particular conditions at a particular time.

The accuracy of plate count methodology has been questioned (Collins, 1984). However as there is still no readily available alternative this technique was used, and as the results depend on measuring differences, it was considered a satisfactory tool. Many workers use OD measurements, which is a simple easy-to-use method of measurement. This method does not distinguish between live and dead cells, so was not considered suitable for these experiments and was simply used as a guide. Actual numbers of organisms were determined by plate count methodology.

For OD measurements to be used for growth rate determinations a calibration against live organisms should be made, therefore interpretation of results from this type of study needs to be done with caution. It is likely that the numbers of

dead organisms increases during the cell cycle and the rate is also likely to differ in different conditions.

The technique of plate counting assumes plates have between 30 and 300 colonies for reliable results. This was done wherever possible. In some cases for these experiments, the dilution was such that no growth at all was recorded on some plates. These plates have been ignored for the calculations. It is likely that this treatment will give a distortedly high value (e.g. if only 1 sample in 3 gave a countable number of colonies, using that value as an average gives a higher result). Most commonly these results were for *L. monocytogenes* and therefore this treatment errs on the side of caution, as actual levels of *L. monocytogenes* may be lower.

Difficulties arise with detecting levels of organisms in mussel samples. Contaminating organisms will tend to reside on the surface and therefore levels are diluted by stomaching whole mussels, as was done here in the final experiments. Effects may therefore be more pronounced than measured here. The method of excising surface flesh was trialled initially but proved to be difficult and time-consuming and hence was not continued. Comparison of results between sub-samples shows adequate consistency and statistical analyses could be made. The stomaching step is likely not to give total extraction, as many organisms will be firmly attached to particulate material which remains in the stomacher bag. However this is a practical and common technique and is commonly used in industry. These results therefore cannot be considered actual measurements but changes are expected to be a real representation of what is happening within the system.

Intuitively 3 effects may be seen from the graphs.
When grown in co-culture:

- There may be an increased lag phase of *L. monocytogenes* when the organisms are grown together in broth (see figure 5.6).
- There appears to be decrease in growth rate of *L. monocytogenes* as indicated in all graphs.
- There appears to be a reduction in the numbers of *L. monocytogenes* as indicated in all graphs.

In these experiments regression lines were calculated using the data covering the straight-line parts of the curves.

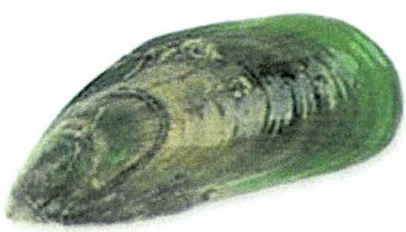
Statistical analyses also provide limitations and a more sophisticated analysis may be useful if future work is done. The linear regression line treatment was used for simplicity as over the early period, the growth rate observed was close to linear. Changes in the lag phases may exist and may not be accounted for by this statistical treatment. Collection of data at more frequent time intervals at the initial stages would be needed to give a more accurate picture of lag phase and this should be done before attempting a more sophisticated statistical analysis.

5.3.4 Summary

These results should be considered as preliminary and further confirmatory work should be done.

They show the organism *C. piscicola* A9b- is capable of inhibiting *L. monocytogenes* in broth, agar and mussel systems and indicate a potential method of assisting in the control of *L. monocytogenes* for the seafood industry.

Chapter 6 Conclusions



6 Conclusions

6.1 Use of an antagonistic organism in the seafood industry

A range of potential antagonists was screened and *C. piscicola* A9b- was selected for detailed study.

C. piscicola A9b- shows promise as a control agent for *L.monocytogenes* in mussel products. At 10°C, it showed effective inhibition of a wild type strain of *L.monocytogenes* obtained from the NZ mussel industry. The wild-type strain of *Listeria* appears more robust than laboratory strains. This was demonstrated in these experiments by the growth of the seafood strain in the media used by other workers to select against *L. monocytogenes.*

The inhibition by *C. piscicola* needs to be tested against a greater range of strains of *L. monocytogenes* and other organisms to demonstrate the breadth of antagonism. If the mechanisms of inhibition are non-specific such as by acetate production and glucose depletion the effect is likely be more general than the effect of a bacteriocin, and be effective against a relatively larger number of target strains. This would enhance the applicability of its use.

Levels of *C. piscicola* A9b- were not reduced in the freeze-thaw trial. Therefore the organism can be applied to a product that is to be frozen and the protection will be conferred when product is thawed, when growth of *L. monocytogenes* may occur.

Sensory tests and safety checks were not done for mussel products. Other workers have shown that there is no flavour change with high levels of *C. piscicola* on cold-smoked salmon (Nilsson et al., 1999). However, this has not been reported for the strain of *C. piscicola* A9b- used here. Confirmation of safety and of sensory attributes would need to be carried out before the antagonist could be used in mussel products.

It should be possible to apply the organism as a spray or a dip prior to freezing of the mussels. The organism demonstrated strong growth in the aerobic conditions used in these experiments and used in the majority of mussel products packed in this country. *C. piscicola* has also shown effective growth in vacuum packaged product (Nilsson et al., 1999), therefore shows good adaptability.

To be used in industry, treatments based on these principles have to be accepted by regulatory authorities. There are likely to be fewer hurdles in accepting a treatment based on naturally-occurring organisms that have been identified as probiotic than in accepting organisms that haven't been so classified. However because *C. piscicola* is naturally occurring on seafood products and is part of the natural flora consumed when these products are eaten, the obstacles should not be too great in obtaining GRAS status.

6.2 Final note

C. piscicola A9b- shows promise as an antagonistic organism to assist in the control of *L. monocytogenes* in mussel products. The organism is hardy, growing well in a variety of conditions where *L. monocytogenes* growth is often unchecked. The organism showed approximately a 1.5 log reduction in *L. monocytogenes* growth in mussels at 121 hours under the conditions used. This is likely to be sufficient to control *L. monocytogenes* in mussel products as the levels occurring are low, provided good hygienic practices are maintained during processing.

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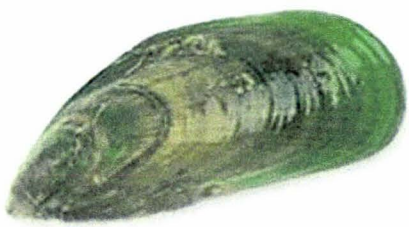

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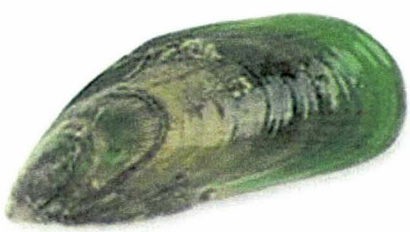
Appendix



Results broth trial 11-May 2005 - high Cp

Date	time (hrs)			count 1	count 2	count 3	mean	dilution factor	cfu/mL	notes
11-May	0:00	Lm	orig broth	83	90	92	88	1.00E-07	8.83E+08	Initial conc in broth
		Lm	as used	12	15	11	13	1.00E-01	1.27E+02	Conc placed into tubes for trial base on 0.1 mL to each plate
		Lm	as used	53	43	59	52	5.00E-01	1.03E+02	conc placed into tubes for trial base on 0.5 mL to each plate
		Lm in Lm+Cp	as used	9	8	10	9	1.00E-01	9.00E+01	conc placed into Lm+Cp tubes for trial based on 0.1 mL to each plate
		Cp in Lm+Cp	as used	112	83	117	104	1.00E-05	1.04E+07	
		Cp	as used	169	153	176	166	1.00E-05	1.66E+07	
12-May	15						0		#DIV/0!	
		Lm rep 1		2	0	1	1	1.00E-04	1.00E+04	Less growth than anticipated, old plates ex 13-5-05 & fridge too warm?
		Lm rep 2		1	1	5	2	1.00E-04	2.33E+04	
		Lm rep 3		0	0	0	0	1.00E-04	0.00E+00	
		Lm in Lm+Cp rep 1		1	0	1	1	1.00E-04	6.67E+03	
		Lm in Lm+Cp rep 2		3	0	2	2	1.00E-04	1.67E+04	
		Lm in Lm+Cp rep 3		3	2	5	3	1.00E-04	3.33E+04	
		Cp in Lm+Cp rep 1		112	75	63	83	1.00E-07	8.33E+08	
		Cp in Lm+Cp rep 2		88	77	73	79	1.00E-07	7.93E+08	
		Cp in Lm+Cp rep 3		123	130	110	121	1.00E-07	1.21E+09	used E-08 readings noted here after multiplying by 10
		Cp rep 1		110	104	103	106	1.00E-07	1.06E+09	agar too wet and plates impossible to count - took 3 clear readings
		Cp rep 2		96	60	460	205	1.00E-07	2.05E+09	where possible plates with 30-300 colonies used.
		Cp rep 3		212	232	230	225	1.00E-07	2.25E+09	
12-May	22.5						0		#DIV/0!	
		Lm rep 1		0	0	0	0	1.00E-06	0.00E+00	Samples too dilute
		Lm rep 2		0	0	0	0	1.00E-06	0.00E+00	
		Lm rep 3		0	0	0	0	1.00E-06	0.00E+00	
		Lm in Lm+Cp rep 1		35	13	21	23	1.00E-02	2.30E+03	
		Lm in Lm+Cp rep 2		10	21	14	15	1.00E-02	1.50E+03	
		Lm in Lm+Cp rep 3		29	20	26	25	1.00E-02	2.50E+03	
		Cp in Lm+Cp rep 1		265	256	215	245	1.00E-07	2.45E+09	
		Cp in Lm+Cp rep 2		190	167	168	175	1.00E-07	1.75E+09	
		Cp in Lm+Cp rep 3		84	101	104	96	1.00E-07	9.63E+08	
		Cp rep 1		104	104	109	106	1.00E-07	1.06E+09	
		Cp rep 2		106	170		138	1.00E-07	1.38E+09	no suitable 3rd reading plates too wet.
		Cp rep 3		124	230	140	165	1.00E-07	1.65E+09	
13-May	41								#DIV/0!	
		Lm rep 1		30	15	19	21	1.00E-07	2.13E+08	
		Lm rep 2		5	17	10	11	1.00E-07	1.07E+08	
		Lm rep 3		19	17	19	18	1.00E-07	1.83E+08	
		Lm in Lm+Cp rep 1		39	21	35	32	1.00E-02	3.17E+03	

Date	time (hrs)		count 1	count 2	count 3	mean	dilution factor	cfu/mL	notes
		Lm in Lm+Cp rep 2	14	25	25	21	1.00E-02	2.13E+03	
		Lm in Lm+Cp rep 3	22	20	32	25	1.00E-02	2.47E+03	
		Cp in Lm+Cp rep 1	71	83	113	89	1.00E-07	8.90E+08	
		Cp in Lm+Cp rep 2	66	92	110	89	1.00E-07	8.93E+08	
		Cp in Lm+Cp rep 3	69	10	10	30	1.00E-07	2.97E+08	
		Cp rep 1	153	153	230	179	1.00E-07	1.79E+09	
		Cp rep 2	77	88	105	90	1.00E-07	9.00E+08	
		Cp rep 3	72	90		81	1.00E-07	8.10E+08	
14-May	67					0		#DIV/0!	
		Lm rep 1	36	36	27	33	1.00E-07	3.30E+08	
		Lm rep 2	245	223	180	216	1.00E-06	2.16E+08	
		Lm rep 3	41	50	52	48	1.00E-07	4.77E+08	
		Lm in Lm+Cp rep 1	151	138	150	90	1.00E-02	8.97E+03	
		Lm in Lm+Cp rep 2	29	14	23	22	1.00E-04	2.20E+05	
		Lm in Lm+Cp rep 3	72	88	109	90	1.00E-02	8.97E+03	
		Cp in Lm+Cp rep 1	52	60	100	71	1.00E-07	7.07E+08	
		Cp in Lm+Cp rep 2	58	67	60	62	1.00E-07	6.17E+08	
		Cp in Lm+Cp rep 3	117	60	60	79	1.00E-07	7.90E+08	
		Cp rep 1	83	64	100	82	1.00E-07	8.23E+08	
		Cp rep 2	79	70	70	73	1.00E-07	7.30E+08	
		Cp rep 3	10	24		17	1.00E-07	1.70E+08	
15-May	91					0		#DIV/0!	
		Lm rep 1	21	13	20	18	1.00E-07	1.80E+08	
		Lm rep 2	19	25	20	21	1.00E-07	2.13E+08	
		Lm rep 3	62	25	53	47	1.00E-07	4.67E+08	
		Lm in Lm+Cp rep 1	206	216	210	211	1.00E-03	2.11E+05	
		Lm in Lm+Cp rep 2	658	836	TNTC	747	1.00E-05	7.47E+07	
		Lm in Lm+Cp rep 3	224	229	242	232	1.00E-05	2.32E+07	
		Cp in Lm+Cp rep 1	74	90	65	76	1.00E-07	7.63E+08	
		Cp in Lm+Cp rep 2	60	66	71	66	1.00E-07	6.57E+08	
		Cp in Lm+Cp rep 3	74	83	75	77	1.00E-07	7.73E+08	
		Cp rep 1	61	55	52	56	1.00E-07	5.60E+08	
		Cp rep 2	115	135	132	127	1.00E-07	1.27E+09	
		Cp rep 3	74	83	75	77	1.00E-07	7.73E+08	
16-May	116								
		Lm rep 1	11	2	13	9	1.00E-07	8.67E+07	
		Lm rep 2	14	13	7	11	1.00E-07	1.13E+08	
		Lm rep 3	21	31	21	24	1.00E-07	2.43E+08	
		Lm in Lm+Cp rep 1	54	73	66	64	1.00E-06	6.43E+07	
		Lm in Lm+Cp rep 2	73	41	66	60	1.00E-06	6.00E+07	
		Lm in Lm+Cp rep 3	34	30	24	29	1.00E-06	2.93E+07	
		Cp in Lm+Cp rep 1	33	35	51	40	1.00E-07	3.97E+08	

Date	time (hrs)		count 1	count 2	count 3	mean	dilution factor	cfu/mL	notes				
		Cp in Lm+Cp rep 2	37	21	33	30	1.00E-07	3.03E+08					
		Cp in Lm+Cp rep 3	23	33	32	29	1.00E-07	2.93E+08					
		Cp rep 1	57	45	47	50	1.00E-07	4.97E+08					
		Cp rep 2	46	50	49	48	1.00E-07	4.83E+08					
		Cp rep 3	39	did not record!!!!		39	1.00E-07	3.90E+08					
17-May	140												
		Lm rep 1	29	31	28	29	1.00E-07	2.93E+08					
		Lm rep 2	18	23	27	23	1.00E-07	2.27E+08					
		Lm rep 3	59	35	49	48	1.00E-07	4.77E+08	3.32E+08				
		Lm in Lm+Cp rep 1	75	89	58	74	1.00E-06	7.40E+07					
		Lm in Lm+Cp rep 2	51	53	55	53	1.00E-06	5.30E+07					
		Lm in Lm+Cp rep 3	76	81	55	71	1.00E-06	7.07E+07	6.59E+07				
		Cp in Lm+Cp rep 1	75	68	69	71	1.00E-07	7.07E+08					
		Cp in Lm+Cp rep 2	64	56	45	55	1.00E-07	5.50E+08					
		Cp in Lm+Cp rep 3	61	51	48	53	1.00E-07	5.33E+08	5.97E+08				
		Cp rep 1	80	82	90	84	1.00E-07	8.40E+08					
		Cp rep 2	93	49	58	67	1.00E-07	6.67E+08					
		Cp rep 3	57	57	75	63	1.00E-07	6.30E+08	7.12E+08				
18-May	164												
		Lm in Lm+Cp rep 1	8.70E+01	8.00E+01	9.30E+01	87	1.00E-07	8.67E+08		8.67E+08			
		Lm in Lm+Cp rep 2	1.44E+02	1.17E+02	1.58E+02	140	1.00E-06	1.40E+08		1.40E+08			
		Lm in Lm+Cp rep 3	8.30E+01	1.04E+02	7.10E+01	86	1.00E-06	8.60E+07	3.64E+08	8.60E+07			
		Cp in Lm+Cp rep 1	5.90E+01	5.00E+01	8.40E+01	64	1.00E-07	6.43E+08		6.43E+08			
		Cp in Lm+Cp rep 2	9.10E+01	6.00E+01	5.40E+01	68	1.00E-07	6.83E+08		6.83E+08			
		Cp in Lm+Cp rep 3	1.03E+02	9.40E+01	1.12E+02	103	1.00E-07	1.03E+09	7.86E+08	1.03E+09			

	Time (hours)	0.00	15.00	18.00	22.50	25.33	67.00	70.00	91.00	95.00	116.00	140.00	164.00
pH	Lm	6.70		6.67		6.68		6.53		6.50		6.51	
pH	Lm+Cp	6.70		6.49		6.53		6.82		6.53		6.68	
pH	Cp	6.70		6.51		6.50		6.59		6.69		6.74	
	Temperature		14.00		13.00	13.50	12.50		12.00	12.00	12.00	10.00	12.00

Results Agar - June 2005

	count1	count 2	count 3	dilution	cfu/ml	
Lm	1.82E+02	1.33E+02	1.83E+02	1.00E-07	1.66E+09	Diluted to 10^{-4} added 2 mL to 98mL B-BHI then took 9 mL and addec
Cp	1.90E+02	1.67E+02	2.07E+02	1.00E-07	1.88E+09	Added 2 mL to 98mL B-BHI then took 9 mL and added 9mL Lm or bu

Lm/plate 1.66E+02
Cp/plate 1.88E+06
Dilute agar from plate with 27mL peptone in stomacher bag & stomach. Ie diln of 27 in 34 mls (assume agar plate = 14mL agar) 13.665
Agar depth (mm) 6,5,5,4,6,4.5,5,5,5,5,4.5,5
Dilution factor 1.26E+00

		ct 1	diln	cfu/cm^2	ct 2	diln	cfu/cm^2	ct 3	diln	cfu/cm^2
Cp	0 hours	2.98E+02	1.00E-02	3.75E+04	3.46E+02	1.00E-02	4.36E+04		1.00E-02	0.00E+00
Cp		4.78E+02	1.00E-02	6.02E+04	4.64E+02	1.00E-02	5.84E+04			
Cp		7.11E+02	1.00E-02	8.95E+04	TNTC					
Lm	from BHA pla	4.40E+01	Nil	1.63E+00	6.10E+01	Nil	2.26E+00	7.50E+01	Nil	2.78E+00
Lm		7.50E+01	Nil	2.78E+00	7.00E+01	Nil	2.59E+00	6.20E+01	Nil	2.30E+00
Cp	16 hours	3.70E+01	1.00E-05	4.66E+06	2.70E+01	1.00E-05	3.40E+06	4.10E+01	1.00E-05	5.16E+06
Cp		2.80E+01	1.00E-05	3.53E+06	3.00E+01	1.00E-05	3.78E+06	3.30E+01	1.00E-05	4.16E+06
Cp		2.90E+01	1.00E-05	3.65E+06	3.11E+02	1.00E-06	3.92E+08	3.06E+02	1.00E-06	3.85E+08
Lm		1.00E+00	1.00E-02	1.26E+02	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm		0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm		0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Cp (w Lm)		4.40E+01	1.00E-05	5.54E+06	4.00E+01	1.00E-05	5.04E+06	3.60E+01	1.00E-05	4.53E+06
Cp (w Lm)		2.74E+02	1.00E-04	3.45E+06	2.73E+02	1.00E-04	3.44E+06	3.13E+02	1.00E-04	3.94E+06
Cp (w Lm)		1.82E+02	1.00E-04	2.29E+06	1.94E+02	1.00E-04	2.44E+06	1.88E+02	1.00E-04	2.37E+06
Lm (w Cp)		4.00E+00	1.00E-02	5.04E+02	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm (w Cp)		0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm (w Cp)		1.00E+00	1.00E-02	1.26E+02	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm	24 hours	1.00E+00	1.00E-02	1.26E+02	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm		0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm		1.00E+00	1.00E-02	1.26E+02	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00

		ct 1	diln	cfu/cm^2	ct 2	diln	cfu/cm^2	ct 3	diln	cfu/cm^2
Lm (w Cp)		1.00E+00	1.00E-02	1.26E+02	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm (w Cp)		0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm (w Cp)		1.00E+00	1.00E-02	1.26E+02	2.00E+00	1.00E-02	2.52E+02	0.00E+00	1.00E-02	0.00E+00
Cp	40 hours	6.00E+01	1.00E-06	7.56E+07	4.40E+01	1.00E-06	5.54E+07	3.40E+01	1.00E-06	4.28E+07
Cp		6.80E+01	1.00E-06	8.56E+07	4.80E+01	1.00E-06	6.04E+07	5.00E+01	1.00E-06	6.30E+07
Cp		6.50E+01	1.00E-06	8.19E+07	6.90E+01	1.00E-06	8.69E+07	8.50E+01	1.00E-06	1.07E+08
Lm		3.00E+00	1.00E-02	3.78E+02	2.00E+00	1.00E-02	2.52E+02	0.00E+00	1.00E-02	0.00E+00
Lm		5.00E+00	1.00E-02	6.30E+02	1.00E+00	1.00E-02	1.26E+02	4.00E+00	1.00E-02	5.04E+02
Lm		2.00E+00	1.00E-02	2.52E+02	3.00E+00	1.00E-02	3.78E+02	3.00E+00	1.00E-02	3.78E+02
Cp (w Lm)		6.80E+01	1.00E-06	8.56E+07	4.30E+01	1.00E-06	5.41E+07	3.80E+01	1.00E-06	4.79E+07
Cp (w Lm)		3.70E+01	1.00E-06	4.66E+07	4.80E+01	1.00E-06	6.04E+07	3.30E+01	1.00E-06	4.16E+07
Cp (w Lm)		4.70E+01	1.00E-06	5.92E+07	7.40E+01	1.00E-06	9.32E+07	5.20E+01	1.00E-06	6.55E+07
Lm (w Cp)		5.00E+00	1.00E-02	6.30E+02	2.00E+00	1.00E-02	2.52E+02	2.00E+00	1.00E-02	2.52E+02
Lm (w Cp)		1.00E+00	1.00E-02	1.26E+02	0.00E+00	1.00E-02	0.00E+00	0.00E+00	1.00E-02	0.00E+00
Lm (w Cp)		0.00E+00	1.00E-02	0.00E+00	3.00E+00	1.00E-02	3.78E+02	3.00E+00	1.00E-02	3.78E+02
Cp	65 hours	8.20E+01	1.00E-07	1.03E+09	9.20E+01	1.00E-07	1.16E+09	1.17E+02	1.00E-07	1.47E+09
Cp		8.00E+01	1.00E-07	1.01E+09	7.70E+01	1.00E-07	9.70E+08	7.10E+01	1.00E-07	8.94E+08
Cp		8.60E+01	1.00E-07	1.08E+09	6.80E+01	1.00E-07	8.56E+08	8.10E+01	1.00E-07	1.02E+09
Lm		1.10E+01	1.00E-03	1.39E+04	1.30E+01	1.00E-03	1.64E+04	1.00E+01	1.00E-03	1.26E+04
Lm		1.10E+01	1.00E-03	1.39E+04	1.20E+01	1.00E-03	1.51E+04	1.20E+01	1.00E-03	1.51E+04
Lm		1.70E+01	1.00E-03	2.14E+04	1.50E+01	1.00E-03	1.89E+04	8.00E+00	1.00E-03	1.01E+04
Cp (w Lm)		5.50E+01	1.00E-07	6.93E+08	5.50E+01	1.00E-07	6.93E+08	6.80E+01	1.00E-07	8.56E+08
Cp (w Lm)		6.10E+01	1.00E-07	7.68E+08	5.60E+01	1.00E-07	7.05E+08	5.40E+01	1.00E-07	6.80E+08
Cp (w Lm)		4.20E+01	1.00E-07	5.29E+08	3.30E+01	1.00E-07	4.16E+08	4.50E+01	1.00E-07	5.67E+08
Lm (w Cp)		4.00E+00	1.00E-02	5.04E+02	8.00E+00	1.00E-02	1.01E+03	1.10E+01	1.00E-02	1.39E+03
Lm (w Cp)		7.00E+00	1.00E-02	8.81E+02	3.00E+00	1.00E-02	3.78E+02	1.00E+00	1.00E-02	1.26E+02
Lm (w Cp)		2.00E+00	1.00E-02	2.52E+02	1.00E+00	1.00E-02	1.26E+02	4.00E+00	1.00E-02	5.04E+02
Cp	89 hours	1.30E+02	1.00E-07	1.64E+09	9.40E+01	1.00E-07	1.18E+09	9.00E+01	1.00E-07	1.13E+09
Cp		1.15E+02	1.00E-07	1.45E+09	6.10E+01	1.00E-07	7.68E+08	1.03E+02	1.00E-07	1.30E+09
Cp		1.85E+02	1.00E-07	2.33E+09	1.88E+02	1.00E-07	2.37E+09	1.89E+02	1.00E-07	2.38E+09
Lm		5.10E+01	1.00E-03	6.42E+04	4.20E+01	1.00E-03	5.29E+04	5.00E+01	1.00E-03	6.30E+04
Lm		1.15E+02	1.00E-03	1.45E+05	6.10E+01	1.00E-03	7.68E+04	1.03E+02	1.00E-03	1.30E+05

		ct 1	diln	cfu/cm^2	ct 2	diln	cfu/cm^2	ct 3	diln	cfu/cm^2
Lm		1.85E+02	1.00E-03	2.33E+05	1.88E+02	1.00E-03	2.37E+05	1.89E+02	1.00E-03	2.38E+05
Cp (w Lm)		1.11E+02	1.00E-07	1.40E+09	1.02E+02	1.00E-07	1.28E+09	1.07E+02	1.00E-07	1.35E+09
Cp (w Lm)		1.62E+02	1.00E-07	2.04E+09	1.04E+02	1.00E-07	1.31E+09	1.27E+02	1.00E-07	1.60E+09
Cp (w Lm)		1.19E+02	1.00E-07	1.50E+09	1.60E+02	1.00E-07	2.01E+09	1.40E+02	1.00E-07	1.76E+09
Lm (w Cp)		1.30E+01	1.00E-02	1.64E+03	1.20E+01	1.00E-02	1.51E+03	1.10E+01	1.00E-02	1.39E+03
Lm (w Cp)		2.20E+01	1.00E-02	2.77E+03	1.50E+01	1.00E-02	1.89E+03	7.00E+00	1.00E-02	8.81E+02
Lm (w Cp)		4.60E+01	1.00E-02	5.79E+03	2.20E+01	1.00E-02	2.77E+03	3.20E+01	1.00E-02	4.03E+03
Lm	113 hours	2.14E+02	1.00E-03	2.69E+05	2.54E+02	1.00E-03	3.20E+05	2.71E+02	1.00E-03	3.41E+05
Lm		2.49E+02	1.00E-03	3.14E+05	2.66E+02	1.00E-03	3.35E+05	2.95E+02	1.00E-03	3.71E+05
Lm		3.40E+01	1.00E-04	4.28E+05	3.00E+01	1.00E-04	3.78E+05	3.50E+01	1.00E-04	4.41E+05
Lm (w Cp)		6.00E+00	1.00E-02	7.56E+02	1.00E+01	1.00E-02	1.26E+03	2.30E+01	1.00E-02	2.90E+03
Lm (w Cp)		1.00E+00	1.00E-02	1.26E+02	1.10E+01	1.00E-02	1.39E+03	0.00E+00	1.00E-02	0.00E+00
Lm (w Cp)		4.70E+01	1.00E-02	5.92E+03	4.30E+01	1.00E-02	5.41E+03	4.70E+01	1.00E-02	5.92E+03
Lm	139 hours	2.32E+02	1.00E-05	2.92E+07	2.41E+02	1.00E-05	3.03E+07	2.19E+02	1.00E-05	2.76E+07
Lm		3.70E+01	1.00E-05	4.66E+06	3.50E+01	1.00E-05	4.41E+06	4.20E+01	1.00E-05	5.29E+06
Lm		6.30E+01	1.00E-05	7.93E+06	6.50E+01	1.00E-05	8.19E+06	6.80E+01	1.00E-05	8.56E+06
Lm (w Cp)		7.20E+01	1.00E-02	9.07E+03	7.40E+01	1.00E-02	9.32E+03	6.00E+01	1.00E-02	7.56E+03
Lm (w Cp)		1.86E+02	1.00E-02	2.34E+04	2.54E+02	1.00E-02	3.20E+04	1.84E+02	1.00E-02	2.32E+04
Lm (w Cp)		2.00E+01	1.00E-02	2.52E+03	1.20E+01	1.00E-02	1.51E+03	1.70E+01	1.00E-02	2.14E+03
Cp	161.5 hours	1.40E+02	1.00E-07	1.76E+09	1.56E+02	1.00E-07	1.96E+09	1.53E+02	1.00E-07	1.93E+09
Cp		1.51E+02	1.00E-07	1.90E+09	1.48E+02	1.00E-07	1.86E+09	1.29E+02	1.00E-07	1.62E+09
Cp		1.79E+02	1.00E-07	2.25E+09	1.98E+02	1.00E-07	2.49E+09	1.98E+02	1.00E-07	2.49E+09
Lm		3.90E+01	1.00E-06	4.91E+07	3.10E+01	1.00E-06	3.90E+07	4.30E+01	1.00E-06	5.41E+07
Lm		1.33E+02	1.00E-06	1.67E+08	1.48E+02	1.00E-06	1.86E+08	1.43E+02	1.00E-06	1.80E+08
Lm				#DIV/0!			#DIV/0!			#DIV/0!
Cp (w Lm)		1.45E+02	1.00E-07	1.83E+09	9.90E+01	1.00E-07	1.25E+09	1.26E+02	1.00E-07	1.59E+09
Cp (w Lm)		1.59E+02	1.00E-07	2.00E+09	1.57E+02	1.00E-07	1.98E+09	1.54E+02	1.00E-07	1.94E+09
Cp (w Lm)		1.97E+02	1.00E-07	2.48E+09	1.72E+02	1.00E-07	2.17E+09	1.98E+02	1.00E-07	2.49E+09
Lm (w Cp)		3.80E+01	1.00E-03	4.79E+04	3.80E+01	1.00E-03	4.79E+04	3.80E+01	1.00E-03	4.79E+04
Lm (w Cp)		2.19E+02	1.00E-02	2.76E+04	2.13E+02	1.00E-02	2.68E+04	1.79E+02	1.00E-02	2.25E+04
Lm (w Cp)		4.80E+01	1.00E-03	6.04E+04	3.00E+01	1.00E-03	3.78E+04	6.40E+01	1.00E-03	8.06E+04

Mussel trial August 2005

Tmt	date	time	temp	bag id	wt bag	wt +mussels	wt +peptone	wt mussel	wt peptone	diln		ct 1	diln on plate	cfu/g	ct 2	diln on plate	cfu/g	ct 3	diln on plate	cfu/g
Lm	30-Jun	0			12.01	50.73		38.72		100	2.58	14	1.00E-01	3.62E+02	5	1.00E-01	1.29E+02	10	1.00E-01	2.58E+02
Lm	17-Aug	0		15	11.88	52.38	146.6	40.5	94.22		2.33	3	1.00E-01	6.98E+01	3	1.00E-01	6.98E+01	4	1.00E-01	9.31E+01
Cp + Lm	17-Aug	0		3	11.54	58.77	151.2	47.23	92.43		1.96	2	1.00E-01	3.91E+01	4	1.00E-01	7.83E+01	2	1.00E-01	3.91E+01
Lm	19-Aug	44.5	10	12	12.01	56.33	149.24	44.32	92.91		2.10	TFTC	1.00E-05	#VALUE!	TFTC	1.00E-05	#VALUE!	TFTC	1.00E-05	#VALUE!
Lm	19-Aug	44.5	10	13	11.9	57.13	149.86	45.23	92.73		2.05	TFTC	1.00E-05	#VALUE!	TFTC	1.00E-05	#VALUE!	TFTC	1.00E-05	#VALUE!
Lm	19-Aug	44.5	10	14	11.94	59.59	151.91	47.65	92.32		1.94	TFTC	1.00E-05	#VALUE!	TFTC	1.00E-05	#VALUE!	TFTC	1.00E-05	#VALUE!
Cp + Lm	19-Aug	44.5	10	4	11.54	60.78	153.81	49.24	93.03		1.89	6	1.00E-02	1.13E+03	4	1.00E-02	7.56E+02	0	1.00E-02	0.00E+00
Cp + Lm	19-Aug	44.5	10	1	11.68	51.59	146.2	39.91	94.61		2.37	6	1.00E-02	1.42E+03	1	1.00E-02	2.37E+02	2	1.00E-02	4.74E+02
Cp + Lm	19-Aug	44.5	10	2	11.45	50.44	143.75	38.99	93.31		2.39	2	1.00E-02	4.79E+02	2	1.00E-02	4.79E+02	0	1.00E-02	0.00E+00
Lm	20-Aug	68.5	10	11	12.03	58.88	152.04	46.85	93.16		1.99	TFTC	1.00E-06	#VALUE!	TFTC	1.00E-06	#VALUE!	TFTC	1.00E-06	#VALUE!
Lm	20-Aug	68.5	10	10	11.35	50.96	143.3	39.61	92.34		2.33	TFTC	1.00E-06	#VALUE!	TFTC	1.00E-06	#VALUE!	TFTC	1.00E-06	#VALUE!
Lm	20-Aug	68.5	10	5	11.41	55	148.12	43.59	93.12		2.14	TFTC	1.00E-06	#VALUE!	TFTC	1.00E-06	#VALUE!	TFTC	1.00E-06	#VALUE!
Cp + Lm	20-Aug	68.5	10	8	11.44	53.31	165.03	41.87	111.72		2.67	2	1.00E-03	5.34E+03	5	1.00E-03	1.33E+04	0	1.00E-03	0.00E+00
Cp + Lm	20-Aug	68.5	10	7	11.57	58.83	149.83	47.26	91		1.93	5	1.00E-03	9.63E+03	4	1.00E-03	7.70E+03	4	1.00E-03	7.70E+03
Cp + Lm	20-Aug	68.5	10	9	11.41	47.57	140	36.16	92.43		2.56	0	1.00E-03	0.00E+00	2	1.00E-03	5.11E+03	1	1.00E-03	2.56E+03
Lm	21-Aug	97.5	10	8	11.31	47.99	142.22	36.68	94.23		2.57	1	1.00E-05	2.57E+05	5	1.00E-05	1.28E+06	1	1.00E-05	2.57E+05
Lm	21-Aug	97.5	10	4	11.49	58.52	152.91	47.03	94.39		2.01	5	1.00E-05	1.00E+06	4	1.00E-05	8.03E+05	4	1.00E-05	8.03E+05
Lm	21-Aug	97.5	10	7	11.41	53.13	146.19	41.72	93.06		2.23	5	1.00E-05	1.12E+06	7	1.00E-05	1.56E+06	7	1.00E-05	1.56E+06
Cp + Lm	21-Aug	97.5	10	6	11.52	51.9	146.04	40.38	94.14		2.33	0	1.00E-04	0.00E+00	0	1.00E-04	0.00E+00	0	1.00E-04	0.00E+00
Cp + Lm	21-Aug	97.5	10	15	11.54	52.34	145.59	40.8	93.25		2.29	1	1.00E-04	2.29E+04	2	1.00E-04	4.57E+04	1	1.00E-04	2.29E+04
Cp + Lm	21-Aug	97.5	10	14	11.49	62.27	156.79	50.78	94.52		1.86	0	1.00E-04	0.00E+00	5	1.00E-04	9.31E+04	0	1.00E-04	0.00E+00
Lm	22-Aug	121	10	3	12.31	62.33	155.72	50.02	93.39		1.87	39	1.00E-05	7.28E+06	45	1.00E-05	8.40E+06	50	1.00E-05	9.34E+06
Lm	22-Aug	121	10	9	11.53	50.77	144.3	39.24	93.53		2.38	30	1.00E-05	7.15E+06	32	1.00E-05	7.63E+06	41	1.00E-05	9.77E+06
Lm	22-Aug	121	10	6	11.51	52.16	145.9	40.65	93.74		2.31	32	1.00E-05	7.38E+06	25	1.00E-05	5.77E+06	26	1.00E-05	6.00E+06
Cp + Lm	22-Aug	121	10	12	11.55	53.84	149.14	42.29	95.3		2.25	3	1.00E-04	6.76E+04	9	1.00E-04	2.03E+05	4	1.00E-04	9.01E+04
Cp + Lm	22-Aug	121	10	13	11.55	42.85	137.92	31.3	95.07		3.04	10	1.00E-04	3.04E+05	7	1.00E-04	2.13E+05	14	1.00E-04	4.25E+05
Cp + Lm	22-Aug	121	10	11	11.55	45.39	138.56	33.84	93.17		2.75	8	1.00E-04	2.20E+05	8	1.00E-04	2.20E+05	12	1.00E-04	3.30E+05
Lm	23-Aug	145	10	1	12.26	64.88	158.84	52.62	93.96		1.79	93	1.00E-05	1.66E+07	110	1.00E-05	1.96E+07	88	1.00E-05	1.57E+07
Lm	23-Aug	145	10	2	12.39	52.61	147.55	40.22	94.94		2.36	78	1.00E-05	1.84E+07	94	1.00E-05	2.22E+07	65	1.00E-05	1.53E+07
Cp + Lm	23-Aug	145	10	10	11.43	55.05	149.14	43.62	94.09		2.16	224	1.00E-03	4.83E+05	252	1.00E-03	5.44E+05	250	1.00E-03	5.39E+05
Cp + Lm	23-Aug	145	10	5	12.48	64.26	157.55	51.78	93.29		1.80	201	1.00E-03	3.62E+05	253	1.00E-03	4.56E+05	228	1.00E-03	4.11E+05

Tmt	date	time	temp	bag id	wt bag	wt +mussels	wt +peptone	wt mussel	wt peptone	diln	ct 1	diln on plate	cfu/g	ct 2	diln on plate	cfu/g	ct 3	diln on plate	cfu/g
Autoclaved	initial 1							29.68	100	3.37	70	1.00E-04	2.36E+06	71	1.00E-04	2.39E+06	70	1.00E-04	2.36E+06
	initial 2							35.94	100	2.78	78	1.00E-04	2.17E+06	61	1.00E-04	1.70E+06	76	1.00E-04	2.11E+06
	final 1				11.44	43.88	137.38	32.44	93.5	2.88	92	1.00E-04	2.65E+06	112	1.00E-04	3.23E+06	117	1.00E-04	3.37E+06
	final 2				12.31	40.1	133.05	27.79	92.95	3.34	96	1.00E-04	3.21E+06	84	1.00E-04	2.81E+06	108	1.00E-04	3.61E+06

	pre-freeze	post-freeze
	2.36E+06	2.65E+06
	2.17E+06	3.21E+06
	2.39E+06	3.23E+06
	1.70E+06	2.81E+06
	2.36E+06	3.37E+06
	2.11E+06	3.61E+06
Av	**2.18E+06**	**3.15E+06**
S.D	**2.63E+05**	**3.57E+05**

Broth Trial August 2005 Low Cp

Tmt	Date	Time (hrs)	diln	ct 1	cfu/mL	ct 2	cfu/mL	ct 3	cfu/mL
Lm	17-Aug	0	1.00E-01	33	3.30E+02	21	2.10E+02	47	4.70E+02
Cp	17-Aug	0	1.00E-03	59	5.90E+04	75	7.50E+04	83	8.30E+04
Lm	18-Aug	25.5	1.00E-02	19	1.90E+03	41	4.10E+03	30	3.00E+03
Lm (wCp)	18-Aug	25.5	1.00E-02	30	3.00E+03	51	5.10E+03	30	3.00E+03
Cp	18-Aug	25.5	1.00E-04	528	5.28E+06	558	5.58E+06	420	4.20E+06
Cp (wLm)	18-Aug	25.5	1.00E-04	758	7.58E+06	722	7.22E+06	616	6.16E+06
Lm	19-Aug	46.5	TFTC		#VALUE!	TFTC	#VALUE!	TFTC	#VALUE!
Lm (wCp)	19-Aug	46.5	1.00E-03	50	5.00E+04	79	7.90E+04	77	7.70E+04
Cp	19-Aug	46.5	1.00E-07	32	3.20E+08	24	2.40E+08	35	3.50E+08
Cp (wLm)	19-Aug	46.5	1.00E-06	246	2.46E+08	296	2.96E+08	301	3.01E+08
Lm	20-Aug	70.5	1.00E-06	1	1.00E+06	0	0.00E+00	1	1.00E+06
Lm (wCp)	20-Aug	70.5	1.00E-04	32	3.20E+05	29	2.90E+05	17	1.70E+05
Cp	20-Aug	70.5	1.00E-07	177	1.77E+09	167	1.67E+09	161	1.61E+09
Cp (wLm)	20-Aug	70.5	1.00E-07	125	1.25E+09	132	1.32E+09	130	1.30E+09
Lm	21-Aug	99.5	1.00E-05	204	2.04E+07	196	1.96E+07	135	1.35E+07
Lm (wCp)	21-Aug	99.5	1.00E-03	150	1.50E+05	145	1.45E+05	113	1.13E+05
Lm	23-Aug	139.5	1.00E-06	23	2.30E+07	15	1.50E+07	17	1.70E+07
Lm (wCp)	23-Aug	139.5	1.00E-04	21	2.10E+05	12	1.20E+05	27	2.70E+05
Cp	23-Aug	139.5	1.00E-07	126	1.26E+09	134	1.34E+09	172	1.72E+09
Cp (wLm)	23-Aug	139.5	1.00E-07	142	1.42E+09	129	1.29E+09	123	1.23E+09