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

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Irene Helen Thomas

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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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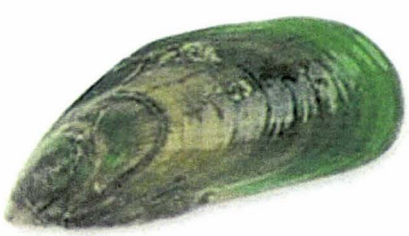
Chapter 1 Introduction

1.1 The Concept

1.2 Objectives and Outcomes



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

2.1 Background

2.2 Purpose of this review

2.3 Concerns with New Zealand Seafood Products

2.4 The problems with Listeria

2.5 Use of microbial control methods

2.6 Mechanisms of antagonism

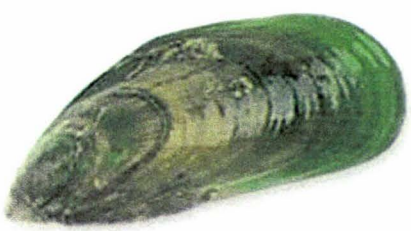
2.7 Bacteriocins

2.8 Challenges on food systems

2.9 Confusing aspects

2.10 Use in industry

2.11 Where to from here?



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.