ASPECTS OF THE GROWTH OF SELECTED COLD-TOLERANT PATHOGENS AND *ESCHERICHIA COLI* ON BEEF

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ABSTRACT

Strains of the cold-tolerant pathogens *Aeromonas hydrophila*, *Listeria monocytogenes*, and *Yersinia enterocolitica* were inoculated onto high-ultimate pH beef slices. Sample steaks were either packaged under vacuum or carbon dioxide atmosphere and stored at temperatures of + 10, 5, 2, 0 and -2°C. In vacuum-packaged meat, *Aeromonas hydrophila* and *Yersinia enterocolitica* grew at all storage temperatures at rates that were similar to or greater than that of the spoilage flora. *Listeria monocytogenes* did not grow in vacuum-packaged meat at -2°C, and at higher temperatures generally grew at growth rates similar to or less than that of the spoilage flora. In samples packaged under carbon dioxide, all three organisms grew at +10°C, but only *Yersinia enterocolitica* grew at +5°C. None of the test organisms grew under carbon dioxide at temperatures of +2°C or lower temperatures.

Storage life of high ultimate-pH beef packaged under carbon dioxide atmosphere was extended by at least a factor of two, and at the lower storage temperatures by up to a factor of three if compared with vacuum-packaging.

The hygienic adequacy of a hot-boning process of beef in a commercial New Zealand meat plant was assessed by a temperature function integration (TFI) technique. The potential proliferation of mesophilic organisms was estimated using the growth of *Escherichia coli* as an example. The growth potential was calculated from 50 temperature histories for the slowest cooling site on a carcass and within a carton. In the first survey of 50 temperature histories the mean calculated growth potential was 9.3 generations of growth, with a mean time of 13.3 hours to reduce the temperature of the meat to +7°C. The mean calculated proliferation for the improved hot-boning process was 7.1 generations of growth of *Escherichia coli* which is comparable to results that had been obtained for conventional cold boning. The mean time to reduce the temperature to +7°C was 12.2 hours with a range from 9 to 16.5 hours. The maximum observed microbial proliferation was less than that found in the conventional carcass cooling operation.

A profile through the geometric centre of a cooling carton of hot-boned meat revealed the location of the point of highest potential proliferation of mesophilic bacteria, i.e. the slowest cooling spot within a carton. In cartons cooling with lids closed over the mass of hot-boned beef this was located at the midway point between the geometric centre and a point halfway between the geometric centre and the surface of the cooling meat mass.

Observed proliferations of inocula of *E.coli* in cooling cartons were compared with the proliferations calculated from the temperature histories obtained from sites adjacent to the inoculas and found to agree within a range of ± 1 generation in 81% of comparisons. In 62% of comparisons the calculated growth potential exceeded the actual observed growth.

The value of TFI evaluation for the purpose of process assurance and regulatory hygienic processing control is described. Suggestions for a need to assess other current meat processes, in particular, offal cooling for its mesophilic growth potential are made. The possibility of replacing some of the current bacteriological sampling regimes with TFI monitoring is also discussed.

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I. GENERAL INTRODUCTION

Prior to adequate scientific knowledge of microbiology and meat science, the empirical approach to meat processing, i.e. trial and error, resulted in practices which were found to provide sufficient safeguard of the consumer from pathogenic agents in meat, and also, allowed the distribution of carcasses or butchered joints of meat from the slaughterhouses to the customer before the onset of spoilage ensued. Quick chilling and freezing gave the greatest extension of storage life and were critical in permitting New Zealand to become an exporter of meat just over 100 years ago.

More recently the knowledge, that pathogenic bacteria in meat are usually mesophiles which cease growth at temperatures above the common refrigeration temperatures, has led to the stipulation of temperature limits for the processing of meat and storage.

The chilling regimes require the processor to attain and maintain certain temperatures before the meat can be further processed. As processors have tried to maximize the throughput of their premises, particularly harsh chilling regimes have evolved; some of the temperature requirements were found to adversely affect the eating quality of meat, by an increase in toughness due to cold-shortening (Locker and Hagyard 1963).

To meet the increased demand by consumers for quality meat, and in particular tender meat (Honikel 1990) processors have tried to establish cooling regimes which meet these demands and also satisfy the hygienic stipulations of the regulatory authorities.

Regulatory authorities have, at the same time, reviewed their regulations with a view to establishing a more scientifically justifiable basis for many of their previous requirements and to providing a more cost-effective service to the meat processing industry (McKenzie and Hathaway 1990).

In a competitive market for the declining numbers of livestock for slaughter new meat processing methods which are either more energy and cost efficient and/or require less input of manpower, are finding their way into the meat industry.

Hot-Boning was introduced into beef processing in New Zealand in 1986 because of its advantages over traditional boning methods with regards to cost and energy efficiency (A.Lowe pers. comm.). With the concerns over the eating quality of meat, and in an attempt to prevent the occurrence of cold shortening, the plant operator aims to achieve the slowest possible cooling regime which is still compatible with hygienic requirements, as laid down by the authorities (anon. 1989).

One tool that meets the need for a scientifically sound, technically justifiable approach to meat processing and hygiene has been developed with the temperature function integration technique (TFI), a method by which temperature product histories of meat and meat products are related to growth data for bacteria. Initially developed to predict spoilage of flesh foods and to allow for the rational management of their storage and distribution thus avoiding unnecessary wastage (Olley and Ratkowsky 1973), it has now found wider use in assessing the hygienic adequacy of food processing. Here it models not the growth of spoilage bacteria, but that of pathogens which may present a health risk to the unsuspecting consumer (Gill et al. 1988). Future applications are in the prediction of aging rates of meat stored at above freezing temperatures (anon. 1991). The latter, combined with an assessment of the hygienic adequacy of the same process should lead to the development of processes that ideally, within the limitations of the process specifications, meet hygienic concerns and the quest for high eating quality foods.

II. FACTORS AFFECTING MICROBIAL GROWTH

II.1. Substrate and initial flora

II.1.1 Substrate:

After death, muscular tissue undergoes changes, leading to the development of *rigor mortis.* Muscles, which are plastic and highly extensible during life, become rigid and inextensible. This stiffening of the musculature is due to the loss of the phosphate compounds, adenosine triphosphate (ATP), and creatine phosphate (CP), which in the living muscle, provide the energy for the engagement and disengagement of cross-links between the contractile proteins of muscle, myosin and actin. In the absence of ATP the muscle becomes rigid because of the irreversible cross-linking between myosin and actin (Huxley 1972).

ATP concentrations are maintained in the living muscle at rest by the aerobic oxidation of the products of glycolysis through the tricarboxylic acid (TCA) cycle and resynthesis of ATP from adenosine diphosphate (ADP) via the electron transport chain. When blood circulation fails after death, the muscle rapidly becomes anoxic and the oxidative metabolism ceases. Initially ATP concentrations are maintained through the transfer of the phosphate group from CP to ADP, and degradation of glycogen to lactic acid via the glycolytic pathway.

When the ATP concentration begins to decline, the ADP which accumulates, is further degraded to the monophosphate (AMP) which in turn is deaminated to inosine monophosphate (IMP). Because AMP is a co-factor to the enzymes phosphorylase and phosphofructokinase, which catalyse rate-determining reactions of glycolysis, the loss of this nucleotide may be one of the factors which cause cessation of glycolysis before the exhaustion of glycogen.

Thus there are, usually, residual concentrations of glycogen, creatine, IMP, glucose and some of its glycolytic intermediates, like glucose-6-phosphate present in low concentration in post-rigor meat (Fischer and Augustini 1977).

Some of these low molecular weight soluble components form the substrate for microbial growth, in particular glucose (Gill 1976; Gill and Newton 1978), and some amino acids, but not the bulk materials, such as protein, water or fat, which make up the muscle (Gill and Newton 1980).

Bacteria grow exponentially, utilizing the low molecular weight soluble components of blood and tissue fluids present at the surface of the meat. The spoilage flora does not form the necessary enzymes to degrade the larger molecules of protein and fat until the growth rate starts to decline (Glenn 1976). Many potent spoilage organisms first utilize glucose, and only when this substrate is exhausted do these bacteria attack amino acids. This results in the release of ammonia and sulphurous compounds that impart the putrid odours and flavours characteristic of spoilage (Gill and Newton 1978, McMeekin 1981). In meat of normal glucose levels (around 0.1 mg/g and higher) (Gill 1983) the growth of spoilage bacteria is maintained by this substrate up to a cell density of approximately 10^8 bacteria/ cm^2 (Gill 1976) before overt spoilage becomes noticeable. In meat of reduced glucose levels, i.e. animals that have been subjected to stress and/or exhaustive exercise prior to slaughter, glucose levels will only support a maximum bacterial density of around 10^6 bacteria/ cm^2 before spoilage ensues (Newton and Gill 1978).

Under aerobic conditions, which prevail on exposed carcass surfaces, the spoilage flora tends to be dominated by *Pseudomonas spp.*. Under close to anaerobic growth conditions, as in vacuum packaged meat, the flora will usually be dominated by facultative anaerobic microorganisms such as species of *Lactobacillus* at tissue pH below 5.8 (Newton and Gill 1978).

II.1.2 Initial flora:

When tissues are removed from a carcass of an animal, previously sterile meat will become contaminated with microorganisms. The contaminating organisms are derived mainly from the hide of the animal and are transferred from this onto the meat during the dressing process, by the slaughtermen, the equipment used during the slaughter process or as a result of cross-contamination from undressed carcasses. The bacteria deposited on the surface of tissues will not penetrate underlying tissues until spoilage is well advanced (Gill et al 1984). Generally, initial contamination in hygienic processing conditions, which are also described as Good Manufacturing Practice (GMP), is low at around 10^2 organisms/ cm^2. The majority of the bacterial species present are gram-positive mesophiles, mainly derived from soil and faeces present on the hide. Only a small fraction are gram-negative psychrotrophic microorganisms originating from soil, water and vegetation and capable of growth at chiller temperatures (West et al 1972; Newton et al 1978). It is members of the psychrotrophic flora which will eventually, after chilled storage, initiate and dominate spoilage.

II.2 pH

The pH of muscle tissue falls from *pre-rigor* levels of around 7.2 to about 5.5 to 5.7 in *post-rigor* meat with adequate levels of muscle glycogen. This decline in pH, due to the anaerobic metabolism of glycogen to lactic acid, ceases, either as a result of depletion of glycogen in the muscle (resulting invariably in an ultimate pH above 6.0), or because of inhibition of the enzymes required for glycolysis and to some degradation of proteins (resulting in pH-levels around 5.5-5.7).

In animals that have been subject to stress, exhaustive exercise or prolonged transport prior to death or slaughter the muscle glycogen reserves have been reduced to much lower than normal levels (Tarrant 1989). Thus, less lactic acid can be produced during the rigor process than in meat with a normal amount of glycogen (Potthast and Hamm 1976); the ultimate pH therefore remains at a higher level, usually above 6.0. Approximately 100 μmol/g lactate is produced in muscle of a low-ultimate pH, whereas only 40 μmol/g lactate would be expected in a muscle of high ultimate pH of around 6.2 (Davey and Gilbert 1976). In the latter situation the concentration of glucose is also lowered so spoilage bacteria, in the absence of glucose will metabolize amino acids at low cell densities, i.e. at a much earlier stage in the spoilage process (Gill and Newton 1978). Because it is the production of breakdown products from the amino acid metabolism that causes meat to appear spoiled and this process commences earlier in low glycogen concentration meat, high ultimate-pH meat will spoil at much lower numbers of spoilage bacteria than normal-pH meat.

Some strains of the gram-negative psychrotrophs, which compose most of the initial flora on meat, are inhibited by the pH of normal post-rigor muscle tissue. *Alteromonas putrefaciens* and *Enterobacter liquifaciens* are particularly sensitive and incapable of initiating growth at pH-values below 6.0 at chiller temperatures, but can cause significant spoilage of high ultimate-pH meat.

Under anaerobic conditions, the growth of *Alteromonas putrefaciens* on meat of high ultimate pH (above 6.0) leads to the production of hydrogen sulphide (H_2S) and results in greening of the meat through the production of sulphmyoglobin. This type of spoilage reduces the normal shelf life of vacuum-packaged meat by half (Taylor and Shaw 1977). Also *Brochothrix thermosphacta* will grow anaerobically on high ultimate-pH meat, but fails to initiate growth on normal-pH meat (Grau 1980). The anaerobic growth of some psychrotrophic species of Enterobacteriaceae, including the cold tolerant pathogens *Yersinia enterocolitica*, but also *Aeromonas hydrophila*, is similarly affected by the pH and lactate concentrations of normal low ultimate-pH meat, but lactate has also been shown to be inhibitive by itself (Grau 1981).

In contrast most strains of *Pseudomonas spp.* under aerobic conditions are unaffected by the pH of the meat (Gill and Newton 1982).

Spoilage bacteria may be inhibited and storage life prolonged if vacuum-packaged meat is treated with small quantities of acid, such as citric acid. The reduced surface-pH inhibits the growth of the spoilage flora long enough for substantial numbers of *Lactobacilli* to develop, thus continuing the inhibition of spoilage bacteria (Newton and Gill 1980). In addition, *Lactobacilli* produce antimicrobial agents that inhibit competing bacteria (Ahn and Stiles 1990; Raccach and

Baker 1978).

From the point of view of extended chilled storage of meat it is the lack of glucose and not the elevated pH as such, that makes high ultimate-pH meat undesirable as it results in low-molecular weight amino acids being metabolised earlier by the *Enterobacteriaceae*, with the resultant release of organo-sulfides and amines (Gill and Newton 1977, Eskin et al. 1971). The increased water holding capacity of high-pH meat, however, can be an advantage for some manufactured meat products, such as sausages, and may even be desirable (Wirth et al 1976).

Apart from their effect on microbial growth high pH-values also affect the appearance of some meat attributes, such as colour, texture and juiciness, characteristically resulting in dark, firm and dry meat (DFD).

II.3 Water activity

Microorganisms require water for their growth. The availability of water to microorganism is commonly expressed as the water activity (a_w), which is defined as the ratio between the equilibrium aqueous vapour pressure of the solution and that of pure water at the same temperature (Scott 1957). This definition takes into account that the availability of water to microorganisms is influenced by the presence of solutes, which decrease the water vapour pressure of a system and thereby its water activity. Most microorganisms able to grow on meat show a marked dependence on water and are generally unable to grow when a_w is below 0.95 (Leistner et al. 1981). Yeasts and moulds are more xerotolerant with the more common spoilage species being able to grow at a_w of 0.90 (Lowry and Gill 1984 a,b). The a_w of muscle tissue, which is around 0.99 if the meat surface is not exposed to any desiccation, does therefore not impose any restriction on bacterial growth.

Drying of meat surfaces occurs during the carcass-cooling period when water evaporating from warm, freshly slaughtered carcasses reduces the a_w of the surface layers of the meat to below that which is required to allow growth of microorganisms. The rate and degree of water loss is greatest at muscle surfaces overlying the thickest areas of tissue where cooling is at its slowest (Scott and Vickery 1939; Hicks et al. 1955).

After the carcass has cooled the carcass surface tissues will tend to rehydrate as a result of diffusion of water from the deeper tissues to the outside, unless airflow over the carcass and a low relative humidity continue to dry the exterior tissues. Weight loss associated with continuous drying is regarded as unacceptable, and thus, with inevitable rehydration occurring, microorganisms will start to grow after a lag of about 24 hours (Hicks et al. 1955; Nottingham and Wyborn 1975). If surface drying is prevented by some impervious cover over the meat surfaces, bacterial numbers will increase rapidly. Bacterial proliferation commences more quickly, the earlier the cover is applied (Scott and Vickery 1939). Bacterial proliferation can also begin early on any site on a carcass where evaporation is limited because of impaired airflow or where early rehydration occurs. The same situation applies were meat is boned, immediately after slaughter while still warm, i.e. at body temperature (Nottingham 1982), as is practised in several meat plants in New Zealand. After boning the meat is packaged in cartons with plastic liners which are immediately closed, thus not allowing for any evaporative drying to occur. A similar situation is encountered with offals which are traditionally bulk packed while still warm, with no provision for drying.

II.4 Temperature

The growth of all microorganisms is dependent on temperature and low temperatures will retard the growth of the bacteria commonly found on meat. Different species of bacteria will grow at different rates at the same given temperatures, and all species have upper and lower limits beyond which they fail to grow. These minimum and maximum temperatures are dependent on other limiting factors, such as pH and water availability, so that marginal conditions of either or both of these, or of other factors will reduce the temperature range for growth. Such synergistic effects are well documented (Ingram and Mackey 1976) and the preservation of many non-sterile food stuffs, e.g. salamis depends upon such synergistic effects (Leistner 1978).

In the case of aerobic growth on meat, the pseudomonads maintain their growth advantage over the competing mesophilic and psychrotrophic flora at temperatures up to around +20°C (Gill and Newton 1980). Under anaerobic storage, psychrotrophic lactobacilli have a growth rate advantage over the competing flora at temperatures below +20°C (Newton and Gill 1978). At +20°C the anaerobic flora is dominated by *Enterobacteriaceae*. This dominance is a result of their greater numbers in the initial flora, rather than an advantage in growth rates. At above +30°C mesophilic species predominate the spoilage flora.

Spoilage of meat by bacteria will not occur if the meat is stored at temperatures below the minimum growth temperature for bacteria. Psychrotrophic bacteria are capable of growth at temperatures below O°C (Larkin and Stokes 1968), thus bacterial spoilage at subzero storage conditions is possible. At temperatures between -2 and -4°C only a small fraction of the water in the meat is frozen as ice, thus not reducing the water activity sufficiently to completely inhibit bacterial growth. At lower temperatures moulds and yeasts, with minimum growth temperatures of -10° to -12°C, may spoil meat (Ingram and Mackey 1976; Leistner et al. 1981).

II.5 Atmosphere

The overwhelming majority of the bacteria constituting the initial spoilage flora are strictly aerobic and are thus unable to grow under close to anaerobic conditions achieved by vacuum-packaging (Grau 1981). In this process meat is sealed in plastic bags of low gas permeability. With freshly slaughtered meat the enclosed muscle tissue still respires and will utilize most of the remaining oxygen, and therefore the pack will finally contain only nitrogen and carbon dioxide, with traces of oxygen. The residual oxygen content depends on the efficiency of the evacuation process and the gas permeability of the packaging film. Maximum shelf life is obtained by strictly maintained anaerobic conditions. With increased oxygen ingress the growth rates and final counts of *Pseudomonas spp.* increase. Undesirable colour changes, due to residual oxygen reacting with the muscle myoglobin can then also be observed (Newton and Rigg 1979).

Part of the effects of vacuum packaging seem to be due to the inhibitory effects of carbon dioxide, with the degree of inhibition increasing with decreasing temperatures. In the main, CO_2 appears to be prolonging the shelf life by increasing the lag phase of the spoilage bacteria, before growth can be initiated (Gill and Penney 1988).

Growth rates of spoilage bacteria are reduced by about 30 to 50% if the atmosphere contains at least 20% of carbon dioxide

(Gill and Tan 1980). Under ideal conditions extensions of the shelf life of up to 50% can be observed (Clark and Lentz 1969, Gill and Tan 1980).

Based on this knowledge, gas flushing with either oxygen, nitrogen or carbon dioxide, or combinations of the three has been developed. Addition of oxygen improves the colour of the packaged meat, but markedly reduces the shelf life (Silliker et al. 1976; Newton et al. 1977) by allowing aerobic growth of spoilage organisms.

A commercial packaging system (CAPTECH), designed to package meat under a carbon dioxide atmosphere within an oxygen-free environment, has been developed; in combination with hygienic production practices and effective temperature control, storage lives of CAPTECH products in excess of six months are achievable (Gill 1988).

PART 1:

III. GROWTH OF COLD-TOLERANT PATHOGENS ON BEEF

III.1. Introduction

III.1.1 Changes to Consumer behaviour and requirements

Food preferences are a reflection of society and its values. Changing attitudes towards the use of time have led to an emphasis on convenience-foods and the associated emphasis on time-savings in the preparation of meals. Ready-to-eat, shelf-stable, refrigerated foods in smaller portions, are being developed to meet this demand (Palmer 1990).

As consumers prefer fresh, chilled meat to frozen meat, various forms of extending the shelf-life of meat and meat products have been developed. Meat producing countries, which are far removed from their main markets have had to develop packaging methods that allow them to deliver fresh, chilled meat at distant market places.

One such method, vacuum-packaging, extends the shelf-life of fresh meat by excluding oxygen from the vacuum-pack, thus restricting the growth of the normal, obligate aerobic spoilage flora, which consists mainly of *Pseudomonas spp.* Because of the lack of oxygen in the pack the muscle pigment myoglobin exists in its de-oxygenated form, which has a purple colour and the meat appears dull. Once exposed to atmospheric oxygen myoglobin is re-oxygenated to Oxymyoglobin and takes on a the bright red colour preferred by consumers.

Controlled atmosphere packaging, especially carbon dioxide packaging, was developed to meet the demand for extended storage life, the ability to pack varying cuts and shapes without concern for residual oxygen left in the pack, and the ready display of a product retaining the fresh, bright colour of meat. Even whole sheep carcasses can be packaged in an aluminium-foil pouch and shipped to distant market places within the extended storage life achieved with this system (Gill 1988a; Bell 1990). The extended storage at chill temperatures also allows the process of ageing to continue until the product reaches the consumer, with a resultant increase in tenderness (Gill 1988a).

III.1.2 Public Health implications

In the case of extended chilled storage, concern has been growing over the commercially achievable shelf-life of more than sixteen weeks of carbon dioxide packaged meat. The potential for growth of cold-tolerant pathogens, such as *Listeria monocytogenes, Aeromonas hydrophila* and *Yersinia enterocolitica*, all of which have frequently been isolated from meat (Lowry 1988; Buchanan and Palumbo 1985; Hanna et al. 1976) and could grow at temperatures commonly used for extended storage, may increase the health risk for consumers of meat and meat product packaged in this way.

An increase in the reported number of cases of food poisoning caused by some of these cold tolerant pathogens has been suggested by some authors (Breer and Breer 1988; McLauchlin 1987). Although this may be related to an improvement in the isolation techniques employed or an increased awareness of these pathogens, it may also reflect a true increase in prevalence due to the changing food preferences of the consuming public.

III.1.3 Aims of the work

With the introduction of controlled atmosphere packaging of meat, the extended chilled storage life has the potential to afford cold-tolerant pathogens additional opportunity to grow to numbers which might present a health risk to the consumer. It was therefore decided to study the growth of three selected pathogens on carbon dioxide packaged beef and compare their respective growth patterns with those recorded on vacuum-packaged beef.

III.2 Selected Pathogens

III.2.1. *Yersinia*

The genus *Yersinia*, named after the French bacteriologist Yersin who first isolated the causal organism of plague in 1894, comprises a group of microorganisms placed in the family Enterobacteriaceae. The genus includes the species *Y. pestis*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *Y. fredericksenii*, *Y. ruckerii*, *Y. kristensenii* and *Y. intermedia*. Within the species several biotypes and serotypes are distinguished (Bercoivier and Mollaret 1984).

Yersinia are straight, gram-negative rods or coccobacilli, about 0.5 to 0.8 µm in diameter, and 1-3 µm in length. They are nonmotile at 37°C, and motile at 25°C, except for *Yersinia pestis* which is always non-motile. *Yersinia* species grow at temperatures between 4 and 42°C, with optimum growth at 28-29°C. They are facultatively anaerobic, thus having both, oxidative and fermentative metabolic pathways. *Yersinia* species can grow within a pH range from 4.6 to 9.0, with optimum growth between 7.2 to 7.4 (Hanna et al. 1977).

III.2.1.1. *Yersinia enterocolitica*

Animal disease and prevalence

Pigs have been implicated as a major reservoir of Y.*enterocolitica* serotypes, and the rate of isolation of the organism from tonsils and tongues of pigs is greater than the rate of isolation from faecal material. Serotypes 0:3; 0:5,27 and 0:8 have been isolated from pigs in Europe and North America, where they appear to be normal inhabitants of the oral cavity of pigs (Wauters 1979, Doyle et al. 1981, Pedersen 1979).

Isolations have also been made from healthy and sick cattle (Davey et al. 1983; Fukushima et al. 1983; Bullians 1987), sheep (McSporran et al. 1984; Bullians 1987; Slee and Button 1990), goats (Slee and Button 1990), deer (Henderson 1984) and other domestic and wild animals. Young animals appear to have a higher rate of carriage of yersinia than older animals. One study reports a prevalence of 0.6% in cull cows, the isolates belonging to Y.*pseudotuberculosis* and *Y. intermedia,* but 28% in lambs, with *Y. enterocolitica* being the predominant isolate, others being *Y. intermedia, pseudotuberculosis* and Y.*frederiksenii* (Bullians 1987). In animals, yersiniosis is characterised by diarrhoea with the formation of microabscesses in the intestinal mucosa.

Several strains of *Y.enterocolitica* have been isolated from vacuum packaged beef and lamb (Hanna et al. 1976) and counts of *Y. enterocolitica*-like organisms have been found in high numbers on vacuum-packaged, high ultimate-pH beef (Seelye and Yearbury 1979; Gill and Newton 1979).

Human Disease and prevalence

Infections in humans usually manifest clinically as gastroenteritis and mesenteric lymphadenitis in infants and children, and generally as abdominal disorders and diarrhoea and arthritis in persons aged 20 to 60 years and erythema nodosum in patients aged 60 years and over (Winblad 1973). The characteristic pain in the right lower abdomen mimics appendicitis and often leads to surgical intervention. The majority of isolations in Europe belong to the serotypes 0:3 and 0:9 (Vandepitte and Wauters 1979), in North America predominantly to the serotype 0:8, with some serotype 0:3 isolations in the eastern provinces of Canada (Bottone 1977). Infections with *Yersinia enterocolitica* are most common in the autumn and winter months.

III.2.2 *Listeria*

Named after Lord Lister, an English surgeon, the genus *Listeria* comprises eight species, *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. murrayi, L. grayi* and *L. denitrificans. Listeria* are short gram-positive rods, 0.4-0.5 µm in diameter and 0.5-2 µm in length. The organisms are motile by means of polar flagellae and grow both aerobically and facultatively anaerobically. The colonies appear grey by normal illumination and show a characteristic blueish sheen when viewed under oblique lighting (Henry 1933). *Listeria spp.* are among the few bacteria which are able to grow at temperatures ranging from +3 to 45°C.

L.monocytogenes and *ivanovii* are the only species known to be naturally pathogenic to animals and man (Seeliger and Jones 1986).

IV.2.2.1 *Listeria monocytogenes*

Animal disease and prevalence

L.monocytogenes is ubiquitous in the environment. It can be isolated from plants, soil and decaying vegetation (Weis and Seeliger 1975). The bacterium has been reported to produce disease in many species of domestic and wild animals, such as sheep, goats, cattle, deer, moose and others (Gray and Killinger 1966) and in these animals it can often be isolated from the faeces (Skovgaard and Morgen 1988).

In infected older animals the central nervous system tends to be involved resulting in encephalitis, but there is also a tendency to infect the organs of the female reproductive system, resulting in abortions. Sheep appear to be particularly susceptible and develop the disease with resultant high mortality. The mode of transmission is presumed to be the oral route and contaminated silage has been shown to be a reservoir for *Listeria* (Fenlon 1985) and implicated in outbreaks.

In cows mastitis caused by *L.monocytogenes* has been reported (Gitter et al. 1980) and the organism has been isolated from milk (Hayes et al. 1986) and soft cheese (Johnston et al. 1986). It has also been frequently isolated from uncooked meat with the prevalence in the test samples reaching as high as 92% in beef mince in New Zealand retail displays (Lowry 1988) and usually lower around 20-40% in minced beef or pork (Kleinlein et al. 1989, Nicolas and Vidaud 1987).
However, the number of cases of human listeriosis that can be attributed to meat-borne transmission is small (Kaczmarski and Jones 1989).

Human disease and prevalence

Human listeriosis is commonly characterised by formation of miliary granulomas and focal necrosis or by suppuration in the infected tissue (Seeliger and Finger 1985). A series of toxins are produced, including haemolytic and lipolytic toxins.

Several disease syndromes can be distinguished in infections of humans. Infection in the pregnant female may lead to "influenza-like" symptoms with infection of the fetus via the transplacental route. This occurs usually late in pregnancy and may result in immediate or delayed abortion or stillbirth. Infection may also occur during delivery and listeriosis of the newborn is often systemic and involves multiple organs. In some cases meningitis may develop (Schlech et al. 1983). Listeriosis can also manifest as meningitis in older patients, and often takes a course with fulminant onset and high fatality rate if left untreated (Seeliger and Finger 1985). Cutaneous listeriosis is seen in adults who have been in contact with infected tissues, for instance farmers and veterinarians. Pinhead sized lesions develop, with the centre soon becoming pustular (Seeliger 1961). Conjunctivitis may also develop from contact with infectious material (Seeliger and Finger 1985).

In Canada the incidence rate per annum is reported to be 2.4 cases per million population and 20 out of the 63 cases of human listeriosis were found in babies under one month of age. In adults the highest rate was found in the 70-79 years age group, followed by those aged 60-69 years. In 88.9% of all cases another, possibly predisposing health condition, including pregnancy, diabetes and neoplastic disease was present (anon. 1991).

In 1989 21 cases of human listeriosis occurred in New Zealand, twothirds of which were maternal and neonatal infections. Of the 21 cases 17 were females. This gives an annual incidence of 7 cases per million with a mortality rate of 33 %. In 33% of adults another underlying disease may have predisposed to the acquisition of infection. The seasonal distribution records the highest monthly incidence in June, with 5 of the human cases recorded in that month. 58% of the isolates belonged to serogroup 4 (Rocourt 1991).

III 2.3. *Aeromonas*

The genus *Aeromonas* in the family *Vibrionaceae* is divided into two groups, the non-motile aeromonads, named *Aeromonas salmonicida* and the motile group, containing the three species *Aeromonas hydrophila, A. caviae* and *A. sobria.* They form rods with rounded ends, 0.3-1.0 μm in diameter and 1.0-3.5 μm in length. Aeromonads grow aerobically and facultatively anaerobically and are Gram-negative. One feature which distinguishes them from other bacteria is their positive oxidase reaction (Popoff 1984), and their ability to grow at +4°C (Palumbo et al. 1985a).

Their toxigenicity is presumed to be based on the production of toxins, enterotoxins, cytotoxins and haemolysins, which, in humans, may induce the gastroenteritis commonly experienced with *Aeromonas* food poisoning, while, in poikilotherm animals, proteases appear to be more important in the pathogenicity (Cahill 1990).

III.2.3.1 *Aeromonas hydrophila*

Animal disease and prevalence

Many aeromonads are pathogenic for cold-blooded animals such as fish and amphibians and are often isolated from water sources (Popoff 1984; Abeyta and Wekell 1988). *A. hydrophila* can also be isolated from faeces of healthy horses, pigs, sheep and cows (Gray 1984) and a variety of fresh meat, seafood and other produce (Buchanan and Palumbo 1985).

Aeromonas hydrophila has been associated with the spoilage of refrigerated meat, i.e. chicken, beef, pork and lamb (Nagel et al. 1960; Enfors et al. 1979; Simard et al. 1984; Palumbo et al. 1985b). In some of these studies *A. hydrophila* could not be isolated if the sample was stored in air, yet under modified atmosphere the organism could be isolated (Simard et al. 1984; Enfors et al. 1979), indicating that *A. hydrophila* may have a growth advantage over the competing spoilage flora under these conditions.

Human disease and prevalence

Aeromonas spp. cause wound infections and septicaemia, particularly in immuno-compromised patients and have been reported to cause diarrhoea (Goodwin et al. 1983). In Australia, *A. hydrophila* has been the most frequently isolated bacterial pathogen in children with diarrhoea (Burke et al. 1983), with the duration of the clinical symptoms lasting much longer than in patients infected with other pathogens. In Japan *Aeromonas* spp. were isolated from 11.1% of diarrhoeal stools and 2.2% of normal stools and were also isolated in high numbers from food samples (Nishikawa and Kishi 1988).

III.3 Materials and Methods

III.3.1 Organisms

The bacteria used were a not serotyped strain of *Yersinia enterocolitica* isolated from vacuum packaged lamb at the Meat Industry Research Institute of New Zealand (MIRINZ), Hamilton, and *Aeromonas hydrophila ATCC 7966* and *Listeria monocytogenes ATCC 19111*, both obtained from the New Zealand Communicable Disease Centre, Porirua.

III.3.2 Preparation of the inocula

Shaken cultures of each organism were cultivated in Brain Heart Infusion Broth (Difco) at +30°C overnight, to reach the stationary phase. Serial tenfold dilutions of 1 ml samples from each culture were prepared and 0.1 ml aliquots of suitable dilutions spread on duplicate plates of Plate Count Agar (Difco). The plates were incubated for 24 hours at +30°C. The concentration of cells in each culture was ascertained from plates containing between 20 and 200 colonies. During that time the cultures were stored at +4°C. For inoculation the cultures were diluted appropriately in 0.1% peptone water to achieve inocula of ca. 10^5 cells per ml.

III.3.3 Meat Samples

Two beef striploins (*M. longissimus dorsi*), from animals that had been slaughtered 48 hours previously, were obtained from a local meat plant for each storage trial, consisting of the specific combination of a packaging atmosphere (vacuum vs. carbon dioxide) and a storage temperature. The pH-values of the striploins were determined by an Orion SA250 pH-meter by direct application of a 91-06 spear glass combination electrode into the muscle. Only striploins of ultimate pH values above 6.0 were selected.

In the laboratory, the fat cover over the striploin was trimmed away from the muscle tissue and discarded. The muscle tissue was then cut into steaks, approximately 10 to 15 mm thick, each weighing between 100 and 150 g.

For each sampling day eight steaks were prepared. Two of these, which were not inoculated with any of the cultures, served as controls, and the other six steaks were inoculated with the three test organisms. Of the six steaks two were each inoculated with *Yersinia enterocolitica*, two with *Listeria monocytogenes* and two with *Aeromonas hydrophila*. For each trial, consisting of a combination of storage temperature and packaging atmosphere, i.e. vacuum or CO_2 , sufficient steaks to withdraw a full complement of samples on at least eight predetermined sample dates were prepared.

The six steaks each received an inoculum of 0.1 ml of one of the diluted cell cultures onto its surface. The inoculum was immediately distributed over the whole surface using a sterile glass spreader. Both control and inoculated steaks were then individually packed in a pouch of co-extruded polyvinylidene chloride (PVDC) of low gas permeability (Cryovac, W.R. Grace, Porirua, New Zealand) which was then evacuated (Multivac, Ag-900, Sepp Haggenmüller KG, West Germany) and sealed. Samples that were to be stored under carbon dioxide were packaged in pouches composed of high gas-permeability polyethylene and then evacuated. Within 30 minutes of their evacuation the polyethylene pouches were further packaged in groups of eight, one group for each of the sampling days, in gas-impermeable, aluminium-foil laminate pouches (CapTech, Printpac-UEB, Auckland, New Zealand) which were evacuated and then filled with 2 litres of carbon dioxide and sealed. Each group of eight consisted of two control steaks and two steaks each inoculated with one of the three pathogens.

III.3.4 Storage of meat samples

Packaged meat samples were stored in insulated , polystyrene boxes that were held in temperature controlled rooms operating at 5 to 10°C below the required storage temperatures of -2, 0, +2, +5 and +10°C. Each insulated box was fitted with a fan, and a temperature controller connected to a light bulb in the box that acted as a heating element. The fan circulated air through the box.

The samples were stored, raised above the box floor on racks and arranged, so that they did not touch the box walls. Sample temperatures were maintained within ± 0.2 °C of the set storage temperature of -2, 0, +2, +5 and +10°C. Storage temperatures were monitored with electronic temperature data loggers (Delphi Instruments, True- Test, Auckland, New Zealand) which were placed with the samples.

III.3.5 Sampling Procedures

Duplicate samples from each series, i.e. each storage temperature, vacuum pack or carbon dioxide packaging for the control packs and each of the three pathogens, were examined at the time of packaging and at subsequent times that were chosen to take account of the varying growth rates at the different storage temperatures. Upon opening of each pack, the odour and colour of each steak were assessed and strong putrid odours presumed to indicate overt spoilage.

To each pack was added 50 ml of 0.1% peptone water, and the meat sample and fluid vigorously massaged for two minutes. At that time the pH of the rinse fluid was measured by immersion of the combination glass electrode and Orion SA250 pH-meter. Samples of the rinse fluid were then ten-fold serially diluted in peptone water and 0.1 ml portions of suitable dilutions spread on duplicate plates of Plate Count Agar (PCA) (Difco) for the assessment of the spoilage flora, and in the case of meat samples inoculated with *Yersinia enterocolitica* also on plates of Cefsoludin-Irgasan-Novobiocin (CIN)- Agar (Appendix, p.138/139). In the case of samples inoculated with *Aeromonas hydrophila* appropriate dilutions were spread on PCA and on Starch- Ampicillin (SA) Agar (Appendix, p.143/144). From the meat which was inoculated with *Listeria monocytogenes* dilutions were spread on PCA and on Lithiumchloride- Phenylethanol- Moxalactam (LPM) Agar (Appendix, p. 140/141). All plates were incubated at 25°C for 48 hours.

The composition of the natural spoilage flora was assessed from PCA plates with at least 100 colonies from the inoculated control samples. Numbers of the two distinctive colony types, i.e. Lactobacilli and Enterobacteria were estimated. At each estimation pinhead or small, white, glossy colonies were assumed to be lactobacilli, and large, opaque or pigmented colonies were assumed to be enterobacteria (Gill and Penney 1988). Numbers of the members of the spoilage flora were determined from PCA plates bearing 20 to 200 colonies.

The numbers of the pathogens were determined from counts on the selective agar plates. At each count, four representative, presumptive colonies were picked from each plate that was counted and transferred onto PCA plates. These were incubated overnight at +25 °C and then stored at +4°C until further tests to confirm their identities were done.

Colonies on CIN-Agar, that were small to medium size, and smooth with a dark red centre surrounded by transparent borders were presumed to be *Yersinia*. Confirmative reactions were gram -ve, catalase +ve, oxidase -ve, Voges-Proskauer +ve, motility at +25°C and non-motility at +37°C (Appendix, p.138,140,142,143).

Colonies on SA- Agar that were amylase-positive, i.e. colonies which were surrounded by a yellow zone against a brown background, after the plates were flooded with Lugol's iodine, were presumed to be *Aeromonas*. Confirmative reactions were gram -ve, catalase and oxidase +ve (Palumbo et al. 1985b)(Appendix, p.140,138,143).

Colonies on LPM Agar that appeared blue with an irregular, lacy surface pattern when viewed under a stereo-microscope, with a fiberoptic cable directing light at the plate at an angle of 45 degrees from underneath, were presumed to be *Listeria*. Confirmative tests were gram and catalase +ve, oxidase -ve, Rhamnose +ve, Mannitol and Xylose -ve, Esculin +ve, Methylred +ve, Nitrate -ve and "umbrella" motility (Lee and McClain 1986) (Appendix, p.137-143).

III.4 Results

Numbers of spoilage organisms were generally similar for inoculated sample steaks and the un-inoculated controls that had been packaged and stored together. In vacuum-packaged steaks the spoilage flora generally consisted of comparable numbers of lactobacilli and enterobacteria. Vacuum-packaged samples were usually spoiled when the total microbial numbers were more than 1 x 10^9 per steak.

The spoilage flora of all CO_2- packaged steaks were dominated by lactobacilli, with the enterobacteria only forming around 1% of the total flora when maximum numbers were attained. Spoilage of CO_2-packaged steaks did not occur until maximum numbers had persisted for some time and when spoilage did occur it was usually associated with an increase of the proportion of enterobacteria. As numbers of enterobacteria were estimated on morphology alone and as these organisms were present only in small numbers on a great number of plates, in particular in the CO_2 stored samples, enterobacteria numbers are expressed as percentages of total counts (Table I a+b, p.36).

The pH of vacuum-packaged samples, as assessed at the microbiological sampling times was in most cases the same as at the time the steaks were cut. In carbon dioxide packaged samples, the pH of the rinse fluid was mainly around 0.2 -

0.3 units lower than at the time when the sample was initially stored (Table II and III, p.37).

Spoilage of the sample packs was evidenced by discoloration of the meat and the putrid odours emanating from the pack upon opening.

Table I: Proportion of enterobacteria as a percentage of the spoilage flora at different sampling days of high ultimate-pH beef.

(a) under carbon dioxide

Temp (°C)	Sampling days							
	0	1	2	3	4	5	6	7
+10	<1	<1	<1	<1	11.5	18.5	ND	ND
+5	<1	<1	2.5	4	2	8.9	ND	ND
+2	<1	<1	<1	2.5	3	15	ND	ND
0	<1	<1	<1	1	1	2	4.3	13
-2	<1	<1	<1	1	<1	4.5	30	ND

(b) and vacuum.

Temp (°C)	Sampling days					
	0	1	2	3	4	5
+10	13.3	30	35	43	37	45
+5	20	28	45	37	45	46
+2	14	23	45	38	34	52
0	15.5	22	34	45	39	46
-2	17	25	23	45	35	35

ND = not done

Table II: pH-values (mean of eight) of CO_2-packaged steaks at five different storage temperatures.

Temp (°C)	Sampling days							
	0	1	2	3	4	5	6	7
+10	6.5	6.18	6.18	6.12	6.18	6.07	ND	ND
+5	6.2	5.8	5.9	5.9	5.8	5.88	ND	ND
+2	6.5	6.22	6.1	6.15	6.22	5.91	ND	ND
0	6.3	6.2	6.2	5.95	6.15	6.01	5.88	6.15
-2	6.4	6.2	6.25	6.33	6.31	6.01	6.0	ND

ND = not done

Table III: pH-values (mean of eight) of vacuum-packaged steaks at five different storage temperatures

Temp (°C)	Sampling days					
	0	1	2	3	4	5
+10	6.3	6.2	6.3	6.4	6.1	6.31
+5	6.17	6.1	6.1	6.1	6.1	6.0
+2	6.4	6.3	6.4	6.3	6.5	6.3
0	6.3	6.4	6.3	6.4	6.3	6.15
-2	6.4	6.3	6.2	6.4	6.3	6.35

A. Storage temperature +10°C

1. Vacuum-packaged samples

Vacuum-packaged samples stored at +10°C were spoiled on the fifth day of the trial. *A. hydrophila* grew without significant lag phase and at a rate of approximately 5.9 hours per generation which was faster than that of the spoilage flora at around 6.9 hours/generation (Because the generation time values were calculated from the graph, they are necessarily inaccurate and should be taken as a guide only). Growth of *A. hydrophila* was apparently little affected by the spoilage flora attaining maximum numbers after 5 days of storage and therefore, becoming the major component of the flora on steaks inoculated with that organism. The total increase of the inoculum was 5.5 log cycles. *Y. enterocolitica* grew, after about one day's lag, at a rate similar to that of the spoilage flora (6.9 hours/gen.) and was also apparently unaffected by the spoilage flora attaining maximum numbers. The total increase in numbers for the inoculum was 3.9 log cycles. The lag before growth of *L. monocytogenes* commenced, appeared greater than for *Y. enterocolitica*. *L. monocytogenes* attained a similar growth rate to that of the spoilage flora, but grew slower at a rate of 8.5 hours/gen. and the maximum numbers of *L. monocytogenes* that were achieved before spoilage ensued were low, with an increase of only 2.9 log cycles (Table IV and Figure 1a, p.40, 41).

2. CO_2-packaged samples

CO_2-packaged steaks stored at 10°C were spoiled after 10 days of storage. There was a significant microbial lag phase before total aerobic numbers increased. The growth rate of the spoilage flora under a CO_2-atmosphere was reduced compared to that in the vacuum-packaged samples (10 hours/gen.). Of the inoculated organisms, only *Y. enterocolitica* (12.4 hours/gen.) showed a corresponding extension of the lag phase. *A. hydrophila* (10.7 hours/gen.) and *L. monocytogenes* achieved growth rates that were similar to that of the spoilage flora, but with *L. monocytogenes* growing slightly slower (13.8 hours/gen.). Compared with their growth under vacuum, growth of all three organisms in a CO_2 atmosphere was greatly reduced and appeared to cease, when the spoilage flora attained maximum numbers. The increase in numbers for the inocula were 3.4 log cycles for *Y. enterocolitica*, 4.1 log cycles for *A. hydrophila* and 3.5 for *L. monocytogenes* (Table V and Figure 1b, p.40, 41).

Table IV: Values (as $\log_{10}$/sample) for the spoilage flora at +25°C, for control (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under vacuum at +10°C (range in brackets).

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.6 (5.5-5.7)	5.6 (5.0-6.1)	4.5 (4.2-4.6)	4.5 (4.4-4.6)	4.2 (4.2-4.2)
1	6.4 (6.2-6.5)	6.4 (6.1-7.0)	4.6 (4.5-4.8)	5.6 (5.5-5.7)	4.4 (4.1-4.6)
2	7.8 (7.6-8.0)	7.7 (7.4-8.2)	5.2 (4.7-5.4)	6.8 (6.5-7.1)	4.7 (4.7-4.7)
3	8.6 (8.3-8.8)	8.7 (8.2-9.2)	6.4 (6.4-6.4)	7.7 (7.4-7.8)	5.3 (5.3-5.3)
4	9.6 (9.6-9.6)	9.6 (8.9-9.8)	7.3 (6.8-7.8)	9.3 (9.3-9.3)	6.4 (6.4-6.4)
5	9.9 (9.8-10.0)	9.8 (8.9-10.5)	8.4 (8.3-8.4)	10.0 (9.9-10.0)	7.1 (7.1-7.1)

Table V: Values (as $\log_{10}$/sample) for the spoilage flora at +25°C, for control (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under carbon dioxide at +10°C (range in brackets).

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.8 (5.4-6.1)	5.8 (5.5-6.4)	4.4 (4.4-4.4)	4.5 (4.4-4.6)	4.5 (4.5-4.5)
1	5.9 (5.8-6.0)	5.9 (5.6-6.5)	4.4 (4.3-4.5)	5.3 (5.3-5.4)	4.7 (4.4-5.0)
2	6.2 (6.1-6.2)	6.2 (5.6-6.7)	4.2 (4.2-4.3)	6.2 (5.7-6.9)	5.3 (5.3-5.3)
5	8.4 (8.2-8.6)	8.4 (7.7-9.5)	5.6 (5.5-5.7)	8.0 (7.8-8.2)	6.8 (6.6-7.0)
8	9.6 (9.5-9.8)	9.6 (9.5-9.8)	7.7 (7.7-7.8)	8.3 (8.0-8.6)	8.0 (8.0-8.1)
10	9.6 (9.5-9.7)	9.6 (9.6-9.7)	7.8 (7.5-8.0)	8.6 (8.5-8.7)	7.9 (7.8-8.0)

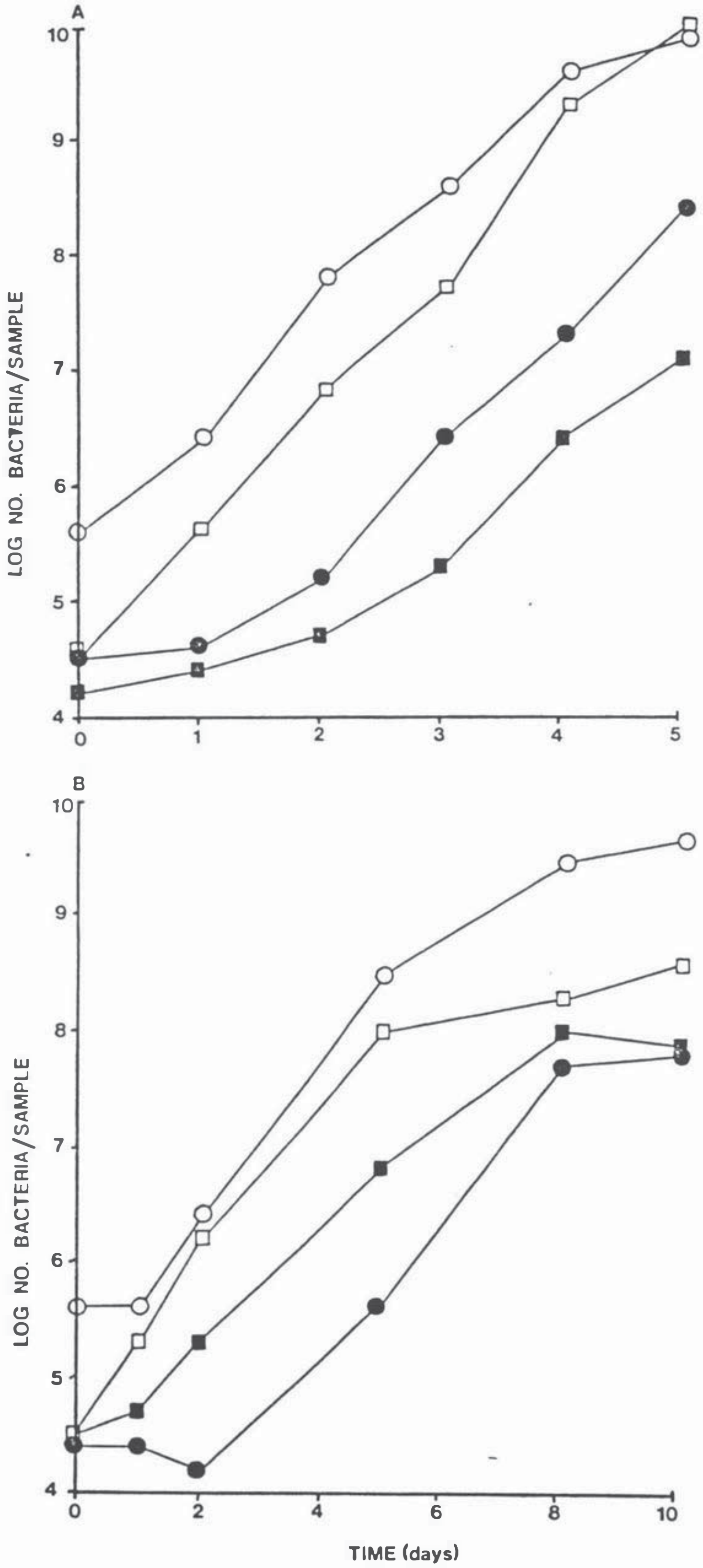


Figure 1: Growth of bacteria in (a) vacuum and (b) CO_2 packs at +10°C. (○) total counts at +25°C, (●) *Y. enterocolitica*, (□) *A. hydrophila*, (■) *L. monocytogenes*.

B. Storage temperature +5°C

1. Vacuum-packaged samples

At +5°C under vacuum, spoilage ensued after 17 days. The duration of the lag phase for *Y.enterocolitica* was not significant and the growth rate at 16.5 hours/gen. was similar to that of the spoilage flora (17 hours/gen.). The maximum increase of the inoculum was 5.3 log cycles. *A.hydrophila* and *L.monocytogenes* showed relatively long lags, with the growth rate of *A.hydrophila* of 18.6 hours/gen. being similar to that of the spoilage flora, and that of *L.monocytogenes* being distinctly slower (23 hours/gen.). The total increase for *A.hydrophila* was 3.4 log cycles and for *L. monocytogenes* 2.9.

As the spoilage flora reached maximum numbers the growth of *Aeromonas* and *Listeria* appeared to be inhibited, whereas the growth of *Yersinia* continued until overt spoilage was evident (Table VI and Figure 2a, p.44, 45).

2. CO_2-packaged samples

Under carbon dioxide, overt spoilage was detected after 35 days of storage at +5°C. Of the test organisms only *Y. enterocolitica* grew (56.2 hours/gen.) after a prolonged lag, which was longer than that of the spoilage flora. The spoilage flora increased at a growth rate of about 39 hours per generation. The total increase of the inoculum of *Y.*

enterocolitica was limited to 2.3 log cycles. None of the other two test organisms grew before spoilage became apparent (Table VII and Figure 2b, p.44, 45).

Table VI: Values (as $\log_{10}$/sample) of the spoilage flora at +25°C, for control (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under vacuum at +5°C (range in brackets).

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.6 (5.2-6.0)	5.6 (5.6-5.6)	4.1 (4.0-4.2)	4.3 (4.1-4.5)	4.3 (4.3-4.3)
3	5.9 (5.7-6.1)	6.1 (6.1-6.2)	4.9 (4.9-4.9)	4.6 (4.6-4.6)	4.3 (4.2-4.3)
7	8.3 (8.1-8.4)	8.3 (8.2-8.3)	6.6 (6.5-6.7)	6.3 (6.3-6.3)	5.0 (4.9-5.1)
11	9.5 (9.5-9.5)	9.5 (9.4-9.5)	8.4 (8.2-8.6)	7.7 (7.2-8.2)	6.0 (5.8-6.2)
14	9.7 (9.7-9.7)	9.7 (9.6-9.7)	9.3 (9.2-9.3)	7.7 (7.7-7.8)	7.2 (7.0-7.4)
17	9.7 (9.7-9.8)	9.7 (9.5-9.8)	9.4 (9.4-9.4)	7.0 (6.9-7.3)	6.8 (6.7-6.8)

Table VII: Values (as $\log_{10}$/sample) for the spoilage flora at +25°C, for controls (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under carbon dioxide at +5°C (range in brackets).

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.7 (5.4-6.0)	5.6 (5.6-5.7)	4.4 (4.3-4.5)	4.3 (4.1-4.4)	4.2 (4.2-4.2)
7	6.2 (6.0-6.4)	5.9 (5.5-6.0)	4.6 (4.4-4.7)	4.2 (4.2-4.3)	4.0 (4.0-4.0)
11	6.8 (6.8-6.8)	6.5 (6.2-6.7)	4.5 (4.4-4.5)	4.3 (4.0-4.6)	4.1 (4.0-4.2)
17	8.6 (8.5-8.6)	7.4 (6.5-7.8)	5.3 (5.3-5.3)	4.1 (4.0-4.2)	4.1 (4.1-4.2)
25	9.3 (9.3-9.4)	8.9 (8.3-9.3)	6.3 (6.1-6.6)	3.9 (3.9-3.9)	4.1 (4.0-4.2)
35	9.6 (9.6-9.7)	9.3 (9.2-9.4)	6.7 (6.6-6.8)	3.6 (3.4-3.8)	4.2 (4.1-4.4)

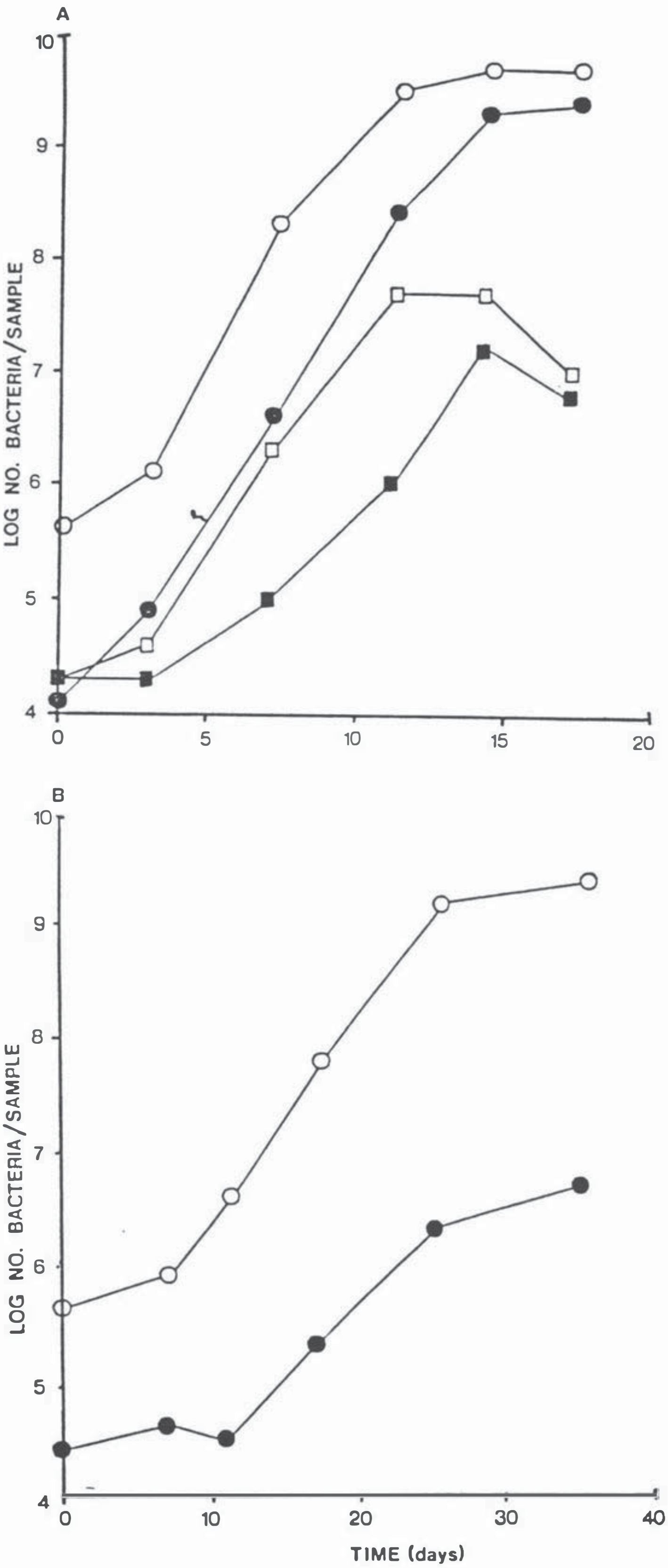


Figure 2: Growth of bacteria in (a) vacuum and (b) CO_2 packs at +5°C. (○) total counts at +25°C, (●)*Y. enterocolitica*, (□) *A. hydrophila*, (■) *L.monocytogenes*

C. Storage temperature +2°C

1. Vacuum-packaged samples

Vacuum-packaged samples, stored at +2°C spoiled after 35 days, with the growth rate of *Yersinia* at 35 hours/gen. being similar to, but somewhat slower, than that of the spoilage flora (28 hours/gen.), with a maximum increase of 5.2 log cycles. *Aeromonas* also grew at rates similar to the spoilage flora (26.6 hours/gen.), but only after a substantial lag, which kept total achieved increases lower than those of the spoilage flora, resulting in only a maximum increase of 4.3 log cycles. *Listeria* grew at a rate less than that of the spoilage flora (53.3 hours/gen.) and showed a substantial lag. The total maximum increase was limited to 2.8 log cycles (Table VIII and Figure 3, p.47, 48).

2. CO_2-packaged samples

Carbon dioxide packaged samples, stored at +2°C spoiled after 70 days (spoilage flora about 57.8 hours/gen.), with maximum spoilage flora numbers having been attained at earlier sampling dates. None of the test organisms grew at this temperature (Table IX, p.47).

Table VIII: Values (as $\log_{10}$/sample) for the spoilage flora at +25°C, for control (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under vacuum at +2°C.

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.2 (5.0-5.4)	5.2 (4.6-5.6)	4.2 (4.1-4.3)	4.1 (4.0-4.2)	4.1 (4.0-4.2)
7	6.0 (5.8-6.1)	6.0 (5.8-6.4)	5.2 (5.1-5.3)	4.2 (4.0-4.3)	4.3 (4.1-4.5)
14	8.0 (7.9-8.0)	8.6 (8.3-8.8)	6.8 (6.7-6.8)	6.0 (5.7-6.3)	5.0 (4.9-5.1)
21	9.6 (9.6-9.7)	9.7 (9.6-9.9)	8.1 (8.0-8.2)	8.0 (7.8-8.3)	5.8 (5.5-6.2)
28	9.7 (9.6-9.7)	10.2 (9.1-11.0)	9.4 (9.3-9.5)	8.4 (8.2-8.6)	6.9 (6.9-6.9)
35	10.0 (9.7-10.3)	9.9 (9.9-10.1)	9.2 (9.2-9.3)	7.1 (6.5-7.7)	6.9 (6.7-7.1)

Table IX: Values (as $\log_{10}$/sample) for the spoilage flora at +25°C, for control (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under carbon dioxide at +2°C (range in brackets).

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.8 (5.4-6.1)	5.6 (5.1-6.0)	4.6 (4.6-4.6)	4.6 (4.4-4.8)	4.5 (4.5-4.6)
14	6.2 (6.1-6.5)	5.7 (5.3-6.2)	4.4 (4.4-4.5)	4.2 (4.1-4.3)	4.3 (4.2-4.3)
28	8.2 (8.0-8.4)	8.2 (7.8-8.6)	4.3 (4.2-4.4)	ND	4.5 (4.5-4.5)
42	9.2 (9.1-9.3)	9.2 (8.4-9.3)	4.2 (4.1-4.3)	4.7 (4.6-4.9)	4.4 (4.4-4.4)
56	9.3 (9.2-9.3)	9.3 (9.0-9.4)	4.0 (3.8-4.2)	4.8 (4.7-4.9)	4.3 (4.3-4.3)
70	9.3 (9.2-9.4)	9.3 (9.2-9.4)	3.8 (3.7-3.9)	ND	ND

ND = not done

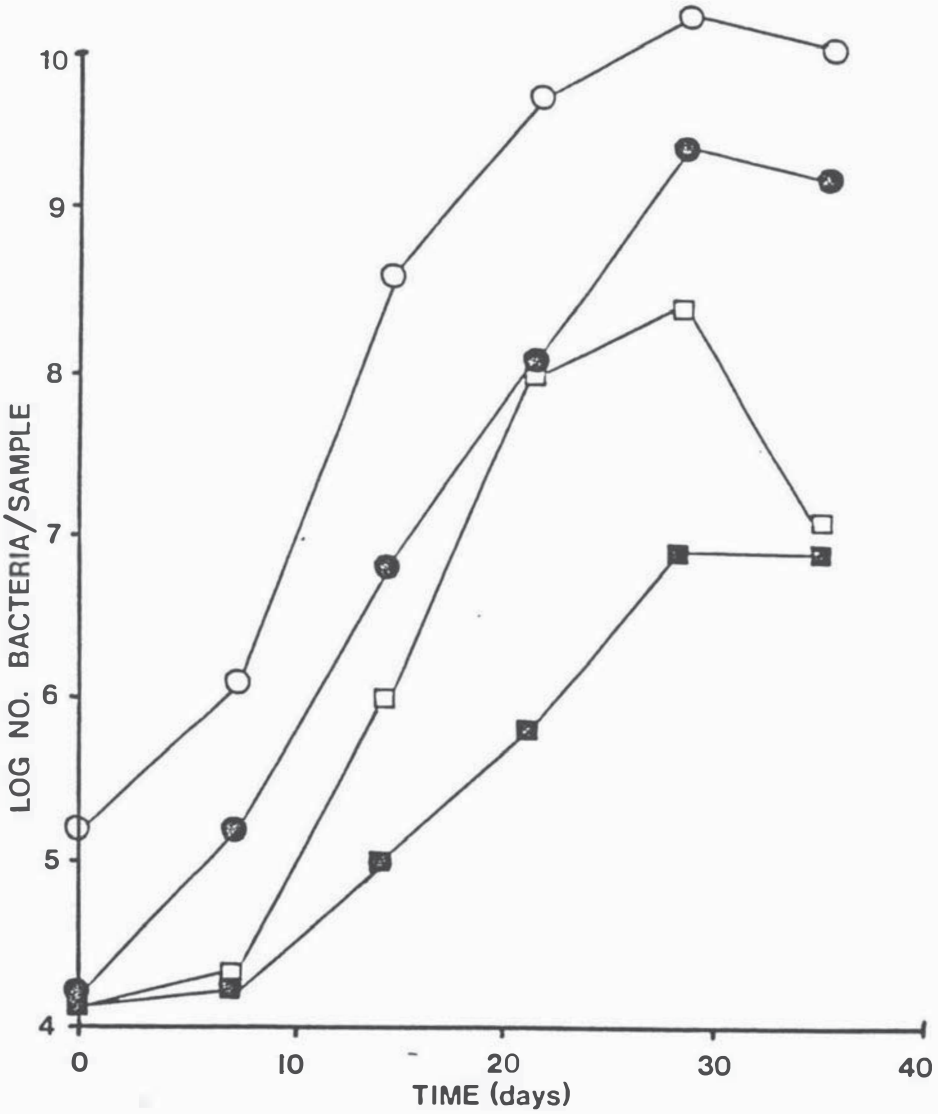


Figure 3: Growth of bacteria in vacuum packs at +2°C. (○) total counts at +25°C, (●)*Y. enterocolitica*, (□) *A. hydrophila*, (■) *L.monocytogenes*.

D. Storage temperature 0°C

1. Vacuum-packaged samples

At 0°C vacuum-packaged samples spoiled after 49 days of storage. After a short lag phase *Yersinia* grew at rates (52 hours/gen.) slower than that of the spoilage flora (44 hours/gen.), with the maximum increase in numbers of 4.7 log cycles. *Aeromonas* grew after a significant lag, and then at growth rates (50 hours/gen.) similar to the spoilage flora, with a maximum increase of 3.9 log cycles. Both test organisms appeared to be inhibited as the spoilage flora attained maximum numbers. *Listeria* grew at a rate much slower (168 hours/gen.) than that of the spoilage flora, after a significant lag, and attained a maximum increase of 1.6 log cycles (Table X and Figure 4, p.50, 51).

CO_2-packaged samples

Samples stored at 0°C under carbon dioxide spoiled after 126 days (growth rate 88 hours/gen.). None of the test organisms grew at this temperature (Table XI, p.50).

Table X: Values (as $\log_{10}$/sample) for the spoilage flora at +25°C, for control (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under vacuum at 0°C (range in brackets).

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.6 (5.3-5.9)	5.7 (5.1-6.1)	4.3 (4.2-4.3)	4.1 (4.1-4.1)	4.1 (4.0-4.1)
14	6.7 (6.6-6.8)	6.7 (6.4-7.1)	5.6 (5.3-5.9)	4.4 (4.2-4.6)	4.3 (4.3-4.4)
28	9.2 (9.1-9.3)	9.0 (8.7-9.6)	7.3 (7.2-7.4)	6.9 (6.9-6.9)	4.8 (4.5-5.2)
35	9.3 (9.1-9.6)	9.6 (9.5-9.6)	ND	ND	5.2 (5.1-5.3)
42	9.4 (9.2-9.6)	9.7 (9.4-9.8)	9.0 (8.9-9.2)	8.0 (7.9-8.2)	5.5 (5.2-5.8)
49	9.6 (9.5-9.7)	9.7 (9.6-9.9)	8.7 (8.5-8.8)	7.8 (7.7-7.9)	5.7 (5.7-5.8)

Table XI: Values (as $\log_{10}$/sample) for the spoilage flora at +25°C, for control (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under carbon dioxide at 0°C (range in brackets).

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.9 (5.8-5.9)	5.6 (5.4-5.9)	4.4 (4.4-4.5)	4.6 (4.6-4.7)	4.1 (4.1-4.1)
14	5.3 (5.1-5.6)	5.6 (5.3-5.8)	4.1 (4.1-4.1)	4.2 (4.1-4.3)	3.8 (3.7-4.0)
28	6.5 (6.5-6.5)	6.4 (6.2-6.6)	4.1 (4.1-4.2)	4.1 (4.0-4.3)	3.6 (3.5-3.7)
42	7.5 (7.3-7.7)	7.9 (7.8-8.0)	3.8 (3.7-3.8)	3.8 (3.6-3.9)	3.8 (3.8-3.8)
70	9.1 (8.7-9.6)	8.6 (8.5-8.7)	3.5 (3.5-3.6)	ND	3.5 (3.4-3.5)
98	9.2 (9.2-9.2)	9.1 (8.9-9.2)	3.5 (3.5-3.5)	3.7 (3.7-3.7)	3.4 (3.4-3.4)
126	9.4 (9.4)	9.3 (9.0-9.5)	3.3 (3.2-3.3)	ND	3.2 (3.2-3.3)
140	9.3 (9.2-9.3)	9.3 (9.3-9.4)	2.8 (2.7-3.0)	3.2 (3.2-3.2)	3.1 (3.1-3.1)

ND = not done

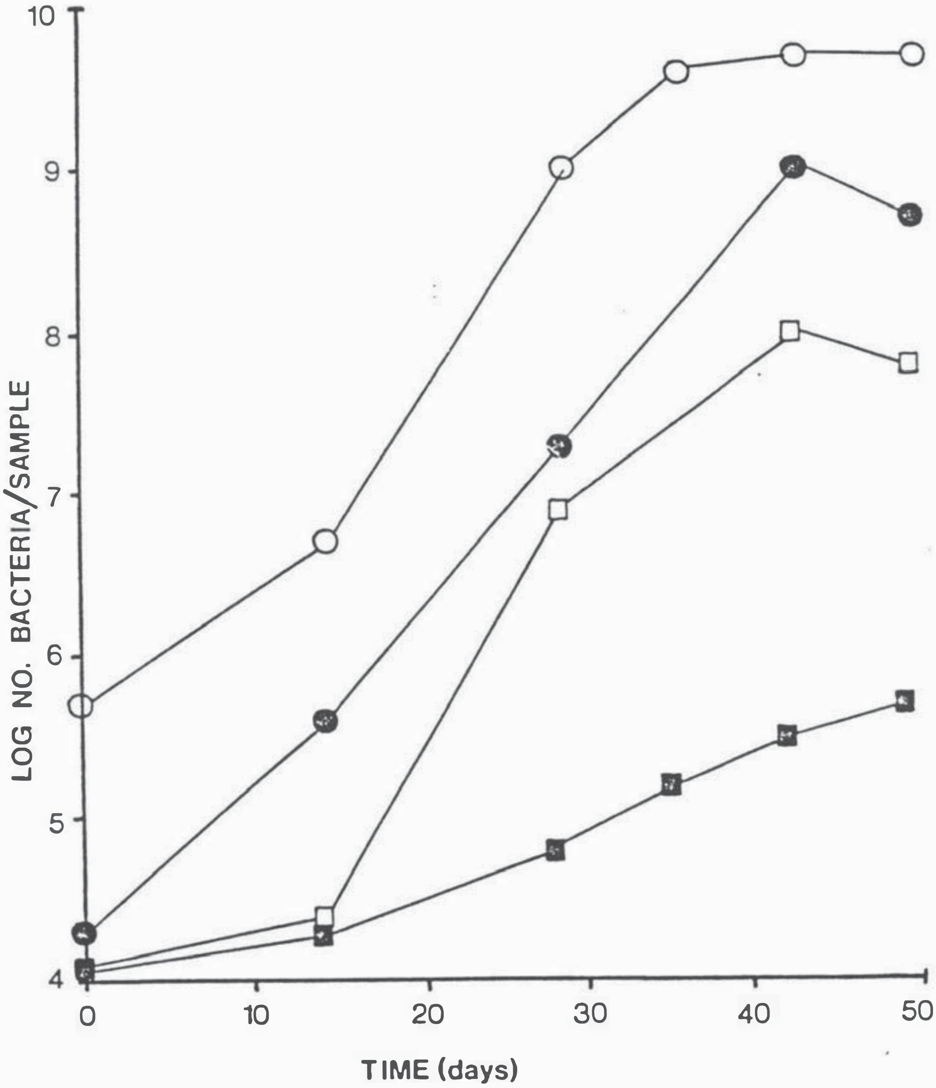


Figure 4: Growth of bacteria in vacuum packs at 0°C. (○) total counts at +25°C, (●)*Y. enterocolitica*, (□) *A. hydrophila*, (■) *L.monocytogenes*.

E. Storage temperature -2°C

1. Vacuum-packaged samples

At -2°C vacuum-packaged samples spoiled after 63 days. *Yersinia* grew after a long lag at a growth rate (84.3 hours/gen.) slower than that of the spoilage flora (59.5 hours/gen.). The maximum increase was limited to 2.6 log cycles. *Aeromonas* grew after an even longer lag at a growth rate of 95 hours per generation and attained a maximum increase of 1.2 log cycles. *Listeria* did not grow at this storage temperature (Table XII and Figure 5, p.53, 54).

2. CO_2-packaged samples

Carbon dioxide stored samples at this temperature spoiled after 182 days of storage, with none of the three test organisms showing signs of growth. The spoilage flora grew at a rate of about 135 hours per generation (Table XIII, p.53).

Table XII: Values (as $\log_{10}$/sample) for the spoilage flora at +25°C, for control (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under vacuum at -2°C (range in brackets).

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.5 (5.4-5.6)	5.3 (5.2-5.5)	4.5 (4.4-4.6)	4.4 (4.2-4.6)	4.4 (4.4-4.5)
14	5.9 (5.8-6.0)	5.9 (5.5-6.3)	4.5 (4.5-4.5)	4.4 (4.4-4.4)	4.4 (4.4-4.4)
28	7.6 (7.4-7.7)	7.6 (7.3-7.9)	4.7 (4.6-4.7)	4.1 (3.9-4.3)	4.3 (4.3-4.3)
242	9.3 (8.9-9.5)	9.3 (8.9-9.5)	6.1 (5.9-6.3)	4.0 (3.7-4.4)	4.6 (3.7-5.6)
56	9.7 (9.5-9.8)	9.7 (9.6-9.9)	7.1 (6.6-7.3)	4.7 (4.7-4.7)	4.2 (4.0-4.4)
63	9.8 (9.6-9.9)	9.8 (9.6-9.9)	6.9 (6.9-6.9)	5.6 (4.8-6.4)	4.2 (3.6-4.8)

Table XIII: Values (as $\log_{10}$/sample) for the spoilage flora at +25°C, for control (mean of two) and inoculated steaks (mean of six), *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* for growth on beef under carbon dioxide at -2°C (range in brackets).

Sample day (days)	Growth of spoilage flora		Growth of pathogens		
	Controls	Steaks	Yersinia	Aeromonas	Listeria
0	5.5 (5.6-5.6)	5.5 (5.4-5.7)	4.3 (4.3-4.3)	4.3 (4.3-4.3)	4.2 (3.9-4.5)
42	8.2 (7.7-8.6)	8.3 (8.1-8.4)	4.0 (3.9-4.1)	4.2 (4.0-4.4)	4.2 (4.2-4.2)
84	9.0 (8.9-9.1)	9.0 (9.0-9.2)	3.7 (3.6-3.8)	3.8 (3.6-3.9)	4.3 (4.1-4.4)
126	9.3 (9.3-9.3)	9.2 (9.0-9.3)	3.7 (3.5-3.9)	3.8 (3.7-3.8)	4.2 (4.1-4.2)
140	9.4 (9.3-9.4)	9.2 (9.2-9.3)	3.5 (3.4-3.6)	3.6 (3.6-3.7)	4.0 (3.8-4.2)
154	9.5 (9.4-9.6)	9.2 (9.1-9.3)	4.3 (3.5-5.0)	3.8 (3.8-3.9)	3.9 (3.9-4.0)
182	9.2 (9.1-9.3)	9.2 (9.2-9.3)	4.2 (4.2-4.2)	3.7 (3.6-3.7)	3.9 (3.9-3.9)

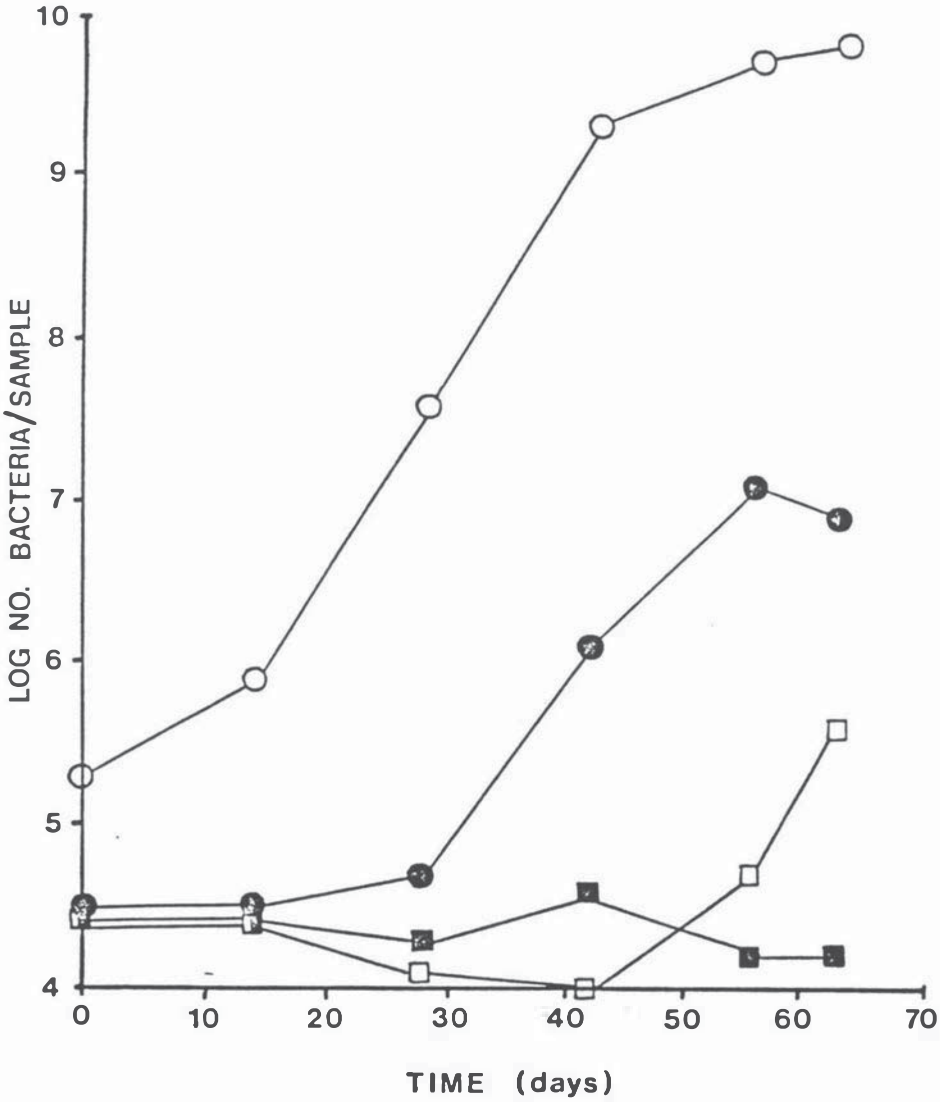


Figure 5: Growth of bacteria in vacuum packs at -2°C. (○) total counts at +25°C, (●) *Y. enterocolitica*, (□) *A. hydrophila*, (■) *L. monocytogenes*

F. Confirmation of pathogen isolates

The isolated colonies, tentatively identified as the test organisms, were confirmed by biochemical tests in 1161 out of 1163 presumptive colonies in the case of *Yersinia enterocolitica*, in 851 out of 877 in the case of *Listeria monocytogenes* and 591 out of 596 for *Aeromonas hydrophila.* The differences in isolate numbers is explained by either insufficient numbers on the counted plates, as numbers declined during storage in trials with no growth of the organisms, or, in particular in the case of presumptive *Aeromonas* colonies with the inadequate suppression of other organisms on the selective plate. This meant that presumptive colonies could only be distinguished after flooding with iodine solution, and therefore, no isolates could be kept.

Table XIV: Summary of generation times (in hours/generation) and time to spoilage (days) for the growth of *Yersinia enterocolitica, Listeria monocytogenes* and *Aeromonas hydrophila* on high ultimate-pH beef at five different temperatures and two different packaging atmospheres.

Storage temperature (°C)	Yersinia	Listeria	Aeromonas	Time to spoilage
Vacuum-pack				
+10	6.9	8.5	5.9	5
+5	16.5	23	18.6	17
+2	35	53.3	26.6	35
0	52	168	50	49
-2	84.3	NG	95	63
Carbon dioxide				
+10	12.4	13.8	10.7	10
+5	56.2	NG	NG	35
+2	NG	NG	NG	70
0	NG	NG	NG	126
-2	NG	NG	NG	182

NG = no growth

III.5 Discussion

It is desirable that a packaging system that extends the storage life of meat not only inhibits the growth of spoilage organisms but also restricts the ability of cold-tolerant pathogens to grow to numbers which are sufficient to pose a risk to human health. At chiller temperatures cold-tolerant pathogens do not grow well on vacuum-packaged normal ultimate-pH meat. However, they grow readily if the pH is at or above a pH of 6.0 (Seelye and Yearbury 1979; Palumbo 1988; Grau 1981; Grau and Vanderlinde 1988). Low ultimate-pH values, which will inhibit the growth of cold-tolerant pathogens in meat, can not always be expected, and in fact some meat, particularly that of animals that have been stressed prior to slaughter, frequently exhibits a high ultimate-pH (Tarrant 1989).

What constitutes the lowest number of bacteria to cause disease in humans is not known for the cold-tolerant pathogens. The infective dose for *Yersinia enterocolitica* necessary to cause disease in a human volunteer has been found to be as high as 3.5×10^9 organisms (Morris and Feeley 1976), in general, however, bacterial numbers sufficient to present an infective dose in the case of some mesophilic pathogens, like *Campylobacter jejuni* can be as low as 5×10^2 (Robinson 1981) and commonly range from 10^5 to 10^6 organisms. In mice the 50% lethal dose requires an infective dose of between 4.5×10^3 to 5×10^9 for infections with *Listeria* *monocytogenes* strains (Baldridge et al. 1988). A recent paper (Low and Donachie 1991), however, reports that even 1 x 10^{10} colony forming units of *Listeria monocytogenes* failed to elicit a clinical response in six-months-old lambs that were dosed with the organism over three days, apart from a transient rise in rectal temperature after 48 hours. The paper does not however report the pre-challenge immune-status of the animals in regards to *Listeria monocytogenes* and thus the high infective dose may be the result of previously acquired immunity.

The uncertainty about the infective dose may be the result of the interaction between the microorganism and the immune-system of the host because all three pathogens are characteristically causing disease in the young or old, and in immuno-compromised individuals (Goodwin et al. 1983; Davis et al. 1978; Marth 1988; Schlech 1988; Palumbo 1986; Swaminathan et al. 1982).

A. Vacuum-packaged samples:

From the results of these experiments it is clear that storage of vacuum-packaged beef at temperatures above 0°C (+2°C for *Listeria monocytogenes*) does allow, potentially, for an increase in the numbers of any of the three pathogens of three or more logarithmic units. This must be considered to be potentially hazardous to consumers, because it would allow just one initial microorganism to proliferate to what could constitute an infective dose. Only the storage at sub-zero temperatures can be considered to be providing a relatively safe packaging environment which precludes the increase of these cold-tolerant pathogens to a level constituting a public health risk. In particular *Yersinia enterocolitica* does show persistently strong growth at all temperatures above 0°C, with high total increases. The highest potential growth however is shown by *Aeromonas hydrophila* at +10°C, with a growth rate that exceeds that of the spoilage flora. Towards the end of the storage life the spoilage flora is dominated by organisms belonging to the species *Aeromonas*.

B. CO_2-packaged samples:

Growth of the three tested pathogens under carbon dioxide may be considered hazardous at temperatures of +10°C, as the total increases still exceed three log units, though the potential health risk to the consumer is much less than that associated with vacuum-packaging. At +5°C the growth of *Yersinia enterocolitica* presents a reduced risk, because of the induced prolongation of the lag phase and the reduced increases in total numbers. The total inhibition of the growth of *Aeromonas hydrophila* and *Listeria monocytogenes* reduces the risks involved with prolonged cold storage under carbon dioxide as compared with vacuum-packaging. At temperatures at and below +2°C none of these pathogens grew. As meat must be held at temperatures of 0°C or below for prolonged chilled storage in order to achieve the intended extended storage life (Gill et al. 1988), CO_2-packaging used in this manner is unlikely to contribute to any rise in the number of food-borne disease outbreaks attributable to the presence of cold-tolerant pathogens.

The effects of the spoilage flora on the growth of the three pathogens appear to be limited to the moment when the spoilage flora attains maximum numbers shortly before spoilage ensues, as in the case of vacuum-packaged samples, or in the case of the carbon dioxide packaged steaks at a much earlier time. As storage temperatures decrease the inhibitory effect of the general spoilage flora becomes more noticeable. At storage temperatures of +10°C under vacuum no inhibitory effect can be observed, but at lower storage temperatures inhibition caused by the spoilage flora attaining maximum numbers becomes more pronounced.

As temperatures in display cabinets in supermarkets often fluctuate (Rose 1986) and probably rise to and above the higher temperature values which were evaluated in this experiment, high increases in the numbers of pathogenic microorganisms may be expected even at the end of an apparently well controlled long-term chilled storage. Total increases after resolution of the lag phase may well exceed the more than one log unit rise per day at +10°C measured in these trials, especially if temperatures rise to much higher than +10°C. When the growth rates of these pathogenic microorganisms exceed the growth rates of the spoilage flora, as already indicated in the case of *Aeromonas* at +10°C, then, as a result of even slight temperature abuse large increases in numbers are possible before spoilage ensues. Thus potentially hazardously high numbers of pathogens may have accumulated before the development of organoleptic changes, such as spoilage odours or flavours, or the formation of slime and discolouration give an indication of the temperature abuse which the meat has experienced. During such chilled storage of meat the risk to the consumer may have increased many-fold.

The effects of CO_2-packaging are said to be reliant on the inhibitory effect of the gaseous portion of CO_2 in the meat pack (Gill and Penney 1988). Measurements of the muscle-pH in the packs that were opened after chilled storage showed a consistently lower pH than at the time when the pack was inoculated and sealed. The lowering of the pH is likely to be a result of the formation of carbonic acid, which in its undissociated form is toxic to microorganisms (Renerre 1989). The lower pH in conjunction with the inhibitory effects of the gaseous phase leads to a prolongation of the lag phase and a reduction in the microbial growth rates which is assumed to be the cause of the extended storage life achieved by CO_2-packaging.

The generation times for the spoilage flora and three pathogens are necessarily a crude approximation, because of the inaccuracies of bacteriological plating techniques (Richardson et al. 1978, Sharpe 1980) and the low number of sample steaks that were examined to obtain the values for each sampling day. They do, however, indicate that the effects of carbon dioxide packaging are a reduction of the growth rates of spoilage bacteria and pathogens to about half the rates of their growth under vacuum, in addition to the effects on lag phase extension and inhibition of the enterobacteriaceae which have been previously described (Gill and Penney 1988). They are also in general agreement with growth rates obtained by other authors (Ryser and Marth 1988), who have been recording a generation time for *L.monocytogenes* at +6°C in whey of between 14 to 21.1 hours compared to the 23 hours recorded in this experiment at +5°C. On meat Grau and Vanderlinde (1988) report growth rates on the fatty tissue of beef at +5.3°C of around 27.5 hours per generation, while their growth at 0°C on high ultimate-pH beef with 168 hours per generation for the meat surface is in agreement with the results presented here. Walker et al. (1990) report generation times of 13-24 hours at +5°C, and 62-131 h at 0°C.

Kaya and Schmidt (1990) describe the growth of *Listeria monocytogenes* at a variety of temperatures and pH-values of meat under vacuum. Their measured growth at combinations comparable to the ones used in this experiment are in general agreement with the results presented here. At temperatures of +10°C inoculum levels increased by about 2.7 log units in three days in their experiments compared to only 1.2 in this experiment. At +4°C they observed growth of *Listeria* of about

1.2 log units per week initially increasing to a maximum of 2 units per week. In this experiment at storage temperatures of +5°C the maximum growth rate for *Listeria monocytogenes* was 2.2 log units per week. At +2°C their measured growth was about one log unit per week, which compares with the 0.8 and 0.9 log units found in this experiment after 14 days of storage.

Growth of *Y. enterocolitica* under CO_2 atmosphere in a simulated milk medium at +7°C is reported to result in growth rates varying from 18.1 to 55.6 hours per generation (Rowe 1988), which is somewhat slower than the growth rates recorded for *Y. enterocolitica* in this experiment. *A. hydrophila* can have a generation time of 12.4 h at +4°C (Palumbo et al. 1985a), but some strains have also been reported to grow slower at 48.3 h per generation (Kirow et al. 1990). Other storage trials with *Aeromonas hydrophila* by Palumbo and co-workers (1985b) suggest generation times of 26.9 to 60.5 hours on beef held at +5°C for seven days. However the pH of the sample was not recorded. On lamb and pork, which could be expected to show higher pH-values than beef, generation times of 8.9 to 14.4 hours at the same temperature are recorded, which is more in line with the results of this experiment.

The confirmation rate for the tested pathogens exceeded 97% for all of the recovered isolates. It can therefore be assumed with a reasonable degree of confidence that the counts (and calculated increases for the three tested pathogens) are indeed reflecting increases of the three test organisms and not that of other contaminating colonies, which may also have grown on the selective plates. This is particularly important in the case of *Aeromonas hydrophila* where presumptive colonies could be distinguished and counted on the selective agar plates, but not isolated, because of heavy growth of contaminants on the selective agar.

A comparison of vacuum-packaging and CO_2-packaging shows that potentially hazardous increases in bacterial numbers for all of the three pathogens occur with vacuum-packaging at all storage temperatures above 0°C (+2°C for *Listeria monocytogenes*). Potentially hazardous increases occur in CO_2-packaging only at temperatures of +10°C.

CO_2-packaging compared to traditional vacuum-packaging results in greater gains of additional storage life at lower temperatures. As the longest possible storage life in chilled storage is of advantage to the processor, commercial practice for CO_2-packaging can be expected to result in storage at sub-zero temperatures. At these temperatures CO_2-packaged meat does not show an increased risk of the proliferation of cold-tolerant pathogens compared with vacuum-packaged meat although the meat is stored at these temperatures for much longer than in the vacuum-packs. Food-borne disease outbreaks resulting from extended low-temperature storage are therefore less likely to occur with carbon dioxide packaged meat.

Compared to vacuum-packaging, there is a wider range of temperatures at which the psychrotrophic microorganisms are unable to grow. The minimum growth temperature for all three tested microorganisms is increased and total possible growth decreased. Extended chilled storage under CO_2 can therefore be considered to offer an additional safety margin over the more traditional method of vacuum-packaging.

PART 2:

IV. Temperature Function Integration

IV.1 Introduction

As the purpose of hygienic measures is to restrain microbial proliferation, and in particular the growth of pathogens, hygiene regulations stipulate cooling regimes which should restrict bacterial numbers to within acceptable limits. This has traditionally been achieved by specifying temperatures to below which meat had to be cooled and then stored. Traditionally +7°C has been used as the reference temperature, because it is generally accepted that growth of mesophilic organisms, *Salmonella spp.* and coliforms in particular, ceases below this temperature (Smith 1985). In some instances a time limit for the reduction of the meat temperature to this level is specified (anon. 1989). However the failure to prescribe continuous time-temperature parameters detailing the shape of the cooling curve makes such specifications an inadequate tool both for overall process control as well as for the estimation of the potential increase of pathogenic bacteria during the cooling regime.

The calculation of bacterial growth from the temperature history of a product and relating it to bacterial growth rates is relatively simple (Olley and Ratkowsky 1973). In the past these calculations have been primarily used for the prediction of storage life of meat and other foods, and the possibility of accurately predicting bacterial growth on meat has been demonstrated (Pooni and Mead 1984). Such an approach is known as temperature function integration (TFI) and various growth models are in use for different purposes, including, but not limited to the control of hygienic processing (Roberts and Jarvis 1983; Zwietering et al. 1991; McMeekin et al. 1988).

For the purposes of control of the hygienic adequacy of a process, it is necessary to identify the point at which the temperature history of a product is monitored. For regulatory purposes the point which would present the worst hygienic outcome, i.e. the highest potential for bacterial growth, should be the one monitored. By definition then, if the bacterial proliferation at that point is deemed to be acceptable, then all other product histories should be acceptable as well (Gill et al. 1988). The region that should be monitored, therefore, should be one that consistently provides the highest temperature and is likely to be contaminated with mesophilic pathogens, and thus presents the highest potential for bacterial growth throughout the cooling process. Traditionally, in regulatory work, sites deep within the bulk of the carcass mass have been used as such a reference point, but because the deeper tissues of healthy carcasses are generally sterile (Gill 1979), they bear no relevance to the temperature function integration (TFI) approach. In contrast, most surface tissues of carcasses will become contaminated with bacteria through the process of slaughter and dressing (Grau 1987) and it is therefore appropriate to measure the temperature history of a meat surface for this approach.

It follows that for process hygiene assurance purposes the relevant region to be monitored is that part of the carcass surface which cools the slowest and therefore provides conditions suitable for the greatest bacterial growth. For whole carcasses this area has been identified by Scott and Vickery (1939) as being the surface of the caudal part of the *M. psoas minor/major* and, for cartoned, bulk-packed product should be a meat tissue surface close to or at the geometric centre of the carton.

To apply TFI efficiently in process assurance work, equipment that is able to conveniently record the temperature history of a process is needed. Once collected these temperature data need to be able to be read by a computer, and applied in a model for bacterial growth that mimics the growth of mesophilic pathogens. Such equipment and bacterial growth model is now available (Gill et al. 1988; Pham 1988) and the description of meat processes by the means of a TFI approach is now possible.

Commercial hot-boning of beef is a relatively new meat process in New Zealand and regulations governing the hygienic aspects of this type of production have only recently been introduced (anon. 1989). Although the microbiological aspects of hot-boning have been described in the past, and hot-boned beef has been found by some authors to be of a higher microbiological quality than traditionally boned meat (Gilbert and Davey 1976, Gilbert et al. 1976, Emswiler and Kotula 1979) but of a lower quality by others (Kennedy et al. 1982), theoretical considerations suggest a greater potential for bacterial growth in hot-boned than in traditionally cold-boned meat (van Laack and Smulders 1989, Lee et al. 1985).

IV. 1.1 Aims of the work

It was felt that the possibility of calculating the potential for bacterial growth during a meat process as is afforded by the TFI approach ,without the often laborious bacteriological sampling regimes, should be evaluated in a commercial beef hot-boning operation in a New Zealand meat plant. This would allow for, in the first instance, the description of a currently accepted meat process in terms of its bacterial growth potential, and secondly, permit the comparison of the hot-boning process (and the potential for bacterial growth it presented) with a traditional cold-boning operation which had previously been described using a TFI approach. From that survey provisional criteria for permissible bacterial growth had been stipulated (Gill et al. 1991). In comparison with the results of the TFI study of the hot-boning operation it was hoped that more wide-ranging criteria, encompassing a greater range of meat processes may be developed. As TFI relies on the collection of objective temperature data, which are related to the growth of indicator organisms, TFI could present an objective tool for process assurance and a valuable aid to traditional regulatory thinking.

IV.2. MATERIALS AND METHODS

IV.2.1 The commercial Hot Boning Process

The meat production system at the Lowe Walker NZ Ltd plant at Paeroa was used to investigate the effect of temperature on the growth of *Escherichia coli*. This plant is designed for the processing of hot boned beef only and stock is processed on a single slaughter line at the rate of 30 to 40 carcasses per hour.

The period of time from the slaughter of an animal until it enters the boning room is approximately 30 minutes at the lower production speed and 20 minutes at the higher one. On entry to the boning room, which is maintained at an air temperature of +10°C, the carcass sides are quartered and the quarters stacked up prior to pre-trimming. The quarters may reside in the stacking area for 5 to 10 minutes, but at tea breaks for up to 30 minutes.

Boning of the quarters commences immediately after pre- trim and most of the meat is quickly packed into standard 27.2 kg cartons for freezing. These cartons have dimensions of 520 x 340 x 175 mm (length x width x height) and are composed of 2 mm thick corrugated cardboard with a polyethylene liner (Figure 6, p.72).

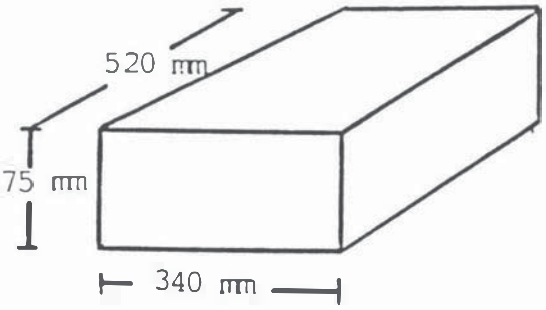


Figure 6: Dimensions of a carton of hot-boned, bulk-packed beef in the commercial meat process at Paeroa.

The filled cartons are passed to the scales, where the weight is adjusted and the cartons sealed with adhesive tape. The cartons are then transferred from the boning room into an assembly area where they are placed onto metal stillages. There are 6 layers to each stillage carrying 6 cartons each. The cartons rest on metal bars which maintain a 5 cm air gap between all sides of one carton and the next one (Figure 7, p.74). Filling of a rack usually takes 8 to 10 minutes when heavier sides are processed and 15 minutes when cull cows are processed. Once a rack is filled it is passed by forklift into one of four blast freezer chambers which each hold 25 racks in 2 rows of 5 racks length, each 2.5 racks high. The half rack arrangement becomes possible through some racks being horizontally divisible into half racks of 3 carton layers each. The time between entry of the quarters into the boning room and entry of the filled and sealed cartons into the blast freezer does not exceed one hour.

In the blast freezers cold air is blown horizontally along the length of the freezing chamber passing through the racks, between the layers of cartons and the cartons within each layer (Figure 8, p.75). The air is cooled down by the refrigeration plant to an off-coil temperature of -30°C and a return air temperature of -20°C. It takes about 4 to 5 hours to fill a freezer chamber and 48 hours to freeze all meat within a chamber to a deep core temperature of -20°C.

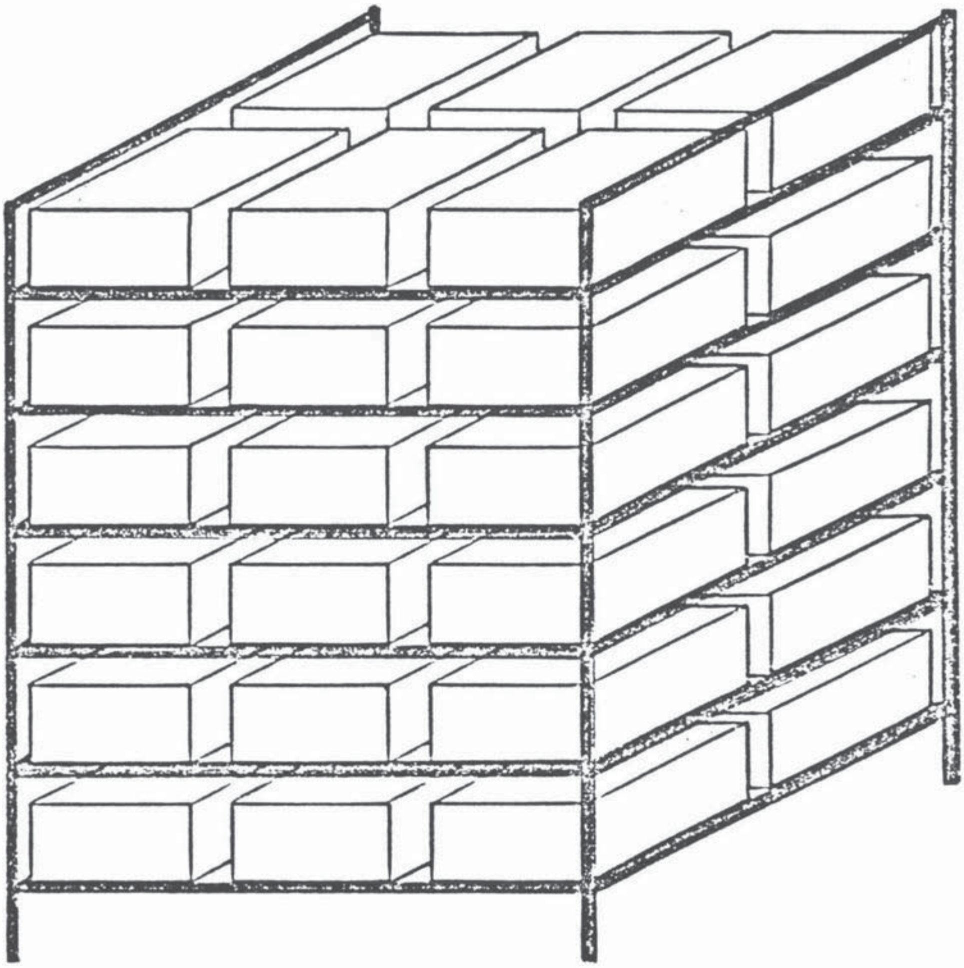


Figure 7: Positioning of cartons of hot-boned, bulk-packed beef on metal stillages for freezing in the commercial meat process in Paeroa

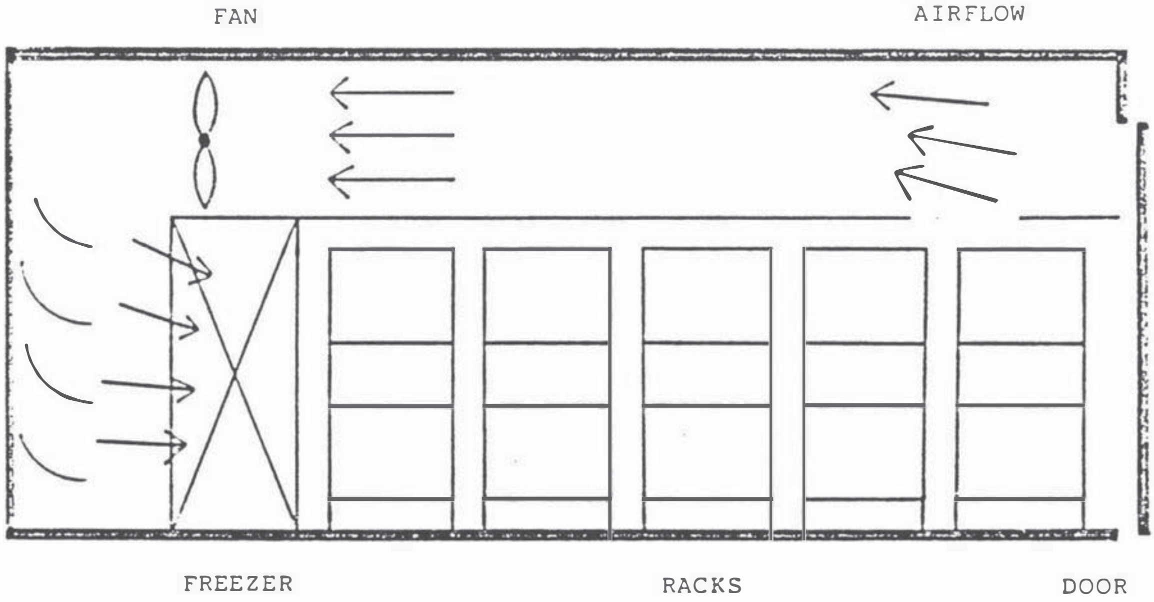


Figure 8: Positions of metal stillages, cooling unit and fan in a freezing chamber in the commercial hot-boning process at Paeroa.

IV.2.2 Collection of the Product Temperature Histories

The temperature histories of the product were collected using MIRINZ-Delphi temperature data loggers (Tru-Test, Auckland, New Zealand), each fitted with an external thermistor probe encased in a tapered teflon sheath. The loggers were set to record temperatures, at intervals of 1.875 minutes, between + 40°C and - 20°C with an accuracy of ± 0.25°C and a resolution of 0.25°C. For recording the surface temperatures of carcasses a disc of stainless steel, 40 mm in diameter was held against the carcass surface by means of a staple passed through holes in the disc. The probe was inserted into a cone- shaped slot running across the disc such that the probe fitted tightly to the inner surfaces of the slot. The logger was attached to the carcass surface by means of a metal skewer passed through a slot in the casing. For recording cartoned product temperature histories, the probe was placed with the product just before the carton was closed and sealed, with the tip next to the region to be monitored and the logger placed between the liner and the carton wall.

IV.2.3 Integration of Temperature History Data to calculate bacterial growth

Models for the growth rate and lag phase of *Escherichia coli* were developed from data for the cultivation of wild-type strains in half- strength Brain Heart Infusion Broth (Gill et al. 1988). The model for aerobic growth has the form:

$y = (0.0513\,x - 0.17)^2$, for $7 < x < 30$;

$y = (0.027\,x + 0.55)$, for $30 < x < 40$;

$y = 2.66$, for $40 < x < 47$;

$y = 0$, for $7 > x$ or $x > 47$;

where x is the temperature in degrees Celsius and y the microbial growth rate expressed as generations per hour. The model is an extension of that used by Lowry et al. (1989) for the estimation of *E.coli* proliferation during thawing of meat.

The model for the lag phase induced in *E. coli* by a shift from aerobic to anaerobic growth conditions has the form of a linear interpolation between values for factors which converts the period during the lag phase at a given temperature to the equivalent fraction of a lag phase at a temperature where the lag is 11 hours (approx. 8°C). The equivalent factor (ELF) has the value:

ELF = Standard lag [11 (h)] / observed lag (at x °C) (h).

The standard lag is thus reduced by an equivalent amount, depending on the temperature in the carton, for every recording interval after the probe is first placed within the carton, until the lag is totally resolved.

The data set used is (Gill and de Lacy, unpublished data):

Temperature (°C)	Equivalent lag factor
<7	0
7	0.95
12	3.2
15	6.6
17	12.8
19	17.6
21	28.8
22	36.8
23	47
24	60
25	76.4
>25	∞

The model for anaerobic growth has the form:

$y = (0.0433\ x - 0.15)^2$, when $7 < x < 30.5$;

$y = (0.0163\ x + 0.676)^2$, when $30.5 < x < 40$;

$y = 1.77$, when $40 < x < 45$;

$y = 0$, when $7 > x$ or $x > 45$;

where x is the temperature in degrees Celsius and y the microbial growth rate in generations per hour. The model is that used by Gill and Harrison (1985) for the evaluation of the hygienic efficiency of offal cooling procedures.

These models were applied in a computer programme, run on an IBM compatible personal computer, that requires that a defined process is specified for each logger before temperature monitoring commences. The process must commence with the recording of carcass or side surface temperatures and the probe be placed within 10 minutes of the carcass leaving the slaughter line. To ensure this, the program does not run if the initial temperature of the process, usually a carcass surface, is below 25°C. If the carcass is then cooled to below 7°C before it is further processed , the process consists of a single phase. If the carcass is further processed while temperatures are still above 7°C, the probe must be removed from the carcass, so that further processing, i.e. boning or packaging may occur and then again placed with the product produced from that carcass within 20 minutes. If packaging occurs bacterial growth may then be following an anaerobic growth model and this part of the process is treated as a separate phase.

When interrogating the logger, the programme requests the times that temperature recording began and ended for the phases of any process. For two-phase processes only, the times of probe removal from the carcass and replacement with the product are requested. For purposes of calculation, the product temperature during that period is assumed to be the higher of the temperatures, at either start or end of that interval.

Any initial lag in microbial growth on the carcass is assumed to be resolved before temperature monitoring starts.

For one-phase processes, growth is calculated from the aerobic growth model during each interval of recording, and the total growth is derived by the summation of the calculated increments.

For two-phase processes, calculation of growth from the start of the record to placement of the probe with the cartoned product proceeds as for the one- phase process. After that, the programme calculates from the lag- phase model the portion of the standard lag time that would be resolved during each record interval and subtracts that from the standard lag time. After resolution of lag, further growth is calculated from the model for anaerobic growth. Total growth is derived from the summation of aerobic and anaerobic growth.

No process, as described by its temperature history, is complete until the product temperature falls below +7°C, because only then will the programme run.

IV.2.4 Proliferation profiles of cooling cartons

Temperature histories were obtained at five points within each of 15 cartons of meat that were closed before being placed in the blast freezer, and of a further 15 cartons that were not closed. The cartons in this series were always placed in the same location on a rack at the rear of one freezer chamber.

Temperature probes were placed with the product as the cartons were filled and the tips placed at five sites along the vertical axis of each carton: at the bottom, at distances of 40, 85 (geometric centre) and 130 mm from the bottom, and at the top (Figure 9, p.82).

The cartons were removed from the freezer after a period of time that was sufficient to allow the temperatures at all five locations monitored within a carton to fall below +7°C.

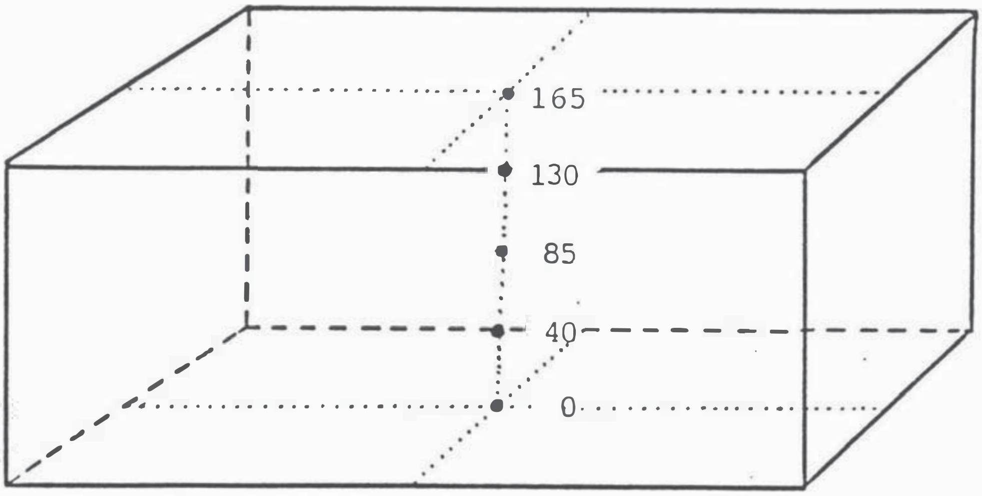


Figure 9: Placement of five temperature logger tips along the vertical axis through the geometric centre of a carton of hot-boned, bulk-packed beef.

IV.2.5 Evaluation of the Hot Boning Process

Temperature histories were collected for product from 50 beef sides, in each of two surveys of the plant.

A logger was placed with the side that was to be monitored within 10 minutes of evisceration, the probe being placed to record the temperature from the consistently warmest region of the surface of the carcass, i.e. the caudal surface of the *Mm. psoas minor/major* complex (Scott and Vickery 1939; Gill et al. 1991). When boning was about to begin, the logger was removed. Later the logger was placed in a carton as it was being filled with meat from the side being monitored, with the tip lying at approximately the thermal centre of the carton (This was earlier determined as described (IV.2.4.) and would record the temperature history at the consistently warmest position, allowing the highest potential for *E.coli* proliferation, within a carton of hot-boned beef). The time between removal of the probe from the carcass and placement with the boned product did not exceed 20 minutes. Loggers were recovered from the cartons on the day after they had been placed with the product.

Within one series, ten loggers were placed with product on each of 5 days. The placements were staggered over the processing day, with approximately equal intervals between placements.

Monitored cartons were placed on racks and the position of the carton and the rack in the blast freezer recorded.

IV.2.6 Verification of the bacterial growth model

IV.2.6.1. Preparation of *E.coli* inocula

A wild-type strain of *E.coli* (Lowry et al. 1989) was cultivated overnight at 30°C in a Minimal Salt Medium (Gill and Phillips 1985; Appendix, p.142) with glucose as the sole carbon source in growth limiting concentrations (0.1 g/l). The numbers of cells per millilitre of the stationary phase culture obtained, were thus determined by the initial concentration of glucose in the medium. Samples of the culture were mixed with half- strength Brain Heart Infusion (BHI) Broth (Appendix, p.137) containing 2.5% agar that was held at 45°C, to give cell suspensions containing approximately 10^4 *E.coli*/ml. The agar suspension was poured into a glass mould 1 mm deep, and a glass plate placed over the mould to expel excess air. The mould was then placed in a refrigerator. After the agar had gelled and cooled to +4°C it was cut into 2 cm x 2 cm squares.

The agar squares were held at a temperature of between 0 and +4°C before being placed with the product within 48 hours of preparation of the agar suspension.

E. coli populations of agar squares were enumerated by homogenizing each square with 3.6 ml of 0.9% saline. This was achieved by repeatedly forcing the sample and the saline between two 10 ml glass syringes connected by a double ended Luer-lok cannula. Samples of 0.1 ml of undiluted or suitably diluted homogenate were incorporated into duplicate pour plates of Violet Red Bile (VRB) Agar (Appendix, p.144). The plates were incubated for 24 hours at +37°C. Crimson coloured round colonies, surrounded by a zone of precipitated bile, were counted on plates containing from 20 to 200 colonies.

IV.2.6.2 Comparison of calculated and observed growth of *E.coli*

Striploins of high ultimate-pH, from beef carcasses chilled before boning were obtained, vacuum-packaged and stored at -35°C until used. When required, each striploin was allowed to warm to ambient temperatures before it was cut into 1.5 cm thick steaks. The temperature probe was placed on the surface of each of 10 steaks and an agar square inoculated with *E.coli* was placed on each steak adjacent to the tip of the logger probe. At that time, the *E.coli* numbers in each of ten of the remaining agar squares were determined, as described above.

After holding each inoculated square at ambient temperatures for approximately one hour, a second steak was placed on top of the first, and the inoculated unit wrapped in cling film (Gladwrap, Borden, Auckland, New Zealand).

The wrapped and inoculated steaks were placed in cartons of hot- boned meat as the cartons were being filled. Five units were dispersed within each carton which was then passed through to the freezer, along with the normal production.

The inoculated steaks were recovered from the cartons after 16 to 20 hours. They were held at 0°C while they were being transferred to the laboratory, where the agar squares were removed and the *E.coli* numbers in each square determined, as described above, and the loggers interrogated.

For each cling-film wrapped unit, the times were recorded when the steaks were inoculated with an agar square; when the second steak was placed over the first, and when the cling-film wrapped unit was placed within the carton. The logger was then interrogated as for a two-phase process.

IV.3. RESULTS

IV.3.1 Plant Survey

In the survey of 50 temperature histories the mean time for the meat temperatures to fall to +7°C, after dressing had been completed, was 13.3 hours (h), with times ranging from a minimum of 9 h to a maximum of 19 h. The maximum temperature was measured shortly after the meat was placed into the cartons, with the mean temperature reaching +33.5°C, and a range from 27 to 39°C (Table XV, p.88/89).

The mean calculated *E.coli* proliferation during the monitored process was 9.3 generations, with 38% of the calculated proliferations equal to and exceeding 10 generations and 2% exceeding 14 generations (Table XV and Figure 10, p.88/89, 90). The mean values for the four different freezer chambers were similar, being 9.5, 8.8, 9.0 and 9.8 generations for chambers one, two, three and four respectively.

The mean values for the five different rows throughout the blast freezers were 8.9 generations for row one, the one closest to the refrigeration unit, 9.1 for row two, 8.4 for row position three and 10.2 generations for both rows four and five. Row position number five was the row closest to the door of each blast freezer chamber (Figure 11 a, p.94).

Tab. XV: 50 *E.coli* proliferation values, maximum temperatures and time taken to reach +7°C, obtained in the first survey of a commercial beef hot-boning process

Chamber	Row	Time to +7°C (h)	Maximum temp(°C)	Growth total	Growth 6 hours
1	4	16.0	34.0	12.1	8.3
1	1	15.2	36.0	11.4	8.2
1	3	10.9	33.0	7.1	6.1
1	4	11.0	32.0	7.4	6.0
1	5	15.2	33.0	10.8	7.7
1	1	12.7	30.0	6.9	5.3
1	5	16.8	38.0	14.9	9.7
1	5	12.0	33.0	9.7	7.3
1	1	14.2	35.0	11.4	8.5
1	2	11.0	27.0	6.8	5.1
1	4	17.0	38.0	10.5	8.0
1	5	14.1	30.0	8.2	6.1
1	2	14.0	33.0	8.6	6.5
2	5	10.0	32.0	6.7	5.7
2	2	14.2	34.0	9.3	7.2
2	1	13.1	33.0	8.0	6.5
2	3	14.0	28.0	7.1	5.3
2	2	12.1	35.0	9.5	7.2
2	2	13.1	32.0	9.1	7.0
2	4	13.0	33.0	10.4	7.7
2	1	10.7	32.0	7.2	5.9
2	4	12.0	34.0	9.0	6.7
2	5	16.2	32.0	11.9	8.0
2	4	17.0	38.0	12.0	8.8
2	5	17.2	34.0	11.5	8.2
2	5	16.0	33.0	9.5	7.2

Chamber	Row	Time to +7°C (h)	Maximum temp(°C)	Growth total	Growth 6 hours
2	5	15.0	35.0	11.2	7.9
2	1	9.0	31.0	3.9	3.2
2	1	11.0	28.0	4.7	3.6
3	2	12.8	35.0	9.7	7.4
3	1	12.0	30.0	7.8	6.0
3	1	14.1	37.0	10.0	7.2
3	2	13.9	33.0	10.7	7.8
3	3	12.8	33.0	8.6	6.6
3	1	11.7	32.0	7.9	6.2
3	4	12.0	29.0	6.9	5.3
4	1	12.0	37.0	10.4	8.3
4	2	12.2	39.0	6.9	5.7
4	2	15.0	36.0	12.2	8.8
4	5	13.7	37.0	10.8	8.2
4	3	14.0	36.0	10.4	7.8
4	2	12.0	32.0	8.3	6.5
4	4	14.9	36.0	10.3	7.5
4	3	19.0	31.0	10.6	7.7
4	2	12.8	32.0	9.2	7.1
4	5	13.8	32.0	9.4	7.0
NR	NR	11.9	33.0	9.6	7.4
NR	NR	10.1	31.0	8.5	6.4
NR	NR	12.0	33.0	9.2	7.1
NR	NR	10.1	33.0	8.5	6.9
Mean:		13.3	33.5	9.3	7.0

NR: Not recorded

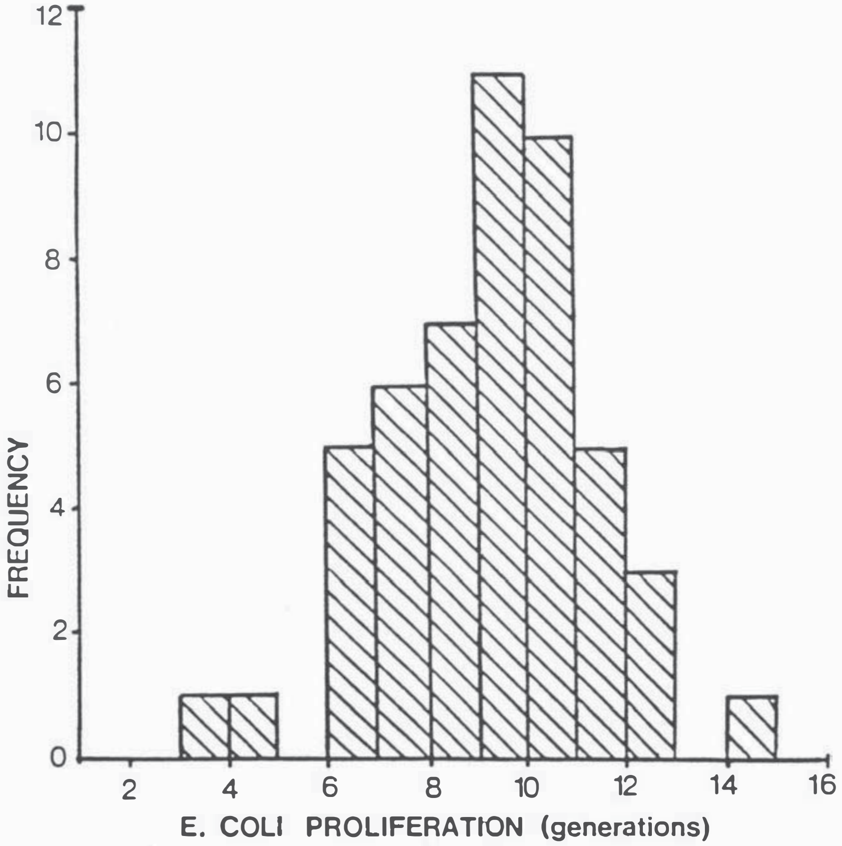


Figure 10: Frequency distribution of calculated *E.coli* proliferation values in 50 bulk-packed cartons during the initial assessment of the commercial hot-boning process.

In a second survey of 50 temperature histories in the same plant, after the refrigeration plant had been altered, through the addition of another compressor, the maximum temperatures for the product were similar to the ones previously recorded. The mean time to reach +7°C, however, was reduced from 13.3 h to 12.2 h, with times ranging from 9 to 16.5 h. The mean maximum temperature measured shortly after the probe had been placed with the meat was +31°C, ranging from 27 to 36°C (Table XVI, p.92/93).

The mean calculated *E.coli* proliferation during the cooling phase was reduced to 7.1 generations, with only 2% of the calculated proliferations now exceeding 10 generations (Table XVI and Figure 12,p. 92/93, 95).

The mean values for *E.coli* proliferation in the second survey was 6.8 generations for blast chamber one, 7.4, 7.2 and 6.9 for blast freezers two, three and four respectively. In respect to the different possible row positions of the cartons, the second survey showed mean values for row one, the closest to the blast unit, of 6.5 generations. The mean values for rows two to five were 6.7, 7.4, 8.1 and 6.5 respectively (Figure 11 b, p. 94).

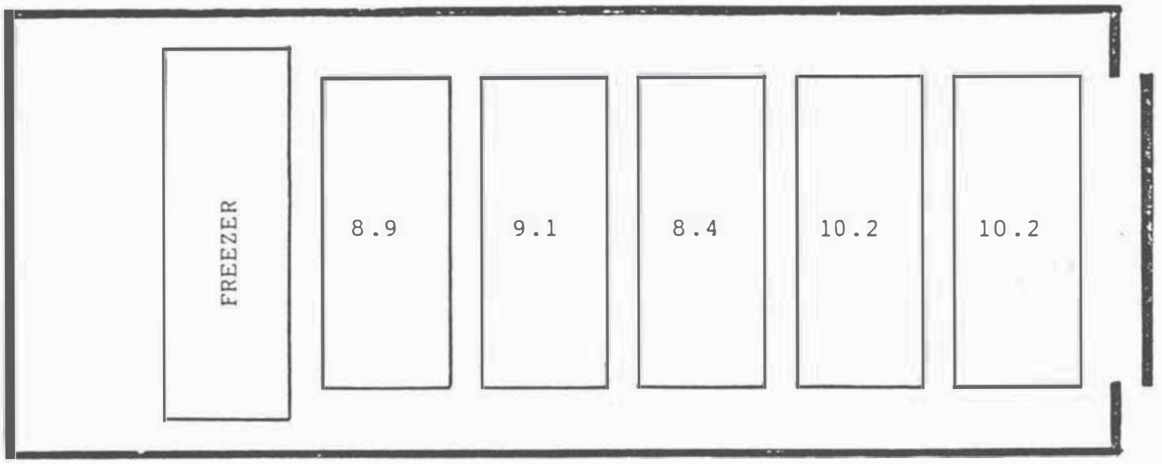
The individual traces show that overall 75% of the potential microbial proliferation was achieved during the first six hours of the cooling phase for the first survey and 76% in

Tab. XVI: 50 *E.coli* proliferation values, maximum temperatures and time taken to reach +7°C, obtained in the upgraded commercial beef hot-boning process

Chamber	Row	Time to +7°C (h)	Maximum temp(°C)	Growth Total	Growth 6 hours
1	3	09.0	32.0	9.7	5.8
1	2	11.8	32.0	8.0	6.4
1	1	12.2	35.0	8.2	6.4
1	5	11.0	27.0	5.0	3.9
1	2	10.7	30.0	6.6	5.4
1	5	14.9	28.0	8.5	5.6
1	3	10.0	30.0	4.7	4.1
1	2	12.0	27.5	5.4	4.2
1	1	10.0	30.0	4.2	3.8
1	3	12.0	31.0	5.4	4.3
1	2	12.5	30.0	7.7	5.9
1	3	14.2	31.0	8.6	6.3
1	4	13.7	30.0	8.0	5.8
1	3	10.5	29.0	6.1	5.1
1	1	10.8	31.0	6.4	5.1
1	3	12.0	29.5	6.8	5.6
1	3	12.1	31.5	6.1	4.7
2	3	14.2	35.0	10.6	8.0
2	1	13.5	31.0	8.3	5.9
2	3	11.5	31.0	8.0	6.4
2	4	12.7	33.0	8.5	6.4
2	2	12.0	29.0	7.0	5.5
2	3	13.1	29.0	7.9	5.6
2	1	11.9	31.0	7.2	5.5
2	2	12.0	36.0	7.9	6.5
2	2	10.1	30.0	4.1	3.3
2	1	12.2	31.0	6.7	5.5

Chamber	Row	Time to +7°C (h)	Maximum temp(°C)	Growth Total	Growth 6 hours
2	2	11.8	35.0	8.7	7.0
2	3	12.0	29.0	6.3	4.5
2	2	12.1	30.0	6.2	5.2
2	1	10.2	33.0	6.7	6.0
3	5	13.2	28.0	5.9	4.9
3	2	12.0	35.0	8.1	6.4
3	4	16.5	33.0	9.5	6.8
3	2	12.5	28.0	6.6	5.1
3	4	14.0	29.0	6.8	4.8
3	4	12.5	31.0	6.9	5.3
3	3	15.1	31.0	8.2	5.7
3	2	11.5	29.0	6.0	5.0
3	3	12.0	30.0	6.8	5.3
4	3	15.1	33.0	9.7	7.0
4	3	13.0	31.0	7.5	5.9
4	2	11.8	34.0	7.3	5.6
4	2	10.1	29.5	5.5	4.9
4	2	10.5	30.0	5.9	4.8
4	1	09.5	30.0	4.6	3.6
4	2	13.1	30.0	6.8	5.1
4	1	12.2	33.0	6.4	5.0
4	4	14.5	32.0	8.7	6.2
4	3	11.8	32.0	6.2	4.9
Mean		12.2	31.0	7.1	5.4

a)



b)

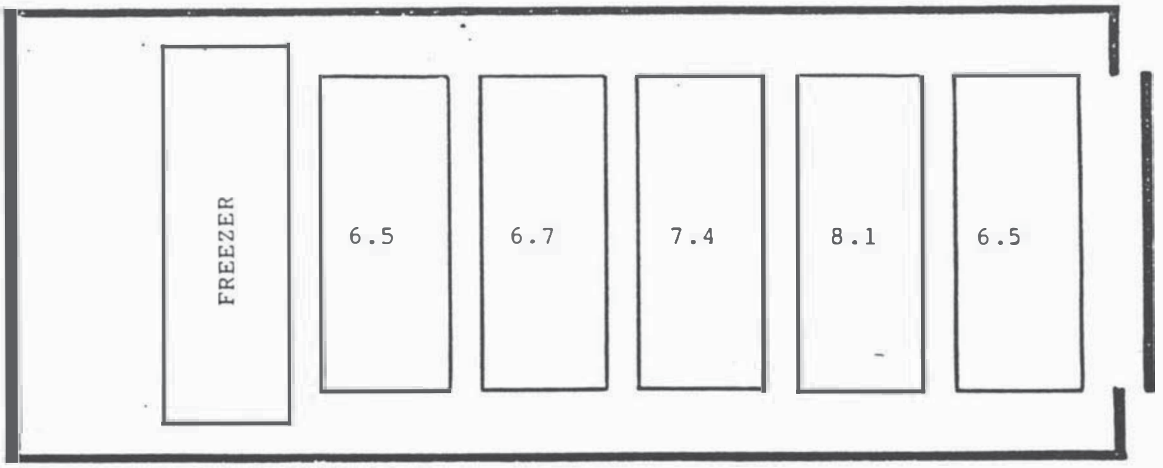


Figure 11: Mean proliferation values for different row positions of cartons in (a) the first plant survey, and (b) the second survey.

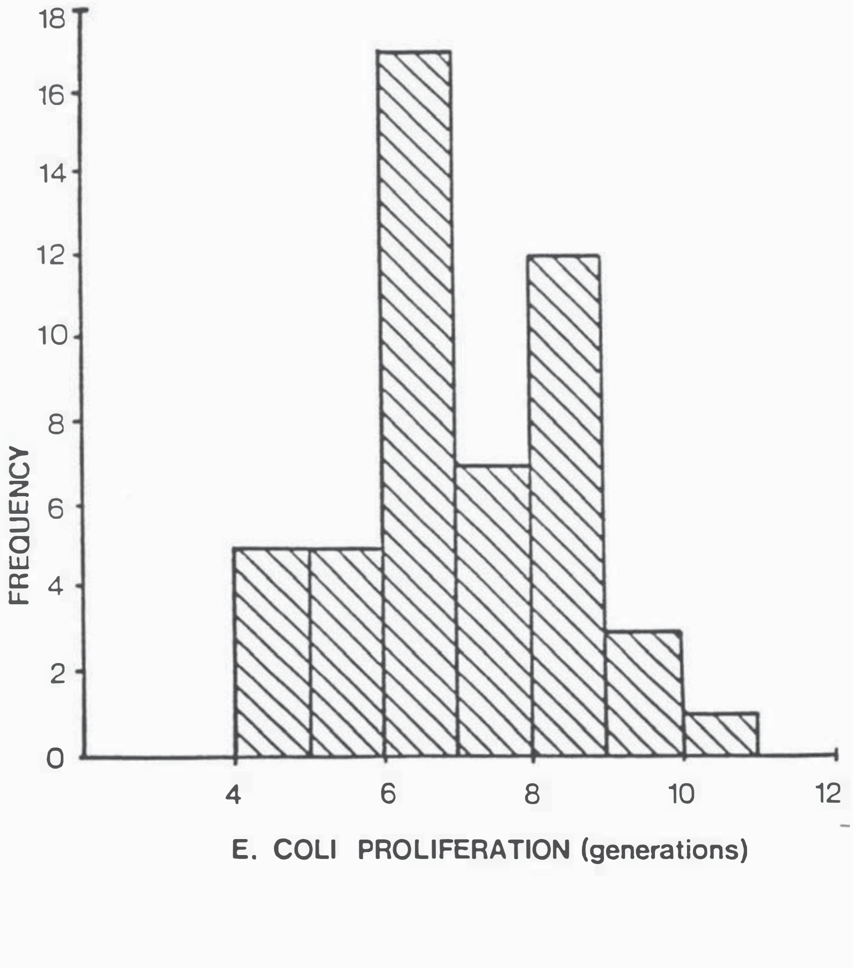


Figure 12: Frequency distribution of calculated *E.coli* proliferation values in 50 bulk-packed cartons during the assessment of the upgraded commercial hot-boning process.

the second survey, with the remaining 25% (or 24%) occurring during the time necessary to complete the cooling phase to +7°C (Table XV, XVI, p.88/89, 92/93).

IV.3.2. Thermal Centre

In cartons that were closed before entering the blast freezer chamber, the highest mean of *E.coli* proliferation values (7.9 generations) was calculated at the geometric centre of the carton. The mean value for the site midway between the geometric centre and the upper surface of the meat was a little lower than the value at the geometric centre (7.7 generations). The mean value for the point midway between the centre and the lower surface was markedly lower (6.0 generations), with the values at the bottom (1.0 generation) and top (3.0 generations) surface only being 13 and 38% respectively of the maximum value at the centre.

However, in only 20% of the individual temperature histories did the geometric centre have the highest proliferation value, or values equal to the highest value. In 60% of the temperature histories the highest proliferation value was presented by a position midway between the geometric centre and the upper surface position. In 20% of the temperature histories point of the highest calculated proliferation value was located at a position midway between the geometric centre and the bottom surface of the meat mass. The cooling profile through the carton appeared to be skewed towards the top surface (Table XVII and Figure 13, p.98, 100).

In the series of recordings in 15 cartons of which the lids were not closed, the mean proliferation at the geometric centre (7.4 generations) was not much lower than that in closed cartons. Similarly, the proliferation values for the base and upper surface positions of the probes were only slightly changed, 1.3 from 1.0 generations for the bottom surface and 2.8 generations from 3.0 for the top surface. The proliferations at the midway point between both the geometric centre and the top surface of the meat, and between the centre and base surface had, however been reduced from 7.7 to 6.3 generations and from 6.0 to 4.7 generations, respectively. The mean value at the midway point between the top surface and the geometric centre in the series of open cartons was significantly lower than that of the geometric centre.

In the individual temperature histories 80% of the highest values were now achieved at the geometric centre, with the remaining 20% achieving their maxima at the midway point between the geometric centre and the upper surface.

The result was a more evenly shaped cooling profile, with the highest point closer to the geometric centre (Table XVIII and Figure 13, p.99, 100).

Table XVII: Proliferation values at five locations along the vertical axis of 15 cartons of hot-boned, bulk-packed beef cooled with their lids closed (Top = upper meat surface, 3/4 = midway between upper meat surface and the centre, Bottom = lower meat surface, 1/4 = midway between centre and lower meat surface).

Top	3/4	Centre	1/4	Bottom
2.0	8.6	8.0	7.5	0.4
0.0	9.4	6.9	5.3	0.6
5.2	7.4	7.2	7.1	1.5
2.0	8.2	8.1	4.1	0.7
4.2	10.7	9.5	7.7	1.4
0.9	5.5	4.3	5.1	0.4
0.5	3.4	5.0	6.1	0.7
4.3	6.6	12.2	0.8	0.6
2.2	8.1	8.4	10.5	3.0
0.7	8.1	10.7	10.5	0.8
3.0	5.8	6.4	8.4	0.3
6.5	10.8	10.3	3.9	1.6
2.9	5.4	6.8	3.9	0.4
4.6	9.3	7.7	5.4	1.7
5.8	8.0	6.4	3.9	0.6
Mean: 3.0	7.7	7.9	6.0	1.0

Table XVIII: Proliferation values for *E.coli* at five locations along the vertical axis of 15 cartons of hot-boned, bulk-packed beef cooled with no lids (Top = upper meat surface, 3/4 = midway between upper meat surface and centre, bottom = lower meat surface, 1/4 = midway between lower meat surface and centre).

Top	3/4	Centre	1/4	Bottom
1.1	5.7	5.3	3.3	0.2
2.4	4.5	9.5	3.1	0.9
1.1	5.2	5.1	5.1	0.0
3.7	7.0	8.3	5.5	2.6
1.6	6.1	7.3	5.6	1.7
1.9	7.8	8.1	6.5	2.0
4.7	9.0	8.6	5.9	2.6
5.8	7.4	7.5	2.8	1.4
6.1	6.0	7.2	6.1	1.0
2.7	5.6	9.1	3.8	1.2
2.1	5.0	6.3	4.4	1.8
2.4	6.9	7.4	2.2	2.0
3.3	6.8	7.4	6.2	0.0
1.0	8.7	8.9	4.8	1.1
1.9	3.0	5.5	5.3	0.6
Mean: 2.8	6.3	7.4	4.7	1.3

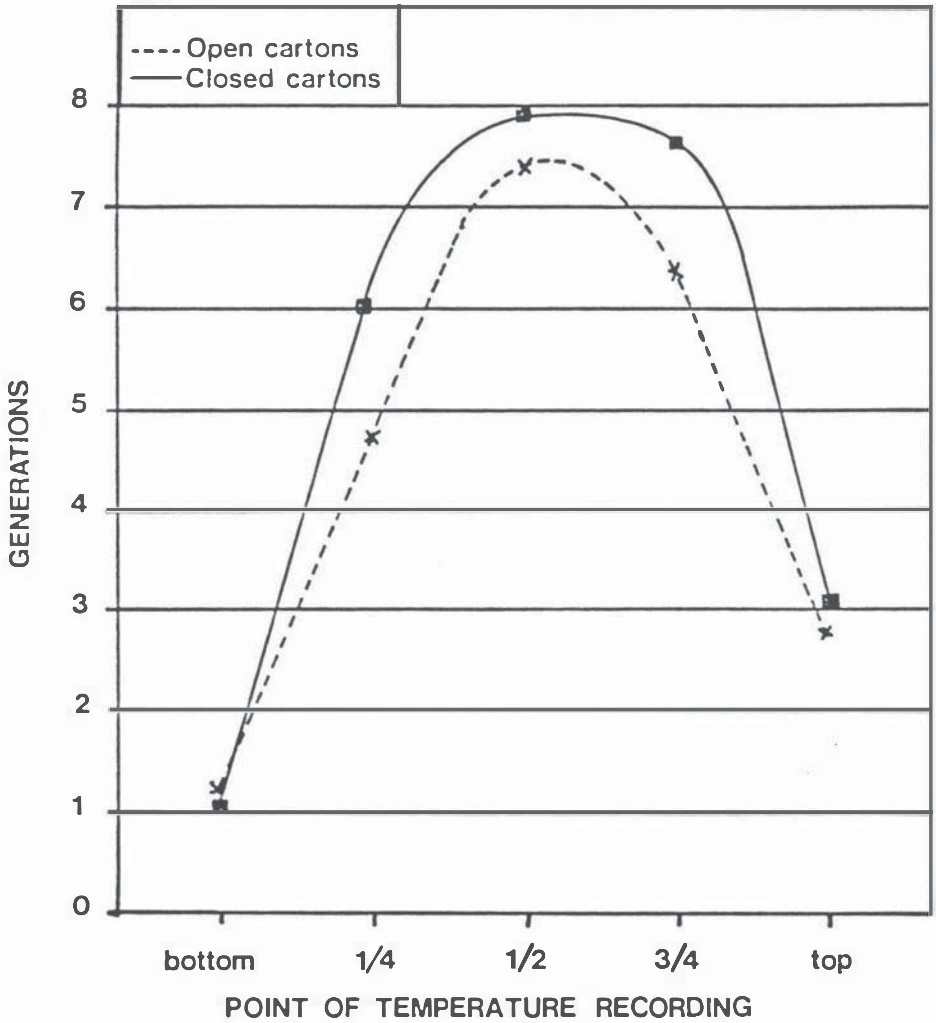


Figure 13: Proliferation values (mean of 15) recorded at five points along the vertical axis through the geometric centre of hot-boned, bulk-packed cartons with lids either closed or left open during the cooling process.

IV.3.3 Comparison of calculated and observed proliferation for *E. coli*

Agar squares inoculated with *E.coli* were prepared and the number of organisms in the initial inoculum estimated from the counts for *E.coli* recovered from squares that were not placed with the meat. The initial inoculum level for squares placed with the meat was assumed to be the same as the mean value of the unplaced squares, which was found to range from 1.6×10^2 to 1.6×10^3 per agar square.

The calculated proliferation was within ± 1 generation of the proliferation in 81 % of the comparisons. In 62 % of the comparisons the calculated proliferation from the computer programme exceeded the observed proliferation from plate counts. For differences in excess of one generation the calculated proliferation value always exceeded the observed counts. Only 7.5 % of differences exceeded 1.5 generations and none exceeded 2 generations (Table XIX and Figure 14, p. 102/103, 104).

Table XIX: Comparison of computer derived proliferation values with observed proliferation in samples of high ultimate-pH beef inoculated with *E.coli.*

Calculated proliferation	Observed proliferation
2.2	1.0
2.8	2.0
2.2	2.6
3.7	3.0
3.9	3.6
5.2	4.0
4.6	3.9
5.2	4.1
5.3	4.6
4.9	4.7
5.5	5.2
5.7	5.6
5.2	5.8
5.2	6.0
6.0	6.1
6.5	6.1
6.7	6.3
6.4	6.6
6.7	6.5
6.8	5.9
7.0	6.1
7.4	5.6
7.3	6.5
7.3	6.9
7.2	6.9
8.4	6.8
8.0	6.9
7.9	7.1

Calculated proliferation	Observed proliferation
8.7	7.8
8.9	7.9
8.7	8.2
8.2	8.4
7.8	8.8
8.1	9.1
8.2	9.2
8.7	9.7
8.7	9.6
9.0	9.1
9.1	9.1
9.3	8.8
9.6	8.7
9.4	9.4
9.3	9.5
9.4	9.8
10.5	9.1
11.2	9.3
11.3	9.7
10.1	9.9
9.6	10.3
9.7	10.2
10.3	10.3
10.2	10.5
LF	11.7
44.3 LF	ND
LF	ND
LF	ND
LF	ND
LF	ND
LF	ND

LF = Logger failure
ND = Not done

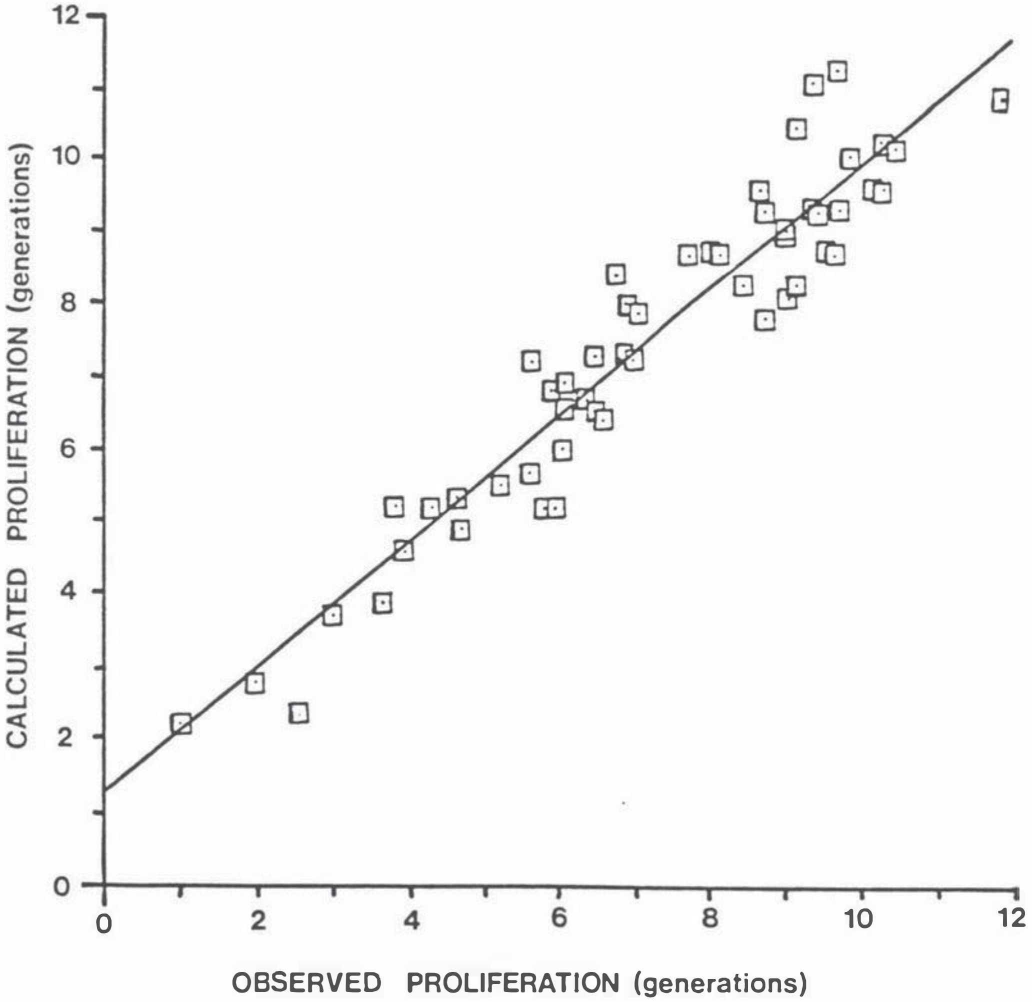


Figure 14: Scatter plot of calculated against observed growth of *E.coli* on inoculated samples of high ultimate-pH beef that were cooled in cartons of hot-boned beef.

IV.4. DISCUSSION

The assessment of the hygienic adequacy of a beef carcass cooling process in a New Zealand meat export plant (Gill et al. 1991), had suggested proliferation criteria for *Escherichia coli* in a process, complying with Good Manufacturing Practice (GMP), which were comparable to the traditionally used temperature/time parameters in beef carcass cooling. The suggested criteria stipulated an average proliferation value of 10 generations for 80% of the temperature histories and a maximum of 14 generations, which should not be exceeded by any temperature history.

This criterion was met in the surveyed hot-boning beef plant after an increase in the refrigeration capacity of the freezing plant, as was shown in the second survey of the chilling phase of the meat process. With only 2% of the temperature histories exceeding the 10 generations-limit, the process was now well within the criterion established from the beef carcass cooling process. However, in order to describe better the distribution of frequencies of generation values, an additional criterion of a mean permissible proliferation for *E.coli* proliferation of 7 generations may be stipulated. This would reject processes which would only just meet the first two criteria of Gill et al. (1991) for maximum and 80th percentile, with most of the individual values concentrating at the higher end of the scale.

With respect to the distribution around the mean of 7.1 generations, the hot-boning process could be considered to be superior to the standard beef carcass cooling process (Figure 15, p.107), because of the more even, and closer distribution of values around the average. This is not an entirely unexpected finding, as one would expect more even freezing behaviour from the uniform hot-boned product which is packaged in cartons of standard size and weight. Beef carcasses tend to vary in weight, size and shape and the airflow over the slowest cooling part is subject to variation, resulting in a wider distribution of proliferation values.

The second survey, and to some degree also the first survey of the hot-boning plant, shows an increase in mean proliferation values, as the cartons are frozen in a rack position further away from the cooling coils. This is true up to the fourth row back from the coils. Cold air from the coils passes through the cartons in the rows and exchanges heat with them. As it passes through the rows to the back of the freezer chamber the air warms up and therefore, this result is not totally unexpected.

The last row, however, shows a small decrease of average proliferation values. This may be explained by cold air by-passing the whole set of racks, on sides, top and bottom and being deflected back into the last row from the door panel at the rear of the freezing chamber. As only 50 data

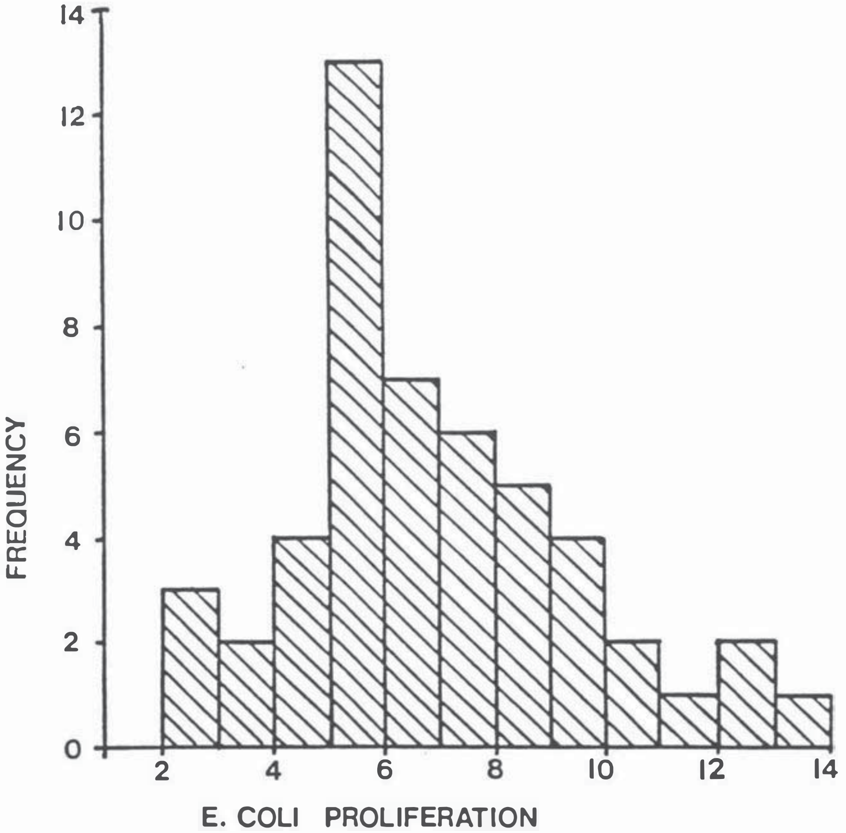


Figure 15: The frequency distribution of *Escherichia coli* proliferations calculated from temperature histories for the aitch-bone pocket sites of 50 beef sides cooling in a commercial chiller (from Gill et al. 1991).

sets were available in each series these results may not be significant, although they agree with theoretical approaches to the freezing of hot-boned beef (Pham 1991).

The mean proliferation values for the four freezing chambers are similar, both numerically and in distribution and this indicates that there is no great difference between the performances of either of the four freezer chambers.

The evaluation of the proliferation potentials at five different sites along the vertical axis through the geometric centre of the cartons shows, in the case of closed cartons, that the slowest cooling point in a carton is situated slightly above the geometric centre, approximately halfway between the geometric centre and the point 130 mm above the bottom of the meat mass. The freezing of closed and sealed cartons is standard commercial practice, and therefore the evaluation of the hygienic adequacy of a hot-boning process should be monitored at this site as a reference point.

It would be difficult in routine monitoring of meat processes to assure the correct placing of the probe of the temperature logger in every single case. It is, therefore, fortunate that the values at the geometric centre, the thermal centre and the midway point between the geometric centre and the surface of the meat mass only differ slightly. Thus the misplacement of the probe by up to 25 mm above or below the ideal reference point would not unduly jeopardise the accuracy of any survey of a hot-boning process.

The proliferation potential values obtained at the five sites when the carton lid was not closed, show a more even cooling profile with most of the highest proliferation values for any series found at the geometric centre. The differences between the geometric centre and the midway sites were more pronounced than in the closed cartons, thus reducing the meat mass within the carton which was likely to have the same high potential for *E.coli* proliferation as the thermal centre. This would make it more difficult in routine monitoring to locate the reference point for a hot-boning process in which freezing or cooling of un-lidded cartons is practised. The thermal and geometric centres are in approximately the same position in the series for open cartons.

Overall, the mean proliferation values for most sites apart from the geometric centre show a marked decrease for cartons frozen with open lids. If freezing cartons with the lids open became commercial practice, the process would, for greater portions of the meat mass result in a reduction in growth potential for mesophilic organisms and therefore be more hygienically acceptable. However, because of the faster cooling rates experienced in parts of the meat mass the risk of toughening through cold-shortening would increase.

A comparison between observed proliferation values of *E.coli* inocula in the agar squares and the calculated potential proliferation based on the temperature history show very good correlation. Given that microbiological plating techniques are necessarily inaccurate (Richardson et al. 1978, Sharpe 1980) differences of ± 1 generation for supposedly identical microbiological samples can be expected. The calculated values for potential increases varied by more than ± 1 generations from the true increases of the inocula only in a minority of comparisons. Thus the calculated values can be assumed to be a realistic estimation of the microbiological proliferation potential in a commercial hot-boning process. As the calculated proliferation values in a majority of cases tended to be greater than the increases found in the agar squares, the use of the computer programme would tend to overestimate the potential for *E.coli* growth. The evaluated process would in practice show lower potential increases and therefore be safer than the computer programme predicts it to be.

For the evaluation of the hygienic adequacy of the cooling process the worst-possible-case is assumed, i.e. that the slowest cooling part of the carton or carcass is contaminated with a mesophilic microorganism. In many situations this may not be the case. Though *Salmonella spp., E.coli* and other mesophilic bacteria are readily isolated from cattle and beef (Grau et al. 1968; Smith and Grau 1973; Stolle 1981), they may not come to rest at the thermal centre of the carton and thus not attain the maximal numbers possible there. The use of temperature function integration (TFI) criteria will therefore tend to err on the high side, i.e. assume higher potential for microbiological increases than may be encountered in a specific single carton.

Traditional regulatory activity has attempted to restrict the growth of mesophilic bacteria by setting temperature/time parameters which were focused on the minimum growth temperature of mesophilic organisms. Thus, for beef carcass cooling, +7°C had to be attained, before further processing in the boning room could commence (anon. 1964). As the growth of these bacteria on carcasses is restricted by surface drying, at least during the first 24 hours (Nottingham 1982), the growth during that period is negligible; after 24 hours surface temperatures may have fallen below the minimum growth temperature for mesophilic growth. This approach, i.e. the setting of parameters of temperatures which have to be achieved after certain times has been applied to hot-boning processes as well and thus, in New Zealand at least, the requirement exists to reduce the temperature at the thermal centre of the carton of hot-boned meat to +7°C in 24 hrs (anon. 1989).

The survey of the hot-boning beef plant presented here indicates that mesophilic organisms like *E.coli* may divide up to 10 times, even in a well controlled process, and thus numerical counts potentially increase at worst by about three

$\log_{10}$ units. The comparison of true increases in the agar squares with those derived from the computer programme show good correlation, and indicate that in a hot-boning situation these increases may be quite realistic. The data presented in this study also indicate that most of the potential to grow exists in the first few hours of the cooling phase, since 75% of the total growth was found to occur in the first six hours of the process. This is to be expected, as higher temperatures provide for faster bacterial growth, and emphasises, that the first few hours of the cooling phase are of critical importance to the outcome of the hot-boning process. Any delays in the product entering the chiller or freezer will allow bacterial numbers to increase to greater numbers. Translated into the practical hot-boning situation this should lead to the condemnation of the practice in some hot-boning plants of defrosting the refrigeration plant while the first of the day's production is being loaded in. Although this practice results, in the eye of the processor, in time and energy savings, it may also allow for the unnecessary proliferation of microorganisms and therefore, microbiological deterioration of the product, because of inadequate cooling of the product during that time.

Hot-Boning of beef or other species is still a relatively uncommon procedure and only a small proportion of the New Zealand beef (and mutton) production is hot-boned. However the same principles apply more or less in any offal cooling procedure. The packaging of hot, i.e. body temperature warm product into an insulated carton which is cooled or frozen without allowing for the evaporation of excess moisture means that temperature becomes the only factor which determines bacterial proliferation. High potential proliferation values for *E,coli* can be expected in a TFI-evaluation of these processes. Because time limits are not commonly specified for offal cooling, the processor's specifications for the refrigeration plant are consequently less stringent; TFI results for offal cooling can be expected to be worse than the first survey of the hot-boning plant. Data obtained by Gill and Harrison (1985) confirm the potential for high increases in numbers of mesophilic organisms in cooling offal.

A description of offal cooling processes in New Zealand, similar to the survey presented here, is desirable and would quantify the possible risks associated with this process. Previous data obtained here (Gill and Harrison 1985) would suggest that one can expect to find similar deficiencies as have been shown in a study in Canada (Gill and Jones 1992), i.e. unacceptably high increases in the numbers of microorganisms if there are deficiencies in the refrigeration plant, in particular in case of an uneven distribution of the refrigeration capacity.

Microbiological limits for the proliferation of mesophiles on meat have not been set, and the view that no proliferation should be possible during any meat process is obviously unrealistic. Fast cooling requirements would result in an expensive and un-desirable product through huge demands on a cooling plant and the negative effects on the eating quality, in particular tenderness, through cold-shortening. Such microbiological limits and cooling requirements have been suggested in Australia, and consequently, hot-boning has not been exploited commercially in that country to the same degree as it has in New Zealand (Anderson et al. 1984).

The proposed limits for carcass cooling and hot-boned cartoned product translate currently accepted processes which comply with GMP into TFI-values and should, at least initially, serve as a guide for the evaluation of any new process. Because the TFI-values represent the calculation from objective measurements they can easily be used for the comparison of quite different processes.

These criteria propose a mean acceptable proliferation of 7 generations, a limit of 10 generations for 80% of traces and an upper limit of 14 generations for a process. A comparison with this standard will allow the evaluation of totally new meat processes which would otherwise be only acceptable after extensive bacteriological sampling.

At present, for example, regulations require meat to be cooled to below +7°C or + 10°C in the meat plant before it can be transferred to a freezing plant or for further processing (anon. 1964). TFI-evaluation may show that a new process, which proposes to transfer meat at temperatures higher than +7°C, in a refrigerated truck or container, yields TFI-values for the whole process which are comparable to the suggested criteria of 7/10/14 generations. The new process would then offer the same assurances for the hygienic adequacy of the process as the traditional process, provided the product entering such a new process was of similar microbiological quality in both instances. Such results can be obtained far more easily using the TFI approach, than could be achieved using traditional microbiological sampling techniques. The results presented here also indicate that the TFI results can be as accurate, if not more, as traditional techniques.

TFI monitors a process of meat production and thus offers a tool for process assurance. Because data are available at "the touch of a button", a process out of control can be remedied much quicker than with bacteriological testing, which is slow and only yield results after days, at which time the final product may have left the control of the regulatory authority and even reached the consumer.

Deviations from the normally accepted values are quickly assessed in terms of their increased (or decreased) potential for bacterial proliferation. Routine monitoring of processes using TFI is therefore helpful in the assessment of situations where process control has broken down, e.g. refrigeration break-down in a hot-boning system or during offal cooling. If only small deviations are evident, TFI obviates the need for extensive bacteriological sampling, which would be necessary where no information is available on the temperature history of the product.

Present regulatory activity centres on product control, i.e. the assurance of a wholesome product at the end of the process. Laborious bacteriological testing is employed at various stages of the process, traditionally of meat on the slaughterboard, after chilling and before packaging. If TFI evaluation was applied, using these criteria which have been shown to result in a safe end product, then the amount of product control could be reduced.

Provided a safe product entered the process, i.e. meat that had been slaughtered and dressed under conditions conforming with GMP, and the process monitored with TFI was shown to be acceptable,i.e. the criteria for bacterial proliferation were met, such process control would ensure a safe end-product.

V. GENERAL SUMMARY

1. Cold-tolerant pathogens:

The purpose of this work was to compare the growth of cold-tolerant pathogens, like *Aeromonas hydrophila*, *Listeria monocytogenes* and *Yersinia enterocolitica* on high ultimate-pH beef packaged under vacuum, the traditional method, with packaging under an atmosphere consisting exclusively of carbon dioxide, as is found with the new CAPTECH packaging technology. Samples which had been inoculated with strains of those three selected pathogens were stored at five different temperatures, which ranged from the low sub-zero temperatures which can be expected to be used in commercial long-term chilled storage to ones which may present a situation of mild temperature abuse. A comparison between the two methods of packaging was expected to establish the relative merits of either packaging in as far as the health risk to the consumer from the growth of the three cold-tolerant pathogens was concerned.

Growth under vacuum-packaging conditions occurred for all pathogens at all temperatures, except for *Listeria* at -2°C, but only at +10°C for all three pathogens under carbon dioxide atmosphere. *Yersinia* also grew at +5°C under CO_2. Growth rates under CO_2 and total increases measured for all three organisms were reduced when compared with the growth under vacuum-packing conditions at the same storage temperature.

Storage life under CO_2 was extended by the factor two at the higher temperatures and by up to the factor three at the lower storage temperature, extending to 182 days before spoilage was evident under CO_2 at -2°C.

Therefore, even with CO_2 packaging extending the storage life for high ultimate-pH beef and thus the time at which pathogens could potentially grow, the conditions were found to be adversely affecting the growth of the three test organisms and thus resulted in a much reduced potential health risk to the consumer. At a storage temperature of around 0°C, which is the commercial practice, carbon dioxide packaging did not lead to any increase in those cold-tolerant pathogens which may have inadvertently contaminated the meat during slaughter and processing. To the contrary, over the very long storage times observed at the lower temperatures, pathogen numbers tended to decrease.

Vacuum-packaged samples permitted the growth of all inocula, except for the one of *Listeria monocytogenes* at -2°C, with higher increases of the inocula than were found with carbon dioxide packaging. Especially at the higher storage temperatures the observed increases were of an order which could have permitted even small initial inocula of these pathogens to grow to final numbers which may present a public health risk.

Carbon dioxide packaging can therefore be deemed to be a safer process than traditional vacuum-packaging and offers, at the same time advantages to the producer and distributor through the increased flexibility which the increased storage life affords them.

With beef of the more commonly encountered low ultimate-pH presenting less favourable conditions to bacterial growth (Grau 1980, 1981), the experimental conditions, which observed growth patterns on high ultimate-pH beef essentially present a worst-possible-case scenario. It can be assumed that under an atmosphere of CO_2 the potential for growth of cold-tolerant pathogens on low ultimate-pH meat will be even further reduced, very much as proposed in Leistner's hurdle theory (1978), i.e CO_2 would present an additional obstacle to bacterial growth. Although a high proportion of meat has been shown to be contaminated with some of the cold-tolerant pathogens (Lowry 1988, Palumbo et al. 1985), in most cases the contamination will either be low in actual microbial numbers or not be present at all, thus further reducing any health risk to the consuming public.

2. Temperature Function Integration:

The modelling of bacterial growth on the example of the growth of *Escherichia coli* on beef has measured the potential increases for a mesophilic microorganism as a function of the temperature history of the product. The results of a survey of a commercial beef hot-boning plant in New Zealand allow an appreciation of the commercially accepted potential for the proliferation for mesophilic microorganisms, in particular *Salmonella spp.*. The data obtained in this survey suggest that hot-boning, as well as the more traditional boning after carcass cooling, could regularly achieve a mean proliferation value of not more than 7 generations of growth for *E. coli*, with not more than 20% of product allowing for more than 10 generations of growth. Whether such proliferation values are acceptable to regulatory authorities has yet to be established. In the past the approach has been to proclaim nil tolerances for pathogens, and in particular *Salmonella spp.*, in meat processing (anon. 1988).

The proliferation data for carcass cooling obtained by Gill et al. (1991) suggest, that a potential for mesophilic growth exists also with traditional meat processing. Traditional carcass cooling procedures, however, have, in general, been found to produce a wholesome product and it would be unreasonable to require a nil tolerance now. The proliferation values for hot-boning in this study present a process which offers the same degree of hygienic adequacy as traditionally accepted processes, i.e. carcass cooling and should therefore be equally acceptable.

Temperature function integration (TFI) techniques present a tool to regulatory authority that has the benefit of analyzing objective data, and thus meets the criteria for a scientifically sound tool. TFI also has the advantage of delivering its data faster than traditional microbiological techniques. Measured at the slowest cooling part, of a carcass or within a carton of packaged meat, TFI presents a worst-possible-case assumption, an approach that regulatory authorities should always take in assessing the hygienic adequacy of meat or food processing. The results presented here also show that, at least in a situation were temperature is the one factor limiting bacterial growth (in hot-boning or offal cooling, for instance) calculated and actual growth are in good agreement and thus can be relied upon in the hygienic assessment of a process. Thus TFI could increasingly substitute for bacteriological sampling in day-to-day process control, the data would be much quicker to hand, and the steps, necessary to correct deviations of the process from the norm could be taken earlier.

Process assurance by means of TFI can then increasingly substitute for product control, through microbiological sampling, which is traditionally carried out. Provided a process does not exceed the TFI parameters the end product will be a wholesome product, if a satisfactory product has been fed into the process at the beginning. Thus there is still a need to control slaughter and dressing to hygienic standards, in order to obtain an acceptable product, i.e. a carcass or joint of meat which can then be further processed under TFI monitoring. Compared to the more traditional end product control which will always be relying on representative sampling, thus does not cover the total product, TFI, through its monitoring of the hygienically worst-possible-case, by definition, guarantees that all product is acceptable if the TFI parameters are being met in the monitored process.

TFI approaches need not be restricted to mesophilic growth models, but could be extended to cold-tolerant pathogens as well. The reasonably fast growth of *Aeromonas* or *Yersinia* at the higher temperatures used in the storage trials suggests that TFI models of those two microorganisms could be used in an evaluation of microbial development during long-term storage under vacuum or carbon dioxide, representing the worst-possible situation for "cold-tolerant" growth. If objective data on the bacterial growth potential are combined with models for the ageing rates of the same product, ideal storage regimes can be devised by computer, maximising the eating quality of the final product, i.e. tenderness, without comprising the health of the consumer.

VI. References

Abeyta,JR,C. and Wekell,M.M. (1988)
Potential sources of *Aeromonas hydrophila*. Jornal of Food Safety, 9, 11-22

Ahn,C. and Stiles, M.E. (1990)
Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. Journal of Applied Bacteriology, 69, 302-310

Anderson,J., Buhot,J.W., Herbert,L.S. and Larnach,W.K. (1984)
Investigation of methods of cooling and freezing hot-boned beef. CSIRO Meat Research News letter. No.12/84

anon., (1964)
Council Directive on health problems affecting intra-Community trade in fresh meat. (64/433/EEC). OJ No 121, p 2012/64. Brussels

anon., (1988)
Proposal for a Council Directive on health problems affecting the production, placing on the market of the Community and importation from third countries of minced meat and meat in pieces of less than 100 grams (88/C 18/10) Chapter V, OJ No. C18/8, 23.01.1988

anon., (1989)
Hot Boning Requirements. New Zealand Ministry of Agriculture and Fisheries. MDC 89/6/2. Government Printer, Wellington, New Zealand

anon., (1991)
Human listeriosis, Weekly Epidemiological Record, No.32, 09 August 1991, Geneva, 236-238

Baldridge,J.R., Thomashow,M.F. and Hinrichs,D.J. (1988)
Induction of Immunity with avirulent *Listeria monocytogenes* 19113 depends on bacterial replication. Infection and Immunity, 56 (8), 2109-2113

Bell,R.G. (1990)
New Zealand chilled lamb in Saudi Arabia.Proceedings 26th Meat Industry Research Conference, Hamilton, New Zealand, 147-153

Bercoivier,H. and Mollaret,H.H. (1984)
Yersinia Van Loghem 1944, 15^{AL}. Bergey's Manual of systematic Bacteriology, Vol.1 (ed Krieg,N.R. and Holt,J.G.), Williams and Wilkins Co., Baltimore. 498-505.

Bottone,E.J (1977)
Yersinia enterocolitica: A panoramic view of a charismatic microorganism. CRC Critical Reviews in Microbiology 5, 211-241

Buchanan,R.L. and Palumbo,S.A. (1985)
Aeromonas hydrophila and *Aeromonas sobria* as potential food poisoning species: a review. Journal of Food Safety, 7, 15-29

Bullians,J.A. (1987)
Yersinia species infection of lambs and cull cows at an abattoir. New Zealand Veterinary Journal 35, 65-67

Burke,V., Gracey,M., Robinson,J., Peck,D., Beaman,J. and Bundell,C. (1983)
The microbiology of childhood gastroenteritis: *Aeromonas* species and other infective agents. Journal of Infectious Diseases, 148, 68-73

Breer,C. and Breer,G. (1988)
The isolation of *Listeria spp.* in meat and meat products. Proceedings 34th. International Congress for Meat Science and Technology, Brisbane, Australia, 520-521

Cahill,M.M. (1990)
A review: Virulence factors in motile *Aeromonas* species. Journal of Applied Bacteriology, 69, 1-16

Clark,D.S. and Lentz,C.P. (1969)
The effect of carbon dioxide on the growth of slime-producing bacteria on fresh beef. Canadian Institute of Food Science and Technology Journal, 2,72

Davey,C.L and Gilbert,K.V (1976)
Thaw contracture and the disappearance of adenosintriphospate in frozen lamb. Journal of Science, Food and Agriculture, 27,1085-1092

Davey,G.M., Bruce.J. and Drysdale,E.M. (1983)
Isolation of *Yersinia enterocolitica* and related species from the faeces of cows. Journal of Applied Bacteriology 55,439-443

Davis,W.A., Kane,J.G. and Garagusi,V.F. (1978)
Human *Aeromonas* infections: A review of the literature and a case report of endocarditis. Medicine, 57,3, 267-277

Doyle,M.P., Hughdal,M.B. and Taylor,S.L. (1981)
Isolation of virulent *Yersinia enterocolitica* from porcine tongues. Applied and Environmental Microbiology, 42, 661-666

Emswiler,B.S. and Kotula,A.W. (1979)
Bacteriological quality of ground beef prepared from hot and chilled beef carcasses. Journal of Food Protection, **42** (7), 561-562

Enfors,S.O., Molin,G. and Ternstrom,A. (1979)
Effect of packaging under carbon dioxide, nitrogen or air on the microbial flora of pork stored at 4°C. Journal of Applied Bacteriology, **47**,197-202

Eskin,N.A.M., Henderson,H.M. and Townsend,A.J. (1971)
Microbiological deterioration of proteins and protein foods. In: Biochemistry of Foods, Academic Press, New York, 183-217

Fenlon,D.R (1985)
Wild birds and silage as reservoirs of *Listeria* in the agricultural environment. Journal of Applied Bacteriology, 59, 537-543

Fischer,K. und Augustini,C. (1977)
Stadien der postmortalen Glykogenolyse bei unterschiedlichen pH-Werten in Schweinefleisch. Fleischwirtschaft, 57, 1191-1194

Fukushima,H., Koichi,S., Misao,T. and Yoshihiro,K. (1983)
Isolation of *Yersinia* species from bovine faeces. Journal of Clinical Microbiology 18 (4): 981-982

Gilbert,K.V. and Davey,C.L. (1976)
Carcass electrical stimulation and early boning of beef. New Zealand Journal of Agricultural Research, **19**, 429-434

Gilbert,K.V., Davey,C.L. and Newton,K.G. (1976)
Electrical stimulation and the hot boning of beef. New Zealand Journal of Agricultural Research, 20, 139-143

Gill,C.O. (1976)
Substrate limitations of bacterial growth at meat surfaces. Journal of Applied Bacteriology,**41**, 401-410

Gill,C.O. (1979)
A review: Intrinsic bacteria in meat. Journal of Applied Bacteriology, **47**, 367-378

Gill,C.O. (1983)
Meat spoilage and evaluation of the potential storage life of fresh meat. Journal of Food Protection, **46**, 444-452

Gill,C.O. (1988a)
CO2 Packaging - Future Possibilities. Proceedings 25th Meat Industry Research Conference, Hamilton, New Zealand, 191-194

Gill,C.O. (1988b)
A Process assurance program for raw meat products. Proceedings 25th Meat Industry Research Conference, Hamilton, New Zealand, 145-148

Gill,C.O. and Harrison,J.C.L. (1985)
Evaluation of the hygienic efficiency of offal cooling procedures. Food Microbiology, 2, 63-69

Gill,C.O. and Jones,S.D.M. (1992)
Evaluation of a commercial process for collection and cooling of beef offals by a temperature function integration technique. International Journal of Food Microbiology. (in press)

Gill,C.O. and Newton,K.G. (1977)
The development of aerobic spoilage flora on meat stored at chill temperatures. Journal of Applied Bacteriology, 43, 189-195

Gill,C.O. and Newton,K.G. (1978)
The ecology of bacterial spoilage of fresh meat at chilled temperatures. Meat Science, 2, 207-215

Gill,C.O. and Newton,K.G (1979)
Spoilage of vacuum-packaged dark firm dry meat at chill temperatures. Applied and Environmental Microbiology 37, 362-364

Gill,C.O. and Newton,K.G. (1980)
Development of bacterial spilage at adipose tissue surfaces of fresh meat. Applied and Environmental Microbiology, 39, 1076-1077

Gill,C.O. and Newton,K.G. (1982)
Effect of lactic acid concentration on growth on meat of Gram-negative psychrotrophs from a meatworks. Applied and Environmental Microbiology, 43, 284

Gill,C.O. and Penney,N. (1988)
The effect of the initial gas volume to meat weight ratio on the storage life of chilled beef packaged under carbon dioxide. Meat Science, 22, 53-63

Gill,C.O. and Phillips, D.M. (1985)
The effect of media composition on the relationship between temperature and growth rate of *Escherichia coli*. Food Microbiology, 2, 285-290

Gill,C.O. and Tan,K.H. (1980)
Effect of carbon dioxide on growth of meat spoilage bacteria. Applied and Environmental Microbiology, 39 (2), 317-319

Gill,C.O., Harrison, J.C.L. and Phillips, D.M. (1991) Use of a temperature function integration technique to assess the hygienic adequacy of a beef carcass cooling process. Food Microbiology, **8**, 83-94

Gill,C.O., Phillips,D.M. and Harrison,J.C.L (1988) Product temperature criteria for shipment of chilled meat to distant markets. Proceedings IIF Conference Refrigeration for Food and People, Brisbane, Australia.

Gill,C.O., Leet,N.G. and Penney,N. (1984) Structural changes developing with rigor that facilitate bacterial invasion of muscle tissue. Meat Science, **10**, 265

Gill,C.O., Phillips,D.M., Loeffen,M.P.F. and Bishop,C. (1988) A computer program for evaluating the hygienic efficiency of meat processing procedures from product temperature history data. Proceedings 34th. International Congress for Meat Science and Technology, Brisbane, Australia, 531-532

Gitter,M., Bradley,R. and Blampied,P.H. (1980) *Listeria monocytogenes* infection in bovine mastitis. The Veterinary Record, **107**, 390-393

Glenn,A.R (1976) Production of extracellular proteins by bacteria. Annual Reviews of Microbiology, **30**, 41

Goodwin,C.S., Harper,W.E.S., Stewart,J.K., Gracey,M., Burke,V. and Robinson,J. (1983) Enterotoxigenic *Aeromonas hydrophila* and diarrhoea in adults. The Medical Journal of Australia, **1**, 25-26

Grau,F.H. (1979) Fresh meats: bacterial association. Archive der Lebensmittelhygiene, **30**, 87-92

Grau,F.H. (1980) Inhibition of the anaerobic growth of *Brochothrix thermosphacta* by lactic acid. Applied and Environmental Microbiology, **40**, 433

Grau,F.H. (1981) Role of pH, lactate and anaerobiosis in controlling the growth of some fermentative Gram-negative bacteria on beef. Applied and Environmental Microbilogy, **42**, 1043-1050

Grau, F.H. (1987) Prevention of microbial contamination in the export beef abattoir. In: Elimination of pathogenic organisms from meat and poultry, Elsevier Science Publishers, B.V. (Biomedical Division).

Grau,F.H. and Vanderlinde,P.B. (1988)
Growth of *Listeria monocytogenes* on vacuum-packaged beef. Proceedings of the 34th International Congress of Meat Science and Technology, Brisbane, Australia, 518-519

Grau, F.H., Brownlie,L.E. and Roberts,E.A. (1968)
Effect of some preslaughter treatments on the *Salmonella* populations in the bovine rumen and faeces. Journal of Applied Bacteriology, 31, 157-163

Gray,S.J. (1984)
Aeromonas hydrophila in livestock: incidence, biochemical characteristics, and antibiotic susceptibility. Journal of Hygiene (Cambridge), 92, 365-375

Gray,M.L. and Killinger,A.H. (1966)
Listeria monocytogenes and listeric infections. Bacteriological Reviews 30, 309-382

Hanna,M.O., Stewart,J.C., Carpenter,Z.L. and Vanderzant,C.(1977)
Effect of heating, freezing and pH on *Yersinia enterocolitica*-like organisms from meat. Journal of Food Protection 40, 689-692

Hanna,M.O., Zink,D.I., Carpenter,Z.L. and Vanderzant,C. (1976)
Yersinia enterocolitica-like Organisms from Vacuum-packaged Beef and Lamb. Journal of Food Science, 41, 1254-1256

Hayes,P.S., Feeley,J.C., Graves,L.M., Ajello,G.W. and Fleming,D.W. (1986)
Isolation of *Listeria monocytogenes* from raw milk. Applied and Environmental Microbiology, 51, 438-440

Henderson,T.G. (1984)
The determination of the Pathogenicity of Members of the Genus *Yersinia* In: Microbiology Seminar, Wallaceville Animal Health Reference Laboratory, Lower Hutt, New Zealand

Henry,B.S. (1933)
Dissociation in the Genus *Brucella*. Journal of Infectious Disease 52, 374-402

Hicks,E.W., Scott,W.J. and Vickery,J.R. (1955)
Influence of water in surface tissues on storage of meat. Bulletin of the International Institut of Refrigeration Annexe 1955-1,72

Honikel, K.O (1990)
The meat Europeans want, Changes and Opportunities in Europe. Proceedings 26th Meat Industry Research Conference, Hamilton, New Zealand, 136-139

Huxley, H.E. (1972)
Molecular basis of contraction in cross-striated muscle. In: Structure and Function of Muscle, Vol.I., 2nd edtn, Academic Press, New York & London

Ingram,M. and Mackey,B.M. (1976)
Inactivation by cold. In: Inhibition and Inactivation of Vegetative Microbes. Academic Press, New York, 111-151

Johnston,M.A., Loit,A., Pervis,U. and Farber,J. (1986)
Surveillance of soft and semi-soft cheese for *Listeria.* Journal of Food Protection, **49**,847

Kaczmarski,E.B. and Jones,D.M. (1989)
Listeriosis and ready-cooked chicken. The Lancet, March 11, 1989, 549

Kaya,M. and Schmidt,U. (1990)
Verhalten von *Listeria monocytogenes* auf vakuumverpacktem Rindfleisch. Fleischwirtschaft, **70**(5), 546-550

Kennedy,J.E., Oblinger,J.L. and West,R.L. (1982)
Microbiological comparison of hot-boned and conventionally processed beef plate cuts during extended storage. Journal of Food Protection, **45** (7), 607-614

Kirow,S.M., Anderson,M.J. and McMeekin,T.A. (1990)
A note on *Aeromonas spp.* from chickens as possible food-borne pathogens. Journal of Applied Bacteriology, 68, 327-334

Kleinlein,N., Untermann,F. and Beissner,H. (1989)
Zum Vorkommen von *Salmonella*-und *Yersinia-Spezies* sowie *Listeria monocytogenes* in Hackfleisch. Fleischwirtschaft, 69 (9),1474-1476

Larkin,J.M. and Stokes,J.L. (1968)
Growth of psychrotrophic microorganisms at sub-zero temperatures. Canadian Journal of Microbiology, **14**, 97

Lee, W.H. and McClain,D. (1986)
Improved *Listeria monocytogenes* selective agar. Applied and Environmental Microbiology, **52**, 1215-1217

Lee,C.Y., Fung,D.Y.C. and Kastner,C.L. (1985)
Computer assisted identification of microflora on hot boned and conventionally processed beef: effect of moderate and slow chilling rate. Journal of Food Science, **50**, 553-567

Leistner,L. (1978)
Hurdle effect and energy saving. In: Food Quality and Nutrition. Applied Science Publishers, London,553-557

Leistner,L., Rodel,W. and Krispien,K. (1981)
Microbiology of meat and meat products in high- and intermediate- moisture ranges. In: Water Activity: Influences on Food Quality. Academic Press, New York, 855-916

Locker,R.H. and Haygard,C.J. (1963)
A cold shortening effect in beef muscles. J.Sci. Food Agriculture, **14**, 787-793

Low,J.C. and Donachie,W. (1991)
Clinical and serum antibody responses of lambs to infection by *Listeria monocytogenes*. Research in Veterinary Science, 51, 185-192

Lowry,P.D. (1988)
The Hysteria over *Listeria*. Proceedings 25th Meat Industry Research Conference, Hamilton, New Zealand, 176-179

Lowry.P.D. and Gill,C.O. (1984a)
Temperature and water activity minima for growth of spoilage molds from meat. Journal of Applied Bacteriology, 56, 193

Lowry,P.D. and Gill,C.O. (1984b)
The development of a yeast microflora on frozen lamb stored at -5°C. Journal of Food Protection, **47**, 309

Lowry,P.D., Gill,C.O. and Pham,Q.T. (1989)
A quantitative method of determining the hygienic efficiency of meat thawing processes. Food Australia, **41**, 1080-1082

Marth,E.H. (1988)
Disease characteristics of *Listeria monocytogenes*. Food Technology, April 1988, 165-168

McKenzie,A.I. and Hathaway,S.C. (1990)
New Post Mortem Inspection Procedures: Vital components in the production and marketing of meat products. Proceedings 26th Meat Industry Research Conference, Hamilton, New Zealand, 313-316

McLauchlin,J. (1987)
Listeria monocytogenes, recent advances in the taxonomy and epidemiology of listeriosis in humans. Journal of Applied Bacteriology, **63**,1-11

McMeekin,T.A. (1981)
Microbial spoilage of meats. In: Developments in Food Microbiology, Vol.1., Applied Science, London, 1-49

McMeekin,T.A., Olley,J. and Ratkowsky,D.A. (1988)
Temperature effects on bacterial growth rates. In: M.J. Bazin and J.I. Prosser (eds.), Physiological models in Microbiology, Vol.1. CRC Press, Boca Raton, Fl., 75-89

McSporran,K.D., Hansen,L.M., Saunders,B.W. and Damsteegt,A. (1984)
An outbreak of Diarrhoea in Hoggets associated with infection by *Yersinia enterocolitica.* New Zealand Veterinary Journal **32**, 38-39

Morris,G.K. and Feeley,J.C. (1976)
Yersinia enterocolitica: a review of its role in food hygiene. Bulletin of WHO, **54**, 79-85

Nagel,C.W., Simpson,K.L., Ng,H., Vaughn,R.H. and Stewart,G.F. (1960)
Microorganisms associated with spoilage of refrigerated poultry. Food Technology, **21** (1),21-23

Newton,K.G. and Gill,C.O. (1978)
Storage quality of dark, firm, dry meat. Applied and Environmental Microbiology, **36**, 375

Newton,K.G. and Rigg,W.J. (1979)
The effect of film permeability on the storage life and microbiology of vacuum-packaged meat. Journal of applied Bacteriology, **47**, 433-441

Newton,K.G., Harrison,J.C.L. and Wauters,A.M. (1978)
Sources of psychrotrophic bacteria on meat at the abattoir. Journal of Applied Bacteriology, **45**, 75

Nicolas,J.A. and Vidaud,N. (1987)
Contribution a l'etude des *Listeria* presentes dans les denrees d'origine animale destinees a la consommation humaine. Recueil de medicine Veterinaire, **163** (3),283-285

Nishikawa,Y and Kishi,T. (1988)
Isolation and characterization of motile *Aeromonas* from human, food and environmental specimen. Epidemiology and Infection, **101**, 213-223

Nottingham,P.M. (1982)
Microbiology of carcass meats. In: Meat Microbiology. Applied Science Publishers, London, 13-65

Nottingham,P.M. and Wyborn,R. (1975)
Microbiology of beef processing II. Chilling and Ageing. New Zealand Journal of Agricultural Research, **18**, 23

Olley,J. and Ratkowsky,D.A. (1973)
Temperature function integration and its importance in the storage and distribution of flesh foods above the freezing point. Food Technology Australia, 25,66-73

Palmer,M.S. (1990)
Retailer and consumer markets overseas.Proceedings 26th Meat Industry Research Conference, Hamilton, New Zealand, 193-196

Palumbo,S.A. (1986)
Is refrigeration enough to restrain foodborne pathogens?, Journal of Food Protection, **49**, 12, 1003-1009

Palumbo,S.A. (1988)
The growth of *Aeromonas hydrophila* K144 in ground pork at 5°C. International Journal of Food Microbiology, 7,41-48

Palumbo,S.A., Morgan,D.R. and Buchanan,R.L. (1985a)
Influence of temperature, NaCl, and pH on the growth of *Aeromonas hydrophila*. Journal of Food Science. 50 1417-1421

Palumbo,S.A., Maxino,F., Williams,A.C., Buchanan,R.L. and Thayer,D.W. (1985b)
Starch-Ampicillin agar for the quantitative detection of *Aeromonas hydrophila*. Applied and Environmental Microbiology,**50**, 1027-1030

Pedersen,K.B. (1979)
Occurrence of *Yersinia enterocolitica* in the throat of swine. in: Contributions to Microbiology and Immunology Vol.5, *Yersinia enterocolitica:* Biology, Epidemiology and Pathology, 335-345. Basel, Karger

Pham,Q.T. (1988)
Computation of temperature and quality changes in food processing. Proceedings 25th Meat Industry Research Conference, Hamilton, New Zealand, 130-139

Pham,Q.T. (1991)
Cooling and freezing of hot-boned meat. Proceedings Hot Boning seminar, April 1991, Huka Village, MAF New Zealand

Pooni,G.S. and Mead, G.C. (1984)
Prospective use of temperature function integration for predicting the shelf-life of non-frozen poultry-meat products. Food Microbiology, **1**, 67-78

Popoff,M.Y. (1984)
Aeromonas Kluyver and Van Niel, Bergey's Manual of systematic Bacteriology, Vol.1 (ed Krieg,N.R. and Holt,J.G.), Williams and Wilkins Co., Baltimore. 545-549

Potthast,K. and Hamm,R. (1976)
Biochemie des DFD-Fleisches. Fleischwirtschaft, **56** (7), 978-982

Raccach,M. and Baker,C. (1978)
Lactic acid bacteria as an antispoilage and safety factor in cooked, mechanically deboned poultry meat. Journal of Food Protection, **41**, 703

Renerre,M. (1989)
Retail storage and distribution of meats in modified atmospheres. Fleischwirtschaft International, (1),51-53

Richardson,K.C., Eyles,M.J. and Murrell,W.G. (1978)
Practical aspects of microbiological testing. CSIRO Australia, Food Research Q., **38**, 51-56

Roberts,T.A and Jarvis,B. (1983)
Predictive modelling of food safety with particular reference to *Clostridium botulinum* in model cured meat systems. In *Food Microbiology, Advances and Prospects* (eds Roberts,T.A. and Skinner,F.A.) pp.85-95 New York, Academic Press

Robinson,D.A. (1981)
Infective dose of *Campylobacter jejuni* in milk. British Medical Journal, 282, 1584

Rocourt,J. (1991)
Human listeriosis 1989. World Health Organisation, Food Safety Unit. Report WHO/HPP/FOS/91.3. Institut Pasteur, Paris, France

Rose,S.A. (1986)
Temperature observations on chilled foods from refrigerated retail displays. Technical Memorandum No.423. Campden Food Preservation Research Association. Gloucestershire. England

Rowe,M.T. (1988)
The effect of carbon dioxide on the growth of *Yersinia enterocolitica* in a simulated milk medium. Letters in Applied Microbiology, **7**, 135-137

Ryser,E.T. and Marth,E.H. (1988)
Growth of *Listeria monocytogenes* at different pH values in uncultured whey or whey cultured with *Penicillium camemberti*. Canadian Journal of Microbiology, **34**,730-734

Schlech,W.F (1988)
Virulence characteristics of *Listeria monocytogenes*. Food Technology, April 1988, 176-178

Schlech,W.F., Lavigne,P.M.,Bortolussi,R.A., Allen,A.C., Haldane,E.V., Wort,A.J., Martin,R.S.,Hightower,A.W., Johnson,S.E., King,S.H., Nicholls,E.S. and Broome,C.V. (1983)
Epidemic listeriosis: Evidence of transmission by food. New England Jornal of Medicine, **308**, 203-206

Scott,W.J. (1959)
Water relations of food spoilage microorganisms. Advances in Food Research, **7**, 84

Scott,W.J. and Vickery,J.R. (1939)
Investigations on chilled beef, Part II. Cooling and storage in the meat works. Council of Science and Industrial Research, Australia, Bull. No. 129.

Seeliger,H.P.R. (1961)
Listeriosis. 2nd edtn, Basel-New York, Karger, 37

Seeliger,H.P.R. and Finger,H. (1985)
Listeriosis. In: Infectious diseases of the Fetus and Newborn Infant. 2nd edtn. ed Remington, J.S.& Klein, J.O., Philadelphia, W.B. Saunders, 264-289

Seeliger,H.P.R. and Jones,D. (1986)
Genus *Listeria* Pirie 1940, 383^{AL} In: Bergey's Manual of Systematic Bacteriology, Vol.2 (ed.: Sneath,P.H.A., Mair,N.S., Sharpe,M.E. and Holt,J.G.). The Williams and Wilkins Co. Baltimore 1235-1245.

Seelye,R.J. and Yearbury,B.J. (1979)
Isolation of *Yersinia enterocolitica*-resembling Organisms and *Alteromonas putrefaciens* from Vacuum-packaged Chilled Beef Cuts. Journal of Applied Bacteriology **46**, 493-499

Sharpe,A.N. (1980)
Shortcomings of microbiological counts. In: Food Microbiology: A framework for the future. C.C.Thomas, Springfield,Illinois, 73-95

Silliker,J.H., Woodruff,R.E., Lugg,J.R., Wolfe,S.K. and Brown,W.D. (1976)
Preservation of refrigerated meats with controlled atmospheres:Treatment and post-treatment effects of carbon dioxide on pork and beef. Meat Science, **1**, 195

Simard,R.E., Zee,J. and L'Heureux,L. (1984)
Microbial growth in carcasses and boxed beef during storage. Journal of Food Protection. **47**, 773-777

Slee,K.J. and Button,C. (1990)
Enteritis in sheep and goats due to *Yersinia enterocolitica* infection. Australian Veterinary Journal **67**, 396- 398

Skovgaard,N and Morgen,C. (1988)
Detection of *Listeria* spp. in faeces from animals, in feeds and in raw foods of animal origin. International Journal of Food Microbiology, **6**, 229-242

Smith, M.G. (1985)
The generation time, lag time, and minimum temperature of growth of coliform organisms on meat, and the implications for codes of practice in abattoirs. Journal of Hygiene. Cambridge. **94**, 289-300

Smith,M.G. and Grau,F.H. (1973)
Occurrence of samonellas on the hides and fleeces of cattle and sheep at slaughter. Australian Veterinary Journal, **49**, 218-219

Swaminathan,B., Harmon,M.C. and Mehlman,I.J. (1982)
A Review: *Yersinia enterocolitica.* Journal of Applied Bacteriology, **52**, 151-183

Stolle,A (1981)
Spreading of samonellas during cattle slaughtering. Journal of applied Bacteriology, **50**, 239-245

Tarrant,P.V. (1989)
Animal Behaviour and Environment in the Dark-Cutting Condition in Beef - A Review. Irish Journal of Food Science and Technology, **13**, 1-21

Taylor, A.A. and Shaw, B.G. (1977)
The effect of meat pH and packaging permeability on putrification and greening of vacuum packaged beef. Journal of Food Technology, **12**, 515

Vandepitte.J and Wauters.G. (1979)
Epidemiological and clinical aspects of human *Yersinia enterocolitica* infections in Belgium. In: Contributions to Microbiology and Immunology Vol.5. *Yersinia enterocolitica: Biology, Epidemiology and Pathology* 150-159. Basel, Karger.

Van Laack,R.L.J.M. and Smulders,F.J.M. (1989)
Quality of 'semi-hot' and cold boned vacuum-packaged fresh pork as affected by delayed or immediate chilling. Journal of Food Protection, **52**, 650-654

Walker,S.J., Archer,P. and Banks,J.G. (1990)
Growth of *Listeria monocytogenes* at refrigeration temperatures. Journal of Applied Bacteriology, **68**,157-162

Wauters,G. (1979)
Carriage of *Yersinia enterocolitica* serotype 3 by pigs as source of human infection. In: Contributions to Microbiology and Immunology Vol.5, *Yersinia enterocolitica:* Biology, Epidemiology and Pathology, 249-252. Basel, Karger

Weis,J. and Seeliger,H.P.R. (1975)
Incidence of *Listeria monocytogenes* in nature. Applied Microbiology **30**, 29-32

West,R.L., Berry,B.W., Smith,G.C., Carpenter,C.L. and Hoke,K.E. (1972)
Microbial studies in a beef distribution centre. Journal of Animal Science, 35, 209

Winblad,S. (1973)
The clinical panorama of human *Yersiniosis enterovcolitica*. In: Contributions to Microbiology and Immunobiology Vol.2. Yersinia, Pasteurella and Francisella. 129-132. Basel. Karger

Wirth,F, Böhm,H. and Salziger,G. (1976)
Technologie bei DFD-Fleisch. Fleischwirtschaft, 56 (7), 988-994

Zwietering,M.H., deKoos,J.T., Hasenack,B.E., deWit,J.C. and van’t Riet,K. (1991)
Modeling of bacterial growth as a function of temperature. Applied and Environmental Microbiology, 57 (4), 1094-1101

VII. APPENDIX

BRAIN HEART INFUSION BROTH

Calf Brain infusion	200 ml
Beef heart infusion	250 ml
Peptone	10 g
Sodium chloride	5 g
Disodium hydrogen phosphate	2.5 g
Glucose	2 g

Dissolve all ingredients and sterilize at 121°C for 15 min.

CARBOHYDRATES

Base medium

Peptone	10 g
Lam lemco	1 g
NaCl	5 g
Distilled water	900 ml

1 ml phenol red pH indicator (360 mg in 20 ml 0.1 N NaOH)

30 ml of filter sterilized (f.s.) sugar solutions (either 5% xylose or rhamnose or 10% mannitol) were added to 270 ml autoclaved base medium. The made-up medium was then dispensed aseptically into sterile 5 ml plastic tubes. These were inoculated with the organism and incubated at +25°C for 48 hrs.

A change in colour to yellow denoted a positive test result.

CATALASE

The culture was selected from a PCA plate and by means of an inoculating needle placed on a clean glass slide. A drop of 30% H_2O_2 was added with a Pasteur pipette onto the culture on the slide. Immediate gas production indicated a positive test.

CEFSOLUDIN-IRGASAN-NOVOBIOCIN (CIN) AGAR

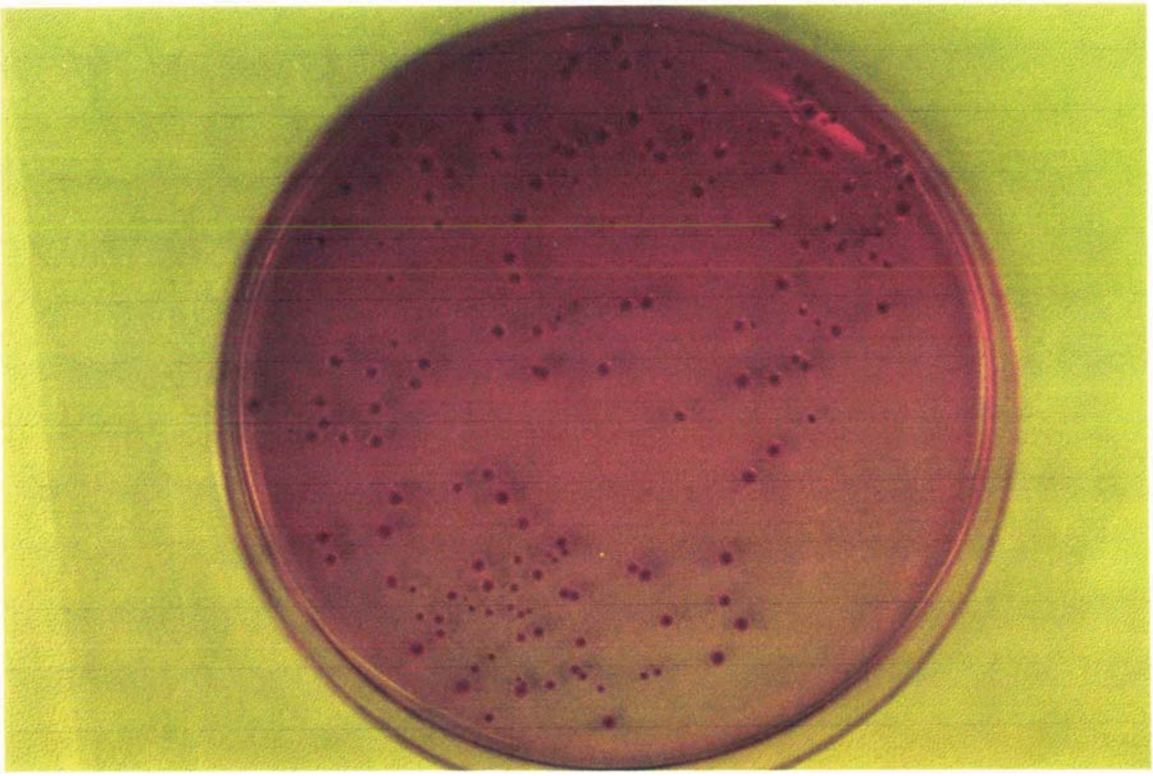
Peptone	20 all g/l
Yeast extract	2
Mannitol	20
Sodium pyruvate	2
Magnesium sulphate	0.01
Sodium desoxylate	0.5
Neutral red	0.03
Crystal violet	0.001
Agar	12.5

Antibiotic supplement (per vial)

Cefsulodin	7.5 mg
Irgasan	2 mg
Novobiocin	1.25 mg

Suspend 29 g in 500 ml distilled water and heat and dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Temper to 50°C and aseptically add the antibiotic supplement from a vial.

Plate 1: Growth of *Yersinia enterocolitica* on CIN-Agar



ESCULIN TEST

Bile Esculin Medium (Difco) was prepared and, dispensed into glass tubes, autoclaved, and allowed to cool in a slanted position. The slanted surface was inoculated with the culture and incubated for 48 hrs at +25°C. Positive samples exhibited a black colour (esculin hydrolysis) on the slant.

Gram stain

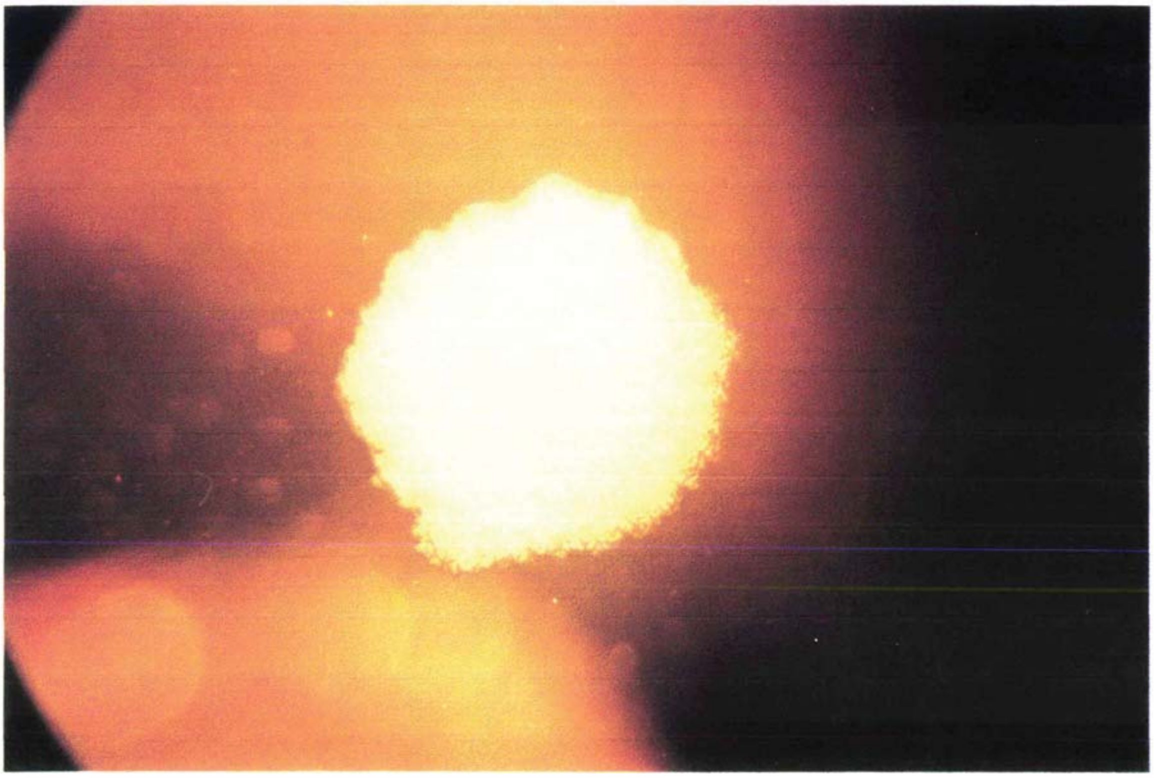
A light suspension of cells of the organism was prepared on a clean glass slide in a drop of distilled water, air-dried and fixed by passing over a bunsen flame. The slide was then flooded with ammonium oxalate- crystal violet solution for 30 seconds. This was washed- off and the slide then covered with Lugol's iodine for 30 seconds. Thereafter the slide was washed again and covered with iodine acetone until the colour started to fade. It was then washed and counter stained with dilute carbol-fuchsin for 30 seconds. Before examination under a microscope the slide was washed and air-dried.

LITHIUM CHLORIDE PHENYLETHANOL MOXALACTAM AGAR

Phenylethanol Agar (Difco)	35.5 g
Glycine anhydride	10 g
Lithium chloride	5 g
Distilled water	1 l

autoclaved at 121°C for 15 minutes and tempered to +50°C, with 2 ml f.s. 1% moxalactam (Sigma Chemical Company: M 1900) added. Poured in thin (10- 12 ml) plates.

Plate 2: Growth of *Listeria monocytogenes* on Lithium Chloride-Phenylethanol-Moxalactam Agar



METHYL RED TEST

MR/VP Broth (Difco) was dispensed into 5 ml tubes and sterilised. The tubes were inoculated with the test organism and incubated for 3 to 5 days at +25°C.

A few drops of methyl red pH indicator (0.1 g of methyl red dissolved in 300 ml of 95% ethyl alcohol) were added and development of a distinct red colour regarded as a positive result.

Minimal Salts Medium

KH_2PO_4	8.6 g
K_2HPO_4	14.6 g
NaCl	1.0 g
$(NH_4)_2SO_4$	1.0 g
Nitrilotriacetic acid	0.1 g
$MgSO_4$	0.04 g
$Fe(NH_4)_2(SO_4)_2$	0.02 g
$CaCl_2$	0.01 g
Glucose	0.1 g

Dissolve in 1 litre of distilled water and sterilize at 121°C for 15 minutes.

MOTILITY MEDIUM

Motility medium (Difco) was dispensed into 10 ml glass tubes and autoclaved and allowed to cool overnight. The culture was then transferred from the plate via an inoculating needle into the centre of the tube by stab inoculation. After 48 hrs of incubation at +25°C *Listeria* showed characteristic "umbrella"- like growth away from the stab and the surface. *Yersinia* exhibited growth centrifugally away from the stab after incubation of the medium at +25°C, but not at +37°C.

NITRATE

I ml of Nitrate Broth (Difco) was sterilised and inoculated with a heavy inoculum of the organism and incubated at +25°C for up to 5 days. 5 drops of reagents A and B (see below) were added and the tube shaken. A positive result was indicated by the development of a red colour and was completed within 2 minutes. To negative sample tubes a pinch of zinc dust was added. A colour reaction completed the test.

Reagent A: 0.6% Dimethyl-α-naphthylamine in acetic acid 30%

Reagent B: 0.8% sulfanilic acid in acetic acid 30%

OXIDASE

A filter paper was placed in a Petri dish. A few drops of Kovacs' reagent (see below) were added at the centre and the organism smeared onto the paper with a platinum loop. A colour reaction occurred in positive samples within seconds.

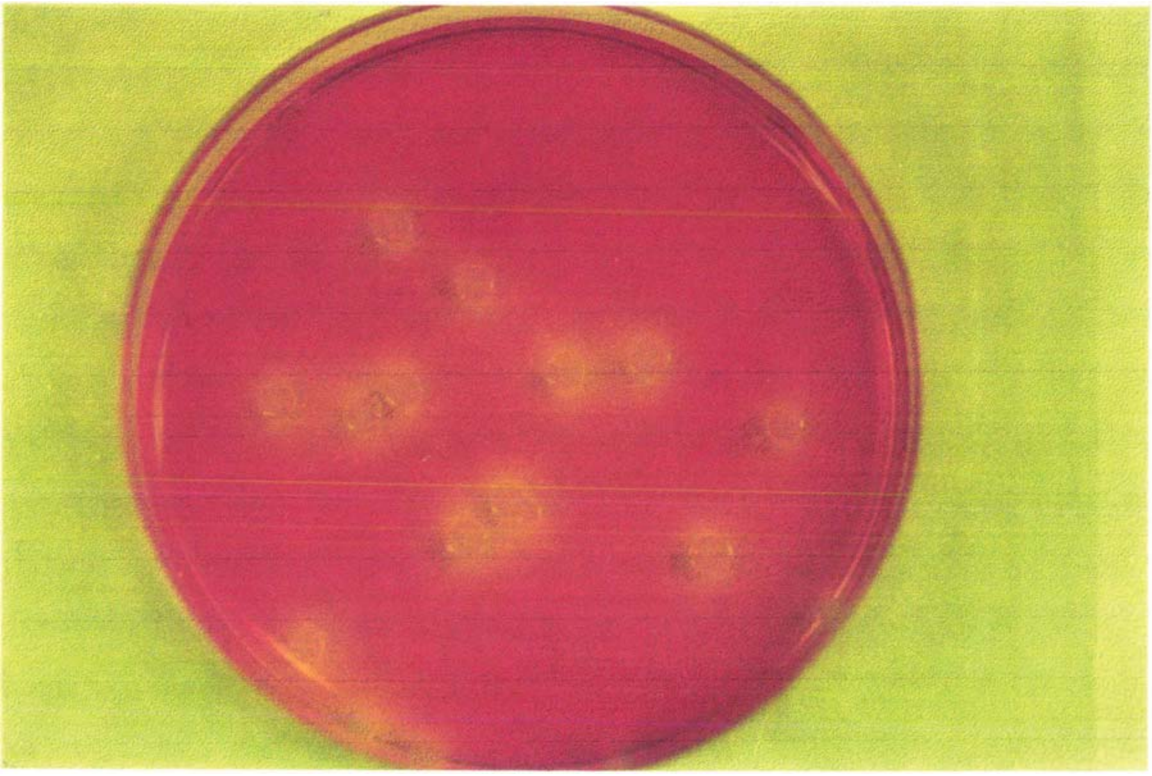
Kovacs'reagent: 0.5% tetramethyl-p-phenylenediamine dihydrochloride

STARCH AMPICILLIN AGAR

Phenol Red Agar Base (Difco)	31 g
Soluble Starch	10 g
Distilled water	1 l

autoclaved at 121°C for 15 minutes and tempered to +50°C, with 10 mg of filter sterilized (f.s.) Ampicillin (Sigma Chemical Company: A 9393) added.

Plate 3: Growth of *Aeromonas hydrophila* on Starch-Ampicillin-Agar



VIOLET-RED BILE AGAR

Yeast extract	3 g
Peptone	7 g
Sodium chloride	5 g
Bile salts No.3	1.5 g
Lactose	10 g
Neutral red (1%)	3 ml
Crystal Violet (0.1%)	2 ml

Add all ingredients to 1 litre distilled water and heat to boiling. Do not sterilize.

VOGES- PROSKAUER TEST

5 mls of MR/VP Broth (Difco) were autoclaved and inoculated with the culture and incubate for 48 hrs at +25°C. 10 drops of reagent A and after that 4 drops of reagent B were added to the tube and then tube then shaken. A red to pinkish ring developed in positive samples within up to 15 minutes.