Microbiological Quality of Toroi: A Maori food delicacy

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Summary

A study was undertaken to determine the food safety of the fermented Maori delicacy, Toroi. Ten batches of Toroi were prepared by a commonly used traditional method that consisted of boiling the vegetable component, either watercress or puha, and combining it with chopped mussel flesh. The mixture was cooled and then stored in a refrigerator for up to eight months to allow natural fermentation to take place. All ingredients were sourced from retail outlets. The Toroi was examined at intervals over eight months for a range of pathogens (seven in all) that have been related to incidents of food poisoning in ready-to-eat foods in New Zealand. The survival of a faecal contamination indicator, the laboratory grown strain *Escherichia coli* NZRM 916, was mapped over eight months. Two strategies to prevent the growth of *Listeria monocytogenes* in Toroi were also investigated.

Only one of the seven pathogens sought was recovered from any sample. This pathogen was *Bacillus cereus*, a spore-former known to be associated with vegetables. All batches contained *B. cereus* on the day of preparation but after two weeks refrigerated storage there was no further recovery from any sample. There was a very low incidence of natural *E. coli* in the Toroi, consistent with levels permitted in mussels sold in retail outlets. The laboratory grown strain, *E. coli* declined substantially over two months and was not recovered from any samples at eight months.

A laboratory grown strain of *Listeria monocytogenes*, (L70) was added to Toroi and grew well with an increase in concentration of about seven-fold, over 19 days storage in a refrigerator. A bacteriocin producing lactic acid bacterium, *Lactobacillus sake* Lb706, was added in combined culture with *L. monocytogenes* to Toroi. It was found that at least 5 $x10^8$ *L. sake* cells were required as an inoculum to ensure elimination of *L. monocytogenes* from the Toroi. When a purified bacteriocin; nisin, was added, a concentration of 10 mg g^{-1} in the Toroi was required to eliminate *L. monocytogenes*. The inhibition study results suggest that unacceptably high inocula or purified bacteriocin would be required to prevent the growth of *L. monocytogenes* in Toroi.

The results of this suggest that Toroi be prepared from mussels either purchased from a retail outlet or harvested from sites known to be free from contamination. Toroi should be safe to eat if prepared carefully, chilled promptly and thoroughly and allowed to ferment for at least two weeks. In addition, care should be taken to maintain Toroi at refrigerated temperatures until it is eaten.

Preface and Acknowledgements

I would like to acknowledge my two boys, Tiniwaata and Tutuki who gave me the "drive" to keep going. I am grateful to have you as my children.

To my extended whanau from Waikato, Pare Hauraki, Ngai Te Rangi, Ngati Tuwharetoa, Ngati Awa, your words of wisdom and visionary phenomenon have led me to this whakatauki:

"Sacrifice me, so my future generation will have whenua FOREVER!

to develop and grow and to better the template that I've started"

I would like to take this opportunity to acknowledge Tainui Raupatu Trust and AgResearch who assisted me financially. I'm so grateful.

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Chapter 1

Literature Review

1.1. Introduction

The literature relating to traditional foods and their historical accounts of traditional culturing and harvesting practices related to food preparation and the scientific literature relating to food safety of wild foods are reviewed.

The New Zealand Food Standards are designed to protect public health (FSANZ, 2001; 2003). Under these Standards there are sets of specified tests for each class of food. On the basis of these test results food is assigned to one of the following categories: acceptable, marginal or unacceptable/unsafe for consumption. Foods that are sold to the public must be either acceptable or at worse marginal.

Toroi is a traditional food for which there are no absolutely defined ingredients or preparation techniques. However, most preparations consist of a mixture of meat or fish and leafy vegetables. The preparation involves some cooking following which Toroi is stored, usually under refrigeration, for several weeks until eaten.

Although little information exists of the food safety of Toroi preliminary investigations have been undertaken by Hudson (*et al*., 2001) and Whyte (*et al.*, 2001). These workers highlighted that Toroi underwent lactic acid fermentation during the storage period providing scientific proof of the appropriateness of the name Toroi in translation means “to brew; to ferment” (Ngata, 1993).

In this thesis I undertook investigations into the food safety of Toroi prepared from mussels sourced from a commercial outlet and leafy vegetables, puha or watercress that were sourced from the wild. In addition I investigated the ability of a natural culture "starter" species of lactic acid bacteria and of a synthetic bacteriocin for their ability to prevent the growth of an important cold-tolerant pathogen, *Listeria monocytogenes*.

The literature review therefore encompasses both the Maori perspective and the published scientific information on Toroi and its ingredients.

1.2. Maori and their relationship with traditional foods

Maori have special traditions concerning planting, harvesting and preparation of foods. As a people they understood the land, the forest, the sea and the foods available to them. The area where food was to be gathered was respected. Maori were conservationists in their own right and kaitiaki (guardians) of the land, water, air and their well-being. Gathering of foods from the wild is still important today as shown in Figure 1.1

Figure 1.1. Harvesting watercress from a clean stream.

Each food product contains a whakapapa (geneology) with Maori geneology. Maori freshwater foods are of value to iwi, for example, fish, eels, lamphrey, fresh water mussels, koura and watercress. All fauna and flora are the offspring of various deities; for example, all sea life is of Tangaroa, forests and animals are of Tane Mahuta and uncultivated foods are of Haumiatiketike. For this reason they have Mana Atua (highest prestige) and are considered tapu (sacred) (Canterbury Regional Council Policy Statement, 1998).

A medical doctor in Gisborne who is strongly promoting the inclusion of traditional foods in the modern Maori diet (D. Tipene-Leach pers. comm.), considers that these foods are not only important in protecting and preserving tikanga associated with harvesting and preparation of foods but also they have good nutritional value.

1.2.1. Mahinga kai

Knowledge of food resources

Tangatawhenua have invaluable knowledge of specific kai resources and its relationship with the environment, much of which will go back over many generations, most of this information exists nowhere else and may be in a form that is completely different from the conventional approach taken by European science. Information may be communicable only in te reo Maori (Maori language) and some information may not be for general dissemination, being either specific to the particular hapu or so sensitive that confidentiality must be protected (eg. Waahi tapu).

Cultural activities are the essential working elements of the tribal culture. They give rise to, and reaffirm, the relationship of Tangata-whenua with the land. The guaranteed availability of these resources has implications that extend beyond the use of a material for any one cultural activity. It extends to preserving tikanga (customary practices) associated with their use.

The knowledge of harvesting is passed down from each generation, and can be different between whanau, hapu or iwi. When gathering food from the land or the sea, special caution is exercised to insure its purity and continued growth. The laws of tapu encouraged conservation also, by taking only what is needed. These laws are carefully considered before harvesting any food from the wild.
Maori tradition is rich in beliefs concerning gathering, preparing, and serving of food. In a survey by Ashcraft (1985), participants aged 50 or older from Ngati

Raukawa (sub-tribe of Waikato) related to a variety of beliefs that they continue to practise today. Out of the 25 members less than 50 years of age who were questioned, 20 could not explain the purpose of the belief other than *"… that's what I was told by my parents"*.

Food also has a strong social and cultural meaning. Manaaki tangata is the custom of being aware of and caring for the needs of your guests. Food is a fundamental way of expressing this ethos. In turn, the Mana of the Tangata-whenua is both upheld and enhanced. The loss of the ability of Tangata-whenua to provide for guests in this way can also be seen as a loss of Mana (Canterbury Regional Council Policy Statement, 1998).

Tradition and modern times.

Traditions specific to Maori foods are considered an event of spiritual importance. As followers of divine laws Maori people follow ceremonial rituals that pertain to agricultural and fishing before these foods are used in daily life. This is evidenced today by the blessed acknowledgements or grace prior to meals at all marae, all Maori functions, and in the home.

Freshwater environments have traditionally been valued by Tangatawhenua as a rich source of mahinga kai (food supplies). In Pond's draft (1997), Tangatawhenua consider traditional sources of food supplies have been degraded. The discharge of waste and human wastes in particular has compromised significant water bodies as sources of traditional food supplies for Maori. In some

locations private ownership of land may restrict access by Tangatawhenua to traditional food source sites.

The gathering of food assumes importance. It is a matter of pride to offer guests food for which the area is renowned. It is equally important to be able to assure guests that the food came from an area free of pollution. The discharge of sewage and industrial waste into traditional Maori mahinga kai sites today destroys this Mana (Whati, 1983).

Environmental concerns

With regard to overall harvesting, the biggest environmental concern is that Maori do not have control over the environment where wild traditional Maori food grows. The main environmental concerns are a) access to our mahinga kai sites b) unknown pollutants at harvesting areas c) black market of Maori kai; desecration of waahi tapu sites.

Sanitation in Maori traditional foods

Today, the sanitation idea takes a role in harvesting and preparation of traditional Maori foods. This is due to the introduction of feral animals and dairy cows polluting waterways where wild foods are harvested with their faeces. Maori will not harvest watercress, puha or mussels when there is evidence of animals and their dung as this is considered as a direct violation of mana kai.

Knowledge of harvesting

In Ngati Porou, to gain knowledge is for the seeker to look for it. It doesn't find you. Knowledge of harvesting is found at the homelands of many Maori. In each hapu Marae have their own knowledge of harvesting where there are specialists of the trade. Whanau of these specialist can retrieve this unique knowledge if they seek it (D. Tipene-Leach pers. comm.).

Traditional Maori foods are harvested from different environments such as fresh waterways, forests, sea and any of these niches can be contaminated by harmful micro-organisms.

Although there has been significant reduction in mahinga kai sites since colonisation and confiscation of Maori lands in the 1800s, Maori still harvest food traditionally as in pre-European times. There is no specific information on food-borne illness among Maori in pre-European times to compare with post-colonial times. However, such illness probably occurred as it is known that Maori had used rongoa (Maori medicine) to cure stomach upsets (Riley, 1994).

There is a worldwide resurgence of interest in the cultures of indigenous peoples, including Maori, and part of this interest is focused on indigenous foods. With this increased interest come new highlights of possible environmental contamination whilst harvesting these foods from the wild. As well as a possibility of new methods leading to the loss of authentic traditional ones, there is a possibility that contemporary adaptations of traditional techniques may negate practices which had produced safe food for centuries (Whyte, 2001).

1.3. Toroi: a traditional Maori food

Toroi, means *to brew; to ferment* (Ngata, 1993). In pre-European times Maori practised a number of food preservation methods through independent invention and/or preparation methods and rituals were passed down through the generations. Numerous accounts in the literature discuss how Maori used preservation methods to maintain their food supply during the off-seasonal harvesting times (Best 1898; Buck, 1974; Colenso, 1880; Dieffenbach, 1843; Fankhauser, 1986; Pomare and Ormsby 1995; Yen, 1959). Preservation methods such as drying, storing, and different types of fermentation processes were used and all details of harvesting and preparation were overseen by a tohunga (a specialist).

In post-European times, Maori adopted some new preparation methods and preserving techniques. Early English settlers introduced "bottling" and this method was widely accepted by Maori due to the glass bottles and screw caps that were readily available. Maori considered this technique useful because it preserved "left-overs" from the mixture of boiled plant material and meat in a pot known as "boil-up" that was a popular meal. Thus Toroi, the most common post-European traditional Maori food delicacy was formed.

1.3.1. Description of Toroi.

Toroi, is a commonly eaten preserved food and it is essentially "Maori food" packed into preserving jars. Popular Toroi ingredients include mussels and puha (*Sonchus* spp.) and watercress (*Nastaurtium officinale*). Other ingredients that are also widely used are freshwater fish (or its contents e.g., eggs, kidney and heart –

te puku o te ika), and seafood (fish and shellfish). The shellfish/fish component is mixed with plant material such as pikopiko, kouka, watercress, dandelion, puha; (just to name a few); the resulting mixture is packed into glass preserving jars and usually stored at refrigerated temperatures to allow fermentation to occur before the Toroi is eaten. As well as plant/fish mixtures, Toroi can be prepared and fermented as individual ingredients that are mixed immediately prior to consumption.

Both selection of ingredients and processing methods depend on the preference of the preparer and these can be closely guarded within a whanau or hapu (sub-tribe).

In this study, Toroi was prepared using procedures that the research of Whyte *et al. (*2001) and Hudson *et al. (*2001) have identified as being common across many hapu particularly Ngai Tahu. These workers identified three methods all of which involved preparation of Toroi from green-lipped mussels (*Perna canaliculis*; kukutai) and either puha (*Sonchus asper*) or watercress (*Nasturtium officinale*). These methods were: Method (1): boiling chopped puha for 30 – 60 minutes, draining, adding chopped mussels which have been steamed open, and packing into preserving jars. Retained mussel juice and/or plant juice is added and the jar overlain with fat or screw-capped. The Toroi mixture is then stored in cool conditions; Method (2): uses a similar method to (1) except that some salt is added to the cooked plant material (puha or watercress) and then stored under cool conditions. Chopped mussels are added just prior to eating. The plant material may be replenished as the existing mixture is consumed; Method (3):

chopped mussels and plant material are boiled together for 20 minutes, placed into containers with air excluded if possible, and stored under cool conditions. The process for method (1) begins with a thorough boiling of the puha (or substitute vegetable). The mussels are steamed to open their shells, the flesh removed, chopped and added to the cooked puha. In method (2), the process involves the preservation of the green vegetable portion only and steamed fresh mussels are added immediately prior to consumption. The container of puha is topped up with more puha as the supply is depleted. In method (3), both puha and mussels are cooked, combined, and then further cooked before bottling and sealing**.**

From a microbiological point of view for all three methods it is likely that vegetative cells on plant material will be inactivated but not spores. Method (1) and (2) steam rather than cook mussels but in method (2) they are eaten very soon after steaming. All three methods involve steps where the food could receive cross-contamination and similarly all three require adequately cleaned and sanitised storage containers. Method (2) involves adding salt with the intention of improving "preservation," but Whyte *et al. (*2001) suggested that the recommended concentration was not high enough to actually achieve this status.

1.3.2. Description of main food ingredients in Toroi

Puha *(Sonchus species)*

Puha or puwha (Sow thistle) (Figure 4) is commonly found in crops, gardens and waste areas (Turner *et al*., 2005). Sow thistle is an annual weed, which can

establish at any time of the year and Maori eat it as a vegetable (Turner *et al*., 2005; Crowe, 2004).

There are four species of puha in New Zealand, and is a favourite green vegetable for Maori with the most popular eating variety being the smoothed leaved *Sonchus asper* (Rauroroa) rather than the prickly sow thistle (*Sonchus oleraceus*; (Rauriki) (Figure 1.2). Puha can grow up to a metre tall and have small dandelion-like flowers and a milky sap. The fluffy seed heads (Figure 1.2) allow the wind to spread the seeds widely. They grow almost anywhere in New Zealand where there is waste or cultivated land, including urban environments (Crowe, 2004).

Traditionally, Maori steamed puha with other food in a hangi (food oven in the ground). Colenso (1880) recorded that it was often cooked with fresh fish. Today the usual method is to boil it with potatoes and meat - usually pork, but also with bacon bones, mutton-birds and sometimes with mutton or beef bones. Puha and pork when cooked together are complementary, with the pork moderating the natural bitter taste of the puha, and the puha adding flavour to the pork. The water the puha is cooked in (with or without meat) can also make a good soup base. While puha is still widely eaten by Maori people it is also eaten by some European New Zealanders - a legacy of recommendations made during the Depression in the 1920s, and later during the Second World War when food was often in short supply (Ministry for the Environment, 2003).

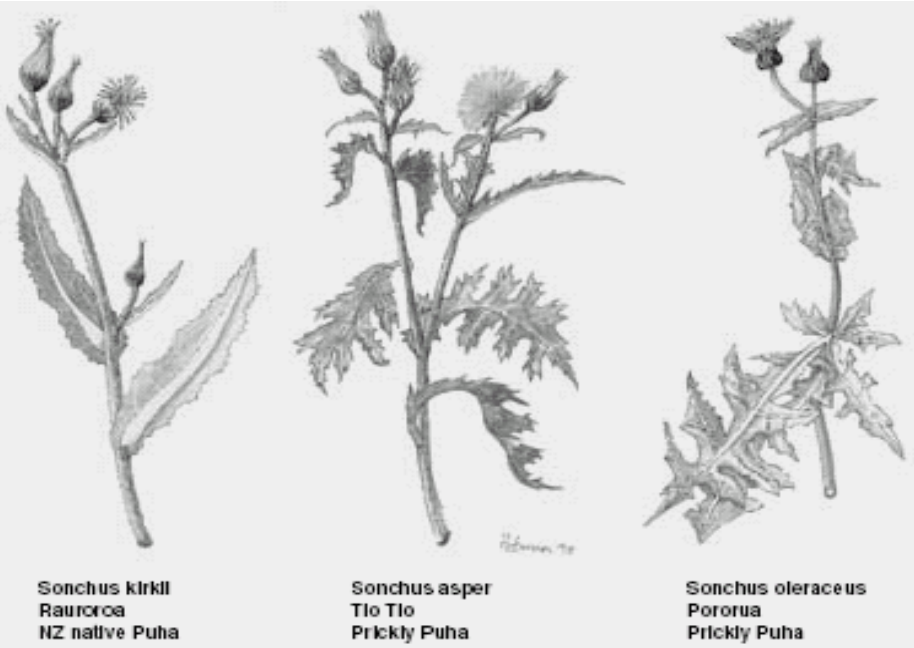

Figure 1.2. Edible *Sonchus* found in New Zealand (Ministry for the Environment, 2003)

In the 1997 National Nutrition Survey puha was reported to be consumed by 0.4% of the randomly selected study respondents, of which 2% reported eating puha one to six times per week. The mean daily intake of puha was calculated as 0.9 g/person/day for the total population (Russel *et al.*, 1999). A concern with the consumption of puha is harvesting from roadsides as these plants can be contaminated with pollutants and pesticides (Turner *et al.*, 2005).

Watercress (*Nasturtium officinale*)

Watercress belongs to the *Cruciferae* or mustard family. Its natural habitat is temperate climates where it grows by springs, flat river banks, and ditches. Watercress, a perennial, grows wild in New Zealand and starts to sprout in the spring. The plant is bright green in colour and leaves grow from nodules along the

main stem. The leaves are round to oval in shape and the white flowers bloom in early summer and grow on tips of stems in loose clusters. The name *Nasturtium* is derived from the Latin words *nasus tortus*, which means convulsed nose, referring to the smell (Webb *et al.*, 1998).

Maori used watercress to cover the hot stones in a hangi (ground oven pit), and boiled it with meat ("boil up"), or blanched and preserved it with mussels and its juices in the dish now known as Toroi. However, in recent times Maori and other New Zealanders are increasingly eating watercress raw. Watercress is sold in retail outlets and market stalls and harvested watercress is available throughout the year. Traditional Maori tikanga around food gathering and food handling in the past may have provided a level of protection from some food safety hazards. A study of traditional methods of watercress harvesting and common methods used today, were shown to reduce bacterial loading by careful selection of harvesting site and careful picking and washing of the plants (Dixon, 2002)

Recent studies have shown that watercress harvested from streams flowing through agricultural land is usually contaminated with high levels of the faecal bacterium *E. coli* (Dixon, 2002). Other workers have demonstrated contamination with the pathogen *Campylobacter* (Edmond and Hawke, 2004). Edmond and Hawke (2004) collected watercress from 11 streams in the greater Wellington region that they classified as; urban, semi-urban and rural. Of these, five were known to be sites where watercress was gathered on a regular basis for commercial sale or personal consumption. These workers measured *E. coli* as an "indicator" of faecal contamination in both watercress and the surrounding water

and in addition they also measured the pathogen *Campylobacter*. *Campylobacter* was recovered from 11% of their watercress samples, and was more common in plants harvested from streams in rural and semi-urban catchments.

Between August 1997 and August 2004, seven cases of gastroenteritis were reported as being possibly linked to consumption of watercress (Turner *et al.*, 2005). Of the two cases of campylobacteriosis, one was recorded on watercress collected from a farm. Giardiasis was identified in three cases, and salmonellosis in a further two cases. Of these, only two cases (one of each microorganism) were identified as definitely harbouring these microorganisms which came from a local stream from which the watercress was sourced (Turner *et al.*, 2005).

Green-lipped mussels (*Perna canaliculus*)

There are many species of shellfish in New Zealand called mussel, all of which are filter-feeders. The green-lipped mussel (*Perna canaliculus*; kukutai) is the most well-known due to its popularity as a commercial species (Greenshell™). The green-lipped mussel is widespread throughout New Zealand and lives on rocky shores below low water to 60 m in harbours, exposed open shores and protected areas (Turner *et al.*, 2005).

To obtain food, shellfish filter from four to 20 litres of water every hour (Meekin, 1998), in the process they accumulate bacteria from the surrounding waters (Greening *et al.*, 2001; Lee *et al.*, 1998). High concentrations of microbes can accumulate within a few hours when shellfish are actively feeding in polluted waters (Greening *et al.*, 2001). Some of this bacterial loading can be reduced by

depuration (transferring shellfish to clean water, usually for one to two days) (Turner *et al.*, 2005), but the rate of depuration depends on the temperature, flow rate, species and initial level of bacterial contamination (Buisson *et al.*, 1981). Faecal coliforms are bacteria that are used as a marker for faecal contamination (Turner *et al.*, 2005; Dufour, 1977) but they do not cause infectious illness.

Contaminated commercially produced mussels have caused listerosis, but there are no reports of illnesses associated with feral shellfish in New Zealand (Turner *et al.*, 2005). However, shellfish collected from sewage-contaminated water has been linked with salmonellosis (Baker and Wilson, 1993). *Salmonella wohlen* was isolated from a cockle in 1992 at Ahuriri estuary (Health Care Hawkes Bay, 1993). As well as bacteria, filter feeding shellfish can also accumulate viruses.

There have been some attempts to characterise people harvesting non-commercial feral shellfish on the basis of ethnicity. It has been estimated that over a one year period, 14% of harvesters were Maori; 2% were Pacific Islanders (Kearney, 1999); and 84% belong to other ethnic groups (Wilson, 1996). Although there is no evidence for Maori specifically, an evaluation of Auckland Pacific Island people found that more than half ignored warning signs saying it was not safe to collect shellfish (Fakalago, 2001).

1.4. Safety of Ready-to-Eat Foods

Toroi is considered as a ready-to-eat food as defined:

> *"food that is ordinarily consumed in the same state as that in which it is sold and does not include nuts in the shell and whole, raw fruits and vegetables that are intended for hulling, peeling or washing by the consumer"* (FSANZ, 2001).

Therefore the investigations of Toroi described in this study were based on the general requirements for food safety for ready-to-eat foods.

1.4.1. Food Standards

There are two standards that apply to ready-to-eat foods in New Zealand.

1. `Microbiological Reference Criteria for Food' (Ministry of Health, 1995).
2. Food Standards Australian New Zealand `Guidelines for the microbiological examination of ready-to-eat foods' (FSANZ, 2001).

Although it needs to be noted that foods gathered outside of normal commercial food production activities are generally not subject to monitoring, food safety programmes, or other risk management measures (Turner *et al.*, 2005).

*Microbiological Reference Criteria for Food***.**

Microbiological Reference Criteria for Food (Ministry of Health, 1995) are formulated as a guide for regulators to plan surveillance programmes that assess whether foods are safe, marginal or unacceptable/unsafe (Table 1.1). They apply until the end of the given shelf life of a particular food. The Microbiological

Reference criteria are not part of a New Zealand Law. They are intended to be used when no standards exist in law to monitor the microbiological safety of a manufacturing process or the safety of a food.

Aerobic Plate Count at 35°C (g^{-1})	n = 5	c = 2	$m = 10^4$	$M = 10^5$
Bacillus cereus (g^{-1})	n = 5	c = 2	$m = 10^2$	$M = 10^3$
Campylobacter (10 g^{-1})	n = 5	c = 0	m = 0	
Clostridium perfringens (g^{-1})	n = 5	c = 2	$m = 10^2$	$M = 10^3$
Coagulase producing staphylococcus (g^{-1})	n = 5	c = 2	$m = 10^2$	$M = 10^3$
Escherichia coli (g^{-1})	n = 5	c = 0	m = 0	
Listeria monocytogenes (25 g^{-1})	n = 5	c = 0	m = 0	
Salmonella (25 g^{-1})	n = 5	c = 0	m = 0	

Table 1.1. Microbiological reference criteria for Foods – cooked, ready-to-eat (or with subsequent minimal heating (<70°C) (Taken from the Microbiological Reference Criteria for Food, Ministry of Health, 1995).

The following terms as used by the International Commission of Microbiological Specification for Foods (ICMSF) are used in the reference criteria for the ready-to-eat foods:

n = the number of sample units which must be examined from a lot of food to satisfy the requirements of a particular sampling plan

c = the maximum allowable number of defective sample units. When more than this number is found, the lot is rejected by the sampling plan.

m = Represents an acceptable level and values above it are marginally acceptable or unacceptable in the terms of the sampling plan.

M = A microbiological criterion which separates marginally acceptable quality from defective quality. Values above M are unacceptable in the terms of the sampling plan and detection of one or more samples exceeding this level would be cause for rejection of the lot.

FSANZ

The "Food Standards Australian New Zealand Guidelines for the microbiological examination of ready-to-eat foods" *(*FSANZ*, 2001)* set out the microorganisms that need to be measured in ready-to-eat foods sold in retail premises (Table 1.2). Although these regulations cannot be enforced in private organisations such as a Marae, the NZFSA wild foods group encourages knowledge of them in these situations (Cherie Flynn pers. comm.). These guidelines identify four categories of microbiological quality for ready-to-eat foods ranging from satisfactory to potentially hazardous. This reflects both the high level of microbiological quality that is achievable for ready-to-eat foods in New Zealand and indicates the level of contamination that is considered to present a significant risk to public health. The guidelines for the microbiological examination of ready-to-eat foods apply to foods sampled at the point of sale or distribution to consumers.

Categories of microbiological quality

Satisfactory - results indicate good microbiological quality.

Marginal - results are borderline in that they are within limits of acceptable microbiological quality but may indicate possible hygiene problems in the preparation of the food. Action: Re-sampling may be appropriate. Premises that regularly yield borderline results should have their food handling controls investigated.

Unsatisfactory - results are outside of acceptable microbiological limits and are indicative of poor hygiene or food handling practices. Action: Further sampling, including the sampling of other foods from the food premises may be required

and an investigation undertaken to determine whether food handling controls and hygiene practices are adequate.

Potentially Hazardous - the levels in this range may cause food borne illness and immediate remedial action should be initiated. Action: Consideration should be given to the withdrawal of any of the food still available for sale or distribution and, if applicable, recall action may be indicated. An investigation of food production or handling practices should be instigated to determine the source/cause of the problem so that remedial actions can commence.

Microbiological Quality (CFU g^{-1})

Test	**S**	**M**	**US**	**P H**
Standard Plate Count	.	.	.	.
Level 1	< 10^4	< 10^5	Greater than or equal to 10^5	.
Level 2	< 10^6	< 10^7	Greater than or equal to 10^7	.
Level 3	N/A	N/A	N/A	.
Indicators	.	.	.	.
Enterobacteriaceae *	< 10^2	10^2-10^4	Greater than or equal to 10^4	.
Escherichia coli	< 3	3 - 100	Greater than or equal to 100	**
Pathogens	.	.	.	.
Coagulase +ve staphylococci	<10^2	10^2-10^3	10^3-10^4	Greater than or equal to 10^4SET +ve
Clostridium perfringens	<10^2	10^2-10^3	10^3-10^4	Greater than or equal to 10^4
Bacillus cereus and other pathogenic*Bacillus* spp	<10^2	10^2-10^3	10^3-10^4	Greater than or equal to 10^4
Vibrio parahaemolyticus #	<3	<3 -10^2	10^2 -10^4	Greater than or equal to 10^4
Campylobacter spp	not detected in 25g	-	-	detected
Salmonella spp	not detected in 25g	-	-	detected
Listeria monocytogenes	not detected in 25g	detected but <10^{2} ++	-	Greater than or equal to 10^{2} ##

Table 1.2. Guideline levels for determining the microbiological quality of ready-to-eat foods (FSANZ, 2001).

Satisfactory = **S**; Marginal = **M**; Unsatisfactory = **US**; Potentially Hazradous = **PH**

1.5. Food safety and Toroi

To determine whether Toroi is safe to eat, tests for food borne bacteria known to be of concern in ready-to-eat foods need to be carried out on sufficient number of samples as described in the two standards set out in Section 1.4.

In this study, all nine bacterial groups (FSANZ, 2001) were investigated on five batches of Toroi (Ministry of Health, 1995). Two of the nine groups are the general indicator Plate Count Bacteria and *E. coli*. Plate Count Bacteria identify the general microbiological status of a food, *E. coli* is measured as an indicator of faecal contamination that can be introduced either on ingredients or during handling. The remaining seven bacteria are specific pathogens of demonstrated importance in ready-to-eat foods.

1.5.1. General Indicators

Plate Count Bacteria

Plate count bacteria are measured in food to indicate the level of microorganisms in a product (USFDA, 2001). Plate count bacteria are not a species grouping but are a physiological grouping of heterotrophic bacteria that grow under the conditions specified for a particular test. Although there are defined test procedures for different foods most use the same microbiological growth medium, Plate Count Agar. Test variations are based on incubation conditions including temperature, incubation time and gaseous atmosphere.

Aerobic plate counts on fish and fishery products generally do not relate to food safety hazards, but can be useful to indicate quality, shelf life and post heat-processing contamination. Fresh fish and fishery products often have an APC of 10^4- 10^5 g^{-1}, although there are examples of sea foods with an APC of 10^6- 10^8 g^{-1} without objectionable quality changes (USFDA, 2001). For many fish and fishery products, a plate incubation temperature of 25ºC produces significantly higher numbers of bacteria than incubation at 35ºC and was the incubation temperature used to measure these bacteria in Toroi in this study.

Because Toroi is a fermented product plate count bacteria that grow in an anaerobic atmosphere were also measured in this study (Hudson *et al*., 2001).

Escherichia coli

As many pathogens are transmitted by the faecal-oral route it is important that food, and water are free from faecal contamination. To ensure food safety, authorities require routine testing for faecal-specific bacteria including: coliform bacteria, faecal coliform bacteria, or *Escherichia coli*. Increasingly *E. coli* measurement is favoured by food protection agencies because it's only known habitat is the intestinal tract of warm-blooded animals (including humans) but coliforms/faecal coliforms can include other species that are not of faecal origin (Dufour, 1977). Although *E. coli* makes up only very small proportion of the total bacterial species that inhabit the intestinal tract it is consistently present in faeces, which, together with the relative ease of recovery in microbiological laboratories makes it an ideal "indicator" of faecal contamination. Measurement of indicators is useful but their recovery only "indicates" a risk (Berg and Metcalf,

1978) and does not necessarily mean that any specific enteric pathogen is present (Grabow, 1996)

Faecal coliforms are found in storm water discharges, and the concentration of these bacteria in cockles was observed to positively correlate with rainfall events in the Whangateau Harbour and Ahuriri estuary (De Luca-Abbott *et al*., 2000; Health Care Hawkes Bay, 1993). Faecal coliforms are commonly isolated from shellfish near sewage outfalls (Lewis *et al*., 1986). Cockles growing in waters near communities or individuals using pit toilets and septic tanks can show irregular contamination events that may be related to malfunctioning or overflowing of sewage treatment systems (Nicholson *et al*., 1989).

There are a number of strains of *E. coli* that are pathogenic. Specifically the Shiga-toxin producing strains such as *E. coli* O157:H7. These *E. coli* are not included among the required organisms for measurement in ready-to-eat foods and were not sought in this study.

1.5.2. Specific Pathogens

Spore Formers

A number of genera of bacteria, including *Bacillus* and *Clostidium* produce structures called endospores within their cells. Endospores are differentiated cells that are very resistant to heat and cannot be destroyed easily by normal cooking processes. Endospore-forming bacteria are commonly found in the soil (Madigan *et al*., 1997), and are therefore also common on plants. Some spores are activated by heating for several minutes at a sub lethal but elevated temperature, or by

storing a spore suspension for weeks or months at 4^{o}C or room temperature (Madigan *et al*., 1997). Activated spores are conditioned to germinate when placed in the presence of specific nutrients.

Foodborne spore formers, including *Clostridium* and *Bacillus* species, are quite common in cooked meat and poultry, cooked vegetables, soups, boiled rice and pasta. However the spores of different species can vary widely in their heat resistance.

1a. Clostridium perfringens

Clostridium perfringens is a spore-forming bacterium that is Gram-positive, non-motile, straight rods with blunt ends that occur singly or in pairs and are 0.6-2.4 x 1.3-19.0 μm (Sneath, 1984). *C. perfringens* is a natural inhabitant of soil and the intestinal tract of many warm-blooded animals and humans (Peck *et al*., 2004). This bacterium and its spores are very wide spread, and is therefore a common problem for the food industry and establishments where large amounts of foods are prepared (Andersson *et al.*, 1995), and this also applies to Marae preparing Toroi.

Food poisoning due to *C. perfringens* is one of the most common types of human foodborne illness (Labbe, 1992). The foods usually involved are cooked meat or poultry products, and infection has been related to contamination with large numbers of viable cells. A heat-labile enterotoxin produced only by sporulating cells induces the major symptom of diarrhoea in perfringens poisoning. *C. perfringens* causes human gastroenteritis, meningitis, appendicitis and gas

gangrene as well as respiratory and urinary tract infection (Hill, 1981). The wide range of infections suggests that *C. perfringens* may be an opportunistic pathogen.

When vegetative *C. perfringens* cells are present in the common Toroi ingredients such puha, watercress and mussels before cooking, they will grow (with sufficient protein sources) at temperatures between 15 and 52^{o}C (Peck *et al*., 2004). Vegetative cells are readily killed by heating but spores are very heat resistant (Ministry of Health, 2001c). Heat-resistant spores generally require high temperatures, such as those of many cooking procedures (10-20 minutes at 75 to 80^{o}C), to activate germination (Labbe, 1992; Murrell, 1989). However, most spores that have undergone chilling and freezing processes cannot germinate and grow under such conditions (ICMFS, 1978). Therefore, food that is cooked and chilled promptly until consumed is not a high risk food. As *C. perfringens* enterotoxin is inactivated by heating for five minutes at 60^{o}C (Ministry of Health, 2001c), any pre-formed toxin on ingredients will be destroyed during the cooking process.

1b. Clostridium botulinum

Clostridium botulinum can be widely distributed in the environment, although the contamination level is generally low (Peck *et al.,* 2004). Nowadays, commercial processing procedures are designed to prevent survival and growth of *C. botulinum* (Hielm *et al.,* 1998; Gill and Penney, 1982; Bremner and Fletcher, 2003; Peck *et al*., 2004) so that most outbreaks of foodborne botulism are associated with home prepared foods. In the USA, approximately 30 cases are reported per year, with most cases associated with home canned vegetables or

fermented marine products (e.g. fermented beaver tail and paw, muktuk (whale meat) prepared in Alaska) (Peck *et al*., 2004). Foodborne botulism is a severe but rare disease caused by consumption of pre-formed botulinum neurotoxin with, for type A, as little as 30 ng of neurotoxin sufficient to cause illness or death (Peck *et al*., 2004). The consumption of as little as 0.1 g of food in which neurotoxin-producing clostridia has grown can result in botulism (Lund and Peck, 2000). There are seven botulinum neurotoxin (A to G), with the type of neurotoxin formed dependent on the producing organism (Hauschild, 1989).

Toroi consumption was the first incidence (of three) of botulism reported in New Zealand (Flacks, 1985). In this incident, mussels had been removed from their shells, brought to the boil and then added to watercress, which had been boiled for two hours. This mixture was dispensed into screw top jars and stored at room temperature. A jar was opened one week later and consumed over the following week during which time intoxication developed and the outcome was one fatality. *C. botulinum* spores may be introduced on the vegetable material (particularly *C. botulinum* type A) or with mussels, as type E is associated with the marine environment (Hielm *et al.*, 1998). Although it is thought that *botulinum* types toxic to human do not occur frequently in New Zealand (Gill and Penney, 1982), *botulinum* types C and D have been detected in aquatic sites in New Zealand and have been associated with disease in wild fowl (Gill and Penney, 1982). A further type, type G has been isolated from soil in Argentina, no outbreaks involving it have been reported (Bremner and Fletcher, 2003).

Recommendations have been produced to prevent a risk of botulism in production of ready-to-eat foods by catering establishments as shown in Table 1.3. (Peck *et al*., 2004).

Storage below 3.0^{o}C,
Storage below 5^{o}C with shelf life restricted to 10 days,
Storage up to 8^{o}C with shelf life restricted to 5 days,
Heating at 90^{o}C for 10 min (or equivalent) with storage below 8^{o}C,
pH below 5.0 with storage below 8^{o}C,
NaCl concentration above 3.5% with storage below 8^{o}C

Table 1.3. Recommendations of inhibition of non-proteolytic *C. botulinum* in *sous-vide*. Table taken from Peck *et al. (*2004).

Analysis of *C. botulinum* was not carried out in this study. This organism is not included in the Guidelines for Ready-to-Eat foods presumably due to its very low incidence in New Zealand.

2. Bacillus cereus

Bacillus cereus belongs to the genus *Bacillus* Group I as defined by Gordon *et al*. (1973). The morphology of *B. cereus* is large (1.0-1.2 μm in width and 3-5 μm in length (Sneath, 1984; Jenson, 1997). It does not have a swollen sporangium; spore shape is ellipsoidal; and spore position is central (Sneath, 1984).

B. cereus is widely distributed in nature including soils containing low levels of organic matter (Jenson, 1997) and can be isolated from a variety of foods. Plants are the major source of *B. cereus* in foods (Jenson, 1997). Consumption of food containing more than 10^5 viable *B. cereus* cells per gram has resulted in outbreaks

of food poisoning (Harmon *et al.,* 1992). It causes two different forms of food poisoning: an emetic illness and a diarrhoeal illness. The emetic illness is mediated by a highly stable toxin that survives high temperatures and exposure to trypsin, pepsin and pH extremes. The diarrhoeal illness is mediated by a heat-and acid-liable enterotoxin (Ministry of Health, 2001a). For both types of food poisoning the food involved has usually been heat treated and surviving spores are the source of the food poisoning. *Bacillus* species find their way into the food chain. *B. cereus* occurs in soils containing low levels of organic matter whilst other species may be found in soils containing higher levels of organic matter (Jenson, 1997). Raw foods of plant origin are the major source of *B. cereus* in foods (Jenson, 1997). Foods incriminated in outbreaks of *B. cereus* poisoning include boiled and fried rice, cooked pasta, cooked meats, cooked vegetables, soups, salads, puddings, and vegetable sprouts.

The optimum temperature of *B. cereus* is 30 – 37^{o}C (NZFSA, 2001; Valero *et al*., 2003; Choma *et al.*, 2000) . Some strains can grow up to 55^{o}C (Ministry of Health, 2001a) while other strains (psychrotrophic) can grow as low as 5-15^{o}C (Valero *et al*., 2003) so that slow cooling of cooked foods would allow *B. cereus* growth (Nauta *et al.,* 2003; Valero *et al*., 2003). The minimum pH for *B. cereus* growth is 4.3 and maximum pH is around 9.3 (Ministry of Health, 2001a). Vegetative cells are readily killed by heat but spores are moderately heat resistant. Spores are more resistant to dry heat than moist heat. Emetic toxins are extremely resistant to heat and can survive for 90 minutes at 126^{o}C. The diarrhoeal toxins are inactivated five minutes at 56^{o}C. *B. cereus* is not a competitive microorganism but grows well after cooking and cooling (<48^{o}C) (Granum and

Lund, 1997). The heat treatment in Toroi will cause spore germination and in the absence of competing flora *B. cereus* grows well. During the subsequent cooling period the bacterial cells would multiply. Incidences of food poisoning due to *B. cereus* have been related to failures to maintain refrigerated temperatures (Choma *et al.*, 2000).

To prevent growth of *B. cereus* including pyschrotrophic strains either the temperature needs to be below 5^{o}C or the pH reduced particularly to minimally processed foods (Valero *et al.*, 2003). For example, acidification at pH 5.0 was sufficient to inhibit *B. cereus* growth in acidified carrot juice (Valero *et al.*, 2000) so it is important to store Toroi in a refrigerator at temperatures below 5^{o}C quickly after cooking. The only reported Maori food study in which *B. cereus* was detected was a preliminary study by Hudson *et al.* (2001). They found 70 *B. cereus* cells g^{-1} in one sample of fresh Toroi stored for approximately two weeks. Analysis for *B. cereus* in Toroi was carried out in this study.

Thermotolerant Campylobacter

The main reservoirs of thermotolerant *Campylobacter* are animals especially birds (Donnison and Ross, 1999), with common source of outbreaks being traced to food, water or un-pasteurised milk. The species *C. jejuni* and *C. coli* are two species implicated in most cases of human disease. Some strains of *C. jejuni* are highly infectious, with the infective dose reported to be as low as 800 cells (Black *et al.*, 1988). More than 12,000 cases of campylobacteriosis were reported to the New Zealand Ministry of Health in 2002 and rates were consistently high across

the country for example with 1,026 cases in 2001 and 1,088 in 2002 in the Waikato Region (Donnison and Ross, 1999).

There is one published report detailing contamination of watercress by *Campylobacter*. Watercress was sampled from 11 streams (urban, semi-urban and rural) in the greater Wellington region. Of these five were known to be sites where watercress was gathered on a regular basis for commercial sale or personal consumption. *Campylobacter* was present in 11% of the watercress samples, and was more common in rural and semi-urban catchments (Edmond and Hawke, 2004). Campylobacteriosis has been associated with the consumption of raw fruits and vegetables (Beuchat, 1998) but is considered rare.

Campylobacter are readily destroyed by cooking, but there is a risk if food is undercooked (particularly chicken). Cross contamination from raw foods is very common.

An Australian study with Sydney rock oysters found that shellfish were capable of accumulating *Campylobacter* and could remain viable after storage at low temperatures (Arumugaswamy *et al*., 1988). Between 1997 and August 2004 there were seven cases of gastroenteritis caused by campylobacteriosis where shellfish was suspected as being the causative agent (Turner *et al.*, 2005). Therefore, although *Campylobacter* would not survive the cooking processes for watercress or puha, they may survive in the mussels so it is possible that this organism may be present in Toroi particularly when mussels are collected from the wild.

Salmonella enterica

The genus *Salmonella* belongs to the family Enterobacteriaceae. The principle site of *Salmonella* infection is the intestinal tract, and the primary route of infection is by ingestion (ICMSF, 1978). In New Zealand infection reported foodborne infection is caused by non-typhoidal species within the genus *Salmonella*, principally serotypes and ecotypes of *Salmonella enterica*. The dose required to cause infection varies with the serotype involved, the nature of the food carrying the *Salmonella* and the health status of the individual (MIRINZ, 2004b). *Salmonella* tolerate a wide range of acidity, temperature, and moisture conditions and therefore can survive well or even grow in foods.

Major inputs of *Salmonella* into the aquatic environment are mostly from effluent, sewage sludge and from farm livestock (including run off from pasture). Factors contributing to the spread of *Salmonella* amongst the Maori population include contamination of soils and waterways where wild foods are traditionally harvested and large centrally located food preparation of wild foods (e.g. on a Marae). Between 1997 and August 2004 there were four cases of salmonellosis where feral shellfish were suspected to be the causative agent (Turner *et al.*, 2005).

Coagulase positive Staphylococcus aureus

Staphylococcus aureus is a Gram-positive, catalse positive, spherical shaped bacterium (Sneath, 1984).

The growth of *S. aureus* in foods presents a potential public health hazard since many strains of *S. aureus* produce an enterotoxin that causes food poisoning. The

most common symptoms are vomiting and diarrhoea, which occur two to six hours after ingestion of the toxin. The illness is relatively mild, usually lasting a few hours to a day, but occasionally the symptoms may be so severe that hospitalisation may be required.

Foods commonly associated with staphylococcal food poisoning are meat and poultry. Foods that poses the greatest risk of staphylococcal food poisoning are those in which the initial flora has been destroyed or inhibited e.g. by cooking, but are then handled during preparation such as salads and sandwiches. The most likely risk factors for Toroi would be in the handling during preparation or subsequently in serving the dish.

Vibrio parahaemolyticus

The genus *Vibro* is Gram-negative, non-spore bearing rods (Charles *et al.*, 1998). The species *Vibrio parahaemolyticus* is a marine vibrio inhabiting temperate and tropical waters around the world (Miwatani and Takeda, 1976). It generally resides in sediments during the winter months, but when water temperatures rise above 15^{o}C during the spring and summer, it spreads with the zooplankton throughout the marine environment (Kaneko and Colwell, 1973). *V. parahaemolyticus* may then be found in shellfish (Fletcher, 1985).

The organism requires NaCl concentrations of between 0.25 and 10% for growth, 2-3% is the optimum (Beuchat, 1982) and grows between 15 and 44^{o}C (optimum is 35 – 37^{o}C) (Beuchat, 1982) and can readily multiply in many foods including raw and cooked seafoods (Kodama, 1967).

It was reported that an infectious dose is between 10^5 and 10^7 cells (Fletcher, 1985) and that a dose of 10^5 cells corresponds to a probability of disease of around 10%, and 10^4 around 1% (Fletcher, 1985). However, Lake *et al. (*2003) consider that the only safe dose is zero.

In a survey of *V. parahaemolyticus* in New Zealand oysters (Fletcher, 1985), it was found that naturally occurring levels are generally low. However, this risk increases in locations where water temperatures increase substantially in summer. Fletcher (1985) also discovered high numbers of *V. parahemolyticus* in shellfish harvested from isolated local areas, in which the organic content of the surrounding water was high.

Infection with *V. parahaemolyticus* is not a notifiable illness in New Zealand (Lake *et al.*, 2003). A total of 32 sporadic cases of infection with *V. parahaemolyticus* have been reported to the national notification database between 1998 and 2003; 19 of these reported seafood as a risk factor, and eight of these 19 implicated Pacific oysters. Although contamination with *V. parahaemolyticus* would not be expected in commercially sourced shellfish, there could be a risk in Toroi preparation from shellfish gathered from the wild.

Listeria monocytogenes

Listeria species are regular, short rods (0.4-0.5 μm in diameter and 0.5-0.2μm in length) with rounded ends. They occur singly, in short chains, or cells may be arranged at an angle to each other to give V forms or in groups lying parallel

along the axis (Sneath, 1984). They are non-spore forming bacteria that prefer microaerophillic conditions but they can also grow aerobically and anaerobically (APHA, 2001).

The species *Listeria monocytogenes* is an important psychrotrophic foodborne pathogen with the ability to grow at temperatures ranging from 1 – 45^{o}C. The optimal growth temperature is in the range of 30 – 37^{o}C (Sneath, 1984; APHA, 2001). The organism is able to grow over this temperatures range due to the ability to modify the fatty acid composition of its cell membrane (Annous *et al.*, 1997). However, *L. monocytogenes* cannot survive heating at 60^{o}C for 30 minutes (Sneath, 1984). *Listeria* can also withstand relatively extreme pH and salt conditions (i.e. pH 4.5–9 and 10% NaCl), although optimal growth occurs at neutral pH and at 0.5% NaCl (McClure *et al.*, 1991). These properties enable *Listeria* to survive harsh environments. Farber and Peterkin (1991), found that the minimum pH that could support initiation of growth was 5.0 to 5.7 at 4^{o}C but this minimum extended to 4.3 (4.3 to 5.3) at 30^{o}C.

Infection by *L. monocytogenes* causes disease of two recognisable forms. One is listeriosis that has implications for pregnant women including spontaneous abortion or stillbirth. The second condition, known as non-invasive febrile gastroenteritis is associated with a high attack rate, and causes typical gastrointestinal symptoms.

L. monocytogenes is ubiquitous in the environment, commonly found in soil and water, and on plant material. *L. monocytogenes* has been reported as the causative

agent in three incidences of seafood-borne listeriosis, the most recent outbreak occurred in New Zealand in 1992 (Brett *et al.*, 1998) and involved smoked mussels. Other outbreaks of listeriosis have been reported in New Zealand; 20 cases in Auckland in 1969 (Flight, 1971) and 22 cases in Auckland in 1980 (Lennon *et al.*, 1984) but the source of infection was not established. However, it was suggested that shellfish and raw fish consumption may have been involved (Lennon *et al.*, 1984). Lovett *et al.* (1990) examined the ability of *L. monocytogenes* to grow in shrimp, crabmeat, surimi and white fish stored at 7^{o}C, and found that when *L. monocytogenes* was inoculated into samples of these products, which had been sterilised prior to inoculation, numbers increased by about five logs within 14 days.

The association of *L. monocytogenes* with shellfish, and the extended storage of Toroi at refrigerated temperatures, provide conditions that could produce a "high risk" food product. Because of this, *Listeria* species and *L. monocytogenes* in particular was sought in this study and various strategies with potential to limit its growth were also investigated.

1.6. Strategies to prevent growth of Cold Tolerant Foodborne Pathogens

Toroi is a fermented product in which fermentation can be carried out at refrigerated temperatures, as was the case with the Toroi prepared in this study. The ability of the cold tolerant pathogen, *L. monocytogenes* to grow at refrigeration temperatures and its association with shellfish were the reasons for a specific study being included to address ways to reduce the risk of Listeriosis being acquired from Toroi.

The physiology of growth at refrigeration temperatures, involves an effect known as homeoviscous adaptation. Cold tolerant microorganisms such as *L. monocytogens* can change the lipid composition of their membranes, allowing them to adapt to temperature changes. Increasing the degree of unsaturation in these membrane lipids decreases their melting point, allowing cell membranes to maintain a fluid nature and thus allows membrane proteins to function (Hazel, 1995). A second cell mechanism for survival at lower temperatures is the production of cold shock proteins (CSPs), which allow for increased environmental adaptability. *L. monocytogenes* has been found to have 12 CSPs that work to stabilize the cells when exposed to low temperatures (Bayles *et al*., 1996). These CSPs are found in nearly all bacteria and act to minimize secondary folding of cellular components, which is one mechanism of decreased cell activity and eventual death (Wemekamp-Kamphuis *et al*., 2002). Production of cold shock

proteins in this instance increased cell survival in the 4°C group, regardless of other treatments employed.

Fermentation is a process in which naturally occurring bacteria grow in food and produce end products that inhibit both spoilage bacteria and pathogens. A very important group of fermenters are the Lactic Acid Bacteria (LAB).

1.6.1. Lactic acid bacteria

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Lactic acid bacteria have been utilized by accident since Biblical times to extend the storage time for foods. The natural reservoir of lactic acid bacteria is green plant material (Vandenbergh, 1993). Early practises such as hand milking led to the involuntary contamination of raw milk with a variety of lactic acid bacteria. These strains converted the carbohydrate lactose in milk into acid, mainly, lactic acid, which prevented the growth of certain undesirable bacteria. Later these applications extended to food products that included: bread, buttermilk, cheese, fermented vegetables, fermented meats and yoghurt. Lactic acid bacteria produce a variety of metabolic products that inhibit the growth of other microbes. All lactic acid producers are micro-aerophilic i.e. that they require small amounts of oxygen to function; they do not grow anaerobically.

1.6.2. Inhibition of other microorganisms

In addition to producing lactic acid which decreases pH and inhibits the growth of many microorganisms, many lactic acid bacteria produce antimicrobial substances such as organic acids, hydrogen peroxide and diacetyl (Lewus and Montville, 1991). In addition, certain lactic acid bacteria produce bacteriocins (Barefoot and Klaenhammer, 1984). Bacteriocins are usually defined as proteins which produce

intra-species antagonistic effects. Some bacteriocins have been well-characterised and commercial production of nisin; produced by *Lactococcus lactis subsp. lactis* has been well documented. In dairy products nisin has been used to suppress spoilage agents and food borne pathogens. In meats, through fermentation (e.g. salami), lactic acid bacteria can also suppress the growth of undesirable bacteria.

Mode of action

The anionic lipid of the cytoplasmic membrane is the primary receptors for bacteriocins of lactic acid bacteria for initiation of pore formation (Chen and Hoover, 2003). Conductivity and stability of pores induced by lantibiotics may be heightened by docking molecules (lipid II, the peptidoglycan precursor), while in the case of class II bacteriocins, receptors in the target membrane act to determine specificity (Venema *et al.*, 1995). Class I bacteriocins induce pore formation according to a wedge-like model, and class II bacteriocins function by creating barrel stave-like pores whereby peptides orient parallel to the membrane surface and interfere with membrane structure (Moll *et al.*, 1999). Lactic Acid Bacteria have two main disadvantages as general anti-microbial species a) bacteriocins produced by protective cultures may inhibit other "desired" cultures and b) are not active against Gram-negative pathogens such as *E. coli* and *Salmonella* because their outer membrane protects them by excluding bacteriocins (Holzapfel *et al.*, 1995). However, manipulation such as the use of chelating agents such as EDTA or citrate, can bind magnesium ions to lipopolysaccharide layer of the outer membrane of Gram-negative bacteria, rendering these organisms susceptible to nisin and other bacteriocins (Stevens *et al.*, 1992).

1.6.3. *Lactobacillus sake*

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The bacteriocin produced by *Lactobacillus sake* LB 706 is sakacin A (Schillinger, 1994) which is classified in class IIa group of bacteriocins (Chen and Hoover, 2003) which includes the non-lanthionine-containing bacteriocins (Jack *et al*., 1995). Production of sakacin A by *L. sake* LB 706 was detected during an investigation of 221 strains of homo-fermentative lactobacilli (Schillinger and Lucke, 1989) which were isolated from different types of meats and meat products (Schillinger, 1994). Sakacin A is active against a variety of bacteria including *Listeria monocytogenes* (Schillinger, 1994; Vandenburg, 1993) although the antilisterial effect differs from strain to strain (Schillinger, 1994; Schillinger *et al.,* 1991). For example, one of the *Listeria* species used in inoculation experiments of minced meat (Schillinger *et al.,* 1991) showed a reduced sensitivity to sakacin A in comparison to three other strains of *L. monocytogenes*. An important property of sakacin A is that it is heat stable (Jack *et al.,* 1995; Schillinger and Lucke, 1989).

The mode of action of the non-lanthionine-containing bacteriocins such as sakacin A is that in order to come in to contact with the cytoplasmic membrane of *L. monocytogenes* the molecules must first pass through the cell wall, and there is evidence that sakacin A molecules may be absorbed on the surface of most Gram-positive bacterial cells, including sensitive and resistant strains (Jack *et al*., 1995). Sakacin A also effects their bactericidal action by destabilising the cytoplasmic membrane of *L. monocytogenes* (Chen and Hoover, 2003; Jack *et al*., 1995; Schillinger and Lucke, 1989). Low concentrations of these bacteriocins increases the permeability of the cytoplasmic membrane, as determined by the increased

influx of small molecules and efflux of UV absorbing materials (e.g. amino acids, K^+) from the cytoplasm. In addition, these bacteriocins dissipated the proton motive force (PMF) of *L. monocytogenes*, as shown by their influence on the uptake of amino acids whose influx is mediated by secondary and phosphate-bond-driven transport systems (Jack *et al*., 1995).

1.6.4. Nisin: an approved antimicrobial food additive

Rogers and Whittier first published on the inhibitory effects of nisin (Rogers and Whittier, 1928). It is a bacteriocin from the class I-type A lantibiotic group (Chen and Hoover, 2003), but the name "nisin" was claimed by Mattick and Hirsch (1947). Nisin's feature polycyclic structures that are very important in the membrane insertion properties of the bacteriocin. These ring structures protect the bacteriocin from proteolytic enzymes and thermal denaturation (Hurst, 1981). Nisin properties that would be useful in fermeted foods include solubility, and stability under acidic conditions. For example, nisin is stable when autoclaving (115.6°C) at pH 2.0, but 40 % of this activity is lost at pH 5.0, and more than 90 % at pH 6.8 (Chen and Hoover, 2003). Loss of nisin activity also occurs during storage particularly at higher pH and higher temperatures. However, nisin was found to be stable over 30 weeks in pasteurized cheese spreads (pH 5.8) at a storage temperature of 20°C (Delves-Broughton, 1990).

Application of nisin in food

Nisin is used as a natural, safe, antibacterial food preservative (Pongtharangkul and Demerci, 2004). In 1969, nisin was approved for use as an antimicrobial in food by the Joint FAO/WHO Expert Committee on Food additives (Broughton, 2005). Nisin is a 34-amino acid peptide. The most established commercially available form of nisin for use as a food preservative is Nisaplin^{TM} , with the active ingredient 2.5% nisin A and the predominate ingredients NaCl (77.5%) and nonfat dry milk (12% protein and 6% carbohydrate).

As previously mentioned, nisin is categorised both as a class I bacteriocin and a type-A lantibiotic (elongated peptides with a net positive charge). These peptides function by disrupting the membrane integrity (Chen and Hoover, 2003; Jack *et al.*, 1995; Pontharangkul and Demecri, 2004). Nisin has no effect on Gram-negative bacteria but only Gram-positive bacteria are affected and these types include LAB, vegetative pathogens such as *Listeria*, *Staphylococcus*, and the sporeforming *Bacillus* and *Clostridium* (Chen and Hoover, 2003). The spore of bacilli and clostridia are more sensitive to nisin than their vegetative cells, although the antagonism is sporostatic rather than sporicidal, thus requiring the continued presence of nisin to inhibit the outgrowth of the spores (Chen and Hoover, 2003). Heat damage of spores increased their sensitivity to nisin, so that nisin is effective against spores in low-acid, heat-processed foods, resulting in its use as a processing aid in canned vegetables (Delves-Broughton, 2005). Nisin at levels of 500 to 10,000 IU per g prevented the growth of *Clostridium botulinum* (Luck and Jager, 1995; Mazzotta *et al.*, 1997).

The mode of action for nisin against *L. monocytogenes* is that it inhibits the biosynthesis of DNA, RNA, protein and polysaccharides, leading to speculation that treated cells no longer have sufficient energy to carry out biosynthesis and that the energy-transducing cytoplasmic membrane may be the primary biochemical target (Sahl, 1985 and 1991). Both the lantibiotic and non-lanthionine-containing bacteriocins seem to affect the membrane permeability barrier by forming water-filled membrane channels or pores, by a barrel-stave mechanism (Klaenhammer, 1993; Benz *et al.,* 1991). Nisin has been shown to induce autolysis (Jack *et al.,* 1995) due to their cationic nature. Synthetic peptides can bind to acids in the cell wall releasing and activating autolytic enzymes that are bound to these substrates (Bierbaum and Sahl 1987, 1988 and 1991; Sahl *et al*., 1985). The amount of autolytic activity depends on the degree of cationicity of the peptides interacting with the cells and enzymes release results from an ion-exchange like process (Bierbaum and Sahl 1985 and 1991). Pre-energized cells, in which pore formation and subsequent deplorization is accelerated, which increases cell lysis (Bierbaum and Sahl, 1987 and 1988). In this study a culture of *L. sake* Lb706 was used to determine its usefulness in preventing growth of *L. monocytogenes* in Toroi. In addition a food approved commercial preparation of the bacteriocin nisin (Sigma, St. Louis, Mo.) was also used.

The objective of this study were to determine whether Toroi; made from marine mussels and either watercress or puha; and prepared by a traditional method, is safe to eat. An objective was to determine whether the addition of approved additives could ensure prevent the growth of the cold-tolerant pathogen, *Listeria*

monocytogenes. To achieve these objectives the following investigations were undertaken:

1) The food safety of Toroi stored for up to eight months was assessed against the criteria in *Guidelines for ready-to-eat food* published by the New Zealand Ministry of Health.
2) The ability of *Escherichia coli*, a widely used faecal indicator bacterium, to survive in Toroi was investigated over an eight month storage period.
3) The effectiveness of an antilisterial species, *Lactobacillus sake*, and also the addition of a purified bacteriocin, nisin (an approved food preservative) to prevent the growth of *Listeria monocytogenes* in Toroi.

Chapter 2

General Materials and Methods

This chapter describes the materials and methods used throughout the study. Methodologies used for specific experiments are described in relevant chapters.

2.1. Selection of Toroi ingredients

Green-lipped Mussels (Perna canaliculus)

All mussels for the study were purchased from a local fishmonger on Toroi preparation day. Mussels that had their shells already opened or cracked were discarded.

Watercress (Nasturtium officinale) and Puha (Sonchus asper)

Watercress and puha were purchased from retail outlets including markets. These plants were stored in a domestic refrigerator for 24 hours prior to Toroi preparation.

2.2. Toroi preparation

For each Toroi batch, 2 kg of green-lipped mussels (*Perna canaliculus)* and approximately 600 g of plant material were used. The Toroi was prepared as summarised in Figure 2.1. This method is widely used throughout New Zealand and is also described by Hudson *et al.* (2001) and Whyte *et al.* (2001).

2.2.1 Preparation of plant material

Plant material (puha or watercress) was washed under the cold tap in the laboratory and the stalks rubbed until they lost their rigidness (to ensure even cooking). Any dead leaves and large stalks were removed and the plant material was chopped on a clean chopping board to approximately 5 cm x 5 cm and placed in a pot containing a litre of tap water and boiled for 30 minutes. The cooked plant material was drained and the liquid from the plant material was stored in a sterile container for further use (if needed).

2.2.2. Preparation of green-lipped mussels

Mussels were prepared as described in the Compendium for the Microbiological Examination of Foods (1992). Briefly, the shells were scrubbed with a stiff brush under running tap water, paying particular attention to the crevices at the junctions of the shells. The mussels were placed on absorbent paper and allowed to dry. For cooking, mussels were placed in the wire basket and steamed without contact with boiling water until the shells opened. Immediately the shells opened mussels were removed from the steamer and the flesh removed using a sterile knife. The water in the pot was retained (approximately 400 ml) for addition to the Toroi. The mussel flesh was chopped on a clean chopping board in pieces to approximately 1 cm x 1 cm.

2.2.3. Assembly of Toroi

Chopped mussels, cooked plant material and the water over which the mussels were steamed were combined to form a batch of Toroi that weighed approximately 1.5 kg. In this study, five batches were prepared using watercress

as the plant material and 5 using puha. To ensure homogeneity, each Toroi batch was blended in a Waring Blender to form a purée consistency. The pureed Toroi was then distributed among screw capped plastic jars (70 or 120 ml). Each jar was filled so as to leave very little head space, and stored in a refrigerator at 4 °C (to allow fermentation) until required.

2.2.4. Analysis of Toroi

For analysis, an aliquot from each of the 10 batches of Toroi that were prepared in this study was analysed immediately after preparation. Fermented Toroi was analysed at the following time intervals: one week, two weeks, one month and two months after preparation. In addition, five batches were analysed eight months after preparation, and three of these 12 months after preparation. For analysis, one jar per batch was removed from the refrigerator.

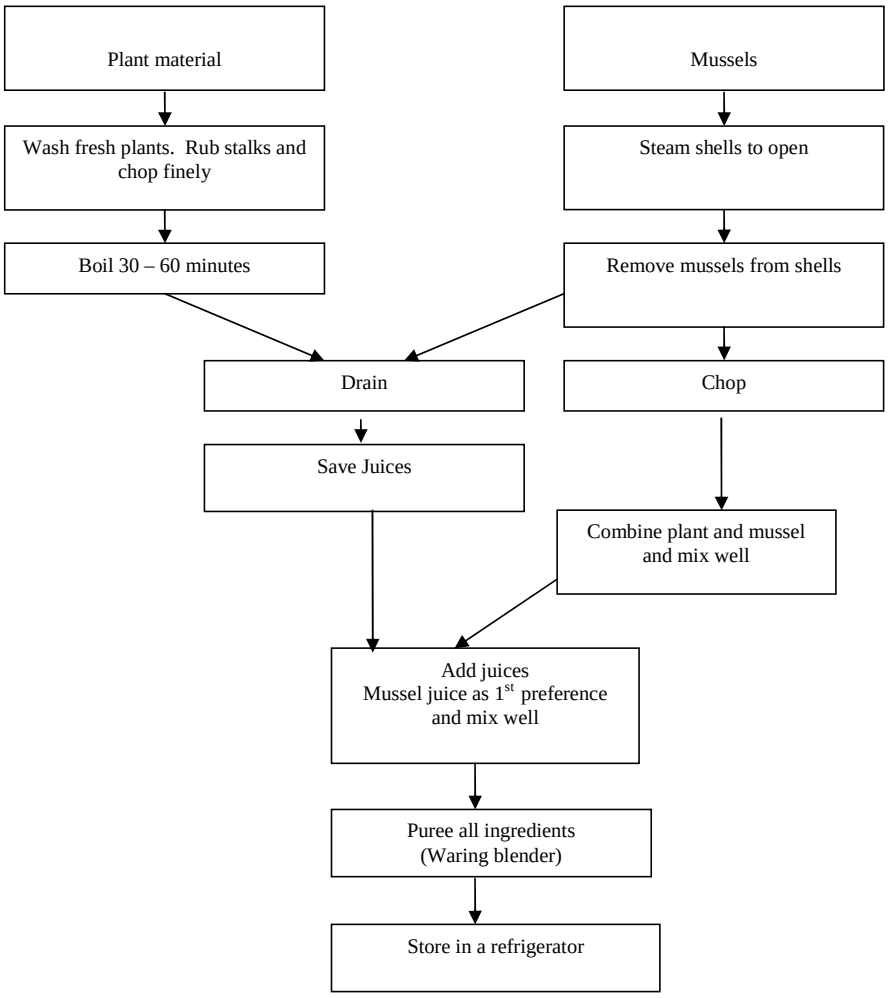


Figure 2.1. Flow chart summarising the preparation of each batch of Toroi.

2.3. pH of Toroi

To take the pH of Toroi, an aliquot (10 ml) from each jar was allowed to return to room temperature. The pH meter (AutoCal pH meter, Radiometer, Copenhagen, Denmark) was fitted with a PHM 83 electrode. Prior to use, the pH meter was calibrated with standard buffers (pH 4; pH 7; pH 10).

2.4. Reference cultures

The reference cultures of bacteria that were used in this study were obtained from the New Zealand Microbiology Reference Culture Collection, Porirua or from AgResearch. *Escherichia coli* NZRM 916, a strain that is widely used in water testing and *Listeria monocytogenes* NZRM 44 were obtained from Porirua. *Listeria monocytogenes* L70 and *Lactobacillus sake* Lb706 were obtained from R. Jones (AgResearch).

These reference cultures were stored at -80°C until required. Working cultures were prepared by streaking from a stored culture to a plate of non-selective medium and checked for purity by culture examination and Gram staining. Pure cultures were streaked over the surface of a slope of non-selective agar in a Universal bottle grown over night at 37°C and used within 48 hours.

2.5. Enumeration of Bacteria

Bacteria were enumerated by either the spread plate technique or the Most Probable Number (MPN) technique. In the spread plate technique, a 0.1 ml aliquot of Toroi, diluted as required was spread evenly over the surface of an agar plate. In the MPN technique, serial dilutions of Toroi were inoculated into replicate tubes (5 per dilution) of appropriate broth. Those tubes that support the growth of the target organism are counted to obtain an MPN score that is referred to published probability tables. The tables used in this study (APHA, 1998) are given in Appendix II. Prior to statistical analysis bacterial concentrations were Log_{10} transformed.

Some pathogenic bacteria were sought in unamended Toroi by presence/absence testing, rather than enumeration, as described below.

2.6. General diluent

The diluent used in this study was 0.1% Peptone (Appendix I).

PART A. NATURAL TOROI

2.7. Recovery of Indicator Bacteria from Toroi

2.7.1. Plate Count Bacteria

Both aerobic and anaerobic bacteria were enumerated on the same non-selective medium, Plate Count Agar (Appendix I), by spread plating. Aerobic Plate Counts (APC) were grown at 25°C for 24 hours and Anaerobic Plate Counts (AnPC) were grown in an anaerobic atmosphere (ANEROGEN, Oxoid, UK) at 25°C for 48 hours. The recovery of bacteria by plate count is summarised diagrammatically in Figure 2.2.

Spread plate technique

This method was suitable for enumerating viable bacteria from Toroi. Ten-fold dilutions of corresponding Toroi samples diluted in 9 ml of Universal Buffer (0.1% peptone and 0.85% NaCl) (see Appendix I) were prepared. 0.1 ml volumes of the appropriated dilution were dispensed onto triplicate, dried plate count agar plates. The inocula were spread over the surface of the agar as evenly and quickly as possible using a disposable plastic spreader. The inoculum was allowed to soak into the agar surface and the plates were incubated at the desired temperature for an appropriate time. The colonies were counted on the triplicate plates of the most suitable dilution. The formulae to calculate the count is described in Appendix II.

Aerobic and Anaerobic Plate Count Technique

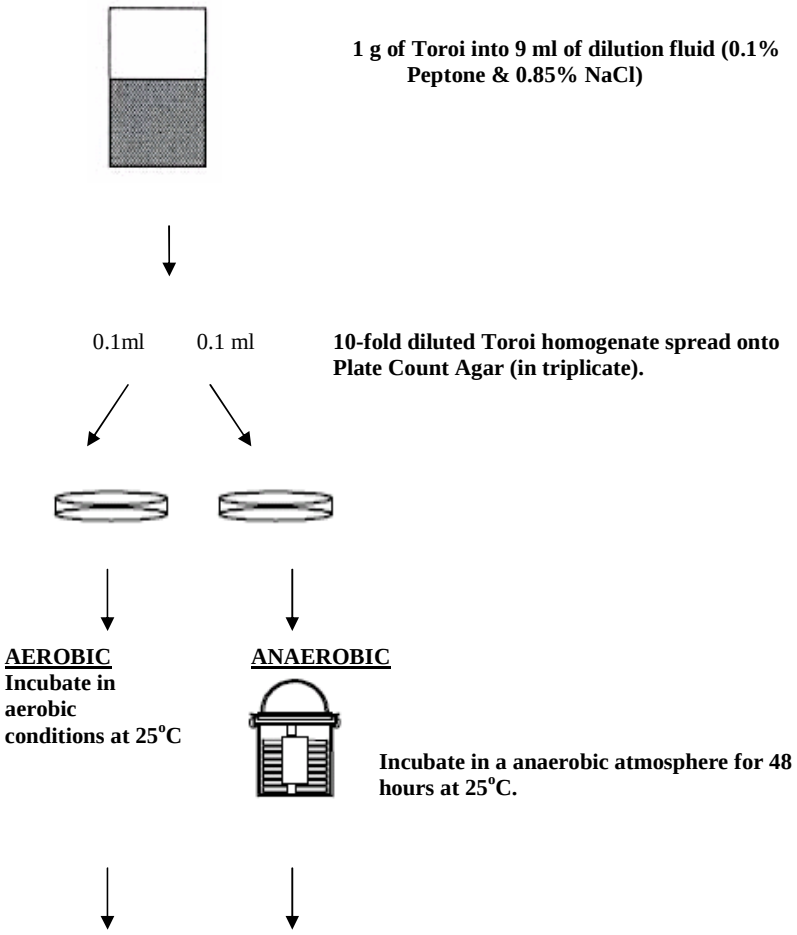


Figure 2.2. Diagram summarising the recovery of bacteria by plate count.

2.7.2. *Escherichia coli*

E. coli were enumerated by the MPN technique using a three-step protocol. Step one was selective enrichment in Lauryl Tryptose Broth (LTB) with tubes incubated at 35°C for 48 hours. All tubes that showed growth (turbidity) were sub-cultured to EC broth. Tubes of EC broth were incubated in a water-bath at 44.5°C for 24 hours (Step two). Tubes that showed growth (turbidity) and gas production (a bubble in the inverted Durham tube) were sub-cultured to vials of EC + MUG. Vials were incubated in a water-bath at 44.5°C for 24 hours. Vials that showed growth (turbidity) were examined under Ultra Violet (UV) light (366 nm) for evidence of purple fluorescence (Step three). Vials with purple fluorescence were scored positive for *E. coli.* All details of media ingredients and preparation are given in Appendix I.

The method for *E. coli* enumeration is a standard method for water (APHA, 1998) but it was necessary to modify the method by adding Step two, as naturally fluorescent substances in the mussels interfered with the identification of *E. coli* if the Lauryl Tryptose positive tubes were sub-cultured directly to EC medium containing 4-methylumbelliferyl-β-D glucuronide (EC + MUG) as given in the standard method. The MUG test depends on the ability of *E. coli* to produce the enzyme β-glucuronidase. *E. coli* is the only member of the Enterobacteriaceae (apart from *Shigella*) that produces this enzyme. The method is summarised diagrammatically in Figure 2.3.

When a non-selective medium was used for *E. coli*, as in maintenance and preparation for the reference strain, the medium used was Tryptic Soy Agar (TSA) (Appendix I).

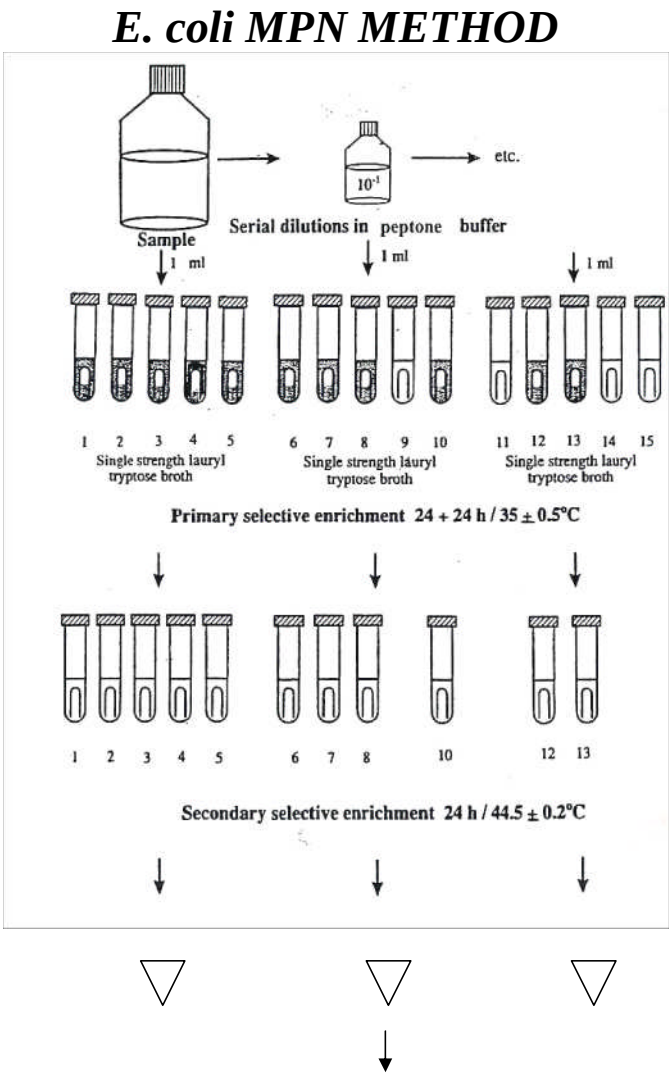


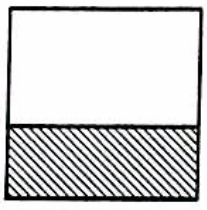
Figure 2.3. MPN method summarising the recovery of *E. coli* from Toroi.

2.8. Recovery of Pathogenic Bacteria from Toroi

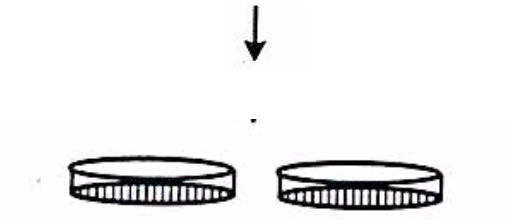
2.8.1. *Listeria monocytogenes*

L. monocytogenes was recovered from Toroi by a presence/absence test not an enumeration test. The conditions of the test were as specified by FSANZ (2001) and set out in MIRINZ (2004a) Briefly, 25 g of Toroi was added to 225 ml of Fraser Broth and incubated at 37°C for 48 hours. A loopful of growth was sub-cultured to duplicate plates of MOX Agar, in a four-phase streak so as to obtain single colonies. Media details are given in Appendix I. Plates were incubated at 37°C for 24 + 24 hours. At each time interval suspect colonies (black) were examined by Gram-stain and under wet mount (under phase contrast microscopy) for motility. Colonies that were Gram-positive rods and motile were further tested using API *Listeria* kits to obtain the species identification. The method is summarised in Figure 2.4.

LISTERIA MONOCYTOGENES



25 g Toroi into 225 ml Fraser broth
Incubate at 37°C for 48 hours.



Subculture Fraser broth to MOX agar and streak the inoculum to obtain single colonies.
Incubate at 37°C for 24-48 hours.

Grey colonies surrounded by black zones on MOX Agar
Confirm presumptive *Listeria* spp. by Gram stain

Confirm *Listeria* using API *Listeria* kits

Figure 2.4. Present/Absent test summarising the recovery of *L. monocytogenes* in Toroi.

2.8.2. Thermotolerant *Campylobacter*

Thermotolerant *Campylobacter* was recovered from Toroi by a presence/absence test (MIRINZ, 2004c) not an enumeration test. Briefly, 25 g of Toroi was added to 225 ml of the primary selective enrichment medium, Exeter broth. Inoculated Exeter broth was incubated at 37°C for 24 hours after which broths were transferred to an incubator at 42°C and incubated for a further 24 hours. A loopful of growth from Exeter broth was subcultured to duplicate plates of modified Charcoal Cefoperazone Deoxycholate (mCCDA) selective agar. Plates were incubated in a microaerophilic atmosphere (using CampyGen, Oxoid, UK to generate a 6% O_2 and10% CO_2 atmosphere) and incubated at 42°C for up to 48 hours. Media details are given in Appendix I. *Campylobacter* colonies were identified by the unique spreading growth and their identity confirmed by examination of a wet mount under phase contrast microscopy. *Campylobacter* is identified by the 'seagull' shape morphology of shells that are highly motile. This method is summarised in Figure 2.5.

CAMPYLOBACTER

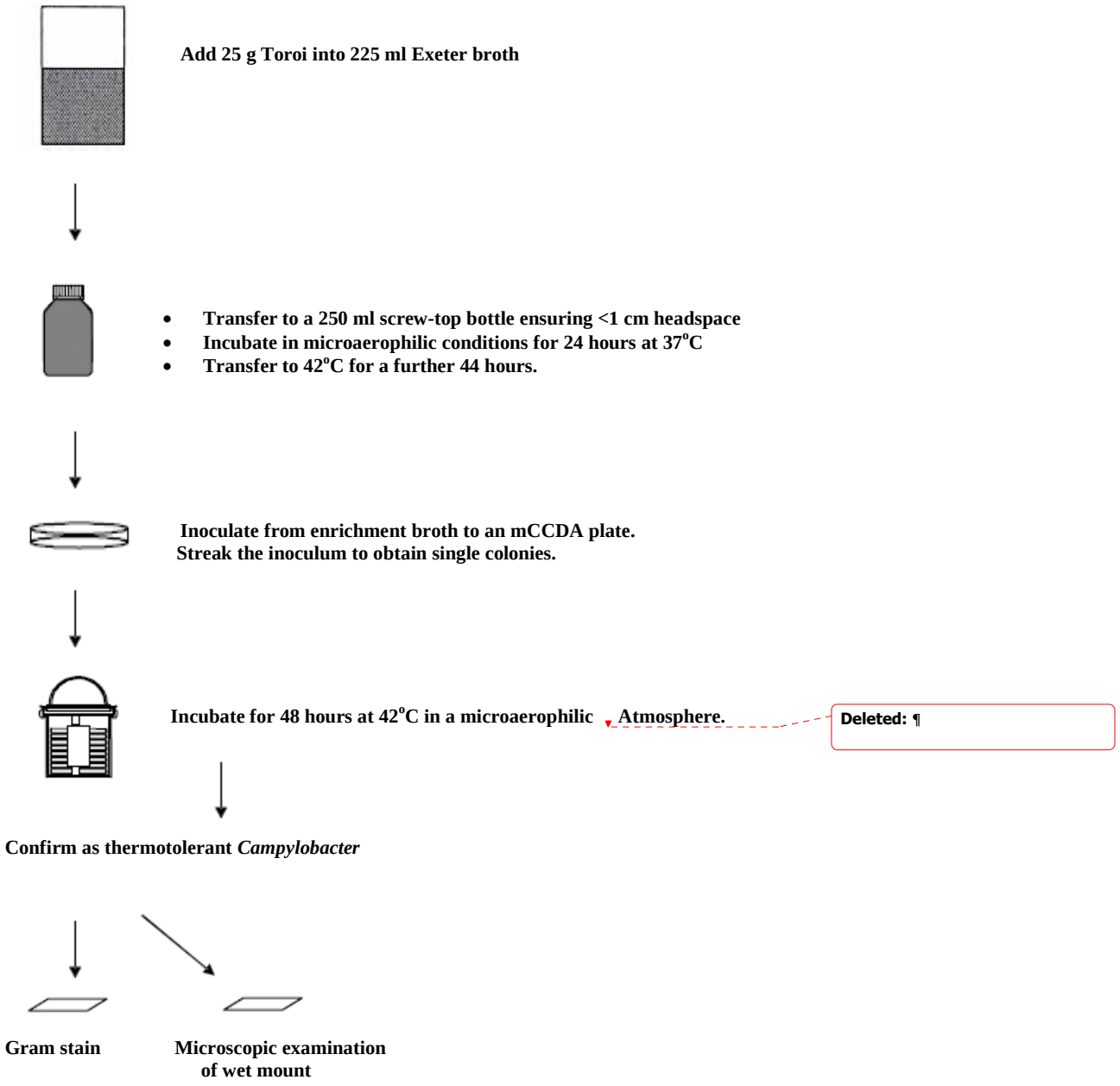
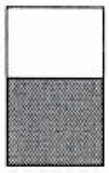


Figure 2.5. Diagram summarising the recovery of thermotolerant *Campylobacter.*

2.8.3. Recovery of *Salmonella*

Salmonella was recovered from Toroi by a presence/absence test (MIRINZ, 2004b) not an enumeration test. Briefly, 25 g of Toroi was added to 225 ml of Buffered Peptone Water (BPW) and the mixture incubated at 37°C for 16-20 hours. 100 µl of growth was transferred to a tube of Rapport-Vassiliadis- (RVS) broth that was pre-warmed to 42°C; inoculated tubes were placed in a water-bath at 42°C and incubated for 24 hours. Tubes that showed evidence of growth (turbidity) were sub-cultured to duplicate Xylose, lysine, desoxycholate (XLD) plates. XLD plates were incubated at 37°C for 24 hours. Colonies that were black and surrounded by a red zone were deemed presumptive *Salmonella* and tested further to confirm the identification. This testing was by Gram-stain (to confirm Gram-negative rods) and Microbact test kits (Microbact, Australia). This method is summarised in Figure 2.6.

SALMONELLA



25 g of Toroi into 225 ml Buffered Peptone Water (BPW).
Incubate at 37°C for 24 hours.

Transfer a loopful of sample homogenated to a selective enrichment medium
(Rappaport-Vassiliadis Soya Peptone (RVS) broth.

Transfer a loopful of selective enrichment medium to
XLD agar.
Streak the inoculum to obtain single colonies.

Check for typical colonies
Black surrounded by a red zone

Confirm *Salmonella* using commercial test kits.

Figure 2.6. Diagram summarising the recovery of *Salmonella.*

2.8.4. Enumeration of *Clostridium perfringens*

C. perfringens was enumerated by spread plating on Toroi samples sent to the analytical laboratory of the Environmental Science Research Institute (ESR), Christchurch Science Centre. Briefly, 0.1 ml aliquots of Toroi, diluted as required were spread to plates of Tryptone-Sulfite-Cycloserine (TSC) Agar and incubated anaerobically for 18 to 24 hours at 35° to 37°C.

2.8.5. Enumeration of Coagulase positive Staphylococci

S. aureus was enumerated by spread plating on Toroi samples sent to the analytical laboratory of the ESR, Christchurch Science Centre. Briefly, 0.4 and 0.3 ml aliquots of Toroi, diluted as required were spread to plates of Baird-Parker agar and for 45 to 48 hours at 35° to 37°C.

2.8.6. Enumeration of *Bacillus cereus*

B. cereus was enumerated by spread plating on Toroi samples sent to the analytical laboratory of the ESR, Christchurch Science Centre. Briefly, 0.1 ml aliquots of Toroi, diluted as required were spread to plates of Pre-dried Mannitol-Egg-Yolk-Polymixin (MYP) agar and incubated for 24 hours at 30°C.

2.8.7. Enumeration of *Vibrio parahaemolyticus*

V. parahaemolyticus was enumerated by spread plating on Toroi samples sent to the analytical laboratory of the ESR, Christchurch Science Centre. Briefly, 0.1 ml aliquots of Toroi, diluted as required were spread to plates of Thiosulphate Citrate Bile Salts Sucrose (TCBS) and incubated for 24 hours at 35°C.

The results obtained for bacterial recovery from Toroi are given in Chapter 3.

PART B. SURVIVAL OF *E. coli* IN STORED TOROI

2.9. Addition of *E. coli* to Toroi

The reference culture *E. coli* NZRM 916 was added to aliquots from each of the 10 batches of Toroi at two different concentrations deemed high and low.

2.9.1. Preparation of the *E. coli* inoculum

The *E. coli* inoculum was prepared by growing the reference culture *E. coli* NZRM 916 on a slope of Tryptic Soy Agar as described in 2.5 above. All the growth was removed from the slope into 100 ml of 0.1% Peptone buffer. This suspension was diluted to obtain the number of *E. coli* required by serial dilution in 9 ml of peptone buffer.

2.9.2. Addition of *E. coli* to Toroi at high level

To add *E. coli* NZRM 916 to Toroi at a high level, the suspension (2.9.1) was diluted to 10^{-4}. This dilution was expected to contain approximately 10^6 cells ml^{-1} and 5 ml of this dilution was added to 500 g of Toroi to give an expected concentration of 10^4 *E. coli* g^{-1} Toroi. Care was taken to ensure thorough mixing of *E. coli* throughout the Toroi. The 500 g of inoculated Toroi was distributed over screw top jars (70 ml capacity) so as to ensure a minimal headspace. The jars of inoculated Toroi were stored at 4°C.

From each batch, at each level of *E. coli* addition, a jar was removed from the refrigerator for analysis of *E. coli* at the following time intervals: one week, two weeks, one month and two months. An aliquot from each batch at each level was analysed prior to refrigeration (time zero). Analysis for *E. coli* was by MPN as described in 2.7.2.

2.9.3. Addition of *E. coli* to Toroi at low level

To add *E. coli* NZRM 916 to Toroi at a low level, the suspension (2.9.1) was diluted to 10^{-6}. This dilution was expected to contain approximately containing approximately 10^4 cells ml^{-1}. One ml of this dilution was added to 100 g of Toroi to give an expected concentration of 10^2 *E. coli* g^{-1} of Toroi. Care was taken to ensure thorough mixing of *E. coli* throughout the Toroi.

PART C. INHIBITION OF *LISTERIA MONOCYTOGENES* IN TOROI

2.10. Maintenance of *Listeria monocytogenes*

L. monocytogenes was recovered by inoculating a vial of BHI broth with a colony of *L. monocytogenes* that had been stored at -80°C and incubating the vial for 24 hours at 35°C. The *L. monocytogenes* culture was Gram-stained to determine culture purity. Slopes of BHI Agar were inoculated with a freshly grown *L. monocytogenes* and slopes incubated at 35°C for 24 hours. Pure cultures of *L. monocytogenes* were enumerated by spread plating of appropriate dilutions to plates of BHI Agar that were incubated for 24 hours at 35°C. The limit of detection of the enumeration method used was 100 *L. monocytogenes* in one gram of Toroi.

2.10.1. Enumeration of *Listeria monocytogenes* in growth inhibition studies

In studies undertaken to determine effective strategies for inhibiting the growth of *L. monocytogenes*, enumeration was by a direct plating method. In this method *L. monocytogenes*, diluted as required, was spread to semi-dried plates of Modified Oxford (MOX) Agar (Oxoid, UK). Plates were incubated at for 48 hours at 37°C. Slightly concave grey colonies with a black halo were counted as *L. monocytogenes* in these pure culture studies.

2.10.2. Growth and preparation of *Lactobacillus sake* Lb706

MRS broth (Merck, Germany) was used to culture *L. sake*. 10 ml of MRS broth (prepared as described in Appendix I) was dispensed into Universal bottles and sterilised (as described in Appendix I). For use, a Universal bottle was inoculated with a loopful of *L. sake* that had been stored at -80°C, and incubated for 24 hours at 30°C (in a 10% CO_2 atmosphere using CampyGEN, Oxoid). Working stocks were prepared by streaking a loopful of growth from an overnight broth to a plate of MRS agar (see Appendix I) that was incubated as described for the broths. Plates were inspected for culture purity and a Gram-stain carried out and then plates were stored in the refrigerator at 4 ± 1°C until required (within 2 weeks).

For use in inhibition studies, only freshly grown 24 hour *L. sake* MRS broth cultures were used. The growth temperature of these inocula was determined experimentally (Chapter 5).

2.11. Microscope Methods

2.11.1. Direct Examination method

Wet mount preparations were used to allow rapid examination of the morphology and motility of microorganisms in a fluid medium.

2.11.2. Gram stain

The principle of the procedure consists of:

- Staining a fixed smear with crystal violet.
- Applying iodine as a mordant.
- Decolourising the primary stain with alcohol acetone; and
- Counterstaining with safranin or basic fuchsin.

Specimen collection and preparation

The test specimen was applied to a clean glass slide in a manner that will yield a thin, uniform smear. Colonies grown from an 18 – 24 hour culture broth were used. The smear was allowed to dry by the heat fix method, by passing the slide through a low flame 2 – 3 times. The slide was cooled to room temperature before staining.

Reagent preparation

The traditional Gram iodine working solution was prepared by adding an entire 2. 5 ml ampule of Gram iodine 100X to 250 ml Gram diluent.

The 4-Step staining procedure was used.

Protocol:

1. Flood the fixed smear with primary stain (Gram Crystal Violet) and stain for 1 minute.
2. Remove the primary stain by gentle washing with cold tap water.

3. Flood the slide with mordant (Gram Iodine or Stabilised Gram Iodine) and retain on the slide for 1 minute.
4. Remove the mordant by gentle washing with tap water.
5. Decolorize (Gram Decolorize) until solvent running from the slide is colourless (30 – 60 seconds).
6. Wash the slide gently in cold tap water.
7. Flood the slide with counter stain (Gram Safranin) and leave for 30 – 60 seconds.
8. Wash the slide with cold tap water.
9. Blot with blotting paper or paper towel or allow air dry.
10. Examine the smear under an oil immersion lens.

Results:

Reaction	4-Step technique using Gram Safranin
Gram positive	Purple-black cells
Gram negative	Pink to red cells

Chapter 3

Microbiological Quality of Toroi

3.1. Introduction

A commonly used preparation technique is to mix boiled green leafy vegetables with chopped mussel flesh, pack the mixture into screw-capped containers that are then stored in the refrigerator for periods ranging from a few days to several weeks.

The food safety of a variety of Toroi preparations was investigated by Hudson *et al.* (2001) and Whyte *et al*. (2001) who reported that the only microorganism of concern they recovered was the spore-former *Bacillus cereus. B. cereus* can grow at temperatures above 5°C so it is important to keep Toroi in a refrigerator and served chilled to prevent bacterial growth.

Nowadays many Toroi makers use mussels purchased from a commercial outlet. As these mussels are required to meet New Zealand Food Safety Standards there is a very little risk that they will be contaminated by microorganisms of health concern. However, there are undoubtedly risks associated with consuming mussels gathered from the wild especially from locations impacted by streams or rivers contaminated by effluents or farm runoff. In addition, as with any pre-prepared ready-to-eat food there is always the possibility that Toroi could be contaminated during its preparation.

To investigate the food safety of Toroi, 10 batches of Toroi were assessed against the Guidelines for ready-to-eat food published by the Ministry of Health in 1995. The Toroi was tested over a storage period of 8 months.

3.2. Results

Ten batches of Toroi ingredients were prepared as described in sections 2.1 and 2.2. All ten batches of Toroi were analysed for the indicator bacteria *E. coli* and aerobic bacteria by Aerobic Plate Counts (APC). Five of the batches were also analysed for anaerobic bacteria by Anaerobic Plate Counts (AnPC), and seven pathogenic bacteria:

- *Listeria monocytogenes* (section 2.8.1);
- Thermotolerant *Campylobacter* (section 2.8.2);
- *Salmonella spp.*(section 2.8.3);
- *Clostridium perfringens* (section 2.8.4);
- Coagulase positive *Staphylococci* (section 2.8.5);
- *Bacillus cereus* (section 2.8.6);
- *Vibrio parahaemolyticus* (section 2.8.7).

3.2.1. pH

Overall, the pH dropped during storage. In freshly prepared Toroi the median pH was 6.70, after 2 months it had declined to 5.78, after 8 months to 5.92 and by 12 months the median pH was 5.84.

The pH of the 5 batches of Toroi that were analysed for pathogens over a 2 month storage period is shown in Table 3.6 together with the recovery of *B. cereus*.

3.2.2. Recovery of Indicator Bacteria

Aerobic Plate Counts

The recovery of aerobic bacteria by plate counts (APC) from all 10 batches of Toroi is summarised in Table 3.1. As shown in $\log_{10}$ values the APC increased from an initial overall average of Log_{10} 3.02 g^{-1} Toroi (wet weight) to an overall average of Log_{10} 7.89 g^{-1} Toroi (wet weight) after 2 months. For all batches the greatest increase occurred over the first week of storage and the numbers remained similar throughout the rest of the storage period; presumably due to the exhaustion of fermentable carbohydrates (Hudson *et al.* 2001).

The APC numbers were also compared on the basis of the plant material used and the results are summarised in Table 3.1. No significant difference was identified on the basis of plant as shown by statistical analysis (of variance) summarised in Table 3.2.

Five batches of Toroi had an extended storage of 8 months with 3 batches further extended for 12 months. The average recovery of APC over the entire 12 months storage is shown graphically in Figure 3.1.

Aerobic Plate Count for Toroi Log_{10} g^{-1} (wet weight)				
Storage Time	Watercress n = 5 Log_{10} Average	Puha n = 5 Log_{10} Average	Overall n = 10	
			Log_{10} Average	s.d
Initial Day	3.54	2.49	3.02	0.98
1 week	7.36	5.03	6.2	1.09
2 weeks	7.92	7.03	7.47	1.04
1 month	7.8	7.93	7.86	1.28
2 months	6.55	7.84	7.89	1.18
8 months			7.44	0.84
12 months			7.44	1.81

Table 3.1. Summary of Log_{10} Average CFU g^{-1} (wet weight) on Toroi storage interval for Aerobic Plate Counts (APC) containing either watercress or puha as the plant material in Toroi. Note: there were only 5 batches analysed for Toroi stored at 8 months and only 3 at 12 months.

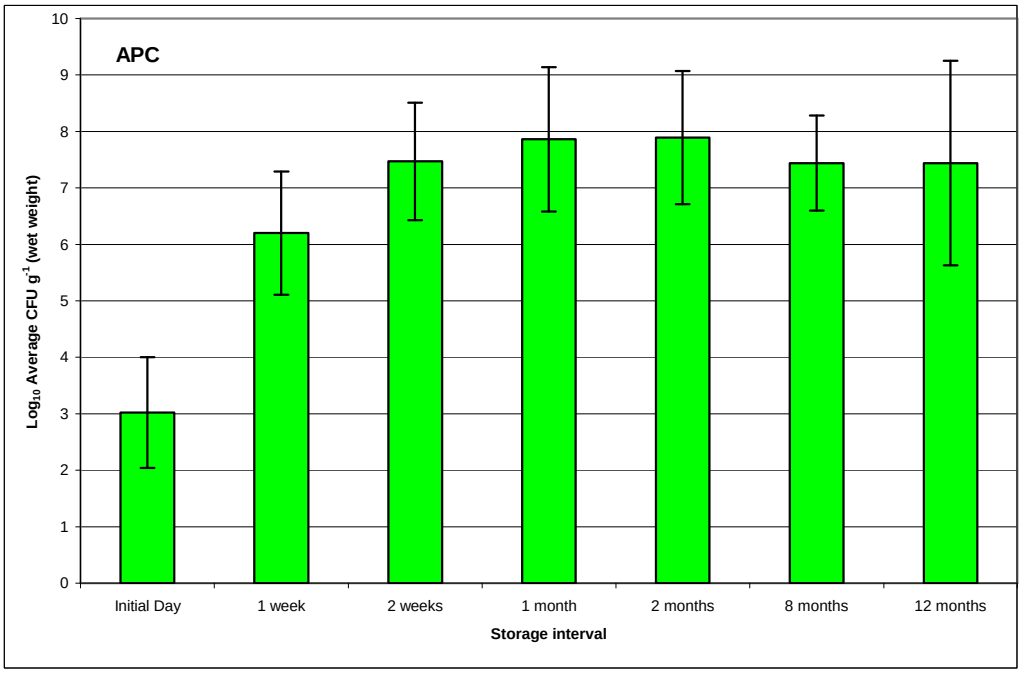


Figure 3.1. Summary of Overall Log_{10} Average CFU g^{-1} (wet weight) on Toroi storage interval for Aerobic Plate Counts (APC) containing either watercress or puha as the plant material in Toroi. Note: there were only 5 batches analysed for Toroi stored at 8 months and only 3 at 12 months.

One-way ANOVA: APC on Toroi prepared from either watercress or puha

```
Source  DF     SS    MS     F      P
Factor   1   0.19  0.19  0.06  0.817
Error   12  40.74  3.40
Total   13  40.93

S = 1.843   R-Sq = 0.46%   R-Sq(adj) = 0.00%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level    N   Mean  StDev  ---------+---------+---------+---------+
w/cress  7  6.823  1.552    (--------------*--------------)
puha     7  6.590  2.093  (--------------*--------------)
                          ---------+---------+---------+---------+
                                 6.0       7.0       8.0       9.0

Pooled StDev = 1.843
```

Table 3.2. Results of One-way analysis of variance carried out to identify any difference in recovery of APC from watercress Toroi and from puha Toroi. There were five batches of each type of Toroi each analysed over two months of refrigerated storage (Minitab statistical package).

Anaerobic Plate Count

The recovery of anaerobic bacteria by plate counts (AnPC) from 5 batches of Toroi is summarised in Table 3.3. As shown in Log_{10} values the AnPC increased from an initial overall average of Log_{10} 2.96 g^{-1} Toroi (wet weight) to an overall average of Log_{10} 8.13 g^{-1} Toroi (wet weight) after 2 months. For all batches the greatest increase occurred over the first week of storage and the numbers remained similar throughout the rest of the storage period. This was presumably also due to the exhaustion of fermentable carbohydrates (Hudson *et al.* 2001). Hudson registered a mean log AnPC of 6.4, suggesting a higher level of carbohydrate in preparations in this study.

Anaerobic Plate Count for Toroi Log_{10} g^{-1}(wet weight)		
Storage Time	Overall n = 5	
	Log_{10} Average	s.d
Initial Day	2.96	0.86
1 week	7.65	0.68
2 weeks	8.42	1.12
1 month	8.14	0.41
2 months	8.13	0.41

Table 3.3. Summary of Anaerobic Plate Counts (AnPC) in Toroi, containing either watercress or puha as the plant material, over two months refrigerated storage. All AnPC are expressed as Log_{10} Average CFU g^{-1} (wet weight).

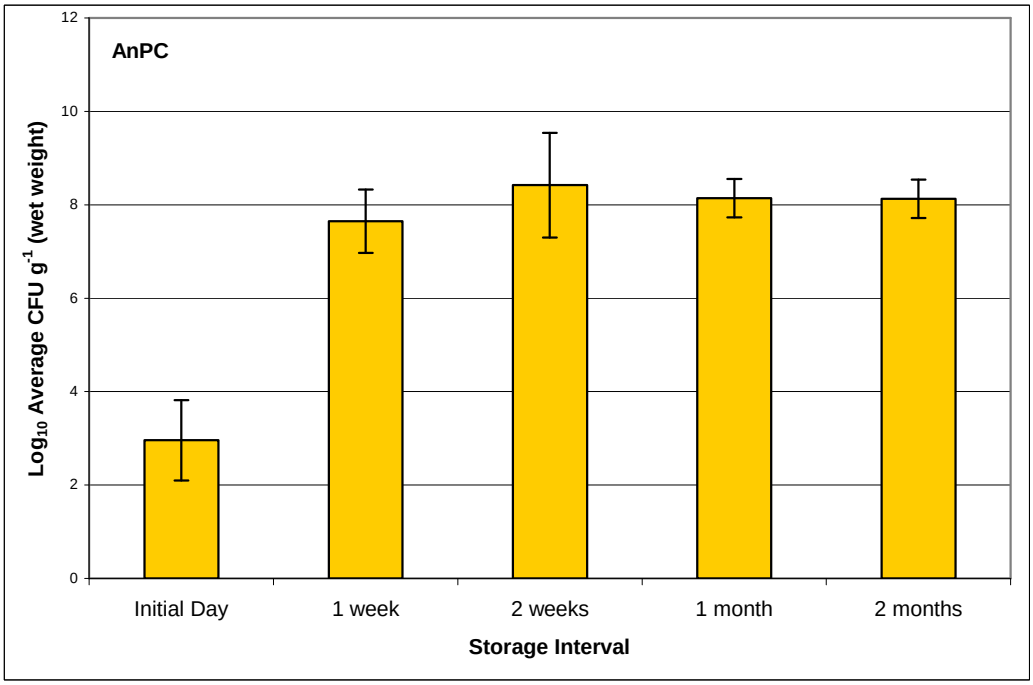


Figure 3.2. Summary of overall Log_{10} Average CFU g^{-1} (wet weight) on Toroi storage interval for Anaerobic Plate Counts (AnPC) in Toroi containing either watercress or puha as the plant material over 2 months refrigerated storage.

Escherichia coli

The recovery of *E. coli* from 10 batches of Toroi is summarised in Table 3.4. *E. coli* were detected in 4 of the 10 batches of Toroi. Recovery was sporadic with only 7 of the 60 samples that were analysed in total being found to contain this organism. The highest level of recovery from any of the Toroi samples was 0. 2 *E. coli* g^{-1} (wet weight), this is considerably less than the criterion of < 3 *E. coli* g^{-1} set out in Guidelines (FSANZ, 2001; NZFSA, 2005a; Ministry of Health, 1995) that are reproduced in Table 3.5; demonstrating that the Toroi was satisfactory. The sporadic recovery of *E. coli* occurred over two months of storage but none were recovered from samples stored for eight months.

Batch number	***E. coli* CFU g^{-1} (wet weight)**				
	Preparation Day	1 week	2 weeks	1 month	2 months
W1	0	0	0	0	0
W2	0	0	0	0	0
W3	0	0	0	0	0.23
W4	0	0	0	0	0.02
W5	0	0	0.02	0	0
P1	0.08	0	0.02	0.04	0.08
P2	0	0	0	0	0
P3	0	0	0	0	0
P4	0	0	0	0	0
P5	0	0	0	0	0

Table 3.4. *E. coli* (CFU g^{-1} (wet weight) detected in Toroi throughout the storage interval. Toroi stored for 8 months had no *E. coli* detectable.

3.2.3. Recovery of Pathogens

The pathogens sought were those specified in the 2001 *Guidelines for ready-to-eat foods* (FSANZ, 2001; NZFSA, 2005a) that also give allowable limits (as summarised in Table 3.5). The Ministry of Health (1995) requirement is that a minimum of five batches of a product be tested to ensure that the assessment is not biased by the results for a single batch.

None of the batches of Toroi contained *Listeria monocytogenes*, *Salmonella* spp., thermotolerant *Campylobacter*; coagulase positive *Staphylococci*, *Clostridium perfringens* or *Vibrio parahaemolyticus*; either when freshly prepared or after storage. All 5 batches were analysed for these bacteria at intervals over 8 months refrigerated storage with 3 batches also analysed after 12 months storage. On one occasion *Vibrio alginolyticus*, a species known to be associated with wound infections after exposure to sea water (Opal and Saxon, 1986; Gomez *et al.* 2003) but is not pathogenic, was recovered from a freshly prepared batch of Toroi. Two of the three 12 month storage samples analysed contained *Listeria ivanovii*, a species that is not considered a foodborne pathogen (Lammerding *et al.* 1992).

Microorganism	Microbiological Quality (cfu g^{-1})			
	Satisfactory	*Marginal*	*Unsatisfactory*	*Potentially hazardous*
Indicator				
Escherichia coli	<3	3-100	≥100	
Pathogens				
Coagulase +ve staphylococci	$<10^2$	10^2-10^3	10^3-10^4	$\geq 10^4$
Clostridium perfringens	$<10^2$	10^2-10^3	10^3-10^4	$\geq 10^4$
Bacillus cereus	$<10^2$	10^2-10^3	10^3-10^4	$\geq 10^4$
Vibrio parahaemolyticus	<3	<3-10^2	10^2-10^4	$\geq 10^4$
Campylobacter spp.	Not detected in 25 g			detected
Salmonella spp.	Not detected in 25 g			detected
Listeria monocytogenes	Not detected in 25 g	Detected but $<10^2$		$\geq 10^2$

Table 3.5. Guideline levels for determining the microbiological quality of ready-to-eat foods (FSANZ, 2001; NZFSA, 2001). The allowable limits for all microorganisms are expressed in terms of colony forming units (CFU).

Bacillus cereus

B. cereus was recovered from all five batches of Toroi in which it was sought. The recovery of *B. cereus* in Toroi is summarised in Table 3.6. All *B. cereus* numbers are expressed as cfu g^{-1}. Five batches of Toroi were detected for *B. cereus* and all batches were positive for *B. cereus* on the initial day. After the initial day of preparation, *B. cereus* was detected sporadically within the first two weeks of storage. In the freshly prepared Toroi *B. cereus* numbers ranged from 70 to12,000 cfu g^{-1}; only one batch met the 'satisfactory' criterion, the other four being 'marginal'. However, after 2 weeks storage *B. cereus* numbers were less than 10 cfu g^{-1} in all batches, so all our Toroi samples were now categorised as 'satisfactory'. One batch (B10) produced an increase of *B. cereus* numbers from 1000 to 15000 cfu g^{-1} within a week of storage. The

vegetable component of this Toroi batch was prepared outside the laboratory and was transported to the laboratory after boiling.

Batch		Storage time				
		Preparation day	*1 week*	*2 weeks*	*1 month*	*2 months*
B 6	*B. cereus*	1200	<10	70	<10	<10
	pH	6.8	6.4	6.3	6.2	5.8
B 7	*B. cereus*	70	<10	<10	<10	<10
	pH	6.7	6.3	6.4	6.2	5.8
B 8	*B. cereus*	1900	<10	<10	<10	<10
	pH	6.9	6.5	6.1	6.0	6.0
B 9	*B. cereus*	380	60	<10	<10	<10
	pH	6.2	6.1	6.0	6.5	6.2
B 10	*B. cereus*	1000	15000	<10	<10	<10
	pH	6.4	6.3	5.9	5.8	5.7

Table 3.6. Recovery of *Bacillus cereus* (as number of colony forming units g^{-1}) from samples of Toroi stored for up to 2 months at 4 – 5°C in a refrigerator.

3.3. Discussion

Over the first week of storage there was a very large increase in bacteria measured by both APC and AnPC, but there was little change after that. The numbers of APC were similar to those reported by Hudson *et al*. (2001) for a Toroi preparation that was broadly similar to that of this study. These workers reported an average value for APC of 3.3 Log_{10} cfu g^{-1} in fresh Toroi and an average value for APC of 7.7 Log_{10} cfu g^{-1} after 2 months storage. The average values in this study were 3.0 and 7.9 respectively. As the samples were analysed progressively throughout the storage period it was possible to identify changes. In this study the APC numbers did not change after two

weeks and for up to eight months; three samples were also analysed after twelve months and the APC population was not significantly different from that at two weeks (see Figure 3.1).

It appears that the APC population grew most quickly early in the study, possibly before the temperature of the Toroi had reached refrigeration level and did not increase beyond that. Because the plant material was subjected to a prolonged boiling period it is likely that only spores survived and those non-spore forming bacteria that contributed to the APC population were only derived from shellfish. Evidence that this might have been the case is provided by the lack of correlation of APC with plant variety.

The changes in pH during storage demonstrate the establishment of fermentation during storage. Hudson *et al*. (2001) identified fermentation by observing both a drop in pH from 6.1 to 5.0, together with an increase in the end products of fermentative metabolites i.e. volatile fatty acids, particularly lactic acid. Lactic acid increased from 0.7 x 10 mol L^{-1} in fresh Toroi to 14.6 x 10 mol L^{-1} after 2 months storage. The drop in pH in this study is somewhat less than that of Hudson *et al*. (2001). These workers suggested that pH 5.0 is not low enough to ensure product safety and recommended that Toroi be held for up to 48 hours at ambient temperature to establish sufficient fermentation to decrease the pH further.

The AnPC numbers in the Toroi analysed in this study were somewhat higher than those reported by Hudson *et al*, (2001). In this study the numbers for AnPC were 2.9 Log_{10} cfu g^{-1} and 8.1 Log_{10} cfu g^{-1} compared to their values of 3.3 and 6.4. AnPC numbers did not change after two weeks and for up to two months (see Figure 3.2). Hudson *et al.* (2001) suggested that the stability in numbers of plate counted bacteria was due to exhaustion of fermentable carbohydrates. However, although the population of AnPC did not increase after two weeks the median pH had dropped by twelve months (pH 5.84) compared to that at two weeks (pH 6.39). The decline in pH over this period suggests that fermentation did continue so presumably the component population contributing to the AnPC at least changed as different carbohydrates were progressively used as substrates.

Bacillus cereus is a spore-former that is widely distributed in nature and some strains produce toxins that cause food poisoning (Rhodehamel and Harmon, 2001). Emetic food poisoning is caused by ingestion of a pre-formed highly stable toxin, cereulide, and is mostly associated with starchy foods. Diarrhoeal disease results from ingestion of *Bacillus cereus* cells (or spores) that multiply within the intestines and produce enterotoxins (designated HBL, HBE or T). The diarrhoeal toxins are heat and acid-labile and are mostly associated with proteinaceous foods such as meat (Whyte and Wong, 2004).

As *B. cereus* is known to be recovered from vegetables (Rhodehamel and Harmon, 2001) it probably originated from the watercress or puha. The

extended boiling would destroy bacterial cells leaving only spores. These spores could include *B. cereus* (although considerable variation in heat resistance is reported for the spores of different strains; Whyte and Wong, 2004). *Bacillus cereus* includes psychrotrophic strains, so that any present could germinate (Dufrenne *et al.* 1994) and grow well during refrigeration storage as there would be little competition for substrates (due to the destruction of bacterial cells). However, the Toroi was made from boiled plant material and lightly cooked marine mussels so that the latter would be able to contribute viable cells. No *B. cereus* were recovered during the later stages of storage but both aerobic plate count bacteria (Fig. 3.1) and anaerobic plate count bacteria increased (Fig. 3.2), supporting their source being the mussels.

The decline of *B. cereus* in Toroi during storage suggests inactivation. Reasons for this are not clear but it could be speculated that both an inability to compete for substrates with other bacteria sourced from mussels and antibacterial compounds produced by some of the competing microorganisms were responsible. It has been reported that lactobacilli greatly decreased the count of *B. cereus* in milk after 10 hours (Kim *et al.* 2000) and that the bacteriocin, nisin inhibited both sporulation and growth (Whyte and Wong, 2004). Inhibition has also been reported for bacteriocin AS-48, produced by *Enterococcus faecalis* (Abriouel *et al.* 2002).

In one batch of Toroi (B 10) a 100-fold increase in *B. cereus* was determined at week one, but after this there was no further recovery of this organism. This finding could be attributed to germination of spores during week one followed by an increase in inhibitory microorganisms (presumably lactobacilli) by week two. Germination of spores can apparently occur at a range of temperatures; including room temperature (Giffel *et al.* 1995) and below the minimal growth temperature of a strain (Dufrenne *et al.* 1994) so that it is likely that all spores had germinated by the end of week one. *Lactobacillus* spp. were not measured specifically but they would be expected to be present as they grow well in fermented products and the establishment of fermentation was demonstrated in the Toroi by the observed reduction in pH.

As not all *B. cereus* strains produce toxin (e.g. only serovar HI produces cereulide toxin) recovery of these bacteria is not a demonstration of toxicity. For example in a recent study only18 of 54 strains were positive for toxin production (Taylor *et al.* 2005). The NZ Food Standards are based on enumeration of *B. cereus* not identification of toxin. There is no routine test for cereulide - only a tissue culture assay of heat-treated culture supernatants (Taylor *et al.* 2005). Commercial test kits are now available for diarrhoeal toxin testing but this was not a done in this study. The numbers of *B. cereus* cells recovered were less than the 10^5 in a gram of food reported to be required for infection.

Establishment of fermentation with the associated production of volatile fatty acids and pH drop are important for the control of *B. cereus* growth. In this study *B. cereus* was recovered from all samples on the day of preparation but only on one occasion on was concentration was high enough to cause concern (B 10, week one). On this occasion the cooked vegetable component was held for several hours before the Toroi was mixed and packed into containers. Hudson *et al*. (2001) and Whyte *et al*. (2001) suggested that Toroi preparation may be safer by allowing the lactic acid fermentation to proceed at a warmer temperature for the first 48 hours before transferring to chilled conditions for storage as has been achieved for kimchi (Lee *et al.* 1995). Results of this study demonstrated that when the boiled vegetable component was held for several hours at room temperature *B. cereus* increased to an unsatisfactory level.

Although the protocol of rapid preparation and refrigeration appeared to be sufficient to prevent the growth of *B. cereus*, it cannot be denied that there is potential for *B. cereus* to grow in Toroi prior to the establishment of fermentation. If a cereulide-producing strain were present and conditions were favourable for growth (reported generation time under optimum conditions is 18 – 27 minutes; Whyte and Wang, 2004) then the concentration could reach the 10^5 or more in a gram of food that has been found to be required to produce sufficient toxin to cause emetic food poisoning. Cereulide toxin is very stable and is unlikely to be affected by the prolonged storage. If *B. cereus* is successfully inactivated during storage, as appeared to be the case

in this study, it is most unlikely that Toroi stored for two weeks or longer would be a source of the enterotoxin-producing strains that can cause diarrhoeal food poisoning.

There were no *Clostridium perfringens* detected in Toroi and this organism may be a helpful indicator of the absence of *Clostridium botulinum*. This organism is significant as the only report of foodborne botulism in New Zealand was associated with Toroi (Flacks, 1985). Although no identified organism was isolated from the two affected patients, a blood sample from one of them contained type A botulinum toxin (Gilbert *et al.* 2006).

Clostridium botulinum is an anaerobic bacterium that produces spores that survive ordinary cooking and are resistant to freezing. There are a number of different strains of *C. botulinum* some of which produce potent neurotoxins (BoNT). The toxins are stable at low pH, resistant to freezing but are inactivated rapidly at temperatures of 85°C or higher. Foodborne botulism in humans is usually caused by Group I (proteolytic) strains of *C. botulinum* - types A, B, or occasionally F - or Group II (non-proteolytic) strains that include type E. Type A is strongly associated with vegetable products while type E associated with seafood including shellfish (Hielm *et al*.1988; MoH, 2001). *Clostridium botulinum* type A grows at neutral pH and at temperatures above 10°C. *Clostridium botulinum* type E produces less heat resistant spores (than type A) but it can grow at refrigeration temperatures (MoH, 2001). Type A toxin is reported to be more potent and have higher lethality

than types B and E (Boerema and Broda, 2004). In spite of extensive surveys there have been no reports of the presence of *C. botulinum* type E in New Zealand (Gilbert *et al.* 2006). Some workers believe that *C. botulinum* types toxic to humans do not occur frequently in New Zealand (Gill and Penney, 1982).

Hudson *et al*. (2001) recommended that if Toroi is to be stored (rather than eaten immediately after preparation) it be held at room temperature for 48 h prior to refrigeration to allow lactic acid fermentation to proceed rapidly so that the pH will be reduced to less than 4; a level at which neither Group I nor II types will grow or produce toxin (MoH, 2001). These workers consider that type E is a particular risk in foods contained shellfish that are subjected to prolonged refrigeration storage. The recommendation to refrigerate Toroi as soon as possible after preparation made with respect to controlling the growth of *B. cereus* would not protect against *C. botulinum* as the lowest pH achieved was pH 5.7 (see Table 3.6).

A species of *Vibrio* was isolated from one of the Toroi batches on preparation day and identified as *Vibrio alginolyticus.* This species is not classed as a food-borne pathogen, although in the marine environment it has been reported as a pathogen associated with infection of open wounds (Opal and Saxon, 1986; Gomez *et al.* 2003). A non-pathogenic *Listeria* species, *L. ivanovii*, was isolated from 2 batches of Toroi after 12 months storage.

Many food-borne pathogens are spread by the faecal-oral route and some of these are common to both animals and humans. *E. coli* is a preferred indicator of faecal microbiological contamination because of its high specificity in the faeces of warm-blooded animals and humans (Dufour, 1977). *E. coli* were recovered sporadically in very low numbers over the storage period. As the plant material was boiled for 30 minutes these *E. coli* would have been sourced from the mussel component. The NZ sanitation requirements for shellfish exported or sold at retail outlets are <230 *E. coli* 100 g^{-1}. In this study recovery was at least 10-fold less than these requirements (Table 3.4), indicating that the Toroi in this study also met the guidelines for ready-to-eat foods (<3 g^{-1}). Traditional Maori tikanga (protocol) around food gathering and food handling in the past may have provided a level of protection from some food safety hazards. In findings presented to the Land Treatment Collective Conference (Dixon, 2005) it was shown that the bacterial loadings of watercress harvested from streams can be reduced by careful selection of harvesting sites and careful picking and washing of watercress plants. Although it might seem there were more incidences of *E. coli* recovery in watercress than puha Toroi (Table 3.4), this is most likely to be simply co-incidental since the mussels were almost certainly the source of this bacterium.

The results obtained in this study were reported to the New Zealand Food Safety Authority (Dixon and Donnison, 2006) and will be presented to the New Zealand Microbiology Society Conference in Hamilton, 2006.

Chapter 4

Survival of *E. coli* in Toroi

4.1. Introduction

Escherichia coli are widely used as an indicator for faecal pollution, and like many food borne pathogens are spread by the faecal-oral route. For this reason it is included among the organisms which are measured to demonstrate that ready-to-eat food is safe to eat. Such food is only classed as satisfactory when less than 3 *E. coli* g^{-1} is detectable. *E. coli* does not grow at refrigeration temperatures (Ingraham *et al*., 1983) and death or injury of *E. coli* caused by chilling is an important reason for recommending refrigerated storage for food (Speck and Cowman, 1969). This chapter describes a study which was carried out to determine whether *E. coli* could survive in Toroi.

The vegetable component of Toroi was boiled for 30 minutes so that only the lightly-cooked mussels would be liable to contain *E. coli*. However, the mussels were purchased from a retail outlet which means that they would meet the New Zealand food safety standard so there was a very low probability that *E. coli* would be naturally present. In order to investigate *E. coli* survival a laboratory-grown culture, *E. coli* NZRM 916, was added to prepared Toroi and its survival followed over an eight month period.

4.2. Methods Used

4.2.1. Amendment of Toroi with laboratory grown *E. coli*

To inoculate Toroi, a culture of *E. coli* NZRM 916 was transferred from -80^{o}C storage into Tryptic Soy Broth (TSB) and grown overnight at 37°C. A loopful of growth was streaked on to a Tryptic Soy Agar (TSA) plate, incubated overnight at 37°C and colonies inspected and a Gram-stain carried out to check culture purity. A colony was then streaked on to a slope of TSA and incubated overnight at 37°C. To prepare an *E. coli* suspension for inoculation into Toroi, all the growth was removed from the slope and resuspended in 100 ml of 0.1% Peptone buffer. Serial dilutions were made in 9 ml aliquots of 0.1% Peptone buffer for use as the inoculum. The actual numbers of *E. coli* cells in the suspension were measured by spread plating (in triplicate) 0.1 ml aliquots of the 10^{-6} dilution on to TSA plates which were incubated overnight at 37°C.

Inoculation of Toroi at high dose

The *E. coli* suspension was diluted to 10^{-4} and 5 ml added to 500 g of homogenised Toroi. Particular care was taken to ensure that the inoculum was mixed thoroughly with the Toroi. The inoculated Toroi was dispensed into screw-capped jars filled so as to leave little or no head-space. The jars were stored in the refrigerator until required for analysis.

Inoculation of Toroi at low dose

The *E. coli* suspension was diluted to 10^{-6} and 5 ml added to 500 g of homogenised Toroi. The inoculated Toroi was dispensed into screw-capped jars

filled so as to leave little or no head-space. The jars were stored in the refrigerator until required for analysis.

A jar of Toroi, at each inoculum level, was removed from the refrigerator at the following intervals; one week, two weeks, one month, two months and eight months and the *E. coli* enumerated by the Most Probable Number (MPN) method described in section 2.5.

4.2.2. Survival of *E. coli* NZRM 916 at refrigeration temperature

The ability of *E. coli* NZRM 916 to survive at refrigeration temperatures was also determined by inoculation of the suspension, prepared as described, above into Peptone buffer. These buffer inoculated samples were also stored in screw-capped jars in a refrigerator and analysed at the following intervals; one week, two weeks, three weeks, four weeks and six weeks.

4.3. Results

Each batch of Toroi was contaminated with a laboratory grown culture of *E. coli* that was diluted to give two levels of contamination designated low and high. For each batch the same overnight culture was diluted and used for the low and high level. The number of *E. coli* in the ten overnight cultures was reasonably similar (mean $\log_{10}$ *E. coli* ml^{-1} = 8.491, sd = 0.171). The results obtained when the Toroi was analysed immediately after the addition of the appropriately diluted *E. coli* culture demonstrate the difficulty of obtaining a homogeneous mixture. For the higher level contamination across all ten batches this variability was reasonably low (mean $\log_{10}$ *E. coli* 100 g^{-1} (wet weight) Toroi = 4.683, sd = 0.294) but at the

lower level of contamination this variability was greater (mean $\log_{10}$ *E. coli* 100 g^{-1} (wet weight) Toroi = 1.655, sd = 0.498)

The recovery of *E. coli* from Toroi contaminated at the low and high level was measured at intervals and the results are shown, without logarithmic transformation, in Table 4.1. As shown in Table 4.1 there were usually no *E. coli* naturally present in the Toroi (on one occasion only, Batch 1, there were 8 *E. coli* 100 g^{-1}).

Batch	**Natural *E. coli* 100 g^{-1}**	**Treatment**	**Prep day**	**1 week**	**2 weeks**	**1 month**	**2 months**	**8 months**
			***E. coli* 100 g^{-1}**					
1	8	Low High	34 30,000	11 8,000	4 1,300	2 1,700	<2 500	<2 <2
2	<2	Low High	80 30,000	11 160,000	4 24,000	<2 8,000	<2 40	<2 <2
3	<2	Low High	8 160,000	30 1,300	13 2,200	2 900	<2 3,000	<2 <2
4	<2	Low High	130 90,000	13 2,400	<2 3,000	2 40	<2 <2	<2 <2
5	<2	Low High	50 30,000	<2 900	<2 3,000	<2 2,400	<2 110	<2 <2
6	<2	Low High	14 30,000	<2 16,000	11 2,400	<2 340	<2 230	<2 <2
7	<2	Low High	9 24,000	2 16,000	4 16,000	<2 2400	<2 230	<2 <2
8	<2	Low High	110 90,000	14 8,000	13 2,400	<2 1,700	<2 400	<2 <2
9	<2	Low High	110 30,000	8 14,000	4 1,300	8 230	<2 300	<2 <2
10	<2	Low High	170 90,000	<2 13,000	4 5,000	<2 3,000	<2 1,100	<2 <2

Table 4.1. Recovery of *E. coli* from Toroi contaminated at both low and high levels with a laboratory grown culture (*E. coli* NZRM 916). Toroi was held at 4°C throughout the 8 month study period.

To evaluate the survival of *E. coli* in the Toroi, statistical analysis was undertaken for all 10 batches and the results are shown graphically by box plot. The survival at a low dose is shown at Figure 4.1 and high dose in Figure 4.2.

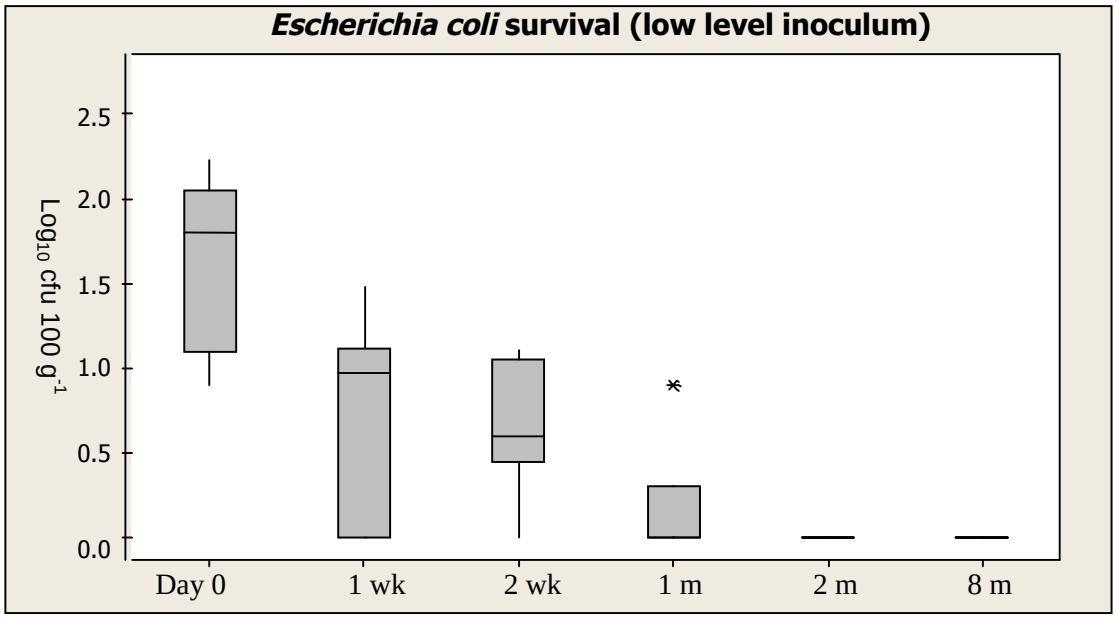


Figure 4.1. Boxplots summarising the survival of *E. coli* over 8 months at 4°C when Toroi was contaminated at the low level. The box bounds the 25% to the 75% confidence limits of the data and the central bar represents the medium. Box extensions represents the 95% confidence limits and outliers beyond this are represented as *.

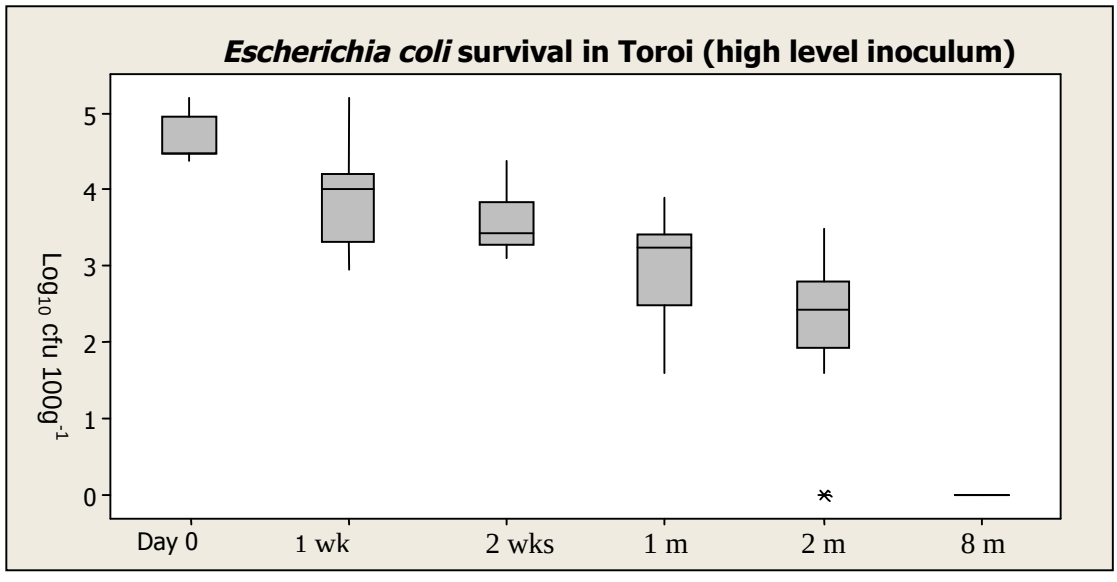


Figure 4.2. Boxplots summarising the survival of *E. coli* over 8 months at 4°C when Toroi was contaminated at the high level. The box bounds the 25% to the 75% confidence limits of the data and the central bar represents the medium. Box extensions represents the 95% confidence limits and outliers beyond this are represented as *.

The variability between batches was high in the low level samples as demonstrated by the length of the boxes. But this was not so pronounced for the high level samples. In the low level samples *E. coli* had declined below the limit of detection after two months but in the high level samples *E. coli* was still recovered after two months. The overall trend was that *E. coli* declined steadily over two months and that none were recoverable after eight months.

In addition, the survival of *E. coli* was compared on the basis of the plant material type used as a Toroi ingredient. No difference was identified on this basis as shown in Figure 4.3.

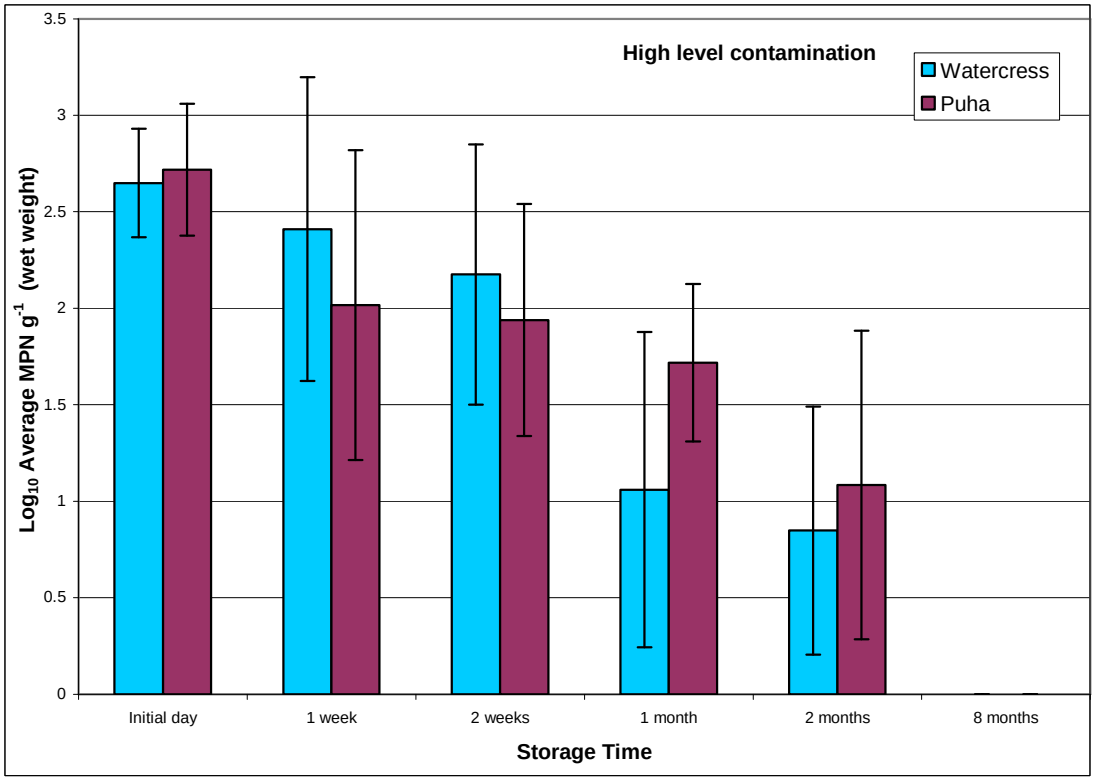


Figure 4.3. Log_{10} Average of *E. coli* (high level) in 5 batches of Toroi containing watercress and 5 batches containing puha as the plant material over 8 months at 4°C.

The survival of *E. coli* NZRM 916 at refrigerated temperatures was also assessed by storage in 0.1% Peptone buffer. The survival rate in buffer was compared to the average survival rate obtained over the 10 batches of Toroi and the results are shown in Figure 4.4. The results of linear regression analysis demonstrated that there was little difference in the inactivation rate of *E. coli* NZRM 916 in Peptone buffer (y = -0.0363x) compared to that in Toroi (y = -0.0394x), in spite of a higher level of inoculum being used for the Peptone buffer experiment. However, there was more variability in recovery from Peptone buffer (R^2 = 0.67%) than from Toroi (R^2 = 92%), which suggests that Toroi provides a consistently favourable environment for *E. coli*.

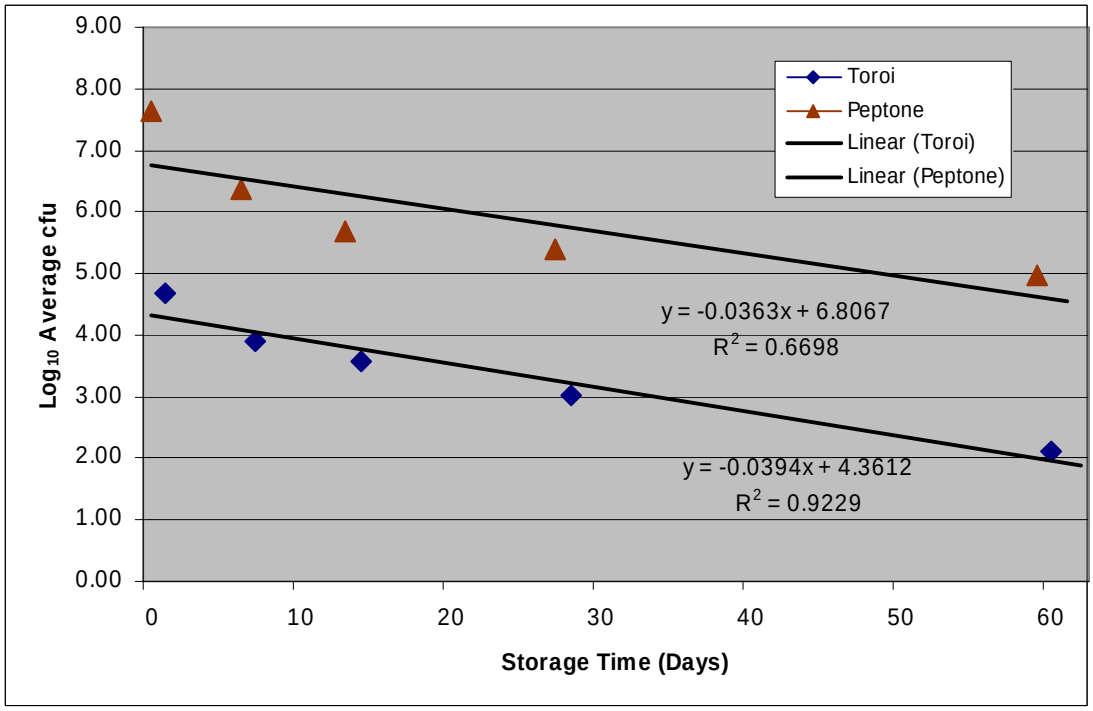


Figure 4.4. The survival of *E. coli* NZRM 916 at 4 °C in 0.1% Peptone buffer (cfu ml^{-1}) and in Toroi (cfu g^{-1})

4.4. Discussion

The preparation of Toroi involved boiling the vegetable component for 30 minutes and mixing this with very lightly cooked chopped mussels. Boiling would likely kill any naturally present *E. coli* on raw vegetables; however, as the mussels were only lightly cooked any naturally present *E. coli* might have survived. In this study natural *E. coli* was only recovered from one of the ten batches of uncontaminated Toroi and then in very low numbers. The absence of *E. coli* in the uncontaminated Toroi demonstrates that the *E. coli* recovered from the contaminated samples was the *E. coli* NZRM 916 that was added in the laboratory. It might be thought that a laboratory-grown strain would not survive as well as a "wild" strain but the recovery of *E. coli* NZRM 916 for up to 2 months, from Toroi contaminated at a high level, demonstrates its suitability for use in this survival study.

Five samples of Toroi were prepared using watercress and five using puha. There was no significant difference in recovery of *E. coli* on the basis of plant material demonstrating that survival was not affected by the choice of plant.

The number of *E. coli* added to the Toroi was reasonably similar between all ten batches but there was more variability in the number of *E. coli* recovered from batches contaminated at a low level. This difference in variability was statistically significant ($t = -16.55$, $p = 0.000$, $df = 14$). Some of this variability is undoubtedly due to the difficulty in mixing the *E. coli* evenly through the Toroi, a difficulty that would be expected to be greater for a lesser number of bacteria. In

spite of the variability in recovery of *E. coli* from the Toroi at low level, the same survival trend was shown as for the high level contamination.

The low level contamination more closely reflects the level of *E. coli* that would be expected in Toroi prepared from mussels collected from a site with an acceptable level of faecal contamination. The average numbers of *E. coli* in the low level samples was 45 *E. coli* 100 g^{-1}, which is less than the allowable level in ready-to-eat food (< 3 g^{-1} i.e. < 300 *E. coli* 100^{-1} gram). The results demonstrate that at the low level of contamination the *E. coli* had declined to barely detectable levels by the end of the first month of storage, suggesting that refrigeration is an important safeguard for Toroi.

At the high level of contamination *E. coli* was about 200-fold higher than the level of acceptability for consumption. This contamination level was chosen to clearly identify the rate of *E. coli* decline and would not reflect a contamination level that would be acceptable for shellfish. It does however, demonstrate that if grossly polluted mussels were used, refrigeration could not be relied on to bring about sufficient decline in *E. coli* in 2 months.

Chapter 5

Inhibition of *Listeria monocytogenes* by *Lactobacillus sake* Lb706 in Toroi

5.1. Introduction

Mussels are widely used as an ingredient in Toroi and these shellfish have been associated with the cold tolerant pathogen *Listeria monocytogenes* (Turner *et al.*, 2005; Bell and Kyriakides, 1998; Brett *et al.*, 1998). The prolonged refrigerated storage of Toroi provides ideal conditions for *L. monocytogenes* to grow and attain high concentrations and therefore high risk. Lactic Acid Bacteria produce a range of bacteriocins (compounds produced by some species of bacteria that are inhibitory to other bacteria), some of which have been used successfully to prevent the growth of *L. monocytogenes* at refrigerated temperatures (Schillinger and Lucke, 1989; Schillinger *et al.*, 2001; Lewus *et al.*, 1991). For example, several investigators (Choi and Beuchat, 1994; Cheigh and Park, 1994; Choi and Park, 1999) have reported the antilisterial activity of different lactic acid bacteria in Kimchi (an Asian fermented food product similar to Toroi). This chapter reports the results of an investigation to determine whether *Lactobacillus sake* Lb706 could prevent the growth of *L. monocytogenes* in Toroi.

5.2. Methods

The effectiveness of *L. sake* Lb706 at inhibiting the growth of *Listeria monocytogenes* was tested as a possible food safety intervention for Toroi. Collection and preparation of Toroi is described in sections 2.1 and 2.2.

5.2.1. Bacterial strains

Two strains of *L. monocytogenes* were used: *L. monocytogenes* NZRM 44, a reference strain commonly used in food safety studies and *L. monocytogenes* L70, an in-house strain (supplied by R. Clemens, AgResearch). A lactic acid bacterium (LAB), *Lactobacillus sake* Lb706 that has known anti-bacterial activity against *L. monocytogenes* was supplied by R. Jones, AgResearch.

Maintenance and enumeration of *L. monocytogenes* is described in sections 2.10 and 2.10.1. Recovery of *L. monocytogenes* after exposure to inhibitors was assessed by the direct plate count method using MOX agar (Oxoid UK) as growth medium. This was done by spreading aliquots of 0.1 ml to triplicate semi-dried MOX agar plates. Inoculated plates were incubated at 37°C for 48 hours.

Growth and preparation of *L. sake* inocula was done in liquid medium as described in section 2.10.2. The number of *L. sake* Lb706 in the inoculum was enumerated by the spread plate technique on plates of MRS agar (see Appendix 1) that were incubated for 24 hours at 30^{o}C in a microaerophilic atmosphere (CampyGen, Oxoid UK).

5.2.2. Detection of inhibition by *L. sake*

Detection of inhibition of *L. monocytogenes* by *L. sake* was identified by either combination culture (Schillinger, 1994), or by the disc diffusion assay described below.

Disc diffusion assay

For this assay the target (in this case *L. monocytogenes,* either NZRM 44 or L70) and inhibitor organism (in this study *L. sake* Lb706) are grown simultaneously and demonstration of antagonism depends upon the release into the medium of a diffusible inhibitor early in the growth phase of the test organism (De Vuyst *et al,* 1994). A freshly grown culture of *L. monocytogenes* (prepared as described in Section 2.10) was spread over the entire surface of a Petri plate containing semi-dried Plate Count Agar. 20 µl of a freshly grown culture of *L. sake* Lb706 (prepared as described in Section 2.10.2) was pipetted onto a 6 mm disc of filter paper and two discs were placed onto each inoculated plate. Plates were incubated at 30°C in a microaerophilic atmosphere (CampyGen, Oxoid UK) for 18 hours. Inhibition was detected by measuring the diameter of the zone of clearing around the 6mm disk (in mm), and subtracting 6mm from this to allow for the diameter of the paper disc. Inhibition zone measurements are reported as the average of the measurements from the two discs on each plate.

5.2.3. Inhibition of *L. monocytogenes* by *L. sake* Lb706 in a soluble matrix

Preliminary experiments were carried out to identify inhibition of *L. monocytogenes* by *L. sake* Lb706 in a soluble matrix; brain heart infusion broth (BHI), prior to the study of inhibition in Toroi. These preliminary experiments were designed to determine an effective growth temperature to prepare an *L. sake* inoculum and to determine the effectiveness of an inoculum grown under the best conditions against different initial concentrations of *L. monocytogenes* cells. These experiments consisted of:

(a) Determining the most suitable temperature for growing the *L. sake* inoculum by:

- Growth of *L. sake* inoculum overnight at 25°C
- Growth of the *L. sake* inoculum overnight at 30°C

(b) Determining the effectiveness of inhibition of a specified inoculum of *L. sake* against different initial concentrations of *L. monocytogenes* by:

- Addition of *L. sake* to 10^2 *L. monocytogenes* cells ml^{-1} BHI
- Addition of *L. sake* to 10^4 *L. monocytogenes* cells ml^{-1} BHI

Inhibition was determined using the disc diffusion assay (as described above) and also by the combined culture technique (in which the target *L. monocytogenes* strain and the inhibitor producing *L. sake* were inoculated together into a test medium and incubated under the conditions required for a particular experiment).

For the combined culture method the *L. sake* inoculum used was 200 µl of the overnight broth culture (expected number of cells ~10^7). The inoculum for the *L.*

monocytogenes test strains was also an aliquot of an overnight broth culture, diluted to obtain the required number of cells: 10^2 and 10^4. This was achieved by diluting the overnight culture to the required level and adding 1 ml of the appropriate dilution. The actual number of cells in the inoculum, both *L. sake* and the two *L. monocytogenes* strains was determined by spread plating as described above.

Both *L. monocytogenes* and *L. sake* were added to ½ strength BHI broth (a medium that supported the growth of both of the target and inhibitor organisms; R. Jones, AgResearch pers. comm.) and the "combined cultures" transferred to a refrigerator at 4°C and examined periodically over a storage interval of 15 days with survival of *L. monocytogenes* determined by spread-plating aliquots to triplicate MOX plates for each dilution (as described above).

The procedure for the combined culture study is summarised diagrammatically in Figure 5.1

- Series a = *L. monocytogenes* NZRM 44
- Series b = *L. monocytogenes* L70

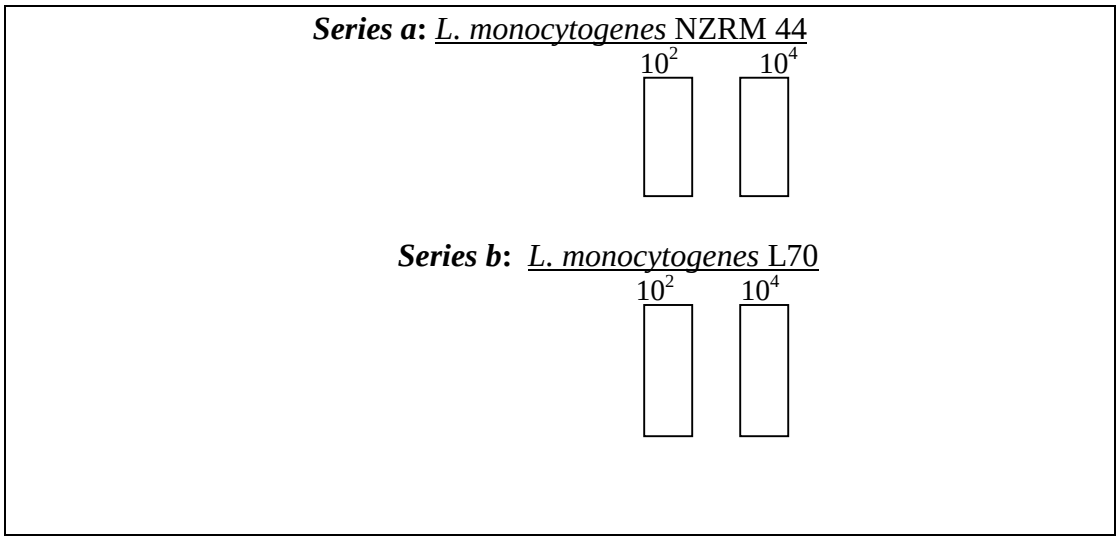


Figure 5.1. Diagram summarising a combined culture study undertaken to evaluate the effectiveness of *L. sake* against two different strains of *L. monocytogenes* used at two different initial concentrations, 10^2 cells and 10^4 cells when incubated at 4 °C for up to 15 days. For all treatments *L. sake* inoculum was 200 µl (~10^7 cells) of an (undiluted) overnight culture.

5.2.4. Inhibition of *L. monocytogenes* by *L. sake* Lb706 in Toroi

Inhibition of *L. monocytogenes* by *L. sake* in Toroi was investigated using *L. monocytogenes* L70, the more susceptible of the two test strains. As done for the preliminary experiments; *L. monocytogenes* L70 cells (obtained from appropriate dilutions of an overnight broth culture (section 5.2.3.) were inoculated into Toroi at two initial levels (10^2 and 10^4 cells).

In addition, the effectiveness of *L. sake* inocula containing different numbers of *L. sake* cells; designated higher (1 ml of an overnight culture – expected to contain 10^8 cells) and lower (200 µl of an overnight culture – expected to contain 10^7 cells) was evaluated. The *L. sake* inoculum was prepared by growing *L. sake* Lb706 on MRS broth in a microaerophilic atmosphere (CampyGen, Oxoid UK) overnight at 30°C.

Three sets of Toroi tubes were prepared and designated: Series A, B or C:

- Series A - No *L. sake* added
- Series B (lower) - 200 µl *L. sake* added
- Series C (higher) – 1 ml *L. sake* added

For each series a pair of Universals was inoculated with *L. monocytogenes* L70 at either 10^2 or 10^4 cells (i.e. 4 Universals per series) as summarised diagrammatically in Figure 5.2.

The number of cells in the inocula was determined by spread plating (as described above).

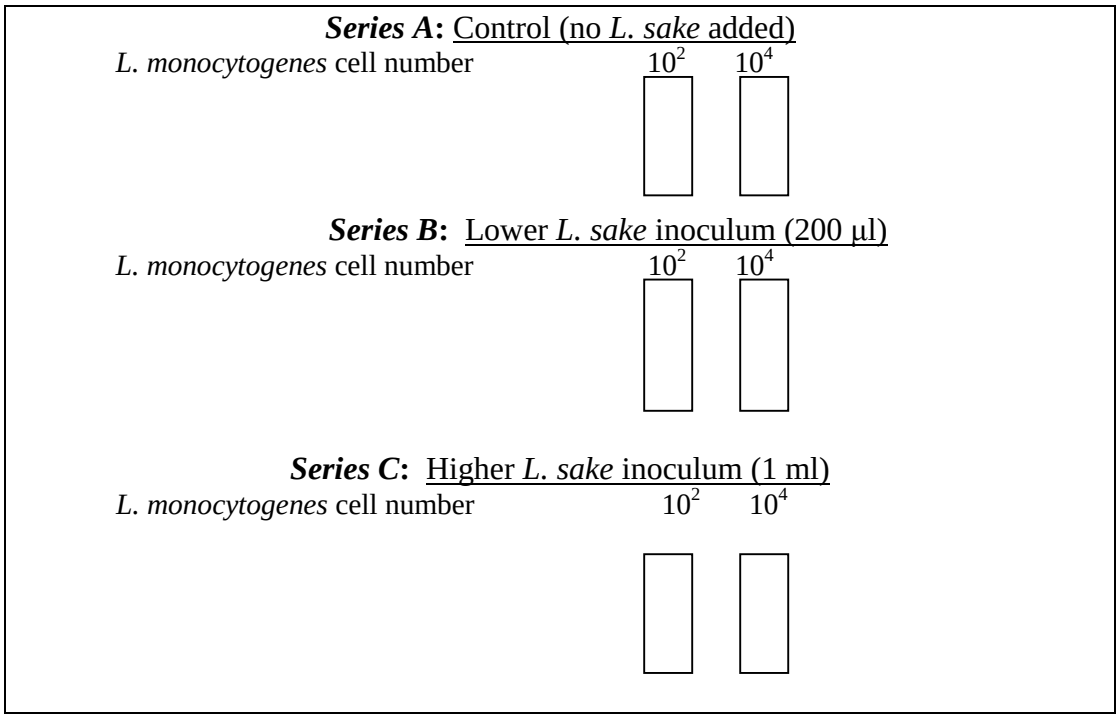


Figure 5.2. Diagram showing three series of combined culture studies to determine the effectiveness of inhibition of *L. monocytogenes* L70 by *L. sake* Lb706. All inocula were from overnight broth cultures. The *L. monocytogenes* cultures were diluted appropriately to achieve 10^2 or 10^4 cells. The lower *L. sake* inoculum was addition of 200 µl of an overnight broth culture (~10^7 cells) and the higher inoculum was an addition of 1 ml of an overnight broth culture (~10^8 cells).

To enable even distribution of the inoculated cells throughout the Toroi, equal quantities of ½ strength BHI broth and pureed Toroi were very thoroughly mixed prior to inoculation. This "diluted" Toroi mixture was added as 20 ml aliquots to sterile Universal bottles and 1 ml of the required initial concentration of *L. monocytogenes* L70 added together with the required amount of *L. sake*. The contents of inoculated Universals were thoroughly mixed and then transferred to a refrigerator at 4^{o}C and stored for up to 26 days with survival of *L. monocytogenes* determined by spread-plating aliquots to triplicate MOX plates for each dilution (as described above).

5.3. Results

When the *L. sake* inoculum was grown at 25°C the overnight broth culture contained 5.6 x 10^8 cells ml^{-1} and when it was grown at 30°C the overnight broth culture contained 4.6 x 10^8 cells ml^{-1} demonstrating the ability of this bacterium to grow equally well at both temperatures. However, the results of the preliminary experiments demonstrated that the ability of *L. sake* to inhibit *L. monocytogenes* was strongly influenced by the temperature at which the inoculum was grown.

As the results of the preliminary experiments demonstrated, when the *L. sake* inoculum was grown at 25°C there was little or no inhibition of either *L. monocytogenes* L70 or *L. monocytogenes* NZRM 44. However, when the *L. sake* inoculum was grown at 30°C there was strong inhibition of *L. monocytogenes* L70 and detectable inhibition of *L. monocytogenes* NZRM 44. This finding was

confirmed by both disc diffusion assay and combined culture as demonstrated by the results presented in Tables 5.1.

To illustrate the effectiveness of growing the *L. sake* inoculum at 30°C the results are also given diagrammatically in Figure 5.3 (NZRM 44) and Figure 5.4 (L70). These two figures describe the results obtained when L. sake inocula grown at either 25°C or 30°C were inoculated into BHI containing 10^4 *L .monocytogenes* cells. These figures also show the greater susceptibility of the L70 strain.

Inhibition zones (mm)				
30°C	*L. monocytogenes* L70		*L. monocytogenes* NZRM 44	
	Disk 1	Disk 2	Disk 1	Disk 2
	24	24	11	11
	25	25	12	18
	24.5	24.5	11.5	14.5
6mm subtracted (paper disk diam.)	− 6	− 6	− 6	− 6
Average	18.5		7	
25°C	*L. monocytogenes* L70		*L. monocytogenes* NZRM 44	
	Disk 1	Disk 2	Disk 1	Disk 2
	7	7	6	6
	7	7	6	6
	6.5	7	6	6
6mm subtracted (paper disk	− 6	− 6	− 6	− 6
Average	1		0	

Table 5.1. Inhibition of *L. monocytogenes* NZRM 44 and *L. monocytogenes* L70 by an *L. sake* Lb706 inoculum grown at either 25°C and 30°C as determined by disc diffusion assay.

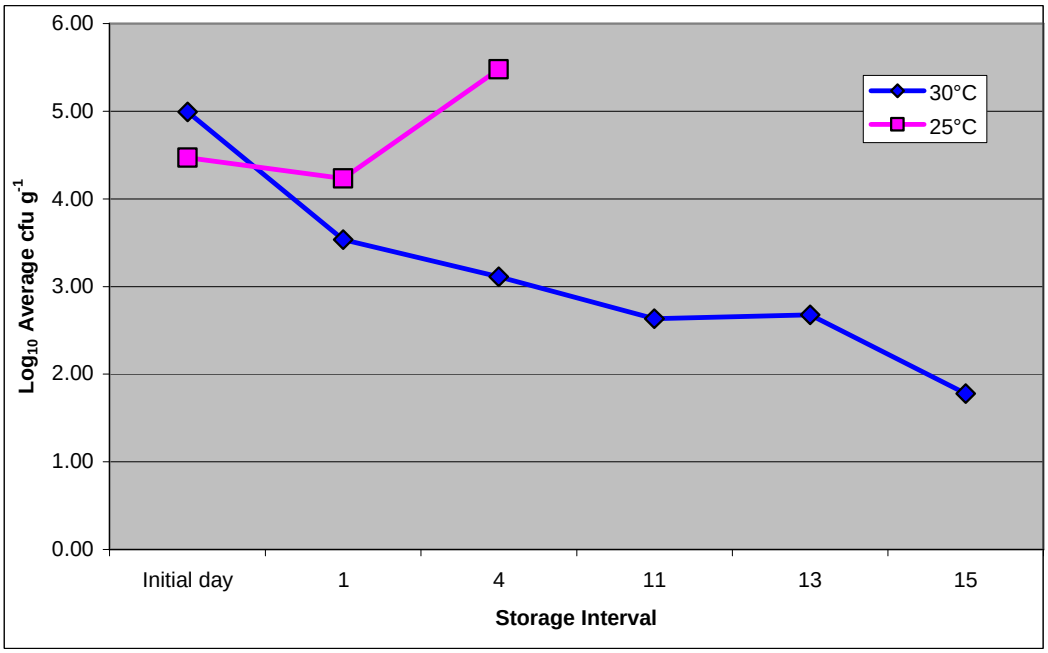


Figure 5.3. Inhibition of *L. monocytogenes* NZRM 44 when an initial concentration of 10^4 cells was exposed to 200µl of *L. sake* Lb706 inoculum grown at 30°C or 25°C. The combined cultures were held at 4°C in ½ strength BHI broth for up to 15 days.

Note. No values are given at 25°C after 4 days as there were too many colonies to count on plates (TNTC).

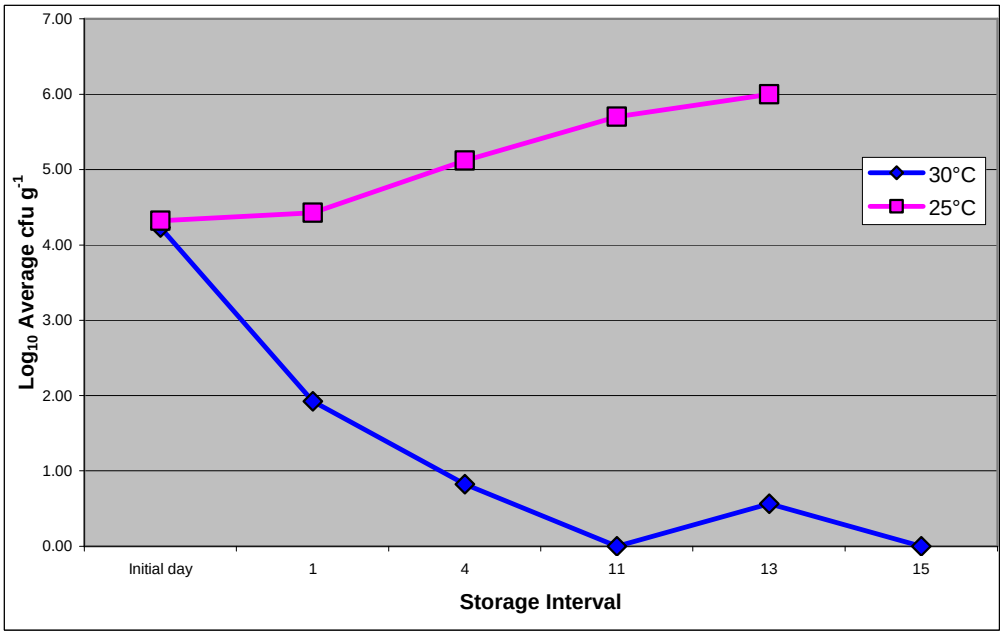


Figure 5.4. Inhibition of *L. monocytogenes* L70 when an initial concentration of 10^4 cells was exposed to 200 µl of *L. sake* Lb706 inoculum grown at 30°C or 25°C. The combined cultures were held at 4°C in ½ strength BHI broth for up to 15 days

Note. No values are given at 25°C for day 15 there were too many colonies to count on plates (TNTC).

As shown in Table 5.2 the inhibition of both strains of *L. monocytogenes* was much greater when the initial inoculum was only 10^2 cells than when it was 10^4 cells. As well as shown diagrammatically in Figure 5.5, when the two strains were compared for susceptibility to *L. sake* the inhibition of L70 was much greater than that of NZRM 44.

L. sake Lb706 inoculum grown at 30°C					
L. monocytogenes NZRM 44			*L. monocytogenes* L70		
No. cells added	9.8×10^4	980	No. cells added	1.7×10^4	170
Days incubated @4°C	Concentraion Log_{10} cfu ml^{-1}		Days incubated @4°C	Concentraion Log_{10} cfu ml^{-1}	
1	3.54	2.54	1	1.92	0.00
4	3.11	1.37	4	0.82	0.00
11	2.63	0.00	11	0.00	0.00
13	2.68	0.00	13	0.56	0.00
15	1.78	0.00	15	0.00	0.00
L. sake Lb706 inoculum grown at 25°C					
L. monocytogenes NZRM 44			*L. monocytogenes* L70		
No. cells added	2.9×10^4	293	No. cells added	2.1×10^4	210
Days incubated @4°C	Concentraion Log_{10} cfu ml^{-1}		Days incubated @4°C	Concentraion Log_{10} cfu ml^{-1}	
1	4.23	2.78	1	4.32	2.18
4	5.48	3.48	4	4.43	3.12
7	TNTC	TNTC	7	5.12	3.70
9	TNTC	TNTC	9	5.70	TNTC
15	TNTC	TNTC	15	TNTC	TNTC

Table 5.2. Two levels of *L. monocytogenes* (NZRM 44 and L70) were combined with 200 µl of *L. sake* Lb706 inoculum grown at either 30°C and 25°C and the combined cultures grown incubated in ½ strength BHI broth at 4°C for up to 15 days (TNTC >Log_{10} 10.48 ml^{-1})

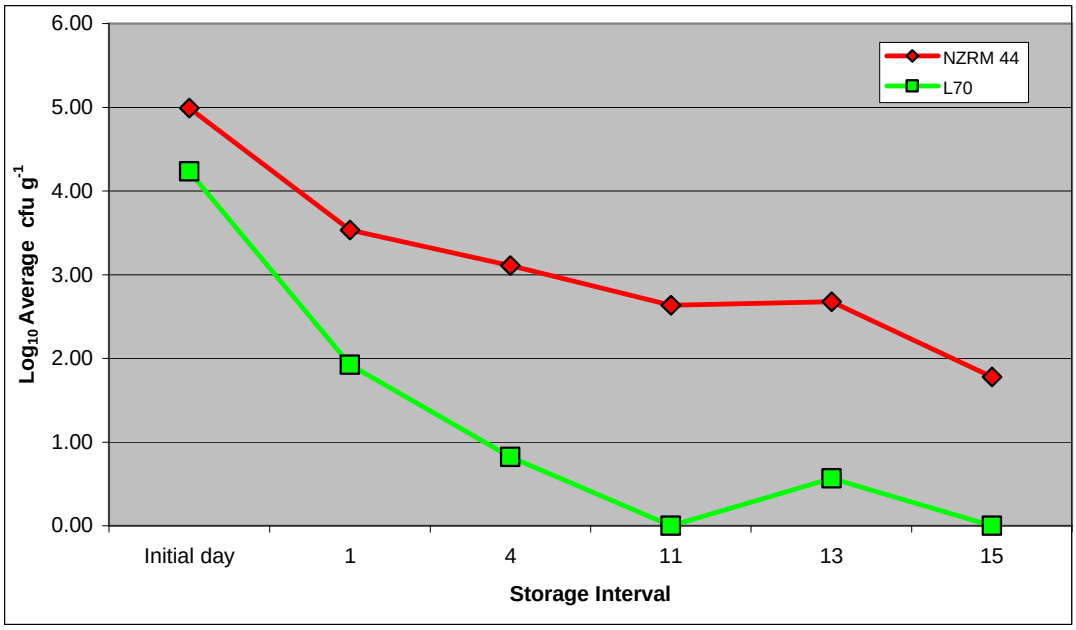


Figure 5.5. Inhibition of *L. monocytogenes* (NZRM 44and L70 with initial target concentrations of Log_{10} 4) by 200 µl *L. sake* 706 (10^8 cfu ml^{-1}) in ½ strength BHI broth at 4°C.

The results of the preliminary studies showed that it was necessary to grow the *L. sake* inoculum at 30°C.

It was decided to use only the wild strain, *L. monocytogenes* L70, in the Toroi study and to add this strain to Toroi at initial concentrations of 10^2 and 10^4, the former being considered more likely to occur naturally, the latter being more likely to illustrate survival trends.

5.3.1. Inhibition of *L. monocytogenes* L70 by *L. sake* Lb706 in Toroi

The *L. monocytogenes* culture that was grown for addition to the Toroi contained 3.6×10^8 cells ml^{-1} (i.e. the actual number in the 10^2 target was 3.6×10^2 cells and in the 10^4 target was 3.6×10^4) The measured number of cells in the "lower" level

L. sake (Series B: 200 µl added) was 1 x 10^8 cells and in the "higher" level (Series C: 1 ml added) was 5.1 x 10^8 cells.

The recovery of *L. monocytogenes* L70 over the 19 day storage from each of the series, A (no *L. sake* added) B (lower dose of *L. sake*) and C (higher dose of *L. sake*), for both initial numbers of *L. monocytogenes* (Log_{10} cfu ml^{-1} Toroi) is summarised in Table 5.3 and shown diagrammatically in Figure 5.7. The degree of inhibition was dependant on the amount of *L. sake* added to the Toroi. Without *L. sake* added, *L. monocytogenes* grew about 7-fold within 19 days of storage at 4°C (Figures 5.6 and 5.7).

As shown, the addition of 1 x 10^8 cells from an overnight culture of *L. sake* to the *Listeria* contaminated Toroi had no inhibitory effect, irrespective of the initial number of *L. monocytogenes* cells present. The viable counts of *L. monocytogenes* were similar to those in Toroi to which no *L. sake* was added. However, addition of 5 x 10^8 cells from an overnight culture of *L. sake* Lb706, which was an increase in *L. sake* inoculum of a half-log, effectively decreased *L. monocytogenes* L70 at both initial cell inoculum levels tested. The rates of decrease were similar (compare Figure 5.6 and 5.7 for series C) and *L. monocytogenes* was no longer detectable (<10 cfu g^{-1}), or barely detectable (10 cells) after 12 days. The results of this study show that at least 5 x 10^8 cells of *L. sake* Lb706 are required for an effective elimination of *L. monocytogenes* L70 from Toroi.

Series A: *L. monocytogenes* L70 Control (no *L. sake* added) Log_{10} cfu g^{-1}		
No. cells added	3.9×10^4	396
Days incubated @ 4°C	Concentration Log_{10} cfu / ml	
2	4.08	2.23
7	5.27	3.28
12	9.40	5.41
19	11.69	6.69
Series B: Low *L. sake* inoculum Log_{10} cfu g^{-1}		
No. cells added	3.9×10^4	396
Days incubated @ 4°C	Concentration Log_{10} cfu ml^{-1}	
2	4.43	1.88
7	5.12	3.13
12	8.56	4.84
19	10.97	6.43
Series C: High *L. sake* inoculum Log_{10} cfu g^{-1}		
No. cells added	3.9×10^4	396
Days incubated @ 4°C	Concentration Log_{10} cfu m^{l-1}	
2	2.81	1.30
7	0.70	0.00
12	1.00	0.00
19	0.00	0.00

Table 5.3. Two levels of *L. monocytogenes* L70 were combined with two levels of *L. sake* inoculum grown at 30°C and the combined cultures grown incubated in Toroi and ½ strength BHI broth at 4°C for up to 19 days. Note: because 1 ml of each dilution of *L. monocytogenes* was added, the initial day is a number of cells; not a concentration.

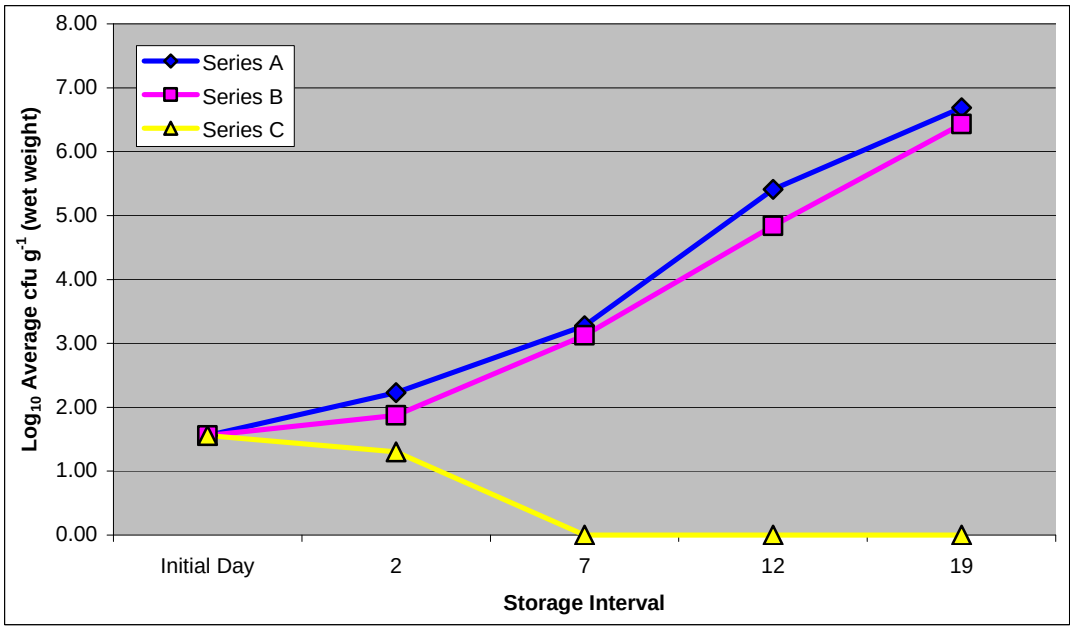


Figure 5.6. Series of *L. sake* added to Toroi with *L. monocytogenes* L70 (initial count of ~ Log_{10} 2) stored at 4°C up to 19 days. Series A: Control series containing no *L. sake*; Series B: diluted *L. sake* series; Series C: 1 mL *L. sake* series.

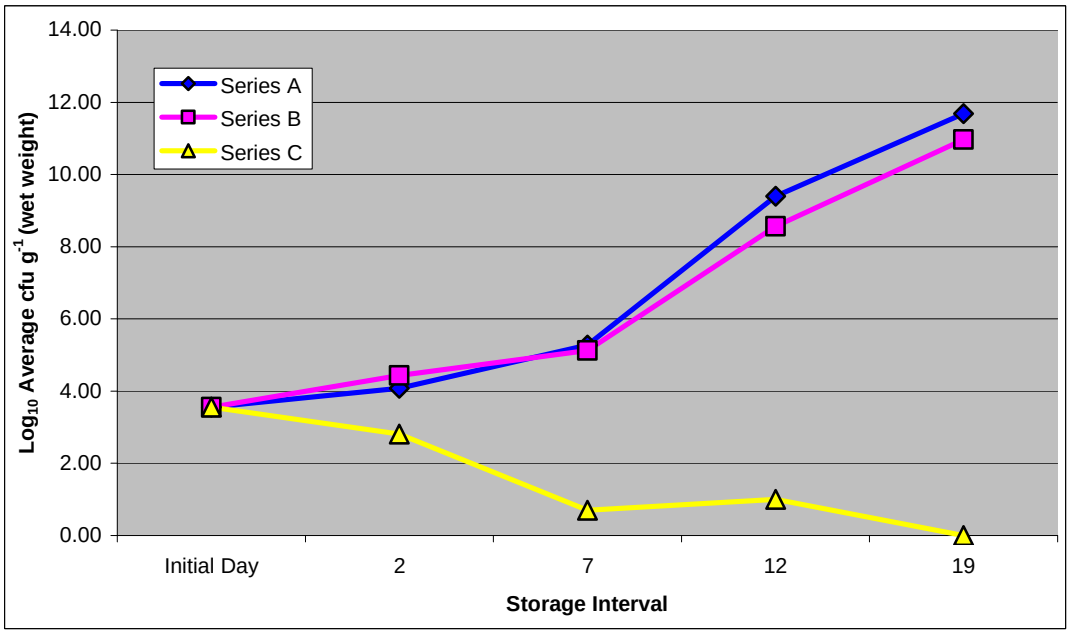


Figure 5.7. Series of *L. sake* added to Toroi with *L. monocytogenes* L70 (initial count of ~ Log_{10} 4) stored at 4°C up to 19 days. Series A: Control series containing no *L. sake*; Series B: diluted *L. sake* series; Series C: 1 mL *L. sake* series.

5.4. Discussion

This study demonstrated that *L. monocytogenes* L70 grew well in Toroi at 4^{o}C. Without *L. sake* added, *L. monocytogenes* grew about 7-fold within 19 days of storage at 4°C. Inhibition of *L. monocytogenes* by *Lactobacillus sake* Lb706 in Toroi stored at 4^{o}C was observed. *L. sake* Lb706 was able to suppress multiplication of *L. monocytogenes* in Toroi. These results have demonstrated that this strain of *L. sake* had an anti-listerial effect on the two strains of *L. monocytogenes* (NZRM 44 and L70) which demonstrated that the bacteriocin that *L. sake* Lb706 contains, reported to be sakacin A (Schillinger, 1994; Chen and Hoover, 2003; Dzung *et al.,* 2000) was an effective inhibitor of *L. monocytogenes* at 4°C. These results have demonstrated that multiplication of *L. monocytogenes* L70 in Toroi was prevented by the addition of higher amounts of *L. sake* Lb706. Schillinger (1994) reported an addition of *L. sake* at a 1:1 ratio in MRS broth resulted in a decrease of *Listeria* viable counts after 5 days at 6°C. At a ratio of 1:100, fewer than 1000 *Listeria* organisms were detected ml^{-1} after 5 days. Thus, relatively high cell numbers are required for an effective elimination of *Listeria.*

Other workers have demonstrated that among *Listeria monocytogenes* strains there were differences in the susceptibility of *L. monocytogenes* to bacteriocins produced by *L. sake* Lb706 (designated sakacin A), and have reported that some strains of *L. monocytogenes* were more sensitive than others (Katla *et al.,* 2003; Schillinger, 1994). In this study, *L. monocytogenes* L70 was found to be more sensitive than *L. monocytogenes* NZRM 44.

This experiment has also shown that the anti-listerial properties of *L. sake*706 are temperature-sensitive, demonstrating that the anti-listerial effect of *L. sake* Lb706 was optimal when grown at 30^{o}C. Dzung *et al*. (2000) reported that *L. sake* Lb706 produced bacteriocin when the growth temperature was kept at 25^{o}C or 30^{o}C, but production was reduced or absent at higher temperatures (33.5 – 35^{o}C). These results have demonstrated that when *L. sake* Lb706 inoculum was grown at 25°C, there was little or no anti-listerial effect on the two strains of *L. monocytogenes* (NZRM 44 and L70) at 4°C.

Chapter 6

Inhibition of *Listeria* with commercial nisin in Toroi

6.1. Introduction

Nisin is a bacteriocin of the lantibiotic type produced by certain strains of Lactic Acid Bacteria belonging to the genus *Lactococcus*. Nisin is a polypeptide (composed of 34 amino acids; Pongtharangkul and Demirci, 2004) with a bacteriocidal activity (Flores *et al.* 2003) due to its action on the cytoplasmic membrane of sensitive cells, particularly *Listeria monocytogenes*. Nisin forms pores in the membrane of sensitive cells that lead to dissipation of the membrane and collapse of the proton motive force.

The bacteriocidal effect of nisin has been exploited in its use as a food preservative. Nisin is the most commercially important bacteriocin and has a relatively long history of safe use (Chen and Hoover, 2003) and is currently approved as a food preservative in approximately 50 countries (Delves-Broughton *et al.* 1996). Biopreservation through the use of bacteriocins like nisin is an alternative to "chemical" preservatives, and as a natural product may be acceptable to Maori.

The antagonistic effects of nisin against *Listeria monocytogenes* was investigated in this study. Inhibition by nisin was tested against two strains of *L. monocytogenes*; L70 and NZRM 44; that were used to determine the effectiveness of *Lactobacillus sake*. This chapter reports studies to investigate nisin effectiveness both in the absence and in the presence of Toroi.

6.2. Methods

Collection and preparation of Toroi is described in sections 2.1 and 2.2. Maintenance and enumeration of *L. monocytogenes* is described in sections 2.10 and 2.10.1.

6.2.1 Preparation of nisin

A commercial preparation of nisin (2.5% nisin concentration balanced with sodium chloride and denatured milk solids) was obtained from the Sigma Chemical Co. For each experiment, a fresh stock solution of nisin was prepared by mixing 1 g of this nisin with 10 ml 0.2 M HCL and stirring until dissolved. The stock solution was serially diluted in sterile distilled water.

6.2.2. Determination of inhibition of *L. monocytogenes* by commercial nisin

Two strains of *L. monocytogenes* were used to determine inhibition by nisin: *L. monocytogenes*: NZRM 44 and *L. monocytogenes* L70. For use these cultures were prepared as described in section 2.10.

Prior to investigating the effectiveness of nisin as an inhibitor of *L. monocytogenes* in Toroi; a preliminary experiment was carried out in ½ strength BHI medium in which the test strain and the inhibitor compound could be thoroughly and easily mixed.

Preliminary Experiment

Universal bottles containing 10 ml of ½ strength BHI broth were inoculated with *L. monocytogenes*, either NZRM 44 or L70 (a total of 12 bottles for each strain) at a target concentration of approximately 10^3 cells. Duplicate Universals for each of the 2 strains received 1 ml of nisin solution, diluted as required to give final concentrations as follows: 10 mg, 1 mg, 100 μg, 10 μg and 1 μg nisin ml^{-1}. One pair of Universals for each strain received 1 ml of sterile distilled water (i.e. minus-nisin control). The contents were thoroughly mixed and the Universals transferred to a refrigerator at 4°C until required. *L. monocytogenes* was enumerated at the following times: 30 minutes after addition of nisin and after 6, 24, 48, and 72 hours.

6.2.3 Determination of the effectiveness of nisin in Toroi

In this experiment the ability of nisin to inhibit *Listeria* in Toroi was investigated using *L. monocytogenes* L70 as the test strain. Universal bottles containing 10 g pureed Toroi were inoculated with *L. monocytogenes* L70 at a target of approximately 10^3 cells. Four series (A, B, C, D) of inoculated Universals were set up so that duplicate Universals could be analysed for each treatment at each time interval. Nisin was added from the stock solution to achieve the following final concentrations of nisin m^{-1} in the Toroi:

- Series A – 1 ml of sterile distilled water (i.e no nisin added)
- Series B – 10 mg nisin
- Series C – 1 mg nisin
- Series D – 100 μg nisin

The contents of each Universal were thoroughly mixed prior to transfer to a refrigerator. Analysis was carried out at the following times: 30 minutes, 6 hours, 24 hours 48 hours, and 72 hours after transfer to the refrigerator.

Data reporting

1. Unless otherwise stated the results for *L. monocytogenes* recovery are expressed as Log_{10} *L. monocytogenes* cfu ml^{-1} for BHI medium and g^{-1} (wet weight) for Toroi.

2. The results for nisin inhibition are given in the results section in terms of the amount of nisin added to each 10 ml aliquot of either BHI or Toroi (rather than the final concentration).

6.3. Results

6.3.1. Inhibition of *L. monocytogenes* by nisin in brain heart medium

Two experiments were carried out to determine the amount of nisin required to inhibit *L. monocytogenes* in a matrix of brain heart infusion broth.

In experiment 1 (which was carried out in ½ strength BHI broth stored at 4^oC), in the absence of nisin, both test strains of *L. monocytogenes* increased in concentration by at least 0.5 Log_{10} over 72 hours as shown in Table 6.1 and Figures 6.1 and 6.2. For *L. monocytogenes* L70 this increase was from Log_{10} 2.26 cfu ml^{-1} to Log_{10} 3.40 after 72 hours (Figure 6.1) and for *L. monocytogenes* NZRM 44

(Figure 6.2) the increase was from an initial count of Log_{10} 2.69 to 3.07 after 72 hours.

Addition of nisin resulted in different responses from the two test strains with *L. monocytogenes* L70 more susceptible than NZRM 44. For *L. monocytogenes* L70 there was effective inhibition in the presence of 1 mg ml^{-1} or more of nisin but for *L. monocytogenes* NZRM 44 there was no inhibition at levels of less than 10 mg ml^{-1} of nisin. Overall, if inhibition occurred evidence was seen in samples analysed 30 mintues after nisin addition. For those samples in which there was no evidence of inhibition after 30 minutes, *L. monocytogenes* recoveries were similar to those for the minus-nisin control. These results demonstrate that storage at 4°C for up to 72 h did not increase inhibition above the response seen after 30 minutes.

Storage Interval	*L. monocytogenes L* 70 Log_{10} CFU ml^{-1}					
	Control L. mono only	*Concentration of nisin (per ml)*				
		10 mg	*1 mg*	*100 µg*	*10 µg*	*1 µg*
1/2 hr	*2.26*	0.00	0.00	2.46	2.40	2.49
6 hrs	*2.43*	0.00	0.78	2.16	2.24	2.29
24 hrs	*2.63*	0.00	0.00	2.18	2.31	2.35
48 hrs	*2.82*	0.00	0.00	2.47	2.58	2.52
72 hrs	*3.40*	0.00	0.00	2.97	3.29	3.22
Storage Interval	*Control L. mono only*	*L. monocytogenes* NZRM 44 Log_{10} CFU ml^{-1}				
1/2 hr	*2.69*	0.00	2.59	2.44	2.67	1.79
6 hrs	*2.68*	0.00	2.90	2.33	2.81	2.77
24 hrs	*3.01*	0.00	2.97	2.41	2.97	3.03
48 hrs	*3.04*	0.00	3.09	2.51	2.96	3.06
72 hrs	*3.07*	0.00	3.22	2.62	2.97	3.09

Table 6.1. The recovery of *L. monocytogenes* L70 and NZRM 44 from BHI broth after addition of nisin at a range of concentrations and storage at 4°C for up to 72 h. Data are the average of 2 results for each strain at each time interval

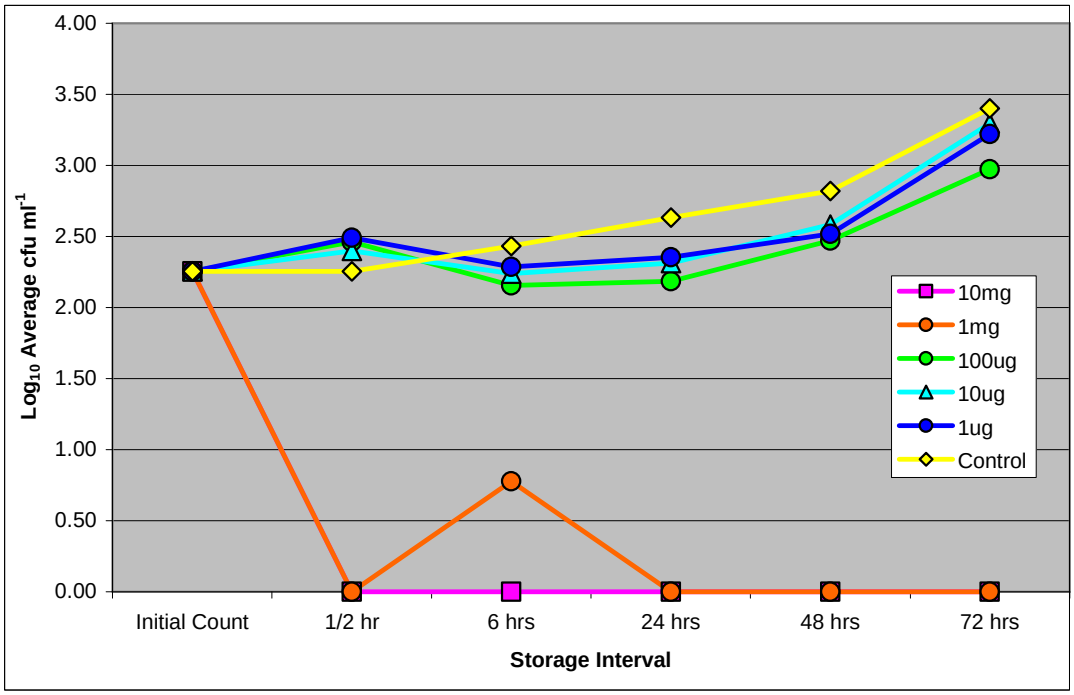


Figure 6.1. Recovery of *L.monocytogenes* L 70 in the presence of varying concentrations of nisin after storage at 4°C for up to 72 h. Each data point is the average of 2 samples

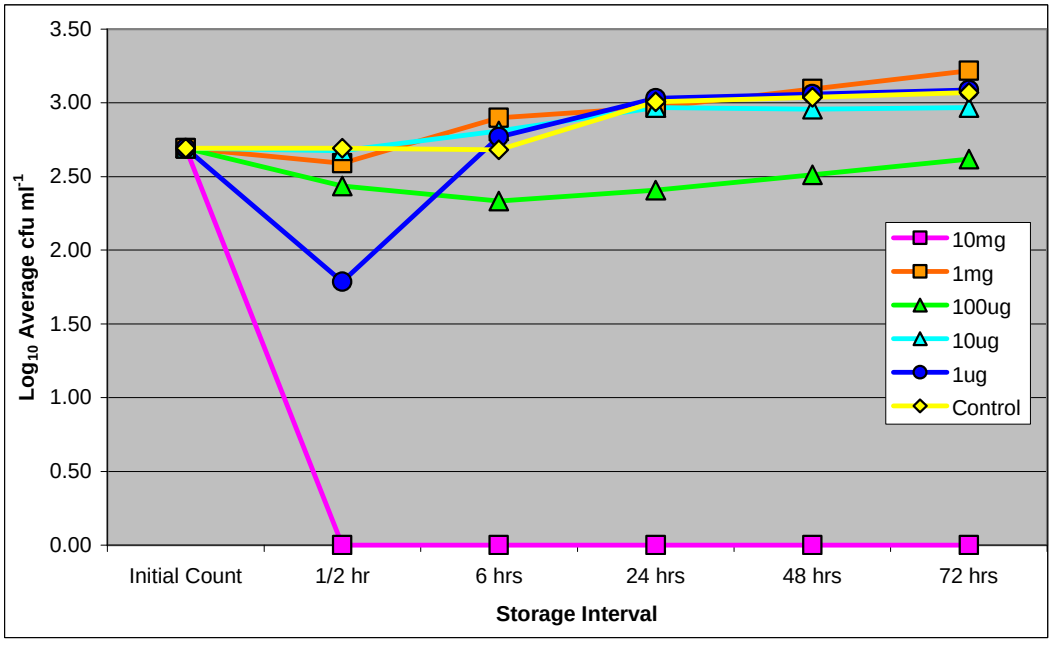


Figure 6.2. Recovery of *L.monocytogenes* NZRM 44 in the presence of varying concentrations of nisin after storage at 4°C for up to 72 h. Each data point is the average of 2 samples

6.3.2. Inhibition of *Listeria monocytogenes* by nisin in Toroi

The effectiveness of nisin as an inhibitor of *L. monocytogenes* L70 in Toroi is summarised in Table 6.2 and shown diagrammatically in Figures 6.3. In contrast to the preliminary experiment in BHI it was found when nisin was present in a concentration of 1 mg ml^{-1} there was no significant inhibition. At a ten-fold higher concentration, 10 mg of nisin ml^{-1}, the growth of *L. monocytogenes* was suppressed but inhibition was not complete. As shown in Figure 6.3 the inhibitory effect of 10 mg nisin ml^{-1} was evident 30 minutes after the addition of nisin; a pattern that was also evident in the preliminary experiment in BHI; but in contrast to BHI, after 72 h the concentration of *Listeria* had started to increase.

	L. monocytogenes L70 Log_{10} CFU g^{-1} (wet weight)			
	Control L. mono only	*Concentration of nisin (per ml)*		
		10 mg	*1 mg*	*100* µg
1/2 hr	2.91	2.17	2.69	2.79
6 hrs	2.84	1.89	2.65	2.68
24 hrs	2.86	1.95	2.68	2.75
48 hrs	3.09	1.91	2.82	2.92
72 hrs	3.46	2.32	3.16	3.24

Table 6.2. Recovery of *L. monocytogenes* L70 from Toroi in the presence of a range of nisin concentrations. Measurements were made in samples stored at 4°C for up to 72 h. Data are the average of results obtained from 2 samples for each treatment at each time interval

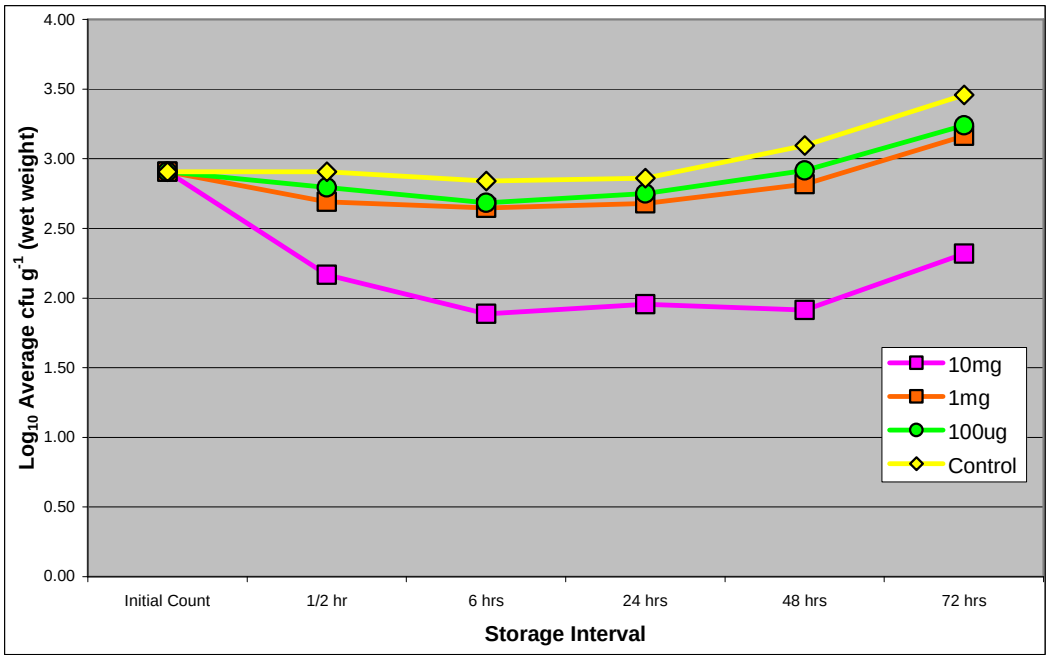


Figure 6.3. Antagonistic effects of diluted nisin against *L. monocytogenes L70* in Toroi sample A

6.4. Discussion

Results obtained from a simple soluble matrix, brain heart infusion broth, demonstrated a ten-fold difference in susceptibility to nisin for the two test strains of *L. monocytogenes*, L70 and NZRM 44. The more susceptible strain, L70, was added to Toroi and it was found that the nisin concentration that effectively inhibited this strain in brain heart broth had no significant effect in Toroi. Suppression of growth at 4°C was evident in the presence of 10 mg ml^{-1} nisin but inhibition was not complete. After 72 h storage at 4°C L70 had again started to grow.

Toroi is a particulate matrix and clearly reduced the effectiveness of nisin inhibition. Schillinger *et al.* (1991) suggested that bacteriocins, such as nisin, may

be absorbed to food and fat particles and then be inactivated. In addition diffusion of nisin may be limited in Toroi compared to that in a liquid medium. Nisin is an ionophore and disruption of membrane permeability depends on contact between the inhibitor and the cell wall of the target bacteria. Budde and Jakobsen (2000) suggested that the reason that nisin is more inhibitory in a soluble system than in a solid and semi-solid system is due to "barrier effects" in a non-soluble system. The physical barrier of food (in this case Toroi) particles and slow diffusion through the food combined with the steric hindrance of the nisin molecules for attacking the cells may, therefore, explain the higher nisin concentration required in foods (Budde and Jakobsen, 2000). Similarly, Scott and Taylor (1981) reported that nisin was much less effective in cooked meat medium containing meat particles, than in brain heart infusion broth and suggest that nisin binds to meat particles. Schillinger *et al.* (1991) discussed another problem which is the loss of activity of the bacteriocin after a certain time. In the Toroi in this study, after 72 hours there was evidence of growth of *L. monocytogenes* L70 in the presence of 10 mg ml^{-1} nisin, an amount that had suppressed growth over the previous 48 h. Muriana (1996) suggested that immediate reduction of target cell populations by 1 to 3 logs upon addition of bacteriocin followed by resumption of growth after storage, could arise when an insufficient quantity of bacteriocin was added to interact with all target cells.

Nisin is widely accepted as a safe food preservative (Riley and Wertz, 2002). Spoilage bacteria include a range of bacteria including spore formers but not pathogens such as *Listeria monocytogenes*. Raju *et al*. (2003) report a 5-fold increase in refrigerated shelf life for fish sausage treated with nisin at 50 μg g^{-1}. In

this study *L. monocytogenes* was suppressed at a nisin concentration of 10 mg g^{-1} but not at a concentration of 1 mg g^{-1}.

The manufacturer of the preparation of nisin used in this study provided information that their product had an activity of $\geq 1 \times 10^6$ IU g^{-1}. In this study the presence of 1×10^4 IU ml^{-1} nisin was demonstrated to be insufficient for complete inhibition of *L. monocytogenes* L70 in Toroi. Preliminary investigations in brain heart infusion demonstrated that L70, a wild type strain, was more susceptible than the food safety reference strain *L. monocytogenes* NZRM 44. Dykes and Moorhead (2002) evaluated inhibition of *Listeria monocytogenes* by nisin on beef and reported that the presence of 5×10^3 IU ml^{-1}, an amount that would be acceptable for addition to a food product, was insufficient to cause inhibition. It is likely that the amount of nisin that would be needed to be added to Toroi to inhibit a range of *Listeria monocytogenes* strains would be unacceptable to consumers, especially Maori.

Chapter 7

Discussion and Conclusion

Toroi is a delicacy food prepared by a method popularly known as "boil-up" amongst Maori. In this study, Toroi was prepared using a procedure that I learned in discussions with senior members of iwi groups to be widely used in the Waikato region. The research of Whyte *et al.* (2201) and Hudson *et al.* (2001) also identified that this method was followed by South Island hapu of Ngai Tahu. Toroi ingredients often include marine mussels and puha or watercress. The first step in Toroi preparation is to boil the puha or watercress. The mussels are steamed (to open their shells), the flesh removed, chopped and added to the cooked vegetable component; the resulting mixture is then packed into screw-top jars and stored in a refrigerator for several weeks to allow low-temperature fermentation to occur before the Toroi is eaten.

From a microbiological point of view, boiling of the puha or watercress for 30 minutes is likely to inactivate bacterial cells but not spores. In this study, the mussels used to prepare the Toroi were purchased from a retail outlet and therefore conformed to New Zealand food standards so there was little of contamination with microorganism of health concern. In practice, Maori often prepare Toroi from mussels harvested from the wild and these may not meet approved standards. The investigations of Toroi described in this study were based on the general New Zealand requirements to ensure food safety of ready-to-eat foods.

Only one of the seven pathogens sought in this study was detected in any of the 10 batches of Toroi. This was *Bacillus cereus*. However, on one occasion a species of *Vibrio* was isolated and identified as *Vibrio alginolyticus.* This species of *Vibrio* is not a food-borne pathogen, but it has been found on seaweed and has been associated with infection of open wounds (Opal and Saxon, 1986; Gomez *et al*., 2003). Commercially produced mussels have been associated with listeriosis (Turner *et al*., 2005) and a *Listeria* species, *L. ivanovii,* was isolated from two batches of Toroi after twelve months storage but this is a non-pathogenic species, however its appearance after prolonged storage demonstrates the ability of *Listeria* species to survive in Toroi during extended refrigeration.

Faecal indicator bacteria, such as *Escherichia coli* are used to indicate the microbiological quality of shellfish. In this study, *E. coli* were recovered sporadically in very low numbers, as the plant material was boiled for 30 minutes these *E. coli* would have been sourced from the mussel component. The New Zealand sanitation programme requirements for shellfish exported or sold at retail outlets are <230 *E. coli* 100^{-1} g. In this study recovery was at least 10-fold less than this level indicating that the Toroi in this study met the guidelines for ready-to-eat foods (<3 g^{-1}) for this indicator.

The only pathogen sought that was regularly recovered from the ten batches of Toroi was *Bacillus cereus.* Toxins produced by some strains of *B. cereus* can cause harm to humans. *Bacillus cereus* concentration limits are imposed to

avoid sufficient numbers of bacteria being present that could produce a toxin at levels sufficient to cause food-poisoning. *Bacillus cereus* was recovered from all batches analysed. This is a concern and care is needed in preparation to prevent their bacterial concentration reaching levels at which toxin production could be of concern. On one occasion, when the vegetable component of the Toroi had had a prolonged cooling period at room temperature, *B. cereus* numbers were only ten-fold less than the concentration at which harmful amounts of toxin could be produced. These results demonstrate that it is extremely important for the vegetable component, the presumed source *B. cereus* and/or the Toroi itself to be cooled quickly and refrigeration temperatures established as soon as possible after preparation. After two weeks of refrigeration, *B. cereus* was no longer recovered from any of the batch. This was presumed due to the effectiveness of cold-temperature fermentation on the survival of *B. cereus*, possibly due to the growth of competing and/or inhibitory species of bacteria derived from the mussels.

To determine the ability of the faecal indicator bacterium *E. coli* to survive during Toroi storage, a laboratory grown strain was added and its survival mapped over eight months. Although concentrations of *E. coli* declined substantially over 2 months but the bacteria could still be recovered. A high level of contamination (of 10^4 *B. cereus* ml^{-1}) was chosen to clearly identify the rate of *E. coli* decline and although it would be hoped that mussels with this level of *E. coli* would not be used in practice. The findings demonstrated that if faecally polluted mussels were used, refrigeration could not be relied on to bring about sufficient decline in *E. coli* in two months. Cheigh and Park (1994)

reported similar results when *E. coli* that was experimentally added to Kimchi, a lactic-acid fermented vegetable product originating from Korea, containing pickled or unpickled seafood (with 5% salt concentration) and fermented at 5°C from Kimchi. *Escherichia coli* were not inactivated completely, but had declined to very low levels by the end of a three to six month fermentation period (Cheigh and Park, 1994).

When laboratory grown strains of *Listeria monocytogenes* were added to Toroi, they were able to grow well, as the concentration increased about seven-fold, over 19 days at 4°C. The known association of *L. monocytogenes* with shellfish, and the extended storage of Toroi at refrigerated temperatures, provides conditions that could produce a "high risk" food product. Two strategies to prevent the growth of *L. monocytogenes* were investigated.

Firstly, a species of lactic acid bacteria known to produce an antilisterial bacteriocin; *Lactobacillus sake* Lb706 was added in combined culture with *L. monocytogenes* to Toroi. It was found that a relatively high number of cells of *L. sake* Lb706 (at least 5×10^8 cells) were required for an effective inoculum to ensure elimination of *L. monocytogenes* L70 from Toroi. It is unlikely that this level of inoculum would be acceptable in practice.

In addition, a purified bacteriocin; nisin, a widely accepted safe food preservative (Riley and Wertz, 2002), was tested for its ability to suppress *L. monocytogenes*. It was found that to suppress *L. monocytogenes* growth in Toroi the concentration of nisin required was 10 mg g^{-1} and there was no

evidence of suppression at 1 mg g^{-1}. Muriana (1996) reported that an immediate reduction of target cell populations by 1 to 3 logs upon addition of bacteriocin that was followed by resumption of growth after storage, was evidence that insufficient bacteriocin had been added to interact with all target cells. Toroi is a particulate matrix and clearly reduced the effectiveness of nisin inhibition. Nisin is an ionophore and disruption of membrane permeability requires contact between the inhibitor and the cell wall of the target bacteria.

The results of this study demonstrated that inhibition of *L. monocytogenes* in Toroi would require large amounts of inhibitor. It is likely that the amount of *L. sake* or purified bacteriocin such as nisin to be added to Toroi that would be needed to inhibit *L. monocytogenes* would be unacceptable to consumers especially to Maori who dislike non-natural.

The recovery of *L. ivanovii* in Toroi in this study demonstrated that cold-temperature fermentation did not lower the pH sufficiently to completely inhibit *Listeria* species in Toroi. Hudson *et al*. (2001) and Whyte *et al*. (2001) suggested that Toroi may be safer with respect to *Listeria* by allowing the lactic acid fermentation to proceed at ambient temperatures for the first 48 hours before transferring to chilled conditions for storage. This regime has been shown to be effective for inhibiting *L. monocytogenes* in Kimchi (Lee *et al.,* 1995). However, as results have demonstrated that *B. cereus* increased to an unsatisfactory level at room temperature and *B. cereus* were constantly present, immediate refrigeration may be preferable.

As a result of my study, I recommend that when people plan to prepare Toroi that raw in-the-shell mussels be purchased from a retail outlet unless a harvesting source is regularly monitored and is known to be free from contamination. As the only known outbreak of botulism was associated with Toroi prepared from cooked mussels it is recommended that the mussels are only lightly steamed to maintain a general bacterial microflora which will be capable of fermentation. I also recommend that the method of Toroi prepared in this study, that the vegetable component and the Toroi itself should be chilled promptly and thoroughly and allowed to ferment for at least two weeks. As a precaution I also recommend that care is taken to maintain Toroi at chilled temperatures (<5°C) until it is actually eaten.

References

Abriouel H, Maqueda M, Galvez A, Martinez-Bueno M & Valdivia E. (2002) Inhibition of bacterial growth, enterotoxin production, and spore outgrowth in strains of *Bacillus cereus* by bacteriocin AS-48. *Applied and Environmental Microbiology, 68,* 1473-1477.

Andersson A, Ronner U, & Granum PE. (1995). What problems does the food industry have with the sporeforming pathogens *Bacillus cereus* and *Clostridium perfringens*. *International Journal of Food Microbiology, 28,* 145-155.

Annous BA, Becker LA, Bayles DO, Labeda DP, & Wilkinson BJ. (1997). Critical role of anteiso-C15:0 fatty acid in the growth of *Listeria monocytogenes* at low temperatures. *Applied Environmental Microbiology, 63,* 3887-3894.

APHA. (1998). *Standard Methods for the Examination of Water and Wastewater* (20th ed.). Washington, DC: American Public Health Association.

APHA. (2001). *Listeria monocytogenes*. In Downs FP & Ito K (Eds.), *Compendium of Methods for the Microbiological Examination of Foods* (4th ed.). Washington DC: APHA.

Arumugaswamy R, Proudford R, & Eyles EJ. (1988). *Campylobacter jejuni* and *Campylobacter coli* in the Sudney rock oyster (*Crassostrea commercialis*). *International Journal of Food Microbiology, 7,* 173-183.

Ashcraft, A. S. (1985). Nutritional beliefs and practices of selected Raukawa tribal members. *Special Report Series / Dept. of Nursing Studies, Massey University, Palmerston North, N.Z, 3*.

Baker M, & Wilson N. (1993). Microbial hazards of seafood: A New Zealand perspective. *Communicable Disease New Zealand, 93*(1), 1-9.

Barefoot SF, & Klaenhammer, T. (1984). Purification and characterisation of the *Lactobacillus acidophilus* bacteriocin lactacin. *Bacterial Antimicrobial Agents Chemoether, 26*, 328-334.

Bayles DO, Annous BA, & Wilkinson BJ. (1996). Cold stress proteins induced in *Listeria monocytogenes* in response to temperature down shock and growth at low temperatures. *Applied Environmental Microbiology, 62*(3), 1116-1119.

Bell C, & Kyriakides A. (1998). *Listeria: a practical approach to the organism and its control in foods.* (1st ed.). London, UK: Blackie Academic & Professional Print, an imprint of Thomson Science.

Benz R, Jung G, & Sahl HG. (1991). Mechanism of channel formation by lantibiotics in black lipid membranes. In Jung G & Sahl HG (Eds.), *Nisin and novel lantibiotics*. Leiden, The Netherlands: Escom Publishers.

Berg G, & T G Metcalf. (1978). Indicators of viruses in waters. In Indicators of Viruses. In: Water and Food. 267-296.

Best E. (1898). Foods of the ancient Maori, Part 1 of sketches from Tuhoeland. *The Canterbury Times, Canterbury, March 1898*.

Beuchat LR. (1982). *V. parahaemolyticus*: Public health significance. *Food technology, 92,* 80-83.

Beuchat LR. (1998). Surface Decontamination of Fruits and Vegetables Eaten Raw: A review. *Food Safety Unit, World Health Organisation WHO/FSF/FOS/98.2*.

Bierbaum G, & Sahl HG. (1985). Induction of autolysis of staphylococci by the basic peptide antibiotics Pep5 and nisin and their influence on the activity of the autolytic enzymes. *Archaeological Microbiology, 141*, 249-254.

Bierbaum G, & Sahl HG. (1987). Autolytic system of *Staphylococci simulans* 22: influence of cationic peptides on activity of *N*-acetylmuramoyl-$_{L}$-alanine amidase. *Journal of Bacteriology, 169*, 5452-5458.

Bierbaum G, & Sahl HG. (1988). Influence of catoinic peptides on the activity of the autolytic endo-*B*-*N*-acetylglucosamidaase of *Staphylococcus simulans* 22. *FEMS Microbiological Letters, 58*, 223-228.

Bierbaum G, & Sahl HG. (1991). Induction of autolysis of Staphylococcus simulans 22 by Pep5 and nisin and influence of the cationic peptides on the activity of the autolytic enzymes. In Jung G & Sahl HG (Eds.), *Nisin and novel lantibiotics*. Leiden, The Netherlands: Escom Publishers.

Black RE, Levine MM, Clements M, Hughes TP, & Blaser MJ. (1988). Experimental *Campylobacter jejuni* infection in humans. *Journal of Infectious Diseases, 157,* 472-479.

Boerema JA & Broda DM. (2004) *Clostridium botulinum.. In*: Editors Jensen W, Devine C & Dikeman M. *Microbiological Safety of Meat Encyclopedia of Meat Sciences*. Elsevier Ltd., B.V. Pp.786-793.

Bremner PJ, & G C Fletcher. (2003). *Clostridium botulinum* in seafood. *New Zealand Institute for Crop and Food Research Limited. Christchurch, N.Z.*

Brett SYM, Short P, & McLauchlin J. (1998). A small outbreak of listeriosos associated with smoked mussels. *International Journal of Food Microbiology, 43*, 223-229.

Buck T-RH. (1974). The coming of the Maori. *Maori Purposes Fund Board, Wellington.*

Budde BB, & Jakobsen M. (2000). Real-Time Measurements of the Interaction between Single Cells of *Listeria monocytogenes* and Nisin on a Solid Surface. *Applied and Environmental Microbiology, 66*(8), 3586-3591.

Buisson D, Fletcher G, & Begg CW. (1981). Bacterial depuration of the Pacific oyster (*Crassostrea gigas*) in New Zealand. *New Zealand Journal of Science, 24*(3/4), 253-262.

Canterbury Regional Policy Statement. (1998). Regional Policy Statement prepared under the Resource Management Act 1991. *Report No: 98/4.*

Charles A, DePaola K, & DePaola Jr A. (1998). *Vibrio cholerae, V. parahaemolyticus, V. vulnificus,* and Other *Vibrio* spp. In FDA (Ed.), *Bacteriological Analytical Manual, Revision A* (8th ed., Vol. Chapter 9.

Substantially rewritten and revised May 2004). USA: FDA, Centre for Food Safety and Applied Nutrition.

Cheigh H-S, & Park K-Y. (1994). Biochemical, Microbiological, and Nutritional Aspects of Kimchi (Korean Fermented Vegetable products). *Critical Reviews in Food Science and Nutrition, 34*(2), 175-203.

Chen H, & Hoover DG. (2003). Bacteriocins and their Food Applications. *Comprehensive Reviews in Food Science and Food Safety, 2*.

Choi MH, & Park YH. (2000). Selective control of lactobacilli in kimchi with nisin. *Letters in Applied Microbiology, 30,* 173-177.

Choi S-Y, & Beuchat LR. (1994). Growth inhibition of *Listeria monocytogenes* by a bacteriocin of *Pediococcus acidilactici* during fermentation of kimchi. *Food Microbiology, 11*, 301-307.

Choma C, Guinebretiere MH, Carlin F, Schmitt P, Velge P, Granum PE, & Nguyen-The C. (2000). Prevalence, characterization and growth of *Bacillus cereus* in commercial cooked chilled foods containing vegetables. *Journal of Applied Microbiology, 88,* 617-625.

Colenso W. (1880). Contributions towards a better knowledge of the Maori. In Hawks Bay Philosophical Institute (Ed.): Kiwi Publishers, Christchurch.

Compendium of Methods for the Microbiological Examination of Foods, &. (1992). *Molluscan shellfish: oysters, mussels and clams* (3rd ed.). Washington DC, USA: American Public Health Association.

Crowe A. (2004). *A field guide to the native plants of New Zealand*. Wellington: Penguin Books.

De Luca-Abbott S, Lewis GD, & Creese RG. (2000). Temporal and spatial distribution of enterococcus in sediment, shellfish tissue, and water in a New Zealand harbour. *Journal of Shellfish Research, 19*(1), 423-429.

Delves-Broughton J. (1990). Nisin and its uses as a food preservative. *Food Technology, 44*, 100-117.

Delves-Broughton J. (2005). Nisin as a food preservative. *Food Australia, 57*(12), 525-527.

Delves-Broughton J, Blackburn P, Evans RJ, & Hugenholtz J. (1996). Applications of bacteriocin, nisin. *Antonie van Leeuwenhoek, 96,* 193-202.

DeVuyst L, & Vandamme EJ. (1994). *Bacteriocins of Lactic Acid Bacteria*: Blackie Academic and Professional.

Dieffenbach E. (1843). Travels in New Zealand. London.

Dixon L. (2005). *Bacterial quality of harvested watercress.* Paper presented at the Land Treatment of Wastewater and Stormwater in Urban Catchments. New Zealand Land Treatment Collective 2005 Annual Conference Proceedings, Auckland.

Dixon L. (2002). *Bacterial quality of harvested and purchased watercress.* Unpublished Master of Science, University of Waikato, Hamilton, NZ.

Dixon L. (phamplet). Bacterial quality of harvested watercress: AgResearch & University of Waikato.

Dixon L, & Donnison A. (2006). *Microbiological quality of a traditional Maori food: Toroi. A Report to the New Zealand Food Safety Authority*: AgResearch, New Zealand.

Donnison AM, & C M Ross. (1999). Animal and Human Faecal Pollution in New Zealand Rivers. *New Zealand Journal of Marine and Freshwater Research, 33*, 119-128.

Dufour AP. (1977). *Escherichia coli:* The Fecal Coliform. In Hoadley AW & Dutka BJ (Eds.), *Bacterial Indicators / Health Hazards Associated with Water* (pp. 48-58): American Society for Testing and Materials.

Dufrenne J, Soentoro P, Tatini S, Day T & Notermans S. (1994) Characteristics of *Bacillus cereus* related to safe food production. *International Journal of Food Microbiology, 23*, 99-109.

Dykes GA, & Moorhead SM. (2002). Combined antimicrobial effect of nisin and a listeriophage against *Listeria monocytogenes* in broth but not in buffer or on raw beef. *International Journal of Food Microbiology, 73*, 71-81.

Dzung BD, Axelsson L, Grefsli C, & Nes IF. (2000). The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three component regulatory system. *Microbiology, 146*, 2155-2160.

Edmond C, & Hawke R. (2004). Microbiological and metal contamination of watercress in the Wellington region, New Zealand - 2000 survey. *Australian New Zealand Journal of Public Health, 28*(1), 20-26.

Fakalogo P. (2001). *Pacific Island home food safety radio campaign: Evaluation report.* Auckland District Health Board, Auckland.

Fankhauser BL. (1986). *Archaeometric studies of Cordyline (ti) based on ethnobotanical and archaeological research.* University of Otago, Otago.

Farber JM, & Peterkin PI. (1991). *Listeria monocytogenes,* a Food-Borne pathogen. *Microbiological Reviews, 55*(3), 476-511.

Flacks L. (1985). Botulism in New Zealand. *New Zealand Medical Journal, 98,* 892-893.

Fletcher GC. (1985). The potential food poisoning hazard of *Vibrio parahaemolyticus* in New Zealand Pacific oysters. *New Zealand Journal of Marine and Freshwater Research, 19*, 495-505.

FSANZ. (2001). Guideline for the microbiological examination of ready-to-eat foods: Food Standards Australian New Zealand.

FSANZ. (2003). Food Standards Australia New Zealand. Australia and New Zealand

Food Standards Code (including amendment 68).

Giffel M, Beumer R, Hoekstra J & Rom bouts F. (1995) Germination of bacterial spores during sample preparation. *Food Microbiology, 12*, 327-332.

Gilbert S, Lake AR, Hudson A & Cressy P (2006) Risk profile: Clostridium botulinum in ready-to-eat smoked seafood in sealed packaging. ESR Client Report FW0625. Christchurch NZ. 51 pages.

Gill CO, & Penny N. (1982). The occurrence of *Clostridium botulinum* at aquatic sites in and around Auckland and other urban areas of the North Island. *New Zealand Veterinary Journal, 30*, 110-112.

Gomez JM, Fajardo R, Patino JF, & Arias CA. (2003). Necrotizing fasciitis due to *Vibrio alginolyticus* in an immunocompetent patient. *Journal of Clinical Microbiology, 41*, 3427-3429.

Gordon RE, Haynes WC, & Nor-Hay PC. (1973). The Genus *Bacillus*. In *Handbook No. 427*. Washington DC: US Department of Agriculture.

Granun PE, & Lund T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Letters, 157*, 223-228.

Greening G, J Dawson, & G Lewis. (2001). Survival of Poliovirus in New Zealand Green-Lipped Mussels *Perna canaliculus*, on Refrigerated and Frozen Storage. *Journal of Food Protection, 64*(6), 881-884.

Harmon SM, Goepfert JM, & Bennett RW. (1992). *Bacillus cereus*. In *Compendium for the Microbiological Examination of Foods.* (pp. 594-604). Washington: APHA.

Hauschild AHW. (1989). *Clostridium botulinim*. In Doyle MP (Ed.), *Foodborne bacterial pathogens* (pp. 12-189). New York: Marcel Dekker.

Hazel JR. (1995). Thermal adaptation in biological membranes: Is homeoviscous adaptation the explanation? *Annual Review Physiology, 57*, 19-42.

Health Care Hawkes Bay. (1993). Ahuriri shellfish and shellfish water bacteriological monitoring programme, April 22 - April 1993. *Health Protection Unit, Community Health, Health Care Hawkes Bay*.

Hielm S, Hyytia E, Anderson AB, & H., K. (1998). A high prevalence of *Clostridium botulinum* type E in Finnish freshwater and Baltic Sea sediment samples. *Journal of Applied Microbiology, 84*, 133 - 137.

Holzapfel WH, Geisen R, & Schillinger U. (1995). A review paper: Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology, 24*, 343-362.

Hudson JA, Hasell S, Whyte R, & Monson S. (2001). Preliminary microbiological investigation of the preparation of two traditional Maori foods (Kina and Toroi). *Journal of Applied Microbiology, 91*, 814-821.

Hurst A. (1981). Nisin. *Advance Applied Microbiology, 27*, 85-123.

ICMSF. (1978). *Microorganisms in Foods 1: Their significance and methods of enumeration represents a major step in establishing a common understanding of, and developing standard methods for important foodborne microorganisms.*: University of Toronto Press, Toronto.

Ingraham JL, Maaloe O, & Neidhardt FC. (1983). *Growth of the bacterial cells.* Sunderland, MA, USA: Sinauer Associates Inc.

Jenson I, & Moir CJ. (1997). *Bacillus cereus* and other *Bacillus* species. In Hocking AD, Arnold G, Jenson I, Newton K & Sutherland P (Eds.), *Foodborne Microorganisms of Public Health Importance* (5th ed., pp. 379-406). Sydney, Australia: Australian Institute of Food Science and Technology Ltd (NSW Branch) Food Microbiology Group.

Kaneko T, & Colwell RR. (1973). Ecology of *V. parahaemolyticus* in Chesapeake Bay. *Journal of Bacteriology, 113*(1), 24-32.

Katla T, Naterstad K, Vancanneyt M, Swings J, & Axelsson L. (2003). Differences in susceptibility of *Listeria monocytogenes* strains to Sakacin P, Sakacin A, Pediocin PA-1, and nisin. *Applied and Environmental Microbiology, 69*(8), 4331-4337.

Kearney M. (1999). *Ecology and management of Austrovenus stutchburi in the Whangateau Harbour.*, University of Auckland.

Kim Y, Ahn Y, Jang Y, Kim H. (2000). A study on the growth inhibition of *Bacillus cereus* by lactobacilli. *Journal of Animal Science and Technology, 42*, 331-338.

Klaenhammer TR. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiological Reviews, 12,* 39-86.

Kodama T. (1967). Growth of *V. parahaemolyticus* in foods and in food processing environment. In Fujino T & Fukumi H (Eds.), *Vibrio parahaemolyticus II*. Tokyo, Japan.

Labbe RG, & Harmon SM. (1992). *Clostridium perfringens*. In *Compendium for the Microbiological Examination of Foods*: 3rd edition, APHA, Washington.

Lake R, Hudson A, & Cressey P. (2003). Risk profile: *Vibrio parahaemolyticus* in Seafood. Client Report FW0348. In New Zealand Food Safety Authority (Ed.): Institute of Environmental Science and Research Limited.

Lammerding AM, Glass KA, Gendon-Fitzpatrick, & Doyle MP. (1992). Determination of virulence of different strains of *Listeria monocytogenes* and *Listeria innocua* by oral innoculation of pregnant mice. *Journal of Clinical Microbiology, 58*(12), 3991-4000.

Lee SH, Kim MK, & Frank JF. (1995). Growth of *Listeria monocytogenes* Scott A during Kimchi fermentation and in the presence of Kimchi ingredients. *Journal of Food Safety, 58*, 1215-1218.

Lee T, Yam WC, Tam T-Y, Bella SW, Ho BSW, Ng MH, & Broom MJ. (1998). Technical Note: Occurrence of Hepatitis A virus in green-lipped mussels (*Perna virdis*). *Water Reserve, 33*(3), 885-889.

Lennon D, Lewus B, Mantell C, Becroft D, Dove B, Farmer K, Tonkin S, Yeates N, Stamp R, & Mickleson K. (1984). Epidemic perinatal listeriosis. *Pediatric Infectious Disease Journal, 3*, 30-34.

Lewis G, Loutit M, & Austin FJ. (1986). Enteroviruses in mussels and marine sediments and depuration of naturally accumulated viruses by green lipped mussels (*Perna cananiculus*). *New Zealand Journal of Marine and Freshwater Research, 20*(3), 431-437.

Lewus CB, Kaiser A, & Montville TJ. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from Lactic Acid Bacteria isolated from meat. *Applied and Environmental Microbiology, 57*(6), 1683-1688.

Lewus CB, & TJ Montville. (1991). Detection of bacteriocin produced by lactic acid bacteria. *Journal of Microbiological Methods, 13*, 145-150.

Lovett J, Francis DW, & Bradshaw GJ. (1990). Outgrowth of *Listeria monocytogenes* in foods. In Miller AJ, Smith JL & Somkuti GA (Eds.), *Foodborne listeriosis*. New York: Society for the Industrial Microbiology, Elsevier Science Publishing, Inc.

Luck E, & Jager M. (1995). Antimicrobial food additives: characteristics, uses, effects. *Springer, London*, 208-221.

Lund BM, & Peck, M. (2000). *Clostridium botulinum*. In Lund BM, Baird-Parker AC & Gould GW (Eds.), *Microbiological safety and Quality of Food*, (pp. 1057-1109). Gaithersburg, MD: Aspen Publishers Inc.

Madigan MT, Martinko JM, & Parker J (Eds.). (1997). *Brock Biology of Microorganisms* (8th ed.). Carbondale: Prentice Hall, USA.

Mattick ATR, & Hirsch A. (1947). Further observation on an inhibitor (nisin) from lactic *Streptococci*. *The Lancet, 2*, 5-8.

Mazzotta AS, Crandall AD, & Montville T J. (1997). Nisin resistance in *Clostridium botulinum* spores and vegetative cells. *Applied and Environmental Microbiology, 63*(7), 2654-2659.

McClure PJ, Kelly TM, & Roberts TA. (1991). The effects of temperature, pH, sodium chloride and sodium nitrite on the growth of *Listeria monocytogenes*. *International Journal of Food Microbiology, 14*, 77-92.

Ministry for the Environment. (2003). Biodiversity on farmland: Good Management Practices. In Ministry for the Environment (Ed.). Wellington: Lincoln University.

Ministry of Health. (1995). New Zealand Microbiological Reference Criteria for Food. Wellington, New Zealand.

Ministry of Health. (2001a). *Bacillus cereus*. In Ministry of Health (Ed.): Environmental Science Research.

Ministry of Health. (2001b). *Clostridium botulinum*. In Minsitry of Health (Ed.): Environment Science Research.

Ministry of Health. (2001c). *Clostridium perfringenes*: Environmental Science Research.

Ministry of Health. (2001d). Clostridium perfringens. In Ministry of Health (Ed.): Environmental Science Research.

Ministry of Health. (2001e). *Listeria monocytogenes*. In Ministry of Health (Ed.): Environmental Science Research.

MIRINZ. (2004a). *Listeria monocytogenes*. In LeRoux G, Donnison A, Johnstone P & Mills J (Eds.), *Microbiological Methods for the Meat Industry* (4th ed.). Hamilton: AgResearch, NZ.

MIRINZ. (2004b). *Salmonella*. In LeRoux G, Donnison A, Johnstone P & Mills J (Eds.), *Microbiological Methods for the Meat Industry* (4th ed.): AgResearch, NZ.

MIRINZ. (2004c). Thermotolerant *Campylobacter*. In LeRoux G, Donnison A, Johnstone P & Mills J (Eds.), *Microbiological Methods for the Meat Industry*: AgResearch, NZ.

Miwatani T, & Y Takeda. (1976). *Vibrio parahaemolyticus: a causative bacterium of food poisoning.*: Saikon Publishing Co. Tokyo (Available from Maruzen Company, Ltd., Import and Export Dept., P.O. Box 5050, Tokyo International 100-31 Japan.).

MoH. (2001) *Clostridium botulinum*. Datasheets produced for the Ministry of Health by ESR Ltd, Issued May 2001. 5 pages.

Moll GN, Konongs WN, & Driessen AJM. (1999). Bacteriocins: mechanism of membrane insertion and pore formation. *Antonie van Leeuwenhoek, 76*, 185-198.

Muriana PM. (1995). Bactericons for control of *Listeria* spp. in Food, 1996 Supplement. *Journal of Food Protection,* 54-63.

Murrell WG. (1989). *Clostridium perfringens.* In Buckle KA, Davey JA, Eyles MJ, Hocking AD, Newton KG & Stuttard EJ (Eds.), *Foodborne Microorganisms of Public health Significance* (4th ed., pp. 209-232). Sydney, Australia: Australian Institute of Food Science and Technology Ltd (NSW Branch) Food Microbiology Group.

Nauta NJ, Litman S, Barker GC, & Carlin F. (2003). A retail and consumer phase model for exposure assessment of *Bacillus cereus*. *International Journal of Food Microbiology, 83,* 205-218.

Ngata HM. (1993). *The H. M. Ngata English-Maori Dictionary*. Wellington: for the Ministry of Education, Wellington, New Zealand by the Learning Media Te Pou Taki Korero Limited.

Nicholson C, Lewis G, & Loutit MW. (1989). Survey of human pathogenic bacteria and viruses in cockle beds at Otakou, Otago Harbour, New Zealand. *New Zealand Journal of Marine and Freshwater Research, 23*(4), 529-532.

NZFSA. (2005a). A guide to calculating the shelf life of foods. Information booklet for the Food Industry. Wellington, New Zealand: New Zealand Food Safety Authority.

NZFSA. (2005b). *Review of Non-Commercial Wild Food in New Zealand. Report to New Zealand Food Safety Authority from the Institute of Environmental Science & Research Ltd*. Wellington, New Zealand.

Opal SM, & Saxon JR. (1986). Intracranial infection by *Vibrio alginolyticus* following injury in salt water. *Journal of Clinical Microbiology, 23*(2), 373-374.

Peck MW, Granum PE, Gould GW, & Mainil JG. (2004). Foodborne clostridia sporulation. In Department of Infectious and Parasitic Diseases (Ed.) (Vol. 4): University of Liege, Belgium.

Pomare E, Keefe-Ormsby V, & Ormsby C. (1995). Hauora. Maori standards of health 1970-1991. In Te Ropu Rangahau Hauora a Eru Pomare (Ed.): Department of Health, Wellington, New Zealand.

Pond W. (1997). The land with all woods and water: Waitangi Tribunal Rangahau Whanui Series. Waitangi Tribunal, 1997.

Pontharangkul T, & Demirci A. (2004). Evaluation of agar diffusion bioassay for nisin quantification. *Applied Microbiological Biotechnology, 65*, 268-272.

Raju CV, Shamasundar BA, & Udupa KS. (2003). The use of nisin as a preservative in fish sausage stored at ambient (28 +/- 2 ° C) and refrigerated 6 +/- 2 ° C) temperatures. *International Journal of Food Science and Technology, 38*, 171-185.

Rhodehamel, E, Harmon S. 2001. *Bacillus cereus*. Chapter 14 In: *Bacteriological analytical manual online*, January 2001. US Food and Drug Administration. http://www.cfsan.fda.gov/~ebam/bam-14.html.

Riley M. (1994). *Maori Healing and Herbal. New Zealand Ethnobotanical Sourcebook.*: Viking Sevenseas.

Riley MA, & Wertz, J. (2002.). Bacteriocins: Evolution, ecology and application. *Annual Review of Microbiology, 56*, 117-137.

Rogers LA, & Whittier EO. (1928). Limiting factors in lactic fermentation. *Journal of Bacteriology, 16*, 211-229.

Russel D, Chisnall BL, Dean TL, & Richardson J. (1999). NZ food, NZ people; key results of the 1997 national nutrition survey. *Ministry of Health, Wellington, New Zealand*.

Sahl HG. (1985). Influence of the staphylococcin-like peptide Pep% on membrane potential of bacterial cells and cytoplasmic membrane vesicles. *Journal of Bacteriology, 162*, 833-836.

Sahl HG. (1991). Pore-formation in bacterial membranes by cationic lantibiotics. In Jung G & Sahl HG (Eds.), *Nisin and novel lantibiotiocs*. Leiden, The Netherlands: Escom Publishers.

Schillinger U. (1994). Sakacin A produced by *Lactobacillus sake* LB 706. In Vuyst L De & Vardamme EJ (Eds.), *Bacteriocins of Lactic Acid Bacteria*: Blackie Academic and Professional.

Schillinger U, Becker B, Vignolo G, & Holzapfel WH. (2001). Efficacy of nisin in combination with protective cultures against *Listeria monocytogenes* Scott A in tofu. *International Journal of Food Microbiology, 71*, 159-168.

Schillinger U, Kaya M, & Lucke F-K. (1991). Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake*. *Journal of Applied Bacteriology, 70*, 473-478.

Schillinger U, & Lucke F-K. (1989). Antibacterial Activity of *Lactobacillus sake* Isolate from Meat. *Applied and Environmental Microbiology, 55*(8), 1901-1906.

Scott VN, & Taylor SL. (1981). Effect of nisin on the outgrowth of *Clostridium botulinum*. *Journal of Food Science, 46*, 117-120.

Sneath PHA, Mair NS, Sharpe ME, & Holt JG. (1984). *Bergy's Manual of Systematic Bacteriology* (Vol. 2). Baltimore/London: Williams and Wilkins.

Speck ML, & Cowman RA. (1969). Metabolic injury to bacteria resulting from freezing. In Nei T (Ed.), *Freezing and drying of microorganisms* (pp. 39-51). Tokyo: University of Tokyo Press, Tokyo, and University Park Press, Baltimore.

Stevens KA, Sheldon BW, Klapes NA, & Klaenhammer TR. (1992). Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. *Applied Environmental Microbiology, 57*, 3613-3615.

Taylor JM, Sutherland AD, Aidoo KE, & Logan NA. (2005). Heat-stable toxin production by strains of *Bacillus cereus*, *Bacillus firmus*, megaterium, simplex and licheniformis. *FEMS Microbiology Letters, 242* (2), 313-317.

Turner N, Cressey P, Lake R, & Whyte R. (2005). *Review of Non-Commercial Wild Food in New Zealand. Report to New Zealand Food Safety Authority from the Institute of Environmental Science & Research Ltd*. New Zealand Food Safety Authority.

USFDA. (2001). Aerobic Plate Count. In *Bacteriological Analytical Manual*: USFDA.

Valero M, Fernandez PS, & Salmeron MC. (2003). Influence of pH and temperature on growth of *Bacillus cereus* in vegetable substrates. *International Journal of Food Microbiology, 82*, 71-79.

Valero M, Leontidis S, Fernandez PS, Matrtinez A, & Salmeron MC. (2000). Growth of *Bacillus cereus* in natural and acidified carrot substrate over the temperature range 5 - 30^{o}C. *Food Microbiology, 17*, 605-612.

Vandenbergh PA. (1993). Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiological Reviews, 12*, 221-238.

Venema K, Venema G, & Kok J. (1995). Lactococcins: mode of action, immunity and secretion. *International Dairy Journal, 5*, 815-832.

Webb CJ, Sykes WR, & Garnock-Jones PJ. (1998). Flora of New Zealand. Naturalised Pteridophytes, Gymnosperms, Dicotyledons. *Botony Divison, Department of Scientific and Indistrial Research, New Zealand*.

Wemekamp-Kamphuis HH, Karatzas AK, Wouters JA, & Abee T. (2002.). Enhanced levels of cold shock proteins in *Listeria monocytogenes* LO28

upon exposure to low temperature and high hydrostatic pressure. *Applied and Environmental Microbiology, 68*(2), 456-463.

Whati T. (1983). Claim by A. Taylor for Te Atiawa Tribe Re: Fishing grounds at Waitara. In Department of Maori Affairs (Ed.): Ministry of Maori Affairs, Wellington.

Whyte, R, Wong, T. 2004 *Bacillus cereus. In*: Editors Jensen W, Devine C & Dikeman M. *Microbiological Safety of Meat Encyclopedia of Meat Sciences*. Elsevier Ltd., B.V. Pp. 825-830.

Whyte R, Hudson JA, Hasell S, Gray M, & O'Reily R. (2001). Traditional Maori food preparation methods and food safety. *International Journal of Food Microbiology, 69*(3), 183-190.

Wilson N. (1996). Review of the surveillance and control of toxic shellfish poisoning from non-commercially gathered shellfish in New Zealand. A report for the Public Health Commission and Ministry of Health, Wellington. *Public Health Commission and Ministry of Health, Wellington.*

Yen DE. (1959). The use of maize by the New Zealand Maoris. *Economic Botany. Devoted to Applied Botany and Plant Utilization, 13*(4), 319-327.

A. Appendix 1

Media Composition

Difco™ Lauryl Tryptose Broth

Approximate Formula* L^{-1}

Tryptose ..... 20.0 g

Lactose ..... 5.0 g

Dipotassium Phosphate ..... 2.75 g

Monopotassium Phosphate ..... 2.75 g

Sodium Chloride ..... 5.0 g

Sodium Lauryl Sulfate ..... 0.1 g

Directions for Preparation from Dehydrated Product

1. Suspend 35.6 g of the powder in 1 L of purified water. Mix thoroughly.

2. Warm slightly to completely dissolve the powder.

3. Dispense required amounts into tubes containing inverted fermentation vials.

4. Autoclave at 121°C for 15 minutes. Cool the broth as quickly as possible.

5. Test samples of the finished product for performance using stable, typical control cultures.

Difco™ EC Medium

Approximate Formula* L^{-1}

Tryptose ..... 20.0 g

Lactose ..... 5.0 g

Bile Salts No. 3 .....1.5 g

Dipotassium Phosphate ..... 4.0 g

Monopotassium Phosphate ..... 1.5 g

Sodium Chloride ..... 5.0 g

Directions for Preparation from Dehydrated Product

1. Dissolve 37 g of the powder in 1 L of purified water. Mix thoroughly.

2. Warm slightly to completely dissolve the powder.

3. Dispense into tubes containing inverted fermentation vials.

4. Autoclave at 121°C for 15 minutes.

5. Test samples of the finished product for performance using stable, typical control cultures.

Difco™ EC Medium with MUG

Approximate Formula* L^{-1}

Tryptose ..... 20.0 g

Lactose ..... 5.0 g

Bile Salts No. 3 ..... 1.5 g

Dipotassium Phosphate ..... 4.0 g

Monopotassium Phosphate ..... 1.5 g

Sodium Chloride ..... 5.0 g

MUG (4-methylumbelliferyl-β-D-glucuronide) ..... 0.05 g

**Adjusted and/or supplemented as required to meet performance criteria.*

Directions for Preparation from Dehydrated Product

1. Dissolve 37.1 g of the powder in 1 L of purified water. Mix thoroughly.

2. Warm slightly to completely dissolve the powder.

3. Dispense into test tubes containing inverted fermentation vials.

4. Autoclave at 121°C for 15 minutes.

5. Test samples of the finished product for performance using stable, typical control cultures.

Oxoid™ Listeria selective Agar Base

Approximate Formula* L^{-1}

Columbia Blood Agar base…… 39.0 g

Aesculin…….. 1.0 g

Ferric ammonium citrate…… 0.5 g

Lithium chloride…… 15.0 g

pH 7.0 ± 0.2

Listeria selective Supplement (Oxford formulation)

Vial contents (each vial is sufficient for 500 ml of medium)

	Per vial
Cycloheximide……..	200 mg
Colistin sulphate…….	10.0 mg
Acriflavin……	2.5 mg
Lefotetan…….	1.0 mg
Fosfomycin…….	5.0 mg

Directions for Preparation from Dehydrated Product

1. Suspend 27.75 of the Listeria Selective Agar Base (Oxford Formulation) in 500 ml of distilled water. Mix thoroughly.

2. Bring gently to the boil to dissolve.

3. Sterilise by autoclaving at 121°C for 15 minutes.

4. Cool to 50°C and aseptically add the contents of one vial Listeria Selective Supplement (Oxford), SR0140E reconstituted with 5 ml of 70% ethanol. Mix well and pour into sterile Petri dishes.

Difco™ Fraser Broth Base

Approximate Formula* L^{-1}

Pancreatic Digest of Casein ..... 5.0 g

Proteose Peptone No. 3 ..... 5.0 g

Beef Extract ..... 5.0 g

Yeast Extract ..... 5.0 g

Sodium Chloride ..... 20.0 g

Disodium Phosphate ..... 9.6 g

Monopotassium Phosphate ..... 1.35 g

Esculin ..... 1.0 g

Nalidixic Acid ..... 0.02 g

Acriflavine HCl ..... 24.0 mg

Lithium Chloride ..... 3.0 g

Difco™ Fraser Broth Supplement

Per 10 mL Vial

Ferric Ammonium Citrate ..... 0.5 g

**Adjusted and/or supplemented as required to meet performance criteria.*

Directions for Preparation from Dehydrated Product

1. Suspend 55 g of the powder in 1 L of purified water. Mix thoroughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes. Cool to room temperature.

4. Aseptically add 10 mL Fraser Broth Supplement. Mix well.

5. Test samples of the finished product for performance using stable, typical control cultures.

Merck™ UVM-Listeria Selective Enrichment broth, modified

Approximate Formula* L^{-1}

Tryptose ……. 10.0 g

Meat extract…… 5.0 g

Yeast extract …… 5.0 g

Sodium Chloride…….. 20.0 g

Disodium hydrogenphosphate…….120.0 g

Potassium hydrogen phosphate…… 1.35 g

Esculin……. 1.0 g

Nalidixic acid…….. 20.0 mg

Acriflavine hydrochloride…… 12.0 mg

Directions for Preparation from Dehydrated Product

1. Suspend 54.4 g of powder in 1 L of purified water: Mix thoroughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes.

Oxoid™ Campylobacter Blood-free selective Medium (Modified CCDA-Preston)

Approximate Formula* L^{-1}

Nutrient Broth No. 2…… 25.0 g

Bacteriological charcoal …….. 4.0 g

Casein hydrolysate…… 3.0 g

Sodium desoxycholate…….. 1.0 g

Ferrous sulphate…… 0.25 g

Sodium pyruvate……... 0.25 g

Agar …….. 12.0 g

pH 7.4 ± 0.2

Cefoperazone Selective Supplement

Vial contents (each vial is sufficient for 500 ml of medium)

Cefoperazone…… 16mg

Directions for Preparation from Dehydrated Product

Suspend 22.75g of Campylobacter Blood-Free selective Agar Base in 500 ml of distilled water and bring to the boil to dissolve. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C. Aseptically add 1 vial of Cefoperazone Selective Supplement SR125 reconstituted with 2ml of sterile distilled water. Mix well and pour into sterile Petri dishes.

Preston Campylobacter Selective Enrichment Broth

Oxoid™ Nutrient Broth No. 2

Approximate Formula* L^{-1}

Lab-Lemco powder…….. 10.0 g

Sodium chloride…… 10.0 g

Peptone……. 5.0 g

Directions for Preparation from Dehydrated Product

Add 25g to 1 litre of distilled water. Mix well, distribute into final containers and sterilise by autoclaving at 121°C for 15 minutes.

Directions for Preparation from Dehydrated Product

1. Dissolve 12.5g of Nutrient Broth No.2 in 475ml of distilled water and sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C or below.

2. Aseptically add 25ml of Lysed Horse Blood SR0048, 1 vial of Preston Campylobacter Selective Supplement SR0117 or SR0204 and 1 vial of Campylobacter Growth Supplement SR0084 or SR0232.

3. Aseptically dispense 5ml volumes in sterile small screw-capped bottles.

Difco™ Plate Count Agar

Approximate Formula* L^{-1}

Pancreatic Digest of Casein ..... 5.0 g

Yeast Extract ..... 2.5 g

Dextrose ..... 1.0 g

Agar ..... 15.0 g

**Adjusted and/or supplemented as required to meet performance criteria.*

Directions for Preparation from Dehydrated Product

1. Suspend 23.5 g of the powder in 1 L of purified water. Mix thoroughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes.

4. Test samples of the finished product for performance using stable, typical control cultures.

Bacto™ Tryptic Soy Broth

Approximate Formula* L^{-1}

Pancreatic Digest of Casein ..... 17.0 g

Enzymatic Digest of Soybean Meal ..... 3.0 g

Sodium Chloride ..... 5.0 g

Dipotassium Phosphate ..... 2.5 g

Dextrose ..... 2.5 g

Directions for Preparation from Dehydrated Product

1. Suspend 30 g in 1 L of purified water: Mix thoroughly.

2. Warm gently until solution is complete.

3. Autoclave at 121°C for 15 minutes.

4. Test samples of the finished product for performance using stable, typical control cultures.

Bacto™ Tryptic Soy Agar

Approximate Formula* L^{-1}

Pancreatic Digest of Casein ..... 17.0 g

Enzymatic Digest of Soybean Meal ..... 3.0 g

Sodium Chloride ..... 5.0 g

Dipotassium Phosphate ..... 2.5 g

Dextrose ..... 2.5 g

Agar……. 30.0 g

Directions for Preparation from Dehydrated Product

1. Suspend 30 g in 1 L of purified water: Mix thoroughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes.

4. Test samples of the finished product for performance using stable, typical control cultures.

Bacto™ Brain Heart Infusion Broth

Approximate Formula* L^{-1}

Calf Brains, Infusion from 200 g ..... 7.7 g

Beef Heart, Infusion from 250 g ..... 9.8 g

Proteose Peptone ..... 10.0 g

Dextrose ..... 2.0 g

Sodium Chloride ..... 5.0 g

Disodium Phosphate ..... 2.5 g

Directions for Preparation from Dehydrated Product

1. Suspend the powder in 1 L of purified water: Bacto™ Brain Heart Infusion – 37 g;

Mix thoroughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes.

4. Test samples of the finished product for performance using stable, typical control cultures.

Difco™ Brain Heart Infusion Agar

Approximate Formula* L^{-1}

Calf Brains, Infusion from 200 g ..... 7.7 g

Beef Heart, Infusion from 250 g ..... 9.8 g

Proteose Peptone ..... 10.0 g

Dextrose ..... 2.0 g

Sodium Chloride ..... 5.0 g

Disodium Phosphate ..... 2.5 g

Agar ..... 15.0 g

Directions for Preparation from Dehydrated Product

1. Suspend the powder in 1 L of purified water: Difco™ Brain Heart Infusion Agar – 52 g; Mix thoroughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes.

4. Before use, agitate gently to distribute the precipitate uniformly throughout the medium.

5. Test samples of the finished product for performance using stable, typical control cultures.

Bacto® Peptone (0.1%)

Approximate Formula* L^{-1}

Peptone…….. 1 g

Directions for Preparation from Dehydrated Product

1. Suspend the powder in 1 L of purified water: Bacto® Peptone (0.1% – 1 g); Mix thoroughly.

2. Warm gently until solution is complete.

3. Autoclave at 121°C for 15 minutes.

Universal Buffer

Approximate Formula* L^{-1}

Peptone……. 1 g

NaCl……. 8.5 g

Directions for Preparation from Dehydrated Product

1. Suspend 0.1% Bacto® Peptone with 0.85% sodium chloride in 1 L of purified water; Mix thoroughly.

2. Warm gently until solution is complete.

3. Autoclave at 121°C for 15 minutes.

Merck™ MRS Broth/Agar (Lactobacilllus broth acc. to De Man, Rogasa and Sharpe)

Approximate Formula* L^{-1}

Peptone from casein…… 10.0 g

Meat extract…… 8.0 g

Yeast extract……... 4.0 g

D9+)-glucose…….. 20.0 g

Dipotassium hydrogen phosphate……... 2.0 g

Tween® 80……. 1.0 g

di-ammonium hydrogen citrate…….. 2.0 g

Sodium acetate…… 5.0 g

Magnesium sulphate…… 0.2 g

Manganese sulphate…… 0.04 g

Agar (not present in MRS broth)…… 12.0 g

Directions for Preparation from Dehydrated Product

1. Suspend 66.2 g MRS Agar or 52.2 g MRS broth

2. For MRS Agar; Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. For MRS broth; stir until dissolved.

3. Autoclave at 121°C for 15 minutes.

4. Before use, agitate gently to distribute the precipitate uniformly throughout the medium. 5. Test samples of the finished product for performance using stable, typical control cultures.

Difco™ or BBL™ Buffered Peptone Water

Approximate Formula* L^{-1}

Peptone ..... 10.0 g

Sodium Chloride ..... 5.0 g

Disodium Phosphate ..... 3.5 g

Monopotassium Phosphate ..... 1.5 g

Directions for Preparation from Dehydrated Product

1. Dissolve the powder in 1 L of purified water: Difco™ or BBL™ Buffered Peptone Water – 20 g; Mix thoroughly.

2. Autoclave at 121°C for 15 minutes.

3. Test samples of the finished product for performance using stable, typical control cultures.

Difco™ Rappaport-Vassiliadis R10 Broth

Approximate Formula* L^{-1}

Pancreatic Digest of Casein ..... 4.54 g

Sodium Chloride ..... 7.2 g

Monopotassium Phosphate ..... 1.45 g

Magnesium Chloride (anhydrous) ..... 13.4 g

Malachite Green Oxalate ..... 36.0 mg

**Adjusted and/or supplemented as required to meet performance criteria.*

Directions for Preparation from Dehydrated Product

1. Suspend 26.6 g of the powder in 1 L of purified water. Mix thoroughly.

2. Warm slightly to completely dissolve the powder.

3. Dispense 10 mL amounts into suitable containers.

4. Autoclave at 116°C (10 psi pressure) for 15 minutes.

5. Test samples of the finished product for performance using stable, typical control cultures.

B. Appendix 11

MPN Tables and Result Sheets

Combination of Positives	MPN Index per g (ml)	95% Confidence Limits Lower	Upper
0-0-0	<3.0	***	9.5
0-0-1	3.0	0.15	9.6
0-1-0	3.0	0.15	11.
0-1-1	6.1	1.2	18.
0-2-0	6.2	1.2	18.
0-3-0	9.4	3.6	38
1-0-0	3.6	0.17	18.
1-0-1	7.2	1.3	18.
1-0-2	11.	3.6	38
1-1-0	7.4	1.3	20
1-1-1	11.	3.6	38
1-2-0	11.	3.6	42
1-2-1	15.	4.5	42
1-3-0	16	4.5	42
2-0-0	9.2	1.4	38
2-0-1	14.	3.6	42
2-0-2	20.	4.5	42
2-1-0	15.	3.7	42
2-1-1	20.	4.5	42
2-1-2	27.	8.7	94
2-2-0	21.	4.5	42
2-2-1	28.	8.7	94
2-2-2	35.	8.7	94
2-3-0	29.	8.7	94
2-3-1	36.	8.7	94
3-0-0	23.	4.6	94
3-0-1	38.	8.7	110
3-0-2	64.	17.	180
3-1-0	43.	9.0	180
3-1-1	75.	17.	200
3-1-2	120.	37.	420
3-1-3	160.	40.	420
3-2-0	93.	18.	420
3-2-1	150.	37.	420
3-2-2	210.	40.	430
3-2-3	290.	90.	1000
3-3-0	240.	42.	1000
3-3-1	460.	90.	2000
3-3-2	1100.	180	4100
3-3-3	>1100.	420	-

Table A.11.1. 3-Tube MPN Probability Table

TABLE 9221.IV. MPN INDEX AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE RESULTS WHEN FIVE TUBES ARE USED PER DILUTION (10 mL, 1.0 mL, 0.1 mL)

Combination of Positives	MPN Index/ 100 mL	95% Confidence Limits		Combination of Positives	MPN Index/ 100 mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<2	—	—	4-2-0	22	9.0	56
0-0-1	2	1.0	10	4-2-1	26	12	65
0-1-0	2	1.0	10	4-3-0	27	12	67
0-2-0	4	1.0	13	4-3-1	33	15	77
				4-4-0	34	16	80
1-0-0	2	1.0	11	5-0-0	23	9.0	86
1-0-1	4	1.0	15	5-0-1	30	10	110
1-1-0	4	1.0	15	5-0-2	40	20	140
1-1-1	6	2.0	18	5-1-0	30	10	120
1-2-0	6	2.0	18	5-1-1	50	20	150
				5-1-2	60	30	180
2-0-0	4	1.0	17	5-2-0	50	20	170
2-0-1	7	2.0	20	5-2-1	70	30	210
2-1-0	7	2.0	21	5-2-2	90	40	250
2-1-1	9	3.0	24	5-3-0	80	30	250
2-2-0	9	3.0	25	5-3-1	110	40	300
2-3-0	12	5.0	29	5-3-2	140	60	360
3-0-0	8	3.0	24	5-3-3	170	80	410
3-0-1	11	4.0	29	5-4-0	130	50	390
3-1-0	11	4.0	29	5-4-1	170	70	480
3-1-1	14	6.0	35	5-4-2	220	100	580
3-2-0	14	6.0	35	5-4-3	280	120	690
3-2-1	17	7.0	40	5-4-4	350	160	820
				5-5-0	240	100	940
4-0-0	13	5.0	38	5-5-1	300	100	1300
4-0-1	17	7.0	45	5-5-2	500	200	2000
4-1-0	17	7.0	46	5-5-3	900	300	2900
4-1-1	21	9.0	55	5-5-4	1600	600	5300
4-1-2	26	12	63	5-5-5	≥1600	—	—

Table A11.2 . 5-Tube MPN Probability Table

Plate Count of Micro-organisms

Sample ……

Enumeration by:…….....

Date of set up: ……..

Date of analysis:……...

Sample description:
……

Treatment	Dilution	Volume	No. of colonies		
			a	b	c

Calculation

Triplicate counts a ……. b …….. c ……..

CFU per ml = $\frac{a + b + c}{\text{vol. a} + \text{vol. b} + \text{vol c}}$ x volume

5-Tube MPN Test for *E. coli* in Toroi

Date_____ Sample No._____

Test	Step 1					Step 2					Step 3					Step 4					Step 5				
Test Portion (V mls)																									
Test Dilution (F)																									
Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
24 hr presumptive ACID + GAS (LT)																									
24-48 hr presumptive ACID + GAS																									
Confirmed at 44.5°C (EC)																									
No. confirmed tubes per step																									

Calculation
MPN value at 95% C.L

MPN Value x $\frac{10}{V}$ x F =

E. coli

EC + MUG																									
No. confirmed tubes per step																									

3-Tube MPN Test for *E. coli*

Date_____ Sample No._____

Test	Step 1			Step 2			Step 3			Step 4			Step 5		
Test Portion (V mls)															
Test Dilution (F)															
Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
24 hr presumptive ACID + GAS (LT)															
24-48 hr presumptive ACID + GAS															
Confirmed at 44.5°C (EC)															
No. confirmed tubes per step															

Calculation
MPN value at 95% C.L

MPN Value x $\frac{10}{V}$ x F =

E. coli

EC + MUG															
No. confirmed tubes per step															