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THE UNIVERSITY OF ALBERTA

Variability of Most Probable Number (MPN) Counts of *Escherichia coli* in Vacuum-Packaged, Fresh Meats

by

L. J. HARRIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

FOOD MICROBIOLOGY

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

FALL 1986

ISBN 0-315-32567-4

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR L. J. HARRIS

TITLE OF THESIS Variability of Most Probable Number (MPN) Counts of *Escherichia coli* in Vacuum-Packaged, Fresh Meats

DEGREE FOR WHICH THESIS WAS PRESENTED MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED FALL 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **Variability of Most Probable Number (MPN) Counts of *Escherichia coli* in Vacuum-Packaged, Fresh Meats** submitted by **L. J. HARRIS** in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE** in **FOOD MICROBIOLOGY.**

Michael E. Stiles
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Supervisor

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Date October 10, 1986

ABSTRACT

This study was done to determine the effect of refrigerated storage of vacuum packaged, fresh meat on the detection of *Escherichia coli* by standard and elevated temperature techniques. Test strains of faecal coliform bacteria were inoculated into fresh ground beef that had been irradiated to reduce the background microflora to $<10^2$ colony forming units per gram. Plate counts on selective media incubated at 35 or 45°C gave highly consistent results, however the necessity for precise control of elevated temperatures became apparent. The standard most probable number (MPN) technique (lauryl sulphate tryptose broth at 35°C followed by EC broth at 45°C) also gave reliable results. In contrast, direct inoculation into broths with incubation at 45°C gave unreliable and highly variable results. While the major factor influencing the variability of the MPN results did not appear to be related to cold temperature storage, it appeared to be strongly influenced by the lactic acid bacteria growing in the meats during storage. Although the effect of lactic acid bacteria on the *E. coli* in the meats was not conclusively demonstrated, addition of a mixed lactic acid bacteria to *E. coli* in nutrient broth had a striking effect on viability of *E. coli*. In addition, several anaerogenic variants of *E. coli* were isolated in the course of the study. The variants also had a deficient nitrate reductase system and were found to be pleiotropic chlorate resistant mutants. The

significance of these mutants to the detection of *E. coli* in meats was not determined. The use of EC and LST broths at 45°C cannot be recommended as direct (rapid) methods for identifying "faecal"-type *E. coli* or coliform bacteria in meats. In the development of modified or rapid methods for the detection of faecal coliforms in food systems, particular attention should be given to the effects of the inherent background microflora, to the selection of media and to the control of incubation temperatures.

ACKNOWLEDGMENTS

I am indebted to Dr. M. E. Stiles, supervisor and friend, for his guidance and understanding throughout the preparation of this thesis. His continuous encouragement and support has inspired the best in my abilities.

I am also thankful to the following people:

N. S. Lee for support and companionship;

M. Cerrone, Department of Food Science, for preparation of diagrams and other creative talents;

L. Steele, Department of Food Science, for preparation of tables and helpful advice in the layout of this thesis;

E. Brose, Department of Medical Microbiology and Infectious Diseases, for guidance on plasmid isolations.

This research was made possible primarily through an NSERC grant to Dr. M. E. Stiles, and a graduate assistantship supplied jointly by the Department of Food Science and the Faculty of Graduate Studies and Research, University of Alberta, Edmonton.

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1. INTRODUCTION

Indicator organisms, particularly the "coliform bacteria", are widely used to monitor the sanitary quality of heat treated foods. Even low levels of coliform bacteria in these foods can indicate inadequate processing or post-process contamination, either of which could affect the keeping quality or safety of the product. However, the ubiquitous nature of the coliform group of bacteria makes them unreliable indicators of "faecal" contamination and of potential hazard in unprocessed foods, such as raw milk, vegetables and meats. Therefore, in these foods, *E. coli* is still considered to be the indicator of choice because of its specificity to the intestinal environment (Mossel, 1982; Shaw and Roberts, 1982). Attempts to develop standards for *E. coli* in raw foods have usually caused controversy, especially regarding standards for meats (Carl, 1975; Winslow, 1975; Goepfert, 1976). However, in quality control programs, high levels of *E. coli* in raw meat can indicate unsanitary handling, processing and storage, and can be useful for monitoring production lines.

The standard tests for coliform bacteria and *E. coli* were originally developed at the turn of the century for testing of water supplies. In the 1920's these tests were adopted for the monitoring of foods, primarily milk and dairy products (Mossel, 1982). Most tests are based upon fermentation of lactose with production of acid (plating media) or acid and gas (broth media). The most probable

number technique (MPN) was originally adopted because of its simplicity and usefulness in detecting low levels of *E. coli* in water and heat processed foods. However, positive identification of *E. coli* in association with the MPN technique requires a protocol that takes as many as 10 days to complete.

In recent years the focus of studies on the enumeration of *E. coli* has been the modification of testing procedures to make them less time consuming. The most popular of these modifications has been the use of elevated incubation temperatures. These tests are based on Eijkman's observation in 1904 that cultures of *E. coli* could ferment glucose at 46°C, while other coliform bacteria could not. Gas and acid production from lactose at 44.0 to 45.5°C, as well as production of indole from tryptophan at 44.0°C, are the most widely accepted criteria in use today.

Although the MPN technique can be useful for determining low levels of contamination of foods and water, many problems have been associated with the test. On the one hand, a false positive test (gas production in the absence of coliform bacteria or *E. coli*) can arise from the growth of non-coliform or non-faecal coliform bacteria in standard or elevated temperature tests, respectively. This could lead to the rejection of an acceptable product. On the other hand, false negative tests (presence of coliform bacteria in the absence of gas production) can arise for a variety of reasons. It is well documented that some strains of *E. coli*,

are either lactose negative, late (slow) lactose fermenters, totally anaerogenic or anaerogenic at elevated temperatures (Edwards and Ewing, 1972; Dufour, 1977). High levels of adventitious bacteria (a situation not uncommon in food) can interfere with growth and gas production of coliform bacteria in MPN tests (Goepfert, 1976; Evans *et al.*, 1981b; Hussong *et al.*, 1981).

It has also been shown that with different types and levels of stress *E. coli* can either totally lose its ability to ferment lactose (Kasweck and Fliermans, 1978) or temporarily or permanently lose its ability to produce gas from lactose at elevated temperatures (Bueschkens and Stiles, 1984; R. Nedd, M. Sc. thesis, University of Alberta, 1984). Food processes or storage conditions such as mild heat treatment or frozen or refrigerated storage can create stressful environments which are injurious, but not lethal, to *E. coli* (Hurst, 1977). Injured coliform bacteria are more sensitive to the bile salts and elevated temperatures used in tests to select them (Ray, 1979). All of these factors can lead to an under-estimation of the coliform population of a food and can result in the acceptance of a potentially hazardous product.

Current trends in meat handling involve the centralized cutting of meats and vacuum packaging for supply to the retail and hotel-restaurant (HRI) trades. Vacuum packaging has been shown to be an effective way of extending the shelf life of fresh meat during refrigerated storage by

suppressing the growth of aerobic spoilage organisms, such as *Pseudomonas* spp. (Roth and Clark, 1972). In combination with storage at 0 - 4°C, the shelf life of fresh meat can be extended from a few days to many weeks. The effects of refrigeration on the viability of bacteria and indicator organisms in foods has not been fully studied (Mackey, 1984). It is not clear whether prolonged cold storage of meats can cause sufficient injury of the bacterial cells to affect the detection of indigenous coliform bacteria in standard testing procedures.

The objective of this study was to determine the effect of refrigerated storage of vacuum packaged fresh meat on the detection of typical coliform bacteria by elevated temperature incubation, with particular emphasis on the reliability of the MPN procedure.

2. REVIEW OF THE LITERATURE

This review focuses on standard tests used to enumerate the coliform group of indicator organisms, with special emphasis on the MPN testing procedure. Particular attention has been given to the causes of false negative MPN results and to the specific cellular reactions involved in the fermentation of lactose to acid and gas.

2.1 Historical Development of "Coliform Bacteria" as Indicator Organisms and Tests for Their Detection

The concept of indicator organisms was introduced independently by Schardinger in Austria in 1892 and by Smith in the United States in 1895 as a means of assessing the sanitary quality of water (Mossel, 1982). At that time, typhoid and paratyphoid fevers, dysentery and cholera were transmitted by drinking water contaminated with raw sewage. These diseases were either spread by infected persons or by asymptomatic carriers of the disease-causing organisms. The principal aim of using indicator organisms was to avoid testing for individual pathogens for which test methods were either too complex or unavailable. The absence of an indicator organism in a water sample was assumed to mean the absence of faecal pollution, whereas its presence was an indication of recent faecal pollution, and the possible presence of intestinal pathogens.

Initially, "*Bacterium coli commune*" (*Escherichia coli*) was chosen as the indicator organism. It was first described

in 1885 by Escherich, who observed large numbers of a "non-typhi" organism in the course of investigations on the faeces of children (Bardsley, 1925). The etiological agent of typhoid fever (*Salmonella typhi*) was discovered at about the same time. For this reason, many of the early studies on these organisms centered around methods for their differentiation (Ewing, Davis and Martin, 1972). The observation that *E. coli* could ferment lactose with the production of acid, while *S. typhi* could not, resulted in this phenotype becoming the major distinguishing characteristic for *S. typhi* and *E. coli*/coliform bacteria.

"*Bacterium coli*" was thus defined as "all gram negative non-spore-forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media" (APHA, 1925). It was found that this definition included other distinct bacterial species, such as "*Bacterium aerogenes*" (now divided between *Enterobacter aerogenes* and *Klebsiella pneumoniae*), so it was renamed the "coli-aerogenes" group. Today these organisms are known as the "coliform" bacteria and, in addition to *E. coli*, they include members of the genera *Citrobacter*, *Enterobacter*, and *Klebsiella* (ICMSF, 1978).

Of the coliform group of bacteria, only *E. coli* is specific to the intestinal environment. The others are found in faeces but they also constitute part of the natural flora of many extraenteral environments, such as soil, vegetation and water (Griffin and Stuart, 1940; Krieg, 1984b). In many

cases, therefore, "coliform bacteria" alone are of little value in assessing "faecal" contamination. When coliform bacteria and *E. coli* were adopted as indicator organisms in foods, problems arose in interpretation of results because, unlike water where *E. coli* dies rapidly, coliform bacteria can multiply in many foods under appropriate conditions. Therefore, large numbers of these organisms in a food do not necessarily indicate either recent or direct faecal contamination.

The MPN method was originally chosen over plating methods because of its sensitivity for enumeration of low numbers of coliform bacteria in water and foods. Isolation of coliform bacteria from natural sources by this method is complicated by the fact that other organisms, alone or synergistically, can produce gas in standard lactose broth. Thus they can give rise to positive tests in the absence of coliform bacteria (Meyer, 1918; Sears and Putnam, 1923). Hussong *et al.* (1981) concluded, however, "that no single specific bacterial group can be identified as being responsible for the false-positive reaction in the presumptive coliform test". The addition of dyes, such as brilliant green, and surface active agents, such as lauryl sulphate, to a confirmatory broth reduced the number of false positive tests due to gram positive bacteria, by inhibiting their growth. Addition of bile salts favoured the growth of the coliform bacteria.

When testing requires the positive identification of *E. coli*, colonies isolated from the confirmatory MPN broth must be subjected to the so-called IMViC tests (indole, methyl red, Voges-Proskauer and citrate) (Parr, 1936, 1938). *E. coli* biotype I was identified by a ++-- reaction. Thus the time required to analyze a sample for *E. coli* increased from 2 days to as many as 10 days.

In the 1930's and 1940's attempts were made to increase the specificity of the testing procedures and thus reduce the time required for analysis. Most of these modifications were based on Eijkman's observation in 1904, that cultures of *E. coli* ferment glucose with acid and gas production at 46°C, while other coliforms could not. This observation was largely ignored until the late 1920's when it was reconsidered as a tool for differentiating faecal *E. coli* from other coliform bacteria. Batty-Smith (1942) reviewed the literature on the subject and concluded that, although early opinions about the validity of the test varied, the Eijkman test could be used successfully to reduce the number of false positive results due to non-*E. coli* coliform bacteria in the MPN test.

The recommended method involves inoculation into MacConkey broth tubes with incubation at 37°C for 48 h, followed by subculture of positive tubes into MacConkey broth at 44°C. Gas positive tubes at the elevated temperature were considered indicative of "faecal *coli*". A temperature of 44°C <u>in the medium</u> was stressed. To achieve

this, circulating water-baths with accurately controlled temperature regulators were used. It was suggested that non-standardization of incubator temperatures was the most likely explanation for the conflicting information reported in the early literature.

Perry and Hajna (1944) increased the sensitivity of Eijkman's broth by substituting lactose for glucose and adding buffer and bile salts. They named the new medium "*Escherichia coli*" (EC) broth, and an incubation temperature of 45.5°C was recommended. In a further attempt to increase the specificity of the tests, it was proposed that the ability of organisms to produce indole from tryptophan in broth at 44.0°C should be added to the testing criteria (MacKenzie, Taylor and Gilbert, 1948). It was suggested that this would eliminate all faecal coliform bacteria other than *E. coli* biotype I. Reports continue to indicate that the specificity of elevated temperature tests for *E. coli* is very good (Geldrich *et al.*, 1958; Burman, 1961; Tennant and Reid, 1961; Geldreich, Kenner and Kabler, 1964; Geldreich and Bordner, 1971; Stiles and Ng, 1981a; Weiss *et al.*, 1983). In most of these studies greater than 90% of strains of *E. coli* produce gas at elevated temperatures while the majority of non-faecal coliform bacteria do not. However, in recent years, there have been increasing numbers of reports of non-faecal coliform bacteria, particularly *K. pneumoniae*, *Citrobacter freundii* and *Enterobacter* spp., causing false positive MPN tests (Mishra, Joshi and Panicker, 1968;

Hendricks, 1970; Splittstoesser *et al.*, 1980; Caplanas and Kanarek, 1984).

Depending upon the intended purpose, *Enterobacteriaceae*, coliforms, faecal coliforms and *E. coli* have all been used or proposed as indicators of faecal pollution. Two most probable number (MPN) methods for the enumeration of coliforms, faecal coliforms and *E. coli* are recommended by ICMSF (1978). In Europe, MacConkey broth at 35 to 37°C is used for enumeration of coliforms, while brilliant green lactose bile broth as well as tryptone broth are used at 44°C for the detection of *E. coli* biotype I. In the United States, presumptive coliforms are enumerated in lauryl sulphate tryptose broth at 35 to 37°C followed by inoculation into EC broth at 44.5°C. The Canadian Health Protection Branch recommends the use of 45.0°C as the incubation temperature, which is a compromise between greater sensitivity (44.5°C) and greater selectivity (45.5°C) (Weiss *et al.*, 1983).

In hopes of reducing the test procedure to 48 h or less researchers have suggested the direct incubation of MPN tubes at elevated temperatures for testing food and water (Perry and Hajna, 1944; Mossel, 1962; Fishbein *et al.*, 1967; Kamplemacher, Leussink and Van Noorle Jansen, 1976; Warren, Benoit and Jesse, 1978). These authors show good correlation of results between direct inoculation into tubes incubated at 37°C and at 44 to 45.5°C. They argue that in addition to saving time and media the number of false positive tubes is

reduced. However, these tests have not been accepted as standard methods. In attempts to decrease testing time for *E. coli* in meats, Ng and Stiles (1978) found that direct inoculation into EC broth incubated at 45.0°C could not be used as a reliable alternative to the standard MPN procedure. Therefore, modifications of MPN and of plating procedures at elevated temperatures continue to be developed in attempts to detect and confirm *E. coli* in rapid, one-step techniques (Anderson and Baird-Parker, 1975; Klein and Fung, 1976; Andrews *et al.*, 1979; and Feng and Hartman, 1982).

2.2 Influence of Phenotype on Reliability of MPN Tests

The standard MPN procedure for determination of *E. coli* requires the ability to ferment lactose with production of gas at 37°C, as well as 44-45.5°C. Several variants of *E. coli* have been reported that have temporary or permanent alterations in one or both of these phenotypes. Such variants can lead to false negative MPN results and can have a significant effect on the results.

2.2.1 Lactose Negative *E. coli*

The existence of lactose negative or "slow lactose" fermenting strains of *E. coli* has been well documented (Hershey and Bronfenbrenner, 1936; Ziegler, 1939; Stuart, Mickle and Borman, 1940). Strains were isolated from a variety of sources and it was postulated that factors such as chlorination, storage or presence of antagonistic

organisms could account for slow or weak lactose fermenters (Stuart *et al.*, 1940). It is also well documented that some strains of *E. coli* lose their ability to ferment lactose during normal laboratory storage (Kriebel, 1936; Stuart *et al.*, 1940). These cultures can often regain the ability to ferment lactose rapidly upon repeated subculture in broth containing lactose (Kriebel, 1936).

Ewing *et al.* (1972) conducted one of the first large scale studies of the biochemical reactions of *E. coli*. They characterized 1,887 strains that had been isolated from various environments, including stool specimens, water and environmental sources. Most of the strains (91 to 92%) were active lactose fermenters, however, some were delayed lactose fermenters (4 to 5%) and others were unable to ferment lactose under any condition. Increasing the concentration of lactose from 1 to 10% in the growth medium can sometimes reduce the time required for late fermentation to appear (Chilton and Fulton, 1946). The existence of lactose negative strains of *E. coli* may in part be due to environmental stress. Kasweck and Fliermans (1978) reported the emergence of lactose negative variants from a lactose positive population when the latter was placed in modified diffusion chambers and submerged in a lake receiving reactor effluent waters.

2.2.2 Anaerogenic (Gas Negative) *E. coli*

Ewing *et al.* (1972) reported that 8% of *E. coli* strains that they tested were unable to produce gas from glucose. However, they did not include the Alkalescens Dispar (A-D) group in these calculations. The A-D group is now considered to be an anaerogenic, lactose negative and non-motile biogroup of *E. coli* (Krieg, 1984b). In early studies it was reported that the appearance of anaerogenic variants from gas producing strains upon laboratory storage (Stuart *et al.*, 1940; Parr, 1936). Some of these strains could revert to the gas positive phenotype upon subculture (Ordal and Halvorson, 1939).

The effect of temperature on gas production is more pronounced than on lactose utilization. Some strains of *E. coli* which produce gas at 35°C are unable to do so at elevated temperatures, so that they would be eliminated in the "faecal" coliform test. Injury due to stressful environments can result in temporary or permanent loss in gas producing ability. During cold storage of *E. coli*, Bueschkens and Stiles (1984) isolated variants which had permanently lost their ability to produce gas at 44.5°C. Variants were isolated with greater frequency from media of poor nutrient composition or lacking cryoprotective agents. Daubner (1975) isolated variants that were unable to produce gas at temperatures above 43°C after storage in water.

2.2.3 Pathogenic Anaerogenic Strains of *E. coli*

It is now known that some strains of *E. coli* are human pathogens that cause foodborne gastroenteritis. *E. coli* has also been identified as a major cause of traveller's diarrhea that is associated with food consumed in developing countries (Kornacki and Marth, 1982). As a result the detection of coliform bacteria in foods has assumed even greater importance.

In 1971, a widespread outbreak of gastroenteritis in the United States was traced to the consumption of imported, soft cheeses contaminated with *E. coli* (Marier *et al.*, 1973). The predominant strain of *E. coli* isolated from this outbreak was anaerogenic, failing to produce gas in lactose broth within 48 h. Some isolates failed to grow at 45.5°C. This was the first confirmed outbreak of foodborne illness caused by enteropathogenic *E. coli* in the United States. Since this strain was anaerogenic, standard testing procedures failed to detect it. The outbreak generated substantial interest in the response of pathogenic *E. coli* in standard isolation procedures.

Hill *et al.* (1985) reported that 30% of human isolates of *E. coli* failed to survive the MPN procedure, even when added in large numbers and without competition. In a study of 50 pathogenic and 50 non-pathogenic strains of *E. coli*, Mehlman and Romero (1982) reported that the percentage of pathogens and non-pathogens fermenting lactose with gas production at 35 and 44°C was 78 and 90%, respectively. A

greater proportion of pathogenic strains was anaerogenic. At 35°C all strains of pathogenic and non-pathogenic *E. coli* grew in the three-tube MPN technique. Corresponding values for tests incubated at 44°C were 75 and 87%, and at 45.5°C were 31 and 60%, respectively.

Several modifications to procedures for detection of enteropathogenic strains have been suggested (Mehlman *et al.*, 1974; Fantasia *et al.*, 1975). Most of them include at least a partial substitution of glucose for lactose and a reduction in elevated incubation temperature to 41.5°C. It is interesting to note that the genus *Shigella* comprises highly pathogenic strains which are typically anaerogenic. They are considered by some workers as metabolically inactive biogroups of *E. coli* (Krieg, 1984b).

2.3 Influence of Competitive Microflora on Reliability of MPN Tests

The influence of competitive microflora on survival of foodborne pathogens, indicator organisms and spoilage microorganisms has been well documented and was reviewed by Hurst (1973). Various studies have implicated specific non-coliform genera as being "antagonistic" towards coliform bacteria, especially *E. coli*. These include species from the genera *Sarcina*, *Micrococcus*, *Flavobacterium*, *Pseudomonas* and *Acinetobacter* as well as some actinomycetes (D. Hutchinson, R. H. Weaver, and M. Scherage, Abstr. 44th Gen. Meet. Soc. Am. Bacteriol. 1943, G34, p.29; Wolford, 1954; Reitler and

Seligmann, 1957).

Some strains of the genera *Pseudomonas* and *Acinetobacter* isolated from water, could either reduce or injure a population of *E. coli* when they are grown together. There was a significant correlation between initial non-coliform level and rate of coliform decline (LeChevallier and McFeters, 1985). When high levels of competitive flora and coliform bacteria are present silmultaneously in the MPN broth, growth or gas production of *E. coli* and other coliform organisms can be suppressed as a result of nutrient competition or the production of bacteriocin-like substances (Wolford, 1954; Levine *et al.*, 1955; Evans *et al.*, 1981b; Means and Olson, 1981).

Suspensions of various antagonistic organisms added to lactose broth simultaneously with serial dilutions of an *E. coli* suspension resulted in reduced MPN values ranging from 28 to 97% depending upon the antagonistic organism (Hutchinson *et al.*, Abstr. 44th Gen. Meet. Soc. Am Bacteriol. 1943, G34, p.29). Large numbers of non-*E. coli* coliform bacteria in MacConkey broth can result in inhibition of growth of *E. coli* both at 37 and 42°C (Etinger-Tulczynska, 1958). Similar results were reported by Levine *et al.* (1955) in presumptive tests using lactose broth. Both situations result in a lower estimation of *E. coli* by the MPN technique.

2.4 Influence of Bacterial Injury on Reliability of MPN Tests

Reversible bacterial injury can cause increased sensitivity of cells to selective agents in growth media (Busta and Jezeski, 1963; Stiles and Witter, 1965). Since the late 1950's there has been ongoing study of the effect of injury on the detection of many bacteria. Extensive reviews of the topic have been published (Hurst, 1977; Speck and Ray, 1977; Mackey, 1984; Mossel and Van Netten, 1984). Specific aspects of the practical implications of injured microorganisms in foods were considered in the reviews cited above, and by Busta (1976), Read (1979) and more recently by Ray (1986).

Most food processes designed to eliminate or retard bacterial growth have deleterious effects on bacteria that result in injury to the cells. Apart from heat, disinfection and irradiation which are intended to kill microorganisms, other food processing and handling treatments of foods such as refrigerated and frozen storage, drying and freeze drying and the addition of preservatives and acidulants, that are not intended to kill, can cause cellular injury. The primary focus in this study is the injury of enteric indicator bacteria caused by refrigerated storage.

In a review of the lethal and sublethal effects of refrigeration on microorganisms Mackey (1984) cites Sherman and Albus (1923) as the first to describe the phenomenon of "cold shock" in *E. coli*. The phenomenon involves loss of

viability both immediately and during subsequent holding at the cold temperature. Several factors affect injury of cells which undergo cold shock. The most important factor is rapid cooling through the temperature zone in which the membrane lipids undergo phase transition from liquid crystalline to gel states. Slow cooling allows an ordered separation of lipids and proteins in the cytoplasmic membrane, whereas rapid cooling causes lipids and proteins to become fixed in a random way. This results in a leaky cellular membrane. Damage to DNA and ribosomes may also occur which can affect the production of enzymes required for growth and maintenance of the cells (Mossel and Van Netten, 1984). Some workers have suggested that chromosomal damage could decrease the genetic stability of the cells (Busta, 1976).

Cells which do not show immediate effects of chilling, for example, stationary phase or slowly cooled log phase cells, may die during prolonged storage below their minimum growth temperature. This phenomenon is pronounced in water and dilute solutions (Zaske, Dockins and McFeters, 1980). In vacuum packaged meats stored at 1.7°C a slow decrease in viability of pathogenic bacteria was observed over 28 days of storage, the most rapid decrease in viability occurred during the first week of storage (Kennedy, Oblinger and West, 1980).

Cold temperature stress of *E. coli* and its effect on enumeration has been studied extensively for its implications in water microbiology (Bissonette *et al.*, 1975;

Daubner, 1975; McFeters, Cameron and LeChevallier, 1982; Rhodes, Anderson and Kator, 1983). Other workers have studied the effects of typical food processing treatments on *E. coli* such as heat (Roth, Stiles and Clegg, 1973) freezing (Ray and Speck, 1973) exposure to chlorine and sodium chloride (Maxcy, 1970) as well as the effects of cold temperature storage (Jackson, 1974; Patterson and Jackson, 1979), The principal manifestation of injury in *E. coli* cells is increased sensitivity of stressed cells to selective agents, such as bile salts (sodium desoxycholate) (McFeters *et al.*, 1982). Injured organisms are also more fastidious in their nutritional requirements (Straka and Stokes, 1959; Postgate and Hunter, 1963), more sensitive to a variety of selective agents other than bile salts (Mossel and Van Netten, 1984) and more sensitive to incubation temperatures above 42°C (Allen, Pasley and Pierce, 1952; Rowley *et al.*, 1979).

The effects of all types of stress are most pronounced for cells in the exponential growth phase (Sherman and Albus, 1923; Meynell, 1958; Patterson and Jackson, 1979; Mackey, Derrick and Thomas, 1980) but this can vary between organisms and with treatment (Mackey, 1984). Gram negative bacteria are more sensitive to environmental stress than gram positive bacteria. In general, injured bacteria are unable to grow under many conditions that allow the growth of uninjured cells. This is particularly significant for the monitoring of the sanitary quality of food products, and it

can result in the incorrect assessment of food safety, by under-estimating population levels of indicator or pathogenic microorganisms.

Modifications to enumeration techniques have been proposed that should enable injured bacteria to be detected in foods and other environments (Speck and Ray, 1977; Hartman, 1979; Andrews, 1986). Successful resuscitation of injured cells has been achieved by "liquid-repair" or "solid-repair" techniques (Ray, 1979). "Liquid-repair" usually involves inoculation into a non-selective nutrient-rich medium and incubation at 25 to 35°C for varying lengths of time before enumeration by selective plating or MPN techniques (Ray and Speck, 1973; Warseck, Ray and Speck, 1973; Ray, 1979). This can cause erroneous results due to multiplication of uninjured or repaired cells (Mackey *et al.*, 1980). Problems caused by cell multiplication can be avoided in the MPN technique by using a non-selective MPN broth, followed by the addition of selective agents after a suitable period of incubation for repair of injured cells (Lanz and Hartman, 1976).

"Solid-repair" involves inoculation onto a non-selective solid medium and incubation at 25 to 35°C for varying lengths of time before overlayering with selective media (Hartman, Hartman and Lanz, 1975; Speck, Ray and Read, 1975; Ray and Speck, 1973) or before transfer to a selective medium on membrane filters (Holbrook and Anderson, 1982). This type of resuscitation is preferred because the problems

associated with cell multiplication are avoided. Since injured populations are usually heterogenous, they may require 1 to 6 hours to recover from sublethal injury (Ray, 1979; Mackey *et al.*, 1980). Most recuscitation procedures involve a 1 to 2 hour recovery period which is usually sufficient for most cells to resuscitate. It is not known whether injury caused by refrigerated storage of meats is sufficient to warrant a resuscitation procedure (Mackey, 1984).

Although most injured populations require a nutritionally complex medium in order to grow, several reports have indicated that some cold-stressed cells recover better on minimal media (Tang and Jackson, 1979; Mackey and Derrick, 1986). Mackey and Derrick (1986) showed that this phenomenon was due to increased cell sensitivity to very low (micromolar) levels of hydrogen peroxide found naturally in autoclaved complex media but not in minimal media. The significance of this observation for resuscitation procedures for injured cells in foods is not known.

Studies on pure cultures of *E. coli* that have not undergone sublethal stress indicate that the addition of more than one selective agent can have a striking effect on the organism's ability to grow and produce gas (Meadows, Anderson and Patel, 1980). Skinner and Njoko-Obi (1958) illustrated that the effect of either boric acid or elevated temperature (43°C) alone did not have a significant effect on the growth of *E. coli*. However the combination of boric

acid and elevated temperature was inhibitory.

Injured (or stressed) pathogenic and indicator organisms have significant implications in food microbiology. Injured cells are capable of recovering their full viability, and organisms which can cause foodborne illness are able to repair and regain virulence and toxin production when returned to a suitable growth environment (Collins-Thompson, Hurst and Kruse, 1973; Mossel and Van Netten, 1984). Some foods constitute an excellent medium for repair of injured cells given the proper growth temperatures. It is considered that standard techniques for detection of pathogens and indicator organisms in foods should include a resuscitation step so that they detect both injured and uninjured cells (Read, 1979).

2.5 Influence of Plasmids on Reliability of MPN Tests

Plasmids are extrachromosomal DNA elements that are capable of replicating autonomously. They can directly affect the phenotype of the host bacterium by carrying genes mediating traits such as antibiotic resistance, bacteriocin production, virulence factors and various catabolic functions. Many plasmids also carry genes that encode for their transmissibility to other strains and species (Lin, Goldstein and Syvanen, 1984). The transfer of genetic material between strains of *E. coli* and other *Enterobacteriaceae* has been significant in the spread of antibiotic resistant strains in the environment, especially with the

use of subtherapeutic antibiotic feeding of animals (Novick, 1981).

'Atypical' characteristics demonstrated in some *Enterobacteriaceae* can be linked to the the presence of a plasmid. Antibiotic resistant strains are more likely to demonstrate these atypical phenotypes (Wolin, Bevis and Thomas, 1962). Hill *et al.* (1985) reported that *E. coli* isolates from ground meats are more likely to be resistant to antibiotics than isolates from other foods. Uncharacteristic strains of *E. coli* have been described which produce hydrogen sulfide (Layne *et al.*, 1971), produce urease (Wachsmuth, Davis and Allen, 1979) or utilize citrate as a sole carbon source (Wolin *et al.*, 1962). Non-coliform *Enterobacteriaceae* such as *Salmonella* spp. and *Proteus* spp. have been isolated which carry lac^+ plasmids, enabling them to ferment lactose (LeClerc *et al.*, 1977; Krieg, 1984b). Plasmids which alter the phenotype of an organism make it difficult to interpret data and can lead to both false positive or false negative results.

Plasmids may also play a significant role in the detection of pathogenic strains of *E. coli*. Several different forms of enteropathogenic *E. coli* have been reported, most of which have plasmid encoded virulence factors involved in their pathogenesis (Levine and Edelman, 1984). In general, enteropathogenic strains carry a greater plasmid complement than non-pathogenic strains (Mehlman and Romero, 1982). This can affect the detection of a pathogenic

strain because cells with greater amounts of total plasmid DNA may have longer generation times (Nordström *et al.*, 1977; Zünd and Lebek, 1980; Hill *et al.*, 1985). Therefore, during enrichment in broth culture, pathogens would be overgrown by non-pathogenic strains.

Hill and Carlisle (1981) demonstrated that detergents such as lauryl sulphate and the high temperatures used in the standard MPN procedure (LST broth at 37°C followed by EC broth at 44.5°C) could result in the loss of plasmids. Hill *et al.* (1985) showed that, after the MPN procedure, plasmid losses from human isolates occurred in 20 to 95% of the population, depending upon the strain tested. These authors estimated that for a 90 to 95% probability of recovering a pathogenic strain of *E. coli* during routine testing, 40 to 50 colonies should be characterized per food sample. A procedure in which plasmids are lost decreases the probability of recovering pathogenic strains. Although specific levels of enteropathogenic strains in food are not normally determined, Sack *et al.* (1977) observed that enterotoxigenic strains may make up 8% of the total strains of *E. coli* isolated from food.

2.6 Plasmid Profiles as a Tool in Strain Identification

Wild-type bacteria carry plasmids which differ in size and number. The component plasmids of a bacterial strain can be demonstrated using agarose gel electrophoresis (Meyers *et al.*, 1976). This can provide a fairly reliable estimate of

plasmid molecular weight (Meyers *et al.*, 1976; Rochelle *et al.*, 1985). Therefore, it is possible to differentiate two otherwise identical strains by determining their plasmid complement. Plasmid profiling has been used successfully as an epidemiological marker in nosocomial infections (McGowan *et al.*, 1979) and in an outbreak of salmonellosis (Brunner *et al.*, 1983), where standard procedures such as biotyping and antibiotic resistance patterns were unsuccessful for strain differentiation. It has also been suggested as a means of strain identification for lactic streptococci used in cheese starters (Davies, Underwood and Gasson, 1981).

Plasmids of the same molecular size can be further characterized by using restriction endonuclease analysis (Farrar, 1983). Restriction endonucleases are enzymes which cleave DNA at specific recognition sites to generate a series of DNA fragments (Lin *et al.*, 1984). The number and size of the fragments are characteristic of that plasmid. Bezanson *et al.* (1985) used gel electrophoresis and restriction endonuclease analysis in the study of a multiprovinical outbreak of salmonellosis. They were able to establish cheese as the source of the outbreak, and that the outbreak was caused by two distinct but related bacteria.

2.7 Production of Energy by *Enterobacteriaceae*

Bacteria generate energy for cell growth and maintenance in the form of energy-rich phosphate bonds of ATP. *Enterobacteriaceae* produce ATP by two mechanisms:

substrate level and oxidative phosphorylation. Substrate level phosphorylation involves the formation of ATP from ADP by transfer of a high energy phosphate group from an intermediate of a metabolic pathway. In oxidative phosphorylation or respiration, formation of ATP is coupled to the oxidation of various metabolites via the electron transport chain. The generation of energy in this way is based upon the chemiosmotic theory proposed by Mitchell (1961) (cited by Ingraham, Maaløe and Neidhardt, 1983, p. 144).

Electron transport complexes are located in the cytoplasmic membrane in such a way that each succeeding component is capable of reducing the next component in the chain. The overall effect is that hydrogen ions are pumped out of the cell, creating a proton motive force. Bringing the hydrogen ions back into the cell through ATPases located at specific entry ports results in the generation of energy (Ingledew and Poole, 1984). Under aerobic conditions the terminal electron acceptor is oxygen. Anaerobic respiration occurs when an alternate terminal electron acceptor is used. A variety of compounds such as nitrate, sulphate or fumarate can serve as alternate electron acceptors. They are less powerful than oxygen as oxidizing agents, therefore, less ATP is generated for each electron that passes through the chain.

A facultative anaerobe can grow in the presence or absence of oxygen by altering its method of generating ATP.

This allows aerobes that are capable of nitrate reduction to grow anaerobically, e.g. *Pseudomonas* spp. (Blazevic and Ederer, 1975). All *Enterobacteriaceae* except some strains of *Erwinia* and *Yersinia* and certain biotypes of *Enterobacter agglomerans* are capable of reducing nitrate (Krieg, 1984b, p. 409; MacFaddin, 1980, p. 441). This is an advantage since the reducing potential of nitrate is relatively high. Almost the same amount of energy is generated when nitrate is used instead of oxygen as the terminal electron acceptor (Ingledew and Poole, 1984).

2.8 Fermentation of Glucose by *Enterobacteriaceae*

By definition, all *Enterobacteriaceae* ferment glucose. Many other carbohydrates and related compounds are also fermented with the production of acid and often gas (Krieg, 1984b). *Enterobacteriaceae* are categorized by their biochemical activity patterns. Sugars are fermented to pyruvate using the Embden-Meyerhof (glycolytic) pathway. Differences occur in the enzymes produced for metabolism of pyruvate. In general, two pathways of pyruvate metabolism exist in members of the family *Enterobacteriaceae*: (i) the butanediol fermentation, characterized by production of a large amount of nonpolar butanediol and neutral ethanol with little or no production of acids; and (ii) the mixed acid fermentation, characterized by the production of a large amount of acid as end product. *E. coli* ferments pyruvate using the mixed acid fermentation to produce lactate,

succinate, acetate, formate and ethanol in significant amounts, which causes a rapid decrease in pH of unbuffered media (MacFaddin, 1980).

The methyl red test ("M" of the IMViC tests) identifies those organisms capable of producing large quantities of acid. A positive test is usually indicative of a mixed acid fermentation. The Voges-Proskauer test ("V" of the IMViC tests) is based on the detection of acetoin, a precursor of butanediol. *Klebsiella* and *Enterobacter* species are generally V-P positive, while *E. coli* is negative (MacFaddin, 1980).

2.9 Fermentation of Lactose by *E. coli*

Fermentation of lactose by *E. coli* is controlled by the well-characterized lactose (*lac*) operon. The operon is inducible so that the gene products are only produced in the presence of a recognized substrate, in this case lactose or β-galactoside analogues. The *lacI* gene represses transcription of the operon in the absence of a substrate. In the presence of substrate, the repressor is inactivated and the operon is expressed. Two gene products are responsible for the transportation and hydrolysis of lactose into its component sugars. The *lacY* gene codes for a β-galactoside permease which catalyzes the transport of lactose and other β-galactosides across the cell membrane. A single membrane protein (M protein) is responsible for the recognition and translocation of β-galactoside (Andrews and Lin, 1976). The

permease requires energy in the form of a pH or electrical gradient, such as that created by the electron transport chain. Under growth conditions, where abundant supplies of energy are available, the binding site of the M protein has a high affinity for the substrate on the outside of the cell, and a low affinity on the inside of the cell (Andrews and Lin, 1976). The *lacZ* gene encodes for the enzyme β-galactosidase, which hydrolyzes lactose into its two component sugars, glucose and galactose, which can then be metabolized via the glycolytic pathway.

Late or slow lactose fermenters lack a functional β-galactoside permease, yet they produce β-galactosidase. Lactose non-fermenters are devoid of both the permease and β-galactosidase enzymes and therefore lactose cannot enter the cell nor can it be degraded. Slow lactose fermenters eventually break down lactose over a long period of time (48 h, several days or weeks). It is thought that as the population multiplies, a certain number of mutants carrying a functional permease grow and that this results in late, but visible, lactose fermentation (Lowe, 1962).

There may be a physiological basis for the ability of faecal coliform organisms to ferment lactose at elevated temperatures, while non-faecal coliforms do not. Dockins and McFeters (1978) reported that at elevated temperature non-faecal coliform bacteria experience a reduction in cell membrane function and glucose uptake and a decrease in β-galactosidase activity. The β-galactosidase of non-faecal

coliform bacteria also has a lower thermal inactivation temperature, which would impair the growth and metabolism of these organisms at elevated temperatures.

2.10 Utilization of Formate by *E. coli*

Once *E. coli* has metabolized a carbohydrate to an acid end product, it can create more energy and increase the pH of the growth medium by utilizing one of several electron transport chains. In particular, formate can be oxidized to carbon dioxide and water by one of three electron transport systems involving the enzyme formate dehydrogenase. Under aerobic conditions, oxygen is the terminal electron acceptor which results in the conversion of formate to carbon dioxide and water.

Under anaerobic conditions, *E. coli* metabolizes formate by one of two pathways. In the presence of nitrate, a system is induced which combines formate dehydrogenase with nitrate reductase. Formate is oxidized to carbon dioxide and water, while nitrate is reduced to nitrite. This electron transport system enables *E. coli* to grow on a variety of non-fermentable substrates, such as D-lactate as a sole source of carbon (Haddock and Jones, 1977). In the absence of nitrate and at pH levels of 6 or less, membrane bound formate-hydrogenlyase is produced which consists of formate dehydrogenase, hydrogenase and intermediate carriers resulting in the oxidation of formate to equal volumes of carbon dioxide and hydrogen (Gest and Peck, 1955; Davis *et*

al., 1973).

All indicator tests which rely on gas production from the fermentation of lactose or other sugars depend on the presence of a functional formate-hydrogenlyase system. The temperature maxima for activity, and especially for cellular production of formate-hydrogenlyase is much lower in non-faecal coliform bacteria than in faecal coliform bacteria (Wolf, Stickland and Gordon, 1954; Quist and Stokes, 1969). This is thought to be an explanation for the anaerogenic nature of non-faecal coliform bacteria in elevated temperature MPN tests. The activity of formate-hydrogenlyase is often reduced and sometimes entirely supressed under environmental conditions that do not favour the survival of coliform bacteria in water (Leclerc *et al.*, 1977).

Variant strains of *E. coli* obtained under adverse storage conditions, that were anaerogenic at elevated temperatures, were considered to have decreased formate dehydrogenase activity or a temperature sensitive formate-hydrogenlyase system (Daubner, 1975; Bueschkens and Stiles, 1984). Although molecular oxygen represses the formation of both formate-hydrogenlyase and nitrate reductase electron transport chains, the reverse does not apply under anaerobic conditions. Oxygen-dependent cytochrome oxidation, proton translocation and transport can all be demonstrated in anaerobically grown cells (Gray *et al.*, 1966; Haddock and Jones, 1977).

Opinions differ about the occurrence of a single formate dehydrogenase in all three of these systems, or whether each system involves an unique formate dehydrogenase. The evidence so far is inconclusive. In general, hydrogenlyase activity correlates with benzyl viologen reductase, while the formate oxidase system utilizes phenazine methosulphate (PMS) or methylene blue as artificial electron acceptors in preference to benzyl viologen (Peck and Gest, 1957; Cox, Edwards and DeMoss, 1981). Some workers believe, however, that this difference is due to the presence or absence of a hydrogenase rather than the production of a distinct formate dehydrogenase (Ingledew and Poole, 1984).

Formate dehydrogenase and nitrate reductase are both molybdenum containing iron-sulphur proteins located in the cytoplasmic membrane of *E. coli*. Formate dehydrogenase also requires selenium in order to be functional. Formate dehydrogenase has been purified and characterized, and it appears to consist of three peptide subunits, A, B, and C. Selenium is associated with subunit A. The functional enzyme also requires a cytochrome (Ingledew and Poole, 1984). Cytochromes are heme proteins and each has a characteristic absorption band in the reduced state. Five classes of mutants have been isolated, which are specifically deficient in formate dehydrogenase activity, and are known as *fdhA* - *fdhE* (Ingledew and Poole, 1984).

The nitrate reductase of *E. coli* is composed of three subunits: α, β, and γ. The α subunit is thought to contain the catalytic site, while the β subunit is responsible for attachment to the membrane. The γ subunit is presumed to be equivalent to cytrochrome b (Haddock and Jones, 1977). This cytochrome is distinguishable from the other cytochromes produced by *E. coli* and is referred to as cytrochrome b^{NR}.

2.11 Formate Dehydrogenase Defective Mutants of *E. coli*

Mutants defective in the formate dehydrogenase and/or nitrate reductase system have been selected by several techniques (Venables and Guest, 1968; Glaser and DeMoss, 1972; Mandrand-Berthelot, Wee and Haddock, 1978; Ruiz-Herrera, Showe and DeMoss, 1969). The technique most commonly used for the the selection of chlorate resistant mutants (*chl*) was first described by Piéchaud *et al.* (1967). The principle behind this technique is simple. Wild-type strains of *E. coli* are sensitive to chlorate under anaerobic conditions, because a functional nitrate reductase system reduces chlorate to chlorite which is toxic to the cells. Therefore, only cells which lack a functional nitrate reductase are able to grow anaerobically in the presence of chlorate. This has generated considerable interest because of the ease with which a variety of mutants can be isolated. The mutants have proven ideal for the study and characterization of the factors regulating the biochemistry and genetics of anaerobic electron transport systems (Gibson and

Cox, 1973).

For the isolation of mutants, cells are usually treated with nitrosoguanidine before being plated onto chlorate agar. Eight different mutants (*chlA* - *chlG*, *chlI*) have been isolated. They map at 4 distinct regions of the chromosome (Figure 2.1; Bachmann and Low, 1980). A diagram of the genes and their proposed gene products is given in Figure 2.2. Five of these mutants (*chlA*, *chlB*, *chlD*, *chlE* and *chlG*) are pleiotropic for nitrate reductase and formate dehydrogenase. They are, in one way or another, responsible for synthesis, processing and insertion of the molybdenum cofactor into the formate dehydrogenase and nitrate reductase enzymes. Concomitant expression of at least 3 genes (*chlA*, *chlB* and *chlD*) is required for transport and incorporation of molybdenum into formate dehydrogenase and nitrate reductase. Haddock and Jones (1977) suggested that the genetic and biochemical makeup of the anaerobic electron transport systems may be far more complex than is now believed. They cited data which suggest that *chlA*, *chlB* and *chlE* may in fact be subdivided into 2, 3 and 2 complementation groups, respectively.

Casse (1970) stated that the frequency of spontaneous *chl* mutations is 10^{-9} to 10^{-6}, and that the majority of these mutations (99%) results in the loss of both nitrate reductase and formate-hydrogenlyase activities. Only 1% are non-pleiotropic, i.e. specific in the loss of nitrate reductase, and they map in the *chlC* region. Among the other

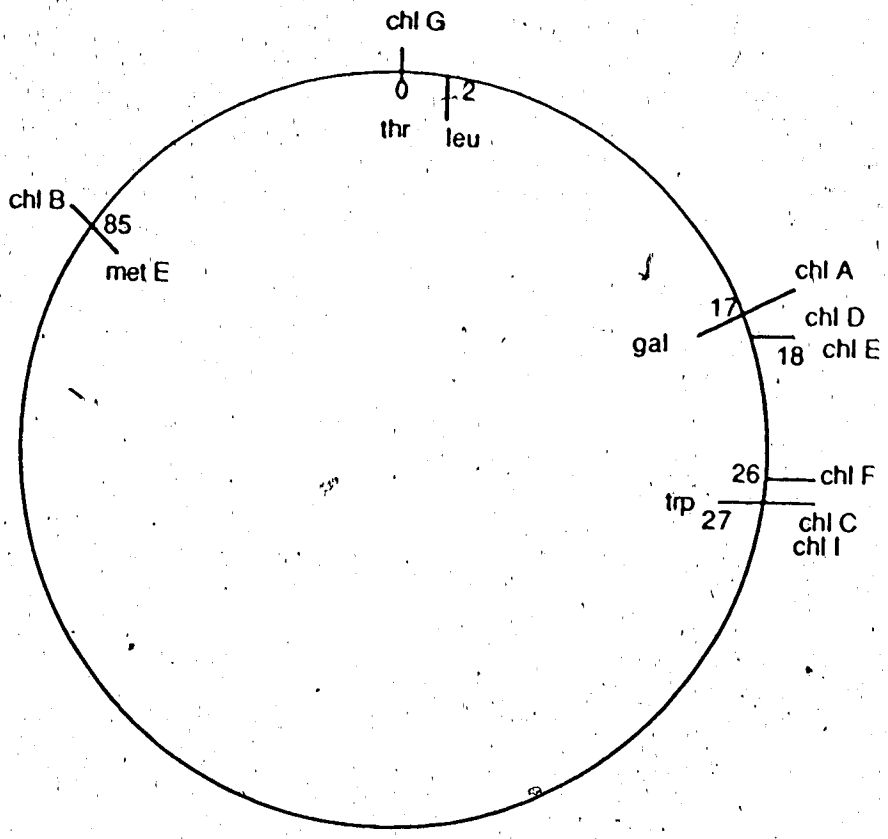


Figure 2.1. Gene map of *E. coli* chromosome showing different locations of determinants for chlorate (*chl*) resistance.

(Numbers indicate the map position of the markers in minutes; gal = galactose, leu = leucine, met = methionine, thr = threonine, trp = tryptophan).

Taken from Glaser and DeMoss (1972), and updated from Bachmann and Low (1980).

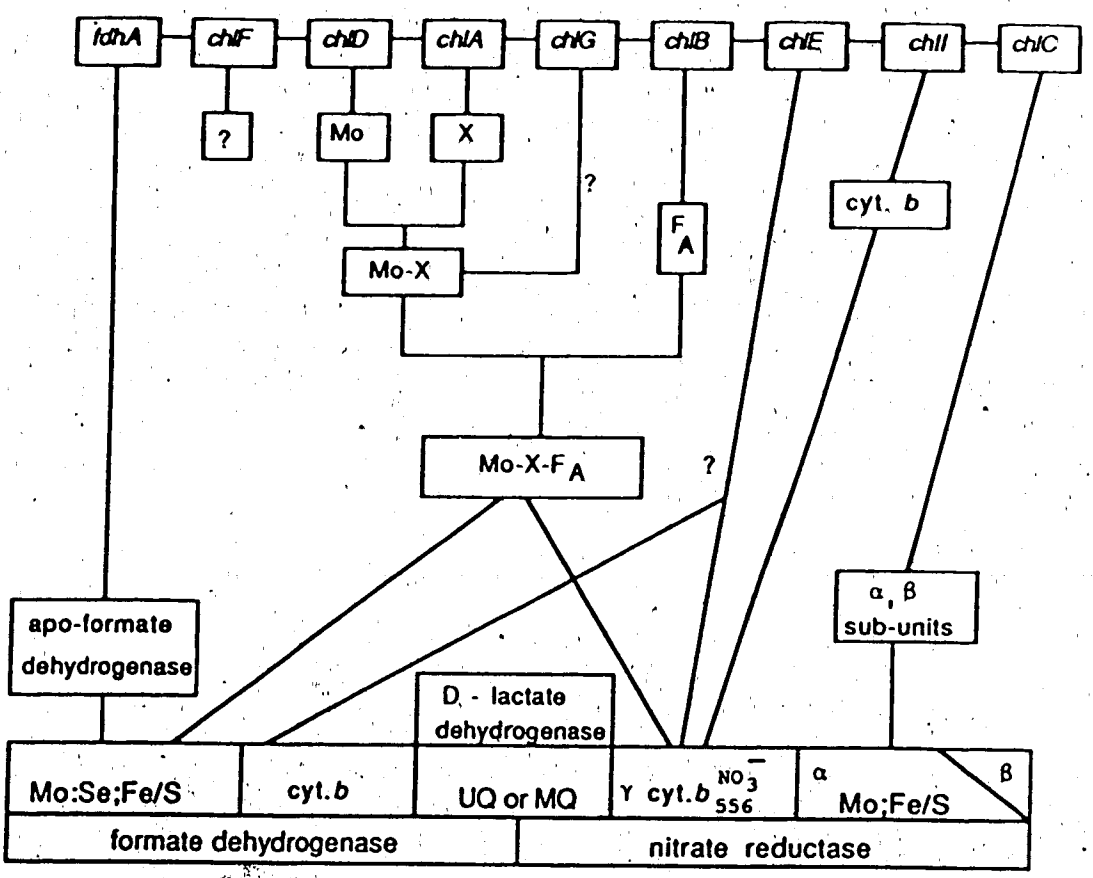


Figure 2.2. Genes and their proposed products responsible for formate-dependent nitrate reductase activity in anaerobically grown *E. coli*.

Abbreviations: cyt = cytochrome; MQ = menaquinone; UB = ubiquinone; X = cofactor; F(A) = association factor.

Taken from Begg, Whyte and Haddock (1977), and updated from Bachmann and Low (1980).

99% of the mutants, 93% map in the *gal* region and they are *chlA*, *chlD* or *chlE* mutants; only 6% map in the *met* region for *chlB* mutants. The distribution of mutants is strongly influenced by the method of selection. Glaser and DeMoss (1972) showed that chlorate resistance selects for more severe alterations in nitrate reductase than other techniques. Approximately 98% of the mutants that they isolated by this method were pleiotropic for nitrate reductase and formate dehydrogenase. However, when mutants were selected for inability to link nitrate reduction to formate oxidation, 48% of the isolates were of the *chlC* class, and 2 new classes of mutants were identified, *chlF* and *chlG*.

To reduce confusion in the identification and characterization of *chl* mutants, Begg, Whyte, and Haddock (1977) described a simple and rapid classification scheme utilizing two indicator plates which divides mutants into those which: (a) lack formate dehydrogenase activity; (b) lack D-lactate dependent nitrate reductase activity; or (c) pleiotropically lack both activities. Table 2.1 shows the formate dehydrogenase and nitrate reductase activities of various chlorate resistant mutants.

2.12 The Requirement for Molybdate and Selenite in Functional Anaerobic Electron Transport Systems

All molybdoenzymes, except nitrogenase, share a common cofactor (Johnson, 1980). Pinsent (1954) was the first to

Table 2.1. Formate dehydrogenase and nitrate reductase activities of various chlorate resistant (chl) mutants.

Mutant	Formate dehydrogenase activity	Nitrate reductase activity
chlA	−	−
chlB	−	−
chlC	+	−
chlD	−(+)[1]	−(+)[1]
chlE	+	−
chlG	+	−
chlI	+	−

[1] Under high molybdate concentrations (10^{-4} M), *chlD* mutants show phenotypic restoration of both enzyme activities.

Taken from Begg, Whyte and Haddock (1977).

report that, in addition to iron, both molybdate and selenite are required during growth of the organism for production of a functional formate dehydrogenase. Maximum stimulation was obtained with selenite concentrations of 3×10^{-8} to 1×10^{-7} M and a molybdate concentration of approximately 1×10^{-8} M. She also demonstrated that in media containing nitrate, tungstate competitively antagonizes the effect of added molybdate, but it has no effect on growth in nitrate-free media.

Glaser and DeMoss (1971) showed that for *chlD* mutants, phenotypic restoration of both formate dehydrogenase and nitrate reductase to wild-type levels was possible by addition of 10^{-4} M molybdate to the growth medium. It has been speculated that the *chlD* gene product is either involved in molybdenum transport or that it is required for ensuring that molybdenum is in the form necessary for both electron transport chains.

Lester and DeMoss (1971) observed that selenite and molybdate are not required for an active formate-oxidase, but they are essential for production of functional formate-nitrate reductase in simple, defined media. Active nitrate reductase only requires the addition of molybdenum to the medium, however Giordano, Haddock and Boxer (1980) indicated that intracellular molybdenum is more important for production and stability of a functional formate dehydrogenase than it is for a functional nitrate reductase. In the presence of tungstate or in the absence of molybdate

they demonstrated high levels of inactive demolybdo-nitrate reductase in both wild-type and *chlD* mutants. However, in the absence of molybdenum, *chlD* mutants do not produce formate dehydrogenase. It is interesting to note that of 100 Alkalescens-Dispar bacteria studied by Ewing *et al.* (1972), 100 (100%) were negative for reduction of nitrate to nitrite. It could be speculated that these organisms were, in fact, *chl* mutants.

3. METHODS AND MATERIALS

3.1 Bacterial Cultures

The enteric cultures used in this study were obtained either from the American Type Culture Collection (ATCC): *Escherichia coli* ATCC 11775; or from a previous study on meat (Stiles and Ng, 1980): *E. coli* #1840 and *Klebsiella pneumoniae* #2. All cultures are faecal coliforms, that is, they ferment lactose to produce acid and gas at elevated temperatures (44.0-45.5°C); the *E. coli* cultures also produce indole from tryptophan at elevated incubation temperatures.

Plasmid marker strains containing single plasmids of known molecular weight including *E. coli* strains DT433 (60 Mdal), DT77 (38 Mdal), DT369 (23 Mdal) and DT370 (5.5 Mdal) were kindly donated by Dr. D. E. Taylor, Department of Medical Microbiology and Infectious Diseases, University of Alberta.

Lactic acid bacteria used in this study were isolated from irradiated, vacuum packaged meat samples plated onto MRS agar adjusted to pH 5.6 (de Man, Rogosa and Sharpe, 1960) and incubated at 20°C. All cultures were gram positive coccus-shaped bacteria that grew in pairs, chains or clusters and were catalase and benzidine negative.

3.2 Media Preparation

Broth and agar media used in this study are listed in Table 3.1. Media were prepared using manufacturers' directions or by following the directions given in the reference cited. pH adjustment of MRS and Rogosa SL agar (RA) was done with lactic acid (85%). The pH of other media was adjusted with 0.1 or 1.0N NaOH or HCl.

3.3 Maintenance of Stock Cultures

Enteric cultures were maintained on tryptic soy agar (TSA) slants and stored at 4°C. New stock cultures were prepared monthly by growing two overnight subcultures in trypticase soy broth (TSB) at 35°C, before streaking the culture onto TSA plates. An isolated colony was selected from the TSA plate and inoculated onto a fresh TSA slant. The biochemical characteristics of the cultures were checked periodically with BBL Minitek Enteric Identification System (Becton-Dickinson, Mississauga, Ontario) using the 20 biochemical disks recommended for identification of enteric bacteria: nitrate, phenylalanine, hydrogen sulphide, indole, Voges-Proskauer, citrate, ONPG, urea, lysine, arginine, ornithine, dextose, malonate, adonitol, arabinose, inositol, raffinose, sorbitol, lactose, rhamnose and sucrose (BBL Minitek, 1979).

Lactic cultures were maintained in Cooked Meat Medium (CMM) at 4°C. Stock cultures were prepared in the same manner as the enteric organisms, except that cultures were

Table 3.1. Selective and nonselective media used in this study, and their sources.

Name	Abbreviation and Supplier[1] or Reference
Culture Media	
APT Broth	APT, Difco
Cooked Meat Medium	CMM, Difco
L-broth	---, Lennox (1955)[2]
Trypticase Soy Broth	TSB, BBL
Differential Media	
Nitrate Broth	---, MacFaddin (1980)[2]
Tryptone Broth	TB, Difco
MPN Broths	
2% Brilliant Green Bile Broth	BGB, Difco
EC Broth	EC, Difco
EC without bile salts	EC-, Difco ingredients[2]
Non-Selective Plating Media	
Plate Count Agar	PCA, Difco
Tryptic Soy Agar	TSA, Difco
Selective Plating Media	
Cephaloridin, Fucidin, Cetrimide Agar	CFC, Mead and Adams (1977)[2,3]
MRS Agar (pH 5.6)	MRS(5.6), Difco
MRS Agar (pH 6.5)	MRS(6.5), Difco
Rogosa SL Agar (pH 5.85)	RA, Difco
Streptomycin, Thallous acetate, Actidione Agar	STAA, Gardner (1966)[2,3]

[1] Difco - Difco media, Difco Laboratories, Detroit, Michigan;
BBL - Becton-Dickinson, Mississauga, Ontario.

[2] Prepared from Difco ingredients and chemicals from Fisher Scientific or J.T. Baker Scientific.

[3] Antimicrobial agents: cephaloridin, fucidin (fusidic acid, sodium salt), cetrimide (trimethyl ammonium bromide), actidione (cycloheximide) and streptomycin sulfate from Sigma Chemicals; thallous acetate from Fisher Scientific.

grown in APT broth at 25°C, streaked onto MRS (pH 6.5) agar plates and incubated in a "candle jar" (see below) at 25°C.

3.4 Sample Preparation

For inoculation into meat or broth, stock cultures were subcultured in APT or TSB and incubated at 25 or 35°C (respectively) for 24 h on 2 successive days. A 10 mL aliquot of an 18 h culture was centrifuged at 3,000 x g for 10 min. The cell pellet was resuspended in an equal volume of sterile, 0.1% peptone water.

Freshly prepared, lean ground beef was obtained from a local supermarket. In the laboratory, the meat was packaged in 11 g portions in "Whirl-Pac" ("Nasco", Systems Plus, Waterloo, Ontario) bags and, where required, the samples were irradiated for 5.5 h at a rate of 0.87 Krads per minute (total 290 Krads). This gave a residual aerobic colony count of <500 colony forming units (CFU)/g of meat, and reduced the coliform count to <10 CFU/g. The prepared culture was inoculated into the ground beef to give 10^5 - 10^7 CFU/g. The inoculum was mixed thoroughly into the sample by manipulating the meat in the sealed bag. The meat was flattened out to an area of 4 x 10 cm to ensure even temperature distribution in the sample during storage. A set of uninoculated samples was prepared as controls. The inoculated samples were vacuum packaged in tri-layer "Vacpac" bags (Cryovac, O_2 transmission 30-50 $cm^3/m^2/h$, 22°C, 1 atm) and stored at 4°C in a refrigerated incubator.

In one trial, 11 mL of sterile nutrient broth (NB) in 16 x 150 mm test tubes was used as a storage menstruum. The test cultures were inoculated into the broth, in the same manner as the meat, except that the culture was mixed by vortex and overlayered with 2 mL sterile mineral oil (Sigma), to mimic the oxygen deficient atmosphere in the Vacpac bags.

3.5 Sample Testing

Samples were tested on day 0, immediately after inoculation, and every 1 or 2 days thereafter for 7 to 14 days. Sterile, 0.1% peptone water (99 mL) was added directly to the meat sample in the Whirl-Pac bag and blended for 1 min using a Colworth Stomacher (Model 400, A. J. Seward, 3, Cavendish Rd., Bury St. Edmund, Suffolk, U. K. IP33 3TE).

Most probable number (MPN) determinations were done using a three-tube MPN technique (ICMSF, 1978, p. 126-133) by inoculating 0.1 or 1.0 mL of appropriate dilutions of the samples into tubes containing 10 mL broth and an inverted Durham vial (10 X 75 mm). Single or duplicate sets of six serial dilutions were prepared. Single sets were incubated at 35°C. When duplicate sets of tubes were prepared, one set was incubated at 35°C, the other was incubated in a circulating water bath at 45 ± 0.2°C. Tubes were scored after 48 h incubation for gas in the Durham tube or for signs of effervescence upon gentle agitation of the tube. The highest dilution showing three positive tubes, and the

two greater dilutions, were used to convert the score to a most probable number using standard tables (ICMSF, 1978).

Plate counts were also determined for each sample using four plating media: TSA, "repair agar", violet red bile agar (VRB) and tryptone bile agar (TBA). Duplicate plates were surface inoculated with 0.1 mL of an appropriate dilution to give 30 - 300 colonies per plate. Inoculated VRB plates were overlayered with 5 mL VRB that had been tempered to 45°C. Plates were incubated at 35°C and 45 ± 0.5°C for 18 h, in stacks not more than 2 plates high in a circulating air incubator.

Tryptic soy agar was used to enumerate the total coliform population, including injured cells unable to grow on selective media. The "repair" medium included resuscitation on TSA for 2 h at 25°C, followed by an overlay of 12 mL VRB (Speck *et al.*, 1975). Repair agar also allowed injured cells to grow, but it had the advantage of inhibiting the background microflora. Violet red bile agar was used to enumerate coliform organisms, only colonies with a zone of bile precipitate larger than 0.5 mm were enumerated (ICMSF, 1978, p. 131). Tryptone bile agar was used to enumerate organisms that produce indole from tryptophan at elevated temperatures.

Indole production was determined on TBA plates using a modification of the procedure of Anderson and Baird-Parker (1975). Samples were plated directly onto TBA, instead of using cellulose acetate filter membranes (Bueschkens and

Stiles, 1984). After 18 h incubation at 35 or 45°C, plates were flooded with TBA stain (2.0 g *p*-dimethylaminobenzaldehyde, 92.0 mL distilled water, and 8.0 mL concentrated HCl). After 1 minute, plates were viewed against a black background: indole positive colonies stained reddish-pink; and indole negative colonies became a creamy-white colour.

Uninoculated control samples were plated on TSA and VRB (35°C for 18 h), MRS (5.6) (25°C for 72 h) and plate count agar (PCA; 20°C for 72 h).

3.6 Microbiological Analysis of Vacuum Packaged Meat

Changes in the adventitious flora of irradiated and unirradiated meats were determined in three separate trials. Uninoculated meat samples were prepared as described above and stored at 4°C. Samples were tested on day 0 and every second day for 14 days. Total aerobic counts were determined on PCA; pseudomonads on cephaloridine fucidin cetrimide agar (CFC, Mead and Adams, 1977); and *Brocothrix thermosphacta* on streptomycin thallous acetate actidione agar (STAA, Gardner, 1966). All plates were incubated at 20°C for 72 h. Coliform bacteria were counted on VRB plates incubated at 35°C for 18 h.

A small number of pseudomonads are able to grow on STAA (Gardner, 1966); therefore, after counting, STAA plates were flooded with 5 mL of a 1% (w/v) solution of tetramethyl-*p*-phenylene diamine dihydrochloride (oxidase reagent) in distilled water. Oxidase positive contaminants stained blue

after 15 sec. These colonies were enumerated and subtracted from the total to give a presumptive *B. thermosphacta* count.

Lactic acid bacteria were enumerated by plating on MRS (pH 5.6) and RA (pH 5.8) and incubating the plates at 20°C for 3 days in anaerobic jars using "Gas Pac" envelopes (BBL, Becton-Dickenson, Mississauga, Ontario) to create a H_2/CO_2-enriched atmosphere. The reduced O_2 tension created by lighting a candle in the anaerobic jar was shown to be sufficient to stimulate the growth of the lactic acid bacteria, and both MRS and RA inhibited other background microflora. Therefore, in subsequent experiments and for culture maintenance, MRS and RA plates were incubated in "candle jars".

3.7 Isolation of Lactic Acid Bacteria

Lactic acid bacteria were isolated during microbial analysis of vacuum packaged ground beef. Colonies were picked from MRS plates of irradiated and unirradiated meat samples that had been plated after 6 and 14 days storage at 4°C. The isolates were subcultured into ATP broth and incubated at 25°C overnight on 2 successive days, before characterization (gram stain, benzidine and catalase tests) and storage in cooked meat medium. Six cultures (2 from each trial) isolated from irradiated ground beef were chosen as test cultures for use in the MPN study.

3.8 pH Measurement

pH of the meat samples blended in 0.1% peptone water (pH 7.0) was measured after microbiological analyses had been completed, using an Orion combination pH electrode (Orion Research Inc., Cambridge, MA)

3.9 Temperature Measurement

Because of the importance of accurate temperature measurement in this study, thermometers were checked against a quartz thermometer (Department of Chemistry, University of Alberta) for the temperature range 35 to 50°C. The thermometers were found to be accurate in this range. Waterbath and incubator temperatures were checked daily to ensure that incubation temperatures remained stable.

3.10 Isolation of Culture Variants

E. coli variants that were negative for gas and/or indole production at elevated temperatures were isolated from the meat and NB storage experiments by selecting atypical colonies from TSA and VRB plates incubated at 35°C. Colonies were picked using a sterile wire and inoculated into TSB. After overnight incubation at 35°C, a loopful of culture was inoculated into tubes containing TSB, EC, lauryl sulphate tryptose broth (LST) and tryptone broth (TB). One TSB tube was incubated at 35°C, all other tubes were incubated in a waterbath at 45 ± 0.2°C for 24 h. Isolates were scored for growth, gas and indole production. Indole

production was determined by adding 0.2 to 0.3 mL of Kovak's indole reagent (ICMSF, 1978, p. 135) to the tube. Production of a dark red colour was scored as a positive reaction, and an orange colour as an intermediate (±) reaction. Variants failing to produce gas or indole at 45°C were tested for stability of the variant phenotype, by successive subculturing in the appropriate broth (LST for gas negative variants, TB for indole negative variants). Variants were also characterized biochemically using the Minitek Enteric System. Nitrate reduction was determined as a separate test using the broth method of MacFaddin (1980).

3.11 Plasmid Profiles of Culture Variants

Plasmid profiles of the parent strain *E. coli* #1840 and three variants were determined. Plasmid profiles were also determined for stock cultures *E. coli* ATCC 11775 and *K. pneumoniae* #2. Size markers were obtained by isolating single plasmids of known molecular weight (60, 38, 23 and 5.5 Mdal) from the four marker strains. Plasmid DNA was isolated using a modification of the method described by Birnboim and Doly (1979).

Cultures were grown overnight in 1.5 mL of L-broth (Lennox, 1955) in an Eppendorf tube. Cells were pelleted by centrifugation at 10,000 x g for 15 sec in an Eppendorf microcentrifuge. The broth was decanted and the cell pellet resuspended in 100 µL of lysozyme solution (25 mM Tris-HCl, pH 8.0; 50 mM glucose; 10 mM EDTA, pH 8.0; 2 mg/ml

lysozyme). This solution was prepared daily from crystalline lysozyme and stock solutions of the other components. After 10 min at 0°C, 200 µL of alkaline sodium dodecyl sulphate (SDS, Calbiochem; 0.2N NaOH; 1% SDS) was added and mixed by inversion. This mixture was held at 0°C for 5 min.

After the lysozyme-SDS treatment, 150 µL of "high salt solution" (3 M sodium acetate, pH 4.8) was added and the mixture was held at 0°C for 10 min. The precipated chromosomal DNA, proteins and high molecular weight RNA were pelleted by centrifugation for 5 min. The pellet was removed with a sterile toothpick. The preparation was purified by extraction with 2 volumes of phenol:chloroform (1:1). This mixture was gently agitated to form an emulsion which was broken by centrifugation for 3 min. The upper aqueous layer was transferred to a clean Eppendorf tube using a micro-pipette, being careful not to disturb the fluid interface.

Plasmid DNA was precipitated by adding 2 volumes of cold (-20°C) 95% ethanol. This solution was held -20°C for 20 min before pelleting by centrifugation for 3 min. The ethanol was decanted, and the pellet was resuspended in 200 µL of 70% ethanol. After 20 min at -20°C the pellet was collected by centrifugation for 3 min. The ethanol was decanted, and ethanol residue was removed in a vacuum chamber. The pellet was resuspended in 20 µL TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) before being loaded onto the gel.

Plasmid DNA (20 μL) was mixed with 5 μL loading dye (40% w/v sucrose in H_2O, 0.25% bromophenol blue) and loaded into wells of a 0.70% agarose gel (Seakem ME., Marine Colloids FMC Corp., Rockland, Maine). The gel was made up and run in Tris/borate buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0). Agarose was melted (3 - 5 min) in a microwave oven and cooled to 45°C before pouring. Gels (0.4 cm x 10 cm x 15 cm) were run at 50 V for 1.5 h, stained in 0.4 μg/ml ethidium bromide for 40 min at room temperature and destained in distilled water overnight. Gels were illuminated using an ultraviolet transilluminator (Fotodyne Inc., New Berlin, Wisconsin) and photographed using Polaroid type 57 or Kodak Tri-X Pan film.

3.12 Selective Isolation of Chlorate Resistant Variants

Chlorate resistant variants were isolated from inoculated NB and meat storage samples using a modification of the method of MacGregor and Schnaitman (1971). A 2 mL aliquot of the sample was pour plated with 10 mL chlorate agar (0.2% ClO_3^-), allowed to solidify, overlayered with an additional 10 mL of chlorate agar, and incubated at 35°C for 24 h in anaerobic jars containing Gas Pac envelopes. Colonies appearing on these plates were isolated and purified by streaking onto chlorate agar plates, which were overlayered with 10 mL of chlorate agar, and incubated anaerobically for 24 h. An isolated chlorate-resistant colony from each of these plates was inoculated into LST

broth to check for gas production. All variants and stock cultures were typed for formate dehydrogenase and nitrate reductase activities, lactose fermentation and utilization of galactose as a sole source of carbon, as follows:

(1) Formate dehydrogenase and (2) nitrate reductase activities were determined by the method of Begg *et al.* (1977). Cultures grown in LST broth were streaked onto glucose-nutrient agar (G-N) plates and incubated aerobically at 35°C for 24 h. Sterile Millipore filters (0.45 μm, 47 mm diameter, Millipore Ltd., London) were placed onto two G-N plates and onto an additional two G-N plates containing 10^{-4} M sodium molybdate (G-N/Mo; Glaser and DeMoss, 1971). Single colonies from the overnight plates were inoculated onto the filters in a systematic manner, using the grid pattern on the filters to organize the inoculation of the isolates. After incubation at 35°C for 24 h, filters were transferred to the surface of a sterile, plain agar plate (1.5% w/v Difco agar) for 15 min. After this, one of the filters incubated on G-N agar and one incubated on G-N/Mo agar were placed onto separate formate dehydrogenase and nitrate reductase indicator plates (Begg *et al.*, 1977).

On the formate dehydrogenase indicator plates, cells with a functional formate dehydrogenase metabolize formate absorbed into the cells, thereby keeping the intracellular pH close to neutrality. These cells turn a red colour as they accumulate phenol red through the filter. Cells lacking a functional formate dehydrogenase system are unable to

metabolize formate. As a result, the intracellular pH in these cells rapidly becomes acidic and the colonies turn a yellow colour. Differentiation of colony types was possible within 15 to 20 min.

Detection of nitrate reductase activity was done by a two step procedure. Filters were incubated on a nitrate reductase indicator plate at room temperature for 15 min. Plates were cooled on ice and overlayered with sloppy agar (0.75% effective concentration) containing the nitrite reagents. The sloppy agar was prepared by mixing equal volumes of 1.5% w/v agar and colour reagent (equal parts of 0.8% w/v sulphanilic acid in 5 N acetic acid and 0.5% w/v α-naphthylamine in 5 N acetic acid; MacFaddin, 1980). Colonies accumulating nitrite (nitrate reductase positive) stained orange shortly after adding the sloppy agar/reagent overlayer. The test was read within 15 min.

(3) Ability to ferment lactose. MacConkey agar base with 1% added lactose was prepared to determine the ability of the culture to ferment lactose.

(4) Utilization of galactose as the sole source of carbon. Growth on minimal agar (Difco) with 0.1% galactose was used to determine this characteristic.

4. RESULTS

4.1 Verification of Identity of Test Organisms

The biochemical characteristics of the stock cultures were determined using the BBL Minitek Enteric Identification system. Biochemical characteristics, Minitek identification numbers and the certainty of identification for each of the stock cultures are given in Table 4.1. All biochemical characteristics remained stable throughout the study.

4.2 Comparison of Most Probable Number (MPN) and Plate Count Methods for Enumeration of *Escherichia coli* in Vacuum Packaged Ground Beef

4.2.1 Changes in Coliform Counts

In the first experiment *E. coli* #1840 was inoculated into irradiated ground beef samples to give an initial load of 10^6 CFU/g. Samples were inoculated into EC broth (0.1 mL/tube) and onto repair and VRB agars. Inoculated media were incubated at 35 and 45°C. At all sampling times and at both incubation temperatures, the plate counts gave consistent results (see Table 4.2). There was no injury on the VRB selective agar, and only minor loss in viability (0.3 to 0.5 log cycle) over the 14-day trial period on both plating media. MPN counts determined at 35°C were relatively stable, but counts were frequently 0.5 - 1 log cycle lower than comparable counts on agar media. At 45°C, MPN counts

Table 4.1. Biochemical characteristics and identity of stock cultures determined using the BBL Minitek Enteric Identification System.

	E. coli ATCC 11775	*E. coli* #1840	*K. pneumoniae* #2
Nitrate reductase	+	+	+
Phenylalanine deaminase	-	-	-
Hydrogen sulfide	-	-	-
Indole	+	+	-
Voges-Proskauer	-	-	+
Citrate	-	-	+
ONPG	+	+	+
Urease	-	-	+
Lysine decarboxylase	+	+	-
Arginine deaminase	-	-	-
Ornithine decarboxylase	+	+	-
Dextrose	+	+	+
Malonate	-	-	+
Adonitol	-	-	+
Arabinose	+	+	+
Inositol	-	-	+
Raffinose	-	+	+
Sorbitol	+	+	+
Lactose	+	+	+
Rhamnose	+	+	+
Sucrose	-	+	+
gas from lactose (45°C)	+	+	+
Minitek ID Profile	4453116	4453137	4361777
% Certainty of Identity[1]	99.90	99.90	99.71

[1] Percentage certainty of identification based on the BBL Minitek Enteric System (BBL Minitek, 1979).

Table 4.2. Counts of *E. coli* #1840 in vacuum packaged ground beef determined on different growth media during storage at 4°C for 14 days (Trial 1).

Growth Media	Incubation Temp. (°C)	Counts (log CFU or MPN/g)				
		Initial Count	Time of Storage (days)			
			1	3	7	14
Repair agar	35	6.53	6.37	6.53	6.43	6.19
	45	6.48	6.56	6.41	6.41	6.16
VRB	35	6.58	6.47	6.47	6.36	6.05
	45	6.54	6.43	6.53	6.49	6.12
EC broth	35	5.36	5.36	5.36	5.36	5.63
	45	4.63	4.87	4.59	2.36	>4.04

were variable between samples during storage, as shown in Table 4.2. MPN counts were as much as 1.5 to 4.5 log cycles lower than agar plate counts. In all cases, at 35 and 45°C, lower counts resulted from the absence of growth and not growth without gas production.

A second experiment was designed to determine whether the variability in MPN counts observed in the first experiment was the result of injury to growth due to the bile salts in EC broth or to growth at 45°C. *E. coli* #1840 was inoculated into irradiated meat samples at 10^7 CFU/g. Samples were inoculated (1.0 mL/tube) into EC and EC without bile salts (EC^-), and onto TSA, repair agar and VRB. Plate counts were extremely consistent throughout the 7-day storage period. MPN values did not vary to the same extent as those observed
in the first experiment (see Table 4.3). MPN counts at 35°C in EC and EC^- were 0.25 to 1.0 and 0 to 0.5 log cycles lower than plate counts, respectively. At 45°C MPN counts in both EC and EC^- were 0.5 to 1.0 log cycle lower than plate counts.

Subsequent trials with irradiated meat were designed to determine whether inoculum size (1.0 mL or 0.1 mL in EC broth), level of test organism inoculated into the meat samples (10^6 or 10^7 CFU/g) or strain of coliform organism (*E. coli* #1840, *E. coli* ATCC 11775, *K. pneumoniae* #2) was responsible for the large variations in MPN counts observed in the initial trial. No differences were seen in

Table 4.3. Counts of *E. coli* #1840 in irradiated, vacuum packaged ground beef determined on different growth media during storage at 4°C for 7 days (Trial 2).

Growth Media	Incubation Temp. (°C)	Counts (log CFU or MPN/g) Initial Count	Time of Storage (days) 1	2	3	5	7
TSA	35	7.56	7.58	7.61	7.64	---	7.57
	45	7.58	7.50	7.60	7.57	---	7.62
Repair agar	35	7.56	7.50	7.60	7.46	7.50	7.66
	45	7.60	7.50	7.60	7.53	7.50	7.51
VRB	35	7.48	7.52	7.45	7.48	7.43	7.41
	45	7.49	7.44	7.45	7.45	7.36	7.40
EC broth	35	6.38	6.63	7.36	6.63	7.36	6.63
	45	7.04	6.36	6.36	6.36	6.36	6.63
EC- broth	35	>7.04	7.18	7.63	7.63	7.36	7.38
	45	>7.04	6.63	7.18	6.63	6.88	6.97

variability of the MPN counts at 35 or 45°C with different inoculum size, with level of inoculation of test organism, or between the test strains used to inoculate the meat.

During these trials, however, it was observed that the pattern of growth of the adventitious background microflora in the irradiated meat samples differed markedly between experiments. Two distinct patterns of growth emerged, as shown in Figure 4.1. In one case no lag period was observed; in the other, a lag period of 3 to 4 days was observed before a noticeable increase in bacterial counts was recorded.

In all trials, plate counts for *E. coli* were extremely stable and consistent with the data presented in Tables 4.2 and 4.3. Considerable variation in colony size was noted after 5 days storage of the meat at 4°C when samples were plated onto both selective and non-selective agar plates at 45°C. Many colonies on VRB plates at 45°C were smaller than those on VRB plates at 35°C. However, most colonies exceeded the required 0.5 mm diameter and actual plate counts on all media determined at 45°C were not markedly lower than those determined at 35°C. The adventitious background microflora was inhibited on the selective and repair media, but it grew on TSA plates at 35°C. After 18 h incubation, colonies of *E. coli* were sufficiently larger than those of the background microflora that they could be readily differentiated. At 45°C the adventitious background microflora was inhibited on all of the plating media.

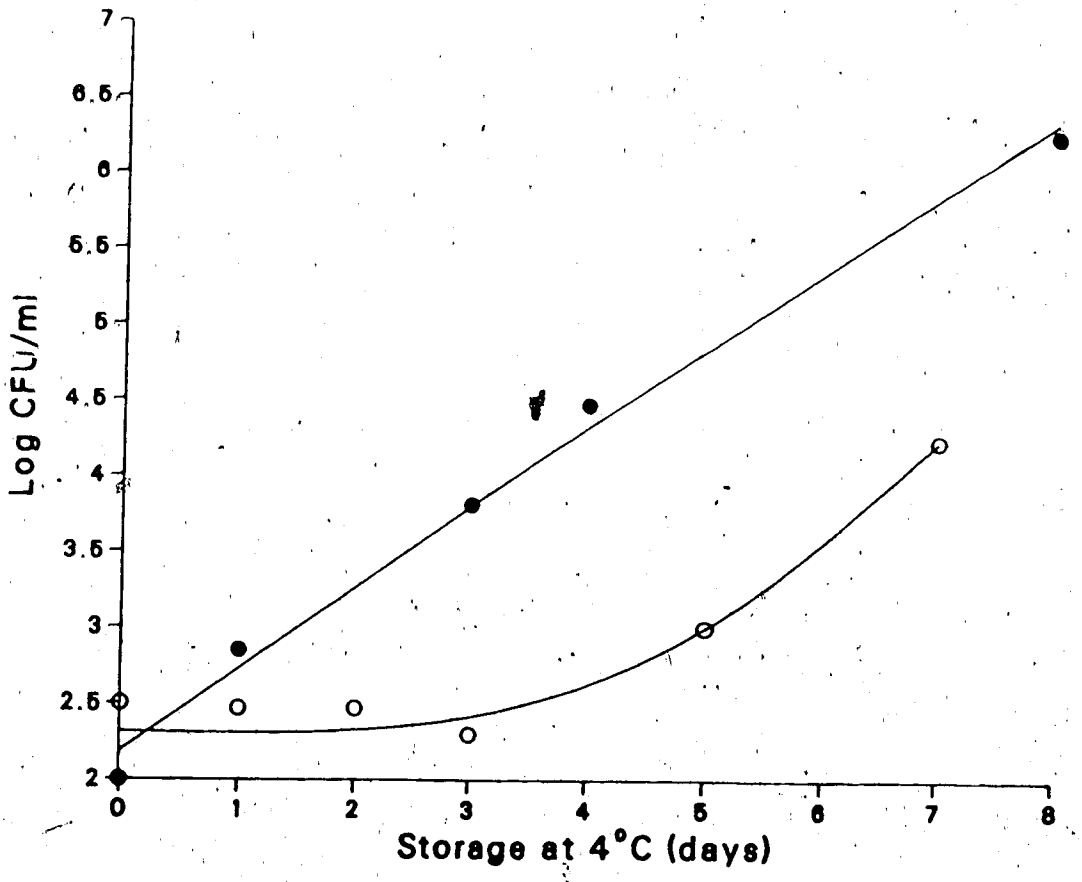


Figure 4.1. Growth patterns of the adventitious background microflora in irradiated ground beef samples.

Key: • growth without lag phase; o growth with lag phase.

Accurate and reliable temperature settings were critical in obtaining consistent plate counts at 45°C. Temperature increases of greater than 0.5°C (>45.5°C) resulted in inhibition of *E. coli* #1840 on the selective media (VRB, TBA). Colonies on TSA plates at >45.5°C were variable in size but they were sufficiently large for accurate counts to be obtained. Colonies on VRB agar incubated at >45.5°C were pinpoint in size and were, technically, too small to be counted as coliform bacteria (ICMSF, 1978). Small colonies on TBA plates at >45.5°C were either weakly positive or negative when tested for indole production. Similar observations were noted for fresh overnight broth cultures of *E. coli* #1840, *E. coli* ATCC 11775 and *K. pneumoniae* #2 inoculated onto the plating media and incubated at >45.5°C.

When MPN counts were determined in EC broth two patterns of variability occurred which appeared to correlate with the different growth patterns observed in the adventitious background microflora of the irradiated meat samples. In trials where there was no lag in initial growth of the adventitious microflora, an increase in variability of the MPN counts at 45°C was observed with increasing length of storage at 4°C. This was similar to the effect seen in Trial 1 (Table 4.2) and is represented by the data shown in Figure 4.2. Since plate counts on selective and non-selective media were essentially the same (Tables 4.2 and 4.3), counts on TSA at 35°C represent all plate counts

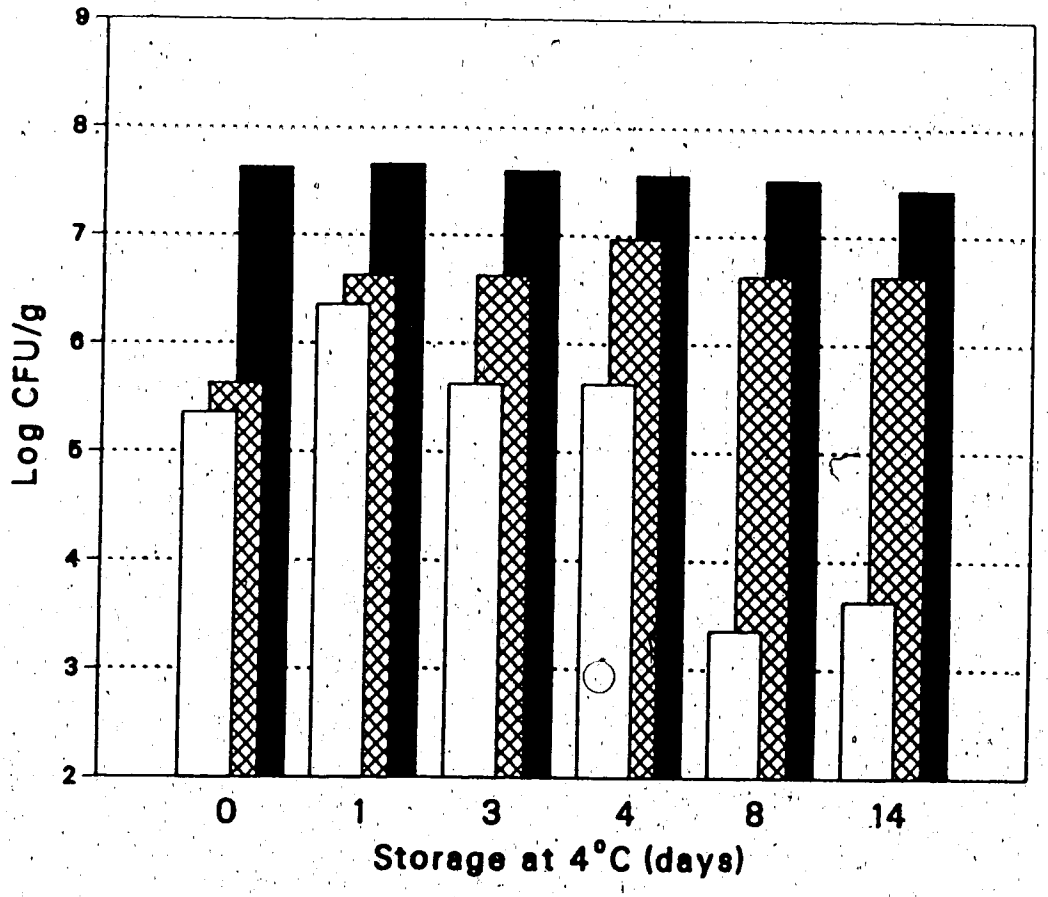


Figure 4.2. Stability of plate and MPN counts (EC broth) for *E. coli* #1840 in irradiadiated meat stored at 4°C in vacuum packages, without a lag phase in the growth of the adventitious flora.

Key: ▭ MPN in EC at 45°C;
▩ MPN in EC at 35°C;
■ Count on TSA at 35°C.

and are referred to collectively as the "plate counts". MPN counts in EC broth at 35°C were generally 0.5 - 1.0 log cycle lower than the plate counts. This did not change with increased time of storage of the meat samples at 4°C. MPN counts in EC broth at 45°C were initially 1.0 - 2.0 log cycles lower than corresponding plate counts from the meat samples stored at 4°C for up to 4 days. However, the differential increased to 4.0 log cycles after 8 and 14 days of storage. When the adventitious background microflora increased rapidly there was a general decrease in MPN counts at 45°C with length of storage.

In contrast, in trials where there was a lag phase of 2 to 4 days in the initial growth of the adventitious background microflora, there was no increase in variability of MPN counts at 45°C with increasing length of storage at 4°C (Figure 4.3). MPN counts in EC broth incubated at both 35 and 45°C were stable throughout the 7-day trial period, but they were consistently lower than the plate counts by approximately 0.5-1.0 log cycle. This pattern was similar to that observed in the second trial (Table 4.3).

Since it appeared that the adventitious background microflora of the meats might be an important factor affecting the variability of the MPN results, a series of experiments was designed to characterize the changes in the adventitious flora of the meat. It was expected that the major components of the adventitious background microflora of vacuum packaged raw beef would be lactic acid bacteria,

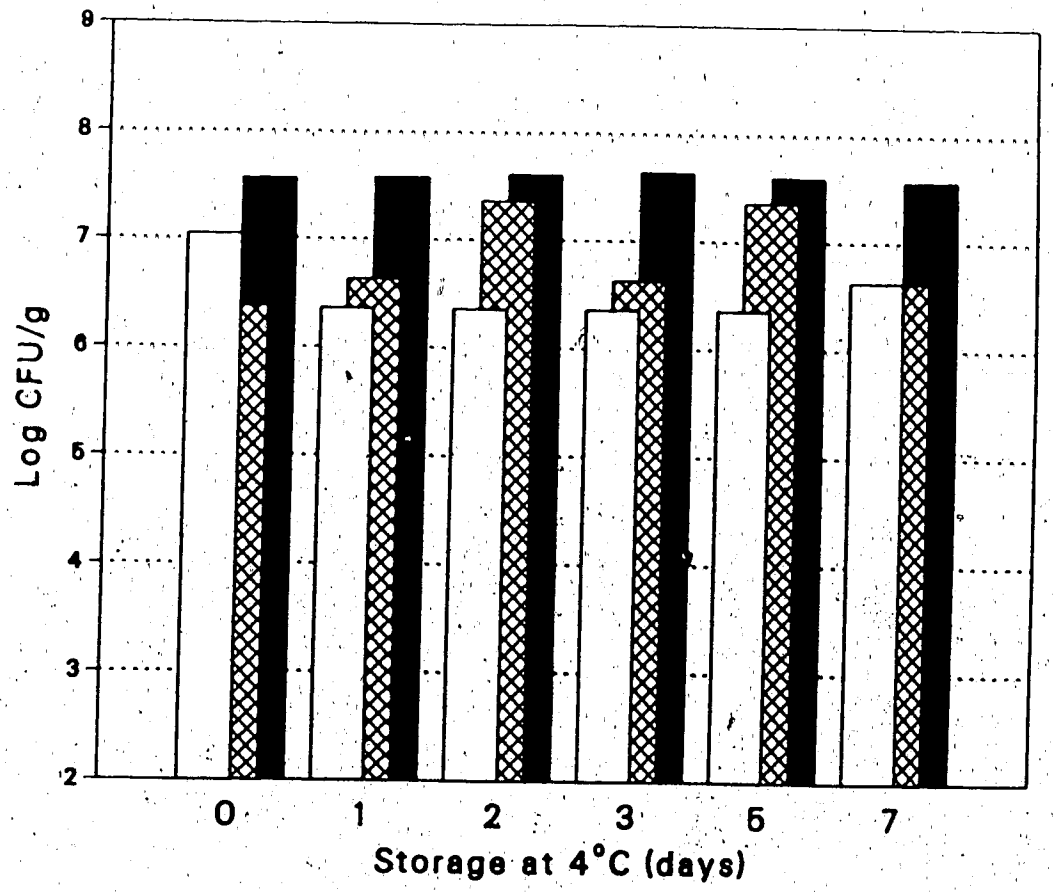


Figure 4.3. Stability of plate and MPN counts (EC broth) for *E. coli* #1840 in irradiated meat stored at 4°C in vacuum packages, with a lag phase in the growth of the adventitious flora.

Key: ☐ MPN in EC at 45°C;
▩ MPN in EC at 35°C;
■ Count on TSA at 35°C.

pseudomonads, *B. thermosphacta*, and coliform bacteria. Plating media were chosen which would selectively differentiate these organisms, so that the changes in the different bacterial populations could be monitored. PCA incubated at 20°C for 3 days was chosen to estimate the total aerobic bacterial count. Unirradiated meat was also used in this experiment to compare the population changes that would be expected in vacuum packaged ground beef under practical conditions with those occurring in irradiated meat.

Meat samples were diluted and plated onto the agar media. Highly consistent growth patterns were observed for all bacterial parameters for both irradiated and unirradiated meat samples. The data in Figures 4.4 and 4.5 are the means for three trials. In both irradiated and unirradiated meat samples, presumptive lactic acid bacteria counts on MRS(5.6) and RA were essentially identical, therefore, only the data from the MRS(5.6) plates are shown.

In irradiated meat samples there were no detectable counts on STAA, VRB or CFC agars in the meats stored for 14 days. During the 14-day trial, counts on PCA increased from 10^2 to 10^7 CFU/g, and were almost the same as counts on MRS(5.6) (Figure 4.4). A lag phase of 2 to 3 days occurred before growth was initiated, and proceeded exponentially for 8 to 9 days. After 12 to 14 days the growth curve appeared to plateau at a count of 10^7 CFU/g, suggesting that the population had reached a maximum and that the stationary

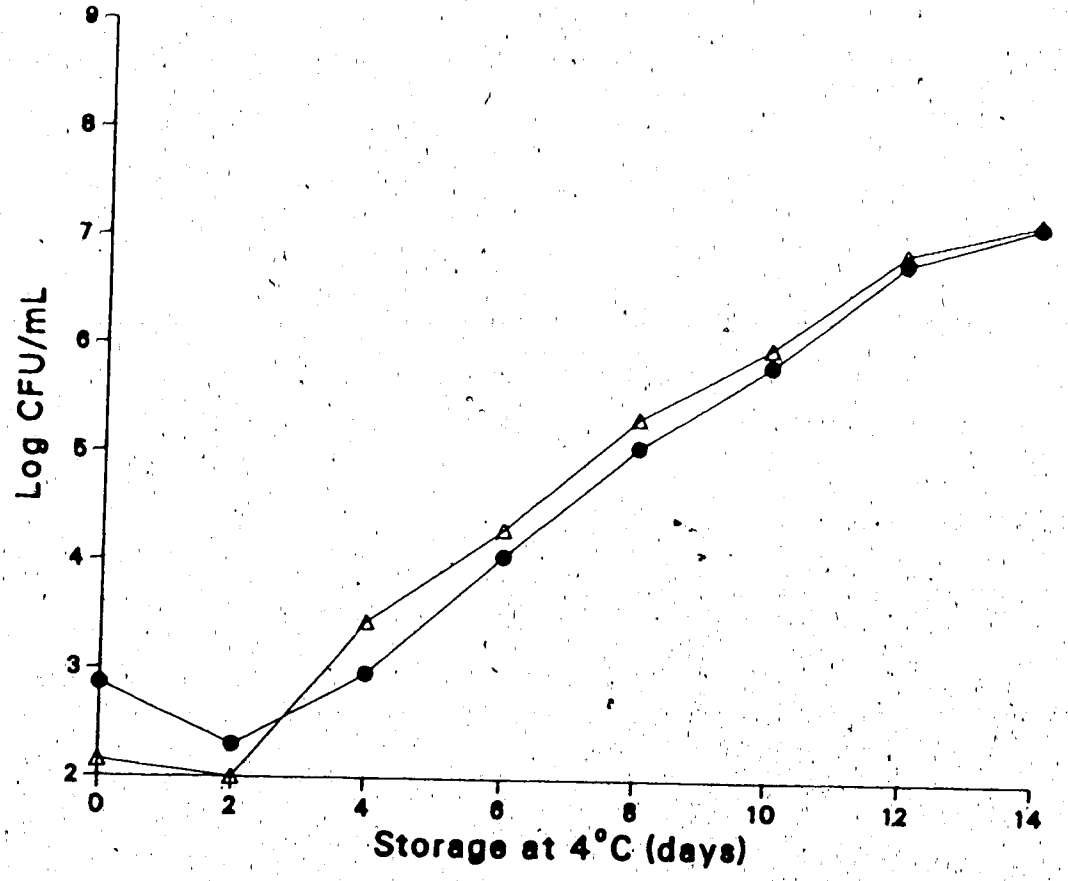


Figure 4.4. Growth of adventitious background flora of irradiated vacuum packaged ground beef during storage at 4°C determined on plate count (PCA) and MRS agar.

Key: • = counts on PCA agar;
Δ = counts on MRS agar.

phase had been reached. From the bacterial parameters measured, it appeared that the presumptive lactic acid bacteria made up virtually 100% of the adventitious flora of the irradiated meat samples.

In contrast, in the unirradiated meat samples there was no lag phase, as shown in Figure 4.5. Final counts on PCA after 14 days storage at 4°C reached markedly higher levels than had been achieved in the irradiated meat samples (5 x 10^8 CFU/g). The lactic acid bacteria made up a large part of this count (1 x 10^8 CFU/g, 20%), however, the remaining 80% of the adventitious microflora was not described by the microbial parameters used. Even so, there were substantial levels of pseudomonads (1 x 10^7 CFU/g), coliform bacteria (5 x 10^6 CFU/g) and *B. thermosphacta* (5 x 10^6 CFU/g). STAA plates, used to determine presumptive *B. thermosphacta* were flooded with oxidase reagent to identify oxidase positive pseudomonads. No oxidase positive organisms were detected on STAA plates in any of the trials.

A trial was run in which *E. coli* #1840 was inoculated into irradiated and unirradiated ground beef at 10^5 CFU/g. Two additional MPN broths (LST and 2% Brilliant Green Bile; BGB) were added for comparison with EC broth at 35°C. The growth of the adventitious background flora in irradiated and unirradiated ground beef samples was similar to the data shown in Figures 4.4 and 4.5, respectively. A lag phase occurred in the irradiated meat samples, and final counts on day 8 reached 10^5 CFU/g on PCA agar. Initially, and on day

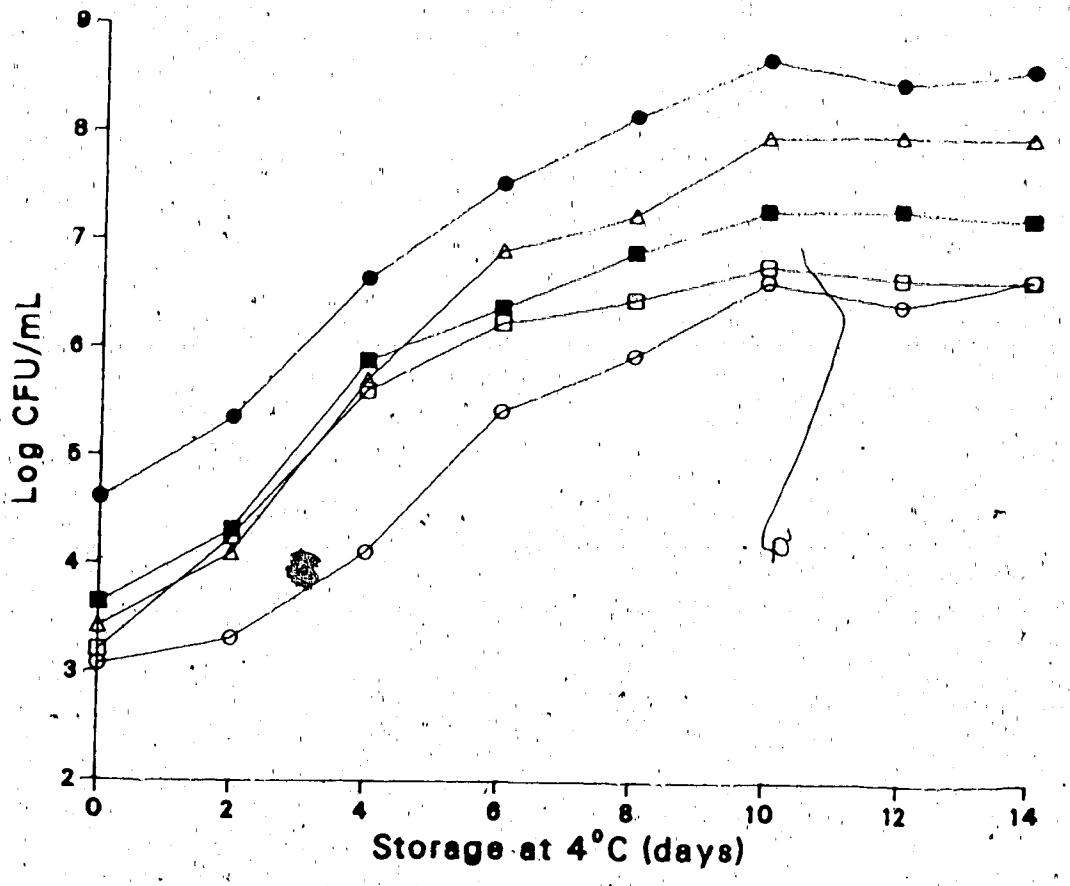


Figure 4.5. Growth of adventitious background flora of unirradiated vacuum packaged ground beef during storage at 4°C for 14 days.

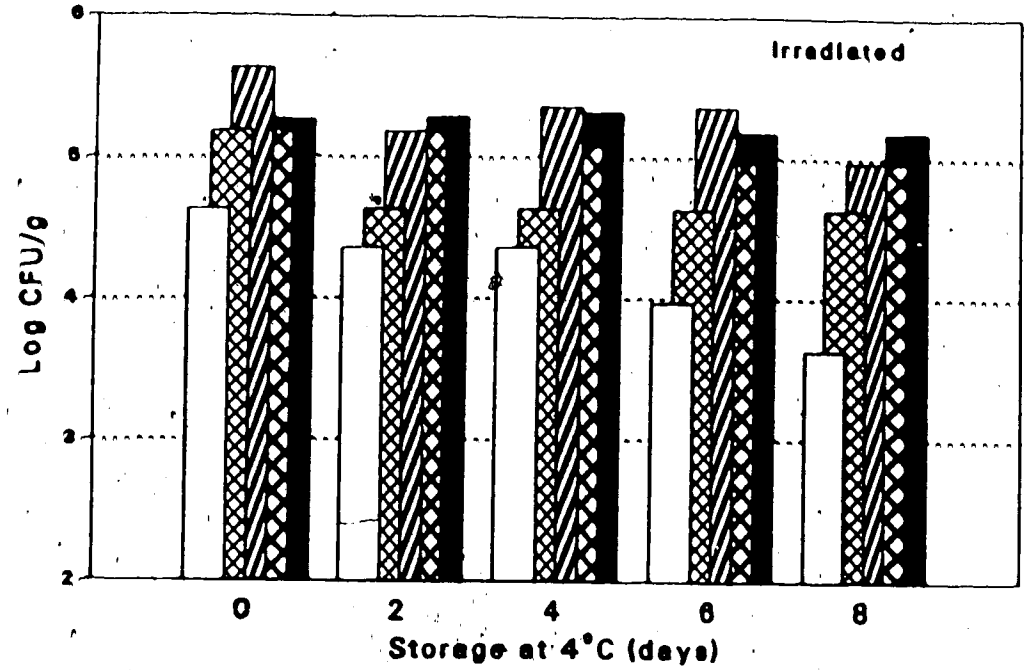
Key: counts determined on • PCA, Δ MRS(5.6), ▪ CFC, □ STAA, o VRB agars.

2, counts on MRS(5.6) agar were $<10^2$ CFU/g, by day 4, counts on MRS exceeded those on PCA by approximately 1.0 log cycle. In contrast, in unirradiated meat, PCA counts were consistently greater than those on MRS(5.6) by approximately 1.0 log cycle. Initial PCA counts in unirradiated meat were 10^5 CFU/g. Final counts exceeded 1×10^8 CFU/g.

Despite the marked difference in growth curves and levels of the adventitious flora seen between the irradiated and unirradiated meat samples, there was little difference in the MPN results for *E. coli* #1840 at either 35 or 45°C (Figure 4.6). In general, for both irradiated and unirradiated meat samples stored at 4°C, MPN counts determined in EC, LST and BGB incubated at 35°C did not differ from plate counts by more than ±0.5 log cycle (Figure 4.6), except in irradiated meat samples where MPN counts determined in EC broth at 45°C were always 1.0 to 1.5 log cycles lower than plate counts.

4.2.2 MPN Variability With High Levels of Lactic Acid Bacteria Inoculated Into Ground Beef Samples

This experiment was designed to study the effect of lactic acid bacteria on the variability of the MPN count of *E. coli* in vacuum packaged ground beef. Six strains of lactic acid bacteria, that had been isolated from a previous experiment as described in Methods section 3.1, were inoculated into meat samples at the same level as the test strain of *E. coli*. Two sets of irradiated ground beef were



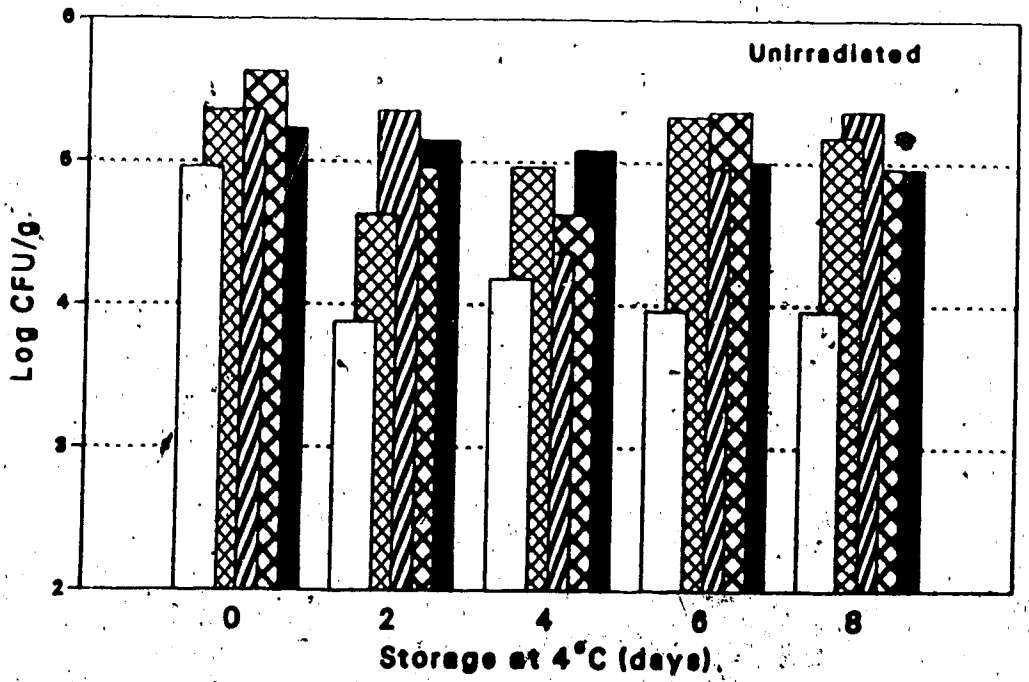


Figure 4.6. Comparison of counts of *E. coli* #1840 inoculated into irradiated and unirradiated ground beef stored at 4°C for 14 days.

Key: Count on TSA at 35°C (irradiated)
at 45°C (unirradiated) ■;

MPN count in EC at 45°C ▭; in EC at 35°C ▨;

MPN count in LST at 35°C ▥; in BGB at 35°C ▩.

inoculated with *E. coli* #1840 at 10^5 CFU/g. One of the sets was also inoculated with the mixture of lactic acid bacteria. These are referred to as samples "with" and "without" lactic acid bacteria. A third set of irradiated ground beef samples was used as an uninoculated control. Parallel samples were prepared with nutrient broth (NB) as the storage menstruum. Samples were stored at 4°C and counts were determined initially and every second day for 14 days. Samples were inoculated into EC and LST broths and onto TSA, VRB and TBA plates, and incubated at 35 and 45°C.

LST was included as a testing broth because of the good correlation between MPN counts determined in LST broth and plate counts in the previous experiment. After 48 h a loop-ful of culture from gas positive LST tubes at 35°C was transferred to EC broth and incubated at 45°C for an additional 48 h, based on the standard MPN procedure for determination of faecal coliform counts recommended by ICMSF (1978) and Standards for Examination of Water and Wastewater (APHA, 1971).

Plate counts at 45°C had to be excluded because of unstable temperatures in the circulating air incubator. All plate counts determined at 35°C for *E. coli* #1840 in meat with and without lactic acid bacteria were stable throughout this experiment. However, in NB with and without lactic acid bacteria, injury occurred for growth of *E. coli* on VRB and TBA agars (Figures 4.7 and 4.8). After 2 days of storage at 4°C, counts on VRB and TBA were consistently 1.0 and 0.5 log

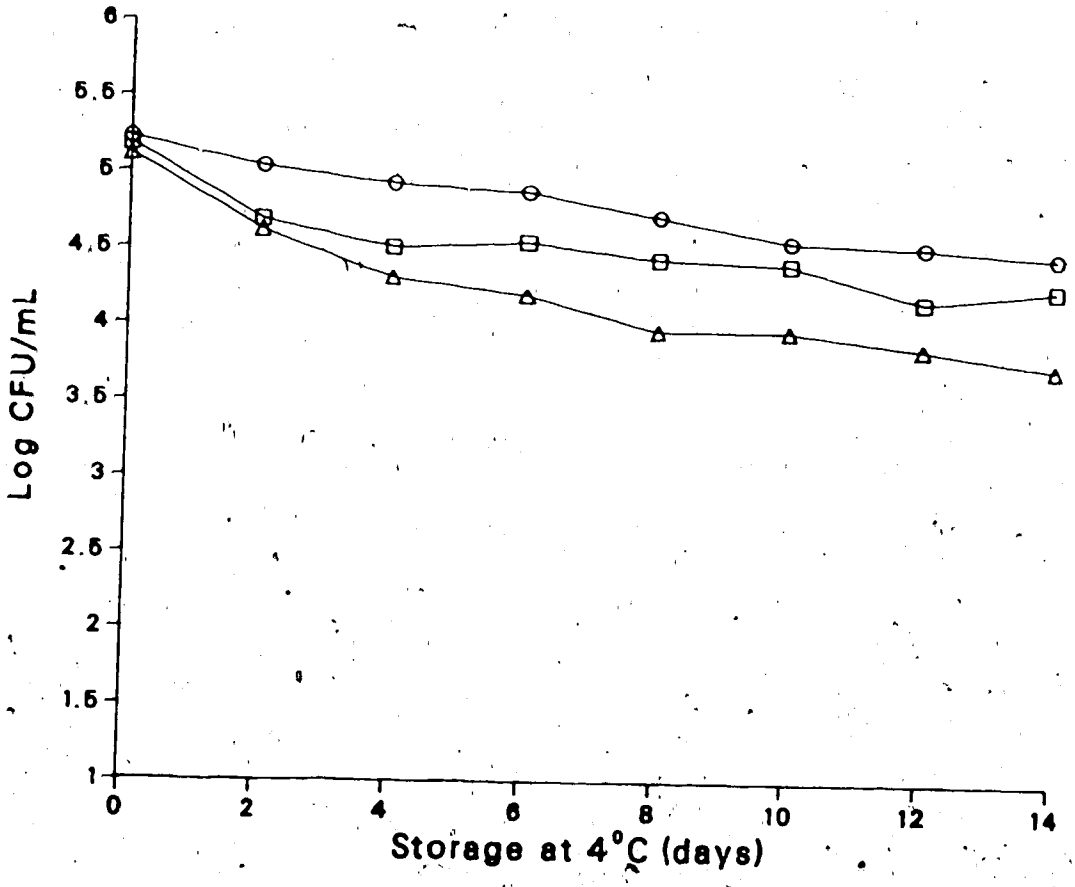


Figure 4.7. Survival of *E. coli* #1840 stored in nutrient broth (NB) at 4°C without added lactic acid bacteria.

Key: plated on o TSA; Δ VRB; □ TBA.

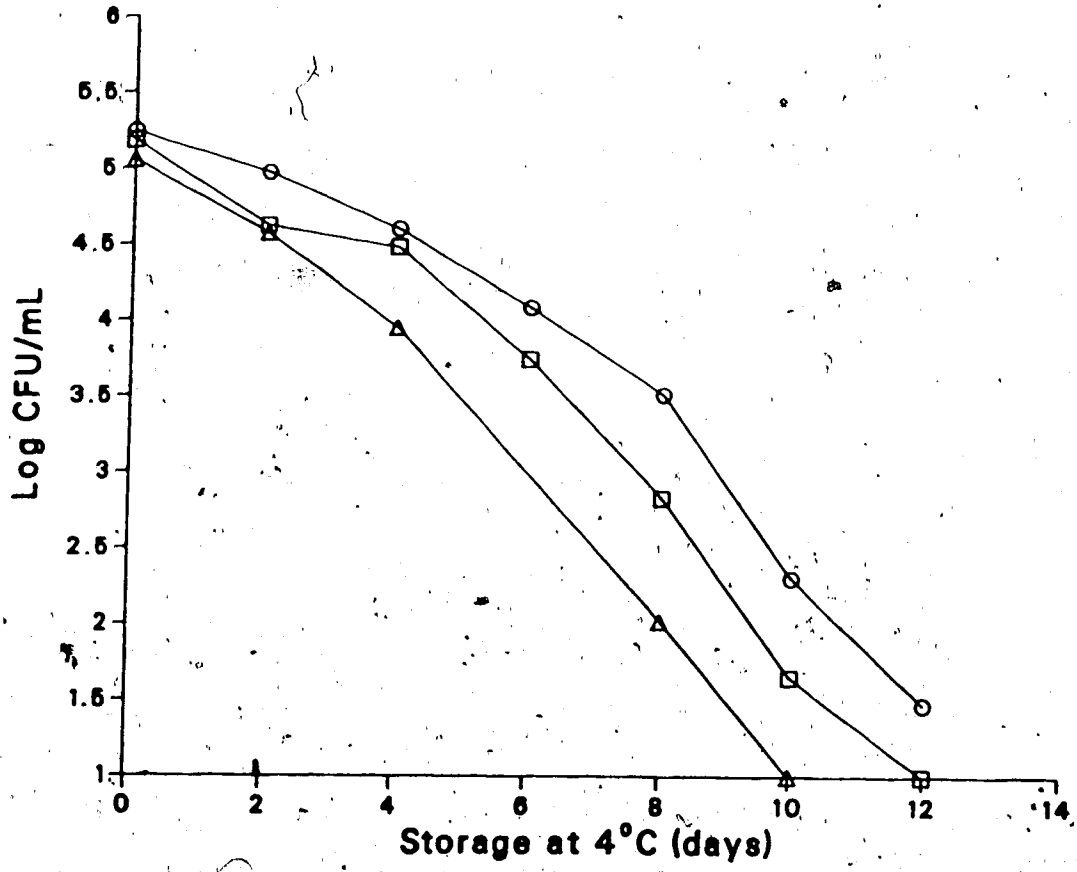


Figure 4.8. Survival of *E. coli* #1840 stored in nutrient broth (NB) at 4°C with added lactic acid bacteria.

Key: plated on o TSA; Δ VRB; □ TBA.

cycles lower than counts on TSA, respectively.

During 14 days of storage at 4°C in meat with and without added lactic acid bacteria, the viability of the test organism decreased slightly (0.2 to 0.3 log cycle) (Figure 4.9a,b). For *E. coli* #1840 stored in NB without added lactic acid bacteria, a similar decrease in viability was observed to that observed in the meat samples, as shown in Figures 4.9c. However, in NB with added lactic acid bacteria, there was a striking loss of viability of *E. coli* #1840. After 14 days of storage at 4°C the *E. coli* had decreased 4 log cycles to less than 10 CFU/g (Figure 4.9d). This effect was somehow negated in the ground beef samples inoculated with lactic acid bacteria.

In all but one sample, MPN counts in LST broth incubated at 35°C corresponded well with plate counts on TSA. LST counts were not more than ±0.5 log cycle of counts on TSA at 35°C (see Figure 4.9a-d). In contrast, MPN counts determined in EC broth at 35°C were lower than counts on TSA by 0.5 to as much as 3.0 log cycles, see Figure 4.10a-d. Direct inoculation into either LST or EC broths incubated at 45°C resulted in counts that were 1.5 to 3.0 log cycles lower than the corresponding plate counts, shown in Figures 4.9a,c and 4.10a,c. It should be noted that in all cases LST gas positive tubes inoculated into EC broth and incubated at 45°C resulted in an identical 'confirmed' MPN count.

The levels of lactic acid bacteria in both the meat with and without added lactic acid bacteria were essentially

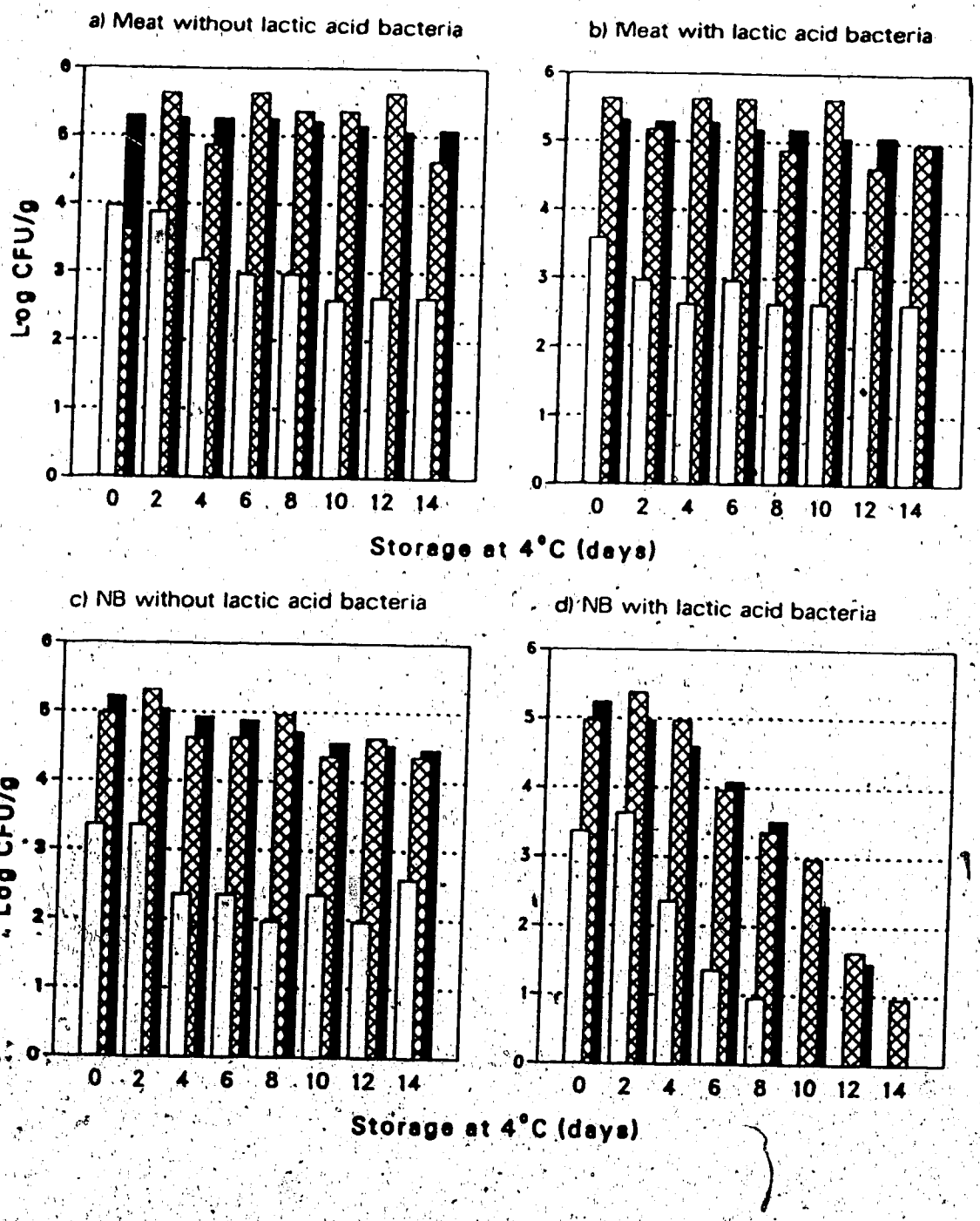


Figure 4.9. Survival of *E. coli* #1840 in irradiated ground beef and nutrient broth (NB) with and without lactic acid bacteria determined in LST broth.

Key: ▭ MPN in LST at 45°C;
▩ MPN in LST at 35°C;
■ Plate counts on TSA at 35°C.

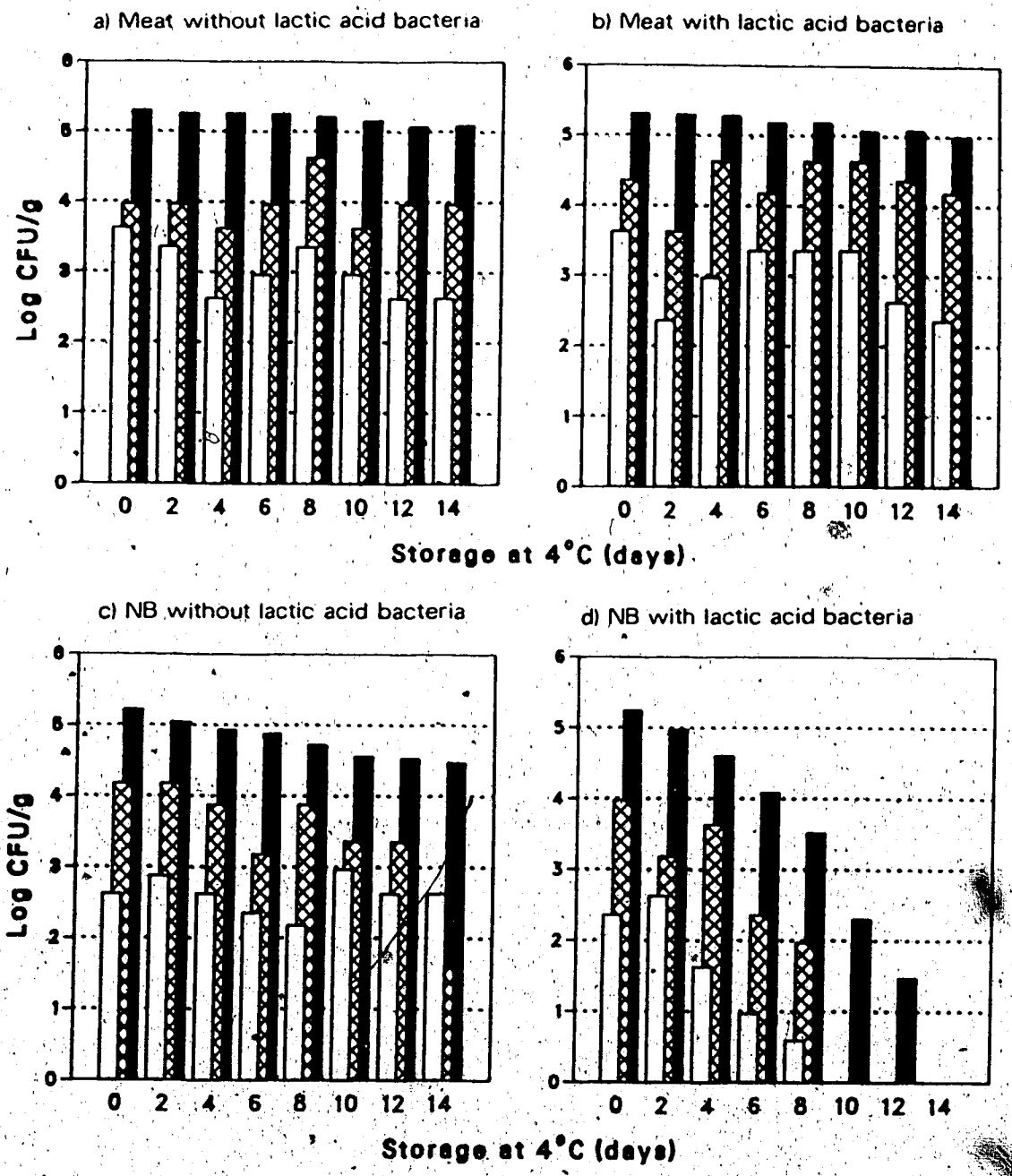


Figure 4.10. Survival of *E. coli* #1840 in irradiated ground beef and nutrient broth (NB) with and without lactic acid bacteria determined in EC broth.

Key: ☐ MPN in EC at 45°C;
▩ MPN in EC at 35°C;
■ Plate counts on TSA at 35°C.

the same, see Table 4.4. The initial adventitious background microflora was relatively high in comparison with previous experiments. Initial levels of lactic acid bacteria in samples in this experiment were 10^5 CFU/g. These levels reached 10^7 CFU/g after 8 days of storage, at which time the counts stabilized. Unfortunately, this did not allow a comparison of MPN counts in meat with low and high levels of initial adventitious background microflora.

4.2.3 Effect of pH on Variability of MPN *E. coli* Counts in Ground Beef

pH of ground beef samples did not seem to have an effect on the variability of the MPN counts. As seen in Table 4.5, initial pH levels in the ground beef were 5.7 to 6.2. This level decreased in unirradiated ground beef samples to 5.3 to 5.5 after 8 days and to 5.2 after 14 days. In irradiated meat samples, pH levels did not fall below 5.7 even after 14 days storage. pH levels were not affected by inoculation of *E. coli* #1840 into the meat sample (Table 4.6, experiment B and C).

When initial levels of adventitious lactic acid bacteria were high, as seen in the final experiment, a decline in pH occurred that was similar to that observed in unirradiated samples (Table 4.6). pH levels decreased from pH 5.9 to pH 5.3 - 5.5 over the 14 days of the experiment. Initial pH levels in NB with and without added lactic acid bacteria were 6.8, and the pH did not change over the 14

Table 4.4. Counts of presumptive lactic acid bacteria in vacuum packaged ground beef during storage at 4°C for 14 days determined on MRS plates incubated at 20°C for 72 h.

Sampling Day	Counts (log CFU/g)		
	E. coli #1840	*E. coli* #1840 + lactic culture[1]	Uninoculated control
0	4.69	5.08	4.56
2	4.13	5.48	6.02
4	5.56	6.37	7.29
6	5.81	6.80	---
8	7.50	7.45	7.87
10	7.58	7.43	7.69
12	7.29	7.34	7.60
14	7.53	7.43	7.78

[1] Mixture of 6 lactic acid bacteria strains isolated from ground beef in previous experiments.

Table 4.5. pH of vacuum packaged irradiated and unirradiated ground beef during storage at 4°C for up to 14 days.

Sampling Day	pH[1]					
	Irradiated			Unirradiated		
	A	B	C	A	B	C
0	6.20	5.75	5.75	6.20	5.75	5.72
2	5.75	5.80	5.80	5.75	5.78	5.80
4	5.72	5.82	5.88	5.72	5.70	5.78
5	5.72	5.80	5.88	5.64	5.52	5.58
8	5.72	5.85	5.88	5.32	5.50	5.52
10	5.72			5.30		
12	5.72			5.12		
14	5.75			5.18		

[1] Results of three experiments. Experiment A, a 14-day uninoculated trial; experiments B and C, uninoculated and inoculated with *E. coli* #1840, respectively.

Table 4.6. pH of vacuum packaged ground beef inoculated with *E. coli* #1840 and a mixed lactic culture during storage at 4°C, for 14 days.

Sampling Day	pH		
	E. coli #1840	*E. coli* #1840 + lactic culture[1]	Uninoculated control
0	5.92	5.98	5.90
2	5.90	5.88	5.90
4	5.85	5.85	5.98
6	5.80	5.75	5.85
8	5.60	5.60	5.72
10	5.40	5.50	5.48
12	5.30	5.28	5.40
14	5.30	5.28	5.48

[1] Mixture of 6 lactic acid bacteria strains isolated from ground beef in previous experiments.

days of the experiment.

4.3 Isolation of *E. coli* Variants

Culture variants that were negative for gas production at 45°C were isolated on three separate occasions from VRB plates that had been used to enumerate *E. coli* #1840 stored in NB at 4°C. Subculturing of these isolates at 35°C revealed that these variants were also negative for gas production at the lower incubation temperature. The variants of *E. coli* #1840 that did not produce gas at 35 or 45°C were identical to the parent strain in all respects, except that they were nitrate reductase negative in the BBL Minitek Disk test (see Table 4.1). The variants were also shown to be nitrate reductase negative in the standard broth test (MacFaddin, 1980). The Minitek ID profile 0453137 compared with 4453137 of the parent strain was confirmed as *E. coli* with 99.99% probability (BBL Minitek Enteric II Identification System, Becton Dickinson, Mississauga). The three variants were subcultured daily in LST broth, at 35°C for 30 days. During this time none of the three variants reverted to the wild type gas-producing phenotype.

4.3.1 Plasmid Isolation

From an earlier study (D. H. Bueschkens, M.Sc. thesis, University of Alberta, 1982) it was known that the serotype of *E. coli* #1840 was O?:K?:H2 meaning that a specific serotype could not be established with available antisera.

Therefore, it was not practical to confirm a relationship between parent and variant strains in this manner. Plasmid profiles were determined in an effort to confirm the identity of the variant strains and to rule out the possibility that the loss in nitrate reductase and formic-hydrogenlyase (gas production) activities observed in the variant was due to the loss of a plasmid. Plasmid DNA from the parent strain *E. coli* #1840 and the three variants was visualized using a rapid plasmid extraction procedure and gel electrophoresis. Plasmid DNA was also isolated from test strains *E. coli* ATCC 11775 and *K. pneumoniae* #2 for comparison with *E. coli* #1840. As seen in Plate 4.1., *E. coli* #1840 and variant strains all showed a single plasmid band. The approximate molecular size of this plasmid was estimated as 57 Mdal by comparison with the mobility of standard plasmids run in the same gel. *E. coli* ATCC 11775 appeared to have a single plasmid band of approximately 68 Mdal and *K. pneumoniae* #2 had 2 plasmid bands of approximately 58 Mdal and 76 Mdal.

4.3.2 Isolation and Characterization of Chlorate Resistant Variants

Since gas negative variants isolated from VRB plates were also deficient in a functional nitrate reductase system, it was possible to use a simple technique available for isolation of nitrate reductase deficient mutants to select simultaneously for gas negative variants. Using

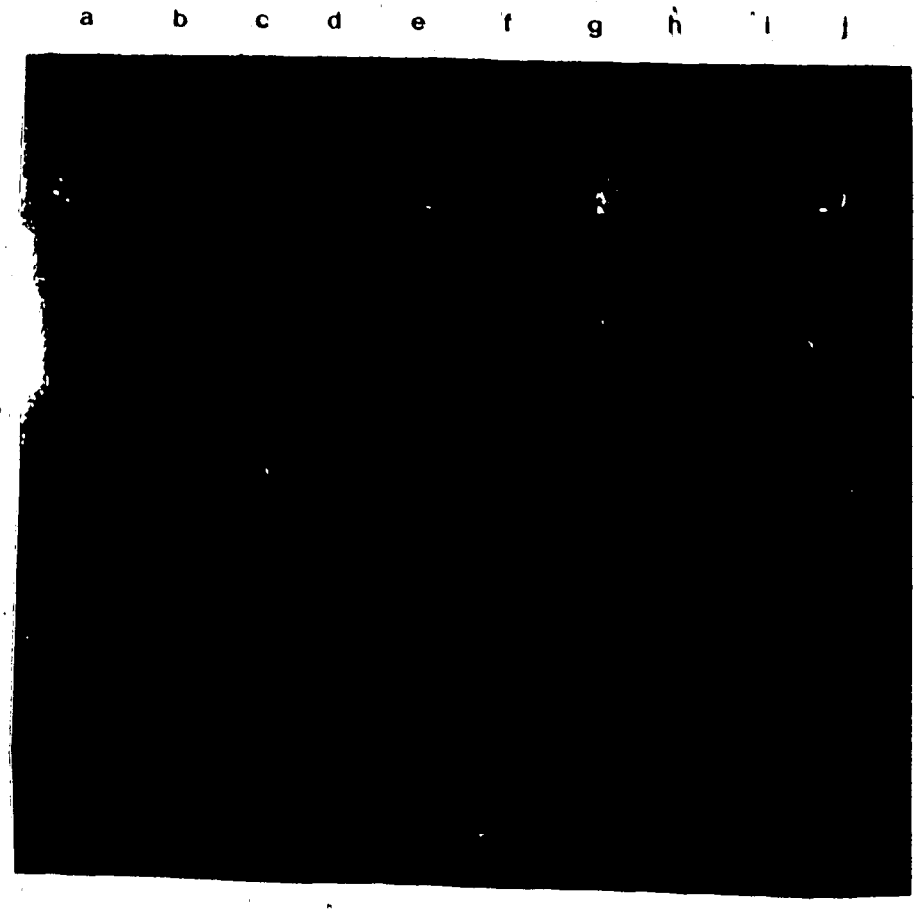


Plate 4.1. Plasmid profiles for *E. coli* #1840 and gas negative variants compared with profiles for *E. coli* 11775 and *K. pneumoniae* #2.

Key: a, DT433 (60Mdal); b, DT77 (38 Mdal);
c, DT369 (23 Mdal);
d, DT370 (5.5 Mdal); e, *E. coli* #1840;
f-h, anaerogenic variants of *E. coli* #1840;
i, *E. coli* ATCC 11775; j, *K. pneumoniae* #2.

chlorate resistance under anaerobic conditions as a selective marker, 95 gas negative variants were isolated from meat and NB samples inoculated with *E. coli* #1840 with and without lactic acid bacteria. All variants were negative for gas production from lactose when incubated at 35°C for 48 h in LST broth. All variants were gas negative, including those originally isolated from colonies on VRB plates, however they were able to ferment lactose on MacConkey agar. In addition, all variants were able to utilize galactose as a sole source of carbon, which was demonstrated by growth on aerobically incubated minimal-salts-galactose agar.

All variants were negative for formate dehydrogenase and nitrate reductase activity when grown on G-N agar. However, one variant isolated from a meat sample (inoculated with *E. coli* #1840 and lactic acid bacteria) was able to revert to the wild-type phenotype in both enzyme systems when grown on agar containing elevated levels of molybdate (10^{-4} M). Thus 1 out of 98 variants (1%) displayed the characteristics of a *chlD* mutant, while the other 97 are either *chlA* or *chlB* mutations.

Using chlorate resistance as a selection technique, four variants of *E. coli* ATCC 11775 were isolated from a stock culture slant. Of these, one displayed the characteristics of a *chlD* mutant, while the other three were either *chlA* or *chlB* mutations. All variants had lost the ability to produce gas.

5. DISCUSSION AND CONCLUSIONS

This study was done to determine the effect of refrigerated storage of vacuum packaged fresh meat on the detection of coliform bacteria, in particular, detection of *E. coli* by the MPN technique at elevated incubation temperatures. Vacuum or "tight" packaging of wholesale meat cuts in gas impermeable wraps has proven to be an effective method of extending the shelf life of fresh beef during refrigerated storage, by limiting the growth of aerobic spoilage bacteria such as psychrotrophic pseudomonads and *B. thermosphacta* (Roth and Clark, 1972; Pierson, Collins-Thompson and Ordal, 1970). Vacuum packaging (VP) also allows for larger, centralized packing plant operations which are more efficient, economical and which may produce a product of higher microbiological quality (Shoup and Oblinger, 1976).

E. coli is considered to be the most valid indicator of unsanitary handling of raw meats, because of its specificity to the enteral environment. The applicability of *E. coli* as an indicator of unsanitary handling of raw meats has been challenged because of its ability to grow in meat. If, however, raw meats are vacuum packaged and stored at refrigerated temperatures of 4°C or below, further contamination and growth of *E. coli* should not occur. The *E. coli* load of the meat, therefore, should reflect the sanitary condition (handling) in which the meat was prepared as wholesale cuts.

Various members of the family *Enterobacteriaceae*, including some coliform bacteria, are able to grow below 4°C and may constitute a significant portion of the microflora of VP meat after extended storage (Genigeorgis, 1985). In a study of *Enterobacteriaceae* at different stages of the meat-processing chain, Stiles and Ng (1981b) detected *E. coli* type I and *S. liquefaciens* in meats at all stages of the system. *Enterobacter agglomerans* and *S. liquefaciens* were the predominant *Enterobacteriaceae* at the retail level. Therefore, in raw meat systems, it is important to distinguish between *E. coli* and other coliform bacteria.

In the 1970's standards for *E. coli* in ground beef were proposed in Canada and were actually implemented in some of the United States (Goepfert, 1976; Pivnick *et al.*, 1976). The necessity and validity of standards for *E. coli* in ground beef have been questioned (Goepfert, 1976; Tompkin, 1983), and in Canada standards were never implemented. However, standards for *E. coli* in raw ground beef may still be useful for quality control monitoring at industry and wholesale levels.

The MPN technique is accepted for the detection of coliform bacteria in meats (HPB, 1974). Although the method is very sensitive, it is laborious and requires further testing for confirmation of faecal coliform bacteria or *E. coli* biotype I. Reports have indicated that direct plating techniques are preferred to the MPN technique, because of lower variability and shorter testing time (Rayman *et al.*,

1979; Rayman and Aris, 1981). However, plating techniques are only useful when levels of bacteria are greater than 10 or 100 CFU/g. There have also been many proposals for direct incubation of MPN tests at elevated temperatures as a rapid alternative for determining *E. coli* in foods. Stiles and Ng (1980) demonstrated that this gave unreliable results for detection of *E. coli* in meats.

In this study, the reliability of MPN counts in EC broth incubated at 45°C differed markedly between experiments. Although many factors were evaluated, the variability of the MPN counts could best be explained by differences in the inherent background microflora. Because meat is a biological system in which both chemical and microbiological changes are occurring, it is impossible to eliminate all of the incidental factors. In an attempt to control the background microflora of the meats, with minimal changes in chemical properties; samples were irradiated with a low dose of γ-irradiation (*ca.* 300 Krad). This was effective in reducing the background population to close to 1×10^2 CFU/g in all but one of the trials (Table 4.4).

Reports have indicated a possible secondary bactericidal effect from radiolytic products in irradiated media. Dickson and Maxcy (1984) showed that radiation end products could inhibit some organisms on experimental media containing very low nutrient concentrations. However, no inhibition could be detected when *E. coli* was incubated at 5°C on the surface of meat irradiated with doses up to 1,500

Krad. This indicates that levels of irradiation used in this study should not have a deleterious effect on the test organisms.

The different patterns observed in the growth of the adventitious microflora could possibily be explained by differences in the initial microflora of the meats. In irradiated meats, a microflora that is particularly resistant to irradiation might not have a long lag phase. In the presence of a slower growing background population, consistent MPN results were observed, whereas a rapidly increasing background population seemed to correlate with increased variability of the MPN results.

In a vacuum package respiration of the meat and metabolism of the inherent microbial flora reduces the oxygen tension, and increases the concentration of carbon dioxide within the package (Ingram, 1962). Growth of the aerobic spoilage flora is generally inhibited and a lactic acid population usually predominates (Roth and Clark, 1972). It is generally accepted that total counts in aerobically packaged ground beef can reach levels of $>10^9$ CFU/g. However, in vacuum packaged fresh meats, the total bacterial population increases at a slower rate and does not attain the levels reached in aerobically packaged meat stored at the same temperature (Erichsen and Molin, 1981; Pierson *et al.*, 1970). In this study, the maximum population of bacteria achieved in irradiated meat was approximately 1×10^7 CFU/g, while levels in unirradiated meat reached 1×10^8

CFU/g. It is assumed that the higher total counts in the unirradiated meat were due to the non-lactic spoilage microflora.

In this study, in unirradiated meat, lactic acid bacteria predominated the microflora after 6 days. Counts of all other bacterial parameters also increased, although at a lower rate. Other investigators have reported findings which both contradict and confirm this observation. Pierson *et al.* (1970) did not detect growth of pseudomonads in VP meats. In fact, counts of *B. thermosphacta* and "gram negative/oxidase negative bacteria" actually declined. Roth and Clark (1972) and Foegeding, Naumann and Stringer (1983) did not detect a change in the numbers of either pseudomonads or *B. thermosphacta* during storage of VP beef. However, Dainty *et al.* (1979) found that levels of *B. thermosphacta* and gram negative bacteria steadily increased over the first 2 weeks of storage. Sutherland, Patterson and Murray (1975) reported similar results. Contradictions in the literature may be due to differences in microbial flora at the time of packaging or to the use of different selective media, especially in the case of the pseudomonads.

Irradiation was effective in reduction of all bacterial parameters tested except for the lactic acid bacteria. After irradiation, the predominant organisms remaining in the meats grew on MRS(5.6) agar and they were gram positive coccus-shaped organisms. This differs from the report of Welsh and Maxcy (1975) who found that the residual

microflora after irradiation of ground meat was predominantly gram negative coccobacilli which they classified as *Moraxella* spp. In contrast, Wolin, Evans and Niven (1957) reported that *B. thermosphacta* became the dominant organism after exposure of meat to low levels of irradiation. All isolates from this study could be distinguished from both *Moraxella* spp. and *B. thermosphacta* by their growth conditions, gram stain and catalase reaction (Buchanan and Gibbons, 1974; Krieg, 1984a) and they could be loosely classified as lactic acid bacteria. In most reports, it is claimed that the lactic acid bacteria developing in VP meats are largely lactobacilli (Pierson *et al.*, 1970; Roth and Clark, 1972), however, Foegeding *et al.* (1983) found that the majority of lactic acid bacteria isolated from VP meats were coccus-shaped bacteria.

Lactic acid bacteria are known to have an inhibitory effect on the growth of many other microbes and as a consequence they frequently dominate the microflora of a food (Hurst, 1973). The inhibitory effects of the lactic acid bacteria are thought to be due to the production of lactic acid, antimicrobial substances and hydrogen peroxide (Hurst, 1973; Nassos, King and Stafford, 1985). The development of gram negative spoilage bacteria in ground beef stored aerobically at 7°C was shown to be inhibited by the addition of high levels of a mixed culture of *Streptococcus lactis* and *Streptococcus citrovorum* (Reddy, Henrickson and Olson, 1970; Reddy and Chen, 1975). Gilliland

and Speck (1975) also reported an inhibition of the spoilage microflora of ground beef when it was inoculated with high levels (1×10^9 CFU/g) of lactobacilli and pediococci. Species of *Serratia* and *Enterobacter* isolated from meats were shown to be inhibited on plating media by a variety of lactic acid bacteria (Dubois *et al.*, 1979). Members of the genus *Streptococcus* were particularly inhibitory. Although strains of *E. coli* were not tested by these authors, it is possible that *E. coli* would also be affected in the same manner.

When lactic acid bacteria were added with *E. coli* to NB there was a striking effect on the viability of *E. coli*. After 14 days of storage in NB at 4°C the number of *E. coli* was reduced to <10 CFU/g. This effect could not be attributed to the production of acid because the pH of the NB did not change during the course of the experiment. In the meat, the addition of lactic acid bacteria did not result in a decrease in viability of the test strain of *E. coli*. However, it is possible to speculate that the "antagonistic" effects were manifested in injury of the cells resulting in increased sensitivity of *E. coli* to direct incubation at 45°C.

A major observation in this study was the reproducability of the plate counts of *E. coli* stored in irradiated meat when determined on selective growth media and incubated at both 35 and 45°C. For plate counts of unirradiated meats determined at 35°C, results are unsatisfactory because of

interference, even on selective media, from the background microflora. In fresh meats *E. coli* biotype I may make up only 10% of the "coliform" colonies isolated from VRB incubated at 35°C (Pierson, Emswiler and Kotula, 1978). At 45°C, all media are more specific for *E. coli*. However, this temperature is close to the maximum for growth of the *E. coli* organisms, so that, extremely accurate temperature control is necessary during incubation. At incubation temperatures slightly above 45°C variable colony sizes of the *E. coli* organisms occurred. This was often to the extent that they would not be considered "typical" *E. coli* or faecal coliform bacteria in terms of the standard definition and would, therefore, give rise to erroneous results. The data indicated that the decreased colony size was not attributable to cold injury, because colonies of unchilled *E. coli* gave the same response. This phenomenon may be even more significant in the natural situation because of the presence of mixed strains of *E. coli* and their different physiological states.

Injury of *E. coli* to growth on selective media was not observed in the ground meat experiments. However, during storage in NB with or without added lactic acid bacteria, injury on both VRB and TBA occurred. Since injury occurred to the same extent in the presence or absence of lactic acid bacteria, the effect may be attributed to cold storage rather than the competing microflora. Differences in viability and injury of *E. coli* noted in NB, as opposed to

meat, suggest that studies of bacterial injury in broths may not be directly appliciable to food systems. Where possible, such studies should be conducted in foods.

There are many reports of differences in MPN counts that have been attributed to growth of *E. coli* without gas production. This phenomenon was reported for *E. coli* grown at both 35 and 45°C, however it was more pronounced at 45°C (Anderson *et al.*, 1980; Meadows, Anderson and Patel, 1980a). This effect is most often observed in media that are not buffered (Hajna, 1937; Anderson *et al.*, 1979; Meadows *et al.*, 1980b). Effects are increased when more than one selective factor is used such as the combination of chemical inhibitors and elevated incubation temperature (Meadows *et al.*, 1980a). In this study, lower MPN counts at 45°C were due to the absence of growth, rather than growth without gas production. This may be explained in either of two ways: only a portion of the *E. coli* population is able to initiate growth at elevated temperatures, or greater than one cell per tube is required to initiate growth. Cultures generally grew normally on plating media at 45°C, therefore it would seem that the latter suggestion is more applicable to these data. It is not uncommon for a selective medium to support growth when heavy inoculations are made, such as the inoculation of loops from slants or overnight broth cultures. These same broths may be inhibitory when only a few cells are inoculated.

The importance of media selection was also demonstrated in that LST broth incubated at 35°C, followed by incubation in EC broth at 45°C, gave counts equivalent to the plate counts. Inoculation of EC broth incubated at 35°C gave reduced MPN counts by as much as 1.5 log cycles compared with plate counts. It therefore appears that EC broth does not support initiation of growth at 35°C as well as LST broth. Meadows *et al.* (1980a) showed that lower counts in EC broth at elevated temperatures were due to a combination effect of bile salts and elevated temperatures. They observed higher counts of *E. coli* in EC⁻ broth at elevated temperatures. However in this study, although higher counts were recorded in EC⁻ broth than in EC broth at 35°C, at 45°C identical counts were obtained in EC and EC⁻ broths. This suggests that bile salts were the cause of lower counts at 35°C, but they did not necessarily have an effect at 45°C.

Permanent gas negative variants were isolated among *E. coli* #1840 colonies growing on VRB after storage in NB at 4°C. These variants were also nitrate reductase negative. This enabled other gas negative variants to be isolated from meat and NB stored at 4°C using the chlorate resistance technique. The fact that equal levels of variants were found in NB and meat indicated that the original culture inoculated into the sample might have contained the *chl* mutants. It would seem desirable to study the incidence of these organisms throughout storage in NB and meats before the practical significance of this observation can be

determined.

From these studies it is difficult to conclude whether there was an effect of cold storage on the reliability of the MPN counts for *E. coli* at elevated temperatures, because of the great variability observed between trials. This study confirmed that *E. coli* in VP meats at refrigerated temperatures do not die and do not become injured for growth on selective plating media, such as VRB and TBA, or to growth in broths used for standard MPN techniques. The use of EC broth incubated at 35°C or the use of EC or LST broth at 45°C cannot be recommended as alternative testing procedures to the standard MPN technique. In developing modified or rapid methods for detection of *E. coli* or faecal coliform bacteria in meats, particular attention should be given to the effects of the inherent background microflora on the reliability of detection and to the precise control of incubation temperatures.

Further studies arising out of this research should be directed toward the effects of added lactic acid bacteria to meats with a low background microflora, the characterization of the lactic acid bacteria isolated in this study and the antagonistic property(ies) they exibit toward *E. coli* when stored in NB as opposed to meat. It would also be appropriate to determine the effect of storage of meats on the appearance of chlorate resistant mutants. To this time, the appearance of anaerogenic variants in meats and water have been detected by chance isolation and observation.

Screening for mutants lacking a functional formic-hydrogenlyase system using a technique such as that described by Mandrand-Berthelot *et al.* (1978) should allow for easy detection and quantification of anaerogenic variants occurring upon refrigerated storage in meats.

Although techniques are now available to test for a wide variety of pathogens in foods it is unlikely that it will ever become practical to do so. Distribution of pathogens in foods is generally uneven and isolation from the abundance of other food organisms is difficult. Apart from the classical enteric pathogens, such as species of *Shigella* and *Salmonella*, many other potentially harmful organisms may also be spread by foods. Testing a food lot for all potential pathogens is therefore unrealistic. For these reasons indicator organisms will continue to be used to monitor the safety and quality of food products. *E. coli* appears to be the most reliable indicator for raw meats.

Despite the fact that nearly 10% of *E. coli* strains are late or non-lactose fermenters and others may be anaerogenic, the aerogenic lactose positive phenotype remains the basis of the majority of tests used to identify coliform organisms and *E. coli* in food and water. This is a problem inherent in the definition of a coliform organism, however, it is highly unlikely that a phenotype that is inclusive of, and exclusive to *E. coli* will ever be found.

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