Predicting the Growth of *Pseudomonas* in Minced Beef

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L. Kamperman
December, 1994

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ABSTRACT

Predictive microbiology offers an alternative to traditional microbiological methods of assessing the quality and safety of foods. The development of mathematical models enables prediction of the rate of growth (or decline) of microorganisms under a given set of environmental conditions.

The spoilage of minced beef at refrigeration temperatures is mainly due to *Pseudomonas* spp. Strains of *Pseudomonas* isolated from minced beef purchased from retail outlets in Tasmania were studied together with strains isolated from pork in Victoria. Growth rates of all strains were compared at ~10°C and the fastest growing strains selected for further study to represent the 'worst-case' scenario. The growth characteristics of these strains were modelled in nutrient broth in the temperature range ~0° to 30°C, using turbidimetric measurements. Data were analysed and fitted to square root - type models (Ratkowsky *et al.* 1982).

Models were calibrated by comparing the turbidimetric data with data obtained by viable counting, the latter being the standard method of enumerating microorganisms in foods. The relationship between growth rates obtained from turbidimetric methods and those obtained from viable counting was found to be constant, therefore models constructed by turbidimetry were calibrated using a simple factor.

Models developed in the laboratory were validated in minced beef. Mince from retail outlets had initial levels of pseudomonads ranging from 10^5 to 10^7 cfu/g. These levels were too high to enable the true growth rates to be monitored. To achieve the desired starting level (10^4 cfu/g or less) mince, aseptically prepared in the laboratory, was inoculated with individual strains or blended with retail products. Evaluation of the model, using the bias and precision indices (Ross 1993a), revealed a difference of less than 10% on average between predicted and observed values. At temperatures below 10°C the observed rates of pseudomonad proliferation in meat were as predicted by the model in broth. However, above 10°C rates were slower than predicted indicating that some other factor may be involved.

No lag phase was detected when sterile meat was inoculated with pseudomonad broth suspension pre - incubated at 25°C for 48 h, or with minced beef purchased from a local butcher.

The effect of pH on the rate of growth of several *Pseudomonas* strains was also examined, using lactic acid as the acidulant. The strains grew at similar rate between pH 5.4 and 8.6, with no growth below 5.0. As the initial pH of fresh meat is generally ~5.5 - 6, pH will not be a critical factor affecting the growth rate of pseudomonads.

At refrigeration temperatures, the fastest growing *Pseudomonas* strains will dominate the meat microbiota and outcompete all the other organisms. To examine if the growth of other organisms can inhibit pseudomonads, *Pseudomonas* strains were grown (~4 - 20°C) in mixed cultures with microorganisms isolated from mince. Isolates tested included Enterobacteriaceae, *Lactobacillus* spp., *Brochothrix thermosphacta*, and *Staphylococcus aureus*. No interactions were evident as *Pseudomonas* grew at the rate predicted in a cocktail containing the above organisms.

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"Glory to God in the highest" (Luke 2:14).

CONTENTS

PUBLICATIONS

Refereed Paper

Dalgaard, P., Ross, T., Kamperman, L., Neumeyer, K. and McMeekin, T.A. (1994). Estimation of bacterial growth rates from turbidimetric and viable count data. *International Journal of Food Microbiology*, **23**: 391-404.

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Refereed Abstract

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Non-refereed Paper

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Conference Paper

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Conference Posters

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1 LITERATURE REVIEW

1.1 INTRODUCTION

"Meat is the post-mortem aspect of the three hundred or so anatomically distinct muscles of the body" (Lawrie 1975). "It continues to be a major food commodity, and is likely to be important in the human diet for as long as can be foreseen in the future. It provides both satisfaction in eating and sound nourishment for a significant proportion of the world's population" (Cole and Lawrie 1975).

The major components of lean meat are: water (~75%), proteins (~20%), lipid (~3%), soluble small molecular weight compounds (~1-5 %) and soluble carbohydrates (< 0.2%) (Gill 1983, Lambert *et al.* 1991, Macrae *et al.* 1993). Proteins and lipids must be degraded before they can be utilised by bacteria. Most are insoluble and are therefore not readily available for microbial attack. Minor components include vitamins, enzyme pigments, flavour compounds and soluble organic compounds such as amino acids, lactic acid, glycogen, glucose, glucose-6-phosphate, creatine, nucleotides and their degradation products and peptides (carnosine and anserine) (Gill 1983).

Unexposed muscle tissues of healthy animals are free from spoilage bacteria. However the soiled hides, hooves, claws and expelled faeces of slaughtered animals are sources of carcass contamination during slaughter. Bacterial contamination from these sources is spread to the carcass surface via contact with knives, saws and meat handlers during carcass dressing and cooling. Additional contamination is due to air, water and soil at various stages of processing (Ayres 1963, Newton *et al.* 1978). Handling during carcass breaking and transport also contributes to the level of contamination (Greer 1984).

Spoilage of fresh meat is determined by temperature, degree of access of oxygen, available moisture, pH, the structural condition of meat, indigenous enzymes and the presence and type of microorganisms (Ingram and Dainty 1971, Mossel *et al.* 1975).

1.2 MEAT SPOILAGE

1.2.1 Processes

The bulk composition (water / protein / fat) of meat is unaffected by the development of rigor. Large changes occur in the concentrations of some low molecular weight soluble components. When the blood circulation fails at death, the muscle rapidly becomes anoxic and oxidative metabolism ceases. Glycogen is broken down to glucose, which is then converted to pyruvate through glycolysis. In the absence of oxygen, lactic acid accumulates causing a fall in pH. The final muscle

pH is determined by the amount of lactic acid accumulating. In muscle with a large initial store of glycogen, an ultimate pH of 5.5 can be attained (from ~7.2 pre - rigor) (Gill 1982).

Microorganisms cannot penetrate into muscle tissues until proteolytic enzymes are produced during late exponential phase, therefore growth occurs mainly at the meat surface (Gill and Penney 1977, Ingram and Dainty 1971). Substrates utilised preferentially by the spoilage biota can become depleted at the surface if the rate of diffusion of the substrate from within the meat is slower than the rate at which it is utilised by the bacteria.

Initially bacteria use simple sugars such as glucose as energy source. When bacterial cell density increases to ~10^8 cm^{-2}, the concentration of glucose at the surface decreases. The rate of diffusion from within the meat to the surface is too slow to meet the demand and the microorganisms attack the secondary substrates (Gill 1983). Amino acids are degraded, volatile substances are produced and the pH of meat rises. Undesirable products formed cause off-odours and flavours. These include organic amines (cadaverine, putrescine, isobutylamine) and sulphides (dimethylsulphide and dimethyldisulphide).

Organoleptic spoilage and slime are noticeable on meat when bacterial numbers reach 10^7 cm^{-2} and 10^8 cm^{-2} respectively (Ayres 1951, Ayres 1960, Dainty *et al.* 1985, Dainty and Mackey 1992). The final bacterial density (>10^9 cm^{-2}) appears to be limited by the accumulation of inhibitory products of metabolism or by the rate of oxygen transfer into the slime layer formed on meat (Gill 1976).

1.2.2 Fat tissue

As carcass surfaces tend to lose moisture during chilling, fat surfaces are often too dry to support bacterial growth. Microbial attack is confined to the moist surfaces of fat tissue. Low molecular weight substances are utilised. Nutrients in fat, however, are present in lower concentrations than in the muscle and substrates are therefore exhausted more rapidly on fat surfaces, final bacterial numbers being lower. Attack on triglycerides plays no part in the onset of spoilage (Nottingham *et al.* 1981, Gill 1983).

1.2.3 Ground meats

The process of comminution has major effects on the microbiology of meat. Bacteria originally present only on the surface product are distributed throughout. The greater surface area favours the growth of aerobic bacteria as the total mass of any substrate is readily available. Cells are broken down and the contents are released, acting as a readily available source of nutrients for bacteria. In addition, ground meats often consist of trimmings from various cuts which have been

handled excessively and consequently contain more organisms than uncomminuted meats (Sutherland and Varnam 1982, Jay 1992).

Many have reported high initial counts of bacteria on ground meats, varying from 10^5 to 10^8 depending on the season (Sumner *et al.* 1981, Nychas *et al.* 1991). Sumner *et al.* (1981) surveyed the bacteriological status of minced beef purchased in the Melbourne area (Australia). Counts were higher in summer compared with those in winter. In summer counts exceeded 10^7 / g in 83% of samples from supermarkets.

Although only permitted in ground meat labelled 'sausage meat', sulphur dioxide was found at levels up to 750 mg / kg in unlabelled ground meat meat. SO_2 enhances the storage life by inhibiting the growth of Gram negative microorganisms, improves retention of colour and confers a weaker and less objectionable odour (Sumner *et al.* 1981).

1.3 ENVIRONMENTAL FACTORS

Assuming sufficient nutrients are available, microbial growth in foods is primarily controlled by temperature, pH, water activity and gaseous atmosphere. Therefore, by monitoring storage conditions (temperature and gaseous atmosphere) and intrinsic properties of the food (water activity and pH), food quality and safety may be estimated (McMeekin *et al.* 1993).

Much attention has been focussed on predicting the shelf life of aerobically stored products on the basis of temperature history (McMeekin and Ross 1993). Temperature is probably the most important single factor influencing the growth of bacteria on meat (McMeekin 1982, Nottingham 1982).

1.3.1 Temperature

Temperature not only determines bacterial numbers, but influences the nature of the biota which becomes dominant. The rate of development of the spoilage biota is principally determined by the temperature history experienced by the microbially contaminated product. The maximum storage life is obtained when meat is maintained at -1°C, that being the minimum temperature that can be sustained indefinitely without freezing of the muscle tissue (Pooni and Mead 1984).

There are critical steps in meat processing influencing the microbiological quality of the product. These include: husbandry practices under which the animals are raised, transport and holding conditions between farm and slaughter, hide removal, evisceration, carcass chilling, boning, freezing or chilling of the boneless product and temperature control in transport (Grau 1987). In addition, storage in retail outlets is also likely to influence the quality of products.

The meat processing steps in which the growth of psychrotrophs is most likely to occur are chilling of carcasses prior to boning, transport of carcasses to retail outlets or export, and retail storage.

1.3.1.1 Boning

The cooling of carcasses from body to chiller temperatures should be well controlled, to limit the rapid proliferation of bacteria on warm carcasses. In the conventional processing of meat (cold boning), carcasses are chilled after slaughter for 24 hours and processed in the chilled state (post-rigor). In Australia, the temperature of boning rooms must operate at a temperature not above 10°C for not more than 2 hours and must not rise above 12°C. These temperatures may effectively inhibit the growth of pathogens but it is possible that such requirements are too stringent and waste energy maintaining an unnecessarily low temperature. It is also unpleasantly cold for plant operators to work in these rooms (Smith 1987).

Hot boning involves the processing of meats generally within 1 - 2 hours after slaughter (pre-rigor) while the carcass is still "hot". In general, the microbiology of hot-boned meats is comparable to that of cold-boned meats (Jay 1992, Gill and Jones 1992a). Some report that hot boning operations can be better controlled than the cold boning process as cooling of the product is more uniform (Reichel *et al.* 1991). However, concerns over the hygienic adequacy of hot processes exist as all carcass surfaces inevitably become contaminated with microorganisms that may include pathogenic types.

Large increases in numbers of *E. coli* and salmonellae can occur when hot-boned meat is cartoned and blast frozen as meat at the centre of the carton may remain above 8°C for long periods (Grau 1987). Electrical stimulation of carcasses prior to hot-boning accelerates the post-mortem fall in muscle pH. This could have some effect in reducing the growth of *E. coli* and salmonellae (Grau 1983).

1.3.1.2 Transport of carcasses

Increasingly, carcasses are being broken down at the slaughtering plant, and are wholesaled as vacuum packaged product or bulk packed in 'combo' bins. The latter are heavy duty cardboard cases fitted with a plastic lining bag which hold up to one tonne of product. For the transport of carcasses as hanging meat, quarters or sides are suspended from rails during storage and transport. It is desirable that the surfaces of hanging beef experience the lowest chiller temperatures. However, little information exists on temperature control during transportation.

Gill and Phillips (1993) evaluated the storage efficiencies achieved during transportation of carcasses by rail or road in North America. Their findings suggest that, in railway wagons, chilled air is distributed effectively to all surfaces of

the meat and that the refrigeration successfully reduces the temperature of the warm product within about 24 hours. In refrigerated road trailers, an envelope of chilled air is maintained around the carcass. There is little air movement and the temperature of the warm product decreases slowly. Therefore, this method of transport can only be used, if products are equilibrated to the desired storage temperature prior to loading.

1.3.1.3 Retail display

Temperatures experienced during retail display affect the product storage life. As contamination of raw meat with pathogens is inevitable, there is concern that these organisms may proliferate to hazardous numbers during periods of temperature abuse in display cases (Greer *et al.* 1993, Grau 1987). Greer *et al.* (1993) found that the surface temperature of steaks in commercial display cases in Canada varied from 1.7 to 4.5°C in the coldest portion of the cases to 6.1 to 10°C in the warmest parts.

The growth of *E. coli* on display meat is likely to be negligible when average surface temperatures are $\leq$ 4°C. However, temperatures of $\leq$ 4°C cannot be maintained through existing retail cabinets. Greer *et al.* (1993) suggest routine monitoring of display cases to identify the maximum, average temperature experienced by the product. Specifications could then be set in place to indicate the maximum time that can be tolerated in a case by reference to the maximum, average product temperature. However, by using the maximum average temperature, gross temperature abuses which could occur are not taken into account as they would then be compensated by periods of very cold temperatures. More studies are required to propose good recommendations for the control of meat temperatures during display storage.

1.3.2 pH

The ultimate pH of muscle tissue can vary between 5.5 and 7, the value being largely dependent upon the amount of glycogen present in the tissues at slaughter. In well rested animals, post-rigor muscle pH is between 5.5 and 5.8 and there is usually a reserve supply of glycogen. If glycogen becomes depleted in the muscle of the live animal as a result of stress, starvation or fright prior to slaughter, the amount of lactic acid produced is reduced. The final pH is much higher (> 6.0) and there is a low residual glucose concentration. As the final pH is high, the water holding capacity of the meat remains high, resulting in the muscle having a dark, firm and dry appearance (DFD meat) (Lambert *et al.* 1991).

Most meat spoilage organisms can grow at their maximum rate in the pH range 5.5 - 7.0 (Gill and Newton 1977). Gill (1982) report that *Pseudomonas* spp. dominate the biota of DFD meat in aerobic conditions. However, as glucose is

absent, amino acids are used without delay and spoilage occurs at lower cell densities (> 10^6 cm^{-2}). Microbial degradation of refrigerated beef in an aerobic environment produces a gradual elevation in pH. Fresh meat increases to over 8.0 after two weeks of storage at 5°C (Shelef 1981).

In anaerobic conditions lactobacilli, *Shewanella putrefaciens*, *Enterobacter* spp. and *Yersinia enterocolitica* are the dominant organisms of DFD meat (Lambert *et al.* 1991).

1.3.3 Water activity (a_w)

Water is the major component of meats. Water activity is determined by the nature and concentration of the dissolved chemical species. Fresh beef has a_w of 0.99 - 0.98 (Chirife and Fontan 1982) but on the meat surface this value is frequently reduced by evaporation during chilling and storing. The rate of desiccation of the surface tissue will depend on the differences between the rates of evaporation and diffusion of moisture from the deeper layers of tissue (Scott and Vickery 1939). Fresh beef retains its full water content when stored at 99.3% relative humidity (Scott 1936).

The a_w of meat varies during storage. The rate of airflow during chilling, the air velocity and relative humidity during storage, the presence or absence of moisture - proof wrapping and the addition of salt or other humectants are all contributing factors (Rosset 1982).

When the cold meat surface is exposed to warm air, condensation takes place, and the meat is said to 'sweat'. This condition favours the microbial proliferation as most psychrotrophic microorganisms require high levels of moisture for proliferation. Initially, pseudomonads often appear in damp pockets, such as those between the foreleg and the breast of the carcass or between the hind quarter and flank. When these become dry, microbial activity is reduced and eventually halted (Ayres 1963). Growth of most gram negative organisms is inhibited at a_w below 0.95 (Sperber 1983).

During conventionally cooled carcasses, reduction of a_w by surface drying may be a significant environmental factor in addition to temperature (Scott and Vickery 1939). However, evaporation of water can be restricted by spray chilling carcasses, hot boning and offal chilling (Gill and Phillips 1990).

Packaging of retail cuts is also important as unpacked meat readily loses moisture by evaporation to the atmosphere, becoming dark and unattractive (Nottingham 1982). One of the most common forms of packaging of meat for chilled storage is the use of rigid trays over - wrapped with clear plastic film. A thin film tightly permeable to oxygen, but impermeable to moisture is often used for fresh meat to be stored only 2 or 3 days (Troller and Christian 1978, Shay and Egan 1987).

Water activity manipulation is a major food preservation procedure that is now less favoured because of requirements for reductions in the levels of salts and sugars in foods. Curing and smoking are commonly used to reduce the a_w of meat. The growth of aerobic spoilage organisms of fresh meat is substantially inhibited even in very lightly cured meats having a_w levels near 0.97 (5% NaCl)(Troller and Christian 1978). As pseudomonads are inhibited, cured meat does not putrefy. The biota is mainly composed of micrococci and lactic acid bacteria (Ingram and Dainty 1971).

1.3.4 Gaseous atmosphere

Atmospheres containing increased levels of CO_2 are used commercially to delay or prevent the growth of psychrotrophic pseudomonads and thereby extend the shelf life of meats. At low concentration levels (e.g. in air), carbon dioxide stimulates the growth of many species, however at higher concentrations it becomes inhibiting. Carbon dioxide has been shown to be effective for foods whose spoilage is dominated by Gram - negative, aerobic, psychrotrophic bacteria. The sensitivity varies according to the species. Gill and Tan (1980) report that *Enterobacter* and *Brochothrix thermosphacta* were both unaffected by CO_2. However the rates of *Pseudomonas, Acinetobacter, Yersinia enterocolitica* and *Shewanella putrefaciens* were reduced in the presence of CO_2. Concentrations of carbon dioxide as low as 10% (v/v) can reduce the growth rate of pseudomonads (Eyles *et al.* 1993, Willocx *et al.* 1993).

"The first practical use of modified atmosphere containing elevated levels of carbon dioxide as a preservative in the handling of fresh beef was in the shipment of whole chilled beef carcasses from Australia and New Zealand to Great Britain in the 1930s. By 1938, 26% of Australian beef and 60% of that from New Zealand was being commercially shipped under CO_2 atmosphere" (Silliker and Wolfe 1980).

Gas mixtures used in modified atmosphere packaging usually consist of various combinations of nitrogen, carbon dioxide and oxygen. The commonest combination used is a mixture of CO_2 (20 - 40%) and oxygen (60 - 80%). Carbon dioxide inhibits microbial growth whilst oxygen allows the red colour of the meat to persist for a week or more (Dainty and Mackey 1992).

Eyles *et al.* (1993) studied the effects of modified atmospheres on psychrotrophic pseudomonads in a model system simulating conditions on surfaces of packaged chilled foods. The atmospheres were microaerobic, due to the small amount of residual oxygen present in the finished packages. Although they are aerobic organisms, pseudomonads grew in modified atmospheres with oxygen concentrations as low as 0.1%. The final population density was reduced for cultures

grown in modified atmospheres. This was also observed by Clark and Burki (1972). If cultures that had grown to stationary phase in a modified atmosphere were placed in air at the same temperature, growth resumed quickly and the counts increased to levels achieved by cultures incubated only in air.

In addition to lack of oxygen, the inhibition of pseudomonads on fresh meats vacuum - packaged in oxygen - impermeable plastic films may be due to the accumulated carbon dioxide (forming 10 - 30 % of the gas). Carbon dioxide was shown to be effective against the proliferation of aerobic psychrotrophic Gram - negative bacteria by principally increasing the lag phase and to a lesser extent the generation time (Willocx *et al.* 1993). Eyles *et al.* (1993) suggest that the modified atmospheres have two effects, the presence of CO_2 reduces the growth rate while the limited availability of oxygen reduces the final population.

Studies by Willocx *et al.* (1993) show that there is a synergistic effect of CO_2 at lower temperatures upon the lag time, the maximum growth rate and the maximum population density. The effectiveness of CO_2 inhibition increases with decreasing temperatures. This may be due to the fact that at lower temperatures, the solubility of CO_2 into the medium is increased. Therefore, modified atmosphere storage becomes less effective at higher temperatures as the CO_2 solubility decreases (Ogrydziak and Brown 1982).

1.3.5 Combination of environmental factors

Temperature, pH, water activity and gaseous atmospheres are the major factors controlling the growth of microorganisms. However other factors such as presence of nitrites, sulphites, organic acids and other preservatives may also be important.

Leistner (1992) describes the interaction of environmental factors, combined to prevent growth. This approach has been termed 'hurdle technology'. In most foods, several factors (hurdles) contribute to stability and safety. Environmental factors can be modified and used in combination to inhibit bacterial growth without changing qualities of products. Often, each of the factors by themselves would be considered not capable of preventing growth, but in combination they have an 'additive or even synergistic effect' (Leistner 1992).

Some examples are the inhibition of growth of *Clostridium botulinum* by a combination of pH, a_w and nitrite and the inhibition of *Shigella flexneri* by combining low temperatures with low pH and high NaCl contents (Roberts and Ingram 1973, Zaika *et al.* 1989). In addition, the reduction of a_w can often be minimised without loss of preservation by combining it with other factors, usually reduced pH, and pasteurising heat treatments.

Environmental factors likely to influence the growth of spoilage bacteria on meat carcasses are temperature, a_w, pH and gaseous atmosphere (McMeekin 1982, Nottingham 1982). On minced beef stored aerobically, temperature and pH are the sole factors likely to determine the growth. Although drying may occur on some surfaces of minced beef, water activity may not require consideration as there will be invariably areas where drying is not sufficient to retard bacterial growth. Under these circumstances, the 'worst case scenario' is appropriate.

1.4 MEAT SPOILAGE BIOTA

On freshly dressed carcasses the initial biota is very diverse and is mainly mesophilic (Dainty and Mackey 1992). However, once the temperature of the surface falls below ~10°C, growth of mesophiles will no longer occur and the psychrotrophic biota will gradually displace the micrococci and coliforms. Therefore mesophilic contamination is highest on the slaughter floor and psychrotrophic contamination increases during cutting and boning (Nottingham 1982).

The most common psychrotrophs found on chilled meat are the strict aerobes *Pseudomonas*, *Moraxella* (now known as *Psychrobacter*, Dainty and Mackey 1992), *Acinetobacter*, and the facultative anaerobes *Lactobacillus*, *Brochothrix thermosphacta*, *Shewanella putrefaciens* and certain genera of Enterobacteriaceae. *Flavobacterium*, *Alcaligenes*, *Vibrio*, *Aeromonas* and *Arthrobacter* are found less commonly (Table 1.1).

Although the common occurrence of *Pseudomonas* spp. as spoilage agents of fresh meats stored in air is well documented, the role of other gram-negative genera is uncertain (Eribo and Jay 1985).

1.4.1 Pseudomonads

Pseudomonads are the dominant organisms in meat stored under aerobic conditions, at chill temperatures. They account for >50% of the biota, sometimes up to 90% and grow faster than any other bacteria at temperatures from 2 to 15°C (Gill and Newton 1977, Dainty *et al.* 1983, Dainty and Mackey 1992, Muermans *et al.* 1993). At 2°C, rates of *Enterobacter*, *Brochothrix* and *Acinetobacter* are 1.4, 1.5 and 2 times, slower than those of *Pseudomonas*, respectively. At 15°C, rates of the same organisms are 1.2, 1.4 and 1.6 times slower (Gill and Newton 1977).

For *Pseudomonas*, the hypothetical minimum temperature below which no growth can occur (T_{min}, see 1.6.3.1.1) calculated from an extensive search of the literature is - 7.65°C (95% confidence interval = 0.67, Neumeyer *pers. comm.*).

TABLE 1.1 Scheme proposed by Dainty *et al.* (1983) to differentiate the Gram negative bacteria commonly found on chilled meat.

	Motility	Oxidase reaction	Glucose metabolism	Ornithine decarboxylase
Pseudomonas	+	+	o	-
S. putrefaciens	+	+	o / i	+
Acinetobacter	-	-	o / i	NA
Moraxella spp.	-	+	i	NA
Moraxella-like spp.	-	+	o	NA
Aeromonas spp.	+	+	f	NA
Enterobacteriaceae	+ or -	-	f	NA

o = oxidative, f = fermentative, i = inert, NA = not applicable

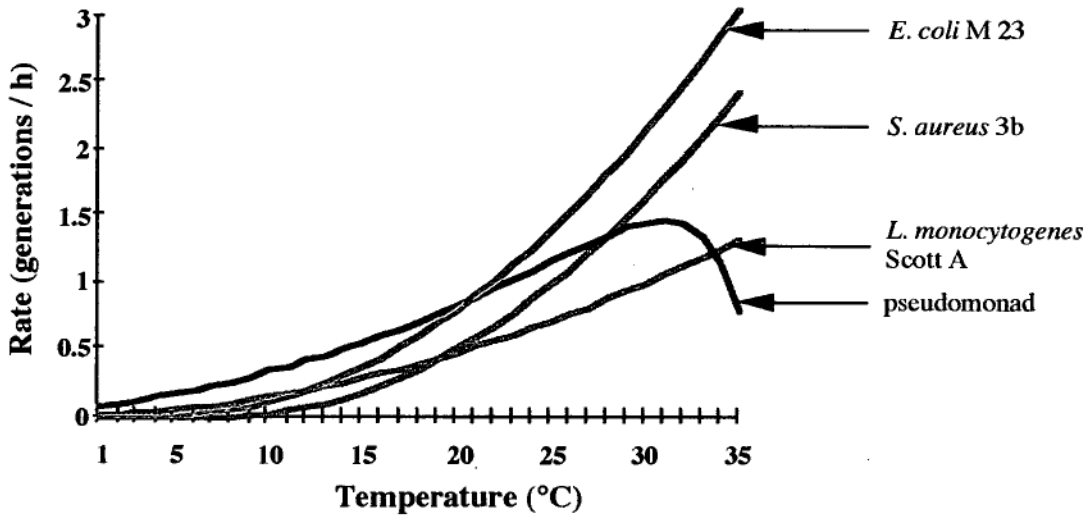
Ross (*pers. comm.*) compared rates of psychrotrophic pseudomonads with those of mesophilic pathogens which are likely to co - exist, including *E. coli, S. aureus* and *L. monocytogenes.* At water activity levels of 0.99 (NaCl - humectant), pseudomonads were predicted to dominate at temperatures below 18°C, however at a_w values of 0.96, pseudomonads were not the dominant organisms. *L. monocytogenes* dominated below ~13°C and *S. aureus* above that temperature (Figure 1.1).

Glucose is utilised preferentially as a substrate by pseudomonads and degradation of amino acids is strongly suppressed until glucose is exhausted. However, most amino acids can be used for growth (Gill and Newton 1977).

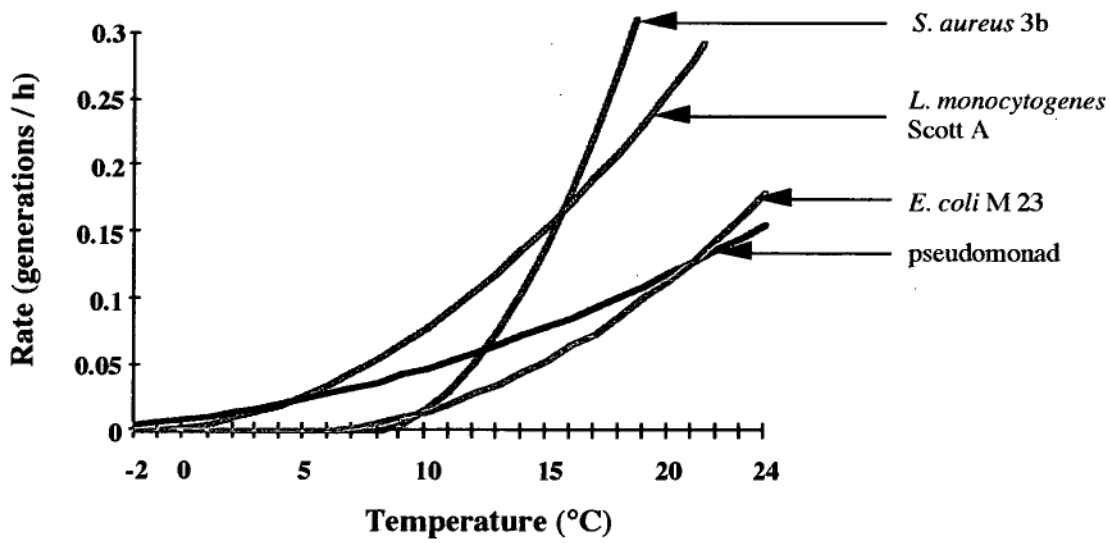
In the range occurring in meat, pH does not affect the growth of pseudomonads (Gill and Newton 1982, Gill 1986, Coates *pers. comm.*). Therefore, in food environments where nutrients, oxygen, water availability and pH are non limiting, growth rates are determined solely by temperature.

FIGURE 1.1 Comparison of growth rates at water activities of 0.99 (a) and 0.96 (b) using NaCl as a humectant.

a



b



Within the pseudomonad group, various subgroups and biovars exist. Their incidence, however, is relatively unknown. This may be partly due to an inadequacy of the commercial kits widely used for bacterial identification which rely mainly upon detection of acid production from specific sugars. There are 3 major groups of pseudomonads present on meat, two of which can be identified with known species, *Pseudomonas fragi* and *Pseudomonas fluorescens*. The third group has been proposed as a new species, *Pseudomonas lundensis* (Dainty and Mackey 1992). The non-fluorescent pseudomonads (sub-group within *Pseudomonas fragi* strain) are typically dominant on chilled meats (Dainty and Mackey 1992). Of the fluorescent pseduomonads, Gennari and Dragotto (1992) found that *P. lundensis* was the most frequently isolated (44%) from chilled spoiled meat, followed by *P. fluorescens* V-1 (16%) and *P. fluorescens* biovar I-1 (12%). Whereas, in fresh meat *P. fluorescens* V - 1 was dominant (26%) followed by *P. fluorescens* I - 1 (12%) and *P. lundensis* (10%).

Psychrotrophic pseudomonads also dominate the spoilage biota of poultry carcasses and of milk stored aerobically at chilled temperatures (Gill and Newton 1977, Pooni and Mead 1984, Chandler and McMeekin 1985 a and b, Chandler and McMeekin 1989a, Neumeyer *pers. comm.*).

1.4.2 *Psychrobacter* and *Acinetobacter*

Strains of the *Psychrobacter / Acinetobacter* group differ markedly from the pseudomonads in their metabolic properties. Many strains are inhibited by the low pH of normal meat. However, they may form a larger part of the population on meat of higher pH or, as the pH effect is less pronounced, on meat held at ambient temperatures (Gill 1983).

Psychrobacter and *Acinetobacter* do not utilise hexoses and must obtain energy from the oxidation of organic or amino acids (Gill and Newton 1977; Dainty and Mackey 1992). Highly offensive by-products do not appear to be produced and therefore these bacteria contribute little to the spoilage process in a biota dominated by pseudomonads (Gill 1983). However, they may enhance spoilage activities of pseudomonads and of *Shewanella putrefaciens* by restricting the availability of oxygen to these organisms. Newton and Rigg (1979) suggest that when oxygen is limiting, pseudomonads grow at submaximal rates, however they may not be subject to catabolite repression and may be able to degrade amino acids in the presence of glucose, producing malodorous substances. Under oxygen limiting conditions, *S. putrefaciens* generates H_2S, resulting in sulfmyoglobin formation and greening of meat (Gill 1983).

1.4.3 Facultative anaerobes

1.4.3.1 Brochothrix thermosphacta

Brochothrix thermosphacta, previously known as ***Microbacterium thermosphactum***, is a gram positive, facultative anaerobe commonly found in soil and animal faeces. It is usually a minor component of fresh meat biota, but is of major importance in vacuum-packaged meat containing residual oxygen (Lambert *et al.* 1991). In some cases it has been involved in the spoilage of fresh meat over - wrapped for display. A slight built-up of CO_2 may have been created, inhibiting the competing pseudomonads (Dainty and Mackey 1992, Gill 1983, Newton *et al.* 1978).

B. thermosphacta utilises glucose as a substrate for growth. Acetic acid and acetoin are produced from aerobic glucose metabolism and give the meat a sweet off-odour (Dainty and Hibbard 1980). Lactic acid is the major spoilage end product of anaerobic metabolism (Lambert *et al.* 1991). After the depletion of glucose, *B. thermosphacta* utilises glutamate which remains abundant at the meat surface. No other amino acids can be used for growth (Gill and Newton 1977).

1.4.3.2 Enterobacteriaceae

Enteric bacteria have slower growth rates than strict aerobes at chill temperatures. However, they have a higher spoilage potential at temperatures above 20°C (Gill and Newton 1977, McMeekin 1982, Lambert *et al.* 1991). At these temperatures, pseudomonads no longer dominate the spoilage biota of aerobically stored meat and are displaced by mesophilic species of *Acinetobacter* and Enterobacteriaceae (Gill 1982). Enteric bacteria utilise glucose, glucose-6-phosphate and then amino acids, producing malodorous by - products.

Alteration of gaseous environment, in which meat is stored by enclosing with gas impermeable wrapping, results in marked changes of the microbiota. Patterson and Gibbs (1977) found that psychrotrophic Enterobacteriaceae constituted a high proportion of the biota from lean and fat surfaces of vacuum packaged meat stored at 4°C for 8 weeks. These enteric organisms were thought to be *Klebsiella, Enterobacter, Hafnia and Serratia.*

Gill and Tan (1980) found that aerobic growth of *Enterobacter* was unaffected by the presence of CO_2. When *Enterobacter* was grown on meat at 3°C, in an atmosphere of 20% CO_2 - 80% air, the growth rates were the same as in air.

1.4.3.3 Lactobacilli

Constituting only a small proportion of the initial spoilage biota of meat, these organisms become dominant when meat is stored under reduced oxygen tension or is vacuum-packed. They produce lactic acid from glucose and when carbohydrates are exhausted, amino acids are utilised with the production of volatile

fatty acids with 'dairy' or 'cheesy' odours (Gill and Newton 1977). High numbers of lactobacilli may inhibit the growth of other microorganisms in meat, through the production of antimicrobial substances (Lambert *et al.* 1991).

In vacuum packaged meat stored at 4°C, growth rates of *Lactobacillus* sp. were 1.5, 1.25 and 1.21 times faster than *Pseudomonas* sp., *B. thermosphacta* and Enterobacteriaceae respectively. At 0°C growth rates were 2.37, 1.17 and 1.20 times faster (Zamora and Zaritzky 1985).

1.4.3.4 Shewanella putrefaciens

Shewanella putrefaciens, previously known as *Alteromonas putrefaciens,* produces organic sulphides under aerobic conditions and hydrogen sulphide under anaerobic conditions (Lambert *et al.* 1991). *S. putrefaciens* dominates DFD meat. It is responsible for the green discolouration as a result of the action of H_2S on myoglobin. The rate of growth and H_2S production are determined by the pH of the meat and by the oxygen permeability of the packaging film. *S. putrefaciens* is unable to grow on normal pH meat (5.4 - 5.8) and, under aerobic conditions, H_2S is not produced, thus greening does not occur. Vacuum packaged meat with a high pH (>6.2) spoils rapidly (Newton and Rigg 1979, Nottingham 1982, McMeekin 1982).

1.4.4 Mesophiles

Mesophilic bacteria of public health significance found in meat include *Salmonella, Staphylococcus aureus, Yersinia enterocolitica, Clostridium botulinum, Clostridium perfringens, Campylobacter, Aeromonas hydrophila,* and *Listeria monocytogenes*. These organisms may play an important role during storage and transportation when temperature abuse may take place (Buchanan and Palumbo 1985).

Contribution of any group of organisms to meat spoilage depends on the initial numbers in the population, with the fastest organism ultimately dominating. The relative rates of growth are affected by the storage conditions of the product.

1.4.5 Microbial interactions

Little information exists regarding microbial interactions during spoilage of meats. Gill and Newton (1977) reported that interactions between any two species occurred only when one organism had attained its maximum cell density. Therefore, if all the species had similar initial cell density, the species with the fastest growth rate dominated the spoilage biota. Substrate exhaustion at the meat surface did not limit bacterial growth and the maximum cell density of aerobic spoilage cultures was determined by the rate at which oxygen became available to the cells.

At low temperature (0 - 15°C), under aerobic conditions *Pseudomonas* is the dominant organism. The presence of other organisms (*Enterobacter* or *B. thermosphacta*) did not affect its growth rate. However, final densities of the pseudomonads were only half those attained with pure cultures (Gill and Newton 1977). The facultative anaerobes did not affect growth rates presumably because they could not reduce the oxygen concentration to a level affecting growth. However, by utilising a portion of the total oxygen available, they reduced the maximum cell density.

Determination of shelf life often depends on predicting the growth of the dominant spoilage biota. However, the prediction of growth of pathogens in foods involves different situations. The organisms of concern are often present in low numbers initially and must grow in the presence of a large excess of competing organisms composing the biota of the food.

Mackey and Kerridge (1988) monitored the growth rates of salmonellae in minced beef at temperatures between 10°C and 35°C. They reported that small numbers of salmonellae were able to compete successfully with the natural spoilage biota of minced beef. These findings agree with those of Gill and Newton (1980) where there was no inhibition of *Salmonella typhimurium* by a range of spoilage organisms on meat stored at 20°C or 30°C.

Studies of the growth of *Staphylococcus aureus* on prawns, smoked and rebrined salmon revealed good agreement between predicted rates and those observed in the product (Ross and McMeekin 1991). This finding indicated that no interactions were evident between *S. aureus* and other organisms on prawns and salmon. Slower rates than predicted were observed only on rebrined salmon at 12.6°C (a_w ~0.92) which was explained by the large numbers of other organisms competing with and inhibiting the growth of *S. aureus* under those conditions close to its limits for growth. Microorganisms do not appear to be greatly affect the growth of one another, except where conditions are growth limiting and when population densities are high. Such population densities occur only after spoilage, and are therefore of no interest in the prediction of shelf life.

However antagonistic effects towards salmonellae have been reported with species of *Lactobacillus* especially under anaerobic conditions (Gill and Newton 1980). In raw milk, *Lactobacillus* sp. have been reported to produce various substances that inhibit the growth of pathogenic organisms, including lactic or acetic acids, H_2O_2, bacteriocins and other uncharacterised antimicrobial substances. On the other hand, growth of *Listeria monocytogenes* has been found to be enhanced by the presence of *Pseudomonas fluorescens*. *L. monocytogenes* grew faster in milk after it had been incubated for 2 days with *Pseudomonas* sp. (Farrag and Marth 1992).

1.5 DETECTION OF SPOILAGE

Numerous methods exist for the evaluation of spoilage of flesh foods. To be suitable for routine purposes, the technique should be rapid, reproducible and not require elaborate equipment (McMeekin 1982). The usual method of determining bacterial density, by counting colony-forming units on agar, is of limited predictive value as at least 1 to 2 days are required for colony development on plates. This is unsatisfactory as the spoilage status of the food could alter considerably in the time required to complete the test.

More rapid, indirect methods are needed for the estimation of bacterial numbers. Rapid methods include chemical, physical, direct bacteriological, and physiochemical methods and are based on the determination of bacterial adenosine triphosphate (ATP), measurement of decreases in electrical impedance resulting from microbial growth, detection of $^{14}CO_2$ released by metabolism of radioactive substrates, and automated photometry (McMeekin 1982, Wood and Gibbs 1982, McClure *et al.* 1993a). However, most methods require specialised and costly equipment. They remain limited in their ability to provide an early warning of spoilage or the presence of dangerous levels of pathogens and their toxins.

A new analytical procedure (contamination index) has been introduced to evaluate the extent of contamination of beef carcasses with psychrotrophic *Pseudomonas* spp. and Enterobacteriaceae during slaughter, chilling, and cutting (Gustavsson and Borch 1993). The contamination index compares the composition of the spoilage bacteria found on meat samples to that in the environment (e.g. chillers). Therefore, it is a reflection firstly of the contamination by spoilage bacteria by contact and aerosols occurring during processing, and secondly of the increase in bacterial numbers due to growth during storage in the chillers.

This technique could be used in conjunction with the temperature function integration technique (1.6.5.1), where estimation of the potential proliferation of microorganisms during various processing steps can be assessed. As the latter method does not take into account any additional contamination that may occur in e.g. cold storage rooms, the two techniques could complement each other.

1.6 PREDICTIVE MICROBIOLOGY

1.6.1 Introduction

"Traditional bacteriological methodology can be replaced by something more rapid, i.e. be mechanised, so that we obtain essentially the same information more quickly, or with less manpower, and therefore more cheaply" (Roberts and Jarvis 1983).

Mathematical modelling provides faster microbiological analysis and offers an alternative to traditional microbiological assessment of quality and safety of

food products. Models are constructed by measuring the growth rate response of microorganisms to the main controlling factors such as temperature, pH, gaseous atmosphere, chemical preservatives and water activity, singly and in combination. The models are then used to predict the extent of growth of the microorganisms under conditions that were not specifically tested in the original testing protocol. In addition, modelling can be used to predict the effect of alterations in product formulation on storage life and product safety (Ross and McMeekin 1991).

Predictive microbiology can also be applied to assess the hygienic efficiency of various meat processing operations (McMeekin and Ross 1993).

1.6.2 Modelling the bacterial curve

Monod (1949) described the study of the growth of bacterial cultures as being a basic method of microbiology. Traditionally, the lag time and the exponential rate of growth have been determined by drawing a line through the exponential phase of the curve or by drawing a tangent to the steepest part of it. More recently, however, data obtained in the laboratory has been fitted to sigmoid functions. The 'line of best fit' is defined mathematically with nonlinear regression techniques, and estimates of generation time, lag phase duration or time to a specified increase in bacterial numbers (kinetic parameters) are derived.

A number of mathematical functions have been used including the Verhulst equation (Broughall and Brown 1984), the logistic function (Jason 1983, Zwietering *et al.* 1990), the Stannard function (Stannard *et al.* 1985) and the Gompertz function (Gibson *et al.* 1988, Gibson and Roberts 1989). The latter function has been reviewed and modified by Garthright (1991) and Zwietering *et al.* (1990). New functions have recently been described to allow modelling of all four phases of the bacterial growth curve (Whiting and Cygnarowicz-Provost 1992, Jones and Walker 1993).

1.6.2.1 Gompertz function

The Gompertz function is based on an exponential relationship between the specific growth rate and the population density (McMeekin *et al.* 1993). Gibson *et al.* (1987) modified the original function (Gompertz 1825) to describe the curves of log of cell density *versus* time. The 'modified Gompertz' function can be written as:

$$Log\ N_{(t)} = A + C\ exp\ \{ - exp\ [- B\ (t - M)]\} \qquad (1.1)$$

where t = time, $N_{(t)}$ = population density at time t, A = value of the lower asymptote [i.e. $Log\ N_{(-\infty)}$], C = difference in value of the upper and lower asymptote [i.e. $Log\ N_{(\infty)}$ - $Log\ N_{(-\infty)}$], M = time at which the exponential growth rate is maximal and where B is related to the slope of the curve at M (BC / e = slope of tangent at M).

The 'modified Gompertz' function has been widely used. Many report that in most cases this function can be regarded as the best model to describe the growth data, in terms of goodness - of - fit and ease of use (Gibson *et al.* 1987, Zwietering *et al.* 1990). In the study by Dalgaard *et al.* (1994) the Logistic model, when log transformed, was found to fit the growth curves better than the 'modified Gompertz' model. However, the data obtained in the studies described later will be analysed using the latter model not only because of its ease of use but also to allow comparison with data similarly analysed in this research group.

1.6.2.2 Lag phase

The lag time is of special interest to the food industry as it is desirable that this time be extended to infinity with respect to the shelf life of the product concerned. Failing this, it needs to be as long as possible.

The lag time is generally taken to represent a period of adjustment by cells to a new environment. Factors influencing lag time include change in nutrition and in physical environment, presence of an inhibitor, spore germination, and state of the inoculum culture. The lag time of a contaminant on food will depend greatly also on the temperature at which the contaminant was previously incubated, i.e. the source and the physiological state are important (Hudson 1993).

Measurements of the variation of the lag phase showed that the temperature response of the rate of the lag phase resolution and the rate of the growth of the culture were qualitatively the same. If all cells in the population resolved the lag phase simultaneously one would expect an abrupt transition between lag phase and the exponential growth phase. An abrupt transition does not usually occur, therefore Ross (1993a) concluded that the times for the resolution of lag by individual cells within the population are represented by a distribution. The observed 'lag time' must represent an average response of the entire population.

The lag phase has been extensively studied (Buchanan and Cygnarowicz 1990, Zwietering *et al.* 1992a, Baranyi *et al.* 1993). McMeekin *et al.* (1993) derived the duration of the lag phase (LPD), from the 'modified Gompertz' function (Gibson *et al.* 1987):

$$LPD = M - (1 / B) \{1 - exp [1 - exp (BM)] \} \quad (1.2)$$

where M and B are as for equation 1.1.

To estimate the end of the lag phase, the above relationship uses the tangent at the maximum absolute growth rate to extrapolate back to the lower asymptote. However if the LPD is short and the exponential growth rate is slow, difficulties can arise.

Buchanan and Cygnarowicz (1990) proposed an alternative method of calculating LPD. They defined the end of the lag phase as the time point where the second derivative of the logarithm of the actual growth curve has its maximum, i.e. where the third derivative of the logarithm of the number of organisms with respect to time is zero. However, Zwietering *et al.* (1992a) state that it is unnecessary to use complicated mathematical equations when lag phase duration can be calculated from the simple parameters of the 'modified Gompertz' function. McMeekin *et al.* (1993) also commented that the concept of Buchanan and Cygnarowicz requires additional computational time and power, and that it has no greater theoretical validity than the traditional tangent method.

Gibson *et al.* (1988), Buchanan and Cygnarowicz (1990) and Zwietering *et al.* (1992a) all used an empirical solution to calculate the lag phase. Empirical models simply describe the data in a convenient mathematical relationship, often with little or no insight into the underlying process (McMeekin *et al.* 1993). With the use of more and more exact methods in microbiology, the demand for less empirical growth models is increasing (Whiting 1992). Baranyi *et al.* (1993) used a more mathematically formalised (mechanistic) approach to model the lag phase. Mechanistic models are built up from theoretical bases, and may allow the interpretation of the modelled response in terms of known phenomena and processes. The models developed by Baranyi *et al.* (1993) describe the lag as being independent of the shape of the growth curve. The effect of the previous environment is separated from that of the present environment. In this way the model separates the effects of the present from the pre - inoculation environment.

1.6.3 Model construction

1.6.3.1 Effect of temperature

Parameters derived from sigmoid functions can be fitted by mathematical models. To be of predictive value, models must predict accurately and reliably microbial behaviour in foods. A prerequisite for the development of a reliable model is the collection of sufficient good quality data (McMeekin *et al.* 1993). Ross and McMeekin (1991) concluded that kinetic models are best developed by measuring response rates in laboratory liquid media to gather sufficient data. The

model can then be validated in the food product to which it is to be applied and can be modified and refined as and if necessary.

Spencer and Baines (1964) constructed a linear model to relate spoilage rate and storage temperature:

$$k_{\phi} = k_{O} (1 + c\phi) \tag{1.3}$$

where k_{ϕ} is the rate of spoilage at a given temperature ϕ (°C); k_{O} is the standard spoilage rate (at 0°C) and c is the linear temperature response.

Olley and Ratkowsky (1973a and b) found that the constant c had a value of 0.24 at temperatures up to 6°C but above this temperature the value increased and became more variable. They used a model which followed the Arrhenius law:

$$k = A \exp [-\mu / RT] \tag{1.4}$$

where k is the spoilage rate; μ is the apparent activation energy (cal mol^{-1}); R is the universal gas constant; T is the storage temperature (K) and A is a constant (sometimes interpreted as the collision factor).

The above model was adequate in describing the spoilage of meat, poultry and fish at temperatures in the range from 3°C to 16°C. At higher and lower temperatures, however, the model seemed less appropriate as the experimental curves deviated markedly from those predicted by the model.

When the logarithm of both sides of the equation is taken, a plot of $ln\ k$ versus the reciprocal of the absolute temperature ($1 / T$) would be expected to yield a straight line if the activation energy μ and the collision factor A were unchanging with temperature. However, this plot appears to be curvilinear, implying that the activation energy decreases in a systematic manner with increasing temperature.

The Arrhenius-type relationship most widely used to describe bacterial growth is the Schoolfield model (Sharpe and DeMichele 1977, Schoolfield *et al.* 1981). This type of model assumes that growth is governed by a single rate - limiting enzyme-catalysed reaction, the rate of which may be described by the Arrhenius equation with additional thermodynamics terms to describe the rate - modifying effects of high and low temperature inactivation of the rate-limiting enzyme. Adair *et al.* (1989) reparameterised the Schoolfield model. The dependent variable was expressed as the logarithm of time, rather than the rate, to improve numerical

stability and increase the speed of convergence when data is fitted to the model by a nonlinear regression procedure.

1.6.3.1.1 Square root models

Ohta and Hirahara (1977) developed a model describing the effects of temperature on nucleotide degradation. Ratkowsky *et al.* (1982) adapted that model to relate the square root of the bacterial specific growth rate (r) to the storage temperature (T in K):

$$\sqrt{r} = b\,(T - T_{min}) \qquad (1.5)$$

where b is the regression coefficient and T_{min} is a hypothetical temperature below which no growth can occur. It is of no metabolic significance but is considered to be an intrinsic property of the organism being studied.

The square root model is a Bělehrádek - type model and differs from the Arrhenius equation by the way in which the data is transformed. In the Arrhenius equation, the dependent variable is transformed using the logarithm and the independent variable using the reciprocal. In Bělehrádek's model the exponent is obtained from a log - log plot of rate versus temperature. Further, Bělehrádek introduced the concept of a 'biological zero' rather than relating biological activity to absolute zero (Bělehrádek 1926).

The square root model (equation 1.5) could only be applied to the low temperature region. Therefore, Ratkowsky *et al.* (1983) expanded the model to describe growth across the full biokinetic range:

$$\sqrt{r} = b\,(T - T_{min})\,\{1 - exp\,[c\,(T - T_{max})]\} \qquad (1.6)$$

where T_{max} is the theoretical maximum temperature for growth and c is a regression coefficient.

The square root model has been applied to various food systems and also to data originating from the literature. It accurately describes the effect of temperature on the growth rate of bacteria. In contaminated raw milk, experimental rates were as predicted by the model up to temperatures of 12 - 14°C. Thereafter experimental rates were higher than predicted (Chandler and McMeekin 1985a). This deviation has also been observed with the spoilage of poultry (Pooni and Mead 1984) and meat products Scott (1937). It is most likely due to the growth of mesophilic bacteria, which have higher T_{min} values, and hence a mesophilic rate curve may be more appropriate at elevated temperatures.

1.6.3.1.2 Model comparison

The potential benefits of predictive microbiology depend entirely on the selection of appropriate models to describe the effects of environmental factors on microbial growth rate. The choice of model to be used should depend upon: the accuracy of predictions compared to observed results, the ease of use and the usefulness in aiding the understanding of the processes involved in microbial growth (Ratkowsky *et al.* 1991). These characteristics of the model are intimately related to a number of other properties which are considered by Ratkowsky (1992).

An important question is how well the model describes the data. Of even greater importance is how accurately the model predicts, that is, how well it models observations not used to estimate the values of the parameters of the model (Ross 1993a). One of the most effective means of comparing modelling systems is to compare the predicted values of the models against the actual observed data points (Adair *et al.* 1989).

There have been many publications critically comparing the two major classes of models, the Arrhenius and Bělehrádek models (Stannard *et al.* 1985, Adair *et al.* 1989, Davey 1989a, Ratkowsky *et al.* 1991, Alber and Schaffner 1992). Although controversy continues to exist, the square root-type models (Bělehrádek) have many favourable features including parsimony, good parameter estimation properties and ease of use (Ross 1993b, McMeekin *et al.* 1993).

Adair *et al.* (1989) argued that the square root model does not fit data at low temperatures as well as the Schoolfield model. However, discrepancies between the two models are particularly pronounced only at low temperatures. At temperatures close to the minimum for growth there is a marked increase in the variance of generation times (Ratkowsky 1992, section 1.6.6). The range of the observed responses is greatly in excess of any discrepancy between their mean value and the predicted values from any of the models (McMeekin *et al.* 1993).

McMeekin *et al.* (1993) report that the two - parameter square root model has proven to be an "excellent expression for modelling the temperature dependence of bacterial growth provided that the data are confined to the region between the lowest temperature for which growth is observed and a temperature just below the optimum temperature for growth". Above this temperature the relationship between $\sqrt{\text{rate}}$ and temperature starts to become curvilinear, therefore the 'expanded' square root model (equation 1.6) needs to be applied.

1.6.3.2 Combined effects of limiting environmental factors

In foods such as fish, meat, poultry and milk, as the water activity is high and the pH is near neutral, temperature is the major factor controlling microbial

growth rates. In many products, however, temperature is only one of a series of factors affecting growth rates (McMeekin *et al.* 1993).

The combined effects of temperature, pH, water activity and other physical and chemical properties of foods have been modelled with low - order polynomials of the form below (Gibson *et al.* 1988):

$$Ln\ Y = a + b_1S + b_2T + b_3P + b_4S^2 + b_5T^2 + b_6P^2 + b_7ST + b_8SP + b_9TP + e \qquad (1.7)$$

where T = temperature, S = salt concentration, P = pH, Y = maximum exponential growth rate or time at which rate is fastest, e = random error, coefficients a, b_1, b_2 .....b_9 are determined in this linear regression model by use of least of squares.

Thé model above takes into account the possibility of interactions among the environmental factors. However, many studies were not specifically designed to test the presence or absence of synergistic effects among the environmental factors. They merely describe the effect of various combinations of factors on the growth of organisms (Gibson *et al.* 1988, Buchanan and Phillips 1990, McClure *et al.* 1993a, Wijtzes *et al.* 1993).

Adams *et al.* (1991) produced a model for the effect of temperature and pH on the growth of *Yersinia enterocolitica*. Chandler and McMeekin (1989b) modelled the growth response of *Staphylococcus xylosus* to changes in temperature and water activity. Davey (1989b) constructed a model for the combined effect of temperature and water activity on the growth rate of several microorganisms. Cole *et al.* (1990) modelled the effect of temperature, pH and water activity on growth rates of *Listeria monocytogenes*. Quintavalla and Parolari (1993) studied the effects of temperature, water activity and pH on the growth of *Bacillus* cells and spores. All the above studies indicate that pH and water activity, temperature and water activity, and temperature and pH act in an additive manner, with no synergistic effects between them.

Therefore, a Bělehrádek - type model of the form below was proposed to describe the rate response to a combination of temperature, water activity and lowered pH values (McMeekin *et al.* 1992):

$$\sqrt{k} = f\sqrt{\left(a_w - a_{w_{min}}\right)\left(pH - pH_{min}\right)}\left(T - T_{min}\right) \qquad (1.8)$$

where f is a constant, $a_{w_{min}}$ is the theoretical minimum a_w for growth and pH_{min} is the theoretical minimum pH for growth.

The effect of the three major factors controlling microbial growth on foods may then be described by the accurate determination of T_{min}, $a_{w_{min}}$, pH_{min} and f(constant).

However, the effects of factors can be interactive rather than independent at the lower limits of growth. In the region of growth / no growth interface, the limiting level of one constraint may be decreased as a result of an increase in the level of another constraint.

Figure 1.2 shows that the effect of sequential reduction in water activity on the growth of *Staphylococcus xylosus* is proportionally the same at any temperature, and as temperature decreases the magnitude of growth rate reduction due to lowered water activity is also decreased (McMeekin *et al.* 1987). The effect is also represented by:

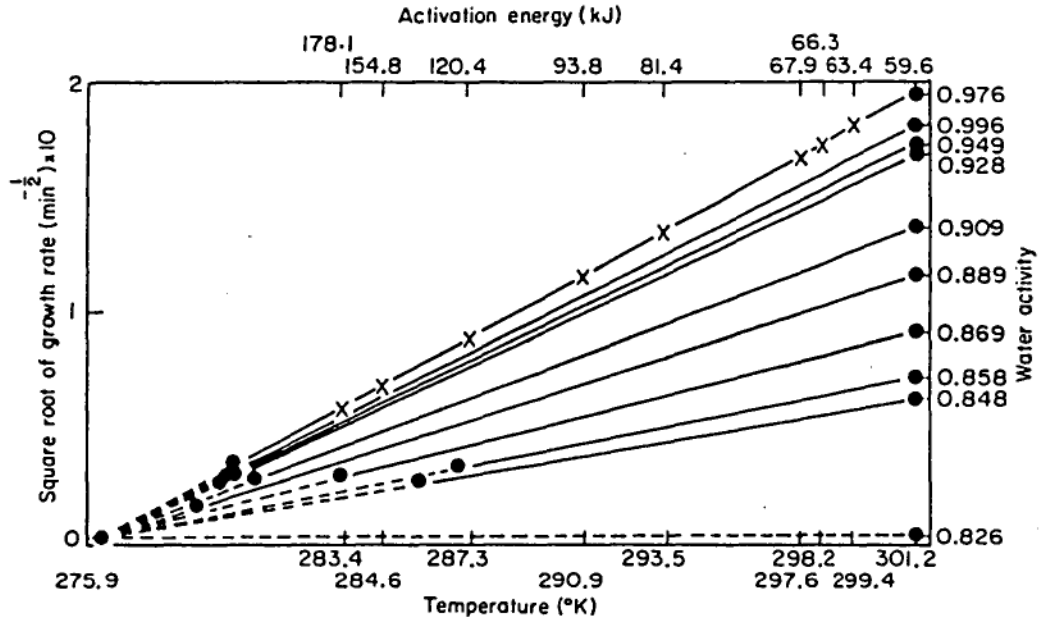
$$\Delta E_{app} = \frac{2RT^2}{(T - T_{min})} \tag{1.9}$$

This equation indicates that the apparent activation energy (ΔE_{app}) depends only on the difference between temperature, T, and T_{min}.

The effect of the two factors is interactive rather than independent at the point where growth ceases, that is, although T_{min} is fixed, the actual temperature at which growth ceases increases with decreasing water activity. The actual minimum temperature at which growth was measured at the optimum a_w (0.976) was 7.4°C whilst at a_w = 0.848 the minimum temperature was 12.6°C (McMeekin *et al.* 1987). At optimum a_w, the ΔE_{app} from Eqn. 1.9 was 343 kJ mol^{-1}. At a_w = 0.848 the energy barrier to growth that the cells were able to overcome was reduced to 134 kJ mol^{-1}.

McMeekin *et al.* (1993) postulated that the combined effects of temperature and water activity on *S. xylosus* (Figure 1.2) might be explained by the diversion of energy to the production of compatible solutes thereby reducing the energy available to overcome the activation energy barrier presented by temperature. Therefore, in the example above (Figure 1.2), approximately 200 kJ mol^{-1} of the energy available to the cell was diverted to the production of compatible solutes to counteract the effects of lowered water activity.

FIGURE 1.2 Fitted lines for strain CM21/3 at nine different water activities at temperatures below T_{opt} using generation time data. Growth did not occur at $a_w = 0.826$ at temperatures below 28°C (301.2 K). The minimum temperature at which growth was measured at each a_w is indicated by • [Adapted from McMeekin *et al.* 1987)].



1.6.3.3 Specific effects of lactic acid

In the studies described below lactic acid was considered the acidulant of choice. It is the predominant acid in muscle tissue, beef muscle containing 9 g / kg (Smulders 1987), and is also used as a preservative in foods. The salts (sodium and potassium) of lactic acid have demonstrated significant antimicrobial properties in meat products (Giese 1994). In countries such as in the United States, where lactate is listed as 'generally recognised as safe' (GRAS), its use for the prolongation of the shelf life of fresh meat (pork sausage, turkey breast and chicken fillet) and other meat products (including ham, bacon, corned beef and turkey salami) is permitted (Belitz and Grosch 1987, Houtsma *et al.* 1993). The U.S. Department of Agriculture allows the use of up to 2% of lactates for flavour enhancement in meat and poultry products, up to 2% of the total formulation for products covered under 9 CFR 319.180 (frankfurter, bologna and others) and up to 4.8% in cooked, hermetically sealed products not covered under 9 CFR 319.180 (Giese 1994).

Organic acids such as lactic acid have also been used in some countries for the decontamination of fresh meat. Washing and sanitising meat carcasses after slaughter appears to reduce the levels of microorganisms, including potential pathogens (Anderson and Marshall 1989, Greer and Dilts 1992). At appropriate pH values, organic acids have bactericidal properties while not aversely affecting the sensory attributes of food. In an Australian study conducted by Eustace (1981), treatment of lamb carcasses with 1.5% acetic acid at 68 -70°C resulted in a 99.8% reduction in bacterial numbers. The average decrease within the body cavity was 98.7%. Discolouration of the meat was negligible and the residual odour of acetic acid after overnight chilling was minimal. Therefore, treatment of carcasses with organic acids immediately after dressing can decrease the large bacterial numbers and reduce cross - contamination occurring in the boning rooms.

The inhibitory effect of weak acids depends on the concentration of the acid and on the pH level. Most recent studies have aimed at distinguishing between the effects of the undissociated molecule and the lactate ion. Antimicrobial activity has usually been ascribed to the undissociated fraction of the organic acid as weak acids appear to be much more active in an acid than in a neutral environment (Baird-Parker 1980). Eklund (1989) reported that lactate and acetate penetrate the cell by means of diffusion in their undissociated form. They then dissociate resulting in the acidification of the 'cell interior'. Differences in antimicrobial actions of various organic acids may be related to the potency to penetrate a cell, the part of the cell which is attacked and the chemical nature of that attack. Buchanan *et al.* (1993) report that lactic acid was more effective than acetic acid in the inactivation of *Listeria monocytogenes* when expressed in terms of concentration of undissociated acid. These results were observed at neutral pH values indicating further that the inhibition was attributable to the undissociated acid.

Studies by Houtsma *et al.* (1993) to established the minimum inhibitory concentration of lactate required to inhibit the growth of organisms, were performed at pH 6.5. At this pH value, less than 1% of the added sodium lactate occurred as undissociated lactic acid. Therefore, a possible role in the inhibition of microbial growth by the dissociated molecule, the lactate ion, can not be excluded. The toxicity of weak organic acids may be a function of the concentration of undissociated and dissociated ions as well as pH. Eklund (1983) presented a mathematical model taking into account both dissociated and undissociated acid into consideration and showed that this model gave a good description of the actions of sorbic, propionic and benzoic acids.

Most published data have been presented as reductions in bacterial numbers following treatment of products with organic acids. Houtsma *et al.* (1993) screened a wide range of pathogenic and spoilage organisms likely to be found in

meat for their sensitivity towards lactate. De Wit and Rombouts (1990) reported that the effect of sodium lactate on microbial growth rises above that which would be expected from its water activity lowering property. This effect was found for a number of lactic acid bacteria, *S. aureus* and *S. typhimurium*. *E. coli* appeared to be an exception as its growth rate was hardly affected by 5% sodium lactate.

1.6.4 Model validation

Once a model has been constructed, it requires validation. Firstly, the 'goodness of fit' of a model has to be determined. There are a number of methods that can be used to assess the predictive ability of a model using data used to construct it, and also to compare the fit of different models to the same data set. The complete data set can be divided into subsets according to some rational criteria. Part of the data can be used to build a model. That model can then be used to predict the remaining part of the data set. The predictions can be compared to the actual data obtained (McMeekin *et al.* 1993, Draper and Smith 1981).

Secondly, the ability of the model to predict accurately the behaviour of microorganisms of concern in foods has to be assessed. Validation is conducted in the food systems, preferably under fluctuating temperature conditions that simulate those likely to be encountered in commercial practice (Spencer and Baines 1964, Langeveld and Cuperus 1980, Smith 1987). Gibson *et al.* (1988) constructed a model in laboratory broth describing the effect of pH, sodium chloride and storage temperature on the growth of salmonellae. There was good agreement between generation times predicted by the model and those from the literature. Model validation was also conducted by comparing generation times predicted from the model with those observed in pork slurries. Ross and McMeekin (1991) constructed a model for the growth of *Staphylococcus aureus* in response to temperature and water activity, and validated it by inoculating the strain in prawns and salmon. The model's predictions were generally in good agreement with the experimental observations.

Within the UK Predictive Microbiology Programme (Walker and Jones 1994), models are validated by comparing predictions from the model with data from inoculated food studies and from published literature. Firstly, both the actual viable counts for a particular combination of factors and the associated predicted population sizes are graphically represented. Second and third types of comparisons involve the actual and predicted maximum generation times or actual and predicted times for a thousand - fold increase in the population size. Walker and Jones (1994) report that a 20% error margin is placed on the predicted value and provided that the observed value is within this margin, the comparison is considered acceptable.

Reichel *et al.* (1991) assessed the validity of a model for *E. coli* growth during a hot boning process. There appeared to be agreement within one generation in 76% of comparisons between calculated and directly determined growth of *E. coli*. The model was 'fail safe' (i.e. over - predicting the extent of proliferation) in 60% of cases.

Ross (1993a) introduced factors to assess the deviation of predicted generation times from those observed. The ratio of the predicted and observed generation time was chosen to standardise the deviation of the data. The logarithm of the ratio was used to give equal weight to over - and under - predictions in determining the average deviation. Thus the geometric mean of the ratio was calculated:

$$\text{BIAS factor} = 10^{\frac{\sum \log(GT_{observed}/GT_{predicted})}{n}} \qquad (1.10)$$

A value less than one indicates that the predicted generation time is, on average, greater than the observed generation time and the model is, therefore, 'fail dangerous'. Conversely, a value greater than one indicates that the model is 'fail safe'. However, this equation will 'cancel out' over - and under - predictions, giving no indications of the average precision of estimates. Therefore, Ross (1993a) also introduced a precision factor by taking the average of the sum of the *absolute* values of the logarithm of the ratio. The antilogarithm of this factor will always be greater or equal to one.

$$\text{PRECISION factor} = 10^{\frac{\sum \left|\log(GT_{observed}/GT_{predicted})\right|}{n}} \qquad (1.11)$$

The larger the value, the less precise is the average estimate.

In general comparisons between data from inoculated food studies and the model produce more favourable results than comparisons with data from the literature. The cause could be attributed to the fact that careful measurements of the factors present are taken when constructing a model, but details are often missing from literature studies and factors often need to be estimated.

Ross (1993a) suggested that there may be a limit to the precision of model predictions to independent data. When models for *Staphylococcus aureus* and *Listeria monocytogenes* were compared to literature data, the highest degree of precision of any of the models was $\sim\pm$ 35%. The quantity and quality of data used for construction of a model together with appropriate validation procedures, ensures confidence in the use of predictive models (Walker and Jones 1994).

1.6.5 Model application

1.6.5.1 Temperature - time function integration (TTFI)

Temperature function integration provides an alternative to the traditional bacteriological and biochemical approaches to estimating product deterioration. It is based on the fact that temperature is the cardinal factor controlling the rate of microbial development and hence the rate of spoilage.

The relative rate function was proposed by Nixon (1971) to predict the shelf life of fish. The relative spoilage rate was defined as the ratio of the actual spoilage rate at any temperature to the actual rate at 0°C. The function enabled integration of temperature history to give equivalent time on ice.

Olley and Ratkowsky (1973a) constructed a relative spoilage rate curve over the temperature range 0 - 25°C. This curve was incorporated into the circuitry of an electronic temperature function integrator by Nixon (1975). The temperature of the product could then be monitored to provide an estimate of the equivalent number of days at 0°C. From a knowledge of the expected shelf-life of the product under a defined set of conditions, the remaining shelf-life could be calculated.

This concept was developed to describe the spoilage of fish but several additional attempts have been made to predict the shelf-life of aerobically stored products on the basis of the temperature history. The hygienic efficiency of various meat and meat products processing operations has been assessed by Gill and colleagues and reviewed by McMeekin and Ross (1993) and McMeekin *et al.* (1993)(Table 1.2). Models based on square root kinetics were developed for mesophilic organisms (Enterobacteriaceae including coliforms, *E. coli*, and pathogens such as *Salmonella*). Fewer studies have concentrated on psychrotrophic organisms (Gill *et al.* 1988, Gill and Jones 1992a, Gill and Phillips 1993).

The first application of temperature function integration was reported by Gill (1984). A temperature model for *E. coli* was used to monitor the spoilage of livers. To allow application of the technique to any cooling procedure, the concept of a 'bacterial growth number' i.e. the number of generations occurring during a process, was introduced (Gill 1984). Smith (1985) examined the growth of coliforms on meat to assess the hygiene of commercial abattoirs. Derived models were tested (Smith 1987) by comparing results obtained by plate counts for *E. coli* and *Salmonella typhimurium* , with numbers of coliforms present on mutton carcasses processed in a commercial abattoir. No significant differences were found between results obtained by calculation and those obtained by plate counts. Mackey and Kerridge (1988) studied the growth of salmonellae in minced beef and confirmed the conclusions of Smith (1987).

Lowry *et al.* (1989), in examining meat thawing procedures, also had to take into account the damage caused by freezing and thawing, variable rates of

warming at different parts of the cartons and the development of anaerobic conditions as a result of accumulation drip from thawed surfaces. To make a correct interpretation of the hygienic adequacy of the process, it is important to define exactly the environmental factors limiting growth during a particular processing operation.

TABLE 1.2 Temperature function integration techniques predicting the behaviour of organisms during meat processing (adapted from McMeekin and Ross 1993).

Gill (1986)	Development of anaerobic model for *E. coli* growth. Validation in offal cooling procedures.
Smith (1987)	Validation of model for coliforms on blended mutton.
Mackey and Kerridge (1988)	Model for *Salmonella* in minced beef.
Lowry *et al.* (1989)	Aerobic model for *E. coli* lag phase and growth. Validation for meat thawing procedures.
Reichel and Gill (1990)	Validation for hot - boning procedures.
Gill *et al.* (1991b)	Validation for spray chilling of beef carcasses.
Gill *et al.* (1991a)	Validation for conventional beef carcass cooling.
Reichel *et al.* (1991)	Validation for hot - boning processes.
Gill and Jones (1992a)	Validation for aerobic *E. coli* model in pig carcasses cooling.
Gill and Jones (1992b)	Validation for anaerobic *E. coli* model in beef offal.
Gill and Phillips (1993)	Validation for pseudomonad model during transportation of beef sides and quarters.

The studies published by Gill and coworkers have a standard format with detailed description of product temperature history. For each type of process, the temperature histories are collected from the slowest cooling, contaminated area of a random sample of units passing through the process. That temperature history data is then integrated with respect to models describing the dependency of the growth of *Escherichia coli* and psychrotrophic pseudomonads on temperature. The model for pseudomonads was derived by Gill and Jones (1992a) from published data describing the growth of these organisms on meat, milk or rich synthetic media.

Application of the criteria proposed for the traditional side cooling process was shown to be applicable also to cooling of beef sides by spray chilling (Gill *et al.* 1991b) and to cooling of cartons of hot boned beef in a blast freezer (Reichel *et al.* 1991). The studies performed by Gill and colleagues demonstrate clearly the potential of temperature function integration to provide rapid, practical assessment of the hygienic efficiency of meat processing operations.

Temperature function integration criteria have been also applied to poultry (Daud *et al.* 1978, Pooni and Mead 1984) and milk products (Langeveld and Cuperus 1980, Chandler and McMeekin 1985a and b, Chandler and McMeekin 1989a, Fu *et al.* 1991). Langeveld and Cuperus (1980) studied the effect of temperature on bacteria in pasteurised milk. A computer program was developed from the Arrhenius equation and a given temperature profile. Growth rates of strains in milk were similar to rates of the same strains grown separately as predicted by the program. Chandler and McMeekin (1985a and b, 1989) applied temperature function integration to milk spoilage data from the literature and from their laboratory. The technique was based on the ability of the square root model to predict the growth of psychrotrophic pseudomonads over the temperature range 0°C to 15°C. A good correlation between pseudomonad numbers and TTFI readings was shown.

1.6.5.2 Time - temperature monitoring devices

Temperature histories can be integrated in devices to monitor food spoilage. The simplest devices are disposable chemical indicators (Fu *et al.* 1991, Taoukis *et al.* 1991). They are based on the diffusion of a dye through a porous medium. The migration of the coloured fluid is governed by the laws of diffusion, and the Arrhenius kinetics of diffusion therefore apply to the system (McMeekin *et al.* 1993). These devices have found useful application within the food industry. Time - temperature monitors (3M Monitor Mark ®) were used by workers at the Victorian Institute of Animal Science to assess levels of contamination on pig carcasses. Carcasses were tagged with these monitor strips three hours after slaughter, the strips remaining in place during transport and chiller storage at the boning room. As carcasses had a consistently low initial level of contamination, the

numbers of bacteria at boning could be correlated to the extent of dye migration. Shelf life of fresh meat can, therefore, be improved by combining this type of carcass management with better boning room hygiene (Widders and Coates *pers. comm.*). McMeekin *et al.* (1993) also reported another successful use of 3M monitor strips outside the food industry. Vaccine storage was monitored as part of the World Health Organisation's Expanded Programme on Immunisation. The strips allowed simultaneous monitoring of the viability of several vaccines including polio and measles.

Electronic devices can be programmed to integrate the predicted rate over time (from a constructed model), and this information can be used to predict the microbiological safety or the remaining shelf life of the product. Three commercially available electronic devices, which can be regarded as true predictive systems, are: the Don Whitley 'time temperature function integrator' (Owen and Nesbitt 1984), the Remonsys 'Smartlog' (Remonsys Sys. Ltd. Keynsham, Bristol, UK) and the 'Delphi' temperature logger (Phillips and Gill 1990).

The time temperature function integrator is an electronic clock which runs at real time at the optimum storage temperature for the product (e.g. 0°C for fresh fish). At temperatures above this reference temperature, the clock runs more quickly, while at those below, it runs more slowly. The rate of the clock is the same as the bacterial growth response to that temperature. The device displays the 'elapsed storage life' at the reference temperature.

The 'Smartlog' is a temperature logger which displays an indication of the product's storage conditions (McMeekin *et al.* 1993). The 'Temperature History Index' (THI) is defined as:

$$\text{THI} = \frac{\text{Elapsed Shelf Life}}{\text{Elapsed Time}} \times 100 \qquad (1.12)$$

A THI value greater than 100 indicates that the product has been subject to temperatures which have overall shortened its expected shelf life, relative to that at the reference storage temperature. A THI value less than 100 indicates that the product has been handled under better than expected conditions and will achieve a longer than expected shelf life (McMeekin *et al.* 1993). The elapsed shelf life is calculated by the 'Smartlog' using a predictive microbiology model, allowing the reference storage temperature to be reset to suit different food products.

The 'Delphi' temperature logger has accompanying software to provide a more detailed assessment of the effects of temperature history on product quality. Temperatures are recorded at selected intervals. Temperature histories are then analysed with specific computer software to predict the extent of microbial growth at

any time, or over any time interval in that product's history. This allows elapsed shelf-life, or pathogen growth, or effects of a particular part of that history on product quality to be estimated.

Integrators have the advantage of providing a more immediate response, however with loggers, temperature histories can be analysed and lapses of temperature control can be specifically identified. The 'Delphi' temperature logger is a compact unit powered by a lithium cell. It has a temperature sensor mounted with a Teflon or stainless steel probe. The instrument is accessed through a personal computer, with software enabling both shelf life prediction and assessment of the hygienic efficiency of processing operations of red meat products. Temperature profiles for *E. coli* (Phillips and Gill 1990, Jones 1990, Gill *et al.* 1991 a and b) and *Pseudomonas* (Gill and Jones 1992b, Gill and Phillips 1993) were incorporated into a computer program to calculate proliferation of these organisms for given temperature histories.

Ross (1993a) described applications software to interpret environmental histories. Prototype applications incorporating *E. coli* and *Pseudomonas* models for temperature were developed in Microsoft Excel 4.0® (Microsoft Corporation, Redmond, WA, USA). The operations are in an easy to use format. Data files are pasted into a template file in which the data processing occurs. The user is presented with a number of information screens, enters the requested information on the input screens, and selects the temperature history file. Processing occurs in 'background' windows so the user is presented only with the outcome.

1.6.5.3 Application software, databases and expert systems

Electronic technology enables the monitoring and integration of the effects of environmental factors on bacterial growth. Cost, however, has limited the use of such devices to consignments, processes and systems, rather than to individual items of product. Technology is most developed for temperature function integration, with devices already available (1.6.5.2). Any of the electronic temperature loggers could be combined with predictive microbiology computer programs to yield estimates of the extent of growth of any microorganism of interest. This information would be invaluable to identify problem areas in processing and distribution chains, to identify mishandling and to evaluate cost *versus* benefit of changed processing protocols (McMeekin *et al.* 1993).

Recent advances in microbiology have led to the development of mathematical models that can be used to reliably predict the growth and death responses of microorganisms under a wide range of conditions relevant to food safety and spoilage. In addition, advances in the development of software and hardware have led to the construction of expert systems (Adair and Briggs 1993).

Expert systems are computer programs that attempt to emulate the performance of human experts. These systems are created to allow non - experts to access the relevant information. Mathematical models describing the growth of bacteria are combined with information on chemical and physical characteristics of food and likely conditions of storage. Expert systems can be interrogated to estimate the effect of changes in storage conditions or product formulation on microbial development.

The development of user - friendly application software has made it possible and easy for non - research personnel to employ mathematical models. In the USA, the United States Department of Agriculture (USDA) has distributed free of charge the 'Microbial Food Safety Pathogen Modeling Program' (Buchanan 1993). The software is based on a spreadsheet program (Lotus 1-2-3) and automates the use of response surface models that were developed by the Microbiological Food Safety Research Unit at the USDA, at the ARS Eastern Research Center. Models for *Listeria monocytogenes*, *Shigella flexneri*, *Aeromonas hydrophila*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* spp. are included.

In the UK, 'Food Micromodel', a food microbiology advisory service, is based on a database incorporating mathematical models describing the growth response of food - borne pathogens to environmental factors. The program includes models constructed by AFRC Institute of Food Research, Campden Food and Drink Research Association, Flour Milling and Baking Research Association, Leatherhead Food Research Association, Torry Research Station and Unilever Research, with also some data from universities (Walker and Jones 1992).

In Europe, the Food - Linked Agro - Industrial Research Program (FLAIR) is a database including data for food spoilage organisms and food pathogens. The program includes 7 countries (Cole 1991). Zwietering *et al.* (1992 b) developed a system combining quantitative and qualitative information to predict the growth of organisms in foods. Two databases were built, one with physical variables of foods and one with organisms and their growth limits for the same physical variables. In addition, an information base is used to add qualitative information concerning products and microorganisms. The presence of microorganisms can be determined, and changes in growth rates when physical properties are altered can also be estimated from the data base.

1.6.6 Concerns regarding the true potential of predictive microbiology

Concerns still exist among food microbiologists regarding the usefulness of predictive microbiology. These include, the effect of initial numbers of contaminant organisms, the complexity of food systems, the effect of non - constant conditions, identification of suitable models, the inherent distrust of empirical

models, the inherent variability of biological systems, and collection of appropriate environmental information.

Firstly, useful predictions depend upon the initial numbers of microorganisms present, however, rapid methods to enumerate the initial microbial load are not yet rapid or cost effective. To overcome this problem, Gill *et al.* (1991b) suggest to base the predictions upon an 'assumed' starting inoculum. The values chosen may be based upon a 'worst case'. Therefore, processes are defined by the product of poorest hygienic condition that the process yields, rather than the average product condition.

In many situations it may not be necessary to have knowledge of absolute rates under each set of conditions, predictions may be based on relative rates. In this, approach models are used to predict the growth rate under a particular set of storage conditions relative to that under conditions for which the shelf life of the product is known. For example, milk has a shelf life of 8 - 10 days at 4°C. If the temperature of storage is found to be 10°C, the shelf life can be calculated without reference to the absolute growth rate of the organisms of concern under those conditions. The growth rate of *Pseudomonas* at 10°C is ~ 1.5 times that at 4°C, thus the shelf life at 10°C is 5 - 7 days (Ross *et al.* 1993).

Secondly, the complexity of food systems may have led to doubts that models derived from experimental systems may not reliably predict the growth of the modelled organism in a food. However, there is much good evidence supporting the predictive ability of models based on data generated in laboratory - based experiments for both constant conditions (Daud *et al.* 1978, Gibson *et al.* 1988, Ross and McMeekin 1991, Wijtzes *et al.* 1993, Neumeyer, *pers. comm.*) and conditions of fluctuating temperatures (Langeveld and Cuperus 1980, Reichel *et al.* 1991). In addition, there is evidence suggesting that microorganisms do not greatly affect the growth of one another, except where population densities are very high (Gill and Newton 1977, Mackey and Kerridge 1988). Therefore, microbial interactions do not appear to be a case for concern.

Thirdly, difficulty exists in predicting the lag time and the presence of microenvironments caused by the inhomogeneity of some foods. These problems can be overcome by a 'worst case' strategy. Gill *et al.* (1991b) determined the position of the slowest cooling part of a carcass contaminated with spoilage or pathogenic organisms, and based the predictions on that 'worst case'. In the absence of reliable data for the duration of the lag phase, one can again assume a 'worst case' i.e. no lag.

Fourthly, concerns have arisen regarding the inherent variability of the growth responses of microorganisms. The question of variability was addressed by Ratkowsky (1992) who indicated that response variables of interest in predictive microbiology are typically non - normally distributed. Using replicated data sets,

variances were shown to be proportional to the squares or cubes of their means. The distribution of the data was 'gamma' or 'inverse Gaussian' respectively, both of which show a strong right hand tail.

The data of Neumeyer (1992) for the growth of *Staphylococcus aureus* strain 3b provides an example of an inverse Gaussian distribution of time as a response variable (Table 1.3). Clearly, at temperatures close to the minimum for growth there is a marked increase in variability, and confidence in obtaining correct predictions must decrease.

Under these circumstances a better approach may be to determine the probability of achieving a certain generation time based on the distribution of data (Ratkowsky 1992). For example, Table 1.4 illustrates that only once in 10,000 events would the generation time of *S. aureus* strain 3b exceed 69 minutes, i.e. if the generation time is chosen for predictive modelling purposes the chance of a 'fail dangerous' event is one in 10,000.

Figure 1.3 shows that the shape of the distribution of θ (mean predicted generation time) at 12.5°C, where the average growth rate ($k = 1 / \theta$) is low, is highly asymmetric, there being a very long right - hand tail. On the other hand, at 30°C, where the growth rate is high, the distribution of θ is much more symmetrical, and much closer to that of a normal distribution (McMeekin *et al.* 1993).

Many models describing the growth of bacteria have been developed. Preceding sections in this review indicate that reliable models are available. However, there seems to be mistrust of empirical models. Cole (1991) asserts that researchers in the field of predictive microbiology are working towards mechanistic models but that currently there are none which deal with the many parameters of interest. McMeekin *et al.* (1993) derived a new mechanistic model by using as a starting the point the assumption that bacterial growth is determined by a single rate limiting enzyme, the concentration of which is determined by reversible inactivation due to temperature. The model is based upon previously published thermodynamic models. It is able to simulate well a wide range of bacterial temperature - growth rate curves. However, Ross (1993a) reported that the model may need further parameters to accurately describe the irreversible cessation of growth of the organisms at both high and low temperatures. Mechanistic models whose parameters cannot be determined experimentally cannot be considered to be truly mechanistic (Heitzer *et al.* 1991).

TABLE 1.3 Predicted generation time (θ) and its variance (V). Data of Neumeyer (1992) for growth of *Staphylococcus aureus* strain 3b in Brain Heart Infusion broth.

Temperature (°C)	θ (min)	V*
12.5	887.76	571600
15	388.62	47950
20	138.23	2158
27	56.43	146.8
28	51.03	108.6
30	43.32	61.9
35	28.28	18.5

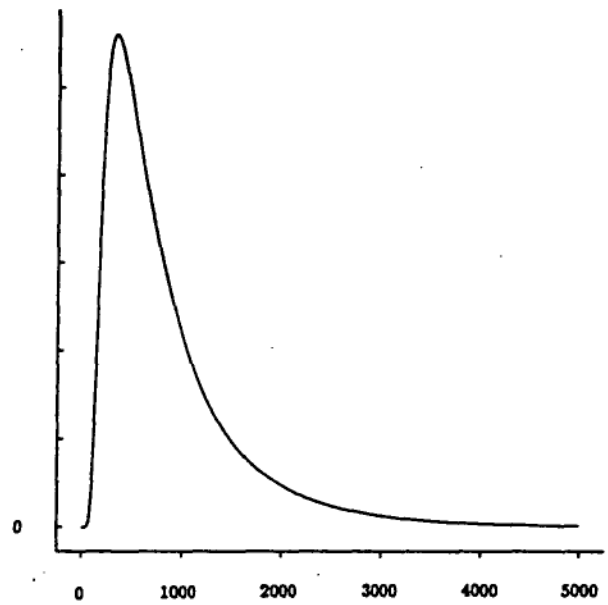
$V^* = 0.000817\ (\theta)^3$

TABLE 1.4 Values of generation times (θ) corresponding to specified levels of confidence in predictions being 'fail safe'. Data of Neumeyer (1992) for growth of *Staphylococcus aureus* strain 3b in Brain Heart Infusion broth at 12.5°C.

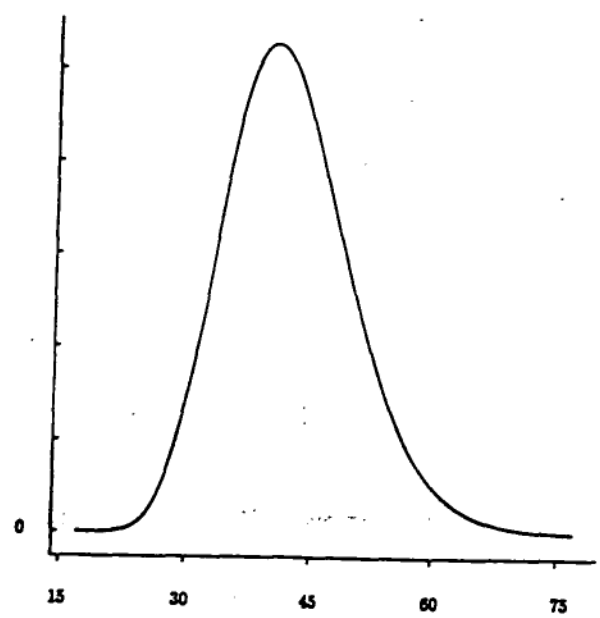
θ (min)	Probability
46	0.000001
55	0.00001
69	0.0001
92	0.001
136	0.01
168	0.025
203	0.05
657	0.5
2356	0.95
2951	0.975
3782	0.99
6038	0.999

FIGURE 1.3 Estimated probability density distribution function for inverse Gaussian distribution of generation time for θ at (a) 12.5°C and (b) 30°C for parameter values in Table 1.4 [Data of Neumeyer (1992)].

a



b



As discussed above (1.6.5), models can be successfully applied to food processing operations. Temperature function integration techniques can be used to evaluate the hygienic efficiency of a process. However, they cannot be used to assess the absolute hygienic status of individual units leaving the process. Processing adequacy will ensure that time and temperature conditions experienced by the product do not cause unacceptable degradation of product hygiene

"It should be remembered that predictive microbiology is perhaps more a philosophical approach than a specific method or technology, and while it may be successfully applied to many problems, it cannot solve all. The potential advantages of predictive microbiology are numerous, but the full realisation of that potential will depend upon a conscientious and rigorous approach to data gathering and modelling, ingenious solutions and strategies for the application of data and models, and willingness on the part of the industry to trial the approach and think in terms of the premises upon which predictive microbiology is based" (Ross *et al.* 1993).

1.7 SUMMARY

Predictive microbiology has been used for many years to evaluate the extent of growth of bacteria in foods. Scott (1937) stated that "a knowledge of the rates of growth of certain micro - organisms at different temperatures is essential to studies of the spoilage of chilled beef. Having these data it should be possible to predict the relative influence on spoilage exerted by the various organisms at each temperature. Further it would be feasible to predict the possible extent of the changes in populations which various organisms may undergo during the initial cooling of sides of beef in the meatworks when the meat surfaces are frequently at temperatures very favourable to microbial proliferation".

Scott's insight is the basis of the practical application of predictive microbiology. The relationship between temperature and bacterial growth response can be quantified by an appropriate function. Microbial responses are measured under defined, controlled conditions, and the results are summarised in the form of mathematical equations (model construction). Models are then validated in the food products. Time - temperature function monitoring devices can be used in conjunction with expert systems to apply the model to particular food processing operations and formulations.

Predictive microbiology can be used not only to minimise the risk of food - borne disease outbreaks, but to estimate product quality and shelf life. It can provide means of identifying and quantifying the effect of deficiencies in processing and distribution systems which cause rejection of products.

2 AIM AND EXPERIMENTAL DESIGN

The aim of this study is to develop a mathematical model for the growth of pseudomonads isolated from meat and to validate it in minced beef. The environmental factors likely to determine the growth of pseudomonads are temperature, pH, water activity and gaseous atmosphere. In minced beef stored aerobically, temperature and pH will be variable and will require consideration. Water activity will be non - limiting and will therefore not be studied. This study is part of a large collaborative project and the effect of water activity on the growth of pseudomonads will be considered by others.

To develop reliable models, large amounts of data need to be collected. The process can be laborious and time consuming. Therefore, models will be initially developed in broth systems, using turbidimetric methods to monitor growth. Minced beef will be used for model validation as bacteria can be uniformly distributed in the system. In addition, it is of great commercial significance especially in North America as the consumption of sausage products and minced beef in the USA is just under 6000 million Kg per year (Macrae *et al.* 1993) The presence of interactions between pseudomonads and other organisms likely to coexist in mince, will also be examined.

2.1 EFFECT OF TEMPERATURE ON THE GROWTH OF *PSEUDOMONAS*

As temperature is probably the most important factor influencing the growth of organisms on meat (McMeekin 1982), its effect on the growth of *Pseudomonas* will be studied extensively in this thesis. For model development, strains will be isolated from fresh and spoiled minced beef as laboratory kept strains may have lost important characteristics. Bacterial cells may be irreversibly damaged by sudden drops in temperature, ice formation and increase solute concentration occuring during freezing and thawing (Rosset 1982). Lowry *et al.* (1989) found that the length of the lag phase of *E. coli* after freezing / thawing was about 2.5 times longer than that after chilling. In addition, as repetitive subculture may alter the phenotype of cells, laboratory kept strains will not be used in this study.

The bacteriological status of retail minced beef will be assessed by measuring core temperatures of the retail product, initial numbers of pseudomonads and total numbers of organisms. Previous studies have shown that pseudomonads are present in high numbers in fresh meat and are the dominant organisms in meat stored under aerobic conditions, at chill temperatures (Gill and Newton 1977, Dainty and Mackey 1992). In this thesis these findings will be examined in detail.

Fast growing pseudomonads, representing the 'worst case scenario', are required for reliable model development. To determine the fastest growing strains,

growth rates of all strains isolated from minced beef will be measured at ~10°C. This temperature is chosen as it is in the mid range of the temperature models to be developed and is often experienced by pseudomonads during meat processing and storage. Growth curves of each strain at ~10°C will be analysed using Eqn. 1.1. For data to be analysed correctly, observations need to be evenly spread throughout the growth curves. 'False' parameters can be obtained if there are only few observations in the region of fastest growth.

To begin temperature model development, the fastest growing, best characterised *Pseudomonas* strain will be chosen. A strain isolated from pork, provided by VIAS laboratory (Victoria), will be modelled for comparison with minced beef strains. A mixture of pseudomonad strains ('cocktail') will also be modelled as it may represent more closely what happens in minced beef. However, no interactions between pseudomonad strains are expected as the nutrients in the medium are non - limiting. Square root models (Eqn. 1.5) will be developed for the growth of strains between ~ 0°C and 30°C, in laboratory broth using turbidimetric techniques. Growth between 0°C and 10°C will also be closely studied as this temperature region is the most relevant for the storage of meat products Other researchers taking part in this collaborative project will model growth of pseudomonads across the whole temperature range (0 - 45°C). Square root functions will be used as they accurately describe the effect of temperature on the growth rate of bacteria and have been applied to various food systems (Adams *et al.* 1991, McMeekin *et al.* 1993, Wijtzes *et al.* 1993).

To test the hypothesis that growth profiles of strains are similar, regardless the source of isolation, models of pseudomonads developed in this study will be compared to data obtained from similar studies conducted in other laboratories in Australia and to data from the literature .

2.2 EFFECT OF pH ON THE GROWTH OF *PSEUDOMONAS*

Changes in pH will be monitored as minced beef is allowed to spoil at chill temperatures. In addition, the effect of pH on the growth of pseudomonads will be studied in nutrient broth using lactic acid as an acidulant. Lactic acid is the predominant acid in muscle tissue formed by glycolysis (Belitz and Grosch 1987). It is also often added to meat because of its flavour enhancing properties. The humectant activities of lactic acid contribute to its water holding capacity (Papadopolous *et al.* 1991, Shelef and Yang 1991, Houtsma *et al.* 1993). Lactic acid can also be used to decrease the bacterial numbers on carcasses following dressing (Greer and Dilts 1992).

Considering that ~9 g / kg of lactic acid (m.w. = 90.1) are present in beef (Smulders 1987) and that moisture, in which the lactic acid is available, is ~75% of the

weight of lean meat (Macrae *et al.* 1993), the concentration of acid in the water component is equal to ~0.13M. To exagerate the situation and clearly see the effect of lactic acid on the growth of pseudomonads, 0.2M lactic acid will be used in this study.

Growth rates of pseudomonads will be measured at pH values between ~ 4.5 and 8.5, with temperature remaining constant. As the pH of muscle tissues can vary between 5.5 and 7 depending on the amount of glycogen present prior to slaughter (Gill and Newton 1977), the 4.5 - 8.5 range is chosen to include pH values commonly found in minced beef. A detailed study at low pH will allow the determination of the lowest value allowing growth (pH_{min}). To examine the effect of sudden drops in pH, the inoculum will be grown in broth at pH close to pH_{min} prior to inoculation in low pH broths. Results will be compared to those obtained when inocula were prepared normally (pH ~7). Growth rates will be determined at ~10°C and 20°C to confirm previous findings indicating the effects of pH and temperature to be additive (Cole *et al.* 1990, Adams *et al.* 1991).

2.3 INTERACTION STUDIES

Despite *Pseudomonas* being the dominant organism on meat stored aerobically at chill temperatures, other microorganisms may be present in lower numbers. Interactions may occur and the growth of *Pseudomonas* may be affected. To test this hypothesis, organisms other than pseudomonads will be isolated from minced beef. These organisms will then be combined with pseudomonads to form a 'cocktail'. The growth rates of *Pseudomonas* in the 'cocktail' will be measured at various temperatures. If interactions are absent, rates observed in the 'cocktail' are expected to be similar to those observed in the pure culture.

2.4 MODEL CALIBRATION AND VALIDATION

As growth rates measured with turbidimetric methods differ from those obtained with viable counting methods, models developed for temperature and pH in nutrient broth need to be adjusted. Previous findings show that, rates calculated with the two methods differ by a constant factor regardless the type of microorganism or the temperature (Dalgaard *et al.* 1994). Therefore in this study, the ratio of rates obtained from viable counting and turbidimetric methods will be calculated to enable the calibration of the developed models.

Once a model has been developed and calibrated, it needs to be validated in the food product to assess its true predictive ability. Models in this study will be validated in minced beef from a local butcher. Growth rates of pseudomonads on mince, stored at temperatures between ~ 0°C and 25°C, will be compared to those predicted by the model.

3 MATERIALS AND METHODS

3.1 ISOLATION AND MAINTENANCE OF *PSEUDOMONAS* STRAINS

Minced beef (300 - 500 g, topside) was purchased from local supermarkets and butchers. In supermarkets, meat was wrapped and stored in retail display cabinets. Mince at butcher stores was stored in open retail cabinets, the turn - over being, supposedly, faster than in supermarkets. The core temperature of the mass of mince was measured immediately outside the store with a thermometer with a flexible probe (Appendix 3). The meat was stored in a water - tight container with a large volume of atmosphere in comparison to the meat volume. It was allowed to spoil at 4 $\pm$ 0.1°C in a waterbath (Appendix 3). Ten grams of mince (fresh and spoiled samples) were diluted with 90 ml of sterile 0.1% peptone (Appendix 1.9), stomached for 2 min (Appendix 3) and further diluted with 0.1% peptone. Appropriate dilutions (100 μl) were spread plated on *Pseudomonas* Agar (Appendix 1.1). Plates were incubated at 25°C for 48 h. *Pseudomonas* strains were isolated from fresh mince initially and at spoilage.

Pseudomonas strains isolated from fresh and spoiled pork were provided by Dr Phillip Widders (Department of Agriculture, Victorian Institute of Animal Science, VIAS; Attwood, Victoria).

Gram stains (Appendix 2.2), oxidase tests (Appendix 2.3), oxidative and fermentative reactions (Appendix 2.4) and API 20NE strips (Appendix 3) were used to characterise each isolate. In addition to maintenance on glass beads at -70°C (Appendix 2.1), strains were stored on slopes (Plate count agar - Appendix 1.2) at 4°C (short term storage) and subcultured at 2 monthly intervals.

3.2 EFFECT OF TEMPERATURE ON THE GROWTH OF *PSEUDOMONAS*

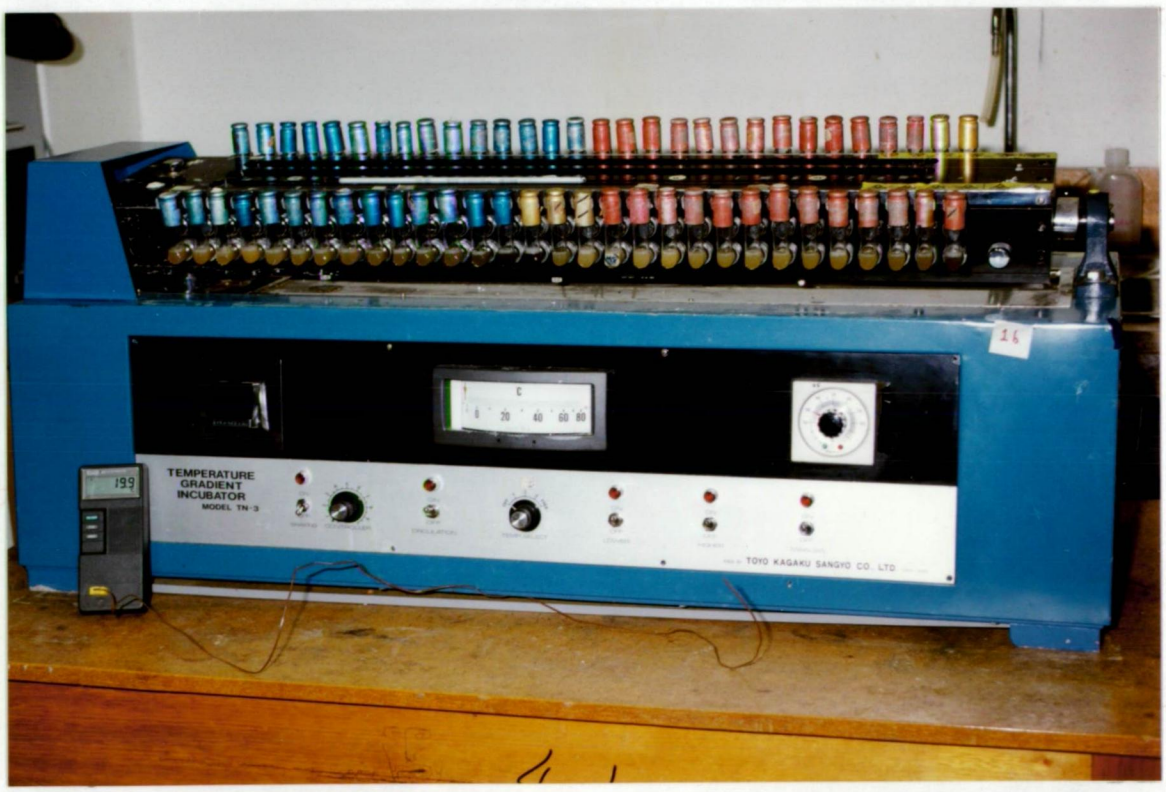
3.2.1 Generation times of strains at ~10°C

All strains isolated were grown on plate count agar (25°C / 48 h). Thirty ml of sterile nutrient broth (Appendix 1.3) in 200 ml conical flasks were inoculated with single colonies and incubated (25°C / 48 h, Appendix 2.7). A 'shaking' temperature gradient incubator (Appendix 3, Figure 3.1) was set up isothermally in a 10°C constant temperature room. L - shaped spectrophotometric tubes, 25 ml in volume, containing 9 ml of sterile nutrient broth were equilibrated at 10°C overnight. Tubes were then inoculated with sufficient bacterial suspension so that the % transmittance of the broth decreased from 100 % to ~80 - 85 %. Between 300 μl and 1000 μl of suspension were required.

Changes in % transmittance (λ = 540 nm) were measured with a spectrophotometer (Appendix 3). At least 12 - 15 readings were taken to construct each growth curve, however, whenever possible, as many as 20 readings were taken. Observations were evenly spread throughout the curve. At least 4 growth curves were constructed for each strain.

As growth terminated in each tube, temperatures were measured using a thermometer with a flexible probe (Figure 3.1, Appendix 3). Measurements were taken 5 times, and the average temperature recorded (Details in Appendix 6).

FIGURE 3.1 Temperature gradient incubator (Appendix 3) used to describe the effect of temperature on the growth of pseudomonads. The incubator can be used with or without a temperature gradient. L - shaped tubes with metal caps contain the bacterial suspension in nutrient broth. A digital thermometer with a thermocouple bead probe is displayed in the foreground. The thermometer was used to measure the temperature of each of the tubes at the end of the experiments.



3.2.1.1 Data analysis

For each replicate of individual strains, changes in % transmittance with respect to time were measured. The steepest tangent of the growth curve was calculated from the parameters of Eqn 1.1 fitted by a nonlinear regression using a SAS PROC NLIN routine written by Dr. Glen McPherson, Mathematics Department, University of Tasmania (SAS: Statistical Analysis System, SAS Institute Incorporated, SAS Circle, Cary, N. Carolina, USA).

Kinetic parameters such as generation time and lag time were then calculated by the method of McMeekin *et al.* (1993):

$$\textit{generation time} = \textit{time taken for population to double}$$

$$= \frac{\log_{10} 2}{(BC \,/\, e)} \tag{3.1}$$

where B is a parameter related to the slope of the curve and C is the maximum increase in population density. BC / e is the exponential growth rate.

and simplifying

$$= \frac{0.8183}{BC} \tag{3.2}$$

Equation 3.2 can be used for viable count data. By introducing a calibration factor, the generation time can be estimated from the Gompertz parameters fitted to % transmittance data (McMeekin *et al.* 1993):

$$\textit{generation time} = \frac{24.5 * e}{BC} \tag{3.3}$$

$$= \frac{66.6}{BC} \tag{3.4}$$

3.2.2 Temperature model development

Pseudomonas strain 3 (isolated from minced beef) was chosen to begin model development as it was among the fastest strains at ~10°C and it had been well characterised. Strain 5 (minced beef) and strain v4 (pork) were also modelled. To test the presence of interactions between pseudomonads, a 'cocktail', composed of strains 3, 5, 8 (minced beef), v1 and v6 (pork) was also used. A gradient incubator was set up with a range from ~0° to 30°C was set up in a 20°C constant temperature room. L - tubes were prepared as described in 3.2.1 and equilibrated in the incubator

overnight. A 48 - hour bacterial suspension of each strain (stationary phase, incubated at 25°C, as described in Appendix 2.6) was used as the inoculum .

For the cocktail studies, a mixture of equal amounts of strain suspensions was used as the inoculum (each suspension prepared as in Appendix 2.7).

Changes in % transmittance were monitored for each tube over a period of time. Between 15 and 20 readings were taken for each growth curve. To develop a model for each strain, ~30 data points (growth curves) were obtained, therefore each model was made up of at least 450 - 600 observations. Temperatures were recorded as growth ceased in each tube. Data were analysed as in 3.2.1.1 and Eqn. 3.4 was used to calculate generation times.

Experiments were carried out in the temperature ranges shown in Appendix 4.1. For gradients ~0 - 10°C and 0 - 20°C the temperature gradient incubator was set up in a constant 10°C room.

The effect of temperature on the generation time of strains was described by constructing square root models (Eqn. 1.5). The square root of rate (1 / generation time) was plotted against temperature. Data was fitted by linear regression (CA - Cricket Graph™ 1.3.2. Computer Associates Intl., Malvern, PA, USA). By extrapolation to y = 0, T_{min} values were calculated for each strain. Slopes of each plot were also calculated. T_{min} values and slopes were compared.

Residual plots (Cook and Weisberg 1982) were drawn where there was striking non - linearity of residuals. The homogeneity - of - slopes model (SAS / STAT, PROC GLM) was used to assess the difference between models developed for a single strain at separate times.

3.3 EFFECT OF pH ON THE GROWTH OF *PSEUDOMONAS*

3.3.1 pH changes in minced beef

Changes in pH were monitored at regular intervals as seeded minced beef was allowed to spoil at 2°C, 4°C and 10°C (3.5.2.2). A pH meter with a flat - surface probe (Appendix 3) was used.

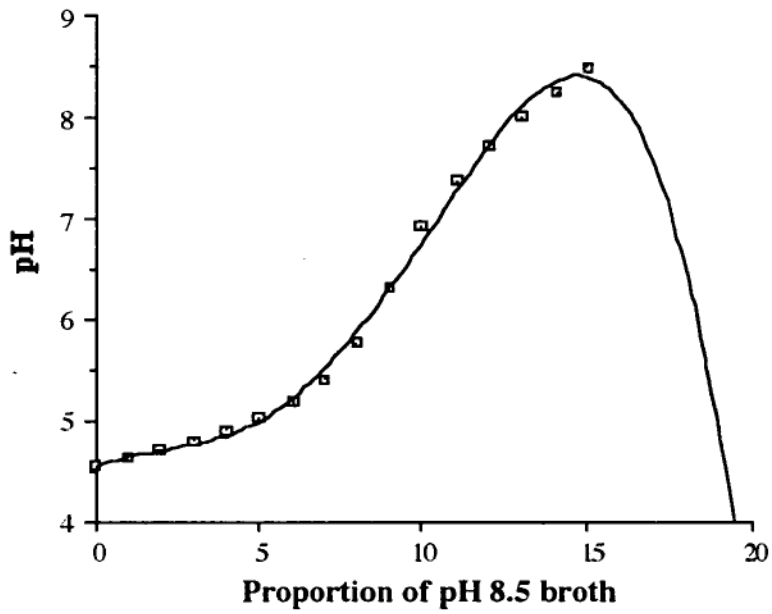
3.3.2 pH model development in broth

The effect of pH on the growth of *Pseudomonas* was studied (between pH ~4.5 and 8.5). Lactic acid (0.2M, Appendix 1.10) was used as an acidulant. Two stock solutions of nutrient broth with lactic acid were prepared. Following autoclaving, one batch was adjusted to pH 4.5 (Appendix 3) and the other to pH 8.5 with sterile NaOH. Batches were made up to final volume with sterile water. Broths of varying pH values were prepared by combining the two stock media in varying proportions in sterile L - tubes. A standard curve was prepared (Figure 3.2) to

determine the proportion of pH 8.5 stock solution to be added to obtain desired pH values. As the total volume of all the tubes was 15 ml, tube 1 contained 0 ml of pH 8.5 broth and 15 ml of pH 4.5 broth; tube 2 contained 1 ml of pH 8.5 broth and 14 ml of pH 4.5 broth ..... and so on.

Autoclaving was performed prior to mixing as volumes and pH were found to alter through sterilisation. Aliquots (0.5 ml) were aseptically removed immediately after inoculation and the pH was determined. The water activities of nutrient broth with and without 0.2M lactic acid were measured (Appendix 3).

FIGURE 3.2 Standard curve representing the proportion of pH 8.5 stock solution (nutrient broth + 0.2 M lactic acid) to be added to pH 4.5 solution to obtain desired pH values.



y = 4.5523 + 0.13832x - 4.7201e-2x^2 + 9.3863e-3x^3 - 3.8078e-4x^4
R^2 = 0.997

L - tubes were equilibrated overnight in a 'shaking only' gradient incubator (set at a constant temperature). Inocula of *Pseudomonas* strains (3 and v4) were pre - incubated (48 h, 25°C) in nutrient broth (no lactic acid added) at normal pH (approximately 7) and at a lower pH (5.6) to evaluate the effect of sudden drops in pH. Tubes were inoculated and changes in % transmittance were monitored. Data were analysed as in previous experiments (3.2.1).

The effects of pH were monitored in the 4.5 to 8.5 range. Detailed studies in the lower region (4.5 - 6.0) were conducted to determine the minimum pH allowing growth.

To allow comparison of rates calculated from the studies at 10°C and 20°C, rates were standardised to an equivalent rate at a designated temperature (10°C). The following equation was used to calculate the factor required to correct all measured rates (McMeekin *et al.* 1988):

$$\text{correction factor} = \left(\frac{T_{obs} - T_{\min}}{T_{ref} - T_{\min}} \right)^2 \qquad (3.5)$$

where T_{obs} is the temperature observed (i.e. ~20°C or ~10°C), T_{min} is the minimum theoretical temperature for growth (-7.4°C for *Pseudomonas*) and T_{ref} is the designated temperature for comparison (10°C).

Analysis of variance was conducted using $\sqrt{}$ *rate* and **ln** *rate* as the response variates.

3.4 INTERACTION STUDIES

3.4.1 Isolation of microorganisms other than *Pseudomonas*

Selective media were prepared to isolate microorganisms commonly found in minced beef. Gardner's medium (Appendix 1.4) was used to isolate *Brochothrix thermosphacta* (Gardner, 1966). Plates were incubated at 25°C (48 h). Violet Red Bile Agar (VRBA, Appendix 1.5) was used to isolate coliforms. Plates were incubated at 4°C (10 days) to identify psychrotrophic coliforms and at 37°C (24 h) for mesophiles. De Man, Rogosa, Sharpe medium (MRS, Appendix 1.6) was prepared to isolate lactic acid bacteria. Plates were incubated at 25°C (3 days) for psychrotrophic bacteria and at 37°C (48 h) for mesophiles. Samples of minced beef were also plated on plate count agar (Appendix 1.2) and incubated at 25°C for psychrotrophs and 37°C for mesophiles.

Colonies from each selective medium were identified by Gram, oxidase, catalase and oxidative / fermentative reactions and by motility (Appendix 2). Organisms were also enumerated in fresh and spoiled mince by plating dilutions on selective agars and incubating at 10°C and 20°C.

3.4.2 Interaction studies in broth and minced beef

The following isolates were selected (from 3.4.1) to investigate the possibility of interactions between *Pseudomonas* strain 3 and other organisms:

1. *Brochothrix thermosphacta* - isolate B
2. Lactic acid bacteria - isolate T
3. Lactic acid bacteria - isolate C_e
4. *Citrobacter freundii* - isolate G
5. *Staphylococcus aureus* - isolate H

All organisms were isolated from fresh minced beef, with the exception of isolate C_e which was from spoiled mince.

Each organism (including *Pseudomonas* strain 3) was grown separately in nutrient broth for up to 4 days, aerobically, at its optimum temperature. Nutrient broth was used as it is of similar composition to meat. All organisms were grown aerobically to simulate the mince environment. Despite lactobacilli being micro - aerophilic, they were able to grow aerobically in nutrient broth within 4 days. To constitute a 'cocktail', organisms previously grown to their stationary phase were mixed in a sterile container (Table 3.1).

L - tubes containing sterile nutrient broth were equilibrated on a temperature gradient incubator (overnight, at the temperature of subsequent incubation, 4 - 20°C range) and were then inoculated with the 'cocktail' suspension. Generation times of *Pseudomonas* strain 3 were measured by viable counting methods (3.5.2.1 and Appendix 2.8) using *Pseudomonas* agar (Appendix 1.1). Generation times were calculated using Eqn. 3.2

Sterile mince was also inoculated with the 'cocktail' suspension (as in 3.5.2.2), and generation times were measured by viable counting methods and calculated as above.

To assess the existence of interactions, rates (1 / generation time) of *Pseudomonas* strain 3 were compared to the model developed for the pure culture. Bias and precision factors (Eqns. 1.10 and 1.11) were used to evaluated the deviation of observed rates from those predicted.

TABLE 3.1 Initial numbers of organisms present in the cocktail used for interaction studies.

Organism	Numbers (cfu / ml cocktail)
S. aureus	2.5×10^4
C. freundii	6.1×10^4
B. thermosphacta	4.7×10^4
Lactic acid bacteria	4.9×10^5
Lactic acid bacteria	1.2×10^5
Pseudomonas strain 3	3.6×10^5

3.5 MODEL CALIBRATION AND VALIDATION

3.5.1 Model calibration

Strains 3, 5 and strain v4 were used to derive a factor to calibrate models constructed in broth. L - tubes and strains were prepared as above (3.2.1). Tubes were inoculated with stationary phase - bacterial suspension (Appendix 2.6) to give an approximate concentration of 1×10^5 cfu / ml. Tubes were incubated on a shaking temperature incubator at several temperatures between 0°C and 30°C.

Viable counting was used to measure increases in cell concentration over the entire growth curve. Limitations of spectrophotometric methods permitted changes in % transmittance to monitor only the portion of the curve between approximately 10^6 and $10^{8.5}$ cfu / ml (Dalgaard *et al.* 1994). Changes in % transmittance data were fitted to Eqn. 1.1 and generation times calculated (Eqn. 3.4).

To monitor changes in numbers by viable counting methods, 100 μl of suspension was removed from each L - tube and diluted serially in 0.1% peptone (duplicate samples, Appendix 1.9). The final dilution (100 μl) was spread plated on plate count agar plates (duplicates from each dilutions, Appendix 1.2). Plates were incubated for 48 h at 25°C and those with 30 - 300 colonies were counted (Appendix 2.8). Eqn. 1.1 was fitted to the data and generation times were calculated (Eqn. 3.2).

Generation times calculated from % transmittance data were divided by those obtained using viable counting methods. An average of the ratios calculated was then used to calibrate models developed (3.2.2).

3.5.2 Model validation

3.5.2.1 Validation in mince stored in bijoux and bags

Calibrated models were validated in minced beef purchased from a local butcher. Portions of the product (2 g) were placed firstly, in sterile 5 ml bijoux which were stored in plastic boxes in waterbaths at various temperatures (± 0.1°C, Appendix 3). To sample, the contents of duplicate bijoux were removed, placed into sterile stomacher bags (Appendix 3) containing 18 ml of sterile 0.1% peptone (Appendix 1.9) and stomached (2 min, Appendix 3). Appropriate dilutions were spread plated in duplicate on plate count and *Pseudomonas* agar (Appendices 1.2 and 1.1). Plates were incubated at 25°C for 48 h and those with 30 - 300 colonies were counted (Appendix 2.8).

Secondly, 2 g portions of minced beef were placed in stomacher bags which were then folded and packed in large waterproof bags in waterbaths (Appendix 3). To sample, duplicate portions were removed, 18 ml of 0.1% peptone (Appendix 1.9) were added and the contents were stomached (2 min, Appendix 3). Appropriate dilutions were plated (plate count and *Pseudomonas* agars, Appendices 1.2 and 1.1). Plates were incubated (25°C, 48 h) and those with 30 - 300 colonies were counted (Appendix 2.8).

Large portions of minced beef (~300g) were assessed visually prior and during storage at 4°C and 20°C. Any changes in the colour and appearance of the mince were recorded.

3.5.2.2 Validation by seeding sterile mince

As initial numbers of *Pseudomonas* on minced beef were too high to allow growth to be monitored, sterile mince was inoculated with the retail product to reduce the numbers.

Large pieces of topside beef (1 - 1.5 kg) were purchased from a local butcher. After freezing, the pieces were thawed slightly to facilitate the removal of the external surfaces. Large sterile knives were used to remove the surfaces and to cut the meat in pieces. The inner sterile portions were then minced with a sterilised metal mincer and were subsequently frozen. Portions of the sterile mince were thawed when required and checked to ensure freedom from contamination.

In some experiments, sterile mince was inoculated with *Pseudomonas* suspension (strain 3 in nutrient broth, stationary phase culture, Appendix 2.7). Mincing (2 - 3 times) allowed uniform distribution of bacteria. Appropriate amounts of bacterial suspensions were added to give initial levels of 1×10^4 cfu / g.

In other experiments, sterile mince portions were minced 2 - 3 times with retail product (minced beef from local butcher with *Pseudomonas* levels between 1×10^5 and 1×10^7 cfu / g) to give initial levels of 1×10^4 cfu / g.

In both methods (inoculating with bacterial suspension or retail product), 1g portions of seeded mixture were placed in individual wells of multi - welled culture plates (Figure 3.3, Appendix 3), which were then sealed in moist water - tight boxes and immersed in waterbaths (Appendix 3). When sampling, portions were removed (duplicate) and placed in small stomacher bags (10 x 16 cm, Appendix 3). Nine ml of sterile 0.1% peptone were added (Appendix 1.9). After stomaching appropriate dilutions were prepared with 0.1% peptone. Spread plates were incubated at 25°C for 48 h. When seeding with bacterial suspension, plate count agar was used as, from preliminary studies, the only microorganism present was *Pseudomonas*. When seeding with retail product, both plate count and *Pseudomonas* agars were used (Appendices 1.2 and 1.1).

Data obtained with model validation procedures were analysed using Eqn. 1.1. When not enough data points were taken in the region of exponential growth, Eqn. 1.1 fitted the data poorly (> 10 iterations). This occured in < 5% of growth curves. In these instances the sigmoidal curve was fitted by eye and the line of best fit through the steepest part of the graph was estimated. Generation times were calculated using Eqn. 3.2. Twenty one growth curves were constructed, each one developed with duplicate samples (~630 observations)

3.5.2.3 Comparison of predicted and observed data

Generation times calculated in the model validation studies (3.5.2.1 and 3.5.2.2) were compared with those predicted by the calibrated model constructed in broth. Strain 3 model was used for comparison as this strain was studied most intensively.

To evaluate the deviation of observed generation times from those predicted by the calibrated strain 3 model, bias and precision indices were calculated (Eqns. 1.10 and 1.11).

Data obtained from these studies were also compared with that from other laboratories and from the literature.

FIGURE 3.3 Multi - well plates (Appendix 3) used to incubate sterile mince inoculated with *Pseudomonas* strain 3 suspension or retail mince. Plates were stored in waterbaths in moist water - tight containers.

4 RESULTS

4.1 EFFECT OF TEMPERATURE ON THE GROWTH OF *PSEUDOMONAS*

4.1.1 Temperature and bacteriological status of minced beef

Core temperatures of portions of minced beef purchased from supermarkets and butchers ranged from 3.7°C to 10.6°C. Log numbers of pseudomonads present on mince, just after purchase, ranged from 5.13 to 6.00. Total log numbers of viable organisms ranged from 6.00 to 7.11 (Table 4.1).

Pseudomonas strains (n = 13) isolated from fresh and spoiled minced beef are presented in Table 4.2. Strains from pork were provided by VIAS laboratory (n = 4).

TABLE 4.1 Core temperatures and bacteriological status of fresh minced beef purchased from supermarkets and butchers.

	Core Temperature (°C)	*Pseudomonas* numbers (log no / g)	Total viable numbers (log no / g)
supermarket 1	6.3	5.13	6.00
supermarket 2	10.5	5.36	6.18
supermarket 3	8.3	5.80	7.11
butcher 1	3.7	6.00	6.00
butcher 2	10.6	NM	NM
butcher 3	6.7	NM	NM

NM = not measured

TABLE 4.2 *Pseudomonas* isolates from minced beef and pork.

Strain	API Identification	Isolated from
1	*P. fluorescens* (62.6)	mince, not spoiled
2	*P. fluorescens* (86.7)	mince, spoiled 4°C
3	*P. fluorescens* (99.2)	mince, not spoiled
4	*P. fluorescens* (83.1)	mince, not spoiled
5	*P. fluorescens* (87)	mince, not spoiled
6	*P. fluorescens* (75.1)	mince, spoiled 4°C
8	*P. fluorescens* (78.8)	mince, spoiled 4°C
11	*P. fluorescens* (83.1)	mince, spoiled 4°C
12	*P. fluorescens* (73.3)	mince, spoiled 4°C
13	*P. fluorescens* (79.3)	mince, spoiled 12°C
14	*P. fluorescens* (74.9)	mince, spoiled 10°C
15	*P. putida* (97.9)	mince, spoiled 4°C
16	*P. putida* (92.9)	mince, spoiled 4°C
v1*	*P. fluorescens* (73.3)	pork, spoiled 4°C
v3*	*P. chlororaphis* (74.2)	pork, not spoiled
v4*	*P. putida* (95.4)	pork, spoiled 4°C
v6*	*P. fluorescens* (98.3)	pork, not spoiled

All strains are Gram negative, oxidase positive and oxidative (Appendix 2)
Identifications performed with API 20NE, in brackets: percentage probability of presumptive identification
*Strains obtained from VIAS (Victoria)

4.1.2 Generation times of strains at ~10°C

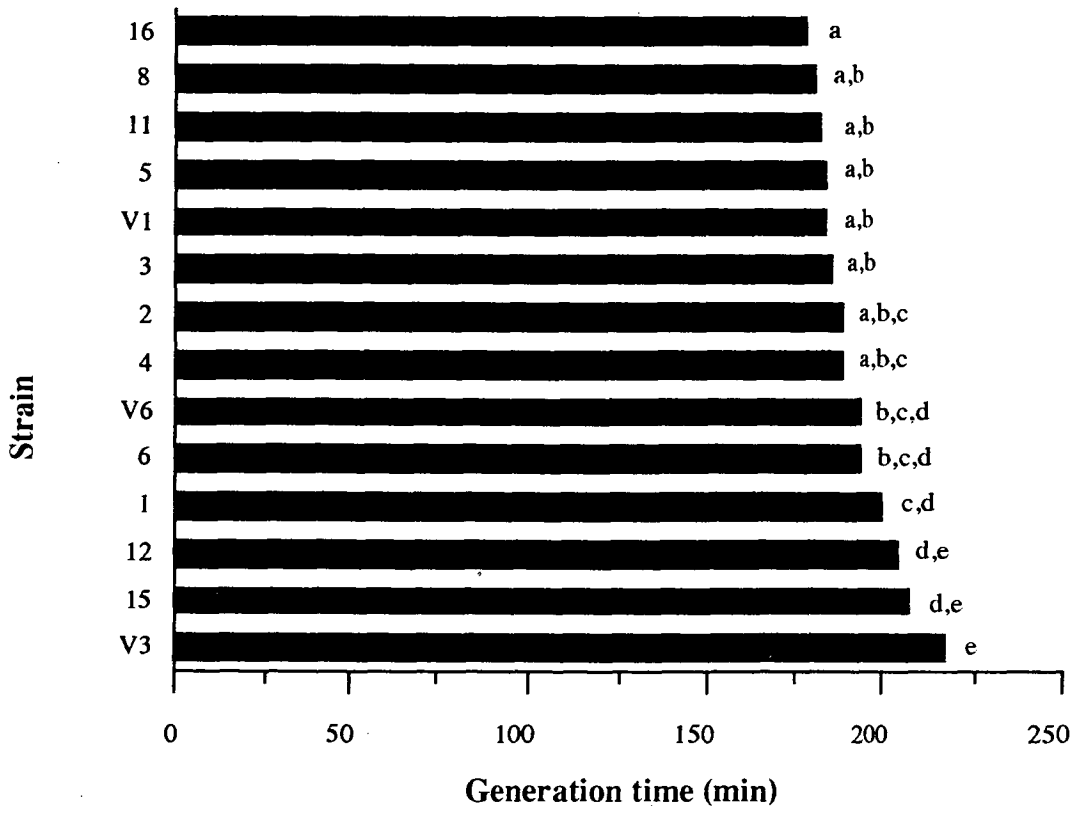
Generation times of strains incubated at ~10°C, calculated using Eqn. 3.4, are shown in Figure 4.1. The range of times calculated was 176 - 217 min (Appendix 5.1). Analysis of variance revealed that generation times of fourteen pseudomonad strains tested were significantly different (F value = 4.73, probability level = 0.0001). The strains could be grouped into 5 groups (a - e in Figure 4.1). Within each group, generation times were not significantly different. On the basis of generation times, >50% of the strains were part of a single group (group a), including the 8 fastest strains from strain 16 to strain 4 in Figure 4.1. The generation time at 10°C of strain v4, obtained at a later stage from VIAS laboratories, was 272 min.

4.1.3 Temperature model development

Square root models, describing the effect of temperature on the growth of pseudomonads, are shown in Figures 4.2 - 4.5 (raw data in Appendices 5.2 - 5.8). *Pseudomonas fluorescens* strains 3 and 5 were modelled as they were among the fastest growing strains at ~10°C and had been well characterised. These strains may represent a 'worst case scenario'. A *Pseudomonas putida* strain isolated from pork (v4) was also modelled. A cocktail containing minced beef strains 3, 5 and 8 and pork strains v1 and v6 was modelled as it represents more closely the situation likely to occur in the product where several strains may co - exist.

Growth rates of pseudomonads were measured between ~0°C and 30°C. Temperatures recorded are shown in Appendix 4.1. Strain 3 was also modelled extensively at lower temperatures using gradients of ~0 - 22°C and 0 - 10°C (Appendix 4.1). The cocktail was modelled using gradients of ~0 - 30°C and 0 - 10°C (Figures 4.5 a and b).

FIGURE 4.1 Generation times of *Pseudomonas* strains isolated from minced beef and pork at ~10°C. Isolates can be statistically grouped into 5 groups (a - e) on the basis of their generation times.

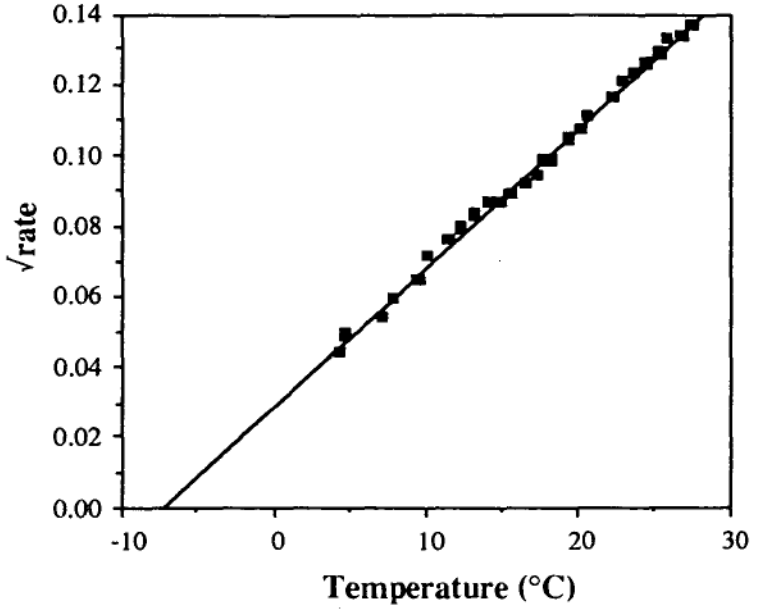


Strains v1, v6, and v3 - pork strains, the remaining - minced beef strains

Generation times of strains 8, 5, v1, 3, v6 and 6 are each averages of 8 replicates (2 experiments, 4 replicates each). Generation times of other strains are averages of 4 replicates (one experiment)

Organisms with the same superscript are not significantly different

FIGURE 4.2a Square root plot of the effect of temperature (4 - 28°C) on the rate of growth of *Pseudomonas* strain 3 (minced beef isolate). Note small run of positive residuals between ~10°C and 15°C.

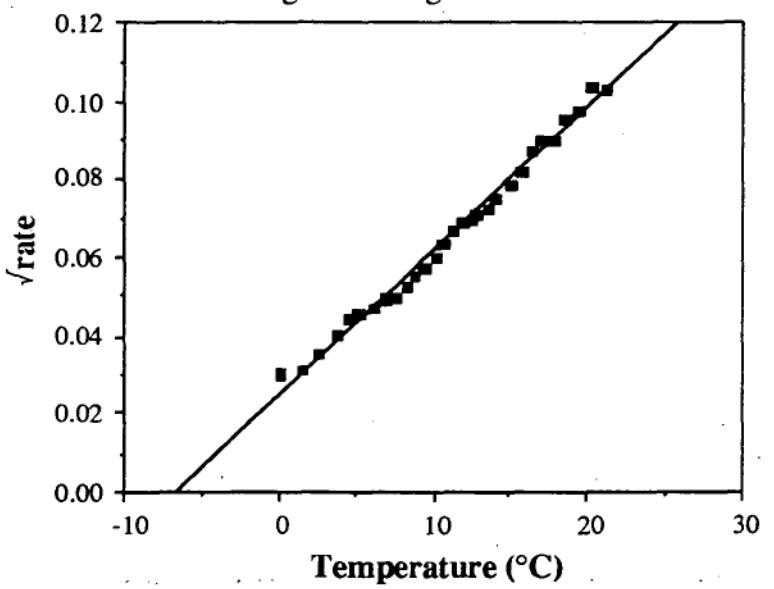


$\sqrt{rate} = 0.003955\ (T - T_{min})$

$R^2 = 0.996$

$T_{min} = -7.4$ °C (265.8 K)

FIGURE 4.2b Square root plot of the effect of temperature (0 - 21°C) on the rate of growth of *Pseudomonas* strain 3 (minced beef isolate). Note long run of negative residuals between ~7°C and 17°C.

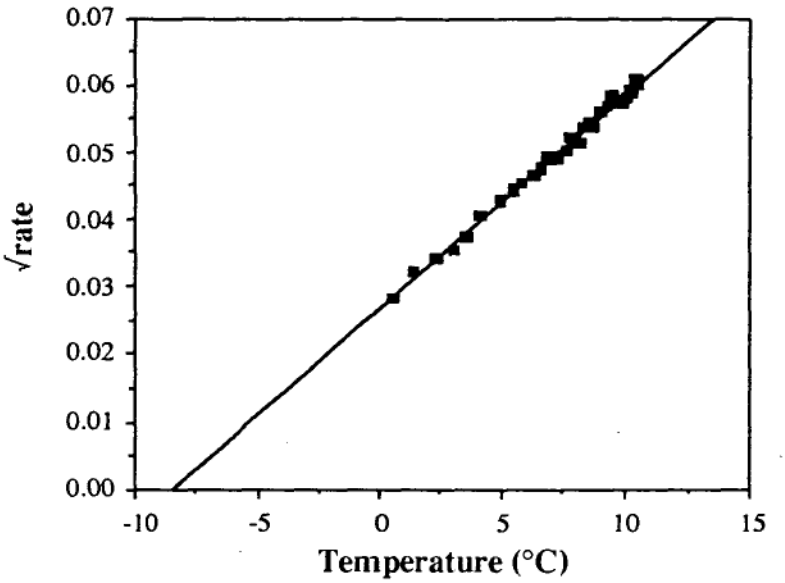


$\sqrt{rate} = 0.0036867\ (T - T_{min})$

$R^2 = 0.991$

$T_{min} = -6.7$ °C (266.5 K)

FIGURE 4.2c Square root plot of the effect of temperature (0 - 10°C) on the rate of growth of *Pseudomonas* strain 3 (minced beef isolate).

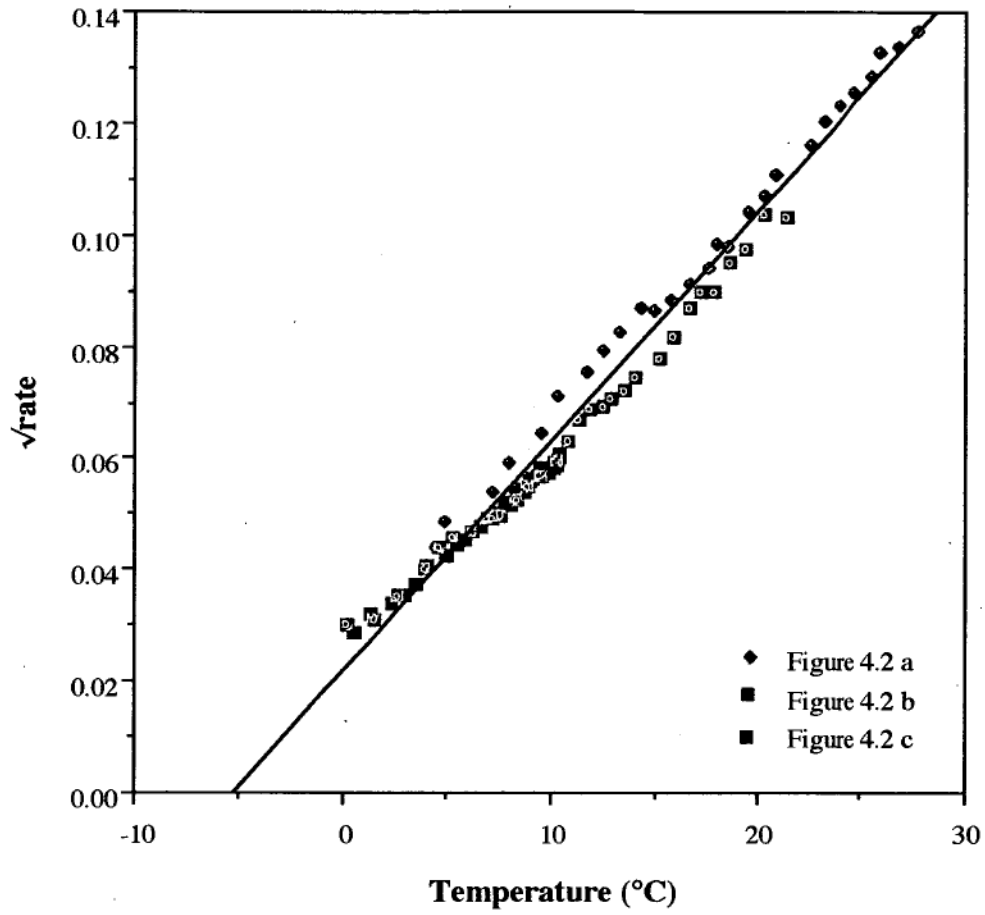


$\sqrt{rate} = 0.003201\ (T - T_{min})$

$R^2 = 0.994$

$T_{min} = -8.4$ °C (264.8 K)

FIGURE 4.2d Combined square root plot (Figures 4.2a - c) of the effect of temperature on the rate of growth of *Pseudomonas* strain 3.



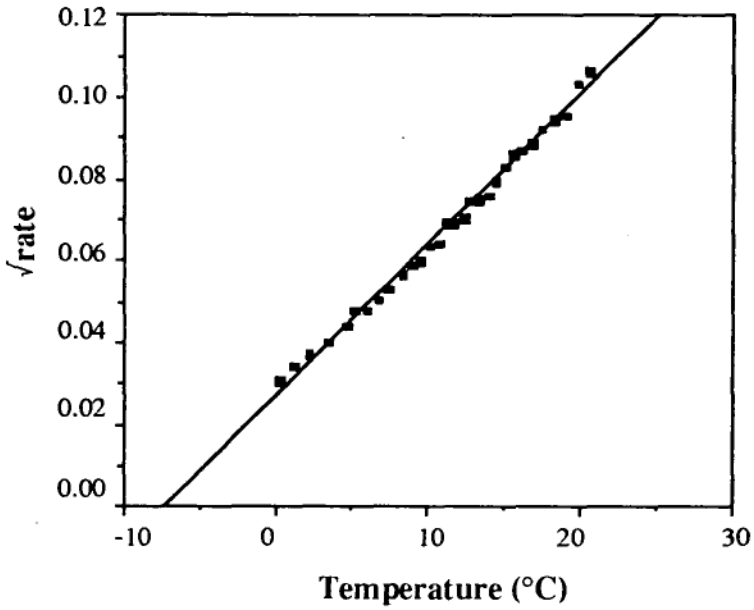
Line of best fit through all data points combined:

$\sqrt{rate} = 0.004125\ (T - T_{min})$

$R^2 = 0.979$

$T_{min} = -5.3\ °C\ (267.9\ K)$

FIGURE 4.3 Square root plot of the effect of temperature (0 - 20°C) on the rate of growth of *Pseudomonas* strain 5 (minced beef isolate). Note long run of negative residuals between ~7°C and 17°C.

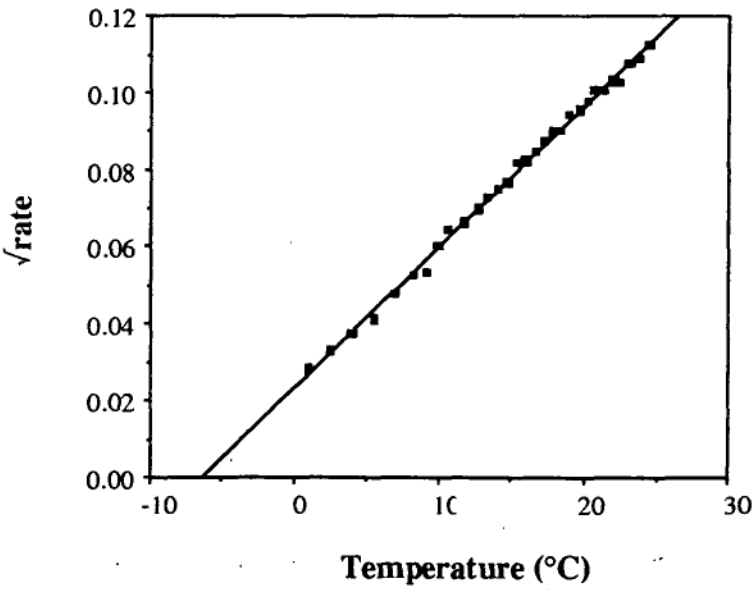


$\sqrt{rate} = 0.003684\ (T - T_{min})$

$R^2 = 0.994$

$T_{min} = -7.3$ °C (265.9 K)

FIGURE 4.4 Square root plot of the effect of temperature (0 - 24°C) on the rate of growth of *Pseudomonas* strain v4 (pork isolate).

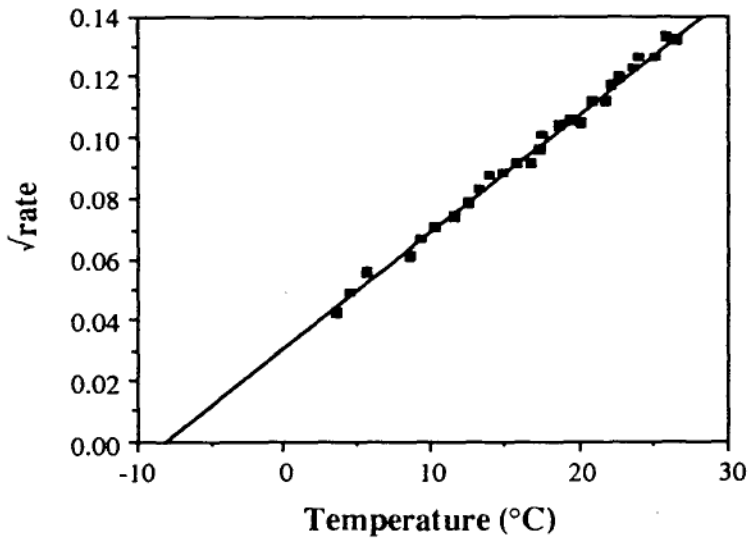


$\sqrt{rate} = 0.003666\ (T - T_{min})$

$R^2 = 0.997$

$T_{min} = -6.5$ °C (266.7 K)

FIGURE 4.5a Square root plot of the effect of temperature (3 - 26°C) on a cocktail of 5 *Pseudomonas* strains.

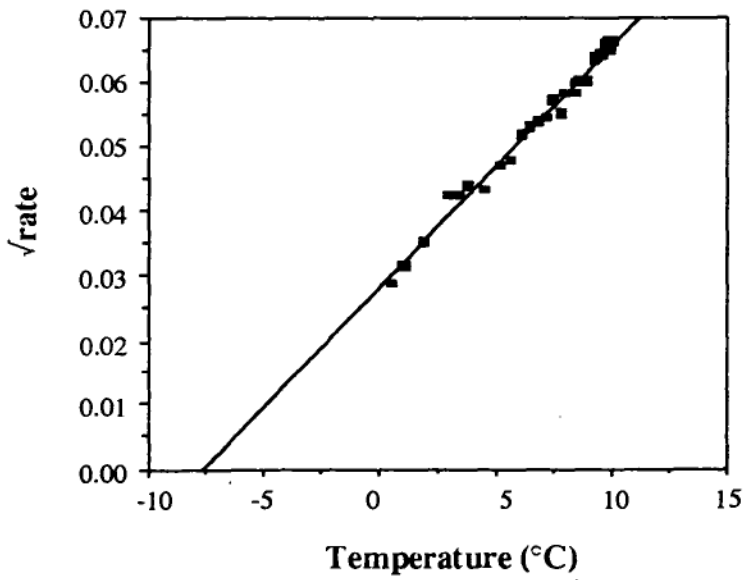


$\sqrt{rate} = 0.003871\ (T - T_{min})$

$R^2 = 0.994$

$T_{min} = -8.2$ °C (265.0 K)

FIGURE 4.5b Square root plot of the effect of temperature (0 - 10°C) on a cocktail of 5 *Pseudomonas* strains.



$\sqrt{rate} = 0.003733\ (T - T_{min})$

$R^2 = 0.998$

$T_{min} = -7.8$ °C (265.4 K)

4.1.4 Model comparison

The hypothetical temperatures below which no growth can occur (T_{min}, Eqn. 1.5) and the slopes calculated from each square root model developed are presented in Table 4.3. Generation times predicted by the models at 11°C are also presented in Table 4.3.

TABLE 4.3 Comparison of T_{min}, slope values and generation times (GT) at 11°C obtained from square root models (Figures 4.2 - 4.5).

Strain	Slope (x 10^{-3})	T_{min} (°C)	T_{min} (K)	GT 11°C (min)	Figure
3 (4-28°C gradient) [a]	3.95	- 7.4	265.8	189.9	4.2 a
cocktail (3-26 °C gradient) [a]	3.87	- 8.2	265.0	182.0	4.5 a
3 (0 - 21°C gradient) [b]	3.69	- 6.7	266.5	236.2	4.2 b
5 (0 - 20°C gradient) [b]	3.68	- 7.3	265.9	221.1	4.3
3 (0 - 10°C gradient) [c]	3.20	- 8.4	264.8	260.7	4.2 c
cocktail (0 - 10 °C gradient) [c]	3.73	- 7.8	265.4	204.1	4.5 b
v4 (0 - 24°C gradient)	3.66	- 6.5	266.7	244.4	4.4

All generation times in model development were expressed in minutes.

Strain v4 was isolated from pork (VIAS), strains 3 and 5 from minced beef. Cocktail contained strains 3, 5, 8, v1 and v6 grown to stationary phase.

Simultaneous experiments are marked with the same superscript.

Figure 4.2d (combination of Figures 4.2 a - c) shows that the three experiments conducted separately with strain 3 cannot be successfully overlapped. On close examination of Figure 4.2b, a long run of negative residuals is evident between ~7°C and 17°C. A similar run was noted in Figure 4.3 (strain 5). These two experiments were conducted simultaneously on opposite sides of the temperature gradient incubator. By plotting Figures 4.2b and 4.3 together (Figure 4.6) it is evident that the two graphs have similar shapes. A residual plot of these two experiments reveals non - linearity of the error variance (Figure 4.7). The mean deviation of the data from each model was 1.7 x 10^{-3} for strain 3 and 1.4 x 10^{-3} for strain 5.

FIGURE 4.6 Comparison of the square root plots of strain 3 (Figure 4.2b) and strain 5 (Figure 4.3) .

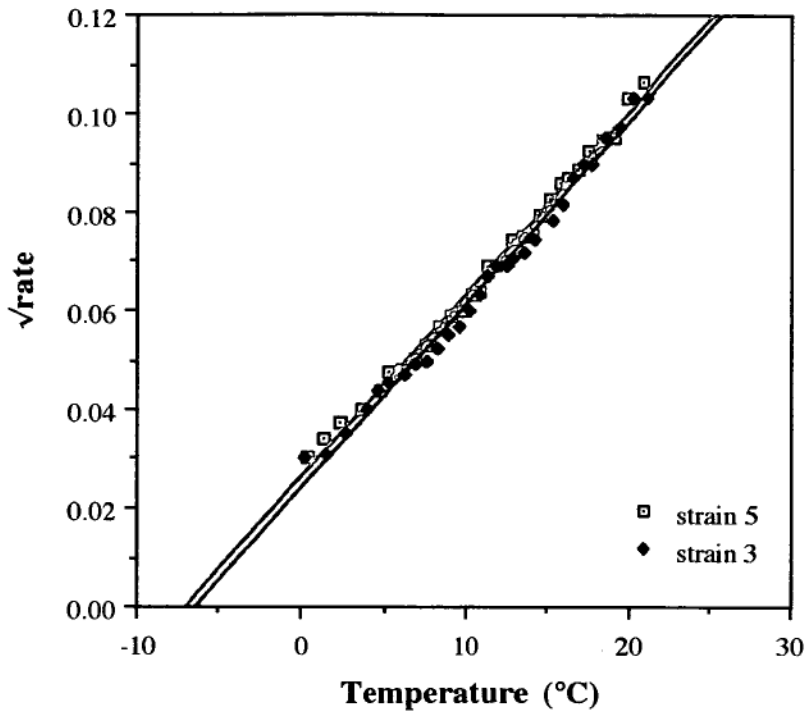
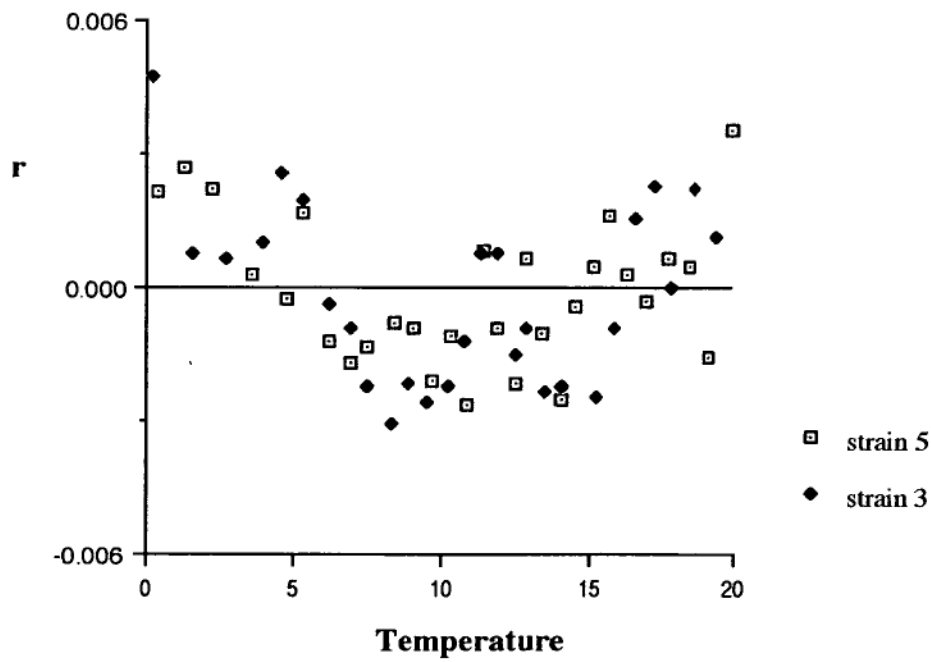


FIGURE 4.7 Residual plots of data from Figures 4.2b and 4.3 indicating non - linearity of error variance.



Despite problems encountered when overlapping Figures 4.2a - c, and negative residuals evident in Figure 4.2b, the average value of T_{min} calculated for strain 3 was - 7.5°C (265.7 K, standard deviation = 0.9). This compares with the average T_{min} of the cocktail (- 8.0°C, 265.2 K ± 0.3) and that of strain 5 (- 7.3°C, 265.9 K, Table 4.3). Pork strain v4 had a slightly higher T_{min} of - 6.5°C (266.7 K). The average slope for strain 3 was 0.00361 (± 0.00038), that of the cocktail was 0.00380 (± 0.00010), that of strain 5 was 0.00368 and that of strain v4 was 0.00366. The average T_{min} from all models developed was - 7.5 (265.7 K ± 0.7) and the average slope was 0.00368 (± 0.00024).

4.2 EFFECT OF pH ON THE GROWTH OF *PSEUDOMONAS*

4.2.1 pH changes in minced beef

The initial pH of sterile mince seeded with strain 3 or retail product (1 x 10^4 cfu / ml, section 4.4.2.2) was in the range 5.5 - 6. A sharp increase from pH ~7 to 9 occurred only when bacterial numbers reached 1 x 10^7 - 1 x 10^9 cfu / ml (Figure 4.8, Appendices 5.13 - 5.14). This indicated that pH increased sharply only when the mince was spoiled. Increases in pH were independent of the temperature at which meat was allowed to spoil.

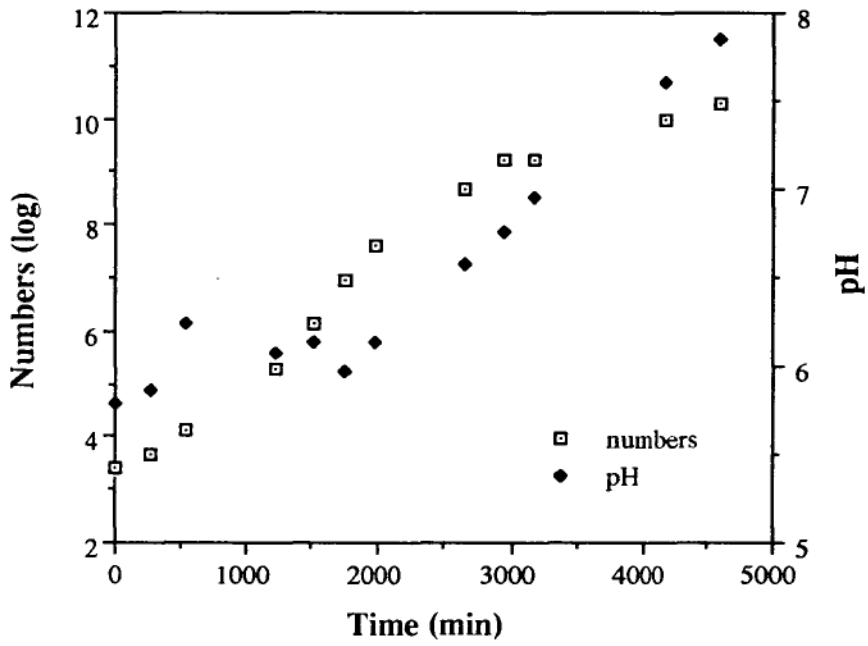
4.2.2 pH model development in broth

The effects of pH on the growth of *Pseudomonas* are presented in Tables 4.4a - 4.4d and Figures 4.9a and 4.9b. Growth rates of *Pseudomonas* strains 3 and v4 were monitored over a range of initial pH values (pH 4.9 - 8.6 for strain 3 and 5.0 - 7.9 for strain v4). The temperature of each tube varied slightly (± 0.5°C) during the experiment. To allow comparisons of experiments performed at two temperatures (~10°C and 20°C), rates were corrected to a reference temperature (10°C, Eqn. 3.5, Appendices 5.9 - 5.12). The water activity of nutrient broth without lactic acid was 0.996 and that of nutrient broth with 0.2M lactic acid was 0.992.

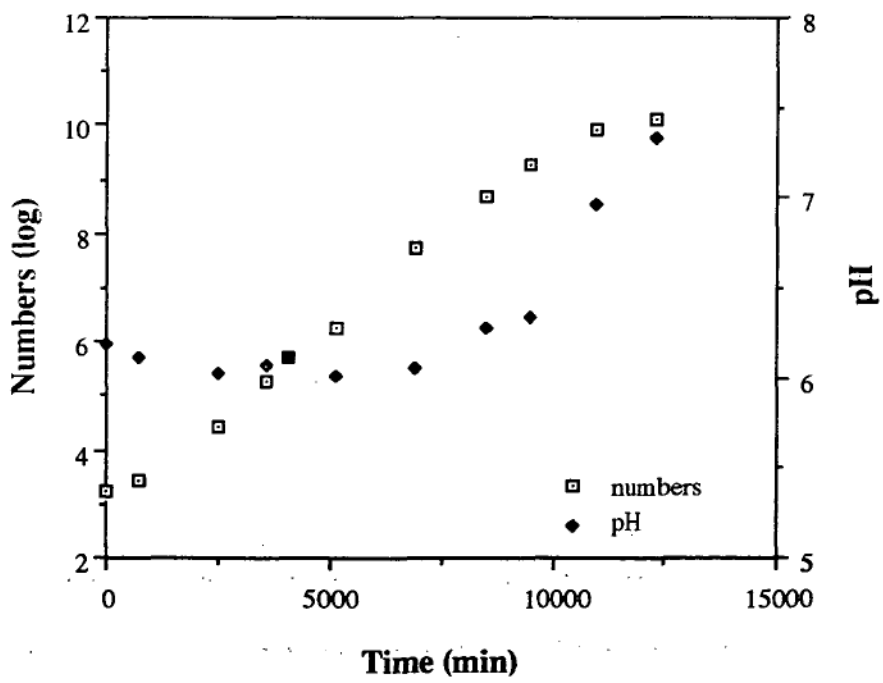
From Table 4.4a and Figure 4.9, it can be seen that, there was no effect of pH on the growth rate of strain 3 in the range 5.35 to 8.56 and of strain v4 between pH 5.26 and 7.89. The variance of the $\sqrt{}$ *rate* and ln *rate* between pH 5.5 and 8.5 were 9.416 x 10^{-6} and 0.0106 respectively. The limit for growth appeared to be between pH 4.97 and 5.17 for strain 3 and between 5.03 and 5.09 for strain v4. Between pH 5.17 and 5.28 rates of strain 3 were slower than expected. Tables 4.4b and 4.4c represent experiments concentrating in the region close to the limit for growth (pH range 5.1 - 5.9). Generation times appeared to be variable in this region.

FIGURE 4.8 Changes in pH of minced beef with respect to increasing numbers of *Pseudomonas*. a) and b): seeded mince (with bacterial suspension) allowed to spoil at 10°C and 2°C respectively. c) and d): seeded mince (with retail product) allowed to spoil at 4°C and 10°C respectively.

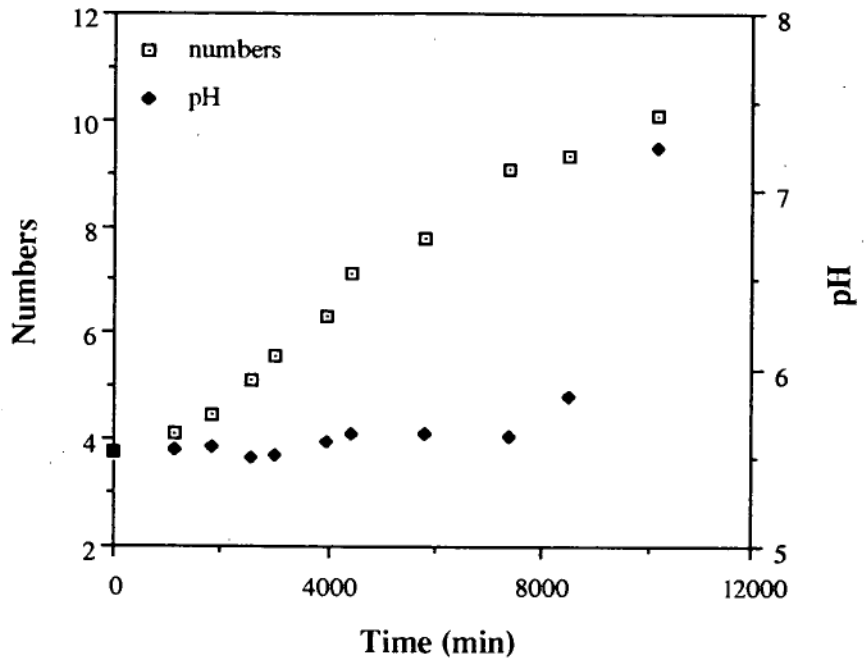
a



b



c


numbers
pH
Numbers
pH
Time (min)
12
10
8
6
4
2
8
7
6
5
0
4000
8000
12000

d

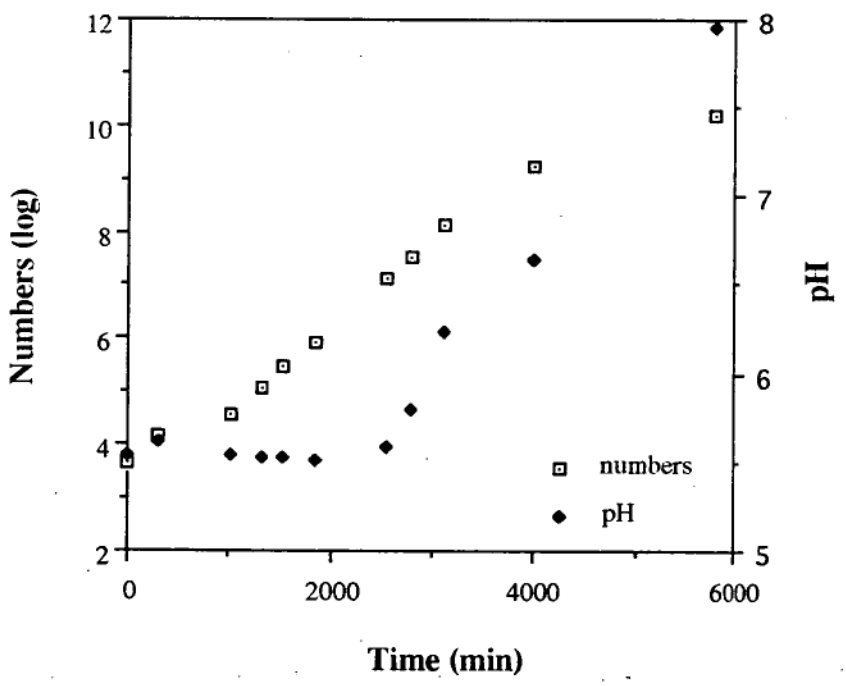

Numbers (log)
pH
Time (min)
numbers
pH
12
10
8
6
4
2
8
7
6
5
0
2000
4000
6000

Table 4.4d presents the effect of pre - inoculating strain 3 in pH 5.6 broth prior to inoculation at lowered pH values. Although the duration of the lag phase was not measured, it was noted that the time taken for the cultures to reach maximal growth rates were considerably shorter than those observed in other experiments. Growth rates appeared variable and were slightly faster than in other experiments (Table 4.4b and 4.4c).

TABLE 4.4 a Effect of initial pH (4.97 - 8.56 range) on the growth of *Pseudomonas* strains 3 and v4.

Experiment	pH range in which rates were unaffected	pH at which slower rates were observed	pH at which no growth occured
1 (strain 3)	5.56 - 8.10	5.17	4.97
2 (strain 3)	5.50 - 8.56	5.25	5.05
3 (strain 3)	5.38 - 7.82	5.25	5.07
4 (strain 3)	5.39 - 7.82	5.28	5.02
5 (strain 3)	5.35 - 7.88	5.24	5.10
6 (strain v4)	5.26 - 7.89	5.09	5.03

Experiments 1-2 performed at ~20°C, experiments 3-6 at ~10°C.
Raw data in Appendices 5.9-5.12.

TABLE 4.4b Effect of initial pH (5.12 - 5.99 range) on the growth of *Pseudomonas* strain 3. Generation times corrected to 10°C using Eqn. 3.5.

Initial pH	Generation time (min)	Temperature (°C)	Correction factor	Corrected generation time (min)	Rate
5.12	121.4	18.5	2.22	269.49	0.0037
5.21	120.2	18.5	2.22	266.83	0.0037
5.46	115.6	18.5	2.22	256.61	0.0039
5.26	98.45	19.3	2.36	232.28	0.0043
5.74	108.17	19.3	2.36	255.21	0.0039
5.99	96.933	19.3	2.36	228.70	0.0044

No growth observed at initial pH values 5.03 and 5.10.

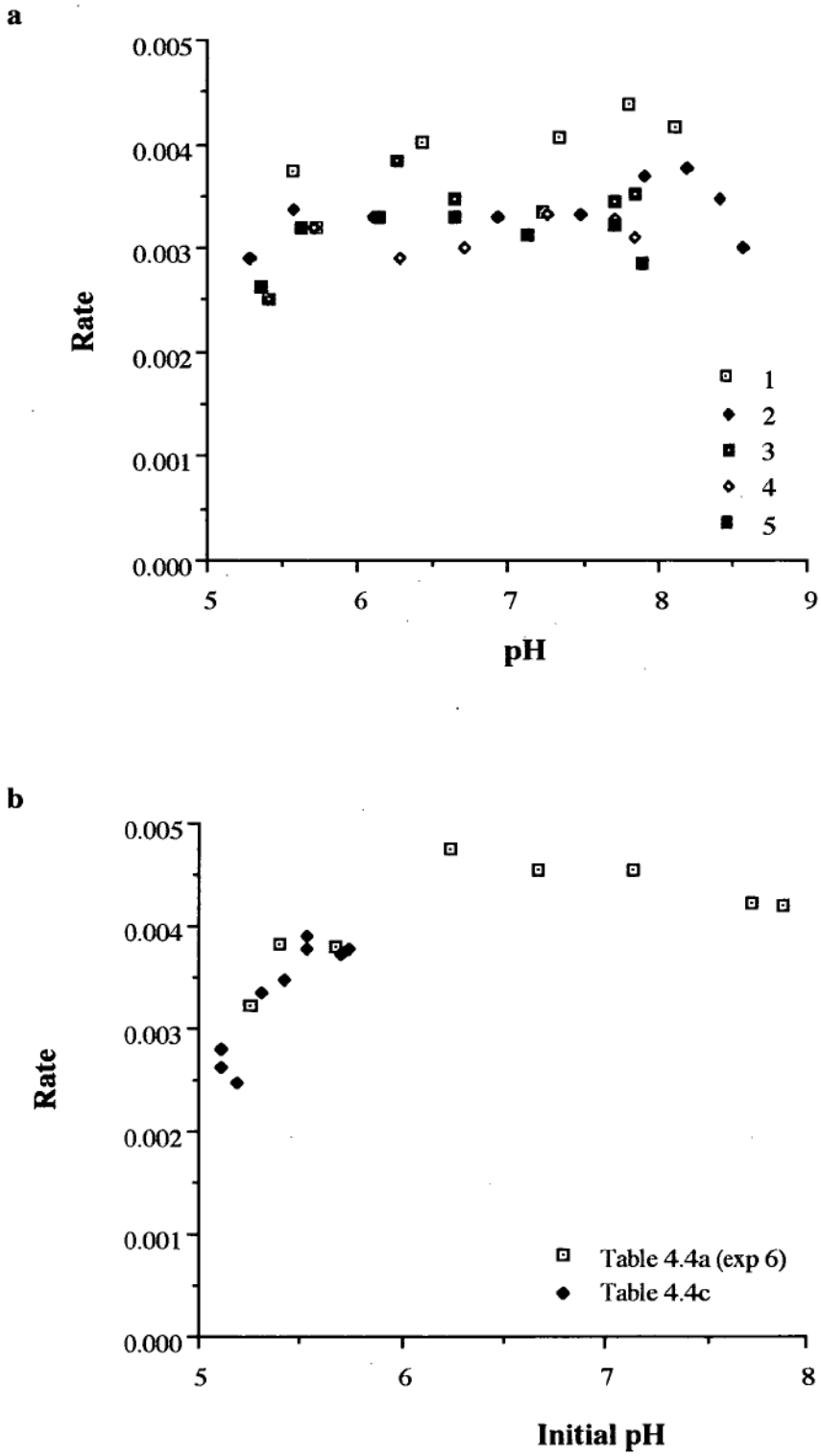
TABLE 4.4c Effect of initial pH (5.11 - 5.74 range) on the growth of *Pseudomonas* strain v4. Generation times corrected to 10°C using Eqn. 3.5.

Initial pH	Generation time (min)	Temperature (°C)	Correction factor	Corrected generation time (min)	Rate
5.11	142.10	19.5	2.49	353.62	0.0028
5.11	152.19	19.5	2.49	378.73	0.0026
5.19	160.32	19.5	2.49	398.96	0.0025
5.30	119.30	19.5	2.49	296.88	0.0034
5.42	114.90	19.5	2.49	285.93	0.0035
5.54	105.93	19.5	2.49	263.62	0.0038
5.54	102.36	19.5	2.49	254.72	0.0039
5.70	107.24	19.5	2.49	266.86	0.0037
5.74	105.79	19.5	2.49	263.25	0.0038

TABLE 4.4d Effect of initial pH (5.23 - 5.59 range) on the growth of *Pseudomonas* strains 3 following pre - incubation at pH 5.6. Times corrected to 10°C using Eqn. 3.5.

Initial pH	Generation time (min)	Temperature (°C)	Correction factor	Corrected generation time (min)	Rate
5.23	146.97	19.1	2.32	341.57	0.0029
5.23	105.99	19.1	2.32	246.33	0.0041
5.32	118.29	19.1	2.32	274.91	0.0036
5.32	131.31	19.1	2.32	305.17	0.0033
5.42	96.10	19.1	2.32	223.34	0.0045
5.45	86.76	19.1	2.32	201.64	0.0050
5.57	75.36	19.1	2.32	175.15	0.0057
5.59	81.46	19.1	2.32	189.32	0.0053

FIGURE 4.9 Effect of initial pH on growth rate of (a) *Pseudomonas* strain 3 (experiments 1-5) and (b) *Pseudomonas* strain v4. All data adjusted to 10°C (Eqn. 3.5).



4.3 INTERACTION STUDIES

No interactions appear to be present between pseudomonad strains growing in the same medium. Temperature models of a cocktail of 5 *Pseudomonas* strains were similar to models of the single strains (4.1.3). It is proposed that the fastest growing strain dominated the cocktail.

In this section of the study, the presence of interactions between *Pseudomonas* and other organisms was tested.

4.3.1 Isolation of microorganisms other than *Pseudomonas*

Microorganisms other than *Pseudomonas* isolated from fresh and spoiled minced beef are presented in Tables 4.5 a and b. The main organisms isolated were lactic acid bacteria, coliforms, *Staphylococcus aureus* and *Brochothrix thermosphacta*. The numbers of organisms present in fresh and spoiled mince are shown in Table 4.6. *Brochothrix* composed 53% of the initial psychrotrophic population, *Pseudomonas* 34% and lactic acid bacteria 20%. When mince was allowed to spoil at 10°C and 20°C, the final population comprised mainly of *Pseudomonas* (86 and 100% respectively).

4.3.2 Studies in broth and minced beef

The growth rate of *Pseudomonas* strain 3 in broth appeared to be unaffected by the presence of other organisms. Growth rates were similar to those predicted by the model of the pure strain (Figure 4.10, Appendix 5.15). Bias and precision indices (Eqn. 1.10 and 1.11) were 1.11 and 1.22 respectively (n = 11). These factors indicate that observed values do not deviate greatly from predicted values.

Growth rates of *Pseudomonas* strain 3 were unaffected by the presence of other organisms, also when grown in minced beef. At 4°C the predicted generation time was similar to that observed (predicted 305 min and observed 297 min). At 20°C the observed generation time was greater (~ 25%) than predicted (predicted 54 min and observed 70 min, Figure 4.10).

TABLE 4.5a Microorganisms isolated from fresh minced beef on selective and non - selective media.

	Media utilised for isolation	Colony description	Gram stain	O x	Ca t	O/F	Presumptive Identification
A	Gardner's	small, round, white	+ short rods	-	+		*Brochothrix*
B	Gardner's	small, round, white	+ short rods	-	+		*Brochothrix*
C	Gardner's	small, round, white	+ short rods	-	+		*Brochothrix*
E	MRS 37°C	brown, small, deep	+ cocci grps	-	-		Lactic acid bacteria
F	VRBA 37°C	large, deep	- rods	+	+	f + g	*Aeromonas* (?) *
G	VRBA 37°C	white, surface	- rods	-	+	f	*C. freundii* #
H	PCA 37°C	very orange, small	+ cocci clusters	-	+	f	*S. aureus*
I	PCA 37°C	orange / brown light	+ cocci clusters	-	+	f	*S. aureus*
J	PCA 37°C	irreg white edges	- rods thick	-	-		Lactic acid bacteria
K	PCA 37°C	very faint, white	- rods faint	-	+	f	coliform
L	VRBA 42°C	large	- rods small	-	+	f	coliform
M	VRBA 42°C	large	- rods small	-	+	f	*E. coli* ø
O	MRS 42°C	white surface	+ cocci / oval	-	-		Lactic acid bacteria
P	PCA 25°C	slimy, large	- rods	+	+	o	*Pseudomonas*
R	PCA 25°C	very small, white	+ cocci / oval	-	-		Lactic acid bacteria
T	MRS 25°C	white, soft surface	+ rods chain	-	-		Lactic acid bacteria
U	MRS 25°C	white, soft surface	+ rods chain	-	-		Lactic acid bacteria
W	MRS 25°C	white, small	+ rods	-	-		Lactic acid bacteria

Ox = oxidase reaction, Cat = catalase reaction, O / F = oxidative / fermentative test (f = fermentative, o = oxidative, g = gas production) (Appendix 2)
* gave poor identification on API 20NE suggesting *Aeromonas*. # was identified with API 20E. ø had a dubious API 20E identification, however it was motile, grew on MacConkey agar (Appendix 1.7) and colonies had a green sheen on EMB agar (Appendix 1.8), confirming *E. coli* profile

TABLE 4.5b Microorganisms isolated from minced beef allowed to spoil at 20°C.

	Media utilised for isolation	Colony description	Gram stain	Ox	Cat	Identification
A_e	PCA 25°	small, white	+ short rods		-	Lactic acid bacteria
B_e	PCA 25°	medium white	+ short rods		-	Lactic acid bacteria
C_e	PCA 25°	very small, white	+ cocci / rods		-	Lactic acid bacteria
D_e	PCA 37°	small, white	+ cocci / rods	-	+	Unidentified

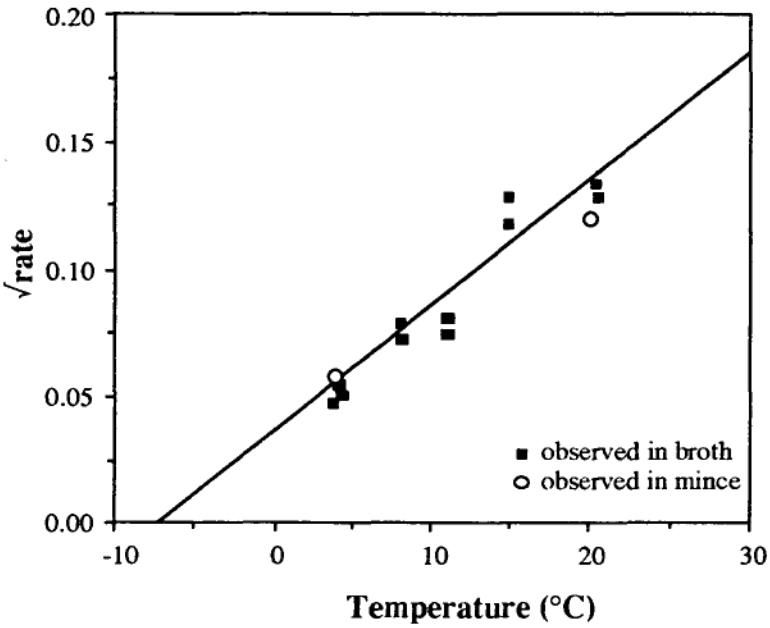
Ox = oxidase reaction, Cat = catalase reaction
Isolate D_e was not able to grow on STAA agars

TABLE 4.6 Proportion of organisms present on fresh minced beef initially and at spoilage.

	fresh mince			mince spoiled at 10°C			mince spoiled at 20°C		
	initial numbers cfu / ml	% total population (incubation at)		final numbers cfu / ml	% total population (incubated at)		final numbers cfu / ml	% total population (incubated at)	
		25°C	37°C		25°C	37°C		25°C	37°C
Enterics (VRBA)	1.9×10^3		10	5.1×10^7		100			
Lactics (MRS)	3.0×10^4	20		2.4×10^7	0.6		1.0×10^6	0.007	
	6.0×10^3		32	1.7×10^7					
Brochothrix (STAA)	8.0×10^4	53		4.8×10^8	13		1.0×10^7	0.07	
Pseudomonads (PSA)	5.1×10^4	34		3.1×10^9	86		1.4×10^{10}	100	
Total (PCA)	1.5×10^5			3.6×10^9			1.4×10^{10}		
	1.9×10^4			4.1×10^7					

VRBA = Violet Red Bile Agar; MRS = De Man, Rogosa, Sharpe Medium; STAA = Gardner's Medium containing streptomycin sulphate, thallous acetate and actidione agar (Appendices 1.4 - 1.6)
PSA = *Pseudomonas* Agar (Appendix 1.1); PCA = Plate Count Agar (Appendix 1.2)

FIGURE 4.10 Comparison of the observed growth rates of *Pseudomonas* strain 3 in the presence of other organisms and growth rates predicted by the model of the single strain. Studies were performed in broth and sterile minced beef.



Solid line represents predicted model

Cocktail includes *Pseudomonas* strain 3, *Brochothrix thermosphacta*, 2 strains of lactic acid bacteria, *Citrobacter freundii* and *Staphylococcus aureus*

4.4 MODEL CALIBRATION AND VALIDATION

4.4.1 Model calibration

4.4.1.1 Correlation between turbidimetric and viable counting data

From the comparison of generation times calculated using % transmittance methods and those calculated by viable counting methods, a constant relationship was found. Ratios, shown in Table 4.7, were similar, independently of strain or temperature. The average ratio obtained was 1.58 ± 0.2. The models constructed with were calibrated using this factor.

TABLE 4.7 Correlation between generation times obtained with turbidimetric and viable counting methods.

Strain	Temperature (°C)	GT % T (min)	GT VC (min)	Ratio GT (%T / VC)
3	19.7	82.62	57.74	1.43
	19.7	95.08	59.45	1.60
	7.5	309.11	226.97	1.36
	7.6	313.88	228.21	1.38
	3.8	911.16	478.47	1.90
	3.5	1112.20	523.45	2.12
	9.9	268.66	157.15	1.71
	9.8	276.92	155.39	1.78
	15.3	136.00	80.92	1.68
	15.2	117.60	78.31	1.50
	2.6	903.09	620.59	1.46
	2.6	979.05	612.94	1.60
v4	4.3	517.99	364.08	1.42
	4.2	517.43	331.70	1.56
5	28.2	53.44	38.03	1.41
	28.2	53.64	38.34	1.40

GT = generation time, %T = % transmittance (turbidimetric method), VC = viable counting method

4.4.1.2 Calibrated models

Models constructed with turbidimetric data required for later comparisons with viable counting data (Figures 4.2a and 4.4) were calibrated by adjusting the *b* value (Eqn. 1.5) with the factor calculated above (1.58). Calibrated square root models, are presented below.

Pseudomonas strain 3 (from minced beef):

Model $\sqrt{rate} = 0.00395\ (T - T_{min})$ (4.1)

Calibrated model $\sqrt{rate} = 0.00497\ (T - T_{min})$ (4.2)

where $T_{min} = -7.4°C$

Pseudomonas strain v4 (pork):

Model $\sqrt{rate} = 0.00367 (T - T_{min})$ (4.3)

Calibrated model $\sqrt{rate} = 0.00461 (T - T_{min})$ (4.4)

where T_{min} = - 6.5°C

4.4.2 Model validation

Visual assessment of large portions of minced beef revealed that, when stored at 4°C, mince retained a bright red colouration both on the surface and throughout the 'ball' for at least 4 days. However, upon storage at 20°C, a brown colouration appeared within 2 - 3 h.

4.4.2.1 Validation in mince stored in bijoux and bags

Generation times for *Pseudomonas* and for total counts observed in minced beef stored in bijoux were longer than predicted by the calibrated strain 3 model (Eqn. 4.2, Figure 4.11 and Table 4.8). Initial numbers of pseudomonads on the retail product ranged from 5 x 10^4 to 3 x 10^7 cfu / ml, with total numbers of organisms ranging from 5 x 10^5 to 3 x 10^7 cfu / ml.

Bias and precision indices (Eqns. 1.10 and 1.11) were both 1.37 (n = 3, Table 4.9). These factors give an indication of the deviation of predicted generation times from those observed. The indices were equal as the observed generation times were all higher than the predicted values. An index of 1.37 indicates that the observed data differs from the model on average by 37%.

When retail mince was placed in bags and incubated at different temperatures, the generation times measured for both *Pseudomonas* and total counts were always much greater than predicted by the model and appeared variable at similar temperatures. Initial levels of organisms on the retail product were high, as described above. Bias and precision indices were both 2.53 (n = 7, Fig. 4.11 and Tables 4.8 and 4.9) indicating that the observed data deviated greatly from the predicted model.

4.4.2.2 Validation by seeding sterile mince

As generation times observed in retail product (4.4.2.1) were slower than predicted, and as the initial numbers of organisms on the product were high, validations were undertaken using seeded sterile mince.

Comparison of generation times observed by seeding sterile mince with pure bacterial suspension (strain 3) and predicted generation times (Eqn. 4.2) gave bias and precision indices of 0.86 and 1.16 respectively (Table 4.9). This indicated good agreement between observed and predicted values. The T_{min} observed was - 7.0°C (266.2 K), being similar to that predicted from the model (- 7.4°C, 265.8 K,

Fig. 4.12a). The slope of the observed data was 0.00549, this was slightly faster than the predicted 0.00497 value.

Generation times observed on sterile mince seeded with retail product, were similar to those predicted by the calibrated model (Eqn. 4.2) at temperatures below 10°C. Bias and precision indices were both 1.13 (n = 12, Table 4.9). However, at temperatures greater than 10°C observed generation times were greater than predicted (Fig. 4.12b, Table 4.8). By including all the data obtained, bias and precision indices were both 1.21 (n = 16, Table 4.8). The T_{min} value calculated from the data below 10°C was - 8.3°C (264.9 K, n = 12) and the slope 0.00433 (n = 12) (predicted T_{min} = - 7.4°C, slope = 0.00395).

Lag phases were not detected as *Pseudomonas* numbers increased immediately following inoculation of sterile mince with strain 3 culture (stationary phase) or with retail product.

FIGURE 4.11 Comparison of *Pseudomonas* growth rates observed in minced beef stored in bijoux and bags (4.4.2.1) and rates predicted by the calibrated strain 3 model constructed in broth (Eqn. 4.2).

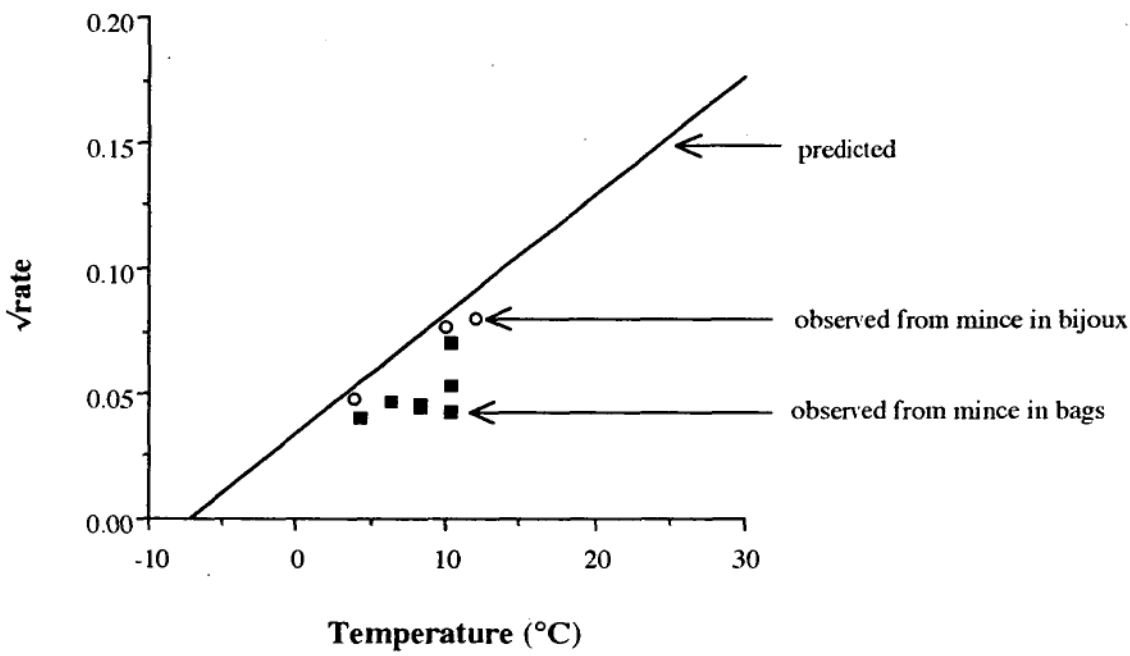
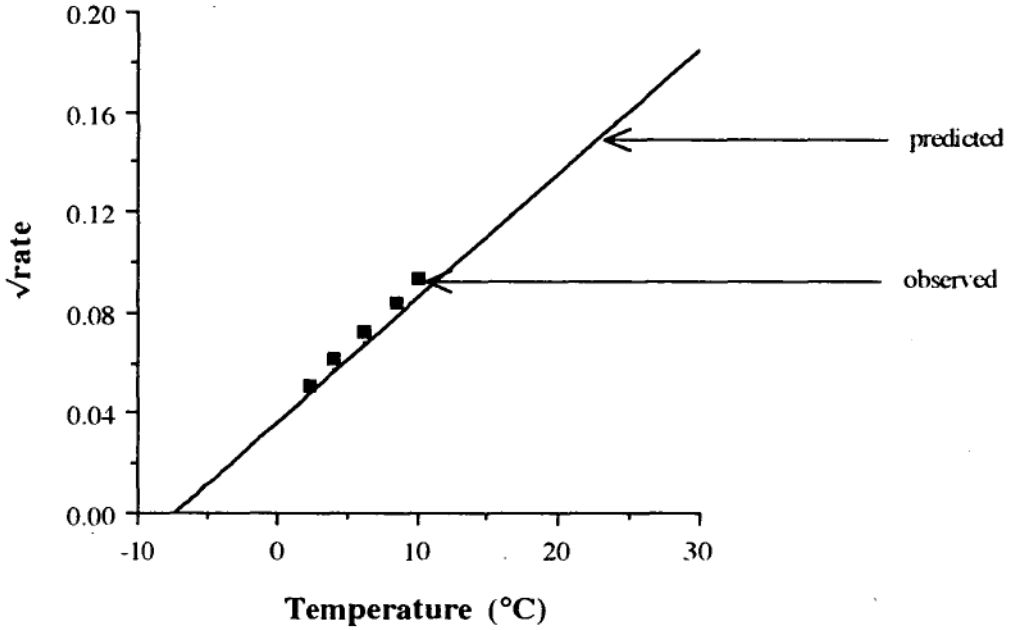


FIGURE 4.12 Comparison of growth rates observed in sterile minced beef seeded with *Pseudomonas* strain 3 (a) and retail product (b) with rates predicted by the calibrated model in broth (Eqn. 4.2).

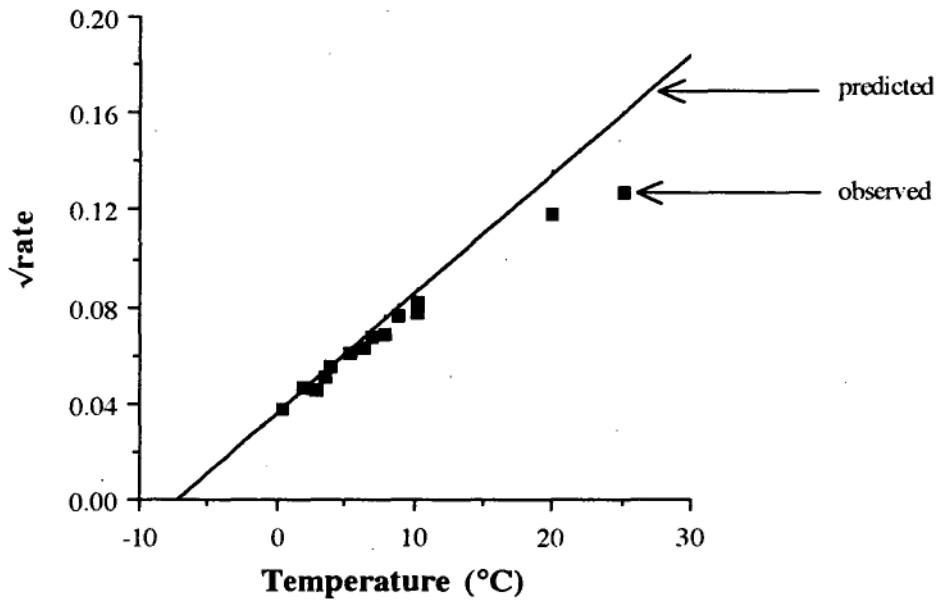
a



T_{min} (observed) = - 7.0 °C (266.2 K)

T_{min} (predicted) = - 7.4 °C (265.8 K)

b



T_{min} (observed) = - 8.3 °C (264.9 K) (n = 12; not including 20 and 25°C data)

T_{min} (predicted) = - 7.4 °C (265.8 K)

TABLE 4.8 Generation times (GT) of *Pseudomonas* predicted from calibrated strain 3 model (Eqn. 4.2) and observed in validation experiments (4.4.2.1 and 4.4.2.2).

Minced beef stored in	Temperature (°C)	Predicted GT (min)	Observed GT (min)
bijoux	4.0	316.34	440.70
(4.4.2.1)	10.0	135.05	172.80
	12.0	108.52	155.36
bags	4.3	302.81	602.40
(4.4.2.1)	6.3	218.46	464.00
	8.4	165.00	487.70
	8.4	165.00	498.00
	10.0	135.05	202.90
	10.4	129.75	351.40
	10.4	129.75	553.83
seeding with	2.3	438.16	391.36
bacterial suspension	4.2	305.44	259.80
(4.4.2.2)	6.3	218.46	189.88
	8.5	161.89	139.99
	10.1	133.50	113.14
seeding with	0.5	663.41	716.53
retail product	2.0	466.86	471.19
(4.4.2.2)	3.0	380.69	484.59
	3.5	346.28	394.84
	4.0	316.34	326.17
	5.4	250.49	274.21
	6.3	218.46	252.95
	7.0	197.61	217.41
	8.0	172.64	213.30
	9.0	152.11	171.52
	10.2	131.98	166.95
	10.2	131.98	151.23
	20.0	54.25	71.82
	20.3	53.08	73.43
	24.9	**39.00**	**61.60**
	25.0	38.76	61.84

TABLE 4.9 Bias and precision indices calculated by comparing observed rates in minced beef to rates predicted by the calibrated strain 3 model (Eqn. 4.2).

Validations in	Bias	Precision	n
bijoux (4.4.2.1)	1.37	1.37	3
bags (4.4.2.1)	2.53	2.53	7
seeding with bacterial suspension (4.4.2.2)	0.86	1.16	5
seeding with retail product (4.4.2.2)	1.21 1.13	1.21 1.13	16 (all data) 12 (< 10°C)

Bias and precision indices (equations 1.10 and 1.11 respectively)

5 DISCUSSION

The work presented in this study supports the literature findings that *Pseudomonas* dominates the biota of meat stored aerobically at chilled temperatures and is responsible for the spoilage of meat products under these conditions (Gill and Newton 1977, Dainty and Mackey 1992). Therefore, predicting the growth of *Pseudomonas* using mathematical models is useful in determining the quality and safety of meat. Models describing the effect of temperature and pH on the growth of pseudomonads were developed in this study. These models will be a powerful tool in estimating the bacterial load at any given time for any given temperature and pH profile. Widders and Coates (*pers. comm.*) evaluated the application of modified management practices and predictive protocols to improve the accuracy and uniformity in defining 'use - by' dates for fresh pork at supermarket boning rooms. Similar techniques will also be able to be applied to red meat and other food products.

5.1 BACTERIOLOGICAL STATUS OF MINCED BEEF: DO INTERACTIONS BETWEEN ORGANISMS EXIST?

The bacteriological status of minced beef in this study revealed high levels of organisms immediately after purchase. Total numbers of viable organisms ranged from $1x10^6$ cfu / g to $1.3x10^7$ cfu / g and pseudomonad counts from $1.4x10^5$ cfu / g to $1x10^6$ cfu / g (Table 4.1). Minced beef was composed initially of pseudomonads (34%) and *Brochothrix thermosphacta* (53%, Table 4.6). However, when it was allowed to spoil aerobically at 10°C and 20°C, the final population was composed entirely of pseudomonads. Dainty *et al.* (1985) found *Brochothrix* to be present in high numbers initially on fresh beef and to compose 20% of the pseudomonad - dominated population on beef allowed to spoil at 5°C.

Reports in the literature also indicate that the bacteriological status of mince purchased from markets, supermarkets and butchers is poor. In the UK, Nychas *et al.* (1991) found that *Pseudomonas* spp., *Brochothrix thermosphacta*, lactic acid bacteria, enteric bacteria and yeasts were present on minced beef at the time of purchase in this order of importance. Bacterial counts were always larger in summer than in winter. Total viable counts ranged from $5x10^6$ cfu / ml to $6.3x10^9$ cfu / ml in summer to $2x10^5$ cfu / ml to $1.6x10^9$ cfu / ml in winter, with *Pseudomonas* numbers ranging from $1x10^5$ cfu / ml to $1.6x10^9$ cfu / ml in winter. In a survey carried out in Australia by Sumner *et al.* (1981) counts were also higher in minced beef purchased in summer compared with that in winter. In winter counts exceeded 10^7 cfu / g in 32% of samples from the Victorian Markets, in 50% of retail butcher shop samples and in 44% of supermarket samples, compared with 43%, 73% and 83% respectively, in summer samples.

High bacterial numbers on minced beef are expected as low grade cuts are often used. Trimmings from primal - cut production and frozen flaked beef are often the ingredients of mince (Kraft 1992). The comminution process has major effects on the microbiological status of meat. Bacteria on the outer surfaces of meat are redistributed over the surfaces of the small particles formed and consequently have greater access to nutrients as they are distributed along with natural juices (Brown 1982). However, Dainty *et al.* (1985) reported high initial numbers also on fresh non comminuted meat. Bacterial numbers on stewing and braising steak ranged from 10^5 cfu / g to 10^7 cfu / g, *Pseudomonas* and *Brochothrix thermosphacta* dominating the flora.

High levels of contamination emphasize the need for good temperature control throughout meat processing operations and storage as growth of spoilage organisms and pathogens will give rise to undesirable and unsafe meat. Although meat should be kept at refrigeration temperatures, it may occasionally become exposed to room temperature. Under aerobic conditions, pseudomonads are expected to constitute at least 60% of the population at 20°C (Gill and Newton 1980). However, at 30°C mesophilic organisms, mainly *Acinetobacter* and Enterobacteriaceae, will be present (Gill and Newton 1980).

As the biota of fresh minced beef is diverse, studies were conducted to determine if interactions were likely to occur among pseudomonad strains, and between pseudomonads and other organisms. No interactions were apparent among pseudomonads as rates observed in a cocktail of strains were similar to those of a single strain (Figure 4.5a and b and Table 4.3). There appeared to be no interactions between *Pseudomonas* and other organisms in broth and on minced beef <20°C, as growth rates observed in a cocktail of organisms, were as predicted by the model of the individual strain (Eqn. 4.2, Figure 4.10). At 20°C the observed generation times were ~25% longer than predicted. However, reasons for the deviation are likely to be due to effects other than interactions as similar findings are reported in the validation studies where sterile mince is inoculated with *Pseudomonas* strain 3 suspension or retail product (4.4.2.2, discussed in 5.3.4). Equal numbers of organisms were difficult to achieve when preparing the cocktail in the interaction studies (Table 3.1). However, the experiment was an exaggeration of the 'real' situation as numbers of *Citrobacter*, *S. aureus*, *Brochothrix* and lactic acid bacteria are not likely to be present on fresh meat in levels as high as in this study. Therefore, as *Pseudomonas* grew at the expected rate in the presence of high numbers of other organisms, it can be concluded that no interactions are likely to occur in retail fresh minced beef, at least below <20°C.

There are many reports in the literature of the absence of interactions among organisms. Gill and Newton (1980) observed no inhibition of *Salmonella*

typhimurium by a selection of spoilage organisms on meat incubated at 20°C or 30°C. Mackey and Kerridge (1988) also found that growth rates of salmonellae in minced beef at temperatures between 10°C and 35°C were unaffected by the presence of the natural spoilage biota. Ross and McMeekin (1991) reported that growth of *S. aureus* on prawns and salmon was as predicted by a model developed from pure culture studies, indicating the absence of interactions with other organisms present in the food products. Buchanan and Phillips (1990) and Ross (1993a) found that observed data for the growth of *L. monocytogenes* on various foods compared well with models constructed in the laboratory. Gill and Newton (1977) reported that microorganisms may affect the growth of one another only when population densities are high. Such population densities usually occur after putrifactive spoilage, or toxigenic / infectious dose levels are reached.

Some exceptions have been noted in the literature by Hudson and Mott (1993). Observed generation times of *Listeria monocytogenes, Aeromonas hydrophila* and *Yersinia enterocolitica* on pâté were much slower than predicted by models developed in the laboratory and taken from the literature. Possible explanations were that the pâté used may have contained inhibitory compounds to the test organisms, or that competing bacteria may have produced inhibitors. The pâté included herbs, spices, white wine and flavouring in addition to the basic ingredients, any of which may have inhibited the growth of the bacteria. There are reports of substances, for example lactic and acetic acids, H_2O_2 and bacteriocins produced by lactic acid bacteria, inhibiting the growth of pathogens (Farrag and Marth 1992). Conversely, pseudomonads have been noted to promote the growth of some bacteria including *L. monocytogenes*. The latter organism appears to grow faster in milk that has been incubated for 2 days with *Pseudomonas* spp. than in untreated milk (Farrag and Marth 1992). Although some exceptions do exist, in most applications the effect of the environmental history on each component of the microbiota may be modelled and calculated independently without the need to consider interactions (Ross *et al.* 1993).

5.2 IDENTIFICATION OF PSEUDOMONADS

The majority of pseudomonads (85%) isolated in this study from fresh and spoiled minced beef were identified as *Pseudomonas fluorescens* with only 2 *Pseudomonas putida* (Table 4.2). Dainty and Mackey (1992) reported that pseudomonads typically isolated from aerobically spoiled chilled meat were *P. fragi* (biotypes 1 and 2), *P. lundensis, P. fluorescens* and *P. putida*. Gennari and Dragotto (1992) found that 65% of organisms isolated from fresh meat (n = 58) were *P. fluorescens* (various biovars), 10% were *P. lundensis*, 3% were Group B3 pseudomonads, 7% were *P. putida* and 15% were unidentified. On spoiled meat

(n = 61), 41% of organisms were *P. fluorescens*, 44% were *P. lundensis*, 3% were group B3 pseudomonads and 12% were unidentified.

Much confusion still exists on the best method of identifying pseudomonads, and the incidence of various subgroups and biovars within this group of bacteria is relatively unknown. This low certainty may be partly due to the frequent use of API systems. These commercially available kits were developed originally for bacteria of medical origin involving a completely different habitat to foods, and presumably, different pseudomonad types. These systems rely mainly upon detection of acid production from specific sugars (Gennari and Dragotto 1992). As rapid identification methods for the identification of pseudomonads have not yet been produced, the *Pseudomonas* strains isolated from minced beef and from pork in this study were identified with API 20NE strips. The percentage probability of presumptive identification given by the system ranged from 62.6% to 99.2% (Table 4.2).

Correct identification to species and subgroup level is not necessary in this study as all organisms are psychrotrophic, having similar growth characteristics and causing similar types spoilage. This thesis is mainly concerned with modelling the effect of temperature on the growth of psychrotrophic pseudomonads as it is the response of the organisms to temperature that determines their spoilage potential.

5.3 MODEL DEVELOPMENT

5.3.1 Modelling the effect of temperature on the growth of *Pseudomonas*

The rate of growth of microorganisms on meat is determined, to a large extent, by the temperatures to which the product becomes exposed. In this study, temperatures of mince from supermarkets and butchers, ranged from 3.7°C to 10.6°C (Table 4.1). Temperatures were lower than those of the Australian survey conducted by Sumner *et al.* (1981), which ranged from 6.5°C (Victorian Market) to 8.5°C (retail shops) in winter and from 9.3°C (supermarkets) to 12.1°C (Victorian Market) in summer. In the latter survey 150 samples were taken, whereas in this study temperatures were recorded only on 6 occasions.

In this study, monitoring the growth of pseudomonads at ~10°C revealed that >50% (8 / 14) of the strains isolated had similar generation times and belonged to a single group (group a, Figure 4.1). The group included a pork isolate (strain v1), two minced beef isolates 3 and 5 which were subsequently modelled, and other mince strains. The other strains (6 / 14) were statistically different and belonged to other groups. No clear explanation was found for the groupings as neither source of isolation, nor species type, nor previous history could explain the variation in generation times. In fact, many reports in the literature indicate that exponential growth

rates are independent of previous temperature histories and inoculum size (Jason 1983, Pooni and Mead 1984, Gill and Harrison 1985, Gill 1986, Mackey and Kerridge 1988, Buchanan and Phillips 1990).

Growth rates of *Pseudomonas* strains in this study were calculated using the modified Gompertz function (equation 1.1). Its goodness - of - fit and ease of use make this function the best model to describe the growth data of this study. The Gompertz equation is an asymmetrical sigmoid curve which fits bacteriological growth data well in most situations, with poor agreement only when growth conditions are near limiting or if little growth occurs (Gibson *et al.* 1988, Buchanan *et al.* 1989, Zwietering *et al.* 1990, Sutherland *et al.* 1994). Whereas the Gompertz is not symmetrical around the inflection point M, the logistic functions is. Comparison studies on the ability of the Gompertz and logistic functions to fit growth data of *Pseudomonas fluorescens* revealed noticeably smaller 95% confidence intervals with the former function (Willocx *et al.* 1993). In addition, the residual variation of the data points of the same study modelled with the Gompertz equation showed a random distribution in each part of the growth curve indicating a good non linear regression.

Among the requirements of the Gompertz function for a good fit, a large number of data points (15-20) are needed and these points need to be distributed fairly evenly throughout the curve, especially around the inflection point M (Bratchell *et al.* 1989, Buchanan and Cygnarowicz 1990). If there are insufficient data on either side of the inflection point, the fitted curve will not be forced towards the upper or lower asymptotes as there is insufficient information from which to evaluate them. Therefore, the resulting growth parameter estimates derived from the fitted curve are likely to be unrealistic (McMeekin *et al.* 1993). To avoid errors and misinterpretations, in this study each growth curve was made up of at least 15 observations.

The effect of temperature on the growth rate of pseudomonads was modelled in this study using the square root function (Eqn. 1.5). This function fitted the data well as the correlation coefficients (R^2) were always greater than 0.991 (Figures 4.2 - 4.5). The square root function has been used by many researchers to model bacterial growth in laboratory media and in food systems. Stannard *et al.* (1985) found that analysis of data with the square root model gave consistently higher correlation coefficients than analysis with the Arrhenius model. The square root model has many favourable features including good parameter estimation properties and ease of use (McMeekin *et al.* 1993, Ross 1993b). There are numerous reports indicating that this function describes accurately the effect of temperature on rates of growth (Pooni and Mead 1984, Chandler and McMeekin 1985a, Smith 1985, Adams *et al.* 1991, Ross and McMeekin 1991).

The confidence of the temperature models developed in this study lies firstly in the amount of data used to construct them. As at least 15 - 20 observations

were recorded for each growth curve, and as each model was made up of ~30 points, it contained 450 - 600 observations. Secondly, model development was performed in a highly nutritious medium (nutrient broth) resembling meat. Therefore, the substrate was not a limiting factor for the growth rate. Thirdly, interactions between pseudomonad strains, which could have complicated model development, were absent as rates observed in a cocktail of pseudomonad strains were similar to those of a single strain. Workers in the past have used cocktails to model organisms as they have argued that it is much more representative of the situation likely to occur when foods are contaminated (Gibson *et al.* 1987, Gibson *et al.* 1988). However, in this study it is thought that the fastest growing strain dominated the cocktail, representing the situation likely to occur in foods.

In this study, strains were modelled from ~0°C to approximately their optimum temperature for growth (30°C, Neumeyer, *pers. comm.*). Models for *Pseudomonas* growth across the full biokinetic range (Eqn. 1.6) were developed in our laboratory by Neumeyer (*pers. comm.*). The maximum temperature for growth (T_{max}) was 40.7°C for *Pseudomonas putida* 1442, and 36.4°C for *Pseudomonas fragi* 1412. Minimum temperatures for growth (T_{min} values) calculated in this study were found to be similar for different pseudomonads (Table 4.3). The T_{min} value from the strain 3 model (- 7.5°C) and the average T_{min} value calculated from all models developed in this study (-7.5°C $\pm$ 0.7), are similar to the average obtained from an extensive search of the literature, -7.7°C (95% confidence interval = 0.67, Neumeyer *pers. comm.*). The literature studies included growth characteristics of pseudomonads isolated from meat, poultry and milk.

To enable comparisons of results in this study, predictions of generation times at 11°C were calculated from each model developed (Table 4.3). This temperature was chosen as the generation times reported for strains 3 and 5 in Figure 4.1 were actually measured at 11°C. The strain 3 model (Figure 4.2a, Eqn. 4.1) predicts a generation time of 189.9 min which is very similar to 182 min predicted by the cocktail (Figure 4.5a). The generation time observed from the cocktail is likely to be a reflection of the growth of the fastest strains, in this case strains 3 and 5. The strain 3 model (Figure 4.2, Eqn. 4.1) predicts closely the generation times observed at 11°C for strain 3 (184.3 min) and strain 5 (182.7 min) in Figure 4.1 (Appendix 5.1). The predictions are also similar to those of *Pseudomonas* Predictor. The latter is a software package developed in this laboratory (section 5.4.1), predicting a generation time of 189.0 min at 11°C. In addition, the model of strain 3 (Figure 4.2b) is identical to that of strain 5 over the same temperature range (Figure 4.6). These findings indicate that different psychrotrophic pseudomonads display similar growth characteristics.

From Table 4.3 and Figure 4.2d, variation between the three models developed for strain 3 (Figures 4.2a - c) is evident. The generation time predicted by Figure 4.2a model (4 - 28°C range) at 11°C is faster than that predicted by Figure 4.2b model (0 - 21°C range) and even faster than the time predicted by Figure 4.2c model (0 - 10°C range). Decreasing the temperature range used to develop the model appears to increase the generation time. Interestingly, a similar observation can be made with the two models developed for the cocktail. The model over the 0 - 10°C range predicts a longer generation time than that from the 3 - 26°C model. In the latter case the difference in generation time is not as significant as with the strain 3 models.

As the homogeneity - of - slopes model (SAS / STAT, PROC GLM) indicates a highly significant level of difference among the models developed for strain 3 (p value <0.0001), the three separate experiments cannot be combined to form a single model. The slopes of Figures 4.2a and 4.2b become significantly similar (p value = 0.219) if only data above 4.3°C is compared. The slopes appear to be parallel above this temperature (Figure 4.2d). The data of Figure 4.2b seem no longer parallel to Figure 4.2a model below 4°C, but appear to almost fall on the extrapolated predicted Fig 4.2a line. Reasons for the variation between Figures 4.2a - c are not fully understood. Technical problems were encountered with the thermometer used to measure temperatures of each L-tube during model development (Appendix 3). The instrument appeared to have an intermittent fault, requiring calibration more frequently than expected. Therefore, incorrect calibration of the thermometer during the development of Figure 4.2b model below 4°C and Figure 4.2c model may have been the underlying cause of variation.

The problems described above could have also been the cause of the observed runs of negative residuals between ~7°C and 17°C in Figures 4.2b and 4.3, giving non linearity of error variance (Figure 4.7). From the strain 3 and strain 5 models it can be calculated that a 0.5°C fluctuation in temperature would give rise to ~1.8 x 10^{-3} variation in the $\sqrt{k}$ (slope = $\Delta\sqrt{k}$ / ΔT, where k = rate and T = temperature). This deviation is in fact similar to the calculated mean deviation of the data from the models (strain 3 = 1.7 x 10^{-3} and strain 5 = 1.4 x 10^{-3}, section 4.1.4). As the temperature gradient incubator does indeed fluctuate in temperature (~0.5°C) over periods of time, the negative residuals are in fact a result of this temperature variation. As a result of the technical problems encountered in this study, a protocol was designed for subsequent users of a temperature gradient incubator in conjunction with a thermometer with a flexible probe (Appendix 6).

By comparing three strain 3 models, it is clear that for the determination of T_{min} as a characteristic of a specific strain, only a model using the biokinetic temperature range from T_{min} to close to T_{opt} can be used. In this study, the effect of temperatures below 10°C were examined more closely as this is the range in which

minced meat is stored in commercial practice. To the author's knowledge, Figure 4.2c is the first attempt to model in depth pseudomonad growth at chill storage temperatures (28 data points between 0°C and 10°C). The change in slope below 4°C (Figure 4.2d) is compatible with the predicted thermodynamic model of McMeekin *et al.* (1993) and Ross (1993a). This model, based on the change of specific heat capacity during the denaturation of enzymes, actually predicts this change in slope and has been confirmed by the data of Hayward (1990) for *Aeromonas hydrophila* and Neumeyer (*unpub.*) for *Pseudomonas fluorescens*.These findings further emphasise the importance of gathering data over as full a range of response variable as possible (McMeekin *et al.* 1993). For subsequent work in this study, the strain 3 (Figure 4.2a) model was chosen above other models. The confidence in this model lies firstly in the broad temperature range it covers (4-26°C), secondly in the amount of data used to construct it (497 observations), and thirdly in the closeness its predictions are to the observations from other experiments as mentioned above.

The strain 3 model (Figure 4.2a, Eqn. 4.2) can also be compared to the model suggested by Gill and Jones (1992a). The latter is derived from published data between 0°C and 25°C and predicts a generation time of 149.7 min at 11°C using viable count data. By using the calibration factor of 1.58 (section 4.4.1.2), a generation time of 236 min differs by ~20% from that of the strain 3 model (189.9 min). The difference between the T_{min} value (- 8.2°C) calculated from Gill and Jones (1992a) model and that of the strain 3 model (- 7.4°C) is most likely due to the former model being a compilation of literature reports.

Several literature reports on the growth of pseudomonads isolated from meat products are compared to the calibrated strain 3 model (Eqn. 4.2) developed in laboratory broth in this study (Table 5.1 and Figure 5.1). All growth rates in the literature were determined from viable count data. Figure 5.1a represents literature studies conducted in laboratory broth compared with Eqn. 4.2 and Figure 5.1b studies in meat products compared also with Eqn. 4.2. Pooni and Mead (1984) determined, in heart infusion broth, the growth rates of pigmented and non - pigmented pseudomonads isolated from poultry. As seen in Figure 5.1a, the rates observed are similar to those predicted by the *Pseudomonas* strain 3 model constructed in nutrient broth (Eqn. 4.1). T_{min} values of - 7.2°C and - 7.3°C, are similar to the T_{min} of strain 3 (- 7.4°C). Rates deviated slightly only at 25°C. As discussed in more detail later, at this temperature factors other than temperature may play a significant role.

Stannard *et al.* (1985) studied a non - pigmented pseudomonad turkey isolate. Growth profiles of this strain were represented in the publication in the form of a graph. Enlarged copies of their published figures enabled the growth rates to be calculated by drawing lines of 'best fit' in the fastest portions of the growth curves. Errors may have been introduced, contributing to the higher T_{min} and to the deviation

of the observed rates from those predicted by the model in this study (Figure 5.1a and Table 5.1). All - Purpose Tween (ATP) broth was the culture medium used by Stannard *et al.* (1985) to monitor the growth of pseudomonads. ATP broth (Difco) is composed of yeast extract tryptone and other chemicals, whereas nutrient broth used in this study is composed of meat extract (Lab Lemco powder, Oxoid). Although differing in composition, both types of broths are very rich in nutrients. Therefore, growth rates and T_{min} values were expected to be similar. In addition, the experimental system of Stannard *et al.* (1985) included 20 ml of broth in flasks of 100 ml capacity, differing from the procedure of this study where 9 ml of nutrient broth were placed in L - tubes of ~25 ml capacity. The faster rates observed by Stannard *et al.* (1985) could have been due to greater oxygen availability as a result of the increased surface area of the flasks. However, this is unlikely as in this study, L - tubes were continually shaken to create an unlimited oxygen supply. The reason for generally faster rates being observed by Stannard *et al.* (1985) can not be easily understood.

Apart from the results published by Stannard *et al.* (1985), there appears to be a remarkable degree of consistency in the growth response of spoilage pseudomonads to temperature. In the development of a *Pseudomonas* temperature model in this laboratory >8000 growth observations were used. The model was validated by comparison with 68 growth curves in milk and 31 in minced beef, incorporating >1500 data points. When the T_{min} values obtained were combined with the data sets from the literature, the T_{min} value obtained was -7.7 + 0.67 °C as described previously in section 5.3.1.

Growth rates of pseudomonads seeded on meat observed by Scott (1937) and Gill and Newton (1977), were similar to the strain 3 model (Eqn. 4.2) below 10°C (Figure 5.1b). Above this temperature, observed rates were slower than predicted. The findings of Scott (1937) and Gill and Newton (1977) are in fact similar to those of this study, as growth rates observed in minced beef above 10°C were slower than those predicted by Eqn. 4.2 (sections 4.4.2.2. and 5.3.4). It is possible that above 10°C, the growth of pseudomonads may be affected by factors other than temperature. Chandler and McMeekin (1985a) report that in contaminated raw milk, observed growth rates of pseudomonads were as predicted by the model up to temperatures of 12 - 14°C. Above these temperatures, experimental rates were higher than predicted. The deviation from the predicted rates may be due to the growth of mesophilic bacteria having higher T_{min} values, as seen in Figure 1.1. T_{min} values calculated from the data of Scott (1937) are variable (- 12.1°C to - 7.2°C). Figure 5.1b includes only one set of the observed growth rates. It can be seen that the rate at 0°C is higher than predicted. This point on the graph caused the T_{min} to be shifted to a lower value. The deviation from the prediction at 0°C may be due to other factors affecting the growth of pseudomonads.

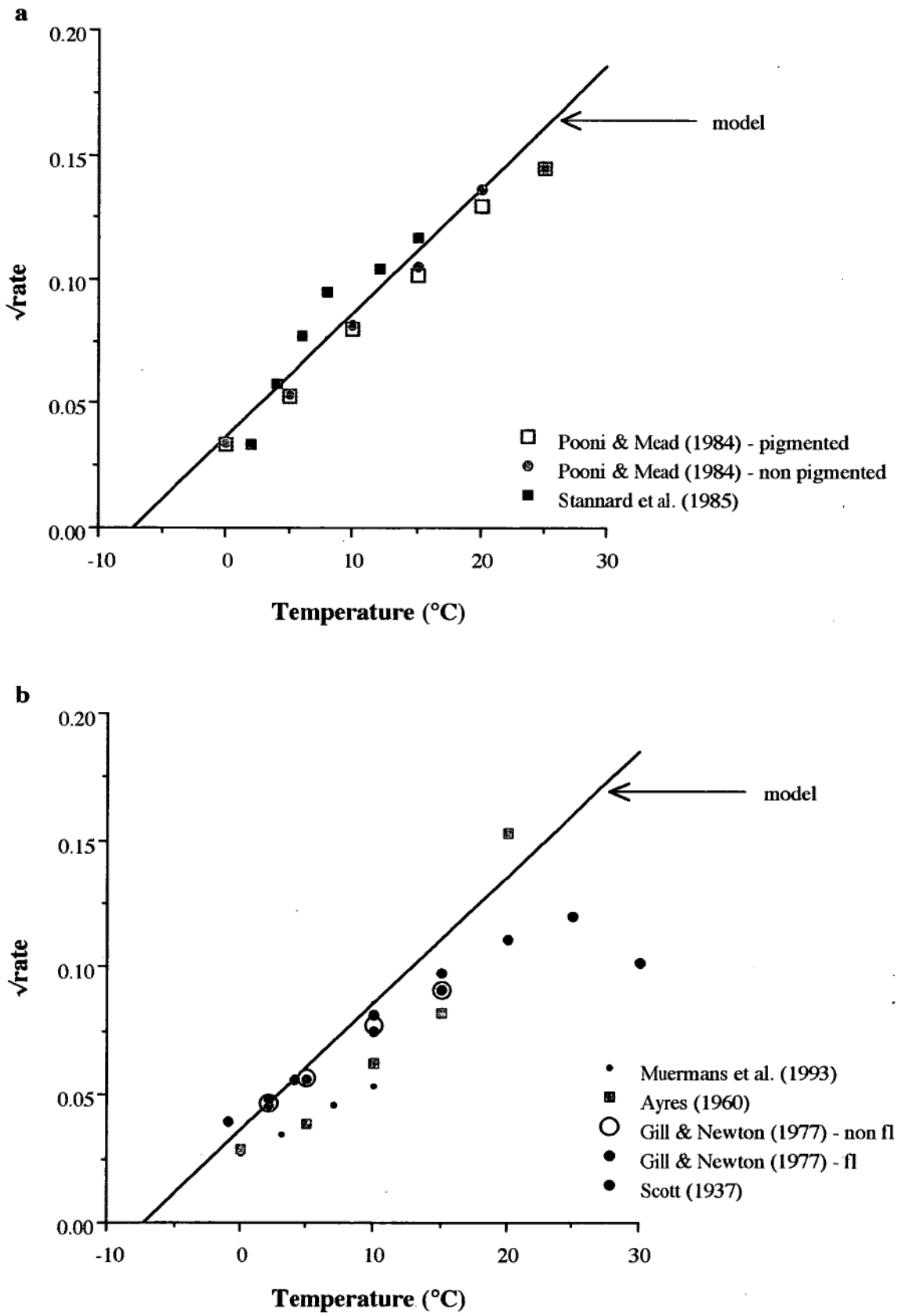
In the studies by Ayres (1960) and Muermans *et al.* (1993) presented in Figure 5.1b, growth rates in retail products were measured by viable counting with non - selective agar. Although *Pseudomonas* is dominant in meat, initially other organisms may be present in high numbers. Therefore, total counts may not be representing solely the pseudomonad population. In addition, the results by Ayres (1960) are presented in the publication in the form of a graph. Growth rates were calculated by enlarging the published figure and drawing the line of 'best fit' by hand. This process may have introduced errors, adding to the deviation from the predicted strain 3 model.

TABLE 5.1 Literature studies of pseudomonads isolated from meat products

Studies in	Reference	Organism	T_{min} (K)	T_{min} (°C)
broth	Pooni and Mead (1984)	pigmented	265.9	- 7.2
		non pigmented	265.8	- 7.3
	Stannard *et al.* (1985)	non - pigmented	267.5	- 5.7
meat	Scott (1937)	*Achromobacter* 7	261.0	- 12.2
		Achromobacter 483	261.0	- 12.2
		Achromobacter 5	264.0	- 9.2
		Pseudomonas 451	266.0	- 7.2
	Ayres (1960)	total counts	266.2	- 7.0
	Gill and Newton (1977)	fluorescent	263.0	- 10.1
		non fluorescent	263.0	- 10.1
	Muermans *et al.* (1993)	total counts	262.8	- 10.3

Note that *Achromobacter* isolates are now classified as *Pseudomonas*

FIGURE 5.1 Comparison of literature studies conducted in broth (a) and meat (b) with *Pseudomonas* strain 3 model (Eqns. 4.1 and 4.2 respectively).



Bias and precision indices (Eqns 1.10 and 1.11) were used to assess the deviation of data obtained for various studies from the strain 3 model (Table 5.2 and Appendix 7.1). Comparison between strain 3 (Eqn. 4.1) and other minced beef strain models developed in this study in broth, gave bias and precision indices ranging from 1.04 to 1.16, indicating <16% deviation. Comparison with a milk strain model developed also in broth by others in this laboratory, gave bias and precision indices of 1.06 and 1.08 respectively (<10% deviation). Comparison, with a pork strain model developed in broth in this study, indicated slightly higher variation (indices = 1.29, <30% deviation). Comparing strain 3 model (Eqn. 4.2) with literature studies conducted in broth confirms the findings discussed above as <20% variation is evident (0.93 - 1.19 bias range, Appendix 7.2a). The results indicate a close similarity between models developed for a range of pseudomonad strains.

Strain 3 model (Eqn. 4.2) can also be used successfully to predict the growth of pseudomonads in food products as comparisons of predicted rates with observed rates on minced beef (data from these studies) and in milk (data from Neumeyer, in this laboratory) indicated deviation of <21% (Table 5.2, Figure 5.2, Appendix 7.1, Appendix 8). The strain 3 model can also predict growth of pseudomonads on pork (data from Coates, *pers. comm.*) as observed values deviated from the predictions by <12% (Table 5.2 and Appendix 7). Comparing Eqn. 4.2 with the growth rates of pseudomonads on meat products found in the literature gives rise to <26% variation. The analysis of data mentioned above indicates that the *Pseudomonas* temperature model developed in broth in this study can be used to predict the growth rates of other pseudomonads in a range of food products. Other researchers have also found good agreement between growth rates of organisms predicted by models constructed in the laboratory and rates observed in the food products (Gibson *et al.* 1988, Ross and McMeekin 1991, Reichel *et al.* 1991).

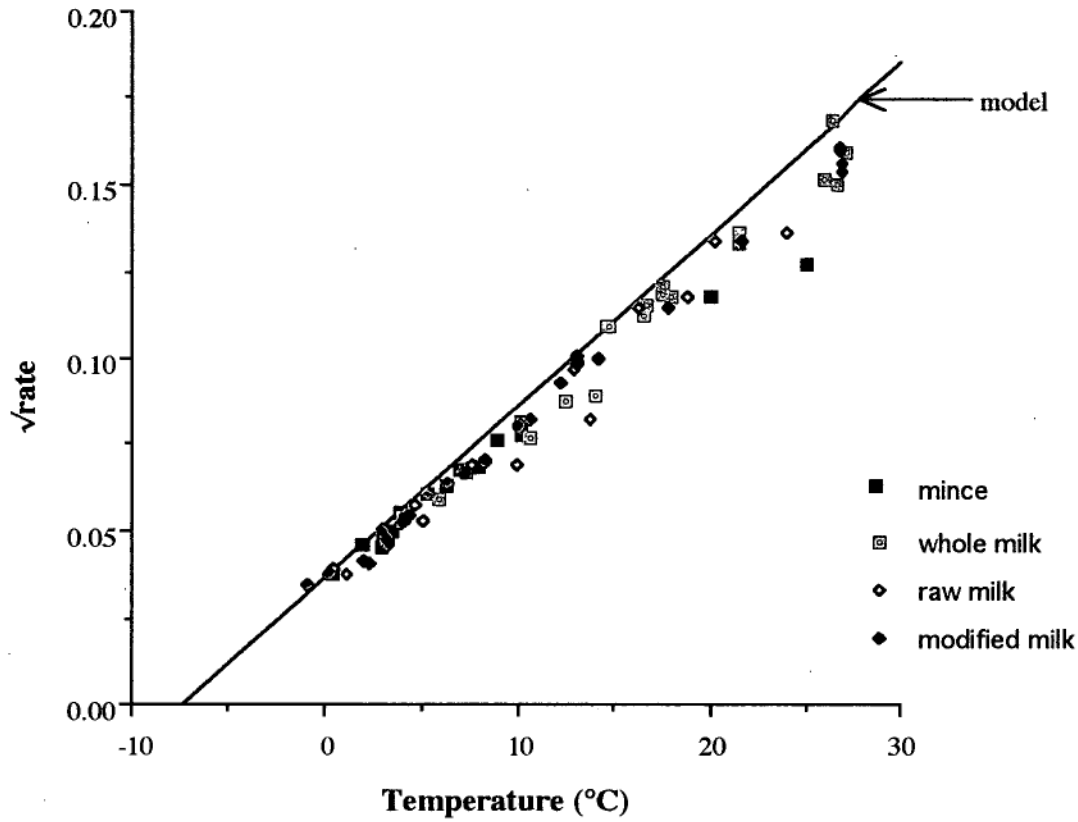
Walker and Jones (1994) report that if the observed value is within a 20% margin of the predicted value, the comparison is acceptable and the predictive model can be successfully used. Comparisons in this study mainly fall within this margin. Ross (1993a) in fact, observed up to 35% deviation between literature data and models developed in the laboratory for *S. aureus* and *L. monocytogenes*. Sutherland *et al.* (1994) also found that, although the model for the growth of *S. aureus* developed in the laboratory was fail safe, great amount of variation existed among literature data used to validate it. Comparisons between models constructed in the laboratory and data obtained from the literature will often show a larger variation than comparisons with data from inoculated food studies in the same laboratory. The major cause of this deviation is probably due to the difficulty in obtaining all the relevant information from published reports.

TABLE 5.2 Comparison of *Pseudomonas* strain 3 model (Eqns. 4.1 and 4.2) with observations from other laboratories and the literature, using bias and precision indices (Eqns. 1.10 and 1.11).

	Comparison with	Bias	Precision	Appendix
Broth studies	Minced beef strains	1.04 - 1.16	1.04 - 1.16	7.1
	Milk strain	1.06	1.08	7.1
	Pork strain	1.29	1.29	7.1
	Literature strains	0.93 - 1.19	1.13 - 1.34	7.2 a
Meat studies	Validations in mince	0.86 - 1.21	1.13 - 1.21	7.1
	Validations in pork	0.88 - 1.02	1.14 - 1.17	7.1
	Literature validations	0.83 - 1.26	1.20 - 1.26	7.2 b
Milk studies	Validations in milk	1.10	1.12	7.1

Pork strain validations performed at VIAS (courtesy of K. Coates and P. Widders); milk strain modelling and validation performed by K. Neumeyer (University of Tasmania). Details in Appendix 7

FIGURE 5.2 Comparison of strain 3 model (Eqn. 4.2) with rates observed in mince and milk (modified from Neumeyer and Kamperman, Appendix 8).



5.3.2 Effect of environmental factors other than temperature

Provided oxygen is not limiting, temperature, water activity (a_w) and pH are the major environmental factors affecting the growth of organisms on foods. The effect of temperature on the growth rate of pseudomonads was studied extensively as it is the factor of most importance on aerobically stored meat. The effect of water activity on the growth rate of pseudomonads was studied by other researchers taking part in the large collaborative project of which this study is a part. Although some surface drying may occur on meat carcasses, there will always be regions where water activity is non limiting to the growth of pseudomonads (Gill and Phillips 1990). Drying of retail minced meat is unlikely to occur as the product is usually stored in rigid trays over-wrapped with a plastic film. Therefore, water activity may not need to be included in the model describing the growth of pseudomonads in minced beef as a worst case scenario assuming non-limiting conditions may be appropriate.

5.3.2.1 Modelling the effect of pH

The effect of pH on the growth of pseudomonads was studied using lactic acid as an acidulant. Growth rates of two *Pseudomonas* strains were measured over a range of initial pH values (4.9 - 8.6). The pH value at which no growth occurred was between 4.97 and 5.17. Growth rates were similar in the pH range ~ 5.3 - 8.6, with slower rates between pH ~ 5.1 and 5.3 (Tables 4.4a - 4.4c). Although, most rates were between 0.0030 min^{-1} (generation time = 330 min) and 0.0040 min^{-1} (generation time = 250 min), variation between experiments was evident (Figure 4.9a and Tables 4.4a - 4.4c). Rates from experiments 2 - 5 appeared scattered throughout whereas those from experiment 1 were constantly higher. The values for the variance with $\sqrt{}$*rate* and **ln** *rate* as the variates were 9.4 x 10^{-6} and 0.0106 respectively (4.2.2). The magnitude of the variance from these experiments is in fact no greater than that which can be calculated from generation times of pseudomonads belonging to 'group a' in Figure 4.1 ($\sqrt{}$*rate* = 1.78 x 10^{-5} and **ln** *rate* = 0.0126).

It is not fully understood which response variate is most appropriate. However, Ross (1993a) suggests that the square root transformation homogenises the variance at least as well as the logarithmic transformation. From this pH study the variance with $\sqrt{}$*rate* as the variate is also similar to that calculated by others. Ross (1993a) reports a variance of ~5.5 x 10^{-5} for *S. aureus* and *L. monocytogenes* data. Ratkowsky *et al.* (1991), using data of Smith for *E. coli* (*pers. comm.*), calculated a variance of ~1.0 x 10^{-6}. Therefore, the variation evident in Figure 4.9 of this study is in fact no greater than expected, but is visually enhanced by the rate scale which is only for one temperature.

Rates observed when *Pseudomonas* strain 3 was pre - incubated at pH 5.6 appeared faster than those observed from other experiments (Table 4.4d). The

reason for this variability is not clearly understood. On close examination of the growth curves, a lag phase appeared to be absent when strains were pre - incubated at a lower pH. However, a lag phase was present and its length was variable in other experiments. Although the lag phase duration should not ultimately affect the growth rate (Neumeyer 1992), it may actually affect the measurement of the maximal growth rate (Ross, *pers. comm.*). Incorrect measurements of the slopes through the fastest point of the curves will lead to 'false' growth rates. Variations between experiments could have been introduced as a result of the variation in the duration of the lag phase.

Rates observed in the pH studies (0.003 - 0.004 min^{-1}) were slower than predicted by the strain 3 temperature model (0.0047 min^{-1}, Eqn. 4.1). Slower rates were expected as the addition of lactic acid to nutrient broth will not only reduce the pH but also lower the water activity. However, the slower growth rates could not be solely due to the reduction in water activity (from 0.996 to 0.992, section 4.2.2) as the rate predicted by *Pseudomonas* Predictor (section 5.4.1) at 10°C with a_w of 0.992 was still faster than that observed (predicted rate = 0.00436 min^{-1}). The deviation may be due to temperature fluctuations of the gradient incubator and / or problems encountered with the thermometer as described above for the development of the temperature models. In addition, lactic acid may have been inhibiting the growth of *Pseudomonas* causing slower rates to be measured.

There are reports in the literature describing the effect of lactic acid on the growth of microorganisms. Houtsma *et al.* (1993) found that meat spoilage organisms (lactic acid bacteria, *B. thermosphacta* and *Pseudomonas*) and bacteria of public health significance (*Y. enterocolitica*, *S. aureus* and *L. monocytogenes*) are inhibited more by sodium lactate than by sodium chloride at particular water activities. De Wit and Rombouts (1990) also reported that sodium lactate has an antimicrobial effect which rises above that expected from its water activity lowering effect. Five percent sodium lactate increased the lag phase, decreased the growth yield and somewhat decreased the growth rate. Various lactic acid bacteria, *S. aureus* and *S. typhimurium* were affected, with *E. coli* being an exception. The latter finding is surprising as *E. coli*, being physiologically similar to *S. typhimurium*, is expected to behave similarly.

The inhibitory effect of weak acids depends on the concentration of the acid and on the pH level, with antimicrobial activity usually been ascribed to the undissociated fraction of the organic acid (Baird-Parker 1980). However, Eklund (1983) reported that both undissociated and dissociated sorbic acid have antimicrobial activity. Since then, many have attempted to explain the antimicrobial actions of a number of organic acids (Anderson and Marshall 1989, Adams *et al.* 1991, Greer and Dilts 1992, Buchanan *et al.* 1993, Houtsma *et al.* 1993). Much confusion still exist as different microorganisms appear to behave differently. The specific inhibitory effect of

sodium lactate probably depends on how microorganisms can cope with this compound.

Weak acids are believed to penetrate the cell by means of diffusion in the undissociated form (lipophilic) and then dissociate in accordance with the internal pH (Warth 1986). The driving forces behind the diffusion process are the concentration of undissociated acid in the medium and the permeability of the membrane (Warth 1986, Cassio *et al.* 1987). Cassio *et al.* (1987) found that the diffusion constant for undissociated lactic acid through the membrane of *Saccharomyces cerevisiae* increases exponentially by a factor of 40 over the pH range 3.0 - 6.0. Therefore the passive diffusion of undissociated lactic acid across the plasma membrane is subject to opposing pH influences. With decreasing pH diffusion increases as the amount on undissociated acid increases, and decreases as the permeability of the membrane decreases. Dissociation of the acid inside the cell, will lead to energy consumption to eliminate the $[H^+]$ continuously entering as the undissociated acid (Warth 1986).

In addition to diffusion, lactic acid may enter the cell via a carrier system. Cassio *et al.* (1987) found that *Saccharomyces cerevisiae* transported lactate by an accumulative electroneutral proton - lactate symport with a proton - lactate stoichiometry of 1:1. The lactate system was inducible and was subject to glucose repression. Therefore, cells are able to avoid high intracellular concentration of acid by allowing the anion to escape from the cell. The process is at the expense of energy, used to eliminate the hydrogen ions that continuously enter the cell in the form of undissociated acid (Warth 1986). The electrochemical proton gradient is generated by the translocation of protons from the cytoplasm to the extracellular medium. It is coupled with ATP hydrolysis, electron transfer chain activity or end product secretion (Ten Brink and Konings 1980, Ten Brink *et al.* 1985). As both diffusion and carrier - mediated transport of organic acids will normally require energy, less can be spent on synthesis and growth resulting in reduced growth rate and possibly growth yield (de Wit and Rombouts 1990). These events are likely to have occured in the pH experiments of this study.

The effect of lactate on the growth of *Pseudomonas* has not been widely reported. Houtsma *et al.* (1993) found that the minimum inhibitory concentration of sodium lactate for the growth of pseudomonads ranged from 714 mM to 982 mM. The pKa value of lactic acid (pKa = the pH at which 50% of the total acid is undissociated) at 25°C, is 3.86 (Cooper 1969). Therefore, as the experiments of Houtsma *et al.* (1993) were conducted at pH ~ 6.4, the equivalent undissociated acid concentration ranged from 2.06 mM to 2.83 mM. In this study, at pH 6.4 with 0.2M lactic acid, the concentration of undissociated acid can be calculated as 0.58 mM, the value being well below the inhibitory range. The above statements assume that the undissociated acid is indeed the major cause of inhibition. As the reports in the literature remain varied, a

conclusion cannot be drawn. The effect of lactate on *Pseudomonas* will be governed by the conditions (pH and concentration), the permeability of the cells and the presence or absence of transport systems.

Although growth rates observed in this study were variable close to the limit for growth, it can be concluded that rates were similar between pH ~ 5.30 and 8.56. These findings confirm those of Gill and Newton (1977) and Coates and Widders (*pers. comm.*). The latter found that the initial pH of pork varied between 5.4 and 6.3. However, the growth of *Pseudomonas* pork strains were not affected in this pH range. In addition, there appeared to be no effect of pH on consumer acceptance of cooked pork. In this study, pH changes were monitored as minced beef was allowed to spoil. Initial pH values ranged from 5.5 to 6.0. Increases in pH to > 7.5 occurred only when numbers of organisms reached 1 x 10^7 - 1 x 10^9 cfu / g (Figure 4.8). At this level of bacterial numbers, the minced beef is expected to be already spoiled. Therefore, pH effects will no longer be important. These results are similar to those reported by Shelef (1981), in which the pH of fresh meat increased from 5.6 - 5.8 to 8.0 after 2 weeks at 5°C. Incipient spoilage was detectable at pH ~ 6.2 (~ 10^8 cfu / g). Coates and Widders (*pers. comm.*) similarly observed rapid rises in pH on pork when numbers were ~10^7 cfu / g. Nychas *et al.* (1991) also reported that there was no correlation between the pH of minced beef (in the range 5.75 - 6.05) and levels of contamination with most members of the microbial association. It can be concluded that, as the ultimate pH of muscle tissue, after slaughter, varies between 5.5 and 7 (Gill and Newton 1982), and as the growth of pseudomonads is not affected in this pH range, pH does not appear to be an environmental factor requiring consideration.

From the studies conducted it can also be concluded that the effects of temperature and pH on the growth of pseudomonads are solely additive. Growth rates observed at 20°C for a wide range of pH values, subsequently corrected to 10°C (Eqn. 3.5), were in fact similar to those actually observed at 10°C. Many reports in the literature indicate that the effects of temperature, pH and water activity on the growth of organisms are indeed purely additive at non - limiting conditions (Cole *et al.* 1990, Adams *et al.* 1991, Quintavalla and Parolari 1993, Wijtzes *et al.* 1993). Adams *et al.* (1991) reported that the growth rate of *Yersinia enterocolitica* under varying conditions of sub - optimal temperature and pH can be modelled by the square root function:

$$\sqrt{r} = c\sqrt{(pH - pH_{min})}(T - T_{min}) \qquad (5.1)$$

where c is a constant; pH_{min} is the theoretical minimum pH for growth and T_{min} is the theoretical minimum temperature for growth

The square root model used by Adams *et al.* (1991) was similar to Eqn. 1.8 without the water activity factor. T_{min} values were unaffected by any of the combinations of acidulant and pH levels examined.

5.3.3 Model correction

The shelf life of food products has been estimated in the past using data obtained with viable counting methods. Many kinetic models have been developed by fitting growth curves of viable count data obtained from cultures grown in liquid media (Gibson *et al.* 1987, Zwietering *et al.* 1990, Wijtzes *et al.* 1993). To create reliable models, large numbers of growth rate estimates are required. Therefore, methods that are less time and labour intensive than viable counting are desirable. Relatively few predictive models related to food microbiology have been derived from optical density measurements. Furthermore, for those which are (Chandler and McMeekin 1989b, Adams *et al.* 1991, McClure *et al.* 1991, Hudson 1993) an exact ratio between maximum growth rate determined from turbidimetric and viable counting data has not been indicated.

There are some difficulties inherent in the use of turbidimetry. Firstly, for measurements on dense cultures, there is a deviation from the response predicted by Beer's Law, resulting in falsely low estimates of cell density. Secondly, the lower sensitivity limit of turbidity measuring devices is such that, in general, they are unable to detect populations of less than about 10^7 cfu / ml. If, at those high cell concentrations, the specific growth rate is significantly lower than the specific maximum growth rate, a more sensitive method, such as viable counting might yield higher estimates of growth rate (Dalgaard *et al.* 1994). McClure *et al.* (1993b) used Bioscreen (automated turbidimetric instrument) to measure the growth response of *Listeria monocytogenes*. This technique allowed many data points to be recorded with less expenditure of effort. To fit a sigmoid growth curve to optical density data, a calibration curve and a curve fitting function were included. Optical density data was converted to equivalent cell numbers. There was good agreement between the numbers estimated from viable counts taken from Bioscreen plates, and numbers estimated from optical density measurements.

To assess the potential of turbidimetric methods, Dalgaard *et al.* (1994) examined the relationship between maximum specific growth rates of organisms determined from viable counts and turbidimetric methods. Although the turbidimetric estimates of growth rate were not the same as those estimates from viable counting data, the deviation was found to be systematic. Therefore, turbidimetric estimates may be equated with viable count estimates by multiplication with a calibration factor. Dalgaard *et al.* (1994) found that the calibration factors were similar for different organisms. The average ratio of generation times estimated from change in

% transmittance and viable counting data was 1.57 ± 0.33. Organisms tested included *Listeria monocytogenes*, *Staphylococcus aureus*, *Shewanella putrefaciens*, *Photobacterium phosphoreum* and *Pseudomonas spp.* Calibration factors were also found to be independent of the maximum specific growth rate, the inoculation level and temperature.

In this study, generation times of *Pseudomonas* obtained with turbidimetric methods (% transmittance data) differed from generation times obtained with viable counting methods by a constant factor. The average factor was 1.58 ± 0.2, being very similar to that calculated by Neumeyer (*pers. comm.*) with pseudomonads isolated from milk (=1.50). The factor (=1.54) reported for *Pseudomonas* by Dalgaard *et al.* (1994) was calculated by combining the data from this thesis with the findings of Neumeyer. It can be concluded that the difference between turbidimetric and viable counting methods is due solely to the methods themselves and not the type of organism, or the temperature.

5.3.4 Model validation

To evaluate their predictive ability, models needed to be tested in the 'real life situation' i.e. in the food product. In this study, models developed in broth (Eqn. 4.2) were compared with growth rates observed in minced beef (Table 4.8). It was found that initial numbers of pseudomonads on mince were as high as 3.2×10^7 cfu / g and that the observed growth rates of pseudomonads were slower than predicted (4.4.2.1). As the meat purchased in this study was close to spoilage, the maximal rate of growth of pseudomonads had most likely occurred prior to commencing the experiments. This could explain the reason for observing growth rates slower than predicted. As discussed in section 5.1, retail fresh meat often has high bacterial counts (Sumner *et al.* 1981, Dainty *et al.* 1985, Nychas *et al.* 1991).

As the initial numbers of organisms on retail minced beef were too high to allow model validation, sterile mince was inoculated with *Pseudomonas* strain 3 or with retail minced beef. Rates observed on mince seeded with strain 3, and incubated in the range 2 - 10°C, were similar to those predicted by the model constructed in broth (Eqn. 4.2). When the square root equation was applied to the observed data, the T_{min} value was - 7.0°C, being similar to the predicted value of - 7.4°C. Bias and precision factors were 0.86 and 1.16 respectively (Table 4.9), indicating that the observed rates were slightly faster than predicted. When rates observed on seeded mince were compared with a model developed on pork by Coates (*pers. comm.*), the bias factor was 1.02 (Appendix 7.1), indicating <5% difference between the two studies. When sterile mince was inoculated with retail mince (with high numbers of organisms), rates observed below 10°C were as predicted. Bias and precision indices were 1.18 indicating <20% deviation. As bias indices indicated that observed data were within

20% of the predictions, it can be concluded that the model described successfully the growth of pseudomonads in minced beef below 10°C. However, rates above 10°C were slower than expected.

To prevent the increase in numbers of pathogenic organisms which may be present on the raw meat, the mandatory codes of practice applied in commercial abattoirs indicate that the temperature of the boning rooms should be kept at or below 10°C (Gill and Phillips 1990). Models for the growth of mesophilic coliforms on blended mutton tissue reveal that the minimum temperature of growth of coliforms is 8°C (Smith 1985). However, if chilled meat is allowed to warm up to 12°C, it would have to be held at this temperature for 15 h before one division of any salmonella cells that may be present could take place (Smith 1987). As meat should be kept below 10°C - 12°C during transport, storage and processing, the most important portion of the temperature model is below 10°C.

Reasons for the observed growth rates deviating from the predicted rates above 10°C could be explained by limited availability of oxygen. For model validation, small 'balls' (1 g) of minced beef were used, the pseudomonads being distributed throughout the entire portion of mince as mixing was performed several times. Oxygen may not have been as available to the pseudomonads in the inner portion of the 'ball' as to those on the surface. When large portions of minced beef (~300 g) were stored at 20°C, the colour changed from red to brown within a few hours. Whereas when mince was stored at 4°C, a bright red colouration was retained throughout. Colour of meat is dependent on the presence of oxygen. Myoglobin becomes oxidised to metmyoglobin and oxygenated to oxymyoglobin. In low O_2 concentrations, myoglobin is present in the form of metmyoglobin (brown colour), and in high concentrations of oxygen, it is present as oxymyoglobin (red colour). Where no O_2 is present, myoglobin is in the reduced state and is purple in colour (Enfors and Molin 1981, Shay and Egan 1987). The brown colour of mince stored at 20°C in this study, indicated that there was not enough oxygen for the myoglobin to be fully oxygenated. Therefore, if not enough oxygen was available to oxygenate the myoglobin, it is likely that not enough was present for the pseudomonads to grow at maximal rates.

Tissue respiration is confined to the surface layer into which oxygen diffuses. The depth of penetration depends on a balance between the oxygen concentration at the surface driving oxygen inward, and the tissue respiration which consumes the oxygen as it becomes available. At lower temperatures, depth of penetration is greater because oxygen consumption is less and solubility is greater (Powell and Cain 1987). The solubility of oxygen at 1°C is about 60% higher than at 22°C (Clark and Burki 1972). Therefore, in this study, oxygen may have diffused to a lesser extent in the mince at higher temperatures, becoming limiting for the organisms distributed throughout the mince. However, both Clark and Burki (1972) and Eyles *et*

al. (1993) reported that *Pseudomonas* could grow at its maximum rate at oxygen tensions as low as 0.1%. Therefore, the cause of slower growth rates could have been the accumulation of CO_2 in pockets of trapped gas rather than insufficient availability of oxygen (Clark and Burki 1972, Willocx *et al.* 1993). Eyles *et al.* (1993) suggest that the presence of CO_2 reduces the growth rate while the limited availability of oxygen reduces the final population. As the effect of CO_2 inhibition increases with decreasing temperatures, growth rates measured at low temperatures in this study would have been expected to be lower than predicted. These were not the observed findings as possibly not enough CO_2 had accumulated. To avoid oxygen stresses in validation experiments, thin strips of meat could be 'surface' inoculated with bacterial suspension. However, this procedure could not be easily performed with minced beef.

Other factors influencing the growth of *Pseudomonas* on mince at higher temperatures may be the presence of preservatives or other substances on the mince. For example, the addition of SO_2 to minced meat enhances the storage life, improves the retention of colour and gives a weaker and less objectionable odour. The antimicrobial properties of SO_2 include increases in the lag and generation times of microorganisms. Although prohibited by regulation, high levels of sulphur dioxide were found in minced beef samples in the Australian survey conducted by Sumner *et al.* (1981). Levels were up to 750 mg SO_2 / Kg meat in about 50% of Victoria Market, 35% of retail shops and 10% of supermarket samples. In the current study the presence of SO_2 or other additives was not tested. The butcher supplying the minced beef assured the absence of additives.

In this study, the lag phase was too short to be apparent when sterile minced beef was inoculated with bacterial suspension or retail product. A drop from the incubation temperature of the inoculum (25°C) to experimental temperatures as low as 2°C, did not appear to be large enough to produce an apparent lag phase. Similar findings have been reported by Dalgaard *et al.* (1993) when measuring the growth rates of fish spoilage bacteria during product storage experiments. A stationary phase culture of *Pseudomonas* strain 3 in nutrient broth (25°C, 48 h) was used to inoculate the sterile mince in this study. As nutrient broth is very similar in composition to minced beef, the pseudomonads were expected to adapt very quickly to the new environment, displaying no measurable lag phase. In addition, as strain 3 is able to grow well at chill temperatures, a large drop from 25°C to 2°C does not induce a long lag period.

The retail product used to inoculate the sterile mince in the validation studies carried a high number of pseudomonads (up to 3×10^7 cfu / g). As maximum population density had not been reached, the pseudomonads were in their exponential phase. Therefore, the cells did not require adjustment and were able to increase in numbers immediately following inoculation of sterile mince. In contrast to the above

reports, Widders and Coates (*pers. comm.*) found that pseudomonads on retail pork displayed a consistent lag phase. This phase was not as pronounced when sterile pork was inoculated with pseudomonad suspension in the laboratory. The longer lag phase on the retail pork may be due to damaged bacterial cells, needing to recover prior to growth at their maximal rate. Bacterial cells grown in the laboratory under constant, favourable conditions are likely to be more healthy than those found on the product.

5.4 MODEL APPLICATION

5.4.1 Software

Extensive research has been conducted to develop models describing the growth of pseudomonads in meat and milk products. The studies presented in this thesis have been part of a large collaborative project of several Australian laboratories. At the University of Tasmania *Pseudomonas* Predictor, a user - friendly spreadsheet designed to predict the generation time of psychrotrophic pseudomonads, has been developed (Ross 1993a, '*Pseudomonas* Predictor' Users Manual). It can interpret 'temperature history' files e.g. collected by a data logger and then calculate the increase in the number of spoilage pseudomonads in a product exposed to those temperatures. The generation time of pseudomonads under specified conditions is calculated, and estimation of the remaining shelf life of the product can be made.

The prototype software includes a model for the rate of growth of *Pseudomonas* as a function of temperature (0°C to 38°C range) and water activity (0.955 to 0.995 range). Although the model covers a temperature range up to 38°C, limitations included in the Users Manual state that above 10°C the growth of other organisms may play an important role. The model was developed in laboratory media and validated in meat and dairy products. *Pseudomonas* Predictor is currently undergoing industry validation trials including milk handling procedures on farm, in transit to the factory and in storage silos at the factory.

The software is flexible and can serve as a template which can incorporate models for other organisms and will accept temperature history data from several types of loggers. In the present format, it is a research and development tool enabling the operator to simulate the effect of modified product formulation or storage conditions. It can examine the effect of alternative process conditions or distribution schemes on product quality. A hypothetical product temperature history to represent a new set of processing conditions can be entered. The *Pseudomonas* Predictor will then compare the new set of conditions with the existing conditions, displaying the difference in growth on the screen and enabling the difference in product shelf life and microbiological quality to be made. The *Pseudomonas* Predictor can also be used to assess the effects of individual processing, storage and distribution stages on the overall quality of the product and to identify where the need for improvement is

greatest. In addition, it can be used as an educational tool for food handlers and operators to better understand the need for temperature control.

5.4.2 Time - temperature monitors

Models developed for the growth of pseudomonads on pork have been used at the Victorian Institute of Animal Science in conjunction with time - temperature indicators (3M Monitor Mark 5 - I) to assess the efficiency of processing. The indicators were supplied with a reference card stating that the time taken for the dye to reach the end of the strip is 50 h at 7°C and 30 h at 21°C. Coates and Widders (*pers. comm.*) found that dye migration correlated with increase in pseudomonad numbers.

The Arrhenius kinetics of diffusion apply to the dye migration (Taoukis and Labuza 1989) and between ~5 and 15°C the relationship between $1 / T$ and $ln\ rate$ is linear (Olley and Ratkowsky 1973b). However, above 15°C the relationship is no longer linear and the migration of the dye does not obey the Arrhenius laws. From the Arrhenius plot of the data from the strain 3 model (between ~ 5°C and 15°C) an activation energy of ~ 19 kcal mol^{-1} can be calculated (Appendix 9). This is similar to that of 18 kcal mol^{-1} reported in Olley and Ratkowsky (1973b). As Appendix 9 shows that the slope of the line through the reference rates of 3M monitors is very different to that of the strain 3 model, it can be concluded that the indicator strips are useful in detecting temperature abuse but cannot be used for predicting the growth of pseudomonads.

5.5 CONCLUSION

In this study, a model was developed for the growth of pseudomonads in minced beef with respect to temperature. As pH values, in the range commonly found on meat, do not appear to affect the growth of pseudomonads, pH does not have to be included in the model development. The model constructed in laboratory broth using transmittance measurements was calibrated to enable comparisons with viable counting data. It was then successfully validated in minced beef at temperatures below 10°C. At higher temperatures, observations deviated from the model as other environmental factors may have become involved. The model developed could be validated further by comparing growth rates predicted with those observed on carcasses during transport from slaughter to boning, during boning and during storage in the retail outlets. By using temperature loggers in conjunction with application software such as the *Pseudomonas* Predictor, temperature histories of a product could be recorded and the extent of proliferation of pseudomonads predicted successfully.

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APPENDICES

APPENDIX 1 MEDIA

Sterilisation of media involves autoclaving at 121°C for 15 min, unless stated.

1.1 *Pseudomonas* Agar

Pseudomonas Agar Base (Oxoid CM559)	24.2 g
Glycerol	5 ml
Distilled water	500 ml

Bring to the boil, autoclave. Allow the medium to cool to 50°C. Add Cetrimide-Fucidin-Cephaloridine (C-F-C) Supplement (SR103) rehydrated with 2 ml of sterile distilled water and mix well. Pour into sterile petri dishes.

1.2 Plate Count Agar

Plate Count Agar (Amyl Media AM144)	22.5 g
Distilled water	1000 ml

Sterilise by autoclaving.

Composition of Amyl Media AM144:

Moisture	6%
Ash	1.8%
Acid insoluble ash	<0.1%
Calcium	110ppm
Magnesium	40ppm

1.3 Nutrient Broth

Nutrient Broth No. 2 (Oxoid CM67)	25 g
Distilled water	1000 ml

Mix well and sterilise.

1.4 Gardner's Medium

(STAA = Streptomycin sulphate - thallous acetate - actidione agar)

	(% w/v)
peptone (Oxoid L37)	2
yeast extract	0.2
glycerol	1.5
K_2HPO_4	0.1
$MgSO_4.7H_2O$	0.1
Bacteriological Agar (Amyl Media RM 250)	1.3

Dissolve in distilled water, adjust pH to 7.0. Dispense in 100 ml bottles and autoclave. To the molten basal medium add:

	(μg / ml)
streptomycin sulphate (Sigma S6501; St Louis, MO, USA)	500
actidione (cyclohexamide)(Sigma C6255)	50
thallous acetate (BDH 30395; Poole England)	50

Each solution is made up with 2 ml of sterile distilled water.
Pour agar into petri dishes. Spread dilutions on STAA medium and incubate (22°C, 48 hr). As some strains of *Pseudomonas* can occasionaly grow on this medium, flood plates with 5 ml oxidase reagent (Appendix 2.3) to identify the oxidase positive pseudomonads.

1.5 Violet Red Bile Agar (VRBA)

Violet Red Bile Agar (Oxoid CM 107)	35.5 g
Distilled water	1000 ml

Bring to the boil to dissolve completely. No further sterilisation is required. Once cooled to 47°C, it is to be utilised within 3 hr. Pour plates are prepared with the additions of an overlay with a thin layer of cooled medium.
Organisms attacking lactose produce purple colonies surrounded by purple haloes. Non - lactose fermenters appear as pale colonies with greenish zones.

1.6 De Man, Rogosa, Sharpe Agar (MRS)

De Man, Rogosa, Sharpe Agar (Oxoid CM 361)	62 g
Distilled water	1000 ml

Boil medium to dissolve completely. Dispense into bottles and autoclave. Pour plates are prepared with the addition of an overlay and incubated in 5% CO_2.

1.7 MacConkey Agar

MacConkey Agar (Oxoid CM 7)	52 g
Distilled water	1000 ml

Dissolve and sterilse by autoclaving.

1.8 Eosin Methylene Blue Agar

Eosin methylene blue agar (Oxoid CM 69)	37.5 g
Distilled water	1000 ml

Dissolve and sterilse by autoclaving. Cool to 60°C and shake the medium in order to oxidise the methylene blue and to suspend the precipitate.

1.9 Bacteriological Peptone

Oxoid L37

1.10 Lactic acid

Min. 88% w/w. Univar AR. Ajax Chemicals Ltd., Auburn, Australia.
Lactic acid (m.w. = 90.1) was added to Nutrient Broth (Appendix 1.3) prior to autoclaving to obtain a 0.2M solution.

APPENDIX 2 PROCEDURES AND COMMON PROCEDURES

2.1 Maintenance of cultures at -70°C

Plastic (3 mm) beads are washed in tap water with detergent, followed by dilute HCl, then rinsed several times in tap water and in distilled water, and finally dried. Twenty beads are placed in screw-cap glass vials of 5 ml capacity and autoclaved (121°C, 15 min).

15% (v/v) glycerol in Nutrient Broth is prepared and sterilised (= suspending medium). Bacteria are grown on Plate Count Agar and 1 ml of suspending medium is added to each plate. After emulsifying with a wire loop, the bacterial suspension is dispensed into each of the vials. The vials are agitated several times to remove air bubbles. The excess suspension is removed and the vials stored at -70°C.

2.2 Gram stain

Prepare and heat fix smear. Add crystal violet (1 min), wash; add iodine (1 min), wash; add acetone / alcohol (5 sec), wash; add safranin (1 min); wash and dry.

If bacteria appear red = Gram negative

If bacteria appear blue = Gram positive

2.3 Oxidase reaction

NNNN-tetramethyl-P -phenylenediamine di HCl	1 g
Distilled water	100 ml

Strips of filter paper are soaked in reagent, air dried and stored in the dark or immediately used. With a platinum loop some bacterial growth is smeared onto the filter paper.

Positive = blue / purple colour within 5-10 sec

Negative = absence of colour

2.4 Oxidative / Fermentative reaction

Bacteriological peptone (Oxoid L37)	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Agar	6 g
Bromothymol blue (1% alcohol solution)	3 ml
Distilled water	990 ml

Add components and bring to the boil. Autoclave and cool to 60°C. Add filter sterilised glucose (10 g in 10 ml water) and aseptically dispense into tubes. Stab inoculate duplicate tubes and to one add paraffin oil to the surface (anaerobic).
Oxidative = yellow at the top of aerobic tube only
fermentative = yellow in both tubes (+ / - gas)
alkaline = blue at the top of aerobic tube only

2.5 Catalase reaction

With a loop smear a small portion of a colony on the lid of a petri plate. Add 2 drops of hydrogen peroxide (3% H_2O_2) to the smear. Observe gas bubble (O_2) production.

2.6 Bacterial motility

Place a few drops of bacterial suspension on a slide, gently lower a coverslip and observe under the microscope with phase contrast.

2.7 Inocula preparation

Strains were grown on plate count agar (25°C / 48 hr). Thirty ml of sterile nutrient broth (Appendix 1.3) in 200 ml conical flasks was inoculated with single colonies and incubated (25°C / 48 hr with frequent agitation).

2.8 Viable counting

The number of microorganisms obtained on replicate agar plates from a single sample is averaged as below, to give a plate count value.
Where possible, plates having between 30 and 300 colonies were counted. For each sample, the number of microorganisms per gram or ml of sample is equal to:

$$\frac{C}{V(n_1 + n_2)f}$$

where C is the total number of colonies counted, V is the volume of inoculum applied to each plate, n_1 is the number of plates counted at the lower dilution, n_2 is the number of plates counted at the higher dilution and f is the dilution factor of the higher dilution.

(Taken in part from Anon 1990).

APPENDIX 3 EQUIPMENT

Thermometer:	Fluke 51 K / J. John Fluke Manufacturing Co., Illinois, USA.
Thermocouple:	Bead Probe 80PK-1. John Fluke Mfg. Co. Inc., Everett, Western Australia.
Waterbath:	Lauda DR. R. Wobser GMBH & Co., K.G., Lauda - Könighofen, West Germany.
Stomacher bags:	Disposable Products, Technology Park, South Australia.
Stomacher:	Colworth Stomacher 400, A.J. Seward, London, UK.
API 20NE strips:	BioMérieux SA, France.
Temperature Gradient Incubator:	Model TN3, Advantec, Toyo Roshi Int., California, USA.
Spectrophotometer:	Spectronic 20, Milton Roy Co., USA.
pH meter:	Model 250A, Orion, Boston, MA, USA.
pH electrode:	Surface electrode, 12 x 120 mm, Activon AEP 433, NSW, Australia.
Wacter activity meter:	Aqualab Model CX2, Decagon Devices Inc., Washington, USA.
Multi - welled plates :	Falcon 3047, flat - bottom, 24 - well tissue culture plates, Becton Dickinson, New Jersey, USA.

APPENDIX 4 TEMPERATURE GRADIENTS

APPENDIX 4.1 Gradients created during model development (section 4.1.3, Figures 4.2 - 4.5).

Appendix 4.1a Gradient (~ 0 - 30°C) created with shaking incubator in constant 20°C. Strain 3 on left side (Figure 4.2a) and cocktail on opposite side (Figure 4.5a).

Temperature (°C)		Temperature (°C)
4.3		3.5
4.7		4.6
7.0		5.5
7.8		8.5
9.4		9.4
10.1		10.2
11.5		11.5
12.3		12.4
13.1		13.2
14.1		13.9
14.8		14.9
15.6		15.8
16.5		16.7
17.3		17.3
17.8		17.6
18.3		18.7
19.3		19.3
20.1		20
20.6		20.8
NM		21.8
22.3		22.1
22.9		22.6
23.7		23.7
24.4		24
25.3		25.1
25.8		25.9
26.7		26.7
27.6		28
28.6		28.7

Appendix 4.1b Gradient (~ 0 - 20°C) created with shaking incubator in constant 10°C. Strain 3 on left side (Figure 4.2b) and strain 5 on opposite side (Figure 4.3).

Temperature (°C)		Temperature (°C)
0.20		0.38
1.54		1.26
2.66		2.22
3.91		3.59
4.57		4.70
5.28		5.27
6.18		6.14
6.88		6.88
7.50		7.46
8.27		8.39
8.84		9.00
9.50		9.64
10.21		10.28
10.76		10.84
11.31		11.33
11.85		11.82
12.46		12.46
12.85		12.84
13.50		13.39
14.02		13.99
NM		14.47
15.17		15.12
15.82		15.66
16.52		16.28
17.14		16.90
17.16		17.61
18.50		18.35
19.35		19.05
20.22		19.89
21.22		20.78

Appendix 4.1c Gradient (~ 0 - 10°C) created with shaking incubator in constant 10°C. Strain 3 on left side (Figure 4.2c) and cocktail on opposite side (Fugure 4.5b).

Temperature (°C)		Temperature (°C)
-0.03		0.49
1.30		0.99
2.14		1.93
3.31		2.91
3.62		3.38
4.01		3.81
4.30		4.55
4.86		5.21
5.24		5.68
6.09		6.13
6.48		6.53
6.78		6.85
7.22		7.20
7.41		7.54
7.63		7.74
7.84		7.97
8.20		8.30
8.33		8.42
8.78		8.54
8.95		8.79
9.11		8.84
9.19		9.16
9.39		9.342
9.55		9.50
9.60		9.62
9.69		9.77
9.90		9.90
9.93		9.97
9.96		9.99
10.04		10.07

Appendix 4.1d Gradient (~ 0 - 25°C) created with shaking incubator in constant 20°C. Strain v4 only (Figure 4.4).

Temperature (°C)		
0.92		
2.53		
4.06		
5.61		
6.87		
8.18		
9.13		
10.03		
10.76		
11.76		
12.66		
13.20		
13.94		
14.65		
15.27		
15.98		
16.65		
17.17		
17.74		
18.32		
18.90		
19.62		
20.15		
20.69		
21.36		
21.94		
22.48		
23.07		
23.71		
24.41		

APPENDIX 5 DATA SETS USED FOR MODEL DEVELOPMENT

APPENDIX 5.1 Generation times of strain at ~10°C (4.1.2, Figure 4.1).

Strain	Generation time (min)		Standard deviation
v3	212.4	210.6	0.14
	217.2	228.6	
15	204.0	206.4	0.05
	204.6	211.2	
12	204.0	210.0	0.11
	206.4	194.4	
1	189.0	223.2	0.26
	193.2	196.2	
6	199.2	208.8	0.15
	195.6	196.2	
	181.2	186.0	
	186.6	190.2	
v6	205.2	208.2	0.29
	207.0	213.0	
	175.2	173.4	
	186.0	172.8	
4	188.4	183.0	0.06
	186.6	192.0	
2	189.0	179.4	0.08
	187.8	190.2	
3	207.6	195.0	0.33
	198.0	201.6	
	180.0	148.2	
	169.2	174.6	
v1	200.4	195.6	0.24
	200.4	186.0	
	175.8	169.2	
	170.4	167.4	
5	190.2	191.4	0.16
	198.6	179.4	
	174.6	177.0	
	178.2	171.0	
11	169.2	183.0	0.14
	181.0	190.2	
8	182.4	183.6	0.06
	181.2	181.2	
	177.0	181.2	
	172.2	177.0	
16	176.4	175.2	0.02
	175.8	178.2	

APPENDIX 5.2 Strain 3 (4-28°C) (Figure 4.2a).

Temperature (°C)	√rate
4.3	0.0443
4.7	0.0490
7.0	0.0542
7.8	0.0596
9.4	0.0651
10.1	0.0716
11.5	0.0759
12.3	0.0800
13.1	0.0833
14.1	0.0873
14.8	0.0868
15.6	0.0889
16.5	0.0917
17.3	0.0946
17.8	0.0987
18.3	0.0984
19.3	0.1045
20.1	0.1074
20.6	0.1112
22.3	0.1164
22.9	0.1209
23.7	0.1238
24.4	0.1259
25.3	0.1292
25.8	0.1334
26.7	0.1342
27.6	0.1370

APPENDIX 5.3 Strain 3 (0 - 21°C) (Figure 4.2b).

Temperature (°C)	√rate
0.2	0.0300
1.5	0.0309
2.7	0.0352
3.9	0.0400
4.6	0.0438
5.3	0.0457
6.2	0.0468
6.9	0.0493
7.5	0.0499
8.3	0.0523
8.8	0.0549
9.5	0.0568
10.2	0.0597
10.8	0.0631
11.3	0.0668
11.9	0.0690
12.5	0.0692
12.9	0.0707
13.5	0.0721
14.0	0.0745
15.2	0.0781
15.8	0.0816
16.5	0.0870
17.1	0.0899
17.8	0.0899
18.5	0.0951
19.4	0.0975
20.2	0.1035
21.2	0.1031

APPENDIX 5.4 Strain 3 (0 - 10°C) (Figure 4.2c).

Temperature (°C)	√rate
0.6	0.0284
1.3	0.0321
2.3	0.0341
3.1	0.0355
3.5	0.0373
4.1	0.0406
4.9	0.0427
5.5	0.0444
5.8	0.0454
6.3	0.0468
6.6	0.0477
6.9	0.0492
7.2	0.0491
7.6	0.0503
7.8	0.0521
8.1	0.0515
8.3	0.0538
8.5	0.0545
8.7	0.0538
9.0	0.0561
9.1	0.0564
9.3	0.0570
9.4	0.0583
9.6	0.0575
9.9	0.0572
10.1	0.0581
10.2	0.0590
10.2	0.0594

APPENDIX 5.5 Strain 5 (0 - 20°C) (Figure 4.3).

Temperature (°C)	√rate
0.4	0.0302
1.3	0.0340
2.2	0.0370
3.6	0.0402
4.7	0.0438
5.3	0.0478
6.1	0.0481
6.9	0.0503
7.5	0.0528
8.4	0.0568
9.0	0.0589
9.6	0.0601
10.3	0.0635
10.8	0.0640
11.3	0.0692
11.8	0.0693
12.5	0.0704
12.8	0.0746
13.4	0.0750
14.0	0.0757
14.5	0.0796
15.1	0.0829
15.7	0.0860
16.3	0.0870
16.9	0.0886
17.6	0.0922
18.4	0.0947
19.1	0.0953
19.9	0.1035

APPENDIX 5.6 Strain v4 (0 - 24°C) (Figure 4.4).

Temperature (°C)	√rate
0.9	0.0280
2.5	0.0327
4.1	0.0378
5.6	0.0415
6.9	0.0476
8.2	0.0528
9.1	0.0534
10.0	0.0606
10.8	0.0647
11.8	0.0662
12.7	0.0697
13.2	0.0731
13.9	0.0752
14.7	0.0765
15.3	0.0818
16.0	0.0823
16.7	0.0848
17.2	0.0877
17.7	0.0901
18.3	0.0902
18.9	0.0942
19.6	0.0955
20.2	0.0980
20.7	0.1009
21.4	0.1006
21.9	0.1033
22.5	0.1027
23.1	0.1075
23.7	0.1087

APPENDIX 5.7 Cocktail (3-26°C) (Figure 4.5a).

Temperature (°C)	√rate
3.5	0.0430
4.6	0.0489
5.5	0.0560
8.5	0.0613
9.4	0.0670
10.2	0.0706
11.5	0.0742
12.4	0.0788
13.2	0.0835
13.9	0.0880
14.9	0.0884
15.8	0.0918
16.7	0.0920
17.3	0.0961
17.6	0.1013
18.7	0.1037
19.3	0.1056
20.0	0.1054
20.8	0.1122
21.8	0.1119
22.1	0.1170
22.6	0.1200
23.7	0.1229
24.0	0.1260
25.1	0.1265
25.9	0.1330

APPENDIX 5.8 Cocktail (0 - 10°C) (Figure 4.5b).

Temperature (°C)	√rate
0.5	0.0288
1.0	0.0316
1.9	0.0352
2.9	0.0426
3.4	0.0426
3.8	0.0441
4.6	0.0434
5.2	0.0469
5.7	0.0479
6.1	0.0517
6.5	0.0532
6.9	0.0539
7.2	0.0545
7.5	0.0570
7.7	0.0552
8.0	0.0582
8.3	0.0582
8.4	0.0598
8.5	0.0603
8.8	0.0602
8.8	0.0601
9.2	0.0634
9.3	0.0638
9.5	0.0644
9.6	0.0644
9.8	0.0662
9.9	0.0665
10.0	0.0653
10.0	0.0650
10.1	0.0662

APPENDIX 5.9 Effect of intial pH on the generation time of strain 3 (experiment 1). Times are corrected to 10°C using Eqn. 3.5.

Initial pH	Generation time (min)	Temperature (°C)	Correction factor	Corrected generation time (min)	Rate
5.17	150.00	19	2.31	346.50	0.0029
5.56	115.86	19	2.31	267.23	0.0037
6.43	107.72	19	2.31	248.47	0.0040
7.33	106.84	19	2.31	246.44	0.0041
7.79	99.12	19	2.31	228.62	0.0044
8.10	103.86	19	2.31	239.55	0.0042

APPENDIX 5.10 Effect of intial pH on the generation time of strain 3 (experiment 2). Times are corrected to 10°C using Eqn. 3.5.

Initial pH	Generation time (min)	Temperature (°C)	Correction factor	Corrected generation time (min)	Rate
5.25	160.38	18	2.13	342.38	0.0029
5.55	138.07	18	2.13	294.76	0.0034
6.09	140.52	18	2.13	299.98	0.0033
6.90	141.13	18	2.13	301.27	0.0033
7.46	139.68	18	2.13	298.18	0.0034
7.89	126.03	18	2.13	269.05	0.0037
8.16	123.83	18	2.13	264.35	0.0038
8.38	134.36	18	2.13	286.83	0.0035
8.56	154.40	18	2.13	329.61	0.0030

APPENDIX 5.11 Effect of intial pH on the generation time of strain 3 (experiments 3-5). Times are corrected to 10°C using using Eqn. 3.5.

Initial pH	Generation time (min)	Temperature (°C)	Correction factor	Corrected generation time (min)	Rate
5.27	>424.50	9.4	0.93	>393.29	<0.0025
5.38	424.50	9.4	0.93	393.29	0.0025
5.70	331.55	9.4	0.93	309.01	0.0032
6.24	269.81	9.6	0.96	258.27	0.0039
6.62	296.00	9.7	0.96	285.01	0.0035
7.20	303.28	9.8	0.98	296.33	0.0034
7.67	292.17	9.9	0.99	287.97	0.0035
7.82	281.58	10.0	1.00	281.58	0.0036
5.39	400.00	10.3	1.04	416.00	0.0024
5.69	299.56	10.3	1.04	310.89	0.0032
6.26	331.61	10.3	1.03	341.24	0.0029
6.69	322.58	10.2	1.03	331.00	0.0030
7.23	292.66	10.2	1.02	298.59	0.0033
7.68	300.26	10.1	1.01	302.86	0.0033
7.82	319.71	10.0	1.00	318.79	0.0031
5.24	>348.80	10.8	1.09	>379.61	<0.0026
5.35	348.80	10.8	1.09	379.61	0.0026
5.61	290.44	10.7	1.08	312.62	0.0032
6.14	291.72	10.3	1.04	302.75	0.0033
6.63	293.75	10.3	1.03	303.99	0.0033
7.12	311.55	10.2	1.03	319.69	0.0031
7.70	305.72	10.2	1.02	311.03	0.0032
7.88	346.67	10.1	1.01	350.68	0.0029

APPENDIX 5.12 Effect of intial pH on the generation time of strain v4 (experiment 6). Times are corrected to 10°C using using Eqn. 3.5.

Initial pH	Generation time (min)	Temperature (°C)	Correction factor	Corrected generation time (min)	Rate
5.09	>343.50	9.2	0.91	>310.90	<0.0032
5.26	343.50	9.2	0.91	310.90	0.0032
5.39	283.82	9.4	0.92	261.83	0.0038
5.67	279.11	9.5	0.94	262.40	0.0038
6.23	221.99	9.6	0.95	210.67	0.0047
6.67	227.79	9.7	0.96	219.56	0.0046
7.13	225.13	9.8	0.98	220.37	0.0045
7.73	238.56	9.9	0.99	236.39	0.0042
7.89	237.51	10.0	1.00	238.23	0.0042

APPENDIX 5.13 Changes in pH of minced beef with respect to increasing numbers of *Pseudomonas* (Figures 4.8a and b).

Time (min)	Log numbers	pH
0	3.40	5.79
262	3.69	5.87
536	4.13	6.24
1229	5.26	6.07
1509	6.15	6.13
1752	6.93	5.97
1975	7.59	6.14
2665	8.66	6.58
2953	9.21	6.75
3169	9.23	6.95
4195	10.00	7.60
4609	10.30	7.85

Time (min)	Log numbers	pH
0	3.26	6.20
761	3.45	6.13
2491	4.41	6.04
3607	5.27	6.08
4074	5.73	6.13
5100	6.24	6.03
6912	7.73	6.07
8462	8.70	6.29
9448	9.30	6.35
10913	9.93	6.98
12318	10.12	7.35

APPENDIX 5.14 Changes in pH of minced beef with respect to increasing numbers of *Pseudomonas* (Figures 4.8c and d).

Time (min)	Log numbers	pH
0	3.77	5.53
1130	4.13	5.54
1847	4.45	5.56
2540	5.14	5.49
2997	5.58	5.52
3987	6.31	5.59
4448	7.12	5.63
5774	7.76	5.64
7394	9.08	5.62
8470	9.35	5.85
10156	10.10	7.25

Time (min)	Log numbers	pH
0	3.68	5.55
282	4.17	5.62
1016	4.54	5.54
1308	5.07	5.53
1518	5.44	5.53
1843	5.92	5.52
2545	7.11	5.59
2778	7.54	5.80
3111	8.16	6.23
4003	9.23	6.65
5786	10.20	7.95

APPENDIX 5.15 Growth rates of *Pseudomonas* strain 3 in nutrient broth and in minced beef in the presence of other organisms (Figure 4.10).

	Temperature (°C)	√rate observed	√rate predicted
broth	3.9	0.0474	0.0555
	4.2	0.0544	0.0570
	4.4	0.0498	0.0580
	8.1	0.0727	0.0764
	8.0	0.0789	0.0761
	11.0	0.0803	0.0911
	11.0	0.0749	0.0910
	14.8	0.1289	0.1099
	14.8	0.1177	0.1099
	20.5	0.1279	0.1383
	20.4	0.1333	0.1379
mince	4.2	0.0581	0.0572
	20.2	0.1196	0.1368

APPENDIX 6 EXPERIMENTAL PROTOCOL FOR THE USE OF A TEMPERATURE GRADIENT INCUBATOR IN CONJUNCTION WITH A FLUKE THERMOMETER

The following protocol was prepared for the benefit of researchers using equipment and methods similar to those of this study. It is very important to be confident with methods to be used as 'false' growth profiles may be otherwise obtained. Section 5 of this appendix (measurements of temperatures) arose as a result of the problems encountered in this study. As explained in section 5.3.1, runs of negative residuals were observed in Figures 4.2b and 4.3, giving non linearity of error variance. These runs may have been due to fluctuations of the equipment used for temperature measurements

1. Preparation of strains

Prepare bacterial suspension of strain(s) in liquid media (Nutrient Broth). Allow suspension to reach stationary phase.

2. Preparation of L - tubes

Place 9 ml of appropriate broth in each tube (prepare a few spares). Place cap. Autoclave (121°C, 15 min). Allow pressure to go down slowly to prevent broth sucking into caps.

3. Using a Temperature Gradient Incubator (Model - Appendix 3)

Set the incubator in a constant temperature room as fluctuations of outside temperature will affect the gradient.

Place a temperature logger in the room and preferably one also in a spare hole of the temperature incubator to ensure no major temperature fluctuations have occurred during the experiment.

Turn on all switches. Set desired gradient. Do not trust the dials, place tubes with water at each end and measure their temperature.

Allow at least 6-10 hours for gradient incubator to reach desired gradient.

4. Experimental procedure

At least 30 min prior to commencing, place all tubes to be inoculated in their place in the gradient incubator to allow them to equilibrate (usually leave overnight).

Turn on spectrophotometer at least 30 min prior to commencement. Set at 540 nm. Use a reagent blank to set the 100% transmittance.

Monitor changes in % transmittance.

Inoculate the 1st L - tube with enough bacterial suspension to bring the % transmittance down to 80 - 85% (from 100%). Always mix tubes (with vortex mixer) before taking a reading to disperse bacteria.

As the bacterial suspension is in stationary phase, a small amount is needed to cause the drop from 100% to 80% (usually 200μl-500μl)

Record the time of inoculation, and the % transmittance at this time.

Start the timer.

Inoculate the rest of the tubes with the same amount of bacterial suspension (particularly if interested in lag times), noting the time and % transmittance.

It is useful to take a complete second set of readings immediately after finishing the inoculation of all tubes. This gives a confirmation of the first reading, after thorough mixing.

Monitor the change in % transmittance with time (take 15 - 20 readings for each tube if possible, i.e. aim to take readings every 5 - 10% transmittance drop).

Continue taking readings until % transmittance reaches 5 - 7%

IMPORTANT

Wipe tubes very well before taking readings (especially prior to the first reading).

If the tubes are at temperatures very much lower than ambient, there will be a lot of condensation when taking the reading. Therefore, it is essential to be meticulous in cleaning the tubes. Wipe with tissues well (use a towel if necessary, but last wipe with tissue). In addition, place tubes in spectrophotometer in exactly the same way each time and take readings a couple of times to convince yourself you have correct reading. Also, need to fabricate a light-proof cover for the spectrophotometer (upside-down box)

Keep gradient incubator rocking at fast speed (oscillations / minute) when not taking readings. Reduce speed when taking readings to avoid scratching or breaking L-tubes.

Never remove or add tubes in the incubator while an experiment is running

5. Measurements of temperatures

On completion, measure the temperature of each tube at least 5 times. An average temperature for each tube is required.

Firstly calibrate the Fluke thermometer against a certified glass thermometer

Check temperature reading at 0°C (ice-water)

Check thermometer also at one or two other temperatures. If measuring tubes in a 0-30°C gradient, check at say 10°C and at 30°C

Begin measuring temperatures of the tubes only when satisfied that the thermometer is reading correctly.

Check thermometer every time that a set of readings is taken.

As the incubator goes through cycles, the temperature will fluctuate (~ +/- 0.4°C). Therefore, measure temperatures at random times so not to coincide with the regular cycles of the incubator.

As growth in tubes at colder temperatures may end days later than growth at warmer temperatures, the 5 temperature readings for the warmer tubes would have already been taken when the time comes to measure temperatures of the colder tubes. Therefore, a check that the warmer tubes are still at the same temperature is essential and will confirm consistency of the thermometer.

It is advisable to coat the thermocouple with a very thin layer of epoxy resin so as to avoid contact with corrosive liquids, such as nutrient broth.

APPENDIX 7 BIAS AND PRECISION INDICES FOR MODEL COMPARISON

Appendix 7.1 Bias and precision analysis (Eqns. 1.10 and 1.11) with data from collaborating laboratories.

Comparing	Observed tested in	Bias	Precision	n
Strain 3 model (Eqn. 4.1) with other models (nutrient broth)	strain 5	1.16	1.16	30
	strain v4 (pork)	1.29	1.29	30
	cocktail (5 strains)	1.04	1.04	27
	Pp 1442 (milk strain)	1.06	1.08	56
Strain 3 model (Eqn. 4.2) with validations experiments	mince seeded with strain 3	0.86	1.16	5
	mince seeded with product (< 10°C)	1.13	1.13	12
	(include all temps.)	1.21	1.21	16
Strain 3 model (Eqn. 4.2) with VIAS seeding experiments	pork seeding	0.88	1.17	5
Strain 3 seeded mince (4.4.2.2) with VIAS seeding	pork seeding	1.02	1.14	5
Strain 3 model (Eqn. 4.2) with UT validations	milk validations	1.10	1.12	68

VIAS = Data obtained curtesy of VIAS, Victoria.
UT = milk models and validations by K. Neumeyer, University of Tasmania

Appendix 7.2a Comparing strain 3 model (Eqn. 4.2) to literature data.

Reference	Bias	Precision	n
Pooni & Mead (1984)			
Heart Infusion broth			
chicken spoilage isolates			
non - pigmented	1.13	1.13	6
pigmented	1.19	1.19	6
Stannard *et al.* (1985)			
All-Purpose Tween (APT) broth			
turkey isolate	0.93	1.34	6

Appendix 7.2b Comparing strain 3 model (Eqn. 4.2) and validations (4.4.2.2) to literature data.
(Bias and precision factors from Eqns. 1.10 and 1.11 respectively)

Reference	**Bias** (comparing with Eqn. 4.2)	**Precision** (comparing with Eqn. 4.2)	**n**	**Bias** (comparing with mince seeded with suspension)	**Precision** (comparing with mince seeded with suspension)	**Bias** (comparing with mince seeded with retail product)	**Precision** (comparing with mince seeded with retail product)
meat slices seeded - Scott (1937)	0.83	1.20	5	1.09	1.27	0.87	1.19
fresh beef slices - Ayres (1960) Nutrient Agar	1.61	1.78	5	1.86	1.92	1.93	1.4
meat slices seeded (mutton isolates) Gill & Newton (1977)							
non - pigmented	1.20	1.21	4	1.38	1.38	1.05	1.09
pigmented	1.26	1.26	4	1.46	1.46	1.09	1.12
beef pieces-Muermans *et al.* (1993) Plate count agar	2.04	2.04	4	2.34	2.34	1.81	1.81

APPENDIX 8 Poster presentation at the Annual Scientific Meeting of the Australian Society for Microbiology. Abstract published in *Australian Microbiologist*, **14** (1993).

Predicting the Growth of Psychrotrophs in Milk and Meat

KARINA NEUMEYER and LAURA KAMPERMAN
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Predictive microbiology offers an alternative to traditional microbiological assessment of food quality and safety. The growth response data for microorganisms can be summarised in the form of mathematical models, which can then be used to

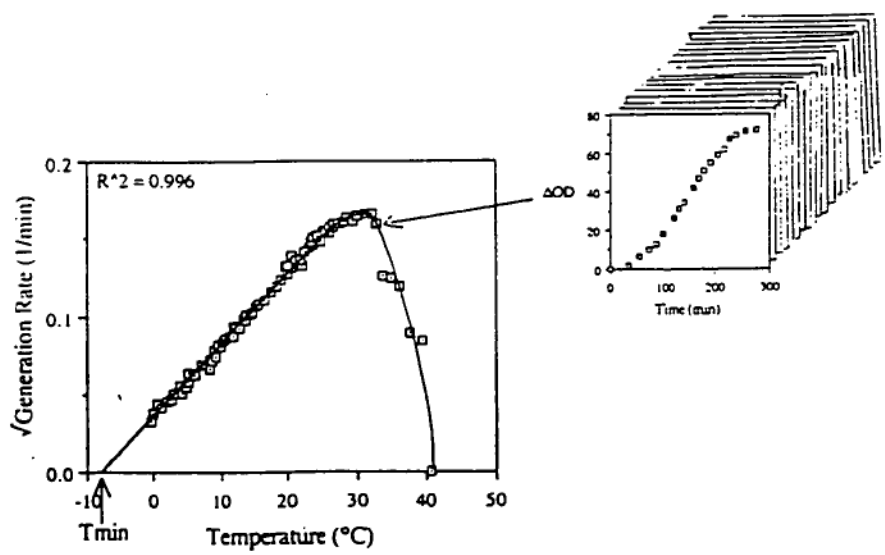
- predict shelf life
- assess the hygenic efficiency of processing and distribution
- determine the effect of lapses in storage conditions
- determine the microbiological quality and safety of a product

Psychrotrophic pseudomonads were isolated from both milk (n=22) and meat (n=16) products at spoilage. Growth rates in artificial broth media were determined using optical density (OD) readings. By fitting a sigmoidal curve (Gompertz function) to the data, generation time can be calculated (Gibson *et al.* 1987). The fastest growing strain isolated (determined in artificial broth media at 10°C) was used to construct the model i.e., a Worst-Case-Scenario approach was adopted. Another factor to consider is that generation times (GT) calculated by OD and viable count (VC) measurements differ. Growing *Pseudomonas* at various temperatures and monitoring both OD and VC of each sample, showed that the ratio between the two methods is constant with respect to temperature and can be incorporated into the modelling process. For *Pseudomonas* a calibration factor of 1.50 ± 0.16 was obtained.

i.e., GT(by OD) = 1.5 * GT(by VC)

This calibration factor needs to be built into the model so that all generation times are expressed as those calculated by VC.

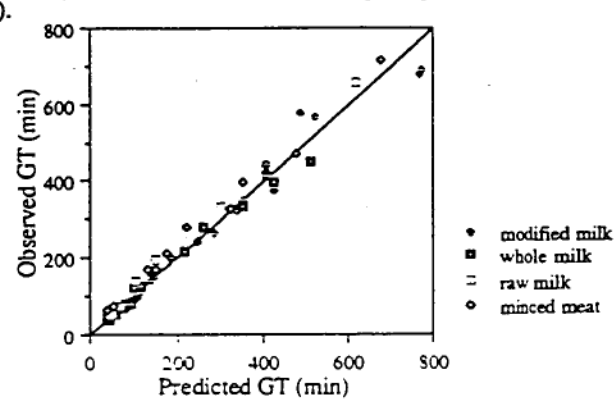
A Square Root Model (Ratkowsky *et al.* 1982) was developed for psychrotrophic pseudomonads in artificial broth media by determining the generation time, of the fastest strain, at 0.5°C intervals from 0 to 50 °C and shown as a square root plot.



The model is based on >1300 individual observations (~90 data points each of which is derived from a growth curve containing a minimum of 15 data readings).

Both our results and those from the literature showed (using F- and t-tests) that the theoretical minimum temperature (T_{min}) for the growth of psychrotrophic *Pseudomonas* is -7.8 °C (265.2 K) ± 1.93 (n=35) regardless of the technique used to determine growth rate and the source of the organism. This implies that only one model is needed for *Pseudomonas* regardless of the food product of concern.

The model was validated in modified milk, whole, pasteurised, homogenised milk, raw milk and minced beef. The figure below shows that the model accurately predicts the growth of *Pseudomonas* in milk and meat. It contains validations from all the products studied. A direct comparison of observed and predicted generation times have been superimposed over a line of slope = 1.0 (i.e., a perfect correlation).



Results indicate that the square root model constructed successfully predicts the growth of *Pseudomonas* in milk and meat. Reports in the literature suggest that predictions from models based on data generated under constant conditions can reliably predict growth under fluctuating conditions (Ross *et al.* 1993). Therefore, it can be concluded that with a validated model, one requires only a temperature profile to predict the extent of growth of the organism of concern.

The *Pseudomonas* model has been incorporated into prototype computer software and evaluation of the model, in the dairy industry, has commenced.

Predictive Modelling is

P recise
R apid
O bjective
V ersatile
E asy to use
N o sweat!

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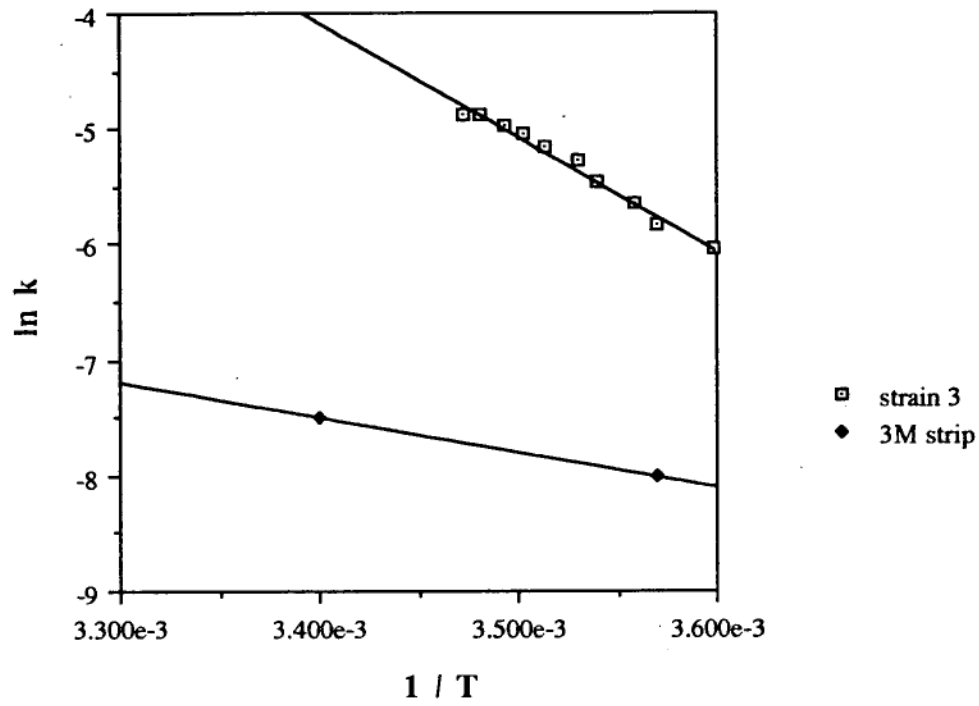
APPENDIX 9 Arrhenius plot comparing strain 3 temperature model (Figure 4.2a, data between 4.7°C and 14.8°C) and reference rates of 3M Monitor Mark temperature history indicator strips (5 - I).



The line of best fit through data of strain 3 model gives the following equation:

$\ln k = 29.409 - 9848.5\ (1 / T)$

where k = rate and T is temperature in K

From Eqn 1.4, the following can be written:

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

where A = collision factor, E_a = activation energy and R = universal gas constant (8.31 J mol^{-1} K^{-1})

For the strain 3 data:

$E_a / R = 9848.5$

$E_a = 9848.5 \times 8.31$

$= 81{,}841$ J mol^{-1}

$= 19{,}551$ cal mol^{-1}