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TO MARIE AND GORDON

MICROBIAL SPOILAGE

OF

POTATO TOP PIES

BY

FIONA MASTERS

A THESIS SUBMITTED IN PARTIAL
FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

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PALMERSTON NORTH

MARCH 1986

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S U M M A R Y

The microbial spoilage of Potato Top pies was investigated to try to provide a view of the events taking place during the spoilage process which renders these pies unsuitable for public consumption. Pies used in this study were obtained from a commercial pie manufacturer and were stored at $4^{o}C$, $25^{o}C$ and $37^{o}C$, and the effects of storage at these temperatures studied. During the examination biochemical techniques were used in conjunction with microbiological methods.

Aspects gained from this study can be summarised as below:

- Major flora of all pie component parts (meat filling, pastry surround, potato topping) of freshly cooked pies and of pies stored at the three temperatures consisted primarily of gram positive rods and cocci.
- The origins of these organisms could be traced directly back to various stages of manufacture.
- Within 24-36 hours of storage at $25^{o}C$ and $37^{o}C$ numbers of gram positive organisms could reach above 10^{7}/gram of pie component.
- No obvious organoleptic spoilage took place.
- Lack of off odours (NH_3; H_2S); the presence of amylase producing *Bacillaceae*; pH decreases in spoiled pies - suggested that the utilisation of low molecular weight compounds (such as glucose) resulted in saccharolytic spoilage of the pies.
- Bacterial numbers in the pies stored at $4^{o}C$ did not rise above 10^{5}/gram within 28 days of storage.
- Isolated dominant bacterial species were identified as *Bacillus*, *Streptococcus*, *Staphylococcus* and *Micrococcus*, with the *Bacillus* species and *Streptococcus* species inhibiting the growth of the other organisms.

INTRODUCTION

Studies of cooked meat products, such as that of Bell and Gill (1982), on chubs, have resulted in some knowledge of the spoilage process which renders cooked meat unacceptable - however, there is a lack of information on cooked meat pies. Because of the popularity and accessibility of cooked meat pies, it seems appropriate to investigate various spoilage aspects of pies stored at a variety of temperatures.

Such a study should include an examination of the origin and development of spoilage flora which develop for a range of storage temperatures, the metabolic processes leading to spoilage and a study of inter-relationships between bacterial species found in spoiled pies.

This thesis, therefore, examines aspects of the spoilage process which takes place within cooked individual Potato Top pies stored at $4^{o}C$, $25^{o}C$ and $37^{o}C$. Biochemical and microbiological techniques were used to gain an understanding of the process which leaves the pies unacceptable for consumption.

LITERATURE REVIEW

INTRODUCTION

Although there has been a number of studies carried out on the spoilage of meats (Gill and Newton 1977, 1980; Gill, 1979; Ingram and Dainty, 1971) and meat products (Bell and Gill, 1982; Hall and Angelotti, 1965) there has been a marked lack of information on the spoilage of cooked meat pies. Information, therefore, that will give some idea of the processes occurring during the spoilage of meat pies can only be obtained indirectly, by applying knowledge in parts from the studies done on meat and other products.

This review will, therefore, examine various aspects of contamination of meats, and meat spoilage; as well as aspects of food-bourne illness in relation to these products.

Topics to be discussed will include:

(1) Spoilage flora of meat

(2) Spoilage flora of meat products

(3) Biochemical changes associated with the growth of micro-organisms in meat.

(4) A quick note on the spoilage microflora of potatoes and pastry

(5) Public Health aspects of spoiling meats.

2.1 Spoilage of Meat

When heavy consumption of meat (and meat products) occurs, knowledge of both biochemical and microbiological aspects of this spoilage is important. This knowledge can prevent mass food-borne illness due to the consumption of incorrectly prepared and stored meats - and foods in general.

Bacteria found associated with meats are present as either deep tissue contaminants or as surface associated contaminants. The surface

associated contaminants are present as a result of post-slaughter contamination of meat from external sources (Ingram, 1949). Gill (1979) examined deep tissue bacteria and found that the most likely type of organism present in the tissue of healthy normal animals were small numbers of the genus *Clostridia* (these organisms commonly occur as soil organisms and are also commonly found in the animal gut). Gill concluded that further work was required to determine the nature, incidence and spoilage potential of deep tissue contaminants.

Surface associated microflora of meats, however, vary because the numbers and types of bacteria present are dependent upon environmental conditions - nutritional value of the meat, storage temperature and conditions of storage. Several authors (Carse and Locker, 1974; Dainty, 1982; Gill and Newton, 1977, 1978; Grau, 1981; Ingram and Dainty, 1971; McMeekin, 1981; Newton and Gill, 1978) have looked at various aspects of meat stored at chill temperatures. The aerobic spoilage flora on meat stored at chill temperatures consists primarily of pseudomonads (Gill and Newton, 1978; Ingram and Dainty, 1971) with *Acinetobacter* spp, *Enterobacter* spp and *Microbacterium thermosphactum* involved to a lesser extent (Gill and Newton, 1977). These species preferentially utilise glucose (Gill and Newton, 1977, 1978, Ingram and Dainty, 1971) as their nutrient source, subsequently utilising amino acids, lactic acid and possibly nucleotides (Dainty, 1982), when the growth of these bacteria exhausts the glucose supply. Utilisation of these nitrogen and sulphur containing substrates soon leads to the production of malodourous end-products such as ammonia and hydrogen sulphide, with a subsequent increase in pH because of the release of ammonia (Gill, 1983). Under anaerobic conditions (usually when meats are vacuum packaged), however, lactobacilli are the main spoilage flora when the storage temperature is 0-15°C; as well as *Microbacterium thermosphactum* and Enterobacteriaceae (Grau, 1978, 1981; Newton and Gill, 1978).

As with the aerobic spoilage flora, these organisms preferentially utilise glucose as a nutrient source (Newton and Gill, 1978), however spoilage does not occur as rapidly as it does under aerobic conditions, and is detectable only after maximum cell density has been attained (Sutherland *et al*, 1976). When lactobacilli dominate the anaerobic spoilage flora, it is the accumulation of short chain fatty acids that

is indicative of spoilage occurring (Gill, 1983). Because all of the above species utilised glucose, competition for this substrate arose under the glucose limiting conditions at the surface of the meat, leading to *Enterobacter* limiting the growth of *M. thermosphactum*, and lactobacilli limiting the growth of both, apparently by producing an inhibiting substance (Grau, 1981; Newton and Gill, 1978). Growth of these bacteria soon ceases, however, because the diffusion of fermentable substrates to the surface is not rapid enough to support further growth (Gill and Newton, 1978).

At the higher storage temperatures (above 10°C) pseudomonads are once again the dominant spoilage flora under aerobic conditions (Gardener, 1965), but lose this dominance to *Acinetobacter* spp and the Enterobacteriaceae at temperatures near 30°C (Gill and Newton, 1980). It is assumed that once again glucose is preferentially utilised, and amino acids used only once this nutrient source is depleted (Gill, 1983). This would once again lead to the production of malodourous end-products and an ultimate increase in pH.

Under anaerobic conditions, lactobacilli and Enterobacteriaceae are the dominant spoilage flora up to temperatures of 20°C, but Enterobacteriaceae decrease, and species of *Clostridium* increase when temperatures rise above 20°C. Further data is required as to which substrates are utilised under anaerobic conditions at these higher temperatures, but presumably glucose is again preferentially utilised as it is under chill temperatures.

In an experiment carried out by Gill and Newton (1980), it appeared that when meat was artificially innoculated with pathogens, the only species not inhibited by normal microflora were *Escherichia coli* and *Salmonella typhimurium*. Their growth was only inhibited at 20°C under anaerobic conditions. It would therefore seem important to store meats at refrigeration temperatures, because the growth of these pathogens is limited at chill temperatures, so do not therefore present a food-bourne hazard.

It is important to note that spoilage of meats is largely dependant upon the microflora initially present on the meat, and that the growth of these organisms depends upon substrate utilisation and storage temperature.

2.2 Spoilage of Meat Products

Meat products show different patterns of spoilage to that of meat, which is largely due to differences in processing and the inclusion of additives. The total count of freshly made meat products therefore reflects the overall quality of the ingredients, handling, and storage (Jay, 1978). This includes the handling the raw meat undergoes to form the meat product. Often, as in the case of luncheon meat, the meat is cooked, so the flora present in the final product will include those heat resistant bacteria and spore-forming bacteria that have survived the cooking process.

Bell and Gill (1982) found the initial flora of luncheon meats to be mainly *Bacillus* and *Micrococcus* spp. When "chubs" were stored at 10°C, bacterial numbers changed little. However, when the chubs were stored at 25°C, surface growth of *Streptococcus* spp and *Bacillus* spp occurred, with the streptococci replacing the *Bacillus* spp after 14 days storage. Streptococci were able to grow then because of the de-nitrifying capabilities of the *Bacillus* spp. This surface growth was accompanied by a fall in pH, and an increase in lactic acid. The glucose concentration varied with the changes in the *Bacillus* population, indicating that glucose converted to lactic acid was largely replaced by hydrolysis of the starch portion of the luncheon meat, mediated by the amylases produced by the *Bacillus*. Under the more anaerobic conditions in the centre of the chub, little change occurred when the chub was stored at 25°C and 10°C, where *Enterobacter* spp dominated the flora. No evidence of spoilage occurred under these anaerobic conditions, but at the surface when the chub was stored at 25°C, spoilage was evident by day 14 when the casings were distended by gas production, surface softening and discolouration was evident and the meat had a distinctly sour smell.

Grau (1978) found that the predominant organisms developing during storage of vacuum-packaged (and therefore essentially anaerobic), cured cooked products are lactic acid bacteria, which cause slime production and sourness of the product, as spoilage indicators. There is, however, debate on the relationship of the bacterial count of the product to spoilage (Grau, 1978). Hamburgers are essentially minced beef, which spoils in the same manner as does whole muscle tissue (Gill, 1983), but often commercially made hamburger patties contain additives, which therefore increases the variability of the microflora present. In Tamminga *et al's* (1982) study of hamburgers, the experiments

showed that the raw minced meat used for hamburgers contained large numbers of a wide range of micro-organisms, and these included potential pathogens such as *Salmonella* spp. Cooked samples differed little in their microbial load and also contained salmonella, and this was due to the prior cooking time these hamburgers received - often the centre of the pattie was uncooked. It therefore seems appropriate to set correct cooking standards for uncooked meat products, in order to decrease the microbial load and therefore reduce the presence of hazardous bacterial types. Just as important is the storage temperature.

Hall and Angelotti (1965) found that proliferation of *Clostridium perfringens* in meat and meat products did not occur at temperatures of 5-15°C, but rapid growth occurred at higher temperatures, especially around 45°C. Since this seems to be the case in most examples quoted, it seems appropriate to store meat products, as well as meats, at chill temperatures.

2.3 Biochemical changes associated with the growth of micro-organisms in meat

As Ingram and Dainty (1971) have already noted, there is a scarcity of information regarding the nature of biochemical changes that occur with meat during spoilage. The most obvious change that can occur in spoiling meats and meat products is the production of malodourous gases such as NH_3 and H_2S. These form from the utilisation of amino acids and other N & S containing compounds (Doelle, 1969). However, although these are obvious indicators of spoilage, other biochemical changes occur to the meats which are also indicative of bacterial spoilage, even though they may all occur after microbial proliferation (Ingram and Dainty, 1971).

Changes in levels of glucose and related compounds; end products of glycolysis such as lactic acid; and end products of amino acid utilisation such as NH_3, have been measured by various authors (Bell and Gill, 1982; Gill, 1976; Gill and Newton, 1977, 1978) as well as changes in pH. Because these changes are often easier and quicker

to measure than bacterial growth, it has been suggested that these be used to assess bacterial spoilage (Ingram and Dainty, 1971). Thus, a high pH could be indicative of bacterial spoilage, due to the production of NH_3. However, there has been some dispute as to the validity of this (Gardner, 1965; Turner, 1960) because the pH does not necessarily rise with bacterial proliferation - Bell and Gill's (1982) study on chubs, for example, where the pH decreased with bacterial proliferation.

As a quick summary of the data from authors quoted in sections 1 and 2, the biochemical changes occurring in spoiled meats and meat products; are as follows:

Under aerobic conditions of storage, the bacteria present preferentially utilise glucose as their main nutrient source. Unless this source is quickly depleted (when the cell density rises above $10^8/cm^3$ - Gill, 1983) the bacteria continue utilising this, resulting in an accumulation in lactic acid and subsequent pH decrease, with no obvious outward changes (Bell and Gill, 1982). Once this glucose is depleted, however, amino acids and lactic acid (as well as other low molecular weight compounds) are used, resulting in the production of malodourous compounds such as NH_3 and H_2S and a rise in pH. Distention of the meat can also result (because of gas formation). These are obvious organoleptic signs that spoilage is taking place. Breakdown of protein and lipids occurs only in the very late stages of spoilage, by which time the meat/meat product is unsightly (Dainty, 1982).

Under anaerobic conditions, a similar situation applies, but often the bacteria cannot survive when the diffusion of fermentable substrates to the surface is not rapid enough to support further growth (Gill and Newton, 1978).

Usually by the time malodourous end-products are produced, the bacterial population has reached numbers above $10^8/cm^3$ (Gill, 1983), so if pathogenic species are present, food poisoning may result from the consumption of the product. Although biochemical changes do occur with changes in the bacterial load of the food, there has not been proven to be a direct link between the two parameters, such that measurement of one parameter can give information about the microbiological quality of the meat, especially since inherent decay occurs in the food (Ingram and Dainty, 1971).

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2.4 The spoilage microflora of potatoes and pastry

Because potato top pies are made up of potato and pastry, it is appropriate to review spoilage aspects of these products.

Some authors (Duran *et al*, 1982; Notermans *et al*, 1985; Snyder *et al*, 1983; Surkiewicz *et al*, 1967) have looked at various aspects of bacterial contamination of potatoes and have concluded that because of the sheer mechanics of producing potato products, the sanitary conditions under which these products are produced are poor. Build up of potato slices and juice on the machines provides ideal conditions for bacterial proliferation, which leads to the contamination of the end product. However, the final cooking process (for example, deep fried chips, dehydrated potato) the product goes through effectively sterilises the product (Duran *et al*, 1982; Surkiewicz *et al*, 1967). However, *Clostridium botulinum* has been isolated from reconstituted dehydrated potato (Notermans *et al*, 1985), the growth of which can be effectively stopped when the product is stored at chill temperatures - thereby stopping the production of the fatal toxin. *Bacillus cereus* has been implicated as the causative agent of food poisoning due to the consumption of incorrectly held whipped potatoes (in Snyder *et al*, 1985) due to improper temperature of storage. It seems, therefore, that potato products must be treated correctly in order to eliminate bacteria, and must be stored at the correct temperatures to prevent bacterial proliferation. Little information is available on the spoilage process that occurs when bacteria proliferate on potato.

There is a distinct lack of information regarding the microbiological quality of pastry itself, although pastry products have been investigated. (Hyatt and Guy, 1981 a, b) In these investigations *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella typhimurium* were isolated from pumpkin pies, but it was not stated whether the pastry itself contained these bacteria, and other investigations give similar non-specific results. Flour (Silliker *et al*, 1980) does contain bacteria, but these are not specified.

2.5 Public Health aspects of spoiling meats

From a Public Health point of view, it is important to have some knowledge of food handling and food storage conditions, in order to prevent food-borne illness occurring as a result of mishandling of foods. Data, with respect to these points, has been previously well documented (Hobbs and Gilbert, 1978 for example). However, food-borne illnesses do occur, and although details have not previously been well documented, attempts are now being made to collate incidences and the types of organisms and foods involved (Hobbs and Christian, 1973; Hobbs and Gilbert, 1978). Food-borne illnesses resulting from the consumption of meat, and meat pies specifically, have not been very well reported, although some cases have been documented. Hutchinson and Taplin (1977) found *Bacillus cereus* in meat loaf and meat dishes, and salmonella (Hobbs and Gilbert, 1978), *Staphylococcus, Clostridium welchii* (Hobbs and Christian, 1973), streptococci (Cory *et al*, 1938) and *Bacillus cereus* (Lechowich, 1978) have all been implicated in food poisoning incidences directly attributable to meat products. The illness due to food poisoning varies with the bacteria involved, these have been outlined by several authors (e.g. Archer and Kvenberg, 1985; Bryan, 1978; Lechowich, 1978; Todd, 1978) - it is important to note the different types of bacterial food poisoning that can occur, because these affect the way foods can be handled (Bryan, 1978).

Because meats do not always show obvious organoleptic changes as they spoil (based on their microbiological load) there is a danger present to the public, because consumption of contaminated meats (and foods in general) can occur, and if pathogens are present, under the correct conditions, food poisoning may result.

The growth of micro-organisms in meat products (with meat pies in mind) is distinctly possible, and, under elevated temperatures of storage, organisms present in the products can multiply to levels high enough to cause food poisoning. In the case of a product that is handled a lot (such as a meat pie) the possibly of potential food poisoning organisms such as staphylococci contaminating the product is high, so hygiene is very important to prevent this from occurring.

Failure to cook the product correctly (thereby eliminating the bacteria present) and cooling it quickly (preventing elevated temperatures, and therefore ideal growth temperature) will lead to the danger of these potential food poisoning organisms growing to such numbers as to cause poisoning.

MATERIALS AND METHODS

3.1 Samples

Freshly cooked potato top pies were obtained from a commercial pie manufacturer. Each pie was wrapped in a sealed cellophane bag and kept in these bags throughout the storage period. Samples of pre-cooked pie components (meat filling, pastry surround, potato topping) were also obtained from the same manufacturer.

3.2 Storage Experiments

Pies were placed on trays and stored at 4, 25 and 37°C for up to 28 days; 12 days and 4 days respectively. Duplicate pies were sampled at regular time intervals and examined as below.

3.3 Isolation and Enumeration of Bacterial Flora of Fresh and Spoiled Pies

Fresh and stored pies were examined as follows: Duplicate 10 gram samples of the meat filling, pastry surround and potato topping were homogenised in 90 mls of peptone water (0.1% w/v) using a model 400 Colworth Stomacher. Aliquots (0.1 ml) of suitable dilutions of each homogenate were then spread in duplicate onto the surface of selective and non-selective microbiological growth media. Inoculated plates of Nutrient agar (NA) (Difco) were incubated anaerobically and aerobically at 37°C for 48 hours, and bacterial counts per gram sample determined. Similarly, coliforms were enumerated on MacConkey agar (Mac) (Difco); gram positive aerobic cocci on Mannitol Salt agar (MSA) (Gibco); *Staphylococci* on Baird-Parker agar (B-P) (Difco); faecal *Streptococci* on Membrane-Enterococcus agar (MEA) (Slanetz and Bartley, 1957); *Bacillus cereus* on Phenol Red Egg Yolk Polymyxin agar (PREYP) (Gibco) and *Clostridium* on Reinforced Clostridial agar (RCA) (Difco). All plates were innoculated and incubated aerobically at 30°C for 24-48 hours, with further overnight incubation at 37°C. Innoculated plates of RCA agar were incubated anaerobically at 37°C for 48 hours.

Colonies of micro-organisms which grew on any of the above media were selected at random; isolated on plates of NA and maintained on slopes of

NA at $4^{o}C$. Presumptive streptococci were isolated on plates of NA and MEA and were maintained on slopes of MEA at $4^{o}C$.

3.4 Characteristics of Isolates

Samples of bacteria isolated from all storage temperatures were identified to genus level by assessing their gram reaction; catalase reaction and oxidase reaction. They were then identified using schemes outlined by several people (A.C. Baird-Parker, 1963; Isenburg *et al*, 1970; Sherman, 1937 and Wolf and Barker, 1968).

Bacterial cultures maintained on slopes of NA were used to assess gram reaction, morphology, oxidase reaction and oxidative/fermentative utilisation of glucose (Hugh and Leifson, 1953). Production of catalase was tested by dropping 3% (w/w) hydrogen peroxide on to a pure culture of the isolate. Motility was examined by phase contrast microscopy of overnight nutrient broth cultures of the isolate in question. Presumptive streptococci isolated were examined as above from cultures held on slopes and broths of MEA.

3.5 Chemical Analysis

Duplicate 1 gram samples of the meat filling, pastry surround and potato topping homogenised in diluents, as below, were used to determine pH, glucose content, amylase enzyme activity and lactate content.

Lactate content was estimated using a method outlined by S.B. Barker (Appendix 1). Samples (1 gram) were homogenised in 10 mls of distilled water and 5 mls of water, the latter homogenates' pH being measured on a pH meter.

The glucose-oxidase-peroxidase method (Sigma Technical Bulletin, No. 510) was used to measure the glucose content of samples. Samples (1 gram) were first homogenised in 10 mls perchloric acid (6% w/v), neutralised with potasium hydroxide (20% w/v) and precipitates removed by centrifugation. 0.5 mls of this supernatant (or standard glucose solution, 0.05 mg/ml) was added to 0.5 mls of the enzyme colour reagent

(Appendix 2) and incubated for 30 minutes in a 30°C water bath. The absorbance was read at 450 nm using distilled water as a blank.

Amylase activity (measured as maltose monohydrate formed from starch) was estimated by the method by A. Danielsson (1974). Samples (1 gram) were homogenised in 10 mls NaK phosphate buffer (0.05 M, pH 6.9) and the mixture centrifuged to remove suspended solids. To 0.5 mls of this supernatant ("enzyme"), and standard maltose monohydrate (100 - 1000 ug/ml) was added 0.5 mls starch solution (Appendix 3). The tubes were incubated for 20 minutes in a 37°C water bath, 3, 5-dinitro-salicylic acid (DNS) (1 ml), added to stop enzyme activity and the tubes boiled for 10 minutes (to allow colour development). After cooling, 10 mls distilled water was added to the tubes, and read at 530 nm (DNS as blank).

3.6 Bacterial Inhibition Patterns

Representative isolates of bacteria from pre-cooked, freshly cooked and spoiled potato top pies (at all storage temperatures) were tested for production of growth inhibitor substances active against other unrelated isolates.

Adaptation of the widely used technique for an antibiotic testing (Mayr-Harting, 1972) and for detecting colicins (Fredericq, 1957) were used, relying on the ability of the "inhibitive affect" (colicins) to diffuse through agar.

0.1 mls of bacterial isolates (overnight broth cultures) were spread onto Plate Count agar (PCA) and allowed to dry for 5-10 minutes. 20 μl of the supernatant of centrifuged broth cultures of another unrelated organism were then dropped on to these plates and incubated at 30°C for 48 hours. Zones of inhibition were then allowed to develop. The combination of bacterial cultures were tested as below:

(1) Seeding plates of PCA with streptococci, tested against "bacteriocins" of *Bacillus*, *Staphylococcus*, *Micrococcus*.

(2) Seeding of PCA with *Bacillus*, tested against "bacteriocins" of *Streptococcus*, *Staphylococcus*, *Micrococcus*.

(3) Seeding of PCA with staphylococci/micrococci, tested against "bacteriocins" of *Streptococcus* and *Bacillus*.

RESULTS

4.1 Examination of Pre-cooked Pie Components

Examination of the bacterial flora present in the pre-cooked components of potato top pies - the meat filling, pastry surround and potato topping - indicated the pre-dominant bacterial types present were gram positive organisms. These were identified as *Bacillus* spp., *Streptococcus* spp., *Micrococcus* spp. and *Staphylococcus* spp. (Table 1). Anaerobes and gram negative bacteria were either absent or in very low numbers. *Bacillus* spp. were the predominant types found in all componant parts, with fewer micrococci and staphylococci.

4.2 Spoilage of Pies

Pies stored at 37°C and 25°C spoiled more rapidly than those stored at 4°C. Microscopic examination of stained smears prepared from pie contents indicated that the spoilage was predominantly bacterial, although pies kept for extended periods supported fungal growth. However, apart from estimates of numbers of bacteria present, no visual parameter indicated the extent of bacterial spoilage at any of the storage temperatures. Pies stored at 37°C and 25°C did not develop detectable spoilage odours until at least 2 and 4 days of storage respectively; at which time total viable counts exceeded 10^8 bacteria per gram of meat or potato (Figure 1).

Pies stored at 4°C did not form detectable odours even after a 28 day trial storage and numbers of bacteria did not exceed 10^5/gram, although fungal growth was evident after 14 days of storage.

4.3 Isolation and Enumeration of the Bacterial Flora from Fresh and Spoiled Pies

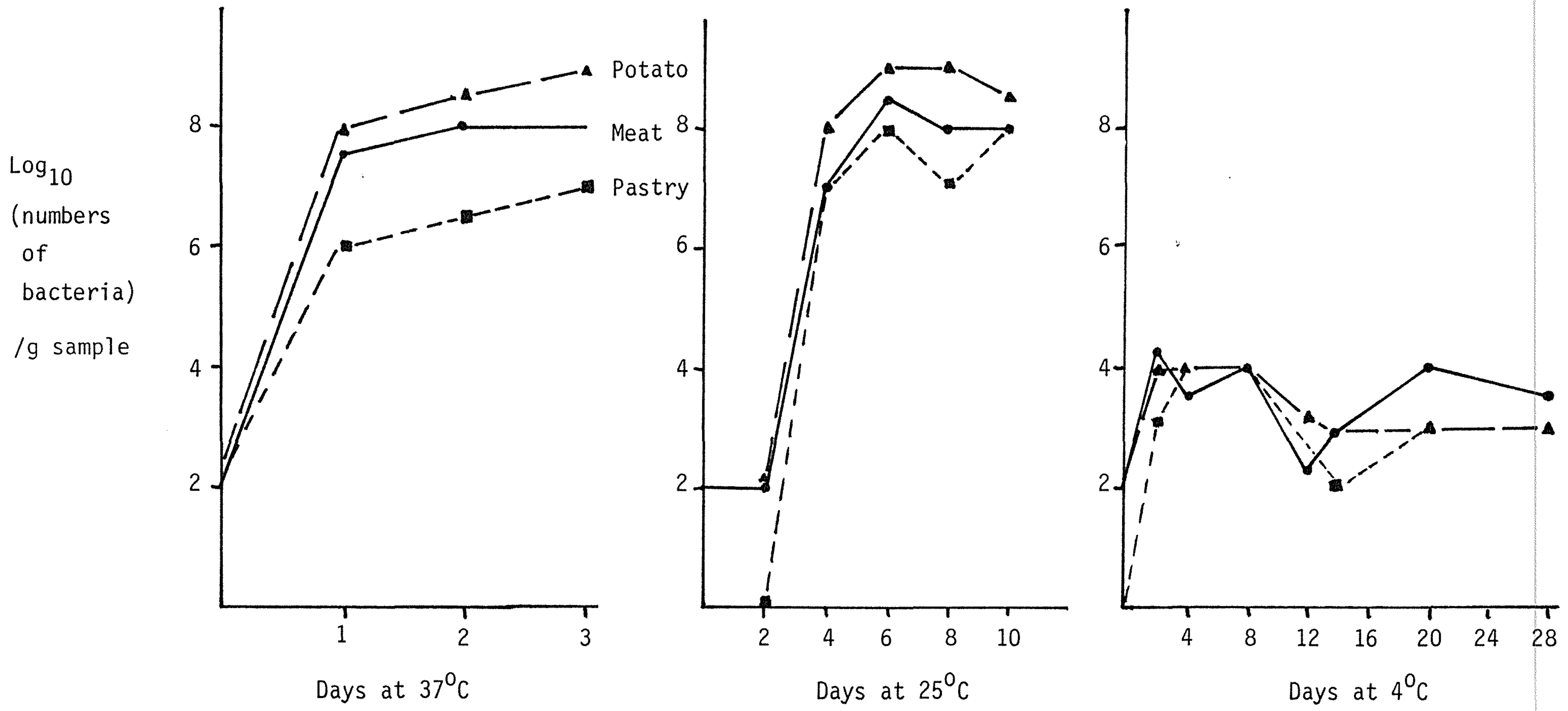
The predominant bacterial types isolated from freshly cooked and spoiled potato top pies were gram positive rods and cocci. These were isolated from all component parts (meat, pastry, potato) of the pies. These genera consisted of: *Bacillus* spp. *Streptococcus* spp., *Staphylococcus* spp. and *Micrococcus* spp.

Fresh Pie Component	Gram Positive Rods	Gram Positive Cocci	
	Bacillus spp.	*Streptococcus* spp.	*Staphylococcus*/ *Micrococcus* spp.
Meat Filling	3×10^2	2×10^2	10^2
Pastry Surround	10^4	10^3	10^4
Potato Topping	5×10^4	3×10^4	10^2

measured as numbers of bacterial cells /g sample

Figure 1

Changes in total viable count of Potato Top pies during storage at 37, 25 and 4°C.


Log10 (numbers of bacteria) /g sample
8
6
4
2
1
2
3
Potato
Meat
Pastry
Days at 37°C
8
6
4
2
2
4
6
8
10
Days at 25°C
8
6
4
2
4
8
12
16
20
24
28
Days at 4°C

Some coliforms were isolated from freshly cooked pies, but these were in very small numbers. Over the successive days of storage at all temperatures, these gram negative bacteria did not increase in numbers, or they decreased to 0/gram. Some anaerobes were found in the pies, but because they were identified as *Bacillus* spp. (facultative anaerobes) it was decided to isolate and enumerate them aerobically.

Counts of *Bacillus*, *Streptococcus* and *Staphylococcus*/*Micrococcus* in pie components stored at $37^{o}C$ indicated potato supported the best growth of these groups compared to the meat and pastry components (Figure 2). The *Bacillus* spp. consistently grew more rapidly and achieved high final counts (10^9/gram) than either *Streptococcus* or *Staphylococcus*/*Micrococcus* in the pastry and potato components (although the *Bacillus* spp grew rapidly in meat, the counts fell to 10^7/gram). Similarly, *Streptococcus* grew more rapidly than the *Staphylococcus* and *Micrococcus* in all pie components.

The pattern of development of the microbial flora of pies stored at $25^{o}C$ was similar to that described for pies at $37^{o}C$ (Figure 3). The meat and potato components both supported extensive growth of *Bacillus* spp. and streptococci. *Bacillus* spp., however, consistently grew more rapidly than the *Streptococcus* spp. and *Staphylococcus*/*Micrococcus*, and achieved counts of 10^9/gram. Similarly, the growth of the streptococci was greater than the staphylococci/micrococci.

By contrast, pies stored at $4^{o}C$ supported little or no growth of any bacteria and total viable counts rarely exceeded 10^5/gram of pie (Figure 4). Nevertheless, *Bacillus* spp. were the predominant type found at all stages of storage, than was the growth of *Streptococcus* and *Staphylococcus*/*Micrococcus*. The potato component of the potato top pies supported the best growth of all bacterial species, with both *Streptococcus* and *Staphylococcus*/*Micrococcus* reaching 10^5/gram.

It was found that pure cultures of two distinct colony types consistently grew on NA, from the homogenates of all component parts of the pies. Repeated identification of these lead to the conclusion that they were *Bacillus* spp., so NA was used for easy enumeration of this bacteria - because the colony types were distinct, and formed pure cultures on the NA plate, it was easy to identify them as *Bacillus* spp., based on previous identifications.

Figure 2

Changes in numbers of various groups of bacteria in the meat (a), potato (b) and pastry (c) fractions of potato top pies stored at 37°C.

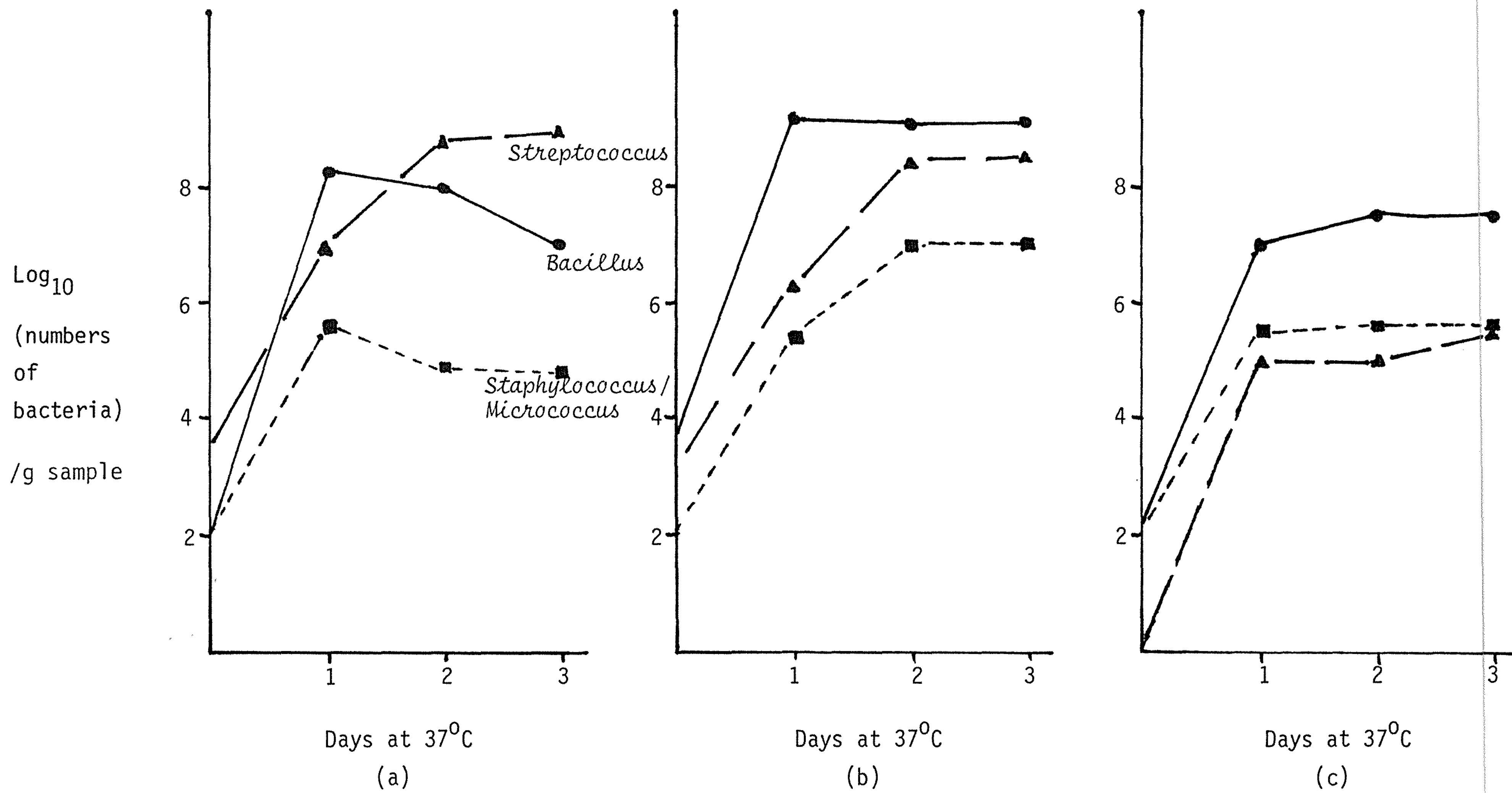

Log10
(numbers
of
bacteria)
/g sample
8
6
4
2
1
2
3
Streptococcus
Bacillus
Staphylococcus/
Micrococcus
Days at 37°C
(a)
(b)
(c)

Figure 3

Changes in numbers of various groups of bacteria in the meat (a), potato (b) and pastry (c) fractions of potato top pies stored at 25°C.

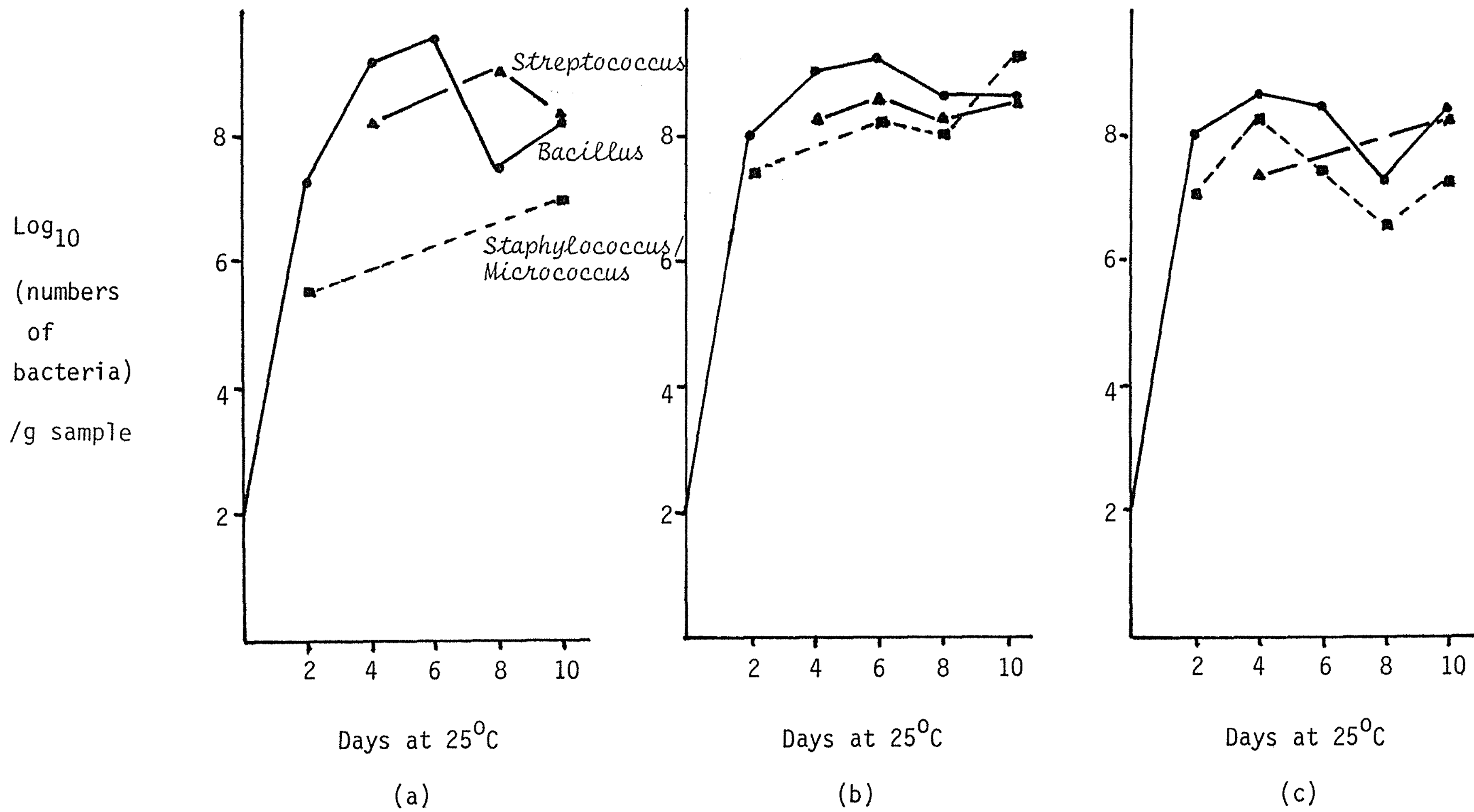

Log10
(numbers
of
bacteria)
/g sample
Streptococcus
Bacillus
Staphylococcus/
Micrococcus
2
4
6
8
10
Days at 25°C
(a)
(b)
(c)

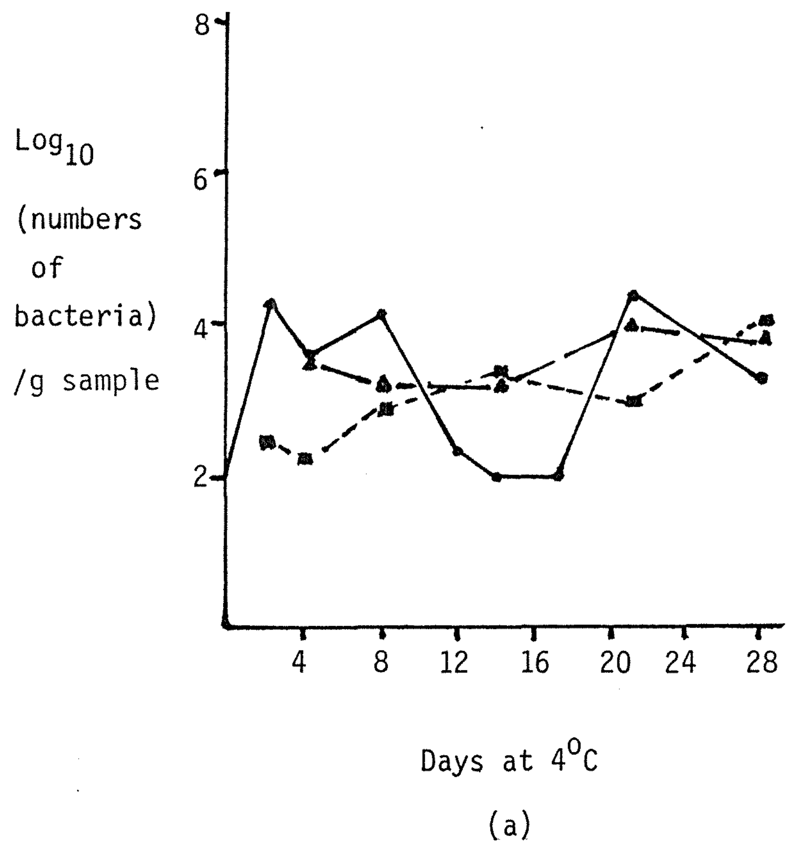
Figure 4

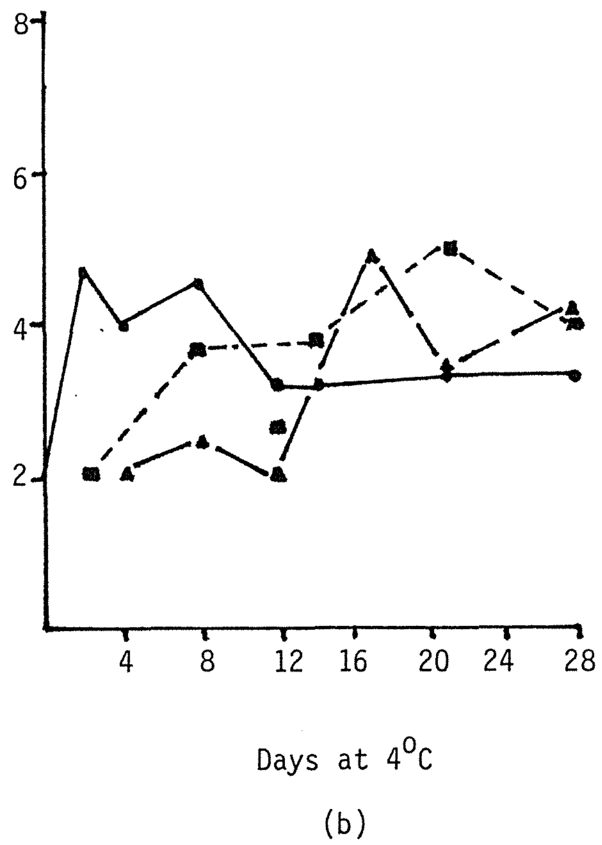
Changes in numbers of various groups of bacteria in the meat (a), potato (b) and pastry (c) fractions of potato top pies stored at 4°C.

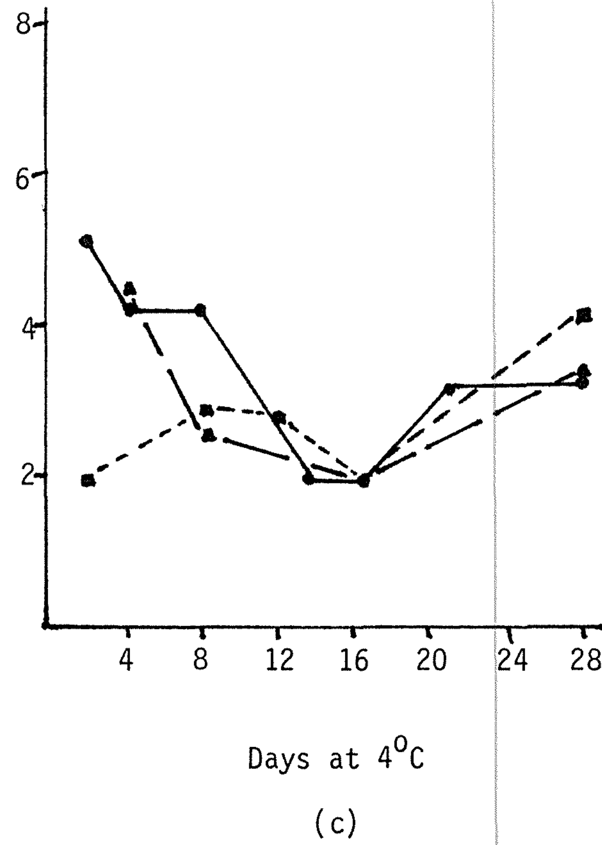
1. *Staphylococcus/Micrococcus* ■---■

2. *Streptococcus* ▲—▲

3. *Bacillus* ●—●


Log10 (numbers of bacteria) /g sample
8
6
4
2
4 8 12 16 20 24 28
Days at 4°C
(a)


8
6
4
2
4 8 12 16 20 24 28
Days at 4°C
(b)


8
6
4
2
4 8 12 16 20 24 28
Days at 4°C
(c)

4.4 Characterisation of Isolates from Spoiled Pies

Isolates of *Bacillus*, *Streptococcus*, *Staphylococcus* and *Micrococcus* spp. were further identified to species level (Table 2). The *Bacillus* species identified were: *Bacillus licheniformis*, *B. cereus*, *B. subtilus* and *B. megaterium*, with *B. licheniformis* being the predominant species isolated.

The *Streptococcus*, isolated as *Str. faecium*, *Str. faecalis*, *Str. faecium* var *casseflavius*, *Str. durans*, and *Str. equinus* were dominated by both the *Str. faecium* species and the *Str. equinus* species.

The *Micrococcus* and *Staphylococcus* species were differentiated according to a scheme presented by Baird-Parker (1963) -

Micrococcus groups 4, 5, 6 and 7
Staphylococcus groups III, IV and *St. aureus*

with the *Micrococcus* species dominated by number 6 and *Staphylococcus* species being dominated by type III.

These isolates were taken from all component parts of the pies stored at all temperatures. Not enough trials were done to establish whether any bacterial species dominated in any pie component at any time or temperature.

4.5 Chemical Analysis of Potato, Meat and Pastry from Pies Stored at 37°C, 25°C and 4°C

Levels of glucose, amylase activity, lactic acid and pH of potato, meat filling and pastry of pies stored at 37°C, 25°C and 4°C are all presented in Appendices 8 - 10 and summarised in Figures 5 - 13.

(i) The pH of all pie components stored at 37°C (Figures 5-7) decreased during storage, with the greatest decrease found in the meat component. The pH of the potato component dropped from 5.95 to 5, and the pH dropped from 6.15 to 5.3 in the pastry component over the 3 day storage period.

Table 2

Bacterial species isolated from spoiled Potato top pies.

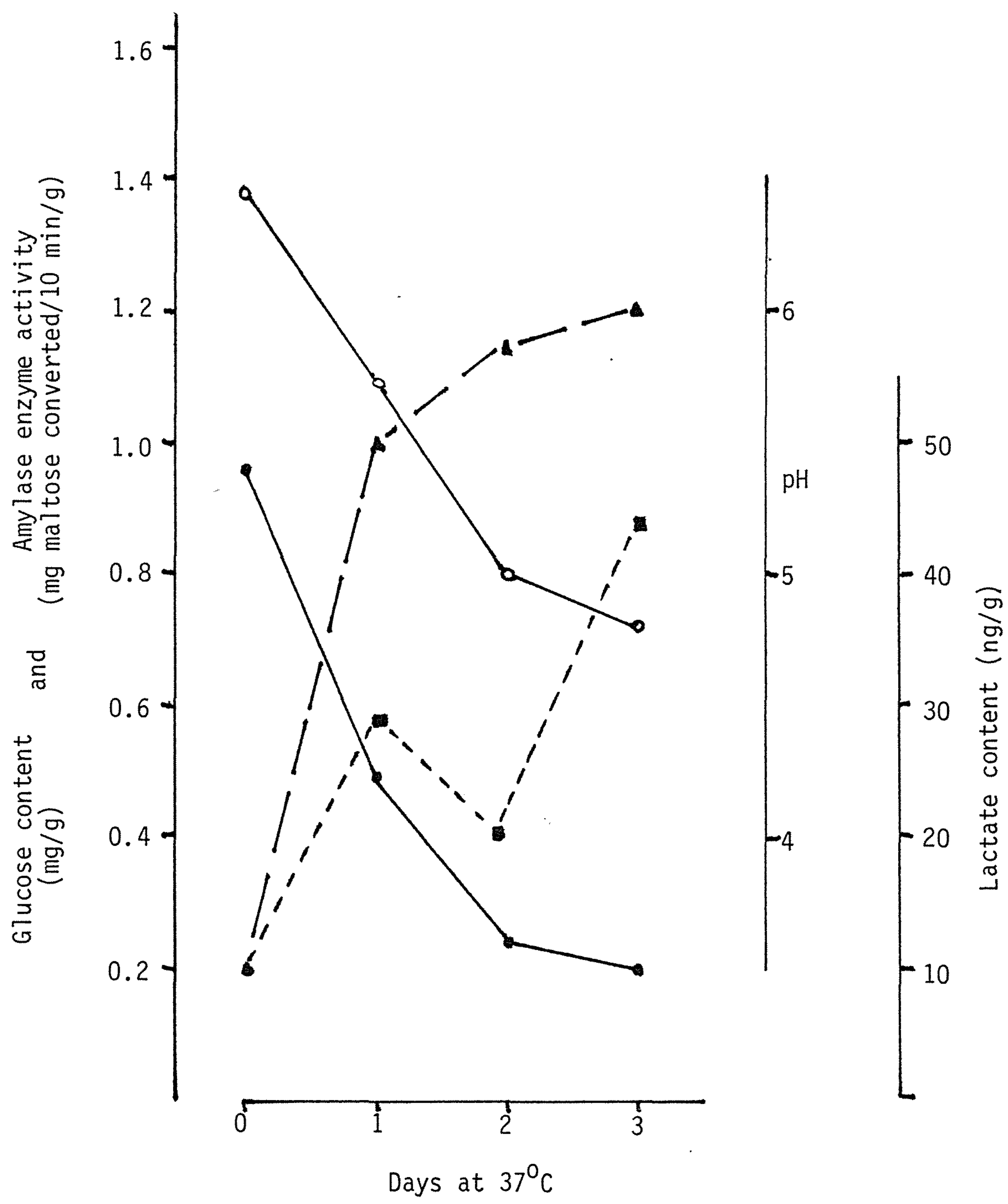
Isolates	Number Identified	N	%
Bacillus licheniformis	11	16	68.75
B. cereus	3	16	18.75
B. subtilus	1	16	6.25
B. megaterium	1	16	6.25
Streptococcus faecalis	1	10	10
Str. faecium	3	10	30
Str. equinus	3	10	30
Str. durans	2	10	20
Str. faecium var *casseflavius*	1	10	10
Micrococcus			
group 4	1	13	7.7
5	4	13	30.0
6	7	13	54.6
7	1	13	7.7
Staphylococcus aureus	1	4	25
type III	2	4	50
IV	1	4	25

N = Total number identified.

% = Isolates expressed as a percentage of N.

Figure 5

Changes in pH (o - o), glucose content (● - ●), lactate content (■ - ■) and amylase activity (▲ - ▲) in the meat component of potato top pies stored at 37°C.



Amylase enzyme activity
(mg maltose converted/10 min/g)
and
Glucose content
(mg/g)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
pH
6
5
4
Lactate content (ng/g)
50
40
30
20
10
0
1
2
3
Days at 37°C

Figure 6

Changes in pH (○ - ○), glucose content (● - ●), lactate content (■ - ■) and amylase activity (▲ - ▲) in the potato component of potato top pies stored at $37^{o}C$.

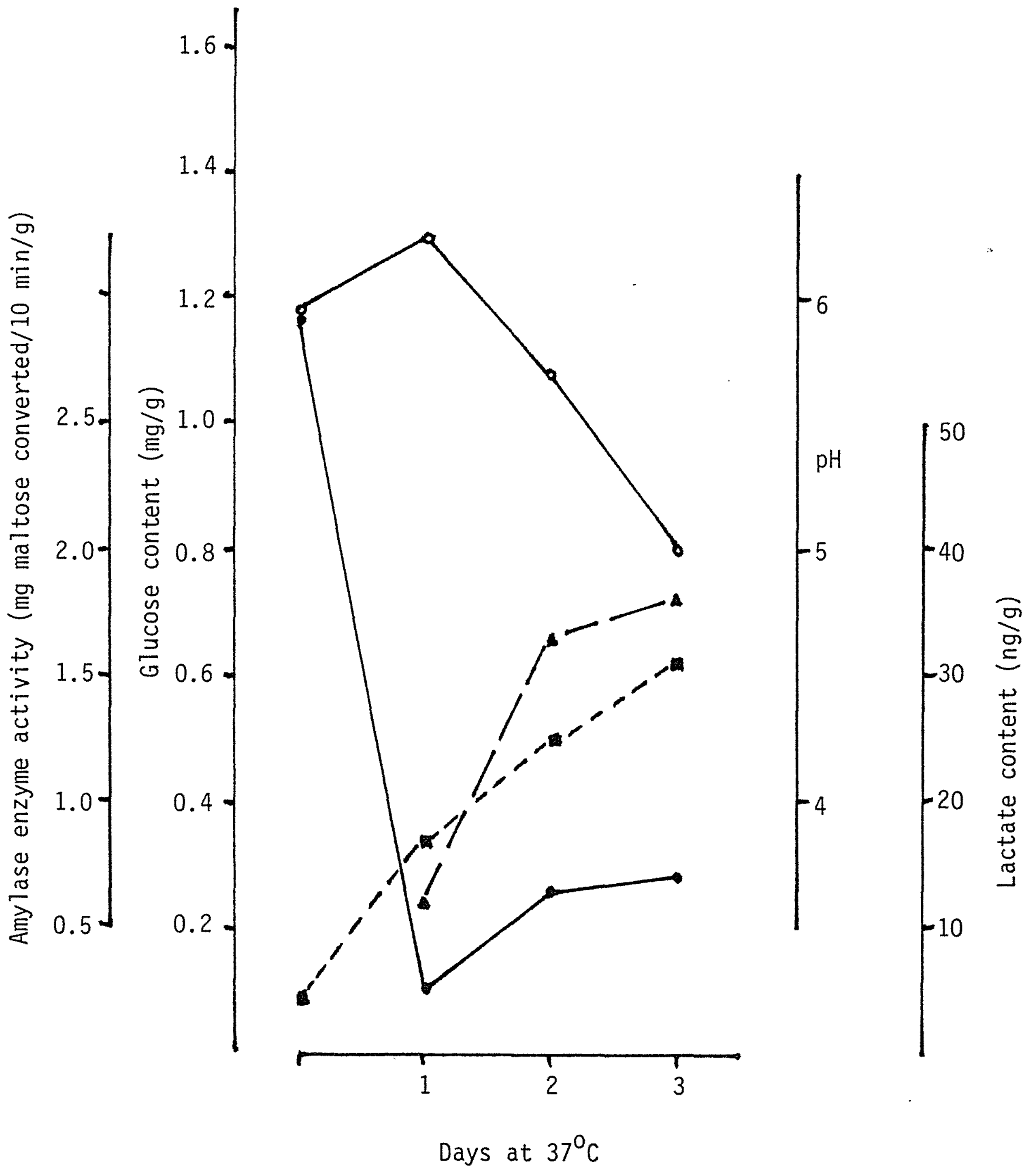
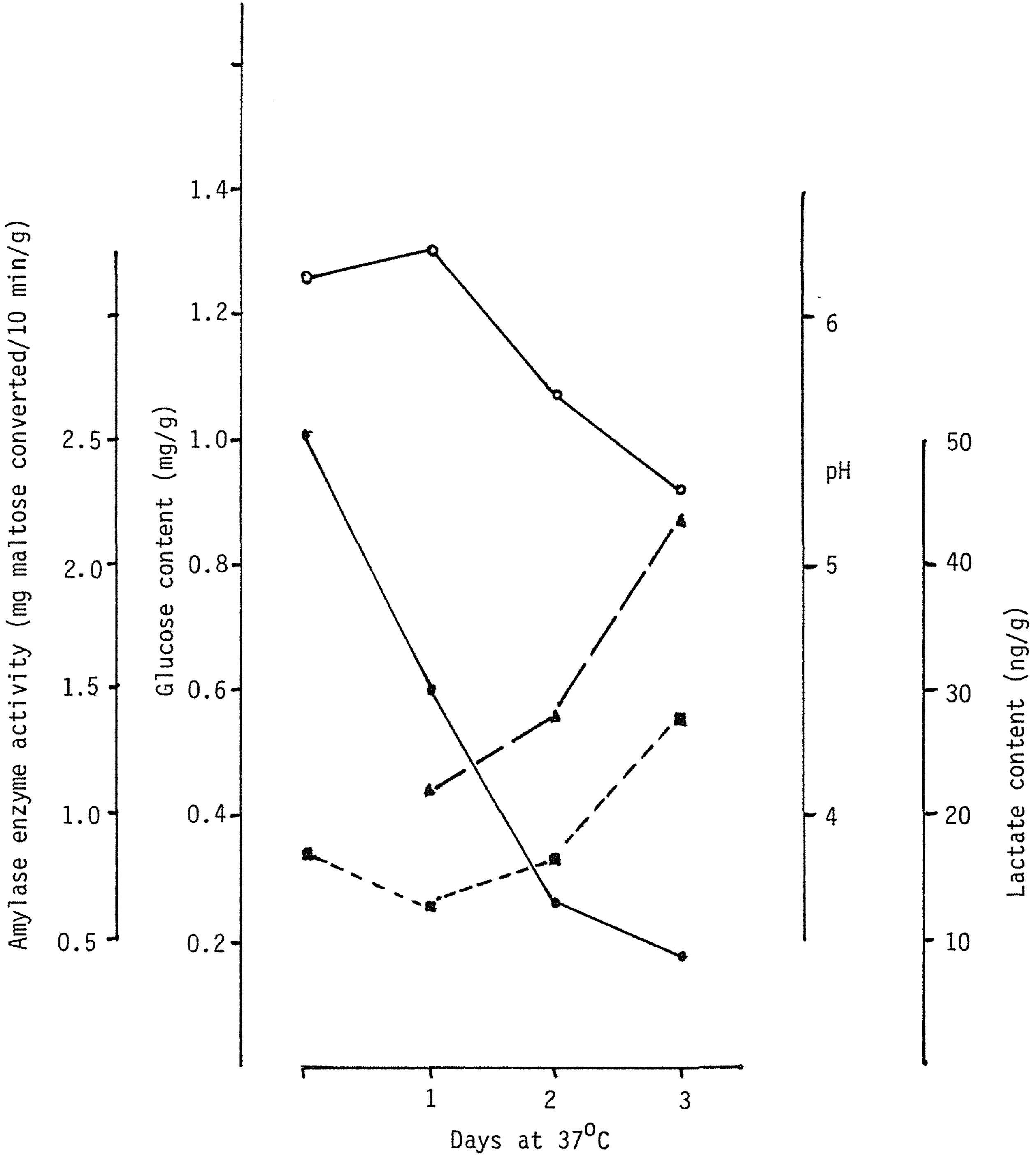

Amylase enzyme activity (mg maltose converted/10 min/g)
2.5
2.0
1.5
1.0
0.5
Glucose content (mg/g)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
pH
6
5
4
Lactate content (ng/g)
50
40
30
20
10
1
2
3
Days at 37°C

Figure 7

Changes in pH (○ - ○), glucose content (● - ●), lactate content (■ - ■) and amylase activity (▲ - ▲) in the pastry component of potato top pies stored at $37^{O}C$.



Amylase enzyme activity (mg maltose converted/10 min/g)
0.5
1.0
1.5
2.0
2.5
Glucose content (mg/g)
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1
2
3
Days at 37°C
4
5
pH
6
10
20
30
40
50
Lactate content (ng/g)

The pH of the pastry component of pies stored at 25°C (Figures 8-10) rose from 5.65 to 6.33, while the pH of the meat and potato component dropped from 6.13 to 6.06 and 5.8 to 5.22 respectively.

Little pH change occurred in the components of pies stored at 4°C (Figures 11-13), although the pH of the meat component decreased from 6.18 to 5.95, and the pH of the pastry increased from 5.93 to 6.14. The biggest pH change occurred in the potato component, where it increased from 5.81 to 6.88.

(ii) Changes in glucose levels (measured as mg glucose formed 1/g substrate) in pie components stored at 37, 25 and 4°C all shown in Figures 5-13.

The over-riding pattern that can be seen in the glucose concentration in pie components stored at 37°C and 25°C is a decrease followed (in some cases) by a small increase in concentration, during the latter stages of the storage trial.

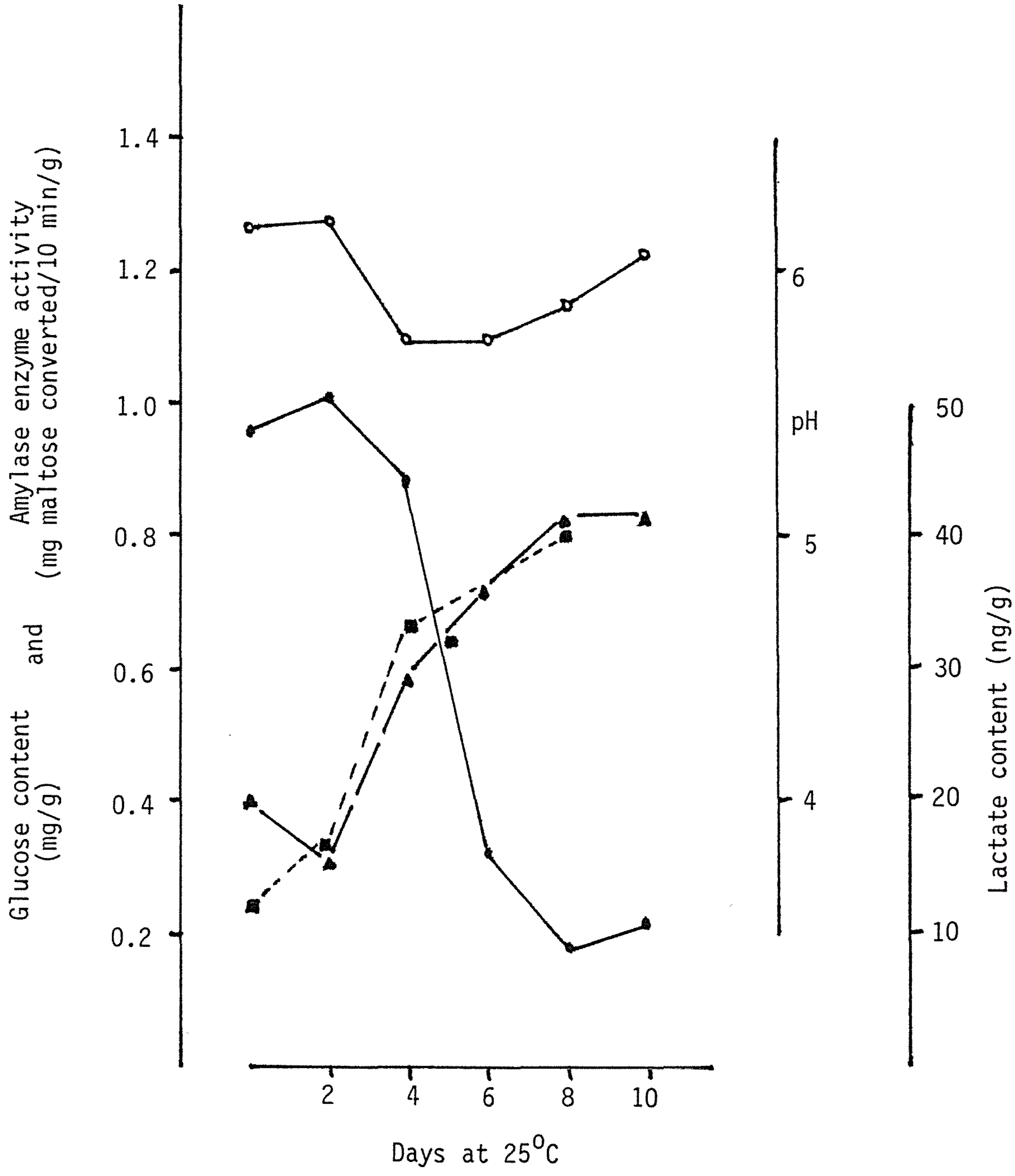
The glucose concentration in the potato component of pies stored at 37°C (Figures 5-7) decreased from 1.18 mg/g potato to 0.1 mg/g (this later increased to 0.28 mg/g on the first day of storage). Similar changes were observed for the glucose concentrations found in the pastry and meat components.

Glucose concentrations of all component parts of pies stored at 25°C (Figures 8-10) decreased, so that by the eighth day of storage the glucose concentration of the meat, potato and pastry components had decreased from 0.96 - 0.18 mg/g; 1.0 - 0.16 mg/g and 1.18 - 0.12 mg/g respectively. These concentrations did however increase, so that the final concentrations were 0.21 mg, 0.66 mg, 0.44 mg/g in the meat, potato and pastry components.

The overall glucose content in the meat and pastry components of pies stored at 4°C (Figures 11-13) decreased from 0.79 mg/g to 0.58 mg/g and 0.89 mg/g to 0.83 mg/g respectively.

Figure 8

Changes in pH (○ - ○), glucose content (● - ●), lactate content (■ - ■) and amylase activity (▲ - ▲) in the meat component of potato top pies stored at 25°C.



Amylase enzyme activity (mg maltose converted/10 min/g)
and
Glucose content (mg/g)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
6
pH
5
4
50
40
30
20
10
Lactate content (ng/g)
2
4
6
8
10
Days at 25°C

Figure 9

Changes in pH (○ - ○), glucose content (● - ●), lactate content (■ - ■) and amylase activity (▲ - ▲) in the potato component of potato top pies stored at 25°C.

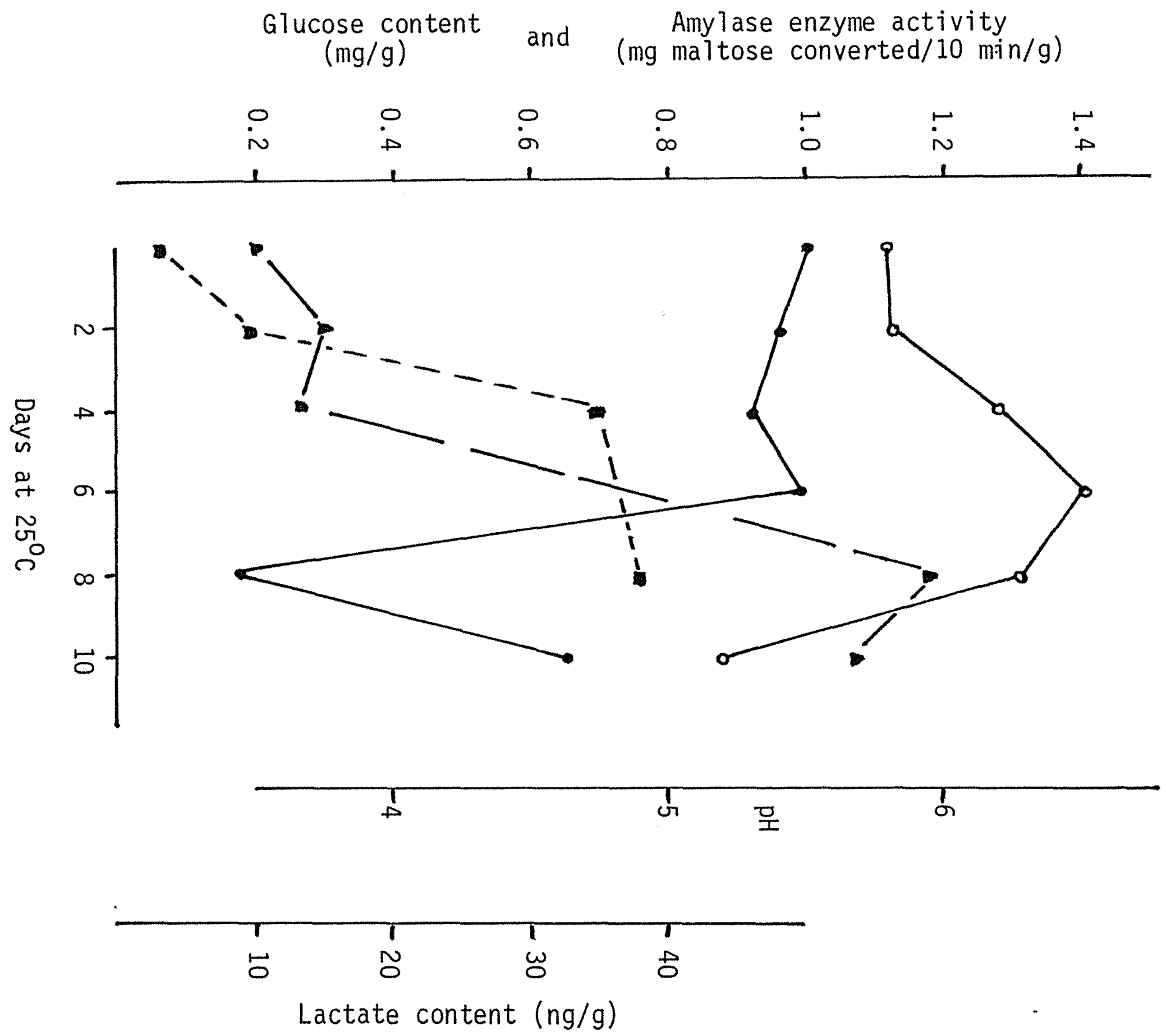
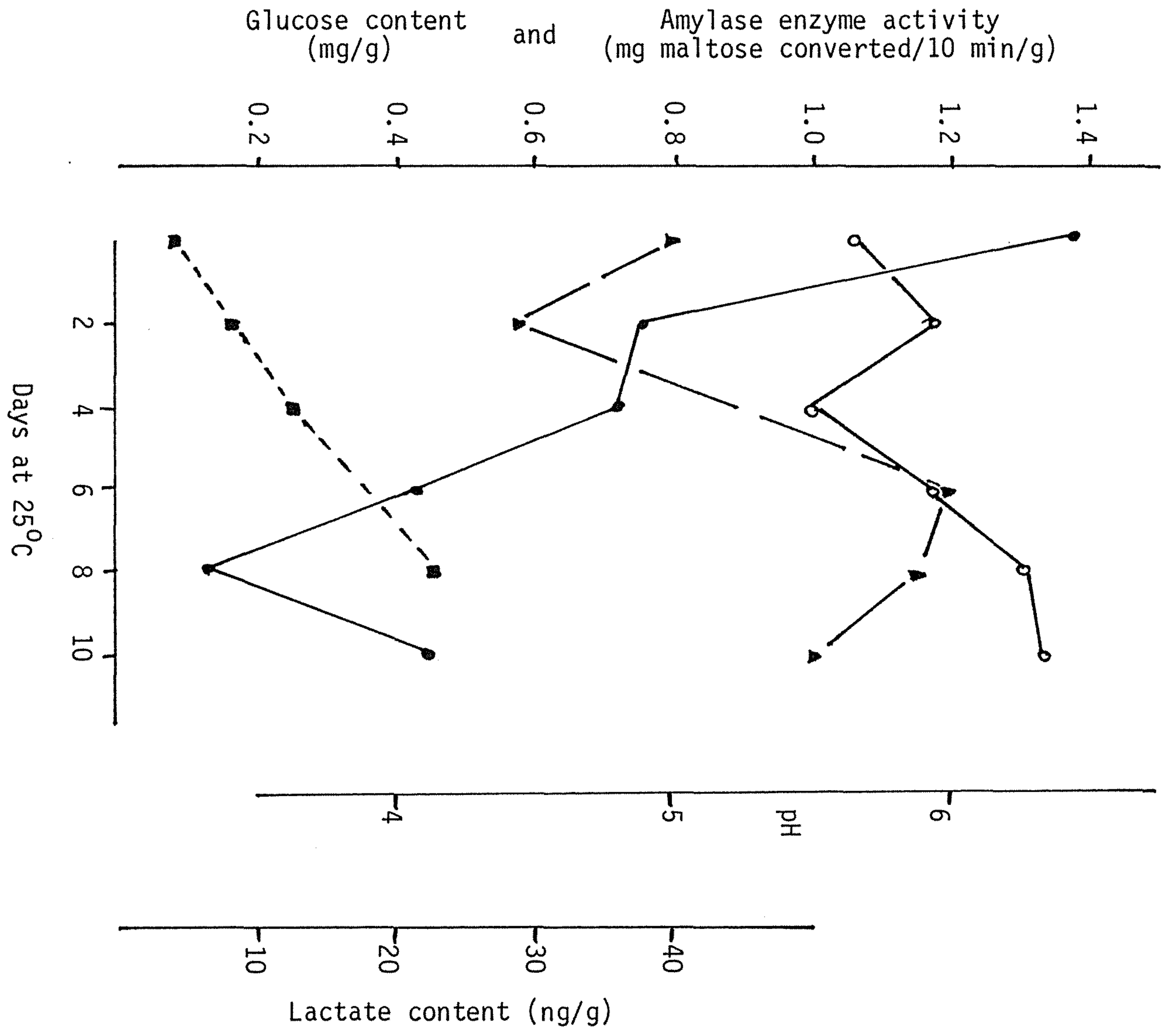

Glucose content
(mg/g)
and
Amylase enzyme activity
(mg maltose converted/10 min/g)
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Days at 25°C
2
4
6
8
10
4
5
pH
6
10
20
30
40
Lactate content (ng/g)

Figure 10

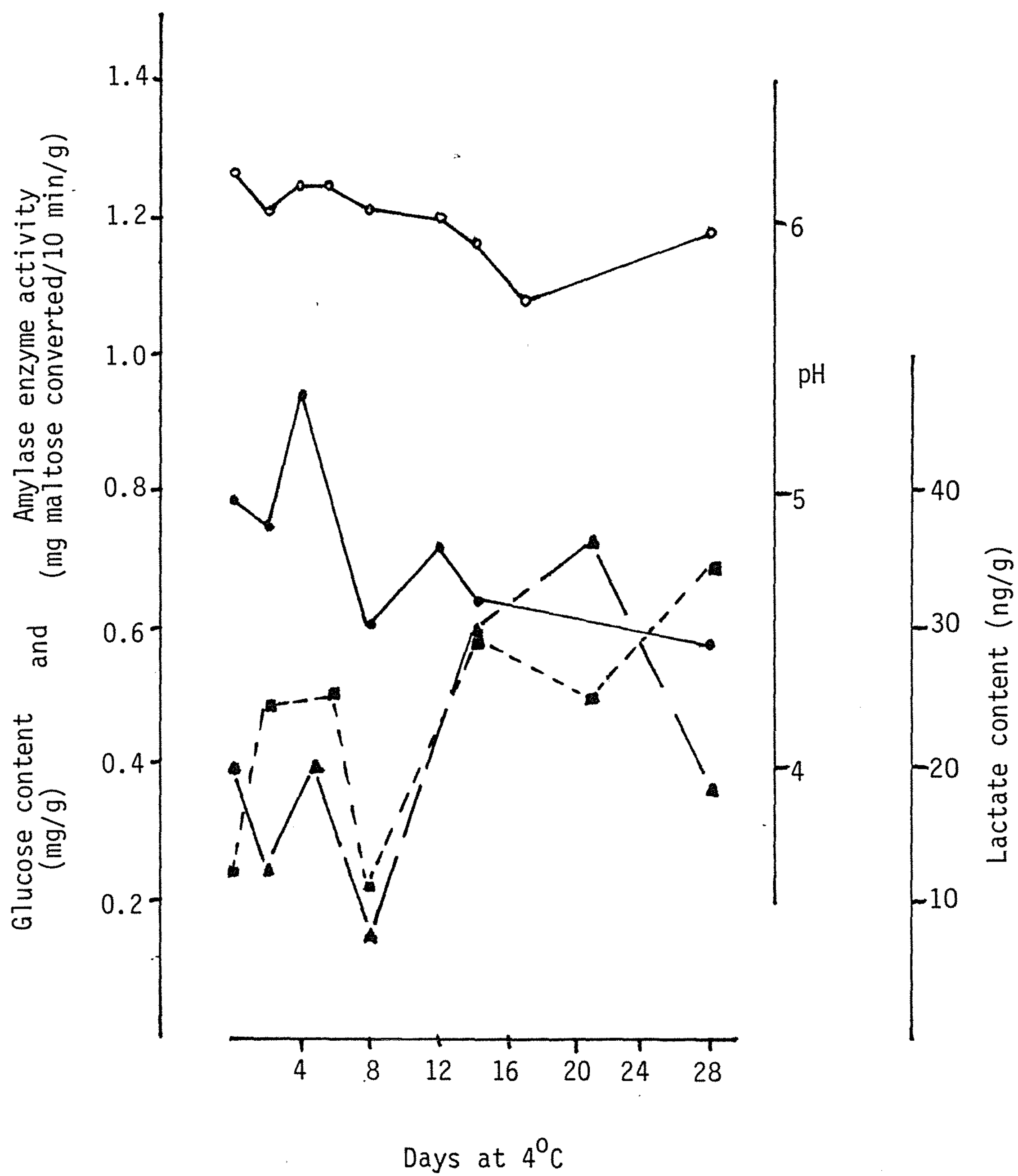
Changes in pH (○ - ○), glucose content (● - ●), lactate content (■ - ■) and amylase activity (▲ - ▲) in the pastry component of potato top pies stored at 25°C.



Glucose content
(mg/g)
and
Amylase enzyme activity
(mg maltose converted/10 min/g)
0.2
0.4
0.6
0.8
1.0
1.2
1.4
2
4
6
8
10
Days at 25°C
4
5
pH
6
10
20
30
40
Lactate content (ng/g)

Figure 11

Changes in pH (○ - ○), glucose content (● - ●), lactate content (■ - ■) and amylase activity (▲ - ▲) in the meat component of potato top pies stored at 4°C.



1.4
1.2
1.0
0.8
0.6
0.4
0.2
Amylase enzyme activity
(mg maltose converted/10 min/g)
and
Glucose content
(mg/g)
6
5
4
pH
40
30
20
10
Lactate content (ng/g)
4
8
12
16
20
24
28
Days at 4°C

Figure 12

Changes in pH (○ - ○), glucose content (● - ●), lactate content (■ - ■) and amylase activity (▲ - ▲) in the potato component of potato top pies stored at 4°C.

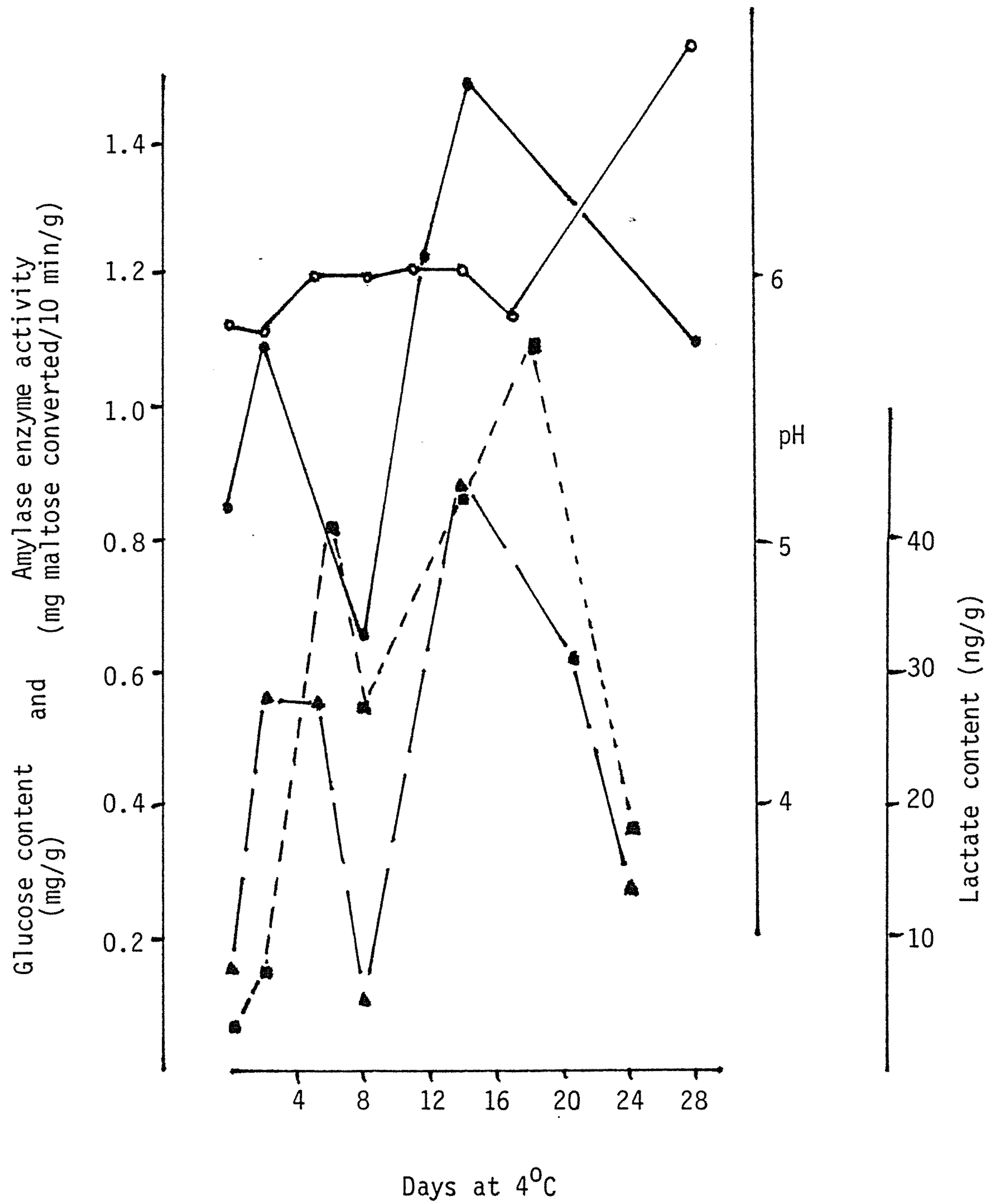
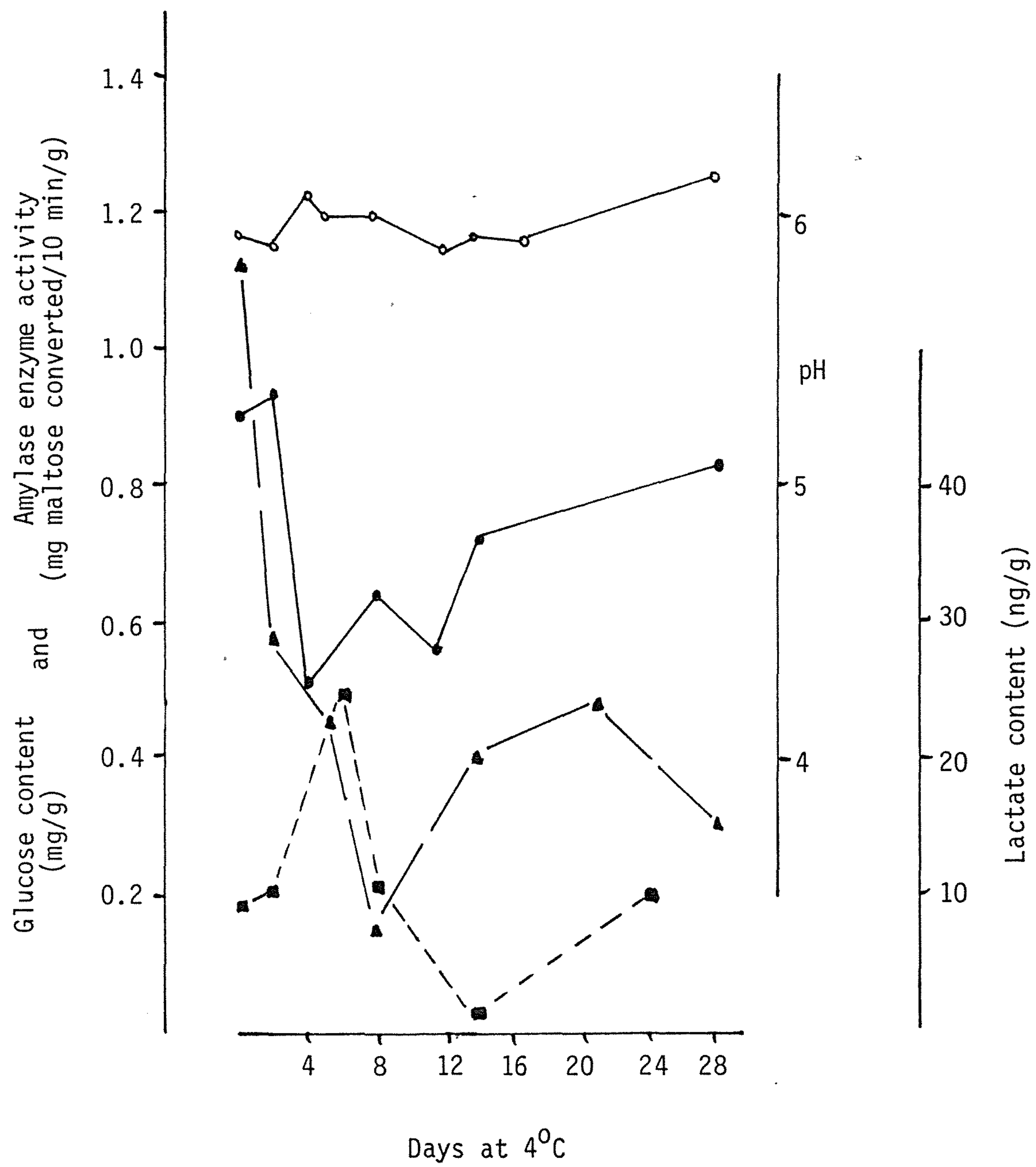

Amylase enzyme activity
(mg maltose converted/10 min/g)
and
Glucose content
(mg/g)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
pH
6
5
4
Lactate content (ng/g)
40
30
20
10
4
8
12
16
20
24
28
Days at 4°C

Figure 13

Changes in pH (○ - ○), glucose content (● - ●), lactate content (■ - ■) and amylase activity (▲ - ▲) in the pastry component of potato top pies stored at 4°C.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
Glucose content (mg/g) and Amylase enzyme activity (mg maltose converted/10 min/g)
6
pH
5
4
40
30
20
10
Lactate content (ng/g)
4
8
12
16
20
24
28
Days at 4°C

There is, however, an increase in glucose concentration in the potato component from 0.84 to 1.1 mg/g.

(iii) Amylase activity (measured in mg maltose converted per 10 min per gram substrate) in pies stored at 37°C and 25°C is large, and increases in all pie components (Figures 5-10).

In all pie components of pies stored at 37°C (Figures 5-7) the amylase activity increased over the 3 day storage period. In the meat component the activity increased from 0.2 mg maltose converted per 10 min/g meat to 1.2 mg/g. The activity in the potato and pastry components was greater, where the activity increased from 0.6 - 1.7 mg/g potato, and 1.1 - 2.2 mg/g pastry.

Activity in the pie components of pies stored at 25°C increased within 8 days of storage (Figures 8-10). However the activity in the potato and pastry components had decreased slightly by the last day of storage (day 10). The activity in the meat component had increased from 0.4 - 0.82 mg/g, and the activity in the potato and pastry components increased from 0.2 - 1.08 mg/g potato and 0.8 - 1.00 mg/g pastry.

The greatest amylase activity found in pies stored at 4°C (Figures 11-13) was in the fresh pastry component, where the activity was 1.12 mg maltose converted per gram pastry initially. This activity, however, decreased to 0.32 mg/g on the final day of storage (day 28).

Activity in the meat and pastry components varied, but the overall activity increased. In the meat portion, activity increased from 0.4 - 0.56 mg/g and the activity increased from 0.13 - 0.27 mg/g maltose converted per 10 mins/g of potato.

(iv) Lactate levels (measured as ng formed/gram substrate) measured in component parts of pies stored at 37, 25 and 4°C can also be seen in Figures 5-13. Lactate concentrations in all components of pies incubated at 37°C and 25°C steadily increased during storage (Figures 5-10).

Concentrations of lactate found in pie components of pies stored at 4°C (Figures 11-13) varied, but the overall trend was for the concentration to increase. The greatest increase in lactate concentration can be found in the meat component where the concentration increased from 12 - 34.5 ng/g. Little overall change in concentration was recorded in the pastry component over the 28 day storage period, and the lactate concentration only increased from 9 - 10 ng/g overall. A larger increase can be seen in the potato portion where the concentration increased from 3 - 18 ng/g.

4.6 Inhibition Patterns

Representative isolates of *Bacillus*, *Streptococcus*, *Staphylococcus* and *Micrococcus* were tested for production of bacteriostatic or bacteriocidal substances effective against other pie isolates. The results of these inhibition studies are presented in Tables 3-5.

Of the *Streptococcus* isolates tested, only *Str. faecium* and *Str. durans* were inhibited by other isolates. Growth of *Str. faecium* was inhibited by *B. subtilus*, *B. licheniformis*, *Staph.* type III and *Micrococcus* group 6; while *Str. durans* was inhibited only by *B. subtilus* (Table 3).

Only *Bacillus subtilus* was inhibited in its growth, by *Str. faecalis*, *Str. faecium* var *casseflavius* and *Micrococcus* group 6 (Table 4).

By comparison, the growth of all of the *Staphylococcus* and *Micrococcus* species tested was inhibited by *Streptococcus* spp. or *Bacillus* spp. (Table 5).

The growth of *Micrococcus* group 4 was inhibited by *B. subtilus*, *B. licheniformis* and *Str. equinus*.

Micrococcus group 5 was inhibited by *B. subtilus*, *B. licheniformis*, *Str. faecalis*, *Str. faecium*, *Str. faecium* var *casseflavius* and *Str. durans*.

Micrococcus group 6 was inhibited by *B. cereus* and *B. licheniformis*.

Finally, the growth of both *Staphylococcus* spp., *St. aureus* and *Staph.* type III, was inhibited by *B. subtilus* and *B. licheniformis*.

Table 3

Inhibition of Streptococcal species by *Bacillus*, *Micrococcus* and *Staphylococcus*.

Bacteria producing "inhibitive" factor / Bacteria inhibited	*B. subtilis*	*B. cereus*	*B. licheniformis*	*B. megaterium*	*Micrococcus 4*	*Micrococcus 5*	*Micrococcus 6*	*Staph. III*	*St. aureus*
Str. faecalis	-	-	-	-	-	-	-	-	-
Str. faecium	+	-	±	-	-	-	+	±	-
Str. faecium var *casseflavius*	-	-	-	-	-	-	-	-	-
Str. durans	+	-	-	-	-	-	-	-	-
Str. equinus	-	-	-	-	-	-	-	-	-

+ Inhibition

± Slight inhibition

- No inhibition

Table 4

Inhibition of *Bacillus* spp. by *Streptococcus*, *Micrococcus* and *Staphylococcus*.

Bacteria producing "inhibitive" factor / Bacteria inhibited	*Str. faecalis*	*Str. faecium*	*Str. faecium* var *casseflavius*	*Str. equinus*	*Str. durans*	*Micrococcus 4*	*Micrococcus 5*	*Micrococcus 6*	*Staph. III*	*St. aureus*
B. *subtilus*	+	-	+	-	-	-	-	+	-	-
B. *licheniformis*	-	-	-	-	-	-	-	-	-	-
B. *megaterium*	-	-	-	-	-	-	-	-	-	-
B. *cereus*	-	-	-	-	-	-	-	-	-	-

Table 5

Inhibition of *Micrococcus* spp. and *Staphylococcus* spp. by *Bacillus* and *Streptococcus*.

Bacteria producing "inhibitive" factor / Bacteria inhibited	*B. subtilus*	*B. cereus*	*B. lichenifiormis*	*B. megaterium*	*Str. faecalis*	*Str. faecium*	*Str. faecium* var *casseflavius*	*Str. durans*	*Str. equinus*
Micrococcus 4	+	-	+	-	-	-	-	-	±
Micrococcus 5	+	-	+	-	±	+	±	+	-
Micrococcus 6	-	+	+	-	-	-	-	-	-
Staph. III	+	-	+	-	-	-	-	-	-
St. aureus	+	-	+	-	-	-	-	-	-

D I S C U S S I O N

1. Introduction

The purpose of this study was to examine microbiologically induced changes which occur in Potato Top pies during storage at refrigeration and ambient temperatures. This included a study of changes in the bacterial flora of the spoiling pie, and factors affecting microbial growth. In addition, the Public Health significance of spoiled pies was examined.

2. Public Health Aspects of Potato Top Pies

The process of Potato Top pie manufacture leads to the exposure of all pie component parts to the open environment of the factory, and easy contamination of the pre-cooked pie components (meat filling, pastry surround, potato topping) can result. The pies are cooked for 15 minutes at 325°C to achieve a theoretical internal temperature of 80°C; cooled; and packed in oxygen permeable cellophane wrappings.

The predominant flora isolated; *Bacillus*, *Streptococcus*, *Staphylococcus* and *Micrococcus* spp., were all found in both pre-cooked and cooked pie components - leading to the assumption that the cooking process is insufficient and/or that post-cooking contamination by these same bacterial types occurs. Because a balance must be reached between cooking the pie adequately and manufacturing an aesthetic product, quite possibly the cooking process is not sufficient to kill all of these organisms. In particular, the cooking process does not eliminate gram positive organisms, but does practically eliminate gram negative organisms such as *Salmonella, Shigella* and *Pseudomonas*.

The gram positive organisms that were isolated have been previously cited in incidences of food poisoning. Staphylococcal food poisoning is common, and *St. aureus* in particular has been cited as the causative agent of food poisoning incidences (Archer and Kvenberg, 1985; Hobbs and Gilbert, 1978). *Bacillus cereus* has been implicated (Hobbs and Gilbert, 1978; Wyatt and Guy, 1981), and so too have *Str. faecalis* and *Str. faecium* (also known as enterococci) (Moore, 1956), although there has been some debate as to whether enterococci are capable of causing food poisoning (Deibel and Silliker, 1963). Therefore, since

Staphylococcus, *Streptococcus* and *Bacillus* spp. form the predominant flora isolated from pre-cooked; fresh; and spoiled pies, a significant potential for food poisoning exists if temperature abuse occurs.

As *Bacillus*, *Micrococcus*, *Staphylococcus* and *Streptococcus* spp. were isolated from the pre-cooked pie components, these ingredients are likely to be primary sources of contamination. The meat component contained similar numbers of *Bacillus*, *Streptococcus*, *Staphylococcus* and *Micrococcus* spp., which is not at all surprising, considering the amount of handling the meat received. *Bacillus* spp. isolated could well have come from the fillers used to make the meat filling. The ability of *Bacillus* spp. to produce heat resistant endospores leads to the speculation that any initial contamination of the fillers by these organisms could lead to the eventual contaimination of the meat used as the filling of the Potato Top pies. Contamination of this meat by *Streptococcus*, *Staphylococcus* and *Micrococcus* spp. is most probably due to contamination through handlers, because these organisms are common skin and intestinal organisms (Hobbs and Gilbert, 1978). Group D streptococci, which include those species isolated from the Potato Top pies, have been found as meat contaminants before (Stiles, 1979).

The dried potato used contained large numbers of *Bacillus* spp., a genera commonly implicated with potato (Duran *et al*, 1982).

Pastry was contaiminated with large numbers of *Staphylococcus* and *Bacillus* spp.; *Bacillus cereus* having been previously implicated in pastry products (Wyatt and Guy, 1981). Once again, this contamination is probably due to the ability of *Bacillus* to produce endospores. Presence of *Staphylococcus* spp. probably reflects the contamination from handlers, since these bacteria are not usually isolated from flour and shortening used as ingredients in the pastry (Silliker *et al*, 1980).

The presence of the *Bacillus*, *Streptococcus*, *Staphylococcus* and *Micrococcus* organisms, the first three of which have been implicated in food-borne illness incidences, indicates the possibility of danger to the public if consumption of these pies occurs under adverse conditions. High bacterial numbers (10^9/g) in conjunction with the absence of

obvious organoleptic changes, leads to a subtle danger to the Public Health.

3. Spoilage of Potato Top Pies

Pies stored at 37 and 25°C demonstrated rapid microbial growth to such an extent that by the first and third day of storage respectively, numbers of bacteria isolated had reached above 10^8/g of meat and potato. The bacterial flora at this stage consisted primarily of *Bacillus* and *Streptococcus* spp., both bacterial types of which dominated the spoilage flora of all component parts of pies stored at 25 and 37°C. *Staphylococcus* and *Micrococcus* spp. were also present, but their growth was not so rapid and only rarely reached numbers of 10^8/g. In all component parts of pies stored at 37, 25 and 4°C, the growth of *Bacillus* spp. was more rapid than the growth of the other bacterial types. This rapid growth was closely matched by the growth of *Streptococcus* spp., which, although the growth rate was slower, inevitably grew to numbers equalling *Bacillus*, especially in those pies stored at 25 and 4°C. The cooking process was quite probably sufficient to eliminate psychotrophic organisms because the dominant microflora of these pies stored at 4°C consisted of those species found in the pies spoiling at 25 and 37°C - no psychotrophic organisms grew on the pies stored at 4°C.

The only indication that spoilage was occurring in the pies was by direct estimation of the microbial load - no organoleptic changes took place in the pies stored at 25 and 37°C until the fourth and second days of storage respectively, when fungi started appearing in the potato topping. By this time, the bacterial numbers (especially of *Bacillus* spp.) had reached above 10^8/g meat and potato. Until this stage no changes in the texture of the pie were visible from the outside, but when the pies were opened a subtle change could be seen in the meat of the pie - especially at the meat-potato and meat-pastry interface, where the meat portion was becoming tacky. Presumably a polysaccharide was being produced by the bacteria present. No odours were present at this stage, or at any other time at any storage temperature. In those pies stored at 4°C, fungi would start to appear on the pie after storage for 14

days, but no spoilage odours were detected throughout the storage period, nor was there any changes in the texture of the pie. However, the pie did dry out by the end of storage (28 days).

This lack of obvious organoleptic change contrasts with the spoilage of meats under the same storage temperatures. When meat is stored under ambient conditions (25 - 40^oC) obvious changes in the meat occur, indicating spoilage is taking place. Malodorous gases such as H_2S and methanethiol can be produced, giving a distinct "off" smell (Gill, 1983), and changes in meat colour can indicate that spoilage is occurring (Ingram and Dainty, 1971).

It is interesting to note that of all the predominant species isolated, *Bacillus* spp. were the first to grow in all pies stored at 37, 25 and 4^oC, even though all genera were isolated in approximately equal numbers. This fact could be due to a variety of reasons - the pH, for example, of all component parts of the pie, is around pH 6.0, which is slightly lower than the pH found at meat surfaces (Carse and Locker, 1974). The *Bacillus* spp. growing could perhaps prefer this lower pH value. The fact that all pie component parts are composed of substrates which seem to contain large amounts of free available nutrients (glucose for example) would imply that all parts are ideal growth medias for all bacterial genera, but *Bacillus* spp. could somehow have an advantage over the bacteria in utilising these substrates. Alternatively, the *Bacillus* spp. could be producing inhibitory substances in the initial stages of growth - leading to the delay in the growth of other bacteria.

The most likely answer to this growth of *Bacillus* spp., however, is the fact that *Bacillus* spp. are well known producers of amylase enzyme (Chiang *et al*, 1979; Medda and Chandra, 1980; Nagata *et al*, 1974) which converts starch into more readily utilisable substrates such as glucose and maltose. The initial growth of the *Bacillus* spp could be due to this ability to use the starches present in the pie (as fillers for the meat, in the pastry and the potato topping) and convert them to more readily available nutrients, which other bacterial genera could then use, and thereby proliferate. If this is indeed the case, then it is likely that predominantly saccharolytic spoilage is taking place in the pies. Spoilage of this kind involves the preferential utilisation of readily

available low molecular weight compounds such as glucose and maltose as a nutrient source. This results in the production of some acidic end-products such as lactate, and consequently a pH decrease. In the pies stored at 25 and 37°C, glucose content in all component parts rapidly decreases, and results in the consequent accumulation of lactate; and hence a recorded drop in pH occurs. It seems likely, therefore, that saccharolytic spoilage is occurring in these pies, but not in those pies stored at 4°C, since no pattern of this kind can be seen.

Contrary to the situation in Potato Top pies, where glucose concentration falls, the study by Bell and Gill (1982) on luncheon meat "chubs" showed that the glucose concentration rose when chubs were stored at similar elevated temperatures. This increase in glucose correlated with the increase in numbers of *Bacillus* spp., which dominated the aerobic spoilage flora until maximum numbers of 9×10^7/g were reached on day 7. From this day on, however, the numbers of *Bacillus* declined, and so too did the glucose concentration. Accompanying these decreases was an increase in the *Streptococcus* numbers, which then dominated the spoilage flora from day 10 onwards. Lactate concentration rose over the whole storage period (28 days), and accompanying this was a decrease in pH. One can draw comparisons between the study of spoilage patterns occurring in Potato Top pies and chubs, where the predominant spoilage flora is similar, and similar changes occur in the lactate concentration and pH measurements. The difference in glucose concentrations could be explained by the fact that the numbers of bacteria isolated from pies exceeded those isolated from chubs, and hence these numerous bacteria could be utilising glucose as quickly as it is formed from starch, via α-amylase activity. In both meat products, storage at refrigeration temperatures showed little overall change in microflora and associated effects such as pH.

It is well known that the spoilage microflora of meats stored aerobically preferentially utilise glucose as a nutrient source (Gill, 1985; Gill and Newton, 1977-78) at both elevated and refrigerated temperatures (Gill, 1981; Ingram and Dainty, 1971). However, because the glucose availability is rapidly depleted when the cell density reaches $10^8/cm^3$ (Gill, 1983) substrates such as amino acids and lactic acid are then used as nutrient sources (Gill, 1976) forming malodourous end-products

which are very indicative of spoilage; such as H_2S and methyl sulphide (Gill, 1983; Gill and Newton, 1978). An associated rise in pH usually occurs as a result of NH_3 production.

There is a readily available source of glucose and other readily utilisable carbohydrates in the Potato Top pie, so there is no need for the bacteria present to use amino acids and other nitrogenous low molecular weight compounds, and hence no off odours are produced. This fact is once again borne out by the results gained from this study, especially when considering the results for those pies stored at 25 and 37°C. No overall change occurs in those pies stored at 4°C, barring the fact that fungal growth occurs and the pie dries out after 2-3 weeks of storage.

A possible explanation for the dominance of *Bacillus* and indeed *Streptococcus* over the *Staphylococcus* and *Micrococcus* spp. is the inhibition of these latter genera by the *Bacillus* and *Streptococcus*. Bactericidal substances can be produced by bacteria which are active against some other strains of the same or closely related species (Ivanovics, 1962; Nomura, 1967). These are commonly named bacteriocins. Streptococci are known to produce bacteriocins, a well known one being nisin, produced by *Streptococcus lactis* (Hurst, 1967), which is active against other closely related species, for example, *Staphylococcus* spp. *Strep. zymogenes* also produces a bacteriocin named lysin, which inhibits the growth of some strains of *Strep. faecalis*, *Str. faecium*, and *Str. liquefaciens* by lysing their cell walls (Jackson, 1971). It is also effective against other gram positive bacteria (Reeves, 1965). Reeves (1965) also noted that *Str. liquefaciens*, *Str. faecalis* and *Str. faecium* produce bacteriocins, called enterococcins, which are active against other related strains of enterococci.

The tests carried out in this study of pies demonstrated this ability of *Str. faecalis*, *Str. faecium* and *Str. faecium* var *casseflavius* to inhibit other gram positive organisms, in this case, *B. subtilus* and *Micrococcus* (type 5). *Str. equinus* could inhibit the growth of *Micrococcus* (type 4). It is not surprising to note that the streptococci could not inhibit the growth of the *Staphylococci*, because the reverse situation applies more often than not. It has been noted by several authors (Clawson and Dajani, 1970; Dajani and Wannamaker, 1969, 1973; Dajani *et al*, 1970; Tagg *et al*, 1976) that bactericidal substances from

staphylococci and *St. aureus* in particular, inhibit the growth of related bacteria, including streptococci. However, in this study of the pies, the strain of *St. aureus* isolated did not inhibit the growth of neither *Bacillus* nor *Streptococcus* spp. isolated. *St. aureus* itself was, however, inhibited in its growth by *B. subtilus* and *B. licheniformis*, these species of which have been found to produce bacteriocin-like inhibitors before (Tagg *et al*, 1976), the most extensively sutided bacteriocin of the *Bacillus* spp. being megacin produced by *B. megaterium* (Holland, 1961). Little is known about the ability of *Micrococcus* spp. to produce bacteriocins, although Su (1948) noted the production of micrococcin from one strain of *Micrococcus*, which was effective against many gram positive organisms, including *Strep. faecalis* and *Bacillus subtilus*. One strain isolated from the pies in this study, identified as *Micrococcus* 6, also demonstrated this ability, and inhibited the growth of *B. subtilus* and *Str. faecium*.

It has, therefore, been noted that many gram positive bacteria can produce substances called bacteriocins which can inhibit the growth of other, usually closely related, species of bacteria. In the tests carried out for this thesis, similar situations to those found by other authors were demonstrated, and it was noted that the *Bacillus* and *Streptococcus* spp. could inhibit the *Staphylococcus* and *Micrococcus* spp. more than the other way around.

This study has shown that the stability of Potato Top pies when stored at chill temperatures (4°C) is adequate, and the growth of micro-organisms at this temperature is such that food poisoning, due to the consumption of pies stored at this temperature, is not a great possibility. At the elevated temperatures of 25 and 37°C, however, the danger of food-borne illness due to the consumption of pies stored at these temperatures is a distinct possibility. The growth of the predominant bacteria isolated affected the surrounding medium markedly, but not in such a manner to cause obvious organoleptic changes, which would indicate that spoilage was occurring. The predominant flora of freshly cooked and spoiled pies was the same as that found in pre-cooked pie components, indicating that the bacteria were surviving the cooking process, the problem being the fact that the manufacturer must reach a balance between the aesthetics of the product and its microbiological

quality. The handling the pie components undergo during pie manufacture is thought to be one of the major sources of contamination of these pies. If subjected to adverse storage temperatures (25 and 37°C) the microbiological load increases to such an extent that consumption of these pies could quite possibly result in food poisoning, especially when one considers the fact that the bacterial types isolated from spoiling Potato Top pies have previously been implicated in food-borne illness incidences.

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APPENDICES

APPENDIX 1

Measurement of Lactate Content

Based on acetaldehyde production (S.B. Barker, 1955).

ρ-hydroxydiphenyl (colour reagent)

Dissolve 1.5 grams ρ-hydroxydiphenyl in 100 mls 0.5% NaOH to give a final concentration of 1.5%.

Add 1 ml of sample or lactate standard containing 20-100 μg lactate/ml to 1 ml 20% $CuSO_4$ (w/v) (to remove glucose and other interfering substances). Add water to make final volume of 10 mls, then add approximately 1 gram powdered $Ca(OH)_2$, cap with parafilm and shake vigorously. Allow to stand at room temperature for at least 30 minutes, then centrifuge.

Formation of Acetaldehyde

Transfer duplicate aliquots of the above supernatant fluid to a test tube (20 mm inside diameter) and add 0.05 mls 4% $CuSO_4$ (w/v). Cool the tube in an ice and water bath. Add exactly 6.0 mls concentrated H_2SO_4 as the tube contents are mixed, then place tubes in a boiling water bath for 5 minutes, remove and cool to below 20^oC.

Development of Colour

Add 0.1 mls of ρ-hydroxydiphenyl solution and distribute the resultant precipitate evenly throughout the acid. Place in a beaker of water held at 30^oC and allow to stand for at least 30 minutes. (Shake at least once during this period.)

The absorbance of the above mixture is read at 560 nm after the tubes have been held in boiling water for 90 seconds, then cooled.

Use concentrated H_2SO_4 as blank.

APPENDIX 2

Determination of Glucose by the Oxidase-Peroxidase Method

Enzyme Solution

1 capsule (containing glucose oxidase, 500 units; peroxidase, 500 units and buffer salts) per 100 mls distilled water.

Colour Solution

50 mg o-dianisidine dihydrochloride to 20 mls distilled water.

Enzyme Colour Reagent

Add 100 mls enzyme solution to 1.6 mls colour solution, and mix.

APPENDIX 3

Determination of Amylase Activity

(A. Danielsson, 1974)

Enzyme Inhibitor - 3,5 dinitrosalicylic Acid (DNS)

1 gram DNS dissolved in 20 mls (2 M) N OH. Add 50 mls distilled water, then 30 grams Rochelle Salt (Nak tartrate). Make up to 100 mls with distilled water, and keep free from CO_2.

Starch Solution

200 mg soluble starch dissolved in 10 mls Nak phosphate buffer (0.05 M, pH 6.9). Heat to 100°C, then add NaCl to give final concentration of 4 mM. Make fresh daily.

One unit of amylase is defined as the enzyme activity liberating reducing groups corresponding to 1 mole maltose/min at 37°C.

APPENDIX 4

Viable counts taken from component parts of pies stored at 37°C, 25°C and 4°C

(Refers to Figure 1)

measured as numbers of bacterial cells /g sample

Storage Temp.	Length of storage (days) \ Component	Meat	Potato	Pastry
37°C	0	10^2	3×10^3	10^2
	1	5×10^7	10^8	10^6
	2	10^8	5×10^8	5×10^6
	3	10^8	9×10^8	10^7
25°C	2	10^2	10^2	0
	4	10^7	10^8	10^7
	6	5×10^8	10^9	10^8
	8	10^8	10^9	10^7
	10	10^8	5×10^8	10^8
4°C	0	10^2	10^2	0
	2	3×10^4	10^4	4×10^3
	4	5×10^3	10^4	10^4
	8	10^4	10^4	10^4
	12	3×10^2	2×10^3	-
	14	10^3	10^3	10^2
	21	10^4	10^3	10^3
	28	5×10^3	10^3	10^3

APPENDIX 5(a)

Viable counts of different types of bacteria found in the meat component of pies stored at 37°C

(Data refers to Figure 2)

measured as numbers of bacterial cells /g sample

Bacterial type / Length of storage (days)	*Bacillus*	*Staphylococcus* and *Micrococcus*	*Streptococcus*
0	10^2	2×10^2	4.6×10^3
1	1.4×10^8	6×10^5	10^7
2	10^8	9×10^4	8.1×10^8
3	10^7	8×10^4	9×10^8

APPENDIX 5(b)

Viable counts of different types of bacteria found in the potato component of pies stored at 37°C

(Data refers to Figure 2)

measured as numbers of bacterial cells /g sample

Bacterial type / Length of storage (days)	*Bacillus*	*Staphylococcus* and *Micrococcus*	*Streptococcus*
0	5.4×10^3	10^2	1.4×10^3
1	2.3×10^9	4×10^5	5×10^6
2	1.6×10^9	10^7	4×10^8
3	1.5×10^9	10^7	5×10^8

APPENDIX 5(c)

Viable counts of different types of bacteria found in the pastry component of pies stored at 37°C

(Data refers to Figure 2)

measured as numbers of bacterial cells /g sample

Bacterial type / Length of storage (days)	*Bacillus*	*Staphylococcus* and *Micrococcus*	*Streptococcus*
0	10^2	2×10^2	0
1	10^7	5×10^5	10^5
2	5×10^7	10^5	10^5
3	5×10^7	10^5	5×10^5

APPENDIX 6(a)

Viable counts of different types of bacteria found in the meat component of pies stored at 25°C

(Data refers to Figure 3)

measured as numbers of bacterial cells /g sample

Length of storage (days) \ Bacterial type	*Bacillus*	*Staphylococcus* and *Micrococcus*	*Streptococcus*
0	1×10^{2}	0	0
2	3×10^{7}	5×10^{5}	0
4	2×10^{9}	-	2×10^{8}
6	5×10^{9}	-	6×10^{8}
8	5×10^{7}	-	10^{9}
10	2×10^{8}	10^{7}	2×10^{8}

APPENDIX 6(b)

Viable counts of different types of bacteria found in the potato component of pies stored at 25°C

(Data refers to Figure 3)

measured as numbers of bacterial cells /g sample

Bacterial type / Length of storage (days)	*Bacillus*	*Staphylococcus* and *Micrococcus*	*Streptococcus*
0	10^2	0	0
2	10^8	4×10^7	0
4	10^9	-	2×10^8
6	1.5×10^9	2×10^8	6×10^8
8	6×10^8	10^8	2×10^8
10	6×10^8	2×10^9	5×10^8

APPENDIX 6(c)

Viable counts of different types of bacteria found in the pastry component of pies stored at 25°C

(Data refers to Figure 3)

measured as numbers of bacterial cells /g sample

Length of storage (days) \ Bacterial type	*Bacillus*	*Staphylococcus* and *Micrococcus*	*Streptococcus*
0	10^{2}	0	0
2	10^{8}	10^{7}	0
4	6×10^{8}	2×10^{8}	3×10^{7}
6	4×10^{8}	4×10^{7}	-
8	3×10^{7}	5×10^{6}	-
10	3×10^{8}	2×10^{7}	2×10^{8}

APPENDIX 7(a)

Viable counts of different types of bacteria found in the meat component of pies stored at $4^{o}C$

(Data refers to Figure 4)

measured as numbers of bacterial cells /g sample

Bacterial type / Length of storage (days)	*Bacillus*	*Staphylococcus* and *Micrococcus*	*Streptococcus*
0	10^2	0	0
2	3×10^4	0	5×10^2
4	6×10^3	5×10^3	3×10^2
8	1.4×10^4	3×10^3	9×10^2
12	3×10^2	-	-
14	10^2	3×10^3	3×10^3
17	10^2	-	-
21	4×10^4	10^4	10^3
28	3×10^3	9×10^3	10^4

APPENDIX 7(b)

Viable counts of different types of bacteria found in the potato component of pies stored at 4°C

(Data refers to Figure 4)

measured as numbers of bacterial cells /g sample

Bacterial type / Length of storage (days)	*Bacillus*	*Staphylococcus* and *Micrococcus*	*Streptococcus*
0	10^2	0	0
2	7×10^4	10^2	0
4	10^4	-	10^2
8	5×10^4	7×10^3	5×10^3
12	2.2×10^3	7×10^2	10^2
14	2×10^3	8×10^3	-
17	-	-	10^5
21	3×10^3	10^5	2×10^3
28	3×10^3	1.5×10^4	3×10^4

APPENDIX 7(c)

Viable counts of different types of bacteria found in the pastry component of pies stored at 4°C

(Data refers to Figure 4)

measured as numbers of bacterial cells /g sample

Bacterial type / Length of storage (days)	*Bacillus*	*Staphylococcus* and *Micrococcus*	*Streptococcus*
0	-	0	0
2	1.4×10^5	10^2	0
4	3×10^4	4×10^2	6×10^4
8	3×10^4	10^3	7×10^2
12	-	10^3	-
14	10^2	-	-
17	10^2	10^2	10^2
21	2×10^3	-	-
28	3×10^3	2×10^4	5×10^3

APPENDIX 8(a)

Glucose content in component parts of pies stored at 37°C

(Data refers to Figures 5-7)

(Measured as mg glucose/g sample)

Length of storage (days) \ Component	Meat	Potato	Pastry
0	0.96	1.184	1.056
1	0.496	0.112	0.6
2	0.24	0.26	0.26
3	0.2	0.28	0.18

APPENDIX 8(b)

Amylase enzyme activity found in component parts of pies stored at 37°C

(Data refers to Figures 5-7)

(Measured as mg maltose converted/g sample in 10 mins)

Component / Length of storage (days)	Meat	Potato	Pastry
0	0.2	-	-
1	1.0	0.6	1.1
2	1.15	1.65	1.4
3	1.2	1.7	2.2

APPENDIX 8(c)

Lactate content in component parts of pies stored at 37°C

(Data refers to Figures 5-7)

(Measured as ng lactate formed/g sample)

Length of storage (days) \ Component	Meat	Potato	Pastry
0	11.0	4.0	17.0
1	29.7	17.5	13.0
2	20.5	25.0	16.7
3	40.3	31.0	28.0

APPENDIX 8(d)

pH of component parts of pies stored at 37°C

(Data refers to Figures 5-7)

Length of storage (days) \ Component	Meat	Potato	Pastry
0	6.45	5.95	6.15
1	5.75	6.25	6.25
2	5.0	5.7	5.7
3	4.8	5.0	5.3

APPENDIX 9(a)

Glucose content in component parts of pies stored at 25°C

(Data refers to Figures 8-10)

(Measured at mg glucose/g sample)

Length of storage (days) \ Component	Meat	Potato	Pastry
0	0.960	1.050	1.180
2	1.100	0.970	0.750
4	0.880	0.935	0.725
6	0.320	1.010	0.440
8	0.180	0.160	0.120
10	0.215	0.660	0.460

APPENDIX 9(b)

Amylase enzyme activity found in component parts of pies stored at 25°C

(Data refers to Figures 8-10)

(Measured as mg maltose converted/g sample in 10 mins)

Component / Length of storage (days)	Meat	Potato	Pastry
0	0.4	0.2	0.8
2	0.3	0.31	0.58
4	0.58	0.26	0.86
6	0.71	0.72	1.2
8	0.82	1.2	1.16
10	0.82	1.08	1.0

APPENDIX 9(c)

Lactate content in component parts of pies stored at 25°C

(Data refers to Figures 8-10)

(Measured as ng lactate formed/g sample)

Length of storage (days) \ Component	Meat	Potato	Pastry
0	12	2.5	4
2	7.5	0	8
4	34	34.8	12.5
5	32	36	-
7	41.5	38	23

APPENDIX 9(d)

pH of component parts of pies stored at 25°C

(Data refers to Figures 8-10)

Component / Length of storage (days)	Meat	Potato	Pastry
0	6.15	5.80	5.65
2	6.17	5.85	5.95
4	5.73	6.23	5.50
6	5.74	6.52	5.93
8	5.85	6.30	6.27
10	6.06	5.22	6.33

APPENDIX 10(a)

Glucose content in component parts of pies stored at $4^{o}C$

(Data refers to Figures 11-13)

(Measured as mg glucose/g sample)

Length of storage (days) \ Component	Meat	Potato	Pastry
0	0.79	0.84	0.89
2	0.75	1.1	0.935
4	0.935	0.97	0.5
8	0.6	0.64	0.64
12	0.72	1.24	0.56
14	0.64	1.5	0.72
28	0.58	1.1	0.83

APPENDIX 10(b)

Amylase enzyme activity found in component parts of pies stored at 4°C

(Data refers to Figures 11-13)

(Measured as mg maltose formed/g sample in 10 mins)

Length of storage (days) \ Component	Meat	Potato	Pastry
0	0.4	0.15	1.12
2	0.24	0.56	0.57
5	0.4	0.56	0.46
8	0.14	0.1	0.14
14	0.59	0.89	0.4
21	0.73	0.61	0.48
28	0.56	0.27	0.32

APPENDIX 10(c)

Lactate content in component parts of pies stored at 4°C

(Data refers to Figures 11-13)

(Measured as ng lactate formed/g sample)

Length of storage (days) \ Component	Meat	Potato	Pastry
0	12	2.5	9
2	24.5	7.3	10.5
5	25	41	25
8	11	17.6	10.5
14	29	55	1
21	25	-	-
28	34.6	18	10

APPENDIX 10(d)

pH of component parts of pies stored at 4°C

(Data refers to Figures 11-13)

Length of storage (days) \ Component	Meat	Potato	Pastry
0	6.18	5.81	5.93
2	6.04	5.77	5.86
4	6.13	5.92	6.08
5	6.11	6.0	5.98
8	6.05	6.0	5.99
12	6.00	6.08	5.89
14	5.92	6.03	5.94
17	5.7	5.85	5.9
28	5.95	6.88	6.14