The bacteria involved in meat spoilage and the effect of environmental factors on their growth

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Declaration

I declare that all the work presented in this thesis is my own performed under the supervision of Dr Heather Craven and Prof. Margaret Britz. The contribution and collaboration of others has been specifically acknowledged. All sources of information used have been referenced. None of the information in this thesis has previously been submitted for any other degree.

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Abbreviations

ADH	Arginine dehydrogenase
ADP	Adenosine diphosphate
APT	All purpose tween
ATP	Adenosine triphosphate
BHIA	Brain heart infusion agar
°C	Degrees Celsius
cfu	Colony forming units
CHEF	Contour clamped homogeneous electrical field
cm	Centimeter
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid disodium salt
g	Grams
GC	Guanine and cytosine
h	Hours
HCl	Hydrochloric acid
HHD	Heterofermentative and homofermentative differentiation media
Kb	Kilo bases (10^3 bases)
kDa	Kilo Dalton
kPa	Kilo Pascal
L	Litre
LA	Lactic acid
LAB	Lactic acid bacteria
M	Moles per litre
MAP	Modified atmosphere packaging
Mb	Mega base (10^6 bases)
meso-DAP	*meso*-diamino pimelic acid
mg	milligrams
µL	Microlitre
µm	Micrometer
μ_{max}	Maximum specific growth rate
mL	Millilitre
MRSA	deMann Rogosa Sharpe agar
MRSB	deMann Rogosa Sharpe broth
N_2	Nitrogen
NA	Nutrient agar
NaOH	sodium hydroxide
NB	Nutrient broth
O_2	Oxygen
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PCA	Plate count agar
PSA	*Pseudomonas* selective agar
PSE	pale soft exudative
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SAHN	sequential, agglomerative, hierarchical and nested clustering
STAA	Streptomycin thallous acetate agar
t_{90}	time taken to reach 90% of the maximum population
TAE	Tris acetate EDTA buffer
TBE	Tris boric acid EDTA buffer

TE	Tris EDTA buffer
TEMED	N,N,N',N'-tetramethylenediamine
TLC	Thin layer chromatography
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TYGA	Tryptone yeast extract glucose agar
TYGB	Tryptone yeast extract glucose broth
VRBGA	Violet red bile glucose agar
v/v	Volume to volume
w/v	Weight to volume
UPGMA	Unweighted pair-grouping method of arithmetic averages

Abstract

The contamination of meat surfaces with microorganisms begins at slaughter then continues throughout the butchering and beyond. The development of the microflora on the meat surface depends on the storage conditions, in particular, on gaseous atmosphere during storage. Beef chicken, lamb and pork were packaged in air or under 30% CO_2/70% N_2 then stored at either one or 10°C until considered visually unacceptable. The meat stored at 10°C had a shorter shelf-life than the meat stored at 1°C, with the air-packaged meat deteriorating before MAP meat. In general, the shelf-life for beef and chicken was greater than for lamb and pork.

Brochothrix, *Enterobacteriaceae*, lactic acid bacteria (LAB) and *Pseudomonas* species from the meats were enumerated using both non-selective and selective media. The microflora of air-packaged meats isolated on the non-selective media was dominated by *Pseudomonas* species, with *Brochothrix, Enterobacteriaceae* and LAB also detected but in lower numbers. *Acinetobacter* was also isolated from lamb. There was one exception, on lamb stored at 10°C there were more *Enterobacteriaceae* than *Pseudomonas.* On the MAP meat, LAB dominated the microflora, some *Brochothrix* and *Enterobacteriaceae* were also detected. Again there was one exception, at 10°C on pork there were more *Enterobacteriaceae* than LAB.

A similar pattern emerged with the selective media. *Pseudomonas* and LAB were the most numerous groups isolated from air-packaged and MAP meats, respectively. The advantage of selective media was that it enabled the detection of bacterial species that were present in numbers that were 100-fold less than the dominant organisms. Using selective media, *Pseudomonas* could be detected on the MAP, and more *Brochothrix* and *Enterobacteriaceae* isolates could be detected on MAP meats as compared to the numbers of these isolates detected using non-selective media.

For *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* two isolates which were deemed to be the most prevalent based on colony morphology were identified to species level. All the *Brochothrix* isolates were identified as *B. thermosphacta*. A majority of the 17 *Enterobacteriaceae* species were identified as *H. alvei*, three were identified as *S. liquefaciens*, one as *S. marcescens*, one as *E. vulneris* and one was not successfully identified. Of the LAB isolates nine isolates were identified at *Lb. sake*, three as *C. divergens*, three as *Lb. curvatus* and two a *Lb. farciminis*. Three of the eight *Pseudomonas* isolates were identified as *P. fluorescens* and the remainder as *P. fragi*.

The organisms important in the spoilage of MAP meats, *Brochothrix*, *Carnobacterium* and *Lactobacillus*, were fingerprinted using pulsed field gel electorphoresis (PFGE). All fingerprints were produced using *Sma*I and running gels with pulse times of one to 35 second for 24 hours. For the *B. themopshacta* strains no two fingerprints were identical. The genome size of the *B. thermosphacta* isolates ranged from 1.85 to 3Mb. For the *C. divergens* strains, two isolates had identtical fingerprints while the third isolate had one band that was 10kb larger. The genome sizes of the *C. divergens* strains were 2.52 to 2.53kb. The PFGE fingerprinting of the *Lactobacillus* species was not as successful as for *B. thermosphacta* or *C. divergens.* Only *Sma*I restricted the *Lactobacillus* DNA but it produced a large number of small bands. Although this was undesirable and prohibitive to genome sizing, it was still possible to see some patterns in the fingerprints. Some of the isolates identified as *Lb. sake* had the same fingerprints as isolated identified as *Lb. curvatus* and *Lb. farciminis*. The PFGE fingerprint results for *Lactobacillus,* brought the validity of the speciation results into question.

The development of the microflora into a spoilage population was dependent upon the environmental conditions. Temperature, pH, sodium chloride, atmosphere and preservative all had a role in determining the ultimate microflora. For each species identified the effect of pH 4.5-7.0, 0-5% sodium chloride, 0-1% potassium sorbate and 0-0.5% methyl- and 0-0.1%propyl-paraben on growth was determined. For representative isolates, *B. thermosphacta* A13B03, *H. alvei* M23En02, *Lb. sake* M11L03 and *P. fluorescens* A11P04, the effect of temperature (0-15°C) and carbon dioxide (0-100%) was examined. Information from these studies provided the background for assessing the impact of the environmental factors alone and in combination on a mixed population and the individual members of that population.

In general, there was no significant difference among the strains within each group. There were however, two exceptions. Firstly, *B. thermosphacta* A13B03 at pH 5.0 had a significantly higher specific growth rate than the other two *B. thermosphacta* strains. Secondly, *C. divergens* had a higher specific growth rate than the *Lactobacillus* strains at one and 3% sodium chloride. As the pH increased the specific growth rate of each bacterial species increased, except for *Lb. curvatus* and *Lb. sake* where there was a decrease in growth rate as the pH increased to 6.5 and 7.0. As the levels of sodium chloride, potassium sorbate and methyl- and propyl-paraben increased, the specific growth rates of *B. thermosphacta* and *H. alvei* strains and LAB and *Pseudomonas* species decreased. Propyl-paraben was more inhibitory than either methyl-paraben or potassium sorbate. In turn, methyl-paraben was more inhibitory than potassium sorbate for *H. alvei* strains and LAB, but the reverse was true for *B. thermosphacta.* For *Pseudomonas* methyl-paraben and potassium paraben had similar inhibitory effects at the same concentrations.

The effects of temperature and atmosphere, in terms of carbon dioxide, were examined on representative strains. At 15°C, all bacterial species were able to increase to $>10^7$cfu/cm^2. The initial number of bacteria was not significantly influenced by either temperature or carbon dioxide. Temperature affected μ_{max}, time to μ_{max}, the rate parameter, time to 90% of the maximum population size (t_{90}) and the increase in cell density of *B. thermosphacta* and *Lb. sake*. Only t_{90} was significantly affected by either temperature or carbon dioxide for *H. alvei*. Although *H. alvei* did not grow at either zero or 5°C. Carbon dioxide did not affect as many aspects of the growth as temperature had.. Only μ_{max} and the rate parameter of *P. fluorescens* were significantly decreased by carbon dioxide while μ_{max}, the rate parameter, and increase in cell density of *B. thermosphacta* were affected by carbon dioxide. The significance of the interaction between temperature and carbon dioxide could not be determined statically due to insufficient data but it was possible to infer an interaction. The effect of temperature and carbon dioxide combined could be described as follows: as the temperature decreased the effect of carbon dioxide increased by slowing the growth rate of all bacteria species only *Lb. sake* was unaffected by increased carbon dioxide levels.

Environmental factors can combine to inhibit or retard bacteria at levels that were otherwise non-inhibitory. The effect of temperature, pH, carbon dioxide and preservatives (sodium chloride, potassium sorbate and propyl-paraben), on a mixed population and each member of the population was determined. The increase in the number of the total population reflected the growth exhibited by the dominant organism. In air, *P. fluorescens* dominated the population while under 25 and 100% CO_2 *Lb. sake* was dominant. *P. fluorescens* and *H. alvei* both failed to grow under carbon dioxide in a mixed population although they had been able to grow in the absence of other organisms. Factors that influenced the population as a whole did not necessarily influence the individual members of that population. *Lb. sake* was the least affected by the environment while *B. thermosphacta* was the most sensitive. It was not possible to examine the impact of the environmental factors on the growth of *H. alvei* due to lack of growth.

List of Publications

Baxter, N.J., Craven, H.M. and Britz, M.L. (1994). Microorganisms involved in the spoilage of refrigerated meat packaged in air of modified atmosphere. *Proceedings of the Australian Society for Microbiology*. A111.

Baxter, N.J., Craven, H.M. and Britz, M.L. (1995). A comparative study of microorganisms involved in spoilage in air and modified atmosphere. *Proceedings of the Eighth Australian Food Microbiology Conference*. P14.

Baxter, N.J., Craven, H.M. and Britz, M.L. (1996). A log-logistic model of *Lactobacillus sake* under CO_2 at 5, 10 and 15°C. *Proceedings of the Second International Conference on Predictive Microbiology*. P6.3.

Baxter, N. J., Craven, H.M. and Britz, M.L. (1998). Biochemical and pulsed-field gel electrophoresis analysis of *Brochothrix* species isolated from aerobically and modified atmosphere packaged beef and lamb. *Food Australia*. **50**: 249

Aims of this thesis

This thesis is composed of two main bodies of work. The first encompassing the spoilage of air-packaged and modified atmospherically packaged meat and the second the effect of environmental factors on the growth of meat spoilage bacteria.

1 To study the distribution of spoilage bacteria on beef, chicken, lamb and pork from the one source stored at 1 or 10°C after packaging in air or under modified atmosphere and isolate the bacteria present using selective and non-selective media.

2 To identify to species level the most prevalent bacteria, determined on the basis of colony morphology.

3 To fingerprint bacteria which are significant in the spoilage of modified atmosphere packaged meat using pulsed-field gel electrophoresis.

4 To determine the effect of pH and the concentration of preservative (sodium chloride, potassium sorbate and methyl- and propyl-paraben) on the growth rate of representative strains of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* on solid media.

5 To determine the effect of temperature and carbon dioxide on the growth parameters of *B. thermosphacta, H. alvei, L. sakei* and *P. fluorescens*

6 To determine the effect of environmental factors alone and in combination on the growth of an artificially inoculated mixed population and the individual members of the population on the surface of solid medium.

Chapter 1

General Introduction

1.1 FROM MUSCLE TO MEAT

In order for meat to be produced, animals must be slaughtered. The processes through which the animals are slaughtered depends on the species. Cattle and sheep are slaughtered in a similar process which differs from both pigs and poultry.

1.1.1 Slaughter of cattle and sheep

The process for the slaughter of cattle and sheep is demonstrated in Figure 1.1. Prior to slaughter the animals are rested and inspected to ensure that they are healthy (Parry, 1989; Anonymous, 1993; Varnam and Sutherland, 1995). The cattle are washed prior to slaughter to remove dirt and faecal matter from the hide before entering the slaughter house (Anonymous, 1993). If the sheep are flyblown they should be rested, trimmed and treated prior to slaughter or if the sheep are wet they should be allowed to dry out prior to slaughter (Anonymous 1993).

Inside the slaughter house the animals are stunned, cattle by captive bolt (Anonymous, 1993; Varnam and Sutherland, 1995) and sheep with electricity (Anonymous, 1993). The hindquarters are then shackled, the animal is stuck with a knife to sever the anterior and posterior aorta and bled (Anonymous, 1993). After bleeding, the head is removed for inspection, then any edible parts are removed for further processing. The hide and pelt are removed by pulling the hide away from the carcass from the hindquarters down toward the forequarters, thus preventing the outer surface of the

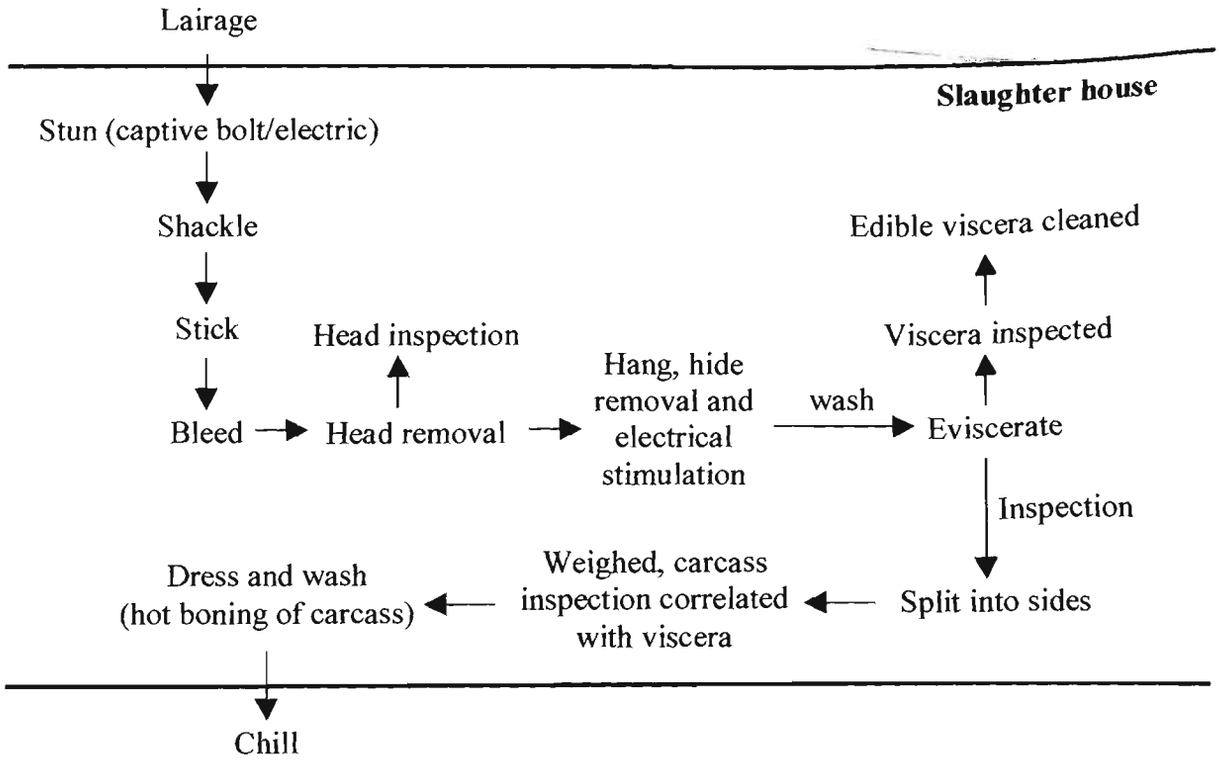


Figure 1.1 A flow diagram of the stages in the slaughter of cattle and sheep (adapted from Anonymous, 1993 and Varnam and Sutherland, 1995).

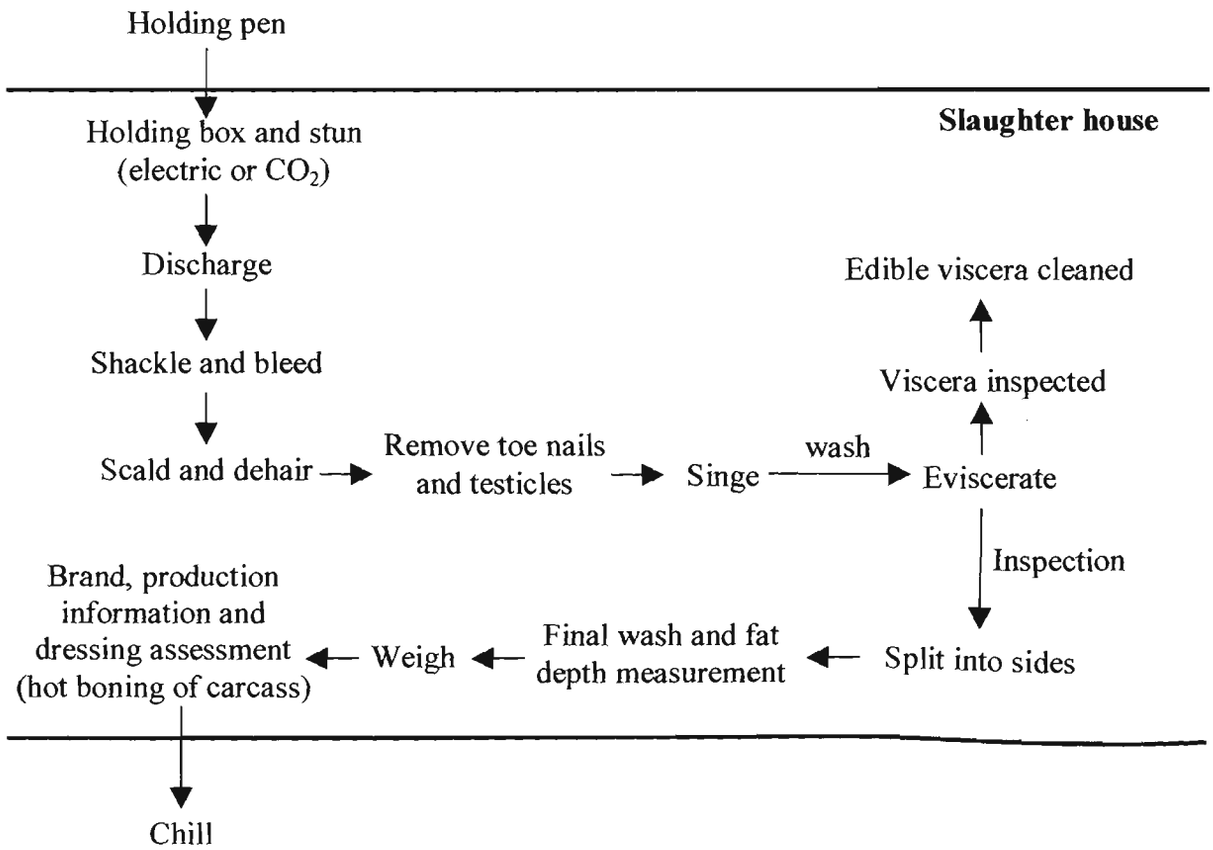


Figure 1.2 A flow diagram of the stages in the slaughter of pigs (adapted from Anonymous, 1993 and Varnam and Sutherland, 1995).

hide coming into contact with the muscle (Anonymous, 1993). Prior to evisceration, the carcasses can be electrically stimulated. This causes a decrease in the muscle pH reducing the risk of cold shortening (Kotula and Emswiler-Rose, 1981; Varnam and Sutherland, 1995; Pisula, and Tyburcy, 1996). Cold shortening is the irreversible contraction of the muscle (Davey *et al.*, 1967). The viscera of the animals are removed and inspected, then any edible organs are taken for further processing (Anonymous 1993; Varnam and Sutherland, 1995). The carcass and viscera move through the processing chain at the same rate to facilitate correlation during inspections (Anonymous, 1993). Finally the carcasses are split into sides, weighed, dressed and washed with water (Anonymous, 1993; Varnam and Sutherland, 1995). Prior to chilling the carcass can be boned which is termed hot-boning (Kotula, 1981; Varnam and Sutherland, 1995), or the carcass can be chilled and matured for 48 hours to one week prior to boning referred to as cold-boning (Kotula, 1981; Varnam and Sutherland, 1995).

1.1.2 The slaughter of pigs

The slaughter of pigs (Figure 1.2) differs from cattle and sheep in that the skin of the animal is not usually removed (Anonymous, 1993; Varnam and Sutherland, 1995). Pigs need to be handled carefully as they are more prone to stress which can result in undesirable pale soft exudative (PSE; Varnam and Sutherland, 1995) meat. A pig is ushered into a holding box where the animal is electrically stunned to render it unconscious. Alternatively, carbon dioxide can be used for stunning as this can reduce the occurrence of PSE because rigor does not occur as quickly (Varnam and Sutherland, 1995). The animal is removed from the box, the hindquarters are shackled then the animal bled by severing the blood vessels in the neck (Anonymous, 1993). The toenails and testicles are then removed (Anonymous, 1993). The carcasses can be de-haired by scalding in water at 62°C, where the carcass remains in the water until the hair and scuff can be easily removed (Grau, 1986; Anonymous, 1993; Varnam and Sutherland, 1995). The carcasses are singed then given a pre-evisceration scrape and wash in water (Grau, 1986). The viscera is removed, inspected and the parts for human consumption are further processed. The carcass is washed, fat depth measured, weighted, branded and dressed. Similar to cattle and sheep, pork can be hot-boned then chilled or chilled prior to boning (Anonymous, 1993; Varnam and Sutherland, 1995).

1.1.3 The slaughter of poultry

Despite the physiological difference between poultry and the animals already discussed, the slaughtering process follows a similar process (Figure 1.3). The birds are brought into the processing plant and electrically stunned prior to the jugular vein being cut (Parry, 1989; Varnam and Sutherland, 1995). The birds are bled prior to scalding and de-feathering. The scalding facilitates the removal of

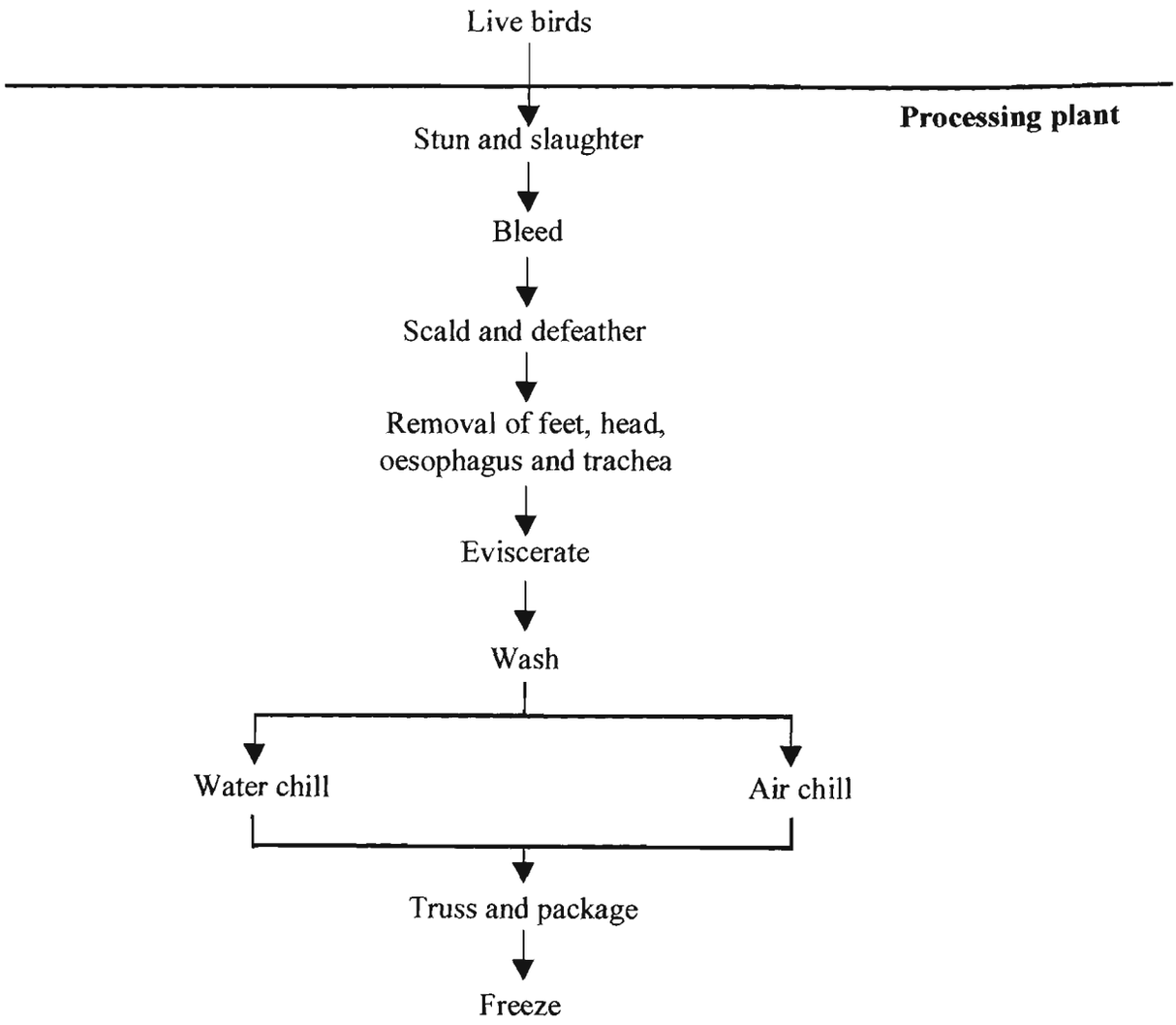


Figure 1.3 A flow diagram of the stages in the slaughter of poultry (From Varnam and Sutherland, 1995).

the feathers (Grau, 1986; Parry, 1989; Varnam and Sutherland, 1995). The temperature at which the bird is scalded depends on whether the bird will be sold fresh or frozen (Parry, 1989). If the bird is to be sold fresh, the scalding temperature is 50-51.5°C to ensure that the epidermis remains intact, while those for freezing are scalded at 56-60°C (Parry, 1989). The birds are de-feathered mechanically by passing the carcass through as series of rubber paddles with "fingers" attached (Parry, 1989; Varnam and Sutherland, 1989). The carcass is washed in water, then the feet and head removed. The head, oesophagus and trachea are removed in one action, this assists in the later removal of the viscera (Parry, 1989). After evisceration, the viscera are inspected, edible parts of the viscera are removed and washed in water prior to chilling (Parry, 1989). The chilling of the carcass can be conducted using chilled water or air. Water chilling involves immersing the carcasses in water that is chilled either by refrigeration or by the addition of ice and the carcasses are agitated to move in the opposite direction of water flow (Parry, 1989). The immersion of carcasses in chilled water and ice was found to be the major source of bacterial contamination by psychrotrophic bacteria (Thomas and McMeekin, 1980), *Staphylococcus aureus* (Cunningham, 1982) and possibly *Salmonella* contamination (Parry, 1989). Consequently, the immersion-chill method is considered undesirable by some retailers, so that chiller rooms or on occasions air blast tunnels are used alternatively to chill carcasses (Parry 1989). As with the other animal types, poultry can also be hot-boned prior to chilling.

1.2 MICROBIAL CONTAMINATION OF CARCASSES

Microbial contamination of meat is a random and inevitable occurrence during slaughter (Lasta and Fonrouge, 1988; Dickson and Anderson, 1992). Contamination comes from hooves, feathers, fleece, intestines, hide, faeces, personnel, slaughtering equipment, air, water and soil (Empey and Scott, 1939; Ayers, 1955; Lasta and Fonrouge; Nortjé *et al.*, 1990; Widders *et al.*, 1995). Lairage slurry, hair and rumen contents have been found to contain up to 10^{10} bacteria per gram (Patterson and Gibbs, 1978). Bacterial numbers as high as 10^7 colony forming units (cfu)/cm^2 have been found on the floor of slaughter houses (Mettler and Carpentier, 1998).

Contamination of carcasses surfaces is not uniform and there can be considerable variation in the microbial load within a carcass, within slaughter runs, between slaughter runs and from abattoir to abattoir (Simard *et al.*, 1984; Nortjé and Naudé, 1987; Nortjé *et al.*, 1990; Prieto *et al.*, 1991; Knudtson and Hartman, 1993; Prieto *et al.*, 1993; Renwick *et al.*, 1993; Jericho *et al.*, 1994; Kotula and Pandya, 1995; Rahkio and Korkeala, 1997; Untermann *et al.*, 1997). The rates of contamination can fluctuate with the seasons (Newton *et al.*, 1978; Ingram and Simonsen, 1980; Simard *et al.*, 1984; Grau, 1986; Rao and Ramesh, 1992; Renwick *et al.*, 1993), with higher microbial loads occurring in the winter months. Seasonal variation will depend on the geographical area: for example Rao and

Ramesh (1992) found no seasonal variation in Indian abattoirs where summer temperature was 30°C ±2°C and winter 27°C ±2°C.

Contamination occurs at each stage of the slaughter process. In general, contamination was restricted to the surface, the deep tissues were not contaminated during the slaughter process (Mackey and Derrick, 1979). But contamination can occur whenever the muscle surface has been damaged (Rodrigues-Szule *et al.,* 1990), thus allowing further penetration into the muscle. Marker organisms have been used by Mackey and Derrick (1979) to assess the possible contamination of deep tissues by organisms on captive bolts for cattle and stick knives for sheep. They found that the marker bacteria could be detected in the spleen and musculature of the slaughtered cattle and sheep.

A significant amount of microbial contamination of carcasses occurs when the hide, fleece or feathers are removed (Patterson and Gibbs, 1978; Nortjé and Naudé, 1981; Grau, 1986; Anand *et al.,* 1989; Rao and Ramesh, 1992; Bell *et al.,* 1993; Geornaras and von Holy, 1994; Gill *et al.,* 1996; Bell, 1997; Untermann *et al.,* 1997). The hide and fleece can contain large numbers of bacteria accumulated from the environment and faecal matter (Ingram and Simonsen, 1980). Bacteria are carried from the hide or fleece to the meat by the knives used to make the first incision (Grau, 1986). Carcasses produced from lamb which still had their fleece at slaughter had higher microbial loads than shorn lambs (Biss and Hathaway, 1996). Lambs that were washed prior to slaughter had a higher carcass microbial load than those that were not (Biss and Hathaway, 1996). The hands of personnel are also important in the spread of bacteria from hide to meat (Grau, 1986): bacterial numbers of 10^4-10^5 cfu/cm^2 have been detected on workers' hands (Patterson and Gibb, 1978). The mechanical removal of hides can improve the microbial quality of the carcass by reducing hand contact (Field *et al.,* 1991). The highest microbial counts in slaughterhouse condensates were found near hide removal, head inspection and evisceration areas (Worfel, *et al.,* 1995). The area around a hide-puller in a beef slaughter house was found to have Gram-negative psychrotrophic bacteria widely spread around it, while *Pseudomonas* was the dominant airborne organism behind the hide-puller (Gustavsson and Borch, 1993). The microbial load of pigs is decreased following scalding and singeing (Dockerty, *et al.,* 1970). Contamination occurs instead during polishing (Knudtson and Hartman, 1993; Schaefer-Seidler, *et al.,* 1984) and small increases in mesophile counts can occur during evisceration (Dockerty *et al.,* 1970). For poultry, the mechanical removal of feathers was shown to increase the microbial contamination of carcasses through cross contamination and the creation of aerosols (Kotula and Kinner, 1964; Patterson 1973; Bryan, 1980; Anand *et al.,* 1989; Varnam and Sutherland, 1995).

Microbial contamination continues as the carcass continues down the processing line. Contamination can increase during evisceration (Cunningham, 1982; Rao and Ramesh, 1992) particularly if the intestinal organs are ruptured (Rao and Ramesh, 1992), although this tends to be a rare occurrence

(Grau, 1986). The number of *Salmonella* and *Enterobacteriaceae* can increase on the carcass surface during evisceration (Grau, 1986). Aerial contamination during boning was found to be insignificant, only the large scale movement of personnel increased the number of bacteria detected (Newton *et al.*, 1975). Instead there was a connection between air-borne bacteria and meat contamination in the packaging room, particularly when personnel moved from a more contaminated area (Rahkio and Korkeala, 1997). Cross-contamination during the boning process occurs primarily on the cutting boards (Widders *et al.*, 1995).

Some sites on the carcass are more prone to contamination than others, the degree to which an area of a carcass is handled will determine contamination (Sheridan and Lynch, 1980; Nortjé and Naudé, 1981; Cunningham, 1982; Schaefer-Saidler *et al.*, 1984; Simard *et al.*, 1984; Lasta and Fonrouge, 1988; Nortjé *et al.*, 1989; Kotula and Pandya, 1995; Untermann *et al.*, 1997). The hide is removed from the hindquarters down to the forequarters, as this minimises the chance of hide rolling onto the carcass (Anonymous, 1993), a major source of contamination (Grau, 1986). The forearms and the brisket (forequarter) have higher bacterial loads than the hindquarters (Nortjé *et al.*, 1990; Bell, 1997; Untermann *et al.*, 1997). Beef carcasses were shown to have higher microbial loads on the neck than on the rest of the carcass (Sheridan and Lynch, 1979). The mechanical removal of pelts from lambs decreased the microbial load on the leg but not on the shoulder, as hand contact was still required at the beginning of mechanical pelt removal (Field *et al.*, 1991).

A decrease in bacterial numbers on carcasses occurs during the chilling process, a decrease due to chilling. When beef carcasses were chilled, the number of bacteria present decreased to $<10^2$ cfu/cm^2 (Nortjé and Naudé, 1981). Microbial numbers on chicken (Cunningham, 1982; Anand *et al.*, 1989; Kotula and Pandya, 1995) and pork (Ockerman, *et al.*, 1977) carcasses were decreased by chilling. Primarily, the decrease in bacterial numbers is due to the death of bacteria from cold shock and the reduction in water activity on the meat surface (Nortjé and Naudé, 1981; Gill, 1986). In addition, the numbers of yeasts and moulds in the chiller are less than in other areas of pork processing plants (Kotula and Emswiler-Rose, 1988).

1.3 MINIMISING BACTERIAL CONTAMINATION AND CARCASS DECONTAMINATION

There are several steps through which microbial contamination can be minimised. Contamination of chicken flocks can be reduced by cleaning and disinfecting transport crates and vehicles (Mead *et al.*, 1994). Washing knives between carcasses during bleeding and hide/pelt removal will reduce cross contamination (Childer *et al.*, 1973; Anonymous, 1993). Cleaning of cutting boards also will minimise cross contamination (Coates *et al.*, 1995; Widders *et al.*, 1995).

Attempts to decontaminate carcasses by cold water washing were ineffective at removing bacteria, instead there was a redistribution of bacteria over the carcasses (Childers *et al.,* 1973; Bell *et al.,* 1996a; Bell, 1997). Microbial numbers were decreased on sheep carcasses washed with water/steam or water at 82.2°C (Dorsa *et al.,* 1996). If an acidified spray, in particular a hot acid spray, was used there was a decrease in the bacterial contamination of beef carcasses (Mashall *et al.*, 1977; Cudjoe, 1988). Although Ockerman *et al.* (1974) found that there was no significant decrease in the number of bacteria on sheep carcasses following spraying with six to 24% acetic acid, this can be attributed to the sampling method employed. Ockerman *et al.* (1974) used swabs to sample microflora, which has been found to be inefficient at removing bacteria from sanitised carcasses (Anderson *et al.*, 1987). When acetic acid was sprayed onto the surface of meat, the reduction in microbial load was greater than seen for hypochlorite and quaternary ammonium (Marshall *et al.,* 1977).

The anti-microbial properties of acid sprays are influenced by concentration, flow rate, pressure of spray and temperature of acid when sprayed: the higher the acid concentration, the greater the bactericidal effect (Cudjoe, 1988). Marshall *et al.* (1977) also demonstrated that there was an interaction between flow rate and pressure: the lower the pressure, the more effective the low flow rate and vice versa (Marshall *et al.,* 1997). There appears to be a correlation between temperature and acid concentration: the higher the acid concentration and temperature, the more inhibitory the spray or dipping treatment (Anderson *et al.,* 1987b; Anderson *et al.,* 1988; Cudjoe, 1988; Anderson and Marshall, 1989; Anderson and Marshall, 1990). Acid sprays exert their bactericidal effects by decreasing the pH of the meat surface (Woolthus and Smulders, 1985; Zeitoun and Debevere, 1990). The effect of pH on bacteria is discussed in more detail in section 1.11.2. Washing carcasses with acid sprays is no substitution for good hygiene practices.

1.4 HOT-BONING VERSES COLD-BONING

Hot-boning is where the unchilled carcass is segmented into cuts (Kotula, 1981; Varnam and Sutherland, 1995) while in conventional processing, cold-boning, a whole carcass is hung for at least 48 hours prior to boning (Kotula, 1981; Varnam and Sutherland, 1995). The advantages of hot-boning over conventional boning are listed in Table 1.1. Hot-boning reduces refrigeration, capital, labour and transport costs and increases yield (Kotula and Emswiler-Rose, 1981; Pisula and Tyburcy, 1996). However, there are some disadvantages as there needs to be an initial investment either to build appropriate facilities or to refurbish old ones, and retrain staff (Pisula and Tyburcy, 1996).

Pisula and Tyburcy (1996) suggested hot-boning caused changes in colour, tenderness and microbiology, but these perceived problems can be overcome. Several authors have reported that

Table 1.1 Advantages and disadvantage of the hot boning of meats (Pisula and Tyburcy, 1996).

Advantage of hot boning	Disadvantages of hot boning
Decreased weight loss	Increased distortion of cuts and muscles
Decreased drip loss during vacuum packaging	Changes required in commercial trading
Decreased chiller space requirements	Synchronising of slaughter, boning and processing operation
Decreased refrigeration costs	Increased hygiene standards required
Increased turn over meat in plant	New building or refit and re-training of staff
Decreased capital costs	Changes in tenderness
Increased final yield	
Decreased labour costs	
Decreased transport costs	

Table 1.2 Comparison of the number of phenotypes on conventionally processed (CP) and hot-boned (HB) beef after slow (16 hours to 21°C) and moderate (9 hours to 21°C) chilling at fabrication (T_0), at 21°C for hot-boned beef and after 14 days storage (Lee *et al.*, 1985).

Organisms	Sampling point	Slow chill rate		Moderate chill rate	
		CP	HB	CP	HB
Mesophiles					
	Fabrication	23	22	20	28
	At 21°C	-	17	-	22
	After storage	20	15	19	12
Psychrotrophs					
	Fabrication	11	15	9	16
	At 21°C	-	14	-	12
	After storage	8	11	11	8

there was improved colour satiability (van Laak *et al.*, 1989; van Laak and Smulders, 1990; Pisula and Tyburcy, 1996), brighter purplish-red colour in hot-boned meats (Claus *et al.*, 1985) and higher colour measurements in sausages made from hot-boned meats (Bentley *et al.*, 1987) compared to conventionally processed meat. The problems associated with tenderness relate to the pH of the muscle at chilling. If the pH is above 6.2 then cold shortening will occur (Locker and Haggard, 1963), which increases toughness (Pisula and Tyburcy, 1996). The pH of the muscle can be decreased to below 6.2 by electrical stimulation (Chrystal and Devine, 1978; Kotula and Emswiler-Rose, 1981; Varnam and Sutherland, 1995; Pisula and Tyburcy, 1996) thereby increasing tenderness.

Perhaps the greatest obstacle to hot-boning was the perceived microbiological problem. Boning increases the surface area available for microbial contamination (Bell *et al.*, 1996b). Hot-boning was done when the carcass was above 15-20°C (Varnam and Sutherland, 1995). Initially there was no difference in the number of bacteria on the hot-boned and conventionally processed meat (Emswiler and Kotula, 1979; Sheridan and Sherington, 1982). Lee *et al.* (1985) examined the number of phenotypes on slow chilled (11.3 hours to 21°C) and moderately chilled (9 hours to 21°C) hot-boned beef. The results are shown in Table 1.2. At fabrication (T_0), there were equal numbers of mesophiles but more psychrotrophs on the slow chilled hot-boned beef, while on the moderately chilled beef there were more mesophile and psychrotrophs for the hot-boned beef (Lee *et al.*, 1985). After 14 days storage, there were fewer mesophiles but more psychrotrophs on the hot-boned beef, and more mesophiles and psychrotrophs on the conventionally processed beef after moderate chilling (Lee *et al.*, 1985). There were less phenotypes on the beef sides that were chilled at the faster rate (Lee *et al.*, 1985). Kotula *et al.* (1987) found that there was no difference in the mesophile numbers between hot-boned and conventionally processed meat but found more psychrotrophs on the hot-boned. The chill rate was considered important in determining the microflora of the hot-boned beef (Fung *et al.*, 1981; Lee *et al.*, 1985). A great variety of microflora was found on sausages made from hot-boned pork (Bentley *et al.*, 1987). Initially it was found that there was a greater microbial load on the hot-boned beef (Follet *et al.*, 1974; Heinz, 1975). This was attributed to the need to condition the meat at temperatures ranging from 5 to 15°C for 16 to 24 hours (Follet *et al.*, 1974; Heinz, 1975) to prevent cold-shortening, however, the advent of electrical stimulation has removed this need (Sheridan and Sherington, 1982). Sheridan and Sherington (1982) found that there was no difference in numbers for aerobic flora and *Brochothrix thermosphacta* between hot-boned and conventionally processed beef after vacuum-packaging, while *Enterobacteriaceae* and *Lactobacillus* species were more numerous on the hot-boned beef. Kastener *et al.* (1976) did not find any difference in the number of mesophiles and psychrophiles between hot-boned and conventionally processed meat. Although there are advantages to hot-boning over conventional boning, and the perceived problems of hot-boning can be rectified, there is limited commercial use of hot-boning (Varnam and Sutherland, 1995).

1.5 BACTERIAL ATTACHMENT TO MEAT SURFACES

In order to remain on the surface of meat, bacteria have to attach themselves. Bacteria have two stages of attachment: the first is reversible attachment, where bacteria are trapped within a film of water, and the second is irreversible, where the bacteria become physically attached (Butler *et al.*, 1979). Confocal microscopy of microbial attachment of bacteria to meat fibres demonstrated that bacteria colonise muscle fibres by extending down the fibres rather than from fibre to fibre (Rodrigues-Szule *et al.*, 1990). Bacteria, both Gram-positive and negative, attach themselves preferentially to collagen and elastin fibres rather than to actin, myosin or fibrin (Rodrigues-Szule *et al.*, 1990). Attachment rates can differ amongst bacterial species. Butler *et al.* (1979) found that the attachment of *Pseudomonas putrefaciens* was slightly greater than seen for *Escherichia coli* and *Erwinia herbicola* and notably greater than seen for either *Lactobacillus* or *Staphylococcus* species. In addition, attachment of *P. putrefaciens*, *E. coli* and *E. herbicola* increased during the first 30 minutes of exposure while attachment of *Lactobacillus* and *Staphylococcus* species did not increase after the first minute of exposure (Butler *et al.*, 1979). The number of bacteria attaching to a surface increased in conjunction with bacterial numbers (Notermans and Kampelmacher, 1974; Butler *et al.*, 1979; Firstenberg-Eden, 1981; Thomas and McMeekin, 1981).

There have been variations in reports on the effects of temperature and bacterial physiology on bacterial attachment. Butler *et al.* (1979) found that there was no significant effect of temperature on the attachment of bacteria, while Delaquis and McCurdy (1990) found that greater attachment occurred at 4°C than at 25°C. Notermans and Kampelmacher (1974) and Butler *et al.* (1979) considered that flagella were important in attachment of bacteria to a surface finding that flagellated bacteria attached at a greater rate, while McMeekin and Thomas (1978) and Notermans *et al.* (1980) found that attachment was independent of flagella. The attachment of bacteria to a surface needs to be considered when undertaking bacteriological sampling (Selgas *et al.*, 1993).

1.6 THE INITIAL MICROFLORA OF FRESH MEAT

The initial microflora on a meat carcass is diverse (Brownlie, 1966; Lee *et al.*, 1985) containing more mesophiles than psychrotrophs (Nortjé and Naudé, 1981; Schaefer-Seidler *et al.*, 1984). The total microbial count on fresh meat can range up to 10^5 cfu/cm^2 (Dockerty *et al.*, 1970; Brune and Cunningham, 1971; Enfors *et al.*, 1979; Gill and Penney 1988; Holley *et al.*, 1994, Jericho *et al.*, 1994; Bell *et al.*, 1996b). The microflora of meats can be composed of *Achromobacter*, *Acinetobacter/Moraxella*, *Aeromonas*, *Bacillus* species, *B. thermosphacta*, *Clostridium*, *Flavobacterium/Cytophaga*, *Enterobacteriaceae* (including *E. coli* and enterics), lactic acid bacteria (LAB), *Listeria*, *Micrococcus*, *Pseudomonas* (both fluorescent and non-fluorescent), *Shigella*, *Staphylococcus* and *Streptococcus* species (Pierson *et al.*, 1970; Patterson and Gibbs, 1977; Newton *et*

al., 1978; Enfors *et al.*, 1979; Gill and Newton, 1980; Thomas and McMeekin, 1980; Simard *et al.*, 1984; Lee *et al.*, 1985; Gill and Penney, 1988; Rao and Ramesh, 1988; Lawrie, 1990; Nortjé *et al.*, 1990; Greer *et al.*, 1993; Knudtson and Hartman, 1993; Bell *et al.*, 1997). The number of bacteria and the composition of the microflora will determine the shelf-life of the product (Gustavsson and Borch, 1993).

1.7 DEVELOPMENT OF SPOILAGE MICROFLORA

Proteinaceous foods are vulnerable to the activities of microorganisms because of their nutrient and water content and slightly acidic pH (Huis in't Veld, 1996). The microflora that develop on fresh meats will depend on the method of packaging that is used for the product. Fresh meat can be either packaged in: air, where the meat is wrapped in a gas permeable plastic; vacuum-packaging, where all the air is removed from within the package so that the gas-impermeable packaging material forms a skin around the meat and minimises the transmission of oxygen (Seidman and Durland, 1982); or modified atmosphere packaging (MAP), where the air within the package is removed and generally replaced with carbon dioxide and air or oxygen or nitrogen in various combinations.

1.7.1 Microbiological aspects of air-packaged meat spoilage

Aerobic spoilage is generally accepted to occur when bacterial numbers reach 10^8 cfu/cm^2 on the basis of odour even though bacterial numbers may increase above 10^9 cfu/cm^2 (Ingram, 1962). When the microbial load reaches 10^8 cfu/m^2, glucose levels are exhausted so the bacteria switch to other components such as amino acids, this coincided with organoleptic spoilage (Ingram 1962; Gill, 1976). *Pseudomonas* species are the dominant microbial group in the spoilage microflora of aerobically packaged meats, regardless of meat species. In humid conditions, the growth and slime production by *Pseudomonas* and *Achromobacter* species was responsible for meat spoilage (Ingram and Shewan, 1960). Gardner *et al.* (1967) found that *Pseudomonas* and *Achromobacter* were numerically significant in the microflora of aerobically packaged pork. *Achromobacter* has subsequently been reclassified as non-pigmented *Pseudomonas*, *Alcaligenes* and *Acinetobacter* species (Ingram and Dainty, 1971). *Pseudomonas* species were the dominant bacteria group on normal and high pH beef (Erichsen and Molin, 1981b), on hung beef cuts and carcasses (Pierson *et al.*, 1970; Simard *et al.*, 1984). In some cases, *Pseudomonas* can comprise up to 96% of the population (Bailey *et al.*, 1979b; Enfors *et al.*, 1979; Asensio *et al.*, 1988). Of the members of the genus *Pseudomonas*, *P. fragi* was the dominant species on spoiled meat (Molin and Ternström, 1982; Shaw and Latty, 1982; Shaw and Latty, 1984), but prior to spoilage the commonly found pseudomonad was *P. fluorescens* (Gustavsson and Borch, 1993). *P. fragi* grown on crab meat stored at seven and 11°C increased in number by 6.76 and 7.13 $\log_{10}$ cfu/cm^2,

respectively, within six days rendering it unacceptable (Ingham *et al.*, 1990a). *P. putrefaciens* together with pigmented and non-pigmented *Pseudomonas* species composed a significant proportion of the microflora of spoiled poultry (Barnes and Melton, 1971). Although *Pseudomonas* species are the most prevalent, there are other bacterial groups associated with spoilage in air.

Commonly found in association with *Pseudomonas* are *Acinetobacter* and *Moraxella* species, both organisms are Gram-negative aerobes. This group is thought to enhance the dominance of *Pseudomonas* species by restricting the amounts of oxygen available to competing organisms (Gill, 1986). *Acinetobacter* LD2, *Moraxella osolensis* and non-fluorescent pseudomonads decreased the attachment of *Brochothrix* to meat surfaces (Farber and Idzaik, 1984). Aside from *Pseudomonas*, *Acinetobacter* were found on spoiled chicken and turkey in significant numbers (Barnes and Melton, 1971) and were dominant on tetracycline-treated chickens after storage at 1°C (Barnes and Shrimpton, 1958). The *Acinetobacter/Moraxella* group was found to dominate the psychrotrophic microflora of hot-boned beef carcass after 14 days refrigerated storage (Lee *et al.*, 1985).

The storage temperature plays an important role in the composition of the microflora. Larger numbers of *Enterobacteriaceae* (Ingram, 1962; Barnes and Thornley, 1966) were isolated at abuse temperatures, 15-25°C (Ingram and Dainty, 1971). Gardner *et al.* (1967) found that *Kurthia* species, *Enterobacter* and *Hafnia* species were prevalent in the microflora of pork stored at 16°C while none were detected at 2°C. On meat stored at 20°C, *Pseudomonas* species still dominated the microflora but when the temperature was increased to 30°C *Acinetobacter* and *Enterobacteriaceae* dominated the microflora (Gill and Newton, 1980). The development of facultative anaerobes in the microflora is assisted by the increase in carbon dioxide produced as a result of meat tissue respiration and *Pseudomonas* growth (Ingram, 1962; Enfors and Molin, 1984).

Although Gram-negative bacteria are the principal group in aerobic spoilage, Gram-positive bacteria also have a role. *Brochothrix* are important in the spoilage process as acetoin, acetic acid, formic acid, isobutyric acid and isolvaleric acid are end-products of metabolism (Borch and Molin, 1989). The microflora of lamb packaged in air then stored at 0°C overnight was found to be dominated by *Microbacterium thermosphactum*, later renamed *B. thermosphacta* (Sneath and Jones, 1976), with the remainder of the population composed of *Micrococcus* (33%), non-pigmented pseudomonads (9.5%), *Achromobacter* (4.8%) and *Staphylococcus* (4.8%) (Barlow and Kitchell, 1966). The number of *Brochothrix* increased to 100% of the population after three days at 5°C (Barlow and Kitchell, 1966). During the storage of lamb carcasses at 3°C, *Brochothrix* was second only to staphylococci in number at spoilage (Prieto *et al.*, 1993), while on pork it was the only group other than *Pseudomonas* detected (Asensio *et al.*, 1988). *Brochothrix* were found commonly in the microflora on lamb surfaces (Barlow and Kitchell, 1966) and pork fat (Blickstad and Molin, 1983a). The spoilage flora of lamb carcasses

was found to be composed primarily of *Brochothrix* (Grau *et al.*, 1985). On cured pork, *Brochothrix* was demonstrated to grow to higher numbers than *Pseudomonas* (Blickstad and Molin, 1983b) which could be attributed to *Brochothrix* being able to withstand high salt concentrations (Talon *et al.*, 1988). Within the dominant populations of *Pseudomonas,* both *Brochothrix* and LAB could be found among the spoilage flora of beef (Erichsen and Molin, 1981a,b). LAB, another Gram-positive group, increased on beef from 10^3 to 10^8 cfu/cm^2, however, this only comprised 1% of the population (Pierson *et al.*, 1970).

1.7.2 Microbiological aspects of vacuum-packaged meat spoilage

During storage of vacuum-packaged meats the numbers of both aerobes and anaerobes increases (Hodges *et al.*, 1974) with a greater period of time required for spoilage to occur compared to air-packaged (Erichsen and Molin, 1981a; Bell *et al.*, 1996c). Often, the package is flushed with carbon dioxide prior to the application of a vacuum (Asensio *et al.*, 1988). Not all the gas is removed by the vacuum, leaving some carbon dioxide within the package which assists the antimicrobial process. Vacuum levels have no significant effect on microbial count (Bailey *et al.*, 1979a,b). The microbial load of vacuum-packaged meats rarely exceeds 10^8 cfu/cm^2 (Ingram, 1962). Higher numbers of bacteria have been isolated from vacuum-packaged dark, dry, firm beef (high pH, low glucose) than normal pH beef (Erichsen and Molin, 1981a).

The microflora of vacuum-packaged meats is more diverse than air-packaged meat. The number of pseudomonads on vacuum-packaged beef was less than on retail, aerobically, packaged beef (Greer and Jones, 1991). In high oxygen transmission polyethylene, 78% of the microflora on pork spoiled after vacuum packaging was *Pseudomonas* with 22% *B. thermosphacta* (Asensio *et al.*, 1988). There is some inhibition of *Pseudomonas* species by the carbon dioxide that evolves within vacuum-packages (Ingram, 1962; Gardner *et al.*, 1967; Shay and Egan, 1987). For example facultative anaerobes can dominate the microflora: *Enterobacter* dominated *Pseudomonas* species on chicken surfaces under vacuum-packaging (Cunningham, 1982). There was a slight increase in *Hafnia alvei* and *Serratia liquefaciens* numbers on artificially inoculated vacuum-packaged round steaks (Hanna *et al.*, 1983). *Enterobacteriaceae* comprised 30% of the population of pork following packaging with low oxygen transmission film (Asensio *et al.*, 1988). On high pH beef, *Enterobacteriaceae* comprise a great portion of the microflora (Gill and Penney, 1988). The number of *Enterobacteriaceae* on vacuum-packaged meats is dependent on the temperature, with numbers diminishing as temperature declines (Sheridan *et al.*, 1997). However, more frequently Gram-positive bacteria have a greater prevalence.

Gram-positive bacteria comprised 60% of the microflora at day five on vacuum packaged chicken, then continued to increase and finally reached above 95% of the population (Bailey *et al.*, 1979b). The largest

increase in bacterial counts on vacuum-packaged meat has been observed to occur between seven and 14 days (Christopher *et al.,* 1980). The anaerobic conditions that develop in vacuum-packaged meats can enhance the growth of LAB (Pierson *et al.,* 1970). The microflora of vacuum-packaged beef was found to contain atypical lactobacilli, whose identity could not be determined and *Leuconostoc mesenteroides* (Hitchener *et al.,* 1982). On normal pH beef LAB were the only bacterial group found while on high pH beef they were only 60% of the bacterial flora (Erichsen and Molin, 1981b). With very low oxygen transmission lactobacilli and *B. thermosphacta* were dominant at a ratio of 2:1 (Asensio *et al.,* 1988). LAB numbers increased by 1.5 $\log_{10}$ cfu/g between one and six days, finally increasing to 7.2 $\log_{10}$ after 18 weeks in vacuum-packages (Seman *et al.,* 1988). On beef which had been vacuum-packaged, LAB were again the dominant organisms (Hanna *et al.,* 1981; Bell *et al.,* 1996b, c).

The development of LAB on vacuum-packaged meats is advantageous as they tend to reach maximum numbers before spoilage is detected. Lactobacilli produce sour or acid odours rather than putrid ones (Gill and Penney, 1988). On luncheon meat inoculated with lactobacilli, numbers reached 10^8 cfu/cm^2 and remained at this level for 21 days before any off-odour was detected, whereas inoculation with *B. thermosphacta* resulted in off-odours being detected when counts reached 10^8 cfu/cm^2 (Egan *et al.,* 1980). *Brochothrix* were the dominant bacterial group on lamb (62.5%) after overnight storage at 0°C, this number increased to 72.8% after six days with the remainder composed of unidentified Gram-positive bacteria (Barlow and Kitchell, 1966). *Brochothrix* are more prevalent if the oxygen transmission rate of the packaging film is high (Newton and Riggs, 1979). Beef with a high pH was found to have 40% *B. thermosphacta* within the microflora (Erichsen and Molin, 1981b).

An additional bonus to the development of *Lactobacillus*, *Carnobacterium* and *Leuconostoc* species is production of antagonist substances called bacteriocins which inhibit the growth of other bacterial species by these groups (Newton and Gill, 1978; Ahn and Stiles, 1990a,b; Stoffels *et al.*, 1992; Garver and Murinana ,1993; Schillinger *et al.*, 1993; Saucier *et al.*, 1995; Casla *et al.,* 1996; Jack *et al.*, 1996).

1.7.3 Microbiological aspects of modified atmosphere packaged fresh meat

MAP is defined as " the enclosure of food products in high gas-barrier materials, in which the gaseous environment has been changed to slow respiration rates, reduce microbiological growth and retard enzymatic spoilage with the intent of extending shelf life" by Young *et al.* (1988). In MAP, the atmosphere within the package will change over time due to the permeability of the packaging materials, evolution or absorption of gases and integrity of the seal (Leeson, 1987). This differs from controlled atmosphere packaging, in which the atmosphere is precisely maintained. Carbon dioxide is commonly used in MAP because of its anti-microbial properties. The anti-microbial properties of carbon dioxide were first described by Pasteur and Joubert in 1877, and first used in meat preservation

by Kolbe in 1882 (Enfors *et al.*, 1979). Carbon dioxide was used commercially for the first time to ship meat from Australia and New Zealand to Great Britain in the 1930s (Lawrie, 1974). MAP is important to the meat industries of Australia and New Zealand export industry in that it provides a greater extension of shelf-life than either air or vacuum-packaging without freezing (Gill, 1988). The modes of inhibition by carbon dioxide are discussed in section 1.11.4.

The number of bacteria present on meat at spoilage following MAP is less than the number on air- or vacuum-packaged meats (Pierson *et al.*, 1970; Silliker *et al.*, 1977; Christopher *et al.*, 1980; Erichsen and Molin, 1981b; Blickstad and Molin, 1983a; Bell *et al.*, 1996b; Sheridan *et al.*, 1997), with the number of aerobic bacteria being significantly lower than the number of facultative anaerobic bacteria (Huffman *et al.*, 1975). A greater time frame is required for bacterial numbers to reach their maximum under elevated carbon dioxide. The shelf-life of MAP meats can be seven times that of air-packaged meat (Enfors *et al.*, 1979). The greatest increase in bacteria on beef, lamb and pork occurred between days 14 and 21 (Christopher *et al.*, 1980). A combination of 50% CO_2/50% N_2 resulted in pork products that were acceptable after 21 days (Holley *et al.*, 1994). Huffman *et al.* (1975) noted that within a 27 day period the number of LAB and anaerobic bacteria did not increase. Similarly, Bell (1996b) noted that there was no increase in bacteria numbers during the first four weeks of storage.

Gram-positive bacteria dominate the microflora when the growth of Gram-negative bacteria is suppressed. The growth of pseudomonads was inhibited when the level of carbon dioxide was increased while the numbers of *Brochothrix* and *Lactobacillus* increased (Leward *et al.*, 1970). LAB and other members of the *Lactobacillaceae* family are the most resistant to elevated carbon dioxide (Ingram, 1962). At temperatures between two and 15°C under anaerobic conditions *Lactobacillus* outgrew and inhibited *B. thermosphacta* and *Enterobacter* (Newton and Gill, 1978). Within 15 days, 90% of the microbial population of beef consisted of LAB (Pierson *et al.*, 1970). After 35 days storage, there were equal numbers of *Lb. plantarum* and heterofermentative lactobacilli under 100% CO_2 (Enfors *et al.*, 1979). After storage in 100% CO_2 the entire microbial population on meats can be composed of LAB (Erichsen and Molin, 1981b; Erichsen and Molin, 1981a; Gill and Jones, 1994). LAB increased from undetectable to a maximum of 6.18-7.89 $\log_{10}$ cfu/g on venison in 18 weeks depending on the packaging material after flushing with 100% CO_2 (Seman *et al.*, 1988). On pork loins and loin chops under 100% CO_2 the only bacterial group to increase in number were LAB (Blickstad and Molin, 1983a; Greer *et al.*, 1993). Even under lower concentrations of carbon dioxide LAB still dominate. On chicken injected with 20 or 65% CO_2 more than 90% of the bacteria were *Lactobacillus* (Bailey *et al.*, 1979b).

The presence of LAB as the dominant members of the spoilage population is beneficial in terms of shelf-life extension, as these bacteria are slow growing (Leeson, 1987), produce anti-microbial agents

(Newton and Gill, 1978; Ahn and Stiles, 1990a,b; Garver and Murinana ,1993; McMullen and Stiles, 1993; Casla *et al.*, 1996) and produce less pungent by-products. Odours produced tend to be described as sour or buttery or sickly-sweet (Nortjé and Shaw, 1989) rather than putrid. However some H_2S production has been noted with extended storage after numbers have reached a maximum (Borch and Agerham, 1992).

In the microflora of MAP meats, *Brochothrix* can be isolated in numbers approaching 10^5 cfu/cm^2 provided the level of carbon dioxide is below 100% (Erichsen and Molin, 1981a). A small number of *Brochothrix* were isolated from uncured pork after storage at 4°C (Blickstad and Molin, 1983a). As storage time continued the frequency of isolation of *B. thermosphacta* increased (McMullen and Stiles, 1993). The significance of *Brochothrix* in a MAP population increased if the oxygen transfer rate of the package was high. Under an atmosphere of 40% CO_2/60% N_2, if the oxygen was increased to 4% *Brochothrix* dominated the population (McMullen and Stiles, 1991). A gas mixture of 20% CO_2/78%N_2/2%O_2 allowed *B. thermosphacta* numbers to increase at the same rate as the total counts, which was higher than LAB (Erichsen and Molin, 1981b; Newton and Jones, 1994). Although appearing in low numbers at spoilage, the significance of *Brochothrix* is increased by its end-products from glucose fermentation. Following the anaerobic metabolism of glucose, *Brochothrix* has been shown to produce L-lactate, acetate, formate and ethanol (Grau, 1983) which can render products organoleptically unacceptable. Another group which causes similar problems are the *Enterobacteriaceae*.

Members of the *Enterobacteriaceae* family are the only Gram negative bacteria of consequence in high CO_2 MAP meat spoilage, because they are facultative anaerobes. Carbon dioxide does not inhibit their growth but it causes an increase in lag phase (Gill and Penney, 1988), thus giving the slower growing LAB time to establish dominance. *Enterobacteriaceae* numbers have a tendency to increase towards the end of storage because of the increased lag phase (Gill and Penney, 1988). *Enterobacteriaceae* are significant because if they comprise 10% of the spoilage population they will render a product unacceptable due to the production of putrid odours (Gill and Penney, 1988). *Enterobacter* on meat slices at 10°C out grew and inhibited *B. thermosphacta* under anaerobic conditions (Newton and Gill, 1978). When grown under glucose limited conditions anaerobically, *Enterobacter* out competed both *Lactobacillus* and *B. thermosphacta* (Newton and Gill, 1978).

The antibacterial effect of carbon dioxide is influenced by temperature (Baker *et al.*, 1986), concentration (Bailey *et al.*, 1979a; Finne, 1982; Gill and Penney, 1988) and oxygen transmission rate of the packaging material (Bailey *et al.*, 1979a). The ability of carbon dioxide to inhibit microbial growth is a function of temperature in that the higher the temperature the less effective is a carbon dioxide enriched environment. Baker *et al.* (1986) showed that inhibition by 80% CO_2 was greater at

2°C than 13°C for *S. aureus*, *S. typhimurium* and *P. fragi*. The level of carbon dioxide within the package significantly influenced the number of bacteria: chicken packaged in 65% carbon dioxide had less bacteria after 15 days storage at 4°C than under 20% CO_2 (Bailey *et al.*, 1979a). Gill and Penney (1988) discovered that as the volume of carbon dioxide added increased, the number of *Enterobacteriaceae* decreased. Low oxygen transmission rates affected microbial growth at day 12, where lower numbers of bacteria were achieved on chickens packaged with low oxygen permeable film (Bailey *et al.*, 1979a). Furthermore it was shown that the higher the concentration of carbon dioxide, the longer the shelf-life of chicken (Bailey *et al.*, 1979b) and beef (Gill and Penney, 1988). The inhibition of carbon dioxide is more complex than just the removal of oxygen because bacteria are inhibited by environments that have 20% CO_2 and 80% O_2 (Asensio *et al.*, 1988). For meat it is generally accepted that levels of at least 20% CO_2 produce the desired extension of shelf-life (Clark and Lentz, 1972) with nitrogen ad the balance gas.

Carbon dioxide has not been the only gas examined for bacterial growth control. Nitrogen has also been examined as a preservative gas, but inhibition of bacterial growth and subsequent shelf-life enhancement was inferior to carbon dioxide (Blickstad and Molin, 1983b; Holley *et al.*, 1994). Interestingly, the growth of bacteria under nitrogen is not dissimilar to that observed with air-packaged meats: *Pseudomonas* species dominate the microflora but growth rates are slower, with the remaining microflora comprised of *Kurthia zopfilii*, *Aeromonas hydrophilia* and *Lb. plantarum* (Enfors *et al.*, 1979). Mould growth is inhibited in the presence of nitrogen, while at the same time lipid oxidation is prevented (Manu-Twiah *et al.*, 1991).

1.7.4 Pathogenic bacteria associated with fresh meats

The pathogens of most concern to food microbiologists are: *S. aureus*, *L. monocytogenes*, *Campylobacter jejuni*, *Salmonella* species, *Cl. perfringens*, *A. hydrophilia*, *B. cereus*, *E. coli* 0157:H7 and *Yersinia enterocolitica*. A major source of these pathogenic bacteria is faecal matter, which contaminated meat surfaces during slaughter (Siragusa and Cutter, 1995). Some pathogens are associated with a particular animal: both *A. hydrophilia* (Barnhart *et al.*, 1989) and *Campylobacter* species (Lior, 1994) are commonly associated with poultry and pigs are thought to be a significant reservoir for *Y. enterocolitica* (de Fernardo *et al.*, 1995). *L. monocytogenes*, however, is ubiquitous in the environment (de Fernardo *et al.*, 1995). Childers *et al.* (1973) found that *E. coli* contamination was high on cattle (59%), swine (54%) and sheep (60%) carcasses, while no *Salmonella* were found on cattle, with moderate numbers on swine (20%) and low numbers on sheep (7%). Knives were found to be a source of carcass contamination by *E. coli* and *Salmonella* (Childers *et al.*, 1973). *S. aureus* (7.4%) was the most common pathogen isolated on pork followed by *Salmonella* (1.7%), *L. monocytogenes* (1.5%), *Y. enterocolitica* (0.4%) and *Cl. perfringens* (0.4%) (Saide-Albornoz *et al.*,

1995). A survey of pork and beef found that pork had more *S. aureus* than beef, while neither meats were found to contain *Salmonella* (Scriven and Singh, 1986). The initial number of pathogenic bacteria does not affect the maximum growth rate or the duration of lag time (Mackey and Kerridge, 1988).

The maximum growth rate of *A. hydrophilia*, *B. cereus*, *L. monocytogenes* and *Y. enterocolitica* increased as carbon dioxide concentration increased irrespective of the presence of 1.5 or 21% O_2 (Bennik *et al.*, 1995). *Cl. perfringens* inoculated onto cooked roast beef then stored under a modified atmosphere of 75% CO_2/15% N_2/10% O_2 was unable to grow (Hintlain and Hotchkiss, 1987), nor could it grow under 80% CO_2 in ground chicken meat or synthetic broth (Baker *et al.*, 1986). Neither *S. typhimurium* or *S. aureus* were able to grow on cooked roast beef under 75% CO_2/15% N_2/10% O_2 at 4.4°C over a 42 day period (Hintlain and Hotchkiss, 1987). The inclusion of oxygen combined with low temperature has been demonstrated to prevent the outgrowth of *Cl. perfringens* (Hintlain and Hotchkiss, 1987). *S. typhimurium* did not grow on crab meat stored at 7°C in air or MAP (50% CO_2/10% O_2) but did increase by 4.78 and 2.91 $\log_{10}$ cfu/cm^2 in air and MAP respectively after storage at 11°C (Ingham *et al.*, 1990a). Final numbers of *A. hydrophilia*, *L. monocytogenes* and *Y. enterocolitica* were lower under carbon dioxide saturation than vacuum-packaging (Hudson *et al.*, 1994). No growth of these pathogens occurred at -1.5°C under carbon dioxide saturation (Hudson *et al.*, 1994). Different combinations of carbon dioxide and oxygen are ineffective at inhibiting pathogen growth if temperature is increased (Hintlain and Hotchkiss, 1987).

1.8 MICROBIOLOGICAL SAMPLING AND ISOLATION

1.8.1 Sampling methods for determining bacterial numbers and identification

The microbial load on a meat surface can be determined through swabbing an area of known size (Favero *et al.*, 1968; Baldock , 1974), by direct contact (Favero *et al.*, 1968; Baldock , 1974), rinsing a sample (Favero *et al.*, 1968; Baldock , 1974), or excising a sample of known size (Baldock , 1974; Anderson *et al.*, 1987a; Prieto *et al.*, 1991). With the exception of the excision technique these techniques are also applicable to equipment (Favero *et al.*, 1968; Baldock , 1974).

The swabbing method involves a moist cotton or alginate swab which is wiped over a known surface area, placed into sterile diluent, diluted and plated out onto agar surfaces (Favero *et al.*, 1968; Baldock, 1974). The primary advantage of the swabbing method is that it is quick, non-destructive and single cells can be enumerated (Baldock, 1974; Rodrigues-Szule *et al.*, 1990). However, only a fraction of the bacteria present on a surface is detected by swabbing when compared to the excision method and the technique is significantly influenced by user variation (Favero *et al.*, 1968; Rodrigues-Szule *et al.*, 1990). Although the excision technique yields a more accurate estimation of bacterial

numbers, the differences between the two methods may become insignificant when the variation in microbial load across a carcass is taken into account (Untermann *et al.*, 1997). If large numbers of samples are required, the swabbing technique will prove satisfactory if an appropriately trained person conducts the sampling (Untermann *et al.*, 1997).

The contact method involves pressing solid nutrient agar directly against the surface of meat, then aseptically removing and incubating the agar slice (Favero *et al.*, 1968; Baldock, 1974). The advantage of this method is that it is non-destructive and gives results correlatable to the excision method (Nortjé *et al.*, 1982). The disadvantage is that if the surface is heavily contaminated or there are spreading colonies present, enumeration can be obscured as clumps of cells cannot be distinguished from single cells (Favero *et al.*, 1968; Baldock, 1974).

The rinse method is generally applied to poultry carcasses. The carcass is submerged in sterile diluent which is then agitated in order to detach bacteria from the surface (Favero, 1968). The microbial mass on the carcass is calculated based on a mass to surface area ratio (Baldock, 1974). This method has the advantage that it is not destructive, however, counts are not as great as for the excision method (Lazarus *et al.*, 1977).

The excision technique is considered to be the most accurate microbiological sampling method as more bacteria are recovered with this method (Baldock, 1974; Snijders *et al.*, 1984; Anderson *et al.*, 1987a; Prieto *et al.*, 1991). Meat tissue of a known surface area is removed, combined with sterile diluent then either blended or macerated. The blending or maceration of the meat facilitates the removal of more firmly attached bacteria (Butler *et al.*, 1979). Dilutions of the resulting homogenate are then plated out. From this, counts are made to determine the number of bacteria per surface area or millilitre. Different sampling techniques exert different forces on meat surfaces and therefore remove bacteria from the surface at different rates (Butler *et al.*, 1979).

1.8.2 Selective media

Selective media can be used to detect bacterial species that are present only in low numbers. *Brochothrix* species can be isolated using selective medium developed by Gardner (1966). The basal medium contains peptone, yeast extract, glycerol, di-potassium hydrogen orthophosphate, magnesium sulphate and agar with anti-microbial agents. Streptomycin is used to inhibit Gram-negative and some Gram-positive bacteria, thallous acetate to inhibit yeast and some aerobic and facultative aerobic bacteria, and actidione to inhibit yeast (Skovgaard, 1985). A selective medium was developed for *Pseudomonas* species by Mead and Adams (1977) which contained cephaloride, fucidin and centrimide. This medium was further developed to combat problems of *Enterobacteriaceae* which are

occasionally capable of growth on this medium. The incorporation of arginine and a pH indicator, phenol red, into the media enabled the distinction. As the pseudomonads grow they utiltise arginine, producing urea which increases the pH around the colony so that a pink coloration develops in the medium which does not occur for *Enterobacteriaceae* (Stanbridge and Board, 1994). *Enterobacteriaceae* can be selected on a MacConkey style medium modified to include glucose instead of lactose (Mossel *et al.*, 1962). *L. monocytogenes* can be isolated from heavily contaminated beef if moxalactam and increased lithium chloride are incorporated into McBrides agar base (Lee and McLain, 1986). Enrichment of bacteria by incubating in nutrient-rich medium for a period of time prior to plating onto selective media can enhance detection (Donnelly and Baigent, 1986).

Some bacteria can even be sensitive to the very agents used to select for them (Lee, 1977; Curtis *et al.*, 1989). Selective media can also be detrimental to injured bacteria (Busta, 1976; Ray, 1986). Injury can be caused by conditions experienced during food processing through heat, refrigeration, dehydration, irradiation or preservative agents (Busta, 1976), or sampling, if samples are frozen for transport to the laboratory for analysis. Injuries to bacteria cells can result in a bacteria becoming sensitive to the selective agents used to select for that particular organism (Busta, 1976). Bacterial cells that have been injured have been demonstrated to repair within two hours if incubated in nutrient rich media (Ray, 1986; Busch and Donnelly, 1992). Therefore it is recommended that a resuscitation step is included when using selective agents, especially for pathogens (Ray, 1986). The inclusion of glucose, yeast extract, Fe^{+} or pyruvate within the medium during a resuscitation step will increase the number of bacteria recovered on selective media (Busch and Donnelly, 1992). The detection of viable but non-culturable bacteria will be discussed in Section 1.10.4.

1.9 CHARACTERISTICS OF THE PRINCIPAL BACTERIA IN THE MEAT SPOILAGE PROCESS

The spoilage process is complicated as it involves many bacteria species and during the development of a microbial population there is a succession process occurring (Ingram, 1962). Although the final population is generally dominated by one particular bacterial group (e.g. *Pseudomonas* for air-packaged meat and LAB for vacuum-packaged and MAP meats), there are other bacterial groups involved as previously discussed. Although pathogens do occur on meat, they are present in relatively low numbers (Hudson *et al.*, 1994; Saide-Albornoz *et al.*, 1995) The main bacterial groups involved in meat spoilage are *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* species.

1.9.1 Characteristics of the members of the genus *Brochothrix*

Brochothrix species are Gram-positive, catalase positive, non-spore forming, non-motile, heat sensitive, rod-shaped bacteria (McLean and Sulzbacher; 1953; Sneath and Jones; 1976; Sneath and Jones, 1986; Talon *et al.*, 1988). McLean and Sulzbacher (1953) described this Gram-positive organism which was isolated from meat and originally named it *Microbacterium thermosphactum*. In a taxonomic investigation into members of the genus *Microbacterium*, Collins-Thompson *et al.* (1972) found that *M. thermosphactum* was distinctly different from the other members of this genus, with a lower guanine and cytosine (GC) content, higher catabolic activity for converting glucose to lactic acid, different protein profiles and esterase activity (Collins-Thompson *et al.*, 1972). Sneath and Jones (1976) proposed the new genus *Brochothrix*, in which the only species *M. thermosphactum* was placed and renamed *B. thermosphacta*. *Brochothrix* was tentatively placed in the family *Lactobacillaceae* as it shared characteristics with other family members even though it had a cytochrome which the others do not (Skovgaard, 1985). Apart from the functioning cytochrome *B. thermosphacta* also has catalase activity and an iron requirement (Thomson and Collins-Thompson, 1986). In a taxonomic survey of *Listeria* and related bacterial species *Brochothrix* clustered with *Propionibacterium* and only in part with *Lactobacillus* and *Streptococcus* (Wilkinson and Jones, 1977).

In 1988, a second member was added to the genus, *B. campestris*, which was isolated from soil and grass (Talon *et al.*, 1988). The two species are distinguished by the ability of *B. thermosphacta* to grow in eight and 10% sodium chloride and reduce 0.05% potassium tellurite, while *B. campestris* hydrolyses hippurate and produces acid from rhamnose (Talon *et al.*, 1988). Additional information on the new species is limited except for bacteroicin production. *B. campestris* produces a bacteriocin, brochocin-C, which was found to inhibited *B. thermosphacta* and *L. monocytogenes* (Siragusa and Cutter, 1993).

Brochothrix species have a maximum growth rate at 24-25°C but produce larger number of cells at the lower temperatures of 3 and 7.5°C (Papon and Talon, 1988). Growth at 30°C had been found to be diphasic with no growth occurring above 30°C (Gil *et al.*, 1992). When growing in meat extract at a pH less than 5.9, *B. thermosphacta* utilised glucose, glycerol, glycerol-3-phosphate and inosine as growth substrates when grown under aerobic conditions but only glucose under anaerobic conditions (Grau, 1988). When the pH of the meat extract was increased to 6.6-6.8, *B. thermosphacta* utilised glycerol and ribose during rapid growth and glycerol-3-phosphate and inosine during slow growth under aerobic conditions but ribose and glucose under anaerobic conditions (Grau, 1988). When either glucose or glycerol are used as a substrate the major end-product of metabolism is acetoin (Macaskie *et al.*, 1984). Acetoin, acetic acid, formic acid, isobutyric acid and isovaleric acid are all by-products of metabolism by *B. thermosphacta* (Dainty and Hibbard, 1980; Borch and Molin, 1989). These end-products will render meat products organolepically unacceptable. As *Brochothrix* is a facultative anaerobic organism it causes significant problems on meat (Dainty and Hibbard, 1980).

Microbial lipases are responsible for off-flavour production and subtle changes in flavour (Alford *et al.*, 1964), and lipase production has been observed in *B. thermosphacta* (Papon and Talon, 1988; Papon and Talon, 1989; Talon *et al.*, 1992). The lipolytic activity was associated with the cell (Papon and Talon, 1988), in particular in the soluble fraction (Papon and Talon, 1989). Lipase production by *Brochothrix* has two maxima, the first during exponential growth when at pH 5.7 with the second at stationary phase, but only if the pH is about 5.2 (Papon as Talon, 1988). Lipase production is greatest at 24°C (Papon and Talon, 1988) but the greatest lipase activity occurs at 37°C (Papon and Talon, 1989). Lipase activity occurs over a pH range of 6.0-8.0, with 75% activity at pH 9.0 (Talon *et al.*, 1992).

1.9.2 Characteristics of the members of the family *Enterobacteriaceae*

Enterobacteriaceae are a large family of Gram-negative bacteria which are ubiquitous in the environment (Brenner, 1986). The phenotypic characteristics shared by all members of the family *Enterobacteriaceae* are: Gram-negative, oxidase negative, catalase positive, non-spore forming, non-acid fast, rod shaped morphology and they are facultative anaerobes (Brenner, 1981). A majority of the genera are motile although *Shigella* (Rowe and Gross, 1986) and *Klebsiella* (Ørstov, 1986a) are non-motile. Furthermore, some members of the genera *Escherichia* (Ørstov, 1986b) and *Salmonella* (Le Minor, 1986) are also non-motile. *Enterobacteriaceae* do not commonly dominate the microflora of refrigerated food although they are present. Their importance stems from the olfactory unacceptable organo-sulfides that they produce (Gill, 1986) and some are pathogenic. Not all members of the family are important in food microbiology, therefore only those genera of significance with regard to food, in particular meat, are considered here.

Members of the genus *Escherichia* are characterised by an optimal growth temperature of 37°C, the ability to utilise a wide range of carbon sources, the production of lactic, acetic and formic acid from the fermentation of sugars and failure to produce hydrogen sulphide (Ørstov, 1986b). The GC content of this genus is 48-52% (Ørstov, 1986b). *E. coli* includes the serotypr 0157:H7 that is a significant pathogen (Böhm and Karch, 1992; Barrett *et al.*, 1994; Johnson *et al.*, 1995). *Yersinia* species are of public health significance with phenotypic characteristics varying with temperature (Bercovier and Mollaret, 1986). *Yersinia* are motile at 30°C but not at 37°C, have optimum growth temperature of 28-29°C, reduce nitrate to nitrite, ferment sugars but do not produce gas and have a GC content of 46-50% (Bercovier and Mollaret, 1986). Another pathogenic genus which is also the largest genus in the family *Enterobacteriaceae* is *Salmonella*. *Salmonella* species produce hydrogen sulfide, grow on citrate, are lysine and ornithine decarboxylase positive, are urease negative, fail to ferment either sucrose, salcin, inositol or amygalin and have a GC content of 50-53% (Le Minor, 1986). *Hafnia*, which can also be pathogenic, has only one species, *H. alvei*. Some strains of *H. alvei* have been implicated in diarrhea (Ridell *et al.*, 1995). *H. alvei* has been transferred back and forth between the genera *Hafnia* and *Enterobacter*, after being originally proposed in 1954 as *Bacillus paratyphi-alvei* (Sakazaki, 1981).

Hafnia does not produce hydrogen sulfite, gelatinase or lipase, is phenylalanine deaminase negative, has a GC content of 48-48.7% and utilise citrate, acetate and lactate as sole carbon and energy sources (Sakazaki, 1986). *Enterobacter* species produce gas and acid from the fermentation of glucose, are gelatinase positive, utilise citrate and malonate, are Voges-Proskauer positive, methyl red negative and have a GC content of 52-60% (Richard, 1986). Non-clinical strains grow optimally at 30°C while clinical strains grow optimally at 37°C (Richard, 1986). *Serratia* species differ from most of the members of the family *Enterobacteriaceae* in that they produce coloured pigments of white, pink or red (Grimont and Grimont, 1981). *Serratia* species produce acetoin from pyruvate, utilise citrate, maltose, mannitol and trehalose, are gelatinase positive, do not produce hydrogen sulfide, are s-nitrophenyl-b-D-galactopyranoside positive and a have GC content of 52-60% (Grimont and Grimont, 1986). *Klebsiella* species are different from other enterobacteria in that they are encapsulated (Ørstov, 1986a). In addition, *Klebsiella* species are citrate positive, Voges-Proskauer positive, hydrolyse urea, are ornithine decarboxylase negative, do not produce hydrogen sulphide and they have a GC content of 53-58% (Ørstov, 1986a).

1.9.3 Characteristics of the members of the species *Lactobacillus* and *Carnobacterium*

The genus *Lactobacillus* represents a diverse group of bacteria and over 40 species are listed in Bergey's Manual for Systematic Bacteriology. *Lactobacillus* are: Gram-positive, rod shaped non-spore forming, microaerophillic, catalase negative bacteria (Stamer, 1979; Kandler and Weiss, 1986). *Lactobacillus* species grow over a wide temperature range from two to 53°C with maxima around 30-40°C, are aciduric (growing optimally at pH 5.5-6.2) and have complex nutrient requirements which are species specific (Kandler and Weiss, 1986). Not all of the members of the genus *Lactobacillus* are important in meat spoilage, the ones that are include: *Lb. sakei*, *Lb. curvatus*, *Lb. alimentarius*, *Lb. farciminis*, *Lb. plantarum* and *Lb. halotolerans* (Shaw and Harding, 1984; Korkeala and Mäkelä, 1989; Grant and Patterson, 1991; Montel *et al.*, 1991; Klein *et al.*, 1996). *Lb. sakei* originally called *Lb. sake* (Trüper and De Clari, 1997), was first isolated from rice wine but can be found in fermented products, silage, pre-packaged dough products and meat products, as can *Lb. curvatus* (Kandler and Weiss, 1986). *Lb. sakei* and *Lb. curvatus* are the two principal isolates found in meat products (Shaw and Harding, 1984; Grant and Patterson, 1991; Klein *et al.*, 1996). Although closely related, *Lb. sakei* and *Lb. curvatus* are distinct species (Kagermeier-Calloway and Lauer, 1995). DNA-DNA homology revealed that *Lb. curvatus* was not related to other members of genus *Lactobacillus* with the exception of *Lb. sakei,* to which it is only 40-50% homologous (Kandler and Weiss, 1986). Within each species there is high sequence homology, 84-98% for *Lb. sakei* and 84-102% for *Lb. curvatus* (Klein *et al.*, 1996). Physiologically both species are homofermentative and produce both D- and L-lactic acid from glucose. *Lb. sakei* is distinguished from *Lb. curvatus* by possessing arginine deaminase and fermenting melibose (Kandler and Weiss, 1984; Montel *et al.*, 1991).

Table 1.3 Characteristics which differentiate members of the genus *Carnobacterium* into the species of *C. divergens*, *C. gallinarum*, *C. mobile* and *C. piscicola*, on the basis of acid production, motility and fatty acid content (from Collins *et al.*, 1987).

	Carnobacterium			
	divergens	***gallinarum***	***mobile***	***piscicola***
Acid production from[a]:				
Amygdalin	+	+	-	+
Gluconate	+(-)	+	-	+
Inulin	-	-	+	+
Mannitol	-	-	-	+
Melezitose	v	-	-	+(-)
α-methyl-D-glucoside	-	+	-	+
α-methyl-D-mannoside	-	+	-	+
D-tagatose	-	+	-(+)	-
D-turanose	-	+	-	+(-)
D-Xylose	-	+	-(+)	-
Voges-Proskauer	+	+	-(+)	+
Motility	-	-	+	-
9,10-methyloctadecanoic acid	+	-	-	-

V variable
+(-) occasional positive
-(+) occasional negative
[a] reading after seven days
>15% of total cellular fatty acid

Like *Lb. sakei*, *Lb. farcinimis* is homofermentative and hydrolyses arginine but it is different in that it only produces L-lactic acid from glucose (Montel *et al.*, 1991). Similarly, *Lb. alimentarius* is homofermentative and produces L-lactic acid but does not hydrolyse arginine. In contrast, *Lb. halotolerans* is heterofermentative, produces D- and L-lactic acid and hydrolyses arginine (Montel *et al.*, 1991). The absence of arginine dihydrolase separates *Lb. viridescens* from *Lb. halotolerans* (Montel *et al.*, 1991). *Lb. plantarum* was originally isolated from plants, it requires calcium panthothenate and niacin for growth, does not grow at 45°C, produces both D- and L- lactic acid from glucose and does not hydrolyse arginine (Kandler and Weiss, 1986; Montel *et al.*, 1991). The major difference between *Lb. plantarum* and other lactobacilli is the presence of meso-diaminopimelic acid (*meso*-DAP) in cell walls of *Lb. plantarium* which is absent in the others (Montel *et al.*, 1991). In general, the absence of *meso*-diaminopimelic acid distinguishes *Lactobacillus* from *Carnobacterium*.

Two members of the genus *Carnobacterium* were originally classified as atypical *Lactobacillus* species. *C. divergens* and *C. piscicola* were initially called *Lb. divergens* (Holzapfel and Gerber, 1983) and *Lb. piscicola* (Hiu *et al.*, 1984), respectively. These two species were re-classified in 1987 by Collins *et al.* together with two unclassified isolates from chicken which were designated *C. mobile* and *C. gallinarum*. *Carnobacterium* species are: Gram positive rods occurring singly or in pairs, are catalase negative, heterofermentative, produce L(+) lactic acid, do not grow on acetate agar (Rogosa *et al.*, 1951), posseses *meso*-DAP acid in their cell wall and have a GC content of 33-37.5% (Collins *et al.*, 1987). Most *Carnobacterium* are capable of growth at 0°C, all grow at 10°C but none at 45°C (Collins *et al.*, 1987). Characteristics which distinguish the different members of the *Carnobacterium* genus are listed in Table 1.3. *C. mobile* is the only member of the genus which is motile (Collins *et al.*, 1987). *C. divergens* does not utilise either α-methyl-D-glucoside or α-methyl-D-mannoside while *C. piscicola* and *C. gallinarum* do (Collins *et al.*, 1987). *C. piscicola* can be differentiated from *C. gallinarum* by the production of acid from inulin and mannitol by *C. piscicola* (Collins *et al.*, 1987). Sequencing of the 16S ribosomal RNA (rRNA) of *Carnobacterium* revealed that the four species are closely related to each other but distinct from other LAB (Wallbanks *et al.*, 1990).

1.9.4 Characteristics of members of the species *Pseudomonas*

Many Gram-negative bacteria which did not fit into any other genera were traditionally placed in the genus *Pseudomonas* (Holloway and Morgan, 1986). Consequently the genus *Pseudomonas* is large and contains a diverse range of species which includes human and plant pathogens (Palleroni 1981; Stolp and Gadkari, 1981), species involved in bioremediation (Lipski *et al.*, 1992) and species important in food spoilage (Molin and Ternström, 1982; Shaw and Latty, 1982; Shaw and Latty, 1984). *Pseudomonas* is ubiquitous in the environment, being isolated from soil, fresh water, marine environments and food (Barnes and Thornley, 1966; Stolp and Gadkari, 1981; Shaw and Latty, 1982;

Molin and Ternström, 1982; Molin and Ternström, 1986; Lipski *et al.*, 1992). *Pseudomonas* was first described in 1894 by Migula but it was not until the 1960s that a systematic biochemical characterisation of the genus was conducted (Pelleroni, 1981). The common characteristics of *Pseudomonas* species are that they are Gram-negative rods which are either straight or slightly curved, with polar flagellation and do not form spores (Palleroni, 1986). They are chemoorganotrophs which utilise a large range of carbon compounds (Palleroni, 1986).

The genus *Pseudomonas* was divided into five rRNA groups (De Vos and De Ley, 1983; Palleroni, 1986) although not all members of the genus fall into these groups (Palleroni, 1986). The groups included the fluorescent pseudomonads (*P. fluorescens*, *P. putida*, *P. aeruginosa*), biochemically active pseudomonads (*P. cepacia*, *P. mallei*), moderately active *Pseudomonas* (*P. acidovorans*, *P. alcaligenes*), *P. vesicular* and *P. maltophila* (Sneath *et al.*, 1981; De Vos and De Ley, 1983; Palleroni, 1986). Further investigations have resulted in members of the genus *Pseudomonas* being reclassified into other genera. *P. acidovorans* and *P. testosteroni* have been re-classified in the genus *Comamonas* (Tamaoka *et al.*, 1987).

The fluorescent pseudomonads, *P. fluorescens*, *P. putida*, *P. aeruginosa*, produce a characteristic fluorescent pigment, pyroverdin (Bergen, 1981; Palleroni, 1986). The species *P. fluorescens* can grow at 4°C but not at 42°C, it grows optimally at 25-30°C (Bergen, 1981). *P. fluorescens* can be divided into five biotypes, biotype I is the reference strain for the species, biotype II contains the saphorphytic strains, biotype III can be further subdivided into two groups based on carboxylic acid utilisation, biotype IV contains the re-named *P. lemonnieri* and biotype V are the miscellaneous *P. fluorescens* (Palleroni, 1986). There were two other *P. fluorescens* biotypes but these have been re-named as *P. aureofacines* and *P. chororaphis* (Palleroni, 1981). The GC content of *P. fluorescens* ranges from 59.4 to 61.3% (Palleroni, 1986). The fluorescent pseudomonads can be separated by the following attributes: *P. fluorescens* can be distinguished from *P. putida* and *P. aeruginosa* by the production of levan from sucrose and lethinase activity, while *P. putida* and *P. aeruginosa* can be separated by the production of gelatinase, which is positive for *P. aeruginosa* (Palleroni, 1986).

P. putida can grow at 42°C with most strains also able to grow at 4°C and it has a GC content of 60.7 to 62.5% (Bergen, 1981; Palleroni, 1986). *P. putida* can be divided into two biotypes, A and B, which can be separated by biotype A not being able to utilise either L-tryptophan or L-kynurenine (Palleroni, 1986).

P. aeurginosa is both a human and plant pathogen, there are two colony morphologies. The first a large, smooth colony with a raised centre is characteristic of clinical isolates, while a small, rough

convex colony type is characteristic of environmental isolates (Palleroni, 1986). *P. aeruginosa* grows optimally at 37°C and some strains have been known to produce a red-pigment (Palleroni, 1986).

P. fragi is one of the members of the genus that does not fall within one of the five rRNA groups. Although it is widely distributed in the environment and is important in meat spoilage, *P. fragi* is poorly characterised and was not described in the eighth edition of Bergey's Manual of Determinative Bacteriology (Molin and Ternström, 1982). Originally called *Bacterium fragi* by Eichholz in 1902, it was isolated as a psychrotrophic organism from spoiled milk. A characteristic of this bacterium is that it produced an odour which resembles strawberries, hence the name *fragi* coming from fragum meaning strawberry (Molin and Ternström, 1982). Eventually *P. fragi* was transferred to the *Pseudomonas* genus. *P. fragi* can be distinguished from the fluorescent pseudomonads in that it does not produce a fluorescent pigment, and it utilises a greater range of carbon substrates (Molin and Ternström, 1982; Drosinos and Board, 1994). The absence of fluorescent pigment combined with the absence of gelatinase differentiates *P. fragi* from *P. fluorescens* (Molin and Ternström, 1982; Craven and McAuley, 1992).

1.10 METHODS IN BACTERIAL IDENTIFICATION

In order to be classified into a species an organism must be characterised and the more information that can be accumulated about an organism's morphology, both cellular and colonial, biochemical pathways and genetics the more accurate is its subsequent identification and classification into a species. Bacterial species can be identified using biochemical methods which rely on the phenotypic expression of their genomes or by molecular biological approaches which examines the genome directly. The results of the biochemical tests indicate the phenotype of a bacteria which can be influenced by the conditions under which it has been grown. Methods which examine bacteria at the molecular level can give more detailed information about the bacteria, not only determining species but also strain type. In addition to taxonomic information, molecular typing methods can provide valuable epidemiological information to the food microbiologist (Skovgaard, 1985). The following sections selectively review traditional taxonomic approaches based on biochemical analysis plus more recent genetic approaches.

1.10.1 Chemotaxonomic techniques

1.10.1.2 Biochemical

Bacteria can be identified by morphological and physiological characteristics through biochemical tests. The Gram stain forms the basis for the selection of all subsequent biochemical tests conducted

on unknown bacteria in order to determine identity (Halebain *et al.*, 1981; Bourgault and Lamothe, 1988). The identification of an unknown bacteria isolate relies on a combination of information about morphology (both colony and cellular), motility, spore formation, oxidase (Kovacs, 1956), catalase, acid and gas production from sugars (Shaw and Latty, 1984), utilisation of amino acids (Shaw and Harding, 1984; Schillinger and Lück, 1987; Montel and Champomier, 1989), mode of metabolism (Cowan and Steel, 1964; McDonald *et al.*, 1987), growth at different temperatures (Wilkinson and Jones, 1977; Talon *et al.*, 1988; Prieto *et al.*, 1992) or pH (Shaw and Harding, 1984) or salt concentrations (Wilkinson and Jones, 1977; Talon *et al.*, 1988; Prieto *et al.*, 1992) and end-product formation (Garvie, 1967). This can produce a vast quantity of information.

Large quantities of morphological and physiological data can be integrated through numerical taxonomy (Sneath, 1986). Numerical taxonomy developed in conjunction with the advent of computers (Sneath, 1986). In general, numerical taxonomy is conducted on a particular group of organisms, for example a group of Gram-negative oxidase positive bacteria (Sneath *et al.*, 1981; Molin and Ternström, 1982; Shaw and Latty, 1982; Molin and Ternström, 1986; Prieto *et al.*, 1992). The unknown organisms are combined with a group of reference strains which undergo analysis in tandem with the unknowns. Each result is given a value often one for a positive result and zero for a negative and results which are all positive or all negative are often discarded as they give little or no information about the strains (Sneath, 1986). The results are fed into a computer program which assesses the relationship of the unknowns and reference cultures, producing a dendrogram indicating the degree of similarity between isolates. Ideally, each cluster of unknowns should group with one reference strain. Numerical taxonomy has been used to identify groups of Gram-negatives (Thornley, 1967; Sneath *et al.*, 1981; Molin and Ternström, 1982; Shaw and Latty, 1982; Molin and Ternström, 1986; Shaw and Latty, 1988; Prieto *et al.*, 1992) and Gram-positives (Wilkinson and Jones, 1977; Shaw and Harding, 1984; Hastings and Holzapfel, 1987; Borch and Molin, 1988; Ferseu and Jones, 1988; Korkeala and Mäkelä, 1989; Grant and Patterson, 1991; Mauguin and Novel, 1994; Samelis *et al.*, 1994).

Not all bacteria will exhibit exactly the characteristics which are associated with a particular bacterial species (Shaw and Harding, 1984; Grant and Patterson, 1991; Mauguin and Novel, 1994). Some of the unclassified organisms can be identified with the benefit of hind-sight. Shaw and Harding (1984) described a distinct large cluster of Gram-positives which did not include a reference stain. These isolates were rod shaped, rarely formed chains and did not grow on acetate agar (Shaw and Harding, 1984), which fits the description of *Carnobacterium* given by Collins *et al.* (1987). Shaw and Harding (1984) had not included *Lb. divergens* or *Lb. piscicola* as reference strains which were later transferred to the genus *Carnobacterium.*

The analysis of unknown bacterial isolates by a large number of morphological and physiological characteristics is exceedingly labour intensive and time consuming. This has lead to the advent of commercial identification kits designed to speed up identification using the same principals as numerical taxonomy. All the substrates required for the differentiation of bacterial species are contained within one compact unit backed up by a computer database to interpret the results (Bochner, 1989a,b). An unknown bacterial isolate can be identified within 24 hours by using commercially available kits (Cox *et al.*, 1977; Bochner, 1989a,b; Miller and Rhoden, 1991; Klinger *et al.*, 1992). A couple of preliminary tests, generally Gram-staining, are required to ensure that the correct kit is used for the identification.

1.10.1.2 Fatty acid analysis

Fatty acids, which are primarily located in the cytoplasmic membrane, can be utilised for bacterial identification (Busse *et al.*, 1996). Bacteria possess a wide range of fatty acids which vary in chain length from eight to 20 carbons, which can be saturated or unsaturated, branched or unbranched, or can have cyclopropane groups or hydroxyl groups (Brondz and Olsen, 1991; Busse *et al.*, 1996). The composition is also affected by the growth environment. The fatty acid composition of *L. monocytogenes* increased when grown in the presence of exogenous fatty acids (Juneja and Davidson, 1993). Therefore when using fatty acid analysis for bacterial identification, it is essential that the experimental conditions are standardised, as the results can vary with incubation temperature, phase of growth and growth medium (Stead, 1992; Busse *et al.*, 1996; Hatab *et al.*, 1997). *Deleya* species and marine *Alcaligenes* exhibited fatty acid profiles that made them distinct from terrestrial *Alcaligenes*, *Achromobacter*, *Agrobacteria* and *Rhizobium* species (Akagawa and Yamasoto, 1989). *Carnobacterium* can be differentiated from lactobacilli by fatty acid analysis, as *Carnobacterium* synthesize oleic acid instead of *cis*-vaccenic (Wallbanks *et al.*, 1990). *Bacteriodes fragilis* could be differentiated from other *Bacteriodies*, *Wolinella*, *Campylobacter*, *Prevottela* and *Porphyromas* by fatty acid composition (Brondz and Olson, 1991). *B. fragilis* contained 3-hydroxy-15-methylhexadecanoic acid, methyltetradecanoic acid and 13-methyl tetradecanoic acid but not dodecanoic acid or unsaturated fatty acids (Brondz and Olson, 1991). Seventy-nine corynebacteria could be divided into two groups each of which could be divided into a further three groups on the basis of fatty acid composition (Bendinger *et al.*, 1992). The first group of corynebacteria contained saturated and unsaturated fatty acids while the second was characterised by the presence of *iso*- and *anteiso*-branched fatty acids (Bendinger *et al.*, 1992). Fatty acid analysis of 31 Gram-negative, non-fermenting bacteria isolated from biofilters showed that these fell into three main groups, which could be further divided, with some isolates being clustered with reference strains (Lipski *et al.*, 1992). Although not all the isolates could be assigned to a known bacteria, five had identical fatty acid profiles to *Al. faecalis* and another five with *P. dimunuta* (Lipski *et al.*, 1992). Although fatty acid

analysis of dairy strains of *Bacillus* separated the *B. cereus* group from other *Bacillus* species, it was unable to separate them further into *B. cereus*, *B. mycoides* and *B. thuringiensis*, as their profiles were identical (Väisänen *et al.*, 1991). Nor could fatty acid analysis separate strains of *P. stutzeri* (Roselló-Mora *et al.*, 1994).

Not only can the presence or absence of fatty acids be used to differentiate bacterial isolates but the ratio of various fatty acids can also be used to distinguish closely related organisms (Klausner, 1988), *Lb. sakei* and *Lb. curvatus* could not be distinguished on the basis of fatty acid content but there were differences in the proportions of fatty acids (Rementizis and Samelis, 1996). *Lb. curvatus* contained less tetradecanoic and hexadecanoic acid than *Lb. sakei* while *Lb. sakei* had more octadecanoic acid, which enabled the differentiation of the strains (Rementizis and Samelis, 1996). There are instances where the fatty acid profile for a bacterial group is homogenous therefore another profiling method, such as protein profiling, is required (Rosselló-Mora *et al.*, 1994).

1.10.1.3 Diamino acid and protein profiling

The determination of the cell wall protein components also assists in the identification of bacterial isolates (Bousfiled *et al.*, 1985). The peptidoglycan layer of Gram-positive and Gram-negative bacteria contains diamino acids as a major component (Busse *et al.*, 1996). *Meso*-DAP, LL-diaminopimelic, L-ornithine, L-lysine and L-diamino acids are found at position three of the peptide (Busse *et al.*, 1996). The presence of *meso*-DAP acid in the cell wall of *Carnobacterium* species and *Lb. plantarum* enables them to be distinguished from other lactobacilli (Montel *et al.*, 1988). Envelope proteins in Gram-negative *Moraxella* species isolated from the slime on the surface of fish had a broad spectrum of polypeptides ranging from 14 kilodalton (kDa) to 64 kD, where most Gram negative bacteria have only a narrow range of outer membrane proteins (Chai, 1981).

A whole bacterial cell contains about 2000 proteins and the information that can be derived from these proteins is invaluable in the separation and characterisation of bacterial strains (Jackman, 1985). Whole cell protein profiles can be determined using sodium dodecyl polyacrilamide gel electrophoresis (SDS-PAGE), which separates out proteins according to the molecular size. SDS-PAGE differentiates bacteria on the basis of the presence or absence of proteins and when combined with densitometry, the profile can be used in numerical analysis (Jackman, 1985; Busse *et al.*, 1996), where the presence of major proteins is indicated by the intensity of the band. SDS-PAGE of soluble proteins differentiated strains of pseudomonads involved in biodegradation and enabled duplicated isolates to be located because of their identical profile (Busse *et al.*, 1989). *Eryispelothrix rhusiopathiae* and *E. tonsilarum* could be differentiated on the basis of dominant proteins (Tamur *et al.*, 1993). Approximately 60% of LAB from a traditional fermentation could be identified to species

level using SDS-PAGE by comparing protein profiles to those of reference strains (van den Berg *et al.*, 1993). Numerical analysis of SDS-PAGE protein profiles generated a dendrogram with 16 clusters of rRNA group II pseudomonads (Li and Haywood, 1994). Each cluster comprised strains of the same species while *P. solanacearum* was divided in three clusters (Li and Haywood, 1994). The SDS-PAGE profile of all four *Carnobacterium* species demonstrated that each species was distinct from the other (Dick *et al.*, 1995).

Protein profiles have been correlated to the biochemical results for *Enterococcus* (Merquier *et al.*, 1994) and serological results for *Erysipelothrix* (Tamura *et al.*, 1993). In general, whole cell protein profiles are used for the discrimination of bacteria at the sub-generic level and protein profiling needs to be combined with other chemotoxonomic techniques to assign a genus (Jackman, 1985).

1.10.2 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PGFE) was first described in 1984 by Schwartz and Cantor. DNA fragments larger than 20 kilobases (kb) cannot be resolved by conventional electrophoresis, where the electrical field is continuous (Mathew *et al.*, 1988a,b,c; Smith and Condimine, 1990). If, however, there is an alternating perpendicular electrical field, then fragments greater than 20kb can be size fractionated (Schwartz and Cantor, 1984). As a molecular typing tool PFGE is discriminatory and highly reproducible (Johnson *et al.*, 1995; Matushek *et al.*, 1996; Talon *et al.*, 1996).

1.10.2.1 Theory of PFGE fingerprinting

Washed whole bacterial cells are embedded within agarose, the cell wall is stripped away and cellular components are largely degraded enzymatically, leaving the genomic DNA, which is protected from shearing by the agarose. The DNA is linearised into fragments by a restriction enzyme which recognises rare sites in the genome, ideally producing between 15 and 30 fragments. The fragments are separated on an agarose gel according to their molecular size by an alternating electrical field which transverses the gel east/west then north/south. The electrical field causes the lineralised DNA to align parallel with the electrical current (Åkerman *et al.*, 1985; Stellwagen, 1985) i.e. east/west. When the field switches the DNA re-orientates in the new direction i.e. north/south. The length of time that the electrical field runs in one direction is referred to as a pulse time. As the electrical field runs diagonally across the gel, the DNA moves through the gel in a zigzag pattern (Mathew *et al.*, 1988a) which in relation to the origin is a straight line. The gel is then stained with ethidum bromide which binds to the DNA and fragments are visualised under UV light. The resulting pattern of the fractionated fragments is described as a fingerprint. There are several factors which affect PFGE fingerprinting.

1.10.2.2 Factors affecting PFGE

Agarose concentration, temperature, pulse duration, DNA topology, electrical field shape and enzyme concentration all influence PFGE fingerprinting (Cantor *et al.*, 1988; Mathew *et al.*, 1988 a,b,c). One of the primary factors affecting the movement of DNA is the size of the pores within the agarose matrix through which the DNA migrates. The concentration of agarose determines pore size, where the higher the agarose concentration, the smaller is the pore size (Serwer and Hayes, 1986). In PFGE the changes in mobility associated with agarose concentration are not the same as continuous electrophoresis (Mathew *et al.*, 1988a). Resolution of DNA fragments has been found to be greater at 1.2% agarose than 0.9% but when the concentration is increased above 1.2% the pore size becomes restrictive to DNA movement (Mathew *et al.*, 1988a).

The pulse time determines the size of DNA fragments separated. As mentioned previously, the DNA orientates itself in alignment with the direction of the electrical field (Åkerman *et al.*, 1985; Stellwagen, 1985). The fragments of DNA that progress through the gel are those that can re-orientate themselves during the pulse time (Schwartz and Cantor, 1984; Mathew *et al.*, 1988b). The longer the pulse time the larger the fragment that can be resolved and vise versa (Schwartz and Cantor, 1984; Hightower *et al.*, 1987; Mathew *et al.*, 1988b). More than one pulse time can be programmed during one run, thus increasing the number of bands resolved.

The electrical field running across the gel increases the temperature. Temperature control is essential during the electrophoresis, as the mobility of the DNA is temperature sensitive (Mathew *et al.*, 1988a). Therefore, a circulating refrigerated water bath is connected to the PFGE unit. The effect of temperature in PFGE cannot be fully explained by changes in the viscosity of the buffer, and it is thought that there are thermal effects involved which affect the DNA topology, ultimately determining movement through the gel (Mathew *et al.*, 1988a). The influence of topology on the migration of DNA through the gel is complex (Mathew *et al.*, 1988c). The movement of linear DNA is totally dependent on the pulse time (Schwartz and Cantor, 1984; Hightower *et al.*, 1987; Mathew *et al.*, 1988b). Supercoiled DNA (plasmids) move through the gel matrix independent of pulse time at a constant rate (Hightower *et al.*, 1987). Relaxation of the supercoiled DNA decreases mobility through the gel matrix, with the migration of nicked open circular DNA retarded even further (Hightower *et al.*, 1987).

The migration of the DNA fragments is also dependent on the electrical field across the field. In early PFGE apparatus, the electrical field across the gel was not uniform (Chu *et al.*, 1986) which resulted in curved lanes meaning that only a portion of a gel could be used and that comparisons between gels could be difficult (Schwartz and Cantor, 1984; Schwartz *et al.*, 1988). The development of the contour clamped homogeneous electrical field (CHEF) overcame the problem of variation in electrical

field (Chu *et al.*, 1986). With the CHEF system the electrical field is generated through multiple electrodes which are arranged along a polygonal contour which is clamped to a predetermined electrical potential (Chu *et al.*, 1986). Excellent resolution is achieved when the angle between the two perpendicular electrical fields is 120° (Chu *et al.*, 1986; Cantor *et al.*, 1988).

Finally, the number of bands produced depends on the restriction enzyme used. Enzyme selection is dependent on the GC content of the organism under investigation. For a definitive fingerprint, between 15 and 30 fragments are desirable. Therefore if an organism has a GC content of greater than 50%, an enzyme which restricts adenosine and thyamine (AT) rich sites will most likely produce the desired number of bands. Conversely, if the organism has a GC content of less than 50% an enzyme which restricts GC-rich sites is preferable. Some nucleotide sequences are rare within the genome, CTAG sites are extremely rare (McClelland *et al.*, 1987), therefore enzymes with this site in their recognition sequence will cut infrequently. In general, enzymes which have recognition sites of six to eight bases in length are used (Tanskanen *et al.*, 1990; Taylor *et al.*, 1992; Chervallier *et al.*, 1994; Daniel, 1995; Eremeeva *et al.*, 1995; Tsen *et al.*, 1997).

1.10.2.3 Bacterial differentiation by PFGE fingerprinting

PFGE fingerprinting can be utilised to differentiate between bacterial species and strains, as bacteria are considered to be different if they differ by one or more bands (Skov *et al.*, 1995; Tsen *et al.*, 1997). PFGE fingerprints enabled the differentiation of pathogenic and non-pathogenic *Listeria* (Brosch *et al.*, 1994) and *E. coli* (Böhm and Karch, 1992; Harseno *et al.*, 1993; Barrett *et al.*, 1994). Of 33 *Helicobacter pylori* strains, 32 had unique PFGE fingerprints (Taylor *et al.*, 1992). PFGE profiling with *Sma*I showed that unique fingerprints were obtained for all 20 methicillin-sensitive *S. aureus* strains while only seven were obtained for 27 methicillin-resistant strains (Carles-Nurit *et al.*, 1992). Eight different PFGE fingerprints occurred among enterotoxcin A producing *S. aureus* following digestion with *Sma*I (Tsen *et al.*, 1995). For *Bifidobactium* species, stains of *B. animalis* produced four different PFGE fingerprints, *B. bifidum* and *B. infanus* strains five while *B. brevis* and *B. lonum* strains produced three patterns (Roy *et al.*, 1996). Twenty-five strains of *Neisseria meningitidis* could be divided into 18 groups after PFGE fingerprinting with *Spe*I (Yakubu and Pennington, 1995). Again using *Spe*I 26 isolates of *Burkholderia picketti* were divided into nine groups (Chetoui *et al.*, 1997). PFGE fingerprinting of *Lb. helveticus* showed that a majority of strains, 18 out of 22, had unique patterns (Lortal *et al.*, 1997). Considerable genetic diversity was also displayed among Taiwanese non-enterotoxigenic *S. aureus*, where there were 54 distinct PFGE fingerprints for 81 isolates (Tsen *et al.*, 1997).

When two different restriction enzymes are utilised for PFGE analysis the number of different fingerprints produced are not necessarily identical. *Listeria* species digested with *Asc*I or *Apa*I produced 63 and 72 restriction fingerprints respectively for 176 isolates (Brosch *et al.*, 1994). Of 60 isolates of *Y. enterocolitica,* 36 and 33 different PFGE fingerprints were produced following digestion with *Not*I and *Xba*I, respectively (Buchrieser *et al.*, 1994). When these results were combined, *Y. enterocolitica* had 34 unique fingerprint patterns (Buchrieser *et al.*, 1994). Of 27 strains of *Propionibacterium jensenii*, 17 different PFGE fingerprints were produced with *Xba*I and 18 with *Ssp*I (Gautier *et al.*, 1996). A total of 78 and 86 different PFGE profiles were obtained following digestion of 141 *Campylobacter* species with *Sal*I and *Sma*I, respectively (Steele *et al.*, 1998). When the fingerprints for *Campylobacter* were combined, they gave rise to 98 distinct profiles (Steele *et al.*, 1998).

As bacterial stains are considered to be different if they differ by more than one band, PFGE fingerprinting can be used in epidemiological investigations. In epidemiological investigations, genotypic characteristics have proven useful in deciphering the relationship between clinical isolates and tracing the dispersion of particular strains (Yukau and Pennington, 1995). Bacteria that are related to each other serologically have a tendency to have common PFGE patterns (Buchrieser *et al.*, 1994; Hall *et al.*, 1996). PFGE fingerprinting has also been used in epidemiological investigations on *P. cepacia* (Anderson *et al.*, 1991), *Enterobacter cloacae* (Haertl and Bandlow, 1993), *Legionella bozemanii* (Lück *et al.*, 1995), *L. monocytogenes* (Proctor *et al.*, 1995), *N. meningitidis* (Yukau and Pennington, 1995) and *E. coli* 0157:H7 (Böhm and Karch, 1992; Barrett *et al.*, 1994; Johnson *et al.*, 1995).

Differences and similarities in the PFGE fingerprints of isolates can be used in examinations of bacterial strains from different countries. The entomopathogen *B. popillae* from America produced different PFGE fingerprints from New Zealand strains, however, there were some similar bands and their presence will facilitate investigations into the world wide spread of this bacterium (MacDonald and Kalmakoff, 1995). A dendrogram of PFGE fingerprint patterns showed that *Candida albicans* strains from Singapore were randomly distributed among those from America and Europe (Clemons *et al.*, 1997), indicating that geographical origin of a *C. albicans* strains could not be determined by PFGE fingerprinting. The origins of toxin producing strains of *S. aureus* could be determined by PFGE fingerprinting, as strains from Taiwan exhibited fewer restriction patterns than American strains (Tsen *et al.*, 1997) indicating that there is greater genetic diversity among American stains of *S. aureus* than Taiwanese stains, possibly due to the greater geographical size of America (Tsen *et al.*, 1997). PFGE can also be used to track genetic traits associated with bacteria in the environment (Eid and Sherwood, 1995) and has been used to trace the survival of *Leuconostocs* species used in wine

Table 1.4 Published estimation of the genome sizes of a variety of bacteria determined by PFGE.

Bacteria	Genome size (Mb)	Enzymes	Reference
B. popilliae	2.6-3.5	*Pme*I, *Sfi*I	MacDonald and Kalmakoff, 1995
B. animalis	1.2-1.5	*Xba*I, *Spe*I	Roy *et al.*, 1996
B. bifidum	1.3-2.2	*Xba*I, *Spe*I	Roy *et al.*, 1996
B. breve	1.0-2.0	*Xba*I, *Spe*I	Roy *et al.*, 1996
B. infantis	1.0-2.0	*Xba*I, *Spe*I	Roy *et al.*, 1996
B. longum	1.0-2.0	*Xba*I, *Spe*I	Roy *et al.*, 1996
C. divergens	2.950-3.410	*Sma*I	Daniel, 1995
Cl. botulinum	4.039±0.40	*Mlu* I, *Sma* I, *Rsr* I	Lin and Johnson, 1995
E. faecalis JH	2.6	*Apa*I, *Sma*I	Le Bourgeouis *et al.*, 1989
E. coli K12	4.7	*Not*I, *Sfi*I	Smith *et al.*, 1987
H. pylori	1.6-1.73	*Not* I and *Nru* I	Taylor *et al.*, 1992
L. lactis subsp. *cremoris* 187	2.6	*Apa*I, *Sma*I	Le Bourgeouis *et al.*, 1989
L. lactis subsp. *cremoris* BK5	2.6	*Apa*I, *Sma*I	Le Bourgeouis *et al.*, 1989
L. lactis subsp. *cremoris*	2.0-2.7	*Sma*I	Tanskanen *et al.*, 1990
L. lactis subsp. *cremoris* C2	2.5	*Apa*I, *Sma*I	Le Bourgeouis *et al.*, 1989
L. lactis subsp. *cremoris* F7/2	2.5	*Apa*I, *Sma*I	Le Bourgeouis *et al.*, 1989
L.lactis subsp. *lactis*	2.0-2.7	*Sma*I	Tanskanen *et al.*, 1990
Lc. oenos	~1.8	*Not*I, *Sfi*I	Kelly *et al.*, 1993
Lb. helveticus	1.85	*Sma*I	Lortal *et al.*, 1997
Lb. helveticus	2.0	*Sgr*I	Lortal *et al.*, 1997
Lb. plantarum	2.7-2.905	*Asc*I	Daniel, 1995
Lb. CCM 1904	3.321	*Sfi*I	Chevallier *et al.*, 1994
Lb. plantarum CCM 1904	3.394	*Asc*I	Chevallier *et al.*, 1994
Lb. plantarum CST 11031	3.758	*Sfi*I	Chevallier *et al.*, 1994
Lb. plantarum CST 11031	3.584	*Asc*I	Chevallier *et al.*, 1994
Lb. plantarum CST 11019	3.387	*Sfi*I	Chevallier *et al.*, 1994
Lb. plantarum CST 11019	3.930	*Asc*I	Chevallier *et al.*, 1994
Lb. plantarum CST 11023	3.883	*Sfi*I	Chevallier *et al.*, 1994
Lb. plantarum CST 11023	4.162	*Asc*I	Chevallier *et al.*, 1994
Lb. plantarum LP85-2	2.984	*Sfi*I	Chevallier *et al.*, 1994
Lb. plantarum LP85-2	3.164	*Asc*I	Chevallier *et al.*, 1994
Lb. paracasei	2.17	*Sfi*I	Ferrero *et al.* 1996
R. sphaeroides	4.4 ± 0.112	*Ase*I, *Spe*I, *Dra*I, *Sma*I	Suwanto and Kalpan, 1989
P. acidilactici	1.56	*Sma*I	Daniel, 1995
P. pentosaceus	1.2	*Sma*I	Daniel, 1995
P. fluorescens ATCC 1325	5.0	*Spe*I, *Dra*I, *Xba*I, *Asn*I	Grothues and Tümmler, 1991
P. putida DSM 50291	4.1	*Spe*I, *Dra*I, *Xba*I, *Asn*I	Grothues and Tümmler, 1991
P. aeruginosa DSM 1707	5.9	*Spe*I, *Dra*I, *Xba*I, *Asn*I	Grothues and Tümmler, 1991
P. cepacia DSM 50180	6.5	*Spe*I, *Dra*I, *Xba*I, *Asn*I	Grothues and Tümmler, 1991
P. aureofaciens DMS 50082	5.0	*Spe*I, *Dra*I, *Xba*I, *Asn*I	Grothues and Tümmler, 1991
S. thermophilus ST1	1.7	*Apa*I, *Sma*I	Le Bourgeois *et al.*, 1989
S. thermophilus	1.8-2.43	*Sma*I	Boutrou *et al.*, 1995
X. campestris DSM 1049	5.0	*Spe*I, *Dra*I, *Xba*I, *Asn*I	Grothues and Tümmler, 991

production (Kelly *et al.*, 1993). Further information about bacteria other than the fingerprint can be obtained from PFGE as described in the following section.

1.10.2.4. Genome size estimation by PFGE

Genome size can be estimated following PFGE fingerprinting by comparing the distance that each well separated band has moved with the distance moved by the molecular size markers run at the same time (Crété *et al.*, 1991). From this the molecular size of each band on a PFGE gel can be calculated and the sum of the fragments estimates the genome size. The genome sizes of a range of bacteria which are clinically and industrially significant as estimated by PFGE are listed in Table 1.4. PFGE showed that genome sizes ranged from 1.0 to 6.5 megabases (Mb)

In some cases, variation in the genome size for the same bacterial species has been seen (Suwanto and Kalpan, 1989; Tanskanen *et al.*, 1990; Chevallier *et al.*, 1994; Boutrou *et al.*, 1995; Daniel, 1995; Lin and Johnson *et al.*, 1995; MacDonald and Kalmakoff, 1995; Roy *et al.*, 1996). This could be explained by the presence or absence of a plasmid which may or may not contain sites recognised by the restriction endonuclease used. This would lead to errors in genome size estimation due to their movement through agarose (Tanskanen *et al.*, 1990) as previously discussed.

1.10.2.5 Some problems associated with PFGE

There are three main problems associated with using PFGE for genome size estimation. Firstly fragments ≤8kb can be difficult to detect especially as staining of DNA with ethidium bromide is size dependent (Smith and Condemine, 1990) and small fragments are able to diffuse out of the agarose matrix (Smith and Condemine, 1990) or they can run off the end of the gel. However, fragments of ≤8kb will not significantly influence the overall estimation of the genome size (Tanskansen *et al.*, 1990). Secondly, errors can be introduced when measuring the distance moved for genome size estimation, in particular, when there are two bands of similar size because bands which differ by <5% are difficult to differentiate (Crètè *et al.*, 1991). Thirdly, the presence of plasmids, which has already been discussed.

Not all bacterial species are suitable for PFGE analysis. Some bacteria cannot be prepared in sufficient quantity or quality for PFGE. Cells can contain indigenous DNases that degrade the DNA during lysis thus rendering the DNA unacceptable for analysis (Johnson *et al.*, 1995; Samore *et al.*, 1996). Degradation of the DNA during removal of the cell wall was prevented in *Actinomycetes* by either treating the cells with solvent prior to embedding or substituting HEPS for Tris (Beyazova *et al.*, 1995).

1.10.3 Ribotyping

Ribotyping is a technique which is used to detect polymorphisms using DNA probes. Chromosomal DNA is digested with restriction enzymes and electrophoresed before being transferred to a nylon membrane generally by Southern transfer. The DNA is then probed with ribosomal DNA (rDNA), the genes for rRNA, which are either radioactively or chemiluminescently labelled to enable detection on X-ray film. The probe can bind in one to several places within one digest, creating a banding pattern referred to as a ribotype. Probing 12 field strains and five reference strains of *Campylobacter* with 16S and 23S rRNA produced 15 different ribotypes following *Hae*II digestion (Hernandez *et al.*, 1991). Bacterial species can be further divided into sub-groups by ribotyping (Bouvet *et al.*, 1991). Sub-species separation could be achieved by ribotyping *Lactococcus* using *Eco*RI and *Hind*III (Rodrigues *et al.*, 1991). Ribotyping has been used to determine the source of nosocomal outbreaks (Blanc *et al.*, 1993) and food poisoning (Samadpour *et al.*, 1993). Ribotyping of bacteria can also be done using a functional gene as a probe instead of rRNA (Zheng and Kathroius, 1995).

The number of ribotypes will vary for the same organism depending on the enzyme used to cleave the DNA. For example when Blanc *et al.* (1993) examined 66 strains of *P. aeruginosa,* eight ribotypes were produced with *Bam*HI, 12 with either *Cla*I or *Eco*RI and 14 with *Pst*I. Both *Eco*RI and *Hind*III gave six different ribotypes for 26 isolates of *B. picketti* (Chetoui *et al.*, 1997). One disadvantage of ribotyping in epidemiological investigations is that ribotypes have been found to change over time (Rodtong and Tannock, 1993).

1.10.4 Polymerase chain reaction and randomly amplified polymorphic DNA

Polymerase chain reaction (PCR) is a highly sensitive and rapid technique (Olive, 1989; Furrer *et al.*, 1991; Wernars *et al.*, 1991; Brooks *et al.*, 1992; Tsuchiya *et al.*, 1992) which exponentially amplifies DNA (Saki *et al.*, 1985; Jones, 1992; Tsuchiya *et al.*, 1992). Primers are oligonucleotides which are designed to complement the boundaries of the target DNA (Tsuchiya *et al.*, 1992), the forward and reverse primers annealing to opposite ends of the target DNA (Saki *et al.*, 1988). The primers and DNA are combined with buffers, deoxynucleotides and a DNA polymerase prior to cycling through three phases: denaturation, annealing and extension (Saki *et al.*, 1985; Jones, 1992; Tsuchiya *et al.*, 1992). The DNA is denatured by heating the DNA above 90°C (Saki *et al.*, 1985; Jones, 1992; Tsuchiya *et al.*, 1992). The reaction mix then cools to a temperature which permits the annealing of the primers to their specific target sequences (Jones, 1992; Tsuchiya *et al.*, 1992). The temperature is then increased to facilitate the extension of the DNA from the primers by the DNA polymerase (Saki *et al.*, 1988; Jones, 1992; Tsuchiya *et al.*, 1992). After the extension the DNA is again denatured and the whole process occurs again with the newly formed DNA fragments also becoming templates for the primers (Saki *et al.*, 1985; Saki *et al.*, 1988; Jones, 1992; Tsuchiya *et al.*, 1992). The original

DNA polymerase, the Klenow fragment of *E. coli*, had to be replenished after each cycle as it is heat labile (Saki *et al.*, 1988). This has now been replaced by a heat stable DNA polymerase, *Taq*I, from *Thermus aquaticus* (Saki *et al.*, 1988).

Randomly amplified polymorphic DNA (RAPD) is a PCR based technique where there is only one primer used which has an arbitrary nucleotide sequence with a GC content greater than 40% (Welsh and McClelland, 1990; Williams *et al.*, 1990). The advantages of RAPD are: firstly, no prior sequence information is required, secondly, little preliminary work is necessary and thirdly, the primers can be utilised for a wide range of species (Welsh and McClelland, 1990; Williams *et al.*, 1990). RAPD has been used to discriminate between *Streptococcus* species (Welsh and McClelland, 1990), *Lactococcus lactis* (Cancilla *et al.*, 1992), *Listeria* species (Czajka *et al.*, 1993), *S. baureus* (Saulnier *et al.*, 1993), "*Haemophilus*" species (Myers *et al.*, 1993), *C. albicans* (Howell *et al.*, 1996), *Lb. sakei* (Björkroth *et al.*, 1996) and *Cl. difficile* (Samore *et al.*, 1996). RAPD analysis of *H. alvei* could separate strains which had an attachment efficiency gene (which was implicated in diarrhea) from strains that did not contain the gene (Ridell *et al.*, 1995). Incorrectly classified strains have been corrected following RAPD analysis (Czajka *et al.*, 1993). RAPD analysis of *Lb. sakei* and *Lb. barvarius* by Torriani *et al.*, (1996) supported the suggestion by Kagermeier-Calloway and Lauer (1995) that these two species were synonymous.

In addition to using random primers, specific primers can be designed for the detection and identification of bacteria. PCR primers have been specifically designed to detect the thermotolerant *Campylobacter* species, *C. jejuni*, *C. coli* and *C. lari,* based on 16S rRNA sequences (Giesendorf *et al.*, 1992). *C. coli and C. jejuni* have been detected by PCR with primers designed to the flagella gene (Oyofo *et al.*, 1992). Using a combination of five primers based on: universal, *Listeria* species and *L. monocytogenes* sequences, within one PCR reaction mix yielded three products, one for any bacteria present, one for any *Listeria* species present and another for any *L. monocytogenes* present (Border *et al.*, 1990). The combination of primers was able to detect *Listeria* species and *L. moncytogenes* within both purified DNA and cell lysates (Border *et al.*, 1990). The histidine carboxylase gene of *Cl. botulinum* and *Lactobacillus* has been detected with specific primers (Le Jeune *et al.*, 1995). Gene specific primers have been designed for toxin genes in toxigenic strains of *A. hydrophilia* (Pollard *et al.*, 1990), *A. salmonica* (Gustafson *et al.*, 1992), *Cl. botulinum* (Szabo *et al.*, 1992), *E. coli* (Olive, 1989; Lampel *et al.*,1990; Brian *et al.*, 1992; Gannon *et al.*, 1992), *L. monocytogenes* (Deneer *et al.*, 1991; Furrer *et al.*, 1991), *S. flexeri* (Lampel *et al.*, 1990), *S. aureus* (Wilson *et al.*, 1991), and *Y. enterocolitica* (Kwaga *et al.*, 1992; Nakajima *et al.*, 1992).

PCR can be used to identify bacteria in pure and mixed culture and in addition, PCR can be used to identify bacteria contaminating foods (Niederhauser *et al.*, 1992). *Carnobacterium* were detected with

a set of genus specific PCR primers in DNA extracted from meat homogenates (Brooks *et al.*, 1992). PCR could potentially be used to determine the microbial load of food homogenates by measuring the concentration of the product by electochemiluminescence (Venkitanarayanan *et al.*, 1997). The concentration of the final product was found to be related to the initial number of bacteria and the number of reaction cycles (Venkitanarayanan *et al.*, 1997). Care needs to be taken when using PCR to detect pathogens in foods as the number of bacteria present and compounds from within food can inhibit PCR reactions (Olive, 1989; Wernars *et al.*, 1991; Brooks *et al.*, 1992; Wang *et al.*, 1992).

PCR can be particularly useful when there are bacteria present in viable but not culturable forms or when present in very low numbers (Bej *et al.*, 1991; Tsuchiya *et al.*, 1992; Turpin *et al.*, 1993; Thornhill *et al.*, 1995). PCR could overcome the need to allow time for the enrichment or repair of bacteria, although enrichment does assist in detection (Gannon *et al.*, 1992). PCR products can be further analysed by sequencing (Wallbanks *et al.*, 1990; Martinez-Mucria and Collins, 1990; Collin *et al.*, 1991; Giessendorf *et al.*, 1992) or by restriction fragment length polymorphism analysis (Akopyanz *et al.*, 1995; Johansson *et al.*, 1995).

1.10.5 DNA-DNA and DNA-RNA hybridization and probes

The hybridization of DNA to DNA is considered to be the superior method for determining the relationship of one bacterial strain to another (Stackebrandt and Goebel, 1994). If there is less than 15% base mismatching, two single stands of DNA will reassociate forming a hybridized DNA which can be measured (Ullmann and McCarthy, 1973). A species generally can be considered to contain members whose DNA-DNA relatedness is approximately 70% or greater, with a melting temperature difference of 5°C or less (Stackebrandt and Goebel, 1994). If the DNA-DNA similarity is greater than 70% then the DNA sequence homology will be above 97% (Stackebrandt and Goebel, 1994). The formation of the DNA-DNA duplex is dependent on the degree of sequence homology, concentration of DNA, liquid volume and incubation temperature (Johnson and Ordal, 1968). DNA-DNA or DNA-rRNA hybridization has been used to assess the relatedness of *Brochothrix* (Talon *et al.*, 1990), *Carnobacterium* (Dicks *et al.*, 1995), *Lactobacillus* (Johansson *et al.*, 1995; Klein *et al.*, 1996) and *Pseudomonas* (Goor et al., 1984) species.

The principles of DNA hybridization can be applied to probes. DNA probes are short single DNA strands designed to detect a specific sequence of DNA or RNA (Betts *et al.*, 1995; Barry *et al.*, 1990). As with PCR, bacteria that have not been cultivated can be identified using probes (Giovannoni *et al.*, 1988). The method of probe utilisation can be summarised as follows: DNA which is bound to a nylon membrane is probed with oligonucleotides, designed to detect a specific gene, that are labelled either chemoluminscently or radioactively. The temperature for the hybridization and the stringency

of the wash will determine the degree of binding of the probe. Bacteria can be identified using species-specific probes that have been designed to hybridize with the variable regions of 16S rRNA (Giovannoni *et al.*, 1988; Barry *et al.*, 1990). Specific probes have been described for species of *Acinetobacter* (Ludwig *et al.*, 1994), *Alcaligenes* (Ludwig *et al.*, 1994), *Aeromonas* (Ludwig *et al.*, 1994), *Cl. botulinum* (Szabo *et al.*, 1992), *C. coli* (Oyofo *et al.*, 1992), *Lactobacillus* (Petrick *et al.*, 1988; Pilloud and Mollet, 1990; Cocconcelli *et al.*, 1991; Lonvaud-Funel *et al.*, 1991; Pot *et al.*, 1993; Vogel *et al.*, 1993; Quere *et al.*, 1997), *Lactococcus* (Salama *et al.*, 1993), *Leuconostocs* (Lonvaud-Funel *et al.*, 1991), *Nannocystis* (Ludwig *et al.*, 1994), *Pediococcus* (Cocconcelli *et al.*, 1991; Lonvaud-Funel *et al.*, 1991), *Pseudomonas* (De Vos and De Ley, 1983; Goor *et al.*, 1984; Ludwig *et al.*, 1994), *Salmonella* (Fitts *et al.*, 1983) and *Xanthomonas* (De Vos and De Ley, 1983). Probes can also be designed to detect functional genes (Zheng and Kathroius, 1995).

DNA probes can be used to detect bacteria within food homogenates (Brooks *et al.*, 1992). Detection of bacteria by DNA probes can be enhanced by pre-enrichment in non-selective media prior to detection (Fitts *et al.*, 1983).

1.10.6 Comparison of PFGE with other molecular typing techniques

Bacteria with the same PFGE patterns have demonstrated identical morphology, plasmid profile and phage sensitivity (Gautier *et al.*, 1996). PFGE results have been found to correlate well with flagella antigens (Brosch *et al.*, 1994), serotypes (Hall *et al.*, 1996), ribotypes (Anderson *et al.*, 1991; Björkroth *et al.*, 1996) and 16S rRNA data (Eremeeva *et al.*, 1995). PFGE has been found to be a more sensitive fingerprinting technique than RAPD for *V. angeullarium* (Slov *et al.*, 1995), *S. aureus* (Saulnier *et al.*, 1993) and *Lb. sakei* (Björkroth *et al.*, 1996). However, for *Cl. difficile* strains PFGE was considered less discriminatory than either restriction enzyme analysis or RAPD due to DNA degradation (Samore *et al.*, 1996).

There is no one definitive method for the identification of bacterial isolates. The identification of bacteria involved the culmination of a series of meticulous investigations which examine the physiological capabilities of a species and the genomic information contained within the cell. Molecular methods cannot be satisfactorily applied without the initial information provided by biochemical tests. Molecular methods could be invaluable in identifying non-culturable bacteria with food homogenates and are essential for further subtyping of bacterial strains in taxonomic investigations.

1. 11 ENVIRONMENTAL FACTORS AFFECTING BACTERIAL GROWTH

The types of bacteria found in a particular environment differ with the environmental conditions present as previously discussed. There are a series of environmental parameters which play an important role in the growth of bacteria in an environment. In food microbiology the environmental factors which are of most importance are: temperature, pH, water availability, the atmosphere composition and preservative. These factors can be manipulated to increase shelf-life through inhibition of bacterial growth. The term inhibition, in food microbiology refers to both the bacteristatic and bactericidal activities of the preservative method. The environmental factors discussed here are done so with reference to meat.

1.11.1 Temperature

Temperature is considered to be the most important of the environmental factors affecting the growth of bacteria (Olsen and Nottingham, 1980; Zwietering *et al.* 1990). In terms of the temperature range over which bacteria grow, they can be divided into four groups: psychrophiles, psychrotrophs, mesophiles and thermophiles. Psychrophiles are bacteria that have optimal growth temperatures ≤15°C and a maximum growth temperature at ≤20°C. True psychrophiles are found in environments that are permanently cold (Gounot, 1991), for example, the ocean depths and the polar ice caps. Mesophiles are bacteria which have an optimum growth temperature between 20 and 40°C. Psychrotrophs are capable of growth over the temperature ranges of both psychrophiles and mesophiles: they grow at a reduced rate under temperatures associated with psychrophiles and have maximum growth rates at temperatures associated with mesophiles. The environments which psychrotrophs are found range from water, soil, air and food (Gounot, 1991). While thermophiles have a maximum growth rate above 45°C, these bacteria are found in a number of hot or super-heated (>100°C) environments such as hot spring. Psychrotrophs, a group which encompass many pathogens, are the significant groups in meat spoilage. This is primarily because temperature influences the microflora composition (McMullen and Stiles, 1993).

Temperature will influence each phase of the growth cycle. Lag phase will increase as temperature decreases (Olsen and Nottingham, 1980; Walker *et al.*, 1990; Buchanan and Klatwitter, 1991; Bhaduri *et al.*, 1995). When *B. cereus* was pre-incubated at 7°C, the lag phase lasted 1.5 days but if pre-incubated at 37°C, lag phase lasted approximately one week when incubated at 7°C (Dufrenne *et al.*, 1997). Bacterial growth rates are reduced at lower temperatures (Jones *et al.*, 1987; Walker *et al.*, 1990; Adams *et al.*, 1991; Williamsky *et al.*, 1992; Bhaduri *et al.*, 1995). Shifting bacteria growing at a higher temperature to a lower one will induce a lag phase followed by growth at a lower rate (Broeze *et al.*, 1978; Jones *et al.*, 1987). The final number of bacteria at stationary phase has been shown to decrease with temperature (Bailey *et al.*, 1979a; Walker *et al.*, 1990). The uptake of solutes is reduced

at low temperatures and for mesophiles uptake can cease completely (Rose, 1968). The production of proteinases is decreased when *Pseudomonas* species are grown at low temperatures (Juffs, 1970). *P. fluorescens* will digest litmus milk when stored at 10°C but will not at either two or 5°C (Juffs, 1970). Changes caused by temperature can also be observed in phospholipids. Gill (1975) demonstrated that carbon limited cells at decreased temperatures had an increased proportion of phosphatidyl ethanolamine and decreased phosphotidyl glycerol and cardiolipin, but under nitrogen limitation there was a significant increase in cardiolipin. However, this variation is not significant as a precise phospholipid composition is not required for growth at a low temperature (Gill, 1975). Cells grown at low temperature increased the synthesis of unsaturated fatty acids enabling the membrane to remain fluid at low temperature and continue membrane activity (Rose, 1968). Coccoid forms of *C. jejuni* formed at 4°C had significantly different fatty acid profiles from those formed at 25°C (Hazeleger *et al.*, 1995). In addition the levels of adenosine triphosphate (ATP) within cells were higher if incubated at four and 12°C than at 25°C (Hazeleger *et al.*, 1995).

The inability of mesophiles to survive at low temperatures was thought to be linked to their inability synthesize to proteins (Broeze *et al.*, 1978). A mesophilic *E. coli* transferred from 37°C to 5°C ceased to grow, protein synthesis was negligible but respiration rate was not inhibited to the same extent, suggesting that protein synthesis was the controlling factor (Broeze *et al.*, 1978). When psychrotrophic *E. coli* and *P. fluorescens* were shifted from 37°C to 10 and 4°C, respectively, a lag phase of fours hours was induced (Broeze *et al.*, 1978; Jones *et al.*, 1987) but no lag phase was observed for *B. subtilis* (Williamsky *et al.*, 1992). Immediately after the shift, the production of polypeptides by *E. coli* was reduced, however, over time the production of polypeptides increased again (Jones *et al.*, 1987) while *P. fluorescens* was found to continue to synthesize proteins but at a new rate (Broeze *et al.*, 1978). A single protein was found to be over-expressed when *E. coli* was transferred from 37°C to 10°C (Jones *et al.*, 1987), suggesting the involvement of a cold shock gene. A cold shock protein, CspB, was found to be involved in the acquired cold tolerance of *B. subtilis* (Williamsky *et al.*, 1992). The gene for CspB, *csp*B, can be disrupted without affecting other cellular phenotypes (Zeng and Katharous, 1994). *B. subtilis* mutants with a disrupted *csp*B gene were found to be sensitive to freezing, however, if the cells were pre-incubated at 4°C prior to freezing, there was an increase in the survival rate (Williamsky *et al.* 1992). This suggests the involvement of more than one protein in cold tolerance (Williamsky *et al.*, 1992). A study of protein synthesis over time in *E. coli* after a down-shift in temperatures showed that there were 13 proteins that were synthesized between zero and 60 minutes after the shift. (Jones *et al.*, 1987). These proteins corresponded to proteins involved in transcription, translation and possibly messenger RNA degradation (Jones *et al.*, 1992). Genes have also been demonstrated to be involved in the ability of *L. monocytogenes* to grow a 4°C (Zheng and Kathariou, 1994). Rose (1968) commented that there were no cold tolerant pathogens,

however, 20 years later *E. coli, L. monocytogenes* (Walker *et al.*, 1990; Zheng and Kathariou, 1994), *A. hydrophilia* and *Y. enterocolitica* (Little *et al.*, 1992b) were all found to grow at low temperatures.

1.11.2 pH

Microorganisms can survive over a wide range of external pH, from 1 to 11, however no bacteria can grow over this entire range (Corlett and Brown, 1980). Bacteria are divided into three broad categories depending on the pH ranges over which they grow. Acidophiles grow in acid environments and have an internal pH of 6.5-7; neutrophiles grow in neutral environments and have an internal of pH 7.5-8, while alkalophiles grow in alkaline environments and have an internal pH of 8.4-9 (Booth, 1985). All of the major meat spoilage bacteria belong to the neutrophile group, therefore it will be the response of this group to environmental pH change which will be reviewed in the following section. The internal pH directly affects cell growth and metabolism (Zeng *et al.*, 1990).

There is a direct relationship between the internal pH of a bacteria and the environment (Gould and Measures, 1977; O'Sullivan and Condon, 1997). As the pH of the environment decreases the internal pH decreases (McDonald *et al.*, 1990; O'Sullivan and Condon, 1997). Neutrophilic bacteria are able to maintain pH homeostasis at pH levels ranging from pH 5-8, depending on the organism, with the internal pH being higher than the external (Booth, 1985). The internal pH of *E. coli* remains at 7.8 as the pH varies from pH 6-8 (Pandan *et al.*, 1976). *S. faecalis* in a medium of pH 6.5-7.8 has an optimum growth rate and a cytoplasmic pH of 7.5-7.7 (Kobayashi, 1985). The internal pH of glycolysing *Streptococcus* species, *S. lactis* and *S. cremoris,* remains at pH 7.5 as the external environment varies between pH 5-7.5, while *S. lactis* metabolising arginine has an internal pH level between 7.1-7.2 (Poolman *et al.*, 1987). As the pH continues to decrease, bacteria will no longer be able to maintain pH homeostasis and the cytoplasm will become acidified. When the internal pH of *L. lactis* subsp. *cremoris* decreased to pH 5, growth ceased and as the internal pH continued to decrease as the cells died (O'Sullivan and Condon, 1997). Similarly, *Lc. mesenteroides* ceased growing when the internal pH reached 5.4-5.7, while *Lb. plantarum* did not cease growth until the internal pH reached 4.6-4.8 (McDonald *et al.*, 1990). The maintenance of internal pH is achieved by cytoplasmic buffers, the production of acids or bases and, more significantly, by the transport of ions (Booth, 1985)

The relationship between the internal and external pH in neutrophiles is that the internal pH is alkaline in relation to the external environment (Booth, 1985). The difference between the two pH levels produces the proton gradient, ΔpH. In addition, neutrophilic bacteria have a negatively charged cytoplasm compared to the external environment: the difference in electrical charge is known as the transmembrane electrical potential, $\Delta\Psi$ (Kashket, 1985). The maintenance of these two factors is of major biological significance. The combination of the ΔpH and $\Delta\Psi$ produces the proton motive force

which drives the movement of protons into the cell (Kashket *et al.*, 1980; Kashket, 1985). When protons move into the cell, adenosine-diphoshphate (ADP) is converted into ATP by ATPase which produces energy for the cell (Mitchell, 1961, 1966). As the bacterial membrane is relatively impermeable to protons, they pass instead through protein complexes situated within the cell wall (Raven and Beardall, 1980). It is the activity of the respiratory pathway which maintains the internal pH of aerobes (Booth, 1985). In anaerobes, pH homeostasis is maintained by H^+-ATPase (Kobayashi *et al.*, 1986).

In *S. faecalis* H^+-APTase has been demonstrated to be involved in pH homeostasis (Kobayashi *et al.*, 1986). When there is a down-shift in the cytoplasmic pH caused by an external pH of less than seven, there is an increase in the quantity of H^+-APTase in the cytoplasm (Kobayashi *et al.*, 1986). Conversely, when cells were transferred from pH5.9 to 8.7, there was a decrease in the amount of H^+-APTase in the cytoplasm (Kobayashi *et al.*, 1986).

The minimum pH at which a bacteria will grow is dependent on the acidulant used and the media composition (Poolman *et al.*, 1987; O'Sullivan and Condon, 1997; Ruis and Lorén, 1998). The proportion of dissociated to undissociated acid is significant, as the active portion is the undissociated acid. Weak acids, for example acetic, citric and lactic acids, are more effective at reducing the internal pH of bacteria than stronger acids, for example hydrochloric (O'Sullivan and Condon, 1997). The weak acids have a higher pKa than strong acids, which means that more undissociated acid is present in the environment (Young and Forgeding, 1993). The undissociated acid passes freely through the cell wall into the cytoplasm where it can then dissociate and decrease the internal pH (Eklund, 1989; Young and Forgeding, 1993). As strong acids exist in the dissociated form, which does not move as freely across the membrane, they are therefore not as efficient at reducing the internal pH (O'Sullivan and Condon, 1997).

Although both the dissociated and undissociated forms of organic acids have inhibitory activity (Skirdal and Eklund, 1993), the more significant active anti-microbial component is the undissociated acid (Eklund, 1985; Zeng *et al.*, 1990). The numbers of *L. monocytogenes*, *Y. enterocolitica* and *A. hydrophilia* were found to decrease on lean pork after dipping into 3% lactic acid (Greer and Dilts, 1995). The addition of sodium lactate caused the ΔpH in *L. mesenteroides* to decrease and eventually collapse, while sodium acetate only decreased the ΔpH (McDonald *et al.*, 1990). When the sodium lactate concentration increased there was a decrease in the aerobic plate count of cooked beef (Papadopoulous *et al.*, 1991) Of the organic acids, acetic acid has been shown to be more inhibitory than lactic or citric acid (Faber *et al.*, 1989; Brocklehurst and Lund, 1990; Adams *et al.*, 1991; Karapinar and Gönöl, 1992). Acetic acid, even if produced by the organisms itself, has a strong inhibitory effect on the growth of *E. aerogens* (Zeng *et al.*, 1990).

The ability of bacteria to withstand lethal pH can be induced by exposing the bacterial cells to sublethal pH for a period of time before exposing to a lethal pH: this is known as the acid tolerance response (O'Sullivan and Condon, 1997). The acid tolerance response has been observed in *E. coli* (Goodson and Rowbury, 1989), *L. monocytogenes* (Kroll and Patchett, 1992), *Lc. mesenteroides* and *Lb. plantarum* (McDonald *et al.,* 1990), and *L. lactis* subsp. *cremoris* (O'Sullivan and Condon, 1997). Acid tolerance is particularly important in pathogenic organisms and it is thought to be linked to the low infective dose of pathogenic bacteria such as *Listeria, E. coli* 0157:H7 and *Shigella* (Datta and Benjaman, 1997).

The normal pH range of meat is 5.5 to 6.5 (Ledward *et al.,* 1970; Carse and Locker, 1974; Gill and Penney, 1985) which is well within the growth ranges of all meat spoilage bacteria. By its nature, meat is a highly buffered system (personal communication, Barry Shay, Division of Food Science and Technology, CSIRO, Cannon Hill, Queensland) therefore it is difficult to change the pH of meat. However, a temporary reduction in surface pH can be achieved with acidic sprays (Woolthus and Smulders, 1985; Anderson and Marshall, 1989; Zeitoun and Debevere, 1990; Greer and Jones, 1991).

Combining an organic acid with reduced temperature or MAP or low pH will enhance the anti-microbial activities of the organic acid. The growth of *B. thermosphacta* was suppressed in liquid media when exposed to lactic acid and anaerobic conditions (Grau, 1980). An increase in shelf-life of chicken legs can by achieved by combining lactic acid and sodium lactate at pH 3.0 with MAP (Zeitoun and Debevere, 1991). After 24 hours the number of bacteria on lactic acid treated beef was significantly lower than control samples (Greer and Jones, 1991). In laboratory media, lactic acid and hydrochloric acid produced the same inhibitory effects on the growth of meat microflora at pH 5.5 and 2°C, while acetic acid produced more severe inhibitory actions: only *Pseudomonas* and *Alteromonas* grew (Gill and Newton, 1982). When the temperature was increased to 25°C however, *Enterobacteriaceae* were no longer inhibited (Gill and Newton, 1982). *L. monocytogenes* was found to be more resistant to a 2% lactic acid at pH 2.6 and 21°C than *S. typhimurium* and *C. jejuni* (van Netten *et al.,* 1994). The bactericidal activities of lactic acid are reduced if the pH of the solution is increased (van Netten *et al.,* 1994) as this would affect the ratio of dissociated to undissociated acid.

1.11.3 Water availability

The availability of water for the growth of bacteria is described as water activity, which is defined as the ratio of the water vapour pressure of the environment, in this case food, to that of pure water, which is defined as one (Christian, 1980). As the solute concentration increases, the water activity decreases. Some bacteria are able to survive in environments where the water activity is low: halophiles are able to grow in environments where the salt concentration is high and these bacteria

often cannot grow unless salt is present. Osmophiles can grow in environments where the osmotic pressure is high, for instance in environments where there is a high amount of sugar such as jams. The third group are xerophiles, which are able to grow in desiccated environments. However, due to the nature of these bacteria and the environments in which they thrive, they are probably not of any real significance in terms of meat spoilage.

In general, Gram-positive bacteria are more resistant to reductions in water activity than Gram-negative bacteria. For example, *S. aureus* can tolerate the lowest water activity of bacteria ranging from 0.86 to 0.83 (Christian, 1980). For non-halophilic bacteria, as water activity decreases, the length of the lag phase increases (Blickstadt, 1984; Li and Torres, 1993b), growth rate reduces (Calhoun and Frazier, 1966; Sperber, 1983; Prior *et al.,* 1987), cell mass decreases (Roller and Anagnostopoulos, 1982; Blickstadt, 1984) and intracellular amino acid content increases (Gould and Measures, 1977; Sperber, 1983; Prior *et al.,* 1987). When actively growing cells are transferred to an environment with high sodium chloride and hence reduced water activity, there is a drop in absorbance due to plasmolysis (Gould and Measures, 1977; Prior *et al.,* 1987) and growth stops. Plasmolysis is osmotic shock, where the cells rapidly lose water and decrease in size (Sperber, 1983), hence the decrease in absorbance. Directly after plasmolysis the cell goes into a lag phase. The removal of water from the cell increases the potassium concentration in the cell so that the cell produces more glutamate (Sperber, 1983; Prior *et al.,* 1987), which can be further synthesized into γ-aminobutyric acid or proline (Gould and Measures, 1977; Sperber, 1983). This reduces the water activity within the cell to the equivalent in the environment, consequently enabling the cell to regain turgor (Gould and Measures, 1977; Sperber, 1983). Cells that can produce α-aminobutyric acid or proline are able to grow at a lower water activity than those that can only produce glutamate (Sperber, 1983). Once the cell regains turgor, it can continue to grow at a reduced rate (Sperber, 1983; Prior *et al.,* 1983). The transfer of amino acids across the cellular membrane then increases in efficiency, enabling the cell to maintain the required high intracellular amino acid concentration (Gould and Measures, 1977). Upon returning cells to a higher water activity, the intracellular amino acid content decreases rapidly (Gould and Measures, 1977).

The minimum water activity that a bacterium can tolerate will depend on the solute involved and the bacterial species. Generally, sodium chloride, potassium chloride, glucose and sucrose all have a similar inhibitory effect at about the same water activity, while when glycerol is used as a solute bacteria can tolerate a lower water activity (Sperber, 1983). Glycerol is regarded as a "compatible solute" because it can accumulate within the cell, reducing water activity without inhibiting the cell. The amounts of amino acids produced by the cell is independent of the water activity and dependent

Table 1.5 Changes in the lag phase (LP), generation rate (GR) and cell mass (CM) of aerobic, microaerophillic, facultative anaerobic and anaerobic bacteria in the presence of carbon dioxide.

Bacteria	CO_2: O_2:N_2 (%)	Temp (°C)	↑LP	↓GR	↓CM	Reference
A. hydrophila	5:0:95	25		✓		Molin, 1983
	100:0:0	25		✓		Molin, 1983
	50:1.5:48.5	8	x	✓	✓	Bennik *et al.*, 1995
	50:21:29	8	x	✓	✓	Bennik *et al.*, 1995
B. cereus	50% CO_2:air	5		✓		Enfors and Molin, 1981a
	50% CO_2:air	35		✓		Enfors and Molin, 1981a
	20:1.5:78.5	8	x	✓	✓	Bennik *et al.*, 1995
	20:21:59	8	x	✓	✓	Bennik *et al.*, 1995
	50:1.5:48.5	8	x	✓	✓	Bennik *et al.*, 1995
	50:21:29	8	x	✓	✓	Bennik *et al.*, 1995
B. thermosphacta	5:0:95	25		✓		Molin, 1983
	100:0:0	25		✓		Molin, 1983
Cl. sporogenes[a]	100:0:0	25		✓		Molin, 1983
E. coli	5:0:95	25		✓		Molin, 1983
	100:0:0	25		✓		Molin, 1983
Lactobacillus sp.	5:0:95	25		✓		Molin, 1983
	100:0:0	25		✓		Molin, 1983
L. viridescens	5:0:95	25		✓		Molin, 1983
	100:0:0	25		✓		Molin, 1983
L. monocytogenes	50:1.5:48.5	8	x	✓	✓	Bennik *et al.*, 1995
	50:21:29	8	x	✓	✓	Bennik *et al.*, 1995
P. fragi	50 CO_2:air	5	x	✓		Enfors and Molin, 1981a
	50 CO_2:air	35	x	✓		Enfors and Molin, 1981a
P. fluorescens	50% CO_2:air	24	✓	✓	✓	King and Nagel, 1967
	20:0:80	5		✓	✓	Eyles *et al.*, 1993
	40:0:60	5		✓	✓	Eyles *et al.*, 1993
	100:0:0	15		✓	✓	Eyles *et al.*, 1993
P. putida	20:0:80	5		✓	✓	Eyles *et al.*, 1993
	20:0:80	15		✓	✓	Eyles *et al.*, 1993
	40:0:60	5		✓	✓	Eyles *et al.*, 1993
	40:0:60	15		✓	✓	Eyles *et al.*, 1993
	100:0:0	15		✓	✓	Eyles *et al.*, 1993
S aureus	5:0:95	25		✓		Molin, 1983
	100:0:0	25		✓		Molin, 1983
S. faecalis	5:0:95	25		x		Molin, 1983
	100:0:0	25		✓		Molin, 1983
Y. enterocolitica	5:0:95	25		✓		Molin, 1983
	100:0:0	25		✓		Molin, 1983
	50:1.5:48.5	8	x	✓	✓	Bennik *et al.*, 1995
	50:21:29	8	✓	✓	✓	Bennik *et al.*, 1995
Y. frederiksenii	5:0:95	25		✓		Molin, 1983
	100:0:0	25		✓		Molin, 1983

[a] = *Cl. sporogenes* generation rate per hour decreased as compared to 5% CO_2/95% N_2 (Molin, 1983).
x = No significant change in parameter
✓= significant change in parameter

on the solute. *P. fluorescens* grown in laboratory media with a water activity of 0.980 produced 310-380nmol/mg dry mass of amino acid when sodium chloride was the solute but only 50nmol/mg dry mass when glycerol was the solute (Prior *et al.*, 1987). This amount of amino acid produced in the presence of glycerol was comparable to the control (Prior *et al.*, 1987). The effect of reducing the water activity on end-product formation depends on the bacteria involved. When the water activity was reduced to ≤0.98, *B. thermosphacta* produced small amounts of 2,3-butanediol under anaerobic conditions and increased amounts of acetic acid in aerobic conditions when compared to a higher water activity (Blickstad, 1984). There can be differences in the effect of water activity reduction among the same genus. When the water activity was decreased, *Lactobacillus* sp. SMRICC produced decreased amounts of D-lactic acid while under the same conditions there was no effect on end-product formation by *Lb. viridescens* (Blickstad, 1984).

1.11.4 Atmosphere composition

Bacteria can be aerobic, microaerophilic, facultative anaerobes or anaerobic, depending on their ability to grow in the presence or absence of oxygen or carbon dioxide. The most common gas used in the preservation of fresh meat is carbon dioxide either alone or in combination with air, oxygen and/or nitrogen. Carbon dioxide can have a multitude of effects or no effect at all, depending on the organism involved (Clark and Takács, 1980). There is not relationship between resistance to carbon dioxide and Gram-reaction, i.e. cell wall structure (Gill and Tan, 1980). Three of the most common effects of carbon dioxide are increased lag phase, increased generation time and decreased final cell yields (Enfors and Molin, 1981b; Molin, 1983; Johnson and Ogrydziak, 1984; Eyles *et al.*, 1993, Drosinos and Board, 1995). These effects occur for aerobic, microaerophillic, facultative anaerobic and anaerobic species as exhibited in Table 1.5, but the effect is greater for the aerobic bacteria.

The effects of carbon dioxide on the growth of bacteria depend on the stage of growth when it is applied. If the bacteria have begun to grow then the extension in shelf-life that can be expected is proportional to the percentage reduction in growth rate (Gill and Tan, 1980), which is only slightly reduced from that of air. This phenomenon was illustrated by Clark and Lentz (1969) who inoculated sliced meat with psychrotrophs then stored it at either five or 10°C with carbon dioxide applied at zero, 24 or 48 hours after inoculation (Figure 1.4). When the carbon dioxide was applied after 24 or 48 hours, the reduction in growth of the psychrotrophs was greater at 5°C than at 10°C. These experiments also demonstrated the relationship between temperature and carbon dioxide, which is further discussed below. The strain of bacteria can also influence the degree of inhibition by carbon dioxide. Non-fluorescent *Pseudomonas* strains were more resistant to carbon dioxide than fluorescent

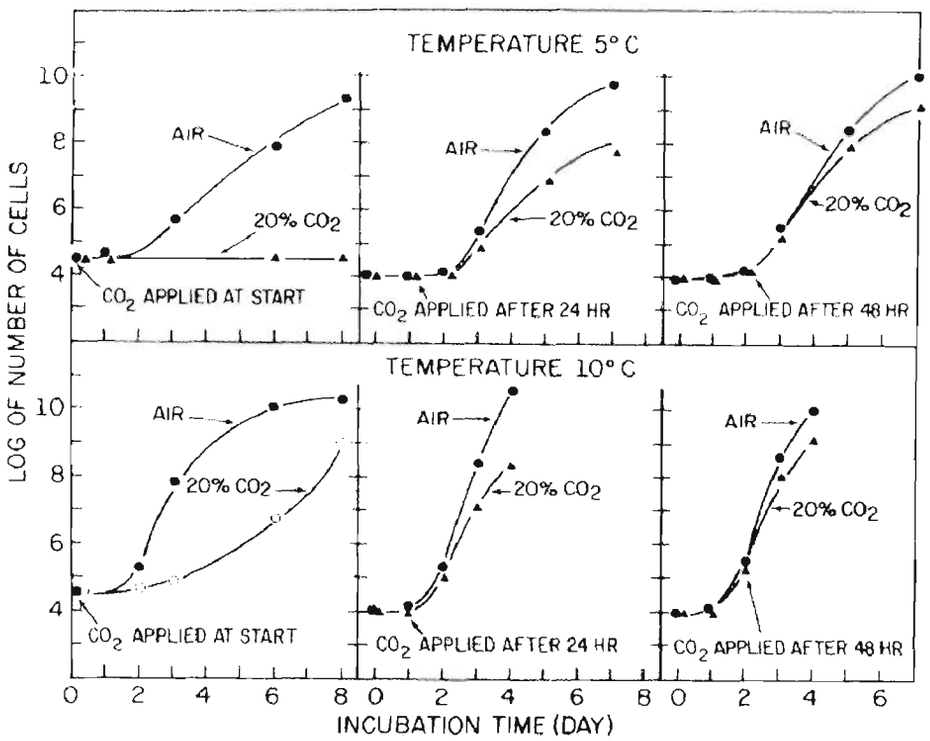


Figure 1.4 Psychrotrophs were inhibited by the application of carbon dioxide at time zero at 5°C relative to growth in air. If the carbon dioxide was added at 24 hours after incubation commenced, the inhibitory effect was reduced when compared to time zero. Similarly, when carbon dioxide was added 48 hours after the commencement of incubation, the effect was diminished compared to that observed at time zero and 24 hours. The effect of carbon dioxide was always greater at five than at 10° C (Clark and Lentz, 1969).

Pseudomonas (Gill and Tan, 1980) and *P. fluorescens* was more susceptible to carbon dioxide than *P. putida* (Eyles *et al.*, 1993).

For facultative anaerobes, the increases in lag phase and generation time and decrease in cell density could relate to the efficiency of the anaerobic metabolic pathways. Anaerobic pathways which metabolise arginine do not produce the same amount of ATP molecules that are produced by aerobic pathways using glucose (Gill, 1986), therefore the bacterial numbers cannot increase at the same rate as when the more efficient aerobic pathways are employed. The mechanisms through which carbon dioxide exerts it effects have not been elucidated (Gill and Tan, 1980; Enfors and Molin, 1981a,b). It is possible that there is more than one mechanism occurring at any one time, plus the effects depend on the general environmental conditions in which an organism resides and the effects differ for different species (Tan and Gill, 1982; Daniels *et al.*, 1985).

Originally it was thought that the effects of carbon dioxide were due simply to the removal of oxygen, however, this has subsequently been discounted (Daniels *et al.*, 1985; Hintlain and Hotchkiss, 1987). The shelf-life of meat and fish products have been extended in atmospheres of elevated carbon dioxide where the balance gas was air or oxygen (Johnson and Ogrydziak, 1984; Wang and Ogrydziak, 1986; Ingham *et al.*, 1990a; Bennik *et al.*, 1995). *B. cereus* was completely inhibited by 50% CO_2 in the presence of either 1.5 or 21% O_2 (Drosinos and Board, 1994). The storage of beef in 75% O_2/25% CO_2 resulted in the development of *Leuconostoc* as the dominant organism rather than *Pseudomonas* (Hanna *et al.*, 1981). Furthermore, growth in the presence of 100% N_2 only slightly decreased the growth rate and total number when compared to air grown cells (Eyles *et al.*, 1993). Carbon dioxide is thought to interfere with the enzymes involved in oxidative metabolism. Under anaerobic conditions, *P. fragi* was unable to catabolise either creatine or creatinine. The uptake of glucose, aspartate and glutamate were inhibited in the presence of carbon dioxide (Tan and Gill, 1982).

The respiration rates of non-fluorescent and fluorescent *Pseudomonas*, *Y. enterocolitica*, *Acinetobacter* and *A. putrefaciens* strains were reduced in the presence of carbon dioxide while the respiration of the facultative anaerobes *Enterobacter* and *B. thermosphacta* were not (Gill and Tan, 1980). The main enzymes that are thought to be affected by carbon dioxide are isocitrate dehydrogenase and malate dehydrogenase (King and Nagel, 1975; Tan and Gill, 1982). *Pseudomonas* species were unable to metabolise glucose-6-phosphate under anaerobic conditions (Drosinos and Board, 1994). Carbon dioxide has also been shown to inhibit succinate dehydrogenase plant mitochondria (Ranson *et al.*, 1957) but not in *P. aeruginosa* (King and Nagal, 1975). In addition, in the plant mitochondria 20% CO_2 reduced the metabolism of fumarate and malate (Ranson *et al.*, 1957).

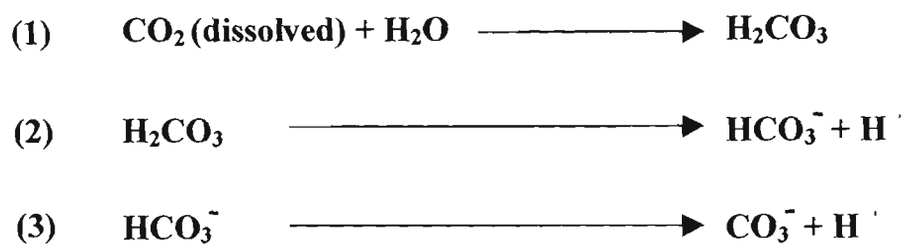


Figure 1.5 The formation of carbonic acid by CO_2 and H_2O and the subsequent dissociation into bicarbonate, carbonate and hydrogen ions (Daniels *et al.*, 1985).

The inhibitory mode of action of carbon dioxide on enzymes could be caused by carbon dioxide acting directly on sites within the cell or by the decreased pH caused by the formation of carbonic acid. The formation of carbonic acid in aqueous solutions by carbon dioxide occurs as shown in Figure 1.5. The first step involves the hydration of the carbon dioxide into carbonic acid (Silliker, 1981; Daniels *et al.*, 1985), where this reaction proceeds slowly (Daniels *et al.*, 1985). The second stage involves the dissociation of carbonic acid into bicarbonate and hydrogen ions, which occurs quickly with a pKa of $4.3x10^{-7}$, while the second dissociation into carbonate and hydrogen ions is negligible with a pKa of $5.61x10^{-11}$ (Silliker, 1981; Daniels *et al.*, 1985).

The solubility of carbon dioxide into the aqueous medium is known to increase as temperature decreases and inhibitory activities subsequently increase (Clark and Lentz, 1967; Clark and Takács, 1980; Enfors and Molin, 1981b; Johnson and Ogrydziak, 1984; Shay and Egan, 1987; Gill, 1988; Eyles *et al.*, 1993). The relationship between carbon dioxide solubility and temperatures from -1.5 from 10°C for beef, lamb and pork muscle appears to be linear (Enfors and Molin, 1981b; Gill, 1988), with solubility of carbon dioxide decreasing by 19 mL/kg for each degree increase (Gill 1988). This relationship is only an estimate, as solubility will depend on: the muscle tissue, the amount and composition of fat (because fat has a vastly different solubility to muscle), pH, and the surface area of the meat (Gill, 1988). Therefore theoretically in meat, as the solubility of the carbon dioxide increases, the pH of the tissue would be expected to decrease. However, the decrease that does occur is not significant enough to explain the decrease in microbial growth observed (Silliker, 1981). Perhaps carbon dioxide causes a greater change in the internal pH of the bacteria, as it can readily dissolve into the cell (Daniels *et al.*, 1985), rather than external environment, in this case muscle tissue. Tan and Gill (1982) found internal pH decreases 0.03 units for each 1mM increase in carbon dioxide for *P. fluorescens*. Haines (1939) and Coyne (1939) both demonstrated that carbon dioxide inhibition was specific and not solely reliant on decrease in pH (Ingram, 1962).

It was thought that there was a residual effect of carbon dioxide on the growth of meat spoilage bacteria. This effect is now thought to be related to the bacteria rather than the carbon dioxide, either through an induced lag phase (Silliker, 1981) or alteration in growth rates or possibly a combination of both, due to the change in the atmospheric environment. When the bacteria are removed from an anaerobic environment, the metabolism would be adapted to that environment, therefore it would take time for the metabolism to switch to the more economic aerobic pathways. In regard to growth rate, the bacteria that are predominant in carbon dioxide enriched environments, for example *Lactobacillus*, have higher growth rates in air (Molin, 1983). Wang and Ogrydziak (1986) demonstrated that when rock cod was transferred from a carbon dioxide enriched environment to air, *Lactobacillus* which had dominated the population was surpassed in number by *Pseudomonas* within a six of days the transfer to air.

1.11.5 Preservatives

Traditionally sulphur dioxide, sodium nitrate and sodium nitrite were commonly used as food preservatives but have subsequently been found to have properties which are adverse to health so that they are now no longer considered to be acceptable, although they are still used. In most countries around the world there are legislative restrictions dictating what preservatives can be used and in what food stuffs they can be applied (Pollard, 1991). The chemical preservatives that are weak acids encompassing sorbic, propionic and benzoic acids and their salts plus the phenolic esters of *p*-hydroxybenzoic acids or parabens are the most commonly used chemical preservatives. Sugars and sodium chloride can also be considered preservative agents, however, these have already been discussed in terms of their actions as water activity reducing agents. There are other preservative agents, for example the alkylcinniminic acids, which are potent inhibitors of microbial growth comparable with parabens (Baranowski and Nagel, 1982) but they are currently not permitted in foods.

Preservatives have three main effects on the growth of bacteria (i) increasing lag phase, (ii) reducing growth rate and (iii) reducing the maximum number of bacteria (Robach, 1978; Chung and Lee, 1982; Restanio *et al.*, 1981; Gray *et al.*, 1984; Zamora and Zaritzky, 1987; El-Shenawy and Marth, 1988; Mendonca *et al.*, 1989; Tsay and Chou, 1989). The degree to which bacteria are retarded or inhibited by a preservative depends on several factors: the organism itself, the number of bacteria, water activity, pH, acidulant used, composition of the medium, presence of other preservatives, storage temperature, storage duration, atmospheric composition and packaging conditions (Elliott *et al.*, 1985; El-Shenawy and Marth, 1988; Sofos *et al.*, 1986). For the weak acid preservatives; benzoic, propionic and sorbic acids, the pH of the medium is an essential parameter in determining the extent of anti-microbial activity.

1.11.5.1 Potassium sorbate

Sorbates were first discovered by Gooding in the 1940s, and they are probably the most widely used chemical preservative (Dziezak, 1986). The outgrowth of spores of *Cl. botulinum* (Sofos *et al.*, 1986), *B. cereus*, *Cl. sporogenes* and *Sporolactobacillus* species can be inhibited by potassium sorbate (Botha and Holzapfel, 1987). Sorbic acid or its salt, potassium sorbate, have been shown to increase the lag phase and generation time of *B. thermosphacta* (Zamora and Zaritzky, 1987), *Enterobacteriaceae* (Zamora and Zaritzky, 1987; Mendonca *et al.*, 1989), *L. monocytogenes* (El-Shenawy and Marth, 1988), *Pseudomonas* species (Robach, 1978; Chung and Lee, 1982; Zamora and Zaritzky, 1987), *Salmonella* species (Restanio *et al.*, 1981; Gray *et al.*, 1984), and *Y. enterocolitica* (Restanio *et al.*, 1981).

On chicken drum sticks that had been dipped in either five or 10% potassium sorbate, there was an increase in shelf-life with no sensory difference between treated and the water dipped controls (Cunningham, 1979). Increased shelf-life and a reduction in the number of inoculated *Salmonella* on chicken pieces was observed after dipping in 10% potassium sorbate (Cunningham, 1980). At 4°C, the shelf-life achieved with 5% potassium sorbate was equivalent that observed with 10%, although this was not observed at 22°C (Cunningham, 1980).

In some cases the lag phase was increased but the growth rate was not affected. Greer (1982) found that the lag phase of psychrotrophs was extended by potassium sorbate in both beef extract medium and on steaks, however the generation time was not significantly different from the control. This could be explained by a difference between this and other investigations in the growth medium and strain used. In addition, there have been some variations in sensitivities to potassium sorbate for some members of the genus *Pseudomonas* (Chung and Lee, 1982) and *Y. enterocolitica* strains (Tsay and Chou, 1989). Therefore it is probable that some species will experience an increase in lag phase but not a decrease in growth rate, such as observed above.

There have also been examples of a reduction the final numbers attained following growth in the presence of potassium sorbate. *L. monocytogenes* had final counts that were lower than the control in the presence of potassium sorbate (El-Shenawy and Marth, 1988). The final numbers of *Pseudomonas* have been demonstrated to decrease in the presence of potassium sorbate (Ingram, 1962; Robach, 1978; Cunningham, 1979). The final numbers of *Y. enterocolitica* also decreased in the presence of potassium sorbate (Tsay and Chou, 1989).

Sorbic acid is a weak acid preservative therefore the anti-microbial activity increases with decreasing pH, (Eklund, 1983; Russell, 1991; Skirdal and Eklund, 1993), however, the acidulant used can influence the inhibitory action. The type of acidulant used can also affect the impact of preservatives on the growth of bacteria. *P. fluorescens* experienced longer lag phases in the presence of potassium sorbate following acidification with citric and lactic acids compared to hydrochloric-acidified media (Restiano *et al.,* 1981). This potentiation effect of the acids can vary within a genus. A *Lactobacillus,* isolated from hotdogs, did not exhibit any difference in response to potassium sorbate in media acidified with either citric, lactic, phosphoric or hydrochloric acid. However *Lb. plantarum* had greater inhibition following acidification with citric, lactic or phosphoric acids compared with hydrochloric acid (Restiano *et al.,* 1981).

Other factors can combine with potassium sorbate and pH to influence inhibition. Combining potassium sorbate with lower temperature will increase the inhibitory capacity. *L. monocytogenes* was inhibited by lower concentrations of potassium sorbate at 4°C than at 13, 21 or 35°C (El-Shenawy and

Marth, 1988). The shelf-life of pork chops was further extended by combining potassium sorbate and vacuum-packaging (Mendonca *et al.*, 1989). Combining carbon dioxide and 2.5% potassium sorbate had synergistic effects on the inhibition of *S. aureus* which was not observed with vacuum-packaging, however, this effect was diminished at the higher pH of 6.0 (Elliott *et al.*, 1982; Gray *et al.*, 1984). A similar trend was noted with *S. enteriditis* (Gray *et al.*, 1984). The shelf-life of chicken thighs could be extended by a further two days by combining carbon dioxide and potassium sorbate rather than carbon dioxide alone (Elloitt *et al.*, 1985). The absence of putrid odours on the spoiled chicken thighs with carbon dioxide and potassium sorbate indicated that the *Enterobacteriaceae* family was inhibited by the combination. *Lactobacillus* are known to be one of the most resistant bacteria to food preservatives (Lück, 1980). The combination of *Lactobacillus* species and potassium sorbate are more inhibitory to psychrotrophs than either one alone (Gilliland and Ewell, 1983).

1.10.5.2 Benzoate and propionate

For both benzoic and propionic acids, it is the undissociated molecule that is responsible for the anti-microbial activity (Chipley, 1983; Eklund, 1989). As the presence of undissociated form increases with decreasing pH, benzoic acid is most effective at pH ranging from 2.5-4.0 (Davidson and Branden, 1981) and propionate at pH ≤6.0. The minimum inhibitory concentrations of benzoic and propionic acids against a selection of yeasts (*S. cerevisiae*, *Kluyveromyces fragilis*, *Pichia ohmeri*, *C. kruiseii*, *Schizosaccharomyces pombe*, *Z. bailii*, *Z. bisporus* and *Z. rouxii*) were ≤1.3 and ≤13.5 g/L respectively (Warth, 1989). Benozic acid has been shown to increase the lag phase of *P. fragi* (Moustafa and Collins, 1969) and to increase both lag and generation times of *L. monocytogenes* (El-Shenawy and Marth, 1989). Propionic bacteria could not reliably inhibit the growth of *P. aeruginosa* (Eklund, 1985). The dependence of these preservatives on low pH renders benzoate and propionate useless in meat microbiology, as the pH is in the range of 5.5-6.5.

1.10.5.3 Parabens

Para-hydroxybenzoic acid or parabens, are esters of organic acids (Davidson and Branden, 1981). The effects of parabens tends to be independent of the pH of the medium, this gives them advantages over other preservatives as they can be used over a wider pH range (Thomson *et al.*, 1993). Methyl- and propyl-parabens became permitted preservative agents in 1988 (Pollard, 1981). Methyl-paraben can be used to inhibit yeasts (*S. cerevisiae*, *K. fragilis*, *P. ohmeri*, *C. kruiseii*, *S. pombe*, *Z. bailii*, *Z. bisporus* and *Z. rouxii*) with minimum inhibitory concentrations ≤1.11g/L (Warth, 1989). *Penicillium* and *Fusarium* species have been inhibited with propyl-paraben over a range of pH levels (Thompson *et al.*, 1993). Methyl- and propyl-paraben have been shown to inhibit spore germination in *Cl. botulinum*, with propyl-paraben inhibition occurring at a lower concentration (Robach and Pierson,

1978). Propyl-paraben inhibited the secretion of proteases and reduced the growth of *A. hydrophila* (Venugopal *et al.*, 1984), increased the lag phase of *L. monocytogenes* (Payne *et al.*, 1989) and inhibited the growth of *P. fragi* (Moustafa and Collins, 1969). There are different sensitivities to parabens among members of the same genus: *B. cereus* was found to be almost twice a sensitive to parabens as *B. subtilis* (Eklund, 1985). As a general rule, the inhibitory potential of parabens on Gram-positive bacteria increases with increases in the length of the ester chain (Eklund, 1980; Davidson and Branden, 1981), however, this does not necessarily apply to Gram-negative bacteria (Davidson and Branden, 1981).

Gram-positive bacteria are more susceptible to non-polar phenolic compounds than Gram-negative (Davidson, 1983). This could be a result of the difference in cell wall structure between the two groups. Gram-positive bacteria generally have cell walls comprised of rigid macromolecular material of N-acetylglucosaimde polymers and N-acetylmuremic acid, with little or no lipids (Hugo, 1967). Conversely, Gram-negative bacteria have a rigid cell wall which contains a high proportion of lipid up to 25% (Hugo, 1967). Parabens are thought to be absorbed into the cell *via* a non-specific physiochemical mechanism (Bargiota *et al.*, 1987). Bargiota *et al.* (1987) suggested that bacterial resistance to the inhibitory effects of methyl- and propyl-paraben was related to the lipid layer within the cell wall, noting increasing resistance with increases in lipid content. A *S. aureus* strain with elevated levels of lipids in the form of phosphatidyl glycerol exhibited the greatest resistance to methyl- and propyl-paraben (Bargiota *et al.*, 1987). In addition, *L. monocytogenes* cultured in the presence of exogenous saturated fatty acids, tetradecanoic and octadecanoic, had increased minimum inhibitory concentrations as compared to a control (Juneja and Davidson, 1993). However, if unsaturated fatty acid ($C_{18:1}$) was present in the culture medium, *L. monocytogenes* exhibited significantly lower minimum inhibitory concentrations than the control (Juneja and Davidson, 1993). Therefore, cells that have high concentrations of unsaturated fatty acids are more vulnerable to these preservatives (Juneja and Davidson, 1993).

1.10.5.4 Mode of inhibition by preservatives

Essentially the mode of action of the different preservative groups can be attributed to (i) reaction with the cell wall, influencing permeability and transport across the membrane, (ii) inactivation of enzyme systems and (iii) interference with genetic material (Eklund, 1980; Davidson and Branden, 1981; Nes and Eklund, 1983; Sofos *et al.*, 1986; Eklund 1989).

The weak acids, benzoic, propionic and sorbic acid have similar modes of interaction. The inhibitory effects of benzoic, propionic and sorbic acid is attributed to both the dissociated and undissociated acid. The amount of undissociated acid present is strongly dependent on the pH (Eklund, 1983). The

inhibitory effect is greater at the lower pH levels because weak acids are less dissociated than strong acids, therefore they are able to pass through the cell wall *via* passive diffusion more readily (Young and Forgeding, 1993). For these weak acids, the lower the pH, the greater the bactericidal or bacteristatic effect.

Parabens are thought to interact with several targets within a cell (Kabara and Eklund, 1991) but primarily with those contained in the cell wall. Eklund (1980) found that there was a strong correlation between growth and amino acid uptake, which lead him to postulate that the parabens increased membrane permeability subsequently neutralising both the chemical and electrical component of the proton motive force. The variations among species in response to preservatives suggests that care must be taken when making generalisation and this also applies to the mode of action (Eklund, 1985).

1.11.6 Combined effects of environmental factors

Each of the environmental factors is independent of the others, however, the impact of the factors can have a synergistic effect in the inhibition of bacterial growth (Scott, 1989; Thomas *et al.*, 1991). As discussed already, there is an interaction between temperature and carbon dioxide and pH and preservative (potassium sorbate) or organic acids. As temperature decreases, the minimum inhibitory pH increases (Hughes and McDermott, 1989; Faber *et al.*, 1989; McClure *et al.*, 1989; Buchanan and Phillips, 1990; Brocklehurst and Lund, 1990; Cole *et al.*, 1990; Little *et al.*, 1992b; Thomas *et al.*, 1992). Similarly, with water activity, as the temperature decreases the minimum water activity which will facilitate growth increases (Gibson *et al.*, 1988; McMeekin *et al.*, 1987; McClure *et al.*, 1989; Chandler and McMeekin, 1989; Cole *et al.*, 1990). Temperature, pH and water activity can also interact to influence microbial growth (Bhaduri *et al.*, 1995; Hughes and McDermott, 1989). For *E. coli*, as temperature and pH decreased the minimum water activity permitting growth increased, with a greater inhibitory effect occurring when nitrite was added (Hughes and McDermott, 1989). Interactions between environmental factors have stimulated further developments in predicting the activities of a particular microorganism under a given set of environmental conditions.

1.12 PREDICTIVE MICROBIOLOGY

Predictive microbiology involves the application of mathematical models to predict the growth of bacteria, given a particular range of growth parameters which were not necessarily specifically determined (Baird-Parker and Kilsby, 1987; McMeekin *et al.*, 1993). Predictive microbiology is not new, one of the earliest models was to 'botulinum cook', which was described in 1922 (Baird-Parker

and Kilsby, 1987). Interest in predictive microbiology increased in the 1980s for three principle reasons. Firstly, there was a rise in the number of food poisoning out breaks, secondly, the realisation that the traditional and rapid methods were of little predictive value and, thirdly, an increase in the use of computer technology (Buchanan 1991; Ross and McMeekin, 1994). Models that have been developed for biotechnology cannot simply be adopted into food microbiology (Baranyi and Roberts, 1994). Food microbiology models should describe the behaviour of bacteria under different conditions of temperature, pH, water activity (Zwietering *et al.*, 1991). The interest in predictive microbiology has resulted in models for the growth of *A. hydrophilia* (Hudson, 1992; McClure *et al.*, 1994), *B. thermosphacta* (McClure *et al.*, 1993; Baranyi *et al.*, 1995; Baranyi *et al.*, 1996), *L. monocytogenes* (Buchanan and Phillips, 1990; Wijtze *et al.*, 1993), *Salmonella* species (Gibson *et al.*, 1988), *S. flexneri* (Zaika *et al.*, 1992), *S. xylosus* (Chandler and McMeekin, 1987) and *Y. enterocolitica* (Adams *et al.*, 1991; Little *et al.*, 1992a,b; Davey, 1994).

Models for bacterial growth in food can be divided into kinetic and probabilistic. Kinetic models are used to model the rate or extent of microbial growth while probabilistic models are used to indicate the probability of an event occurring for example spore germination (Ross and McMeekin, 1994). Probability models will not be considered here. Kinetic models include: Arrhenius-type, Bělehrádek square root, modified Arrhenius and polynomial or surface response models (McMeekin *et al.*, 1993; Ross and McMeekin, 1994). The first models to be developed and validated regarded the growth temperature. The Arrhenius-model describes the temperature dependence of chemical reactions and was first described in 1889 (Mohr and Karwiec, 1980; Ratkowsky *et al.* 1982; Li and Torres, 1993a). The Arrhenius function is as follows:

$$\ln k = \ln A - E_a/RT$$

where k is the rate of growth, A is a parameter to be fitted, E_a is a temperature characteristic, R is the universal gas constant and T is temperature in °C (Ross and McMeekin, 1994). Mohr and Krawaiec (1980) and Reichtardt and Mortia (1982) applied the Arrhenius function to microbial growth over a range of temperatures and concluded that there were two linear temperature characteristics present. However Johnson *et al.* (1974) demonstrated that the natural log of rate (ln k) was not proportional to the inverse of temperature (1/T), which is the basis of the Arrhenius function (McMeekin *et al.*, 1993). The Arrhenius function was designed to describe the temperature dependence of simple chemical reactions therefore it was not adequate for the description of complex microbiological systems (Ratkowsky *et al.*, 1982; Stannard *et al.*, 1985).

The square root model was proposed as an alternative to the Arrhenius model for the description of microbial growth as a function of temperature. The square root model is derived from the Bělehrádek

function and is described as a Bělehrádek-type function (McMeekin *et al.*, 1993). Ratkowsky *et al.* (1982) proposed the square root model to describe microbial growth at sub-optimal temperatures:

$$\sqrt{k} = b(T-T_{min})$$

where k is rate of growth, b is a coefficient to be fitted, T is temperature and T_{min} is the theoretical minimum temperature in °C (Ratkowsky *et al.*, 1982; Ross and McMeekin, 1994; McMeekin *et al.*, 1993). The square root model produced a high correlation between the fitted curves and the data points (Stannard *et al.*, 1985). There was less variability in T_{min} values of the square root plot than for E_a values in Arrhenius plot (Stannard *et al.*, 1985). The square root model was further developed to incorporate the entire biokinetic temperature range (Ratkowsky *et al.*, 1983). The model gave a good fit for 30 strains of bacteria over the whole temperature range (Ratkowsky *et al.*, 1983). Other adaptations of the square root model for sub-optimum temperatures have incorporated water activity (Chandler and McMeekin, 1987) and pH (Adams *et al.*, 1991).

Although the Arrhenius function was deemed unacceptable for complex biological systems Schoolfield *et al.* (1981) and Davey (1989) undertook the reparameterisation of the model, producing non-linear and linear Arrhenius-type models respectively. The model developed by Schoolfield has received a significant amount of attention (Ross and McMeekin, 1994). Schoolfield *et al.* (1981) analysed the reparameterisation of the Arrhenius and Eyring equations that had been conducted by other workers to give a modified description of growth in the following equation:

$$R(T) = \frac{\rho_{(25°C)} * T/298 * \exp[\Delta H_A^{*}/R * (1/298-1/T)]}{1+ \exp[\Delta H_L/R * (1/T_{1/2L}-1/T)]+\exp[\Delta H_H/R * (1/T_{1/2H}-1/T)]}$$

where r(T) is the mean development rate per unit time at T, T is absolute temperature, R is the universal gas constant, ρ(25°C) is the development rate at 25°C assuming no enzyme inactiviation, "ΔH_A^{*} is enthalpy of activation of the reaction that is catalysed by the enzyme" Schoolfield *et al.* (1981), $T_{1/2L}$ is the absolute temperature at which the enzyme is half active due to low temperature, "ΔH_H is the change in enthalpy associated with low temperature inactivation of the enzyme" Schoolfield *et al.* (1981) and $T_{1/2H}$ is where the enzyme is half active due to high temperature (Schoolfield *et al.*, 1981). The Schoolfield model was further developed by Broughall *et al.* (1983) to encompass water activity. The linear-Arrhenius model developed by Davey (1989) described the effect of temperature and water activity on microbial growth. The equation is as follows:

$$\ln k = C_0+C_1/T+C_2/T^2+C_3a_w+C_4a_w^2$$

where k is the rate coefficient, C_0 to C_4 are parameters that need to be fitted, T is absolute temperature and a_w is water activity (Davey, 1989). If the water activity was non-limiting, the water activity term could be removed (McMeekin *et al.*, 1993). Davey (1994) modified the model to include pH rather than water activity.

The final group of models used in predictive microbiology are the polynomial or surface response models. The surface response model was found to be more reliable at predicting the growth of *Y. enterocolitica* than the square root model (Little *et al.*, 1992a). Of this group, the modified-Gompertz function is the most frequently used (Ross and McMeekin, 1994). A Gompertz-like equation was introduced by Gibson *et al.* (1987) which described log of cell density versus time growth curves in terms of exponential growth rate and lag phase duration (McMeekin *et al.*, 1993). This has become known as the modified-Gompertz equation:

$$\log N_{(t)} = A + D \exp\{-\exp[-B(t-M]\}$$

where t is time, $N_{(t)}$ is the population density at t, A is the value of the lower asymptote, D is the difference between the upper and lower asymptote, M is the point at which the exponential growth is maximal and B is the slope of the curve at M (McMeekin *et al.*, 1993; Ross and McMeekin, 1994). When the logarithm of cell density is plotted against time, the growth curve changes, resulting in a sigmoidal curve with a lag phase just after time zero, an exponential phase and a stationary phase (Zwietering *et al.*, 1990). This is the advantage of the modified-Gompertz equation: it does not assume a continuous growth rate (Gibson *et al.*, 1988). The Gompertz function was superior to the logistic, linear, quadratic and tth power models for modelling the growth of *Lb. plantarum*, *C. parapsilosis*, *P. putida*, *E. agglomerans*, *Nocardia* sp., *S. heidelberg*, *S. aureus* and *L. monocytogenes* (Zwietering *et al.*, 1990).

In order to create and validate a model, a large amount of growth information is required (McMeekin and Ross, 1994). For the modified-Gompertz model between 10-15 data points are required (Gibson *et al.*, 1988; McMeekin *et al.*, 1993). Bratchell *et al.* (1989) demonstrated the effect of removing data from models thus simulating an inadequate data set. Reducing the number of data points resulted in a poor estimation of lag time, generation time and growth rate (Bratchell *et al.*, 1989). Although the idea of predictive microbiology is to predict the growth of bacteria under a set of conditions that have not been specifically tested, it is unwise to predict outside the limits of the data base (Gibson *et al.*, 1988; McClure *et al.*, 1993; Davey 1994). It is essential to know the limits of a model. Small fluctuations in temperature during exponential growth had no significant effect (Li and Torres, 1993; Baranyi *et al.*, 1995) but steep changes, five to 25°C, resulted in the model being no longer accurate (Baranyi *et al.*, 1995).

When devising predictive models it is important to establish which criteria are important (Gibson *et al.*, 1988; McClure, 1994). The inoculum size has been found to have no effect on the lag phase, exponential growth rate or generation time (Zaika *et al.*, 1992; Bhaduri *et al.*, 1995). The pre-incubation temperature will impact on the duration of the lag phase, however this will vary for different bacterial species (Buchanan and Klatwitter, 1991; Dufrenne *et al.*, 1997). The growth rate of *L. monocytogenes* was shown to vary with pre-incubation temperature (Grau and Vanderlinde, 1993) while for *Salmonella* it was unaffected (Dufrenne *et al.*, 1997). Therefore, it is important to standardise pre-incubation treatment. When incorporating pH into a model it is important to keep in mind the type of acidulant used, as organic acids have different inhibitory effects due to the dissociated acid (Adams *et al.*, 1991). Nutrients are generally not a consideration, as it is assumed that there will be more than sufficient substrate for microorganisms to reach unacceptable levels (Zwietering *et al.*, 1990). The advantage of deriving models from laboratory media is that the models will provide a worst case scenario (Buchanan and Phillips, 1990).

Chapter 2

Materials and methods

2.1 SOURCES OF MATERIALS

2.1.1 Bacteriological media and Chemicals

All media were purchased from Oxoid (Melbourne, Australia) with the exception of all purpose tween agar (APT) which was purchased from Amyl Media (Melbourne, Australia). Anaerobic gas kits were purchased from Oxoid (Melbourne, Australia) and used according to manufacturer's instructions unless otherwise stated. Oxidative/fermentative basal media was purchase from Becton Dickinson (Cockeyville, USA). The API 20E kits were purchased from bioMèrieux (Lyon, France) and Biolog GN from Biolog Inc. (Haywood, USA).

All chemicals used were of analytical grade or higher. Chemicals purchased from Sigma Chemical Co. (St Louis, USA) included: ammonium persulfate, boric acid, brilliant blue R (Coomassie brilliant blue), diaminopimelic acid, dimethyl-*p*-phenylenediamine hydrochloride, ethidum bromide, glycerophosphate disodium salt, L-lysine dihydrochloride, L-ornithine dihydrochloride, lysozyme, N,N,N',N'-tetramethylenediamine (TEMED), phenylmethylsulfonyl fluoride, sarkosyl, Tween 80 and tergitol. Chemicals purchased from Ajax Chemicals (Auburn, Australia) included: glacial acetic acid, L-arginine mono hydrochloride, lysine-dihydrochloride, ornithine-dihydrochloride, magnesium sulphate, di-potassium hydrogen phosphate, sodium chloride, sodium hydroxide and tri-ammonium citrate. Chemicals purchased from BDH Chemicals Ltd. (Poole, England) included: ethanol, ethylenediaminetetra-acetic acid disodium salt (EDTA), glycerol, hydrocholoric acid (HCl), hydrogen peroxide, isopropanol, manganous sulphate, methanol, potassium tellurite, sulphuric acid, Tris-base and

xylene. Sucrose, glucose and glycine were purchased from BDH Chemicals (Kilsyth, Australia). Chemicals purchases from Merck (Munich, Germany) included: bromocresol purple, bromocresol green, hippursäure natrumsalz and thionin. Chemicals purchased from Boehringer Mannheim GmBH (Mannheim, Germany) included: lysozyme and proteinase K. Potassium sorbate was purchased from Langdon and Co. (Melbourne, Australia). Methyl- and propyl-paraben were gift from San Fu Chemical Co. (Sydney, Australia).

Enzymes and molecular weight markers purchased from New England Biosciences (Brisbane, Australia) included: *Dra*I, *Eco*RI, *Hae*II, *Hae*III, *Hind*III, I-*Ceu*I, *Nhe*I, *Pst*I, *Sfi*I, *Sma*I, *Spe*I, *Xba*I, *Xho*I, low-range molecular marker, mid-range molecular marker, Lambda ladder. Bovine serum albumin (BSA) was also purchased from New England Biosciences (Brisbane, Australia). The enzyme *Not*I was purchased from Progen Industries (Brisbane, Australia) while *Bam*HI was purchase from Bresatech Pty. Ltd. (Burnley, Australia). The low range protein standard was purchased from BioRad Laboratories (USA).

UHT milk was purchased from a local supermarket. Protect beads for the storage of cultures at -80°C were purchased from Microdiagnostics (Lancaster, England). Millipore filters (0.22μm) were purchased from Millipore Australia Pty. Ltd. (North Ryde, Australia). Sterile bags for maceration and Petri dishes (92x16mm) were purchased from Labsupply (Melbourne, Australia). Thin layer chromatography aluminium plates pre-coated with cellulose (no. 5552) were purchased from Merck (Darmstadt, Germany). Gases were purchased from BOC (Melbourne, Australia). All films were purchased from Polaroid.

2.1.2 Equipment

The Venus® bag sealer was purchased from Venus Hartung Pty. Lt. (Abbotsford, Australia). Packaging bags, RA454, were purchased from Cryovac (Melbourne, Australia). The packaging machines, Webomatic type E50.G and Mulitvac R7000 were purchased from Werner Bonk (Mauegatt, Germany) and Multivac (Germany) respectively. The gas mixer was purchased from Witt-Gasetechnik (Germany). Gas analysis was conducted on either a Shimadzu 8A gas analyser (Shimadzu, Japan) or a M.A.P. Test 4000 (Hitech Instruments Ltd., Luton, England).

The Cermatrix® chamber, the U shaker and glass ampoules were purchased from Crown Scientific (Burwood, Australia). The spiral plater from Spiral systems Inc. (USA) was used to plate out bacterial suspensions from meat homogenates while the remainer of the spiral plating was conducted with a

W.A.S.P. spiral plater from Don Whitely Scientific (England). The Chemitrix 20A colourimeter was purchased from Chemtrix Inc. (USA) and the Pharmacia LKB Novaspec II from Pharmacia (Melbourne, Australia). Orion pH electrode was purchased from Lindbrook (Mt Waverley, Australia) and the flat tip comb glass pH probe purchased from Activon Scientific (Carlton, Australia). The Beckman centrifuge was purchased from Beckman (Mt Waverley, Australia), a bench top centrifuge from Hettic Universal (Melbourne, Australia) and the Sorvall RT2 bench top centrifuge from Amrad (Boronia, Australia).

PFGE gel CHEF apparatus Pharmacia LBK Gene Navigator was purchased from Pharmacia (Melbourne, Australia). The Protean II apparatus was purchased from BioRad Laboratories (USA). The B. Braun homogeniser and 1mm glass beads were purchased from Braun (Mesurgen, Germany).

Computer programs used for data analysis were Microsoft Excel 5.0a licensed from Microsoft (Delaware, USA), NTSYS version 1.7 (Applied Biostatistics Inc., New York, USA) and Genstat 5 release 3.2 (Laws agricultural Trust, Rothamstead Experimental Station, Harpenden, England).

2.1.3 Bacterial cultures

B. thermosphacta ATCC11509 was purchased from the Queensland University Culture Collection (Brisbane, Australia). *C. divergens* was kindly donated by M.-C. Montel, Institut National de la Recherche Agronomique, Theix, Saint Genès Champanelle, France. *C. piscicola* JG126 was kindly donated by Dr John Coventry and *Lb. curvatus* by Dr Heather Craven both of the Australian Food Industry Science Centre, Werribee, Victoria, Australia.

2.2. MEDIA PREPARATION

2.2.1 Autoclaving and general microbiological requirements

All media were sterilized at 121°C under 150kPa. The duration of autoclaving was determined from a chart based on thermocouple results specifically for the autoclaves in the Australian Food Industry Science Centre Microbiology Department. Autoclaving times for media were: 20 minutes for ≤100mL, 25 minutes for 500mL and 30 minutes for 1L. All media was cooled to 45°C in an incubator before dispensing. All media was stored in a refrigerated coldroom at 2°C until required. Broths were warmed to room temperature and agar plates dried inverted at 45°C prior to use. All bacteriological wastes

were autoclaved for 40 minutes before discarding. Filter sterilizing was done with 0.22μm Millipore filters.

All glassware, metal syringes, corers, knives, stainless steel filters, wooden sticks, centrifuge tubes and Eppendorfs were sterilized for 25 minutes, then dried prior to use.

All commercially purchased media were made according to the manufacturer's instructions unless stated otherwise. These media included: APT, Brain Heat Infusion Broth (BHIB), de Mann Rogosa Sharpe Agar (MRSA), de Mann Rogosa Sharpe Broth (MRSB), Nutrient Agar (NA), Nutrient Broth (NB), Oxidative/Fermentative basal media (OF media), Plate Count Agar (PCA), *Pseudomonas* Selective Agar (PSA), Streptomycin Sulphate Thallous Acetate Agar (STAA), Tryptose Soya Agar (TSA), Tryptose Soya Broth (TSB) and Violet Red Bile Glucose Agar (VRBGA). In all cases the diluent used was 0.01% peptone at pH 7.0 ±0.1, made using Bacteriological Peptone. After sterilizing the peptone was cooled to room temperature then dispensed into nine, 9.5mL or 500mL aliquots.

The pH measurements of broths were done with a an Orion pH electrode, while agar pH measurements were done using a flat tip comb glass pH probe. All pH adjustments were made with 1M HCl or NaOH.

2.2.2 APT broth, pH 3.9 without phosphate

APT minus phosphate: 12.5g tryptone, 10.0g dextrose, 7.5g yeast extract, 5.0g sodium citrate, 0.0001g thiamin hydrochloride, 5.0g sodium chloride, 0.14g manganese chloride, 0.8g magnesium sulphate, 0.04g ferrous sulphate and 1.0g Tween 80 per litre. The pH was adjusted to 3.9 prior to then autoclaving. Filter sterilized glucose was added to the cooled APT broth to give a final concentration of 0.2% (w/v), then 5mL aliquots were dispensed into sterile test tubes (Shaw and Harding, 1984).

2.2.3 Arginine, Lysine or Ornithine broths

These broths were used for determining arginine, lysine or ornithine hydrolysis and were prepared as described in the following. Broths contained: 5.0g peptone, 5.0g yeast extract, 5.0g di-potassium hydrogen phosphate, 3.0g L-arginine mono-hydrochloride or lysine-dihydrochloride or ornithine-dihydrochloride, plus 0.5g glucose per litre. This was dispensed in 5mL aliquots into test tubes and autoclaved. After the tubes had cooled, 0.25mL of 0.4% bromocresol purple was added. Controls were prepared in a similar manner without the amino acids (Anonymous, 1995).

2.2.4 Buffered media

Buffered-BHIA contained: 37.0g BHIB, 10.0g Bacteriological Agar and 19.0g glycerophosphate disodium salt per litre distilled water, with or without one or 2% sodium chloride. Prior to autoclaving, the pH was adjusted to 5.5 or 6.0 ±0.01. Buffered-BHIA media without sodium chloride at both pH levels were dispensed into three 600mL batches when cooled to 50°C. The agar without the sodium chloride was dispensed either unaltered or with potassium sorbate added at a final concentration of 0.4% or with propyl-paraben at a final concentration of 0.02% (w/v). The agar with sodium chloride was dispensed without further addition. All media were dispensed in 10mL aliquots in Petri dished subsequently. Media containing the preservatives, potassium sorbate and propyl-paraben, were prepared on the day the experiment was setup.

Buffered-MRSA for *Lactobacillus* contained: 10.0g Bacteriological Peptone, 8.0g Lab-Lemco powder, 4.0g yeast extract, 20.0g glucose, 1.0g Tween 80, 2.0g tri-ammonium citrate, 0.2g magnesium sulphate, 0.05g manganous sulphate, 10.0g Bacteriological Agar and 19.0g glycerophosphate disodium salt per litre distilled water. The pH buffered-MRSA was adjusted to 6.2, as the pH dropped by 0.2 units after autoclaving, giving a final pH of 6.0.

Buffered-NA for *Enterobacteriaceae* and *Pseudomonas* contained: 1.0g Lab-Lemco powder, 2.0g yeast extract, 5.0g bacteriological peptone, 5.0g sodium chloride, 10.0g Bacteriological Agar and 19.0g glycerophosphate disodium salt per litre. The pH was adjusted to 6.0 then the medium autoclaved. After cooling, buffered-NA was dispensed into 10mL aliquots.

Buffered-TSA for *Brochothrix* contained: 15.0g tryptone, 5.0g neutralized soya peptone, 5.0g sodium chloride, 15.0g Bacteriological Agar and 19.0g glycerophosphate disodium salt per litre. The pH was adjusted to 6.0 then the medium autoclaved. After cooling, buffered-TSA was dispensed into 10mL aliquots.

Buffered-TYGA for *Carnobacterium* contained: TYGA plus 19.0g glycerophosphate disodium salt per litre. The pH was adjusted to 6.0 then the medium autoclaved. After cooling, buffered-TYGA was dispensed into 10mL aliquots.

2.2.5 Buffered pH media

Buffered-MRSA, -NA, -TSA and -TYGA media was used for determining the effect of pH 4.5-7.0 on the growth of meat spoilage bacteria. The buffered-NA, buffered-TSA and buffered-TYGA, were adjusted to final pH values of: 4.5, 5.0, 5.5 6.0, 6.5 and 7.0, prior to autoclaving. Buffered-MRSA

was adjusted to pH 4.7, 5.2, 5.7, 6.2, 6.7 and 7.2 to allow for the 0.2 unit decrease in pH that occurred during autoclaving. After cooling, the media was dispensed into 10mL aliquots in Perti dishes. The pH of each medium batch was measured before use. Media at pH 4.5 was set but not solidly.

2.2.6 Buffered preservative media

Buffered-MRSA, -NA, -TSA, and -TYGA media was used for examining the effects of potassium sorbate and methyl- and propyl-paraben on the growth of meat spoilage bacteria. Buffered media was prepared as described above. After cooling, the buffered media was divided into 100mL aliquots, placed into sterile Schott bottles and stored at 50°C until dispensed into plates. Immediately prior to dispensing agar, either potassium sorbate, methyl-paraben or propyl-paraben were added to final amounts ranging from 0.05 to 1% (w/v) potassium sorbate, 0.05 to 0.5% (w/v) methyl-paraben or 0.005-0.1% (w/v) propyl-paraben. All media were made and used in the same day.

2.2.7 Buffered sodium chloride media

Buffered-MRSA, -NA, -TSA and -TYGA were used to determine the effect of sodium chloride on the growth of meat spoilage bacteria. Sodium chloride was added to each buffered agar to concentrations of 0, 1, 2, 3, 4 or 5% (w/v) then autoclaved. After cooling, the media was dispensed into 10mL aliquots in Perti dishes.

2.2.8 Gelatin media

Gelatin media was prepared according to the manufacturer's instructions.

2.2.9 Heterofermentative and homofermentative determination broth

Heterofermentative and homofermentative determination (HHD) broth contained: 2.5g fructose, 10.0g tryptone, 1.5g phytone peptone, 3.0g casein hydrolysate, 1.0g yeast extract, 1.0g Tween 80 per litre. The pH was adjusted to 7.0 ±0.02. Following sterilization, cooling and aliquoting 5mL into sterile test tubes, 20μL bromocresol green (0.1g in 30mL 0.01M NaOH) was added to each tube (McDonald *et al.*, 1987).

2.2.10 Hippurate hydrolysis

Simplified basal medium (SBM) broth contained: 15.0g peptone, 1.0g yeast extract, 1.0g Tween 80, 2.0g di-potassium hydrogen phosphate, 0.2g magnesium sulphate, 0.5g manganous sulphate and 1% (w/v) hippursäure natrumsalz per litre. After autoclaving and cooling to room temperature filter sterilised glucose was added to give a final concentration of 0.1% (w/v) glucose. The hippurate-SBM was then dispensed into 10mL aliquots into sterile McCartney bottles. Control broths were prepared in the same fashion minus the hippurate (Willkinson and Jones, 1977).

2.2.11 King's B medium

King's B agar contained: 20g proteose peptone number 3, 1.5g dipotassium-hydrogen phosphate, 1.5g magnesium sulphate, 10.0g glycerol, 15.0g Bacteriological Agar per litre. The King's B medium was then autoclaved and dispensed in Petri dishes (Harrigan and McCance, 1976).

2.2.12 La-broth

La-broth contained: 10.0g peptone, 4.0g yeast extract, 20.0g glucose, 1.0g Tween 80, 3.0g sodium citrate, 5.0g sodium acetate, 0.2g tri-ammonium citrate, 0.2g magnesium sulphate and 0.5g manganous sulphate per litre. The pH was adjusted to 6.8 (±0.01), the La-broth was dispensed into 10mL aliquots then autoclaved (Shaw and Harding, 1984).

2.2.13 NA supplemented with 5% sucrose

NA was prepared according to manufacturer's instructions. After autoclaving then cooling, 20% sucrose which had been filter sterilised was added to give a final concentration of 5%, then agar was dispensed into Petri plates (Harrigan and McCance, 1976).

2.2.14 Nitrate peptone water

Nitrate peptone water contained: 0.2g potassium nitrate, 0.1g peptone in per litre. Aliquots of 10mL were dispensed into test tubes containing inverted Durham tubes and autoclaved (Anonymous, 1995).

2.2.15 OF basal medium.

OF basal medium was made according to the manufacturer's instruction and dispensed in 5ml aliquots.

2.2.16 Rogosa agar

Rogosa agar was made according to the manufacturer's instructions

2.2.17 Potassium tellurite agar

Potassium tellurite media was made from basal medium (BM) agar with tellurite added (tellurite-BMA). BM agar contained: 10.0g peptone, 8.0g 'Lab-Lemco' powder, 4.0g yeast extract, 20.0g dextrose, 1.0g Tween 80, 2.0g di-potassium hydrogen phosphate, 0.2g magnesium sulphate, 0.05g manganous sulphate and 10.0g Bacteriological Agar per litre. This was then autoclaved. When cooled, aqueous potassium tellurite (10%) which had been autoclaved separately was added to give a final concentration of 0.05% (w/v). The agar was then dispensed into Perti dishes in 10mL aliquots. Controls were prepared in the same manner but without tellurite (Wilkinson and Jones, 1977).

2.2.18 Sodium chloride broths

Eight and 10% sodium chloride in TSB or APT was prepared by adding either 7.5g and 9.5g sodium chloride (w/v) to 100mL TSB or APT to give a final concentration of eight and 10% sodium chloride respectively. The broths were then autoclaved and 5mL aliquots were dispensed into sterile test tubes when cool (Talon *et al.*, 1988).

2.2.19 Sugar fermentation broths

Glucose, inulin, mannitol, melibiose and rhamnose fermentation broths were prepared as follows: filter sterilised inulin, mannitol, melibiose or rhamnose was added to 5mL of sterile diluent to give a final concentration of 1% (w/v). To this, 10µL of 0.4% (w/v) bromocresol purple was added. For the glucose broth, inverted Durham tubes were added for the detection of gas production. The control broth was prepared in the same manner but without the sugars (Anonymous, 1995).

2.3.20 Tryptone yeast glucose broth for growth of *Carnobacterium*

As *Carnobacterium* did not grow well in MRSB, tryptone yeast extract glucose broth (TYGB) was used instead for routine culture of these strains and this contained: 10.0g tryptone, 10.0g yeast extract, 5.0g dextrose, 0.2g Tween 80, 0.05g magnesium sulphate and 0.05g manganous sulphate per litre. This was solidified with 1% Bacteriological Agar for preparing plates (TYGA).

2.2.21 Urea agar

Urea agar contained: 1.0g peptone, 5.0g sodium chloride, 2.0g potassium dihydrogen phosphate, 20.0g Bacteriological agar per litre. The agar was dissolved by heat and the pH adjusted to 6.8 prior to autoclaving. Once cooled to 50°C, filter sterilized glucose was added to give a final concentration of 0.1% (w/v), filter sterilized aqueous urea was to give a final concentration of 2% (w/v) and 6mL of aqueous 0.2% (w/v) phenol red was added to the agar before dispensing into sterile test tubes as 10mL aliquots (Barrow and Feltham, 1993).

2.3 CHEMICAL SOLUTIONS

All chemical solutions were stored at room temperature. All stock chemical solutions are listed in Appendix 1.

2.3.1 Carbol thionin stain

Carbol thionin stain for direct microbial count contained: 0.2g thionin dissolved in 5% aqueous phenol. For staining the carbol thionin was diluted 1:3 with water.

2.3.2 Coomassium brilliant blue R250

Coomassium brilliant blue R250 contained: 0.25g Coomassie brilliant blue in 90mL methanol:water (1:1 v/v) (Sambrook *et al.*, 1989).

2.3.3 Griess-Illosvay's Reagents

Reagent 1 contained: 1.0g sulphanilic acid in 100mL acetic acid and Reagent 2 contained: 1.0g naphthol dissolved on 100mL 95% ethanol (Harrigan and McCance, 1976).

2.4 MAINTENANCE AND GROWTH OF BACTERIAL STRAINS

2.4.1 Bacterial nomenclature

The majority of the strains used in this investigations were isolated from locally purchased meat that had been packaged in either air or 30% CO_2/70% N_2 and subsequently stored at one or 10°C then allowed to spoil. The isolation procedures for these bacteria are fully described in section 2.5. The

bacteria were named according the conditions from which they were isolated: atmosphere (A= air and M= modified atmosphere), temperature (1= 1°C and 2= 10°C), the meat type (1= beef, 2= chicken, 3= lamb and 4= pork), bacterial group (B= *Brochothrix*, En= *Enterobacteriaceae*, L= *Lactobacillus* or *Carnobacterium* and P= *Pseudomonas*) and finally there were arbitrarily designated a number (01, 02, 03...) to distinguish isolates. An example of the nomenclature is: A13B03 was isolated from air-packaged lamb stored at 1°C and was subsequently identified as belonging to the bacterial group *Brochothrix* and was isolate number three of this type.

2.4.2 Culture maintenance

Cultures stocks were stored at 2°C on the appropriate agar and renewed every three months, except *Lactobacillus* and *Carnobacterium*, which were renewed every six weeks. *Brochothrix* was maintained on TSA, *Carnobacterium* was maintained on TYGA, *Enterobacteriaceae* and *Pseudomonas* were maintained on NA and *Lactobacillus* was maintained on MRSA. All freezer stocks were stored on Protect Bacterial Preservers and in NB containing 16 % glycerol at -80°C. Fresh agar stocks were prepared by placing a Protect Bacterial Preservation bead or the glycerol stock into 5mL of broth, then incubated overnight at the temperatures described in section 2.4.4.

2.4.3 Selective media and selection conditions

Brochothrix was selected on STAA incubated at 25°C for 48 hours. The selective media for *Enterobacteriaceae* was VRBGA, which was incubated anaerobically at 30°C for 24 hours. The method of VRBGA incubation differed from the manufacturer's instructions because the colonies were going to be differentiated on the basis of morphology then cultured into nutrient broth. If the agar had been overlaid this would not have been possible. *Lactobacillus* and *Carnobacterium* were selected on MRSA incubated anaerobically at 30°C for 48 hours. *Pseudomonas* was selected on PSA incubated at 25°C for 48 hours. Total viable count were enumerated on PCA incubated at 30°C for 72 hours.

2.4.4 Cultivation methods

Overnight cultures were prepared by aseptically placing a loop-full of growth from agar stocks into 5mL of sterile broth then incubating at the optimum temperature for 16 hours, unless stated otherwise. *Brochothrix* was cultured at 25°C in TSB, *Enterobacteriaceae* was cultivated at 30°C in NB, *Lactobacillus* and *Carnobacterium* were cultivated at 30°C on MRSB or TYGB respectively. *Pseudomonas* was cultivated at 25°C in NB and isolates from PCA plates were cultivated at 30°C on

NB. When agar was required for cultivation or enumeration, all culture were incubated on the cultivation media's agar equivalent for 48 hours.

2.4.5 Direct microbial count

An overnight culture was diluted one in 100 and 10μL of culture was placed onto a washed slide that had been stored in 95% ethanol. A drop of sterile UHT milk was added to the culture and mixed so that the solution covered $1cm^2$. The slide was allowed to dry then was heat fixed. The slide was then placed in methanol for less than five minutes and drained. The slide was then placed into xylene for three minutes then drained again. The side was then dipped into 95% ethanol twice, drained then flooded with Carbol thionin stain for 30 seconds, drained, rinsed and allowed to dry. Under the microscope, 10 fields under 100x magnification were counted, averaged and multiplied by 625,000 to give the number of cfu/mL.

2.5 ISOLATION OF MEAT SPOILAGE BACTERIA USING NON-SELECTIVE AND SELECTIVE MEDIA

2.5.1 Cuts used

Fresh beef steaks, chicken breasts, lamb fillets and pork fillets were purchased from a local supermarket one day prior to packaging under specific atmospheres. The meat had been placed in the retail display case that morning and stored aerobically on polystyrene trays covered with cling film. The meats were aseptically divided into equal proportions to ensure that there was sufficient surface area on the meat for microbiological sampling. The meats were refrigerated at 4°C prior to packaging. This experiment was not repeated as meat spoilage had been well characterized by traditional techniques.

2.5.2 Packaging of meat under specific atmospheres

Each meat type was divided into two groups; the first group was to be packaged in air and the second group under an atmosphere of 30% CO_2/70% N_2. Each piece was aseptically placed into an individual semi-ridged, pre-formed molded plastic package. The oxygen transmission rates for the packages were unable to be obtained from the company that supplied the material, as the transmission rate would be altered by the molding process and would vary over the package. The packages were then passed through a Multivac R7000 packaging machine, the air within the package was replace with air or 30% CO_2/70% N_2 then sealed. The square semi-rigid packages measured $25x25x10cm^3$. The gas

Table 2.1 Meat spoilage bacteria were characterised according to Gram reaction, catalase reaction, oxidase reaction, glucose metabolism and growth on selective media.

Bacterial Groups	Gram reaction	Oxidase reaction	Catalase reaction	Glucose metabolism	Selective media
Acinetobacter	-	-	+	-[a]	NA
Brochothrix	+	-	+	F[b]	STAA
Enterobactericaeae	-	-	+	F	VRBGA
LAB	+	-	-	F	MRSA
Pseudomonas	-	+	+	O[c]	PSA

[a] Glucose not fermented
[b] Fermentation of glucose
[c] Oxidation of glucose

combination of 30% CO_2/70% N_2 was mixed with a Witt-Gastechnik gas mixer. Once packaged, the meats were stored in refrigerators at either 1 or 10°C (±0.5°C). The packages were checked daily by two senior microbiologist for signs of visible deterioration, such as the appearance of cloudy exudate and slimy shiny surfaces.

2.5.3 Isolation of spoilage microflora and preliminary characterisation

The microbial composition of the meat was determined immediately after packaging and upon visible deterioration, according to Australian Standard 1766.1.4.1979. Three 25cm^2 cores of meat were cut from the meat and placed into a sterile bag with 300mL of diluent. The bags were then macerated in a stomacher for one minute and stored in ice water. Samples of homogenate were serially diluted in diluent and 50µL was plated out in duplicate using a Spiral Systems Inc. spiral plater onto selective media (STAA, VRBGA, MRSA and PSA) and non-selective media (PCA). The number of bacteria were enumerated on each media type. Identification of isolates commenced immediately, all plates were stored at 2°C in case required. Individual colonies with different colony morphologies were selected from the PCA plates as representative isolates for identification.

2.5.4 Characterisation of isolates from non-selective and selective media

Isolates from the PCA plates were classified into *Acinetobacter*, *Brochothrix*, *Enterobacteriaceae*, LAB or *Pseudomonas* species. From PCA, colonies were sub-cultured into NB for 16 hours and purified on NA incubated at 30°C for 48 hours. Differentiation was based on: Gram reaction, catalase reaction, oxidase reaction, oxidation or fermentation of glucose and growth on selective media, as listed in Table 2.1. Catalase was determined by mixing a colony with hydrogen peroxide the immediate formation of bubbles indicated a positive reaction. A drop of oxidase reagent was placed on a piece of Whatman filter paper, a colony was wiped onto the filter paper with a sterile tooth pick: the formation of a pink colour within seconds indicated a positive reaction. Oxidation or fermentation of glucose was determined using OF medium. Colonies were stabbed into the OF medium then incubated at 25°C. Yellowing occurring at the top of the agar indicated oxidation; yellowing along the stab-line indicated fermentation and blue colouration indicted that glucose was not utilized.

The same process was repeated for isolates from selective media, to confirm the identity of single colonies as belonging to *Brochothrix*, *Enterobacteriaceae*, LAB or *Pseudomonas*. Identification of *Acinetobacter* species was not included as no selective media was available. From selective media, isolates were sub-cultured into NB and purified on NA for presumptive *Enterobacteriaceae* and *Pseudomonas* species;

Brochothrix isolates were sub-culture in TSB then TSA, and LAB in MRSB then MRSA. The tests listed in Table 2.1 were used to confirm identity.

2.5.5 Gas analysis

The gas composition within the packages was measured on the day of packaging and at spoilage prior to opening, using a Shimadzu 8A gas analyzer. One mL of gas was removed from the package through a silicon patch with a needle and syringe, and immediately injected into the gas analyzer. All gas analyses were conducted in triplicate. The gas chromatograph was standardized with 48.7 % CO_2/10.4% O_2/40.9 %N_2.

2.6. BIOCHEMICAL METHODS FOR THE IDENTIFICATION OF ISOLATES

2.6.1 Acid and gas production from sugars

An emulsion of bacteria was prepared in 5mL of sterile water for colonies that had been grown on agar for 48 hours at the appropriate temperature. A sample (0.1mL) of culture emulsion was added to broths containing glucose, inulin, melibiose, mannitol or rhamnose, and the control lacking in sugars. The tubes were then incubated at 25°C and examined daily for up to 30 days. A positive reaction was indicated by the broth changing from purple to yellow in colour due to the production of acid. For gas production, a positive reaction was indicated by the formation of a bubble in the Durham tube (Anonymous, 1995).

2.6.2 Arginine, lysine and ornithine hydrolysis

Overnight cultures were added in 0.1mL aliquots to both the control and arginine, lysine or ornithine broths. The test tubes were incubated at 25°C for up to seven days with the colour noted daily. If the colour changed from purple to yellow, then back to purple, the culture hydrolysed arginine, but if the broth remained yellow then the culture did not hydrolyse arginine, lysine or ornithine. Glucose fermentation yields acids which decreases the pH and changes the indicator to yellow; the return to the purple colour occurs when the arginine is hydrolysed and ammonia is produced increasing the pH (Anonymous, 1995).

2.6.3 Gelatin hydrolysis

Cultures from agar plates were stabbed into commercially available nutrient gelatin and incubated at 25°C for seven days. Following incubation, the gelatin tubes were plunged into ice water, a positive reaction was recorded if the medium remained liquid.

2.6.4 Growth at four, 30, 35 and 45°C

To 5mL of TSB, 0.1mL of an overnight culture was added. The broths were then incubated at four, 30, 35 or 45°C for seven days. If growth was visible after that time, this was considered to be positive result (Talon *et al.*, 1988).

2.6.5 Growth on acetate agar

Cultures were streaked onto Rogosa agar and at 30°C for 48 hours. If no growth was evident after this time, a negative result was recorded (Rogosa *et al.*, 1951).

2.6.6 Growth at pH 3.9

To APT broth at pH 3.9, 0.1mL of an overnight culture was added then incubated for seven days. If visible growth occurred, the result was positive (Shaw and Harding, 1984).

2.6.7 Growth in eight and 10% sodium chloride

To each TSB tube containing either eight or 10% sodium chloride, 0.1mL of an overnight culture was added and incubated at 25°C for seven days. After this time the tubes were removed, any visible growth was recorded as positive (Talon *et al.*, 1988).

2.6.8 Growth and reduction of potassium tellurite

An overnight culture was streaked onto both tellurite-BMA and control plates then incubated at 25°C for seven days. The appearance of black colonies was considered to be a positive result (Wilkinson and Jones, 1979).

2.6.9 Heterofermentation and homofermentation

Three drops of an overnight culture were added to HHD broth and incubated at 30°C for up to seven days. Broths which turned green and had blue-green cell sediments contained homofermentative organisms and those that remained blue and had white cell sediments were heterofermentative (McDonald *et al.*, 1987).

2.6.10 Hippurate hydrolysis

To both hippurate-SBMB and control, 0.1mL of an overnight culture was added then incubated at 25°C for seven days. Each culture was then centrifuged in a Beckman centrifuge at 2,600*g* for 10 minutes in clean 10mL centrifuge tubes. One mL of supernatant was mixed with 1mL of 50% (v/v) sulphuric acid: the formation of a white precipitate indicated a positive reaction (Wilkinson and Jones, 1979).

2.6.11 King's B medium

Overnight cultures were streaked onto King's B and incubated at 25°C for one to five days. During this time, the plates were examined under UV-light for the presence of fluorescent pigments, which appear blue or green (Harrigan and McCance, 1976).

2.6.12 Nitrate reduction

A sub-culture of an isolate from agar was inoculated into the nitrate peptone water and incubated at 25°C for seven days. One mL of each Griess-Ilosvay's reagents (Harrigan and McCance, 1976) was added to each test tube. A red colour was formed if the nitrate had been reduced to nitrite. If the result was negative, zinc powder was added which reduced the nitrate present to nitrite. If no colour change occurs following the addition of zinc, then the nitrate had been reduced beyond nitrite to N_2 which was indicated by the presence of gas in the Durham (Anonymous, 1995).

2.6.13 Production of D- and L-lactic acid

The formation of D- and L-lactic acid form glucose was determined using a commercially available kit from Boehringer Mannheim GmBH according to manufacturer's instructions. The minimum detection limit for the production of isomer of D- and L-lactic acid was 0.01g/L.

2.6.14 Production of polysaccharides from sucrose

Overnight cultures were streaked onto NA supplemented with 5% sucrose and incubated at 25°C for seven days. Synthesis of polysaccharides was indicated by mucoidal growth (Harrigan and McCance, 1976).

2.6.15 Terminal pH

A 0.1mL aliquot of 16h broth culture was added to La-broth and incubated at 25°C for seven days. At the conclusion of incubation, the culture was centrifuged in a Beckman J2-21M/E at 2,680*g* for 10 minutes at 25°C. The pH of the cell-free supernatant was determined with an Orion pH electrode. Strains were differentiated on the basis of the pH being above or below 4.15 (Shaw and Harding, 1984).

2.6.16 Urea hydrolysis

A colony was taken from an agar plate and stabbed into the Urea agar then incubated at 25°C for seven days. If the colour changed from colourless to pink due to the formation of ammonia, urease was present; if no colour change occurred, the test was negative (Barrow and Feltham, 1993).

2.7 POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) PROTEIN PROFILING

2.7.1 Preparation of whole cell proteins

A 24h broth culture was inoculated into 10mL of TSB and grown for another 24h at 25°C. The optical density at 610nm (OD_{610nm}) was measured in a Pharmacia LKB Novaspec II. Cultures were centrifuged at 2,600*g* for 10 minutes in a Hettich bench top centrifuge. The cells were washed once in sterile double de-ionised water. A 4% (v\v) aqueous solution of tergitol and 1.0mm glass beads were added to the pellet in a 1:2:1 ratio (tergitol: glass beads: pellet). These were then homogenised in a B. Braun MSK cell homongeniser for 30 seconds, then immediately placed on ice. The suspension was centrifuged at 4,000rpm in a Sorvall RT7 bench top refrigerated centrifuge at 4°C. The supernatant was carefully removed so as not to disturb the pellet, placed into a sterile Eppendorf and stored at -20°C until required.

2.7.2 Preparation of acrylamide gel

The method of Sambrook *et al.* (1989) was used for SDS-PAGE gel preparation. The running gel was prepared by combining 6.4mL acrylamide stock solution with 6.18mL distilled water and 3.2mL Tris-HCl (pH 8.8) which was then degassed for 15 minutes. The following were added in order to the degassed acrylamide solution: 160µL 10% SDS, 8µL TEMED and 54µL of 10% ammonium persulphate. The gel was poured immediately into a slab gel mold with 1mm spacers. A mixture of 1:1 water and butanol was layered over the gel while it polymerised to sharpen the interface. Once the gel had polymerised the water/butanol mixture was removed and the gel was rinsed with water then drained. The stacking gel was prepared in the same manor as the running gel. An aliquot of 1.6mL acrylamide stock solution was added to 7.2mL distilled water and 1mL Tris-HCl (pH 6.8), and degassed for 15 minutes. The following were then added in order: 100µL 10% SDS, 18µL TEMED and 44µL of 10% ammonium persulphate. The gel was poured immediately over the top of the running gel and a 16-well comb inserted. When the stacking gel had polymerised the comb was removed and the wells rinsed to remove any unpolymerised gel debris in the wells.

2.7.3 Final sample preparation

The supernatants of cell lysates which had been stored at -20°C were thawed and a 50µL aliquot removed and placed into a sterile Eppendorf tube. To each tube 50µL of 0.5M Tris-HCl (pH6.8) containing: 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.001% bromophenol blue, was added, giving a total volume of 100µL. The samples were then heated at 100°C for 30 seconds in a heating block.

2.7.4 Loading and running of SDS-PAGE

The electrophoresis was conducted in a BioRad Protean II apparatus with a running buffer of 1x Tris acetate EDTA buffer (TAE; 1x: 4.48g Tris-base, 1.142mL glacial acetic acid and 2mL 0.5M EDTA [pH 8.0]). The samples were prepared as described above were loaded into the gel so that 60µL (for sample the isolates with the lowest OD_{610nm}) was the highest amount loaded, other loading volumes were determined in regard the OD_{610nm} at the time of harvesting the isolates so that a constant protein loading was obtained (equivalent to OD_{610nm}=0.596 at harvest). The gel was run at 40mA until the dye front reached the bottom of the gel. The molecular marker used was an unstained SDS-PAGE standard-Low range.

2.7.5 Staining of the SDS-PGE gel

The gel was placed in Coomassie brilliant blue stain R250 for one hour at room temperature with gentle shaking. The gel was rinsed with distilled water for 10 minutes to remove the excess stain. The gel was then placed in de-staining solution containing 90mL methanol:water (1:1 v/v) with 10mL acetic acid then gently shaken for 30 minutes at room temperature. The de-staining procedure was conducted three times. The gel was transferred to distilled water and allowed to stand overnight. The gel was then photographed by illuminating with white light and photographed with black and white positive (667) or positive/negative (665) films.

2.8 DETECTION OF MESO-DIAMINOPIMELIC (*meso*-DAP) ACID BY THIN LAYER CHROMATOGRAPHY (TLC).

2.8.1 Preparation of whole cell wall hydrolysate

Whole cell wall hydrolysates were produced according to the method of Bousfield *et al.* (1985). Ten mL of overnight MRSA or TYCB culture grown at 30°C, was centrifuged at 12,900*g* for 10 minutes at 4°C in a Beckman J2-21M/E centrifuge. The supernatant was discarded and the pellet washed twice in distilled water. The pellet was then suspended in 95% ethanol and re-centrifuged. The supernatant was discarded and the pellet air dried in an oven at 55°C for approximately one hour. Once dry, the pellet was resuspended in 1mL 6M HCl then transferred into 5mL glass ampoules and sealed. The suspension was hydrolyses at 105°C for 18h. The hydrolysate was removed using a clean plastic syringe and filtered through Whatmann N°1 filter paper, cut to fit a 13mm Swinny stainless steel filter, into a clean bijou bottle. The filter paper was washed with a few drops of distilled water. The hydrolysate was evaporated to dryness in an oven at 100°C. The residue was taken up in 1mL distilled water then evaporated to dryness. Following the second evaporation, the residue was taken up in 20µL distilled water and stored at -20°C until required.

2.8.2 Running TLC plates

One dimensional chromatography was performed on Merck aluminium plates pre-coated with 0.1mm cellulose (no. 5552) which had been activated at 100°C for one hour prior to use. Two µL of sample or *meso*-DAP standard (1% (w/v) diaminopimelic acid dissolved in 10% (v/v) isopropanol) were loaded on the plates. The plates were developed in a solvent system of methanol-pyridine-10M HCl-water (80:10:2.5:17.5; Rhuland *et al.*, 1955) for two to three hours at room temperature (Bousfield *et al.*, 1985). The plate was removed, air dried, then sprayed with acidified ninhydrin (465mL 1-butanol,

Table 2.2 The differentiation of the two *Brochothrix* species, *B. thermosphacta* and *B. campestris* on the basis of the ability to grow in the eight and 10% sodium chloride, reduction potassium tellurite, hydrolysis of hippurate and fermentation of rhamnose (Talon *et al.*, 1988).

Species	Sodium chloride		Potassium tellurite	Hippurate	Rhamnose
	8%	10%			
B. thermoshacta	+	+	+	-	-
B. campestris	-	-	-	+	+

35mL acetic acid and 2.5g ninhydrin) and heated at 100°C for a few minutes (personnel communication, Dr N. Weiss, Deutche Sammlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig, Germany). The presence of *meso*-DAP was characterized by the appearance of an olive green spot near the origin (Bousefield *et al.*, 1985). *C. divergens* was run as a positive control.

2.9 IDENTIFICATION OF DOMINANT BACTERIAL ISOLATES

The number of different colony morphologies were observed then a representative of the dominant types of colonies were selected for identification This resulted in two to four isolates from *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* being selected from beef and lamb for each of the four storage conditions. It was not possible to identify all colonies.

2.9.1 Identification of isolates identified as members of the genus *Brochothrix*

Currently there are only two species of *Brochothrix* characterized and these can be differentiated by five biochemical tests, as described by Talon *et al.* (1988) and listed in Table 2.2. *B. thermosphacta* is able to grow in the presence of eight and 10% sodium chloride and potassium tellurite, but is unable to hydrolyze hippurate or ferment rhamnose, while the reverse applies to *B. campestris* (Table 2.2). A reference strain *B. thermosphacta* ATCC11509 was included in the identification. Prior to testing, *Brochothrix* strains were grown for 16 hours in TSB or for 48h on TSA at 25°C. *Brochothrix* isolates were also subjected to tests for: growth at four, 30, 35 and 45°C, growth at pH 3.9, production of slime from sucrose-supplemented NA, terminal pH in La-broth, urea hydrolysis and protein profiling on SDS-PAGE. A type strain of *B. campestris* was unavailable for comparison.

2.9.2 Identification of isolates identified as members of the family *Enterobacteriaceae*

Bacteria identified as belonging to the family *Enterobacteriaceae* was identified to species level using Biolog (Biolog Inc., Haywood, USA) and 20E API strips (bioMérieux, Lyon, France) according to manufactures' instructions. Prior to testing, *Enterobacteriaceae* were grown at 30°C in NB for 16h then on NA for 48h. A singe colony was suspended in 5mL sterile distilled water, then added to the wells. Differentiation of the *Serratia* species was done by gas production from glucose and acid production for rhamnose using sugar fermentation media.

Table 2.3 Differentiation of *Lactobacillus* and *Carnobacterium* species from meat sources on the basis of the presence of *meso*-DAP (mDAP) in the cell wall, growth on acetate agar (Ace.), heterofementation (Hetero) or homofermenatation (Homo), the production of D-, L- or DL-lactic acid (lactic acid) arginine hydrolysis (ADH) and the production of acid from inulin (Inu.), mannitol (Man.) or melibose (Mel.) (Montel *et al.*, 1991).

Species	mDAP	Ace.	Hetero/ Homo	Lactic acic	ADH	Inu.	Man.	Mel.
C. divergens	+	-	NT	L[a]	+	-	-	NT
C. piscicola	+	-	NT	L	+	+	+	NT
Lb. plantarum	+	+	NT	DL[b]	-	NT	NT	NT
Lb. halotolerance	-	NT	Hetero	DL	+	NT	NT	NT
Lb. viridescencs	-	NT	Hetero	DL	-	NT	NT	NT
Leuconostoc	-	NT	Homo	D[c]	NT	NT	NT	NT
Lb. sakei	-	NT	Homo	DL	+	NT	NT	+
Lb. curvatus	-	NT	Homo	DL	-	NT	NT	-
Lb. farciminis	-	NT	Homo	L	+	NT	NT	NT
Lb. alimentarius	-	NT	Homo	L	-	NT	NT	NT

NT= not tested
[a] produces L-lactic acid
[b] produces D-lactic acid
[c] produces DL-lactic acid

Table 2.4 Differentiation of species of *Pseudomonas* associated with meat spoilage on the basis of fluorescent pigment production (Fluor.), the production of levan from sucrose (Levan), lethinase acitivity, gelatin hydrolsysis (Gelatinase) and acid production from lactose (adapted from Pelleroni (1981) and Craven and McAuley (1992))

Species	Fluor.	Levan	Lethinase	Gelatinase	Lactose
P. fluorescens	+	+	+	+	NT
P. fragi	-	NT	-	-	NT
P. putida	+	-	-	NT	-
P. aeruginosa	+	-	-	NT	+

2.9.3 Identification of isolates identified as members of the genus *Carnobacterium* and *Lactobacillus*

A simplified method for the identification of *Lactobacillus* and *Carnobacterium* species isolated from meat was devised by Montel *et al.* (1991). *Lactobacillus* strains were cultured on MRSB and MRSA and *Carnobacterium* were cultured on TYGB and TYGA, both at 30°C. *Carnobacterium* and *Lactobacillus* were identified according to: the presence or absence of *meso*-DAP, growth on Rogosa agar, heterofermentation or homofermentation, production of D- and L-lactic acid, arginine hydrolysis, acid production from inulin, melibiose or mannitol, as shown in Table 2.3. The presence of *meso*-DAP in the cell wall was determined by TLC. Growth on acetate agar was done using Rogosa agar. Mode of fermentation (heterofermentative or homofermentative) was determined in HHD. The production of D- and L-lactic acid was completed using a Boehringer Mannheim D/L-lactic acid kit. Arginine hydrolysis was determined in arginine broths. Acid production from inulin, mannitol or melibiose was determined in sugar fermentation media containing the appropriate sugar.

2.9.4 Identification of isolates identified as members of the genus *Pseudomonas*

The method of Craven and McAuley (1992) were used to determine the species of the *Pseudomonas* isolates. Prior to testing, *Pseudomonas* species were grown in NB for 16 hours or on NA for 48h at 25°C. Table 2.4 demonstrates the difference between the *Pseudomonas* strains. The ability to produce slime was demonstrated on NA supplemented with 5% sucrose; fluorescent pigment production on King's B medium; conversion of nitrate to nitrite in nitrate broth and hydrolysis of gelatin in gelatin agar.

2.10 PULSED-FIELD GEL ELECTROPHORESIS OF *Brochothrix*, *Carnobacterium* AND *Lactobacillus* SPECIES

2.10.1 Growth of cultures for PFGE

Brochothrix isolates were grown in TSB at 25°C and *Carnobacterium* and *Lactobacillus* in TYGB at 30°C. Cultures were incubated in 5mL of the appropriate broth for 24h, then 1mL was inoculated into 40mL of the same broth and incubated for 16h. A 20% (w/v) aqueous glycine solution which had been filter sterilized was added to give a final concentration of 0.5% (w/v) then incubation continued for a further three hours: this was found to assist in cell lysis (personal communication, Mohomid Mohideen, Centre for Bioprocessing and Food Technology, Victoria University of Technology, Werribee, Australia).

Table 2.5 The random combination of groups of plates for Experiment 1 containing agar only (none), sodium chloride (NaCl), potassium sorbate (PS) or propyl-paraben (PP) at both pH levels under either air, 25% CO_2 and 100% CO_2 and stored at 1 or 10°C for the first replicate. Treatment combinations were as follows: 1= pH 5.5 + none, 2= pH5.5 + NaCl, 3=pH5.5 + PS, 4= pH 5.5+PP, 5= pH 6.0 + none, 6= pH6.0 + NaCl, 7=pH6.0 + PS, 8=pH6.0 + PP.

Bag No.	Plate	Temp (°C)	Atmos.	Treatment	pH	Preservative
1	1	10	25% CO_2	8	6.0	PP
1	2	10	25% CO_2	5	6.0	None
1	3	10	25% CO_2	2	5.5	NaCl
1	4	10	25% CO_2	4	5.5	PP
2	1	10	25% CO_2	6	6.0	NaCl
2	2	10	25% CO_2	1	5.5	None
2	3	10	25% CO_2	3	5.5	PS
2	4	10	25% CO_2	7	6.0	PS
3	1	1	Air	4	5.5	PP
3	2	1	Air	2	5.5	NaCl
3	3	1	Air	3	5.5	PS
3	4	1	Air	6	6.0	NaCl
4	1	1	Air	8	6.0	PP
4	2	1	Air	7	6.0	PS
4	3	1	Air	1	5.5	None
4	4	1	Air	5	6.0	None
5	1	10	Air	3	5.5	PS
5	2	10	Air	2	5.5	NaCl
5	3	10	Air	1	5.5	None
5	4	10	Air	5	6.0	None
6	1	10	Air	6	6.0	NaCl
6	2	10	Air	8	6.0	PP
6	3	10	Air	7	6.0	PS
6	4	10	Air	4	5.5	PP
7	1	1	100% CO_2	4	5.5	PP
7	2	1	100% CO_2	5	6.0	None
7	3	1	100% CO_2	3	5.5	PS
7	4	1	100% CO_2	7	6.0	PS
8	1	1	100% CO_2	6	6.0	NaCl
8	2	1	100% CO_2	2	5.5	NaCl
8	3	1	100% CO_2	1	5.5	None
8	4	1	100% CO_2	8	6.0	PP
9	1	1	25% CO_2	7	6.0	PS
9	2	1	25% CO_2	2	5.5	NaCl
9	3	1	25% CO_2	8	6.0	None
9	4	1	25% CO_2	3	5.5	PP
10	1	1	25% CO_2	1	5.5	PS
10	2	1	25% CO_2	5	6.0	NaCl
10	3	1	25% CO_2	6	6.0	PP
10	4	1	25% CO_2	4	5.5	PS
11	1	10	100% CO_2	6	6.0	NaCl
11	2	10	100% CO_2	5	6.0	None
11	3	10	100% CO_2	3	5.5	PS
11	4	10	100% CO_2	8	6.0	PP
12	1	10	100% CO_2	4	5.5	PP
12	2	10	100% CO_2	1	5.5	None
12	3	10	100% CO_2	7	6.0	PS
12	4	10	100% CO_2	2	5.5	NaCl

Table 2.6 The random combination of groups of plates for Experiment 2 containing agar only (none), sodium chloride (NaCl), potassium sorbate (PS) or propyl-paraben (PP) at both pH levels under either air, 25% CO_2 and 100% CO_2 and stored at 1 or 10°C for the second replicate. Treatment combinations were as follows: 1= pH 5.5 + none, 2= pH5.5 + NaCl, 3=pH5.5 + PS, 4= pH 5.5+PP, 5= pH 6.0 + none, 6= pH6.0 + NaCl, 7=pH6.0 + PS, 8=pH6.0 + PP.

Bag No.	Plate	Temp (°C)	Atmos.	Treatment	pH	Preservative
1	1	1	100% CO_2	4	5.5	PP
1	2	1	100% CO_2	2	5.5	NaCl
1	3	1	100% CO_2	5	6.0	None
1	4	1	100% CO_2	1	5.5	None
2	1	1	100% CO_2	3	5.5	PS
2	2	1	100% CO_2	7	6.0	PS
2	3	1	100% CO_2	6	6.0	NaCl
2	4	1	100% CO_2	8	6.0	PP
3	1	10	100% CO_2	3	5.5	PS
3	2	10	100% CO_2	8	6.0	PP
3	3	10	100% CO_2	1	5.5	None
3	4	10	100% CO_2	2	5.5	NaCl
4	1	10	100% CO_2	4	5.5	PP
4	2	10	100% CO_2	6	6.0	NaCl
4	3	10	100% CO_2	5	6.0	None
4	4	10	100% CO_2	7	6.0	PS
5	1	1	Air	5	6.0	None
5	2	1	Air	7	6.0	PS
5	3	1	Air	6	6.0	NaCl
5	4	1	Air	2	5.5	NaCl
6	1	1	Air	3	5.5	PS
6	2	1	Air	8	6.0	PP
6	3	1	Air	1	5.5	None
6	4	1	Air	4	5.5	PP
7	1	1	25% CO_2	6	6.0	NaCl
7	2	1	25% CO_2	1	5.5	None
7	3	1	25% CO_2	5	6.0	None
7	4	1	25% CO_2	3	6.0	PS
8	1	1	25% CO_2	2	5.5	NaCl
8	2	1	25% CO_2	7	6.0	None
8	3	1	25% CO_2	4	5.5	None
8	4	1	25% CO_2	8	6.0	PS
9	1	10	25% CO_2	6	6.0	NaCl
9	2	10	25% CO_2	8	6.0	PS
9	3	10	25% CO_2	1	5.5	PP
9	4	10	25% CO_2	4	5.5	PP
10	1	10	25% CO_2	7	6.0	NaCl
10	2	10	25% CO_2	2	5.5	PP
10	3	10	25% CO_2	5	6.0	None
10	4	10	25% CO_2	3	5.5	PS
11	1	10	Air	2	5.5	NaCl
11	2	10	Air	7	6.0	PS
11	3	10	Air	6	6.0	NaCl
11	4	10	Air	1	5.5	None
12	1	10	Air	3	5.5	PS
12	2	10	Air	4	5.5	PP
12	3	10	Air	8	6.0	PP
12	4	10	Air	5	6.0	None

2.10.2 Preparation of DNA

The cultures were placed into sterile 50mL centrifuge tubes and centrifuged at 2,600*g* for 5 minutes in a Hettich Universal bench top centrifuge. The supernatant fluid was discarded and the pellet was washed three to four times in 10mL PIV (Tris-Cl [pH 7.6] and 1M sodium chloride). Finally, the pellet was suspended in PIV and the OD_{610nm} determined. A final volume of PIV was added to give a cell density of 10^7-10^8 cfu/mL. The cell suspension was incubated on ice for 30 minutes then transferred to 37°C for a further 30 minutes. Aliquots of cell suspension were mixed in a 1:1 ratio with molten 1% agarose in PIV and dispensed into 2mm x 5mm x 10mm molds. These were then allowed to solidified on ice for one to two hours. The blocks of agarose containing cells were suspended in fresh EC-lysis (6mM Tris-Cl [pH7.6], 1% sarkosyl, 100mM EDTA [pH 8.0], 1M sodium chloride and 20µg/mL DNase free RNase) solution containing 10mg/mL lysozyme then incubated overnight at 37°C to remove the cell wall.

The blocks were transferred into ESP (1mg/mL protinease K and 1% sarkosyl) and incubated at 45°C for 24-48h. The ESP solution was removed by washing the blocks at 45°C in 10mL Tris-EDTA (TE: 10mM Tris-CL pH8.0 and 1mM EDTA pH 8.0) buffer for 1.5 hours containing 1mL of 0.175% phenylmethylsulfonyl fluoride (PMSF) in isopropanol. The TE-PMSF was removed by rinsing the blocks three times in TE at 45°C for 30 minutes. The blocks were stored until required at 4°C in sufficient TE buffer to just cover the blocks, noting that excessive amounts of TE buffer can cause the DNA to dissipate out of the agarose in prolonged storage.

2.10.3 Digestion of agarose blocks with restriction endonucleases

The agarose blocks were cut in half with a sterile scalpel both and halves were placed into separate lots of 1mL of TE in a sterile Eppendorf tubes. One half was returned to the coldroom for later use and the other was placed at 45°C for 15 minutes prior to digestion with restriction endonuclease. The total volume of the digestion reaction was 150µl: 116-118µL sterile double distilled water, 15µL nuclease-free bovine serum albumin, 15µL 10x restriction buffer (according to the manufacturer's instruction) and two to 4µL of restriction enzyme. Enzymes screened included: *Bam*HI, *Drd*I, *Eco*RI, *Hae*II, *Hae*III, *Hind*III, I-*Ceu*I, *Nhe*I, *Not*I, *Pst*I, *Sfi*I, *Sma*I, *Spe*I, *Xba*I and *Xho*I. The agarose block was added to the mixture just prior to the addition of the restriction enzyme. Blocks were digested for 16h at appropriate temperatures for specific enzymes. The agarose blocks were washed in TE buffer twice at 45°C to remove the enzyme before electrophoresis.

2.10.4 Preparation of electrophoresis gel

The 1.2% agarose in 0.5x Tris-Boric acid-EDTA (TBE; 1x: 10.8g Tris-base, 5.5g boric acid, 4mL EDTA [pH 8.0]) gel was poured into a 20cm x 20cm rubber mold placed on a perspex tray. A comb was placed over the mold prior to the addition the molten agarose to produce 2mm x 5mm x 5mm wells. The blocks were loaded into the wells using two small, clean, heat sterilised spatulas. The gel was run in a Pharmacia CHEF apparatus with a hex electrode, with 1x TBE as the running buffer. The PFGE unit was attached to a circulating water bath to prevent over heating. The gels were run using pulse times varying from one to 35 seconds over a 20 to 24h period at 170mv with 10mA field strength at 12°C. Low range (0.13-194kb) and mid-range I (15.0-291.0kb) markers and a lambda ladder (48.5-1018.5kb) were run with each gel in order to estimate genome size and aid in the differentiation of isolates.

2.10.5 Visualisation of bands

The gels were placed in 0.001 mg/mL ethidium bromide for four hours and rinsed under running water. The bands were illuminated with UV-light and photographed with black and white positive (667) or positive/negative (665) films.

2.10.6 Determination of molecular weight and relatedness of species

For each molecular marker or sample from *Brochothrix* and *Carnobacterium* species, the distance each band had migrated from the origin in millimetres was measured by hand from the photograph. A standard curve was fitted using Microsoft Excel version 5.0a and the resulting formula for the curve used to calculate the molecular weight of each band. The sum of the molecular weights was calculated to give the genome size. The genome size for *Lactobacillus* species could not be estimated due to the large number of small fragments generated by the endonucleases used.

A dendrogram was produced to determine the relatedness of the *Brochothrix* isolates. Over the areas measured for the two, five and 10 second gels, each isolated was given a value of one for the presence of a band and a zero for the absence of a band for each millimetre. The dendrogram was performed using the NTSYS version 1.7 programs. The data was transformed into a simple square matrix. The similarity measure was performed using a simple matching coefficient and the clustering was performed using a sequential, agglomerative, hierarchical and nested (SAHN) clustering with an unweighted pair-grouping method of arithmetic averages (UPGMA).

2.11 EFFECTS OF ENVIRONMENTAL PARAMETERS ON THE GROWTH OF MEAT SPOILAGE BACTERIA

A member of each species from the four bacterial groups was selected to examine the impact of environmental factors on their growth.

2.11.1 Growth rate determination in broth cultures

A representative of each group was selected as follows: *B. thermosphacta* A13B03 for *Brochothrix*, *H. alvei* M23En02 for *Enterobacteriaceae*, *Lb. sakei* M11L04 and *C. divergens* M11L03 for LAB and *P. fluorescens* A11P04 for *Pseudomonas*. The growth curve for each bacterial isolate was determined aerobically in the appropriate broth at the optimum growth temperatures: 25°C for *B. thermosphacta* A13B03 in TSB and *P. fluorescens* A11P04 in NB and 30°C for *C. divergens* M11L03 in TYGB, *H. alvei* M23En02 in NB and *Lb. sakei* M11L04 in MRSB. Overnight cultures were grown at optimum temperature. Aliquots of culture were diluted to give an optical density of 0.01 in 10mL of broth in a 50mL sidearm flask. The cultures were then incubated in a Certomat® chamber HK and U shaker at optimum temperature and 90 rpm, except for *Lb. sakei* M11L04 and *C. divergens* M11L03, which were not agitated. The OD_{610nm} of each culture was measured every hour using a Chemtrix 20A colourimeter until the culture had reach maximum stationary phase, which was indicated by three constant sequential readings.

2.11.2 Growth rate determination on agar surfaces

The same representative strains that were used in section 2.11.1 were used to determined bacterial growth rates on agar. The growth curve for each bacterial isolate was determined aerobically on the appropriate agar at the optimum growth temperatures: 25°C for *B. thermosphacta* A13B03 and *P. fluorescens* A11P04 and 30°C for *C. divergens* M11L03, *H. alvei* M23En02 and *Lb. sakei* M11L04. An aliquot of an overnight culture was diluted to give an OD_{610nm} of 0.01 then incubated at the optimal temperature until late log phase as indicated by the OD_{610nm} determined in section 2.11.1 For each strain, the relationship between viable count and OD_{610nm} reading had been determined and the late log cultures were diluted to give 10^4 cfu/cm^2 then 0.1mL of this was spread onto replicates of the appropriate buffered agar: MRSA for *Lb. sakei* M11L04, NA for *H. alvei* M23En02 and *P. fluorescens*, STAA for *B. thermosphacta* A13B03 and TYBG for *C. divergens* M11L03. The plates were incubated at the optimum temperature for each organism. Every three hours over a period of up to 36 hours, three plates were sacrificed at each time interval.

2.11.3 Determination of bacterial number on agar surfaces

All bacteria growing on agar surfaces were enumerated in this manner. Bacterial numbers on the agar surface were established using the method of Eyles *et al.* (1993): the agar was placed aseptically into a sterile bag with 50mL of diluent and macerated for 30 seconds using a stomacher. The homogenate was serially diluted and 50μL plated onto appropriate media using a W.A.S.P. spiral plater. Plates were incubated for 48 hours at the appropriate temperature then enumerated.

Growth rates were calculated from the data according to the following equation:

$$\text{Specific growth rate } (\mu) = (\log b_1 - \log b_0)/t_1 - t_0 * 2.013$$

where : b_1= the number of bacteria at the end of the incubation period t_1,

b_0= the number of bacteria at t_0

t_1= the time incubation was terminated

t_0= the moment the plates placed in the incubator (Stainer *et al.*, 1979)

For *Brochothrix* and *Carnobacterium*, t_1 was 15 hours, *Enterobacteriaceae* t_1 was 12 hours, and for LAB 16h and *Pseudomonas* t_1 was 18 hours.

2.11.4 Effects of pH, sodium chloride and preservatives on bacterial growth

Broth cultures were prepared as described in section 2.11.2. The diluted cultures were plated in 0.1mL aliquots onto buffered agar at different pH levels or at pH 6.0 for media containing either sodium chloride, potassium sorbate, methyl-paraben or propyl-paraben. All plates were prepared in triplicate then incubated at optimum temperature: 25°C for *Brochothrix* and *Pseudomonas* and 30°C for *Carnobacterium*, *Enterobacteriaceae* and *Lactobacillus* 30°C. The experiment was terminated when bacterial numbers were estimated to be at 90% of maximum population density as determined by the section 2.11.2 results. Bacteria were enumerated in duplicate and specific growth rate (μ) calculated as described in section 2.11.3.

2.11.5 The preparation, packaging and enumeration of pure cultures exposed to different temperatures and carbon dioxide concentrations

For determining the effect of temperature and carbon dioxide on bacterial growth a representative of each group was selected as follows: *B. thermosphacta* A13B03 for *Brochothrix*, *H. alvei* M23En02 for *Enterobacteriaceae*, *Lb. sakei* M11L04 for LAB and *P. fluorescens* A11P04 for *Pseudomonas*. Cultures

were prepared as described in section 2.11.2. One hundred μL of culture was plated onto the appropriate buffered agar: MRSA for *Lb. sakei* M11L04, NA for *H. alvei* M23En02 and *P. fluorescens*, STAA for *B. thermosphacta* A13B03 and TYBG for *C. divergens* M11L03. The lid of the Petri dish was propped open by a sterile stick cut to the width of the Petri dish lid, allowing greater gas diffusion. The plate and lid were secured together with a rubber band.

Duplicate plates were placed into RA454 bags then gas flushed with either 25, 50, 75 or 100% CO_2 with N_2 as the balance gas or 100% N_2 using a Webomatic type E50.G packaging machine. The gas combinations were mixed with a Witt-Gastechnik Type KM100-M gas mixer. A series of air packaged control plates were prepared by sealing the bags with a Venus® bag sealer model VHIB-400. During the packaging process all plates were kept at ≤2°C. After packaging the bags were randomly divided into four groups of eight bags then stored at 0, 5, 10 or 15°C (±1°C). Bags were removed over a period of time ranging from 12 to 2678 hours. In general, six bags were removed over the experimental time course for each temperature and atmosphere combination.

At the time of packaging and prior to opening, the head-space gas composition was determined with a M.A.P Test 4000 packaging atmosphere analyser. Any package with an oxygen content greater than 1% was discarded and another bag removed from the incubator. Bacterial number were determined as in section 2.11.3.

2.11.6 Combined effect of environmental factors on a mixed population

The same representative cultures were used as in section 2.11.5 for determining the combined effects of environmental factors on a mixed population. Cultures were prepared as described in section 2.11.2 except the cultures were diluted to give: 10^5 cfu/mL for *B. thermosphacta*, *Lb. sakei* and *P. fluorescens* and 10^4 cfu/mL for *H. alvei*, before mixing in a sterile schott bottle. When 0.1mL was spread onto an buffered-BHIA, the final cell density was: 10^3 to 10^4 cfu/cm^2 of *B. thermosphacta*, *Lb. sakei* and *P. fluorescens* and 10^1 to 10^2 cfu/cm^2 *H. alvei*.

The cultures were plated out on to buffered-BHIA prepared as described in section 2.2.4. For the second experiment the concentration of sodium chloride was increased from 1% to 2%, as 1% sodium chloride had no impact on microbial growth. Once plated onto the agar surface, a sterile stick was placed along the inside of the Petri dish lid then the plate and lid secured together with a rubber band. Plates were grouped together according to Tables 2.5 and 2.6 to reduce bias, before being placed into RA454 plastic bags. Prior to and during the packaging process, the plates were stored at ≤2°C.

The plates were under 25% CO_2/75%N_2 or 100% CO_2 using a Webomatic type E50.G packaging machine with gas mixtures made with a gas mixer Type KM100-2M. The same grouping procedure was used for the control, however, these were heat sealed with a Venus® bag sealer model VHIB-400 in the laboratory. At the completion of packaging, the plates were stored at 1 or 10°C (±0.5°C). Time zero and at 24, 48, 108, 156, 324, 660, 1334 and 2678h. The gas atmosphere was determined with a M.A.P Test 4000 packaging atmosphere analyser, if the oxygen content was above 1% the package was discarded and another removed. Bacteria growing under each atmosphere at both temperatures were enumerated according to the method in section 2.11.3. In order to isolate each member of the population selective media (MRSA, PSA, STAA and VRBGA) and the total viable count was determined on PCA.

2.12 ANALYSIS OF THE EFFECT OF ENVIRONMENTAL FACTORS ON THE GROWTH OF MEAT SPOILAGE BACTERIA

2.12.1 Analysis of the effects of temperature and carbon dioxide on *B. thermosphacta, H. alvei, Lb. sakei* and *P. fluorescens*

A modified-Gompertz equation was used to fit sigmodal curves to growth data achieved in the atmosphere and temperature using a Genstat 5 release 3.2 program written by Dr John Renyolds of the Australian Food Industry Science Centre. For the temperature and atmosphere experiments the significance of effects of both factors: time to the point of inflection or time to μ_{max}, time 90% of the maximum population, the maximum slope or μ_{max}, the rate parameter and the final population number were determined. All programs used are listed in Appendix 2.

2.12.2 Analysis of the effect of environmental factors alone and in combination on the growth of a mixed population

For the analysis of the effects of environmental factors alone and in combination on the growth of a mixed population a second Genstat program was written by Dr John Renyolds of the Australian Food Industry Science Centre. Sigmoidal curves were fitted to the growth data for the total population and *B. thermosphacta H. alvei, Lb, sakei* and *P. fluorescens. H. alvei* experienced limited growth under the conditions used in the investigation as a consequence curves could not be fitted to the data. The fitted curves provided information on the effects of each environmental factor: temperature, pH, preservatives (sodium chloride, potassium sorbate and propyl-paraben), and the interaction of these factors in combinations of two, three or four, on microbial growth. The parameters for which most information

could be obtained for was the slope of the curve or μ_{max}, time to 90% of the final population number and maximum population number. All programs used are listed in Appendix 3.

Chapter 3

Meat spoilage bacteria found on beef, chicken, lamb and pork after refrigerated storage air or modified atmosphere

3.1 INTRODUCTION

Contamination of meat with microorganisms is unavoidable during the slaughter process. Microorganisms come from a variety of sources including: air, water, the animals, equipment and personnel (Newton *et al.*, 1978; Patterson and Gibbs 1978; Nortjé *et al.,* 1990). As the carcass proceeds along the processing line, the level of contamination increases although once enclosed in the chiller microbial numbers decrease due to water activity on the carcass surface (Nortjè and Naudè, 1981; Gill. 1986). Once the carcass leaves the chiller, the contamination process continues as the carcass is divided into smaller pieces, providing additional fresh surfaces on which bacteria can establish.

As storage time continues, the number of bacteria increases and the nature of the dominant bacteria is determined by storage temperature and atmospheric gas composition within the package. *Pseudomonas* is the primary genus involved in the spoilage of refrigerated meats that are stored in air (Bailey *et al.,* 1979a). Of this genus, both fluorescent and non-fluorescent types have been found to comprise between 70 and 100% of the spoilage population respectively (Bailey *et al.,* 1979a; Erichsen and Molin, 1981b; Gill, 1986). In conjunction with pseudomonads, two types of strictly aerobic bacteria, *Acinetobacter* and *Moraxella,* are found as a part of the microflora (Gill, 1986). Two groups of facultative anaerobes are frequently found in

the spoilage population, *Enterobacteriaceae* (Gardner *et al.*, 1967; Gill and Newton, 1980) and *Brochothrix* (Barlow and Kitchell, 1966; Blickstad and Molin, 1983a), but in numbers substantially below *Pseudomonas* species.

Enterobacteriaceae, a Gram-negative family, are of concern because some members of this group are pathogenic (Bercovier and Mollaret, 1986; Le Minor, 1986; Johnson *et al.*, 1995). The source of this bacterial group in meat tends to be faecal in origin and the presence of *Enterobacteriaceae* in a spoilage population is results in putrid odours (Gill, 1986). The production of off odours by the facultatively anaerobic genus *Brochothrix* (Egan *et al.*, 1980), gives this genera an important role in the spoilage of air-packaged meat. *Brochothrix* species are non-pathogenic, Gram-positive, non-motile, non-spore forming bacteria commonly associated with spoiled lamb (Barlow and Kitchell, 1966).

The family *Enterobacteriaceae* and the genus *Brochothrix* are also associated with the spoilage of meat stored under atmospheres with high levels of carbon dioxide. However, the major bacterial group responsible for the spoilage of modified atmosphere packaged (MAP) meats is *Lactobacillus*. These Gram-positive bacteria are preferable to *Pseudomonas* as they are slower growing (Leeson, 1987), produce antibacterial agents which inhibit other bacteria (Garver and Murinana, 1993) and generally do not produce off odours (Gill and Penney, 1988).

The aims of this investigation were to determine the shelf-life duration of beef, chicken, lamb and pork stored in air and under a carbon dioxide-enriched atmosphere by determining: the numbers of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* at spoilage on each of the four meat types using selective media and the proportions of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* in the total population at spoilage on each of the four meat types using non-selective media.

3.2 METHODS

Beef, chicken, lamb and pork were purchased from a local supermarket, individually packaged in either air or 30% CO_2/ 70% N_2 and stored at one or 10°C until visibly deteriorated. The bacteria on the surface were sampled by excising three separate 25 cm^2 cores from the meats then macerating these cores with diluent. The diluent was serially diluted and spiral plated out onto both non-selective (PCA) and selective (MRSA, PSA, STAA and VRBGA) media. The bacteria were enumerated and individual colonies selected for preliminary identification. The plates were then stored at 2°C, with the PCA plates being re-examined after the

Table 3.1 Time taken for each meat type to be considered visually unacceptable at both one and 10°C in air and MAP

Atmosphere	Beef		Chicken		Lamb		Pork	
	1°C	10°C	1°C	10°C	1°C	10°C	1°C	10°C
Air	4[a]	1.5	4	1.5	2.5	1	2.5	1
MAP	7	5	7	5	7	4	7	4

[a] weeks

preliminary identification to ascertain the proportion of *Acinetobacter*, *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* species present.

The preliminary identification of the meat spoilage bacteria from both non-selective and selective media assigned the bacteria to the following groups: *Acinetobacter*, *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas*. Bacteria were characterised depending on the selective media on which they grew, Gram reaction, oxidase and catalase reactions and oxidation or fermentation of glucose.

The gaseous atmosphere within the package was determined at the time of packaging and prior to opening at spoilage. The percentage of each gas was calculated by comparing the peak height with that of the control gas.

3.3 RESULTS

3.3.1 Shelf-life duration in air and modified atmosphere

Visible deterioration was reached by all meat types under each atmosphere and temperature combination within a period of one to seven weeks (Table 3.1). All meats packaged in air visibly deteriorated by week four at one and 10°C. Air-packaged lamb and pork stored at one or 10°C were deemed spoiled at 2.5 and one weeks respectively, while air-packaged beef and chicken stored at one or 10°C were considered unacceptable at four and 1.5 weeks respectively. Under modified atmosphere, the time taken to show visible deterioration ranged between four and seven weeks. At 10°C, lamb and pork showed visible deterioration at four weeks, which was one week prior to beef and chicken spoilage. All meat types were considered visually unacceptable at week seven at 1°C.

3.3.2 Gaseous atmosphere changes during storage

The gas chromatograms produced for beef are shown in Figure 3.1 and provide an example of the traces produced for all meat types. At packaging, the level of carbon dioxide within the air-packaged samples was barely detectable at 1.62%. The levels of oxygen and nitrogen were close to atmospheric concentrations at 19.7 and 74.2% respectively. In MAP, oxygen was 0.2%, carbon dioxide was 30.7% and nitrogen 70.0% at the time of packaging. At spoilage, there were undefined peaks which occurred immediately after the carbon dioxide peak in both the air and MAP meats. These undefined peaks were included with nitrogen as the remaining gases. The putrid odours observed on opening of the packages would contribute to the undefined peaks.

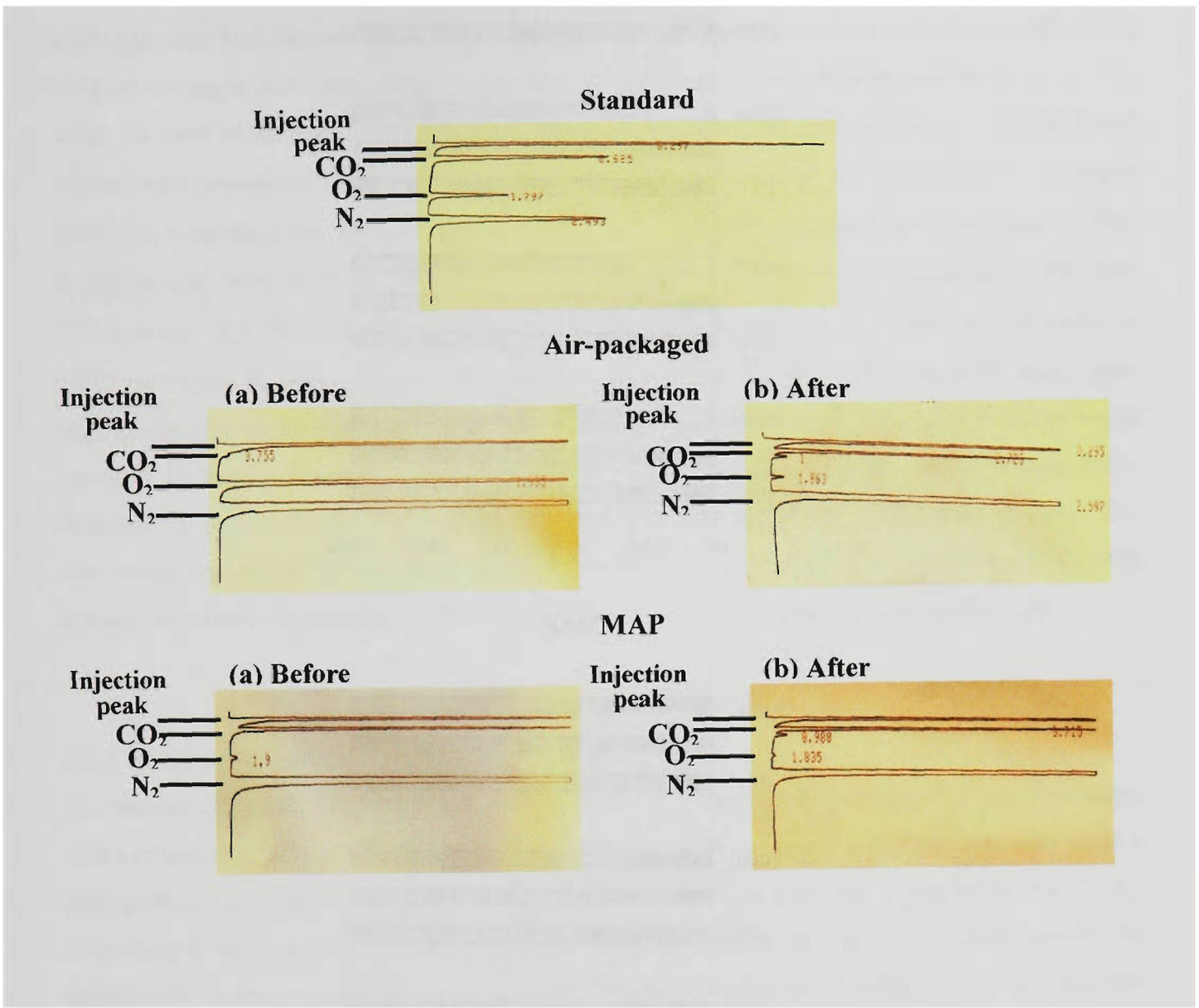


Figure 3.1 Examples of the gas chromatogram traces for the standard gas (48.7% CO_2/ 10.4% O_2/ 40.9% N_2) and air-packaged and MAP beef before and after visible deterioration.

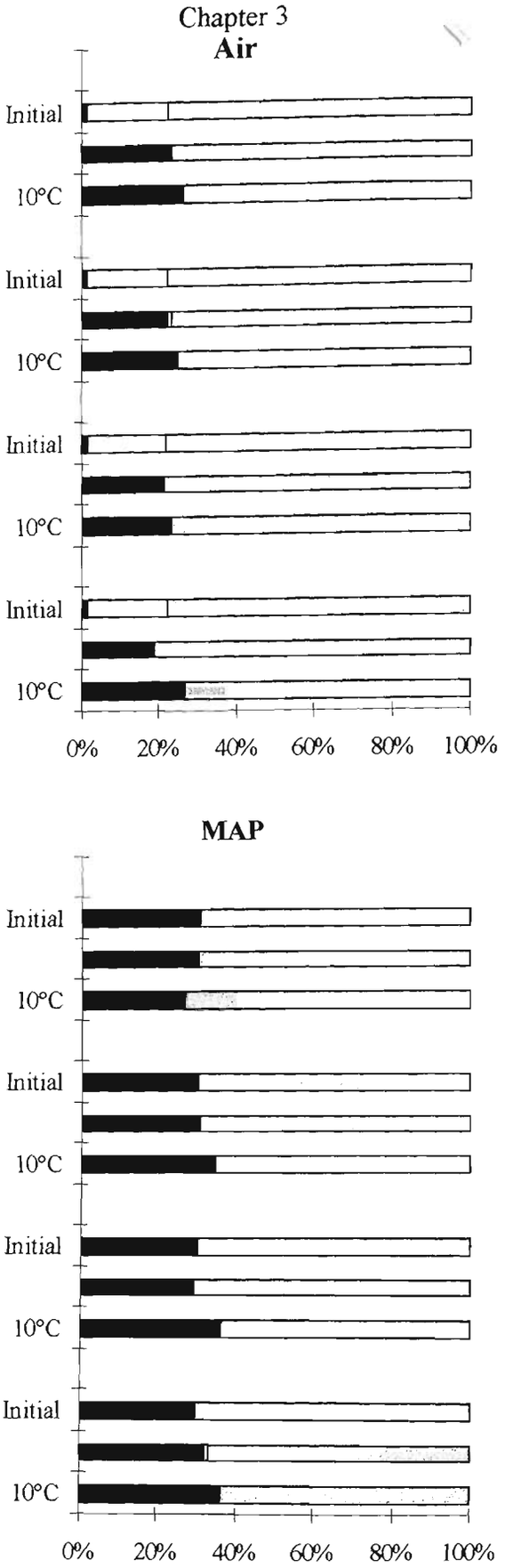


Figure 3.2 The atmospheric concentrations (%) of CO_2 (■), O_2(□) and N_2 plus other undefined gases (▣), within air-packaged and MAP beef, chicken, lamb and pork stored at either one or 10°C at spoilage

The changes in gas atmosphere composition within the packages during storage are shown in Figure 3.2 for the four meat types, when packaged initially and after storage at one or 10°C. When the air-packaged meat had become visually unacceptable, the gas analysis revealed that there had been a reversal in oxygen and carbon dioxide percentages. The level of oxygen decreased from 19 to <1% while the level of carbon dioxide increased from one to 20%, with nitrogen and the undefined gas comprising the balance (Figure 3.2). Interestingly for air-packaged meat, at 1°C the amount of oxygen remaining at spoilage was always higher (average 0.37%) than after storage at 10°C (average 0.20%). In addition, the level of carbon dioxide in the air packages was higher at 10°C (average 23.0%) than 1°C (average 19.85%), with the reverse occurring for the remaining gases. The remaining gases at 10°C were lower proportionally (average 68.80%) than at 1°C (74.05%). For the MAP meats there were limited alterations in the gas composition during the storage period. At 1°C, carbon dioxide levels increased by one to 3%, with nitrogen decreasing by one to 2%. At 10°C, carbon dioxide levels increased by three to 7.6%, with nitrogen and remaining gases decreasing by the same amount. The only exception was for beef at 10°C, where the carbon dioxide concentration decreased by 6% with nitrogen and remaining gases increasing by 6%. The oxygen levels remained unchanged at <1%.

3.3.3 Isolation of bacteria on non-selective media

The number of bacterial isolates selected on the basis of different colony morphology for preliminary characterisation from non-selective media are listed in Table 3.2. The numbers of individual isolates selected from those isolated from air-packaged beef, chicken, lamb and pork ranged from 13 to 22 for air-packaged meat and 11 to 20 for MAP meat. The prevalence of each individual colony type on the non-selective media was determined in order to calculate the proportion of presumptive *Acinetobacter*, *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* in the total viable population. Each isolate exhibited characteristics which were typical of either *Acinetobacter*, *Brochothrix*, *Enterobacteriaceae*, LAB or *Pseudomonas*. Lamb was the only air-packaged meat where *Acinetobacter* species were detected.

3.3.3.1 Isolation of bacteria on non-selective media: air packaged meat

For the air-packaged meats stored at 1°C, the dominant bacterial group detected after storage was *Pseudomonas* for all meat types (Figure 3.3). On the beef, the remainder of the population was composed of LAB which comprised 16.8% of the population. The remaining population on the chicken included LAB (19.3%) and *Enterobacteriaceae* (7.3 %). Of the four meat types stored at 1°C, lamb had the most complex bacterial population, where species of *Pseudomonas* made up 54.8%,

Table 3.2 The number of morphologically distinct colonies chosen from non-selective media for preliminary characterisation from air-packaged or MAP beef, chicken, lamb and pork after storage at either one or 10°C.

Packaging	Meat type	Storage temperature	
		1°C	10°C
Air			
	Beef	13	16
	Chicken	22	20
	Lamb	21	15
	Pork	21	14
MAP			
	Beef	14	14
	Chicken	20	14
	Lamb	11	14
	Pork	14	12

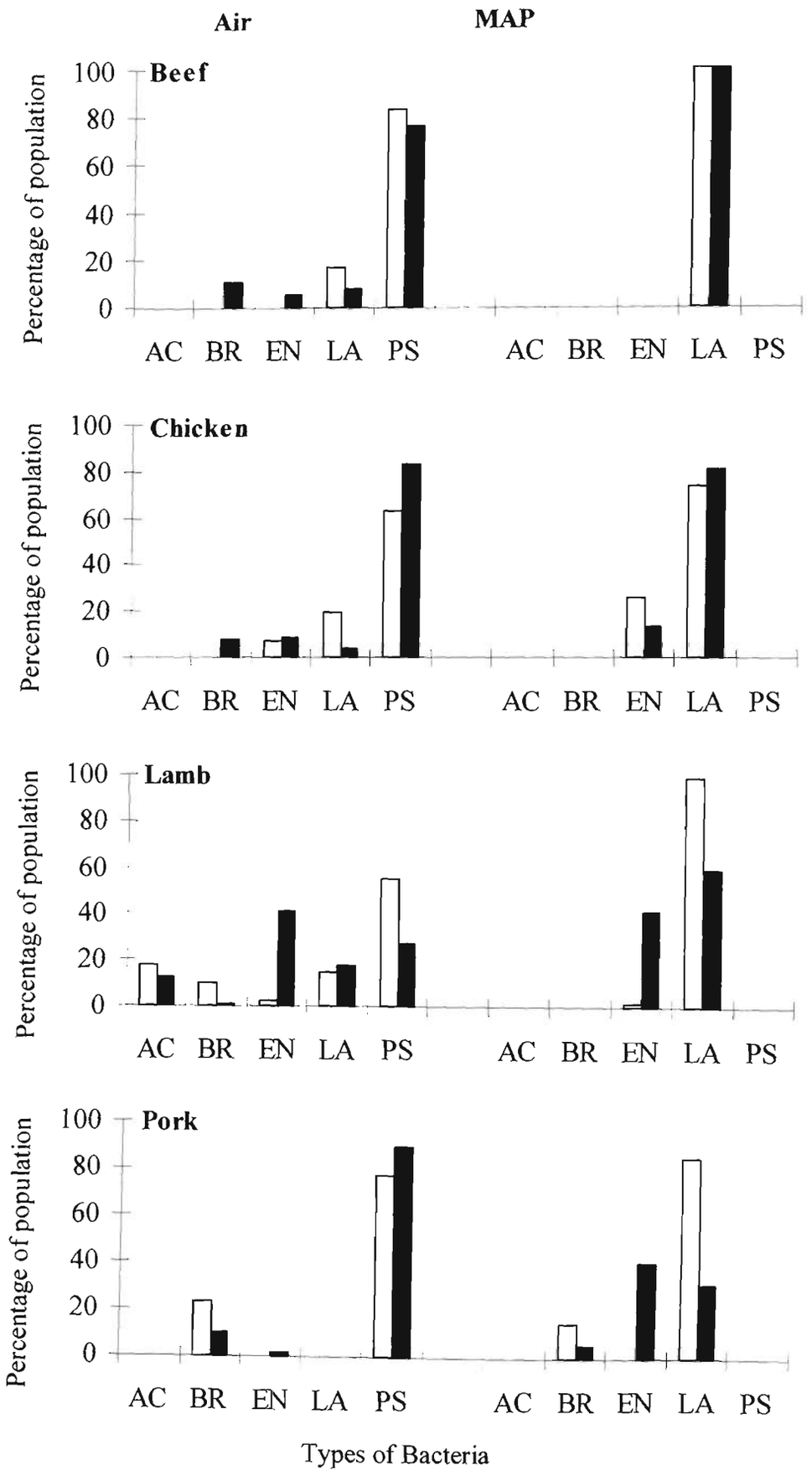


Figure 3.3 Enumeration of *Acinetobacter* (AC), *Brochothrix* (BR), *Enterobacteriaceae* (EN), LAB (LA) and *Pseudomonas* (PS) species at spoilage after storage at one (□) and 10°C (■) using non-selective media.

Acinetobacter 17.6%, LAB 14.4%, *Brochothrix* 9.8% and *Enterobacteriaceae* 2.6%. *Brochothrix* species comprised the remaining 22.9% of the population on the pork.

At 10°C, *Pseudomonas* dominated the spoilage population on the four meat types (Figure 3.3). Lamb was the only exception, where *Enterobacteriaceae* numbers were higher (40.9%) followed by *Pseudomonas* (27.8%), LAB (18.1%), *Acinetobacter* (12.7%) and *Brochothrix* (0.9%) species. The final populations of beef and chicken were similar: *Pseudomonas* species dominated but there were small numbers of *Brochothrix*, *Enterobacteriaceae* and LAB species present. On beef, the final numbers of *Brochothrix*, *Enterobacteriaceae* and LAB were 10.5, 5.2 and 7.6% respectively. On chicken, *Brochothrix*, *Enterobacteriaceae* and LAB were 7.4, 8.9 and 4.2% of the final population respectively. The simplest population combination occurred on pork, which had only three spoilage groups: *Pseudomonas* 88.9%, *Brochothrix* 9.7% and *Enterobacteriaceae* 1.6%.

3.3.3.2 Isolation of bacteria on non-selective media: modified atmosphere packaged meat

On MAP meat stored at 1°C, the dominant bacterial group on all meat types was LAB (Figure 3.3). LAB were the only bacterial group present on the beef. On chicken and lamb after storage, the remaining proportion of the bacterial population was composed of members of the *Enterobacteriaceae* family, which were present at 13.3% on chicken and 1.3% on lamb. On pork, the remaining section of the population was composed of *Brochothrix* species (5.5%).

At 10°C, the LAB group was also the only group present on the beef. For chicken and lamb stored at 10°C, the LAB group were again dominant, while on pork *Enterobacteriaceae* was the major bacterial group. Of the remainder of the population on pork, 31.4% were LAB and 5.5% were *Brochothrix*. *Enterobacteriaceae* species were also an important bacterial group on the chicken and lamb, where they made up 25.5 and 41.3% of the final population respectively. *Brochothrix* were not detected.

3.3.4 Comparison of total numbers of viable bacteria detected following storage in air-packages and MAP using non-selective and selective media

The total counts recorded for meat packaged under modified atmosphere conditions were lower than the air-packaged meat samples at the time of spoilage (Figure 3.4). Total counts for air-packaged meat were in the range of 10^7-10^8 cfu/cm^2 and 10^5-10^7 cfu/cm^2 for MAP meat. For air-packaged and MAP meat, initial counts of the bacterial groups showed that, prior to storage, the bacterial load on the meat was $\leq 1.8 \times 10^5$ cfu/cm^2. *Brochothrix* and *Pseudomonas* numbers were lower on the MAP meats

Table 3.3 The number of morphologically distinct colonies of *Brochothrix* (*Brocho.*), *Enterobacteriaceae* (*Entero.*), LAB and *Pseudomonas* (*Pseudo.*) chosen from selective media for preliminary characterisation from air-packaged or MAP beef, chicken, lamb and pork after storage at either one or 10°C.

Packaging	**Meat type**	**Temp (°C)**	**Storage time (weeks)**	***Brocho.***	***Entero.***	**LAB**	***Pseudo.***
Air							
	Beef	1	4	5	6	3	4
		10	1.5	3	5	4	6
	Chicken	1	4	6	5	5	6
		10	1.5	4	2	4	9
	Lamb	1	2.5	5	4	4	6
		10	1	5	2	6	6
	Pork	1	2.5	2	6	7	6
		10	1	5	2	6	6
MAP							
	Beef	1	7	4	3	4	3
		10	5	4	4	4	4
	Chicken	1	7	5	5	4	5
		10	7	4	3	4	5
	Lamb	1	4	5	3	4	8
		10	7	4	3	5	4
	Pork	1	4	5	0	4	7
		10	7	4	5	4	7

compared to air packaged meats at spoilage. *Enterobacteriaceae* were generally lower on MAP meats and in some cases members of this group were not detected. LAB numbers were generally the same under each packaging condition (Figure 3.4). *Acinetobacter* species were not enumerated on selective media because no selective media were available for this genus.

3.3.5 Isolation of bacteria on selective media

From the microflora of air-packaged meats stored at either one or 10°C, different colony types from the selective media were chosen for preliminary characterisation (Table 3.3). Between two and nine different morphologies were found on each different media. The number of distinct morphology types from the microflora of MAP beef, chicken, lamb and pork, after storage at either one or 10°C, chosen from the non-selective media are listed in Table 3.3. No *Enterobacteriaceae* were isolated from pork at 1°C, but between three and eight different colony morphologies were otherwise observed on the selective media following MAP. The prevalence of each colony type was determined for later use. If the characteristics of the bacteria from the selective media were not typical of the isolate expected (one of *Brochothrix*, *Enterobacteriaceae*, LAB or *Pseudomonas*), they were not further pursued and excluded from the colony count.

3.3.5.1 Isolation of bacteria on selective media: air-packaged meats

For all the meats packaged in air and stored at 1°C, *Pseudomonas* species were present in the largest numbers after storage (Figure 3.4). The decreasing order of the remaining bacterial groups on beef, lamb and pork were *Brochothrix*, LAB and *Enterobacteriaceae*, while chicken had larger numbers of LAB than either *Brochothrix* or *Enterobacteriaceae*.

Beef and pork stored at 10°C in air had a larger number of *Pseudomonas* than any other spoilage group, where these were present at 10^8 cfu/cm^2. The remaining bacterial groups on beef, *Brochothrix*, *Enterobacteriaceae* and LAB, were present in similar numbers, at 10^7 cfu/cm^2. On pork stored at 10°C, the numbers of LAB and *Brochothrix* were similar, both at 10^7 cfu/cm^2, with *Enterobacteriaceae* at 10^6 cfu/cm^2. However, on chicken and lamb no single bacterial group dominated. On chicken, the numbers of *Pseudomonas*, *Brochothrix* and LAB detected were 10^7-10^8 cfu/cm^2. On lamb, 10^7 cfu/cm^2 of LAB and *Pseudomonas* were present, with 10^6 cfu/cm^2 *Brochothrix*.

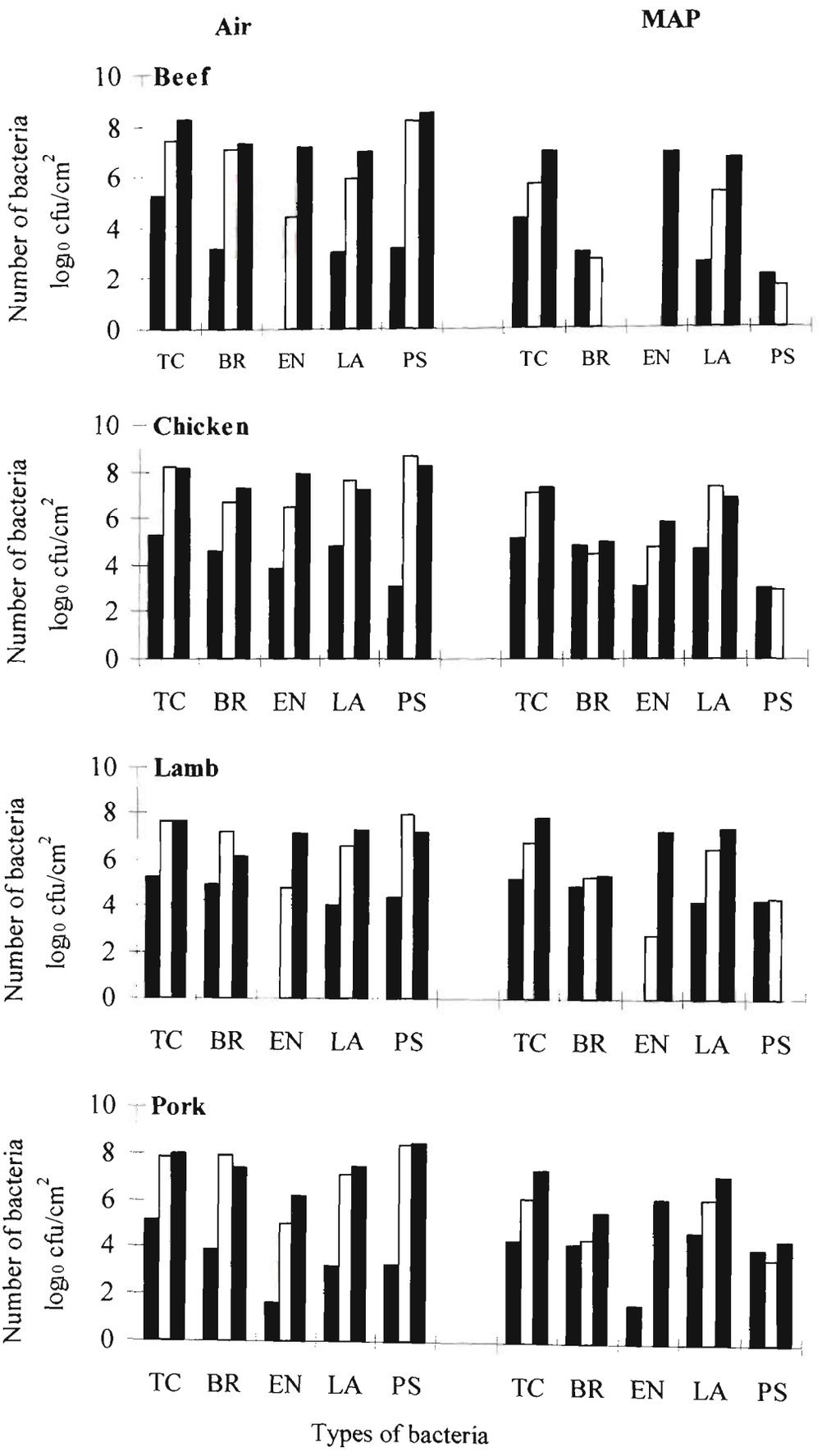


Figure 3.4 Enumeration of total count (TC), *Brochothrix* (BR), *Enterobacteriaceae* (EN), LAB (LA) and *Pseudomonas* (PS) species initially (■) and at spoilage after storage at one (□) and 10°C (■) using selective media for *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas*.

3.3.5.2 Enumeration of bacteria on selective media: modified atmosphere packaged meats

For MAP meat stored at both 1 and 10°C, *Pseudomonas* species failed to make a significant contribution to the final population, as their numbers either remained unchanged (1°C) or decreased (10°C) from initial levels (Figure 3.4). Under modified atmosphere at 10°C, pork was the only meat type to have detectable numbers of *Pseudomonas* species, which were present at 10^4 cfu/cm^2. The level of oxygen within MAP pork was not different to the other 10°C MAP meats. A similar situation was observed for *Brochothrix*, where their numbers remained largely at initial numbers on all meats at both temperatures, with the notable exceptions of pork stored at 10°C, where numbers increased by 10-fold, and beef, where no *Brochothrix* were isolated.

The growth of *Enterobacteriaceae* on the meats varied between meat types. At 1°C, no *Enterobacteriaceae* were detected on beef and pork, while on chicken and lamb, their numbers were between 10^2-10^5 cfu/cm^2. At 10°C, *Enterobacteriaceae* numbers were between 10^5 and 10^6 cfu/cm^2 for all meat types. High numbers of LAB, 10^6-10^7 cfu/cm^2, were found on all meats at both temperatures. There were only two meat types where LAB numbers were not substantially greater than any other bacterial group, both of which were stored at 10°C. One was beef where similar numbers of *Enterobacteriaceae* and LAB occurred (10^6 cfu/cm^2). The other was on lamb, where the numbers of LAB and *Enterobacteriaceae* were ~10^7 cfu/cm^2.

3.4 DISCUSSION

3.4.1 Shelf-life in air and under modified atmosphere

No evidence could be found from the initial microbial load or in the literature to explain why the lamb and pork spoiled before beef and chicken. The atmosphere and temperature combined together to influence the shelf-life. Meat stored in air became visually unacceptable faster than the same type of meat under MAP. In addition, meats stored at 10°C visually deteriorated earlier than the same type of meat stored at 1°C. The elevation of carbon dioxide levels in an enclosed package containing fresh meat has been demonstrated to enhance the shelf-life of beef (Clark and Lentz, 1972; Rousset and Renerre, 1991), chicken (Bailey *et al.*, 1979a,b; Cunningham, 1982), lamb (Christopher *et al.*, 1980; Manu-Twiah *et al.*, 1991) and pork (Blickstad and Molin, 1983a; Gill and Harrison; 1989; Holley *et al.*, 1994). The shelf-life can be increased by up to seven-fold through increasing the carbon dioxide content (Enfors *et al.*, 1979), due principally to the significant reduction in bacterial growth rate (Clark and Lentz, 1969; Blickstad and Molin, 1983a; Johnson and Ogrydzaik, 1984; Gill and Penny, 1988) . Temperature is acknowledged as one of the primary factors controlling microbial growth, as reducing

temperature reduces bacterial growth rates therefore increasing shelf-life (Bailey *et al.*, 1979b; Olsen and Nottingham, 1980; McMullen and Stiles, 1991). In this investigation, combining 1°C with 30% carbon dioxide increased the shelf-life of beef, chicken lamb and pork.

The bactericidal and bacteristatic effects of carbon dioxide have been demonstrated to increase with decreasing temperature (Leeson, 1987; Gill, 1988). The data gathered in this investigation supported this observation, as the final number of bacteria under MAP were generally lower at 1°C than at 10°C for all bacterial groups. There was, however, a notable exception. In the present investigation, the *Pseudomonas* species on beef, chicken or lamb decreased to be below detection limits, of the spiral plater, at 10°C, while at 1°C they remained at approximately the same level at the initial numbers. As the effect of carbon dioxide has been shown to decrease at temperature increases (Gill and Tan, 1980; Eklund and Jarmund, 1983; Hintlain and Hotchkiss, 1987; Holley *et al.*, 1994), it would be expected that the number of *Pseudomonas* that were able to survive in the presence of carbon dioxide would actually be higher at 10°C than at 1°C. The atmosphere composition made a significant contribution to the type of bacteria that were able to grow and the final numbers that they reached.

Shelf-life was independent of the final numbers attained when the meat was deemed visually unacceptable (Erichen and Molin, 1981a,b). In this investigation all meat stored under MAP at 10°C were considered to be unacceptable at seven weeks, however, the final number of viable cells at this point were quite different. The final number of viable bacteria on beef was 10-fold less than lamb, pork and chicken. In addition, the initial numbers of bacteria on chicken, lamb and pork were similar. However, when stored in air at the same temperature, the appearance of slime and cloudy exudate on the chicken was delayed compared to the lamb and pork but the bacterial numbers were 10-fold higher. Furthermore, the MAP meats were spoiled also but the final number of bacteria was significantly lower than the air-packaged meats.

3.4.2 Proportion of bacterial groups and final number on air-packaged and MAP meats

The atmospheric composition appeared to dictate the bacterial composition and final numbers of bacteria on meat, since the meat was randomly distributed into air-packages or MAP. *Pseudomonas* species comprised the greatest proportion of the microflora on meats that were packaged in air. There was only one exception, on air-packaged lamb stored at 10°C, where the number of *Enterobacteriaceae* LAB and *Pseudomonas* species were similar. In contrast, the LAB group dominated the microflora on meats under MAP. The dominance of these two bacterial groups in this investigation was in agreement with previous investigations. On aerobically packaged beef (Clark and Lentz, 1972), chicken (Bailey *et*

al., 1979a,b) and pork (Asensio *et al.,* 1988), *Pseudomonas* species were shown to comprise up to 90% of the spoilage population. In contrast, MAP stored meats were found to have LAB species either as the dominant (Bailey *et al.,* 1979b; Greer *et al.,* 1993) or only bacterial group present (Erichsen and Molin, 1981b). The shelf-life of MAP meats appeared to be enhanced by the growth of this bacterial group. LAB grow more slowly than *Pseudomonas* species (Leeson, 1987) therefore they would take longer to reach levels which cause deterioration of meat through the production of off-odours and slime.

There were two results that were unexpected, the presence of *Pseudomonas* on the MAP pork stored at 10°C and the absence of *Brochothrix* on MAP beef also stored at 10°C [See Figure 3.4]. *Pseudomonas* was only found on pork stored under modified atmosphere packaging stored at 10°C. As the numbers of *Pseudomonas* species present at the conclusion of the experiment (4 weeks) were similar to the initial number, it was concluded that the *Pseudomonas* species present were not growing but presumably managed to remain viable on the pork after four weeks of storage. The number of *Brochothrix* only increased on the pork, while on the remaining two meats *Brochothrix* numbers remained at initial levels. *Brochothrix* species could have disappeared from the beef due to the production of bacteriocins by the LAB which dominated the microflora. Indeed, production of antibacterial substances by the LAB, which may have included bacteriocins, was observed in experiments not reported in this thesis. The effects of bacteriocins are discussed in greater detail later in this chapter.

Not only are the dominant organisms different for the two different atmospheres, but the final numbers on the air-packaged meats were consistently higher than those on MAP meats and the population on air-packaged meats was more diverse. Spoilage is considered to occur for air-packaged meat at 10^8 cfu/cm^2, but on MAP meat it is only 10^7 cfu/cm^2 (Gill, 1986). The principal factor which could be attributed to the observed difference in final bacterial numbers at spoilage between air-packaged and MAP meat appeared to be low oxygen levels. On the air-packaged meats, there was a dramatic change in the concentration of oxygen level, which can be attributed to both the consumption of oxygen by the bacteria and the meat itself. As the concentration of oxygen decreased and that of carbon dioxide increased, the growth rate of *Pseudomonas* species would begin to decrease, as this genera is susceptible the elevated carbon dioxide levels. In contrast, elevated carbon dioxide would assist the facultative anaerobes, *Brochothrix* and *Enterobacteriaceae*, and the microaerophillic *Lactobacillus* species, either by decreasing the competitiveness of *Pseudomonas* or through carbon dioxide-stimulated growth, as in the case of *Lactobacillus* species (Kandler and Weiss, 1986). In addition, *Acinetobacter* species are sensitive to elevated levels of CO_2 (Gill and Tan, 1980), which could account for the absence of this genus under MAP. This would enable more bacterial species to grow thus resulting in a

larger total number of bacteria present on air-packaged meat as compared to MAP meat, where aerobic bacteria are inhibited by carbon dioxide.

A subsidiary contributing factor to the lack of bacterial diversity on MAP meats could be due to the LAB themselves. Some members of the LAB group, particularly *Lactobacillus* and *Carnobacterium* species, have been demonstrated to produce bacteriocins which can inhibit the growth of other spoilage organisms (Garver and Murinana, 1993) and, in the case of *Carnobacterium*, members of the same species can also be inhibited (Schillinger and Holzapfel, 1990). It is also interesting to note that LAB, in this investigation, never increased in numbers above 10^8 cfu/cm^2, regardless of atmosphere or the presence or absence of other bacterial species.

Therefore packaging in a modified atmosphere can decrease the number of bacteria and limit the diversity of bacterial types when isolating bacteria on non-selective media. If selective media are utilised in addition to non-selective media, bacteria which are present on the meat but in significantly lower number can then be detected.

3.3.4 Benefits of using selective media

The utilisation of selective media provided a more diverse picture of the spoilage population in comparison with screening isolates from non-selective plates. Using only non-selective media for the isolation of the microflora would have resulted in the presence of minor groups involved in the spoilage process going undetected. This is particularly obvious when some members of the population are, for example, 100-fold lower in counts than the dominant bacteria. On the MAP meats, LAB and *Enterobacteriaceae* were consistently detected with *Brochothrix* species being detected only once using non-selective media. However, when the selective media was utilised, members of each spoilage population were detected and enumerated. Low numbers in the population does not diminish the significance of the group in the spoilage process because of the possible end-products formed (Dainty and Hibbard, 1979; Gill and Penney, 1988), as they may cause off flavours and odours.

The meat spoilage process is an example of a dynamic ecosystem. Bacteria are growing and competing against each other for nutrients and space. The organisms which are present at spoilage will not necessarily be the ones of importance at the beginning of the process (Dainty and Mackey, 1992). The process of microbial succession has never been fully investigated in meat. Traditional microbiological techniques are inadequate for the detection of all microorganisms present. There is extensive evidence that standard microbiological techniques fail to culture all the organisms present (Head et al., 1998), the

viable non- culturable bacteria. Food microbiology severely lags behind other areas of microbiology (Boddy and Wimpenny, 1992; Fleet, 1999) where the short-comings of traditional techniques have been addressed using molecular biology methods which do not rely on the ability to cultivate microorganisms. An example of an approach which could be utilized in the study of microbial succession in meat spoilage is denaturing gradient gel electrophoresis (DGGE). Using 16S rRNA universal and eubacterial primers with GC clamps the diversity and abundance bacteria of each time interval chosen can be determined

3.5 CONCLUSION

The visual shelf-lives of four meat types, beef, chick, lamb and pork, were extended when low temperature and elevated carbon dioxide were combined. At the lowest temperature, 1°C, under 30% CO_2/70% N_2, the shelf-life was up to seven weeks after packaging, which was three weeks longer than any of the air-packaged meats and the MAP meat stored at 10°C. The composition of the atmosphere and temperature combined together to influence the type of bacteria that were present on the meat surface. In the presence of air, *Pseudomonas* species were generally the dominant bacterial group, with each of the other spoilage groups present in the range of 10^6-10^7 cfu/cm^2. *Acinetobacter* was only detected on lamb which had been packaged in air. Under MAP, LAB species became the dominant organisms with generally only *Enterobacteriaceae* species reaching numbers within 100-fold lower than the maximum number of LAB. There were other bacterial groups present but they were significantly less in number than LAB and they were only detected with selective agar. In order to understand which bacteria were the most significant in the meat spoilage process in air and under MAP, it is important to be able to identify the bacteria and characterize the environmental conditions under which the bacteria do and do not survive.

The information contained within this chapter confirms work that has been published previously, however, it was essential that it was included within the thesis as it provided the basis of all the work that is presented in the remained of this thesis. What is novel about the work is that it takes four different meat types from the same source and examines the spoilage distribution of spoilage bacteria which develops during spoilage. Each meat began with similar numbers of bacteria but the spoilage microflora that developed was not identical across the four meat types.

Chapter 4

Biochemical identification and pulsed-field gel electrophoresis characterisation of a selection of meat spoilage bacteria

4.1 INTRODUCTION

The identification of the bacterial species involved in the meat spoilage process would enhance the understanding of the process and contribute to developing methods for suppressing microbial growth. Bacterial species can be differentiated on the basis of cell wall components (Bousfield *et al.*, 1985), the ability to utilise substrates (Shaw and Harding, 1984; Shaw and Latty, 1984) or on the basis of end-product formation (Garvie, 1967). Information about the relatedness of bacterial strains can be obtained from fatty acid analysis (Akagawa and Yasamoto, 1989; Wallbanks *et al.*, 1990; Brondz and Olson, 1991), and protein profiling (Jackman, 1985; Busse *et al.*, 1996). All of these methods give information about the phenotype, i.e. the expression of the genome. These results can vary depending on the nutrients in the medium (Samelis *et al.*, 1994). There are occasions where these phenotypic methods are unable to provide information on the species or the relatedness of bacteria.

The genome can provide vital information detailing the relationship between bacterial species and strains. PFGE is a highly sensitive and reproducible technique (Johnson *et al.*, 1995; Matushek *et al.*, 1996) which has provided information on the relatedness of bacterial species and strains based

Table 4.1 List of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* isolates identified to species level indicating source in terms of meat type (beef or lamb), storage temperature (one or 10°C) and atmosphere composition (air or modified atmosphere (MA)).

Code	Isolation conditions			
	Spoilage group	Meat Type	Temp. (°C)	Atmosphere
A23B03	*Brochothrix*	Lamb	10	Air
A23B06	*Brochothrix*	Lamb	10	Air
A13B02	*Brochothrix*	Lamb	1	Air
A13B03	*Brochothrix*	Lamb	1	Air
M23B03	*Brochothrix*	Lamb	10	MA
M13B02	*Brochothrix*	Lamb	1	MA
M13B03	*Brochothrix*	Lamb	1	MA
A21B01	*Brochothrix*	Beef	10	Air
A21B02	*Brochothrix*	Beef	10	Air
A11B02	*Brochothrix*	Beef	1	Air
A11B04	*Brochothrix*	Beef	1	Air
M11B01	*Brochothrix*	Beef	1	MA
M11B04	*Brochothrix*	Beef	1	MA
A23En01	Enterobacteriaceae	Lamb	10	Air
A23En02	Enterobacteriaceae	Lamb	10	Air
A13En02	Enterobacteriaceae	Lamb	1	Air
A13En03	Enterobacteriaceae	Lamb	1	Air
M23En02	Enterobacteriaceae	Lamb	10	MA
M23EN03	Enterobacteriaceae	Lamb	10	MA
M13En01	Enterobacteriaceae	Lamb	1	MA
M13En02	Enterobacteriaceae	Lamb	1	MA
A21En08	Enterobacteriaceae	Beef	10	Air
A11En01	Enterobacteriaceae	Beef	1	Air
A11En02	Enterobacteriaceae	Beef	1	Air
A11En03	Enterobacteriaceae	Beef	1	Air
A11En04	Enterobacteriaceae	Beef	1	Air
M21En01	Enterobacteriaceae	Beef	10	MA
M21En02	Enterobacteriaceae	Beef	10	MA
A23L01	LAB	Lamb	10	Air
A23L03	LAB	Lamb	10	Air
A13L02	LAB	Lamb	1	Air
A13L03	LAB	Lamb	1	Air
M23L06	LAB	Lamb	10	MA
M23L07	LAB	Lamb	10	MA
M23L09	LAB	Lamb	10	MA
M13L02	LAB	Lamb	1	MA
M13L03	LAB	Lamb	1	MA
A21L01	LAB	Beef	10	Air
A21L03	LAB	Beef	10	Air
A11L01	LAB	Beef	1	Air
A11L02	LAB	Beef	1	Air
A11L03	LAB	Beef	1	Air
M21L01	LAB	Beef	10	MA
M21L03	LAB	Beef	10	MA
M11L03	LAB	Beef	1	MA
M11L04	LAB	Beef	1	MA
A23P01	*Pseudomonas*	Lamb	10	Air
A23P06	*Pseudomonas*	Lamb	10	Air
A13P03	*Pseudomonas*	Lamb	1	Air
A13P04	*Pseudomonas*	Lamb	1	Air
A21P03	*Pseudomonas*	Beef	10	Air
A21P04	*Pseudomonas*	Beef	10	Air
A11P03	*Pseudomonas*	Beef	1	Air
A11P04	*Pseudomonas*	Beef	1	Air

on the genome (Taylor *et al.*, 1992; Roy *et al.*, 1996; Lortal *et al.*, 1997). Unique fingerprints are produced by enzymatic degradation of chromosomal DNA into fragments which are then size fractionated through an agarose matrix by a transversing, alternating electrical field at different pulse times (Cantor *et al.*, 1988; Mathew *et al.*, 1988a,b,c; Tenover *et al.*, 1995). In addition, PFGE is a valuable epidemiological technique (Anderson *et al.*, 1991; Haertl and Bandlow, 1993; Lück *et al.*, 1995) and can also be used to determine the genome size of an organism (McClellend *et al.*, 1987; Tanskanen *et al.*, 1990; Chevallier *et al.*, 1994; Roy *et al.*, 1996).

The first aim of these series of experiments was to determine the dominant species of the bacteria of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* which had been isolated from spoiled air-packaged and MAP beef and lamb after storage at either one or 10°C. The second aim was to define the conditions required to produce PFGE fingerprints for *Brochothrix*, *Lactobacillus* and *Carnobacterium*. From these fingerprints, the degree of genetic variability among the strains could be determined then correlated with the identification results.

4.2 METHODS

4.2.1 Identification of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* isolates

The focus of the microflora investigation was narrowed from four meat types (beef, chicken, lamb and pork), to two: beef and lamb, following the commencement of funding by the Meat Research Corporation of Australia to support these PhD studies. From *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas*, two isolates which were judged to be the most numerous at spoilage were selected for each meat type, storage temperature and atmosphere combination for identification. The meat, storage temperature and atmosphere origins of each isolate examined are listed in Table 4.1. The additional *Brochothrix* isolates that were also isolated from beef and lamb that were examined are listed in Table 4.2, together with meat, temperature and atmosphere origins.

Identification of the genus *Brochothrix* was conducted according to the method of Talon *et al.* (1988). Further biochemical tests were conducted to further differentiate the species. These tests included: growth at four, 30, 37 and 45°C, terminal pH, decarboxylation of arginine, lysine and ornithine, growth at pH 3.9, slime production from glucose, presence of urease and SDS-PAGE. SDS-PAGE was

Table 4.2 List of additional *Brochothrix* isolates identified to species level indicating source in terms of meat type (beef or lamb), storage temperature (one or 10°C), atmosphere composition (air or modified atmosphere (MA)) and PFGE code numbers.

Code	Isolation conditions			
	Spoilage group	Meat Type	Temp. (°C)	Atmosphere
A23B01	*Brochothrix*	Lamb	10	Air
A23B02	*Brochothrix*	Lamb	10	Air
A23B04	*Brochothrix*	Lamb	10	Air
A23B05	*Brochothrix*	Lamb	10	Air
A13B05	*Brochothrix*	Lamb	1	Air
M23B01	*Brochothrix*	Lamb	10	MA
M23B02	*Brochothrix*	Lamb	10	MA
M13B01	*Brochothrix*	Lamb	1	MA
M13B04	*Brochothrix*	Lamb	1	MA
M13B05	*Brochothrix*	Lamb	1	MA
A21B03	*Brochothrix*	Beef	10	Air
A11B01	*Brochothrix*	Beef	1	Air
A11B03	*Brochothrix*	Beef	1	Air
A11B05	*Brochothrix*	Beef	1	Air
M11B02	*Brochothrix*	Beef	1	Air
M11B03	*Brochothrix*	Beef	1	Air

Table 4.3 Each restriction enzyme used on *Brochothrix*, *Carnobacterium* and *Lactobacillus* species for PFGE fingerprinting is listed together with the recognition site and incubation temperature (A= adenine, C= cytosine, G= guanidine, T= thiamine, Pu= purine, Py= pyridine).

Enzyme type	Enzyme	Recognition site	Incubation temp. (°C)
G+C rich			
	*Hae*II	PuGCGC/Py	37
	*Hae*III	GG/CC	37
	*Sma*I	CCC/GGG	25
	*Not*I	GC/GGCCGC	37
	*Sfi*I	GGCCNNNN/NGGCC	50
CTAG			
	*Nhe*I	G/CTAGC	37
	*Spe*I	A/CTAGT	37
	*Xba*I	T/CTAGT	37
A+T rich			
	*Drd*I	TTT/AAA	37
Miscellaneous			
	*Eco*RI	G/AATTC	37
	*Hind*III	A/AGCTT	37
	*Pst*I	CTGCA/G	37
20 base cutter			
	I-*Ceu*I	TAACTATAACGGTCCTAA/GGTAGCGA	37

performed on the first 13 *Brochothrix* isolates to examine the protein profile of whole cells. A 12.5% acrylamide gel was used to separate the proteins. The gel was stained with Coomassie blue.

Identification of the family *Enterobacteriaceae* was conducted using two commercially available kits. The first was the GN Biolog (Biolog Inc., Haywood, CA.) and the second 20E API (bioMérieux, Lyon, France). Kits were used according to manufacturer's instructions.

The group LAB were identified to species level according to the method of Montel *et al.* (1991). *Lactobacillus* and *Carnobacterium* isolates were differentiated according to the presence or absence of *meso*-DAP, mode of fermentation (homofermentative or heterofermentative), production of D-, L- or DL-lactic acid, arginine hydrolysis, growth on acetate agar and the fermentation of inulin, melibiose or mannitol.

The *Pseudomonas* isolates were identified to species level using the method of Craven and McAuley (1992). *Pseudomonas* isolates were differentiated on their ability to hydrolyse gelatin, reduce nitrate, produce a fluorescent pigment and produce slime from sucrose.

4.2.2 PFGE fingerprinting of *Brochothrix* and LAB

DNA was prepared for PFGE by growing 30mL of culture for 16 hours in TSB at 25°C for *Brochothrix* and TYGB at 30°C for *Carnobacterium* and *Lactobacillus*, with glycine added to the culture three hours before the end of incubation. Cells were washed, suspended in agarose and allowed to set in moulds. Cells were lysed in EC-lysis solution overnight and cell debris was removed by washing in ESP for 24 to 48 hours. Finally, the agarose blocks were washed twice in TE-PMSF in isopropanol for 1.5 hours then TE buffer before being stored at 4°C in TE buffer.

A selection of restriction enzyme with recognition sites of four to 20 bases were used in order to achieve the desired number of fragments, between 15-30 fragments. The enzymes evaluated, recognition sites and incubation temperatures used are listed in Table 4.3. Enzyme digests were conducted at the appropriate temperature for 16 hours. The digestion mix was removed from the agarose blocks by washing in TE buffer prior to electrophoresis.

Table 4.4 Identification *Brochothrix* isolates from beef and lamb according to the ability to grow in the presence of eight and 10% sodium chloride, reduction potassium tellurite, hydrolysis of hippurate and fermentation of rhamnose. *B. thermosphacta* ATCC11509 (ATCC) was used as the positive control.

Isolate	Sodium chloride		Potassium tellurite	Hippurate	Rhamnose	Species
	8%	10%				
ATCC	+	+	+	-	-	*B. thermosphacta*
A23B03	+	+	+	-	-	*B. thermosphacta*
A23B04	+	+	+	-	-	*B. thermosphacta*
A13B02	+	+	+	-	-	*B. thermosphacta*
A13B03	+	+	+	-	-	*B. thermosphacta*
M23B03	+	+	+	-	-	*B. thermosphacta*
M13B02	+	+	+	-	-	*B. thermosphacta*
M13B03	+	+	-	-	-	*B. thermosphacta*
A21B01	+	+	+	-	-	*B. thermosphacta*
A21B02	+	+	+	-	-	*B. thermosphacta*
A11B02	+	+	+	-	-	*B. thermosphacta*
A11B04	+	+	+	-	-	*B. thermosphacta*
M11B02	+	+	+	-	-	*B. thermosphacta*
M11B04	+	+	+	-	-	*B. thermosphacta*

PFGE was conducted using 1.2% agarose in 0.5x TBE, with 1X TBE as the running buffer. The gel was run in a Clamped Homogenous Electric Field (CHEF) apparatus with a hex electrode. Pulse times varied from one to 35 seconds over a 20 to 24 hour period at 170mv with 10mA field strength at 12°C. Molecular weight markers were run on each gel. All gels were stained with ethidium bromide prior to photographing.

The genome size of each *Brochothrix* and *Carnobacterium* isolate was determined by measuring the distance each band had moved from the origin in relation to the molecular weight markers. The dendrogram of the relatedness of the *Brochothix* species was performed with NTSYS.

4.3 RESULTS

4.3.1 BIOCHEMICAL IDENTIFICATION

4.3.1.1 The identification of *Brochothrix* isolates

The method for the identification of *Brochothrix* by Talon *et al.* (1988) required some alterations in order to achieve an adequate result. APT broth was used by Talon *et al.* (1988) for determination of growth in the presence of eight and 10% sodium chloride. However, eight and 10% sodium chloride in APT formed a sediment which resembled a turbid culture although to media had not been inoculated. Instead, TSB was substituted for APT as no sediment was formed at these sodium chloride concentrations and the reference strain, *B. thermosphacta* ATCC11509, was able to grow in the presence of eight and 10% sodium chloride in TSB. According to Talon *et al.* (1988), *B. thermosphacta* should grow on the 0.05% potassium tellurite in two days, however, at this concentration it took up to two weeks for any colony formation and those colonies that formed were small including *B. thermosphacta* ATCC11509. Other authors used ≤0.02% potassium tellurite (Skovgaard, 1985) for the identification of *Brochothrix* species, therefore, it was decided to halve the amount of potassium tellurite used: this still required seven days incubation for colony formation. The bacteria were able to grow on the control plates unhindered, therefore the base medium not was the problem.

Brochothrix isolates chosen for the initial identification were all identified as *B. thermosphacta* (Table 4.4) as they were: positive for growth in eight and 10% sodium chloride and potassium tellurite reduction and negative for hippurate hydrolysis and rhamnose fermentation. The only exception was M13B03, which was negative for growth on potassium tellurite. M13B03 was still

Table 4.5 Identification of additional *Brochothrix* isolates from beef and lamb according to the ability to grow in the presence of eight and 10% sodium chloride, reduction potassium tellurite, hydrolysis of hippurate and fermentation of rhamnose.

Isolate	Sodium chloride		Potassium tellurite	Hippurate	Rhamnose	Species
	8%	10%				
A23B01	+	+	+	-	+	*B. thermosphacta*
A23B02	+	+	+	-	-	*B. thermosphacta*
A23B04	+	+	+	-	-	*B. thermosphacta*
A23B05	+	+	+	-	-	*B. thermosphacta*
A13B05	+	+	+	-	-	*B. thermosphacta*
M23B01	+	+	+	-	+	*B. thermosphacta*
M23B02	+	+	+	-	-	*B. thermosphacta*
M13B01	+	+	+	-	-	*B. thermosphacta*
M13B04	+	+	+	-	+	*B. thermosphacta*
M13B05	+	+	+	-	-	*B. thermosphacta*
A21B03	+	+	+	-	-	*B. thermosphacta*
A11B01	+	+	+	-	-	*B. thermosphacta*
A11B03	+	+	+	-	+	*B. thermosphacta*
A11B05	+	+	+	-	-	*B. thermosphacta*
M11B02	+	+	+	-	+	*B. thermosphacta*
M11B03	+	+	+	-	-	*B. thermosphacta*

considered to be *B. thermosphacta* because it was positive for growth in eight and 10% sodium chloride and negative for hippurate hydrolysis and rhamnose fermentation.

The additional isolates that were included for the PFGE investigation were also all identified as *B. thermosphacta* (Table 4.5), although five isolates (A23B01, M23B01, M13B04, A11B03 and M11B02) were produced acid from rhamnose. However, the reaction was weak and took over three weeks to occur. These isolates were able to grow in eight and 10% sodium chloride and reduce potassium tellurite but could not hydrolyse hippurate, therefore they were considered to be *B. thermosphacta*.

Following analysis of the PFGE patterns (see Section 4.3.7), additional biochemical tests were performed in an attempt to determine biochemical diversity among the *B. thermosphacta* strains (Table 4.6). All strains grew strongly at four and 30°C and weakly at 35°C, while at 45°C strains: A23B03, A23B06, A13B03, M23B03, M13B02, M13B03, A11B04, M11B01, M11B04, M23B01 and M11B02, failed to grow, while the remainder grew weakly by day seven. All *B. thermosphacta* strains were found to decarboxylate arginine, lysine and ornithine. Following growth in LA-broth for seven days, none of the stains had decreased the pH of the supernatant below 4.1: instead they ranged between pH 4.78 (ATTCC11509) and 5.48 (M11B02). A few strains were able to grow weakly at pH 3.9: A23B06, M23B03, M13B02, A11B04, A23B04, A23B05, A13B05, M23B01, M23B02, M13B01, A21B03, A11B01 and A11B03. None of the isolates produced slime from sucrose nor did any of the strains produce urease.

The protein profiles by SDS-PAGE of the initial thirteen *Brochothrix* isolates are shown in Figure 4.1. All isolates had the same protein profile. *Carnobacterium* M11L03 was used at the negative control and had a protein profile that differed from the *B. thermosphacta* strains. The protein concentrations of the samples were not determined therefore the intensity of the bands cannot be interpreted.

4.3.1.2 The identification of *Enterobacteriaceae* isolates

The results generated by both Biolog and API are listed in Table 4.7, where all Biolog results are for 24 hour incubations. Using both identification kits, all except one isolate was identified to at least the genus level, unfortunately it was not the same isolate for both kits that was not identified. Biolog could

Table 4.6 Additional biochemical tests for the differentiation of *Brochothrix* isolates from lamb and beef. In an attempt to further differentiate these isolates the following series of tests were conducted: ability to grow at four, 30, 35 and 45°C, decarboxylation of arginine (Arg), lysine (Lys) and ornithine (Orn), supernatant pH after seven days growth in LA-broth (final pH), ability to grow at pH 3.9, slime production from sucrose and the presence or urease (Urea).

	Growth temperatures (°C)				Final	Decarboxylation			pH 3.9	Slime	Urea
Isolate	4	30	35	45	pH	Arg	Lys	Orn			
ATCC11509[a]	+	+	+	-	4.778	+	+	+	-	-	-
A23B03	+	+	+	-	5.072	+	+	+	-	-	-
A23B04	+	+	+	+	4.902	+	+	+	+	-	-
A13B02	+	+	+	+	4.971	+	+	+	-	-	-
A13B03	+	+	+	-	4.910	+	+	+	-	-	-
M23B03	+	+	+	-	4.904	+	+	+	+	-	-
M13B02	+	+	+	-	5.190	+	+	+	+	-	-
M13B03	+	+	+	-	5.162	+	+	+	-	-	-
A21B01	+	+	+	+	4.920	+	+	+	-	-	-
A21B02	+	+	+	+	5.074	+	+	+	-	-	-
A11B02	+	+	+	+	5.084	+	+	+	-	-	-
A11B04	+	+	+	-	4.940	+	+	+	+	-	-
M11B02	+	+	+	-	4.980	+	+	+	-	-	-
M11B04	+	+	+	-	5.074	+	+	+	-	-	-
A23B01	+	+	+	+	5.282	+	+	+	-	-	-
A23B02	+	+	+	+	5.301	+	+	+	-	-	-
A23B04	+	+	+	+	4.965	+	+	+	+	-	-
A23B05	+	+	+	+	4.909	+	+	+	+	-	-
A13B05	+	+	+	+	4.905	+	+	+	+	-	-
M23B01	+	+	+	-	5.226	+	+	+	+	-	-
M23B02	+	+	+	+	5.249	+	+	+	+	-	-
M13B01	+	+	+	+	4.967	+	+	+	+	-	-
M13B04	+	+	+	+	4.928	+	+	+	-	-	-
M13B05	+	+	+	+	4.971	+	+	+	-	-	-
A21B03	+	+	+	+	4.924	+	+	+	+	-	-
A11B01	+	+	+	+	4.949	+	+	+	+	-	-
A11B03	+	+	+	+	5.205	+	+	+	+	-	-
A11B05	+	+	+	+	4.996	+	+	+	-	-	-
M11B02	+	+	+	-	5.481	+	+	+	-	-	-
M11B03	+	+	+	+	4.944	+	+	+	-	-	-

[a] *B. thermosphacta* ATCC11509 was used as a positive control

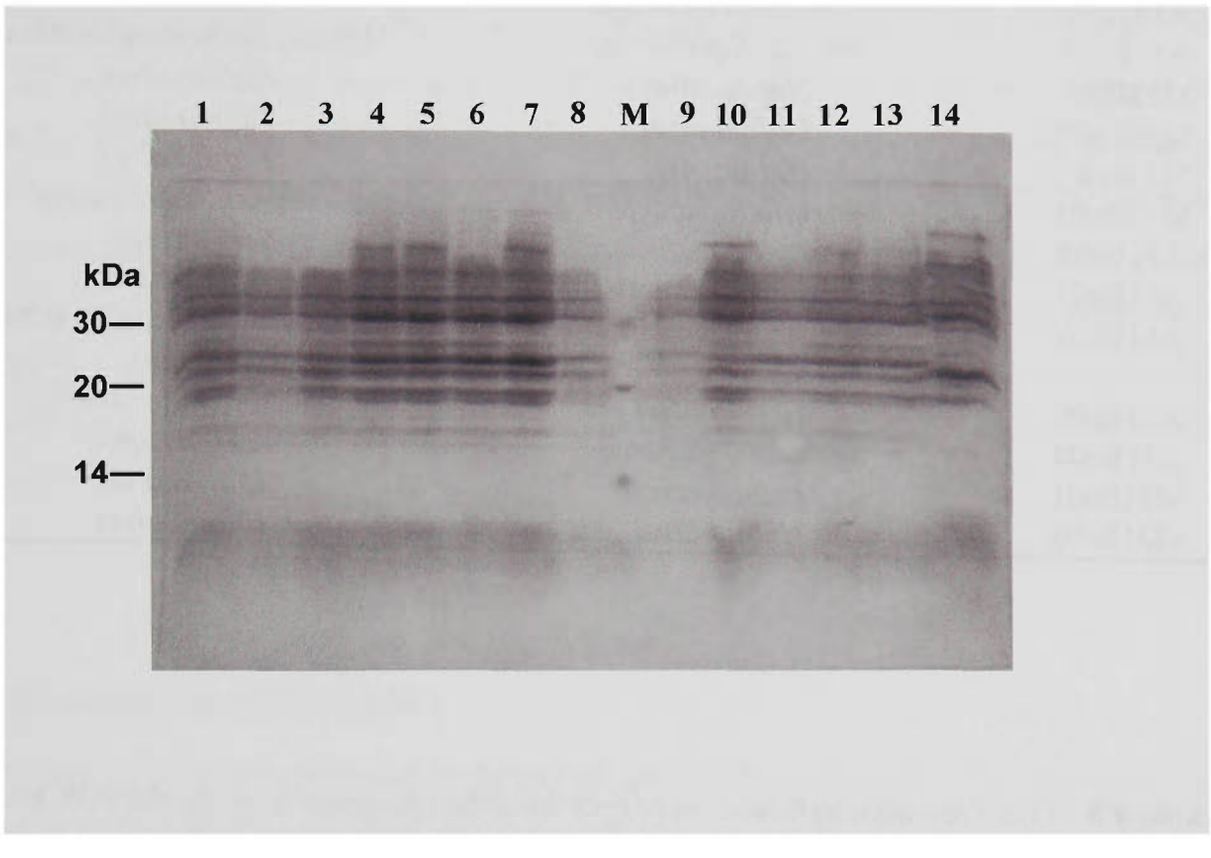


Figure 4.1 Coomassie blue R250 stained SDS-PAGE protein profile of *Brochothrix* isolates A23B01 (1), A23B04 (2), A13B02 (3), A13B03 (4), M23B03 (5), M132B02 (6), M13B03 (7), A21B01 (8), A21B02 (9), A11B02 (10), A11B04 (11), M11B02 (12) and M11B04 (13) with *Carnobacterium* M11L03 (14) as the negative control. A low molecular weight marker (kilo Daltons, kDa) was run in the centre of the gel (M).

Table 4.7 *Enterobacteriaceae* species associated with spoiled beef and lamb as identified with Biolog (Biolog Inc., Haywood, CA, USA) and API (bioMérieux, Lyon, France).

Isolate Id.	Biolog	API
A23En01	*Hafnia alvei*	*Hafnia alvei*
A23En02	*Hafnia alvei*	*Hafnia alvei*
A13En02	*Serratia marcescens*	*Hafnia alvei*
A13En03	*Serratia liquefaciens*	Unsuccessful identification
M23En02	*Hafnia alvei*	*Hafnia alvei*
M23En03	*Hafnia alvei*	*Hafnia alvei*
M13En01	*Hafnia alvei*	*Hafnia alvei*
M13En02	*Serratia liquefaciens*	*Serratia marcesens*
A21En08	*Serratia liquefaciens*	*Serratia spp.*
A11En01	*Buttiauxella agrestis*	*Hafnia alvei*
A11En02	Unsuccessful identification	*Klebsiella pneumoniae* subsp. *ozaenae*
A11En03	*Hafnia alvei*	*Escherichia vulneris*
A11En04	*Serratia liquefaciens*	*Serratia spp.*
M21En01	*Hafnia alvei*	*Hafnia alvei*
M21En02	*Hafnia alvei*	*Hafnia alvei*

Table 4.8 Two *Enterobacteriaceae* were only identified to genus level by the API kit. The production of gas from glucose (Glucose) and acid from rhamnose (Rhamnose) will distinguish between *S. liquefacines* and *S. marcescens*.

Isolate	Glucose	Rhamnose	Species
A21En08	+	+	*S. liquefaciens*
A11En04	+	+	*S. liquefaciens*

not identify isolate A11En02, while API identified this isolate as *Klebseilla pneumoniea* subsp. *ozaenae*. The API could not successfully identify A13En03, while Biolog identified the isolate as *S. liquefaciens*. There were two isolates, A21En08 and A11En04, which were not confidently assigned a species name by API, only to the genus *Serratia*. Additional testing for gas production from glucose and acid from rhamnose indicated that these two isolates were *S. liquefaciens* (Table 4.8), as this species is positive for both (Grimont and Grimont, 1986). In general, there was good agreement between the two identification kits, the identification to genus was identical for nine of 15 (60%) isolates. Both kits identified seven isolates: A23En01, A23En02, M23En02, M23En03, M13En01, M21En01 and M21En02 as *H. alvei*. The two isolates that were designated only to the genus *Serratia* by API were also assigned to this genus by Biolog, which was able to identify a species. Biolog identified both A21En08 and A11En04 as *S. liquefaciens*. For the other *Serratia* strain identified, there were discrepancies between the species name given by the two kits. Isolate M13En02 was considered to be *S. liquefaciens* by Biolog and *S. marcescens* by API. The remaining three isolates were assigned to different genera by the two kits. Isolate A13En02 was identified by Biolog as *S. marcescens,* while API identified it as *H. avlei*. Isolate A11En01 was identified by Biolog as *Buttiauxella agrestis*, while API identified it as *H. avlei*. Finally, A11En03 was identified as *H. avlei* by Biolog but as *Escherichia vulneris* by API.

4.3.1.3 Identification of LAB isolates

Eighteen isolates of LAB belonging to the genus *Lactobacillus* or *Carnobacterium* were identified to species level (Table 4.9). A majority of *Lactobacillus* species, which do not contain *meso*-DAP within the cell wall, can be distinguished from *Carnobacterium* which does contains *meso*-DAP in the cell wall. The presence of *meso*-DAP was determined by thin layer chromatography (Figure 4.2). The solvent phase used in the TLC contained a highly carcinogenic substance, pyridine, therefore another solvent system which is used in the detection of alanine, ornithine and diaminobutyric acid (Bousfield *et al.*, 1985) in the cell wall was assessed. This solvent system of chloroform, methanol, ammonia (specific activity 0.88) and water (8:8:1:3 v/v) was found to be unsatisfactory and the original system of methanol, pyridine, 10M hydrochloric acid and water (80:10:2.5:17.5 v/v) was used.

Meso-DAP was an olive coloured spot slightly above the origin (Figure 4.2). Isolates A23L03, A13L03 and M11L03 were found to contain *meso*-DAP, while the remaining isolates were negative. These three isolates failed to grow on acetate agar and this distinguished them from *Lb.*

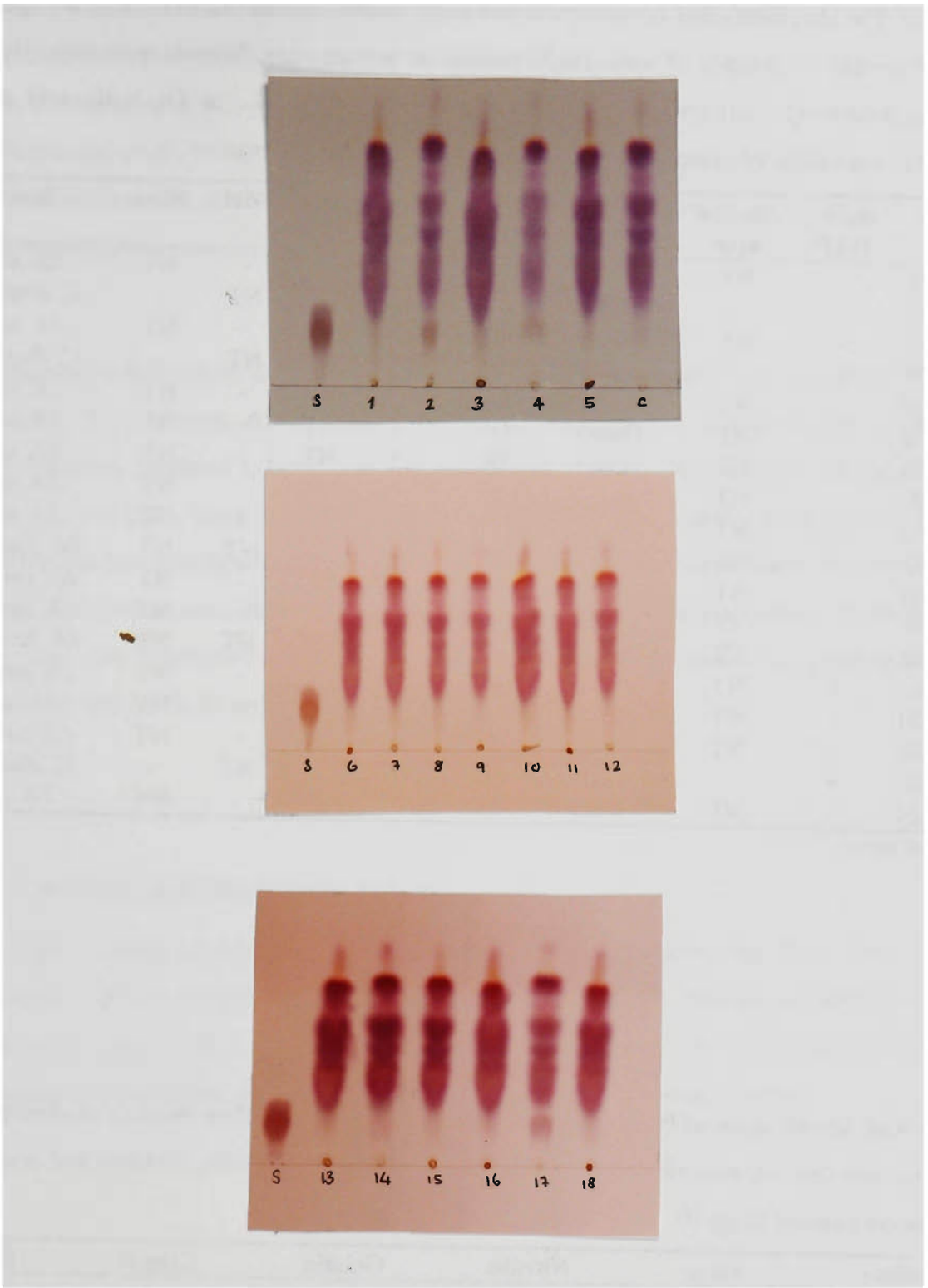


Figure 4.2 Detection of *meso*-DAP by TLC in the cell walls of LAB isolates: A23L01 (1), A23L03 (2), A13L02 (3), A13L03 (4), M23L06 (5), M23L07 (6), M23L09 (7), M13L02 (8), M13L03 (9), A21L01 (10), A21L03 (11), A11L01 (12), A11L02 (13), A11L03 (14), M21L01 (15), M21L03 (13), M11L03 (17) and M11L04 (18). A standard of 1% (w/v) *meso*-DAP (S) and a *C. divergens* reference strain (C) were run as controls. *C. divergens* and isolates A23L03 (2), A13L03 (4) and M11L03 (17) all have the olive coloured spot of *meso*-DAP above the origin.

Table 4.9 The identification of *Lactobacillus* and *Carnobacterium* isolated from lamb and beef on the basis of present or absence of *meso*-DAP, growth on acetate agar, heterofermentative (Hetero) or homofermentative (Homo) glucose metabolism, production of D-, L. or DL-lactic acid and acid production from either of inulin (Inu), melibiose (Mel) or mannitol (Man).

Isolate	*meso*-DAP	Acetate agar	Hetero/ Homo	Lactic acid	ADH	Inu	Mel	Man	Species
A23L01	-	NT	Homo	DL	+	NT	-	NT	*Lb. sakei*
A23L03	+	-	NT	L	+	-	NT	-	*C. divergens*
A13L02	-	NT	Homo	DL	+	NT	-	NT	*Lb. sakei*
A13L03	+	-	NT	L	+	-	NT	-	*C. divergens*
M23L06	-	NT	Homo	DL	+	NT	-	NT	*Lb. sakei*
M23L08	-	NT	Homo	DL	+	NT	-	NT	*Lb. sakei*
M23L09	-	NT	Homo	DL	+	NT	-	NT	*Lb. sakei*
M13L02	-	NT	Homo	DL	+	NT	-	NT	*Lb. sakei*
M13L03	-	NT	Homo	DL	+	NT	+	NT	*Lb. sakei*
A21L01	-	NT	Homo	L	+	NT	NT	NT	*Lb. farcimins*
A21L03	-	NT	Homo	DL	-	NT	-	NT	*Lb. curvatus*
A11L01	-	NT	Homo	DL	-	NT	-	NT	*Lb. curvatus*
A11L02	-	NT	Homo	L	+	NT	NT	NT	*Lb. farcimins*
A11L03	-	NT	Homo	DL	-	NT	-	NT	*Lb. curvatus*
M21L01	-	NT	Homo	DL	+	NT	-	NT	*Lb. sakei*
M21L03	-	NT	Homo	DL	-	NT	-	NT	*Lb. curvatus*
M11L03	+	-	NT	L	+	-	NT	-	*C. divergens*
M11L04	-	NT	Homo	DL	+	NT	-	NT	*Lb. sakei*

NT= not tested

Table 4.10 Identification of *Pseudomonas* isolated from lamb and beef on the basis of slime production from sucrose (Slime), reduction of nitrate (Nitrate), hydrolysis of gelatin (Gelatin) and production of fluorescent pigment (King B).

Isolate	Slime	Nitrate	Gelatin	King B	Species
A23P01	-	-	-	-	*P. fragi*
A23P06	-	-	+	+	*P. fluorescens*
A13P03	-	-	-	-	*P. fragi*
A13P04	-	-	+	+	*P. fluorescens*
A21P03	-	-	-	-	*P. fragi*
A21P04	-	-	-	-	*P. fragi*
A11P03	-	-	-	-	*P. fragi*
A11P04	-	-	+	+	*P. fluorescens*

plantatrum, which also contains *meso*-DAP, subsequently confirming that these isolates belong to the genus *Carnobacterium*. Within the genus *Carnobacterium*, the two species associated with meat spoilage, *C. divergens* and *C. piscicola*, can be differentiated by the production of acid from inulin and mannitol (Montel *et al.*, 1988). *C. piscicola* is positive for both reactions (Montel *et al.*, 1988). None of the three isolates was able to produce acid from these two sugars, therefore A21L03, A13L03 and M11L03 were classified as *C. divergens* strains.

The remaining isolates which did not contain *meso*-DAP were considered to be *Lactobacillus* species (Table 4.9). Two isolates, A21L01 and A11L02, were identified as *Lb. farciminis* as they were homofermentative, produced L-lactic acid and hydrolysed arginine. Four isolates, A21L03, A11L01, A11L03 and M21L03, were homofermentative, produced D- and L-lactic acid, did not hydrolyse arginine nor produce acid from rhamonse. Therefore these isolates were identified as *Lb. curvatus*. The remaining nine isolates were identified as *Lb. sakei* as they were: homofermentative, produced D- and L-lactic acid and hydrolysed arginine. These nine isolates were expected to produce acid from rhamnose but only M13L03 was able to produce acid.

4.3.1.4 Identification of *Pseudomonas* isolates

Of the eight isolates chosen for further identification, it was found that there were only two *Pseudomonas* species present. Three isolate were identified as *P. fluorescens* and the rest were identified as *P. fragi* (Table 4.10). These two species are differentiated by the hydrolysis of gelatin and the production of fluorescent pigment. *P. fluorescens* was positive for both reactions.

4.3.2 PULSED-FIELD GEL ELECTROPHORESIS FINGERPRINTING OF MEAT SPOILAGE ISOLATES

4.3.2.1 Optimising conditions for preparation of DNA from *Brochothrix*, *Carnobacterium* and *Lactobacillus* for PFGE

Growing *Lactobacillus* isolate in TYGB instead of MRSA increased cell mass, evidenced by an increase in OD_{610nm} from 1.1-1.6 to 1.4-1.9. As Gram-positive organisms have a tougher cell wall they can be difficult to lyse. In order to combat this problem, glycine was added to the cultures three hours prior to harvesting the cells. Five different levels of glycine were assessed ranging from zero to 2.5%.

These concentrations were chosen on the basis of glycine concentration used to alter the cell wall structures in *Corynebacterium glutamicum*, *Streptomyces* and *Bacillus* species during preparation of protoplasts (Hopwood, 1981; Best and Britz, 1986). The clearest banding pattern was achieved when 0.5% glycine was added to the broth. In addition, the concentration of lysozyme was manipulated to enhance cell lysis. Three concentrations were assessed, 10, 15 and 20 mg/mL lysozyme, and 10mg/mL lysozyme found to produce satisfactory cell lysis.

The GC content of *Brochothrix* is 36% (Sneath and Jones, 1986) and for *Carnobacterium* and *Lactobacillus* of meat origin 32-44% (Kandler and Weiss, 1986). Consequently, the restriction enzymes that were chosen were those that recognised GC-rich sites, as the recognition sites should occur less frequently. In addition, enzymes which recognised the rare sequence CTAG (McClellend *et al.*, 1987), one enzyme recongising AT-rich DNA and a 20 base cutter were also screened. Of the 13 enzymes tested, *Sma*I was the only enzyme suitable for PFGE fingerprinting of *Brochothrix*, *Carnobacterium* and *Lactobacillus* species. A majority of the restriction enzymes used produced large numbers of small bands, which were unsuitable for PFGE analysis, or they produced smears. There were no *Xba*I restriction sites in *Lactobacillus* species, no *Sfi*I sites in *Carnobacterium* species and no *Bam*HI sites in *Brochothrix* species. *Lactobacillus* species were only partially digested with *Sfi*I, a majority of the DNA remaining in the agarose block. An increase in the concentration of *Sfi*I failed to increase the digestion of the *Lactobacillus* genome.

PFGE was chosen to examine the fingerprints *Brochothrix* and LAB species because it had never been used to examine bacteria involved in food spoilage. For each bacterial species of *Brochothrix*, *Carnobacterium* and *Lactobacillus*, it was necessary to run between three to six gels of different pulse times to separate each individual band for genome size estimation. No single mixture of pulse times was able to separate out all the bands on one gel.

4.3.2.2 PFGE fingerprinting of *B. thermosphacta* strains

The diversity of the *Sma*I restriction patterns in *B. thermosphacta* strains was an unexpected result, as all the isolates were identified as *B. thermosphacta* therefore similar fingerprints were expected (Figures 4.3 to 4.7). In order to separate out all the bands, five gel runs were required at pulse times of two (Figure 4.3), five (Figure 4.4), 10 (Figure 4.5), 15 (Figure 4.6) and 25 (Figure 4.7) seconds for 24 hours. Initially *Brochothrix* isolates A23B03, A23B04, A13B02, A13B03, M23B03, M13B02, M13B03, A21B01, A21B02, A11B02, A11B04, M11B02 and A11B04, were fingerprinted. Analysis

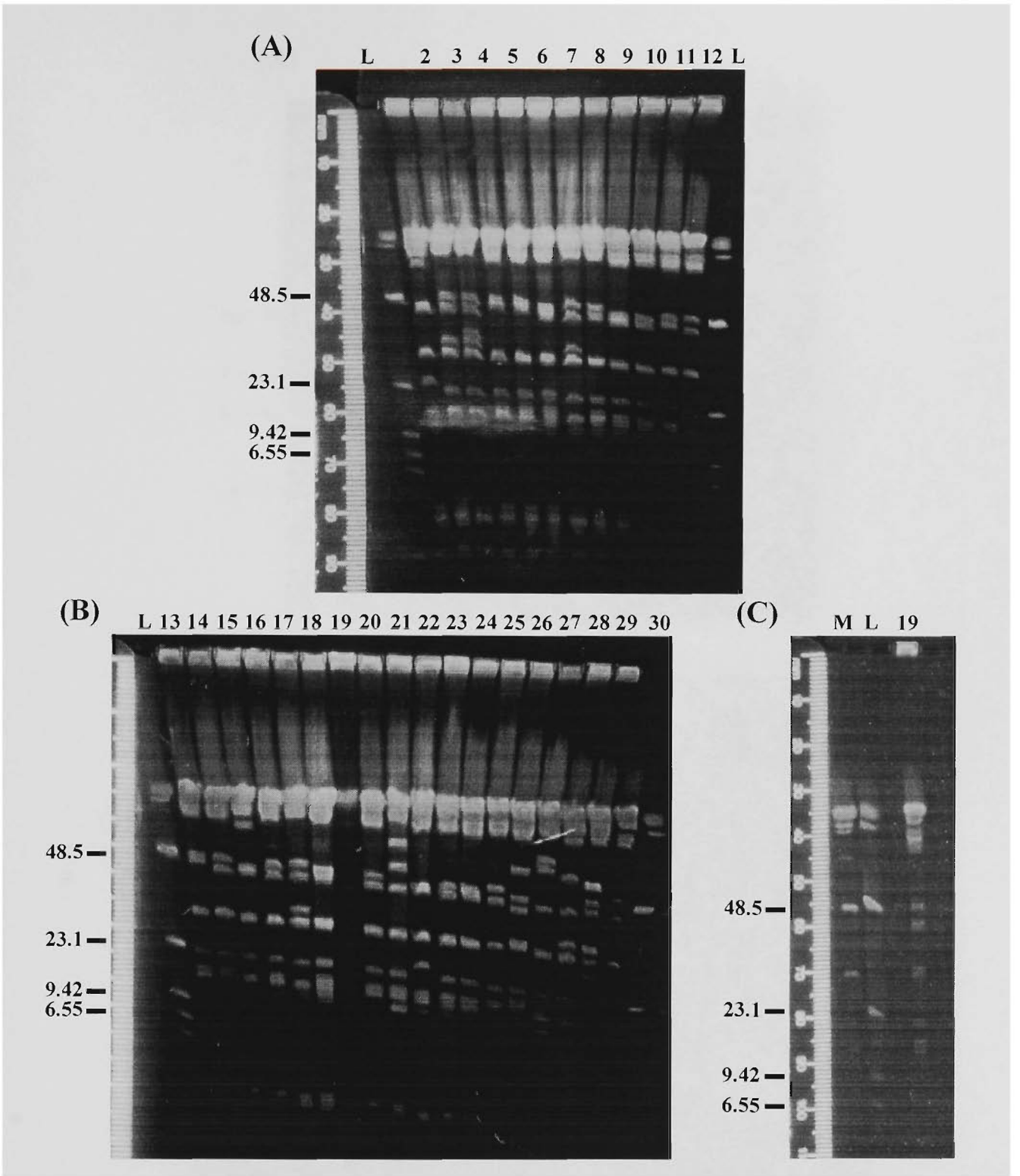


Figure 4.3 PFGE gel of *Sma*I digested *Brochothrix* isolates (**A**) A23B03 (1), A23B06 (2), A13B02 (3), A13B03 (4), M13B02 (5), M13B03 (6), A21B01 (7), A21B02 (8), A11B02 (9), A11B04 (10), M11B01 (11), and M11B04 (12) (**B**) M23B03 (13), A23B01 (14), A23B02 (15), A23B04 (16), A23B05 (17), A13B05 (18), M23B01 (19), M23B02 (20), M13B01 (21), M13B04 (22), M13B05 (23), A21B03 (24), A11B01 (25), A11B03 (26), A11B05 (27), M11B02 (28), M11B03 (29) and *B. thermosphacta* ATCC11509 (30). (**C**) Repeat of isolate M23B01 (19), which did not run well on a previous gel. All gels were run at a pulse time of two second for 24 hours. Low (L) and medium (M) ladders were run as molecular size markers, sizes are in Kilobases (Kb).

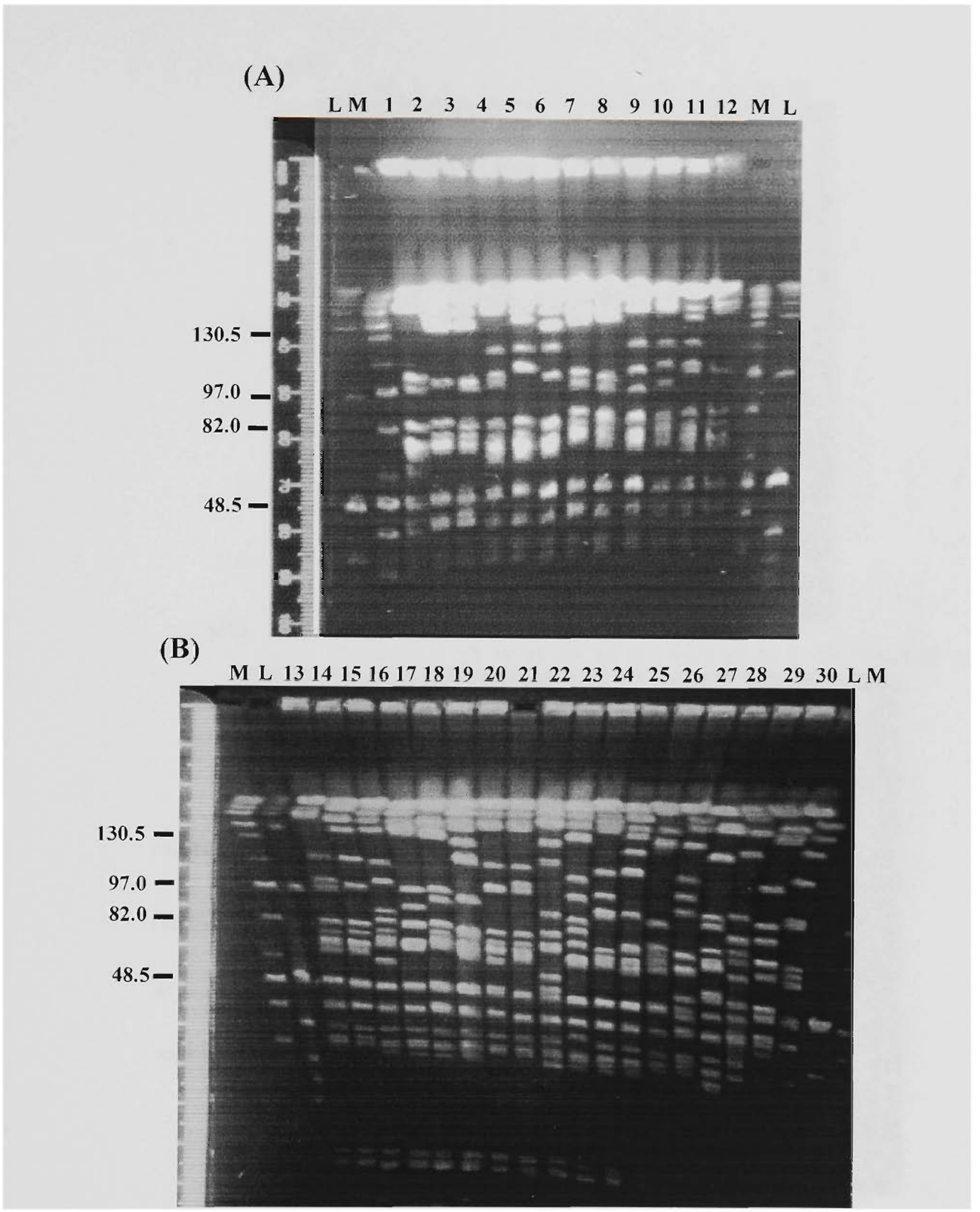


Figure 4.4 PFGE gel of *Sma*I digested *Brochothrix* isolates (**A**) A23B03 (1), A23B06 (2), A13B02 (3), A13B03 (4), M13B02 (5), M13B03 (6), A21B01 (7), A21B02 (8), A11B02 (9), A11B04 (10), M11B01 (11), and M11B04 (12) (**B**) M23B03 (13), A23B01 (14), A23B02 (15), A23B04 (16), A23B05 (17), A13B05 (18), M23B01 (19), M23B02 (20), M13B01 (21), M13B04 (22), M13B05 (23), A21B03 (24), A11B01 (25), A11B03 (26), A11B05 (27), M11B02 (28), M11B03 (29) and *B. thermosphacta* ATCC11509 (30). Both gels were run at a pulse time of five seconds for 24 hours. Low (L) and medium (M) ladders were run as the molecular size marker, sizes are in Kilobases (kb).

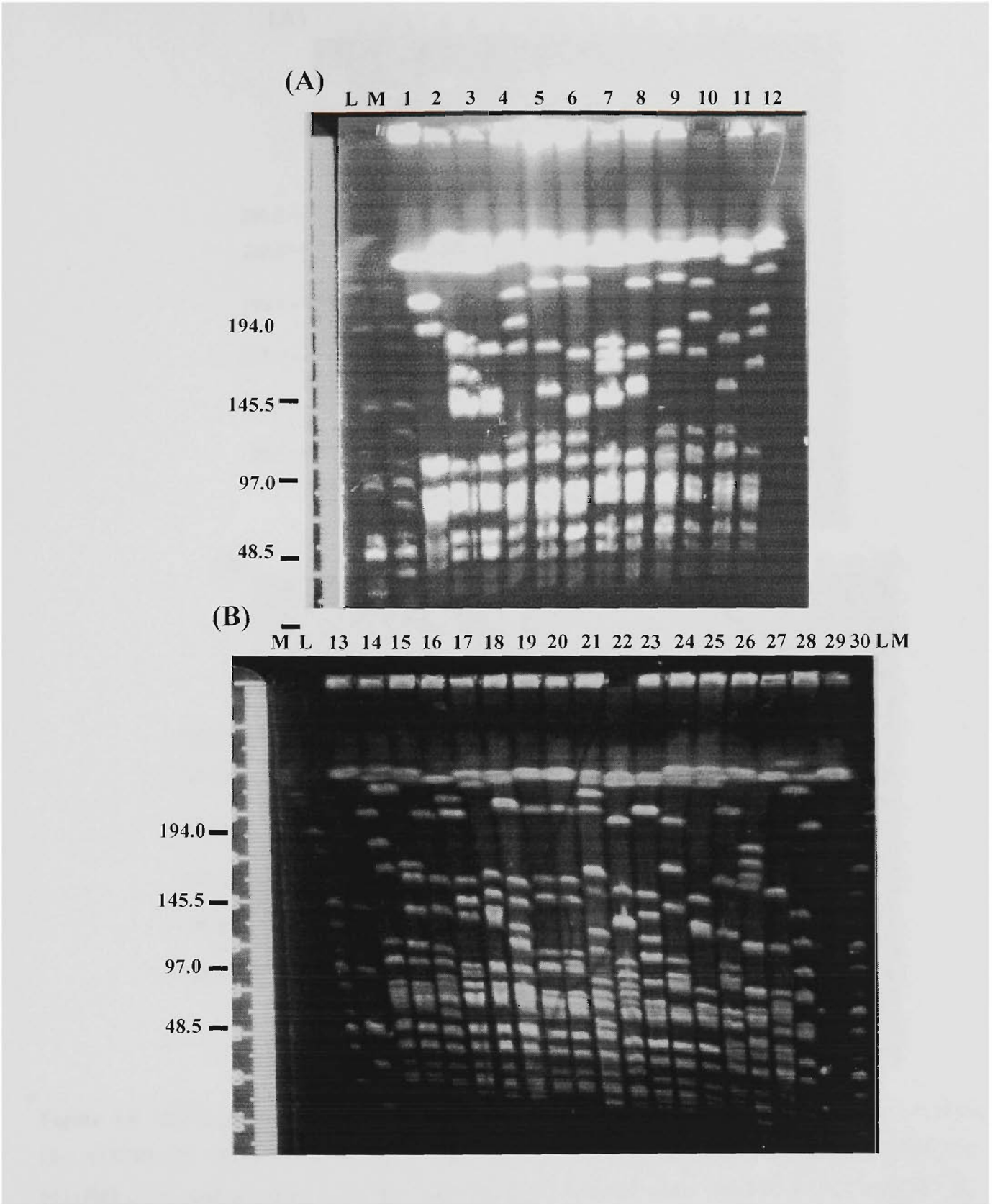


Figure 4.5 PFGE gel of *Sma*I digested *Brochothrix* isolates (**A**) A23B03 (1), A23B06 (2), A13B02 (3), A13B03 (4), M13B02 (5), M13B03 (6), A21B01 (7), A21B02 (8), A11B02 (9), A11B04 (10), M11B01 (11), and M11B04 (12) (**B**) M23B03 (13), A23B01 (14), A23B02 (15), A23B04 (16), A23B05 (17), A13B05 (18), M23B01 (19), M23B02 (20), M13B01 (21), M13B04 (22), M13B05 (23), A21B03 (24), A11B01 (25), A11B03 (26), A11B05 (27), M11B02 (28), M11B03 (29) and *B. thermosphacta* ATCC11509 (30). Both gels were run at a pulse time of 10 seconds for 24 hours. Low (L) and medium (M) ladders were run as the molecular size marker, sizes are in Kilobases (kb).

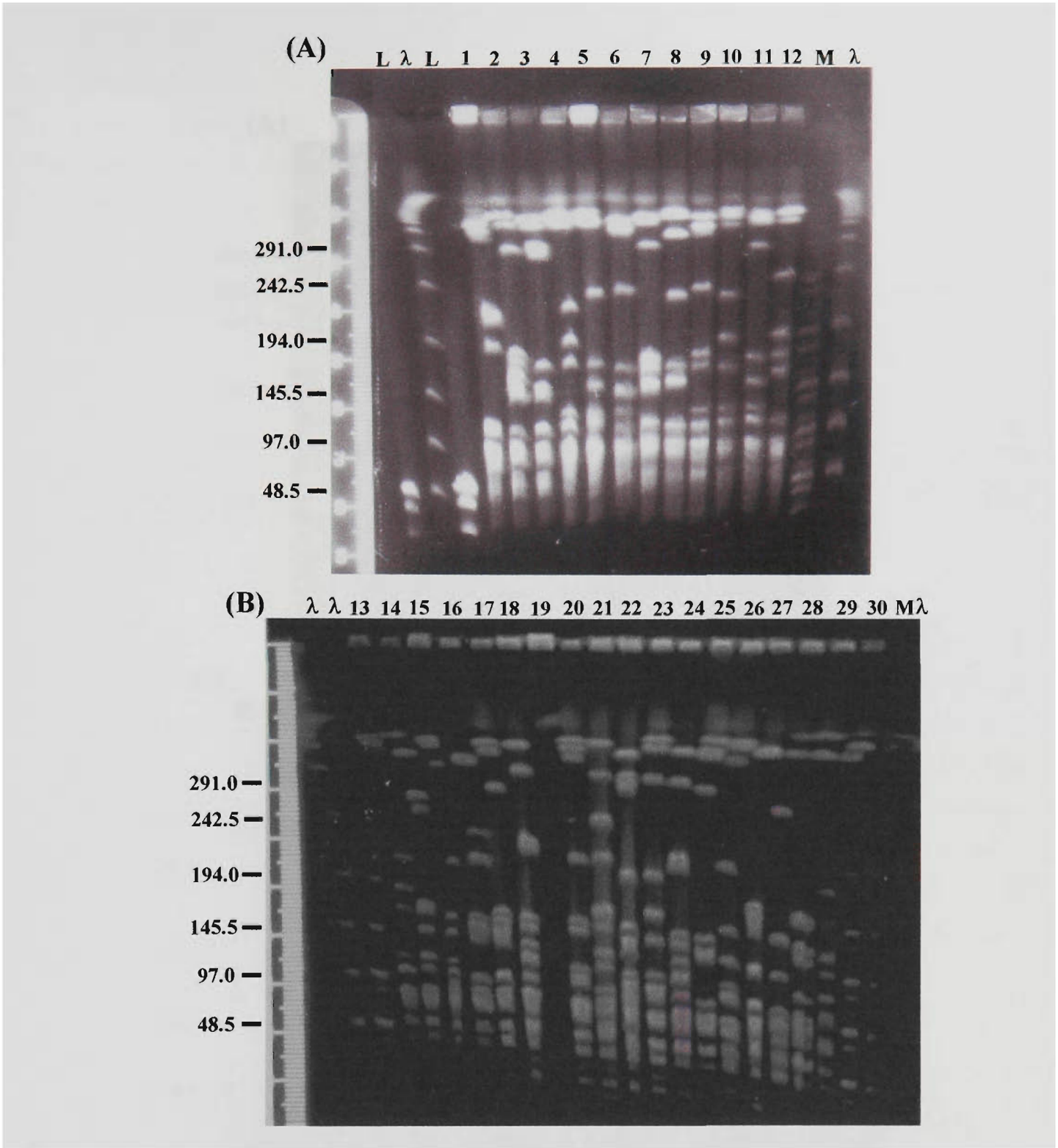


Figure 4.6 PFGE gel of *Sma*I digested *Brochothrix* isolates (**A**) A23B03 (1), A23B06 (2), A13B02 (3), A13B03 (4), M13B02 (5), M13B03 (6), A21B01 (7), A21B02 (8), A11B02 (9), A11B04 (10), M11B01 (11), and M11B04 (12) (**B**) M23B03 (13), A23B01 (14), A23B02 (15), A23B04 (16), A23B05 (17), A13B05 (18), M23B01 (19), M23B02 (20), M13B01 (21), M13B04 (22), M13B05 (23), A21B03 (24), A11B01 (25), A11B03 (26), A11B05 (27), M11B02 (28), M11B03 (29) and *B. thermosphacta* ATCC11509 (30). Both gels were run at a pulse time of 15 seconds for 24 hours. Low (L) medium (M) and Lambda (λ) ladders were run as the molecular size marker, sizes are in Kilobases (kb).

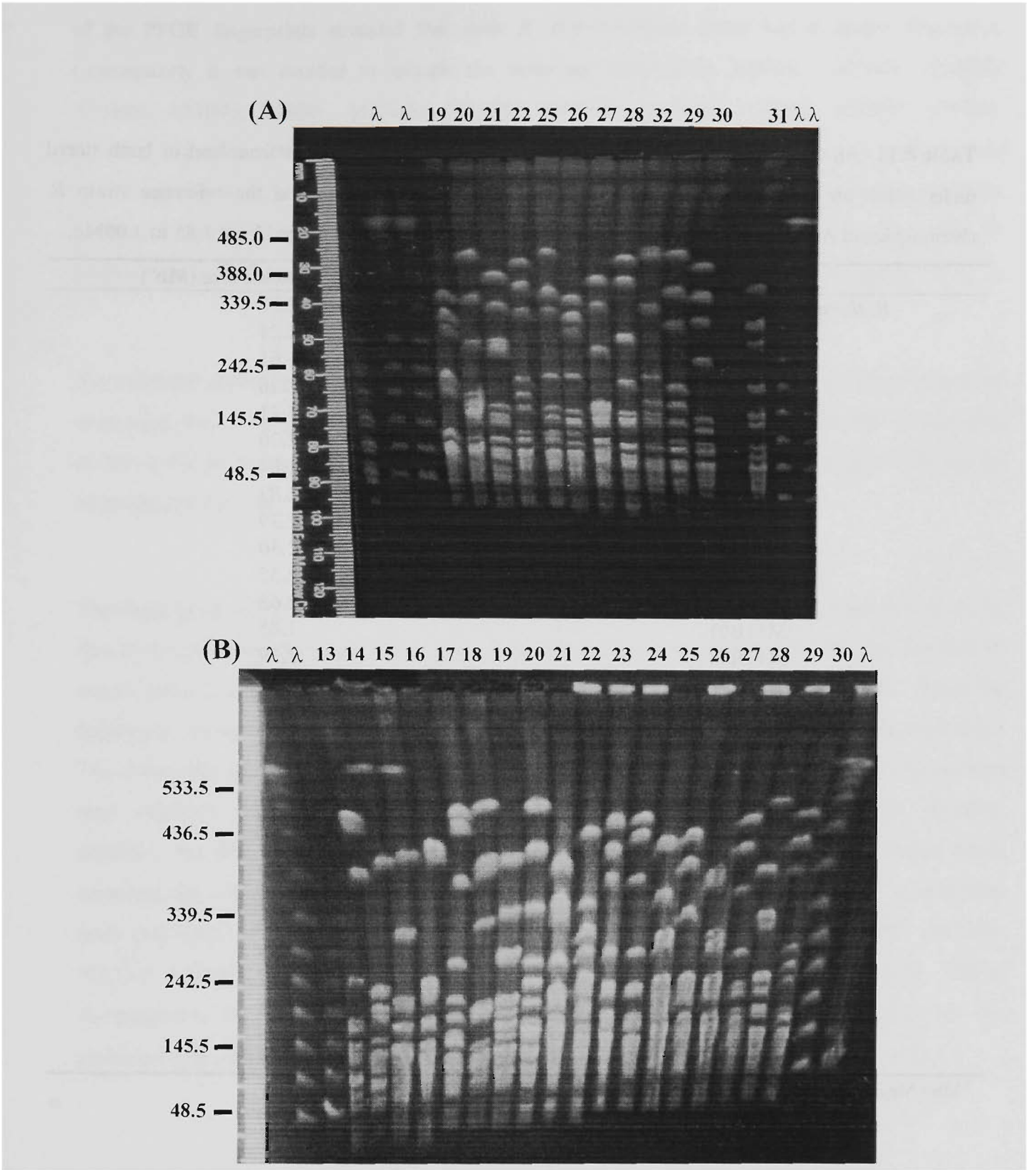


Figure 4.7 PFGE gel of *Sma*I digested *Brochothrix* isolates (**A**) A23B03 (1), A23B06 (2), A13B02 (3), A13B03 (4), M13B02 (5), M13B03 (6), A21B01 (7), A21B02 (8), A11B02 (9), A11B04 (10), M11B01 (11), and M11B04 (12) (**B**) M23B03 (13), A23B01 (14), A23B02 (15), A23B04 (16), A23B05 (17), A13B05 (18), M23B01 (19), M23B02 (20), M13B01 (21), M13B04 (22), M13B05 (23), A21B03 (24), A11B01 (25), A11B03 (26), A11B05 (27), M11B02 (28), M11B03 (29) and *B. thermosphacta* ATCC11509 (30). Both gels were run at a pulse time of 25 seconds for 24 hours. Low (L) medium (M) and Lambda (λ) ladders were run as the molecular size marker, sizes are in Kilobases (kb).

Table 4.11 An estimate of the genome size for the 29 *Brochothrix* isolates from beef or lamb stored under either air or modified atmosphere and at either one or 10°C, and the reference strain *B. thermosphacta* ATCC11509. The estimated genome size of the isolates ranged from 1.85 to 3.00Mb.

Isolate	Estimated genome size (Mb[a])
B. thermosphacta ATCC11509	2.37
A23B03	2.24
A23B06	2.61
A13B02	2.26
A13B03	2.41
M23B03	2.20
M13B02	2.84
M13B03	2.83
A21B01	2.39
A21B02	2.30
A11B02	2.35
A11B04	2.65
M11B01	1.85
M11B04	2.19
A23B01	2.16
A23B02	2.05
A23B04	1.91
A23B05	2.85
A13B05	2.64
M23B02	2.50
M13B01	2.84
M13B04	2.80
M13B05	2.85
A21B03	3.00
A11B01	2.39
A11B03	2.37
A11B05	2.18
M11B02	2.30
M11B03	1.88

[a] Mb= Megabases (10^6 base pairs)

of the PFGE fingerprints revealed that each *B. thermosphacta* strain had a unique fingerprint. Consequently it was decided to include the remaining *Brochothrix* isolates: A23B01, A23B02, A23B04, A23B05, A13B05, M23B01, M23B02, M13B01, M13B04, M13B05, A21B03, A11B01, A11B03, A11B05, M11B02 and M11B03, which had been collected from spoiled air-packaged and MAP beef and lamb following storage at one or 10°C. Again, each strain of *B. thermosphacta* had a unique PFGE fingerprint. These extra isolates were fingerprinted with the control strain, *B. thermosphacta* ATCC11509, whose fingerprint was markedly different from the local strains.

The estimated genome size for each *Brochothrix* isolate is listed in Table 4.11. The estimated genome sized range from 1.85 to 3.00Mb. The genome size for M23B01 was not estimated as the isolate failed to run on the gel pulsed at 15 seconds. The reference strain *B. thermosphacta* ATCC11509 had an estimated genome size of 2.37Mb.

The dendrogram exhibiting the relatedness among the *B. thermosphacta* strains is shown in Figure 4.8. Due to the limitations of the computer program the dendrogram was derived from the two, five and 10 second pulse time gels, as these gels provided common bands as well as unique bands. From the dendrogram it can be seen that the *B. thermosphacta* strains were more than 75% related to each other. The strains fall into two main clusters, the first cluster contained 13 isolates of which 12 came from lamb (A23B01, A23B02, A23B03, A23B04, A23B05, A23B06, A13B02, A13B03, A13B05, M23B01, M13B02 and M13B03) and only one (A11B05) from beef. The second cluster which contained the reference strain *B. thermosphacta* ATCC 11509, was comprised of four strains from lamb (M23B02, M13B01, M13B04 and M13B05) and 11 strains from beef (A21B01, A21B02, A21B03, A11B01, A11B02 A11B03, A11B04, M11B01, M11B03, M11B04 and M11B05). The *B. thermosphacta* strains from the MAP beef and lamb were dispersed among the strains from air-packaged meat, similarly strains from 1°C were intermingled with those from 10°C (Figure 4.8).

4.3.2.3 PFGE fingerprinting of *C. divergens* strains

The *Sma*I PFGE fingerprints obtained for the three *C. divergens* strains and two reference cultures, *C. divergens* and *C. piscicola* JG126, are shown in Figures 4.9 and 4.10 at pulse times ranging from two to 35 seconds. In comparison to the local isolates of *C. divergens* strains, the two reference strains had significantly more bands. The restriction patterns from the isolates A13B03 and M11L03 were identical. Although the majority of the bands were of the same size for the three isolates, there was a

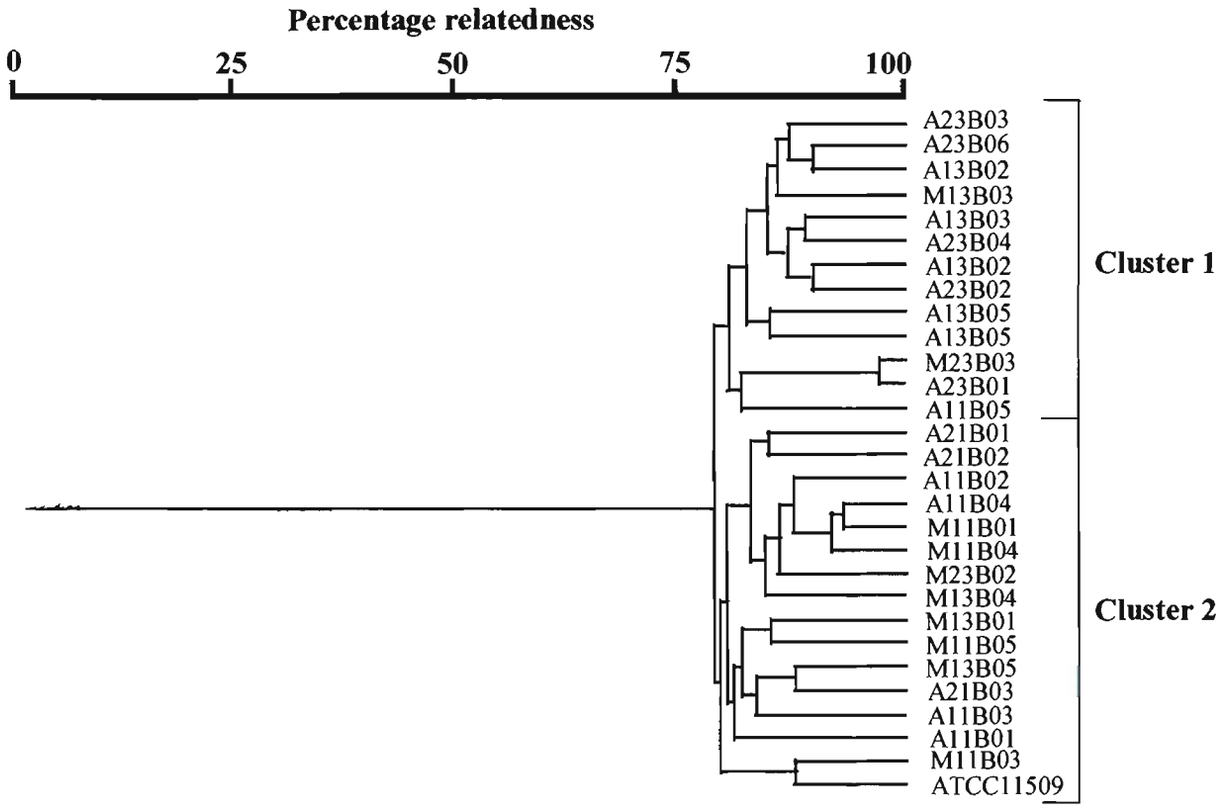


Figure 4.8 Dendrogram demonstrating the relatedness between stains of *B. thermosphacta* isolated from spoiled air-packaged and MAP beef and lamb stored at one and 10°C. The 29 strains could be spilt into two clusters. The first containing 13 isolates, one from beef and remainder from lamb. The second containing 16 isolates, 11 from beef, four from lamb and the reference strain *B. thermosphacta* ATC11509.

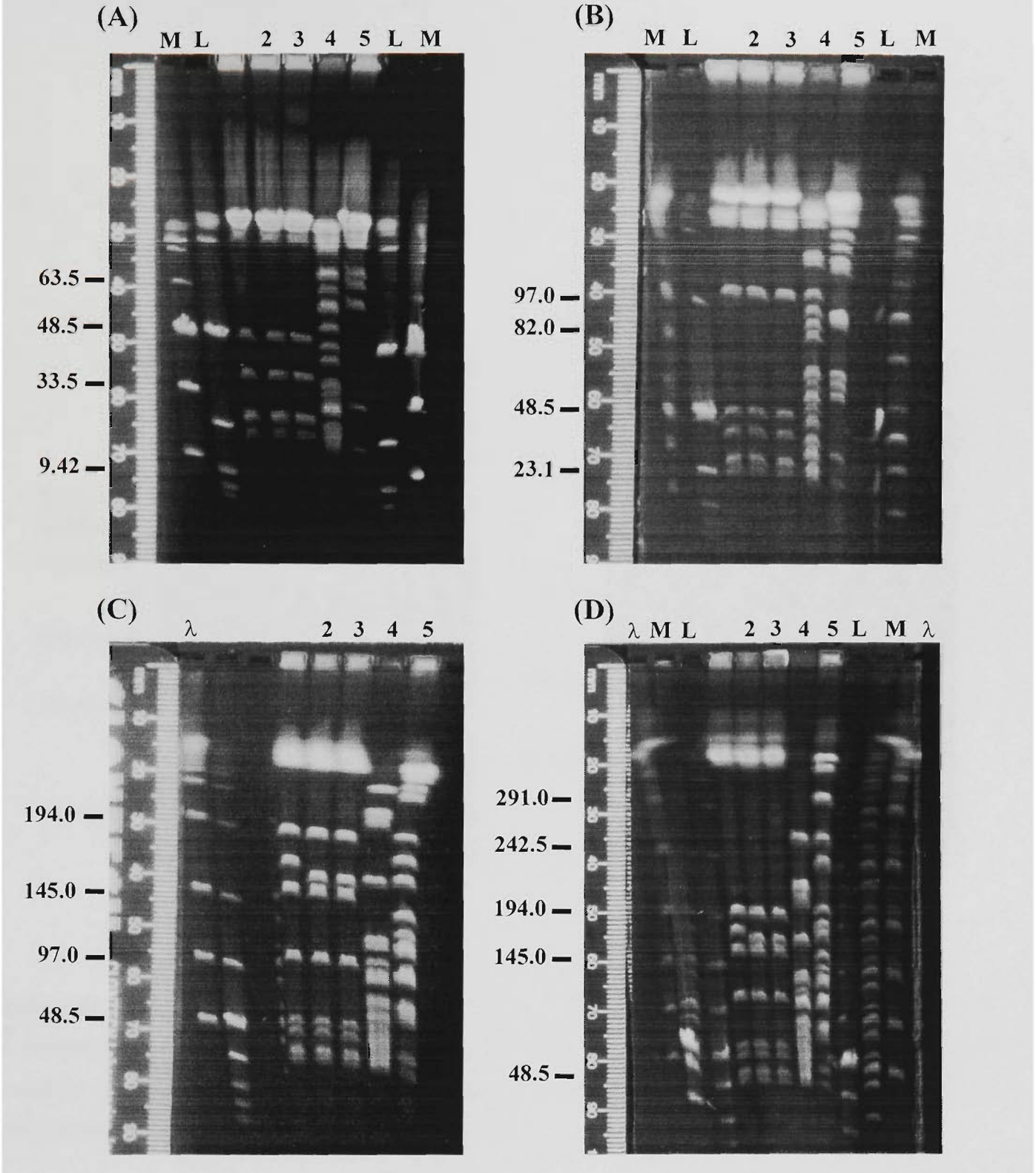


Figure 4.9 PFGE gel of *Sma*I *Carnobacterium* isolates A23L03 (1), A13L03 (2) and M11L03 (3) and reference strains *C. divergens* (4) and *C. piscicola* JG126 (5). Gels were run at pulse times of (**A**) two second (**B**) five seconds (**C**)10 seconds and (**D**) 15 seconds for 24 h. Low (L) medium (M) and Lambda (λ) ladders were run as the molecular size marker, sizes are in Kilobases (kb).

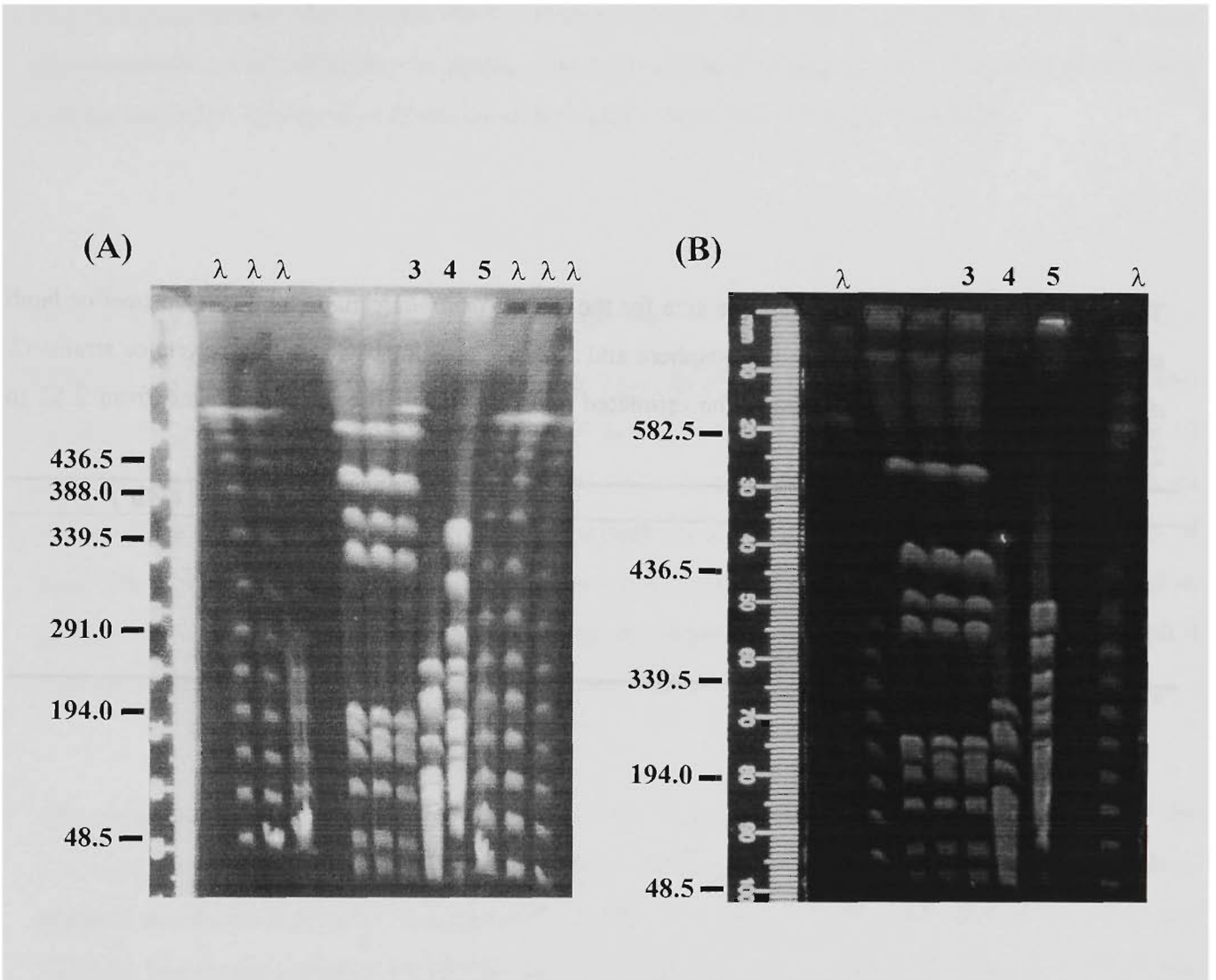


Figure 4.10 PFGE gels of *Sma*I *Carnobacterium* isolates A23L03 (1), A13L03 (2) and M11L03 (3) and the reference strains *C. divergens* (4) and *C. piscicola* JG126 (5). Gels were run at pulse times of (**A**) 25 second for 12h then 30 seconds for a further 12h and (**B**) 35 seconds for 24 h. Lambda ladder (λ) was run as the molecular size marker, sizes are in Kilobases (kb).

Table 4.12 An estimate of the genome size for the three *Carnobacterium* isolates from beef or lamb stored under either air or modified atmosphere and at either one or 10°C, and the reference strains *C. divergens* and *C. piscicola* JG126. The estimated genome size of the isolates ranged from 2.52 to 2.53Mb.

Isolate	Estimated genome size (Mb[a])
A21L03	2.53
A13L03	2.53
M11L03	2.52
C. divergens	2.47
C. piscicola JG126	3.58

[a] Mb= Megabases (10^6 base pairs)

one band difference on the gel pulsed for 15 seconds. Isolate A23L03 had a band that was estimated to be 159kb while the corresponding band in A13L03 and M11L03 was estimated to be 149kb. The estimated genome size of the three *C. divergens* strains, the reference *C. divergens* and *C. piscicola* JG126 are listed in Table 4.12. The estimated genome size of the three *C. divergens* isolates was similar: A23L03 was 2.53Mb, A13L03 and M11L03 were 2.52Mb. The estimated genome size for the two reference strains was 2.47Mb for *C. divergens* and 3.58Mb for *C. piscicola* JG126, indicating approximately a 1Mb difference in genome size between the two species. More examples of the latter species need to be examined to determine if this value is typical for *C. piscicola* strains.

4.3.2.4 PFGE fingerprinting of *Lactobacillus* species

Of all the enzymes tested, only *Sma*I was able to digest the DNA of *Lactobacillus* species isolated from spoiled beef and lamb (Figure 4.11). The PFGE gels were run at a combination of one second for 10 hours then two seconds for 10 hours, two seconds for 20 hours and five seconds for 20 hours. Each gel yielded fingerprints for *Lactobacillus*. Unfortunately the DNA was cleaved into a large number of small fragments. Consequently, it was not possible to estimate the genome size of the *Lactobacillus* isolates with any accuracy, as the largest fragment was approximately 130kb (Figure 4.11), although it was still possible to obtain information about the isolates from the gels.

The pulse times of two and five seconds for 20 hours produced interpretable patterns for the *Lactobacillus* isolates. There were eight different pattern types produced, with the same isolates grouped together on both gels. Isolates A23L01, M23L06, M23L09, M13L03, A21L03, A11L02 and M21L03 had the same pattern and formed the largest group. The second group contained A13L02 and A21L01, while the third group contained isolates M23L08 and A11L03. Isolates M13L02, A11L01, M21L01, M11L04 and the dairy strain *Lb. curvatus* had unique PFGE fingerprints (Figure 4.11). The grouping of PFGE fingerprints did not correlate with either meat type, storage temperature or atmosphere.

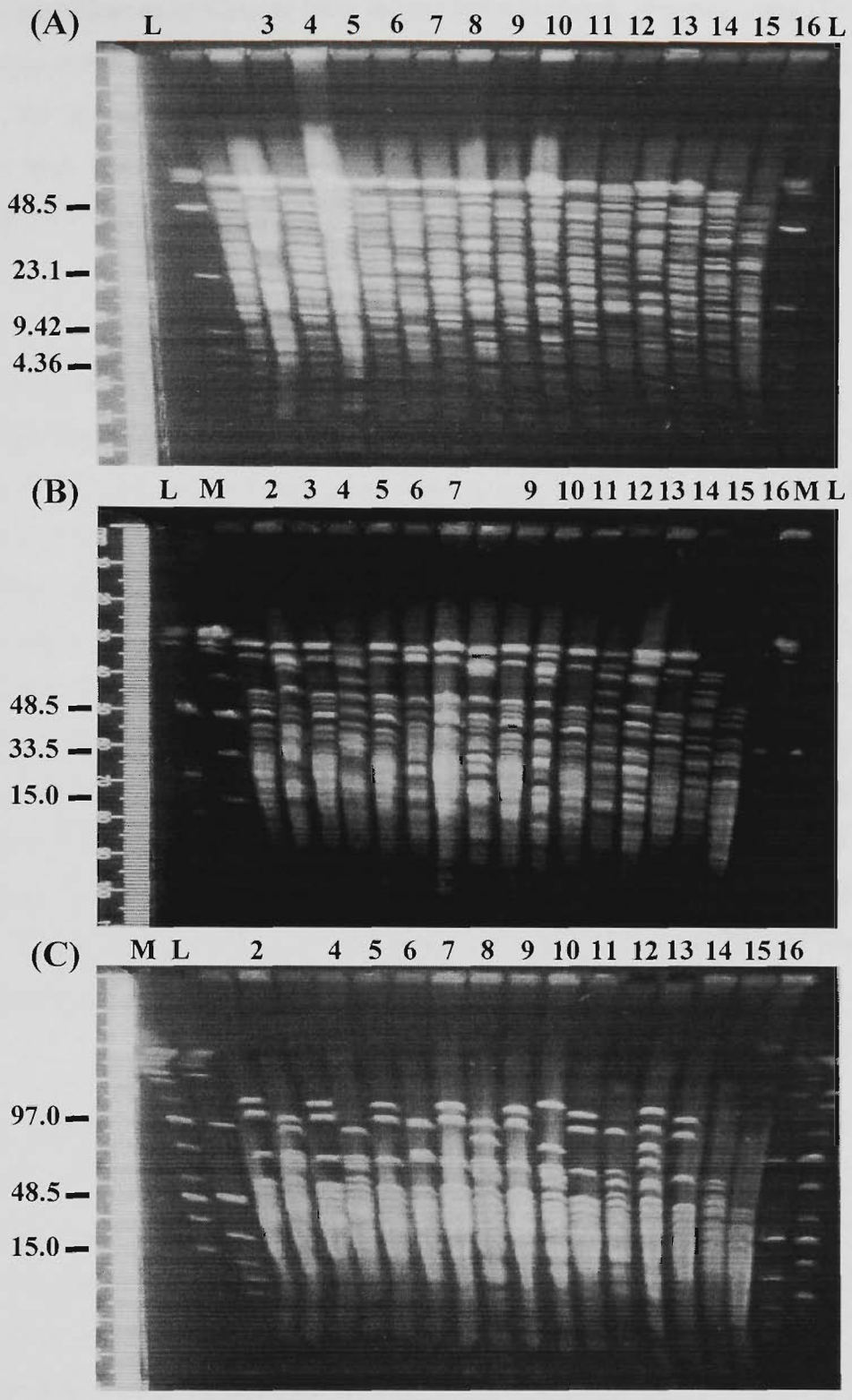


Figure 4.11 PFGE gel of *Sma*I digested *Lactobacillus* isolates A23L01 (1), A13L03 (2), M23L06 (3), M23L08 (4), M23L09 (5), M13L02 (6), M13L03 (7), A21L01 (8), A21L03 (9), A44L01 (10), A11L02 (11), A11L03 (12), M21L01 (13) , M21L03 (14), M11L04 (15) and a dairy isolate of L. curvatus (16). Gels were run at pulse times of (**A**) one second for 10h the 2 second for 10h, (**B**) two seconds for 20h and (**C**) five seconds for 20 hours. Low (L) and medium (M) range markers were run as the molecular size marker, sizes are in Kilobases (kb).range molecular weight markers were run.

4.4 DISCUSSION

Microbiologists continue to strive for new ways to identify bacterial strains definitively (Klausner, 1988). The identification of bacteria from several different family or genus groups from spoiled meat of different types stored at different temperatures and under different atmospheres has not been described previously, nor has the relatedness of *Brochothrix*, *Carnobacterium* or *Lactobacillus* species isolated from meat been determined by PFGE fingerprinting. This proved to be a useful approach in determining the diversity of the bacterial flora at spoilage under different conditions of storage.

4.4.1 BIOCHEMICAL IDENTIFICATION

4.4.1.1 Identification of *Brochothrix* isolates

Of the two *Brochothrix* species which are recognised, only *B. thermosphacta* was isolated from spoiled, air-packaged or MAP beef and lamb following storage at one or 10°C. The ability of this species to grow under conditions of reduced water activity (Skovgaard, 1985; Talon *et al.*, 1988) would account for its prevalence on carcasses. *B. campestris* was originally isolated from soil (Talon *et al.*, 1988) therefore it was not improbable that it would be capable of contaminating meat, particularly if hygiene standards were poor. Originally only 13 isolates of *Brochothrix* were identified, however, due to the diversity of the PFGE fingerprints, an extra 16 *Brochothrix* isolates that had also been collected from the spoiled beef and lamb were fingerprinted. All of these isolates were identified as *B. thermosphacta* biochemically. In addition, the number of biochemical tests was increased to examine a wider range of attributes. There was some variation in the response of the isolates to the different biochemical tests but these variations were not considered to be sufficient to reassign the isolates to another genus or species.

For the test differentiating *B. thermosphacta* and *B. campestris*, M13B03 was unable to reduce tellurite and the five isolates, A23B01, M23B01, M13B04, A11B03 and M11B02, were able to ferment rhamnose but only weakly. Since the other tests were in agreement with those for *B. thermosphacta* (Talon *et al.*, 1988), these six atypical isolates were classified as *B. thermosphacta*. In the limited additional tests, there were only two tests where there was any variation in response. Nineteen of the 29 isolates were able to grow weakly at 45°C. *B. thermosphacta* (by definition) should not be able to grow at 45°C and the name suggests, *thermosphacta* means "heat sensitive" (Sneath and Jones *et al.*, 1986). Thirteen of the isolates were able to grow at pH 3.9, while *B. thermosphacta* ATCC11509 was not. Of the 13 able to grow a pH 3.9, nine had also been able to grow at 45°C. As the number of additional biochemical tests was still limited, it is still possible that further biochemical tests would indicate an even greater diversity among the isolates.

4.4.1.2 Identification of *Enterobacteriaceae*

It is not uncommon for a bacterial isolate not to be aligned to known bacterial species on the basis of biochemical reactions. This was seen with the *Enterobacteriaceae* isolates A13En03 analysed with API 20E and A11En02 with Biolog. These isolates could not be classified as belonging to a particular group. A large number of the bacteria isolated from lamb carcasses during aerobic refrigerated storage could not be identified (Prieto *et al.*, 1993). Of the *Pseudomonas* species isolated from pork, 17% could not be assigned to a particular species (Enfors *et al.*, 1979). Instead, they were classified as either (1) proteolytic and lipolytic or (2) proteolytic but not lipolytic or (3) neither lipolytic or proteolytic (Enfors *et al.*, 1979). The identification of *Enterobacteriaceae* isolates with the API kit involved 21 separate reactions, while the Biolog kit had 95, yet there was one isolate which could not be identified for each kit. Phenotypic characteristics do not always provide sufficient information for the successful identification of a bacterial isolate.

A majority of the *Enterobacteriaceae* strains were identified by both commercial kits as *H. alvei.* From sampling in abattoirs, *H. alvei* was frequently detected in water, soil, air, on carcasses, processing surfaces and hands of personnel (Patterson and Gibb, 1977). Of the oxidase negative bacteria from reconstructed and conventional steaks, 62% were *H. alvei* (Newsome *et al.*, 1987). Similarly, on refrigerated meat *H. alvei* comprised 68% of the microbial population, with *S. liquefaciens* constituting 36% (Ridell and Korkela, 1997). *S. liquefaciens* is the most commonly isolated member of the genus *Serratia* (Grimont and Grimont, 1981). Biolog identified four isolates as *S. liquefaciens* and one as *S. marcescens*, while API identified two isolates as *Serratia* spp., one isolate as *S. liquefaciens* and one as *S. marcescens*. Both kits designated one of the isolates as *S. marcescens*, however, *S. marcescens* is described as having red pigmentation (Grimont and Grimont, 1981; Grimont and Grimont, 1986). None of these isolates produced any red pigment, therefore it is unlikely the either A12En02 (Biolog) or M13En02 (API) were *S. marcescens*. The remaining species identified, *B. agrestis*, *K. pneumoneae* subsp. *ozaenae* and *E. vulneris*, all have been identified amongst the *Enterobacteriaceae* microflora isolated previously from chilled meats (Newsome *et al.*, 1987; Ridell and Korkela, 1997).

API 20E identification kit by bioMérieux is a commonly accepted method for the identification of *Enterobacteriaceae* in food microbiology laboratories. Analysis of the accuracy of API 20E kits in the identification of *Enterobacteriaceae* commenced in the 1970s. Washington *et al.* (1971) found API had an accuracy of 93%, while Smith *et al.* (1971) found the accuracy to be 96.4%. Problems associated with API identification kits are related to the flimsy plastic, the tediousness of filling tubes (Washington

et al., 1971) and occasional difficulty in interpreting secondary tests (personal observation). The advantages of API, however, outweigh the disadvantages (Cox *et al.*, 1977).

Biolog is a newer system. Like API, the test revolves around colourimetric detection, changing from clear to purple within four to 24 hours (Bochner, 1989a,b). Several problems have been noted with the identification of bacterial species with Biolog. One of the main problems for a food microbiologist is that Biolog is primarily aimed at the clinical microbiologist (Miller and Rhoden, 1991). In addition, Biolog can be unreliable at the identification of bacterial strains. Of 41 authentic Gram-negative ATCC cultures, 98% were classified to the correct genus but only 59% were classified to the correct species (Klinger *et al.*, 1992). Biolog identified 266 of 352 known *Enterobacteriaceae* isolates, of the 266 identified isolates, 87.3% assigned to the correct genus but only 75.6% to the correct species after 24 hours (Miller and Rhoden, 1991). Eighty-six percent of *P. cepacia* strains from cystic fibrosis patients were identified correctly with Biolog (Roman *et al.*, 1991). Biolog does not always give the results that are expected. *Azosporillum* isolates were identified as *A. brasilense*, but these isolates utlised α-ketoglutaric acid, *cis*-aconitic acid and D-galacturonic acid, which are normally metabolised by *A. lipoferum* not *A. brasilense* strains (Eid and Sherwood, 1995). The identification of some isolates has been observed to change between the four and 24 hour reading periods (Miller and Rhoden, 1991; Klinger *et al.*, 1992) and some bacteria are consistently difficult to identify with the Biolog system (Miller and Rhoden, 1991). *Klebsiella*, *Enterobacter* and *Serratia* species tend to be too active metabolically, resulting in too many positive reactions which consequently gave erroneous identifications (Miller and Rhoden, 1991; Klinger *et al.*, 1992). During this investigation, there were occasions where the colour of the negative control changed from clear to purple thus making the results invalid. Identification of bacterial isolates with the Biolog kit is based on 95 substrates which include: sugars, carboxylic acids, amine esters, amino acids, peptides, amines, alcohols, aromatics, halogenated compounds, phosphorous- and sulphur-containing compounds (Bochner, 1989b, Stager and Davis, 1992). If motility, oxidase, catalase, and indole were included, perhaps the identification accuracy would increase (Klinger *et al.*, 1992). As the Biolog results tend to be less accurate than API, the identity of the bacteria isolated from beef and lamb was taken from the API results.

4.4.1.3 Identification of LAB isolates

Of the LAB isolates a majority was identified as *Lb. sakei*, which is commonly the most prevalent spoilage bacteria on MAP meat (Hastings and Holzapfel 1987; Grant and Patterson, 1991; Björkroth *et al.*, 1996). The remainder were identified as either *Lb. curvatus, Lb. farciminis* or *C. divergens*, which are also associated with the spoilage of meat (Collins *et al.*, 1987; Hastings and Holzapfel 1987;

Korkela and Mäkelä, 1989; Grant and Patterson, 1991; Montel *et al.*, 1991; Björkroth *et al.*, 1996). Interestingly, *Lb. curvatus* was only identified among the isolates collected from beef. *Lb. sakei* is homofermentative, produces D- and L-lactic acid, hydrolyses arginine and ferments melibiose. *Lb. curvatus* which is related to *L. sakei* (Kandler and Weiss, 1986), is separated from *Lb. sakei* by arginine hydrolysis and melibiose fermentation, for which it is negative. Variations in the sugar fermentation, particularly for melibiose, by *Lb. sakei* and *Lb. curvatus* from those described in Bergey's Manual of Determinative Bacteriology have been observed previously (Hastings and Holzapfel 1987; Korkela and Mäkelä, 1989; Samelis *et al.*, 1994; Klein *et al.*, 1996). In LAB strains, sugar fermentation can be plasmid-mediated therefore sugar fermentation as a criterion for the identification of LAB could be unreliable (Hofer, 1977; Chassy *et al.*, 1978), although Lauret *et al.* (1996) found no difference in carbohydrate fermentation patterns between "wild-type" and plasmid-cured strains of *Lb. sakei*. Also, arginine hydrolysis by *Lb. sakei* is dependent on the level of glucose in the media. If the glucose content is above 2% (w/v), *Lb. sakei* will be negative but if it is less than 2% it will be positive (Samelis *et al.*, 1994). In this investigation the glucose content was below 2% so the arginine hydrolysis should have been detected if present. The failure of arginine hydrolysing *Lactobacillus* to produce acid from melibiose was not considered significant.

4.4.1.4 Identification of *Pseudomonas*

P. fragi was more commonly found to be the dominant *Pseudomonas* species on the surface of spoiled air-packaged and MAP beef and lamb after storage at one or 10°C. Psychrotrophic *Pseudomonas* species isolated from a variety of sources including meat, water and soil have encompassed the following: *P. fragi*, *P. lundensis*, *P. fluorescens*, *P. chloroplasis*, *P. aureofaciens*, *P. aeruginosa*, *P. glathei* and *P. mephitica* (Molin and Ternström, 1986). Numerical taxonomy of these isolates shows the greatest proportion of pseudomonads from a meat origin were *P. fragi* and *P. lundensis* species, with *P. fluorescens* comprising a minor but significant proportion (Molin and Ternström, 1986). Numerical taxonomy of *Pseudomonas* isolates from meat sources have consistently found more isolates cluster with *P. fragi* but there are still a number of isolates that cluster with *P. fluorescens* (Molin and Ternström, 1982; Shaw and Latty, 1984; Molin and Ternström, 1986). The higher occurrence of *P. fragi* on some meat could be attributed *P. fragi* being more metabolically versatile than *P. fluorescens* (Drosinos and Board, 1994) therefore enabling it to utilize more substrates as growth compounds.

4.4.2 PFGE FINGERPRINTING OF MEAT SPOILAGE ISOLATES

PFGE is not suitable for the identification of bacteria (Gautier *et al.*, 1996) but it is instead a powerful molecular typing technique which gives highly reproducible and discriminatory results (Johnson *et al.*, 1995; Matushek *et al.*, 1996; Talon *et al.*, 1996), enabling the degree of genomic relatedness between bacterial strains to be determined (Gothures and Tümmler, 1991). PFGE fingerprinting of bacteria has been largely restricted to bacteria of clinical importance (Buchrieser *et al.*, 1994; Boutrou *et al.*, 1995; Lück *et al.*, 1995; Proctor *et al.*, 1995; Tsen *et al.*, 1995; Yakubu and Pennington, 1995; Samore *et al.*, 1996; Talon *et al.*, 1996; Chetoui *et al.*, 1997) and to a lesser extent industrially important bacteria (Tanskanen *et al.*, 1990; Kelly *et al.*, 1993; Björkroth *et al.*, 1996; Gautier *et al.*, 1996; Roy *et al.*, 1996). PFGE could be a useful epidemiological tool in meat spoilage, enabling the tracing and control of contamination sources. PFGE had not previously been applied to bacteria of meat origin. *Brochothrix*, *Carnobacterium* and *Lactobacillus* species were selected for PFGE fingerprinting because of their importance in the spoilage of MAP meats. *Brochothrix* are capable of propagation at low water activity levels such as those of chilled meat surfaces (Skovgaard, 1985) and produce organolepically unacceptable end-products (Grau, 1983; Borch and Molin, 1989), so they are undesirable. The LAB group which includes *Lactobacillus* and *Carnobacterium* dominate the spoilage microflora of MAP meat (Enfors *et al.*, 1979; Erichsen and Molin, 1981b; Erichsen and Shaw, 1981; Gill and Jones, 1994) thus supporting epidemiological investigation. *Sma*I was the only enzyme of those screened which produced usable PFGE patterns.

4.4.2.1 PFGE fingerprinting of *B. thermosphacta* strains

The most interesting aspect of the *Brochothrix* strains isolated from beef and lamb was discovered when the PFGE fingerprints were examined, as no two fingerprints were identical. Bacterial strains are considered to be different if they differ by more than one band in a PFGE fingerprint (Skov *et al.*, 1995). The patterns for the *B. thermosphacta* strains were stable and reproducible with fresh batches of cells. The diversity that occurred within this species suggests that there is considerable genetic variation among *B. thermosphacta* strains. The prevalence of more than one banding pattern within a species is not uncommon. Between three and five different PFGE fingerprints were observed previously for reference strains of *Bifidobacterium* (Roy *et al.*, 1996). Twenty-seven strains *of P. jensenii* exhibited 17 different patterns (Gautier *et al.*, 1996). There were 18 different restriction patterns for 22 strains of *Lb. helveticus* (Lortal *et al.*, 1997). Only one other bacterial species has been reported to show the diversity of PFGE fingerprints that *B. thermosphacta* displayed. That was *H. pylori* (Taylor *et al.*, 1992). Subsequent mapping of the location of genes in *H. pylori* strains found that there were no

obvious similarities in gene order suggesting significant genetic diversity (Taylor *et al.*, 1992; Jiang *et al.*, 1996). Taylor *et al.* (1992) proposed several hypotheses for the observed variation in the *H. pylori* genome including:

(1) Different patterns were produced because of the movement of short repeated sequences which are ubiquitous in the genome (Lupski and Weinstock, 1992).

(2) Partial amplification of DNA possibly combined with the deletion of DNA. *Streptomyces* has been observed to delete and add DNA to its genome (Birch *et al.*, 1990). Although the loss of DNA from *Streptomyces* was accompanied by the loss of the attributes, up to 800kb could be deleted with *S. glaucescens* still capable of growth the on minimal media (Birch *et al.*, 1990). It is unlikely that this has occurred in *B. thermosphacta*, as *Streptomyces* strains have a genome size of 5.0 to 7.0Mb (Gladek and Zakrzewska, 1984), which is 2.5 to three times that of *B. thermosphacta*, therefore it would not be as significantly affected by the loss of DNA as *B. thermosphacta*. The addition of DNA fragments of between two and 90kb through amplification within the cell has been observed in *Streptomyces* (Birch *et al.*, 1990). This could account for the variations in both PFGE fingerprints and genome size.

(3) Silent point mutations which do not interfere with the phenotype (Tenover *et al.*, 1995) could explain the diversity, although a large number of point mutations would be required given the number of *Brochothrix* isolates tested.

(4) Genome rearrangement following natural transformation has been observed in *Campylobacter* (Wang and Taylor, 1990), *P. aeruginosa* and *P. putida* (Holloway and Morgan, 1986). Holloway and Morgan (1986) found considerable diversity within the genome of *P. aeruginosa* due to natural transformations but isolates still remained within a distinct species regardless of the criteria imposed. The competence of a cell, the ability to uptake naked DNA, is increased when cell numbers are high and in the presence of a secreted competence factor (Smith *et al*, 1981). Competence is only slightly less in late log phase than in early log phase (Wang and Taylor, 1990). This would suggest that genetic transformation in *B. thermosphacta* could occur during the latter stages of meat spoilage when bacterial numbers are high.

(5) Protection of the DNA from restriction enzymes by methylation could prevent enzyme digestion (Taylor *et al.*, 1992). Although *Sma*I is not affected by methylation.

Another suggestion for the diversity associated with the genome of *H. pylori* is that there is some genome rearrangement as a stress response following the infection of a new host (Taylor *et al.*, 1992; Jiang *et al.*, 1992). Inoculation studies in piglets failed to demonstrate any genome reorganisation in *H. pylori* (Akopyanz *et al.*, 1995). Using RAPD fingerprinting, the fingerprints of the *H. pylori* strains remained identical to the ancestral strain after eight and 15 serial passages through the piglets (Akopyanz *et al.*, 1995). A possibility for the diversity seen in *B. thermosphacta* could be due to the presence of plasmids. Dodd and Waites (1988) found that all of the *B. thermosphacta* strains isolated from sausages contained plasmids while the reference strain did not. The plasmid profiles of *B. thermosphacta* strains were similar to *K. zopfi* suggesting that gene exchange could occur between these two organisms thus increasing genetic diversity (Dodd and Waites, 1988). Another possibility is that the strains are actually different species: 16S rRNA sequencing and ribotyping would assist in determining the taxonomic relatedness between the *B. thermosphacta* isolates described in this thesis but it was not feasible to do this as part of this work due to time constraints.

4.4.2.2 PFGE fingerprinting of *Carnobacterium* species

The two isolates with the identical PFGE fingerprint came from different meats and grew under different atmospheric conditions. A13L03 was isolated from lamb that had been stored in air at 1°C while M11L03 came from spoiled beef stored at 1°C under a modified atmosphere of 30% CO_2/70% N_2. Isolate A23L03 came from the same atmosphere composition and meat type as A13L03, air-packaged lamb, but it had been incubated at 10°C instead of 1°C. A23L03 was closely related to the other two *C. divergens* strains isolated from meat, as there was only one band difference (Tenover *et al.*, 1995). This difference could be attributed to a point mutation in the cutting site for the enzyme used (Tenover *et al.*, 1995). The genome sizes of the *C. divergens* strains isolated from meat and the reference *C. divergens* were similar, differing by only 110kb, while the genome size of *C. piscicola* JG126 was 1Mb larger. *C. piscicola* is a bacteriocin-producing member of the *Carnobacterium* species (Ahn and Stiles, 1990a, b; Shillinger and Holzapfel, 1990; Schillinger *et al*; 1993; Saucier *et al.*, 1995). *C. piscicola* JG126 is also a bacteriocin producer (Jack *et al.*, 1996). The production of bacteriocins by *C. piscicola* strains has been demonstrated to be plasmid mediated (Ahn and Stiles, 1990b), and the

presence of a plasmid in *C. piscicola* JG126 could account at least in part for the 1Mb difference in genome size observed in the *Carnobacterium* species.

4.4.2.3 PFGE fingerprinting *Lactobacillus* species

The PFGE fingerprints for *Lactobacillus* from spoiled air-packaged and MAP meats were disappointing. The large number and small sizes of the fragments meant it was not possible to adequately separate all of the fragments, therefore the genome size could not be estimated. PFGE patterns for *Lb. sakei* associated with the production of ropy slime have been obtained using *Sma*I, where a section containing 14 to 18 fragments varying in size from 20 to 130kb was used to distinguish ropy slime producers (Björkroth *et al.*, 1996). Digestion with *Sma*I has also successfully differentiated *Lb. casei* and *Lb. paracasei* using PFGE (Ferrero *et al.*, 1996). Therefore the *Lactobacillus* species isolated from spoiled air-packaged beef and lamb for this investigation should have been successfully fingerprinted. The unexpected number of fragments seen in the *Lactobacillus* isolates could be accounted for by the presence of endogenous enzymes. Some strains of *Cl. difficile* cannot be PFGE fingerprinted as the DNA is degraded by exogenous enzymes (Samore *et al.*, 1996). This was not the case for the DNA of *Lactobacillus* species used in this investigation, as there was no smearing of the lanes on the gels. Alternatively, the genome of the *Lactobacillus* isolates from meat sources could contain a repeated sequence (Lupski and Weinstock, 1992) which harbored the *Sma*I restriction site, although the site should be rare (McClelland *et al.*, 1987). Daniel (1995) found that *Sfi*I digested *Lb. plantatum* into approximately 25 fragments, however, for the meat lactobacilli there was only partial digestion. The DNA for the *Lactobacillus* isolates was not degraded by several other restriction enzymes, although these worked successfully on other genera.

Despite the poor PFGE fingerprints, it was possible to identify some patterns among the isolates on the gels pulsed for two and five seconds. Some isolates possessed similar banding patterns which enabled them to be grouped together. Interestingly, the isolates which shared common patterns on the two-second gel also shared common patterns on the five-second gel. If the isolates that possessed similar patterns were compared to the identification results, some discrepancies were observed. *Lb. sakei* isolates A23L01, M23L06, M23L09 and M13L03, shared common patterns with *Lb. curvatus* isolates A21L03 and M21L03, and with *Lb. farciminis* isolate A11L02,which formed the largest group. *Lb. sakei* isolate M23L08 and *Lb. curvatus* A11L03 shared a common PFGE fingerprint as did *Lb. sakei* isolate A13L03 and *Lb. farciminis* isolate A21L01. These similarities cast some doubt over the validity

of the identification results based on biochemical tests, suggesting that perhaps the amplification and sequencing of the 16S rRNA gene or DNA-DNA hybridization may be required to clarify the identity of these isolates.

4.5. CONCLUSION

A majority of bacterial strains could be successfully identified to a species based on the biochemical tests applied. There were a variety of bacterial species associated with the spoilage of meat. *B. thermosphacta*, *C. divergens*, *H. alvei*, *Lb. sakei* and *P. fragi* were prevalent among the *Brochothrix*, *Carnobacterium*, *Enterobacteriaceae*, *Lactobacillus* and *Pseudomonas* isolates respectively. Although not all characteristics possessed by individual strains were typical of a particular species, a majority of the strains could be confidently designated to a species.

PFGE fingerprinting was to be reproducible (the fingerprinting of the isolates was repeated from the beginning yielding identical profiles) for *Brochothrix* and *Carnobacterium* species. Although the results for *Lactobacillus* were not as successful in terms of genome size measurement, valuable information could still be derived from the results in terms of identifying strains with similar genomic fingerprints. This was considered to be more reliable than the identification achieved through the biochemical analysis. *B. thermosphacta* strains exhibited a highly diverse array of PFGE fingerprints while still being more than 75% related to each other. There tended to be some clustering on the basis of meat origin which could be further pursued, but no clustering based on storage temperature or atmosphere. *C. divergens* isolated from meat were homogenous but distinct from the bacteriocin-producing *C. divergens* JG126 originally isolated from ham in Australia.

Chapter 5

Influence of pH, sodium chloride and chemical preservatives on the growth of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* species isolated from beef and lamb

5.1 INTRODUCTION

Microbial growth is influenced by pH, water availability and preservative concentration. The pH of the external environment will determine the internal pH of a bacterium (Gould and Measures, 1977; O'Sullivan and Condon, 1997). Although *Lactobacillus* are considered aciduric (Kandler and Weiss, 1986), a majority of bacteria involved in meat spoilage are neutrophiles. In relation to their external environment the internal pH of neutrophiles is alkaline (Pandan *et al.*, 1976; Booth, 1985; Poolman *et al.*, 1987) and it is this difference that drives the proton motive force (Kashket *et al.*, 1980; Kashket, 1985) vital for the survival of bacteria. The impact of pH will vary with the acidulant used (Poolman *et al.*, 1987; Brockelhurst and Lund, 1990; O'Sullivan and Condon, 1997). Failure to maintain pH homeostasis will result in a decline in growth rate and eventually loss of viability.

Water availability will also determine the viability of bacterial cells in different environments. Reducing the water availability increases lag phase (Blickstad, 1984; Li and Torres, 1993b), reduces growth rate (Calhoun and Frazier, 1962; Sperber, 1983; Prior *et al.*, 1987) and decreases cell mass (Roller and Anagnostopoulos, 1982; Blickstad, 1984). Water availability can be expressed as water activity (Christian, 1980) and the water activity is controlled by either dehydration or the addition of

Table 5.1 Bacterial isolates from each species identified for *Brochothrix*, LAB, *Enterobacteriaceae* and *Pseudomonas* in chapter 4. These isolates came from both air-packaged and MAP beef and lamb that had been stored at either one or 10°C.

Bacterial group	Isolate code	Temp (°C)	Meat	Atmosphere	Species
Brochothrix	A13B03	1	lamb	Air	*B. thermosphacta*
	A23B06	10	lamb	Air	*B. thermosphacta*
	M13B02	1	lamb	Modified	*B. thermosphacta*
LAB	M11L04	1	beef	Modified	*Lb. sakei*
	M21L03	10	beef	Modified	*Lb. curvatus*
	A11L02	1	beef	Air	*Lb. farciminis*
	M11L03	1	beef	Modified	*C. divergens*
Enterobacteriaceae	M23En02	10	lamb	Modified	*H. alvei*
	M13En02	1	lamb	Modified	*H. avlei*
	M13En03	1	lamb	Modified	*H. avlei*
	M11En01	1	beef	Modified	*H. avlei*
Pseudomonas	A11P04	1	beef	Air	*P. fluorescens*
	A13P03	1	lamb	Air	*P. fragi*

solutes. The response of a bacterium to a particular water activity will be dependent on the solute. Sodium chloride can inhibit bacteria at a higher water activity than glycerol (Sperber, 1987).

Microbial growth is inhibited by the presence of chemical preservatives such as potassium sorbate and para-hydroxybenzoates (parabens). Sorbates which are weak acid preservatives are the most widely utilised chemical preservative (Dziezak, 1986) while parabens are esters of organic acids (Davidson and Branden, 1981). The antimicrobial effects of potassium sorbate are dependent on the pH of the medium and the effects of the parabens are independent of pH. Although the modes of inhibition by sorbates and parabens differ, they will either increase lag phase, reduce growth rate and/or reduce the maximum number of bacteria (Robach, 1978; Chung and Lee, 1982; Restaino *et al.*, 1981; Gray *et al.*, 1984; Zamora and Zaritzky, 1987; El-Shenawy and Marth, 1988; Mendonca *et al.*, 1989; Tsay and Chou, 1989).

The aim of this series of experiments was to determine the effects of pH, sodium chloride, potassium sorbate, methyl-paraben and propyl-paraben on the growth of bacteria isolated from spoiled air-packaged beef and lamb. This series of experiments was designed to provide the required background information for further experiments involving determining the effects of combinations of preservatives, pH, atmosphere and temperature.

5.2 METHODS

5.2.1 Cultures and growth conditions

A member of each bacterial species identified in Chapter 4 was selected for determining the effect of pH, sodium chloride, potassium sorbate and methyl- and propyl-paraben. The isolates selected from *Brochothrix*, LAB, *Enterobacteriaceae* and *Pseudomonas*, and their original source are listed in Table 5.1. Since all of the *Brochothrix* isolates were identified as *B. thermosphacta,* isolates A13B03, A23B06 and M13B02, were chosen at random. The *Enterobacteriaceae* were selected on the basis of the Biolog results, however these strains were subsequently renamed as *H. alvei* for reasons discussed in Chapter 4. For determining the growth curves in liquid and solid media, *B. thermosphacta* A13B03, *C. divergens* M11L03, *H. alvei* M23En02, *Lb. sakei* M11L04 and *P. fluorescens* A11P04 were used as representative strains.

Brochothrix were always grown on TSB or TSA, *Carnobacterium* on TYGB or TYGA, *Enterobacteriaceae* and *Pseudomonas* on NB or NA and *Lactobacillus* on MRSB or MRSA. The

effects of pH, sodium chloride, potassium sorbate and methyl- and propyl-paraben were determined in buffered media. All media were incubated at 25°C for *Brochothrix* and *Pseudomonas* and at 30°C for *Enterobacteriaceae* and LAB. For enumeration, *Brochothrix*, *Enterobacteriaceae* and *Pseudomonas* were incubated for 48 hours and *Carnobacterium* and *Lactobacillus* for 72 hours.

5.2.2 Growth in liquid and solid media

Growth in both liquid and solid media was determined for a representative of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas*. The results were used to standardise culture conditions and set the experimental time course for later experiments.

Overnight cultures were inoculated into fresh broth to give an optical density at OD_{610nm} of 0.01, then cultures were incubated at optimum temperature in a shaking incubator, except for LAB which were not shaken. The OD_{610nm} of the culture was measured every hour until three constant readings were taken, indicating stationary phase.

Alternatively, an aliquot of overnight culture was placed in fresh broth and grown until late exponential phase was reached, as determined from the liquid media growth curve. The cells were diluted to give a cell destiny of 10^3 cfu/mL then 100µL was spread onto 10mL agar plates. Replicate plates were incubated then removed in triplicate at three hourly intervals. The agar was removed, macerated with 50mL diluent and spiral plated onto 16mL agar plates, then incubated before counting.

5.2.3 Effect of pH, sodium chloride and preservative concentration on bacterial growth

For determining the response of growth rates to pH, sodium chloride, potassium sorbate and methyl- and propyl-paraben, cultures were prepared as for the agar growth curve. The cultures were spread plated onto agar plates at pH 4.5-7.0 or plates containing 0 to 5% sodium chloride or 0 to 1% potassium sorbate or 0 to 0.5% methyl-paraben or 0 to 0.1% propyl-paraben at pH 6.0. The plates were incubated until the control sample (which was at pH 6.0 with no additives) was predicated to have reached late exponential phase, as determined by the agar growth curves: 15 hours for *Brochothrix*, 15 hours for *Carnobacterium*, 12 hours for *Enterobacteriaceae*, 18 hours for *Lactobacillus* and 16 hours for *Pseudomonas*. Bacteria were enumerated as for the growth curve on agar. Growth rates were then calculated and expressed as specific growth rate.

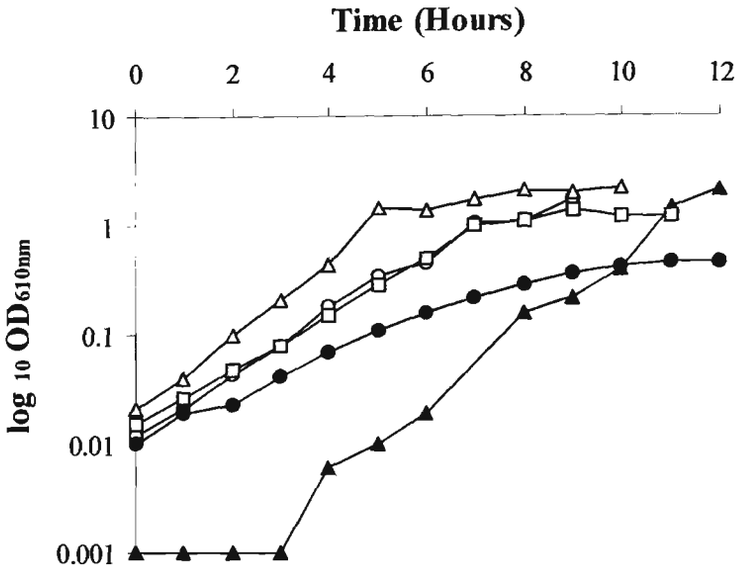


Figure 5.1 Growth of representative cultures in liquid media. *B. thermosphacta* A13B03(○) was grown in TSB, *C. divergens* (●) was grown in TYGB, *Lb. sakei* (△) was grown in MRSB and *H. alvei* M23En02 (▲) and *P. fluorescens* (□) were grown in NB.

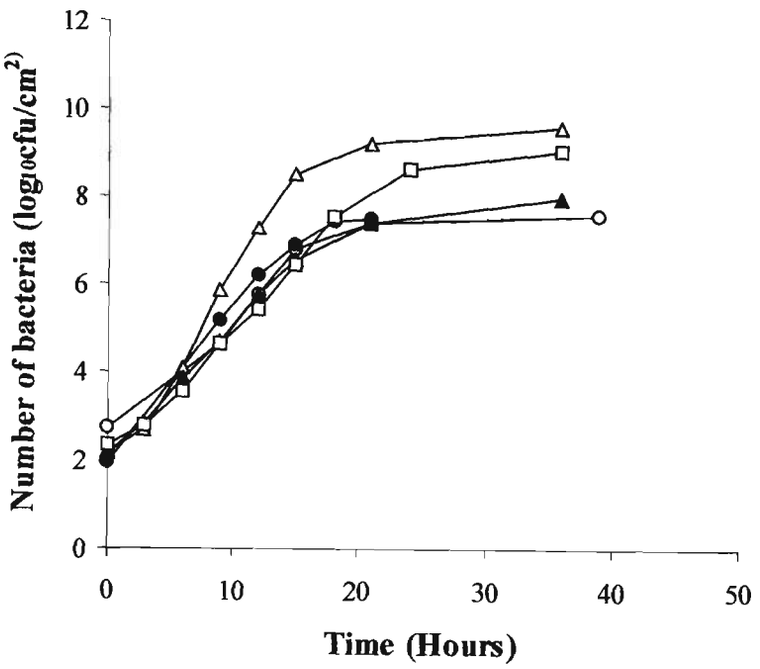


Figure 5.2 Growth of representative cultures on solid media. *B. thermosphacta* A13B03 (○) was grown on TSA, *C. divergens* (●) was grown on TYGA, *Lb. sakei* (△) was grown on MRSA and *H. alvei* M23En02 (▲) and *P. fluorescens* (□) were grown on NA.

5.3 RESULTS

5.3.1 Growth rates in broth and on agar surfaces

The growth of representative isolates from *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* in liquid media is demonstrated in Figure 5.1. The OD_{610nm} of the overnight cultures in 5mL liquid media was determined in order to calculate the volume of culture required to give an OD_{610nm} of 0.01 in 10mL of liquid media. Although calculations indicated that sufficient culture was added to give the required starting OD_{610nm} for *Lb. sakei,* the initial OD_{610nm} measured was 0.001. It is not possible to conclude that *Lb. sakei* was in a lag phase in the first three hours, as the OD_{610nm} was very low during this period. Stationary phase in liquid media was reached by *B. thermosphacta* A13B03 after seven hours incubation, *C. divergens* after 10 hours incubation, *H. alvei* M23En02 after five hours incubation, *Lb. sakei* after 11 hours incubation and *P. fluorescens* after seven hours incubation.

The growth of *B. thermosphacta* A13B03, *C. divergens*, *H. alvei* M23En02, *Lb. sakei* and *P. fluorescens* on solid media is shown in Figure 5.2. No lag phase was observed for any isolate under the experimental conditions used. The time required for each species to reach population numbers of 10^6 to 10^7 cfu/cm^2 were: 15 hours for B. *thermosphacta* A13B03, 15 hours for *C. divergens*, 12 hours for *H. alvei* M23En02, 18 hours for *Lb. sakei* and 16 hours for *P. fluorescens*.

These experiments were conducted to determine the time-frame required for the standardisation of cultures in liquid media, so that each culture was transferred to solid media in the same phase of growth. For solid media, cultures were grown first in liquid media to late exponential phase as indicated by the OD_{610nm}. The incubation time was extended if the culture failed to increase to the desired OD_{610nm} and if the desired OD_{610nm} was exceeded, the culture was discarded. On solid media, cultures were grown until the population density of the control was estimated to be between 10^6-10^7 cfu/cm^2.

5.3.2 Effect of pH on the growth of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* species

The effect of pH levels ranging from 4.5 to 7.0 on the growth of each species of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* isolated from beef and lamb is shown in Figure 5.3. The specific growth rate for *B. thermosphacta* A23B06 and M13B03 increased at a similar rate as the pH increased from 4.5 to 7.0, while *B. thermosphacta* A13B03 had a higher specific growth rate over pH 4.5 to 6.0, but not at pH 6.5 or 7.0. Only at pH 5.0 was the specific growth rate of *B. thermosphacta* A13B03 significantly greater than for the other two *B. thermosphacta* strains. For

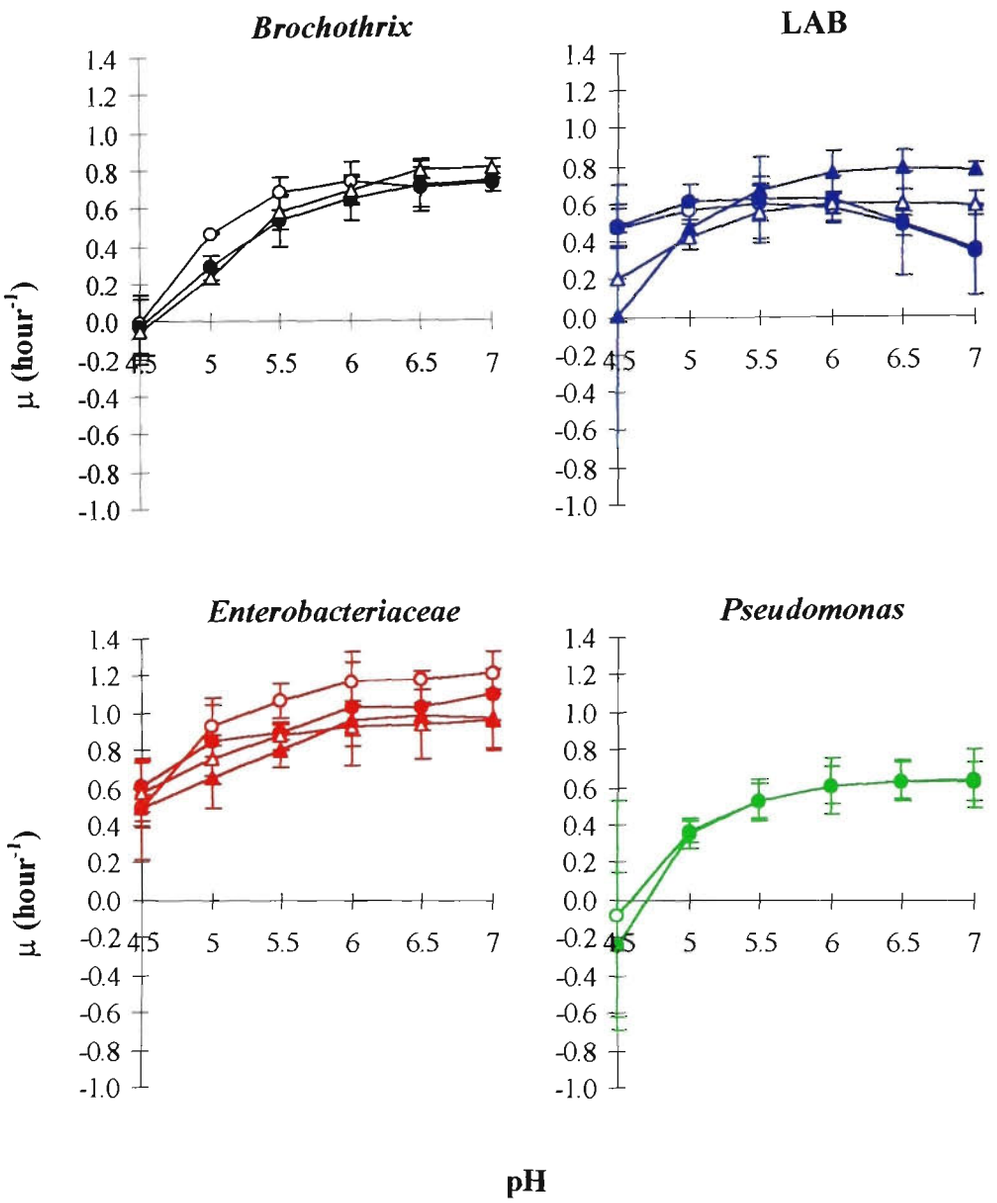


Figure 5.3 The effect of pH 4.5 to 7.0 on the specific growth rate (μ) of *Brochothrix*, LAB, *Enterobacteriaceae* and *Pseudomonas* species on solid media. The strains used were: *B. thermosphacta* A13B03 (○), A23B06 (●) and M13B03 (△); *Lb. sakei* (○), *Lb. curvatus* (●), *Lb. farciminis* (△) and *C. divergens*(▲); *H. alvei* M23En02 (○), M13En02 (●), M13En03 (△) and M11En01 (▲); and *P. fluorescens* (◌) and *P. fragi* (●). *B. thermosphacta* strains were grown on buffered TSA, *Lactobacillus* on buffered MRSA, *C. divergens* on TYGA and *H. alvei* and *Pseudomonas* on buffered NA.

the three *B. thermosphacta* strains, the number of cells declined at pH 4.5, indicating that the bacteria were dying. For *B. thermosphacta* A13B03, the specific growth rate increased reaching a maximum at pH 6.0 of 0.74 h^{-1} which was maintained at pH 6.5 and 7.0. The specific growth rate for *B. thermosphacta* A23B06 and M13B03 increased with pH, reaching a maximum rate at pH 7.0 of 0.75 and 0.81 h^{-1} respectively.

There were significant differences in specific growth rates between the *Lactobacillus* species under the conditions tested (Figure 5.3). The specific growth rates for *Lb. sakei* and *Lb. curvatus* were very similar over the pH range of 4.5 to 7.0. Both were able to grow more rapidly in the lower pH range, particularly pH 5.0 to 6.0, than around neutral, pH 6.5 and 7.0 (Figure 5.3). *Lb. farciminis* was able to grow over the whole pH range, although it had a significantly lower specific growth rate than the other two *Lactobacillus* species at pH 4.5. At pH 6.0, the specific growth rates for *Lb. sakei*, *Lb. curvatus* and *Lb. farciminis* were similar at 0.57-0.62 h^{-1}. From pH 6.0 to 7.0, the specific growth rate of *Lb. farciminis* remained constant while for *Lb. sakei* and *Lb. curvatus* it decreased. *C. divergens* had a significantly higher specific growth rate at pH 7.0 compared to the *Lactobacillus* species. At pH 4.5, *C. divergens* experienced no net increase or decrease in bacterial numbers. From this point there was an increase in specific growth rate up to pH 6.0, where the growth rate remained virtually identical from pH 6.0 to 7.0 (Figure 5.3).

Among the *H. avlei* strains there was no significant difference between the strains in the specific growth rates over pH 4.5 to 7.0 under the conditions used in this investigation (Figure 5.3). The lowest specific growth rate observed was for the *H. alvei* strains ranged from 0.49 to 0.6 h^{-1} at pH 4.5. As the pH increased, the specific growth rate continued to increase, reaching a maximum of 0.94 to 1.22 h^{-1} at pH 6.0, which continued at this over pH 6.5 to 7.0. *H. alvei* M23En02 exhibited the largest increase in growth rate of the four *H. alvei* strains tested under these experimental conditions.

In contrast to the *H. alvei* strains, the other Gram-negative bacteria, *P. fragi* and *P. fluorescens*, were more sensitive to pH 4.5 (Figure 5.3). The viable counts detected decreased at pH 4.5, indicating that the bacteria were dying. At pH 5.0, specific growth rate increased until this reached a plateau at pH 6.0, where the specific growth rate was 0.6 h^{-1}. At pH 6.5 and 7.0 the specific growth rate of the *Pseudomonas* species was approximately half that of the *H. alvei* strains.

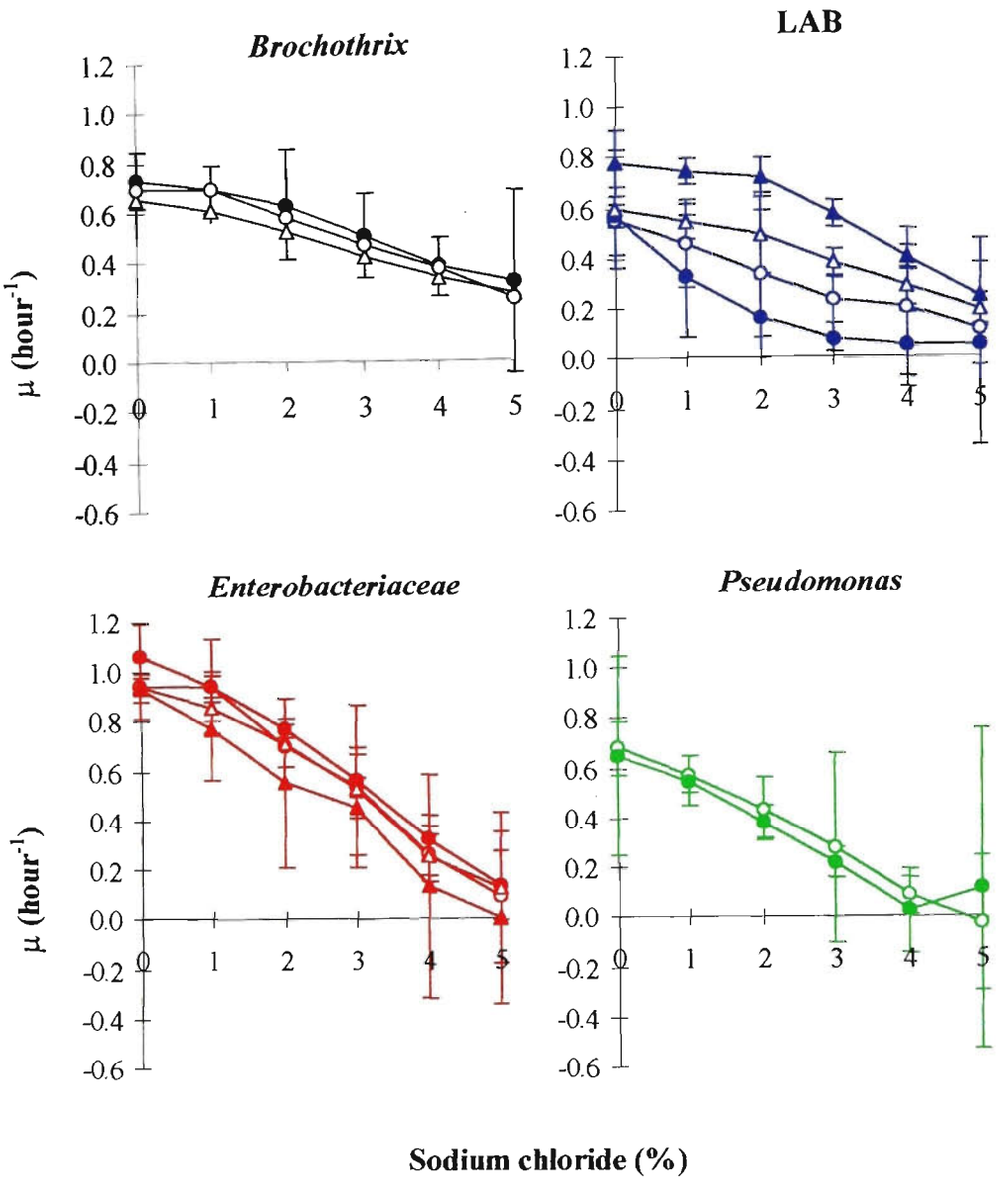


Figure 5.4 The effect of sodium chloride on the specific growth rate (μ) of *Brochothrix*, LAB, *Enterobacteriaceae* and *Pseudomonas* species on solid media. The strains used were: *B. thermosphacta* A13B03 (○), A23B06 (●) and M13B03 (△); *Lb. sakei* (○), *Lb. curvatus* (●), *Lb. farciminis* (△) and *C. divergens*(▲); *H. alvei* M23En02 (○), M13En02 (●), M13En03 (△) and M11En01 (▲); and *P. fluorescens* (○) and *P. fragi* (●). *B. thermosphacta* strains were grown on buffered TSA, *Lactobacillus* on buffered MRSA, *C. divergens* on TYGA and *H. alvei* and *Pseudomonas* on buffered NA.

5.3.3 Effects of sodium chloride on the growth of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* species

The influence of increasing sodium chloride concentration on the growth of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* species isolated from spoiled beef and lamb is demonstrated in Figure 5.4. The final amounts of sodium chloride within the solid media ranged from one to 5%, the control media contained no sodium chloride.

There was no significant difference amongst the *B. thermosphacta* strains. All three *B. thermosphacta* strains exhibited similar responses to elevations in the sodium chloride concentrations. In the absence of sodium chloride, specific growth rate ranged from 0.65 to 0.73 h^{-1} which gradually decreased to 0.26-0.32 h^{-1} at 5% sodium chloride. In general, this gave the *B. thermosphacta* strains the highest specific growth rate at 5% sodium chloride.

The LAB group had the most diverse response of the four groups to the presence of sodium chloride (Figure 5.4). The specific growth rate of *Lb. sakei* and *Lb. farciminis* gradually decreased from 0.55 and 0.60 h^{-1} in the absence of sodium chloride to 0.11 and 0.19 h^{-1} at 5% sodium chloride respectively. The growth rate of *Lb. curvatus* decreased rapidly between zero and 2% sodium chloride (from 0.57 to 0.17 h^{-1}), thus giving it the lowest growth rate of the LAB group. *C. divergens* had the highest growth rate of the LAB over the concentrations tested, which was significantly higher at one and 3% sodium chloride. *C. divergens* also exhibited the most dramatic decrease in specific growth rate after the amount of sodium chloride added increased above 2%. Between the absence of sodium chloride and addition of 2% sodium chloride, the specific growth rate gradually decreased from 0.78 to 0.72 h^{-1}, but when the concentration increased to 3% the specific growth rate decreased to 0.57 h^{-1}, which then continued to decrease with increasing sodium chloride.

The Gram-negative bacteria, *H. alvei* strains and *Pseudomonas* species, experienced a sharp decrease in specific growth rate when exposed to sodium chloride (Figure 5.4). There was no significant difference in the specific growth rates among the *H. alvei* strains, with specific growth rates decreasing from 0.93-1.06 h^{-1} in the absence of sodium chloride to 0-0.13 h^{-1} with 5% sodium chloride. *H. alvei* M23En02 was unaffected by 1% sodium chloride, while the remaining *H. alvei* strains experienced a slight decrease in specific growth rate over the same range. *H. alvei* M11En01 had the lowest specific growth rate of the four strains over the range tested, but this was not significantly different.

The specific growth rates of the two *Pseudomonas* species were virtually identical over the whole range of sodium chloride tested (Figure 5.4). In the absence of sodium chloride, *P. fluorescens* and *P. fragi*

had specific growth rates of 0.68 and 0.65 h^{-1} respectively, which decreased rapidly to 0.09 and 0.03 h^{-1} at 4% sodium chloride. At 5% sodium chloride, *P. fluorescens* declined marginally in number while *P. fragi* exhibited some weak growth.

5.3.4 Effects of preservatives on the growth of *Brochothrix, Enterobacteriaceae,* LAB and *Pseudomonas* species

5.3.4.1 Determining the concentrations of preservative agents required

Before the effect of potassium sorbate, methyl-paraben and propyl-paraben on the growth of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* species could be measured, the concentrations over which growth was able to occur had to be determined. Five sequential concentrations of preservative were evaluated at pH 6.0. This was done to facilitate the selection of preservative concentrations for the later combined experiment of preservatives, pH, atmosphere and temperature. The highest amount of each preservative agent was initially between one and 5% then this decreased until the concentration range which yielded the desired growth effects was achieved. The appropriate concentration ranges were determined for each preservative agent using the representative cultures: *B. thermosphacta* A13B03, *C. divergens*, *H. alvei* M23En02, *Lb. sakei* and *P. fluorescens*, as described in the following. The pH of the media was pH 6.0.

5.3.4.2 Effects of potassium sorbate on the growth of *Brochothrix, Enterobacteriaceae,* LAB and *Pseudomonas* species

The response of the *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* species to the presence of increasing concentrations of potassium sorbate is demonstrated in Figure 5.5. Of the four bacteria groups the *B. thermosphacta* strains were the most sensitive to potassium sorbate (Figure 5.5). There was a sharp decrease in specific growth rate as the potassium sorbate concentration increased to 0.04% (Figure 5.5). When the concentration was increased above this point there were no culturable cells detected.

Each LAB isolate tested was able to grow on 1% potassium sorbate (Figure 5.5). *Lb. sakei* and *Lb. curvatus* were more resistant to potassium sorbate than *Lb. farciminis* or *C. divergens*. The decline in specific growth rate of *Lb. sakei* and *Lb. curvatus* in the presence of potassium sorbate was not significantly different from *Lb. farciminis* but it was significantly different from *C. divergens*. The specific growth rate for *Lb. sakei* and *Lb. curvatus* decreased gradually over the concentration range tested, with specific growth rates at values of 0.24 and 0.23 h^{-1} respectively at 1% potassium sorbate.

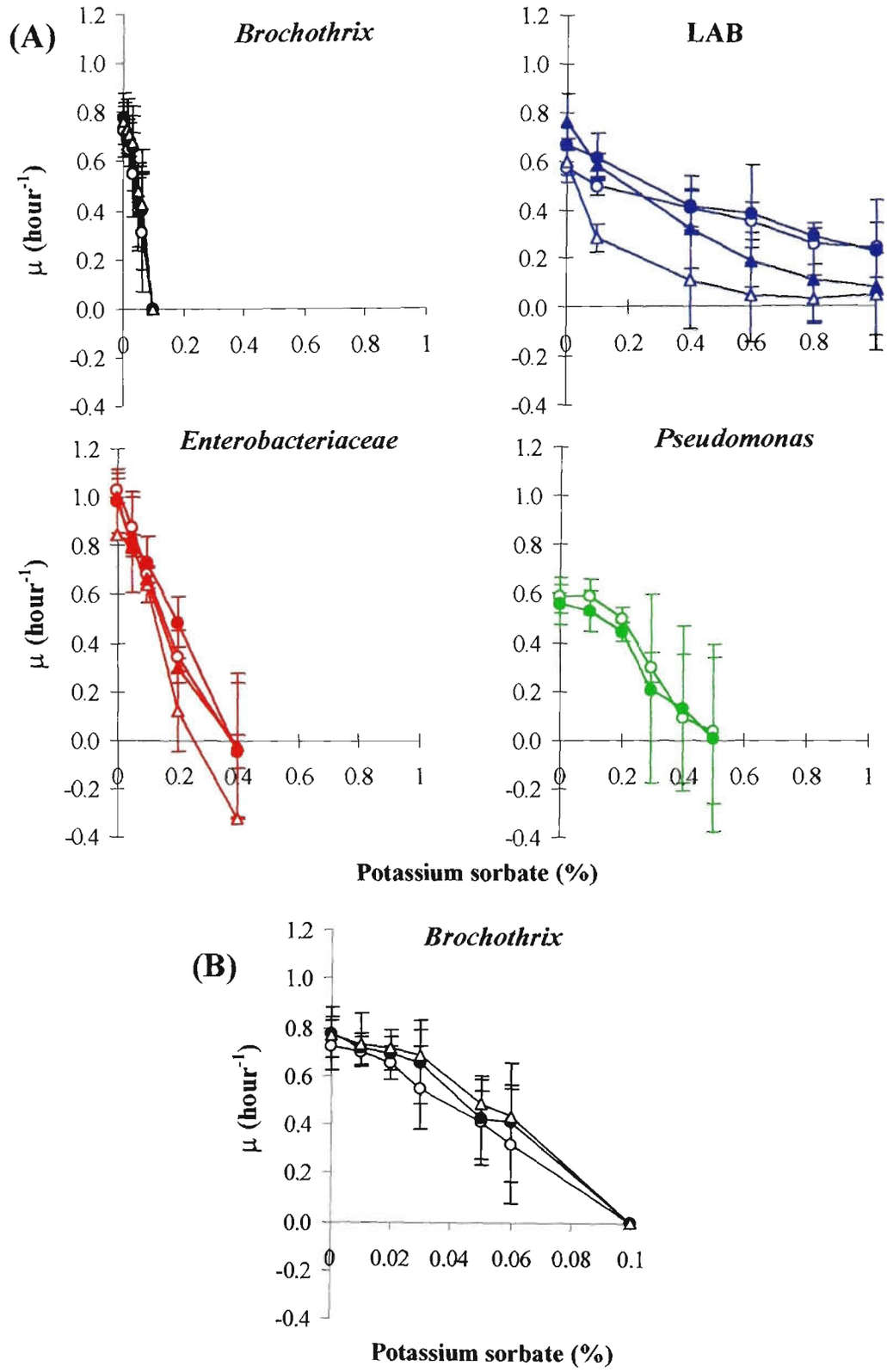


Figure 5.5 (**A**) The effect of potassium sorbate on the specific growth rate (μ) of *Brochothrix,* LAB, *Enterobacteriaceae* and *Pseudomonas* species on solid media. The strains used were: *B. thermosphacta* A13B03 (○), A23B06 (●) and M13B03 (▲); *Lb. sakei* (○), *Lb. curvatus* (●), *Lb. farciminis* (▲) and *C. divergens*(▲); *H. alvei* M23En02 (○), M13En02 (●), M13En03 (▲) and M11En01 (▲); and *P. fluorescens* (○) and *P. fragi* (●). *B. thermosphacta* strains were grown on buffered TSA, *Lactobacillus* on buffered MRSA, *C. divergens* on TYGA and *H. alvei* and *Pseudomonas* on buffered NA.. (**B**) Expansion of the scale for the effect of potassium sorbate on *Brochothrix.*

Lb. farciminis and *C. divergens* followed a similar pattern of inhibition, with *Lb. farciminis* initially having the greater specific growth rate but showing a greater rate of decrease. The difference in specific growth rate between *Lb. farciminis* and *C. divergens* was only significant at 0.1% potassium sorbate, with *C. divergens* having the higher specific growth rate. The specific growth rate for *Lb. farciminis* and *C. divergens* at 1% potassium sorbate was 0.05 and 0.08 h^{-1} respectively.

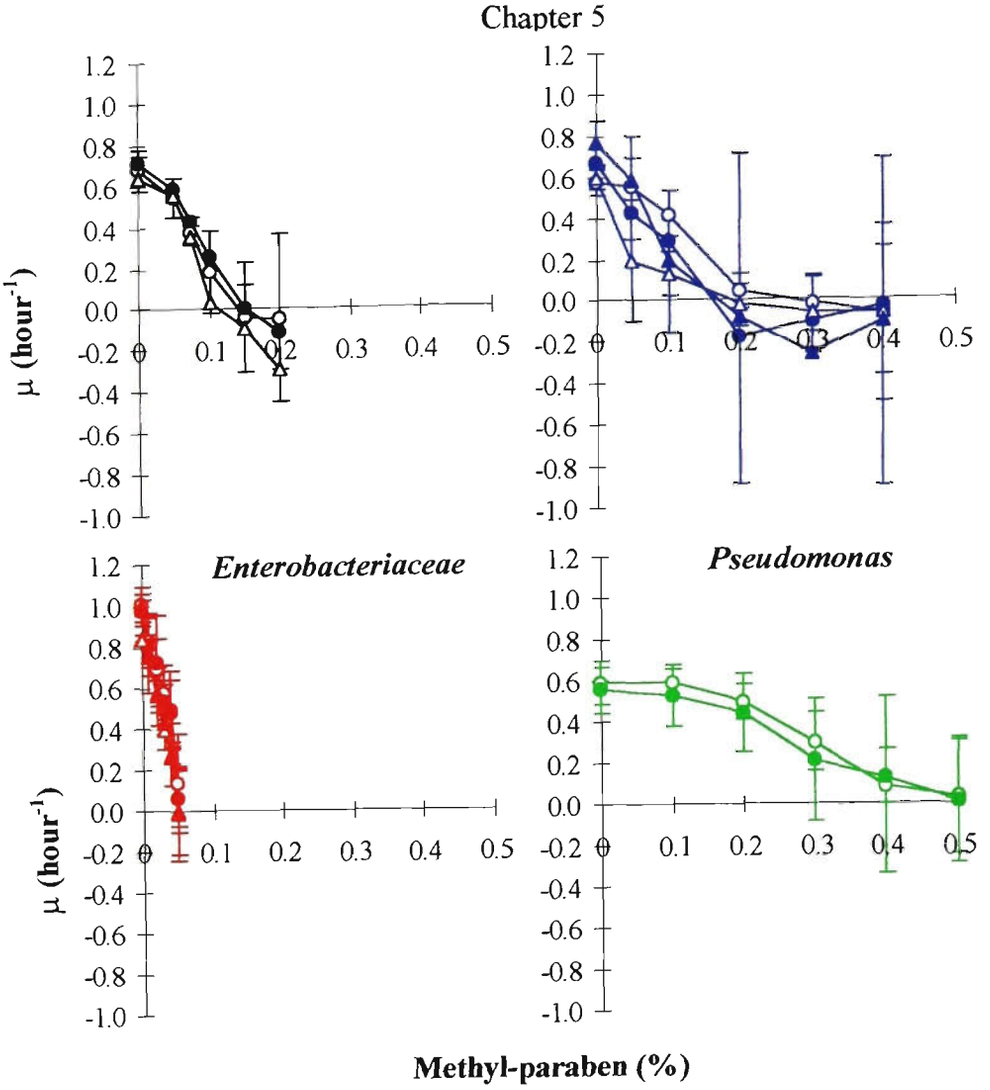
The growth rates of the *H. alvei* strains decreased almost linearly as the concentration of potassium sorbate increased (Figure 5.5). Although not significantly different from each other, the greatest difference in growth time between the strains occurred at 0.2% potassium sorbate. At this point the specific growth rate of each strain had decreased from the control rate by: 34% for *H. alvei* M13En02, 50.3% for *H. alvei* M23En02, 55.5% for *H. alvei* M11En01 and *H. alvei* M13En03 exhibited the greatest decrease of 81.4%. When the level of potassium sorbate increased to above 0.2% potassium sorbate, the number of viable cells of *H. alvei* M13En03 detected decreased from the initial numbers, indicating that this strain was dying. The specific growth rates of the remaining three *H. alvei* strains decreased until there was a slight decline in the numbers of bacteria detected at 0.4% potassium sorbate.

Over the potassium sorbate range of zero to 0.5% the specific growth rates for both *Pseudomonas* species decreased gradually at similar rate, with no significant difference between the species (Figure 5.5). There was no change in the growth rate of *P. fragi* between zero and 0.05% potassium sorbate while the growth rate of *P. fluorescens* decreased slightly. When the level of potassium sorbate was increased above 0.1%, the specific growth rates of *P. fluorescens* and *P. fragi* decreased in tandem, with *P. fragi* having a slightly higher specific growth rate at 0.1 to 0.3% potassium sorbate. At 0.5% potassium sorbate, the specific growth rates of *P. fluorescens* and *P. fragi* were negligible. The large standard deviation was caused by the failure of growth on some plates.

5.3.4.3 Effects of methyl-paraben on the growth of *Brochothrix, Enterobacteriaceae,* LAB and *Pseudomonas* species

The effect of methyl-paraben on the growth rates of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* are demonstrated in Figure 5.6. Within each group there was no significant difference in specific growth rate observed under these conditions.

The growth of the *B. thermosphacta* strains was inhibited at 0.15% methyl-paraben over the 16 hour incubation. In the absence of methyl-paraben specific growth rate ranged from 0.62 to 0.72 h^{-1}. With increasing methyl-paraben concentration, the specific growth rate of the *B. thermosphacta* strains



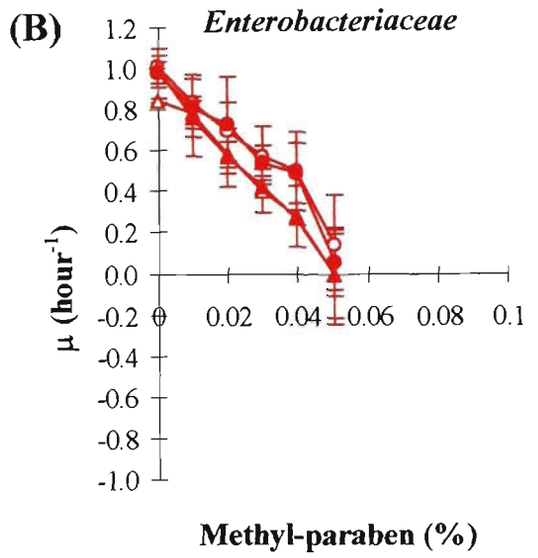


Figure 5.6 **(A)** The effect of methyl-paraben on the specific growth rate (μ) of *Brochothrix*, LAB, *Enterobacteriaceae* and *Pseudomonas* species on solid media. The strains used were: *B. thermosphacta* A13B03 (O), A23B06 (●) and M13B03 (Δ); *Lb. sakei* (O), *Lb. curvatus* (●), *Lb. farciminis* (Δ) and *C. divergens*(▲); *H. alvei* M23En02 (O), M13En02 (●), M13En03 (Δ) and M11En01 (▲); and *P. fluorescens* (O) and *P. fragi* (●). *B. thermosphacta* strains were grown on buffered TSA, *Lactobacillus* on buffered MRSA, *C. divergens* on TYGA and *H. alvei* and *Pseudomonas* on buffered NA.

decreased. The most notable decrease was for *B. thermosphacta* M13B02 which had a specific growth rate of 0.04 h^{-1} at 0.1% methyl-paraben, while *B. thermosphacta* A13B03 and A23B06 had rates of 0.18 and 0.22 per hour respectively. Once the concentration of methyl-paraben increased above 0.15%, the number of viable cells detected declined over the incubation period.

The growth rates of the LAB group declined until the concentration of methyl-paraben increased to 0.2%, at which concentration only *Lb. sakei* was able to grow while the remaining LAB were dying (Figure 5.6). *Lb. sakei* was slightly, but not significantly, more resistant to methyl-paraben than the other LAB species, as no reduction in the growth rate occurred between zero and 0.05% methyl-paraben. However, *Lb. curvatus*, *Lb. farciminis* and *C. divergens* experienced a decrease in growth rate at concentrations of methyl-paraben between zero and 0.05%. The number of viable cells detected for all LAB decreased at 0.3 and 0.4% methyl-paraben.

Each *H. alvei* strain experienced a rapid decline in growth rate as the concentration of methyl-paraben increased from zero to 0.05% (Figure 5.6) At 0.05%, both *H. alvei* M13En03 and M11En01 exhibited no growth while *H. alvei* M23En02 and M13En02 had specific growth rates of 0.14 and 0.06 h^{-1} respectively.

In contrast to *Enterobacteriaceae*, the two *Pseudomonas* species were more resistant to the inhibitory effects of methyl-paraben (Figure 5.6). There was no decline in specific growth rate between zero and 0.01% methyl-paraben. When the methyl-paraben concentration increased further to 0.2%, there was a decrease in specific growth rate which continued until the rate almost reached zero at 0.5% methyl-paraben.

5.3.4.4 Effects of propyl-paraben on the growth of *Brochothrix, Enterobacteriaceae,* LAB and *Pseudomonas* species

The response of *Brochothrix, Enterobacteriaceae,* LAB and *Pseudomonas* to increasing concentrations of propyl-paraben is shown in Figure 5.7. As for methyl-paraben, there was no significant difference among the *B. thermosphacta* strains, *H. alvei* strains, LAB species or *Pseudomonas* species. One exception was at 0.02% propyl-paraben, where *Lb. farciminis* had a significantly lower specific growth rate compared to the other three LAB species.

Although not significant, there were some differences in the responses of the *B. thermosphacta* strains when the concentration of propyl-paraben increased above 0.2% (Figure 5.7). The specific growth rate

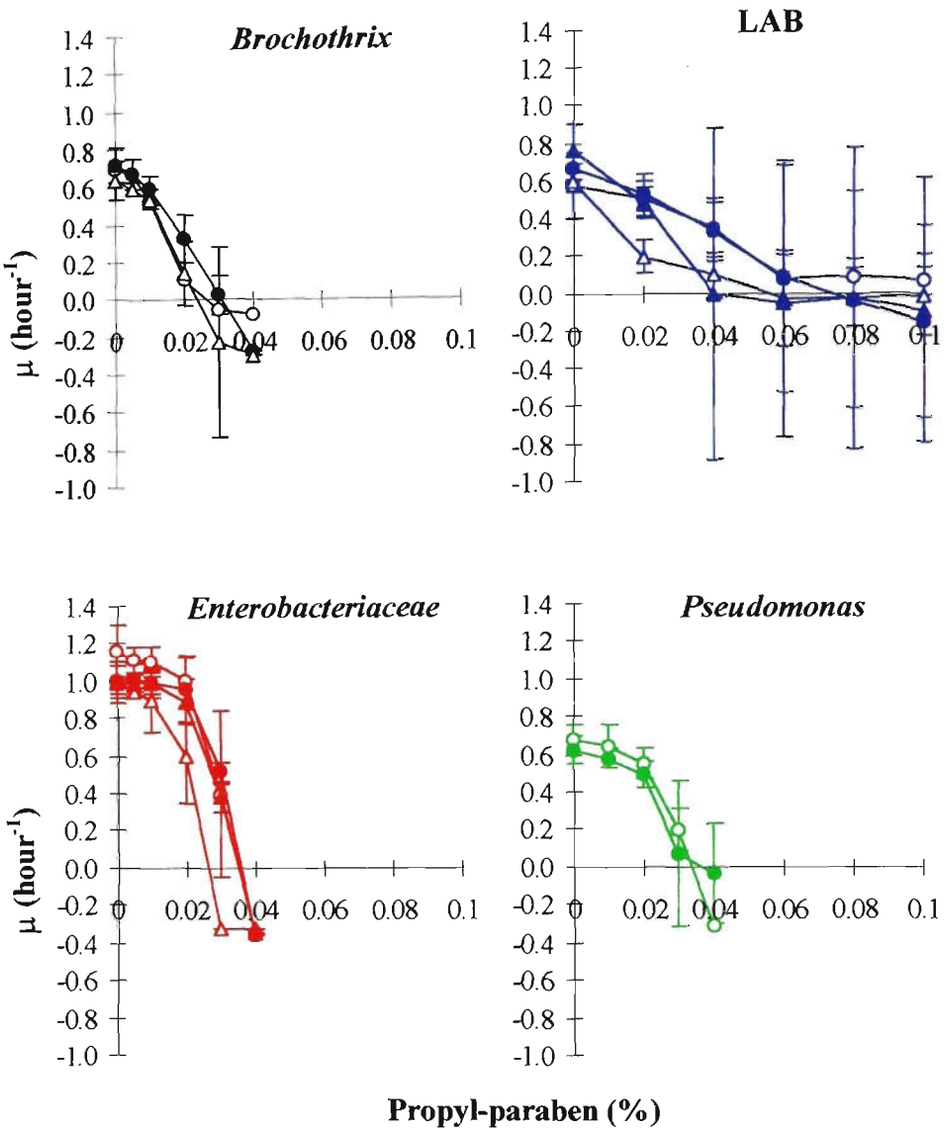


Figure 5.7 The effect of propyl-paraben on the specific growth rate (μ) of *Brochothrix*, LAB, *Enterobacteriaceae* and *Pseudomonas* species on solid media. The strains used were: *B. thermosphacta* A13B03 (○), A23B06 (●) and M13B03 (△); *Lb. sakei* (○), *Lb. curvatus* (●), *Lb. farciminis* (△) and *C. divergens*(▲); *H. alvei* M23En02 (○), M13En02 (●), M13En03 (△) and M11En01 (▲); and *P. fluorescens* (○) and *P. fragi* (●). *B. thermosphacta* strains were grown on buffered TSA, *Lactobacillus* on buffered MRSA, *C. divergens* on TYGA and *H. alvei* and *Pseudomonas* on buffered NA.

began to declining as propyl-paraben concentration increased above 0.01%. For *B. thermosphacta* A13B03 and M13B02, there was a greater decrease in specific growth rate between 0.01 and 0.02% propyl-paraben with the specific growth rates declining from 0.52 to 0.11 and 0.54 to 0.14 h^{-1} respectively. At 0.03% propyl-paraben, *B. thermosphacta* A13B03 and M13B03 were declining in number while *B. thermosphacta* A23B06 was still able to increase, although the specific growth rate was only slightly above zero.

The specific growth rate of the *Lactobacillus* species approached zero over the 18 hour incubation period when the concentration of propyl-paraben was 0.04% (Figure 5.7). Of this group, *Lb. sakei* was slightly more resistant to the inhibitory effects of propyl-paraben over the concentration range tested. *Lb. sakei* and *Lb. curvatus* had the same response to propyl-paraben until the concentration reached 0.08%, where the number of viable *Lb. curvatus* decreased below the initial level. Above 0.06% propyl-paraben, there was no further decrease in the specific growth rate of *Lb. sakei*. *Lb. farciminis* exhibited the sharpest decrease in specific growth rate as the concentration of propyl-paraben increased above 0.02%. Above 0.02% propyl-paraben, the reduction in *Lb. farciminis* growth rate became more gradual, until finally viable numbers detected began to decline at 0.06% propyl-paraben. There was no further reduction in viable count of *Lb. farciminis* as the concentration of propyl-paraben increased. *C. divergens* experienced the greatest reduction in specific growth rate when the propyl-paraben concentration increased from 0.02 to 0.04%. Above 0.04%, the number of viable *C. divergens* cells detected decreased.

The *H. alvei* strains exhibited a dramatic decrease in specific growth rate over concentrations of propyl-paraben between 0.01 to 0.04% (Figure 5.7). *H. alvei* M23En02 experienced a decrease in growth rate as the concentration of propyl-paraben increased from zero to 0.02%, while the other three *H. alvei* strains remained unchanged. As the concentration increased from 0.02% to 0.03% propyl-paraben, there was a sharp decrease in specific growth rate for all *H. alvei* strains. The specific growth rate of *H. alvei* M13En03 decreased from 0.99 to 0.51 h^{-1}, *H. alvei* M23En02 from 1.10 to 0.39 h^{-1}, *H. alvei* M11En01 from 0.99 to 0.37 h^{-1}, while *H. alvei* M13En02 had the largest decrease in specific growth rate from 0.89 to -0.35 h^{-1}. *H. alvei* M13En02 did not exhibit any further decrease in growth rate at the higher propyl-paraben concentrations, while the specific growth rate of the remaining three isolates decreased to the same negative value at 0.04% propyl-paraben.

The *Pseudomonas* species exhibited a similar but less dramatic decline in specific growth rate as the *H. alvei* strains, with the largest decrease occurring between 0.02% to 0.03% propyl-paraben (Figure 5.7). For both *P. fluorescens* and *P. fragi*, specific growth rate began to decrease as the propyl-paraben

concentration increased from zero to 0.005%. A further increase from 0.02% to 0.03% propyl-paraben resulted in decreases in the specific growth rate of *P. fluorescens* from 0.55 to 0.19 h^{-1}, while *P. fragi* declined from 0.49 to 0.07 h^{-1}. The growth rates for both species was negative at 0.04% propyl-paraben.

5.4 DISCUSSION

The specific growth rate of *Brochothrix*, *Enterobacteriaceae*, LAB and *Pseudomonas* were influenced by pH, sodium chloride, potassium sorbate and methyl- and propyl-paraben. Due to the use of different media for the *B. thermosphacta* isolates and LAB species, only the results of the *H. alvei* strains and *Pseudomonas* species can be compared as these were grown on the same media, buffered NA. Variations in the sensitivity to pH, sodium chloride, potassium sorbate or methyl- or propyl-paraben observed among the groups could be attributed to the different media used. Potassium sorbate and methyl- and propyl-paraben are not currently used in fresh meat preparations. These preservatives were included in this investigation because they inhibit microbial growth at low concentrations.

5.4.1 Effect of pH on the growth of meat spoilage bacteria

Hydrochloric acid was chosen as the acidulant rather than lactic acid as this enabled evaluation of the effect of pH, which was the parameter of interest. At the pH levels used, lactic acid would exist in both the dissociated and undissociated forms (Karapinar and Gönül, 1992). The undissociated acid has greater anti-microbial properties (Corlett and Brown, 1980; Eklund, 1983; Faber et al., 1989; Karapinar and Gönül, 1992; Moir and Eyles, 1992). This is discussed further in Chapter 7.

The first observations of the impact of the pH of the surrounding medium on the metabolic activity of microorganisms was made in 1942 by Gale and Epps (Booth, 1985). *B. thermosphacta, C. divergens, H. alvei,* and *Pseudomonas* species can all be regarded as neutrophiles as they had maximum growth rates at pH 6-7. *Lactobacillus* are aciduric bacteria, as they grow optimally over pH 5.5-6.2 (Kandler and Weiss, 1986). The results reported in this chapter support these observations as *Lb. sakei, Lb. curvatus* and *Lb. farciminis* had their highest specific growth rates over this range, although, *Lb. farciminis* continued at the same growth rate at pH 6.5 and 7, where as *Lb. sakei* and *Lb. curvatus* began to decline. These three species demonstrated some variation in response to increased pH among

the *Lactobacillus* species, however, more strains of each species would need to be examined to see whether the ability of *Lb. farciminis* to maintain the same specific growth rate at higher pH was typical of this species.

There is no correlation between Gram reaction and tolerance to low pH (Gould and Measures, 1977), as survival depends on maintaining the internal pH rather than cell surface structure (Booth, 1985). In order to maintain a proton motive force, the pH inside the cell must be higher than the external environment (Kashket *et al.*, 1980; Booth, 1985; Kashket, 1985). *B. thermosphacta, P. fluorescens* and *P. fragi* were all sensitive to pH 4.5, declining in numbers rather than increasing. This would suggest that under these experimental conditions at pH 4.5, these organisms were unable to maintain a higher internal pH. In contrast to the pseudomonads, the *H. alvei* strains were significantly more acid tolerant. Perhaps the ability of *H. alvei* to survive these low pH stems from the source of these bacteria, the gastrointestinal system, where these bacteria may have been exposed to low pH for extended periods. Subjecting bacteria to sub-lethal pH induces an acid tolerance response which enables the bacteria to withstand pH levels that would be lethal normally (Goodson and Rowbury, 1989; McDonald *et al.*, 1990; Kroll and Patchett, 1992; O'Sullivan and Condon, 1997). The acid tolerance response has been observed in *E. coli,* which is a member of this group (Goodson and Rowbury, 1989). If these *H. alvei* strains had been subjected to sub-lethal pH, it would explain their ability to grow at low pH.

The response to pH is also dependent on the acidifying agent used (Poolman *et al.*, 1987; O'Sullivan and Condon, 1997; Ruis and Lorén, 1998). The proportion of undissociated to dissociated acid in an environment influences microbial growth. The undissociated acid is the more active agent as it easily penetrates the cell wall (Young and Forgeding, 1993; O'Sullivan and Condon, 1997). In this investigation hydrochloric acid was used as the acidifying agent, as is has a low pKa it will be present almost entirely in the dissociated form (Corlett and Brown, 1980; O'Sullivan and Condon, 1997), therefore the effects observed are fully dependent on pH and not on the actions of an undissociated acid.

5.4.2 Effect of sodium chloride concentration on the growth of meat spoilage bacteria

Sodium chloride can be considered as a preservative because it reduces water availability therefore inhibiting microbial growth. Sodium chloride increases the solute concentration outside the cell causing water to move from inside to outside the cell, as sodium chloride cannot penetrate the cell (Gould and Measures, 1977). The reduction in water availability induces an extended lag phase (Blickstad, 1984; Li and Torres, 1993; Bhaduri *et al.*, 1995) and reduced the growth rate (Calhoun and Frazier, 1966; Sperber, 1983; Prior *et al.*, 1987). The growth rate of *S. typhumurim* decreased as sodium chloride

concentration increased (Thomas *et al.*, 1991). The observation that Gram-positive bacteria are more resistant to lower water activities than Gram-negative was supported by the data obtained in this investigation. *B. thermosphacta*, *Lb. curvatus*, *Lb. sakei, C. divergens* and, to a lesser extent, *Lb. farciminis,* were all able to reproduce in the presence of 5% sodium chloride. Both Gram-negative groups declined in growth rate rapidly as the sodium chloride concentration increased.

The *B. thermosphacta* strains were resistant to sodium chloride, experiencing a 56 to 61% reduction in growth rate in the presence of 5% sodium chloride. Similar observations of the resistance of *B. thermosphacta* to sodium chloride were made by Talon *et al.* (1988), who showed that *B. thermosphacta* grew in 10% sodium chloride. The response of bacteria to an increase in osmotic stress induced by sodium chloride has been demonstrated in *B. subtilis*. In the presence of 5.9% (or 1M) sodium chloride, a temporary lag phase was induced during which time the bacteria produced large amounts of proline (Gould and Measures, 1977). It may be possible that *B. thermosphacta* produces large amounts of proline or γ-aminobutyric acid, which accumulates in organisms that have a moderate resistance to low water activity such as *S. faecalis* (Gould and Measures, 1977). This was not determined in the species tested here.

5.4.3 Effect of chemical preservatives on the growth of meat spoilage bacteria

Chemical preservatives increase lag phase and reduce bacterial growth rate (Robach, 1978; Robach and Pierson, 1978; Robach, 1979; Greer, 1982; Zamora and Zaritzky, 1987; El-Shenawy and Marth, 1988; Payne *et al.*, 1989; Tsay and Chou, 1989). Among the species tested in this investigation, there was no significant variation in the decline of specific growth rate for either potassium sorbate, methyl-paraben or propyl-paraben. Under the conditions used in this investigation, *Pseudomonas* species were the most resistant to methyl-paraben, the LAB group were the most resistant to propyl-paraben and potassium sorbate. *Enterobacteriaceae* were the most sensitive group to all preservatives.

Propyl-paraben has been demonstrated to be more inhibitory than both potassium sorbate (Davidson and Branden, 1981; Payne *et al.*, 1989) and methyl-paraben (Eklund, 1980; Russell, 1991). The results obtained in this investigation concur with these observations. Propyl-paraben reduces the specific growth rate of the *B. thermosphacta, C. divergens, H. alvei, Lactobacillus and Pseudomonas* strains at lower concentrations than either methyl-paraben or potassium sorbate. In turn, methyl-paraben was more inhibitory than potassium sorbate for the LAB species and the *H. alvei* strains investigated. Conversely, for the *B. thermosphacta* strains investigated, potassium sorbate was more inhibitory than methyl-paraben, while for the *Pseudomonas* species potassium sorbate and methyl-paraben had equally inhibitory effects. The greater

inhibitory effects of the parabens can be explained by the nature of the compounds and that of the bacteria, as described in the following.

In terms of the nature of the compound, the anti-microbial effects of the parabens increase as the length of the side chain increases (Eklund, 1980; Russell, 1991). Therefore, as the side chain length of the paraben increases from methyl to propyl, the anti-microbial activity increases. In general, Gram-negative bacteria are more resistant to chemical preservatives than Gram-positive because of the lipopolysaccharide content in the cell wall of Gram-negative bacteria (Nikado and Vaara, 1985; Bargiota *et al.*, 1987; Juneja and Davidson, 1993). The lipopolysaccharides within the cell wall prevent hydrophobic preservatives from passing to the phospholipid layer in the cell membrane (Russell, 1991). As Gram-positive bacteria generally do not contain lipopolysaccharides (Hugo, 1967), preservatives can move more freely into the cell (Russell, 1991). This however does not explain why the *H. alvei* strains were considerably more sensitive to methyl-paraben (Mendonca *et al.*, 1989) than the *Pseudomonas* species. In addition to the lipopolysaccharide content, *P. aeruginosa* has been demonstrated to degrade methyl-paraben (Hugo, 1964), which could also contribute to the greater resistance of *Pseudomonas* to methyl-paraben than *H. alvei*.

The LAB group's greater resistance to potassium sorbate than either methyl- or propyl-paraben relates to the nature of potassium sorbate. Potassium sorbate is the salt of the weak acid, sorbic acid, therefore its effects are influenced by pH (Moustafa and Collins, 1969; Robach, 1978; Eklund, 1983; Sofos *et al.*, 1986; Zamora and Zaritzky, 1987). The anti-microbial effect of weak acids is linked to the undissociated acid passing in the cell and subsequently dissociating (Eklund, 1980). When sorbate dissociates within the cell, the internal pH of the organism decreases. As LAB have been demonstrated to withstand low internal pH (McDonald *et al.*, 1990; O'Sullivan and Condon, 1997), this is consistent with the results seen for the effect of pH on the specific growth rate, where *Lb. sakei* and *Lb. curvatus* had higher growth rates at the lower pH levels. Although the mode of action of potassium sorbate is more complex than simply the reduction of internal pH, as the dissociated acid outside the cell also had anti-microbial effects which can be observed at a higher pH (Eklund, 1983), the resistance of LAB to low pH could explain their resistance to potassium sorbate at least in part.

5.4.4 Solid media for the measurement of environmental variables

A majority of experiments which examine the response of bacteria to different environmental treatments are conducted in liquid media. However, solid media have also been used. For example, agar surfaces were used to study the response of moulds from prunes to carbon dioxide, water activity and storage temperature (El Halouat and Devevere, 1997). Solid media were used in this investigation to examine

the response of meat spoilage bacteria to pH, sodium chloride and preservatives because the solid surface would better reflect the solid surface of meat than liquid media would. The growth rates of bacteria on solid media are lower than those observed in liquid media [see Figures 5.1 and 5.2]. Optical density measurements can be correlated to bacterial numbers, but this does not indicate the growth response of bacteria on a solid surface, which is what meat is. The environment in which bacteria grow is an ecosystem (Boddy and Wimpenny; Fleet, 1999) regardless of whether it occurs in soil or on a piece of meat. Therefore, when examining the growth or physiological responses of bacteria to environmental factors, the chemical and structural composition of that environment needs to be taken into account in the experimental design (Fleet, 1999).

5.5 CONCLUSION

This investigation took bacterial species isolated from spoiled air-packaged and MAP beef and lamb that had been stored at either one or 10°C and exposed them to a series of different conditions of pH, and the presence of sodium chloride, potassium sorbate and methyl- and propyl-paraben at a series of concentrations. Between the strains and species used in this investigation, there was little significant variation. The sensitivity of a group of bacteria to changes in one environmental factor does not affect the response of that group to changes in another environmental factor. *H. avlei* strains exhibited the fastest growth over pH 4.5-7.0, indicating that they are able to maintain internal pH at these levels. However, in the presence of potassium sorbate, a weak acid, they exhibited marked sensitivity. *Pseudomonas* species were more resistant to methyl-paraben than *H. alvei* strains, although the two groups exhibited equal sensitivity to sodium chloride, propyl-paraben and potassium sorbate. *Brochothrix* exhibited the lowest reduction in specific growth rate over increasing concentrations of sodium chloride but was sensitive to low pH and chemical preservatives. Propyl-paraben was more inhibitory to each species than either methyl-paraben or potassium sorbate, but methyl-paraben was not always more inhibitory than potassium sorbate. The growth of *B. thermosphacta*, *H. alvei*, *Lactobacillus* species and *Pseudomonas* species were therefore differently influenced by pH, sodium chloride, potassium sorbate, methyl-paraben and propyl-paraben.

Chapter 6

The effects of temperature and carbon dioxide on the growth of *B. thermosphacta*, *H. alvei*, *Lb. sakei* and *P. fluorescens*

6.1 INTRODUCTION

The extension of the shelf-life of meats has been achieved by combining low temperature with elevated carbon dioxide levels in MAP (Huffman *et al.*, 1975; Gill, 1988; Holley *et al.*, 1994). The combination of these two factors not only extends the shelf-life but also altered the microflora composition (Pierson *et al.*, 1970; Silliker *et al.*, 1977; Christopher *et al.*, 1980; Erichsen and Molin, 1983a; Bell *et al.*, 1996b; Sheridan *et al.*, 1997) and reduces the final microbial load (Huffman *et al.*, 1975; Gill, 1986).

Temperature is considered to be the most significant environmental factor in the growth of bacteria (Brock and Magidan, 1988; Stainer *et al.*, 1988; Zwietering *et al.*, 1990). Temperature has been demonstrated to influence the movement of molecules into and out of the cell (Rose, 1968; Brock and Magidan, 1988), the duration of lag phase (Olsen and Nottingham, 1980; Walker *et al.*, 1990; Bhaduri *et al.*, 1995), growth rate (Jones *et al.*, 1987; Walker *et al.*, 1990; Adams *et al.*, 1991, Williamsky *et al.*, 1992; Bhaduri *et al.*, 1995) and final microbial number (Bailey *et al.*, 1979a; Walker *et al.*, 1990). Carbon dioxide has been used as a preservative agent for fresh meat products in Australia and New Zealand since the 1930s (Lawrie, 1974). Cardon dioxide has a diverse range of effects on microorganisms (Clark and Takács, 1980) which do not correlate with bacterial cell wall structure (Gill and Tan, 1980). The principal inhibitory effects constitute increased lag phase, decreased

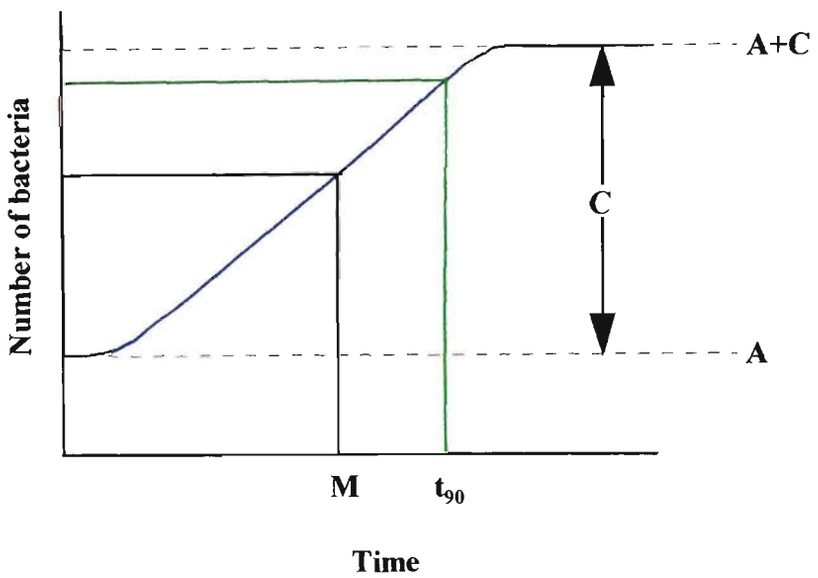


Figure 6.1 A sigmoidal growth curve of a bacterium indicating the initial number of bacteria in cfu/cm^2 (A), the increase in bacteria (C), the time where the growth rate is fastest or the point of inflection or μ_{max} (m) and the time taken to reach 90% of the final population (t_{90}).

generation time and decreased final cell yield (Clark and Takács, 1980; Enfors and Molin, 1981a; Johnson and Ogrydziak, 1984; Molin, 1983; Eyles *et al.*, 1993, Drosinos and Board, 1995). Carbon dioxide has also been demonstrated to reduce the temperature range over which *B. cereus* will grow (Enfors and Molin, 1981a).

The aim of this series of experiments was to examine the impact of different levels of carbon dioxide from zero to 100%, with N_2 as the balance gas, at temperatures ranging from refrigeration (0 and 5°C) to abusive temperatures for meat storage (10 and 15°C) on the growth of bacteria isolated from spoiled beef and lamb.

6.2 METHODS

6.2.1 Selection of cultures, cutlure preparation, packaging and sampling

It was not logistically possible to determine the effects of temperature and carbon dioxide on all the bacterial species identified in Chapter 4, therefore the same reperesentive isolates as those in Chapter 5 were used: *B. thermosphacta* A13B03, *H. alvei* M23En02, *Lb. sakei* M11L04 and *P. fluorescens* A11P04. Cultures were prepared as described in Chapter 5. *B. thermosphacta* was grown on buffered TSA, *Lb. sakei* was grown on buffered MRSA and *H. alvei* and *P. fluorescens* were grown on buffered NA. All media was at pH 6.0. Duplicate plates were packaged in bags with air or 0 (100%N_2), 25, 50, 75 or 100% CO_2 with N_2 as the balance gas. The bags were placed into incubators set at zero, five, 10 and 15°C (±1°C). Over 12, 24, 48, 96, 108, 168, 336, 604, 1344 and 2678 hours, bags were removed in order to enumerate the bacteria on the surface of the agar using the method of Eyles *et al.* (1993). The incubation time of up to 16 weeks was chosen for the conclusion of plates stored at 0°C because it was the duration of storage for published studies of MAP meats. In addition, the time frame enabled the study of microbial growth under high levels of carbon dioxide over an extensive period of time. Prior to opening the bags, triplicate measurements of the gaseous atmosphere compostion were taken.

6.2.2 Statistical analysis of the effects of temperature and carbon dioxide on microbial growth

Log-logistic curves were fitted to the growth data using a modified-Gompertz equation (Figure 6.1). From these fitted curves, an accumulated analysis of variance was conducted to determine the effects of temperature and carbon dioxide on initial bacterial numbers per cm^2 (A), the rate paramenter (B), the time taken to the point of inflection (M) where growth is fastest or μ_{max}, the slope at the point of inflection or μ_{max}, the time taken to reach 90% of the final population (t_{90}) and the final bacterial number per cm^2 (C).

Table 6.1 pH of media with and without buffering, following exposure to either air, 40% CO_2/60% N_2 and 100% CO_2 for three days at 25°C.

Atmosphere	MRSA			NA		
	Commercial	Unbuffered	Buffered[a]	Commercial	Unbuffered	Buffered
Air	6.13	6.08	5.97	7.19	6.39	6.26
40%CO_2/60% N_2	6.12	6.10	6.05	5.97	5.66	6.12
100% CO_2	6.10	5.72	5.97	6.83	5.57	6.02

[a] Contains 1.9% glycerophosphate

6.3 RESULTS

6.3.1 The selection of glycerophosphate as the buffer

As carbon dioxide readily dissolves into aqueous environments (Gill, 1988), a buffer was required to prevent the change in pH caused by the dissociation of carbon dioixde into carbonic acid. Four buffers were tested: glycerophosphate, phosphate, tri-ammonium citrate and sodium acetate. Of these, glycerophosphate was found to be the most suitable as it prevented a decrease in media pH. With the exception of MRSA, all media had the same chemical compostion as Oxoid media made from initial constituents with the addtion of 1.9% glycerophosphate in the buffered media. In the case of MRSA, neither the buffered or unbuffered media contained the sodium acetate or di-potassium hydrogen phosphate which are present in the Oxoid media. Carbon dioxide had little effect on the pH of MRSA, buffered or unbuffered (Table 6.1).

The effect of carbon dioxide on the Oxoid and unbuffered NA under 40% CO_2/60% N_2 and 100% CO_2 was to decrease the surface pH by almost one pH unit, while there was no significant change in the pH for the buffered NA (Table 6.1). The pH of the agar stored under 100% CO_2 did not exhibit the same decreases in pH as the agar stored under 40% CO_2/60%N_2. This result was not expected, as it was anticipated that the pH of the plate stored under the higher carbon dioxide should have experienced a larger decrease in pH. The integrity of the bag was checked prior to opening by measuring the gas atmosphere composition within the package, which was the same as at the beginning of the experiment (100% CO_2). The discrpency could be attributed to the temperature at which the packages were stored, 25°C. The solubility of carbon dioxide increases with decreasing temperature (Clark and Lentz, 1967; Clark and Takács, 1980; Enfors and Molin, 1981b; Johnson and Ogrydziak, 1984; Shay and Egan, 1987; Gill, 1988; Eyles et al., 1993). Therefore, at the temperature used the dissociation of carbon dioxide into carbonic acid may not have been uniform. It would have been more appropriate to examine the changes of pH caused by carbon dioxide at the temperatures used in the subsequent experiments.

As 1.9% glycerophosphate minimised the effect of the dissociation of carbon dioxide into NA while having a comparable buffering capacity to the buffers in Oxoid MRSA, it was incorporated into all media and these were all made from constituent ingredients described in the Oxoid Manual. Although MRSA was relatively unchanged by the presence of carbon dioxide, it was decided to incorporate 1.9% glycerophosphate instead of the buffering agents, sodium acetate or di-potassium hydrogen phosphate, to maintain consistency.

6.3.2 Gas analysis

The gas composition within the packages was determined at the time of packaging and prior to opening the packages at the sampling time for all four organisms (Figure 6.2-6.5). The gas

chromatograph used for this investigation only measured the proportion of oxygen and carbon dioxide and any other gases present, including nitrogen, were termed balance gases.

For *B. thermospahcta* (Figure 6.2) and *Lb. sakei* (Figure 6.4) there was a decrease in the percentage of oxygen at 15°C, while for the other temperatures the oxygen levels remained unchanged. The oxygen content for the air packages samples *of H. alvei* (Figure 6.3) and *P. fluorescens* (Figure 6.5) decreased at each of the storage temperatures, except for *P. fluorescens* at 0°C where the percentage of O_2 remained constant. The failure of the oxygen level to impede *P. fluorescens* would suggest that the integrity of the seal had been compromised rather than the a result of the plastic permeability. If the result was due to the permeability, the decline would have occurred in all experiments. For the modified atmospheres of 0 (100% N_2), 25, 50, 75 and 100% CO_2, the percentage of oxygen within the packages was generally below 1% over the course of the experiment.

The percentage of carbon dioxide within the modified atmosphere packages decreased slightly after packaging for all species, which coincided with an equal increase in the relative level of balance gases (Figures 6.2-6.5). After the initial decrease, the level of carbon dioxide was relatively constant for the duration of the storage period. For the control which contained air, there was an increase in the proportion of carbon dioxide within the package during storage. This was most notable for *H. alvei* (Figure 6.4) at 10 and 15°C, where the concentration of carbon dioxide approached 10%.

After the initial increase in the relative percentage of the balance gas in the modified atmosphere packages, the relative proportion then remained unchanged across all species and temperatures. In the air controls, there was no change in the relative percentage of balance gas.

6.3.3 The effect of temperature and atmosphere on the growth of *B. thermosphacta*

As the temperature increased from 0 to 15°C, the time taken for *B. thermosphacta* to reach stationary phase decreased and the impact of carbon dioxide on the cell number decreased (Figure 6.6). Storage at 0°C produced a notable increase in the lag phase for all atmospheric combinations including the air control. Under 25% CO_2, the increase in cell number followed the same pattern as the air control, with numbers being identical at 350 hours. After 350 hours, the numbers of *B. thermosphacta* in air continued to increase another 3.5 $\log_{10}$ while those under 25% CO_2 only increased 2.5$\log_{10}$ in the same time period. The maximum population density was reached at the same time under these two atmospheric combinations. The lag phase for *B. thermosphacta* stored under 0% CO_2 (100% N_2) was longer than the air control, with bacterial numbers actually decreasing between time 0 and 181 hours. After this time, *B. thermosphacta* increased in numbers to 10^7 cfu/cm^2 after 1261 hours, finally reaching the same maximum number of 10^8 cfu/cm^2 as air-stored *B. thermosphacta*. Under 50% CO_2

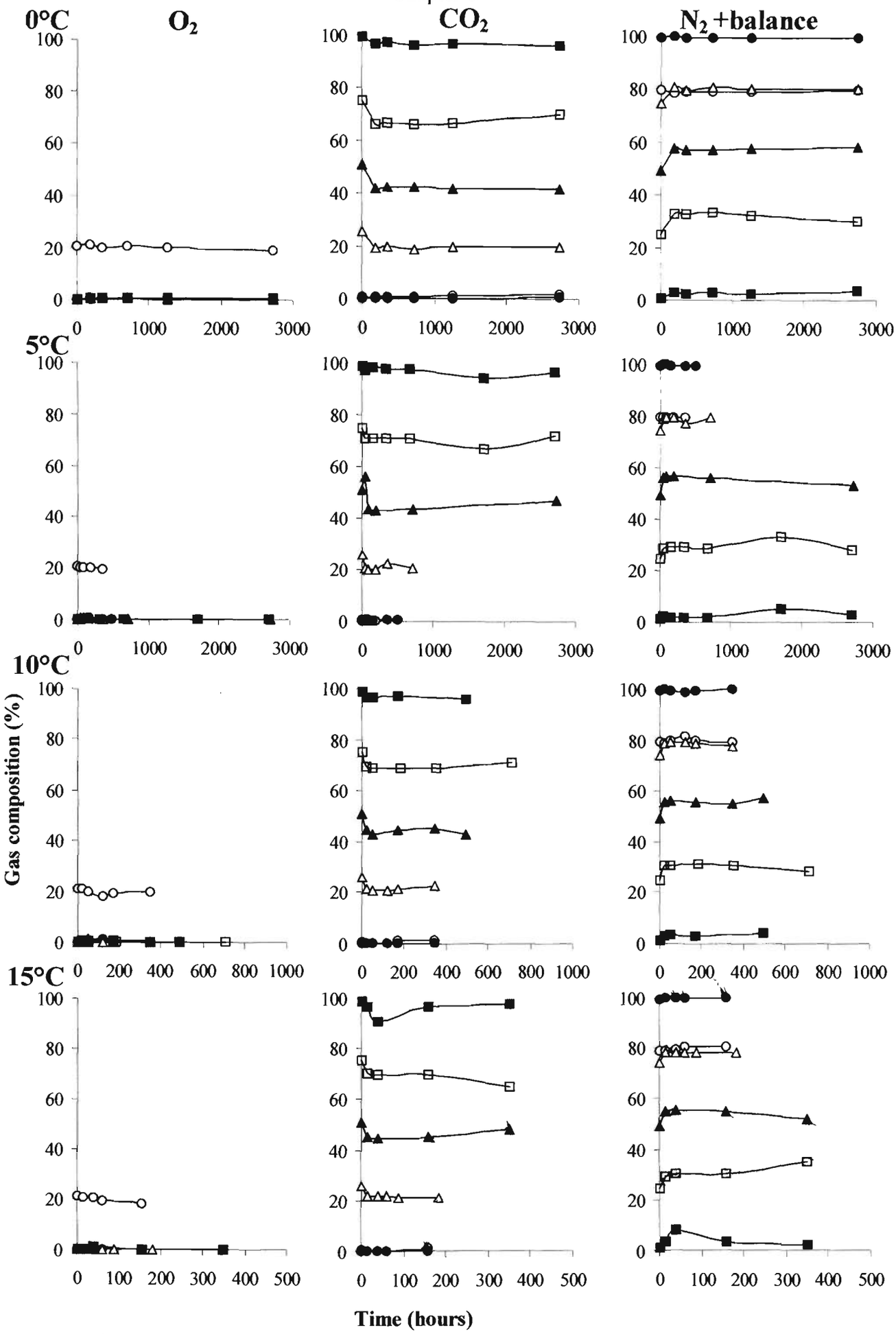


Figure 6.2 Changes in percentage gas composition following growth of *B. thermosphacta* stored at 0, 5, 10 and 15°C in air (○) or in carbon dioxide at the levels of 0 (100% N_2,●), 25 (△), 50 (▲), 75 (□) or 100% (■).

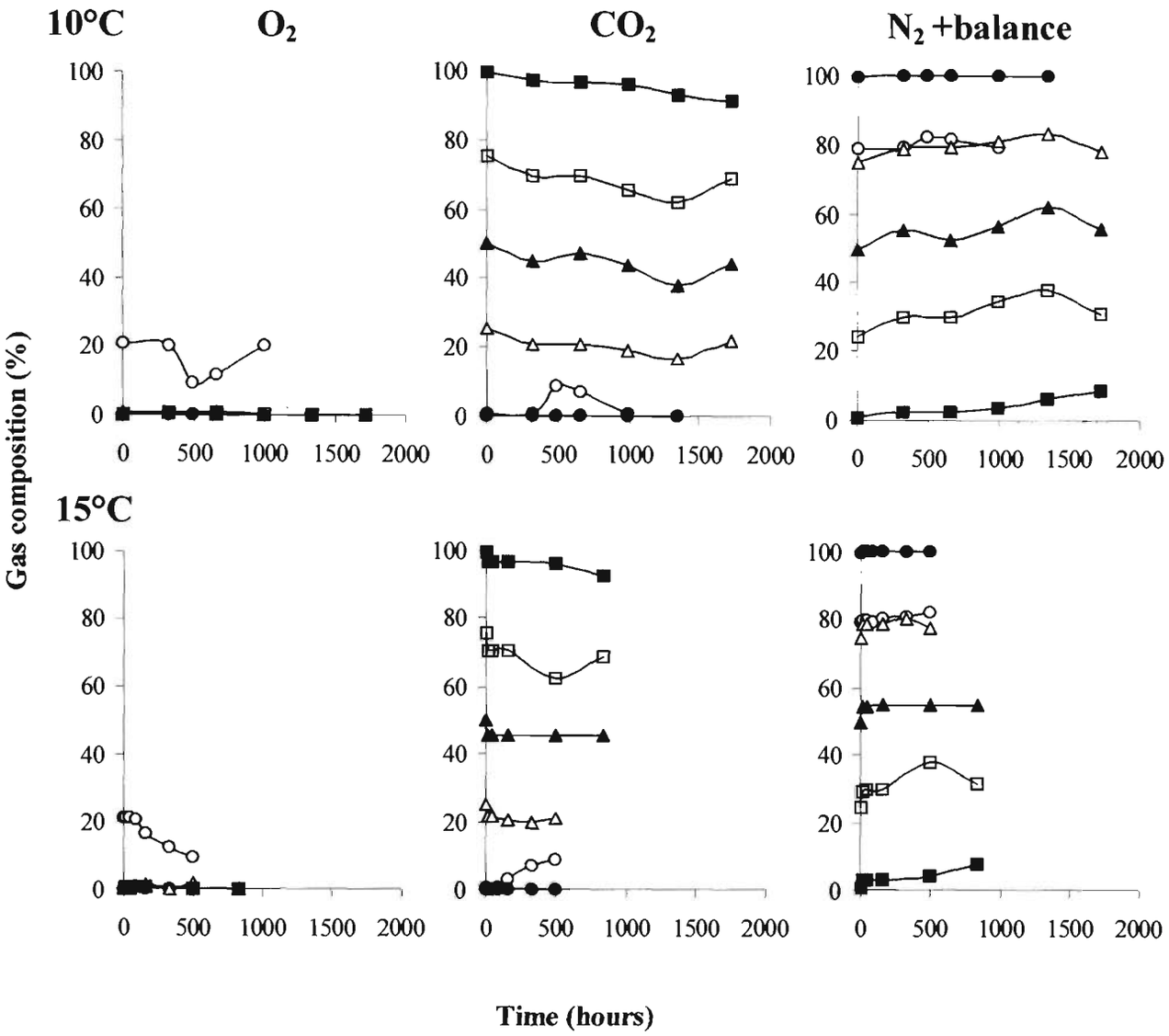


Figure 6.3 Changes in percentage gas composition following growth of *H. alvei* stored at 0, 5, 10 and 15°C in air (○) or in carbon dioxide at the levels of 0 (100% N_2,●), 25 (△), 50 (▲), 75 (□) or 100% (■).

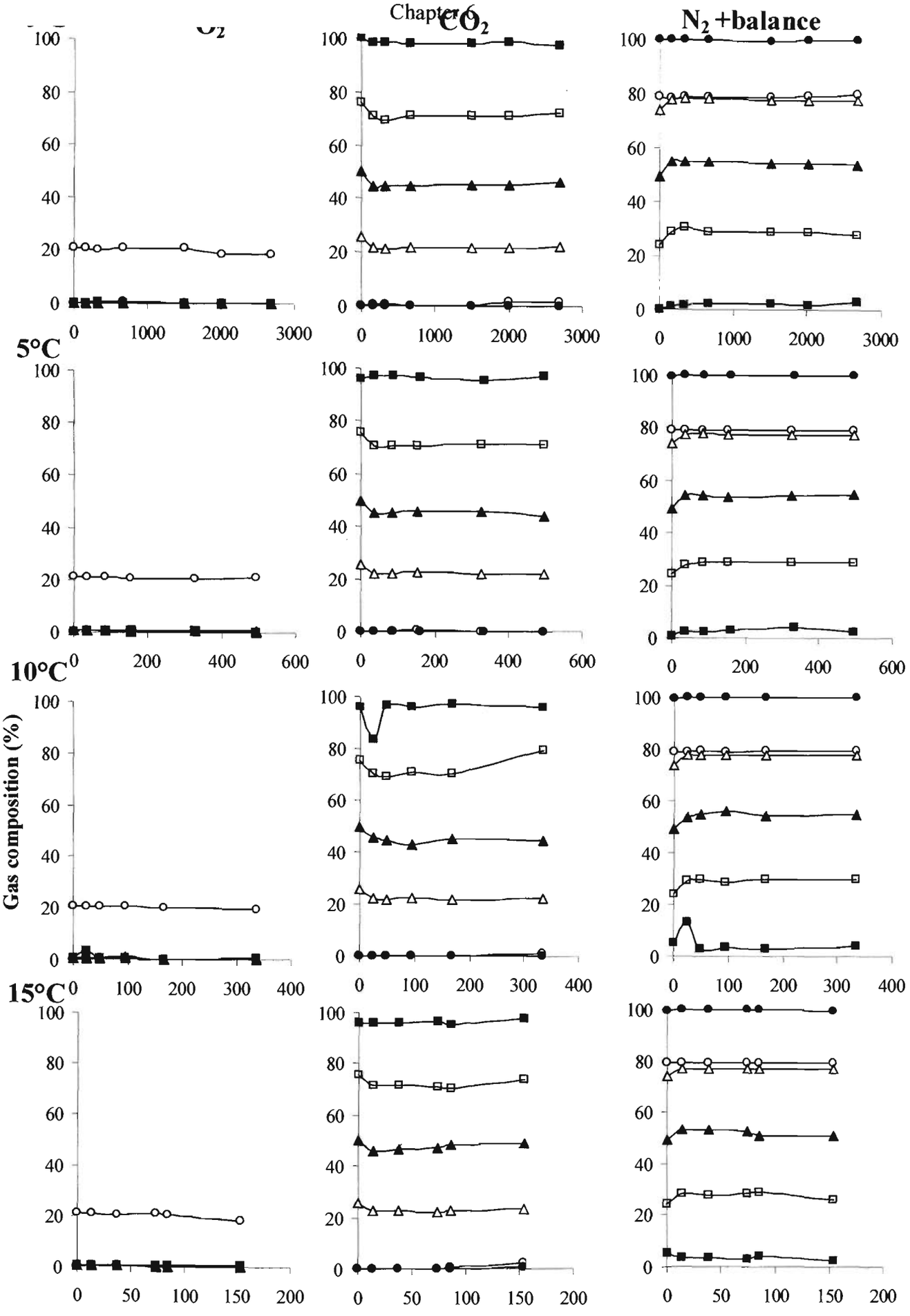


Figure 6.4 Changes in percentage gas composition following growth of *Lb. sakei* stored at 0, 5, 10 and 15°C in air (○) or in carbon dioxide at the levels of 0 (100% N_2,●), 25 (△), 50 (▲), 75 (□) or 100% (■).

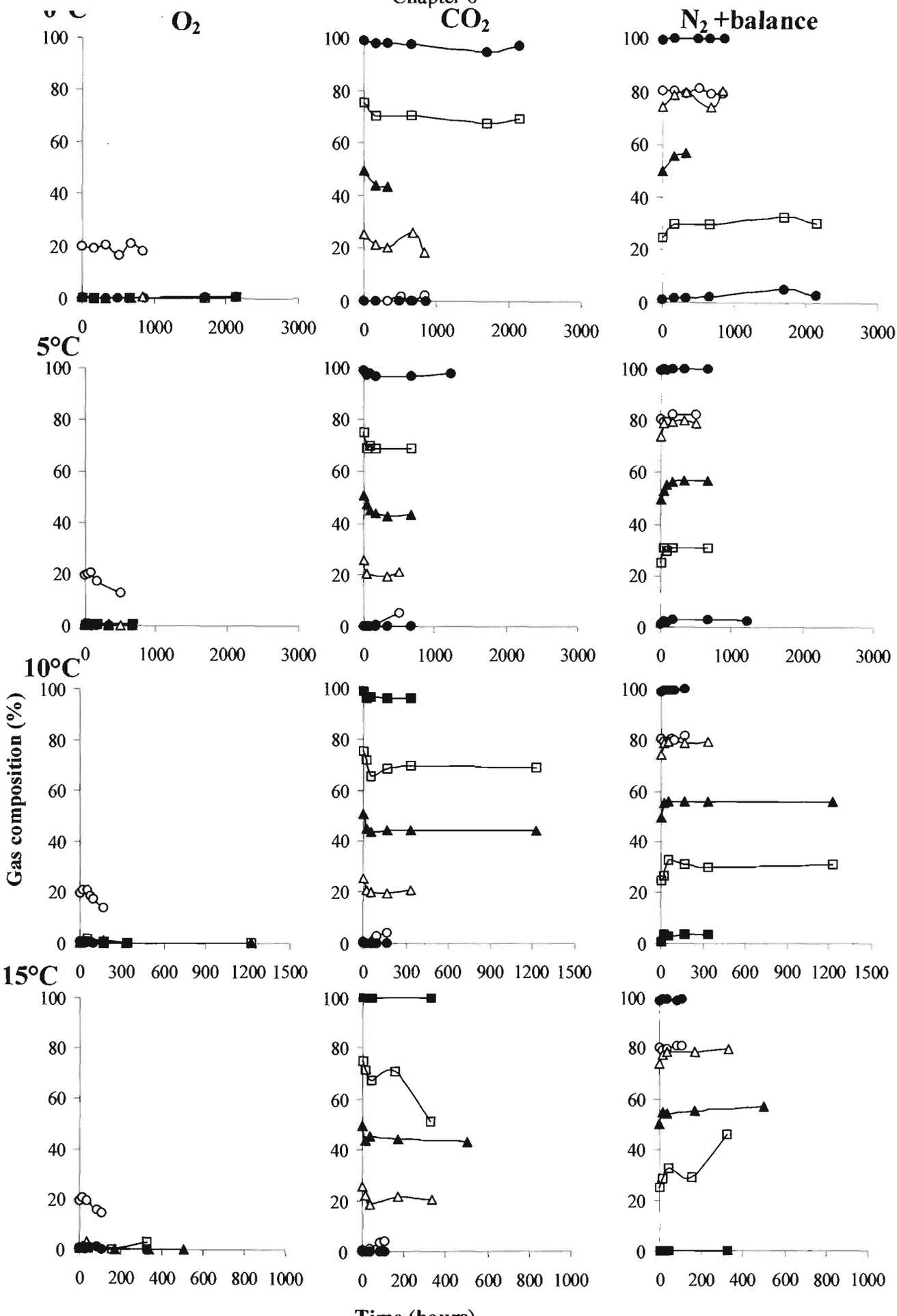


Figure 6.5 Changes in percentage gas composition following growth of *P. fluorescens* stored at 0, 5, 10 and 15°C in air (○) or in carbon dioxide at the levels of 0 (100% N_2,●), 25(△), 50 (▲), 75 (□) or 100% (■).

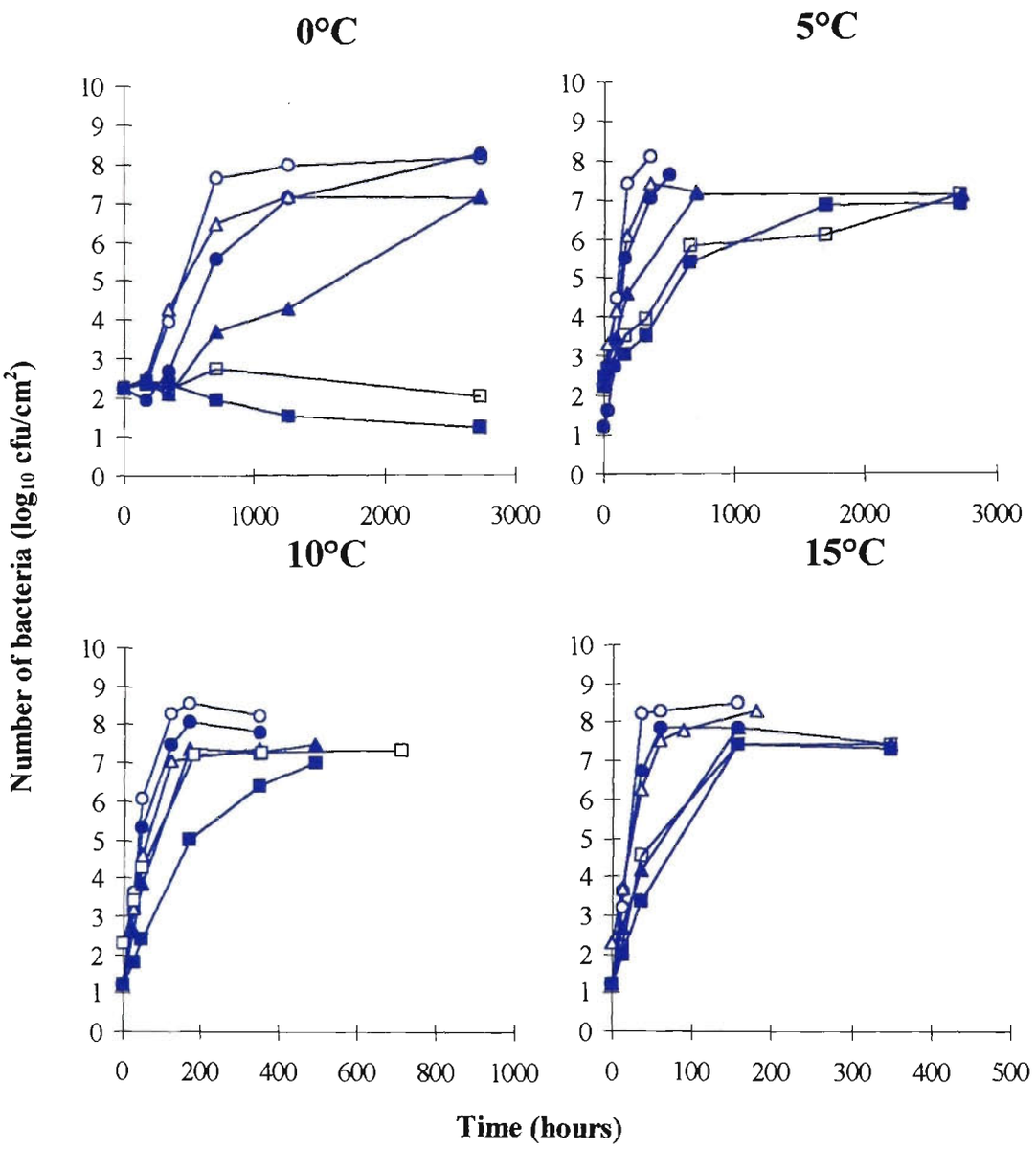


Figure 6.6 Growth of *B. thermosphacta* over time stored in air (○) or carbon dioxide at levels of 0 (100% N_2, ●), 25 (△), 50 (▲), 75 (□) or 100% (■) at 0, 5, 10 and 15°C. Note that the scale of the X-axis has been decreased with increasing temperature to demonstrate the growth kinetics.

at 0°C, there was no increase in cell number until after 350 hours, when *B. thermosphacta* increased and finally reached 10^7 cfu/cm^2, which was the same as for 25% CO_2. Under 75 and 100% CO_2, there was a decrease in *B. thermosphacta* numbers over the 2729 hour time period at 0°C.

At 5°C, the impact of carbon dioxide was markedly reduced compared to that observed at 0°C with final numbers of *B. thermosphacta* reaching 10^7 cfu/cm^2 for all atmospheres except 100% CO_2, where numbers only reached $7.3x10^6$ cfu/cm^2 (Figure 6.6). When incubated in air, 0% CO_2 and 25% CO_2, *B. thermosphacta* numbers increased from 10^2 cfu/cm^2 to 10^7 cfu/cm^2 within 660 hours. Under 50% CO_2 at 5°C, the increase in *B. thermosphacta* numbers was slower than for air, 0 (100% N_2) and 25% CO_2 but faster than incubating under 75 and 100% CO_2. Under 75 and 100% CO_2, the increase in numbers occurred at a similar rate with a higher number of *B. thermosphacta* under 100% CO_2 after 1699 hours incubation at 5°C.

With the exception of 100% CO_2 at 10°C, *B. thermosphacta* increased in cell number at the same rate regardless of carbon dioxide concentration (Figure 6.6). The only impact of carbon dioxide appeared to be on the final number reached at stationary phase. In the presence of air and 0% CO_2 (100% N_2), the final numbers of *B. thermosphacta* were 10^8cfu/cm^2 while those for 25, 50 and 75% CO_2 were 10^7 cfu/cm^2. Under 100% CO_2, *B. thermosphacta* reached 10^7 cfu/cm^2, however, a greater period of time was required.

At 15°C, *B. thermosphacta* had reached 10^7-10^8 cfu/cm^2 within 168 hours Under higher levels of CO_2, 75 and 100%, the final numbers were lower at 10^7 cfu/cm^2 compared to air and 0 (100% N_2), 25 and 50% CO_2 at 10^8 cfu/cm^2 (Figure 6.6).

6.3.4 The effect of temperature and atmosphere on the growth of *H. alvei*

H. alvei did not grow at 0 or 5°C under any of the atmosphere compositions examined. Less time was required of *H. alvei* numbers to reach stationary phase at 15°C compared to 10°C (Figure 6.7), with final numbers being slightly lower at 10°C. During storage at 10°C, the number of *H. alvei* in the presence of carbon dioxide (25, 50, 75 and 100%) was noticably lower than in the absence of carbon dioxide (air or 0% CO_2). The fastest increase in *H. alvei* numbers occurred in the presence of air. Under 0% CO_2 (100% N_2), *H. alvei* numbers followed a similar pattern to the air control, only the numbers were always one $\log_{10}$ lower than those in air. Under the carbon dioxide concentrations of 25, 50, 75 and 100% CO_2, there was an unexpected occurrence. *H. alvei* stored under 75 and 100% CO_2 had a greater increase in cell number than 25 or 50% CO_2 after 327 hours. However, after this time *H. alvei* numbers were always lower at the two higher carbon dioxide levels as compared to the

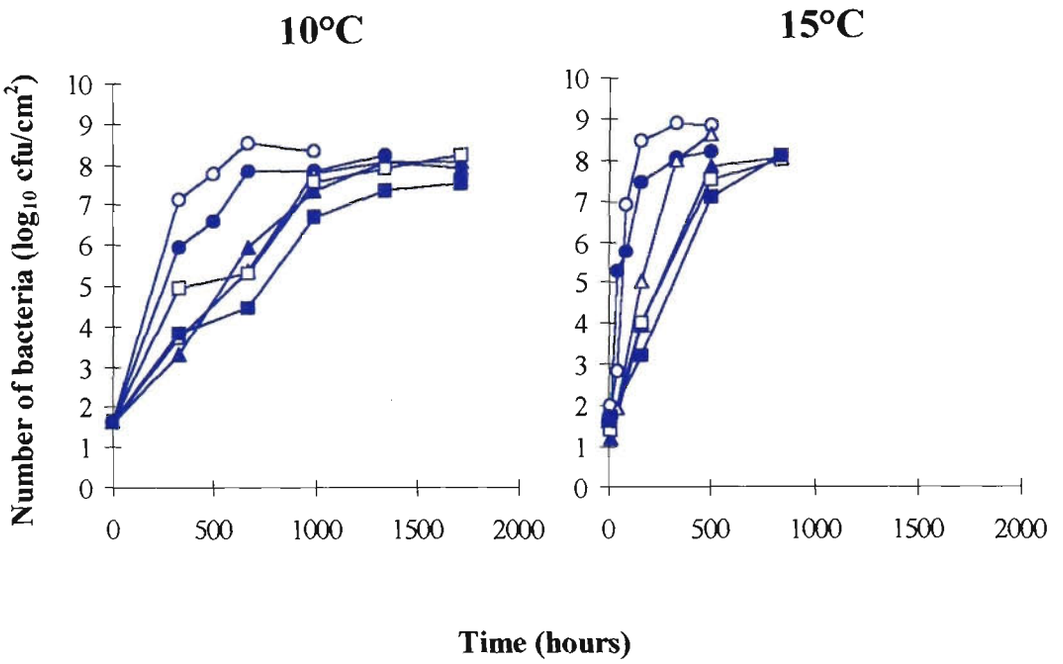


Figure 6.7 Growth of *H. alvei* over time stored in air (○) or in carbon dioxide at the levels of 0 (100% N_2, ●), 25 (△), 50 (▲), 75 (□) or 100% (■) at 10 and 15°C.

lower two levels. All the final population numbers exceeded 10^7 cfu/cm^2 in the presence of carbon dioxide when incubated to 10°C.

Storage at 15°C resulted in a similar growth pattern for *H. alvei* as those observed at 10°C (Figure 6.7). In the presence of air, *H. alvei* numbers increased at the fastest rate and reached the highest cell numbers. There was a similar increase in the *H. alvei* numbers under 0% CO_2 (100%N_2), however, the final number at maximum stationary phase was 0.5 $\log_{10}$ less than in air. Although *H. alvei* stored under 0% CO_2 (100% N_2) and 25% CO_2 reached final numbers in excess of 10^8 cfu/cm^2 at 327 hours, those stored under 25% CO_2 were up to four $\log_{10}$ less at the preceeding time intervals. This indicates that between 158 and 327 hours, *H. alvei* stored under 25% CO_2 had a faster generation time than *H. alvei* stored under 0% CO_2 (100% N_2). In the presence of the three higher carbon dioxide levels, 50, 75 and 100%, *H. alvei* increased in cell number at a similar rate with a maximim population density of 10^7 cfu/cm^2.

6.3.5 The effect of temperature and atmosphere on the growth of *Lb. sakei*

The presence of increasing amounts of carbon dioxide had little impact on the growth of *Lb. sakei* (Figure 6.8). Temperature had more of an impact on of *Lb. sakei* than CO_2 level. As the temperature increased, the time taken to reach 10^7 cfu/cm^2 decreased. Carbon dioxide did, however, have an influence on the increase in bacterial number at 0°C which was most evident at 838 hours. Initially for each atmospheric combination at 0°C there was less than one $\log_{10}$ increase in the number of bacteria within 334 hours, after which time *Lb. sakei* numbers began to increase. At 838 hours, *Lb. sakei* numbers in the presence of air were almost one $\log_{10}$ greater than numbers seen under 0 (100% N_2) or 25% CO_2 and up to 1.5 $\log_{10}$ greater than numbers seen for growth under 50, 75 and 100% CO_2. When stationary phase was reached at 1510 hours under all atmosphere treatments, the final numbers of *Lb. sakei* stored in air were lower than those stored in the modified atmospheres of 0 (100% N_2), 25, 50, 75 and 100% CO_2.

At the conclusion of the experiment the final number of *Lb. sakei* seen after exposure to air was consistently lower than those under 0 (100% N_2), 25, 50, 75 and 100% CO_2 across the whole temperature range studied. At the higher temperatures of 5, 10 and 15°C, the final number of *Lb. sakei* grown in air were lower than those of the modified atmospheres. However, the rate at which the numbers of *Lb. sakei* increased was uniform across all combinations of atmosphere.

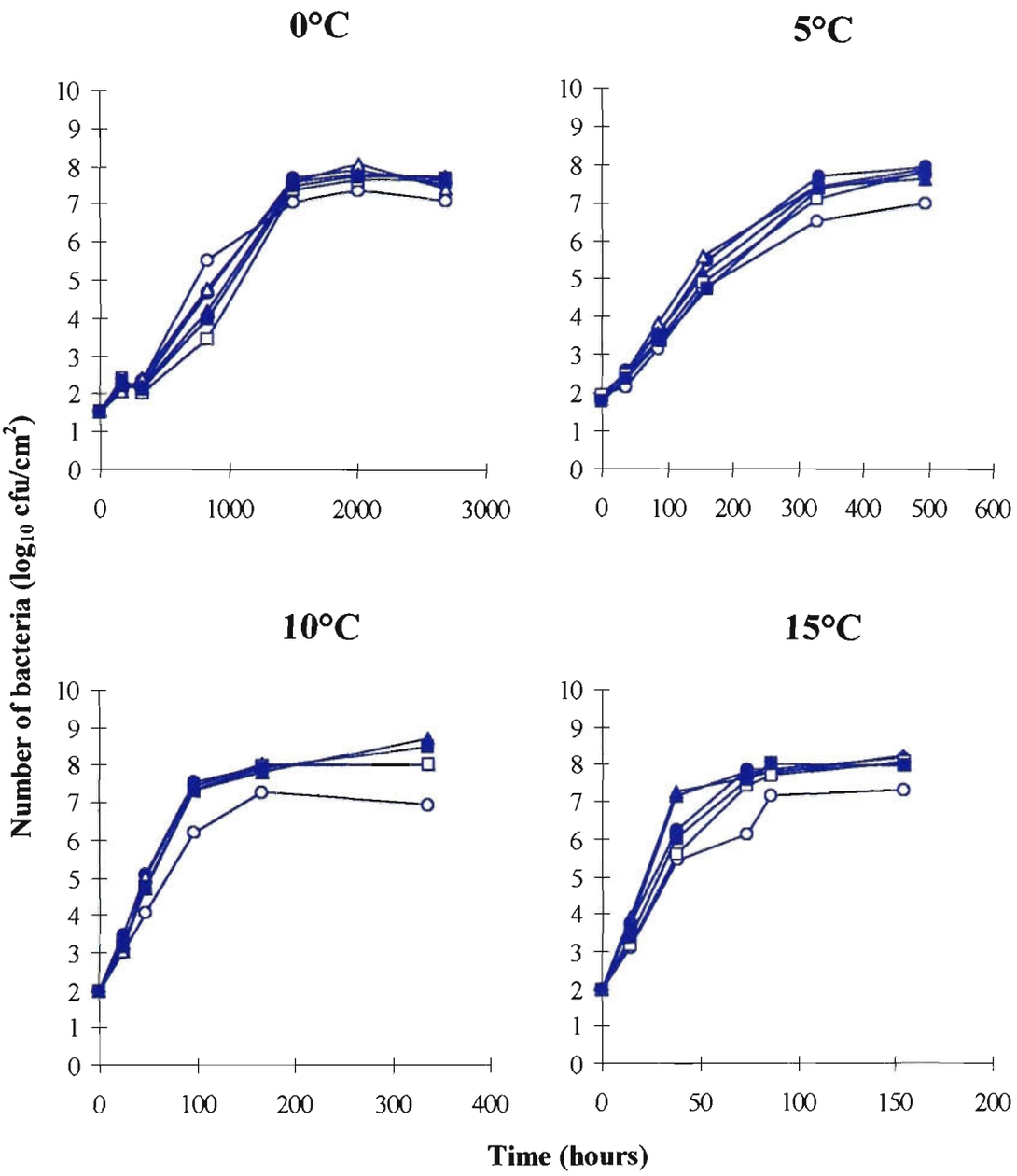


Figure 6.8 Growth of *Lb. sakei* over time stored in air (○) or in carbon dioxide at the levels of 0 (100% N_2, ●), 25 (△), 50 (▲), 75 (□) or 100% (■) at 0, 5, 10 and 15°C. Note that the scale of the X-axis has been decreased with increasing temperature to demonstrate the growth kinetics.

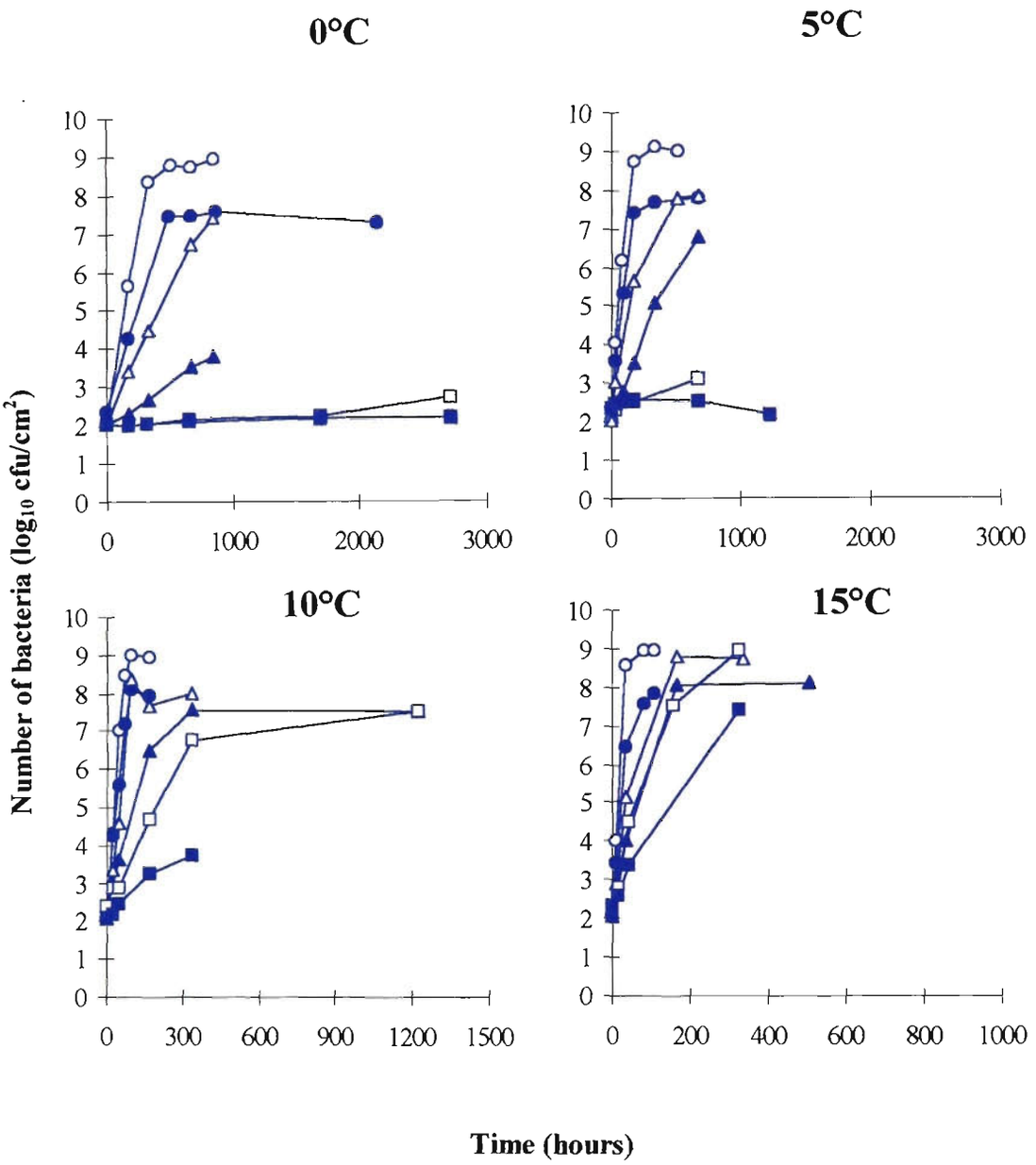


Figure 6.9 Growth of *P. fluorescens* over time stored in air (○) or carbon dioxide at levels of 0 (100% N_2, ●), 25 (△), 50 (▲), 75 (□) or 100% (■) at 0, 5, 10 and 15°C. Note that the scale of the X-axis has been decreased with increasing temperature to demonstrate the growth kinetics.

6.3.6 The effect of temperature and atmosphere on the growth of *P. fluorescens*

As the temperature increased from zero to 15°C, the impact of carbon dioxide on the growth of *P. fluorescens* decreased (Figure 6.9). *P. fluorescens* is increased in number to greater than 10^8 cfu/cm^2 within 336 hours in the presence of air at 0°C. In the absence of O_2, the increase in *P. fluorescens* numbers was significanly retarded, however, if carbon dioxide was also absent (0% CO_2 [100% N_2]), *P. fluorescens* increased to 10^7 cfu/cm^2 within 496 hours. Under 25% CO_2 *P. fluorescens* numbers increased to 10^7 cfu/cm^2 within 840 hours at 0°C, while within the same time-frame *P. fluorescens* stored under 50% CO_2 failed to increase above 10^4 cfu/cm^2. Under 75 and 100% CO_2, there was no increase in the numbers *of P. fluorescens* over 2705 hours incubation at 0°C.

In general, the increase in *P. fluorescens* numbers at 5°C followed a similar pattern to those stored at 0°C, only there was a difference in the final numbers (Figure 6.9). The final numbers for *P. fluorescens* were slightly higher at 5°C for the air control, 0 (100% N_2) and 25% CO_2, while under 50% CO_2 they were three $\log_{10}$ greater than the final numbers observed for the same carbon dioxide concentrations at 0°C. Under 75% CO_2, there was a slight increase in the *P. fluorescens* numbers, while those stored under 100% CO_2 decreased slightly during the 1225 hour storage period at 5°C.

During storage at 10 and 15°C, *P. fluorescens* was able to increase in number to above 10^7 cfu/cm^2 under all carbon dioxide concentrations within 327 hours, with the exception of growth at 10°C when 75 and 100% CO_2 was present. Under 75% CO_2 at 10°C, *P. fluorescens* numbers were 10^6 cfu/cm^2 at 327 hours but did increase to above 10^7 cfu/cm^2 after this time, while under 100% CO_2 *P. fluroescens* did not increase above 10^4 cfu/cm^2 in 336 hours.

6.3.7 Analysis of the impact of temperature and carbon dioxide on aspects of microbial growth

Log-logistic curves were fitted to the growth data using the modified Gompertz equation described by Gibson et al. (1987):

$$\log N(t) = A + C / 1 + \exp [-B (t - M)]$$

where t is time (hours), N (t) is the population density (cfu/cm^2) at t, A is lower asymptote or the initial number of bacteria (cfu/cm^2), C is the difference between the lower and upper asymptote or the increase in population density (cfu/cm^2), M is the time when growth is at is maximum or the time to μ_{max} and B is rate parameter related to the slope of the tangent at M by BC/4= slope of tangent at M (McMeekin *et al.,* 1993). The slope of the tangent at M is the maximum specific growth rate (μ_{max}).

Table 6.2 The R^2 value for the log-logistic curves fitted to *B. thermosphacta*, *H. alvei*, *Lb. sake* and *P. fluorescens*

Species	Temp (°C)	Air	Carbon dioxide (%)				
			0[a]	25	50	75	100
B. thermosphacta							
	0	99.6	95.5	98.5	92.7	*	93.0
	5	99.9	99.0	98.3	99.9	97.6	99.8
	10	99.7	99.5	99.5	99.7	99.8	99.8
	15	99.7	99.6	99.6	97.1	99.9	99.6
H. alvei							
	10	98.5	97.8	94.9	99.7	86.2	93.2
	15	99.8	90.3	99.1	99.0	99.1	100.0
Lb. sakei							
	0	99.7	99.1	98.1	99.2	98.6	99.3
	5	99.8	90.3	99.1	99.0	99.1	100.0
	10	99.1	99.8	100.0	97.9	100.0	99.9
	15	95.3	99.9	99.5	98.9	99.1	99.7
P. fluorescens							
	0	99.9	99.4	99.6	99.6	81.3	70.3
	5	99.4	99.6	99.8	99.8	95.8	41.0
	10	99.9	96.6	100.0	99.9	99.6	99.3
	15	100.0	99.6	99.9	99.5	98.6	100.0

[a] 100% N_2

* = Sigmodial curve could not be fitted

The fitting of log-logistic curves to the growth data enabled an analysis of variance to be conducted to determine the impact of temperature and carbon dioxide concentration of the microbial growth parameters of initial number, time to μ_{max}, t_{90}, increase in population density and μ_{max} to be estimated. The output for the estimates of these parameters are listed in Appendix 4. The analysis of variance for the effect of carbon dioxide was conducted on the growth data for 0 (100% N_2), 25, 50, 75 and 100% CO_2. There was insufficient data to examine the significance of the interaction between temperature and atmosphere statistically.

For the cuves fitted, the R^2 values for the fit of each log-logistic curve to the data of *B. thermosphacta*, *H. alvei*, *Lb. sakei* and *P. fluorescens* are shown in Table 6.2. In general, the R^2 values ranged between 90 and 100%, however, there were some exceptions. These were: *B. thermosphacta* stored at 0°C under 75% CO_2, where a curve could not be fitted due to failure of growth; *H. alvei* at stored at 10°C under 75% CO_2, where R^2 was 86.2, *P. fluorescens* stored at 5°C under 100% CO_2, where R^2 was 41.0, and at 1°C under 75 and 100% CO_2, where R^2 was 81.3 and 70.3, respecitvely. Due to the lack of growth, the fit of these curves was not as good as for the other treatments.

Statistical analysis showed that the growth parameters of *B. thermosphacta* were influenced more by temperature than carbon dioxide (Table 6.3). Temperature significantly ($p<0.05$) affected the time to μ_{max}, the rate parameter, t_{90}, the increase in population density number and μ_{max} of *B. thermosphacta*. As the temperature increased from zero to 15°C, the time to reach both μ_{max} and t_{90} decreased, while the rate parameter, increase in population density and μ_{max} all increased. Carbon dioxide significantly affected the rate parameter, the increase in population density and μ_{max}: in general, as the level of carbon dioxide increased from zero to 100%, the rate parameter, the increase in population density and μ_{max} all decreased.

H. alvei was only able to grow at 10 and 15°C, therefore the analysis could only encompass two temperatures (Table 6.3). Only t_{90} was significantly ($p<0.05$) affected by either temperature or carbon dioxide. The t_{90} decreased as temperature increased from 10 to 15°C, while it increased as the level of carbon dioxide increased from zero to 100%. Increasing the level of carbon dioxide had no significant effect on the growth of *Lb. sakei* (Table 6.3). Temperature, however, had a significant ($p<0.05$) effect on the time to μ_{max}, the rate parameter, t_{90}, the increase in population density and μ_{max} of *Lb. sakei*. As the temperature increased from zero to 15°C, the time to both μ_{max} and t_{90} decreased, while the rate parameter, final population density and μ_{max} all increased.

For *P. fluorescens*, temperature and carbon dioxide both significantly influenced the rate parameter and μ_{max}: as the temperature increased, the rate parameter and μ_{max} both decreased, while as carbon dioxide levels increased, both of these growth parameters decreased.

Table 6.3 The effect of temperature and carbon dioxide on the time to μ_{max} (M), the rate parameter (B), time to reach 90% of the maximum population density (t_{90}), increase in cell numbers (C), and μ_{max}.

Species	Temp / CO_2	Growth parameter				
		M	B	t_{90}	C	μ_{max}
B. thermospacta						
	Temp	✓[a]	✓	✓	✓	✓
	CO_2		✓		✓	✓
H. alvei						
	Temp			✓		
	CO_2			✓		
Lb. sakei						
	Temp	✓	✓	✓	✓	✓
	CO_2					
P. fluorescens						
	Temp		✓			✓
	CO_2		✓			✓

[a] significant ($p<0.05$)

6.4 DISCUSSION

6.4.1 The effect of temperature on the growth of *B. thermosphacta*, *H. alvei*, *Lb. sakei* and *P. fluorescens*

Temperature is considered to be the most significant environmental factor determining microbial growth (Ratkowsky *et al.*, 1982; Stannard *et al.*, 1985; Zwietering *et al.*, 1990). Temperature had a greater impact than carbon dioxide in influencing the growth of *B. thermosphacta*, *H. alvei*, *Lb. sakei* and *P. fluorescens* studied in this investigation. There was an demonstrable lag phase for *B. thermosphacta* and *Lb. sakei* at 0°C that this was not observed for five, 10 or 15°C under the conditions used in these experiments. Lag phase has been demonstrated to increase as temperature is decreased for *E. coli* (Broeze *et al.*, 1978), *L. monocytogenes* (Walker *et al.*, 1990; Buchanan and Klawitter, 1991; Grau and Vanderline, 1993), *P. fluorescens* (Broeze *et al.*, 1978) and *Y. enterocolitica* (Bhaduri *et al.*, 1995). A lag phase can also be induced by a down-shift in temperature (Broeze *et al.*, 1978; Jones *et al.*, 1987; Williamsky *et al.*, 1992).

The μ_{max} and time taken to reach μ_{max} for *P. fluorescens*, *B. thermosphacta* and *Lb. sakei* were decreased as temperature increased. This was in accordance with results found for *E. coli* (Jones *et al.*, 1987), *L. monocytogenes* (Walker *et al.*, 1990) and *Y. enterocolitica* (Adams *et al.*, 1991; Bhaduri *et al.*, 1995). *H. alvei* should have been capable of growth at 5°C, as the minimum growth temperature for this species has been observed as 2.5°C (Ridell and Korkeala, 1997). In addition, other strains of *H. alvei* were isolated from meat stored at 1°C when they had not been detected prior to storage in Chapter 3. Although for *H. alvei* temperature only statistically influenced t_{90}, the absence of growth at either zero or 5°C infers that temperature does have a significant effect on the μ_{max} of *H. alvei*.

As the temperature was increased from zero to 15°C, the number of both *B. thermosphacta* and *Lb. sakei* observed at stationary phase increased. This has been observed for *L. monocytogenes* where it was seen that as the temperature increased, the final population density increased (Walker *et al.*, 1990). In addition, the number of bacteria on the surface of chicken carcasses at spoilage depended on storage temperature, with numbers decreasing with temperature (Bailey *et al.*, 1979a).

6.4.2 The effect of carbon dioxide on the growth of *B. thermosphacta*, *H. alvei*, *Lb. sakei* and *P. fluorescens*

P. fluorescens and *B. thermosphacta* were more significantly affected by the presence of carbon dioxide than either *H. alvei* or *Lb. sakei*. As the level of carbon dioxide was increased, the μ_{max} of both *P. fluorescens* and *B. thermosphacta* decreased. A reduction in growth rate has been observed as one of the primary effects of elevated carbon dioxide on bacterial growth (Clark and Takács, 1980; Enfors and Molin, 1981a,b; Johnson and Ogrydziak, 1984; Molin, 1983; Eyles *et al.*, 1993, Drosinos and Board, 1995). The growth rates of *A. hydrophila*, *L. monocytogenes* and *Y. enterocolitica* decreased in the

presence of 50% CO_2, although there was 21% O_2 also present (Bennik *et al.*, 1995). *B. cereus* also experienced a significant retardation in the generation rate when exposed to 20% CO_2 in the presence of 21% O_2 (Bennik *et al.*, 1995).

It was expected that carbon dioxide would influence *P. fluorescens* growth more than *B. thermosphacta*, but this was not the case. In this investigation, *P. fluorescens* was able to grow under 50% CO_2 even at 0°C, albeit weakly. In contrast, Eyles *et al.* (1993) demonstrated the *P. fluorescens* and *P. putida* were significantly inhibited by the presence of 20 and 40% CO_2 (balance N_2). The variation in these observation could relate to the duration of storage, as *P. fluorescens* and *P. putida* were incubated for only 20 days while *P. fluorescens* in this investigation was incubated for a minimum of 28 days. Also the difference could relate to the buffer used here to negate the pH effects caused by carbon dioxide. Eyles *et al.* (1993) used a buffer containing citric acid, which has been demonstrated to have anti-microbial properties (Faber *et al.*, 1989; Brocklehurst and Lund, 1990; Adams *et al.*, 1991; Karapinar and Gönül, 1992). Alternatively, the difference observed could be due to strain variation. In order to generalise about the ability or inability of *P. fluorescens* to grow in the presence of carbon dioxide, further strains isolated from a variety of sources would need to be examined, and this remains to be undertaken.

Although the effect of carbon dioxide on the increase in population density was only significant for *B. thermosphacta*, *P. fluorescens* does appear to experience a decrease in population density as carbon dioxide concentration increases. The maximum population density of *A. hydrophila*, *L. monocytogenes* and *Y. enterocolitica* were decreased in the presence of 50% CO_2 while for *B. cereus* 20% CO_2 reduced the maximum population density (Bennik *et al.*, 1995), as noted above. Further data are required to determine the significance of carbon dioxide on the final population density of *P. fluorescens*.

There have been conflicting reports on the impact of carbon dioxide on the growth of *B. thermosphacta* at low temperature. Gill and Tan (1980) found that carbon dioxide had no effect on *B. thermosphacta* grown on pieces of meat stored under elevated carbon dioxide at 3°C, while Molin (1983) reported that *B. thermosphacta* was significantly retarded by the presence of carbon dioxide at levels as low as 5%. In the investigation described in this thesis, *B. thermosphacta* was able to grow under 25 and 50% CO_2, however, the rate of the increase in cell number was less than seen for air-stored samples. As *B. thermosphacta* is classified as a facultatively anaerobic bacteria (Sneath and Jones, 1986), it should be able to grow oxygen reduced environments. *Enterobacteriaceae* species are relatively resistant to elevated carbon dioxide, however, this resistance is not as great as that exhibited by *Lactobacillus* species (Molin 1983). This is consistent with the results found in this investigation. Only t_{90} of *H. alvei* was significantly affected by carbon dioxide, while none of the growth parameters of *Lb. sakei* growth was affected by carbon dioxide.

It was particularly interesting that *P. fluorescens* was able to grow in the presence of 50% CO_2 even at 0°C. Although there was a high percentage of CO_2, *P. fluorescens* was still able to maintain metabolic activity. The ability of *P. fluorescens* to grow in the presence of 100% N_2 at rates that were similar to the air control suggests that retardation of microbial growth by carbon dioxide is more complex than simply the removal of air. The gas chromatography results showed that there was less than 1% O_2 within the packages. This is definitely worthy of further investigation and raises several questions. Is the isolate actually a *P. fluorescens* strain? This identification of this isolate was determined twice, one by the methods outlined in this thesis and again with an API kit, both times the results indicated that it was *P. fluorescens*.. A second question, is the organism using another electron acceptor such as NO_3^-? Due to time constraints it was not possible to answer these questions.

6.4.3 The interaction between temperature and carbon dioxide on the growth of meat spoilage bacteria

The advantage of combining refrigeration temperatures and elevated levels of carbon dioxide was first demonstrated in 1922 (Lawrie, 1974). Although there was insufficient data to statistically demonstrate an interaction between temperature and carbon dioxide level, the interaction of these two environmental factors can be inferred from the results. From the output of the estimates of growth parameters for the species examined in this investigation, it was shown that as the temperature was decreased and carbon dioxide levels increased, μ_{max} decreased, time to reach both μ_{max} and t_{90} increased and the final population number decreased.

Other researchers have also found that the effect of carbon dioxide increases with decreasing temperature (Clark and Lentz, 1967; Clark and Takács, 1980; Enfors and Molin, 1981a,b; Johnson and Ogrydzaik, 1984; Shay and Egan, 1987; Gill, 1988; Eyles *et al.*, 1993). In this investigation, none of the species examined were inhibited at 15°C by the presence of carbon dioxide, even when the concentration was 100%. Even *P. fluorescens*, which is an aerobic bacterium normally, was able to increase in cell number to $>10^7$ cfu/cm^2 under 100% CO_2, although growth was at a slower rate than seen for the other atmosphere treatments. When the temperature was decreased to 10°C, the impact of carbon dioxide increase substantially, with *P. fluorescens* numbers failing to reach 10^4 cfu/cm^2 in 336 hours. *B. thermosphacta* was not inhibited by 100% CO_2 when stored at five or 10°C, however, the rate of the increase in cell number was lower than seen for the other atmosphere treatments. *H. alvei* increased to final numbers $>10^7$ cfu/cm^2 under all CO_2 levels at 10°C but the rate of the increase in number under 25, 50, 75 and 100% CO_2 were lower than the air or 0% CO_2 (100% N_2).

As the temperature dropped to zero and 5°C, the effect of carbon dioxide on bacterial growth increased. *P. fluorescens* was inhibited by both 75 and 100% CO_2 at both 0 and 5°C. Eyles *et al.* (1993) observed that neither *P. fluorescens* nor *P. putida* were able to increase in number under 100% CO_2 at 5°C. *B.*

thermosphacta was observed to increase in numbers under the highest levels of CO_2 (75 and 100%) at 5°C, however, the increase was not as rapid as seen for the lower levels of carbon dioxide. *B. thermosphacta* demonstrated no net increase or decrease in bacterial numbers under 75 and 100% CO_2 at 0°C even after 2729 hours.

Perhaps the best example of the combined inhibitory actions of temperature and CO_2 was demonstrated on *Lb. sakei*. Carbon dioxide has been shown to be selective for the slower growing microaerophillic *Lactobacillus* species (Kandler and Wiess, 1986) over the faster growing aerobic species which dominated the micorflora of meat stored in the presence of air (Bailey *et al.*, 1979b; Erichsen and Molin, 1981; Gill, 1986). In this thesis it was shown that the number of *Lb. sakei* was not influenced by carbon dioxide concentration when stored at five, 10 and 15°C. At 0°C, however, carbon dioxide did decrease in the number of *Lb. sakei* observed during the exponential phase at 334 hours. At this time point, the numbers of *Lb. sakei* in the presence of 50, 75 and 100% CO_2 were 1.5 $\log_{10}$ less than the air packaged samples. Enfors (1983) observed that *Lactobacillus* had higher growth rates in air than under 5% CO_2 (balance N_2) or 100% CO_2. This is significant, as it supports the use of elevated CO_2 levels for meat packaging as, although these spoilage bacteria are able to grow, their growth rate is impeded by the high concentration of carbon dioxide.

6.5 CONCLUSION

This investigation took *B. thermosphacta*, *H. alvei*, *Lb. sakei* and *P. fluorescens* from spoiled air-package and MAP beef and lamb, and exposed them to temperatures ranging from zero to 15°C and carbon dioxide levels ranging from zero to 100%. The effect of carbon dioxide on the growth of bacteria has been well documented, however there had been no sequential studied of the effects of temperature and carbon dioxide on the growth of individual bacteria isolated from meat. Log-logistic curves fitted to the growth data provided statistical information on the significance of both factors on the growth of these bacterial species, and this had not been reported in the literature previously. Temperature was found to influence more microbial growth parameters than carbon dioxide. *Lb. sakei* and *H. alvei* were largely unaffected by carbon dioxide while *P. fluorescens* and, in particular, *B. thermosphacta* were sensitive to increasing carbon dioxide. The effects of temperature and carbon dioxide on microbial growth are additive, although further data is required to statistically validate this observation.

Chapter 7

Combined effect of environmental factors on the growth of meat spoilage bacteria

7.1 INTRODUCTION

The microflora on meat at spoilage is complex, even though one bacteria species tends to dominate. *Brochothrix* (Barlow, and Kitchell, 1966; Blickstad and Molin 1983a; Borch and Molin, 1989), *Enterobacteriaceae* (Ingram, 1962; Barnes and Thornley, 1966; Newton and Gill, 1980), LAB (Hanna et al., 1981; Bell et al., 1996b,c) and *Pseudomonas* species (Bailey et al., 1979b; Enfors et al., 1979; Molin and Ternström, 1982; Shaw and Latty 1982; Asensio et al., 1988) all have an important role in determining the shelf-life of meat through both growth and end-product formation.

Environmental factors affect microbial growth and can inhibit the growth of bacteria as seen in previous chapters. Combinations of environmental factors can be used to retard or inhibit bacterial growth at levels which alone have no significant inhibitory effect (Scott, 1989). Temperature (Rose, 1968; Juffs, 1976; Broeze et al., 1978; Olsen and Nottingham, 1980; Jones et al., 1987; Walker et al., 1990), pH (Corlett and Brown, 1980; Booth 1985; Zeng et al., 1990), water activity (Valley and Rettger, 1922; Gould and Measures, 1977; Christian, 1980; Sperber, 1983; Prior et al., 1987), atmosphere composition (Enfors and Molin 1981b; Gill and Tan, 1980; Johnson and Ogrydziak, 1983; Drosinos and Board, 1995) and preservatives (Moustafa and Collins, 1969; Robach, 1978; Eklund, 1985; El-Shenawy and Marth, 1988;

Sofos et al., 1986; Mendonica et al., 1989) all influence bacterial growth. Previous investigators have demonstrated the combined effect of temperature/pH or temperature/water activity/temperature or pH/water activity on microbial growth (McMeekin et al., 1987; Faber et al., 1989; Hughes and McDermott, 1989; McClure et al., 1989; Buchanan and Phillips, 1990; Brocklehurst and Lund, 1990; Little et al., 1992b). However, these investigations tended to centre around studies on one organism, predominantly pathogens (Buchanan and Phillips, 1990; Hudson et al., 1992; Zaika et al., 1992; Wijtze et al., 1993; McClure et al., 1994; Membré and Burlot, 1994).

No study has investigated the impact of environmental factors on a mixed population of meat spoilage bacteria as a whole population or individual members of such a population. The aim of this work was to examine the effects of temperature, pH, sodium chloride, potassium sorbate and propyl-paraben on the growth of a mixed population and the individual members of that population, using *B. thermosphacta*, *H. alvei*. *Lb. sakei* and *P. fluorescens* strains which had originally been isolated from spoiled beef and lamb.

7.2 Methods

7.2.1 Culture preparation

Representative strains (*B. thermosphacta* A13B03, *H. alvei* M23En02, *Lb. sakei* M11L04 and *P. fluorescens* A11P04) were used to simulate the microflora of meat. The cultures were grown until late log phase, then diluted and mixed together to give final concentrations of 10^3 cfu/cm^2 for *B. thermosphacta*, *Lb. sakei* and *P. fluorescens* and 10^1-10^2 cfu/cm^2 for *H. alvei*, when plated onto an agar surface. These concentrations reflected the ratios observed on fresh supermarket meat. Cultures were stored on ice while plating was in progress.

7.2.2 Preparation of media, packaging and sampling of treatments

BHIA which had a pH of 5.5 or 6.0 contained either nothing (control), one or 2 % sodium chloride, 0.4% potassium sorbate or 0.02% propyl-paraben was dispensed in 10mL aliquots into petri dishes. The mixed microflora was spread plated on to the agar surface. A sterile stick which was placed along the inside of the lid of the Petri dish to facilitate the diffusion of the gases. The plates were grouped together in a predetermined fashion to reduce bias, then packaged in either air, 25% CO_2/75% N_2 or 100% CO_2. After packaging, one set of plates was removed for initial microbial count determination, and the remainder were stored at either one or 10°C. Samples were removed at 24, 48, 108, 156, 324, 660, 1334 and 2678

hours after storage commenced. Prior to opening the package, the gas composition atmosphere was analysed: if the oxygen concentration exceeded 1% in MAP, the package was discarded, and a new one removed from the incubator.

7.2.3 Enumeration of bacteria

Bacterial numbers were determined by removing the agar from the Petri dish, macerating with 50mL of diluent, diluting appropriately then spiral plating onto selective and non-selective media. Total plate counts were determined on PCA incubated at 30°C for 72 hours. *H. alvei* and *Lb. sakei* were enumerated on VRBGA and MRSA, respectively. Both sets of plates were incubated anaerobically at 30°C (*H. alvei* for 24 hours and *Lb. sakei* for 48 hours). *B. thermosphacta* and *P. fluorescens* were enumerated on STAA and PSA respectively, incubated at 25°C for 48 hours. Bacterial numbers were determined as cfu/cm^2.

7.2.4 Statistical analysis of the effects of environmental factors on bacteria growth

Log-logistic curves were fitted to the growth data of the total population and for *P. fluorescens*, *Lb. sakei* and *B. thermosphacta*, based on the modified-Gompertz equations listed in Appendix 3. The impact of each environmental factor alone and in combinations, on the slope of the curve at the point of inflection or μmax, t_{90} time to reach 90% of the maximum population and the increase in population density was determined.

7.3 RESULTS

7.3.1 Gaseous atmospheres

Over the storage period, the gaseous environment of the air-packaged samples changed dramatically (Figure 7.1). As for Chapter 6, nitrogen and other gases were included in the balance gases. For the air-packages stored at 10°C, the percentage of oxygen decreased rapidly from 20% to 0.1% within 324 hours. Over the same time frame, carbon dioxide increased from 0.2 to 15.77%. A similar change in the oxygen and carbon dioxide proportions was observed in the air-packages stored at 1°C, however the changes took place over a greater period of time. There was no change in the percentage of either oxygen or carbon dioxide before 156 hours at 1°C. After 324 hours, the percentage of oxygen began to decrease while carbon dioxide increased. Finally by 660 hours the proportion of oxygen had reached a minimum of <1% and carbon dioxide a maximum of 14.21%.The percentage of balance gases in packages stored at one and

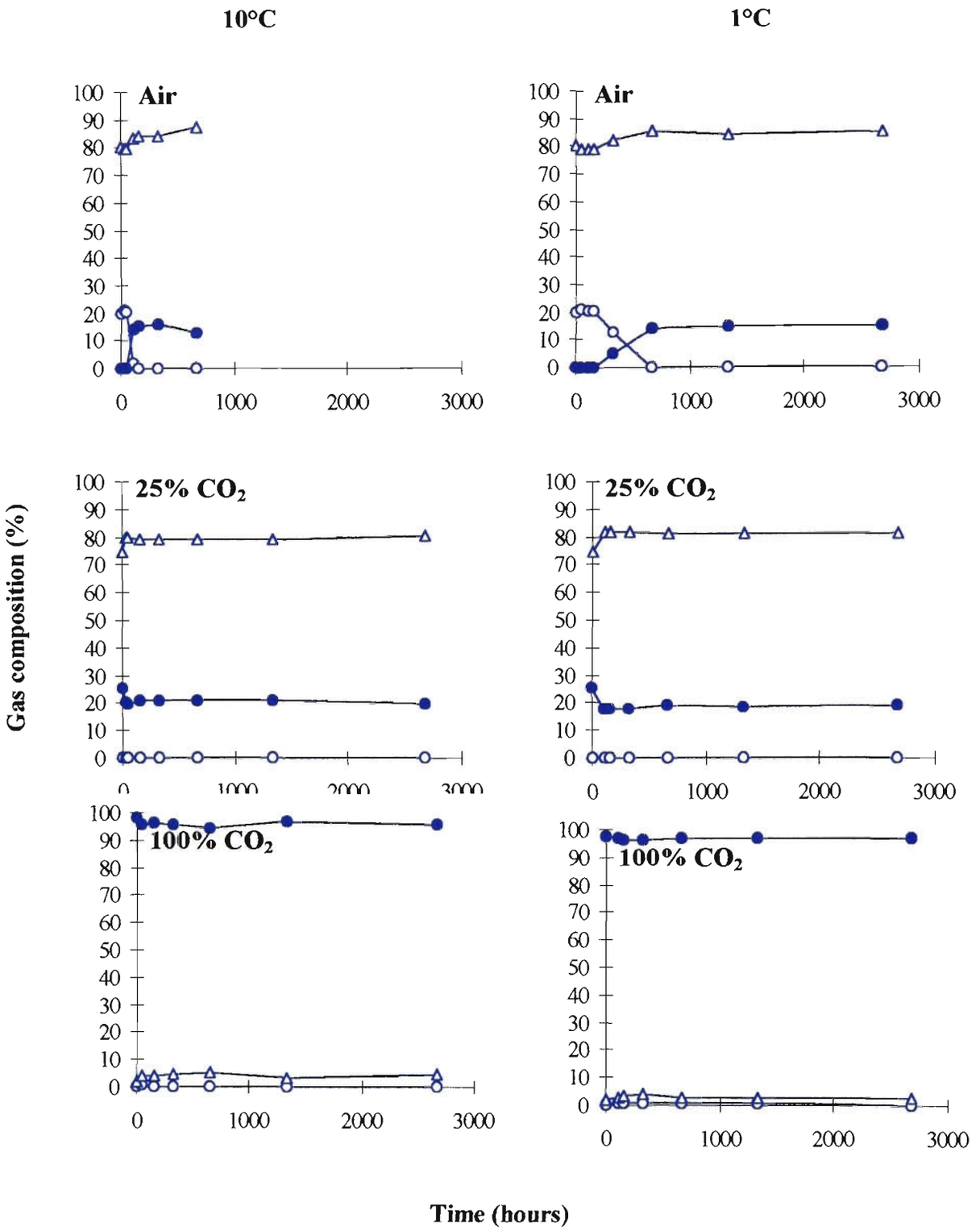


Figure 7.1 Atmospheric concentration of O_2 (○), CO_2 (●) and balance (△) within the packages prior to opening.

10°C increased by three and 7% respectively from their initial values of 80.1%.

For packages flushed with 25% CO_2/75% N_2 there was a reduction in carbon dioxide over time with a greater reduction occurring at 10°C than at 1°C (Figure 7.1). At 10°C, carbon dioxide decreased from 25% to 17%, while at 1°C the decrease was from 25% to 20%. After the initial decrease, there was no further reduction in the proportion of carbon dioxide. As carbon dioxide decreased, balance gases increased: in packages stored at 10°C, balance gases increased from 78.84% to 79.60%, while at 1°C storage this increased from 74.84% to 81.71%. After the initial increase in balance gases, there was no further change. The percentage of oxygen remained unchanged throughout the experiment at <0.3%.

For the packages flushed with 100% CO_2 there was no discernible change in the percentages of any of the three gases (Figure 7.1). Carbon dioxide was at least 96% with one exception, a 10°C package removed at 660 hours where carbon dioxide was 94.54%. Oxygen was always less than 0.6% while balance gases were always less than 4.5% throughout the experiment at either storage temperature.

7.3.2 Growth of the total population under different environmental conditions

Figures of the growth response were drawn to visualise the growth of the total population, *Lb. sakei*, *P. fluorescens*, *B. thermosphacta* and *H. alvei* over the time course of the experiments (Figures 7.2-7.11). Then log-logistic curves were fitted to the data points in order to estimate the effects of environmental factors singly and in different combinations on three aspects of microbial growth.

7.3.2.1 Storage in air at 10°C

For air-packaged samples, the total population at pH 5.5 on the control, one and 2% sodium chloride and propyl-paraben all followed the same growth pattern (Figure 7.2). The maximum population of $>10^8$ cfu/cm^2 was achieved within 108 hours, with both the control and 1% sodium chloride exceeding 10^9 cfu/cm^2. The only exception was the potassium sorbate treatment, which only reached a final number of 10^6 cfu/cm^2, which was almost three $\log_{10}$ less than the control. For the pH 6.0 plates, there was no difference in the growth pattern seen for the five treatments, where population numbers exceeded 10^8 cfu/cm^2 within 108 hours (Figure 7.2).

7.3.2.2 Storage in 25% CO_2 at 10°C

All growth curves followed the same pattern, however, the final numbers attained varied between

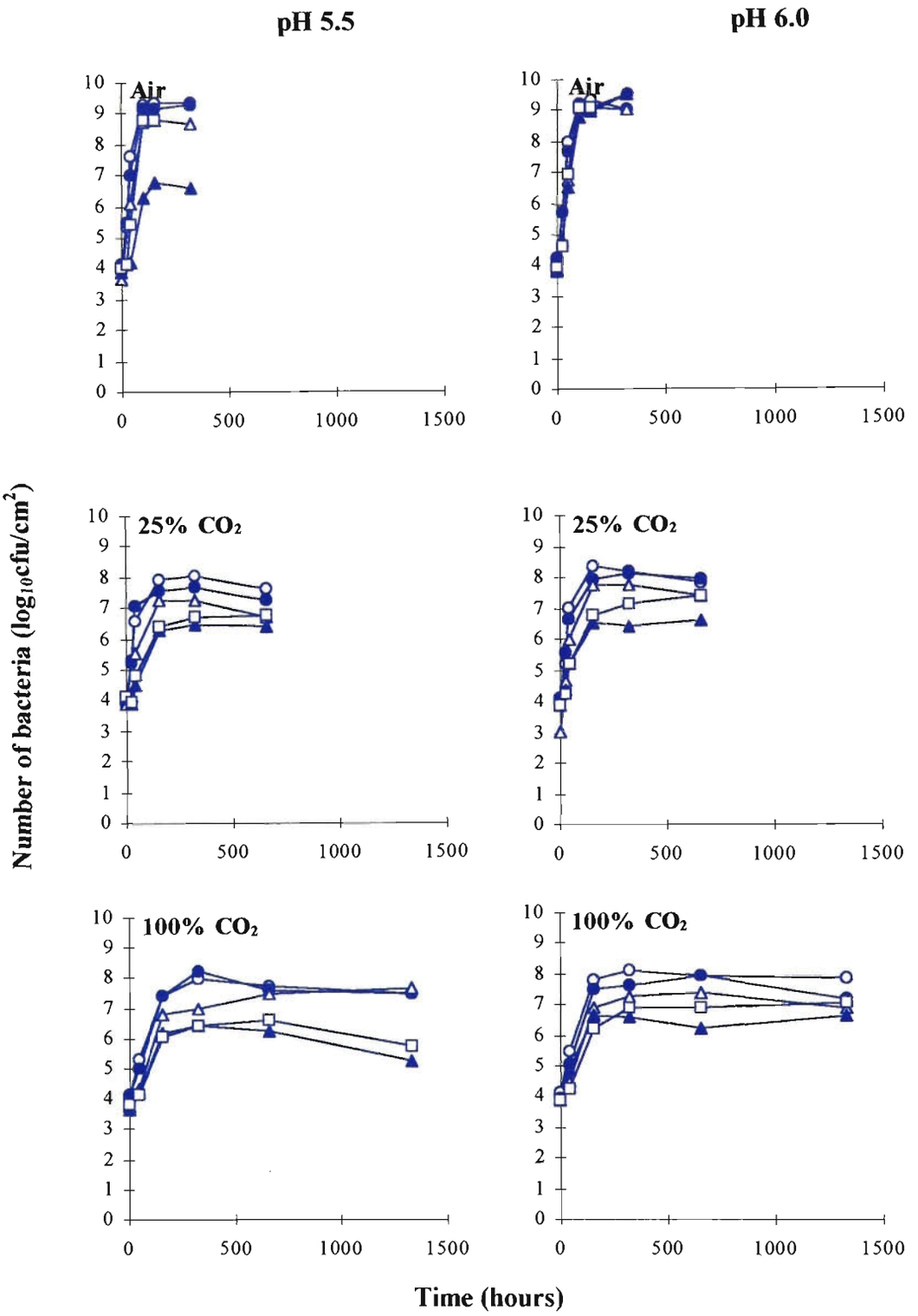


Figure 7.2 The effect storage at 10°C at either pH 5.5 or 6.0 on the growth of the total population when exposed to preservatives (control (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□)) and incubation in different atmospheres (air, 25% CO_2 or 100% CO_2).

treatments (Figure 7.2). At pH 5.5 under 25% CO_2 the control plates had the highest final numbers, followed in decreasing order by one and 2% sodium chloride, all of which were above 10^7 cfu/cm^2. The populations on the potassium sorbate and propyl-paraben treatments had the same growth trend but numbers on the propyl-paraben treatments were higher and neither had total population above 10^7 cfu/cm^2.

The population growth on the pH 6.0 plates mirrored those observed at pH 5.5, however final numbers were greater at pH 6.0. There was a reduced difference in the number of bacteria present on the control and one and 2% sodium chloride treatments. The final numbers on the potassium sorbate treatment were two $\log_{10}$ less than the control while plates with propyl-paraben were only one log10 less.

7.3.2.3 Storage in 100% CO_2 at 10°C

At pH 5.5 there was no difference in bacterial numbers between the control and 1% sodium chloride when grown at 10°C under 100% CO_2 (Figure 7.2). The final numbers for the 2% sodium chloride treatment reached the same final number as the control, except this occurred after a longer period of incubation. Numbers seen in the presence of potassium sorbate and propyl-paraben increased at the same rate, which was lower than the control. These two reached a maximum population of 10^6 cfu/cm^2 at 324 hours, then the numbers began to decline.

When the pH of the plates was 6.0, all five treatments followed the same pattern under 100% CO_2 at 10°C (Figure 7.2). A higher population number was achieved by the control, which reached 10^8 cfu/cm^2 at 324 hours, and for 1% sodium chloride numbers reached a maximum of 10^7 cfu/cm^2 at 156 hours and failed to increase further. For the 2% sodium chloride treatment, numbers reached a final population density of 10^7 cfu/cm^2 at 324 hours. The population for the potassium sorbate treatment was the lowest, at 10^6 cfu/cm2, while the propyl-paraben treatment had final population numbers between those seen for 2% sodium chloride and potassium sorbate. All the final population numbers were higher at pH 6.0 than pH 5.5 (Figure 7.2).

7.3.2.4 Storage in air at 1°C

The pH 5.5 control had the highest number of bacteria at above 10^9 cfu/cm^2, which was reached by 324 hours. The 1% sodium chloride plates had final numbers which were 0.5 $\log_{10}$ less than the control while 2% sodium chloride numbers were one $\log_{10}$ less than the control (Figure 7.3). The total population for the potassium sorbate treatment at pH 5.5 reached stationary phase by 1334 hours, however, the maximum

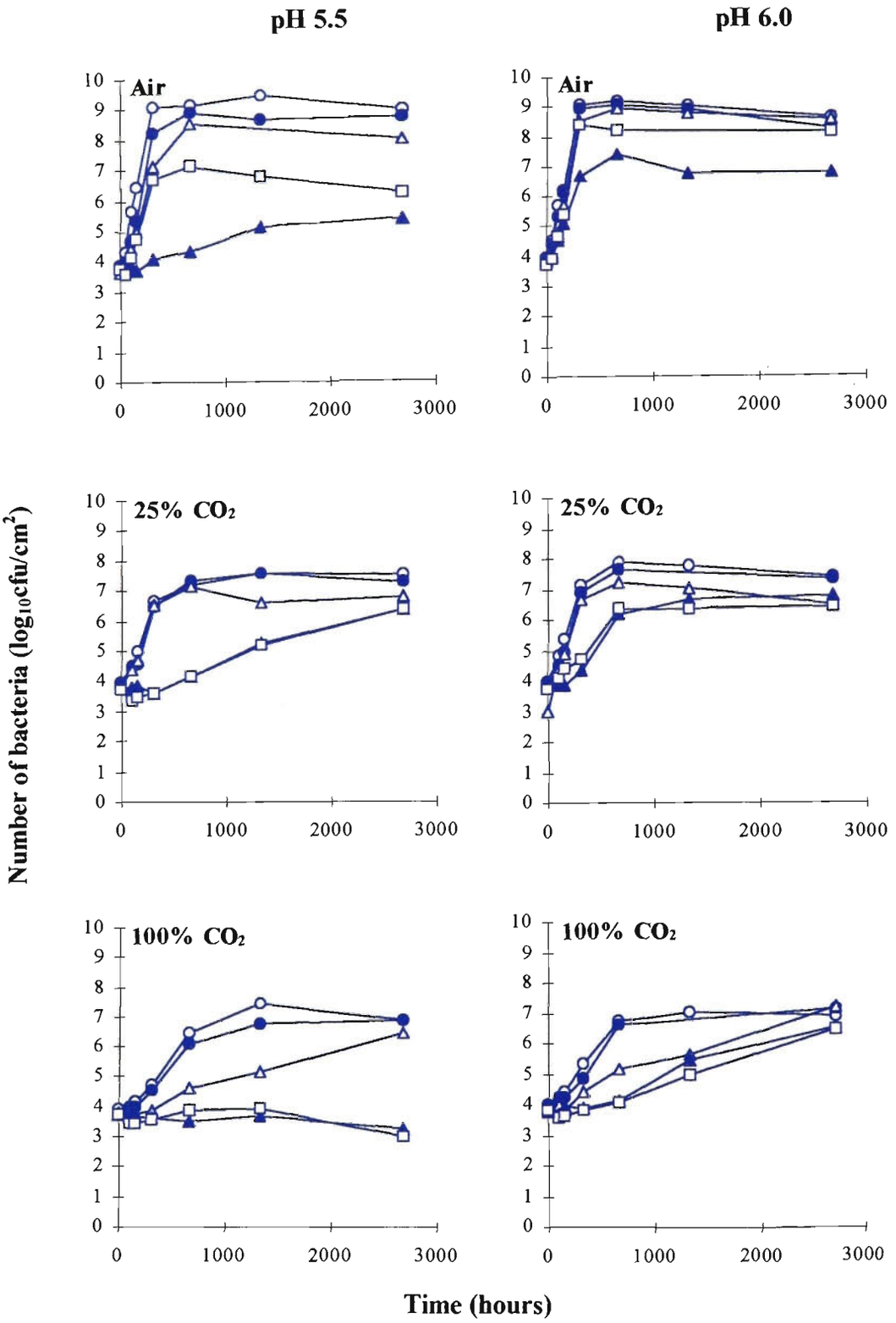


Figure 7.3 The effect storage at 1°C at either pH 5.5 or 6.0 on the growth of the total population when exposed to preservatives (control (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□)) and incubation in different atmospheres (air, 25% CO_2 or 100% CO_2).

population density was only 10^5 cfu/cm^2. This was four $\log_{10}$ less than the control, while the propyl-paraben treatment had a final population of 10^7 cfu/cm^2 after which the number of bacteria began to decrease.

There was less variation in the population growth at pH 6.0, with all treatments reaching maximum numbers within 324 hours, except for potassium sorbate treatment which required 660 hours (Figure 7.3). The control, one and 2% sodium chloride and propyl-paraben treatments all followed the same pattern. The principal difference was that the final numbers for the control was 10^9 cfu/cm^2, while the remaining three were 10^8 cfu/cm^2. Again, the potassium sorbate counts showed the lowest maximum population density at 10^6 cfu/cm^2.

7.3.2.5 Storage in 25% CO_2 at 1°C

For plates incubated under 25% CO_2 at 1°C and pH 5.5, the populations seen for the control and one and 2% sodium chloride followed the same growth pattern until 660 hours, at which point the number of bacteria on the control and 1% sodium chloride treatments continued to increase, finally reaching 10^8 cfu/cm^2 (Figure 7.3). On the 2% sodium chloride treatment, however, numbers began to decrease after reaching 10^7 cfu/cm^2. The same growth pattern was observed for potassium sorbate and propyl-paraben treatments, however, initially there was a lag phase which did not occur for the other treatments at this pH. Growth commenced on these two treatments between 324 and 660 hours. A definite maximum population number was not reached over the experimental time course and the population density at 2678 hours was 10^6 cfu/cm^2.

Under the same conditions at pH 6.0, the control and one and 2% sodium chloride treatments followed the same growth pattern as seen at pH 5.5 with marginally higher final numbers (Figure 7.3). Unlike pH 5.5, there was not a lag phase for either potassium sorbate or propyl-paraben at pH 6.0. The number of bacteria on these plates increased marginally at each time point. In addition, the maximum population density of 10^6 cfu/cm^2 was reached within 660 hours for both treatments.

7.3.2.6 Under 100% CO_2 stored at 1°C

Initially there was no increase in the number of bacteria for any treatments packaged under 100% CO_2 at pH 5.5 stored at 1°C (Figure 7.3). After 156 hours, the number of bacteria on the control and 1% sodium chloride treatments began to increase and reached a maximum of 10^6-10^7 cfu/cm^2 at 660 hours.

The bacterial population on 2% sodium chloride plates did not reach 10^6 cfu/cm^2 until the conclusion of the experiment at 2678 hours. There was no increase in bacterial numbers for either potassium sorbate or propyl-paraben treatments, instead numbers decreased immediately from initial levels.

At pH 6.0 under 100% CO_2 at 1°C, there was a lag in the growth of the total population although it was not as great as that observed at pH 5.5 (Figure 7.3). In addition, all treatments reached maximum population densities above 10^6 cfu/cm^2. The pattern of growth was identical for the control and 1% sodium chloride treatments, with higher numbers on the control. The 2% sodium chloride treatment was the next to increase in cell density, which was closely followed by both potassium sorbate and propyl-paraben. These last three treatments reached population numbers of 10^6 cfu/cm^2 at the conclusion of the experiment at 2678 hours.

The next section describes the individual isolates and their response to temperature, atmosphere and preservatives as individual cultures on plates.

7.3.3 Growth response of *Lb. sakei* to different environmental conditions

7.3.3.1 Storage in air at 10°C

In air at pH 5.5 following storage at 10°C the increase in the number of *Lb. sakei* on the control, potassium sorbate and propyl-paraben treatments followed the same pattern (Figure 7.4). The number of *Lb. sakei* reached 10^6 cfu/cm^2 within 156 hours for the control, 1% potassium sorbate and propyl-paraben. Although both one and 2% sodium chloride treatments achieved a similar final number, more time was required. At 324 hours, these two treatments had a cell density of 10^6 cfu/cm^2 at 156 hours the numbers were only 10^4 cfu/cm^2.

The growth patterns of *Lb. sakei* at pH 6.0 reflected those observed at pH 5.5 (Figure 7.4), except that at pH 6.0, *Lb. sakei* numbers did not increase above 10^6 cfu/cm^2 until 660 hours in the presence of either 1% or 2% sodium chloride.

7.3.3.2 Storage in 25% CO_2 at 10°C

The fastest growth rate and highest population number were achieved on the control at pH 5.5 under 25% CO_2 at 10°C storage temperature (Figure 7.4). The number of *Lb. sakei* on the control

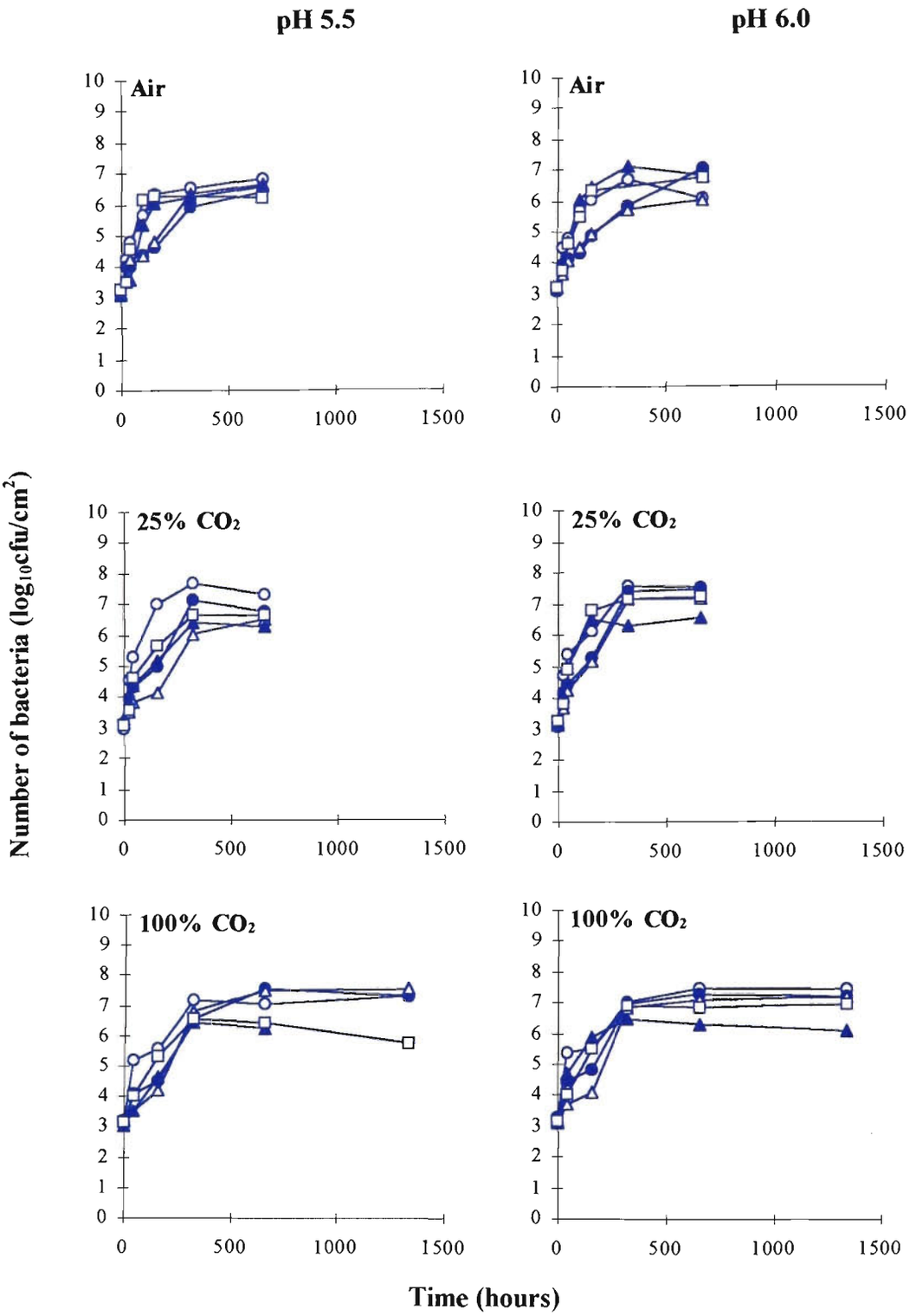


Figure 7.4 The effect storage at 10°C at either pH 5.5 or 6.0 on the growth of *Lb. sakei* when exposed to preservatives (control (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□)) and incubation in different atmospheres (air, 25% CO_2 or 100% CO_2).

was consistently one to two $\log_{10}$ greater than the other treatments throughout the experiment. For 1% sodium chloride, potassium sorbate and propyl-paraben treatments, the same increase in numbers of *Lb. sakei* was seen. Population densities of 10^6 cfu/cm^2 were achieved by these three treatments at 324 hours. Within this time frame, *Lb. sakei* on 2% sodium chloride reached the same population density, however, initiation of growth for this treatment was slower than for others treatments.

At pH 6.0 under 25% CO_2 and 10°C storage, the growth rate seen for the control, potassium sorbate and propyl-paraben plates was similar until 156 hours. At this point, the number of *Lb. sakei* on the potassium sorbate plate remained at 10^6 cfu/cm^2, while on the control and propyl-paraben treatments numbers continued to increase to 10^7 cfu/cm^2. The number of *Lb. sakei* on one and 2% sodium chloride increased to 10^7 cfu/cm^2 by 324 hours, as seen for the other treatments, but at the earlier time points the numbers of *Lb. sakei* on the sodium chloride treatment were always lower than the other treatments.

7.3.3.3 Storage in 100% CO_2 at 10°C

The response of *Lb. sakei* to the five treatment groups was the same at both pH levels following storage under 100% CO_2 at 10°C (Figure 7.4). The number of *Lb. sakei* on all the treatments reach 10^6 cfu/cm^2 by 324 hours. Unlike the other two gas atmosphere treatments, the number of *Lb. sakei* on the sodium chloride treatments was not lower than the other three treatments. At pH 5.5 the final number of *Lb. sakei* on potassium sorbate and propyl-paraben was one $\log_{10}$ less than the control and sodium chloride plates. At pH 6.0, the number of *Lb. sakei* on the potassium sorbate and propyl-paraben treatment was not distinctly different from the other treatment at pH 5.5, although at 324 and 660 hours *Lb. sakei* on the potassium sorbate treatment were one $\log_{10}$ less than the control (Figure 7.4).

In contrast to storage at 10°C, there was a wider variety of response seen for *Lb. sakei* under the different treatments at 1°C. The effect of sodium chloride on growth in air and under 25% CO_2 was not as profound and there appeared to be a lag phase for all treatments under each atmosphere (Figure 7.5).

7.3.3.4 Storage in air at 1°C

The control and 1% sodium chloride had the same growth pattern, where maximum numbers of 10^6 cfu/cm^2 were reached at 1334 hours at pH 5.5 (Figure 7.5). At 660 hours, the number of *Lb. sakei* on the 1% sodium chloride treatment was higher than the control plates. *Lb. sakei* on 2% sodium chloride, potassium sorbate and propyl-paraben treatments increased in number over the duration of the experiment,

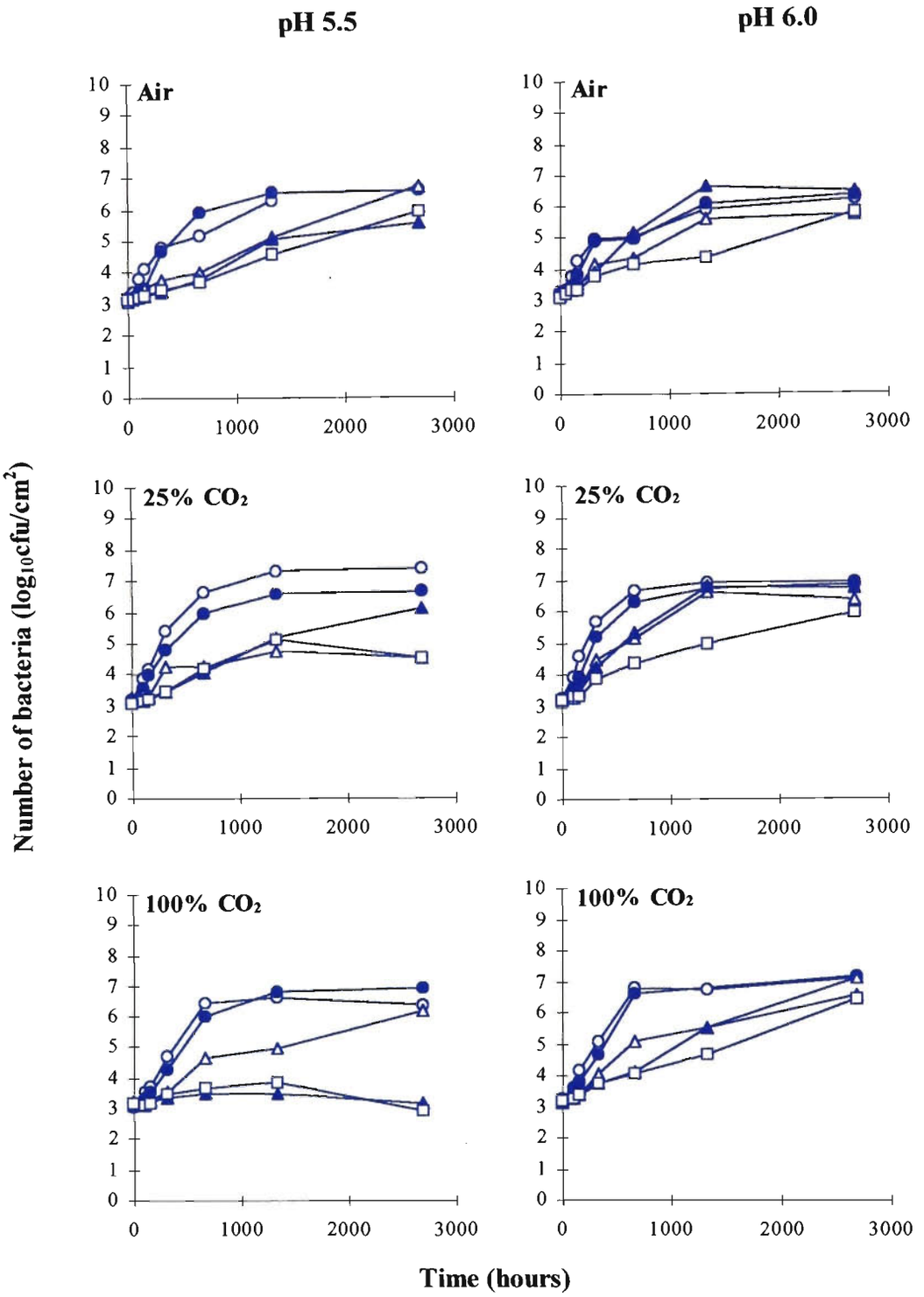


Figure 7.5 The effect storage at 1°C at either pH 5.5 or 6.0 on the growth of *Lb. sakei* when exposed to preservatives (control (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□)) and incubation in different atmospheres (air, 25% CO_2 or 100% CO_2).

with only those on 2% sodium chloride plates reaching 10^6 cfu/cm^2. At this pH, the propyl-paraben treatment always had the smallest population density of *Lb. sakei*.

Lb. sakei at pH 6.0 on the control and 1% sodium chloride treatments increased in numbers between 156 and 324 hours (Figure 7.5). The same occurrence was observed for 2% sodium chloride and propyl-paraben treatments except growth did not commence until after 324 hours, while the potassium sorbate treatment numbers continued to increase over the experimental time course. At 660 hours, *Lb. sakei* on all treatment groups, for except propyl-paraben, reached a maximum population density of 10^6 cfu/cm^2. This number was not reached on the propyl-paraben treatment until 2678 hours.

7.3.3.5 Storage in 25% CO_2 at 1°C

At pH 5.5 under 25% CO_2 after storage at 1°C, the number of *Lb. sakei* on the control and 1% sodium chloride plates followed the same pattern (Figure 7.5). However, the number of *Lb. sakei* on the control plate was increasingly higher than those on 1% sodium chloride. After an initial increase, the number of *Lb. sakei* on the 2% sodium chloride treatment increased at the same rate as those on propyl-paraben. The final numbers of *Lb. sakei* for these two treatments were only one $\log_{10}$ greater than the initial number of cells. The number of *Lb. sakei* on the potassium sorbate plates was slow to increase, however, once growth commenced the population number continued to increase, finally reaching 10^6 cfu/cm^2 at 2678 hours.

As seen for pH 5.5, the control and 1% sodium chloride treated *Lb. sakei* increased at the same rate at pH 6.0, with higher numbers on the control (Figure 7.5). The 2% sodium chloride and potassium sorbate treatments increased at the same rate, where the numbers of *Lb. sakei* on 2% sodium chloride plates were less than on potassium sorbate. There was an initial lag phase seen for the propyl-paraben treatment: after 156 hours, the numbers of *Lb. sakei* began to increase and reached 10^5 cfu/cm^2 at 2678 hours.

7.3.3.6 Storage in 100% CO_2 at 1°C

The response of *Lb. sakei* on the control and 1% sodium chloride plates was similar under 100% CO_2 at pH 5.5 at 1°C (Figure 7.5) and final numbers on the 1% sodium chloride treatment were actually higher than the control. *Lb. sakei* on 2% sodium chloride had increased to 10^4 cfu/cm^2 by 660 hours, finally reaching 10^5 cfu/cm^2 at 2678 hours. There was no increase in the number of *Lb. sakei* on the potassium sorbate and propyl-paraben plates within 2678 hours, instead numbers for both treatments began to

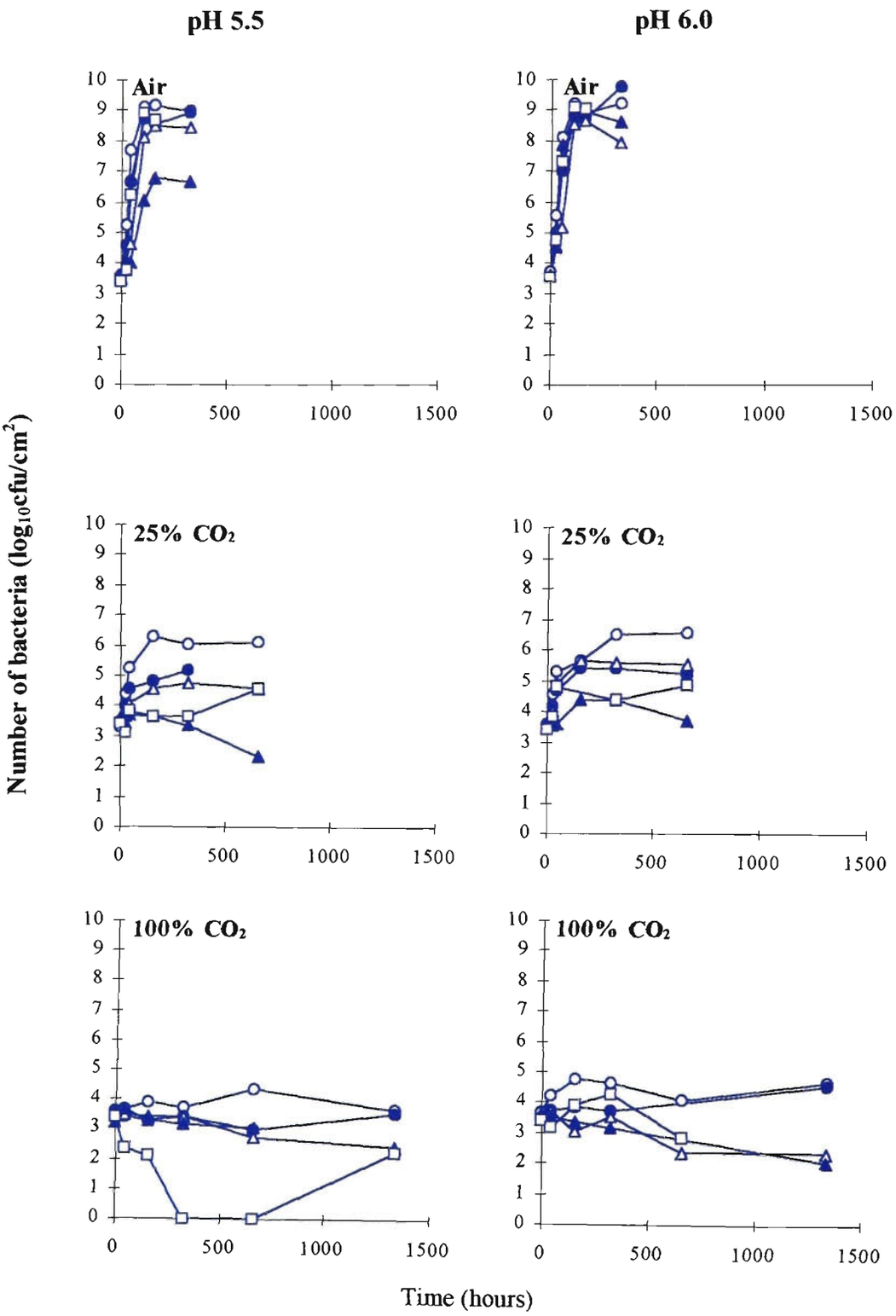


Figure 7.6 The effect of pH (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□) on *P. fluorescens* growth in air, 25% CO_2 and 100% CO_2 at pH 5.5 or 6.0 following storage at 10°C.

decrease from initial inoculum numbers.

At pH 6.0, 10^6 cfu/cm^2 was reached by the control and 1% sodium chloride at 660 hours and both treatments had a similar growth pattern. *Lb. sakei* on the remaining three treatments, 2% sodium chloride, potassium sorbate and propyl-paraben, increased slowly and only increased above 10^6 cfu/cm^2 at 2678 hours.

7.3.4 Response of *P. fluorescens* to different environmental conditions

7.3.4.1 Storage in air at 10°C

In air and stored at 10°C, there was a uniform response of *P. fluorescens* to each of the treatment groups at both pH levels, where final numbers exceeded 10^8-10^9 cfu/cm^2 within 48 to 108 hours (Figure 7.6). There was one notable exception: *P. fluorescens* exposed to potassium sorbate at pH 5.5, where the population density reached a maximum of 10^6 cfu/cm^2 at 108 hours.

7.3.4.2 Storage in 25% CO_2 at 10°C

There was a wider variety of responses to the treatments when *P. fluorescens* was under 25% CO_2 at 10°C at both pH levels (Figure 7.6). At pH 5.5, the number of *P. fluorescens* on the control plate increased to only 10^6 cfu/cm^2. At the two sodium chloride levels, the number of *P. fluorescens* increased only one $\log_{10}$ above the initial level. The number of *P. fluorescens* on the potassium sorbate plates decreased over the experimental period, while the number on *P. fluorescens* on the propyl-paraben plates increased by one $\log_{10}$ by 2678 hours.

The response seen for *P. fluorescens* on the control treatment at pH 6.0 was similar to that observed at pH 5.5, while on the two sodium chloride treatment plates the numbers increased above 10^5 cfu/cm^2, which was not achieved at pH 5.5 (Figure 7.6). When exposed to potassium sorbate, there was a slight increase in cell number but this returned to the initial level by 2678 hours. As seen with the sodium chloride plates, the number of *P. fluorescens* increased to 10^5 cfu/cm^2.

7.3.4.3 Storage in 100% CO_2 at 10°C

The growth of *P. fluorescens* at both of the pHs tested was limited when stored at 10°C under 100% CO_2. There was no change in the number of *P. fluorescens* at pH 5.5 on the control or on one and 2% sodium

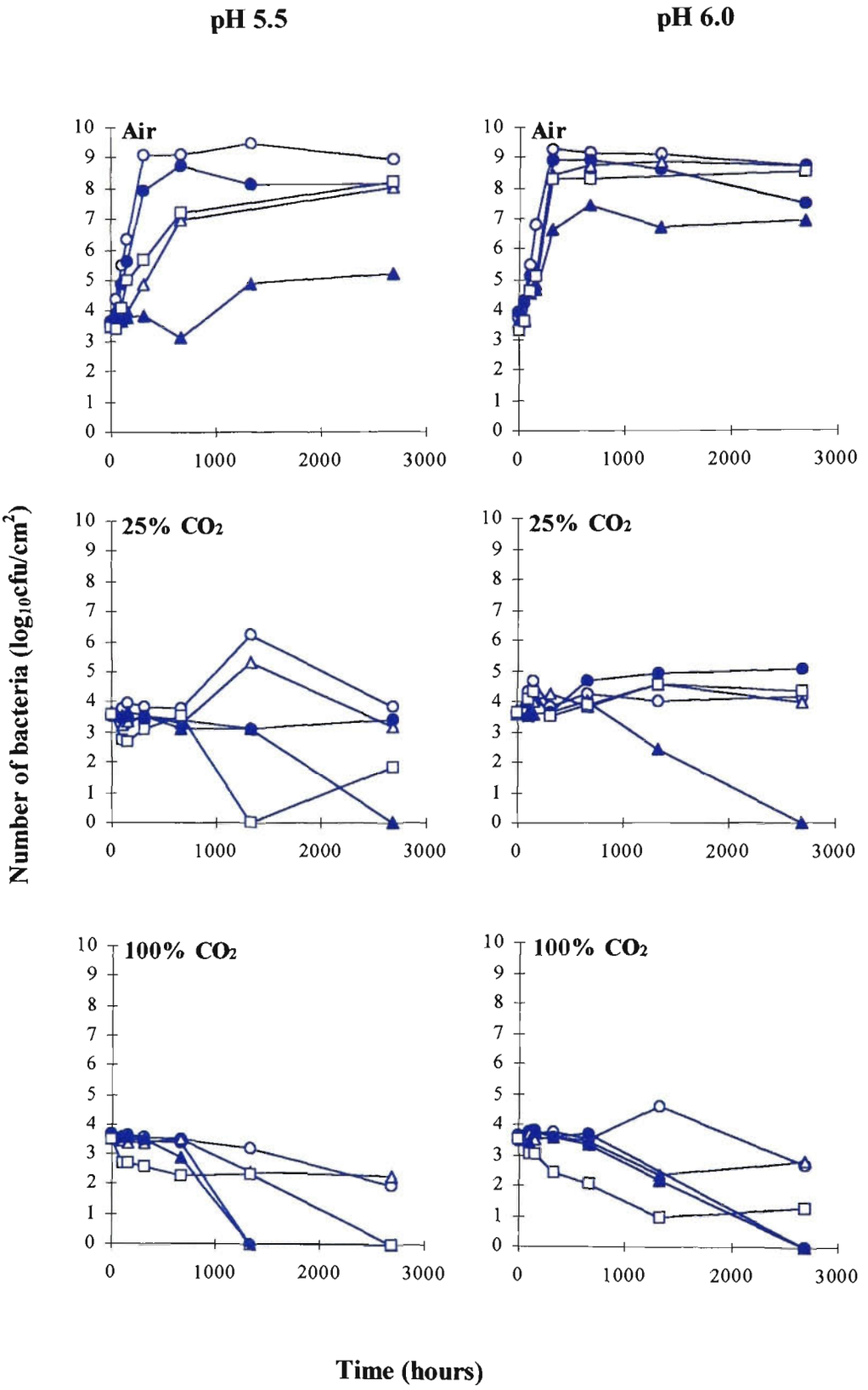


Figure 7.7 The effect storage at 1°C at either pH 5.5 or 6.0 on the growth of *P. fluorescens* when exposed to preservatives (control (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□)) and incubation in different atmospheres (air, 25% CO_2 or 100% CO_2).

chloride. There was a slight decrease in the number of *P. fluorescens* on the potassium sorbate while there were no *P. fluorescens* detected on the propyl-paraben plates at 324 and 660 hours but there was 10^2 cfu/cm^2 detected at 1334 hours at pH 5.5. Under these conditions, *P. fluorescens* did not increase in number and on some plates there was a decline in the number of viable cells. No *P. fluorescens* were isolated from some plates while only a few were isolated from another: this caused the fluctuation in numbers observed."

At pH 6.0, there was a one $\log_{10}$ increase in the number of *P. fluorescens* over the course of the experiment on the control and 2% sodium chloride, while for 1% sodium chloride and potassium sorbate *P. fluorescens* numbers decreased one $\log_{10}$ from initial numbers and on propyl-paraben increased one $\log_{10}$ above the initial number then decreased to one $\log_{10}$ below this.

7.3.4.4 Storage in air at 1°C

P. fluorescens reached maximum population density of 10^9 cfu/cm^2 on the control plate at 324 hours, while numbers on 1% sodium chloride only reached 10^8 cfu/cm^2 at 660 hours at pH 5.5 in air at 1°C (Figure 7.7). The same pattern was seen for 2% sodium chloride and propyl-paraben treatment: numbers increased to 10^7 cfu/cm^2 at 660 hours and reached 10^8 cfu/cm^2 at 2678 hours. After an initial decline in *P. fluorescens* numbers on potassium sorbate, numbers increased marginally to a maximum of 10^5 cfu/cm^2.

7.3.4.5 Storage in 25% CO_2 at 1°C

Under 100% CO_2 at 1°C on pH 5.5, *P. fluorescens* exhibited no significant growth over the 2678 hours (Figure 7.7). At 1554 hours, there was some increase in the number of *P. fluorescens* detected but numbers were again at the initial level by 2678 hours. The number of *P. fluorescens* on the potassium sorbate and propyl-paraben plates decreased to undetectable levels at 1554 and 2678 hours respectively. At 2678 hours, 10^2 cfu/cm^2 of *P. fluorescens* were detected on the propyl-paraben plates.

Under the same storage conditions at pH 6.0, no changes in the number of *P. fluorescens* were seen on the control, one and 2% sodium chloride or propyl-paraben plates (Figure 7.7). On the potassium sorbate plates the number of *P. fluorescens* declined after 660 hours and were finally undetectable at 2678 hours.

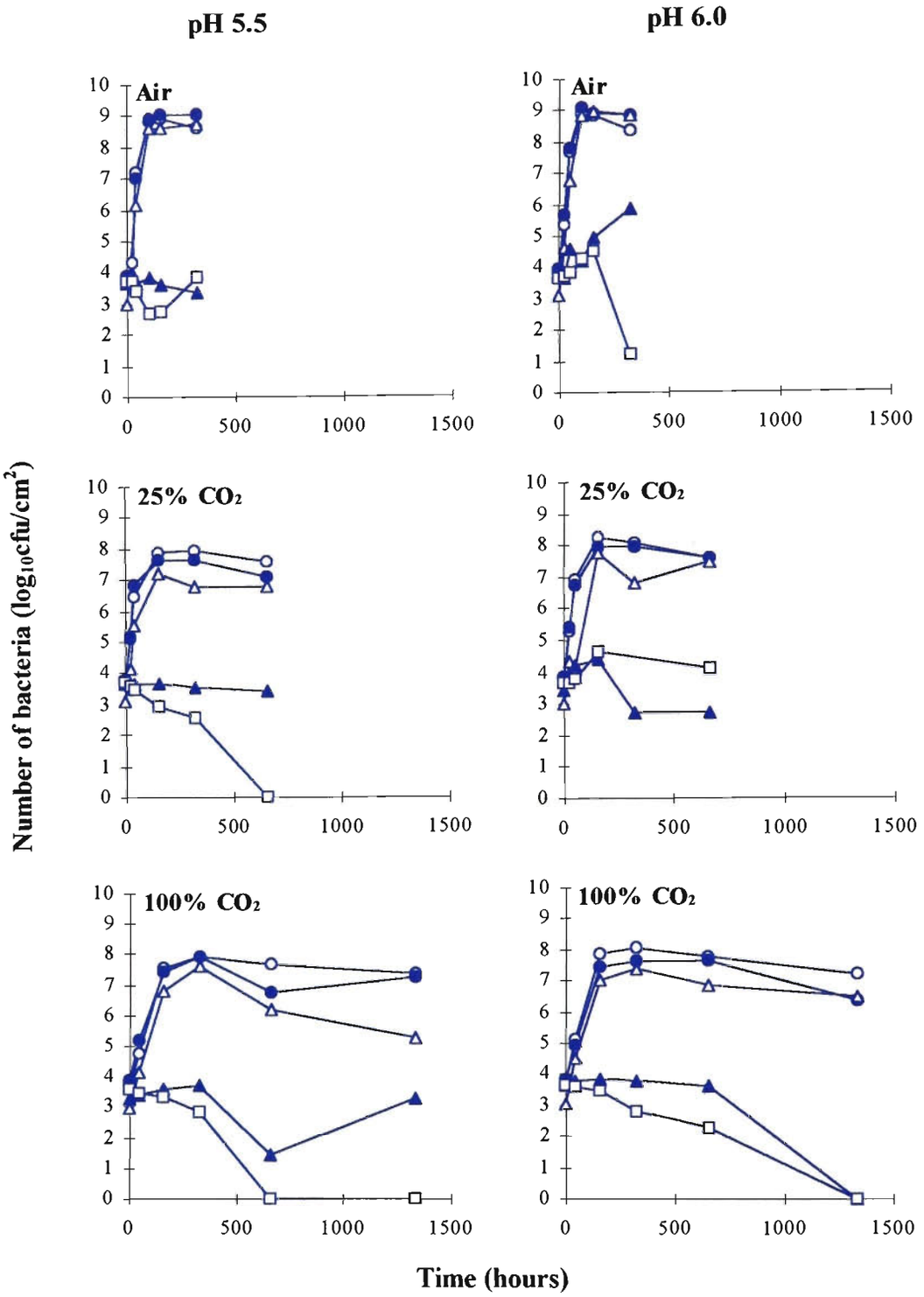


Figure 7.8 The effect storage at 10°C at either pH 5.5 or 6.0 on the growth of *B. thermosphacta* when exposed to preservatives (control (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□)) and incubation in different atmospheres (air, 25% CO_2 or 100% CO_2).

7.3.4.6 Storage in 100% CO_2 at 1°C

There was no increase in the number of *P. fluorescens* for any of the treatments at either pH 5.5 or 6.0 (Figure 7.7). *P. fluorescens* became undetectable on the potassium sorbate and propyl-paraben plates at pH 5.5 by 1554 hours and at pH 6.0 by 2678 hours.

7.3.5 Growth response of *B. thermosphacta* to different environmental conditions

7.3.5.1 Storage in air at 10°C

The numbers of *B. thermosphacta* on the control and one and 2% sodium chloride plates increased rapidly when stored at 10°C in air at pH 5.5 (Figure 7.8): within 108 hours there were $>10^8$ cfu/cm^2. However, on the potassium sorbate and propyl-paraben treatments there was no increase in the number of *B. thermosphacta* within 2678 hours, indicating the efficiency of the concentrations of the preservatives used.

This growth pattern was similar under the same conditions at pH 6.0 for the control and sodium chloride treatments but not for potassium sorbate nor propyl-paraben treatments (Figure 7.8). *B. thermosphacta* had an initial lag phase on the potassium sorbate plates, after which numbers increased to 10^5 cfu/cm^2 at the conclusion of the experiment. For the propyl-paraben treatment, there was a dramatic decrease in *B. thermosphacta* numbers from 10^4 cfu/cm^2 to 10^1 cfu/cm^2 from time zero.

7.3.5.2 Storage in 25% CO_2 at 10°C

The number of *B. thermosphacta* on the control and 1% sodium chloride plates at pH 5.5 under 25% CO_2 increased at the same rate reaching 10^7 cfu/cm^2 at 156 hours. A similar increase was observed for *B. thermosphacta* exposed to 2% sodium chloride but numbers were less than the other two treatments. There was no change in the number of *B. thermosphacta* on the potassium sorbate plates (Figure 7.8). On the propyl-paraben plates there was a decrease in numbers of *B. thermosphacta*, so that eventually no viable cells were detected.

The same growth patterns as pH 5.5 and 6.0 for control and one and 2% sodium chloride treatments, only the number of *B. thermosphacta* on the 2% sodium chloride was higher at pH 6.0 (Figure 7.8).
There was a slight increase in the number of *B. thermosphacta* on the potassium sorbate plates which was followed by a decrease, while on the propyl-paraben treatment there was only a slight increase in cell numbers.

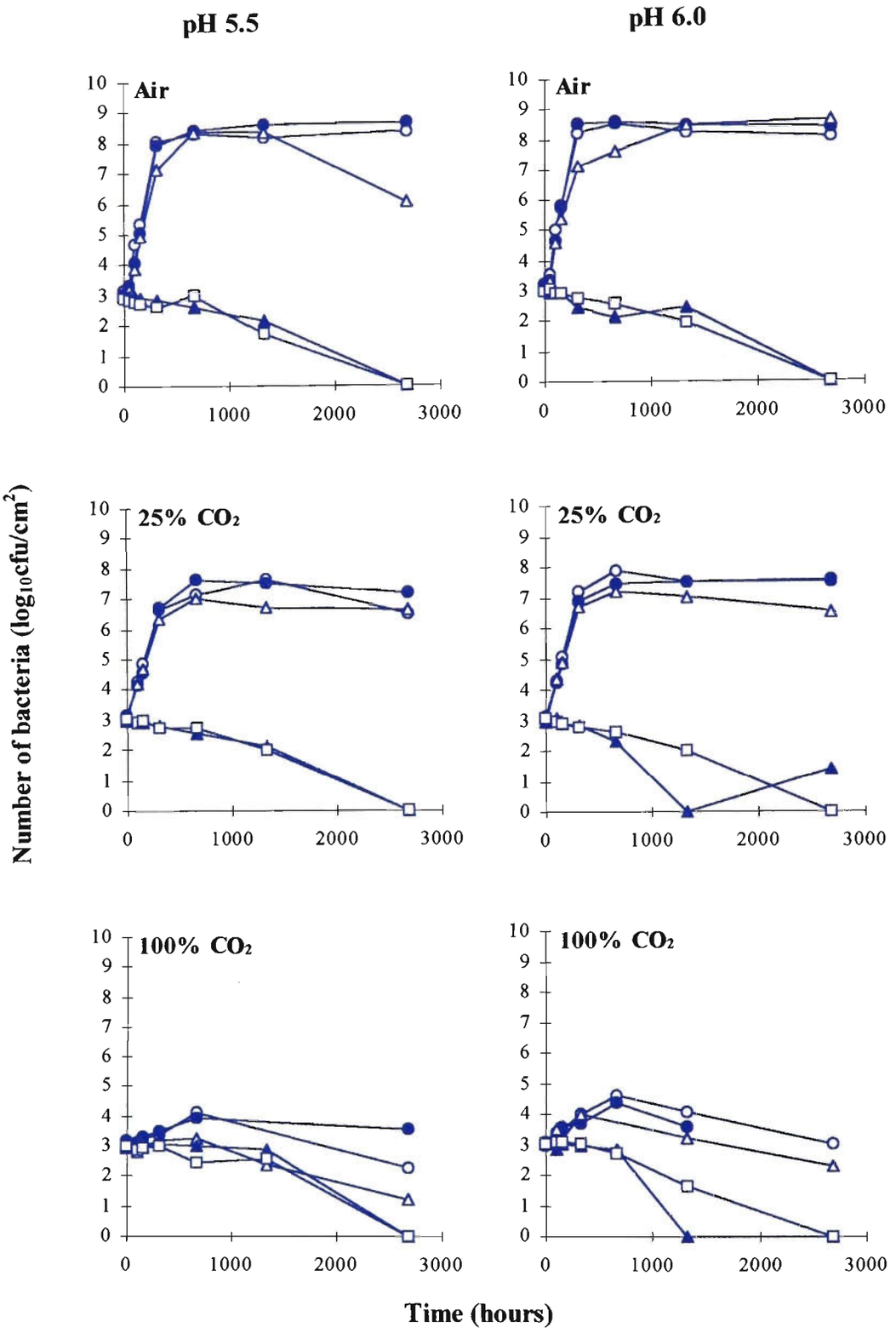


Figure 7.9 The effect storage at 1°C at either pH 5.5 or 6.0 on the growth of the *B. thermosphacta* when exposed to preservatives (control (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□)) and incubation in different atmospheres (air, 25% CO_2 or 100% CO_2).

7.3.5.3 Storage in 100% CO_2 at 10°C

The number of *B. thermosphacta* on the control and one or 2% sodium chloride plates increased at the same rate at pH 5.5 under 100% CO_2 at 10°C, where the number on 2% sodium chloride was consistently less than the control (Figure 7.8). The number of *B. thermosphacta* on the 2% sodium chloride plates decreased dramatically after 324 hours to 10^5 cfu/cm^2 at 1334 hours. The number of *B. thermosphacta* on the potassium sorbate treatment plates decreased two $\log_{10}$ between 324 and 660 hours then increased again to initial numbers at 1334 hours. *B. thermosphacta* numbers on the propyl-paraben plates decreased until being undetectable at 664 hours.

At pH 6.0 under the same conditions, the control and one or 2% sodium chloride exposed *B. thermosphacta* followed the same growth pattern as at pH 5.5, however the decrease in numbers on the 2% sodium chloride plates was not as distinct (Figure 7.8). The numbers of *B. thermosphacta* on potassium sorbate plates remained unchanged until 660 hours, after which time no viable cells were detected. In the presence of propyl-paraben, the numbers of *B. thermosphacta* decreased until they were no longer detectable at 1334 hours.

7.3.5.4 Storage in air and 25% CO_2 at 1°C

The response of *B. thermosphacta* to the treatment groups at pH 5.5 and 6.0 was identical under air and 25% CO_2 (Figure 7.9). The numbers on the control and one or 2% sodium chloride plates increased to 10^7 cfu/cm^2 at 324 hours for the air-packaged treatments, reaching a maximum of 10^8 cfu/cm^2 at 660 hours. There was one exception: on 2% sodium chloride plates at pH 6.0, this number was not reached until 1554 hours.

B. thermosphacta stored under 25% CO_2 exhibited a similar response to those stored in air. The final numbers of *B. thermosphacta* were higher in air than under 25% CO_2. The number of *B. thermosphacta* on the potassium sorbate and propyl-paraben plates declined until none were detected for both air and 25% CO_2 incubation.

7.3.5.5 Storage in 100% CO_2 at 1°C

There was a less than one $\log_{10}$ increase in the number of *B. thermosphacta* on the control and 1% sodium chloride plates under 100% CO_2 at 1° at pH 5.5 (Figure 7.9). For *B. thermosphacta* under the same incubation conditions on 2% sodium chloride, potassium sorbate and propyl-paraben plates, there was a

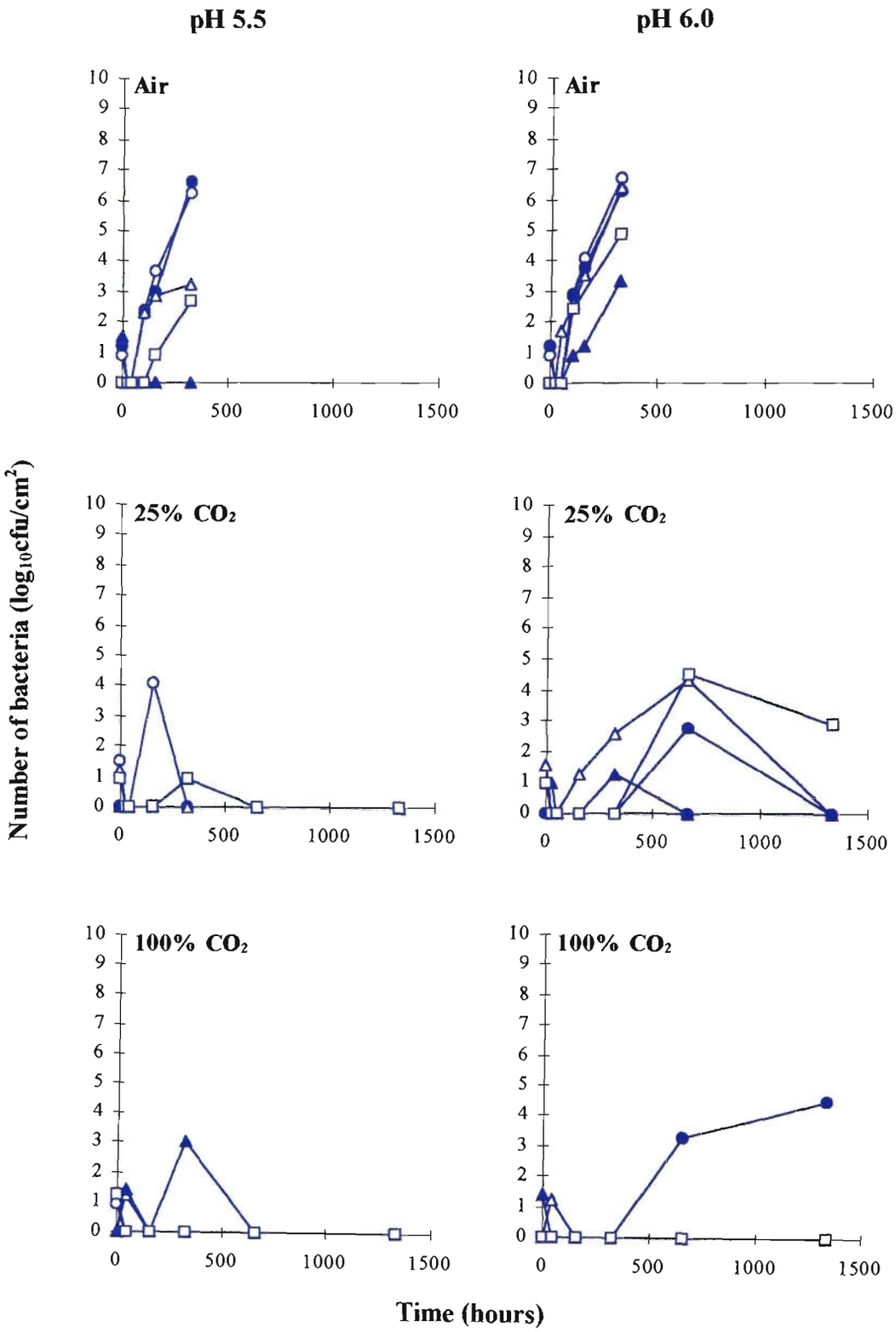


Figure 7.10 The effect storage at 10°C at either pH 5.5 or 6.0 on the growth of *H. alvei* when exposed to preservatives (control (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□)) and incubation in different atmospheres (air, 25% CO_2 or 100% CO_2).

decrease in numbers over time, with only potassium sorbate plates having detectable numbers of cells present.

Under the same conditions at pH 6.0 there was a one $\log_{10}$ increase in the number of *B. thermosphacta* on the control and 1% sodium chloride plates, however, cell numbers then decreased to initial levels. The number of *B. thermosphacta* on the 2% sodium chloride plates did not change significantly over 2678 hours. The number of *B. thermosphacta* on the potassium sorbate and propyl-paraben plates decreased until no viable cells were detected at either 1334 and 2678 hours respectively.

7.3.6 Growth response of *H. alvei* to different environmental conditions

7.3.6.1 Storage in air at 10°C

H. alvei stored in air at 10°C at pH 5.5 increased from undetectable numbers to 10^6 cfu/cm^2 on the control and 1% sodium chloride plates (Figure 7.10). On the 2% sodium chloride plates, the number of *H. alvei* only reached 10^3 cfu/cm^2 where bacterial numbers remained. There was no increase in the number of *H. alvei* in the presence of potassium sorbate. After a lag phase, there was an increase in numbers of *H. alvei* for propyl-paraben treatment and these reached a maximum of 10^4 cfu/cm^2 at 336 hours.

At pH 6.0 under the same conditions, the number of *H. alvei* increased for all treatments (Figure 7.10). On the control and one or 2% sodium chloride, the number of *H. alvei* increased at the same rate and a maximum of 10^6 cfu/cm^2 was reached at 334 hours. On potassium sorbate, the number of *H. alvei* increased after an initial lag phase reaching 10^3 cfu/cm^2 at 334 hours. There was an increase in numbers of *H. alvei* on propyl-paraben which reached a maximum of 10^4 cfu/cm^2 at 334.

7.3.6.2 Storage in 25 and 100% CO_2 at 10°C

Under 25 and 100% CO_2, there was sporadic growth of *H. alvei* for all treatments (Figure 7.10). Under 25% CO_2 on potassium sorbate, there appeared to be an increase in the number of *H. alvei* cells, however, this decreased again to undetectable levels. *H. alvei* also appeared to be growing on the 1% sodium chloride plate under 100% CO_2 however this occurred towards the conclusion of the experiment and could not be measured further.

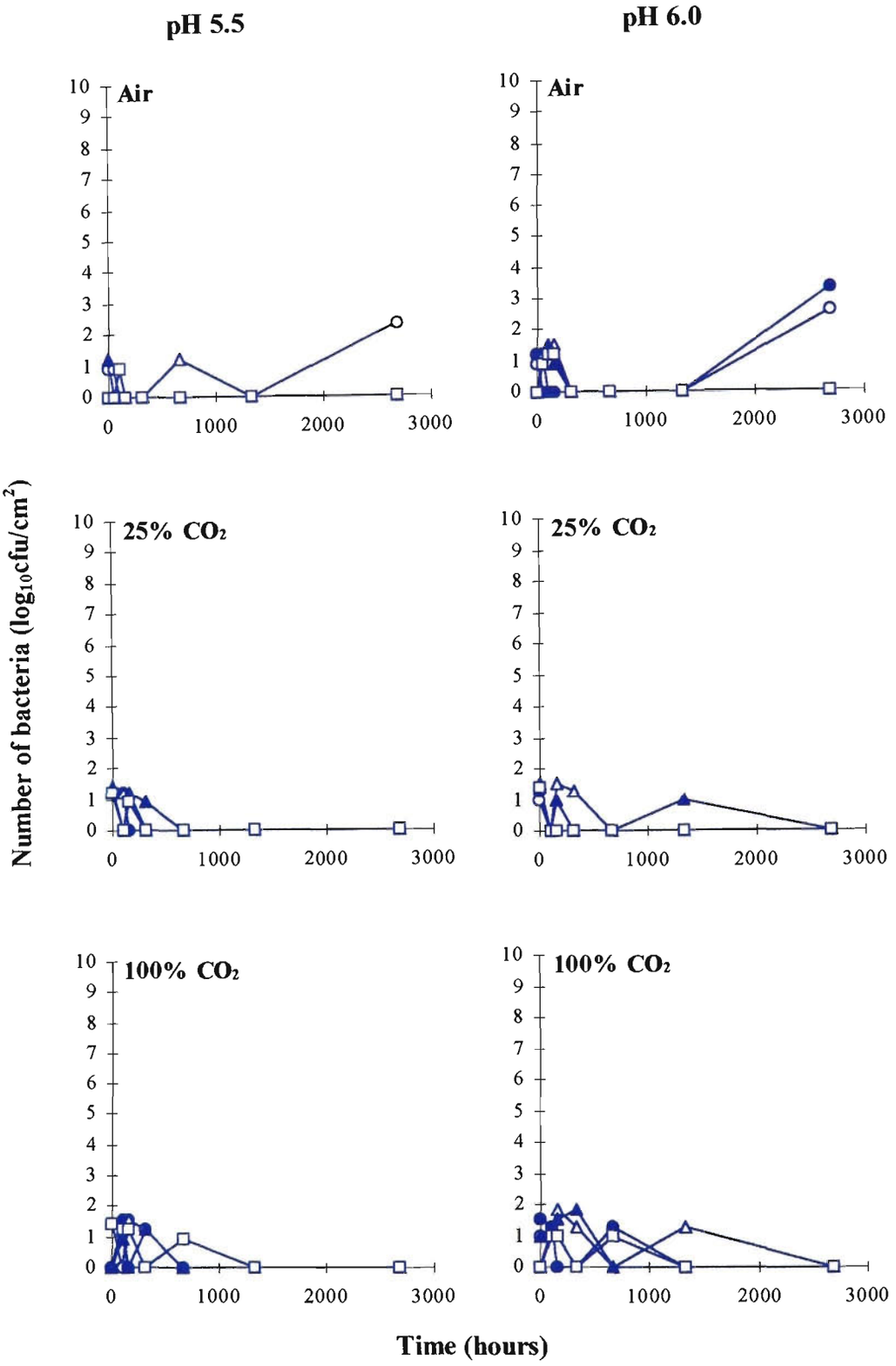


Figure 7.11 The effect storage at 1°C at either pH 5.5 or 6.0 on the growth of *H. alvei* when exposed to preservatives (control (○), 1% sodium chloride (●), 2% sodium chloride (△), potassium sorbate (▲) and propyl-paraben (□)) and incubation in different atmospheres (air, 25% CO_2 or 100% CO_2).

7.3.6.3 Storage in air and 25 and 100% CO_2 at 1°C

The number of *H. alvei* did not increase above 10^3 cfu/cm^2 under any treatment (Figure 7.11). For the majority of the treatments there were no viable *H. alvei* detected over the experimental period.

7.3.7 Fitting of curves to the growth data for the determination of the impact of environmental factors on microbial growth parameters

There was insufficient data for *H. alvei* to examine the effects of the environmental factors on growth, therefore curves were not statistically analysed. In some cases the data needed to be transformed in order to reduce the variance of the residual plot (McMeekin et al., 1993). The parameters that were fitted were μmax, t_{90} and the maximum population density. In cases where the growth was either negative, slow or non-existent (as defined by an increase less than 0.5 $\log_{10}$ in cell numbers over the incubation period), estimates were set as: B=0 indicating a flat profile or no growth phase, M= * indicating no point of inflection, C=0 indicating zero height between asymptotes and A= average of the counts in the sequence indicating a flat profile or no growth phase (personal communication, Dr. J. Reynolds, Biometrician, Australian Food Industry Science Centre, Werribee, Victoria, Australia). The actual output data from the curve fitting are listed in Appendix 5. For the parameters of the fitted curves, the effects of preservatives, pH, temperature and atmosphere and combinations of two, three and all four factors were determined. For analysis, the preservatives were considered to be one or 2% sodium chloride, potassium sorbate and propyl-paraben, while atmosphere referred to carbon dioxide levels.

7.3.8 Total population response to environmental factors alone and in combination

The effect of individual factors and combinations of two, three and four factors on the total population are listed in Table 7.1. Changes in preservatives, pH, temperature, atmosphere, pH/ temperature, pH/ atmosphere, temperature/ atmosphere, preservative/ temperature/ atmosphere and pH/ temperature/ atmosphere significantly influenced the μmax of the total population following $\log_{10}$ transformation. Both potassium sorbate and propyl-paraben decreased the μmax, as did sodium chloride but the effect was not significant. The μmax was lower at pH 5.5 than pH 6.0, similarly at 1°C the μmax was lower than at 10°C. The increase in the percentage of carbon dioxide decreased the μmax significantly. The combination of temperature and pH decreased the μmax with decreasing pH and temperature. The impact of pH on μmax was enhanced when combined with increasing carbon dioxide levels. Likewise, combining carbon dioxide and decreasing temperature resulted in a greater decrease in the μmax.

Table 7.1 A summary of the effects of environmental factors alone and in combination on the growth parameters of a total population. Factors and factor combinations that had a significant ($p \leq 0.05$) effect on growth are indicated by a tick (✓).

Environmental factor	Growth parameter		
	Log_{10} Slope	Log_{10} t_{90}	maximum
One factor			
Pres [a]	✓	✓	✓
pH [b]	✓		✓
Temp [c]	✓	✓	✓
Atmos [d]	✓	✓	✓
Two factors			
Pres/pH			✓
Pres/Temp		✓	✓
pH/Temp	✓		
Pres/Atmos		✓	✓
pH/Atmos	✓		
Temp/Atmos	✓	✓	✓
Three factors			
Pres/pH/Temp			
Pres/pH/Atmos			
Pres/Temp/Atmos		✓	✓
pH/Temp/Atmos		✓	✓
Four factors			
Pres/pH/Temp/Atmos			

[a] Preservative = 1% NaCl, 2% NaCl, 0.2% potassium sorbate or 0.04% propyl-paraben
[b] pH = 5.5 or 6.0
[c] Temperature = 1 or 10°C
[d] Atmosphere = air, 25% CO_2 or 100% CO_2

The combination of all four factors did not significantly influence μmax for the total population. However, trends could be seen in the data for μmax: (max decreased as temperature and pH decreased, carbon dioxide increased and when either one or 2% sodium chloride or potassium sorbate or propyl-paraben were added to the medium. When an increased number of factors was examined, the number of replicates for each data point decreased due to the experimental design, thus making the significance more difficult to establish. Conducting more replicates could have overcome this limitation once the initial data was collected and the above trends established but this experimentation was beyond what was possible in the timeframe.

Preservatives, pH, atmosphere, preservatives/ pH, atmosphere/ pH, temperature/ atmosphere, preservative/ temperature/ atmosphere and pH/ temperature/ atmosphere all significantly influenced t_{90} following $\log_{10}$ transformation (Table 7.1). One percent sodium chloride did not significantly affect t_{90}, however 2% sodium chloride, potassium sorbate and propyl-paraben increased t_{90}. At pH 5.5, t_{90} was greater than at pH 6.0, similarly at 1°C the time required to reach t_{90} was higher than at 10°C. Significantly more time was required to reach t_{90} when carbon dioxide was increased to 25% and then to 100%. When temperature was decreased in combination with potassium sorbate or propyl-paraben, the time required to reach t_{90} was increased. Increasing carbon dioxide together with 2% sodium chloride, potassium sorbate and propyl-paraben increased t_{90}. Decreasing the temperature from 10 to 1°C in conjunction with increasing CO_2 from atmospheric levels to 25 and 100% significantly increased the time to t_{90}. Combining preservatives, atmosphere and temperature increased t_{90}, especially when potassium sorbate and propyl-paraben were combined with increased carbon dioxide and decreased temperature. At pH 5.5, increased carbon dioxide at lower temperature increased t_{90} more than at pH 6.0.

The maximum population density was significantly affected individually by: preservatives, pH, temperature, atmosphere, preservatives/pH, preservatives/temperature, preservatives/atmosphere, temperature/atmosphere, preservatives/temperature/atmosphere and pH/temperature/atmosphere (Table 7.1). Sodium chloride did not significantly influence the maximum population density, however both potassium sorbate and propyl-paraben did, with potassium sorbate having a greater effect. The maximum population density was greater at pH 6.0 than at 5.5 and at 10°C rather than 1°C. The maximum population density decreased as carbon dioxide increased: the population density was lower when carbon dioxide was 100% compared to 25%. Combining potassium sorbate and propyl-paraben with pH 5.5 decreased the maximum population density compared to pH 6.0. A similar trend was observed for sodium chloride but the effect was not significant. Similarly, when preservative and temperature were combined there was a reduction in maximum population density by sodium chloride with decreasing temperature but

Table 7.2 A summary of the effects of environmental factors alone and in combination on growth parameters of *L. sakei*. Factors and factor combinations that had a significant ($p \leq 0.05$) effect on growth are indicated by a tick (✓).

Environmental factor	Growth parameter		
	√slope	Log_{10} t_{90}	Log_{10} maximum
One factor			
Pres [a]			
pH [b]			
Temp [c]	✓	✓	
Atmos [d]			
Two factors			
Pres/pH			
Pres/Temp	✓		
pH/Temp			
Pres/Atmos			
pH/Atmos			
Temp/Atmos			
Three factors			
Pres/pH/Temp			
Pres/pH/Atmos			
Pres/Temp/Atmos			
pH/Temp/Atmos			
Four factors			
Pres/pH/Temp/Atmos			

[a] Preservative = 1% NaCl, 2% NaCl, 0.2% potassium sorbate or 0.04% propyl-paraben
[b] pH = 5.5 or 6.0
[c] Temperature = 1 or 10°C
[d] Atmosphere = air, 25% CO_2 or 100% CO_2

this was not significant, but it was for potassium sorbate and propyl-paraben. Potassium sorbate and propyl-paraben decreased the maximum population density when combined with increased carbon dioxide. A reduction in the maximum population density occurred when carbon dioxide was increased and temperature decreased. Combining potassium sorbate or propyl-paraben with increasing carbon dioxide and decreasing temperature resulted in a decreased maximum population density and the effect was greater for propyl-paraben. Finally, combining decreasing temperature and pH with increasing carbon dioxide decreased the maximum population density.

7.3.9 *Lb. sakei* response to environmental factors alone and in combination

Of the four groups in the total population (*B. thermosphacta*, *H. alvei*, *Lb. sakei* and *P. fluorescens*), *Lb. sakei* was the least affected by the environment (Table 7.2). The μmax (once square root transformed) was only significantly affected by temperature and the combination of preservative/ temperature. Decreasing the temperature from 10°C to 1°C decreased the μmax. The combination of preservative and atmosphere was only significant for potassium sorbate and propyl-paraben. At 10°C, μmax measurements for potassium sorbate and propyl-paraben treatments were greater than the control but when the temperature was reduced to 1°C the μmax for these two preservatives were less than the control. None of the three- or four-factor combinations had any influence on the μmax of *Lb. sakei*, however, similar trends to those observed for the total population occurred in the data but there was insufficient data to establish the significance. Again, this warrants further investigation.

The only other parameter of the fitted curves for *Lb. sakei* to be affected by the environmental factors was t_{90}, which was only influenced by temperature: as temperature decreased, t_{90} increased. The maximum population density of *Lb. sakei* was not affected significantly by any of the environmental factors alone or in combination.

7.3.10 *P. fluorescens* response to environmental factors alone and in combination

The influence of environmental factors individually and in combination on *P. fluorescens* is demonstrated in Table 7.3. *P. fluorescens* failed to increase in number in some instances when exposed to potassium sorbate and propyl-paraben, in such cases the terms were set as described previously.

Preservatives, pH, temperature, atmosphere, pH/ atmosphere and the four factors combined all significantly impacted on the $\log_{10}$ transformation of μmax for *P. fluorescens*. Of the preservatives, only

Table 7.3 A summary of the effects of environmental factors alone and in combination on the growth parameters of *P. fluorescens*. Factors and factor combinations that had a significant ($p \leq 0.05$) effect on growth are indicated by a tick (✓).

Environmental factor	Growth parameter		
	Log_{10} slope	Log_{10} t_{90}	Log_{10} maximum
One factor			
Pres [a]	✓	✓	✓
pH [b]	✓	✓	
Temp [c]	✓	✓	
Atmos [d]	✓	✓	✓
Two factors			
Pres/pH		✓	
Pres/Temp		✓	
pH/Temp			
Pres/Atmos			✓
PH/Atmos	✓	✓	
Pres/pH		✓	✓
Three factors			
Pres/pH/Temp		✓	✓
Pres/pH/Atmos			✓
Pres/Temp/Atmos		*	
pH/Temp/Atmos		*	
Four factors			
Pres*pH*Temp*Atmos	✓	*	✓

[a] Preservative = 1% NaCl, 2% NaCl, 0.2% potassium sorbate or 0.04% propyl-paraben
[b] pH = 5.5 or 6.0
[c] Temperature = 1 or 10°C
[d] Atmosphere = air, 25% CO_2 or 100% CO_2
*= degrees freedom was zero

potassium sorbate significantly decreased the μmax of *P. fluorescens*. The μmax was greater at pH 6.0 than 5.5, likewise at 10°C μmax was greater than at 1°C. Increased concentrations of carbon dioxide decreased μmax with the decrease being greater at 100% than 25%. The reduction in μmax was greater at pH 5.5 when combined with 25 or 100% CO_2, than at pH 6.0. The combination of all four factors had a significant effect of μmax of *P. fluorescens*, however, the effects were complex. In general, decreased pH and temperature and increased carbon dioxide decreased μmax. Only in the presence of air did the preservatives have a significant role in μmax reduction, in which case, only the (max following potassium sorbate exposure at 1°C was significantly different. However, the effect was not significantly different from the control μmax under either 25 or 100% CO_2 at the same temperature and pH.

Preservatives, pH, temperature, atmosphere, preservatives/ pH, preservatives/ temperature, preservatives/ atmosphere, pH/ atmosphere, temperature/ atmosphere and preservative/ pH/ temperature all significantly affected the $\log_{10}$ transformed t_{90} of *P. fluorescens* (Table 7.3). The combinations of preservative/ temperature/ atmosphere, pH/ temperature/ atmosphere and preservative/ pH/ temperature/ atmosphere had zero degrees of freedom which meant they could not be analysed. Of the preservatives, 2% sodium chloride, potassium sorbate and propyl-paraben significantly extended t_{90} and the latter two had a greater effect. The t_{90} was greater at pH 5.5 than 6.0 and at 10°C than 1°C. The t_{90} was not significantly different between air and 25% CO_2 and was only significantly different when carbon dioxide was 100%. At pH 5.5 with 2% sodium chloride, potassium sorbate or propyl-paraben, t_{90} was greater than with the same preservatives at pH 6.0. Both one and 2% sodium chloride and propyl-paraben had a larger t_{90} at 1°C than 10°C, while t_{90} for potassium sorbate was significantly longer at 10°C than seen for the other preservatives; it was not significantly different from that observed at 1°C. For preservatives combined with carbon dioxide, potassium sorbate had an increased t_{90} when carbon dioxide was 25% but was not significantly different from 100% CO_2, while for the other preservatives significant differences in t_{90} were only seen with 100% CO_2, where t_{90} increased. When pH and atmosphere were combined, only 100% CO_2 and pH 5.5 had a significant effect on t_{90}. As temperature decreased and carbon dioxide increased, t_{90} also increased. Decreased temperature and pH together with either 2% sodium chloride, potassium sorbate or propyl-paraben increased t_{90}. The three factors combined, preservative, pH plus atmosphere, increased t_{90} as pH decreased and carbon dioxide increased for all preservatives. Notably, the t_{90} for 1% sodium chloride was less than the control, which was unexpected.

The maximum population density was found to be significantly affected by: preservatives, atmosphere, preservatives/ atmosphere, temperature/ atmosphere, preservatives/ pH/ temperature, preservatives/ pH/ atmosphere and preservatives/ pH/ temperature/ atmosphere (Table 7.3). Potassium sorbate was the only

preservative to significantly decrease the maximum population density of *P. fluorescens*. Increased carbon dioxide decreased the maximum population density. For the remainder of the environmental factor combinations which were found to be significant ($p<0.05$), there was no apparent pattern to the significance of these factors on the maximum population density, except that as carbon dioxide concentration increased the maximum population density decreased. The failure of a pattern to emerge for these factors could be attributed to the sporadic growth of *P. fluorescens* under the conditions used.

7.3.11 *B. thermosphacta* response to environmental factors alone and in combination

The effect of the individual and combinations of environmental factors on the growth of *B. thermosphacta* are summarised in Table 7.4. For the analysis of *B. thermosphacta*, potassium sorbate and propyl-paraben values were set to zero as *B. thermosphacta* failed to grow on these treatments. The term preservatives in this instance only refers to one and 2% sodium chloride. None of the environmental factors, regardless of combination, significantly affected the maximum population of *B. thermosphacta* for the conditions tested.

Preservatives, temperature, atmosphere, preservatives/ pH, preservatives/ temperature, preservatives/ atmosphere, temperature/ atmosphere, preservatives/ pH/ temperature, preservatives/ pH/ atmosphere, preservatives/ temperature/ atmosphere and preservatives/ pH/ temperature/ atmosphere all significantly influenced the square root transformed μmax of *B. thermosphacta*. The effect of increased sodium chloride was diverse: 1% sodium chloride increased μmax while 2% sodium chloride decreased μmax. Both results were significantly different from the control. Reducing the temperature decreased μmax significantly. Increased carbon dioxide decreased μmax, with 100% CO_2 producing a μmax that was significantly lower than for 25% CO_2. When pH was combined with sodium chloride there were significant changes in μmax at low pH (5.5) and low sodium chloride (1%) μmax increased, while low pH (5.5) and high sodium chloride (2%) μmax decreased compared to those at high pH (6.0). Decreased temperature combined with increasing sodium chloride decreased μmax. As carbon dioxide increased and sodium chloride increased, μmax decreased. At pH 5.5 the effect of increased carbon dioxide on μmax was more pronounced than at pH 6.0. The combined interaction of pH and atmosphere produced interesting results in that at pH 5.5 μmax was greater in air than at pH 6.0, at 25% carbon dioxide μmax was lower than air but was not different between pH levels and under 100% CO_2, μmax was greater at pH 6.0 than 5.5. The μmax continued to decrease as carbon dioxide increased and temperature decreased. At pH 5.5, the μmax was greater for 1% sodium chloride than pH 6.0, but the reverse occurred for 2% sodium chloride however, when both factors were combined with temperature, the μmax was less

Table 7.4 A summary of the effects of environmental factors alone and in combination on the growth parameters of *B. thermosphacta*. Factors and factor combinations that had a significant ($p \leq 0.05$) effect on growth are indicated by a tick (✓).

Environmental factor	Growth parameter		
	√slope	Log_{10} t_{90}	maximum
One factor			
Pres	✓	✓	
pH		✓	
Temp	✓	✓	
Atmos	✓	✓	
Two factors			
Pres/pH	✓	✓	
Pres/Temp	✓	✓	
pH/Temp	✓	✓	
Pres/Atmos			
pH/Atmos	✓	✓	
Pres/pH	✓	✓	
Three factors			
Pres/pH/Temp	✓		
Pres/pH/Atmos	✓	✓	
Pres/Temp/Atmos	✓	✓	
pH/Temp/Atmos		✓	
Four factors			
Pres/pH/Temp/Atmos	✓		

[a] Preservative = 1% NaCl, 2% NaCl, 0.2% potassium sorbate or 0.04% propyl-paraben
[b] pH = 5.5 or 6.0
[c] Temperature = 1 or 10°C
[d] Atmosphere = air, 25% CO_2 or 100% CO_2

at 1°C than at 10°C. When carbon dioxide was increased, pH decreased and sodium chloride increased from one to 2%, there was a significant reduction in the μmax. The same situation was observed for carbon dioxide, sodium chloride and temperature. When combining the four factors, the effect can be described in general in that as temperature decreased, pH decreased, sodium chloride increased from one to 2% and carbon dioxide increased, the μmax decreased for *B. thermosphacta*. There was one case where decreased pH did not decrease μmax: 1% sodium chloride treatment in air at 10°C had a higher μmax at pH 5.5 than 6.0.

Preservatives, pH, temperature, atmosphere, preservatives/ pH, pH/ temperature, preservatives/ atmosphere, pH/ atmosphere, temperature/ atmosphere, preservatives/ pH/ atmosphere, preservatives/ temperature/ atmosphere, pH/ temperature/ atmosphere and preservatives/ pH/ temperature/ atmosphere, all significantly influenced the (log_{10} transformed) t_{90} of *B. thermosphacta*. Only 2% sodium chloride significantly increased t_{90} of *B. thermosphacta*. The t_{90} of *B. thermosphacta* was increased by decreasing pH from 6.0 to 5.5 and temperature from 10°C to 1°C. Increasing the carbon dioxide content from that found in air to 25% did not increase t_{90} significantly, however, when increased to 100%, t_{90} was markedly increased. As sodium chloride concentration increased at pH 6.0, t_{90} increased, while at pH 5.5 and 1% sodium chloride, t_{90} decreased but this increased for 2% sodium chloride. When temperature and pH were combined with both decreasing, t_{90} increased. When sodium chloride was combined with carbon dioxide, t_{90} increased when both factors were increased. As pH decreased and carbon dioxide increased, t_{90} also increased. With the combination of sodium chloride, carbon dioxide and pH, t_{90} increased more at pH 6.0 than at 5.5, especially under 100% CO_2. The effect of carbon dioxide on t_{90} was also particularly marked when combined with temperature and preservative, increasing with decreased temperature, increased sodium chloride and carbon dioxide. The t_{90} at pH 5.5 was greater than at pH 6.0 when combined with increased carbon dioxide at 1°C. When the combination of the four factors was applied, the majority of the significance was related primarily to temperature and the presence of 100% CO_2.

7.4 Discussion

The response of bacteria to environmental conditions varies between species and even strains (Eklund, 1983; Buchanan and Klawitter, 1991; Chandrasekharan et al., 1991; Thomas et al., 1992; Houtsma et al., 1994; Skirdal and Eklund, 1993). In the investigation reported in this chapter, rich bacteriological media were used to eliminate variations in response caused by nutrient availability (Buchanan and Phillips, 1990; McClure et al., 1993; McClure et al., 1994). The environmental factors that were chosen for this investigation were those that could be associated with meat for temperature, carbon dioxide and pH. The

levels of preservatives used for sodium chloride, potassium sorbate and propyl-paraben were selected as those that caused a one $\log_{10}$ reduction in the growth of the most resistant organisms. Hydrochloric acid was chosen as the acidulant rather than lactic acid as this enabled evaluation of the effect of pH, which was the parameter of interest. At the pH levels used, 5.5 and 6.0, weak acids would have existed in both the dissociated and undissociated forms (Karapinar and Gönül, 1992), both of which have anti-microbial properties, with the undissociated being more inhibitory (Corlett and Brown, 1980; Eklund, 1983; Faber et al., 1989; Karapinar and Gönül, 1992; Moir and Eyles, 1992). As hydrochloric acid is a strong acid, it would exist entirely in the dissociated form (Corlett and Brown, 1980), therefore the effect would be due to H+ and not undissociated acid. Wild-type strains were used instead of type strains, as type strains have been demonstrated to be more temperature sensitive, have shorter lag times and have faster growth rates (Hudson, 1992).

Given the erratic growth of *P. fluorescens* in the presence of potassium sorbate and propyl-paraben, the data for these should have been removed from the analysis as was done for *B. thermosphacta*. The exclusion of data corresponding to no growth does result in a better fit for the experimental data (Buchanan and Phillips, 1990).

The failure of the *H. alvei* strain used to reach maximum stationary phase or indeed grow under most of the treatment combinations was unexpected. *Enterobacteriaceae* have been demonstrated to increase from almost undetectable numbers to become a significant member of the spoilage population (Ingram, 1962; Barnes and Thornley, 1966; Newton and Gill, 1980). Although this bacterial strain was isolated from meat at 10°C, it failed to grow above 10^6 cfu/cm^2 at this temperature, even though it had previously grown as a pure culture in the temperature and atmosphere experiments (Chapter 6). It is generally thought that although bacteria compete with each other for nutrients, they are generally considered to be indifferent to one another (Gill, 1986), except if bacteriocins are present. LAB have been demonstrated to produce bacteriocins (Ahn and Stiles, 1990a,b; Schillinger and Holzapfel, 1990; Stoffels et al., 1992; Garver and Murina, 1993; Quadri et al., 1995; Saucier et al., 1995; Schillinger et al., 1995; Worobo et al., 1995; Casla et al., 1996) which inhibit the growth of competing bacteria. The *Lb. sakei* strain used in this investigation has been demonstrated to produce a bacteriocin (personal communication, Mohammed Mohideen, Centre for Bioprocessing and Food Technology, Victoria University, Werribee, Australia). *B. thermosphacta* has been demonstrated to be sensitive to bacteriocins (Cutter and Siragusa, 1996; Siragusa et al., 1999). If *Lb. sakei* was producing a bacteriocin under these conditions, then *B. thermosphacta* would also be inhibited but this was not obvious. Therefore, the production of bacteriocin may not account for the failure of *H. alvei* to grow.

It was not surprising that the response of the total population to each treatment [demonstrated in the Figures 7.2 and 7.3] mirrored the responses of the dominant organisms, *P. fluorescens* in air and *Lb. sakei* under carbon dioxide enriched atmospheres. The dominant organisms will be determined by the growth rate: if an organism has a growth rate which is 10% faster than the others in the environment it will become dominant (Gill, 1986; Zwietering et al., 1990). In turn, the dominant organism will be determined by the surrounding environment (Campbell et al., 1979; Erichsen and Molin, 1981a,b; Blickstad and Molin, 1983a,b; Gill, 1986; Gill and Penny, 1988).

Non-limiting environmental factors can combine in an additive fashion to retard or inhibit microbial growth (Sperber, 1983; Gibson et al., 1988; Scott, 1989; Cole et al., 1990, Li and Toreres, 1993b). Of the environmental factors, temperature is the most important (Ratkowsky et al., 1982). The μmax and t_{90} of the total population and that of *P. fluorescens* and *B. thermosphacta* were significantly influenced by temperature. As temperature is decreased the microbial population density has also been reported to decrease (Bailey et al., 1979a; Walker et al., 1990). In this investigation, the maximum population density of the total population decreased as temperature decreased, while the maximum population densities of *B. thermosphacta*, *Lb. sakei* or *P. fluorescens* were not affected.

The combination of decreasing temperature and pH had been well documented to retard or inhibit the growth of bacteria (Carse and Locker, 1984; Brocklehurst and Lund, 1988; El-Shenawy and Marth, 1988; El-Shenawy and Marth, 1989; Hughes and McDermott, 1989; McClure et al., 1989; Brocklehurst and Lund, 1990; Buchanan and Phillips, 1990; Adams et al., 1991; Little et al., 1992b). Interestingly *Lb. sakei*, *P. fluorescens* and *B. thermosphacta* were not significantly affected by this combination, only the total population was. This result is difficult to explain especially since pH alone had a significant ($p<0.05$) impact on both *P. fluorescens* and *B. thermosphacta*, which can be seen in the Figures 7.6-7.9. Little et al. (1992) demonstrated that *Y. enterocolitica* was able to survive inhibitory pH at low temperature, possibly due to a reduced metabolism. If the pH had been decreased further, a significant reduction in growth parameters, μmax and t_{90}, would be expected.

Water activity is reduced by the addition of sodium chloride (Calhoun and Frazier, 1966; Sperber, 1983; Prior et al., 1989; Li and Torres, 1993b). The addition of 1% sodium chloride seemed to have a stimulatory effect on the growth of *B. thermosphacta* which can been seen in Figures 7.8 and 7.9 and the fitted curve data. Growth was greater than the control in the presence of 1% sodium chloride, but increasing to 2% sodium chloride caused a reduction in growth observed. *B. thermosphacta* is resistant to sodium chloride, being capable of growth in sodium chloride to concentrations up to 10% (Talon et al.,

1988). The addition of 2% sodium chloride is sufficient to reduced water activity towards the limits for *P. fluorescens* (Christian et al., 1980). The t_{90} of *P. fluorescens* was affected by 2% sodium chloride but not by 1%. When temperature and sodium chloride are combined, the generation time of bacteria increases (Gibson et al., 1988; McClure et al., 1989; Li and Torres, 1993b). One or 2% sodium chloride and temperature did not affect μmax, t_{90} or maximum population density of the total population or *Lb. sakei*, but 2% sodium chloride and temperature did significantly affect t_{90} of *P. fluorescens*.

In terms of meat spoilage, the effect of atmosphere, specifically carbon dioxide, is perhaps more important than either pH or water activity. The dynamics of the population exposed to carbon dioxide were changed more dramatically than those observed with decreasing temperature. The total population, *B. thermosphacta*, *P. fluorescens* and *H. alvei* were all affected by increased carbon dioxide, while *Lb. sakei* was unaffected. The reduction in maximum population density observed with MAP (Clark and Takás, 1981; Enfors and Molin, 1983; Blickstad and Molin, 1983a,b; Johnson and Ogrydzaik, 1984; Hintlain and Hotchkiss, 1987; Penney and Gill, 1988; Ingham et al., 1990a,b; Drosinos and Board, 1994; Bennik et al., 1995) is a product of the suppression of other members of the population, rather than the stimulation of Lactobacillus. *P. fluorescens* had been demonstrated to be sensitive to carbon dioxide (Huffman et al., 1979; Enfors and Molin, 1981a; Eklund, 1985; Baker et al., 1986; Gill and Penney, 1986). Increased carbon dioxide decreased μmax and maximum population and also increased t_{90} for *P. fluorescens*. The final population number of *B. thermosphacta* was not affected by carbon dioxide in this investigation when it has been demonstrated previously on meat that *Brochothrix* numbers decline in elevated carbon dioxide (Chapter 3 and 6). There can be some inhibitory actions within a food that are as yet unknown, which are not reproducible in laboratory media (Walker et al., 1990; Grau and Vanderlinde, 1993; Wijtze et al., 1993). A reduction in microbial growth is observed when elevated carbon dioxide is combined with temperature (Gill and Tan, 1980; Enfors and Molin, 1981b; Eklund and Jarmund, 1983; Hintlain and Hotchkiss, 1987; Leeson et al., 1987; Holley et al., 1994) or pH (Campbell et al., 1979; Eyles et al., 1993).

Lb. sakei growth parameters were not significantly affected by sodium chloride, potassium sorbate, propyl-paraben, pH or temperature under the conditions tested. However, examining the graphs of the growth data revealed that there does appear to be some interaction between either potassium sorbate or propyl-paraben with either carbon dioxide, temperature or pH. At 1°C, pH 5.5 and increasing carbon dioxide levels, the growth of *Lb. sakei* was retarded. A reduction in growth rate and maximum population can be achieved by combining potassium sorbate and carbon dioxide (Elliot et al., 1982; Gray et al., 1984) or potassium sorbate and low temperature (Tsay and Chou, 1989; Medonica et al., 1989). Potassium sorbate combined with temperature to increase t_{90} for the total population, while the same effect was

observed for propyl-paraben and temperature for t_{90} of *P. fluorescens*.

Potassium sorbate and propyl-paraben alone can increase lag phase and suppress or inhibit growth (Robach, 1978; Robach and Pierson, 1978; Eklund, 1980; Venugopal et al., 1984; Payne et al., 1989). In this investigation, *B. thermosphacta* was totally inhibited by both potassium sorbate and propyl-paraben. The maximum cell density of the total population was decreased by both potassium sorbate and propyl-paraben, but only potassium sorbate has the same effect on *P. fluorescens*, which was consistent with previous investigations (Robach, et al., 1979; El-Shenawy and Marth, 1989). Potassium sorbate has also been demonstrated to decrease the growth rate of *Pseudomonas* (Zamora and Zaritzky, 1987). Potassium sorbate increases in anti-microbial activity when combined with decreasing pH (Moustafa and Collins, 1969; Restaino et al., 1981; Elliot et al., 1982; Eklund, 1983; Sofos et al., 1986; El-Shenawy and Marth, 1988) and, to a lesser extent, the effect of propyl-paraben increases with decreased pH (Thompson et al., 1993). Both t_{90} of *P. fluorescens* and maximum cell density of the total population were affected by pH and preservative in this investigation.

If two environmental factors impair microbial growth alone, the combination of these two factors would be expected to increase inhibition further (Scott, 1989). The effects of the three or four factor combinations on the growth of the total population, *Lb. sakei*. *P. fluorescens* and *B. thermosphacta* did not have as great an impact as expected. Potassium sorbate or proply-paraben in combination with increased carbon dioxide and decreased temperature or pH increased t_{90} of the total population. Sodium chloride, pH and temperature combined to influence *B. thermosphacta*, as did sodium chloride, pH and carbon dioxide. The growth rate (μmax) of *P. fluorescens* and *B. thermosphacta* were the only growth parameters to be affected by the combination of all four environmental factors. Trends in the inhibition of microbial growth could be seen within the data, but the effects were not significant.

7.5 CONCLUSION

The effect of temperature, pH, sodium chloride, potassium sorbate and propyl-paraben alone and in combinations on the growth of a mixed population and the individual species had not been done previously investigated. The factors which affect the entire population do not necessarily influence each individual member of that population. Within the population of meat spoilage bacteria used in this investigation, *B. thermosphacta* and *H. alvei* were the most sensitive to the environment (temperature, pH, carbon dioxide, sodium chloride and preservatives), with *Lb. sakei* the least affected by the environment

under the conditions used in this investigation. Each individual factor, temperature, pH, preservative and carbon dioxide, had a role in influencing the μmax, t_{90} and maximum population density whether singly or in combination. In order to study the impact of combinations of three or four factors together more adequately more data needs to be collected.

There appeared to be some sort of interaction occurring between the bacterial isolates used in this investigation. The failure of both *H. alvei* and *P. fluorescens* to increase in numbers under 25 and 100% CO_2 where previously they had increased when grown as a pure culture (Chapter 6), suggests that the presence of other bacteria was inhibiting their growth. Perhaps with more data the occurrence of interactions between bacterial species within a population can be ascertained.

There was not enough data collected in this investigation to develop a model, as a minimum of 10 data points per growth curve are required to build an accurate model (Gibson et al., 1988; Bratchell et al., 1989; Ratkowsky et al., 1993; McMeekin et al., 1993). Predictive modeling was not the objective of this investigation. The main objective of this investigation was to determine which of the combinations of environmental factors were the most important for minimising growth. It can be concluded for bacteria associated with the spoilage of meat products that, although each environmental factor had a role influencing the parameters of growth, atmosphere primarily in terms of carbon dioxide levels, then temperature were the two critical factors in controlling the population composition, growth rate and final cell density.

Chapter 8

Summary and further work

8.1 SUMMARY

8.1.1 Spoilage of air-packaged and MAP meats

- MAP meat had a longer shelf-life, lower number of bacteria at spoilage and less bacterial species present compared to air-package meats. Meat stored at 1°C had longer shelf-life and a less diverse microflora than meat stored at 10°C.

- *Pseudomonas* species were the dominant bacterial group found on air-packaged meats while LAB were the dominant group on MAP meats. *Enterobacteriaceae* and to a lesser extent *Brochothrix* were also important in the spoilage of meats following both air-packaging and MAP. LAB were also found on air-packaged meats but *Pseudomonas* did not constitute a significant portion of the spoilage population on MAP meats. *Acinetobacter* were only detected on lamb.

8.1.2 Identification of *Brochothrix, Enterobacteriaceae,* LAB and *Pseudomonas* isolates

- *B. thermosphacta* was the only *Brochothrix* species identified in this investigation.

- Due to problems experienced with the Biolog kit the identification of the family *Enterobacteriaceae* was conducted with the API kit. *H. alvei* was the most commonly isolated member of the *Enterobacteriaceae* family, second was members of the genus *Serratia.* One isolate was identified as *Klebseilla pneumonea* subsp. *ozaenae* and another as *Escherichia vulneris.*

- A majority of the LAB isolated from the spoiled air-packaged and MAP beef and lamb were identified as *Lb. sakei.* There were also *Lb. curvatus, Lb. farcininis* and *C. divergens* strains among the LAB population.

- Only two species of *Pseudomonas* were identified, *P. fragi* and *P. fluorescens*, with more isolates identified as *P. fragi.*

8.1.3 PFGE fingerprinting of *Brochothrix, Carnobacterium* and *Lactobacillus*

- *Sma*I was the most suitable enzyme for the PFGE fingerprinting of *B. thermosphacta* and *C. divergens* strains and *Lactobacillus* species isolated spoiled from air-packaged and MAP beef and lamb.

- The PFGE fingerprints of the *B. thermosphacta* strains were diverse, with no two fingerprint being identical, although there were some common bands.

- The PFGE fingerprints of the three *C. divergens* strains isolated from the spoiled beef and lamb were very similar to each other but quite distinct from the *C. divergens* isolated from meat in France and from the bacteriocin producing *C. piscicola* JG126.

- The PFGE fingerprints of the *Lactobacillus* species isolated from beef and lamb in this investigation could not be adequately resolved. However, it was possible to decipher common patterns within the fingerprints, with some isolates having the same patterns. Some for the isolates which exhibited the same PFGE pattern had been identified as different species. This called into question the identification results.

8.1.4 The influence of environmental factors on the growth of meat spoilage bacteria

- As the pH, sodium chloride concentration and preservative concentrations increased the growth rate of *B. thermosphacta* and *H. alvei* strains and LAB and *Pseudomonas* species decreased.

- Meat spoilage bacteria are neutrophiles with the exception of *Lb. sakei* and *Lb. curvatus* which are aciduric, that is they have a higher growth rate at pH levels less than 7.0 but above 5.0.

- The *B. thermosphacta* strains were capable of growth in the presence of 5% sodium chloride, while the other bacterial species were not under the conditions used in this investigation.

- Proply-paraben was more inhibitory than either methyl-paraben or potassium sorbate for all bacterial species examined. Methyl-paraben was more inhibitory than potassium sorbate for both LAB and *H. alvei* strains, while reverse occurred for the *B. thermosphacta* strains. For the *Pseudomonas* species, methyl-paraben and potassium sorbate exerted their inhibitory effects over the same concentration ranges.

- Temperature influences more growth parameters of microbial growth than carbon dioxide.

- As the temperature was decreased the growth rates of *B. thermosphacta, H. alvei, Lb. sakei* and *P. fluorescens* decreased.

- In general, as the level of carbon dioxide increased the growth rates of *B. thermosphacta, H. alvei* and *P. fluorescens* decreased. Only *Lb. sakei* was unaffected by increased carbon dioxide levels.

- When grown in a mixed culture both *H. alvei* or *P. fluorescens* failed to increase in number under conditions which they had when grown as a pure culture.

- When in a mixed population, the growth curve parameters of *Lb. sakei* were the least affected by the environmental conditions while those of *B. thermosphacta* were the most affected.

- The growth patterns of the total population reflects that of the dominant bacterial species or group.

- Although temperature influences more growth parameters aspects of microbial growth than carbon dioxide, when considered in terms of meat spoilage, carbon dioxide was more important than

temperature. As the presence of carbon dioxide will determine the microflora composition which develops which in turn dictates shelf-life because of the growth rates of the bacteria involved.

8.2 FUTURE WORK

8.2.1 Determining the microflora composition on meats using both traditional and modern techniques

Although *Pseudomonas*, *Enterobacteriaceae*, *Brochothrix* and LAB were the dominant members of the population, this investigation failed to look for the presence of pathogens within the bacterial population. In addition, there may have been other bacterial groups present which are not pathogenic but are involved in the meat spoilage process that were not cultured due to the methods used in this investigation.

Combining traditional isolation techniques together with modern molecular biology methods, for example PCR, RFLP or DNA probing, which do not rely on the culturing of bacteria could facilitate the detection of bacteria that are present within the population but for some reason cannot be cultured or are present in very low numbers and are masked by the more prevalent bacteria. Molecular biology techniques could also developed to speed up the laborious and time consuming task of assessing the microbial load of food stuffs.

8.2.2 Further examination of the genus *Brochothrix*

The diversity of PFGE fingerprints for the *B. thermosphacta* strains was unexpected. Only 29 isolates were examined in this investigation which is not a large amount when compared to some of the epidemiological investigations. Therefore, the collection and fingerprinting of more isolates of *B. thermosphacta* from a variety of sources i.e. other supermarkets and butchers in various locations, would enhance the results gathered in this investigation. It would also be interesting to compare the PFGE fingerprints of *B. thermosphacta* to that of *B. campestris* isolates and so see if the same diversity occurs in this species.

Another possible avenue for further work would be to undertake the genome mapping of *B. thermosphacta* to see whether any common patterns in genome organization. This could be achieved by

Southern blotting the PFGE gels and probing with radioactively or chemiluminescently labeled "housekeeping" genes.

8.2.3 The identification and PFGE fingerprinting of *Lactobacillus* species associated with meat spoilage

It can be difficult to assign species names to some members of the genus *Lactobacillus* as evidenced in this and other investigations. Isolates which belong to different species should not form the same PFGE fingerprints. Although the PFGE fingerprints of the *Lactobacillus* species were not ideal the results could still be interpreted, unfortunately they did not correlate with the biochemical identification as expected. Consequently, the identity of the *Lactobacillus* isolates needs to be clarified. The identification of the *Lactobacillus* isolates could be confirmed or corrected by sequencing the 16S rRNA genes from the isolates.

8.2.4 The interactions occurring between bacterial species involved in meat spoilage

There appeared to be some form of interaction occurring in the mixed population as both *H. alvei* and *P. fluorescens* failed to grow under conditions which they had previously when grown as a pure culture. Despite earlier assumptions that there is no interaction among bacterial species within a population, there does appear to be some interactions occurring. In a recent study, *E. coli* 0175:H7 exhibited an extension in the lag phase, a reduction in the generation rate and a decline in the maximum population density when grown in the presence of other bacteria (Duffy *et al.*, 1999). This effect was observed when *E. coli* 0157:H7 was grown in the presence of *P. fragi* but particularly in the presence of *H. alvei* (Duffy *et al.*, 1999).

8.2.5 The influence of environmental factors on a mixed population of meat spoilage bacteria

The results from the experiment examining the effect of the interaction of environmental factors on the growth of a mixed population of meat spoilage bacteria did not provide all the information that was aimed for. The failure of combinations of two or more environmental factors to significantly influences parameters of microbial growth when the factors alone had a significant effect was puzzling. It was

initially hoped that the investigation would highlight the combination of environmental factors which had the greatest impact on the growth of a spoilage population so that these factor combinations could be targeted for the development of a model for predicting meat spoilage. In short more growth data was required, therefore the collection of more growth data would be the natural progression from the point reached in this thesis.

The contamination of meat products by microorganism is unavoidable, but with further a understanding of the microorganisms involved in the spoilage process and the role of environmental factors in microflora development the spoilage and be postponed.

Appendix 1
Chemical stock solutions

All chemical solutions were stored at room temperature unless stated otherwise.

Acrylamide: 29g acrylamide and 1.0g *N,N'*-methylenebisacrulamide distilled water was added to bring the volume to 60mL. The solution was heated at 37°C to dissolved to chemical, then the volume adjusted to 100mL with water. The solution was filter sterilised and stored in a dark bottle.

Ammonium persulfate: 1.0g ammonium persulfate was added to distilled water and made up to 10mL. This could be stored at 4°C for several weeks (Sambrook *et al.*, 1989).

EDTA (0.5M): 186.1g ethylenediaminetetra-acetic acid disodium salt was added to 800mL distilled water. The pH was adjusted to 8.0 (±0.1) by the addition of approximately 20g NaOH pellets (Sambrook *et al.*, 1989).

Ethidium bromide: 1.0g ethidium bromide added to 100mL distilled water in a foil wrapped bottle, then stirred with a magnetic stirrer for several hours until dissolved (Sambrook *et al.*, 1989).

Oxidase reagent: 0.1g dimethyl-*p*-phenylenediamine hydrochloride was added to 10mL distilled water, aliquoted at stored at –20°C until required.

Parabens: 2.5g methyl-paraben or propyl-paraben were dissolved in 100mL ethanol: water (1:1). This was stored at room temperature

Potassium sorbate: 20g potassium sorbate was dissolved in 100mL distilled water then filter sterilised. This was stored at room temperature.

Proteinase K: 20mg/mL proteinase K was dissolved in sterile distilled water and stored at –20°C (Sambrook *et al.*, 1989).

RNase (DNase free): 10mg/mL of RNase A was dissolved in 10mM Tris.HCl (pH 7.5), 15mM NaCl then heated at 100°C for 15 minutes. After this time it was cooled slowly to room temperature then frozen at –20°C (Sambrook *et al.*, 1989).

Sugar solutions: 20g sucrose or 10g of glucose, inulin, mannitol, melibiose or rhamnose were dissolved into 100mL distilled water then filter sterilised.

SDS: 10g of sodium dodecyl sulphate was dissolved in distilled water.

SDS-PAGE gel-loading buffer contained: 50mM Tris.HCl, 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol.. This was stored at –20 until required (Sambrook *et al.*, 1989).

Sodium chloride (5M): 292.2g sodium chloride per litre distilled water (Sambrook *et al.*, 1989).

Tris-HCl (1M): 121.1g Tris base was added to 800mL distilled water. The pH was adjusted at required with conc. HCl (Sambrook et al., 1989).

Urea: 20g of Urea was dissolved in 100mL distilled water, then filter sterilised.

Appendix 2

Genstat programs for analysing the effect of temperature and carbon dioxide on the growth of *B. thermosphacta, H. alvei, Lb. sake* and *P. fluorescens*

The Genstat 3.2 program used to fit sigmoidal curves to the growth data for *B. thermosphacta, Lb. sake* and *P. fluorescens,* are listed below. These programs were written by Dr John Renyolds, Biometrican at the Food Industry Science Centre, Werribee, Victoria, Australia. From the fitted curves the effect of temperature and carbon dioxide (atmosphere in the program) was determined on initial bacterial number, the point of inflection or μmax, the time to μmax, a rate parameter, the time to 90% of the population and the final bacterial number. Although there was insufficient data to determine the statistical significance of the combined effect of temperature and atmosphere a cross product term (TxA) was included within the program, however, this cross product had no significant effect on any of the growth curve parameters.

A2.1 Genstat program for determining the effects of temperature and carbon dioxide on the growth of *B. thermosphacta*

```
"
.....  Brochothrix thermosphacta  (temperature x atmosphere experiment)
"
output [pr=dots; width=80] 1
units [39]
open 'brocho.txt'; ch=2; file=in; width=152
skip [ch=2] 2
read [ch=2; layout=fixed; format=!(3(*),(19(8),*)8,\
                                   3(*),(19(8),*)10,\
                                   3(*),(19(8),*)15,\
                                   3(*),(19(8),*)6); justi=*]\
 t,lac[1,2],a1,lac[3,4],a2,lac[5,6],a3,lac[7,8],a4,lac[9,10],a5,\
   lac[11,12],a6
close ch=2; file=in

factor [lev=4; lab=!t('15deg','10deg','5deg', '0deg')] te;\
 values=!(8(1),10(2),15(3),6(4))
variate [24] b,m,c,a,resdv,rdf,nv,maxslope
scalar [*] star
scalar [0] ind
for d=1...4; dlab='15deg','10deg','5deg','0deg'
print '.....  Curve fitting for this temperature...',dlab
 restrict t,te,a1,a2,a3,a4,a5,a6; te.eq.d
 for y=a1,a2,a3,a4,a5,a6
  calc ind = ind+1
  calc nm = nobservations(y)
  if nm.ge.4
   calc y=log10(y)                "....  We fit log-logistic curves"
   model y
   fitcurve [curve=logistic] t
```

```
   rkeep estim=bhat; dev=rdev; df=resdf
   rfunc [calc=!e(maxs='B'*'C'/4)] maxs
   rgraph [graph=high]
   calc b$[#ind] = bhat$[1]
    &   m$[#ind] = bhat$[2]
    &   c$[#ind] = bhat$[3]
    &   a$[#ind] = bhat$[4]
    &   resdv$[#ind] = rdev
    &   rdf$[#ind] = resdf
    &   maxslope$[#ind] = maxs
  else
   calc b$[#ind] = star
    &   m$[#ind] = star
    &   c$[#ind] = star
    &   a$[#ind] = star
    &   resdv$[#ind] = star
    &   rdf$[#ind] = star
    &   maxslope$[#ind] = star
  endif
  calc nv$[#ind] = nm
 endfor
 restrict t,te,a1,a2,a3,a4,a5,a6
endfor
units [24]
factor [lev=4; lab=!t('15d','10d','5d','0d')] temp;\
 values=!(6(1...4))
factor [lev=6; lab=!t(Air,'0','25','50','75','100')] atm;\
 values=!((1...6)4)
factor [lev=24] curv; values=!(1...24)

"..... Duration time to reach 90% of the maximum (a+c)"
calc t90 = m + (log((0.9*(a+c)-a)/(0.1*(a+c))))/b

"..... Log of the b values"
calc logb = log10(b)

print curv,temp,atm,nv,resdv,rdf,b,m,c,a,t90, maxslope;\
 fieldw=5,5,4,4,6,4,9,10,8,8,8,7; deci=4(0),3,0,5,2,3,3,2,4

tabulate [class=temp,atm; pr=nobs] m

"..... Anovas - All Atmospheres"
treatments temp + atm
for y=b,m,c,a,t90,logb, maxslope
 anova [fprob=y] y; res=rr; fitted=ff
 graph [nrow=20; ncol=60] rr;ff
endfor


"..... Regression Approach - Ignoring the Air treatment"
variate [24] Temp; values=!(6(15,10,5,0))
variate [24] Atm;  values=!((2(0),(25,50,75,100))4)
variate [24] TxA
calc TxA = (Temp-10)*(Atm-50)
restrict curv,temp,Temp,atm,Atm,TxA,b,m,c,a,t90,logb, maxslope; atm.ne.1
for y=b,m,c,a,t90,logb,maxslope
 model y
```

```
 terms Temp + Atm + TxA
 fit [fprob=y; tprob=y; pr=mod,summ,est,acc] Temp + Atm + TxA
 rkeep res=RR; fitted=FF
 graph [nrow=20; ncol=60] RR;FF
 print curv,Temp,Atm,TxA,y,FF,RR; fieldw=4(7),11,12,12; deci=4(0),4,5,5
endfor
restrict curv,temp,Temp,atm,Atm,TxA,b,m,c,a,t90,logb,maxslope


stop
```

A2.2 Genstat program for determining the effects of temperature and carbon dioxide on the growth of *H. alvei*

As *H. alvei* did not grow at either zero or 5°C, only 10 and 15°C were included in the program

```
"
.....  Hafnia alvei  (temperature x atmosphere experiment)
"
output [pr=dots; width=80] 1
units [15]
open 'entero.txt'; ch=2; file=in; width=152
skip [ch=2] 1
read [ch=2; layout=fixed; format=!(3(*),(19(8),*)8,\
                                   3(*),(19(8),*)7); justi=*]\
 t,lac[1,2],a1,lac[3,4],a2,lac[5,6],a3,lac[7,8],a4,lac[9,10],a5,\
   lac[11,12],a6
close ch=2; file=in

factor [lev=2; lab=!t('15deg','10deg')] te;\
 values=!(8(1),7(2))
variate [12] b,m,c,a,resdv,rdf,nv,maxslope
scalar [*] star
scalar [0] ind
for d=1...2; dlab='15deg','10deg'
print '.....  Curve fitting for this temperature...',dlab
 restrict t,te,a1,a2,a3,a4,a5,a6; te.eq.d
 for y=a1,a2,a3,a4,a5,a6
  calc ind = ind+1
  calc nm = nobservations(y)
  if nm.ge.4
   calc y=log10(y)                "....  We fit log-logistic curves"
   model y
   fitcurve [curve=logistic] t
   rkeep estim=bhat; dev=rdev; df=resdf
   rfunc [calc=!e(maxs='B'*'C'/4)] maxs
   rgraph [graph=high]
   calc b$[#ind] = bhat$[1]
    &   m$[#ind] = bhat$[2]
    &   c$[#ind] = bhat$[3]
    &   a$[#ind] = bhat$[4]
    &   resdv$[#ind] = rdev
    &   rdf$[#ind] = resdf
```

```
    &   maxslope$[#ind] = maxs
  else
   calc b$[#ind] = star
    &   m$[#ind] = star
    &   c$[#ind] = star
    &   a$[#ind] = star
    &   resdv$[#ind] = star
    &   rdf$[#ind] = star
    &   maxslope$[#ind] = star
  endif
  calc nv$[#ind] = nm
 endfor
 restrict t,te,a1,a2,a3,a4,a5,a6
endfor
units [12]
factor [lev=2; lab=!t('15d','10d')] temp;\
 values=!(6(1...2))
factor [lev=6; lab=!t(Air,'0','25','50','75','100')] atm;\
 values=!((1...6)2)
factor [lev=12] curv; values=!(1...12)

".....  Duration time to reach 90% of the maximum (a+c)"
calc t90 = m + (log((0.9*(a+c)-a)/(0.1*(a+c))))/b

".....  Log of the b values"
calc logb = log10(b)

print curv,temp,atm,nv,resdv,rdf,b,m,c,a,t90, maxslope;\
 fieldw=5,5,4,4,6,4,9,10,8,8,8,7; deci=4(0),3,0,5,2,3,3,2,4

tabulate [class=temp,atm; pr=nobs] m

".....  Anovas - All Atmospheres"
treatments temp + atm
for y=b,m,c,a,t90,logb, maxslope
 anova [fprob=y] y; res=rr; fitted=ff
 graph [nrow=20; ncol=60] rr;ff
endfor


".....  Regression Approach - Ignoring the Air treatment"
variate [12] Temp; values=!(6(15,10))
variate [12] Atm;  values=!((2(0),(25,50,75,100))2)
variate [12] TxA
calc TxA = (Temp-10)*(Atm-50)
restrict curv,temp,Temp,atm,Atm,TxA,b,m,c,a,t90,logb, maxslope; atm.ne.1
for y=b,m,c,a,t90,logb,maxslope
 model y
 terms Temp + Atm + TxA
 fit [fprob=y; tprob=y; pr=mod,summ,est,acc] Temp + Atm + TxA
 rkeep res=RR; fitted=FF
 graph [nrow=20; ncol=60] RR;FF
 print curv,Temp,Atm,TxA,y,FF,RR; fieldw=4(7),11,12,12; deci=4(0),4,5,5
endfor
restrict curv,temp,Temp,atm,Atm,TxA,b,m,c,a,t90,logb,maxslope
```

```
stop
```

A2.3 Genstat program for determining the effects of temperature and carbon dioxide on the growth of *Lb. sake*

```
"
.....  Lactobacillus sake  (temperature x atmosphere experiment)
"
output [pr=dots; width=80] 1
units [28]
open 'lacto3.txt'; ch=2; file=in; width=152
skip [ch=2] 2
read [ch=2; layout=fixed; format=!(3(*),(19(8),*)6,\
                                   3(*),(19(8),*)6,\
                                   3(*),(19(8),*)9,\
                                   3(*),(19(8),*)7); justi=*]\
 t,lac[1,2],a1,lac[3,4],a2,lac[5,6],a3,lac[7,8],a4,lac[9,10],a5,\
   lac[11,12],a6
close ch=2; file=in

factor [lev=4; lab=!t('15deg','10deg','5deg', '0deg')] te;\
 values=!(6(1,2),9(3),7(4))
variate [24] b,m,c,a,resdv,rdf,nv,maxslope
scalar [*] star
scalar [0] ind
for d=1...4; dlab='15deg','10deg','5deg','0deg'
print '.....  Curve fitting for this temperature...',dlab
 restrict t,te,a1,a2,a3,a4,a5,a6; te.eq.d
 for y=a1,a2,a3,a4,a5,a6
  calc ind = ind+1
  calc nm = nobservations(y)
  if nm.ge.4
   calc y=log10(y)                "....  We fit log-logistic curves"
   model y
   fitcurve [curve=logistic] t
   rkeep estim=bhat; dev=rdev; df=resdf
   rfunc [calc=!e(maxs='B'*'C'/4)] maxs
   rgraph [graph=high]
   calc b$[#ind] = bhat$[1]
    &   m$[#ind] = bhat$[2]
    &   c$[#ind] = bhat$[3]
    &   a$[#ind] = bhat$[4]
    &   resdv$[#ind] = rdev
    &   rdf$[#ind] = resdf
    &   maxslope$[#ind] = maxs
  else
   calc b$[#ind] = star
    &   m$[#ind] = star
    &   c$[#ind] = star
    &   a$[#ind] = star
    &   resdv$[#ind] = star
    &   rdf$[#ind] = star
    &   maxslope$[#ind] = star
```

```
  endif
  calc nv$[#ind] = nm
 endfor
 restrict t,te,a1,a2,a3,a4,a5,a6
endfor
units [24]
factor [lev=4; lab=!t('15d','10d','5d','0d')] temp;\
 values=!(6(1...4))
factor [lev=6; lab=!t(Air,'0','25','50','75','100')] atm;\
 values=!((1...6)4)
factor [lev=24] curv; values=!(1...24)

"..... Duration time to reach 90% of the maximum (a+c)"
calc t90 = m + (log((0.9*(a+c)-a)/(0.1*(a+c))))/b

"..... Log of the b values"
calc logb = log10(b)

print curv,temp,atm,nv,resdv,rdf,b,m,c,a,t90, maxslope;\
 fieldw=5,5,4,4,6,4,9,10,8,8,8,7; deci=4(0),3,0,5,2,3,3,2,4

tabulate [class=temp,atm; pr=nobs] m

"..... Anovas - All Atmospheres"
treatments temp + atm
for y=b,m,c,a,t90,logb, maxslope
 anova [fprob=y] y; res=rr; fitted=ff
 graph [nrow=20; ncol=60] rr;ff
endfor


"..... Regression Approach - Ignoring the Air treatment"
variate [24] Temp; values=!(6(15,10,5,0))
variate [24] Atm;  values=!((2(0),(25,50,75,100))4)
variate [24] TxA
calc TxA = (Temp-10)*(Atm-50)
restrict curv,temp,Temp,atm,Atm,TxA,b,m,c,a,t90,logb, maxslope; atm.ne.1
for y=b,m,c,a,t90,logb,maxslope
 model y
 terms Temp + Atm + TxA
 fit [fprob=y; tprob=y; pr=mod,summ,est,acc] Temp + Atm + TxA
 rkeep res=RR; fitted=FF
 graph [nrow=20; ncol=60] RR;FF
 print curv,Temp,Atm,TxA,y,FF,RR; fieldw=4(7),11,12,12; deci=4(0),4,5,5
endfor
restrict curv,temp,Temp,atm,Atm,TxA,b,m,c,a,t90,logb,maxslope


stop
```

A2.4 Genstat program for determining the effects of temperature and carbon dioxide on the growth of *P. fluorescens*

```
"
.....  Pseudomonas fluorescens  (temperature x atmosphere experiment)
"
output [pr=dots; width=80] 1
units [48]
open 'pseudo.txt'; ch=2; file=in; width=152
skip [ch=2] 2
read [ch=2; layout=fixed; format=!(3(*),(19(8),*)13,\
                                   3(*),(19(8),*)9,\
                                   3(*),(19(8),*)12,\
                                   3(*),(19(8),*)14); justi=*]\
 t,lac[1,2],a1,lac[3,4],a2,lac[5,6],a3,lac[7,8],a4,lac[9,10],a5,\
   lac[11,12],a6
close ch=2; file=in

factor [lev=4; lab=!t('15deg','10deg','5deg', '0deg')] te;\
 values=!(13(1),9(2),12(3),14(4))
variate [24] b,m,c,a,resdv,rdf,nv,maxslope
scalar [*] star
scalar [0] ind
for d=1...4; dlab='15deg','10deg','5deg','0deg'
print '.....  Curve fitting for this temperature...',dlab
 restrict t,te,a1,a2,a3,a4,a5,a6; te.eq.d
 for y=a1,a2,a3,a4,a5,a6
  calc ind = ind+1
  calc nm = nobservations(y)
  if nm.ge.4
   calc y=log10(y)                "....  We fit log-logistic curves"
   model y
   fitcurve [curve=logistic] t
   rkeep estim=bhat; dev=rdev; df=resdf
   rfunc [calc=!e(maxs='B'*'C'/4)] maxs
   rgraph [graph=high]
   calc b$[#ind] = bhat$[1]
    &   m$[#ind] = bhat$[2]
    &   c$[#ind] = bhat$[3]
    &   a$[#ind] = bhat$[4]
    &   resdv$[#ind] = rdev
    &   rdf$[#ind] = resdf
    &   maxslope$[#ind] = maxs
  else
   calc b$[#ind] = star
    &   m$[#ind] = star
    &   c$[#ind] = star
    &   a$[#ind] = star
    &   resdv$[#ind] = star
    &   rdf$[#ind] = star
    &   maxslope$[#ind] = star
  endif
  calc nv$[#ind] = nm
 endfor
 restrict t,te,a1,a2,a3,a4,a5,a6
```

```
endfor
units [24]
factor [lev=4; lab=!t('15d','10d','5d','0d')] temp;\
 values=!(6(1...4))
factor [lev=6; lab=!t(Air,'0','25','50','75','100')] atm;\
 values=!((1...6)4)
factor [lev=24] curv; values=!(1...24)

"..... Duration time to reach 90% of the maximum (a+c)"
calc t90 = m + (log((0.9*(a+c)-a)/(0.1*(a+c))))/b

"..... Log of the b values"
calc logb = log10(b)

print curv,temp,atm,nv,resdv,rdf,b,m,c,a,t90, maxslope;\
 fieldw=5,5,4,4,6,4,9,10,8,8,8,7; deci=4(0),3,0,5,2,3,3,2,4

tabulate [class=temp,atm; pr=nobs] m

"..... Anovas - All Atmospheres"
treatments temp + atm
for y=b,m,c,a,t90,logb, maxslope
 anova [fprob=y] y; res=rr; fitted=ff
 graph [nrow=20; ncol=60] rr;ff
endfor

"..... Regression Approach - Ignoring the Air treatment"
variate [24] Temp; values=!(6(15,10,5,0))
variate [24] Atm;  values=!((2(0),(25,50,75,100))4)
variate [24] TxA
calc TxA = (Temp-10)*(Atm-50)
restrict curv,temp,Temp,atm,Atm,TxA,b,m,c,a,t90,logb, maxslope; atm.ne.1
for y=b,m,c,a,t90,logb,maxslope
 model y
 terms Temp + Atm + TxA
 fit [fprob=y; tprob=y; pr=mod,summ,est,acc] Temp + Atm + TxA
 rkeep res=RR; fitted=FF
 graph [nrow=20; ncol=60] RR;FF
 print curv,Temp,Atm,TxA,y,FF,RR; fieldw=4(7),11,12,12; deci=4(0),4,5,5
endfor
restrict curv,temp,Temp,atm,Atm,TxA,b,m,c,a,t90,logb,maxslope


stop
```

Appendix 3

Genstat programs for analysing the effect of environmental factors alone and in combination on a mixed population.

The Genstat 3.2 program used to analyses fit sigmoidal curves to the growth data for the total viable population and *B. thermosphacta, Lb. sake* and *P. fluorescens,* are listed below. These programs were written by Dr John Renyolds, Biometrican at the Food Industry Science Centre, Werribee, Victoria, Australia. From the curves the effects of: temperature, pH, carbon dioxide (atmosphere) and preservatives (sodium chloride, potassium sorbate and propyl-paraben), alone and in combinations of two, three or four, on the slope (μ_{max}), time to reach 90% of the total population and maximum population number was determined. The results that were analysed were those that did not contain the air control.

A3.1 The effect of environmental factors on the growth of the total viable population

```
".....    PCA - Total Plate Counts for Mega Experiments
          PCA.TXT
          Analysis of Estimated Parameters from
          Fitted Log-Logistic Curves"
output [pr=dots; width=80] 1
units [96]

factor [lev=2] Rep
factor [lev=5; lab=!t(no,Na1,Na2,PS,PP)] Pres
factor [lev=2; lab=!t(D10,D1)] Temp
factor [lev=3; lab=!t(air,c25,c100)] Atmos
factor [lev=2; lab=!t(pH5,pH6)] pH

variate [96] Ind,b,m,c,a,t90,nv,rdev,rdf,max,r,f

open 'pca.txt'; ch=2; file=in; width=120
skip [ch=2] 2
read [ch=2] Ind,Rep,Pres,Temp,Atmos,pH,nv,rdev,rdf,b,m,c,a,t90;\
            frep=*,le,la,la,la,la,8(*)
close ch=2; file=in

"
restrict Ind,Rep,Pres,Temp,Atmos,pH,nv,rdev,rdf,b,m,c,a,t90,max,r,f;\
  Ind.in.!(1,2,3,4, 7,8, 13,14,15,16, 19,20, 25,26, 37,38, 43,44,\
     49,50,51,52, 55,56, 61,62,63,64, 67,68, 73,74, 85,86, 91,92)
"
calc max = a + c
calc lb = log(b + 0.000005)
calc lmax = log(max)
calc lt90 = log(t90)
calc sqrtb = sqrt(b)
calc rt90 = 1/t90

".... Large b value
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90; b.gt.0.1
```

```
print Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90;\
 fieldw=4,4,5,5,6,4,5(9); deci=6(0),5,1,4,4,1
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90
"

histo b
histo m
histo c
histo a
histo t90

for y=b,"m,c,a,"max,t90,lb,sqrtb,lmax,lt90,rt90;\
 ylab='Slope', " 'Inflexion Point','Maximum Increase',\
      'Lower Asymptote', " 'Maximum','Time to get to 90% of Maximum',\
      'Log Slope','Square Root Slope','Log Maximum',\
      'Log Time to get to 90% of Maximum',\
      'Reciprocal of Time to get to 90% of Maximum'
 print '...  Analysis for',ylab
 treatments  Pres*pH*Temp*Atmos
 anova [fprob=y; pse=lsd; fact=4] y; res=r; fitted=f
 graph [nrow=20; ncol=60] r; f
endfor
"
restrict Ind,Rep,Pres,Temp,Atmos,pH,nv,rdev,rdf,b,m,c,a,t90,max,r,f
tabulate [class=Pres,Temp,Atmos,pH; pr=means] b
 &                  max
 &                  t90
"
```

A3.2 The effect of environmental factors on the growth of *B. thermosphacta*

For *B. thermosphacta* the program was altered to exclude potassium sorbate and propyl-paraben as *B. thermosphacta* failed to grow under conditions which contained these preservative agents.

```
".....    STAA - Brochothrix Counts for Mega Experiments
          STAA.TXT
          Analysis of Estimated Parameters from
          Fitted Log-Logistic Curves"
output [pr=dots; width=80] 1

units [96]

factor [lev=2] Rep
factor [lev=5; lab=!t(no,Na1,Na2,PS,PP)] Pres
factor [lev=2; lab=!t(D10,D1)] Temp
factor [lev=3; lab=!t(air,c25,c100)] Atmos
factor [lev=2; lab=!t(pH5,pH6)] pH

variate [96] Ind,b,m,c,a,t90,nv,rdev,rdf,max,r,f

open 'staa.txt'; ch=2; file=in; width=120
skip [ch=2] 2
read [ch=2] Ind,Rep,Pres,Temp,Atmos,pH,nv,rdev,rdf,b,m,c,a,t90;\
            frep=*,le,la,la,la,la,8(*)
```

```
close ch=2; file=in

calc max = a + c
calc lb = log(b + 0.000005)
calc lmax = log(max)
calc lt90 = log(t90)
calc sqrtb = sqrt(b)
calc rt90 = 1/t90

".... Large b value
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90; b.gt.0.1
print Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90;\
 fieldw=4,4,5,5,6,4,5(9); deci=6(0),5,1,4,4,1
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90
"

histo b
histo m
histo c
histo a
histo t90


"
..... ANALYSES FOR THREE LEVELS OF PRESERVATIVE (no, Na1, Na2)
      AND BOTH TEMPERATURES (D10, D1)
"
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,max,t90,\
 lb,lmax,lt90,sqrtb,rt90,r,f; Pres.lt.4

for y=b,"m,c,a,"max,t90,lb,sqrtb,lmax,lt90,rt90;\
 ylab='Slope', " 'Inflexion Point','Maximum Increase',\
      'Lower Asymptote', " 'Maximum','Time to get to 90% of Maximum',\
      'Log Slope','Square Root Slope','Log Maximum',\
      'Log Time to get to 90% of Maximum',\
      'Reciprocal of Time to get to 90% of Maximum'
 print '... Analysis for',ylab
 treatments Pres*pH*Temp*Atmos
 anova [fprob=y; pse=lsd; fact=4] y; res=r; fitted=f
 graph [nrow=20; ncol=60] r; f
endfor

restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,max,t90,\
 lb,lmax,lt90,sqrtb,rt90,r,f


"
..... ANALYSES FOR ALL LEVELS OF PRESERVATIVE (no, Na1, Na2, PS, PP)
      AND ONE TEMPERATURE (D10)
"
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,max,t90,\
 lb,lmax,lt90,sqrtb,rt90,r,f; Temp.eq.1

for y=b,"m,c,a,"max,t90,lb,sqrtb,lmax,lt90,rt90;\
 ylab='Slope', " 'Inflexion Point','Maximum Increase',\
      'Lower Asymptote', " 'Maximum','Time to get to 90% of Maximum',\
      'Log Slope','Square Root Slope','Log Maximum',\
```

```
      'Log Time to get to 90% of Maximum',\
      'Reciprocal of Time to get to 90% of Maximum'
 print '...  Analysis for',ylab
 treatments  Pres*pH*Temp*Atmos
 anova [fprob=y; pse=lsd; fact=4] y; res=r; fitted=f
 graph [nrow=20; ncol=60] r; f
endfor

restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,max,t90,\
 lb,lmax,lt90,sqrtb,rt90,r,f
```

A3.3 The effect of environmental factors on the growth of *Lb. sake*

```
".....    MRSA - Lactobacillus for Mega Experiments
          MRSA.TXT
          Analysis of Estimated Parameters from
          Fitted Log-Logistic Curves"
output [pr=dots; width=80] 1

units [96]

factor [lev=2] Rep
factor [lev=5; lab=!t(no,Na1,Na2,PS,PP)] Pres
factor [lev=2; lab=!t(D10,D1)] Temp
factor [lev=3; lab=!t(air,c25,c100)] Atmos
factor [lev=2; lab=!t(pH5,pH6)] pH

variate [96] Ind,b,m,c,a,t90,nv,rdev,rdf,max,r,f

open 'mrsa.txt'; ch=2; file=in; width=120
skip [ch=2] 2
read [ch=2] Ind,Rep,Pres,Temp,Atmos,pH,nv,rdev,rdf,b,m,c,a,t90;\
            frep=*,le,la,la,la,la,8(*)
close ch=2; file=in

"
restrict Ind,Rep,Pres,Temp,Atmos,pH,nv,rdev,rdf,b,m,c,a,t90,max,r,f;\
  Ind.in.!(1,2,3,4, 7,8, 13,14,15,16, 19,20, 25,26, 37,38, 43,44,\
     49,50,51,52, 55,56, 61,62,63,64, 67,68, 73,74, 85,86, 91,92)
"
calc max = a + c
calc lb = log(b + 0.000005)
calc lmax = log(max)
calc lt90 = log(t90)
calc sqrtb = sqrt(b)
calc rt90 = 1/t90

"....  Large b value
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90; b.gt.0.1
print Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90;\
 fieldw=4,4,5,5,6,4,5(9); deci=6(0),5,1,4,4,1
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90"
```

```
histo b
histo m
histo c
histo a
histo t90

for y=b,"m,c,a,"max,t90,lb,sqrtb,lmax,lt90,rt90;\
 ylab='Slope', " 'Inflexion Point','Maximum Increase',\
      'Lower  Asymptote',  "  'Maximum','Time  to  get  to  90%  of
Maximum',\
      'Log Slope','Square Root Slope','Log Maximum',\
      'Log Time to get to 90% of Maximum',\
      'Reciprocal of Time to get to 90% of Maximum'
 print '...  Analysis for',ylab
 treatments  Pres*pH*Temp*Atmos
 anova [fprob=y; pse=lsd; fact=4] y; res=r; fitted=f
 graph [nrow=20; ncol=60] r; f
endfor
"
restrict Ind,Rep,Pres,Temp,Atmos,pH,nv,rdev,rdf,b,m,c,a,t90,max,r,f
tabulate [class=Pres,Temp,Atmos,pH; pr=means] b
 &                   max
 &                   t90
"
```

A3.4 The effect of environmental factors on the growth of *P. fluorescens*

```
".....    PSA - Pseudomonas Counts for Mega Experiments
          PSA10.TXT
          Analysis of Estimated Parameters from
          Fitted Log-Logistic Curves"
output [pr=dots; width=80] 1

units [96]

factor [lev=2] Rep
factor [lev=5; lab=!t(no,Na1,Na2,PS,PP)] Pres
factor [lev=2; lab=!t(D10,D1)] Temp
factor [lev=3; lab=!t(air,c25,c100)] Atmos
factor [lev=2; lab=!t(pH5,pH6)] pH

variate [96] Ind,b,m,c,a,t90,nv,rdev,rdf,max,r,f

open 'psa10.txt'; ch=2; file=in; width=120
skip [ch=2] 2
read [ch=2] Ind,Rep,Pres,Temp,Atmos,pH,nv,rdev,rdf,b,m,c,a,t90;\
            frep=*,le,la,la,la,la,8(*)
close ch=2; file=in

calc max = a + c
calc lb = log(b + 0.000005)
calc lmax = log(max)
calc lt90 = log(t90)
calc sqrtb = sqrt(b)
calc rt90 = 1/t90
```

```
".... Large b value
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90; b.gt.0.1
print Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90;\
 fieldw=4,4,5,5,6,4,5(9); deci=6(0),5,1,4,4,1
restrict Ind,Rep,Pres,Temp,Atmos,pH,b,m,c,a,t90
"

histo b
histo m
histo c
histo a
histo t90

for y=b,"m,c,a,"max,t90,lb,sqrtb,lmax,lt90,rt90;\
 ylab='Slope', " 'Inflexion Point','Maximum Increase',\
      'Lower Asymptote', " 'Maximum','Time to get to 90% of Maximum',\
      'Log Slope','Square Root Slope','Log Maximum',\
      'Log Time to get to 90% of Maximum',\
      'Reciprocal of Time to get to 90% of Maximum'
 print '... Analysis for',ylab
 treatments  Pres*pH*Temp*Atmos
 anova [fprob=y; pse=lsd; fact=4] y; res=r; fitted=f
 graph [nrow=20; ncol=60] r; f
endfor
"
restrict Ind,Rep,Pres,Temp,Atmos,pH,nv,rdev,rdf,b,m,c,a,t90,max,r,f
tabulate [class=Pres,Temp,Atmos,pH; pr=means] b
 &                  max
 &                  t90
"
```

Appendix 4

Output of Genstat program for the effects of temperature and atmosphere on the growth of *B. thermosphacta, H. alvei, Lb. sakei* and *P. fluorescens*

The effects of temperature and atmosphere on the growth of *B. thermosphacta, H. alvei, Lb. sakei* and *P. fluorescens*. *B. thermosphacta* was grown and enumerated on TSA, *Lb. sakei* on MRSA, and *H. alvei* and *P. fluorescens* on NA. Pure cultures were grown (*B. thermosphacta* and *P. fluorescens* at 25°C and *H. alvei* and *Lb. sakei* at 30°C) until late exponential phase, diluted to 10^3 cfu/cm^2 then plated onto an agar surface before being packaged in either air or 0, 25, 50, 75 or 100% CO_2 (balance N_2) then stored at either 0, 5, 10 or 15°C. At time period of between 12 and 2678 hours plates were removed from the incubators in order to determine bacterial numbers. The gas atmosphere within each package was checked prior to opening to ensure the integrity of the packaging. The number of bacteria on the agar surface was determined by macerating the agar with 50mL diluent then spiral plating homogenate onto the appropriate agar. All plates were incubated for 48 hours, *B. thermosphacta* and *P. fluorescens* at 25°C and *H. alvei* and *Lb. sake* at 30°C.

The tables below contain the data from the Genstat program described in Appendix 2, which fitted log-logistic curves to the growth data for each organisms. From the curves the values for initial number, the increase in bacterial numbers, the time to μ_{max}, μ_{max}, time to reach 90% of the final population density and a rate parameter which is related to μ_{max} were determined. When either temperature or carbon dioxide concentration had a significant effect ($p<0.05$) on a growth parameter is indicated by ^{a} within the table.

A4.1 Output from the Genstat program for *B. thermosphacta*

Table A4.1.1 The initial bacterial numbers (A) for *B. thermosphacta* (log_{10} cfu/cm^2)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature (°C)	**0**	1.8884	1.8323	0.2172	1.8934	1.2415
	5	0.9924	0.8495	0.1943	1.8781	2.1114
	10	-1.2602	-3.8726	-0.5830	0.1770	-3.9857
	15	-2.2343	-0.9216	-2.6291	-0.0276	0.1157

Table A4.1.2 The increase in bacterial numbers (C) for *B. thermosphacta* (log_{10} cfu/cm^2)

		Concentration of CO_2 (%)[a]				
		0	**25**	**50**	**75**	**100**
Temperature[a]	**0**	5.8320	5.2544	8.8144	0.5444	1.1993
(°C)	**5**	6.3487	6.4938	7.3288	4.2402	4.7502
	10	9.1452	11.3161	8.0096	7.5164	11.0269
	15	10.1963	9.0850	10.3253	7.4569	7.3114

[a]= significant ($p \leq 0.05$) effect on parameter

Table 4.1.3 The rate parameter (B) for *B. thermosphacta*

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature[a]	0	0.0061	0.0072	0.0009	-0.0022	-0.0036
(°C)	5	0.0260	0.0145	0.0072	0.0057	0.0407
	10	0.0397	0.0274	0.0302	0.0248	0.0094
	15	0.0739	0.0518	0.0318	0.0555	0.0389

[a]= significant ($p \leq 0.05$) effect on parameter

Table A4.1.4 The time to μ_{max} (M) for *B. thermosphacta* (hours)

		Concentration of CO_2 (%)[a]				
		0	**25**	**50**	**75**	**100**
Temperature[a]	**0**	636.8245	386.7079	1379.4824	2140.2537	
(°C)	**5**	122.4389	80.0436	90.0730	287.3797	490.9356
	10	25.0645	6.7803	39.7025	34.2165	12.1631
	15	8.9466	11.9187	14.5570	28.6888	42.4272

[a]= significant

Table A4.1.5 μ_{max} for *B. thermosphacta*

		Concentration of CO_2 (%)[a]				
		0	**25**	**50**	**75**	**100**
Temperature[a]	**0**	0.0089	0.0094	0.0021	-0.0003	-0.0011
(°C)	**5**	0.0413	0.236	0.0132	0.0060	0.0056
	10	0.0907	0.0776	0.0605	0.0443	0.0260
	15	0.1883	0.1178	0.0822	0.1035	0.0711

[a]= significant ($p \leq 0.05$) effect on parameter

Table A4.1.6 The time take to reach 90% of the maximum population (t_{90}) for *B. thermosphacta* (hours)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature[a]	**0**	944.3590	646.4578	3675.9614	2043.1072	532.1864
(°C)	**5**	200.6165	221.8467	398.9259	600.8451	870.9055
	10	84.5473	103.4934	115.1810	121.9155	296.5313
	15	42.3645	56.5782	93.6642	68.3379	98.4746

[a]= significant ($p \leq 0.05$) effect on parameter

A4.2 Output from the Genstat program for *H. alvei*

Table A4.2.1 The initial bacterial numbers (A) for *H. alvei* (log_{10} cfu/cm^2)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**10**	-12.2552	0.0800	0.9267	-57.4549	-1.6027
(°C)	**15**	-150.7989	0.4661	1.4305	-0.3377	0.4138

Table A4.2.2 The increase in bacteria numbers (C) for *H. alvei* (log_{10} cfu/cm^2)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**10**	609.1077	1147.4255	921.0248	1019.8597	1397.1316
(°C)	**15**	143.4110	288.1011	198.1971	409.4874	538.7089

Table A4.2.3 The rate parameter (B) for *H. alvei*

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**10**	0.0040	0.0032	0.0046	0.0021	0.0022
(°C)	**15**	0.0151	0.0147	0.0605	0.0086	0.0071

[a] = significant ($p \leq 0.05$) effect on parameter

Table A4.2.4 The time to μ_{max} (M) for *H. alvei* (hours)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**10**	-190.6363	458.8369	474.2663	-1077.2904	293.3607
(°C)	**15**	-206.6263	142.5065	165.6138	149.8963	237.8232

Table A4.2.5 μ_{max} for *H. alvei*

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**10**	0.0203	0.0069	0.0082	0.0343	0.0053
(°C)	**15**	0.5985	0.0298	0.0983	0.0179	0.0138

Table A4.2.6 The time take to reach 90% of the maximum population (t_{90}) for *H. alvei* (hours)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**10**	609.1077	1147.4255	921.0248	1019.8597	1397.1316
(°C)	**15**	143.4110	288.1011	198.1971	409.4874	538.7098

A4.3 Output from the Genstat program for *Lb. sakei*

Table A4.3.1 The initial bacterial numbers (A) for *Lb. sakei* (log_{10} cfu/cm^2)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**0**	1.6782	1.5099	1.7734	1.9157	1.7251
(°C)	**5**	0.5364	0.3645	0.9123	0.4308	0.4868
	10	0.8221	0.7462	-0.0648	1.0615	1.1336
	15	-1.9170	1.1698	1.1360	-0.2715	0.1685

Table A4.3 2 The total increase in bacterial numbers (C) for *Lb. sakei* (log_{10} cfu/cm^2)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature[a]	**0**	6.1265	6.3295	6.0648	5.7678	6.1020
(°C)	**5**	7.4945	7.5231	6.8377	7.5399	7.5199
	10	7.2050	7.3198	8.4980	6.9357	6.8309
	15	10.0398	6.6399	6.8114	8.3290	7.8474

[a]= significant

Table A4.3.3 The rate parameter (B) for *Lb. sakei*

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature[a]	**0**	0.0049	0.0041	0.0048	0.0057	0.0039
(°C)	**5**	0.0139	0.0139	0.149	0.0113	0.0116
	10	0.0424	0.0406	0.0298	0.0421	0.0409
	15	0.0512	0.1141	0.1050	0.0487	0.0600

[a]= significant ($p \leq 0.05$) effect on parameter

Table A4.3.4 The time to μ_{max} (M) for *Lb. sakei* (hours)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature[a]	**0**	836.4848	811.7011	915.8152	1009.2809	1149.5110
(°C)	**5**	111.9702	96.9848	118.3052	122.8736	132.5567
	10	38.1502	38.9327	39.0633	44.3624	47.2159
	15	38.8744	17.2296	18.6269	20.6586	20.1280

[a]= significant ($p \leq 0.05$) effect on parameter

Table A4.2.5 μ_{max} for *Lb. sakei*

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature[a]	**0**	0.0074	0.0065	0.0072	0.0082	0.0059
(°C)	**5**	0.0261	0.0261	0.0243	0.0213	0.0217
	10	0.0763	0.0743	0.0633	0.0730	0.0698
	15	0.1286	0.1900	0.1789	0.1013	0.1177

[a]= significant ($p \leq 0.05$) effect on parameter

Table A4.3 6 The time take to reach 90% of the maximum population (t_{90}) for *Lb.sakei* (hours)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature [a]	**0**	1233.1207	1290.7163	1316.7039	1337.2673	1641.8202
(°C)	**5**	264.1197	251.7406	267.7254	312.0447	316.4548
	10	87.1373	90.3563	113.1206	92.7723	96.7326
	15	56.3119	34.8392	37.8996	66.5716	56.3637

[a]= significant effect on parameter

Tables 6.4 Output from the Genstat program for *P. fluorescens*

Table A4.1.1 The initial bacterial numbers (A) for *P. fluorescens* (log_{10} cfu/cm^2)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**0**	2.0702	-4.9703	2.381	1.8542	2.0166
(°C)	**5**	1.5378	-6.8295	1.6314	2.2073	1.8893
	10	-0.2775	-1.2937	0.8293	1.5759	1.0504
	15	0.6375	0.7526	0.1493	-11.4815	1.9043

Table A4.1.2 The increase in bacteria numbers (C) for *P. fluorescens* (log_{10} cfu/cm^2)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**0**	5.3911	14.7975	1.1871	23.5569	0.1276
(°C)	**5**	6.2183	14.7488	5.3745	0.9717	0.6491
	10	8.4523	9.2768	6.7296	5.9452	2.7465
	15	7.0947	8.0052	7.9864	20.2535	5.4736

Table A4.4.3 The rate parameter (B) for *P. fluorescens*

		Concentration of CO_2 (%) [a]				
		0	**25**	**50**	**75**	**100**
Temperature [a]	**0**	0.0801	0.0498	0.0296	0.0131	0.0392
(°C)	**5**	0.0401	0.0231	0.0172	0.0108	0.0120
	10	0.0256	0.0077	0.0073	0.0064	-0.0037
	15	0.0141	0.0021	0.0086	0.0006	0.0068

[a]= significant ($p \leq 0.05$) effect on parameter

Table A4.3.4 The time to μ_{max} (M) for *P. fluorescens* (hours)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature	**0**	194.7905	42.6865	393.9488	8111.8154	430.4283
(°C)	**5**	68.9919	-55.9056	256.4370	255.6941	1143.6029
	10	22.6246	24.2057	68.8430	159.5210	45.2289
	15	17.1059	32.3702	36.5547	-56.0547	64.9989

Table A4.2.5 μ_{max} for *P. fluorescens.*

		Concentration of CO_2 (%)[a]				
		0	**25**	**50**	**75**	**100**
Temperature [a]	**0**	0.0190	0.0076	0.0039	0.0036	0.0002
(°C)	**5**	0.0398	0.0283	0.0098	0.0016	-0.0006
	10	0.0848	0.0535	0.0289	0.0160	0.0082
	15	0.1420	0.0998	0.0591	0.0663	0.0537

[a]= significant ($p \leq 0.05$) effect on parameter

TableA4.4 6 The time take to reach 90% of the maximum population (t_{90}) for *P. fluorescens* (hours)

		Concentration of CO_2 (%)				
		0	**25**	**50**	**75**	**100**
Temperature [a]	**0**	324.4038	1326.3920	547.3920	11537.1143	*
(°C)	**5**	145.0808	318.3029	515.4305	368.3217	1023.3217
	10	78.3270	126.6356	189.1656	338.7214	198.1700
	15	43.3509	74.4389	110.1001	180.2910	112.4026

[a]= significant ($p \leq 0.05$) effect on parameter

*= no increase in bacterial numbers

Appendix 5

Output of Genstat program for the effects of environmental factors on the growth kinetics of the total population and *Lb. sakei, P. fluorescens* and *B. thermosphata*

Effects of environmental factors, alone or in combinations, on the growth kinetics (μ_{max}, t_{90} and maximum population density) of the total population and *P. fluorescens, Lb. sakei* and *B. thermosphacta* were determined. The effects of the environmental factors on the growth kinetics of *H. alvei* could not be determined statistically, as there was insufficient data for this organism.

Each culture was grown until late exponential phase as a pure culture, diluted to 10^{4-5} cfu/cm^2 for *B. thermosphacta, Lb. sakei* and *P. fluorescens* and 10^{2-3} cfu/cm^2 for *H. alvei*, before being added together to produce a mixed culture. A 100μL aliquot was then plated onto containing BHIA which either contained no additives (control), 1% sodium chloride (experiment 1), 2% sodium chloride (experiment 2), 0.4% potassium sorbate or 0.02% propyl-paraben at pH 5.5 or 6.0. The plates were then grouped together in fours before being packaged in either air, 25% CO_2 (balance N_2) or 100% CO_2 and stored at 0, 5, 10 or 15°C. Groups of plates were removed at intervals of 24, 48, 108, 156, 324, 660, 1334 and 2678 hours.

The number of bacteria on the surface of the agar was determined by macerating the agar with 50mL diluent then spiral plating the homogenate. Total viable population was determined on PCA which had been incubated at 30°C for 48 hours, *B. thermosphacta* was enumerated on STAA incubated at 25°C for 48 hours, *H. alvei* was enumerated on VRBGA incubated anaerobically at 30°C for 24 hours, *Lb. sakei* was enumerated on MRSA incubated anaerobically at 30°C for 48 hours and *P. fluorescens* was enumerated on PSA incubated at 25°C for 48 hours.

The growth data generated for the total population and *P. fluorescens, Lb. sakei* and *B. thermosphacta* was analysed using the Genstat programs listed Appendix 3. Firstly, the effect of each environmental factor alone on the growth kinetics was determined, then the effects of combinations of two, three and four environmental factors on the growth kinetics were determined. For each table, only the effect of the environmental factor(s) stated were determined..

The least significant differences (l.s.d.) of the means were calculated to determine whether values from the fitted curves were significantly different. The l.s.d. values were recorded below each table. Numbers that differ by more than the l.s.d values were significantly different ($p \leq 0.05$). As the salt

concentration was increased to 2% for the second experiment the number of replicates for one and 2% sodium chloride were lower than the other treatments, therefore the l.s.d for one and 2% sodium chloride was different from the l.s.d for the other environmental factors. In tables where more than one l.s.d. value was necessary: A indicates the l.s.d value for comparing 1% sodium chloride to 2% sodium chloride, B indicates the l.s.d used for comparing one or 2%sodium chloride to the other environmental factors and C indicated the l.s.d used when comparing the other environmental factors to each other.

The superscripted letters next to each value in the table indicates which numbers were significantly ($p \leq 0.05$) different, firstly from the control and then from the other factors. Numbers that have the same superscript letter are not significantly different. Tables only contain superscripted letters when the environmental factor or combination of environmental factors had a significant ($p \leq 0.05$) effect on the growth kinetic. Note that the tables are independent from one another, there is no relationship between superscripted letters across different tables.

A5.1 The effect of environmental factors on the μ_{max} of total population after log_{10} transformation

A5.1.1 The effect of each environmental factor alone on μ_{max} of the total population

A5.1.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
-3.98^{a}	-3.96^{a}	-4.29^{a}	-4.80^{b}	-4.67^{c}

l.s.d A=0.869, B=0.752, C=0.614

A5.1.1.2 The effect of pH

pH 5.5	pH 6.0
-4.62^{a}	-4.16^{b}

l.s.d=0.434

A5.1.1.4 The effect of temperature

10°C	1°C
-3.34^{a}	-5.44^{b}

l.s.d=0.434

A5.1.1.3 The effect of atmosphere

Air	25% CO_2	100% CO_2
-3.77^{a}	-4.06^{a}	-5.34^{b}

l.s.d=0.532

A5.1.2 The effect of a combination of two environmental factor on μ_{max} of the total population

A5.1.2.1. The effect of preservative and pH

	pH 5.5	pH 6.0
Control	-3.88	-4.07
1% NaCl	-3.98	-3.95
2% NaCl	-4.28	-4.29
PS	-5.56	-4.03
PP	-4.92	-4.42

l.s.d A=1.229, B=1.064, C=0.869

A5.1.2.2 The effect of preservative and temperature

	10°C	1°C
Control	-3.17	-4.78
1% NaCl	-3.27	-4.66
2% NaCl	-3.12	-5.45
PS	-3.22	-6.37
PP	-3.77	-5.57

l.s.d A=1.229, B=1.064, C=0.869

A5.1.2.3 The effect of pH and temperature

	10	1
pH 5.5	-3.27[a]	-5.97[b]
pH 6.0	-3.41[a]	-4.91[c]

l.s.d.=0.614

A5.1.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	-3.58	-3.83	-4.51
1% NaCl	-3.62	-3.98	-4.29
2% NaCl	-6.57	-3.76	-5.53
PS	-4.01	-4.20	-6.18
PP	-3.90	-4.34	-5.77

l.s.d A=1.505, B=1.303, C=1.064

A5.1.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	-3.87[a]	-3.99[a]	-6.00[b]
pH 6.0	-3.67[a]	-4.13[a]	-4.68[c]

l.s.d=0.7.52

A5.1.2.6 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	-2.97[a]	-3.38[a]	-3.67[a]
1°C	-3.67[a]	-4.74[b]	-4.68[c]

l.s.d=0.752

A5.1.3 The effect of the combination of three environmental factors on μ_{max} of the total population

A5.1.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	**1°C**	**10°C**	**1°C**
Control	-3.18	-4.59	-3.16	-4.97
1% NaCl	-3.24	-4.72	-6.29	-4.60
2% NaCl	-3.07	-5.50	-3.18	-5.40
PS	-3.33	-7.79	-3.12	-4.95
PP	-3.43	-6.41	-4.12	-4.72

l.s.d A=1.738, B=1.505, C=1.229

A5.1.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
Control	-3.47	-3.80	-4.38	-3.69	-3.86	-4.65
1% NaCl	-3.60	-3.99	-4.34	-3.64	-3.96	-4.24
2% NaCl	-3.86	-3.72	-5.29	-3.30	-3.80	-5.77
PS	-4.31	-4.14	-8.22	-3.71	-4.26	-4.13
PP	-3.98	-4.18	-6.60	-3.83	-4.51	-4.93

l.s.d A=0.2.128, B=1.843, C=1.505

A5.1.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
Control	-2.66	-3.23	-3.62	-4.50	-4.43	-5.40
1% NaCl	-2.99	-3.23	-6.57	-4.24	-4.72	-5.01
2% NaCl	-2.71	-3.15	-3.51	-4.43	-4.37	-7.55
PS	-2.94	-3.44	-3.29	-5.09	-4.95	-9.07
PP	-3.43	-3.66	-4.23	-4.38	-5.03	-7.30

l.s.d A=2.128, B=1.843, C=1.505

A5.1.3.4 The effect of pH temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
pH 5.5	-2.88	-3.03	-3.91	-4.86	-4.96	-8.10
pH 6.0	-3.06	-3.73	-3.43	-4.29	-4.52	-5.29

l.s.d=1.064

A5.1.4 The effect of all four environmental factors on μ_{max} of the total population

A5.1.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	**25% CO_2**	**100% CO_2**
Control	**pH 5.5**	**10°C**	-2.65	-3.11	-3.79
		1°C	-4.30	-4.49	-4.97
	pH 6.0	**10°C**	-2.68	-3.35	-3.46
		1°C	-4.70	-4.37	-5.84
1% NaCl	**pH 5.5**	**10°C**	-3.11	-3.00	-3.60
		1°C	-4.08	-4.99	-5.09
	pH 6.0	**10°C**	-2.88	-3.46	-3.55
		1°C	-4.40	-4.45	-4.94
2% NaCl	**pH 5.5**	**10°C**	-2.69	-3.00	-3.51
		1°C	-4.98	-4.45	-7.06
	pH 6.0	**10°C**	-2.72	-3.30	-3.51
		1°C	-3.87	-4.30	-8.03
PS	**pH 5.5**	**10°C**	-2.67	-3.08	-4.24
		1°C	-5.96	-5.20	-12.21
	pH 6.0	**10°C**	-3.21	-3.81	-2.33
		1°C	-4.22	-4.70	-5.92
PP	**pH 5.5**	**10°C**	-3.30	-2.94	-4.04
		1°C	-4.66	-5.42	-9.17
	pH 6.0	**10°C**	-3.56	-4.38	-4.42
		1°C	-4.10	-4.63	-5.44

l.s.d A=3.010, B=2.606, C=2.128

A5.2 The effect of environmental factors on the t_{90} of total population after log_{10} transformation

A5.2.1 The effect of each environmental factor alone on t_{90} of the total population

A5.2.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
5.157[a]	5.187[a]	5.536[b]	5.703[b]	5.753[b]

l.s.d. A=0.3420, B=0.2962, C=0.2418

A5.2.1.2 The effect of pH

pH 5.5	pH 6.0
5.579	5.417

l.s.d.=0.1710

A5.2.1.3 The effect of temperature

10°C	1°C
4.572[a]	6.424[b]

l.s.d.=0.1710

A5.2.1.4 The effect of atmosphere

Air	25% CO_2	100% CO_2
5.127[a]	5.363[b]	6.004[c]

l.s.d=0.2094

A5.2.2 The effect of a combination of two environmental factor on t_{90} of the total population

A.5.2.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	5.156	5.193
1% NaCl	5.222	5.153
2% NaCl	5.539	5.533
PS	5.962	5.444
PP	5.817	5.689

l.s.d. A=0.4837, B=0.4189, C=0.3420

A.5.2.2.2 The effect of preservative and temperature

	10°C	1°C
Control	4.340[a]	6.010[b]
1% NaCl	4.365[a]	6.009[b]
2% NaCl	4.482[a]	6.590[b]
PS	4.536[a]	6.870[b]
PP	4.991[a]	6.516[b]

l.s.d. A=0.4837, B=0.4189, C=0.3420

A.5.2.2.3 The effect of pH and temperature

	10	1
pH 5.5	4.606[a]	6.552[b]
pH 6.0	4.539[a]	6.296[b]

l.s.d.=0.2418

A.5.2.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	4.847[a]	4.983[a]	5.694[b]
1% NaCl	4.923[a]	4.987[a]	5.653[b]
2% NaCl	5.020[c]	4.964[b]	6.623[d]
PS	5.420[c]	5.673[b]	6.017[d]
PP	5.269[c]	5.821[b]	6.170[d]

l.s.d A=0.5924, B=0.5130, C=0.4189

A.5.2.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	5.266	5.440	6.031
pH 6.0	4.988	5.286	5.978

l.s.d.=0.2962

A.5.2.2.6 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	4.406[a]	4.403[a]	4.908[b]
1°C	5.848[c]	6.323[d]	7.100[e]

l.s.d.=0.0.2962

A5.2.3 The effect of the combination of three environmental factors on t_{90} of the total population

A5.2.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	4.352	5.960	4.327	6.059
1% NaCl	4.367	6.077	4.364	5.941
2% NaCl	4.499	6.578	4.464	6.601
PS	4.709	7.216	4.363	6.525
PP	4.931	6.704	5.050	6.328

l.s.d. A=0.6840, B=0.5924, C=0.4837

A5.2.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	4.827	4.968	5.674	4.868	4.999	5.713
1% NaCl	4.983	4.978	5.705	4.862	4.995	5.600
2% NaCl	5.162	5.012	6.442	4.877	4.917	6.803
PS	5.808	5.884	6.196	5.033	5.462	5.837
PP	5.358	5.913	6.181	5.180	5.728	6.159

l.s.d A=0.8378, B=0.7255, C=0.5924

A5.2.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	4.046^{a}	4.237^{a}	4.737^{a}	5.648^{b}	5.730^{b}	6.651^{c}
1% NaCl	4.208^{a}	4.060^{a}	4.827^{a}	5.637^{b}	5.913^{b}	6.478^{c}
2% NaCl	4.245^{a}	4.291^{a}	4.908^{a}	5.794^{b}	5.638^{b}	8.337^{d}
PS	4.523^{a}	4.432^{a}	4.653^{a}	6.318^{e}	6.914^{f}	7.380^{f}
PP	4.829^{g}	4.767^{g}	5.376^{g}	5.709^{h}	6.875^{f}	6.964^{f}

l.s.d. A=0.8378, B=0.7255, C=0.5924

A5.2.3.4 The effect of pH, temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
pH 5.5	4.444^{a}	4.290^{a}	5.084^{b}	6.088^{c}	6.590^{d}	6.978^{d}
pH 6.0	4.368^{a}	4.515^{a}	4.733^{a}	5.607^{e}	6.057^{f}	7.223^{d}

l.s.d.=0.4189

A5.2.4 The effect of all four environmental factors on t_{90} of the total population

A5.2.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	25% CO_2	100% CO_2
Control	**pH 5.5**	**10°C**	4.084	4.148	4.824
		1°C	5.570	5.787	6.524
	pH 6.0	**10°C**	4.008	4.325	4.649
		1°C	5.727	5.672	6.777
1% NaCl	**pH 5.5**	**10°C**	4.303	3.910	4.888
		1°C	5.663	6.047	6.522
	pH 6.0	**10°C**	4.114	4.211	4.767
		1°C	5.611	5.779	6.434
2% NaCl	**pH 5.5**	**10°C**	4.275	4.299	4.922
		1°C	6.049	5.724	7.962
	pH 6.0	**10°C**	4.215	4.283	4.895
		1°C	5.539	5.551	8.712
PS	**pH 5.5**	**10°C**	4.565	4.430	5.131
		1°C	7.050	7.337	7.261
	pH 6.0	**10°C**	4.480	4.434	4.175
		1°C	5.586	6.490	7.499
PP	**pH 5.5**	**10°C**	4.839	4.477	5.477
		1°C	5.877	7.349	6.885
	pH 6.0	**10°C**	4.820	5.056	5.276
		1°C	5.541	6.401	7.042

l.s.d. A=1.1848, B=1.0261, C=0.8378

A5.3 The effect of environmental factors on the increase in maximum population density of total population

A5.3.1 The effect of each environmental factor alone on the increase in maximum population density of the total population

A5.3.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
8.159[a]	8.037[a]	7.948[a]	6.361[b]	6.912[c]

l.s.d. A=0.4657, B=0.4033, C=0.3293

A5.3.1.2 The effect of pH

pH 5.5	pH 6.0
7.048[a]	7.664[b]

l.s.d.=0.2328

A5.3.1.3 The effect of temperature

10°C	1°C
7.701[a]	7.011[b]

l.s.d.=0.2328

A5.3.1.4 The effect of atmosphere

Air	25% CO_2	100% CO_2
8.386[a]	7.038[b]	6.644[c]

l.s.d.=0.2852

A5.3.2 The effect of a combination of two environmental factor the increase in maximum population density of the total population

A5.3.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	8.121[a]	8.196[a]
1% NaCl	7.958[a]	8.112[a]
2% NaCl	7.600[a]	8.296[a]
PS	5.683[b]	7.040[c]
PP	6.607[d]	7.216[e]

l.s.d. A=0.6585, B=0.5703, C=0.4657

A5.3.2.2 The effect of preservative and temperature

	10°C	1°C
Control	8.389[a]	7.928[a]
1% NaCl	8.305[a]	7.769[a]
2% NaCl	7.854[a]	8.042[a]
PS	6.759[b]	5.964[c]
PP	7.576[d]	6.247[c]

l.s.d. A=0.6585, B=0.5703, C=0.4657

A5.3.2.3 The effect of pH and temperature

	10	1
pH 5.5	7.490	6.605
pH 6.0	7.911	7.418

l.s.d.=0.3293

A5.3.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	9.193[a]	7.780[b]	7.504[b]
1% NaCl	9.062[a]	7.636[b]	7.412[b]
2% NaCl	8.758[a]	7.132[b]	7.954[b]
PS	7.080[c]	6.76[d]	5.628[e]
PP	8.362[f]	6.614[d]	5.759[e]

l.s.d. A=0.8065, B= 0.69875, C=0.5703

A5.3.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	8.051	6.869	6.223
pH 6.0	8.722	7.208	7.064

l.s.d=0.4033

A5.3.2.6 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	8.869[a]	7.176[b]	7.058[b]
1°C	7.904[c]	6.901[b]	6.229[d]

l.s.d.=0.4033

A5.3.3 The effect of the combination of three environmental factors on increase in maximum population density of the total population

A5.3.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	8.339	7.903	8.440	7.953
1% NaCl	8.243	7.674	8.366	7.864
2% NaCl	7.699	7.501	8.010	8.583
PS	6.231	5.136	7.287	6.792
PP	7.421	5.793	7.730	6.702

l.s.d. A=0.9313, B=0.8065, C=0.6585

A5.3.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	9.259	7.637	7.468	9.126	7.924	7.539
1% NaCl	9.016	7.500	7.359	9.109	7.772	7.464
2% NaCl	8.569	6.984	7.245	8.947	7.279	8.663
PS	60.32	6.192	4.825	8.127	6.560	6.432
PP	8.118	6.406	5.296	8.605	6.821	6.223

l.s.d. A=1.1406, B=0.9878, C=0.8065

A5.3.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	9.229^{a}	8.030^{b}	7.909^{b}	9.156^{a}	7.531^{b}	7.098^{b}
1% NaCl	9.288^{a}	7.786^{b}	7.840^{b}	8.836^{a}	7.486^{b}	6.984^{b}
2% NaCl	8.931^{a}	7.368^{b}	7.264^{b}	8.586^{a}	6.895^{b}	8.645^{a}
PS	7.950^{c}	6.237^{d}	6.089^{d}	6.209^{d}	6.515^{d}	5.167^{e}
PP	9.185^{a}	6.858^{d}	6.684^{d}	7.538^{d}	6.369^{d}	4.835^{e}

l.s.d. A=1.1406, B=0.9878, C=0.8065

A5.3.3.4 The effect of pH temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
pH 5.5	8.530^{a}	6.950^{b}	6.991^{b}	7.571^{b}	6.789^{d}	5.454^{d}
pH 6.0	9.207^{e}	7.401^{b}	7.126^{b}	8.236^{e}	7.014^{c}	7.003^{c}

l.s.d.=0,5703

A5.3.4 The effect of all four environmental factors on the increase in maximum population density of the total population

A5.3.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	25% CO_2	100% CO_2
Control	**pH 5.5**	**10°C**	9.326	7.879	7.813
		1°C	9.193	7.395	7.123
	pH 6.0	**10°C**	9.133	8.181	8.004
		1°C	9.120	7.666	7.073
1% NaCl	**pH 5.5**	**10°C**	9.276	7.552	7.901
		1°C	8.756	7.448	6.818
	pH 6.0	**10°C**	9.300	8.019	7.779
		1°C	8.917	7.525	7.150
2% NaCl	**pH 5.5**	**10°C**	8.774	7.093	7.229
		1°C	8.365	6.875	7.261
	pH 6.0	**10°C**	9.088	7.644	7.298
		1°C	8.806	6.914	10.028
PS	**pH 5.5**	**10°C**	6.639	6.080	5.973
		1°C	5.426	6.305	3.676
	pH 6.0	**10°C**	9.262	6.394	6.205
		1°C	6.992	6.726	6.659
PP	**pH 5.5**	**10°C**	9.131	6.519	6.613
		1°C	7.106	6.294	3.979
	pH 6.0	**10°C**	9.239	7.198	6.755
		1°C	7.971	6.444	5.691

l.s.d. A=1.6131, B=1.3970, C=1.1406

A5.4 The effect of environmental factors on the μ_{max} of *P. fluorescens* $\log_{10}$ after transformation

A5.4.1 The effect of each environmental factor alone on μ_{max} of *P. fluorescens*

A5.4.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
-6.38[a]	-7.23[a]	-8.02[a]	-9.26[b]	-7.54[a]

l.s.d. A=1.920, B=1.663, C=1.358

A5.4.1.2 The effect of pH

pH 5.5	pH 6.0
-8.64[a]	-6.76[b]

l.s.d.=0.960

A5.4.1.3 The effect of temperature

10°C	1°C
-6.16[a]	-9.24[b]

l.s.d.=0.960

A5.4.1.1 The effect of atmosphere

Air	25% CO_2	100% CO_2
-4.16[a]	-7.74[b]	-11.20[c]

l.s.d.=1.176

A5.4.2 The effect of a combination of two environmental factor on μ_{max} of *P. fluorescens*

A5.4.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	-7.37	-5.39
1% NaCl	-7.92	-6.53
2% NaCl	-8.35	-7.69
PS	-10.66	-7.86
PP	-8.39	-6.69

l.s.d. A=2.715, B=2.351, C=1.920

A5.4.2.2 The effect of preservative and temperature

	10°C	1°C
Control	-4.93	-7.83
1% NaCl	-6.32	-8.14
2% NaCl	-6.11	-9.93
PS	-7.70	-10.82
PP	-5.79	-9.29

l.s.d. A=2.715, B=2.351, C=1.920

A5.4.2.3 The effect of pH and temperature

	10°C	1°C
pH 5.5	-6.85	-10.42
pH 6.0	-5.46	-8.07

l.s.d.=1.358

A5.4.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	-3.49	-5.43	-10.21
1% NaCl	-3.69	-5.79	-12.21
2% NaCl	-4.25	-7.60	-12.21
PS	-5.32	-10.26	-12.21
PP	-3.84	-8.60	-10.19

l.s.d. A=3.352, B=2.880, C=2.351

A5.4.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	-4.90^{a}	-9.58^{b}	-11.44^{c}
pH 6.0	-3.42^{a}	-5.91^{d}	-10.96^{c}

l.s.d.=1.663

A5.4.2.6 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	-2.78	-5.50	-10.20
1°C	-5.54	-9.99	-12.21

l.s.d.=1.663

A5.4.3 The effect of the combination of three environmental factors on μ_{max} of *P. fluorescens*

A5.4.3.1 the effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	**1°C**	**10°C**	**1°C**
Control	-5.24	-9.50	-4.62	-6.16
1% NaCl	-6.23	-9.62	-6.41	-6.66
2% NaCl	-6.28	-10.42	-5.94	-9.44
PS	-9.12	-12.21	-6.29	-9.44
PP	-6.81	-9.97	-4.76	-8.62

l.s.d. A=3.325, B=2.880, C=2.351

A5.4.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
Control	-3.83	-7.58	-10.69	-3.16	-3.27	-9.74
1% NaCl	-3.55	-8.00	-12.21	-3.82	-3.58	-12.21
2% NaCl	-5.23	-7.61	-12.21	-3.28	-7.58	-12.21
PS	-7.57	-12.21	-12.21	-3.08	-8.31	-12.21
PP	-3.79	-10.71	-10.67	-3.89	-6.49	-9.70

l.s.d. A=4.703, B=4.073, C3.325

A5.4.3.3 the effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
Control	-3.01	-+3.56	-8.22	-3.98	-7.29	-12.21
1% NaCl	-3.23	-3.52	-12.21	-4.14	-8.06	-12.21
2% NaCl	-3.13	-2.99	-12.21	-5.37	-12.21	-12.21
PS	-2.59	-8.31	-12.21	-8.06	-12.21	-12.21
PP	-2.33	-6.87	-8.17	-5.35	-10.32	-12.21

l.s.d. A=4.703, B=4.073, C3.325

A5.4.3.4 the effect of pH temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
pH 5.5	-2.94	-6.94	-10.68	-6.85	-12.21	-12.21
pH 6.0	-2.61	-4.05	-9.72	-4.22	-7.77	-12.21

l.s.d. 2.351

A5.4.4 The effect of all four environmental factors on μ_{max} of *P. fluorescens*

A5.4.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	25% CO_2	100% CO_2
Control	**pH 5.5**	**10°C**	-3.59[a]	-2.95[a]	-9.17[b]
		1°C	-4.08[a]	-12.21[b]	-12.21[b]
	pH 6.0	**10°C**	-2.44[a]	-4.61[a]	-7.27[b]
		1°C	-3.88[a]	-2.38[a]	-12.21[b]
1% NaCl	**pH 5.5**	**10°C**	-2.67[a]	-3.80[a]	-12.21[b]
		1°C	-4.43[a]	-12.21[b]	-12.21[b]
	pH 6.0	**10°C**	-3.79[a]	-3.23[a]	-12.21[b]
		1°C	-3.85[a]	-3.92[a]	-12.21[b]
2% NaCl	**pH 5.5**	**10°C**	-3.62[a]	-3.01[a]	-12.21[b]
		1°C	-6.84[a]	-12.21[b]	-12.21[b]
	pH 6.0	**10°C**	-2.65[a]	-2.96[a]	-12.21[b]
		1°C	-3.91[a]	-12.21[b]	-12.21[b]
PS	**pH 5.5**	**10°C**	-2.94[a]	-12.21[b]	-12.21[b]
		1°C	-12.21[b]	-12.21[b]	-12.21[b]
	pH 6.0	**10°C**	-2.23[a]	-4.42[a]	-12.21[b]
		1°C	-3.92[a]	-12.21[b]	-12.21[b]
PP	**pH 5.5**	**10°C**	-2.09[a]	-9.21[b]	-9.19[b]
		1°C	-5.49[a]	-12.21[b]	-12.21[b]
	pH 6.0	**10°C**	-2.57[a]	-4.53[a]	-7.19[b]
		1°C	-5.21[a]	-8.44[b]	-12.21[b]

l.s.d. A=6.651, B=5.760, C=4.703

A5.5 The effect of environmental factor on t_{90} of *P. fluorescens* after log_{10} transformation

A5.5.1 The effect of each environmental factor alone on t_{90} of *P fluorescens*

A5.5.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
5.079[a]	5.404[a]	5.630[b]	6.069[c]	6.096[c]

l.s.d. A=0.6100, B=0.5283, C=0.4313

A5.5.1.2 The effect of pH

pH 5.5	pH 6.0
6.116[a]	5.264[b]

l.s.d.=0.3050

A5.5.1.3 The effect of temperature

10°C	1°C
5.188[a]	6.192[b]

l.s.d.=0.3050

A5.5.1.4 The effect of atmosphere

Air	25% CO_2	100% CO_2
5.219[a]	5.349[a]	6.502[b]

l.s.d.=0.3736

A5.5.2 The effect of a combination of two environmental factor on t_{90} of *P. fluorescens*

A5.5.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	5.569[a]	4.590[b]
1% NaCl	5.644[a]	5.164[a]
2% NaCl	6.705[c]	4.555[b]
PS	6.290[c]	5.848[c]
PP	6.432[c]	5.759[c]

l.s.d. A=0.8627, B=0.7471, C=0.6100

A5.5.2.2 The effect of preservative and temperature

	10°C	1°C
Control	4.949[a]	5.210[b]
1% NaCl	4.553[a]	6.254[b]
2% NaCl	4.796[a]	6.465[b]
PS	5.823[b]	6.314[b]
PP	5.307[a]	6.885[b]

l.s.d. A=0.8627, B=0.7471, =0.6100

A5.5.2.3 The effect of pH and temperature

	10	1
pH 5.5	5.703	6.530
pH 6.0	4.674	5.855

l.s.d.=0.4313

A5.5.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	4.857[a]	4.240[a]	6.142[b]
1% NaCl	5.301[a]	5.096[a]	6.084[b]
2% NaCl	6.051[b]	4.495[a]	6.345[b]
PS	5.116[a]	6.421[b]	6.669[b]
PP	5.364[a]	5.941[a]	6.983[c]

l.s.d. A=1.0566, B=0.9150, C=0.7471

A5.5.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	5.421[a]	5.208[a]	7.721[b]
pH 6.0	5.018[a]	5.491[a]	5.283[a]

l.s.d.=0.5283

A5.5.2.1 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	4.318[a]	5.188[b]	6.058[c]
1°C	6.120[d]	5.511[b]	6.946[e]

l.s.d.=0.5283

A5.5.3 The effect of the combination of three environmental factors on t_{90} of *P. fluorescens*

A5.5.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	5.549[a]	5.589[a]	4.348[b]	7.832[b]
1% NaCl	4.785[a]	6.502[ac]	4.321[b]	6.006[c]
2% NaCl	5.378[a]	8.033[d]	4.215[b]	4.896[b]
PS	6.180[ac]	6.399[ac]	5.466[a]	6.230[ac]
PP	6.000[ac]	6.864[ac]	4.613[b]	6.905[c]

l.s.d. A=1.2200, B=1.0566, C=0.8627

A5.5.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	5.078[a]	3.569[b]	8.060[c]	4.635[a]	4.912[a]	4.223[a]
1% NaCl	4.956[a]	5.003[a]	6.972[c]	5.107[a]	5.188[a]	5.196[a]
2% NaCl	7.096[d]	4.930[e]	8.090[cd]	5.006[a]	4.059[a]	4.601[a]
PS	5.399[a]	5.955[a]	7.515[c]	4.833[a]	6.887[f]	5.823[g]
PP	5.179[a]	6.340[h]	7.778[c]	5.548[a]	5.541[a]	6.188[i]

l.s.d. A=1.4942, B=1.2940, C=1.0566

A5.5.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	4.261	4.594	5.991	5.542	3.887	6.293
1% NaCl	4.389	3.977	5.294	5.674	6.214	6.874
2% NaCl	4.826	4.025	5.538	7.276	4.965	7.153
PS	4.313	6.623	6.533	5.919	6.219	6.805
PP	4.092	5.534	6.293	6.635	6.347	7.672

l.s.d. A=1.4942, B=1.2940, C=1.0566

A5.5.3.4 The effect of pH temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
pH 5.5	4.538	5.169	7.402	6.303	5.246	8.040
pH 6.0	4.098	5.208	4.714	5.938	5.772	5.851

l.s.d.=0.7471

A5.5.4 The effect of all four environmental factors on t_{90} of *P. fluorescens*

A5.5.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	25% CO_2	100% CO_2
Control	**pH 5.5**	**10°C**	4.648	3.963	8.037
		1°C	5.508	3.174	8.084
	pH 6.0	**10°C**	3.874	5.225	3.944
		1°C	5.396	4.599	4.501
1% NaCl	**pH 5.5**	**10°C**	4.171	3.969	6.217
		1°C	5.742	6.038	7.727
	pH 6.0	**10°C**	4.607	3.986	4.370
		1°C	5.607	6.391	6.021
2% NaCl	**pH 5.5**	**10°C**	5.282	4.036	6.815
		1°C	8.911	5.824	9.365
	pH 6.0	**10°C**	4.370	4.013	4.261
		1°C	5.642	4.105	4.941
PS	**pH 5.5**	**10°C**	4.681	6.299	7.560
		1°C	6.117	5.611	7.470
	pH 6.0	**10°C**	3.945	6.947	5.560
		1°C	5.722	6.827	6.140
PP	**pH 5.5**	**10°C**	4.098	6.409	7.494
		1°C	6.260	6.270	8.061
	pH 6.0	**10°C**	4.087	4.659	5.093
		1°C	7.010	6.423	7.282

l.s.d. A=2.1132, B=1.8301, C=1.4942

A5.6 The effect of environmental factors on the increase in maximum population density of *P. fluorescens* after log transformation

A5.3.1 The effect of each environmental factor alone on the increase in maximum population density of *P. fluorescens*

A5.6.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
1.818[a]	1.663[a]	2.026[a]	1.477[b]	1.850[a]

l.s.d. A=0.4290, B=0.3715, C=0.3034

A5.6.1.2 The effect of pH

pH 5.5	pH 6.0
1.750	1.745

l.s.d.=0.2145

A5.6.1.3 The effect of temperature

10°C	1°C
1.779	1.716

l.s.d.=0.2145

7.6.4 Atmosphere

Air	25% CO_2	100% CO_2
2.348	1.478	1.416

l.s.d.=0.2627

A5.6.2 The effect of a combination of two environmental factor the increase in maximum population density of *P. fluorescens*

A5.6.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	1.879	1.758
1% NaCl	1.606	1.720
2% NaCl	2.404	1.647
PS	1.364	1.591
PP	1.753	1.947

L.s.d. A=0.6067, B=0.5254, C=0.4290

A5.6.2.2 The effect of Preservative and temperature

	10°C	1°C
Control	2.002	1.635
1% NaCl	1.702	1.624
2% NaCl	1.686	2.365
PS	1.559	1.396
PP	1.862	1.839

l.s.d. A=0.6067, B=0.5254, C=0.4290

A5.6.2.3 The effect of pH and temperature

	10°C	1°C
pH 5.5	1.820	1.681
pH 6.0	1.738	1.751

l.s.d.=0.3034

A5.6.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	2.218^{a}	1.611^{b}	1.626^{b}
1% NaCl	2.196^{a}	1.532^{b}	1.262^{b}
2% NaCl	3.373^{a}	1.456^{b}	1.248^{b}
PS	1.842^{c}	1.344^{a}	1.247^{b}
PP	2.548^{a}	1.465^{b}	1.538^{b}

l.s.d. A=0.7431, B=0.6435, C=0.5254

A5.6.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	2.318	1.415	1.591
pH 6.0	2.378	1.542	1.314

l.s.d.=0.3715

A5.6.2.1 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	5.158	1.609	1.519
1°C	2.538	1.348	1.314

l.s.d.=0.3715

A5.6.3 The effect of the combination of three environmental factors on increase in maximum population density of *P. fluorescens*

A5.6.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	2.152[a]	1.606[a]	1.852[a]	1.663[a]
1% NaCl	1.660[a]	1.552[a]	1.744[a]	1.696[a]
2% NaCl	1.675[a]	3.133[a]	1.698[a]	1.597[a]
PS	1.454[c]	1.274[ac]	1.664[ac]	1.591[ac]
PP	2.007[a]	1.499[a]	1.717[a]	2.178[a]

l.s.d. A=0.8580, B=0.7431, C=0.6067

A5.6.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	2.228[a]	1.556[a]	1.854[a]	2.208[a]	1.666[a]	1.399[ab]
1% NaCl	2.166[a]	1.432[a]	1.220[a]	2.225[a]	1.631[a]	1.303[ab]
2% NaCl	4.596[c]	1.378[a]	1.238[a]	2.150[a]	1.534[a]	1.257[ab]
PS	1.598[a]	1.267[a]	1.226[a]	2.086[a]	1.420[a]	1.267[ab]
PP	2.064[a]	1.430[a]	1.766[a]	3.032[d]	1.500[a]	1.310[ab]

l.s.d. A=1.0509, B=0.9101, C=0.7431

At pH 6.0 100% CO_2 is significantly different from air but not from 25% CO_2.

A5.6.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	2.217	1.827	1.963	2.219	1.395	1.290
1% NaCl	2.208	1.648	1.250	2.183	1.416	1.273
2% NaCl	2.193	1.625	1.241	4.553	1.288	1.254
PS	2.033	1.405	1.237	1.650	1.282	1.256
PP	2.181	1.567	1.837	2.915	1.363	1.238

l.s.d. A=1.0509, B=0.9101, C=0.7431

A5.6.3.1 The effect of pH temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
pH 5.5	2.127	1.552	1.781	2.508	1.278	1.526
pH 6.0	2.189	1.666	1.360	2.568	1.418	1.268

l.s.d.=0.5254

A5.6.4 The effect of all four environmental factors on the increase in maximum population density of *P. fluorescens*

A5.6.4.4 The effect of preservative, pH, temperature and atmosphere

			Air	25% CO_2	100% CO_2
Control	**pH 5.5**	**10°C**	2.237	1.790	2.429
		1°C	2.218	1.322	1.278
	pH 6.0	**10°C**	2.196	1.863	1.496
		1°C	2.220	1.468	1.301
1% NaCl	**pH 5.5**	**10°C**	2.168	1.619	1.194
		1°C	2.614	1.246	1.246
	pH 6.0	**10°C**	2.248	1.676	1.306
		1°C	2.202	1.586	1.300
2% NaCl	**pH 5.5**	**10°C**	2.256	1.531	1.238
		1°C	6.936	1.225	1.239
	pH 6.0	**10°C**	2.313	1.718	1.244
		1°C	2.170	1.350	1.270
PS	**pH 5.5**	**10°C**	1.901	1.260	1.200
		1°C	1.295	1.275	1.252
	pH 6.0	**10°C**	2.166	1.551	1.274
		1°C	2.006	1.290	1.260
PP	**pH 5.5**	**10°C**	2.158	1.581	2.280
		1°C	1.969	1.278	1.251
	pH 6.0	**10°C**	2.204	1.553	1.393
		1°C	3.860	1.448	1.226

l.s.d. A=1.461, B=1.2870, C=1.0509

A5.7 The effect of environmental factors on the μ_{max} of *Lb. sakei* after square root transformation

A5.7.1 The effect of each environmental factor alone on μ_{max} of *Lb. sakei*

A5.7.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
0.0844	0.0811	0.0764	0.1094	0.0894

l.s.d A=0.03286, B=0.02846, C=0.02323

A5.7.1.2 The effect of pH

pH 5.5	pH 6.0
0.0911	0.0900

l.s.d=0.1643

A5.7.1.3 The effect of temperature

10°C	1°C
0.1228^{a}	0.0582^{b}

l.s.d=0.1643

A5.7.1.4 The effect of atmosphere

Air	25% CO_2	100% CO_2
0.0883	0.0960	0.0872

l.s.d=0.02013

A5.7.2 The effect of a combination of two environmental factor on μ_{max} of *Lb. sakei*

A5.7.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	0.0875	0.0814
1% NaCl	0.0869	0.0745
2% NaCl	0.0745	0.0783
PS	0.1105	0.1083
PP	0.0856	0.0933

l.s.d A=0.04647, B=0.04024, C=0.03286

A5.7.2.2 The effect of preservative and temperature

	10°C	1°C
Control	0.1014[a]	0.0675[b]
1% NaCl	0.0940[a]	0.0682[b]
2% NaCl	0.1069[a]	0.0459[c]
PS	0.1679[d]	0.0508[b]
PP	0.1215[e]	0.0574[b]

l.s.d A=0.04647, B=0.04024, C=0.03286

A5.7.2.3 The effect of pH and temperature

	10°C	1°C
pH 5.5	0.1278	0.0543
pH 6.0	0.1178	0.0621

l.s.d.=0.02323

A5.7.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	0.0828	0.0893	0.0811
1% NaCl	0.0599	0.0926	0.0909
2% NaCl	0.0488	0.0985	0.0819
PS	0.1187	0.1103	0.0992
PP	0.0972	0.0890	0.0821

l.s.d A=0.05691, B=0.04929, C=0.04024

A5.7.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	0.0863	0.1050	0.0819
pH 6.0	0.0903	0.0871	0.0925

l.s.d=0.02846

A5.7.2.6 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	0.1175	0.1278	0.1232
1°C	0.0591	0.643	0.0512

l.s.d=0.02846

A5.7.3 The effect of the combination of three environmental factors on μ_{max} of *Lb. sakei*

A5.7.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	0.1106	0.6043	0.0922	0.0706
1% NaCl	0.1039	0.0699	0.0842	0.0666
2% NaCl	0.1006	0.0483	0.1132	0.0435
PS	0.1793	0.0417	0.1566	0.0600
PP	0.1192	0.0520	0.1237	0.0629

l.s.d A=0.06571, B=0.05691, C=0.04647

A5.7.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	0.0717	0.0999	0.0908	0.0940	0.0788	0.0751
1% NaCl	0.0737	0.0973	0.0896	0.0461	0.0879	0.0922
2% NaCl	0.0348	0.1119	0.0767	0.0629	0.0850	0.0870
PS	0.1333	0.1148	0.0834	0.1041	0.1058	0.1149
PP	0.0858	0.1007	0.0703	0.1086	0.0773	0.0940

l.s.d A=0.08048, B=0.06970, C=0.05691

A5.7.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	0.1082	0.1139	0.0821	0.0574	0.0648	0.0801
1% NaCl	0.0638	0.1128	0.1056	0.0561	0.0724	0.0763
2% NaCl	0.0645	0.1169	0.1393	0.0332	0.0801	0.0244
PS	0.1646	0.1583	0.1808	0.0728	0.0621	0.0176
PP	0.1331	0.1240	0.1074	0.0614	0.0540	0.0569

l.s.d A=0.04647, B=0.04024, C=0.03286

A5.7.3.4 The effect of pH temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
pH 5.5	0.1200	0.1447	0.1188	0.0525	0.0653	0.0450
pH 6.0	0.1150	0.1109	0.1275	0.0656	0.0633	0.0575

l.s.d=0.04024

A5.7.4 The effect of all four environmental factors on μ_{max} of *Lb. sakei*

7.7.15 Preservative, pH, temperature and atmosphere

			Air	25% CO_2	100% CO_2
Control	**pH 5.5**	**10°C**	0.1026	0.1397	0.0895
		1°C	0.0408	0.0600	0.0921
	pH 6.0	**10°C**	0.1138	0.0880	0.0747
		1°C	0.0741	0.0696	0.0682
1% NaCl	**pH 5.5**	**10°C**	0.0762	0.1294	0.1060
		1°C	0.0713	0.0652	0.0733
	pH 6.0	**10°C**	0.0513	0.0961	0.1051
		1°C	0.0409	0.0796	0.0792
2% NaCl	**pH 5.5**	**10°C**	0.0515	0.1213	0.1291
		1°C	0.0182	0.1026	0.0243
	pH 6.0	**10°C**	0.0775	0.1124	0.1496
		1°C	0.0483	0.0576	0.0245
PS	**pH 5.5**	**10°C**	0.1910	0.1800	0.1669
		1°C	0.0756	0.0495	0.0000
	pH 6.0	**10°C**	0.1382	0.1369	0.1947
		1°C	0.0701	0.0748	0.0351
PP	**pH 5.5**	**10°C**	0.1225	0.1336	0.1015
		1°C	0.0490	0.0677	0.0391
	pH 6.0	**10°C**	0.1463	0.1143	0.1133
		1°C	0.0737	0.0403	0.0746

l.s.d A=0.11382, B=0.09857, C=0.08048

A5.8. The effect of environmental factors on t_{90} of *Lb. sakei* after log transformation

A5.8.1 The effect of each environmental factor alone on t_{90} of *Lb. sakei*

A5.8.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
5.824	6.174	6.609	5.945	6.292

l.s.d A=0.7378, B=0.6389, C=5.217

A5.8.1.2 The effect of pH

pH 5.5	pH 6.0
6.138	6.088

l.s.d.=0.3869

A5.8.1.3 The effect of temperature

10°C	1°C
5.271 [a]	6.955 [b]

l.s.d.=0.3869

A5.8.1.4 The effect of Atmosphere

Air	25% CO_2	100% CO_2
6.227	5.953	6.159

l.s.d=0.4518

A5.8.2 The effect of a combination of two environmental factor on t_{90} of *Lb. sakei*

A5.8.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	5.781	5.868
1% NaCl	6.113	6.234
2% NaCl	6.789	6.428
PS	6.016	5.873
PP	6.305	6.279

l.s.d. A=1.0434, B=0.9036, C=0.7378

A5.8.2.2 The effect of preservative and temperature

	10°C	1°C
Control	5.149	6.499
1% NaCl	5.838	6.509
2% NaCl	5.817	7.401
PS	4.788	7.102
PP	5.321	7.263

l.s.d. A=1.0434, B=0.9036, C=0.7378

A5.8.2.3 The effect of pH and temperature

	10°C	1°C
pH 5.5	5.305	6.972
pH 6.0	5.238	6.937

l.s.d.=0.5217

A5.8.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	5.828	5.739	5.906
1% NaCl	6.423	5.962	6.137
2% NaCl	7.005	5.959	6.862
PS	5.807	5.898	6.128
PP	6.559	6.215	6.100

l.s.d A=1.2779, B=1.1067, C=0.9036

A5.8.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	6.439	5.897	6.079
pH 6.0	6.015	6.010	6.238

l.s.d.=0.6389

A5.8.2.6 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	5.266	5.160	5.388
1°C	7.188	6.747	6.929

l.s.d.=0.6389

A5.8.3 The effect of the combination of three environmental factors on t_{90} of *Lb. sakei*

A5.8.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	4.999	6.563	5.300	6.435
1% NaCl	5.730	6.496	5.946	6.522
2% NaCl	6.035	7.544	5.599	7.258
PS	4.929	7.102	4.646	7.101
PP	5.408	7.201	5.234	7.324

l.s.d. A=1.4756, B=1.2779, C=1.0434

A5.8.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	5.979	5.650	5.714	5.677	5.828	6.068
1% NaCl	6.151	5.974	6.215	6.695	5.949	6.059
2% NaCl	7.737	5.741	6.890	6.274	6.176	6.835
PS	5.842	6.144	6.061	5.772	5.653	6.195
PP	6.992	5.935	5.988	6.127	6.496	6.213

l.s.d A=1.8072, B=1.5651, C=1.2779

A5.8.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	4.936	4.997	5.515	6.720	6.481	6.267
1% NaCl	6.180	5.565	5.770	6.666	6.358	6.504
2% NaCl	6.075	5.667	5.708	7.936	6.250	8.017
PS	4.804	4.794	4.765	6.810	7.003	7.492
PP	5.198	5.231	5.533	7.921	7.200	6.667

l.s.d. A=1.8072, B=1.5651, C=1.2779

A5.8.3.4 The effect of pH, temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
pH 5.5	5.342	5.076	5.495	7.536	6.717	6.662
pH 6.0	5.190	5.243	5.281	6.840	6.777	7.196

l.s.d.=0.9036

A5.8.4 The effect of all four environmental factors on t_{90} of *Lb. sakei*

A5.8.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	25% CO_2	100% CO_2
Control	**pH 5.5**	**10°C**	5.061	4.604	5.331
		1°C	6.897	6.697	6.069
	pH 6.0	**10°C**	4.810	5.391	5.699
		1°C	6.544	6.266	6.497
1% NaCl	**pH 5.5**	**10°C**	5.859+	5.480	5.852
		1°C	6.442	6.469	6.577
	pH 6.0	**10°C**	6.500	5.651	5.689
		1°C	6.890	6.247	6.430
2% NaCl	**pH 5.5**	**10°C**	6.577	5.760	5.767
		1°C	8.897	5.723	8.013
	pH 6.0	**10°C**	5.573	5.575	5.649
		1°C	6.975	6.777	8.020
PS	**pH 5.5**	**10°C**	4.786	4.893	5.109
		1°C	6.899	7.394	7.014
	pH 6.0	**10°C**	4.823	4.695	4.421
		1°C	6.721	6.611	7.970
PP	**pH 5.5**	**10°C**	5.304	5.186	5.733
		1°C	8.679	6.683	6.242
	pH 6.0	**10°C**	5.092	5.275	5.334
		1°C	7.162	7.718	7.091

l.s.d A=2.5557, B=2.2133, C=1.8072

A5.9 The effect of environmental factors on the increase in population density of *Lb. sakei after* log transformation

A5.9.1 The effect of each environmental factor alone on the increase in maximum population density of *Lb. sakei*

A5.9.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
1.1921	1.945	1.948	1.785	1.961

l.s.d. A=0.3442, B=0.2981, C=0.2434

A5.9.1.2 The effect of pH

pH 5.5	pH 6.0
1.850	1.957

l.s.d.=0.1721

A5.9.1.3 The effect of temperature

10°C	1°C
1.916	1.891

l.s.d.=0.1721

A5.9.1.4 The effect of atmosphere

Air	25% CO_2	100% CO_2
1.919	1.951	1.840

l.s.d.=0.2108

A5.9.2 The effect of a combination of two environmental factor the increase in maximum population density of *Lb. sakei*

A5.9.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	1.926	1.916
1% NaCl	1.923	1.967
2% NaCl	1.983	1.913
PS	1.666	1.904
PP	1.855	2.066

l.s.d. A=0.4868, B=0.4216, C=0.3442

A5.9.2.2 The effect of preservative and temperature

	10°C	1°C
Control	1.955	1.887
1% NaCl	1.983	1.906
2% NaCl	1.971	1.925
PS	1.826	1.744
PP	1.905	2.016

l.s.d. A=0.4868, B=0.4216, C=0.3442

A5.9.2.3 The effect of pH and temperature

	10°C	1°C
pH 5.5	1.902	1.798
pH 6.0	1.930	1.983

l.s.d.=0.2434

A5.9.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	1.849	1.992	1.923
1% NaCl	1.917	1.944	1.973
2% NaCl	2.026	1.814	2.004
PS	1.798	1.845	1.712
PP	2.057	2.087	1.738

l.s.d. A=0.5963, B= 0.5164, C=0.5963

A5.9.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	1.985	1.837	1.728
pH 6.0	1.853	2.064	1.953

l.s.d=0.2981

A5.9.2.6 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	1.098	1.924	1.916
1°C	1.930	1.977	1.764

l.s.d.=0.2981

A5.9.3 The effect of the combination of three environmental factors on increase in maximum population density of *Lb. sakei*

A5.9.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	1.975	1.895	1.953	1.879
1% NaCl	1.943	1.902	2.024	1.909
2% NaCl	2.020	1.947	1.923	1.904
PS	1.781	1.551	1.871	1.937
PP	1.888	1.821	1.922	2.211

l.s.d. A=0.6885, B=0.5963, C=0.4868

A5.9.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	1.892	2.005	1.881	1.805	1.979	1.964
1% NaCl	1.880	1.920	1.968	1.954	1.969	1.977
2% NaCl	2.272	1.696	1.982	1.780	1.933	2.027
PS	1.709	1.809	1.480	1.880	1.881	1.943
PP	2.263	1.727	1.574	1.851	2.447	1.901

l.s.d. A=0.8432, B=0.7303, C=0.5963

A5.9.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	1.862	2.014	1.990	1.836	1.969	1.856
1% NaCl	1.966	1.988	1.996	1.868	1.900	1.949
2% NaCl	1.985	1.936	1.992	2.067	1.692	2.017
PS	1.862	1.823	1.793	1.735	1.866	1.631
PP	1.933	1.896	1.887	2.181	2.278	1.588

l.s.d. A=0.8432, B=0.7303, C=0.5963

A5.9.3.4 The effect of pH temperature and atmosphere

	10°C			1°C		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
pH 5.5	1.910	1.898	1.989	2.060	1.776	1.557
pH 6.0	1.906	1.950	1.934	1.800	2.179	1.971

l.s.d.=0.4216

A5.2.4 The effect of all four environmental factors on the increase in maximum population density of *Lb. sakei*

A5.2.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	25% CO_2	100% CO_2
Control	**pH 5.5**	**10°C**	1.901	1.998	1.971
		1°C	1.883	2.011	1.791
	pH 6.0	**10°C**	1.822	2.013	2.008
		1°C	1.789	1.927	1.920
1% NaCl	**pH 5.5**	**10°C**	1.876	1.947	2.006
		1°C	1.883	1.893	1.930
	pH 6.0	**10°C**	2.055	2.030	1.986
		1°C	1.852	1.907	1.969
2% NaCl	**pH 5.5**	**10°C**	2.160	1.884	2.015
		1°C	2.384	1.508	1.949
	pH 6.0	**10°C**	1.811	1.989	1.969
		1°C	1.750	1.877	2.085
PS	**pH 5.5**	**10°C**	1.790	1.794	1.759
		1°C	1.628	1.823	1.201
	pH 6.0	**10°C**	1.933	1.852	1.826
		1°C	1.842	1.909	2.061
PP	**pH 5.5**	**10°C**	1.930	1.885	1.849
		1°C	2.595	1.568	1.299
	pH 6.0	**10°C**	1.935	1.906	1.925
		1°C	1.767	2.988	1.878

l.s.d. A=1.1925, B=1.0327, C=0.8432

A5.10 The effect of environmental factors on μ_{max} of *B. thermosphacta* after square root transformation

A5.10.1 The effect of each environmental factor alone on μ_{max} of *B. thermosphacta*

A5.10.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
0.1632[a]	0.1817[b]	0.1326[c]	*	*

l.s.d. AA=0.01324, B=0.01146, C=0.00936

A5.10.1.2 The effect of pH

pH 5.5	pH 6.0
0.1573	0.1630

l.s.d.=0.00936

A5.10.1.3 The effect of temperature

10°C	1°C
0.2262[a]	0.0941[b]

l.s.d.=0.00936

A5.10.1.4 The effect of atmosphere

Air	25% CO_2	100% CO_2
0.2038[a]	0.1633[b]	0.1143[c]

l.s.d.=0.01146

A5.10.2 The effect of a combination of two environmental factor on μ_{max} of *B. thermosphacta*

A5.10.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	0.1500[a]	0.1763[b]
1% NaCl	0.2062[c]	0.1572[d]
2% NaCl	0.01231[e]	0.1422[f]

l.s.d. A=0.01872, B=0.01621, C=0.01324

A5.10.2.2 The effect of preservative and temperature

	10°C	1°C
Control	0.2247[a]	0.1061[b]
1% NaCl	0.2671[c]	0.0962[d]
2% NaCl	0.1883[e]	0.0770[f]

l.s.d. A=0.01872, B=0.01621, C=0.01324

A5.10.2.3 The effect of pH and temperature

	10°C	1°C
pH 5.5	0.2265	0.0882
pH 6.0	0.2260	0.1000

l.s.d.=0.01324

A5.10.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	0.1929^{a}	0.1619^{b}	0.1347^{c}
1% NaCl	0.2655^{d}	0.1653^{b}	0.1142^{c}
2% NaCl	0.1639^{b}	0.1641^{b}	0.0699^{f}

l.s.d. A=0.02293, B=0.01986, C=0.01621

A5.10.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	0.2159^{a}	0.1624^{b}	0.0938^{c}
pH 6.0	0.1918^{d}	0.1642^{d}	0.1330^{e}

l.s.d.=0.01621

A5.10.2.6 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	0.2285^{a}	0.2194^{b}	0.1707^{c}
1°C	0.1191^{d}	0.1071^{d}	0.0561^{e}

l.s.d.=0.01621

A5.10.3 The effect of the combination of three environmental factors on μ_{max} of *B. thermosphacta*

A5.10.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	0.2110	0.0891	0.2385	0.1141
1% NaCl	0.3068	0.1056	0.2275	0.0896
2% NaCl	0.1771	0.0691	0.1995	0.0849

l.s.d. A=0.02647, B=0.02293, C=0.1872

A5.10.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	0.1809^{a}	0.1572^{b}	0.1120^{c}	0.2050^{d}	0.1666^{b}	0.1573^{e}
1% NaCl	0.3382^{f}	0.1649^{b}	0.1155^{c}	0.1929^{d}	0.1657^{b}	0.1129^{g}
2% NaCl	0.1634^{a}	0.1703^{ab}	0.0355^{h}	0.1644^{a}	0.1579^{a}	0.1043^{i}

l.s.d. A=0.03242, B=0.02802, C0.02293

A5.10.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
Control	0.2553^{a}	0.2175^{b}	0.2013^{b}	0.1306^{c}	0.1062^{c}	0.0680^{d}
1% NaCl	0.4129^{e}	0.2221^{b}	0.1664^{h}	0.1182^{c}	0.1085^{c}	0.0620^{d}
2% NaCl	0.2308^{a}	0.2206^{ab}	0.1135^{f}	0.0971^{g}	0.1076^{h}	0.0262^{i}

l.s.d. A=0.03242, B0.02808, 0.02293

A5.10.3.4 The effect of pH temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
pH 5.5	0.3087	0.2218	0.1489	0.1230	0.1031	0.0386
pH 6.0	0.2684	0.2171	0.1925	0.1152	0.112	0.0735

l.s.d.=0.02293

A5.10.4 The effect of all four environmental factors on μ_{max} of *B. thermosphacta*

A5.10.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	**25% CO_2**	**100% CO_2**
Control	**pH 5.5**	**10°C**	0.2293^{a}	0.2139^{a}	0.1897^{b}
		1°C	0.1325^{c}	0.1009^{c}	0.0344^{d}
	pH 6.0	**10°C**	0.2813^{e}	0.2212^{a}	0.2130^{a}
		1°C	0.1287^{c}	0.1120^{c}	0.1017^{c}
1% NaCl	**pH 5.5**	**10°C**	0.5515^{f}	0.2238^{a}	0.1451^{g}
		1°C	0.1248^{c}	0.1061^{c}	0.0858^{h}
	pH 6.0	**10°C**	0.2742^{i}	0.2205^{a}	0.1877^{b}
		1°C	0.1116^{c}	0.1109^{c}	0.0381^{d}
2% NaCl	**pH 5.5**	**10°C**	0.2247^{a}	0.2355^{a}	0.0710^{j}
		1°C	0.1021^{c}	0.1051^{c}	0.000^{k}
	pH 6.0	**10°C**	0.2368^{a}	0.2057^{a}	0.1561^{b}
		1°C	0.0920^{l}	0.1101^{c}	0.0524^{d}

l.s.d. A=0.04585, B0.03942, =0.3242

A5.11 The effect of environmental factors on t_{90} of *B. thermosphacta* after log_{10} transformation

A5.11.1 The effect of each environmental factor alone on t_{90} of *B. thermosphacta*

A5.11.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
5.223[a]	5.095[a]	5.831[b]	*	*

l.s.d. A=0.2789, B=0.2415, C=0.1972

A5.11.1.2 The effect of pH

pH 5.5	pH 6.0
5.566[a]	5.120[b]

l.s.d.=0.1972

A5.11.1.2 The effect of temperature

10°C	1°C
4.369[a]	6.317[b]

l.s.d.=0.1972

A5.11.1.4 The effect of atmosphere

Air	25% CO_2	100% CO_2
4.865[a]	4.943[a]	6.221[b]

l.s.d.=0.2415

A5.11.2 The effect of a combination of two environmental factor on t_{90} of the total population

A5.11.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	5.529[a]	4.916[b]
1% NaCl	5.033[b]	5.157[b]
2% NaCl	6.171[c]	5.491[d]

l.s.d. A=0.3944, B=0.3416, C=0.2789

A5.11.2.2 The effect of preservative and temperature

	10°C	1°C
Control	4.261	6.185
1% NaCl	4.230	5.960
2% NaCl	4.722	6.940

l.s.d. A=0.3944, B0.3416, C=0.2789

A5.11.2.3 The effect of pH and temperature

	10°C	1°C
pH 5.5	4.457[a]	6.675[b]
pH 6.0	4.280[a]	5.960[c]

l.s.d.=0.2789

A5.11.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	4.792[a]	4.929[a]	5.947[b]
1% NaCl	4.806[a]	4.873[a]	5.606[b]
2% NaCl	5.070[a]	5.039[a]	7.385[c]

l.s.d. A=0.4830, B=0.4183, C=0.3416

A5.11.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	4.922[a]	4.939[a]	6.836[b]
pH 6.0	4.808[a]	4.946[a]	5.606[c]

l.s.d.=0.3416

A5.11.2.1 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	4.048[a]	4.144[a]	4.877[b]
1°C	5.646[c]	5.741[c]	7.565[c]

l.s.d.=0.3416

A5.11.3 The effect of the combination of three environmental factors on t_{90} of *B. thermosphacta*

A5.11.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	4.354	6.704	4.167	5.667
1% NaCl	4.248	5.818	4.213	6.101
2% NaCl	4.870	7.472	4.574	6.409

l.s.d. A=0.5578, B=0.4830, C=0.3944

A5.11.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	25% CO_2	100% CO_2	Air	25% CO_2	100% CO_2
Control	4.883[a]	4.971[a]	6.734[b]	4.701[a]	4.887[a]	5.159[c]
1% NaCl	4.812[a]	4.882[a]	5.405[a]	4.800[a]	4.865[a]	5.806[d]
2% NaCl	5.110[a]	4.933[a]	8.471[e]	5.031[a]	5.145[a]	6.298[d]

l.s.d. A=0.6831, B=0.5916, C=0.4830

A5.11.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
Control	4.064[a]	4.099[a]	4.620[c]	5.520[b]	5.759[b]	7.272[e]
1% NaCl	3.938[a]	3.973[a]	4.780[c]	5.674[b]	5.779[b]	6.431[f]
2% NaCl	4.271[a]	4.405[a]	5.491[d]	5.869[b]	5.673[b]	9.279[g]

l.s.d. A0.6831, B=0.5916, C=0.4830

A5.11.3.4 The effect of pH temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
pH 5.5	4.177[a]	4.090[a]	5.104[b]	5.667[c]	5.788[c]	8.596[d]
pH 6.0	3.992[a]	4.198[a]	4.651[b]	5.625[c]	5.695[c]	6.560[e]

l.s.d.=0.4830

A5.11.4 The effect of all four environmental factors on t_{90} of the total population

A5.11.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	**25% CO_2**	**100% CO_2**
Control	**pH 5.5**	**10°C**	4.223[a]	4.138[a]	4.703[a]
		1°C	5.543[b]	5.804[b]	8.766[c]
	pH 6.0	**10°C**	3.905[a]	4.060[a]	4.537[a]
		1°C	5.497[b]	5.715[b]	5.782[b]
1% NaCl	**pH 5.5**	**10°C**	3.941[a]	3.927[a]	4.877[a]
		1°C	5.684[b]	5.836[b]	5.933[b]
	pH 6.0	**10°C**	3.936[a]	4.091[a]	4.683[a]
		1°C	5.664[b]	5.711[b]	6.930[d]
2% NaCl	**pH 5.5**	**10°C**	4.321[a]	4.157[a]	6.133[a]
		1°C	5.898[b]	5.708[b]	10.809[d]
	pH 6.0	**10°C**	4.221[a]	4.652[a]	4.849[a]
		1°C	5.840[b]	5.638[b]	7.748[f]

l.s.d. A=0.9661, B=0.8366, C=0.6831

7.12 The effect of environmental factors on the increase in maximum population density of *B. thermosphacta*

A5.12.1 The effect of each environmental factor alone on the increase in maximum population density of *B. thermosphacta*

A5.12.1.1 The effect of preservatives

Control	1% NaCl	2% NaCl	PS	PP
18.8	7.5	10.6	*	*

l.s.d. A=36.92, B=31.97, C=26.11

A5.12.1.2 The effect of pH

pH 5.5	pH 6.0
18.7	9.2

l.s.d=26.11

A5.12.1.3 The effect of temperature

10°C	1°C
8.0	19.8

l.s.d.=26.11

A5.12.1.4 The effect of atmosphere

Air	25% CO_2	100% CO_2
8.6	7.5	25.7

l.s.d.=31.97

A5.12.2 The effect of a combination of two environmental factor the increase in maximum population density of *B. thermosphacta*

A5.12.2.1 The effect of preservative and pH

	pH 5.5	pH 6.0
Control	30.1	7.5
1% NaCl	7.6	7.6
2% NaCl	7.0	14.2

l.s.d. A=52.21, B=45.22, C=36.92

A5.12.2.2 The effect of preservative and temperature

	10°C	1°C
Control	8.1	29.5
1% NaCl	8.1	6.9
2% NaCl	7.9	13.3

l.s.d. A=52.21, B=45.22, C=36.92

A5.12.2.3 The effect of pH and temperature

	10°C	1°C
pH 5.5	8.0	29.3
pH 6.0	8.1	10.4

l.s.d.=36.92

A5.12.2.4 The effect of preservative and atmosphere

	Air	25% CO_2	100% CO_2
Control	8.5	7.7	40.2
1% NaCl	8.8	7.6	6.2
2% NaCl	8.5	7.1	16.2

l.s.d. A=63.94, B=55.38, C=45.22

A5.12.2.5 The effect of pH and atmosphere

	Air	25% CO_2	100% CO_2
pH 5.5	8.6	7.4	40.0
pH 6.0	8.6	7.7	11.4

l.s.d.=45.22

A5.12.2.1 The effect of temperature and atmosphere

	Air	25% CO_2	100% CO_2
10°C	8.8	7.6	7.7
1°C	8.4	7.4	43.7

l.s.d.=45.22

A5.12.3 The effect of the combination of three environmental factors on increase in maximum population density of *B. thermosphacta*

A5.12.3.1 The effect of preservative, pH and temperature

	pH 5.5		pH 6.0	
	10°C	1°C	10°C	1°C
Control	8.1	52.1	8.1	6.9
1% NaCl	8.1	6.7	8.1	7.1
2% NaCl	7.8	6.1	7.9	20.6

l.s.d. A=73.84, B=63.94, C=52.21

A5.12.3.2 The effect of preservative, pH and atmosphere

	pH 5.5			pH 6.0		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
Control	8.5	7.6	74.3	8.5	7.9	6.2
1% NaCl	8.7	7.5	6.0	8.8	7.7	6.4
2% NaCl	8.5	6.9	5.4	8.5	7.3	27.0

l.s.d. A=90.43, B=78.32, C=63.94

A5.12.3.3 The effect of preservative, temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
Control	8.6	7.9	7.8	8.3	7.6	772.7
1% NaCl	9.0	7.6	7.8	8.6	7.5	7.4.6
2% NaCl	8.8	7.1	7.6	8.2	7.0	24.8

l.s.d. A=90.43, B=78.32, C=63.94

A5.12.3.4 The effect of pH temperature and atmosphere

	10°C			1°C		
	Air	**25% CO_2**	**100% CO_2**	**Air**	**25% CO_2**	**100% CO_2**
pH 5.5	8.8	7.5	7.8	8.3	7.3	72.2
pH 6.0	8.7	7.8	7.7	8.4	7.6	15.2

l.s.d.=63.94

A5.12.4 The effect of all four environmental factors on the increase in maximum population density of the total population

A5.12.4.1 The effect of preservative, pH, temperature and atmosphere

			Air	**25% CO_2**	**100% CO_2**
Control	**pH 5.5**	**10°C**	8.8	7.8	7.8
		1°C	8.3	7.4	140.8
	pH 6.0	**10°C**	8.5	7.9	7.8
		1°C	8.4	7.8	4.5
1% NaCl	**pH 5.5**	**10°C**	9.0	7.5	7.9
		1°C	8.5	7.6	4.0
	pH 6.0	**10°C**	8.9	7.8	7.6
		1°C	8.7	7.5	5.2
2% NaCl	**pH 5.5**	**10°C**	8.7	6.9	7.8
		1°C	8.4	6.9	3.0
	pH 6.0	**10°C**	8.9	7.4	7.4
		1°C	8.1	7.1	46.5

l.s.d. A=127.89, B=110.75, C=90.43

References

Adams, M.R., Little, C.L. and Easter, M.C. (1991). Modeling the effect of pH, acidulant and temperature on the growth rate of *Yersinia enterocolitica*. *Journal of Applied Bacteriology.* **71**: 65-71

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

Ahn, C. and Stiles, M.E. (1990b). Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. *Applied and Environmental Microbiology.* **56**: 2503-2510

Ahn, C. and Stiles, M.E. (1992). Mobilization and expression of bacteriocin plasmids for *Carnobacterium piscicola* isolated from meat. *Journal of Applied Bacteriology.* **73**: 217-228

Akagawa, M. and Yamasoto, K. (1989). Synonymy of *Alcaligenes aqamarinus, Alcaligenes faecalis* subsp. *homari,* and *Deleya aesta*: *Deleya aquarmarina* comb. nov. as the type species of the genus *Deleya. International Journal of Systematic Bacteriology.* **39**: 462-466

Akopyanz, N.S., Eaton, K.A. and Berg, D.E. (1995). Adaptive mutation and cocolonization during *Helicobacter pylori* infection of gnotobiotic piglets. *Infection and Immunity.* **63**: 116-121

Åkerman, B., Jonsson, M. and Norden, B. (1985). Electrophoretic orientation of DNA detected by linear dichromism spectroscopy. *Journal of the Chemical Society-Chemical Communication.* **7**: 422-423

Alford, J.A., Pierce, D.A. and Suggs, F.G. (1964). Activities of microbial lipases of natural fats and synthetic triglycerides. *Journal of Lipid Research.* **5**: 390-394

Anand, S.K., Mahapatra, C.M., Pandey, N.K. and Verma, S.S. (1989). Microbial changes on chicken carcasses during processing. *Indian Journal of Poultry Science.* 24: 203-209

Anderson, D.J., Kuhns, J.S., Vasil, M.L., Gerding, D.N. and Janoff, E.N. (1991). DNA fingerprinting by pulse field electrophoresis and ribotyping to distinguish *Pseudomonas cepacia* isolates from nosocomal outbreak. *Journal of Clinical Microbiology.* **29**: 648-649

Anderson, M.E., Huff, H.E., Naumann, H.D. and Marshall, R.T. (1988). Counts of six types of bacteria on lamb carcasses dipped of sprayed with acetic acid at 25°C or 55°C and stored vacuum packaged. *Journal of Food Protection.* **51**: 874-877

Anderson, M.E., Huff, H.E., Naumann, H.D., Marshall, R.T., Damare, J., Johnson, R. and Pratt, M. (1987a). Evaluation of swab and tissue excision methods for recovering microorganisms from washed and sanitized beef carcasses. *Journal of Food Protection.* **50**: 741-743

Anderson, M.E., Huff, H.E., Naumann, H.D., Marshall, R.T., Damare, J.M., Pratt, M. and Johnston, R. (1987b). Evaluation of an automated beef carcass washing and sanitizing system under production conditions. *Journal of Food Protection.* **50**: 562-566

Anderson, M.E. and Marshall, R.T. (1989). Interaction of concentration and temperature of acetic acid solution on reduction of various species of microorganisms on beef surfaces. *Journal of Food Protection.* **52**: 312-315

Anderson, M.E. and Marshall, R.T. (1990). Reducing microbial populations on beef tissue: concentration and temperature of an acid mixture. *Journal of Food Science.* **55**: 903-905, 1990.

Anonymous (1993). Quality assurance manual. No Name Pty. Ltd.

Anonymous (1995). University of Melbourne technical manual. Melbourne University, Australia.

Asensio, M.A., Ordonez, J.A. and Sanz, B. (1988). Effect of carbon dioxide and oxygen enriched atmosphere on the shelf-life of refrigerated pork packaged in plastic bags. *Journal of Food Protection.* **51**: 356-360

Ayers, J.C. (1955) Microbial implications in the handling slaughter and dressing of meat animals. *Advances in Food Research.* **6**: 109-161

Bailey, J.S., Reagan, J.O., Carpenter, J.A. and Schuler, G.A. (1979a). Microbiological condition of broilers as influenced by vacuum and carbon dioxide in bulk shipping packs. *Journal of Food Science.* **44**: 134-136

Bailey, J.S., Reagan, J.O., Carpenter, J.A., Schuler, G.A. and Thompson, J.E. (1979b). Types of bacteria and shelf-life of evacuated carbon dioxide injected and ice packed broilers. *Journal of Food Protection.* **42**: 218-221

Baird-Parker, A.C. and Kirkby, D.C. (1984). Principles of predictive food microbiology. *Journal of Applied Bacteriology Supplement.* **64**: 43S-49S

Baird-Parker, A.C., Kirkby, D.C. and Broughall, J.M. (1985). The use of the computer to aid decision making by food microbiologists. *In*: Rapid methods and automation in microbiology and immunology. *Ed* K.-O. Habermehl. Springer-Verlag, Berlin. **pp** 570-579

Baker, R.C., Qureshi, R.A. and Hotchkiss, J.C. (1986). Effect of elevated level of carbon dioxide containing atmosphere on the growth of spoilage and pathogenic bacteria at 2, 7, and 13°C. *Poultry Science.* **65**: 729-737

Baldock, J.D. (1974). Microbiological moitoring of the food plant: methods to assess bacterial contamination of surfaces. *Journal of Milk Food Technology.* **37**: 361-368

Baranowski, J.D. and Nagel, C.W. (1982). Inhibition of *Pseudomonas fluorescens* by hydroxycinnimanic acids and their alkyl esters. *Journal of Food Science.* **47**: 1587-1589

Baranyi, J. and Roberts, T.A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology.* **23**: 277-294

Baranyi, J., Jones, A., Walker, C., Kaloti, A., Robinson, T.P. and Mackey, B.M. (1996). A combined model for growth and subsequent thermal inactivation of *Brochothrix thermosphacta. Applied and Environmental Microbiology.* **62**: 1029-1035

Baranyi, J., Robinson, T.P., Kaloti, A. and Mackey, B.M. (1995). Predicting growth of *Brochothrix thermosphacta* at changing temperature. *International Journal of Food Microbiology.* **27**: 61-75

Bargiota, E. Rico-Munzo, E. and Davidson, P.M. (1987). Lethal effect of methyl and propyl-paraben as related to *Staphylococcus aureus* lipid composition. *International Journal of Food Microbiology.* **4**: 257-266

Barlow, J. and Kitchell, A.G. (1966). A note on the spoilage of prepackaged lamb chops by *Microbacterium thermosphactum. Journal of Applied Bacteriology.* **29**: 185-188

Barnes, E.M. and Melton, W. (1971). Extracellular enzymic activity of poultry spoilage bacteria. *Journal of Applied Bacteriology.* **34**: 599-609

Barnes, E.M. and Shrimpton, D.H. (1958). The effect of the tetracycline compound on the storage life and microbiology of chilled eviscerated poultry. *Journal of Applied Bacteriology.* **2**:314-329

Barnes, E.M. and Thornley, M.J. (1966). The spoilage flora of eviscerated chicken stored at different temperatures. *Journal of Food Technology.* **1**: 113-119

Barnhart, H.M., Pancorbo, O.C., Dresden, D.W. and Shotts, E.B. (1989). Recovery of *Aeromonas hydrophilia* from carcasses and processing in water in a broiler processing operation. *Journal of Food Protection.* **52**: 649-649

Barrett, J.H., Lior, H., Green, J.H., Khakhria, R., Wells, J.G., Bell, B.P., Greene, K.D., Lewis, J. and Griffin, P.M. (1994). Laboratory investigation of a multi-state foodborne outbreak of *Escherichia coli* 0157:H7. *Journal of Clinical Microbiology.* **32**: 3013-3017

Barrow, G.I. and Feltham, R.K.A. (1993). Cowan and Steel's manual for the identification of medical bacteria. 3rd Edition. Cambridge University Press.

Barry, T., Powell, R. and Gannon, F. (1990). A general method to generate DNA probes for microorganisms. *Biotechnology.* **8**: 233-236

Bej, A.K., Mahbubani, M.H. and Atlas, R.M. (1991). Detection of viable *Legionella pneumophila* in water by polymerase chain reaction. *Applied and Environmental Microbiology.* **57**: 597-600

Bell, R.G. (1997). Distribution and sources of microbial contamination on beef carcasses. *Journal of Applied Microbiology.* **82**: 292-300

Bell, R.G., Harrison, J.C.L. and Rogers, A.R. (1993). Preliminary investigation of the distribution of microbial contamination on lamb and beef carcasses. *Publication, Meat Industry Research Institute of New Zealand.* **927**: 20 (Abstract)

Bell, R.G., Harrison, J.C.L., Rogers, A.R. and le Roux, G.J. (1996a). Distribution and sources of microorganisms on beef carcasses. *Publications,-Meat Industry Research Institute of New Zealand.* **No.963**: 19 (Abstract)

Bell, R.G., Penney, N., Gilbert, K.V., Moorhead, S.M. and Scott, S.M. (1996b). The chilled storage life and retail display performance of vacuum and carbon dioxide packaged hot deboned beef striploins. *Meat Science.* **42**: 371-386

Bell, R.G., Penney, N. and Moorhead, S.M. (1996c). The retail display life of steaks prepared from chill stored chilled vacuum and carbon dioxide-packaged sub-primal beef cuts. *Meat Science.* **42**:165-178

Bell, R.G., Penney, N. and Moorhead, S.M. (1997). Comparison of aerobic and anaerobic methods for the microbiological monitoring of chilled packaged meat during storage. *Letters in Applied Microbiology.* **24**: 265-268

Bendinger, B., Kroppenstedt, R.M., Klatte, S. and Alterndorf, K. (1992). Chemotaxomonic differentiation of coryneform bacteria isolated from biofilters. *International Journal of Systematic Bacteriology.* **42**: 474-486

Bennik, M.H., Smid, E.H., Rombouts, F.M. and Gorris, L.G.M. (1995). Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen. *Food Microbiology.* **12**: 509-519

Bentley, D.S., Reagan, J.O., Cox, N.A. and Bailey, J.S. (1987). Effects of meat type, storage time and temperature on various physical, chemical and microbiological characteristics of ground pork. *Journal of Food Protection.* **59**: 948-951

Bercovier, H. and Mollaret, H.H. (1986). Genus XIV. *Yersinia* van Loghem 1944 15^{AL}. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson, Baltimore London Los Angeles Sydney. **pp** 498-506

Bergen, T (1981). Human- and amimal-pathogenic members of the genus *Pseudomonas. In*: The Prokaryotes Volume I. *Eds*: M.P. Starr, H. Stlop, H.G. Trüper, A. Balows and H.G. Schlegel. Spinger-Verlag, Berlin Heidelberg New York. **pp** 666-700

Best, G.R. and Britz, M.L.. (1986). Facilitated protoplasting in certain auxotrophic mutants of *Corynebacterium glutamicum. Applied Microbiology and Biotechnology* **23**: 288-293

Betts, R., Stringer, M., Banks, J. and Dennis, C. (1995). Molecular methods in food microbiology. *Food Australia.* **47**: 319-322

Beyazova, M.L., Brodsky, B.C., Shearer, M.C. and Horan, A.C. (1995). Preparation of acitonmycete DNA for pulsed-field gel electrophoresis. *International Journal of Systematic Bacteriology.* **45**: 852-854

Bhaduri, S., Buchanan, R.L. and Phillips, J.G. (1995). Expanded response surface model for predicting the effect of temperature, pH, sodium chloride content and sodium nitrite concentrations on the growth rate of *Yersinia enterocolitica. Journal of Applied Bacteriology.* **79**: 163-170

Birch, A., Häusler, A. and Hunter, R. (1990). Genome rearrangement and genetic instability in *Streptomyces* spp. *Journal of Bacteriology.* **172**: 4138-4142

Biss, M.E. and Hathaway, S.C. (1996). Effect of pre-slaughter washing of lambs on the microbiological and visible contamination of the carcasses. *Veterinary Record.* **138**: 82-86

Björkroth, K.J., Ridell, J. and Korkeala, H. (1996). Characterization of *Lactobacillus sake* strains associating with production of ropy slime by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) patterns. *International Journal of Food Microbiology.* **31**: 59-68

Blanc, D.S., Siegrist, H.H., Sahli, R. and Francioli, P. (1993). Ribotyping of *Pseudomonas aeruginosa*: discriminatory power and usefulness as a tool for epidemiological studies. *Journal of Clinical Microbiology.* **31**: 71-77

Blickstad, E. (1984). The effect of water activity on the growth and end-product formation of two *Lactobacillus* spp. and *Brochothrix thermosphacta* ATCC 11509^{T}. *Applied and Microbiology Biotechnology* **19**: 13-17

Blickstad, E. and Molin, G. (1983a). Carbon dioxide as a controller of the spoilage flora of pork, with special reference to temperature and sodium chloride. *Journal of Food Protection.* **46**: 756-763

Blickstad, E. and Molin, G. (1983b) The microbial flora of smoked pork loin and frankfurter sausage stored in different gas atmosphere at 4°C. *Journal of Applied Bacteriology.* **54**: 45-56

Bochner, B.R. (1989a). Sleuthing out bacterial identities. *Nature.* **339**: 157-158

Bochner, B.R. (1989b). "Breathprints" at the microbial level. *ASM News.* **55**:536-539

Boddy, L. and Wimpenny, J.T.W. (1992). Ecology concepts in food microbiology. *Journal of Applied Bacteriology Supplement.* **73**: 23S-38S

Böhm, H and Karch, H. (1992). DNA fingerprinting of *Escherichia coli* 0157:H7 by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology.* **30**: 2169-2172

Booth, I.R. (1985). Regulation of cytoplasmic pH in bacteria. *Microbiological Reviews.* **49**: 359-378

Borch, E. and Agerhem, H. (1992). Chemical, microbial and sensory changes during the anaerobic cold storage of beef inoculated with a homofermentative *Lactobacillus* sp. or a *Leuconostocs* sp. *International Journal of Food Microbiology.* **15**: 99-108

Borch, E. and Molin, G. (1988). Numerical taxonomy of psychrotrophic lactic acid from pre-packaged meat and meat products. *Antonie van Leeuwenhoek.* **54**: 301-323

Borch, E. and Molin, G. (1989). The aerobic growth and end-product formation of *Lactobacillus*, *Leuconostoc*, *Brochothrix* and *Carnobacterium* in batch culture. *Applied Microbiology and Biotechnology.* **30**: 81-88

Border, P.H., Howard, J.J., Plastow, G.S. and Siggen, K.W. (1990). Detection of *Listeria* species and *Listeria monocytogenes* using polymerase chain reaction. *Letters in Applied Microbiology.* **11**: 158-162

Botha, S.J. and Holzapfel, W.H. (1987). Resistance of *Sporolactobacillus* to potassium sorbate and sodium nitrite. *International Journal of Food Microbiology.* **5**: 331-336

Bourgault, A.-M. and Lamothe, F. (1988). Evalutation of the KOH test and antibiotic disk test in routine clinical anaerobic bacteriology. *Journal of Clinical Microbiology.* **26**: 2144-2146

Bousfield, I.J., Keddie, D.E., Dando, T.R. and Shaw, S. (1985). Simple rapid methods of cell wall analysis as an aid in the identification of aerobic coryneform bacteria. *In*: Chemical methods in bacterial systematics. *Eds* M. Goodfellow and D.E. Minnikin. Academic Press, London Orlando San Diego new York Toronto Montreal Sydney Tokyo. **pp** 221-236

Boutrou, R., Thuault, D. and Bourgeois, C.M. (1995). Identification and characterization of *Streptococcus thermophilus* strains by pulsed-field gel electrophoresis. *Journal of Applied Bacteriology.* **79**: 454-458

Bouvet, A., Grimont, F. and Grimont, P.A.D. (1991). Intraspecies variation in nutritionally variant streptococci: rRNA gene restriction patterns of *Streptococcus defectivus* and *Streptococcus adjacens.* *International Journal of Systematic Bacteriology.* **41**: 483-486

Bratchell, N., Gibson, A.M., Truman, M., Kelly, T.M. and Roberts, T.A. (1989). Predicting microbial growth: the consequence of the quantity of data. *International Journal of Food Microbiology.* **8**: 47-58

Brenner, D.J. (1981). Introduction to the family *Enterobacteriaceae*. *In*: The Prokaryotes Volume II. *Eds*: M.P. Starr, H. Stlop, H.G. Trüper, A. Balows and H.G. Schlegel. Spinger-Verlag, Berlin Heidelberg New York. **pp** 1103-1127

Brenner, D.J. (1986). Section 5 Facultative anaerobic Gram-negative rods. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson, Baltimore London Los Angeles Sydney. **pp** 408-420

Brian, M.J., Frosolono, M., Murry, B.E., Miranda, A., Lopez, E.L., Gomez, H.F. and Cleary, T.G. (1992). Polymerase chain reaction for diagnosis of enterohemoragic *Escherichia coli* infection and hemolytic-uremic syndrome. *Journal of Clinical Microbiology.* **30**:1801-1806

Brock, T.D. and Madigan, M.T. (1988). Chapter 9. Growth and its control. *In*: Biology of Microorganisms. 5th Edition. Prentice Hall International Inc. **pp** 315-356

Brocklehurst, T.F. and Lund, B.M. (1988). The effects of pH on the initiation of growth of cottage cheese spoilage bacteria. *International Journal of Food Microbiology.* **6**: 43-49

Brocklehurst, T.F. and Lund, B.M. (1990). The influence of pH, temperature and organic acids on the initiation of growth of *Yersinia enterocolitica*. *Journal of Applied Bacteriology.* **69**: 390-397

Broeze, R.J., Solomon, C.J. and Pope, D.H. (1978). Effects of low temperature on in vivo and in vitro protein synthesis in *Escherichia coli* and *Pseudomonas fluorescens*. *Journal of Bacteriology.* **134**: 861-874

Brondz, I. and Olson, I. (1991). Multivariate analysis of cellular fatty acid in *Bacteroides, Prevotella, Porphyromas, Wolinella* and *Campylobacter* spp. *Journal of Clinical Microbiology.* **29**: 183-189

Brooks, J.L., Moore, A.S., Patchett, R.A., Collins, M.D. and Kroll, R.G. (1992). Use of the polymerase chain reaction and oligonucleotide probes for the rapid detection and identification of *Carnobacterium* species from meat. *Journal of Applied Bacteriology.* **72**: 294-301

Brosch, R., Chen, J. and Luchansky, J.B. (1994). Pulsed-field fingerprinting of Listeriae: identification of genomic divisions for *Listeria monocytogenes* and their correlation with serovar. *Applied and Environmental Microbiology.* **60**: 2584-2592

Broughall, J.M., Anslow, P. and Kilsby, D.C. (1983). Hazard analysis applied to microbial growth in foods: development of mathematical models describing the effect of water activity. *Journal of Applied Bacteriology.* **55**: 101-110

Brownlie, L.E. (1966). Effect of some environmental factors on psyc' microbacteria. *Journal of Applied Bacteriology.* **29**: 447-454.

Brune, H.E. and Cunnignham, F.E. (1971). A review of microbiological aspects of poultry processing. *World's Poultry Science Journal.* **27**: 233-240

Bryan, F.L. (1980). Poultry and poultry meat products. *In*: Microbial Ecology of Foods Volume 2. *Eds* J.H. Silliker, R.P. Elliott, A.C. Baird-Parker, F.L. Bryan, J.H.B. Christian, D.S. Clark, J.C. Olson Jr. and T.A. Roberts. Academic Press, New York. **pp**. 410-458

Buchanan, R.L. (1991). Using spreadsheet software for predictive microbiology applications. *Journal of Food Science.* **11**: 123-134

Buchanan, R.L. and Klatwitter, L.A. (1991). Effects of temperature history on the growth of *Listeria monocytogenes* Scott A at refrigeration temperatures. *International Journal of Food Microbiology.* **12**: 235-246

Buchanan, R.L. and Phillips, J.G. (1990). Response surface model for predicting the effect of temperature pH, sodium chloride content, sodium nitrite concentration and atmosphere on growth of *Listeria monocytogenes. Journal of Food Protection.* **53**: 370-376

Buchanan, R.L., Stahl, H.G. and Whiting, R.C. (1989). Effects and interactions of temperature, pH, atmosphere, sodium chloride and sodium nitrite on the growth of *Listeria monocytogenes. Journal of Food Protection.* **52**: 844-851

Buchrieser, C., Weagant, S.D. and Kaspar, C.W. (1994). Molecular characterization of *Yersinia enterocolitica* by pulsed-field gel electrophoresis and hybridization of DNA to *ail* and pYV probes. *Applied and Environmental Microbiology.* **60**: 4371-4379

Busch, S.V. and Donnelly, C.W. (1992). Development of a repair enrichment broth for resusciation of heat-injured *Listeria monocytogenes* and *Listeria innocula. Applied and Environmental Microbiology.* **58**: 14-20

Busse, H.-J., Denner, E.B.M. and Lubitz, W. (1996). Classification and identification of bacteria: current approaches to an old problem. Overview of method used in bacteria systematics. *Journal of Biotechnology.* **47**: 3-38

Busta, F.F. (1976). Practical implications of injured microorganisms in food. *Journal of Milk Food and Technology.* **39**: 138-145

Butler, J.L., Stewart, J.C., Vanderzant, C., Carpenter, Z.L. and Smith, G.C. (1979). Attachment of microorganisms to pork skin and surfaces of beef and lamb carcasses. *Journal of Food Protection.* **42**: 401-406

Calhoun, C.L. and Frazier, W.C. (1962). Effect of available water on thermal resistance of three non-sporeforming species of bacteria. *Applied Microbiology* **14**: 416-420

Campbell, R.J., Egan, A.F., Grau, F.H. and Shay, B.J. (1979). The growth of *Microbacterium thermosphactum* on beef. *Journal of Applied Bacteriology.* **47**: 505-509

Cancilla, M.R., Powell, I.B., Hillier, A.J. and Davidson, B.E. (1992). Rapid genomic finger printing of *Lactococcus lactis* strains by arbitrarily primed polymerase chain reaction with ^{32}P and fluorescent labels. *Applied and Environmental Microbiology.* **58**: 1772-1775

Cantor, C.R., Gaal, A. and Smith, C.L. (1988). High-resolution separation and accurate size determination in pulsed-field gel electrophoresis of DNA. 3. Effect of electrical field shape. *Biochemistry.* **27**: 9216-9221

Carles-Nurit, M.J., Christophile, B., Broche, S, Gouby, A., Bouziges, N. and Ramuz, M. (1992). DNA polymorphisms in methicillin-susceptible and methicillin-resistant strains of *Staphylococcus aureus.* *Journal of Clinical Microbiology.* **30**: 2092-2096

Carse, W.A. and Locker, R.H. (1974). A survey of pH values at the surface of beef and lamb carcasses, stored in a chiller. *Journal of Food Science and Agriculture.* **25**: 1529-1535

Casla, D., Requena, T. and Gómez, R. (1996). Antimicrobial activity of lactic acid bacteria isolated from goat's milk and artisanal cheese: characteristics of a bacteriocin produced by *Lactobacillus curvatus* IFPL 105. *Journal of Applied Bacteriology.* **81**: 35-41

Chai, T.J. (1981). Usefulness of electrophoresis patterns of cell envelope protein as a taxonomic tool for fishhold slime *Moraxella* species. *Applied and Environmental Microbiology.* **42**: 351-356

Chandler, R.E. and McMeekin, T.A. (1989). Modeling the growth response of *Staphylococcu xylosus* to changes in temperature and glycerole concentation/water activity. *Journal of Applied Bacteriology.* **66**: 543-548

Chandrasekaran, M., Lakshmanaperumalsamy, P. and Chandramohan, D. (1991). Combined effect of environmental factors on spoilage bacteria. *Fishery Technology.* **28**: 146-153

Chassy, B.M., Gibson, E. and Giuffrida, A. (1987). Evidence for plamid-associated lactose metabolismin *Lactobacillus casei. Current Microbiology.* **1**: 141-144

Chetoui, H., Melin, P., Struenlens, M.J., Delhalle, E., Mutro Nigo, M., De Ryck, R. and De Mol, P. (1997). Comparison of biotyping, ribotyping, and pulsed-field gel electrophoresis for investigation of a common-source outbreak if *Burkholderia picketti* bacteremia. *Journal of Clinical Microbiology.* **35**: 1398-1403

Chevallier, B., Hubert, J.-C. and Krammer, B. (1994). Determination of chromosome size and number of *rrn* loci in *Lactobacillus plantarum* by pulse field electrophoresis. *FEMS Microbiology Letters.* **120**: 51-56

Childers, A.B., Keahey, E.E. and Vincent, P.G. (1973). Sources of salmonellae contamination of meat following approved livestock slaughtering procedures. II. *Journal of Milk Food and Technology.* **36**: 365-638

Chipley, J.R. (1983). Sodium benzoate and benzoic acid. *In*: Antimicrobials in foods. *Eds* A.L. Brannen and P.M. Davidson. Marcel Dekker, New York. **pp** 11-35

Christian, J.H.B. (1980). Reducing water activity. *In*: Microbial Ecology of Foods Volume 1. *Eds* J.H. Silliker, R.P. Elliott, A.C. Baird-Parker, F.L. Bryan, J.H.B. Christian, D.S. Clark, J.C. Olson Jr. and T.A. Roberts. Academic Press, New York London Toronto Sydney San Francisco. **pp**. 70-91

Christopher, F.M., Carpenter, Z.L., Dill, C.W., Smith, G.C. and Vanderzant, C.V.(1980). Microbiology of beef, pork and lamb stored in vacuum or modified gas atmospheres. *Journal of Food Protection.* **43**: 259-264

Chrystall, B.B and Devine, C.E. (1978). Electrical stimulation, muscle tendons and glycloysis in bovine *Sternomandibularis. Meat Science.* **2**: 49-58

Chu, G.F., Frank, M. and Olson, M.V. (1986). Separation of large DNA molecules by contour clamped homogeneous electrical field. *Science.* **234**: 1582-1585

Chung, Y.-M. and Lee, J.S. (1982). Potassium sorbate inhibition of microorganisms isolated from seafood. *Journal of Food Protection* **45**:1310-1313

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 Technology Journal.* **2**: 72-75

Clark, D.S. and Lentz, C.P. (1972). Use of carbon dioxide for extending the shelf-life of prepackaged beef. *Canadian Institute of Food Technology Journal.* **5**: 175-178

Clark, D.S. and Takács, J. (1980). Gases as preservatives. *In*: Microbial ecology of foods *Eds* J.H. Silliker, R.P. Elliott, A.C. Baird-Parker, F.L. Bryan, J.H.B. Christian, D.S. Clark, D.S., J.C.J. Olson, and T.A. Roberts. Academic Press, New York London Toronto Sydney San Francisco. **pp**. 170-192

Claus, J.R., Kropf, D.H., Hunt, M.C., Kastner, C.L. and Dikeman, M.G. (1985). Effects of beef carcass electrical stimulation and hot boning on colour of unfrozen vacuum packaged steaks. *Journal of Food Science.* **50**: 881-883

Clemons, K.V., Feroze, F., Holmberg, K. and Stevens, D.A. (1997). Comparative analysis of genetic variability among *Candida albicans* isolation from different geographic locales by three genotypic methods. *Journal of Clinical Microbiology.* **35**: 1332-1336

Coates, K.J., Beattie, J.C., Morgan, I.R. and Widders, P.R. (1995). The contribution of carcass contamination and the boning process to microbial spoilage of aerobically stored pork. *Food Microbiology.* **12**: 49-54

Cocconcelli, P.S. Triban, E., Basso, M. and Bottazzi, V. (1991). Use of DNA probes in the study of silage colonization by *Lactobacillus* and *Pediococcus* strains. *Journal of Applied Bacteriology.* **71**: 296-301

Cole, M.B., Jones, M.V. and Holyoak, C. (1990). The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes. Journal of Applied Bacteriology.* **69**: 63-72

Collins, M.D., Farrow, J.A.E., Phillips, B.A., Ferusu, S. and Jones, D. (1987). Classification of *Lactobacillus divergens, Lactobacillus piscicola,* and catalase negative, asporogenous rod-shaped bacteria for poultry in a new genus, *Carnobacterium. International Journal of Systematic Bacteriology.* **37**: 310-316

Collins, M.D., Wallbanks, S., Lane, D.J., Shah, J., Nietupski, R., Smida, J., Dorsch, M. and Stackebrandt, E. (1991). Phylogenetic analysis of the genus *Listeria* based of reverse transcriptase sequence of 16S rRNA. *International Journal of Systematic Bacteriology.* **41**: 240-246

Collins-Thompson, D.L., Slade, P.J. and Goethals, M. (1991). Use of low molecular mass RNA profiles to identify lactic acid bacteria and related organisms associated with food. *International Journal of Food Microbiology.* **14**: 135-144

Collins-Thompson, D.L., Sørhaug, T., Witter, L.D., and Ordal, Z.J. (1972). Taxonomic condsiderations of *Microbacterium lactum, Microbacterium flavum* and *Microbacterium thermosphactum. International Journal of Systematic Bacteriology.* **22**: 65-72

Corlett Jr., D. A. and M. H. Brown, M.A. (1980). Chapter 5 pH and Acidity. *In*: Microbial Ecology of Foods. Volume 1. *Eds*: J. H. Silliker, R. P. Elliot, A. C. Baird-Parker, F.L. Bryan, J. H. B. Christian, D. S. Clark, J. C. Olsen Jr., T. A. Roberts. Academic Press, New York.. **pp** 38-69

Cowan, S.T. and Steele, K.L. (1964). Comparison of differentiating criteria for staphylococci and micrococci. *Journal of Bacteriology.* **88**: 804-805

Cox, N.A., McHan, F. and Fung, D.Y. (1977). Commercially available minikits for identification of *Enterobacteriaceae*: a review. *Journal of Food Protection.* **40**: 866-872

Coyne, F.P. (1933). The effects of carbon dioxide on bacterial growth. *Proceedings of the Royal Society (London) Series B.* **113**: 196-217

Craven, H.M. and MaCauley, B.J. (1992). Microorganisms in pasteurised milk after refrigerated storage 1. Identification of types. *The Australian Journal of Dairy Technology.* **47**: 38-45

Crété, N., Delabar, J.M., Sinet, P.M. and Creau-Goldberg, N. (1991). Accurate evaluation of the sizes of DNA fragments (from 30 to 4700 kb) in pulse field gel electrophoresis. *Biotechniques.* **11**: 711-717

Cudjoe, K.S. (1988). The effects of lactic acid sprays on the keeping quality of meats during storage. *International Journal of Food Microbiology.* **7**: 1-7

Curtis, G.D.W., Nichols, W.W. and Falla, T.J. (1989). Selective agents for *Listeria* can inhibit their growth. *Letters in Applied Microbiology.* **8**: 169-172

Cunningham, F.E. (1979). Shelf-life and quality characteristics of poultry parts dipped in potassium sorbate. *Journal of Food Science* **44**: 863-864

Cunningham, F.E. (1981). Microbiology of poultry parts dipped in potassium sorbate. *Poultry Science.* **60**: 969-971

Cunningham, F.E. (1982). Microbiological aspects of poultry and poultry products-an update. *Journal of Food Protection.* **45**: 1149-1164

Cutter, C.A. and Siragusa, G.N. (1996). Reductions of *Listeria innocua* and *Brochothrix thermosphacta* on beef following nisin spray treatment and vacuum packaging. *Food Microbiology.* **13**: 229-235

Czajka, J., Bsat, N., Piani, M., Russ, W., Sultana, K., Wiedmann, M. Whitaker, R. and Batt, C.A. (1993). Differentiation of *Listeria monocytogenes* and *Listeria innocua* by 16S rRNA genes and intraspecific discrimination of *Listeria monocytogenes* strains by randomly amplified polymorphic DNA polymorphisms. *Applied and Environmental Microbiology.* **59**: 304-308

Dainty, R.H. and Hibbard, C.M. (1980). Aerobic metabolism of *Brochothrix thermosphacta* growing on meat surfaces and in laboratory media. *Journal of Applied Bacteriology.* **48**: 387-396

Daniel, P. (1995). Sizing of the *Lactobacillus plantarum* genome and other lactic acid bacteria species by transverse alternating field electrophoresis. *Current Microbiology.* **30**: 243-246

Daniels, J.A., Krishnamurthi, R. and Rizvi, S.S.H. (1985). A review of the effects of carbon dioxide on microbial growth and food quality. *Journal of Food Protection.* **48**: 532-537

Datta, A.R. and Benjaman, M.M. (1997). Factors controlling acid tolerance of *Listeria monocytogenes*: effect of nisin and other ionophores. *Applied and Environmental Microbiology.* **63**: 4123-4126.

Davey, C.L., Kuttle, H. and Gilbert, K.V. (1967). Shortening as a factor in meat aging. *Journal of Food Technology.* **2**: 53-56

Davey, K.R. (1989). A predictive model for combined temperature and water activity on microbial growth during the growth phase. *Journal of Applied Bacteriology.* **67**: 483-488

Davey, K.R. (1994). Modeling the combined effect of temperature and pH on the growth rate coefficient for bacterial growth. *International Journal of Food Microbiology.* **23**: 295-303

Davidson, P.M. (1983). Phenolic compounds. *In*: Antimicrobials in foods. *Eds* A.L. Brannen and P.M. Davidson. Marcel Dekker, New York. **pp** 37-74

Davidson, P.M. and Branden, A.L. (1981). Antimicrobial activity of non-halogenated phenolic compounds. *Journal of Food Protection* **44**: 623-632

de Fernando, G.D.D., Nychas, G.J.E., Peck, M.W. and Ordonez, J.A. (1995). Growth/survival of psychrotrophic pathogens on meat packaged under modified atmosphere. *International Journal of Food Microbiology.* **28**: 221-231

De Vos, P. and De Ley, J. (1983). Intra- and intergenic similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. *International Journal of Systematic Bacteriology.* **33**: 487-509

De Vos, P., Kersters, K., Falsen, E., Pot, B., Gillis, M., Segers, P and De Ley, J. (1985). *Comamonas* Davis and Park 1962 gen. nov., nom. rev. emend., and *Comamonas terringena* Hugh 1962 sp. nov., nom. rev. *International Journal of Systematic Microbiology.* **37**: 52-59

Delaquis, P.J. and McCurdy, A.R. (1990). Colonization of beef muscle surfaces by *Pseudomonas fragi. Journal of Food Science.* **55**: 898-902

Deneer, H.G. and Beychuck, I. (1991). Species-specific detection of *Listeria monocytogenes* by DNA amplification. *Applied and Environmental Microbiology.* **57**: 606-609

Dicks, L.M.T., Janssen, B. and Dellaglio, F. (1995). Differentiation of *Carnobacterium divergens* and *Carnobacterium piscicola* by numerical analysis of total soluble cell protein patterns and DNA-DNA hybridizations. *Current Microbiology.* **31**: 77-79

Dickson, J.S. and Anderson, M.E. (1992). Microbiological decontamination of hoof animal carcasses by washing and sanitizing system: a review. *Journal of Food Protection.* **55**: 133-140

Dockerty, T.R., Ockeman, H.W. Cahill, V.R. Knuckle, L.E. and Weiser, H.H. (1970). Microbial load of pork skin as affected by the dressing process. *Journal of Animal Science.* **30**: 884-890

Dodd, C.E.R. and Waites, W. (1988). The use of plasmid profiling to determine strains of *Brochothrix thermosphacta* important in spoilage of sausages. *Journal of Applied Bacteriology.* **65**: XIV

Donnelly, C.W. and Baigent, G.J. (1986). Method for flow cytometric detection of *Listeria monocytogenes* in milk. *Applied and Environmental Microbiology.* **52**: 689-695

Dorsa, W.J., Cutter, C.N., Siragusa, R.G. and Koohmaraie, M. (1996). Microbial decontamination of beef and sheep carcasses be steam, hot water spray washes, and a steam-vacuum sanitizer. *Journal of Food Protection.* **59**: 127-135

Drosinos, E.H. and Board, R.G. (1994). Growth of *Listeria monocytogenes* in meat juice under a modified atmosphere at 4°C with or without members of a microbial association from chilled lamb under a modified atmosphere. *Letters in Applied Microbiology.* **19**: 134-137

Dufrenne, J., Delfgou, E., Ritmeester, W. and Notermans, S. (1997). The effect of previous growth condition on the lag phase time of some foodborne pathogenic micro-organisms. *International Journal of Food Microbiology.* **34**: 89-94

Dziezak, J.D. (1986). Preservatives: Antimicrobial agents. *Food Technology.* **40**: 104-110

Egan, A.F., Ford, A.L. and Shay, B.J. (1980). A comparison of *Microbacterium thermosphactum* and lactobacilli as spoilage organisms of vacuum-packaged sliced luncheon meats. *Journal of Food Science.* **45**: 1745-1748

Eid, M.A.M. and Sherwood, J.E. (1995). Pulsed-field gel electrophoresis fingerprinting for identification of *Azospirillum* species. *Current Microbiology.* **30**: 127-131

Eklund, T. (1980). Inhibition of growth and uptake processes in bacteria by some chemical food preservatives. *Journal of Applied Bacteriology.* **48**: 423-432

Eklund, T. (1983). The antimicrobial effects of dissociated and undissociated sorbic acid at different pH levels. *Journal of Applied Bacteriology.* **54**: 383-389

Eklund, T. (1985). Inhibition of microbial growth at different pH levels by benzoic acid and proponic acids and esters of *p*-hydroxybenzoic acid. *International Journal of Food Microbiology.* **2**: 159-167

Eklund, T. (1989). Organic acids and esters. *In*: Mechanisms of action of food preservation procedures. *Ed* G.W. Gould. Elsevier Applied Science, London and New York. **pp**. 161-200.

Eklund, T. and Jarmund, T. (1983). Microculture model studies on the effects of various gas atmospheres on microbial growth at different temperatures. *Journal of Applied Bacteriology*. **55**: 119-125

El. Halouat, A. and Debevere, J.M. (1997). Effect of water activity, modifided atmosphere packaging and storage temperature on spore gemination of moulds isolated from prunes. *International Journal of Food Microbiology*. **35**: 41-48

El-Shenawy, M.A. and Marth, E.H. (1988). Inhibition and inactivation of *Listeria monocytogenes* by sorbic acid. *Journal of Food Protection* **51**:842-847

El-Shenawy, M.A. and Marth, E.H. (1989). Inhibition of inactivation of *Listeria monocytogenes* by sodium benzoate together with some other organic acids. *Journal of Food Protection*. **52**: 771-776

Elliott, P.H., Tomilins, R.I. and Gray, R.J.H. (1982). Inhibition and inactivation of *Staphylococcus aureus* in sorbate modified atmosphere combination system. *Journal of Food Science*. **45**: 1112

Elliott, P.H., Tomilins, R.I. and Gray, R.J.H. (1985). Control of microbial spoilage on fresh poultry using a combination potassium sorbate/carbon dioxide packaging system. *Journal of Food Science*. **50**: 1360-1363

Empey, W.A. and Scott, W.J. (1939). Investigations on chilled beef. I Microbial contamination acquired in the meat works. *Council for Scientific and Industrial Research, Australia. Bulletin* **No**. 126.

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**: 561-562

Enfors, S.-O. and Molin, G. (1981a). The influence of temperature on the inhibition effect of the carbon dioxide on *Pseudomonas fragi* and *Bacillus cereus*. *Canadian Journal of Microbiology*. **27**: 15-19

Enfors, S.-O. and Molin, G. (1981b). The effects of different gases on the activity of microorganisms. *In*: Psychrotrophic microorganisms in spoilage and pathogenicity. *Eds* T.A. Roberts, G. Hobbs, J.H.B. Christian and N. Skovgaard. Academic Press, London. **pp**. 335-343

Enfors, S.-O. and Molin, G. (1984). Carbon dioxide evolution of refrigerated meat. *Meat Science*. **10**:197-206

Enfors, S.-O., Molin, G. and Termström, A. (1979). Effects of packaging under carbon dioxide, nitrogen or air on the microbial flora of pork stored at 4°C. *Journal of Applied* Bacteriology **47**: 197-208

Eremeeva, M., Balayeca, N., Roux, V., Ignatovich, V., Kotsinjan, M. and Raoutl, D.J. (1995). Genomic and proteinic characteriazation of strain S, a rickettsia isolated form *Rhipicephalis sanguineis* ticks in Armenia. *Journal of Clinical Microbiology.* **33**: 2738-2744

Erichsen, I. and Molin, G. (1981a). The microbial flora of normal and high pH beef stored at 4°C in different gas environments. *In:* Psychrothrophic microorganisms in spoilage and pathogenicity. *Eds* T.A. Roberts, T.A., G. Hobbs, J.H.B. Christian and N. Skovgaard. Academic press, London. **pp**. 359-367

Erichsen, I. and Molin, G. (1981b). Microflora of normal and high pH beef stored at 4°C in different gas environments. *Journal of Food Protection.* **44**: 866-869

Eyles, M.J., Moir, C.J. and Davey, J.A. (1993). The effects of modified atmospheres on the growth of psychrotrophic pseudomonads on a surface in a model system *International Journal of Food Microbiology.* **20**: 97-107

Farber, J.M. and Idziak, E.S. (1984). Attachment of psychrotrophic meat spoilage bacteria to muscle surface. *Journal of Food Protection.* **47**: 92-95

Farber, J.M., Sanders, G.W., Dunfield, S. and Prescott, R. (1989). The effect of various acidulants on the growth of *Listeria monocytogenes. Letters in Applied Microbiology.* **9**: 181-183

Favero, M.S. McDade, J.J., Robertson, J.A., Hoffman, R.K. and Edwards, R.W. (1968). Microbial sampling of surfaces. *Journal of Applied Bacteriology.* **31**: 336-343.

Feresu, S.B. and Jones, D. (1988). Taxonomic studies on *Brochothrix*, *Erysipelothrix*, *Listeria* and atypical lactobacilli. *Journal of General Microbiology.* **134**: 1165-1183

Ferrero, M., Cesena, C., Morelli, L., Scolari, G. and Vescovo, M. (1996). Molecular characterization of *Lactobacillus casei* strains. *FEMS Microbiology Letters.* **140**: 215-219

Field, R.A., Kalchayanand, N., Rozbeh, M. and Andersen, M.K. (1991). Influence of a pelt puller on microbial quality of lamb. *Sheep Research Journal.* **7**: 24-26

Finne, G. (1982). Modified-and controlled-atmosphere storage of muscle foods. *Food Technology.* **36**: 128-133

Firstenberg-Eden, R. (1981). Attachment of bacteria to meat surfaces: a review. *Journal of Food Protection.* **44**: 602-607

Fitts, R. Diamond, M. Hamilton, C. and Neri, M. (1983). DNA-DNA hybridization assay for detection of *Salmonella* spp. in foods. *Applied and Environmental Microbiology.* **46**: 1146-1151

Fleet, G.H. (1999). Microbiology of food ecosystems. *International Journal of Systematic Bacteriology.* **50**: 101-117

Follet, M.J., Norman, G.A. and Ratcliff, P.W. (1974). The ante-rigor excision and air cooling of beef semimembranous muscles at temperatures between -5°C and +15°C. *Journal of Food Technology.* **9**: 509-523

Fung, D.Y.C., Kastner, C.L., Lee, C.-Y., Hunt, M.C., Dikeman, M.E. and Kropf, D.H. (1981). Initial chilling rate effects on bacterial growth on hot-boned beef. *Journal of Food Protection.* **44**: 539-544

Furrer, B. Candrian, U. Hoefelein, C. and Luethy, J. (1991). Detection and identification of *Listeria monocytogenes* in cooked sausage products and in milk by *in vitro* amplification of haemolysin gene fragment. *Journal of Applied Bacteriology.* **70**: 372-379

Gannon, V.P.J., King, R.K., Kim, J.Y. and Golsteyn Thomas, E.J. (1992). Rapid detection and sensitive method for detection of Shiga-like toxin producing *Escherichia coli* in ground beef using the polymerase chain reaction. *Applied and Environmental Microbiology.* **58**: 3809-3815

Gardner, G.A. (1965). The aerobic microflora of stored meat with particular reference to the use of selective media. *Journal of Applied Bacteriology.* **28**: 252-254

Gardner, G.A. (1966). A selective medium for the enumeration of *Microbacterium thermosphactum* in meat and meat products. *Journal of Applied Bacteriology.* **29**: 455-460

Gardner, G.A., Carson, A.W. and Patton, J. (1967). Bacteriology of prepackaged pork with reference to the gas composition within the pack. *Journal of Applied Bacteriology.* 30: 321-333

Garver, K.I. and Murinana, P.M. (1993). Detection, identification and characterization of bacteriocin-producing lactic acid bacteria from retail food products. *International Journal of Food Microbiology.* **19**: 241-258

Garvie, E.I. (1967). The production of L(+) and D(-) lactic acid in culture of some lactic acid bacteria, with a special study of *Lactobacillus acidophilus* NCDO 2. *Journal of Dairy Research.* **34**: 31-38

Gautier, M., deCarvalho, A. and Rouault, A (1996). DNA fingerprinting of dairy propionibacteria strains by pulsed-field gel electrophoresis. *Current Microbiology.* **32**: 17-24

Geornaras, G. and von Holy, A. (1994). Bacterial contamination in poultry processing. *Food Industries.* **47**: 31-34

Gibson, A.M., Bratchell, N. and Roberts, T.A. (1987). The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurized pork slurry. *Journal of Applied Bacteriology.* **62**: 479-490

Gibson, A.M., Bratchell, N. and Roberts, T.A. (1988). Predicting microbial growth: growth response of salmonellae in a laboratory medium as affected by pH, sodium chloride and storage temperature. *International Journal of Food Microbiology.* **6**: 155-178

Giesendorf, B.A.J., Quint, W.G.V., Henkens, M.H.C., Stegeman, H, Huf, F.A. and Niesters, H.G.M. (1992). Rapid and sensitive detection of *Campylobacter* spp. in chicken products using polymerase reaction. *Applied and Environmental Microbiology.* **58**: 3408-3808

Gil, A., Kroll, R.G. and Poole, R.K. (1992). The cytochrome composition of the meat spoilage bacterium *Brochothrix thermosphacta*: identification of cytochrome a_3- and *d*-type terminal oxidases under various conditions. *Archives of Microbiology.* **158**: 226-233

Gill, C.O. (1975). Effect of growth temperature on lipids of *Pseudomonas fluorescens*. *Journal of General Microbiology.* **89**: 293-298

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

Gill, C.O. (1986). The control of microbial spoilage of fresh meats. *In*: Meat and poultry microbiology. *Eds* A.M. Pearson and T.R. Dutson. Macmillan Publishers, Basingstoke, England. **pp**. 49-88.

Gill, C.O. (1988). The solubility of carbon dioxide in meat. *Meat Science.* **22**: 65-71

Gill, C.O. and Harrison, J.C.L. (1989). The storage life of chilled pork packaged under carbon dioxide. *Meat Science.* **26**: 313-324

Gill, C.O. and Jones, T. (1994). The display life of retail packaged pork chops after their storage in master packs under atmospheres of N_2, CO_2 or O_2+CO_2. *Meat Science.* **42**: 203-213

Gill, C.O., McGinnis, J.C. and Badoni, M. (1996). Assessment of the hygienic characteristics of a beef carcass dressing process. *Journal of Food Protection.* **59**: 136-140

Gill, C.O. and Newton, K.G. (1976). Growth of bacteria on meat at room temperature. *Journal of Applied Bacteriology.* **49**: 315-323

Gill, C.O. and Newton, K.G. (1980). Growth of bacteria on meat at room temperatures. *Journal of Applied Bacteriology.* **49**: 315-323

Gill, C.O. and Newton, K.G. (1982). Effect of lactic acid concentration on growth on meat of Gram-negative psychrotrophs from a meat works. *Applied and Environmental Microbiology.* **43**: 284-288

Gill, C.O. and Penney, N. (1985). Modification of intact conditions to extend the storage life of vacuum packaged lamb. *Meat Science.* **14**:43-60

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**: 56-63

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

Gilliland, S.E. and Ewell, H.R. (1983). Influence of combinations of *Lactobacillus lactis* and potassium sorbate of growth of psychrotrophs in raw milk. *Journal of Dairy Science.* **66**: 974-980

Giovannoni, S.J., deLong, E.F., Olsen, G.J. and Pace, N.R. (1988). Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. *Journal of Bacteriology.* **170**: 720-726

Gladek, A. and Zakrzewska, J. (1984). Genome size of *Streptomyces*. *FEMS Microbiology letters.* **24**: 73-76

Goodson, M. and Rowbury, R.J. (1989). Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. *Letters in Applied Microbiology.* **8**: 77-79

Goor, M. De Vos, P., Gillis, M. and De Ley, J. (1984). Taxonomic localization, by DNA:rRNA hybridization of unclassified phytopathogenic *Pseudomonas* species. *Antonie van Leeuwenhoek.* **50**: 302-303

Gould, G.W. and Measures, J.C. (1977). Water relations in single cells. *Philosophical Transactional Royal Society London B* **278**:151-166

Gounot, A.M. (1991). Bacterial life at low temperature: physiological aspects and biotechnological implications. *Journal of Applied Bacteriology.* **71**: 386-397

Grant, I.R. and Patterson, M.F. (1991). A numerical taxonomic study of lactic acid bacteria isolated from irradiated pork and chicken packaged under various gas atmospheres. *Journal of Applied Bacteriology.* **70**: 302-307

Grau, F.H. (1980). Inhibition of the anaerobic growth of *Brochothrix thermosphacta* by lactic acid bacteria. *Applied and Environmental Microbiology.* **40**: 433-436

Grau, F.H. (1983). End product of glucose fermentation by *Brochothrix thermosphacta. Applied and Environmental Microbiology.* **45**: 84-90

Grau, F.H. (1986). Microbial ecology of meat and poultry. *In:* Meat and Poultry Microbiology. *Eds.* Pearson, A.M. and Dunston, T.R.. Macmillan Publishers, Basingstoke, England. **pp**. 1-47.

Grau, F.H. (1988). Substrates used by *Brochothrix thermosphacta* when growing on meat. *Journal of Food Protection.* **51**: 639-642

Grau, F.H., Eustace, I.J. and Bill, B.A. (1985). Microbial flora of lamb carcasses stored at 0°C in packs flushed with nitrogen or filled with carbon dioxide. *Journal of Food Science.* **50**: 482-485

Grau, F.H. and Vanderlinde, P.B. (1993). Aerobic growth of *Listeria monocytogenes* on beef lean and fatty tissue: equations describing the effect of temperature and pH. *Journal of Food Protection.* **56**: 96-101

Gray, R.J.H., Elliott, P.H. and Thomlins, R.I. (1984). Control of two major pathogens on fresh poultry using combination potassium sorbate and carbon-dioxide. *Journal of Food Science.* **49**: 142

Greer, G.G. (1982). Mechanism of beef shelf-life extension by sorbate. *Journal of Food Protection* **45**: 82-83

Greer, G.G and Dilts, B.D. (1995). Lactic acid inhibition of the growth of spoilage bacteria and cold tolerant pathogens on pork. *International Journal of Food Microbiology.* **25**: 141-151

Greer, G.G., Dilts, B.D. and Jerehmiah, L.E. (1993). Bacteriology and retail case life of pork after storage in carbon dioxide. *Journal of Food Protection.* **56**: 689-693

Greer, G.G. and Jones, D.S.M. (1991). Effects of lactic acid and vacuum packaging on beef processed in a research abattoir. *Canadian Institute of Food Science and Technology.* **24**: 161-168

Grimont, P.A.D. and Grimont, F. (1981). The genus *Serratia. In*: The Prokaryotes Volume II. *Eds*: M.P. Starr, H. Stlop, H.G. Trüper, A. Balows and H.G. Schlegel. Spinger-Verlag, Berlin Heidelberg New York. **pp** 1187-1203

Grimont, P.D.A. and Grimont, F. (1986). Genus VIII. *Serratia* Bizio 1823, 288^{AL}. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 477-484

Grothues, D. and Tümmler, B. (1991). New approaches in genome analysis by pulsed-field gel electrophoresis: application to the analysis of *Pseudomonas* species. *Molecular Microbiology.* **5**: 2763-2776

Gustafson, C.E., Thomas, C.J. and Trust, T.J. (1992). Detection of *Aeromonas salmonicida* from fish by using polymerase chain reaction amplification of the virulence surface array protein-gene. *Applied and Environmental Microbiology.* **58**: 3816-3825

Gustavsson, P. and Borch, E. (1993). Contamination of beef carcasses by psychrotrophic *Pseudomonas* and *Enterobacteriaceae* at different stages along the processing line. *International Journal of Food Microbiology.* **20**: 67-83

Haertl, R. and Bandlow, G. (1993). Epidemiological fingerprinting of *Enterobacter cloacae* by small fragment restriction endonuclease analysis and pulsed-field gel electrophoresis restriction fragments. *Journal of Clinical microbiology.* **31**: 128-133

Haines, R.B. (1933). The influence of carbon dioxide on the rate of multiplication of certain bacteria as judged by viable counts. *Journal of the Society of Chemical Industry.* **52**: 13T-17T

Halebain, S., Harris, B. Finegold, S.M. and Rolfe, R.D. (1981). Rapid method that aids in distinguishing Gram-positive bacteria for Gram-negative bacteria. *Journal of Clinical Microbiology.* **13**: 444-448

Hall, L.M.C., Whitely, R.A., Duke, B., George, R.C. and Efstratiou, A. (1996). Genetic relatedness within and between serotypes of *Streptococcus pneumoniae* for the United Kingdom: analysis of multilocus enzyme electrophoresis, pulsed field gel electrophoresis and antimicrobial resistance patterns. *Journal of Clinical Microbiology.* **34**: 853-859

Hanna, M.O., Savell, J.W., Smith, G.C., Purser, D.E., Gardner, F.A. and Vanderzant, C. (1983). Effect of growth of individual meat bacteria on pH, color and odor of aseptically prepared vacuum-packaged round steaks. *Journal of Food Protection.* **46**: 216-221

Hanna, M.O., Vanderzant, C., Smith, G.C. and Savell, J.W. (1981). Packaging of beef loin steaks in 75% O_2 plus 25% CO_2 II. Microbiological properties. *Journal of Food Protection.* **44**: 928-933

Harrigan, W.F. and McCance, M.E. (1976). Laboratory methods in food and dairy microbiology. Academic Press, London.

Harseno, K.D., Kaspar, C.W. and Luchansky, J.B. (1993). Comparison and genome sizing of *Escherichia coli* 0157:H7 isolates by pulsed field gel electrophoresis. *Applied and Environmental Microbiology.* **59**: 3141-3144

Hastings, J.W. and Holzapfel, W.H. (1987). Numerical taxonomy of lactobacilli: surviving radurization of meat. *International Journal of Food Microbiology.* **4**: 33-49

Hatab, M.A. and Gaugler, R. (1997). Growth mediated variations in fatty acids of *Xenorhabdus* sp. *Journal of Applied Microbiology.* **82**: 351-358

Hazeleger, W.C., Janse, J.D., Koenraad, P.M.F.J., Beumer, R.R., Rombouts, F.M. and Abee, T. (1995). Temperature-dependent fatty acid and cell physiological changes in coccoid form of *Campylobacter jejuni. Applied and Environmental Microbiology.* **61**: 2713-2718

Heinz, G. (1975). Warmzerlegung von Rinderfleisch mit anschliessender Reifelagerung. *Fleischwirtschaft.* **55**: 937-942

Hernandez, J. Owen, R.J. and Fayos, A. (1991). Biotypes and DNA ribotyping of thermophilic *Campylobacter* from faeces and seawater in eastern Spain. *Letters in Applied Microbiology.* **13**: 207-211

Hightower, R.C., Metye, D.W. and Santi, D.V. (1987). Plasmid migration using orthogonal-field alternation gel electrophoresis. *Nucleic Acid Research.* **15**: 8387-8389

Hintlain, C.B. and Hotchkiss, J.C. (1987). Comparative growth of spoilage and pathogenic organisms on modified atmosphere-packaged cooked beef. *Journal of Food Protection.* **50**: 218-223

Hitchner, B.J., Egan, A.F. and Rogers, P.J. (1982). Characteristics of lactic acid bacteria isolated from vacuum-packaged beef. *Journal of Applied Bacteriology.* **52**: 31-37

Hodges, J.H., Cahill, V.R. and Ockerman, H.W. (1974). Effect of vacuum packaging on weight loss, microbial growth and palatability of fresh beef wholesale cuts. *Journal of Food Science.* **39**: 143-146

Hofer, F. (1977). Involvement of plamsid in lactose metabolism *Lactobacillus casei* suggested by genteic experiments. *FEMS Microbiology Letters.* **1**: 167-170

Holley, R.A., Delaquis, P., Rodrigue, N., Doyon, G. and Geriepy, C. (1994). Controlled atmosphere storage of pork under carbon dioxide. *Journal of Food Protection.* **57**: 1088-1093

Holloway, B.W. and Morgan, A.F. (1986). Genome organization in *Pseudomonas*. *Annual Reviews of Microbiology.* **40**: 79-105

Holzapfel, W.H. and Gerber, E.S. (1983). *Lactobacillus divergens* sp. Nov. a new heterofermentative Lactobacillus species producing L(+) lactate. *Systematic and Applied Microbiology.* **4**: 522-554

Hopwood, D.A. (1981). Genetic studies with bacterial protoplasts. *Annual Review of Microbiology.* **35**: 237-272

Howell, S.A., Anthony, R.M and Power, E. (1996). Application of RAPD and restriction enzyme analysis to the study of oral carriage of *Candida albicans*. *Letters in Applied Microbiology.* **22**: 125-128

Houtsma, P.C., Kusters, B.M.J., Wit, J.C.D., Rombouts, F.M. and Zwietering, M.H. (1994). Modelling the growth rates of Listeria monocytogenes as a function of lactate concentration. *International Journal of Food Microbiology.* **24**: 113-123

Hudson, J.A. (1992). Variations in growth kinetics and phenotype of *Aeromonas* spp. from clinical, meat processing and flesh sources. *International JournalFood Microbiology.* **16**: 131-139

Hudson, J.A., Mott, S.J. and Penney, N. (1994). Growth of *Listeria monocytogenes*, *Aeromonas hydrophila*, and *Yersinia entrocolitica* on vacuum and saturated carbon dioxide controlled atmosphere-packaged sliced roast beef. *Journal of Food Protection.* **57**: 204-208

Huffman, D.L., Davis, K.A., Marple, D.N. and McGuire, J.A. (1975). Effect of gas atmosphere on microbial growth, colour and pH of beef. *Journal of Food Science.* **40**: 1229-1231

Hughes, A.H. and McDermot, J.C. (1989). The effects of phosphate, sodium chloride, sodium nitrite, storage temperature and pH on the growth of enteropathogenic *Escherichia coli* in a laboratory medium. *International Journal of Food Microbiology.* **9**: 215-223

Hugo, W.B. (1964). Growth of *Pseudomonas aeruginosa* in solutions of esters of p-hydroxybenzoic acid. *Journal of Pharmacy and Pharmaceutics.* **16**: 209

Hugo, W.B. (1967). The mode of action of antibacterial agents. *Journal of Applied Bacteriology.* **30**: 17-50

Hui, S.R., Holt, R.A., Sriranganathan, H., Seilder, R.J. and Fryer J.L. (1984). *Lactobacillus piscicola*, a new species from salmonid fish. *International Journal of Systematic Bacteriology.* **34**: 393-400

Huis in't Veld, J.H.J. (1996). Microbial and biochemical spoilage of foods: an overview. *International Journal of Food Microbiology.* **33**: 1-18

Ingham, S.C., Alford, R.A. and McCown, A.P. (1990a). Comparative growth rates of *Salmonella typhimurium* and *Pseudomonas fragi* on cooked crab meat stored under air and modified atmosphere. *Journal of Food Protection.* **53**: 566-567

Ingham, S.C., Escude, J.M. and McCown, P. (1990b). Comparative growth rates of *Listeria monocytogenes* and *Pseudomonas fragi* on cooked chicken loaf stored under air and two modified atmospheres. *Journal of Food Protection.* **53**: 289-291

Ingram, M. (1962). Microbiological principles in pre-packaged meats. *Journal of Applied Microbiology.* **25**: 259-281

Ingram, M. and Dainty, R.H. (1971). Changes caused by microbes in spoilage of meats. *Journal of Applied Bacteriology.* **34**: 21-39

Ingram, M. and Shewan, J.M. (1960). Introductory reflection on the *Pseudomonas-Achromobacter* group. *Journal of Applied Bacteriology.* **23**: 373-378

Ingram, M. and Simonsen, I. (1980). Meat and meat products. *In*: Microbial Ecology of Foods Volume 2. *Eds* J.H. Silliker, R.P. Elliott, A.C. Baird-Parker, F.L. Bryan, J.H.B. Christian, D.S. Clark, J.C. Olson Jr. and T.A. Roberts. Academic Press, New York. **pp**. 333-409

Jack, R.W., Wan, J., Gordon, J., Harmark, K., Davidson, B.E., Hillier, A.J., Wettenhall, R.E.H., Hickey, M.W. and Coventry, M.J. (1996). Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola. Applied and Environmental Microbiology.* **62**: 2897-2903

Jackman, P.J.H. (1985). Bacterial taxonomy based on electrophoretic whole cell protein patterns. *In*: Chemical methods in bacterial systematics. *Eds* M. Goodfellow and D.E. Minnikin. Academic Press London Orlando San Diego new York Toronto Montreal Sydney Tokyo. **pp** 115-129

Jericho, K.W.F., Bradley, J.A. and Kozub, G.C. (1994). Bacteriological evaluation of groups of beef carcasses before the wash at six Alberta abattoirs. *Journal of Applied Bacteriology.* 77: 631-634

Jiang, Q., Hiratsuka, K. and Taylor, D.E. (1996). Variability of gene order in different *Helicobacter pylori* strains contributes to genome diversity. *Molecular Microbiology.* **20**: 833-842

Johansson, M.-L., Molin, G., Patterson, B., Uhlén, M. and Ahrné, S. (1995). Characterization and species recognition of *Lactobacillus plantarum* stains by restriction fragment length polymorphism (RFLP) of the 16S rRNA gene. *Journal of Applied Bacteriology.* **79**: 525-535

Johnson, A.W. and Ogrydziak, D.M. (1984). Genetic adaptation to elevated carbon dioxide atmospheres by *Pseudomonas*-like bacteria isolated from rock cod. *Applied and Environmental Microbiology.* **48**: 486-490

Johnson, F.H., Eyring, H. and Polissar, M.J. (1974). The theory of rate processes in biology and medicine. John Wiley and Sons Inc., New York. **pp** 99

Johnson, J.M., Weagant, S.D., Jinneman, K.C. and Bryant, J.L. (1995). Use of pulse-field gel electrophoresis for epidemiological study of *Escherichia coli* O157:H7 during a food-borne outbreak. *Applied and Environmental Microbiology.* **61**: 2806-2808

Johnson, J.L. and Ordal, E.J. (1968). Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity. *Journal of Bacteriology.* **95**: 893-900

Jones, D.H. (1992). Polymerase chain reaction (PCR). *In*: Encyclopedia of Microbiology. *Ed* J. Ledweberg. Academic Press Inc. San Diego New York Boston London Sydney Tokyo Toronto. **pp** 443-449

Jones, P.G., van Bogolen, R.A. and Neidhardt, F.C. (1987). Induction of proteins in response to temperature in *Escherichia coli. Journal of Bacteriology.* **169**: 2092-2095

Juffs, H.S. (1976). Effects of temperature and nutrients on proteinase production by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa. Journal of Applied Bacteriology.* **40**: 23-32

Juneja, V.K. and Davidson, P.M. (1993). Influence of altered fatty acid composition on resistance of *Listeria monocytogenes* to antimicrobials. *Journal of Food Protection* **56**: 302-305

Kabara, J.J. and Eklund, T. (1991). Organic acids and esters. *In*: Food Preservatives. *Eds* N.J. Russell and G.W. Gould. Blackie and Sons Ltd., Glasgow and London. **pp**. 44-71.

Kagermeier-Calloway, A.S. and Lauer, E. (1995). *Lactobacillus sake* Katagiri, Kitahara and Fukami 1934 is the senior synonym for *Lactobacillus bavaricus* Stetter and Stetter 1980. *International Journal of Systematic Bacteriology.* **45**: 398-399

Kandler O. and Weiss, N. (1986). Section 14 Regular, non-sporing Gram-negative rods. *In:* Bergey's Manual of Systematic Bacteriology Volume II. *Eds* P.A.H. Sneath, N.S. Mair, M.E. Shapre and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 1208-1234

Karapinar, M. and Gönül, S.A. (1992). Effects of sodium bicarbonate, vinegar, acetic and citric acids on the growth of *Yersinia enterocolitica. International Journal of Food Microbiology.* **16**: 343-347

Kashket, E.R. (1985). The proton motive force in bacteria: a critical assessment of methods. Annual Review of Microbiology. **39**: 219-249

Kashket, E., Blanchard, A. and Metzeger, N. (1980). Proton motive force during growth of *Streptococcus lactis* cells. *Journal of bacteriology.* **143**: 128-134.

Kastner, C.L., Luedecke, L.O. and Russell, T.S. (1976). A comparison of microbial counts on conventionally and hot-boned bovine carcasses. *Journal of Milk Food Technology.* **39**: 684-685

Kelly, W.J., Huang, C.M. and Asmundson, R.V. (1993). Comparison of *Leuconostoc oenos* strains by pulsed-field gel electrophoresis. *Applied and Environmental Microbiology.* **59**: 3969-3972

King Jr., A.D. and Nagel, C.W. (1967). Growth inhibition of a *Pseudomonas* by carbon dioxide. *Journal of Food Science.* **32**: 575-579

King Jr., A.D. and Nagel, C.W. (1975). Influence of carbon dioxide upon the metabolism of *Pseudomonas aeruginosa. Journal of Food Science.* **40**: 362-366

Klausner, A. (1988). New methods aid microbial identification. *Biotechnology.* **6**: 756

Klein, G., Dicks, L.M.T., Pack, A., Hack, B., Zimmermann, K., Dellaglio, F. and Reuter, G. (1996). Emended description of *Lactobacillus sake* (Katagiri, Kitahata, and Fukami) and *Lactobacillus curvatus* (Abo-Elnaga and Kandler): Numerical classification revealed by protein fingerprinting and identification based on biochemical patterns and DNA-DNA hybridizations. *International Journal of Systematic Bacteriology.* **46**: 367-376

Klinger, J.M., Stowe, R.P., Oberhuber, D.C., Grous, T.O., Mishra, S.K. and Pierson, D.L. (1992). Evaluation of the Biolog automated microbial identification system. *Applied and Environmental Microbiology.* **58**:2089-2092

Knudtson, L.M. and Hartman, P.A. (1993). Enterococci in pork processing. *Journal of Food Protection.* **56**: 6-9

Kobayashi, H. (1985). A proton-translocating ATPase regulates pH of bacterial cytoplasm. *Journal of Biological Chemistry.* **260**: 72-76

Kobayashi, H., Suzuki, T. and Unemoto, T. (1986). Streptococcal cytoplasmic pH is regulated by changes in amount and activity of a proton translocating ATPase. *Journal of Biological Chemistry.* **260**: 72-76

Korkeala, H. and Mäkelä, P. (1989). Characterization of lactic acid bacteria isolated from vacuum-packaged cooked ring sausages. *International Journal of Food Microbiology.* **9**: 33-43

Kotula, A.W. (1981). Microbiology of hot-boned and electeostimulated meat. *Journal of Food Protection.* **44**: 545-549

Kotula, A.W. and Emswiler-Rose, B.S. (1981). Bacteriological quality of hot-boned primal cuts from electrically stimulated beef carcasses. *Journal of Food Science.* **46**: 471-474

Kotula, A.W. and Emswiler-Rose, B.S. (1988). Airborne microorganisms in a pork processing establishment. *Journal of Food Protection.* **51**: 935-937

Kotula, A.W., Emswiler-Rose, B.S. and Berry, B.W. (1987). Microbial counts of selected hot boned primals and ground beef. *Journal of Food Protection.* **50**: 915-919

Kotula, A.W. and Kinner, J.A. (1964). Airborne micro-organisms in broiler processing plants. *Applied Microbiology.* **12**: 179

Kotula, K.L. and Pandya, Y. (1995). Bacterial contamination of broiler chickens before scalding. *Journal of Food Protection.* **58**: 1326-1329

Kovacs, N. (1956). Identification of *Pseudomonas pyocanea* by oxidase reaction. *Nature.* **178**: 703

Kroll, R.G. and Patchett, R.A. (1992). Induced acid tolerance in *Listeria monocytogenes. Letters in Applied Microbiology.* **14**: 224-227

Kwaga, J., Iverson, J.O. and Misra, V. (1992). Detection of pathogenic *Yersinia enterocolitica* by polymerase chain reaction and digoxigenin-labelled polynucleotide probes. *Journal of Clinical Microbiology.* **30**: 2668-2673

Lampel, K.A., Jagew, J.A., Truchsess, M. and Hill, N.E. (1990). Polymerase chain reaction for detection of invasive *Shigella flexeri. Applied and Environmental Microbiology.* **56**: 1536-1540

Lasta, J. and Fonrouge, R. (1988). Significance of samples taken for bacterial counts for reduced areas of bovine carcasses. *Journal of Food Protection.* **51**: 214-217

Lauret, R., Morel-Deville, F., Berthier, F., Champomier-Verges, M., Postma, P. and Zagorec, M. (1996). Carbohydrate utilization in *Lactobacillus sake*. *Applied and Environmental Microbiology.* **62**: 1922-1927

Lawrie, R.A. (1974). The spoilage of meat by infecting organisms. *In*: Meat Science. Pergamon Press, Oxford. **pp**. 101-121.

Lawrie, R.A. (1990). Meat quality-the significance of muscle differentiation. *Food Australia.* **42**: 84-86

Lazarus, C.R., Abu-Baker, A., West, R.L. and Oblinger, J.R. (1977). Comparison of microbial counts on beef carcasses by using the moist-swab contact method and secondary tissue removal. *Applied and Environmental Microbiology.* **33**: 217-218

Le Bourgeouis, P., Mata, M. and Ritzenhaler, P. (1989). Genome comparison of *Lactococcus* strains by pulse-field gel electrophoresis. *FEMS Microbiology Letters.* **59**: 65-70

Le Jeune, C. Lonvaud-Funel, A., ten Brick, B., Hofsra, H. and van der Vossen, J.M.B.M. (1995). Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity tests. *Journal of Applied Bacteriology.* **78**: 316-326

Le Minor, L. (1986). Genus III. *Salmonella* Lignieres 1900, 389^{AL}. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 427-458

Ledward, D.A., Nicol, D.J. and Shaw, M.K. (1970). Microbiological and colour changes during aging of beef. *Food Technology in Australia.* **23**: 30-32

Lee, C.Y, Fung, 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

Lee, W.H. (1977). Two plating media modified with Tween 80 for isolating *Yersinia enterocolitica. Applied and Environmental Microbiology.* **33**: 215-216

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

Leeson, R. (1987). The use of gaseous mixtures in controlled and modified atmosphere packaging. *Food Technology in New Zealand.* **22**: 24-25

Li, K.-Y. and Torres, J.A. (1993a). Microbial growth estimation in liquid media exposed to temperature fluctuations. *Journal of Food Science.* **58**: 644-648

Li, K.-Y. and Torres, J.A. (1993b). Water activity relationships for selected mesophiles and psychrotrophs at refrigeration temperatures. *Journal of Food Protection* **56**: 612-615

Li, X. and Hayward, A.C. (1994). Bacterial whole cell protein profiles of the rRNA group II pseudomonads. *Journal of Applied Bacteriology.* **77**: 308-318

Lin, W.-J. and Johnson, E.A. (1995). Genome analysis of *Clostridium botulinum* type A by pulsed-field gel electrophoresis. *Applied and Environmental Microbiology.* **61**: 4441-4447

Lior, H. (1994). *Campylobacter*-epidemiological markers. *Dairy, Food and Environmental Sanitation.* **14**: 317-324

Lipski, A., Klatter, S., Bendiger, B. and Alterdorf, K. (1992). Differentiation of Gram-negative, non-fermentative bacteria isolated from bioifilters in the basis of fatty acid composition, quinone system and physiological reaction profile. *Applied and Environmental Microbiology.* **58**: 2053-2065

Little, C.L. Adams, M.R. Anderson, W.A. and Cole, M.B. (1992a). Comparison of a quadratic response surface model and a square root model for predicting the growth rate of *Yersinia enterocolitica. Letters in Applied Microbiology.* **15**: 63-68

Little, C.L., Adams, M.R. and Easter, M.C. (1992b). The effect of pH, acidulant and temperature on the survival of *Yersinia enterocolitica. Letters in Applied Microbiology.* **14**: 148-152

Locker, R.H. and Haggard, C.J. (1963). A cold shortening effect in beef muscle. *Journal of Food Science and Agriculture.* **14**: 787-793

Lonvaud-Funel, A., Fremaux, C., Biteau, N. and Joyeux, A. (1991). Speciation of lactic acid bacteria from wine by hybridization with DNA probes. *Food Microbiology.* **8**: 215-222

Lortal, S., Rouault, A., Guezenec, S. and Gautier, M. (1997). *Lactobacillus helveticus*: strain typing and genome size estimation by pulsed field gel electrophoresis. *Current Microbiology.* **34**: 180-185

Lück, E. (1980). Antimicrobial food additives. Springer-Verlag, Berlin.

Lück, P.C., Helbig, J.H., Hagedorn, H.-J. and Ehret, W. (1995). DNA fingerprinting by pulsed-field gel electrophoresis to investigate a nosocomial pneumonia caused by *Legionella bozemanii* serogroup 1. *Applied and Environmental Microbiology.* **61**: 2759-2761

Ludwig, W., Dorn, S., Springer, N., Kirchof, G. and Heinz Scheifer, K. (1994). PCR-based preparation of 23S rRNA-targeted group specific polynucleotide probes. *Applied and Environmental Microbiology.* **60**: 3236-3244

Lupski, J.R. and Weinstock, G.M. (1992). Short, interspersed repetitive DNA sequences in prokaryotic genomes. *Journal of Bacteriology.* **174**: 4525-4529

Macaskie, L.E., Sheard, A.G., Dainty, R.H. and Henderson, P.J.F. (1984). Glycerol utilization by *Brochothrix thermosphacta*. *Journal of Applied Bacteriology.* **56**: 137-143

MacDonald, R. and Kalmakoff, J. (1995). Comparison of pulsed-field gel electrophoresis DNA fingerprints of field isolates of the entomopathogen *Bacillus popilliae*. *Applied and Environmental Microbiology.* **61**: 2446-2449

Mackey, B.M. and Derrick, C.M. (1979). Contamination of deep tissues of carcasses by bacteria present in the slaughter instruments or in the gut. *Journal of Applied Bacteriology.* **46**: 355-366

Mackey, B.M. and Kerridge, A.L. (1988). The effect of incubation temperature and inoculum size on growth of salmonellae in minced beef. *International Journal of Food Microbiology.* **6**: 57-65

Manu-Twiah, W., Ammann, L.L., Sebranek, J.G. and Molins, R.A. (1991). Extending the colour stability and shelf life of fresh meat. *Food Technology.* **45**: 94-102

Marshall, R.T., Anderson, M.E., Naumann, H.D. and Stringer, W.C. (1977). Experiments in sanitizing beef with sodium hypochlorite. *Journal of Food Protection.* **40**: 246-249

Martinez-Murcia, A.J. and Collins, M.D. (1990). A phylogenetic analysis of the genus *Leuconostoc* based on reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiology Letters.* **70**: 73-84

Mathew, M.K., Hui, C.-F., Smith, C.L. and Cantor, C.R. (1988c). High-resolution separation and accurate size determination in pulsed-field gel electrophoresis of DNA. 4. Influence on DNA topology. *Biochemistry.* **27**: 9222-9226

Mathew, M.K., Smith, C.L. and Cantor, C.R. (1988a). High-resolution separation and accurate size determination in pulsed-field gel electrophoresis of DNA. 1. DNA size standard and the effect of agarose and temperature. *Biochemistry.* **27**: 9204-9210

Mathew, M.K., Smith, C.L. and Cantor, C.R. (1988b). High-resolution and accurate size determination on pulsed-field gel electrophoresis of DNA. 2. Effect of pulse time and electric field strength and implications for models of the separation process. *Biochemistry.* **27**: 9210-9216

Matushek, M.G., Bonten, M.J. and Hayden, M.K. (1996). Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. *Journal of Clinical Microbiology.* **34**: 2598-2600

Mauguin, S. and Novel, G. (1994). Characterization of lactic acid bacteria isolated from seafood. *Journal of Applied bacteriology.* **76**: 616-625

McClelland, M., Jones, R., Patel, Y. and Nelson, M. (1987). Restriction endonuclease for pulse field mapping of bacterial genomes. *Nucleic Acid Research.* **15**: 5985-6005

McClure, P.J., Baranyi, J., Boogard, E., Kelly, T.M. and Roberts, T.A. (1993). A predictive model for the combined effect of pH, sodium chloride and storage temperature on the growth of *Brochothrix thermosphacta*. *International Journal of Food Microbiology.* **19**: 161-178

McClure, P.J., Cole, M.B. and Davies, K.W. (1994). An example of the stages in the development of a predictive mathematical model for microbial growth: the effects of NaCl, pH and temperature on the growth of *Aeromonas hydrophila*. *International Journal of Food Microbiology.* **23**: 359-375

McClure, P.J., Roberts, T.A. and Oguru, P.O. (1989). Comparison of the effects of sodium chloride, pH and temperature on the growth of *Listeria monocytogenes* on gradient plates and in liquid culture. *Letters in Applied Microbiology.* **9**: 95-99

McDonald, L.C., Flemming, H.P. and Hassan, H.M. (1990). Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. *Applied and Environmental Microbiology*. **56**: 2120-2124.

McDonald, L.C., McFeeters, R.F., Daeschel, M.A. and Fleming, H.P. (1987). A differential medium for the enumeration of homofermentative and heterofermentative lactic acid bacteria. *Applied and Environmental Microbiology*. **53**: 1382-1384

McLean, R.A. and Sulzbacher, W.L. (1953). *Microbacterium thermosphactum* sp. nov., a non heat-resistant bacterium from fresh pork sausages. *Journal of Bacteriology*. **65**: 428-433

McMeekin, T.A., Chandler, R.E., Doe, P.E., Garland, C.D., Olley, J., Putro, S. and Ratkowsky, D.A. (1987). Model for the combined effect of temperature and water activity on the growth rate of *Staphylococcus xylosus*. *Journal of Applied Bacteriology*. **62**: 543-550

McMeekin, T.A., Olley, J.N., Ross, T. and Ratkowsky, D.A. (1993). Predictive microbiology: theory and applications. Research Studies Press Ltd., Somerset.

McMeekin, T.A. and Thomas, C.J. (1978). Retention of bacterial on chicken surfaces after immersion in bacterial suspensions. *Journal of Applied Bacteriology*. **45**: 273-279

McMullen, L.M. and Stiles, M.E. (1991). Changes in microbial parameters and gas composition during modified atmosphere storage of fresh pork loin cuts. *Journal of Food Protection*. **54**: 778-783

McMullen, L.M. and Stiles, M.E. (1993). Microbial atmosphere of fresh pork stored under modified atmosphere at -1, 4.4 and 10°C. *International Journal of Food Microbiology*. **18**: 1-14

Mead, G.C. and Adams, B.W. (1977). A selective medium for the rapid isolation of pseudomonads associated with poultry meat spoilage. *British Poultry Science*. **18**: 661-670

Mead, G.C., Hudson, W.R. and Hinton, M.H. (1994). Use of a marker organism in poultry processing to identify sites of cross contamination and evaluate possible control measures. *British Poultry Science*. **35**: 345-354

Mettler, E. and Carpentier, B. (1998). Variations over time of microbial load and physicochemical properties of floor materials after cleaning in food industry premises. *Journal of Food Protection*. **61**: 57-65

Membré, J.M. and Burlot, P.M. (1994). Effects of temperature, pH, and NaCl on growth and pectinolytic activity of *Pseudomonas marginalis*. *Applied and Environmental Microbiology.* **60**: 2017-2022

Mendonca, A.F., Molins, R.A., Kraft, A.A. and Walker, H.W. (1989). Effects of potassium sorbate, sodium acetate, phosphates and sodium chloride alone and in combination on shelf-life of vacuum packaged pork chops. *Journal of Food Science.* **54**: 302-306

Merquior, V. L.C., Perlta, J.M., Facklam, R.R. and Teixeira, L.M. (1994). Analysis of electrophoresis of whole cell-protein profiles as a tool for characterization of *Enterococcus* species. *Current Microbiology.* **24**: 149-153

Miller, J.M. and Rhoden, D.L. (1991). Preliminary evaluation of Biolog, a carbon source utilization method for bacterial identification. *Journal of Clinical Microbiology.* **29**:1143-1147

Mitchell, P. (1961). *Nature.* **191**: 141-148

Mitchell, P. (1966). Chemiocmotic coupling in oxidative and photosynthetic phosphorylation. *Bacteriological Reviews.* **41**: 445-502

Mohr, P.W. and Krawiec, S. (1983). Temperature characteristics and arrhenius plots for nominal psychrophiles, mesophiles and thermophiles. *Journal of General Microbiology.* **121**: 311-317

Moir, C.J. and Eyles, M.J. (1992). Inhibition, injury, and inactivation of four psychothrophic foodboune bacteria by the preservatives methyl *p*-hydroxybenzoate and potassium sorbate. *Journal of Food Protection.* **55**: 360-366

Molin, G. (1983). The resistance to carbon dioxide of some food related bacteria. *European Journal of Applied Microbiology and Biotechnology.* **18**: 214-217

Molin, G. and Ternström, A. (1982). Numerical taxonomy of psychrophilic pseudomonads. *Journal of General Bacteriology.* **128**: 1249-1264

Molin, G. and Ternström, A. (1986). Phenotypically base taxonomy of psychrotrophic *Pseudomonas* isolated from spoiled meat, water, and soil. *International Journal of Systematic Bacteriology.* **36**: 257-274

Montel, M.-C. and Champomier, M.-C. (1987). Arginine catabolism in *Lactobacillus sake* isolated from meat. *Applied and Environmental Microbiology.* **53**: 2683-2685

Montel, M.-C., Talon, R., Fournaud, J. and Champomier, M.-C. (1991). A simplified key for the identifying homofermentative *Lactobacillus* and *Carnobacterium* spp. from meat. *Journal of Applied Bacteriology.* **70**: 469-472

Moustafa, H.H. and Collins, E.B. (1969). Effects of selected food additives on growth of *Pseudomonas fragi*. *Journal of Dairy Science.* **52**: 335-340

Mossel, D.A.A., Mengerick, W.H.J. and Scholts, H.H. (1962). Use of modified MacConkey agar medium for the selective growth and enumeration *Enterobacteriaceae. Journal of Bacteriology.* **84**: 381

Myers, L.E., Silva, S.V.P.S., Procunier, J.D. and Little, P.B. (1993). Genomic fingerprinting of "*Haeophilus sommus*" isolated by using random-amplified polymorphic DNA assay. *Journal of Clinical Microbiology.* **31**: 512-517

Nakajima, H., Inoue, M., Thori, T., Itoh, K.-I., Arakawa, E. and Watanabe, H. (1992). Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction. *Journal of Clinical Microbiology.* **30**: 2484-2486

Nes, I.F. and Eklund, T (1983). The effect of parabens on DNA, RNA and protein synthesis in *Escherichia coli* and *Bacillus subtilis*. *Journal of Applied Bacteriology.* **54**: 237-242

Newsome, R.L., Langiois, B.E. and Moody, W.G. (1987). Identification and antibiotic resistance of staphylococci and *Enterobacteriaceae* isolated from restructured and conventional steaks. *Journal of Food Science.* **52**: 1169-1173

Newton, K.G. and Gill, C.O. (1978). The development of the anaerobic spoilage flora of meat stored at chill temperatures. *Journal of Applied Bacteriology.* **44**: 91-95

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**:75-82

Newton, K.G., Nottingham, P.M. and Harrison, J.C.L. (1975). Microbial contamination of meat in boning and packing rooms. *Publication, Meat Industry Research Institute of New Zealand.* **452**: 22 (Abstract)

Newton, K.G. and Riggs, 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

Niederhauser, C., Candrian, U., Hofelein, C., Jermini, M., Buhler, H.-P. and Luthy, J. (1992). Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. *Applied and Environmental Microbiology.* **58**: 1564-1568

Nikado, H. and Vaara, M. (1985). Molecular basis of outer membrane permeability. *Microbiological Reviews.* **49**: 1-32

Nortjé, G.I. and Naudé, R.T. (1981). Microbiology of beef carcass surface. *Journal of Food Protection.* **44**: 355-358

Nortjé, G.I. and Shaw, B.G. (1989). The effect of aging treatment on the microbiology and storage characteristics of beef in modified atmosphere packs containing 25% CO_2 plus 75% O_2. *Meat Science.* **25**: 43-58

Nortjé, G.I., Nel, L., Jordaan, E., Badenhorst, K., Goedhart, G., Holzapfel, W.H. and Grimbeek, R.J. (1990). A quantitative survey of a meat production chain to determine the microbial profile of the final product. *Journal of Food Protection.* **53**: 411-417

Nortjé, G.I., Nel, L., Jordaan, E., Naude, R.T., Holzapfel, W.H. and Grimbeek, R.J. (1989). A microbiological survey of fresh meat in the supermarket trade. I. Carcasses and contact surfaces. *Meat Science.* **25**: 81-97

Nortjé, G.I., Swanepoel, E., Naude, R.T., Holzapfel, W.H. and Steyn, P.L. (1982). Evaluation of three carcass surface microbial sampling techniques. *Journal of Food Protection.* **45**: 1016-1017

Notermans, S., Dufrenne, J. and Van Schothrost, M. (1980). The effect of cultural procedures on the attachment of bacteria to chicken breast meat. *Journal of Applied Bacteriology.* **49**: 273-279

Notermans, S. and Kampelmacher, E.H. (1974). Attachment of some bacterial strains to the skin of broiler chickens. *British Poultry Science.* **15**: 573-585

Notermans, S. and Van Schothorst, M. (1977). [Poultry processing: a special problem for factory hygiene.] Die gefluegelverarbeitung: ein besonderes problem der betriebshygiene. *Fleischwirtschaft.* **57**: 248-252 (Abstract)

Ockerman, H.W., Borton, R.J., Cahill, V.R., Parrett, N.A. and Hoffman, H.D. (1974). Use of acetic and lactic acid to control the quantity of microorganisms on lamb carcasses. *Journal of Milk Food and Technology.* **37**: 203-204

Ockeman, H.W., Plimpton, R.F. and Mills, E.W. (1977). Relationship between meat chiller cooler condensation and pork carcasses microbial contamination. *Proceedings of the European Meeting of Meat Research Workers.* **No. 23**, L3:1-L3:20 (Abstract)

O'Sullivan, E. and Condon, S. (1997). Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis. Applied and Environmental Microbiology.* **63**: 4210-4215.

Olive, M.D. (1989). Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. *Journal of Clinical Microbiology.* **27**: 261-265

Olsen Jr., J.C. and Nottingham, P.M. (1980). Temperature. *In*: Microbial Ecology of Foods Volume 1. *Eds* J.H. Silliker, R.P. Elliott, A.C. Baird-Parker, F.L. Bryan, J.H.B. Christian, D.S. Clark, J.C. Olson Jr. and T.A. Roberts. Academic Press, New York London Toronto Sydney San Francisco. **pp**. 1-37

Ørskov, F. (1986a). Genus V. *K.ebsiella* Trevisan 1885, 105^{AL}. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 461-465

Ørskov, F. (1986b). Genus I. *Escherichia* Castellani and Chalmers 1919, 941^{AL}. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 420-423

Oyofo, B.A., Thornton, S.A., Burr, D.H., Trust, T.J., Pavlouski, O.R. and Guerry, P. (1992). Specific detection of *Campylobacter jejuni* and *Campylobacter coli* by polymerase chain reaction. *Journal of Clinical Microbiology.* **30**: 2613-2619

Palleroni, N.J. (1981). Introduction to the family *Pseudomonaceae. In*: The Prokaryotes Volume I. *Eds*: M.P. Starr, H. Stlop, H.G. Trüper, A. Balows and H.G. Schlegel. Spinger-Verlag, Berlin Heidelberg New York. **pp** 655-665

Palleroni, N.J. (1986). Section 4 Gram-negative aerobic rods and cocci. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 141-199

Pandan, E., Zilberstein, D. and Rottenberg, H. (1976). The proton electrochemical gradient in *Escherichia coli* cells. *European Journal of Biochemistry.* **65**: 533-541.

Papadopoulous, L.S., Miller, R.K., Acuff, G.R., Vanderzant, C. and Cross, H.R. (1991). Effects of sodium lactate on microbial and chemical composition of cooked beef during storage. *Journal of Food Science.* **56**: 341-347

Papon, M. and Talon, R. (1988). Factors affecting the growth and lipase production by meat lactobacilli strains and *Brochothrix thermosphacta. Journal of Applied Bacteriology.* **64**:107-115

Papon, M. and Talon, R. (1989). Cell location and partial characterization of *Brochothrix thermosphacta* and *Lactobacillus curvatus* lipases. *Journal of Applied Bacteriology.* **66**: 235-242

Parry, R.T. (1989). Technology development in pre-slaughter handling and processing. *In*: Processing of poultry. *Ed* G.C. Mead. Elserver Applied Science, New York. **pp**. 65-101

Patterson, J.T. (1973). Airborne microorganisms in poultry processing plants. *British Journal of Poultry Science.* **14**: 161-165

Patterson, J.T. and Gibb, P.A. (1977). Incidence and spoilage potential of isolates from vacuum-packaged meat of high pH. *Journal of Applied Bacteriology.* **43**: 25-38

Patterson, J.T. and Gibbs, P.A. (1978). Sources and properties of some organisms isolated in two abattoirs. *Meat Science.* **2**:263-273

Payne, K.D., Rico-Munzo, E. and Davidson, P.M. (1989). The antimicrobial activity of phenolic compounds against *Listeria monocytogenes* and their effectiveness in a model milk system. *Journal of Food Protection.* **52**:151-153

Petrick, H.A.R., Ambrosio, R.E. and Holzapfel, W.H. (1988). Isolation of DNA probe for *Lactobacillus curvatus. Applied and Environmental Microbiology.* **54**: 405-408

Pierson, M.D., Collins-Thompson, D.L. and Ordal, Z.J. (1970). Microbiology, sensory and pigment changes of aerobically and anaerobically packaged beef. Food Technology. **24**: 1171-1175

Pilloud, N. and Mollet, B. (1990). DNA probes for the detection for the detection of *Lactobacillus helveticus*. *Systematic and Applied Bacteriology.* **13**: 345-349

Pisula, A. and Tyburcy, A. (1996). Hot processing of meat. *Meat Science.* **43**: S125-S134

Pollard, D.R., Johnson, W.M., Lior, H., Tyler, S.D. and Rozee, K.R. (1990). Detection of the aerolysin gene in *Aeromonas hydrophilia* by polymerase chain reaction. *Journal of Clinical Microbiology.* **28**: 2477-2481

Pollard, J.A. (1991). Legislative aspects. *In*: Food Preservatives. *Eds* N.J. Russell and G.W. Gould. Blackie and Sons Ltd., Glasgow and London. **pp**. 235-265

Poolman, B., Driessen, A.J.M. and Konings, W.N. (1987). Regulation of solute transport in streptococci bXy external and internal pH values. *Microbiological Reviews.* **51**: 498-508

Pot, B., Hertel, C., Ludwig, W., Descheemaeker, P., Kersters, K.K. and Scheifer, K.-H. (1993). Identification and classification of *Lactobacillus acidophilus, L. gasseri,* and *L. johnsonii* strains by SDS-PAGE and rRNA-targeted oligonucleotide probe hybridization. *Journal of General Microbiology.* **139**: 513-517

Prieto, M., Garcia, M.L., Garcia, M.R., Otero, A. and Moreno, B. (1991). Distribution and evolution of bacteria on lamb carcasses during aerobic storage. *Journal of Food Protection.* **54**: 945-949

Prieto, M., Garcia-Armesto, M.R., Garcia-Lopez, M.L., Otero, A. and Moreno, B. (1992). Numerical taxonomy of Gram-negative, nonmotile, nonfermentative bacteria isolated during chilled storage on lamb carcasses. *Applied and Environmental Microbiology* **58**: 2245-2249

Prieto, M., Garcia-Lopez, M.L., Garcia-Armesto, M.R., Otero, A., Lopez, T.M. and Moreno, B. (1993). Factors affecting spoilage of microflora succession on lamb carcasses at refrigeration temperatures. *Journal of Applied Bacteriology.* **74**: 521-525

Prior, B.A., Kenyon, C.P., van der Veen, M. and Mildenhall, J.P. (1987). Water relations of solute accumulation in *Pseudomonas fluorescens*. *Journal of Applied Bacteriology* **62**: 119-128

Proctor, M.E., Brosch, R., Mellen, J.W., Garret, L.A., Kaspar, C.W. and Luchansky, J.B. (1995). Use of pulse-field gel electrophoresis to link sporadic cases of invasive listeriosis with recalled chocolate milk. *Applied and Environmental Microbiology.* **61**: 3177-3179

Quere, F., Deschamps, A. and Urdaci, M.C. (1997). DNA probes and PCR-specific reactions for *Lactobacillus plantarum*. *Journal of Applied Microbiology.* **82**: 783-790

Rahkio, T.M. and Korkeala, H.J. (1997). Airborne bacteria and carcass contamination in slaughterhouses. *Journal of Food Protection.* **60**: 38-42

Ranson, S.L., Walker, D.A. and Clarke, I.D. (1957). The inhibition of succinic oxidase by high CO_2 concentrations. *Biochemical Journal.* **66**: 57

Rao, D.N. and Ramesh, B.S. (1992). The microbiology of sheep carcasses processed in a modern Indian abattoir. *Meat Science* **32**: 425-436

Ratkowsky, D.A., Lowry, R.K., McMeekin, T.A., Stokes, A.M. and Chandler, R.E. (1983). Model for bacteria culture growth rate throughout the entire biokinetic temperature range. *Journal of Bacteriology.* **154**: 1222-1226

Ratkowsky,. D.A., Olley, J., McMeekin, T.A. and Ball, A. (1982). Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology.* **149**: 1-5

Raven, J. A. and Beardall, J. (1980). The intrinsic permeability of biological membranes to H^+. Significance for the efficiency of low rates of energy transformations. *FEMS Microbiological letters.* **10**: 1-5

Ray, B. (1986). Impact of bacterial injury and repair in food microbiology: Its past, present and future. *Journal of Food Protection.* **49**: 651-655

Reitchardt, W. and Morita, R.Y. (1982). Temperature characteristics of psychrotrophic bacteria and psychrophilic bacteria. *Journal of General Microbiology.* **128**: 565-568

Rementizs, J. and Samelis, J. (1996). Rapid GC analysis of fatty acids for characterizing *Lactobacillus sake* and *Lact. curvatus* strains of meat origin. *Letters in Applied Microbiology.* **23**: 379-384

Renwick, S.A., McNab, W.B., Lowman, H.R. and Clarke, R.C. (1993). Variability and determinants of carcass bacterial load at a poultry abattoir. *Journal of Food Protection.* **56**: 694-699

Restaino, L., Komatus, K.K. and Syracause, M.J. (1981). Effects of acids on potassium sorbate inhibition of food related microorganisms in culture media. *Journal of Food Science* 47:134-138

Rhuland, L.E., Work, E., Denman, R.F. and Hoare, D.S. (1955). The behavior of the isomers of a,Î-diaminopimelic acid on paper chromatography. *Journal of the American Chemical Society.* **77**: 4844-4846

Richard, C. (1986). Genus VI. *Enterobacter* Hermache and Edwards 1960, 72^{AL}, nom cons. Opin. 28, Jud. Com. 1963.38. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 465-469

Ridell, J. and Korkeala, H. (1997). Minimum growth temperatures of *Hafnia alvei* and other Enterobacteriaceae isolated from refrigerated meat determined with temperature gradient incubator. *International Journal of Food Microbiology.* **35**: 287-292

Ridell, J., Sitonen, A., Paulin, L., Lindroos, O., Korkeala, H. and Albert, M.J. (1995). Characterization of *Hafnia alvei* by biochemical test, random amplified polymorphic DNA PCR, and partial sequencing of 16S rRNA gene. *Journal of Clinical Microbiology.* **33**: 2372-2376

Robach, M.C. (1978). Effect of potassium sorbate on the growth of *Pseudomonas fluorescens*. *Journal of Food Science* **43**: 1886-1887

Robach, M.C. and Pierson, M.D.(1978). Influence of para-hydroxybenzoic acid esters on the growth and toxin production of *Clostridia botulinum* 10755A. *Journal of Food Science* **43**: 787-789,792

Rodrigues, U.M., Aguire, M., Facklam, R.R. and Collins, M.D. (1991). Specific and intraspecific molecular typing of lactococci based on polymorphism of DNA encoding RNA. *Journal of Applied Microbiology.* **71**: 509-516

Rodrigues-Szule F, U.M., Ventoura, G., Mackay, B.M. and Payne, M.J. (1990). Rapid physiochemical detachment, separation and concentration of bacteria from beef surfaces. *Journal of Applied Bacteriology.* **80**: 673-681

Rodtong, S. and Tannock, G.W. (1993). Differentiation of *Lactobacillus* strains by ribotyping. *Applied and Environmental Microbiology.* **59**: 3480-3484

Rogosa, M., Mitchell, J.A. and Wiseman, R.F. (1951). A selective medium for the isolate of oral and faecal lactobacilli. *Journal of Bacteriology.* **62**: 132-133

Roller, S.D. and Anagnostopoulos, G.D. (1982). Accumulation of carbohydrate by *Escherichia coli* B/r/1 during growth at low water. *Journal of Applied Bacteriology* **52**: 425-434

Roman, S.B., Carson, L.A., O'Hara, C.M., Pegues, D.A and Miller, M. (1991). Comparison of four identification methods used to identify *Pseudomonas cepacia* isolated from sputum of cyctic fibrosis patients. *Abstracts of the 91st General meeting of the American Society for Microbiology* **pp** 379

Rose, A.H. (1968). Physiology of microorganisms at low temperatures. *Journal of Applied Microbiology.* **31**: 1-11

Ross, T. and McMeekin, T.A. (1994). Predictive microbiology. *International Journal of Food Microbiology.* **23**: 241-264

Rosselló-Mora, R.A., Lalucat, J., Dott, W. and Kampfer, P. (1994). Biochemical and chemotaxonomic characterization of *Pseudomonas stutzeri* genomovars. *Journal of Applied Bacteriology.* **76**: 226-233

Rousset, S. and Renerre, M. (1991). Effect of CO_2 or vacuum packaging on normal and high pH meat shelf-life. *International Journal of Food Science and Technology.* **26**: 641-652

Rowe, B. and Gross R.G. (1986). Genus II. *Shigella* Castenllani and Chalmers 1919, 936^{AL}. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 423-427

Roy, D., Ward, P. and Champagne, G. (1996). Differentiation of bifidobacteria by use of pulsed-field gel electrophoresis and polymerase chain reaction. *International Journal of Food Microbiology.* **29**: 11-29

Ruis, N. and Lorén, J.G. (1998). Buffering capacity and membrane H+ conductance of neutrophilic and alkalophilic Gram positive bacteria. *Applied and Environmental Microbiology.* **64**: 1344-1349

Russell, A.D. (1991). Mechanism of bacteria resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. *Journal of Applied Bacteriology.* **71**:191-201

Saide-Albornoz, J.J., Knipe, C.L., Murano, E.A. and Beran, G.W. (1995). Contamination of pork carcasses during slaughter, fabrication, and chilled storage. *Journal of Food Protection.* **58**: 993-997

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science.* **239**: 487-491

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985). Enzymic amplification of β-globulin genomic sequences and restriction analysis for diagnosis of sickle cell anemia. *Science.* **230**: 1350-1354

Sakazaki, R. (1981). The genus *Hafina*. *In*: The Prokaryotes Volume II. *Eds*: M.P. Starr, H. Stlop, H.G. Trüper, A. Balows and H.G. Schlegel. Spinger-Verlag, Berlin Heidelberg New York. **pp** 1181-1186

Sakazaki, R. (1986). Genus IX. *Hafnia* Møller 1954, 272^{AL}. *In:* Bergey's Manual of Systematic Bacteriology Volume I. *Eds* N.R. Frieg and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 484-486

Salama, M., Sandine, W. and Giovannoni, S. (1991). Development and application of oligonucleotide probes for identification of *Lactococcus lactis* subsp. *cremoris*. *Applied and Environmental Microbiology.* **57**: 1313-1318

Salama, M.S., Sandine, W.E. and Giovannoni, S.J. (1993). Isolation of Lactococcus lactis subsp. cremoris from nature by colony hybridization with rRNA probes. *Applied and Environmental Microbiology.* **59**: 3941-3945

Samadpour, M., Grimm, L.M., Desai, B., Alfi, D., Ongarth, J.E. and Tarr, P.I. (1993). Molecular epidemiology of *Escherichia coli* 0157:H7 strains by bacteriophage λ restriction fragment length polymorphism analysis: application to multistate food-borne outbreak and a day-care centre cluster. *Journal of Clinical Microbiology.* **31**: 3179-3183

Sambrook, J., Fitsch, E.J. and Maniatus, T. (1989). Molecular cloning. A laboratory manual. *Eds*: N. Ford, C. Nolan and M. Ferguson. 2nd Edition. Cold Springs Harbour Press.

Samelis, J., Maurogenakis, F. and Metaxopoulos, J. (1994). Characterization of lactic acid bacteria isolated from naturally fermented Greek dry salami. *International Journal of Food Microbiology.* **23**: 179-196

Samore, M.J., Kristjansson, M., Venkataraman, L., DeGiroami, P.C. and Arbeit, R.D. (1996). Comparison of arbitrarily-primed polymerase chain reaction, restriction enzyme analysis and pulsed-field gel electrophoresis for typing of *Clostridium difficile*. *Journal of Microbiological Methods.* **25**: 215-224

Saucier, L., Poon, A. and Stiles, M.E. (1995). Induction of bacteriocin in *Carnobacterium piscicola* LV17. *Journal of Bacteriology.* **78**: 684-689

Saulnier, P., Bournex, C., Prévost, G. and Andremont, A. (1993). Randomly amplified polymorphic DNA assay is less discriminatory than pulsed-field gel electrophoresis for typing methicillin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology.* **31**: 982-985

Schaffer-Seidler, C.E., Judge, M.D., Cousin, M.A. and Aberle, E.D. (1984). Microbial contamination and primal cut yields of skinned and scalded pork carcasses. *Journal of Food Science.* **49**: 356-358

Schillinger, U. and Holzapfel, W.H. (1990). Antimicrobial activity of carnobacteria. *Food Technology.* **7**: 305-310

Schillinger, U. and Lück, F.-K. (1987). Identification of lactobacilli from meat products. *Food Microbiology.* **4**: 199-208

Schillinger, U., Stiles, M.E. and Holzapfel, W.H. (1993). Bacteriocin production by *Carnobacterium piscicola* LV 61. *International Journal of Food Microbiology.* **20**: 131-147

Schoolfield, R.M., Sharpe, P.H.J. and Magnuson, C.E. (1981). Non-linear regression of biological temperature-dependent rate models based on absolute reaction-rate theory. *Journal of Theoretical Biology.* **88**: 719-731

Schwartz, D.C. and Cantor, C.R. (1984). Separation of yeast chromosome-sized DNA by pulsed field gel electrophoresis. *Cell.* **37**: 67-75

Scott, V.N. (1989). Interactions of factors to control microbial spoilage of refrigerated foods. *Journal of Food Protection.* **52**: 431-435

Scriven, F.M. and Singh, R. (1986). Comparison of the microbial populations of retail beef and pork. *Meat Science.* **18**: 173-180

Seideman, S., Carpenter, Z.L., Smith, G.C., Dill, C.W. and Vanderzant, C. (1979). Physical and sensory characteristics of pork packaged in various gas atmospheres. *Journal of Food Protection.* **42**: 317-322

Seideman, S. and Durland, P.R. (1982). Vacuum packaging of fresh beef: a review. *Journal of Food Quality.* **6**:29-47

Selgas, D., Marin, M.L., Pin, C. and Casas, C. (1993). Attachment of bacteria to meat surfaces: a review. *Meat Science.* **34**:265-273

Seman, D.L., Drew, K.R., Clarken, P.A. and Littlejohn, R.P. (1988). Influence of package methods and length of chilled storage on microflora, tenderness and colour stability of venison loins. *Meat Science.* **22**: 267-287

Serwer, P. and Hayes, J.S. (1986). Exclusion of spheres by agarose gels during agarose-gel electrophoresis-dependence on the sphere radius and the gels concentration. *Analytical Biochemistry.* **158**: 72-78

Shaw, B.G. and Harding, C.D. (1984). A numerical taxonomic study of lactic acid bacteria from vacuum-packaged beef, pork, lamb and bacon. *Journal of Applied Bacteriology.* **56**: 25-40

Shaw, B.G. and Latty, J.B. (1982). A numerical taxonomy study of *Pseudomonas* strains from spoiled meat. *Journal of Applied Bacteriology.* **52**: 219-228

Shaw, B.G. and Latty, J.B. (1984). A study of the relative incidence of different *Pseudomonas* groups on meat using a computer-assisted identification technique employing only carbon source tests. *Journal of Applied Bacteriology.* **57**: 59-67

Shaw, B.G. and Latty, J.B. (1988) A numerical taxonomic study of non-motile, non-fermentative, Gram-negative bacteria from foods. *Journal of Applied Bacteriology.* **65**: 7-21

Shay, B.J. and Egan, A.F. (1987). The packaging of chilled red meats. *Food Technology in Australia.* **39**: 283-285

Sheridan, J.J., Doherty, A.M., Allen, P., McDowell, D.A., Blair, I.S. and Harrington, D. (1997). The effect of vacuum and modified atmosphere packaging on the shelf-life of lamb primals, stored at different temperatures. *Meat Science.* **45**: 107-117

Sheridan, J.J. and Lynch, B. (1979). Effect of microbial contamination of the storage of beef carcasses in an Irish meat factory. *Irish Journal of Food Science and Technology.* **3**: 43-52

Sheridan, J.J. and Lynch, B. (1980). Effect of commercial storage on the microbiology and keeping-quality of beef carcasses. *Irish Journal of Food Science and Technology.* **5**: 71-85

Sheridan, J.J. and Sherington, J. (1982). The microbiology of hot and conventionally deboned vacuum packed beef. *Meat Science.* 7: 245-258

Silliker, J.H. (1981). The influence of atmosphere containing elevated levels of CO_2 on growth of psychrotrophic organisms in meat and poultry. *In*: Psychrotrophic microorganisms in spoilage and pathogenicity. *Eds* T.A. Roberts, G. Hobbs, J.H.B. Christian and N. Skovgaard. Academic Press, London New York Toronto Sydney San Francisco. **pp**. 369-375

Silliker, J.H., Woodruff, R.E., Lugg, J.R., Wolfe, S.K. and Brown, W.D. (1977). Preservation of refrigerated meats with controlled atmospheres: treatment and post-treatment effects of carbon dioxide on pork and beef. *Meat Science* **1**: 195-204

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

Siragusa, G.R. and Cutter, C.N. (1993). Brochocin-C, a new bacteriocin produced by *Brochothrix campestris.* Applied and Environmental Microbiology. 59: 2326-2628

Siragusa, G.R. and Cutter, C.N. (1995). Microbial ATP bioluminescence as a means to detect contamination of artificially contaminated beef carcass tissue. *Journal of Food Protection.* **58**: 764-769

Skirdal, I.M. and Eklund, T. (1993). Microculture model studies on the effect of sorbic acid on *Penicillum chryogenum, Cladosporioides* and *Ulocladium atrum* at different pH levels. *Journal of Applied Bacteriology.* **74**: 191-195

Skov, M.N., Pedersen, K. and Laurits Larsen, J. (1995). Comparison of pulsed-field gel electrophoresis, ribotyping, and plasmid profiling for typing of *Vibrio anguillarum* serobav O1. *Applied and Environmental Microbiology.* **61**: 1540-1545

Skovgaard, N. (1985). *Brochothrix thermosphacta*: comments on its taxonomy, ecology and isolation. *International Journal of Food Microbiology.* **2**: 71-79

Smith, C.L. and Condemine, G. (1990). New approaches for physical mapping of small genomes. *Journal of Bacteriology.* **172**: 1167-1172

Smith, H.O., Danner, D.B. and Deich, R.A. (1981). Genetic transformations. *Annual Reviews in Biochemistry.* **50**: 41-68

Smith, P.B., Tomfohrole, K.M., Rhoden, D.L. and Balows, S.A. (1971). Evaluation of modified R/B system for identification of Enterobacteriaceae. *Applied Microbiology.* **22**: 928-929

Sneath, P.A.H. (1986). Bacterial classification II. Numerical taxonomy. *In:* Bergey's Manual of Systematic Bacteriology Volume II. *Eds* P.A.H. Sneath, N.S. Mair, M.E. Shapre and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 969-971

Sneath, P.A.H. and Jones, D. (1976). *Brochothrix*, a new genus tentatively placed in the family *Lactobacillaceae*. *Journal of Systematic Bacteriology.* **26**: 102-104

Sneath, P.A.H. and Jones, D. (1986). Genus *Brochothrix* Sneath and Jones 1976, 102^{AL}. *In:* Bergey's Manual of Systematic Bacteriology Volume II. *Eds* P.A.H. Sneath, N.S. Mair, M.E. Shapre and J.G. Holt. Williams and Wilkinson Baltimore London Los Angeles Sydney. **pp** 1249-1253

Sneath, P.H.A., Stevens, M. and Sakin, M.J. (1981). Numerical taxonomy of *Pseudomonas* based on published records of substrate utilization. *Antonie van Leeuwenhoek.* **47**: 423-448

Snijders, J.M., Janssen, M.W.H., Greats, G.E. and Corstiaesen, G.P. (1984). A comparative study of sampling techniques for monitoring carcass contamination. *International Journal of Food Microbiology.* **1**: 229-236

Sofos, J.N., Pierson, M.D., Blocher, J.C. and Busta, F.F. (1986). Mode of action of sorbic acid on bacterial cells and spores. *International Journal of Food Microbiology.* **3**: 1-17

Sperber, W.H. (1983). Influence of water activity on foodborne bacteria-a review. *Journal of Food Protection.* **46**: 142-150

Sperber, W.H. and Swan, J. (1976). Hot-loop test for the determination of carbon dioxide production from glucose by lactic acid bacteria. *Applied and Environmental Microbiology.* **31**: 990-991

Stackebrandt, E. and Goebel, B.M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology.* **44**: 846-849

Stainer, R.Y., Adelberg, E.A. and Ingraham, J.L. (1979). General Microbiology. 4th Edition. MacMillan Press Ltd., Basingstoke, England.

Stamer, J.R. (1979). The lactic acid bacteria: microbes of diversity. *Food Technology.* **33**: 60-65

Stanbridge, L.H. and Board, R.G. (1994). A modification of the *Pseudomonas* selective media, CFC, that allows differentiation between meat pseudomonads and *Enterobacteriaceae*. *Letters in Applied Microbiology.* **18**: 327-328

Stannard, C.J., Williams, A.P. and Gibbs, P.A. (1985). Temperature/growth relationships for psychrotrophic food-spoilage bacteria. *Food Microbiology.* **2**: 115-122

Stead, D.E. (1992). Grouping of plant pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. *International Journal of Systematic Bacteriology.* **42**: 281-295

Steele, M., McNab, B., Fruhner, L., DeGrandis, S., Woodward, D. and Odumeru, J.A. (1998). Epidemiological typing of *Campylobacter* isolated from meat processing plants by pulsed-field gel electrophoresis, fatty acid profile typing, serotyping, and biotyping. *Applied and Environmental Microbiology.* **64**: 2346-2349

Stellwagen, N. C. (1985). Orientation of DNA molecules in agarose gels by pulse electric-fields. *Journal of Biomolecular Structure and Dynamics.* **3**: 299-314

Stoffels, G., Nes, I.F. and Guomundsdottir, A. (1992). Isolation and properties of a bacteriocin producing *Carnobacterium piscicola* isolated from fish. *Journal of Applied Bacteriology.* **73**: 309-316

Stolp, H. and Gadkari, D. (1981). Non-pathogenic members of the genus *Pseudomonas*. *In*: The Prokaryotes Volume I. *Eds*: M.P. Starr, H. Stlop, H.G. Trüper, A. Balows and H.G. Schlegel. Spinger-Verlag, Berlin Heidelberg New York. **pp** 719-741

Suwanto, A. and Kaplan, S. (1989). Physical and genetic mapping on the *Rhodobacter sphaeoides* 2.4.1 genome: presence of two unique circular chromosomes. *Journal of Bacteriology.* **171**: 5850-5859

Szabo, E.A., Pemberton, J.M. and Desmarchelier, P.M. (1992). Specific detection of *Clostridium botulinum* type B toxin by using the polymerase chain reaction. *Applied and Environmental Microbiology.* **58**: 418-420

Talon, D., Dupont, M.J., Lesne, J., Thouverez, M. and Michel-Briand, Y. (1996). Pulse-field gel electrophoresis as an epidemiological tool for clonal identification of *Aeromonas hydrophila*. *Journal of Applied Bacteriology.* **80**: 277-282

Talon, R., Champomier, M.-C. and Montel, M.-C. (1990). DNA-rDNA hybridization studies among *Brochothrix* spp. and some other Gram-positive bacteria. *International Journal of Systematic Bacteriology.* **40**: 464-466

Talon, R., Grimont, P.A.D. and Grimont, F. (1988). *Brochothrix campestris* sp. nov. *International Journal of Systematic Bacteriology* **38**: 99-102

Talon, R., Papon, M., Bauchart, D., Duboisset, F. and Montel, M.-C. (1992). Lipolytic activity of *Brochothrix thermosphacta* on natural triglycerides. *Letters in Applied Microbiology.* **14**: 153-157

Tamoka, J., Ha, D.M. and Kemagata, K. (1987), Reclassification of *Pseudomonas acidovorans* den Dooren de Jamg 1926 and *Pseudomonas testeroni*: comb. Nov. with emended description of the genus *Comamonas*. *International Journal of Systematic Bacteriology.* **37**: 52-59

Tamura, Y., Takahashi, T., Zarkasie, K., Nakamura, M. and Yoshimura, H. (1993). Differentiation of *Erysipelothrix rhusiopathiae* and *Erysipelothrix tonsillarum* by sodium, dodecyl-polyacrylamide gel electrophoresis of cell proteins. *International Journal of Systematic Bacteriology.* **43**: 111-114

Tan, K.H. and Gill, C.O. (1982). Physiological basis of CO_2 inhibition of a meat spoilage bacterium, *Pseudomonas fluorescens*. *Meat Science.* 7: 9-17

Tanskanen, E.I., Tulloch, D.L., Hiller, A.J. and Davidson, B.E. (1990). Pulse-field electrophoresis of *Sma*I digests of Lactococcal genomic DNA, a novel method of strain identification. *Applied and Environmental Microbiology.* **56**: 3105-3111

Taylor, D.E., Eaton, M., Chang, N. and Salama, S.M. (1992). Construction of a *Helicobacter pylori* genome map and demonstration of diversity at the genome level. *Journal of Bacteriology.* **174**: 6800-6806

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelson, P.A., Murry, B.E., Persing, D.H. and Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulse-field gel electrophoresis: criteria for bacteria strain typing. *Journal of Clinical Microbiology.* **33**: 2233-2239

Thomas, C.J. and McMeekin, T.A. (1980). Contamination of broiler carcass skin during commercial processing procedures: an electron microscopic study. *Applied and Environmental Microbiology.* **40**: 133-144

Thomas, C.J. and McMeekin, T.A. (1981). Attachment of *Salmonella* spp. to chicken muscle surfaces. *Applied and Environmental Microbiology.* **42**: 130-134

Thomas, L.V., Wimpenny, J.W.T. and Peters, A.C. (1991). An investigation of the effects of four variables on the growth of *Salmonella typhimurium* using two types of gradient gel plates. *International Journal of Food Microbiology.* **14**: 261-275

Thomas, L.V., Wimpenny, J.W.T. and Peters, A.C. (1992). Testing multiple variables on the growth of a mixed inoculum of *Salmonella* strains using gradient plates. *International Journal of Food Microbiology.* **15**: 165-175

Thompson, D.P., Metevia, L. and Vessel, T. (1993). Influence of pH alone and in combination with phenolic antioxidants on growth and germination of mycotoxigenic species of *Fusarium* and *Penicillium*. *Journal of Food Protection.* **56**: 134-138

Thompson, I.Q. and Collins-Thompson, D.L. (1986). Iron requirements of *Brochothrix thermosphacta*. *International Journal of Food Microbiology.* **3**: 299-309

Thornhill, R.H., Burgess, J.G. and Matsunaga, T. (1995). PCR for direct detection of indigenous uncultured magnetic cocci in sediment and phylogenetic analysis of amplified 16S ribosomal DNA. *Applied and Environmental Microbiology.* **61**: 495-500

Thornley, M.J. (1967). A taxonomic study of *Acinetobacter* and related genera. *Journal of General Microbiology.* **49**: 211-257

Torriani, S., Van Reenen, C.A., Klein, G., Reuter, G., Dellaglio, F. and Dicks, L.M.T. (1996). *Lactobacillus curvatus* subsp. *curvatus* subsp. nov. and *Lactobacillus curvatus* subsp. nov. and *Lactobacillus sake* subsp *sake*. nov. and *Lactobacillus sake* subsp. *carnosus* subsp. nov., new subspecies of *Lactobacillus curvatus* Abo-Enaga and Kandler 1965 and *Lactobacillus sake* Katagiri, Kitahara, and Fukami 1934 (Klein *et al.*

1996, Emended descriptions), respectively. *International Journal of Systematic Bacteriology.* **46**: 1158-1163

Trüper, H.G. and De Clari, L. (1997). Taxonomic note: necessary corrections of specific epithets formed as substansives (nouns) "in apposition". *International Journal of Systematic Bacteriology.* **47**: 908-909

Tsay, W.I. and Chou, C.C. (1989). Influence of potassium sorbate on the growth of *Yersinia enterocolitica*. *Journal of Food Protection.* **52**: 723-726

Tsen, H.-Y., Yu, G.-K. and Hu, H.-H. (1997). Comparison of type A enterotoxigenic *Staphylococcus aureus* strains isolated from geographically far distant locations by pulsed field gel electrophoresis. *Journal of Applied Microbiology.* **82**: 485-493

Tsen, H.-Y., Yu, G.-K. and Lin, I. (1995). Plasmid profiles and pulse-field gel electrophoresis for type A enterotoxigenic *Staphylococcus aureus* isolated from foods. *Journal of Food Protection.* **58**:147-153

Tsuchiya, Y., Kaneda, H., Kano, Y. and Koshino, S. (1992). Detection of beer spoilage by polymerase chain reaction technology. *American Society of Brewing Chemists Journal.* **50**: 64-67

Turpin, P.E., Maycroft, K.A., Rowlands, C.L. and Wellington, E.M.H. (1993). Viable but non-culturable salmonellas in soil. *Journal of Applied Bacteriology.* **74**: 421-427

Ullman, J.S. and McCarthy, (1973). The relationship between mismatched base pairs and the theromstablitiy of DNA duplexes. *Biochimica Biophysica Acta.* **294**: 416-424

Untermann, F., Stephan, R., Dura, U., Hofer, M. and Heimann, P. (1997). Reliability and practicability of bacteriological monitoring of beef carcass contamination and their rating with a hygiene quality control program of abattoirs. *International Journal of Food Microbiology.* **34**: 67-77

Väisänen, O.M., Mwaisumo, N.J. and Salkinoja-Saknen, M.S. (1991). Differentiation of dairy strains of *Bacillus cereus* group by phage typing, minimum growth temperature and fatty acid analysis. *Journal of Applied Bacteriology.* **70**: 315-324

Valley, G. and Rettger, L.F. (1927). The influence of carbon dioxide on bacteria. *Journal of Bacteriology.* **14**: 101-137

van den Berg, D.J.C., Smits, A., Pot, B., Ledeboer, A.M., Kresters, K., Verbakel, J.M.A. and Verrips, C.T. (1993). Isolation, screening and identification of lactic acid bacteria from traditional food fermentation process and culture collections. *Food Biotechnology.* **7**: 189-205

van Laak, R.L.J.M. and Smulders, F.J.M. (1990). Colour stability of bovine longissimus and psoas major muscle as addedted by electrical stimulation and hot boning. *Meat Science.* **28**: 211-221

van Laak, R.L.J.M., Eikelenboom, G. and Smulders, F.J.M. (1989). Colour stability of hot and cold boned longissimus and psoas mahor muscle. *Journal of Food Protection.* **52**: 894-897

van Netten, P. Huis in't Veld, J.H.J. and Mossel, D.A.A. (1994). The immediate bactericidal effect of lactic acid on meat-borne pathogens. *Journal of Applied Bacteriology.* 77: 490-496

van Netten, P., Perales, I. and Mossel, D.A.A. (1988). An improved selection and diagnostic medium for isolation and counting of *Listeria* spp. *Letters in Applied Microbiology.* 7: 17-21

Varnam, A.H. and Sutherland, J.P. (1995). Conversion of meat into muscle. *In*: Meat and Meat Products. *Eds* A.H. Varnam and J.P. Sutherland. Chamberland and Hall London. **pp**. 46-120

Venkitanarayanan, K.S., Faustman, C., Crivello, J.F., Khan, M.I., Hoagland, T.A. and Berry, B.W. (1997). Rapid estimation of spoilage bacterial load in aerobically stored meat by quantitative polymerase reaction. *Journal of Applied Microbiology.* **82**: 359-364

Venugopal, R.J., Pansare, A.C. and Lewis, N.F. (1984). Inhibitory effect of food preservatives on protease secretion by *Aeromonas hydrophilia. Journal of Food Science.* **49**: 1078-1081

Vogel, R.F., Lohmann, M., Nguyen, M., Weller, A.N. and Hammes, W.P. (1993). Molecular characterization of *Lactobacillus curvatus* and *Lact. sake* isolated from sauerkraut and their application in sausage fermentations. *Journal of Applied Bacteriology.* **74**: 295-300

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

Wallbanks, S., Martinez-Murcia, A.J., Fryer, J.L., Phillips, B.A. and Collins, M.D. (1990). 16S rRNA sequence determination for members of the genus *Carnobacterium* and related lactic acid bacteria and description of *Vagococcus samloninarum* sp. nov. *International Journal of Systematic Bacteriology.* **40**: 224-230

Wang, R.-F., Kao, W.-W. and Johnson, M.G. (1992). 16S rRNA-base probes and polymerase chain reaction to detect *Listeria monocytogenes* cells added to food. *Applied and Environmental Microbiology.* **56**: 2827-2831

Wang, M.Y. and Ogrydzaik, D.M. (1986). Residual effects of storage in the elevated carbon dioxide atmosphere on the microflora of rock cod (*Sebastes* spp). *Applied and Environmental Microbiology.* **52**: 727-732

Wang, Y. and Taylor, D.E. (1990). Natural transformation in *Campylobacter* species. *Journal of Bacteriology.* **172**: 949-955

Warth, A.D. (1989). Relationship between the resistance of yeasts to acetic, proponic and benzoic acids and to methly paraben and pH. *International Journal of Food Microbiology.* **8**: 343-349

Washington II, J.A., Yu, P.W.K. and Martin, W.J. (1971). Evaluation of accuracy of multitest micromethod system for identification of Enterobacteriaceae. *Applied Microbiology.* **22**: 267-269

Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acid Research.* **18**: 7213-7218

Wernars, K., Heuvelman, C.J., Charkraborty, T and Notermans, S.H.W. (1991). Useful polymerase chain reaction for direct detection of *Listeria monocytogenes* in soft cheese. *Journal of Applied Microbiology.* **70**: 121-126

Widders, P.R., Coates, K.J., Warner, S., Beattie, J.C., Morgan, I.R. and Hickey, M.W. (1995). Controlling microbial contamination on beef and lamb meat during processing. *Australian Vetinary Journal.* **72**: 208-211

Wijtzes, T., McClure, P.J., Zwietering, M.H. and Roberts, T.A. (1993). Modeling bacterial growth of *Listeria monocytogenes* as a function of water activity, pH and temperature. *International Journal of Food Microbiology.* **18**: 139-149

Wilkinson, B.J. and Jones, D. (1977). A taxonomic survey of *Listeria* and related bacteria. *Journal of General Microbiology.* **98**: 399-421

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research.* **18**: 6531-6535

Williamsky, G., bang, H., Fischer, G. and Marahiel, M.A. (1992). Characterization of *csp*B, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperature. *Journal of Bacteriology.* **174**: 6326-6335

Wilson, I.G., Cooper, J.E. and Gilmour, A. (1991). Detection of enterotoxigenic *Staphylococcus aureus* in dried skimmed milk: use of polymerase chain reaction for amplification and detection of staphylococcal enterotoxin genes *ent*B and *ent*C. *Applied and Environmental Microbiology.* **57**: 1793-1798

Worfel, R.C., Sofos, J.N., Smith, G.C., Morgan, J.B. and Schmidt, G.R. (1995). Microbial contamination of condensates formed on superstructures of wood and other materials in meat plants. *Dairy, Food and Environmental Sanitation.* **15**: 430-434

Worobo, R.W., Van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. (1995). A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. *Journal of Bacteriology.* **177**: 3143-3149

Woolthus, C.H.J. and Smulders, F.J.M. (1985). Microbial decontamination of calf carcasses by lactic acid sprays. *Journal of Food Protection.* **48**: 832-837.

Yakubu, D.E. and Pennington, T.H. (1995). Epidemiological evaluation of *Neisseria meingitidis* serogroup B by pulsed-field gel electrophoresis. *FEMS Immunology and Medical Microbiology.* **10**: 185-190

Young, K.M. and Foegeding, P.M. (1993). Acetic, lactic and citric acids and pH inhibition of *Listeria monocytogenes* ScottA and the effects on intracellular pH. *Journal of Applied Bacteriology.* **74**: 515-520

Young, L.L., Reviere, R.D. and Cole, A.B. (1988). Fresh red meats: a place to apply modified atmospheres. *Food Technology.* **42**: 65-69

Zaika, L.L., Phillips, J.G. and Buchanan, R.L. (1992). Model for aerobic growth of *Shigella flexneri* under various conditions of temperature, pH, sodium chloride and sodium nitrite concentrations. *Journal of Food Protection.* **55**: 509-513

Zamora, M.C. and Zaritzky, N.E. (1987). Potassium sorbate inhibition on microorganisms growing on refrigerated packaged beef. *Journal of Food Science* **52**:257-262

Zeitoun, A.A.M. and Debevere, J.M. (1990). The effect of treatment with buffered lactic acid on microbial decontamination and on shelf life. *International Journal of Food Microbiology.* **11**: 305-312

Zeitourn, A.A.M. and Debevere, J.M. (1992). Decontamination with lactic acid/sodium lactate buffer in combination with modified atmosphere packaging effects on the shelf life of fresh poultry. *International Journal of Food Microbiology.* **16**: 89-98

Zeng, A.-P., Biebl, H. and Deckwer, W.-D. (1990). Effect of pH and acetic acid on growth and 2,3-butanediol production of *Enterobacter aerogenes* in continuous culture. *Applied and Microbiology Biotechnology.* **33**: 485-489

Zheng, W. and Kathariou, S. (1994). Transposon-induced mutants of *Listeria monocytogenes* incapable of growth at low temperature (4°C). *FEMS Microbiology Letters.* **121**: 287-292

Zheng, W. and Kathariou, S. (1995). Differentiation of epidemic-associated strains of *Listeria monocytogenes* by restriction fragment length polymorphism in a gene region essential for growth at low temperatures (4°C). *Applied and Environmental Microbiology.* **61**:4310-4314

Zuniga, M., Pardo, I. and Ferrer, S. An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. *International Journal of Food Microbiology* 18:37-42, 1993.

Zwietering, M.H., Jongenburger, I., Rombout, F.M. and van Reit, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology.* **56**: 1875-1881