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UNIVERSITY OF ALBERTA

Practical Application of Hazard Analysis Critical Control Point (HACCP) Analysis to Enhance the Safety of a Cooked Meat Product

by

Connie E. Zagrosh - Miller

A THESIS

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Practical Application of Hazard Analysis Critical Control Point (HACCP) Analysis to Enhance the Safety of a Cooked Meat Product

submitted by **Connie E. Zagrosh - Miller**

in partial fulfillment of the requirements for the

degree of **Master of Science**

in **Food Microbiology**

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Abstract

Meat products provide an excellent medium for the growth of microorganisms. Cooked meat products are susceptible to post-cooking contamination and time/temperature abuse. Extended storage and a lack of a competitive microflora create concern for product safety. Cooked hamburger patties were packaged in thermoform (40% CO_2 with the balance N_2) and 50% CO_2 and 50% air packaging and stored at 4 and 10°C. Thermoform packaging with storage at 4°C was the more effective in repressing microbial growth. Variability in the development of lactic acid bacteria was observed.

Two ways of improving the microbiological safety of cooked ground beef were examined. The first was through the application of Hazard Analysis Critical Control Point (HACCP) analysis to the production of modified atmosphere packaged hamburger sandwiches. The cooking process, raw material quality, time/temperature relationships, hygiene of employees and equipment sanitation were identified as the critical control points that needed to be controlled and monitored. The other was to influence the development of a predictable lactic microflora by the addition of *Carnobacterium piscicola* to hamburger meat. *Carnobacterium piscicola* (UAL 26.CL97) was inoculated into hamburger patties prior to cooking. Due to heat sensitivity of the organism initial survival was only 17% when patties were cooked to temperatures greater than 70°C. Storage at 4°C allowed heat injured cells to resuscitate with 58% of the patties developing a *Carnobacterium piscicola* microflora. Although UAL 26.CL97 did not develop in 100% of the patties, in those where it did, it dominated the microflora. The ability of UAL 26.CL97 to grow and produce bacteriocin in hamburger meat was demonstrated. *Carnobacterium piscicola* was capable of suppressing the growth of *Enterococcus faecium* and *Listeria monocytogenes* in a meat system.

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1. INTRODUCTION

The Hazard Analysis Critical Control Point (HACCP) concept provides an alternative to the traditional control of food quality and safety. HACCP offers a rational approach to the control of microbiological hazards in foods, avoids the many weaknesses in the inspection approach to quality assurance and circumvents the shortcomings of reliance on microbiological testing (ICMSF, 1988). This system can be applied to all segments of a food system, from production to processing, through distribution and to consumption of a food product. HACCP plans must be related to the unique conditions existing for each food product and each process within each establishment, and must be updated as changes are made (Tompkin, 1990).

Meat and meat products are highly sensitive to microbial spoilage and are capable of supporting the growth of pathogenic microorganisms. This has been demonstrated by the frequent involvement of meat products in foodborne illness (Bryan, 1974). The application of HACCP to the production of meat products is a practical approach to improving the microbiological safety of these products. Traditionally, meat has been preserved by drying, salting and fermenting. Fermentation by lactic acid bacteria extends the shelf life and ensures safety of meat products. This is accomplished by the ability of lactic acid bacteria to repress the growth of spoilage and pathogenic microorganisms through the production of antagonistic substances, such as lactic acid, carbon dioxide, hydrogen peroxide, diacetyl and bacteriocins. Refrigeration has also been used to retard microbial growth. The type of microflora developing in refrigerated meats is influenced by the method of packaging. Modified atmosphere packaging of meat products in elevated CO_2 or under vacuum in low gas permeable film inhibits the growth of aerobic spoilage organisms and favors the development of lactic acid bacteria (Christopher

et al., 1979; Egan, 1983). However, it was observed that the development of lactic microflora in cooked hamburger sandwiches does not develop in the same way as in other modified atmosphere packaged meats (M^{c}Mullen and Stiles, 1989).

Lactic acid bacteria isolated from meat have been shown to produce bacteriocins (Schillinger and Luecke, 1989 and 1990; Ahn and Stiles, 1990; Hastings and Stiles, 1991). The inhibitory activity of bacteriocins may aid lactic acid bacteria in extending shelf life of meat products without acidification. Lactic acid bacteria from meat should be the best candidates for improving the microbiological safety of meat products, because they are adapted to the conditions in meats and should therefore be more competitive than lactic acid bacteria from other sources (Schillinger and Luecke, 1989). Antimicrobial activity of lactic acid bacteria has resulted in the study of inhibitory activity in meat systems.

The objective of this study was to look at a practical approach of improving the microbiological safety of cooked hamburger patties intended for the production of modified atmosphere packaged sandwiches. This study is divided into two sections:

1. The application of HACCP analysis to hamburger sandwich production.
2. Evaluation of the survival, growth and bacteriocin production of *Carnobacterium piscicola* (UAL 26.CL97) in hamburger meat.

2. LITERATURE REVIEW

2.1 Hazard Analysis Critical Control Point

2.1.1 History

The Hazard Analysis Critical Control Point (HACCP) system was developed by the Pillsbury Company in cooperation with National Aeronautics and Space Administration (NASA) and U.S. Army Natick Laboratories in an effort to assure safety of food products intended for space travel. The concept of HACCP was first presented at the 1971 U.S. National Conference on Food Protection (ICMSF, 1988; Buchanan, 1990). This concept generated excitement within the food industry and resulted in the incorporation of HACCP as a means of insuring the safety of low-acid canned foods.

2.1.2 Definition of Terms

HACCP is a preventative control system (Bauman, 1974). It includes the assessment of potential hazards, prescribes for the elimination of avoidable hazards and sets tolerances for the hazards that cannot be eliminated in the processing of a food. It defines appropriate control measures, frequency of their application, sampling program, specific tests to be applied and criteria for product acceptance. It gives a rational, systematic, documented procedure which can be used for organizing and implementing the entire quality assurance program (Peterson and Gunnerson, 1974; Munce, 1984).

HACCP can be divided into two parts:

1) Hazard Analysis - which is the identification of sensitive ingredients, critical process points and relevant factors as they affect product safety.
2) Critical Control Points - which are processing factors for which loss of control would result in an unacceptable food safety risk (Bauman, 1974; Munce, 1984).

2.1.3 Principles of HACCP

The implementation of HACCP involves seven principles:

1. Hazard analysis;
2. Identification of critical control points (CCP);
3. Establishment of critical control point limits;
4. The establishment of procedures to monitor control;
5. The development of corrective action for critical control point deviation;
6. Effective record keeping and;
7. Verification.

(ICMSF, 1988; Buchanan, 1990; Tompkin, 1990).

Each of these principles will be discussed below.

1. Hazard analysis. This identifies hazardous materials and potential sources of contamination associated with growing, harvesting, raw materials, processing, marketing, preparing and consuming a food (NACMCF, 1989). Emphasis is placed on potential sources of contamination, temperature/time relationship, pH and water activity. In addition, hygiene of the food preparation area, personal hygiene of employees and cleanliness and sanitation of equipment are important.

Efficient assessment of hazard analysis requires a team of individuals from various disciplines (ie. microbiology, processing, engineering, quality control). Construction of a flow diagram detailing all of the processing steps is valuable for the hazard analysis. Flow diagrams serve several functions:

(a) aid in identifying hazards and their critical control points;

(b) indicate material movement and interrelationships between product lines;

(c) show transportation steps;

(d) point out potential problem areas and delays in production

(e) optimize the use of space and equipment (Smith *et al.*, 1990).

The flow diagram is specific for a food product, a process, equipment and the manufacturing facility. Any changes to any of these specifications requires a new hazard analysis.

Hazard analysis needs to be quantitative to be meaningful (Tompkin, 1990). There are two parts in hazard assessment:

1. ranking the food according to six hazard characteristics - assessment of risk;
2. assigning a risk category based on the ranking - assessment of severity (NACMCF, 1989).

Ranking of a food according to hazard characteristics is based on:

(a) presence of microbiologically sensitive ingredients;

(b) efficiency of process to destroy harmful microorganisms;

(c) risk of post-process contamination;

(d) potential for abusive handling during distribution or at home;

(e) no terminal heat process after packaging or when cooked at home.

Once the food is ranked according to these characteristics, the risk category is assigned depending on the product's hazard characteristics. There is a special risk category (VI) which consists of non-sterile food products which are intended for consumption by individuals at high risk, such as the immunocompromised, the elderly and infants.

2. Identification of critical control points. This is defined as a location, practice, procedure or process at which control can be exercised over one or more factors which, if controlled, could minimize or prevent a hazard (ICMSF, 1988; Tompkin, 1990). The purpose of CCPs is to control product safety. One aspect of control is the control of microbial contamination and proliferation. Thus any steps which destroy or prevent the growth of microorganisms are considered CCPs. These include: heating, refrigeration, sanitation, cross contamination, personal hygiene, environmental hygiene and time/temperature relationships. It is also important to

consider the handling of the product during distribution and handling by the consumer.

ICMSF (1988) proposed a two-class CCP system. CCP1 assures control of a hazard, whereas CCP2 minimizes the hazard but does not assure control. CCP1 refers to an operation where hazards are eliminated or prevented, ie. pasteurization or cooking and CCP2 refers to reduction of hazards, ie. refrigeration or sanitation.

3. Establishment of CCP limits. These are one or more prescribed tolerances that must be met to insure that a CCP effectively controls a microbiological health hazard (NACMCF, 1989). Criteria that may be used for critical limits may include properties such as: pH, time, temperature, water activity, aroma, texture and others depending on the product being processed.

4. Establishment of procedures to monitor control. Monitoring is the scheduled testing or observation of CCP and its limits (NACMCF, 1989). Continuous monitoring is the most effective, but this can be impractical under some circumstances, thus specified periodic monitoring will suffice.

Physical, chemical, visual and microbiological methods can be used to insure that CCPs are under control. Visual observations can be very effective. On-line visual inspection or quick chemical or physical tests are preferred. Individuals involved in inspection need to know what to look for, tc be able to identify deviations and need to know what corrective action to take.

Microbiological testing is not usually done to insure that CCPs are under control because of the time involved in obtaining a result. There are two exceptions to this. Microbiological testing is used as a monitoring procedure for raw ingredients prior to processing and for a finished product that is to be consumed by a sensitive population.

5. Development of corrective action for CCP deviation. When a CCP has deviated from its limit, prompt action is required to correct it. Corrective action for

each CCP has to be developed because CCPs vary between products and processes. Any product produced during the time a CCP is out of control should be held until measures have been taken to insure that the product is safe.

6. Effective record keeping. This involves documentation to show that the HACCP plan is followed as designed. Records should show specifications for ingredients, packaging material, temperature, processing and product shelf life. Any deviation in CCPs must be recorded as well as the corrective action taken. This information verifies that the manufacturer has taken all of the necessary steps to insure that the products are safe. All records should be available for review.

7. Verification. This is the use of supplementary information to check whether the HACCP system is working (Tompkin, 1990). It is the responsibility of both the processor and the regulatory agency to insure that the HACCP system is operating properly. To do this, a review of the existing HACCP plan and the records may be necessary. Additional testing, such as microbiological testing, is also helpful in verifying the HACCP system is functioning properly. Microbiological testing of products at various stages of the process, finished product testing, shelf life studies and verification of proper equipment sanitation all aid in verifying that the HACCP system is functioning as designed.

2.1.4 Application of HACCP to Meat Products

Meat and meat products can be sources of pathogenic microorganisms and have frequently been implicated in foodborne illness (Bryan, 1980; Tompkin, 1990). Application of HACCP to the processing of meat products shifts the emphasis from the microbiology of the finished product to raw material and process control (Smith *et al.*, 1990). There are various potential hazards which may be associated with meat (Tompkin, 1990). These include: raw materials, contamination during processing, potential of microorganisms to survive and(or) grow during storage, distribution and use by the consumer. CCPs that require monitoring in the processing of a meat

product include: ingredient or raw product control, time/temperature control, equipment sanitation and employee hygiene.

Raw material quality influences the quality of the finished product. It is therefore important that high quality raw materials are purchased from reputable suppliers. The purchaser should obtain from the supplier information regarding product composition and microbiological status. Temperature control during shipment is important to assure high quality raw materials on arrival at the processing plant. Upon receipt of the raw materials at the processing plant, it is the responsibility of the plant to verify that the raw materials have not been temperature abused. All materials should be visually examined and promptly transferred to the appropriate storage conditions.

Frozen foods should be stored at -18°C or below, whereas refrigerated foods should be stored at <5°C (Bobeng and David, 1977). Care should be taken to avoid cross-contamination of raw and cooked foods during refrigeration. Materials not requiring refrigeration should be stored in a clean dry area. Regular rotation of all food ingredients is important to maintain optimum freshness of products.

Time/temperature relationships are the major factor that needs to be controlled when handling meat (Tompkin, 1990). Cooking is an important CCP for meat products. Cooking to 63°C or its equivalent at a lower temperature for a specified length of time, assures the destruction of salmonellae in raw meat (Tompkin, 1990). Temperatures of 74°C and greater should destroy most pathogenic vegetative cells but spores may not be killed. If the product is consumed immediately the spores will not have time to germinate and the product will be safe. Extended storage of the product will allow time for spores to germinate and for post-heat treatment contaminants to grow, which may result in a potential hazard. This is where cold storage is important. Meat should be maintained at -1 to 2°C for

optimum storage (Bobeng and David, 1977). In addition, the package type has an important role in the development of the microflora.

Equipment sanitation and personal hygiene are two CCPs where loss of control could lead to foodborne illness. It has been reported (Bryan, 1974; Bobeng and David, 1977) that the major causes of foodborne illness are related to time/temperature abuse, inadequate cleaning and sanitation of equipment, cross-contamination and poor employee hygiene. Rapid methods of assessing CCP are required to assure control. Visual, aroma and touch observations can be used to insure proper sanitation of equipment. Quick chemical methods such as pH, fat and moisture measurements can be done to monitor formulation of products (Tompkin, 1990). The maintenance of records throughout the process will help to pin-point any problems that may arise. Proper documentation gives the processor some backup reference if problems arise with the product once it has left the processing plant.

2.2 Microbial Quality of Ground Beef

2.2.1 Raw Ground Beef Quality

The manufacture of ground beef involves the grinding of whole muscle tissue resulting in the incorporation of microorganisms throughout the meat. Microbial quality of ground beef depends on the quality of the meat used, sanitary conditions, hygienic practices used during preparation and time and temperature of storage (Duitschaever *et al.*, 1973). Microorganisms distributed by the grinding process have an ideal growth environment. Ground beef has a high water activity (0.96-0.97), favorable pH (5.6-5.8), and the availability of virtually all nutrients, growth factors and minerals necessary for optimum microbial growth (Hammes *et al.*, 1990).

Numerous studies on ground beef quality have been conducted (Kirsch *et al.*, 1952; Ayres, 1960; Duitschaever *et al.*, 1973 and 1977; Goepfert, 1976; Westhoff and Feldstein, 1976; Gill and Newton, 1977). The consensus of these studies was that the quality of ground beef is poor. As a result of the study conducted by Duitschaever *et*

al. (1973) and the growing concern about the microbial quality of ground beef, the Health Protection Branch of Health and Welfare Canada undertook their own survey of ground beef quality in Canada. Results of the survey were published by Pivnick *et al.* (1976) with the following proposed microbial guidelines for ground beef quality:

Aerobic plate count (35°C)	$< 10^7$/g (non-frozen)
Aerobic plate cour ' (35°C)	$< 10^6$/g (frozen)
Escherichia coli	$< 10^2$/g
Staphylococcus aureus	$< 10^2$/g
Salmonella	absent in 25 g

2.2.2 Spoilage Microflora of Raw Ground Beef

The predominant spoilage microorganisms associated with refrigerated, aerobically packaged ground beef are psychrotrophs. Researchers (Kirsch *et al.*, 1952; Ayres, 1960; Gill and Newton, 1977; Harrison *et al.*, 1981) have identified *Pseudomonas* species as the predominant bacteria. Other bacteria that may grow include: *Acinetobacter*, *Moraxella*, *Enterobacter*, *Micrococcus* and *Lactobacillus* species.

Pseudomonas species dominate refrigerated, aerobically packaged ground beef because of their ability to grow faster over wider temperature and pH ranges than competing species and their insensitivity to the presence of other species (Gill and Newton, 1977). Growth of these organisms results in the production of off odors (10^7 organisms/g) and slime (10^8 organisms/g), that leads to the putrefaction of the meat.

Ayres (1960) reported that storage temperature affects the type of microflora which predominates on meat. When meat is stored under proper refrigeration, *Pseudomonas* species predominate with *Micrococcus* species the next most numerous. When the meat was temperature abused (> 10°C) there were equal

numbers of *Pseudomonas* and *Micrococcus* species with bacilli and chromogenic bacteria also present.

The growth of pathogenic organisms in temperature abused meat was not reported by Goepfert and Kim (1975). They concluded from their study on the behavior of foodborne pathogens in ground beef, that potential pathogens such as *S. aureus*, *Bacillus cereus*, *Clostridium perfringens* are unable to compete with the natural flora of raw ground beef over a wide range of refrigeration temperatures. They showed that only *E. coli* and enterococci increased in significant number, and only at 12.5°C.

2.2.3 Hamburger Sandwich Quality

Raw product quality has a great influence on the microbial quality of the cooked product. Studies by Mueller (1975) and Duitschaever *et al.* (1977) have shown that, after cooking, the microbial quality of hamburger is satisfactory with the aerobic plate counts being approximately 10^4 organisms/g or less. Foodborne pathogens were either absent or present in very low numbers. To ensure that acceptable microbial quality is achieved, adequate cooking, proper handling after cooking, proper refrigeration and sufficient reheating prior to consumption are required.

2.3 Modified Atmosphere Packaging of Meat

2.3.1 Action of CO_2

The first practical use of modified atmospheres containing elevated levels of CO_2 as a preservative in the handling of fresh meat was in the shipment of whole beef carcasses from Australia and New Zealand to Great Britian in the 1930's (Lawrie, 1974). The ability of CO_2 to extend the shelf life of numerous meat and fish products is well documented (Dixon and Kell, 1989).

Carbon dioxide inhibits the growth of a number of microorganisms, Gram-negative species being the most sensitive (Sutherland *et al.*, 1977; Enfors and Molin,

1980; Wolfe, 1980). The specific mode of action is not clearly understood, but there are several theories about the antimicrobial activity of CO_2 (Genigeorgis, 1985; Dixon and Kell, 1989). Bacterial inhibition by CO_2 involves the extension of the lag phase and generation time, resulting in a decreased growth rate and delay in spoilage of meat (Veranth and Robe, 1979; Finne, 1982). The effect of CO_2 depends on the concentration of CO_2, age and number of microorganisms, storage temperature and type of food (Genigeorgis, 1985; Dixon and Kell, 1989).

Solubility of CO_2 in meat is affected by pH, temperature and the proportion and composition of fat (Gill, 1988). Carbon dioxide is more soluble in meat at higher pH and low temperatures (Enfors and Molin, 1980) and bacterial inhibition with CO_2 increases as the temperature decreases (Adams and Huffman, 1972). All of these factors determine the quantity and the rate at which CO_2 will be absorbed by meat.

There is no agreement as to the optimum concentration of CO_2 required for maximum shelf life, but it seems to be between 10 and 25%. A concentration of greater than 25% has a detrimental effect on the appearance of the raw meat, causing the conversion of myoglobin to metmyoglobin (Ogilvy and Ayres, 1951; Wolfe, 1980).

2.3.2 Development of Lactic Microflora

The growth of aerobic spoilage organisms, such as *Pseudomonas*, is inhibited when meat is packaged in a film of low gas permeability, less than 100 $mL/m^2/24$ h/atm at 25°C (Egan, 1983). Residual O_2 is utilized by the meat and the resident aerobic flora to produce CO_2, while the surface redox potential of the meat becomes negative, resulting in the suppression of the common psychrotrophic aerobic spoilage organisms (Genigeorgis, 1985). The new environment favors the growth of lactic acid bacteria, *Brochothrix thermosphacta*, and psychrotrophic Enterobacteriaceae (Egan, 1983).

Growth of lactic acid bacteria is stimulated by CO_2 and, upon extended storage at low temperature, the lactic acid bacteria become the dominant microflora. The significance of the domination by lactic acid bacteria is that they grow at a slower rate than aerobic psychrotrophs, this in itself extends the shelf life. Lactic acid bacteria cause spoilage due to souring rather than putrefaction. Spoilage by lactic acid bacteria occurs long after they have reached maximum population compared with spoilage by aerobic bacteria at 10^7 organisms/g.

2.3.3 Safety of Modified Atmosphere Packaging (MAP)

Concern about the potential for pyschrotrophic pathogens to grow to high numbers has been expressed (Hanna *et al.*, 1976; Post *et al.*, 1985; Palumbo, 1986) because of the long shelf life of MAP products. Because of anaerobic storage, this includes concern for growth of *Clostridium botulinum*.

There are currently two kinds of MAP products, those that are raw and require cooking, such as raw meat; and those that are prepared, such as low acid foods, that require little or no heating prior to consumption (Farber *et al.*, 1990). Silliker and Wolfe (1980) studied the effect of MAP on the growth of *C. botulinum* in fresh meat and concluded that elevated levels of CO_2 did not increase the hazard of botulism. However, there is concern about using MAP in the fish industry where non-proteolytic *C. botulinum* has the potential to grow in MAP fish and to produce toxin before the development of detectable spoilage (Post *et al.*, 1985). *Yersinia enterocolitica* has been isolated from vacuum packaged red meat (Hanna *et al.*, 1976). The examination of MAP sandwiches (Steele and Stiles, 1981; Farber *et al.*, 1990) for growth of pathogens showed that the sandwiches did not sustain the growth of pathogens at refrigeration temperatures. With the exception of *Listeria* species, severe temperature abuse was necessary for growth of pathogenic organisms to occur.

2.4 Use of Lactic Acid Bacteria as Preservatives for Meat and Meat Products

Traditionally lactic acid bacteria, represented by the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and more recently *Carnobacterium* (Collins *et al.*, 1987) have been associated with the preservation of a variety of foods by fermentation. Lactic starter cultures have been used by the meat industry in the production of fermented meat products, ie. fermented sausage. Fermentation imparts unique aromas, flavors and textures which are not possible through any other means of processing. Not only is spoilage retarded by the lactic acid bacteria, but also the safety of certain foods is enhanced by the inhibition of pathogenic microorganisms. Inhibition is primarily achieved by the accumulation of organic acids (mainly lactic and acetic) with an accompanying reduction in pH (Lindgren and Dobrogosz, 1990).

In addition to organic acids, lactic acid bacteria produce carbon dioxide, diacetyl, hydrogen peroxide and bacteriocins (Raccach and Baker, 1978; Daeschel, 1989; Lindgren and Dobrogosz, 1990). These metabolites can be inhibitory to other microorganisms. Antimicrobial activity of CO_2 has been discussed in section 2.3.1. Diacetyl has been shown to be inhibitory to yeasts, Gram-negative bacteria and non-lactic acid, Gram-positive bacteria (Jay, 1982). Inhibition is believed to be associated with interference of arginine utilization by diacetyl reacting with the arginine binding protein of Gram-negative bacteria (Jay, 1986). Generation of hydrogen peroxide by lactic acid bacteria occurs by several mechanisms (Gotz *et al.*, 1980; Kandler, 1983). Antagonistic activity of hydrogen peroxide has been shown against *S. aureus* (Dahiya and Speck, 1968) and *Pseudomonas* species (Price and Lee, 1970). This activity is attributed to a strong oxidizing effect on the bacterial cell (Foster *et al.*, 1957) and destruction of the basic molecular structures of cell proteins (Sykes, 1965).

Bacteriocin production among the lactic acid bacteria has been observed in most of the genera, this was summarized in the review by Klaenhammer (1988). Examples of bacteriocins produced include nisin from *Lactococcus lactis* subsp. *lactis* (Kaletta and Entian, 1989; Delves-Broughton, 1990), diplococcin from *Lactococcus lactis* subsp. *cremoris* (Davey, 1984), pediocin A from *Pediococcus pentosaceous* (Daeschel and Klaenhammer, 1985), helveticin J from *Lactobacillus helveticus* 481 (Joerger and Klaenhammer, 1986), lactacin F from *Lactobacillus acidophilus* 88 (Muriana and Klaenhammer, 1987), sakacin A from *Lactobacillus sake* Lb 706 (Schillinger and Luecke, 1989), carnobacteriocin from *Carnobacterium piscicola* LV17 (Ahn and Stiles, 1990) and leucocin A from *Leuconostoc gelidum* (Hastings and Stiles, 1991).

Bacteriocins are proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer organism (Tagg *et al.*, 1976) The lactic acid bacteria produce a diverse group of bacteriocins that are inhibitory to other Gram-positive bacteria. Inhibition of Gram-negative bacteria has not been clearly shown (Klaenhammer, 1988). From his review, Klaenhammer (1988) identified two types of bacteriocins produced by lactic acid bacteria (a) bacteriocins that have a narrow range of inhibitory activity, inhibiting only closely related strains; and (b) bacteriocins that have a wide range of inhibitory activity, inhibiting numerous Gram-positive genera. Bacteriocin production has been shown to be associated with both plasmid DNA (Scherwitz *et al.*, 1983; Davey, 1984; Daeschel and Klaenhammer, 1985; Van Belkum *et al.*, 1989 and 1991) and chromosomal DNA (Barefoot and Klaenhammer, 1983; Joerger and Klaenhammer, 1986 and 1990).

Antimicrobial compounds produced by the lactic acid bacteria have provided these organisms with a competitive advantage over other microorganisms. Bacteriocins produced by lactic acid bacteria have been shown to be inhibitory

against pathogenic organisms in culture media: (a) *Listeria monocytogenes* (Carminati *et al.*, 1989; Harris *et al.*, 1989; Raccach *et al.*, 1989; Schillinger and Luecke, 1989); (b) *C. botulinum* (Scott and Taylor, 1981; Okereke and Montville, 1991); (c) *S. aureus* (Spelhaug and Harlander, 1989); and (d) *B. cereus* (Spelhaug and Harlander, 1989). Spelhaug and Harlander (1989) also showed slight inhibition of several Gram-negative organisms.

Antibiosis of lactic acid bacteria against spoilage and pathogenic microorganisms in culture media sparked interest in the use of lactic acid bacteria as food preservatives in meat systems. Antagonistic activity of lactic acid bacteria in meat systems has been demonstrated in two ways: (a) by the addition of bacteriocin, such as nisin (Calderon *et al.*, 1985; Chung *et al.*, 1989) or pediocin PA-1 (Nielsen *et al.*, 1990) to the meat product; and (b) inoculation of meats with a bacteriocin-producing strain which must grow and produce bacteriocin in the meat system, as studied in ground beef (Reddy *et al.*, 1970), deboned poultry meat (Raccach and Baker, 1978), beef steaks (Hanna *et al.*, 1980), emulsion sausage (Nielsen and Zeuthen, 1985) and pork (Schillinger and Luecke, 1987 and 1990).

Lactic acid bacteria originally isolated from meat and meat products are probably the best candidates for improving the microbiological safety of these foods, because they are well adapted to the conditions in meat and should therefore be more competitive than lactic acid bacteria from other sources (Schillinger and Luecke, 1989). In order for lactic acid bacteria to be successful in their preservative role they must be active against undesirable microflora but at the same time they must not alter the meat or impart any toxic effects to the consumer. With the development of methods to transfer genetic material between strains and to expand the inhibitory spectrum of bacteria, the future holds great promise for the enhancement of food safety by the lactic acid bacteria.

2.5 Characteristics of Strains of Carnobacterium piscicola

2.5.1 Carnobacterium piscicola LV17 (UAL 8)

Carnobacterium piscicola LV17 was first isolated from vacuum packaged beef as a nonaciduric "Streptobacterium" (*Lactobacillus carnis* LV17) by Dr. B.G. Shaw (Langford, Bristol, UK). On closer examination of this organism and similar strains (Holzapfel and Gerber, 1983; Hiu *et al.*, 1984; Shaw and Harding, 1985) differences were observed from the *Lactobacillus* species. Collins *et al.* (1987) proposed a new genus *Carnobacterium* for these organisms based on their inability to grow on acetate agar, low G + C content, ability to grow at high pH (8.5 to 9.5) and synthesis of oleic acid instead of cis-vaccenic acid.

C. piscicola LV17 produces bacteriocin early in its growth cycle, which has been characterized by Ahn and Stiles (1990) as proteinaceous, with a bactericidal mode of action, and a relatively narrow inhibitory spectrum (in agreement with Schillinger and Holzapfel, 1990) but includes *Enterococcus* species and *L. monocytogenes*. It is stable over a wide pH range and it is heat resistant. Bacteriocin production is inhibited at pH <5.5, but the organism itself is capable of growth at pH 5.0. Ahn and Stiles (1990) showed that bacteriocin and immunity are associated with two plasmids, pCP40 and pCP49, and that activity of the bacteriocin(s) is directed towards the cytoplasmic membrane of sensitive cells.

2.5.2 Carnobacterium piscicola (UAL 26)

UAL 26 was isolated from vacuum packaged meat by Burns (1987) at the University of Alberta. UAL 26 differs from UAL 8 in that it cannot grow at a pH <5.5, bacteriocin production occurs at the end of the log phase and it is believed to be chromosomally mediated. The antibacterial spectrum includes other lactic acid bacteria, *Bacillus* and *Clostridium* species, *E. faecalis* and *L. monocytogenes*.

2.5.3 Carnobacterium piscicola (UAL 26.CL97)

Carnobacterium piscicola (UAL 26.CL97) was genetically derived by Ahn (1991). This organism contains the gene for bacteriocin production from the plasmid pCP49 cloned into pCaT plasmid which encodes for chloramphenicol resistance (Cm^R). Strain UAL 26.CL97 produces its own chromosomally mediated bacteriocin and the bacteriocin mediated by pCP49. Chloramphenicol resistance provides a useful marker for the selection of UAL 26.CL97.

Characteristics of *C. piscicola* and its antibacterial activity indicate that this microorganism may be applicable as a meat preservative. Therefore this study will look at improving the safety of cooked hamburger through HACCP analysis and by inoculation of the meat with UAL 26.CL97 to obtain a predictable lactic acid microflora in the cooked meat product.

3. HACCP ANALYSIS FOR MANUFACTURE OF HAMBURGER SANDWICHES

3.1 Introduction

Hazard analysis of hamburger sandwiches was conducted at Quality Fast Foods Ltd., 12251 William Short Road, Edmonton, Alberta. Quality Fast Foods is a federally inspected meat processing establishment (number 398) under the jurisdiction of Agriculture Canada. It has been in existence for fifteen years, and it has been located at its present address for the past three and a half years. Quality Fast Foods processes a variety of modified atmosphere packaged (MAP) sandwiches on three packaging lines. Two of the packaging lines incorporate 50% CO_2 into the packages with the balance of the atmosphere air, the third line uses thermoform packaging, in which the air is evacuated and replaced with the required modified gas atmosphere (40% CO_2 with the balance nitrogen). In this study the HACCP analysis was done for hamburger sandwich preparation. The HACCP analysis was based on procedures outlined by ICMSF (1988), NACMCF (1989), Smith *et al.* (1990) and Tompkin (1990).

3.2 Product Description

Frozen raw hamburger patties were supplied by J.D. Sweid & Co., Ltd. of Burnaby, B.C. These patties included a mixture of beef, water, binder (toasted wheat crumbs, spice, hydrogenated vegetable oil) and salt. The patties were processed by combining a percentage of 60/40 of fresh/frozen meat and the patties were formed by running the meat through a Tender-form process (Formax Inc., Mokena, IL), "scored" on both sides and frozen in a mechanical spiral freezer. The tender-form process and "scoring" provide greater surface area; thus more efficient cooking, more efficient freezing and increased product yield. The frozen raw hamburger patties were cooked in an automatic broiler (NIECO - Burlingame, CA)

for 3 min to an internal temperature of approximately 75-80°C and cooled in a 4°C cooler overnight prior to sandwich assembly and packaging.

Sandwiches were packaged in MKS 5200 film - 50 gauge polyester/saran/1 mil polyethylene/1 mil surlyn; thickness 2.6 mils (Curwood Packaging, Georgetown, Ontario) in a gas mixture of approximately 50% CO_2/50% air. The oxygen permeability of the film is 0.5-0.8 cc/100 sq. in./24 h at 23°C, 0% RH with a moisture vapor transmission of 0.3 g moisture/100 sq. in./24 h at 37.8°C and 90% RH.

A complete product description for the hamburger sandwiches is summarized in Table 3.1.

3.3 Flow Diagram

The flow diagram for hamburger sandwich production is outlined in Figure 3.1. It outlines the operations in the production of hamburger sandwiches from the receiving of raw materials, through the cooking process, assembly of sandwiches, to storage and distribution of the finished product.

3.4 Hazard Analysis

3.4.1 Food Hazards

In identifying food hazards it is necessary to consider microbiological, chemical and physical aspects of the product. It is believed that microbiological hazards pose the greatest threat to consumers (Smith *et al.*, 1990). Microbiologically the hamburger patties are the most sensitive food ingredient in the production of the hamburger sandwiches. Raw ground beef has the potential to be a source of a number of spoilage and pathogenic microorganisms. Spoilage organisms pose no real threat to the well-being of the consumer, unless under special circumstances. Potentially pathogenic organisms associated with ground beef include: *S. aureus*, enterotoxigenic or enterohemorrhagic *E. coli*, *C. perfringens* and *L. monocytogenes*.

Table 3.1. Product Description for Hamburger Sandwiches

Product description	Variety of heat-and-serve hamburger sandwiches with a shelf life of 35 days under refrigeration.
Ingredients	Hamburger patties, hamburger bun, cheese, ketchup
Raw Hamburger Composition[a]	Fat (20%), moisture (58%), protein (17%), carbohydrate (2.75%), salt (0.3%)
Packaging	MKS 5200 film. Gas mixture approximately 50% CO_2/50% air.
Processing	Patties cooked 3 minutes to an internal temperature of 75-80°C. Cooled overnight to 4°C.
Labelling	List of ingredients. Type of packaging. Storage instructions. "Best Before" date.

[a]Composition information provided by J.D. Sweid & Co., Ltd.

except for salt result - analyzed at the Food Laboratory,

Alberta Agriculture.

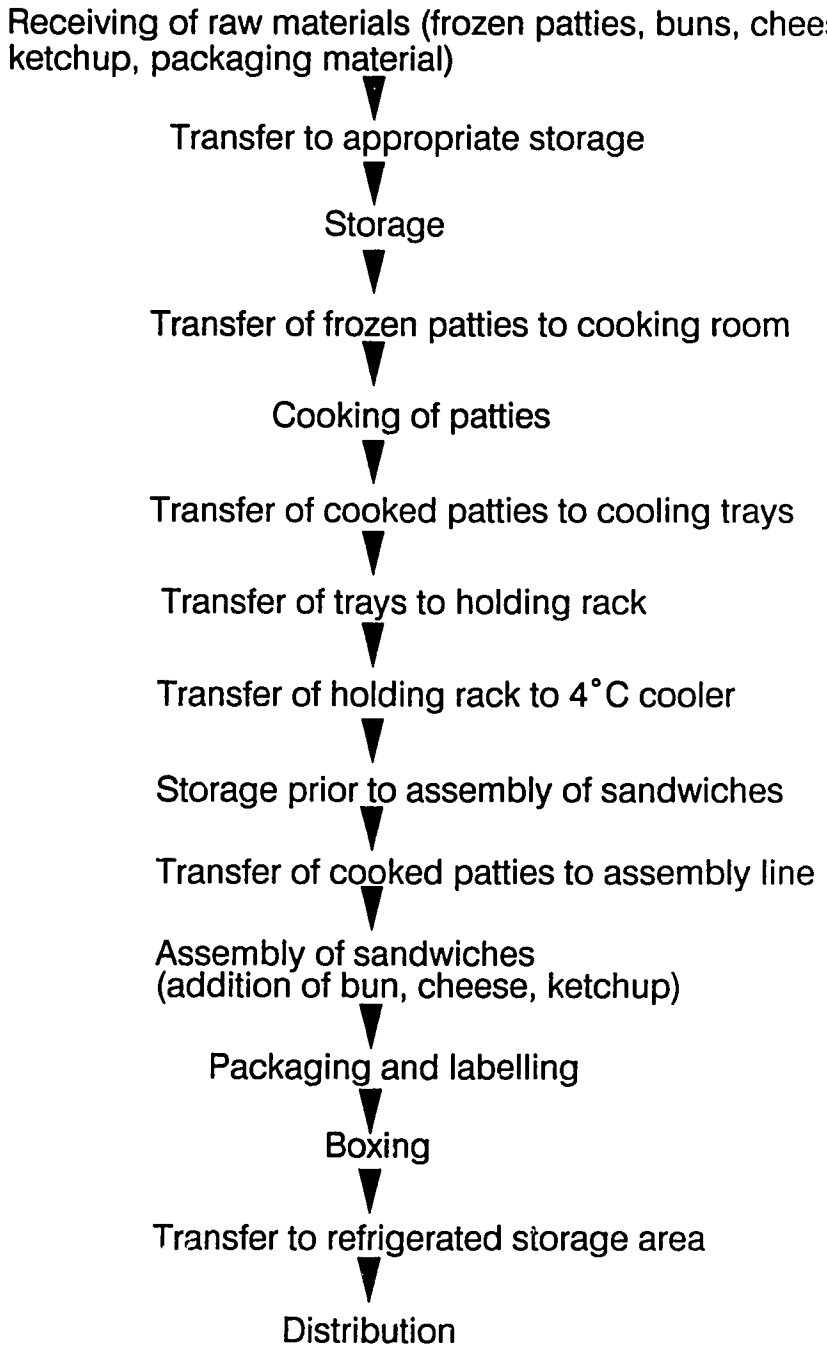


Figure 3.1. Process Flow Diagram for a Hamburger Sandwich

Thorough cooking should eliminate potential hazards of the raw patties. But the cooked patties then become susceptible to cross-contamination, either by contact with raw materials, infected employees and(or) employees practicing poor personal hygiene.

3.4.2 Processing Hazards

Each processing step in Figure 3.1 has the potential to be out of control and pose a potential hazard. The most critical processing hazard which can occur is the inadequate cooking of the hamburger patties. Efficiency of cooking depends on the quality and storage of the raw patties, proper functioning of the broiler and desired internal temperature being reached in the patties. Other factors which are important to recognize are the maintenance of proper working area and storage temperatures, potential for cross-contamination by improperly sanitized equipment or improper hygienic practices by employees. To quantify the hazard analysis, the food ingredients and processes are ranked according to the five hazard characteristics previously listed in the literature review (p. 5). Risk analysis of the hazard analysis for the hamburger sandwiches is shown in Table 3.2.

3.5 Identification of Critical Control Points

Table 3.3 identifies the critical control points for hamburger sandwich production. As can be seen from this table the control of time/temperature is the most important critical control point. As well equipment sanitation and employee hygiene have important roles in preventing potential hazards.

3.6 Monitoring of Critical Control Points

Table 3.4 summarizes the critical operations in hamburger sandwich production and the potential hazards that could be associated with each. In addition, it documents the observations at Quality Fast Foods and indicates monitoring procedures which should be followed.

Table 3.2. Risk Assessment of Hamburger Sandwiches

	Risk Characteristics					
	Sensitive Ingredient	Microorganisns not Destroyed	Post-process Contamination	Potential Abuse	No Heating Prior to Consumption	Hazard Category
Ingredients						
Frozen Hamburger Patty	+	+	+	+	-	IV
Bun	-	+	-	-	-	I
Cheese	+	+	-	-	-	II
Ketchup	-	+	-	-	-	I
Hamburger Sandwich	+	+	+	+	-	IV

Table 3.3. Critical Control Points for a Hamburger Sandwich

Critical Operation	Critical Control Points				
	Raw Ingredient Control	Equipment Sanitation	Employee Hygiene	Time/Temperature Relationship	Packaging
Receiving of raw materials	X				
Storage of raw materials		X	X	X	
Cooking of patties				X	
Transfer of cooked patties to cooling trays		X	X		
Transfer of trays to holding rack				X	
Transfer of holding rack to 4°C cooler		X		X	
Storage prior to assembly of sandwiches				X	
Assembly of sandwiches		X	X	X	
Packaging and labelling				X	X
Boxing				X	X
Transfer to refrigerated storage area				X	
Distribution				X	

Table 3.4. Monitoring of Critical Control Points for a Hamburger Sandwich

Critical Operation	Potential Risk	Critical Control Points	Observation	Control and Monitoring
Raw Hamburger	Presence of spoilage and pathogenic organisms: *Pseudomonas, Acinetobacter, S. aureus, E. coli, C. perfringens*	Supplier and microbiological status of raw patties.	Supplier - J.D. Sweid Co. Obtained compositional analysis. No microbiological results known.	Consider potential contamination of product and monitor supplies for low microbial counts. Rotate supplies.
Receiving and Storage	Growth of spoilage and pathogenic organisms. Cross-contamination to other foods.	Temperature control. Sanitation of storage area.	Received frozen and stored in freezer (-15°C) Raw and cooked products separated.	Ensure that the product is frozen on receipt. Store at less that -18°C and separate from ready-to-eat foods. Note cleanliness of storage area.
Cooking	Survival of pathogens	Time/temperature control	No gloves worn when handling raw hamburger. Patties of 3 sizes cooked (4, 6, & 8 patties/pound). Different rack speeds used to cook the various sized patties. Cooking just based on visual appearance of patty. No temperature measurement taken.	Minimum internal temperature of 74°C should be achieved. Temperature monitoring devices should be periodically used to insure proper cooking temperature.

Table 3.4. Monitoring of Critical Control Points for a Hamburger Sandwich (continued)

Critical Operation	Potential Risk	Critical Control Points	Observation	Control and Monitoring
Transfer of Cooked Patties to Cooling Trays	Contamination by food handlers and equipment.	Good employee hygiene Good equipment sanitation.	Tongs used to handle cooked patties, but required periodic cleaning. Patties were layered four high for cooling.	Ensure that hands are properly washed, that clean utensils are used and that no cross-contamination between raw and cooked product occurs.
Transfer to Holding Rack	Growth of spoilage and pathogenic organisms.	Time/temperature control	Trays of patties put on holding rack just outside the cooking room. High traffic area. No proper temperature control or monitoring in this area.	Important that patties do not remain in this area for an extended period of time Temperature monitoring required. Alternative placement of holding rack.
4°C Storage	Contamination of patties by spoilage/pathogenic organisms. Growth of surviving and contaminating organisms.	Time/temperature control	Cooler (3°C) door left open for extended periods of time. No temperature monitoring observed during the day.	Place in cooler (<5°C) with good air circulation. Ensure temperature drops to less than 20°C within 2 h and <5°C within 4 h. Temperature monitoring required.
Storage Prior to Assembly of Sandwiches	Growth of surviving spoilage/pathogenic organisms.	Time/temperature control	Patties regularly left in 4°C cooler overnight before assembly of sandwiches.	Monitor cooler temperature periodically. Ensure no cross-contamination occurs.

Table 3.4. Monitoring of Critical Control Points for a Hamburger Sandwich (continued)

Critical Operation	Potential Risk	Critical Control Points	Observation	Control and Monitoring
Assembly of Sandwiches	Contamination by food handlers and equipment. Introduction of contaminating ingredients.	Good employee hygiene. Good equipment sanitation. Time/temperature control.	Hazard with bakery racks.[a] Buns were separated after handling bakery racks without hand washing. Cooked hamburger patties always handled with gloves on. Patties were thoroughly cooled. Working area 10°C.	Proper washing of hands. Workers should not have any infectious diseases. Clean and sanitize equipment routinely. Use of gloves. Cool working conditions (10°C or less).
Packaging and Labelling	Contaminated packaging material. Improper seals and punctures. Consumer abuse.	Time/temperature control Proper and clean packaging. Proper labelling.	Triple layer film (saran, surlyn and polyester). 35-day shelf life. Contamination of packaging not known. Packages examined for proper seals and labelling.	Ensure packages are sealed properly and are intact. Labels have storage conditions, best before date and cooking/heating directions.
Boxing	Growth of spoilage and pathogenic organisms.	Time/temperature control Packaging	Work area 10°C. Packaging appeared to be suitable.	Monitoring of work area temperature. Avoid product build up. Prompt transfer to storage area.

[a]Hazard recognized

Table 3.4. Monitoring of Critical Control Points for a Hamburger Sandwich (continued)

Critical Operation	Potential Risk	Critical Control Points	Observation	Control and Monitoring
Refrigerated Storage	Growth of spoilage and pathogenic organisms.	Time/temperature control	Sandwiches packaged and boxed rapidly. Cooler 3°C.	Monitoring and recording cooler temperature. Cooler temperature should be < 5°C.
Distribution	Growth of spoilage and pathogenic organisms.	Time/temperature control	Monitor product temperature. Record any temperature abuse.	

3.7 Verification of the HACCP System

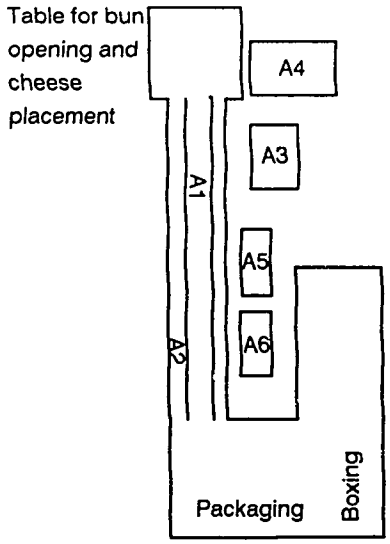
3.7.1 Equipment Sanitation

Monitoring of equipment cleanliness and sanitation should be done on a continual basis. It is however necessary to verify routinely that the equipment is adequately cleaned and sanitized. The efficiency of the sanitizing program is important for optimizing the quality and safety of the hamburger sandwiches and to extend the shelf life of these products.

At the time that HACCP analysis was done at Quality Fast Foods, they did not have in place a HACCP system. Therefore, verification of the efficiency of the established sanitation program was conducted.

Verification of their sanitation program involved taking samples on 4 days over a 9-day period. Areas surveyed are outlined in Figures 3.2 and 3.3. Sampling was done by contact rodac plates (Falcon - Division of Becton, Dickinson & Co., Cockeysville, MD). Total aerobic microbial load, presumptive coliform, presumptive staphylococci and presumptive yeasts and molds were determined. Details on media used, incubation temperatures and incubation times will be discussed later in section 4.2.4 (3). The level of contamination was scored on the basis of the actual count or amount of growth on the rodac plate.

The efficacy of sanitation achieved on Line A (sandwich assembly - Tables 3.5 and 3.7) was variable, especially on the rubber conveyor and stainless steel work surface. On day 1, sanitation was generally satisfactory, but this was not maintained on subsequent testing days. There was a problem with yeast and mold contamination and staphylococci. The other sampling areas on Line A did not present particular problems. The presence of staphylococci is an indication that the equipment has not been adequately sanitized. The quaternary ammonium compound (QAC) used in sanitation should eliminate these organisms. QAC is not as effective against yeasts and molds, as indicated by the results.



Legend

A1 - long rubber conveyor
A2 - stainless steel table
A3 - square plastic trolley
A4 - stainless steel loading trolley
A5 - slicer blade
A6 - slicer blade

Figure 3.2. Sampling Diagram for Processing Line A

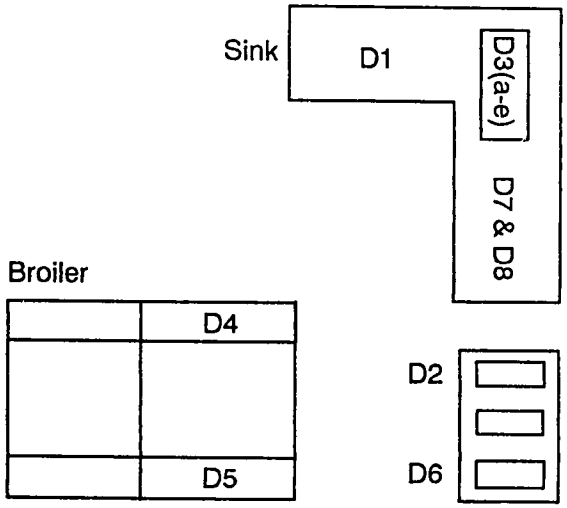
Table 3.5. Evaluation of Sanitation for Line A

Sample Point	Total Count - incubated at 30°C for 48 h.[a]			
	Day 1	Day 2	Day 3	Day 4
A1[b]	++	+++	+++++	++++
A2[b]	++	++++	+++++	-
A3	++	++	++	++
A4	++	++	+	-
A5	++	++	+	+++
A6	-	-	++	+

[a]Growth per rodac plate

-	no growth
+	1-9 colonies
++	10-99 colonies
+++	100-999 colonies
++++	heavy uncountable growth
+++++	confluent growth

[b]See Table 3.7 for differential counts.



Legend

D1 - sink
D2 - inside of slicer hopper
D3(a-e) - trays
D4 - broiler raw surface
D5 - broiler cooked surface
D6 - inside of slicer hopper
D7 - stainless steel bowl
D8 - stainless steel bowl

Figure 3.3. Sampling Diagram for Cooking Room

Table 3.6. Evaluation of Sanitation for Cooking Room

Sample Point	Total Count - incubated at 30°C for 48 h.[a] Day 1	Day 2	Day 3	Day 4
D1	-	++	++	-
D2	++	++++	-	-
D3a	++++	++++	-	++++
D3b	-	++	-	-
D3c	-	++++	-	-
D3d	-	++++	+++	-
D3e	++	-	-	-
D4	-	+	++	+
D5	++	++++	++	++
D6	+	++++	++	+
D7	-	+	-	-
D8	-	+++	-	-

[a]Growth per rodac plate

-	no growth
+	1-9 colonies
++	10-99 colonies
+++	100-999 colonies
++++	heavy uncountable growth
+++++	confluent growth

Table 3.7. Differential Counts from Evaluation of Sanitation for Line A

	Day 1				Day 2				Day 3				Day 4			
Sample Point	Total Count	Coliform	Staph	Y&M[a]	Total Count	Coliform	Staph	Y&M	Total Count	Coliform	Staph	Y&M	Total Count	Coliform	Staph	Y&M
A1	++	+	ND	++	+++	-	+++	++	+++++	+	+++	++++	+++++	-	+++	++
A2	++	+	ND	++	++++	+	++	++++	+++++	++	++++	-	-	+	-	-

ND - Not determined

[a]Growth per rodac plate

-	no growth
+	1-9 colonies
++	10-99 colonies
+++	100-999 colonies
++++	heavy uncountable growth
+++++	confluent growth

Total Count - Tryptic Soy Agar (TSA)	30°C for 48h
Coliform - Violet Red Bile Agar (VRBA)	35°C for 24h
Staph - Baird Parker (BP)	35°C for 48h
Yeast & Mold - Potato Dextrose Agar (PDA)	25°C for 5 days

Variable results were obtained for the sampling points in the cooking room (Table 3.6). Overall, the level of sanitation was good; with the most common exception being the sanitation of the hamburger trays. Samples taken on day 2 indicated that a poor level of sanitation was achieved on that day. This suggested that insufficient care was taken in sanitation after the previous day's work.

The results obtained from the sanitation evaluation indicate that, although good visual sanitation was being achieved, the food contact surfaces were not being adequately sanitized. Either the sanitation process or the QAC (Wunrub 185 - PCP12,234,DIN 460958) was inadequate for the job. The concentration of 2 mL/L was not effective in reducing the microbial load. QACs are known to be effective against Gram-positive organisms but they are relatively ineffective against most Gram-negative organisms, especially *Pseudomonas* (Marriott, 1985; Troller, 1983). Yeasts and molds account for many of the high counts. Although QACs are licensed for use on food premises, their use is not recommended on food contact surfaces because of the film that is left on these surfaces. At concentrations necessary to achieve efficient sanitation, food may be contaminated with residues making it taste bitter (Marriott, 1985).

From this sanitation evaluation it was concluded that the sanitation program required improvement. The high microbial counts on the equipment were evidence that the processing equipment was not being properly cleaned and sanitized. Attention needed to be focussed on improving the efficiency of the sanitation program. Consistent sanitation is required to give a predicted shelf life of 35 days for the hamburger sandwiches.

3.7.2 Raw Hamburger Quality and Consistency

To determine the quality of the incoming raw hamburger patties and the consistency of this quality, patties were sampled on five different occasions. This resulted in sampling of five different code dates. Boxes of patties were randomly

selected from the freezer. Five boxes were sampled per code date and five patties were analyzed per box. Sampling was based on randomized selection of patties using a predrawn sampling plan. For patty size of 4 patties/pound, there were approximately 88 patties/box with 5 stacks of 17-18 patties/stack.

The mean log counts obtained from the sampling of five different code dates are shown in Table 3.8. Details of the statistical analysis are shown in Appendix I (Statview, Abacus Concepts, Inc., Berkeley, CA). There is a significant difference in microbial counts between code dates ($p<0.05$). The data for the code date Aug 28, 1990 accounted for the difference. Comparisons between code dates and boxes (Appendix II) indicated a significant difference ($p<0.05$) between code dates, between boxes and interaction between code dates and boxes. Again results obtained from Aug 28, 1990 caused the variation. Comparisons between code dates and patties (Appendix III) revealed a significant difference between code dates as previously described. There was no significant difference ($p>0.05$) between individual code dates and patties. This indicates that the patties within boxes have the same microbial load between code dates.

3.7.3 Efficiency of Cooking Process Used at Quality Fast Foods

Patties were randomly selected as previously described for raw product evaluation. They were cooked in an automatic broiler for about 3 min to an internal temperature of approximately 75-80^{o}C and cooled overnight in a 4^{o}C cooler. The data in Table 3.9 indicate that the cooking method used by Quality Fast Foods is effective in achieving a desirable microbial quality in the cooked product. Cooked patties had microbial counts of log 2 CFU/g.

3.7.4 Margin of Safety of the Cooking Process

Patties (4 patties/pound) were cooked at various rack speeds. Internal temperature was measured by inserting a dial thermometer into the center of a patty

Table 3.8. Microbial Quality of Raw Hamburger Patties (4 patties/pound)

	Mean Log CFU/g[a]				
Code Date	Box 1	Box 2	Box 3	Box 4	Box 5
May 30, 1990	5.58	5.45	5.49	5.52	5.47
Jun 08, 1990	5.58	5.61	5.64	5.65	5.60
Jul 04, 1990	5.79	5.75	5.71	5.79	5.75
Aug 28, 1990	6.61	5.58	6.62	5.56	6.56
Nov 21, 1990	5.64	5.61	5.57	5.54	5.32

[a]Mean Log CFU/g determined from the mean of the counts of 5 replicates obtained on Tryptic Soy Agar at 30°C

Table 3.9. Microbial Survival after Cooking Hamburger Patties (4 patties/pound)

	Mean Log CFU/g[a]				
Code Date	Box 1	Box 2	Box 3	Box 4	Box 5
May 30, 1990	2.00	2.10	2.25	2.36	2.19
Jun 08, 1990	2.25	2.13	2.03	2.17	2.10
Jul 04, 1990	2.32	2.42	2.28	2.52	2.44

[a]Mean Log CFU/g determined from the mean of the counts of 5 replicates obtained on Tryptic Soy Agar at 30°C

immediately after they dropped from the broiler. Cooking times were determined using a stop watch.

Quality Fast Foods used rack speeds of 20 and 25 to cook patties (4 patties/pound). These rack speeds allow the patties to reach internal temperatures of approximately 78-80°C and 74-77°C, respectively, with corresponding microbial log counts of 2.31 and 2.52 as shown in Table 3.10. The use of these rack speeds achieves satisfactory cooking. With a faster rack speed (ie. 30), an internal temperature of only 66°C was reached. This was not sufficient to provide a finished product that was properly cooked and was not efficient in reducing the microbial count. The data obtained indicates that rack speeds of 20 and 25 are required for patties (4 patties/pound) to reach the desired internal temperature, to reduce the microbial load to the desired level and to achieve an appropriate "cooked" appearance of the finished product.

3.8 Summary of HACCP Analysis

The overall level of sanitation, personal hygiene and time/temperature control at Quality Fast Foods is sound. The sanitation evaluation revealed that the sanitation program required improvement. Several recommendations were made to improve practices and to avoid the development of potential problems. The recommendations were based on the premise that, in order to achieve a 35-day storage life, an excellent level of plant sanitation, hygiene of personnel and temperature control must be achieved.

1. Temperature control
   (a) automatic closing devices or air flow curtains should be installed on cold room doors to prevent operation with open doors for lengthy periods of time, alternatively that practice should be avoided;
   (b) improved awareness of temperature control of products is required.

Table 3.10. Margin of Safety of the Cooking Process Used at Quality Fast Foods to Cook Hamburger Patties (4 patties/pound)

Cooking Setting	Cooking Time (min)	Internal Temperature of Cooked Patties (°C)	Mean[a] Log CFU/g
10	4.06	84	2.19
15	3.36	84	2.21
20	3.06	81	2.31
25	2.46	77	2.52
30	2.30	66	2.81
35	2.14	56	4.07
40	2.00	46	4.85

[a]Mean Log CFU/g determined from 10 replicates

Rationale: the operation of a food plant at or below 10°C allows for plant sanitation at the end of the work shift. This coincides with the lag phase and growth rate of bacteria and allows for good sanitary control. However, at lower temperatures the growth rate, as well as the variety of microorganisms that grow, are reduced. The prompt transfer of products to refrigerated (4°C) storage is desirable. Similarly the efficiency of operation of cold rooms at 4°C is important. Careful control should be exercised over the time that a product is at 10°C as opposed to 4°C. For example, a finished product or cooked hamburger patties should not be left at 10°C over lunch.

2. Hand hygiene

(a) the proper use of plastic gloves should be carefully monitored;

(b) the hand washing procedures should be reviewed.

Rationale: Using plastic gloves can give rise to a false sense of security in food handling. For example, if workers handle garbage with bare hands they are likely to wash their hands before returning to handle food; however, if they handle garbage while wearing plastic gloves the same sense of potential contamination is not conveyed. Disposable plastic gloves need replacement as often as worker would normally be expected to wash their hands. Gloves are not a necessity for food handlers, but they create hygiene awareness, so their use is justified.

3. Cross-contamination

(a) if different meats are cooked on the premises the desirable sequence is beef before pork before poultry; otherwise clean up is necessary between product types;

(b) stacking trays of cooked hamburger patties in vertical racks starting at the top rather than from the bottom - would reduce the possibility of cross-contamination.

Rationale: Cross-contamination is an important aspect of safety assurance. All possible routes of cross-contamination, especially raw to cooked foods, should be monitored and controlled.

4. Clean-up

(a) food residues should be thoroughly removed from all processing equipment to achieve visual cleanliness;

(b) the availability and use of a vacuum system should be investigated to determine its appropriateness for the removal of food particles;

(c) the clean-up process requires more supervision;

(d) the trays for hamburgers need a more reliable cleaning and sanitizing procedure, possibly including draining of the equipment.

Rationale: There are food residues that are not being removed in the cleaning process, and the sanitation efficiency check indicated that the sanitation process has variable results. Proper removal of food residues would reduce the potential for the growth of microorganisms. Proper clean-up would also reduce the need for spot cleaning prior to the commencement of production.

5. Sanitation

(a) the sanitation process needs improvement;

(b) a procedure that gives better control of yeasts and molds needs to be considered;

(c) concentration of the quaternary ammonium compound (QAC) being used needs to be increased or, preferably, an alternative sanitizer should be used.

Rationale: the present sanitation process is not achieving the degree of microbial control that is desirable or can be expected. This is due to the inadequate sanitation process and (or) the inappropriate concentration or type of sanitizer used. Increased concentration of QAC would require rinsing of the work surfaces after sanitation.

The efficacy of alternate sanitizers that have a broader antimicrobial range and that are compatible with the equipment should be tested - eg. an iodophor or hypochlorite.

Raw hamburger patties supplied by J.D. Sweid & Co., Ltd. are of good microbial quality and appear to be relatively consistent. Periodic microbial analysis of raw patties will assure that the raw products are of consistent quality.

According to the data obtained the cooking process used at Quality Fast Foods seems to be effective in reducing the microbial load to an acceptable level. The margin of safety evaluation indicates that care must be taken in the selection of the rack speed used when cooking the hamburger patties. Just a slightly faster rack speed can cause a great reduction in the internal temperature achieved and increase the survival of spoilage/pathogenic organisms. As mentioned previously cooking is based on varying the rack speed for the different sizes of patties cooked and by visual appearance. Periodic temperature monitoring to assure efficient cooking and broiler operation is recommended.

Application of HACCP examined the raw materials and the processes involved in the production of hamburger sandwiches. Monitoring and controlling the identified critical control points puts emphasis on production practices to prevent microbial hazards. Packaging meats in modified atmospheres with increased levels of carbon dioxide prevents the growth of aerobic spoilage microorganisms and favors the dominance by lactic acid bacteria. This was demonstrated in luncheon meat and roast beef sandwiches, but hamburger sandwiches did not develop a predictable lactic acid microflora (M^{c}Mullen and Stiles, 1989). To facilitate the growth of a predictable lactic microflora in the cooked meat, this study further investigated the addition of *Carnobacterium piscicola* (UAL 26.CL97) to raw hamburger meat to achieve this.

4. MICROBIOLOGICAL STUDIES OF HAMBURGER PATTIES INTENDED FOR USE IN MODIFIED ATMOSPHERE PACKAGED SANDWICHES

4.1 Introduction

Modified atmosphere packaging (MAP) has extended the storage life of sandwich products. In MAP, the sandwich products are flushed with a gas atmosphere of specified composition, usually a combination of carbon dioxide, nitrogen and oxygen (Farber *et al.*, 1990). The gas composition within the package changes on storage as a result of meat and bacterial respiration and permeability of the packaging film (Seideman *et al.*, 1979; Wolfe, 1980). Extension of storage life is attributed to the inhibitory effect of carbon dioxide towards spoilage organisms (Enfors and Molin, 1980; Dixon and Kell, 1989). The effectiveness of carbon dioxide is dependent on its solubility in the meat. Refrigeration temperatures enhance the solubility, and the effectiveness of carbon dioxide (Wolfe, 1980). Resistance to carbon dioxide allows lactic acid bacteria to dominate the bacterial population of MAP meat sandwiches. This was observed for luncheon meat and roast beef sandwiches (M^{c}Mullen and Stiles, 1989). In comparison, the lactic development in the hamburger sandwiches was variable.

Even though MAP inhibits spoilage microorganisms, there is some concern that during extended storage pathogenic growth may be stimulated before spoilage is evident (Farber *et al.*, 1990). Mueller (1975) and Farber *et al.* (1990) showed that the majority of MAP sandwiches examined were free of pathogenic microorganisms, *C. botulinum*, *C. perfringens*, *S. aureus* and *Salmonellae*. The presence of *Listeria* appeared to be the major concern. Microbial safety of hamburger sandwiches is difficult to predict. This product has no back-up preservation system and an unpredictable lactic microflora, which may make it susceptible to the growth of pathogenic organisms if temperature abused. Addition of lactic acid bacteria or their bacteriocins to meat systems have been effective in inhibiting spoilage and

pathogenic organisms (Nielsen and Zeuthen, 1985; Nielsen *et al.*, 1990; Schillinger and Luecke, 1990). This section of the study examined microbial development on cooked hamburger patties packaged in different gas atmospheres. Further, it investigated the possible use of *Carnobacterium piscicola* (UAL 26.CL97) as a meat preservative; determining its ability to survive, grow and produce bacteriocin in hamburger meat.

4.2 Methods and Materials

4.2.1 Bacterial Cultures

The bacterial cultures used in this study are listed in Table 4.1. The *Carnobacterium* species were isolated from vacuum packaged meat. Strain UAL 26.CL97 was genetically derived by a series of conjugation, ligation and electrotransformation experiments (Ahn, 1991). UAL 26.CL97 was inoculated into the hamburger patties, while other strains were used as indicator organisms.

4.2.2 Growth Media

The culture media, selective plating media and reagents used for the microbiological assessment of hamburger patties are listed in Tables 4.2, 4.3 and 4.4, respectively. Media were prepared as directed by the manufacture or as indicated by the reference. Soft agar was made by adding 0.75% agar to APT broth, which was used as an overlay agar when testing cultures for antagonistic activity or sensitivity. Sodium chloride (6.5%) was added to Brain Heart Infusion (BHI) broth as a confirmation test for enterococci. Lactic acid bacteria were enumerated on MRS agar adjusted to pH 5.6 with 85% lactic acid. Chloramphenicol (10 μg/mL) was added to APT broth or agar for the growth and enumeration of UAL 26.CL97. Casamino acids (0.5%) was added to APT as a supplement for the growth of UAL 26.CL97 for the experiment on cell yield. The GM17 with casamino acids was prepared by replacing the tryptone with an equal amount of casamino acids. Yeast

Table 4.1. Bacterial Cultures Used in This Study and Their Sources

Bacterial Strain	Source
Carnobacterium spp.	
C. piscicola LV17 (UAL 8)	Shaw[a]
C. piscicola (UAL 26)	Burns[b]
C. piscicola (UAL 26.CL97)	Ahn[c]
Enterococcus faecium (durans)	ATCC 11576[d]
Listeria monocytogenes	ATCC 15313

[a]Isolated from vacuum packaged meat by B.G. Shaw (Institute of Food Research, Langford, Bristol, U.K.)

[b]Isolated by Kim Burns (U of A, Dept. of Food Science)

[c]Genetically derived by Cheol Ahn (U of A, Dept. of Food Science)

[d]American Type Culture Collection

Table 4.2. Culture Media Used in This Study and Their Suppliers or Reference

Culture Media	Supplier or Reference
APT Broth/Agar	APT (Difco)[a]
Brain Heart Infusion Broth	BHI Broth (Difco)
Cooked Meat Medium	CMM (Difco)
M17 Broth	M17, Terzaghi and Sandine (1975)
GM17 Broth	GM17[b]
Tryptic Soy Broth/Agar	TSB, TSA (Difco)
Tryptic Soy Yeast Extract Agar/Broth	TSB-YE (Difco)[c]

[a]Difco media, Difco Laboratories Inc., Detroit, MI

[b]Modification of M17 - replacement of lactose with 1% glucose

[c]0.6% yeast extract added to TSB

Table 4.3. Selective Plating Media Used in This Study and Their Suppliers or References

Selective Media	Supplier or Reference
Baird-Parker Agar Base	BP (Difco)[a]
Glucose Salt Medium	GSM, ICMSF (1978)
KF Streptococcus Agar	KF (Difco)
Lactobacilli MRS Broth/Agar	MRS Broth (Difco)[b] Solidified with 1.5% Agar (BBL)[c]
Potato Dextrose Agar	PDA (Difco)[d]
Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesulin Mannitol Agar	PALCAM (Merck)[e]
Violet Red Bile Agar	VRBA (BBL)
Violet Red Bile Glucose Agar	VRBG, Elliott *et al.* (1978)[f]

[a]5% egg yolk tellurite enrichment added (Difco)

[b]Acidified to pH 5.6 with 85% lactic acid (Fisher Scientific)

[c]BBL - Becton Dickinson Microbiology Systems, Cockeysville, MD

[d]Adjusted to pH 3.5 with 10% tartaric acid (Fisher Scientific)

[e]Developed by van Netten *et al.* (1989)

[f]Prepared from VRBA with addition of 1% D-glucose (BDH)

Table 4.4. Reagents Used in This Study and Their Suppliers

Reagents	Supplier
Chloramphenicol	Cm (Sigma)[a]
Tetramethylparaphenylenediamine dihydrochloride	Oxidase Reagent(BDH)[b]
Sodium Chloride	NaCl (BDH)
30% Hydrogen Peroxide	H_2O_2 (Fisher Scientific)[c]
2,3,5 - Triphenyl Tetrazolium Chloride	TTC (Aldrich)[d]

[a]Sigma, St. Louis, MO

[b]BDH Chemicals, Toronto, Canada

[c]Fisher Scientific Co., Fair Lawn, NY

[d]Aldrich Chemical Co., Inc., Milwaukee, WI

extract (0.6%) was added to tryptic soy broth (TSB) for the growth of *Listeria monocytogenes*.

4.2.3 Maintenance and Growth of Bacterial Cultures

Carnobacterium cultures were maintained in Cooked Meat Medium (CMM) at 4°C and subcultured every three months. Prior to use strains UAL 8 and UAL 26 were subcultured twice in APT broth; strain UAL 26.CL97 was subcultured in APT.Cm broth (APT broth containing 10 *u*g of chloramphenicol per mL) at 25°C. *Enterococcus faecium* 11576 and *Listeria monocytogenes* 15313 were maintained on tryptic soy agar (TSA) slants at 4°C. *E. faecium* was subcultured twice in APT broth and *L. monocytogenes* in tryptic soy yeast extract broth (TSB-YE) at 35°C for 24 h prior to use. Strains were not subcultured more than 5 consecutive times before use in experiments.

4.2.4 Storage of Cooked Hamburger Patties

(1) Hamburger patties

Commerical frozen raw hamburger patties were supplied by J.D. Sweid & Co., Ltd. of Burnaby, B.C. These patties included a mixture of beef, water, binder (toasted wheat crumbs, spice, hydrogenated vegetable oil) and salt. They were "scored" to provide greater surface area and better heat penetration. The frozen raw hamburger patties (4 patties/pound) were cooked in an automatic broiler (NIECO - Burlingame, CA) to an internal temperature of approximately 80°C and cooled to 4°C prior to packaging. For more detailed information see Section 3.2.

(2) Packaging

Cooked patties were packaged under two modified atmosphere conditions, one containing a gas mixture of approximately 50% CO_2 and 50% air and the other approximately 40% CO_2 and 60% N_2. The same packaging film, MKS 5200 (50 gauge polyester/saran/1 mil polyethylene/1 mil surlyn; thickness 2.6 mils - Curwood Packaging, Georgetown, Ontario, Canada), was used for both packaging methods.

After packaging, the hamburger patties were packed into boxes and transported to the laboratory, where they were placed in a 4°C cooler until sorted, labelled and placed at the appropriate storage temperatures (either 4°C or 10°C).

(3) Microbiology

Media used for the microbial analysis of the hamburger patties are listed in Tables 4.2 and 4.3. Whole patties were placed in stomacher bags (Seward Medical, London, England), weighed and blended with 198 mL tryptic soy broth in a Colworth Stomacher (Model 400, A.J. Seward, Bury St. Edmund, Suffolk, UK) for 2 min. Blended samples were held at room temperature for 2 h to allow injured cells to resuscitate.

Dilutions were made with 0.1% peptone water (Difco) and 50 uL was spotted onto the appropriate selective and nonselective prepoured agar plates. Microorganisms were enumerated according to procedures outlined by ICMSF (1978).

(a) Total Count - TSA incubated aerobically at 25°C for 48 h;

(b) Lactic Acid Bacteria - MRS adjusted to pH 5.6 with 85% lactic acid incubated anaerobically (10% CO_2 and 90% N_2) at 25°C for 48 h;

(c) Presumptive Coliform Count - VRBA overlayered with 4-5 mL VRBA before incubation at 35°C for 24 h;

(d) Presumptive Enterobacteriaceae - VRBG overlayered with 4-5 mL VRBG before incubation at 35°C for 24 h. The presence of Enterobacteriaceae were confirmed by the oxidase test and growth in glucose salt medium (GSM). GSM was overlayered with sterile mineral oil and incubated at 35°C for 24 h;

(e) Presumptive *Staphylococcus* Count - Baird-Parker Agar (BP) with egg yolk tellurite enrichment and incubated at 35°C for 48 h;

(f) Presumptive Yeast and Mold Count - Potato Dextrose Agar (PDA) adjusted to pH 3.5 with 10% tartaric acid and incubated at 25°C for 5 days;

(g) Anaerobic Sporeformers - 20 mL of a 10^{-1} dilution was heated in a water bath to 75°C and held for 20 min. Enumeration was done on plates containing approximately 10 mL tryptic soy agar TSA, incubated anaerobically at 35°C for 48 h (Health Protection Branch, 1989).

4.2.5 Characteristics of UAL 26.CL97

(1) Stability of CL97 p

The purpose of t[illegible]ment was to determine the number of Cm^R (chloramphenicol resis[illegible]olonies on APT.Cm and Cm^S (chloramphenicol sensitive) colonies growing on APT. Any difference in counts at the end of 50 generations would indicate the loss of the CL97 plasmid, thus loss of chloramphenicol resistance and bacteriocin production.

UAL 26.CL97 was subcultured twice in APT.Cm broth from Cooked Meat Medium (CMM) before use. A 100 *u*L volume of a 24-h culture was transferred from APT.Cm into 10 mL of APT and APT.Cm broth giving a 1% inoculum. Cultures were incubated at 25°C for 24 h. Enumeration of cultures was done after 24 h (7 generations) on both APT and APT.Cm agar, which were incubated anaerobically at 25°C for 48 h. The 24 h APT culture continued to be subcultured (using a 1% inoculum) into APT broth for 7 days (50 generations). After these 7 successive subcultures, the APT culture was enumerated on APT and APT.Cm agars.

(2) Heat resistance in broth

The test culture was grown in 100 mL of APT.Cm broth at 25°C for 24 h. Supernatant was removed by centrifugation at 8000 x g for 10 min and cells were resuspended in 5 mL of 0.1% sterile peptone water. A 500-mL flask containing 100 mL APT broth and a stir bar was placed in a circulating waterbath set on a stirring

plate. Stirring was initiated and the flask was allowed to temper for 15 min to reach 60°C before the addition of 4 mL of resuspended cells. At 30-s intervals 2 mL samples were withdrawn. One millilitre was placed in 9 mL APT broth; the other 1 mL was placed in a sterile tube, set in a tray of water, to cool to room temperature. Samples were enumerated on APT agar incubated anaerobically at 25°C for 48 h.

(3) Tolerance to NaCl

A 1% inoculum of UAL 26.CL97 was inoculated into APT broth and Cooked Meat Medium (CMM) containing 0.5, 1.0, 1.5 and 2.0 percent NaCl, incubated aerobically at 25°C for 24 h. The presence or absence of growth was recorded.

4.2.6 Cell Yield in Different Culture Media

A 1% inoculum was added to the following culture media APT, M17, GM17, APT with 0.5% casamino acids and GM17 with casamino acids to determine differences in cell yield. Culture broths were incubated at 25°C for 24 h, centrifuged and the cell pellet was resuspended in 10 mL of 0.1% peptone water. Viable counts were determined on APT and APT.Cm agars, incubated anaerobically at 25°C for 48 h.

4.2.7 Reforming of Hamburger Patties

Commerical frozen raw hamburger patties were placed in individual sterile plastic bags to thaw overnight in a 4°C cooler. Thawed patties were formed into a ball, inoculated with 1 mL UAL 26.CL97 culture and then blended in a Colworth Stomacher 400 for 2 min to allow for the extraction of protein to aid in annealing the meat and distribution of bacterial cells. Patties were subsequently reformed using an adjustable hamburger mould (Model AHM-485, Taiwan). Patties were then refrozen in a -30°C freezer.

4.2.8 Cooking of Reformed Hamburger Patties

Reformed patties were cooked in an electrical broiler (CTX Tube Broiler T400, CTX Division Pet Inc., Fenton, MO) at the Alberta Agriculture Food Processing

Development Centre, Leduc. The broiler is equipped with two temperature controllers. One controller monitors the top and the other the bottom heating element. Cooking time is controlled by a conveyor system which is set by a thumbwheel switch. The broiler is also equipped with a flame adjuster (three sizes) which regulates the size of the flame in the cooking chamber. A drip pan filled with water is situated below the flame adjuster to collect the fat drippings.

4.2.9 Monitoring of Internal Cooking Temperature

(1) Calibration of thermometer and data logger

A partial-immersion thermometer (Fisher) was calibrated to an ice-point reference and a steam point reference as outlined in Wise and Soulen (1986), with modifications. Ice cubes made from distilled water were placed in a large beaker with distilled water, so there was no excess water on the surface of the ice. The partial-immersion thermometer was inserted to the immersion line and clamped in place, allowed to equilibrate for 1 to 2 min and stability of the ice point reading was recorded. Steam point reference was determined by gently heating the water and ice mixture with continuous stirring until the boiling point was reached. Upon stabilization the boiling point temperature was recorded. Barometric pressure was measured with a Nova Barometer (Princo, Southampton, PA) and a theoretical boiling point calculated. The two boiling points were compared.

Simultaneously, a data logger (Hanzon Data Inc., Woodinville, WA) was calibrated as outlined in its instruction manual. Adjustments to the data logger were made if the boiling point indicated by the data logger did not correspond to the thermometer reading or theoretical boiling point. The data logger was also checked for accuracy over a range of temperatures from 50 to 100°C, using the calibrated thermometer as the reference. A circulating waterbath was set at selected temperatures and allowed to equilibrate. Thereafter the data logger was activated and the temperature of the eight thermocouples (Type J, Thermo Electric (Canada)

Ltd.) was recorded on a printer (Hewlett Packard Think Jet, Singapore) to see if there was any variation in temperature between the different thermocouples.

(2) Determining internal temperature of cooked patties

Temperature of the cooked patties was recorded using Type J thermocouples attached to the Hanzon data logger. Frozen hamburger patties were cooked in the CTX tube broiler and as the patties dropped from the broiler an alcohol cleaned thermocouple was inserted into the center of the patty. The patty was then compressed with an alcohol flamed spatula until the temperature started to decrease. Temperatures were recorded on a printer (Hewlett Packard Think Jet).

4.2.10 Inoculation of Hamburger Patties with UAL 26.CL97

(1) Bacterial inoculum

Bacterial inocula for seeding hamburger patties were prepared from 24-h cultures grown in APT.Cm broth and centrifuged at 8000 x g for 10 min. Cells were resuspended in various amounts of 0.1% peptone water, depending on the volume of culture grown and the concentration of cells required. The resuspended cells (1 mL) were inoculated into the thawed hamburger patties, blended and refrozen as described in 4.2.7.

In addition, resuspended cells containing approximately 10^{10} CFU/mL were divided into 1 mL aliquots, frozen at - 70^{o}C and subsequently freeze dried (VIRTIS Co., Inc., Gardiner, NY) for 16-18 h. Each patty was inoculated with one vial of freeze dried culture.

(2) Enumeration of UAL 26.CL97 inoculated in frozen raw hamburger patties

Viable counts were determined for raw hamburger patties inoculated with 1 mL of 10^{10} CFU/mL culture and freeze dried culture. Whole frozen patties were weighed, diluted with 198 mL 0.1% peptone water and blended for 2 min. Appropriate dilutions were spotted onto APT and APT.Cm agar plates.

(3) Determining inoculum size required to obtain survivors

Centrifuged UAL 26.CL97 cells were resuspended to give cell concentrations of 10^5 to 10^{11} CFU/mL. A 1-mL portion of each of the dilutions was inoculated into thawed raw hamburger patties (approximately 113 g/patty), frozen at -30°C before cooking to an internal temperature of 70-75°C. Patties were tested for survivors by surface spotting on APT and APT.Cm agar plates with anaerobic incubation at 25°C for 48 h.

(4) Determining cooking temperature at which UAL 26.CL97 survives and grows during storage at 4°C

Thawed hamburger patties were inoculated with 1 mL of a culture containing 10^{10} CFU/mL. Frozen patties were cooked and the internal temperature recorded as described in sections 4.2.8 and 4.9.2 (2). Cooled, cooked patties were divided in half. One half for initial plating and the other half was packaged in MKS 5200 film (Curwood Packaging, Georgetown, Ontario) and gas flushed with 50% CO_2 and 50% N_2 mixture and sealed in a packaging machine (Bizerba Canada Inc., Mississauga, Ontario) and stored at 4°C for 2 weeks. A second storage experiment was done in a similar way, except that the patties were divided into 5 sections. Sections of the patties were plated initially, after 1 week of storage at 10°C, and after 2, 4 and 6 weeks of storage at 4°C. Patties inoculated with the freeze dried cultures were similarly tested to determine the effect of freeze drying on the survival of UAL26.CL97 in cooked hamburger patties.

4.2.11 Confirmation of Identity of Survivors

To confirm that the surviving organisms were indeed UAL26.CL97, a modification of the direct antagonism described by Tagg *et al.* (1976) was used. Colonies were picked from APT and APT.Cm agar plates with sterile toothpicks and streaked onto APT agar. Indicator strains, UAL 8 and UAL 26 were subcultured in APT broth at 25°C for 18-24 h. The agar plates were overlayered

with 10 mL of soft APT agar inoculated with 1% of one of the indicator strains and incubated anaerobically at 25°C for 18 h. Plates were examined for zones of inhibition.

4.2.12 Ability of UAL26.CL97 to Grow at 4 and 10°C in Laboratory Media and Meat

(1) Sample preparation and inoculation

Modifications of methods described by Burns (1987) were used. Cooked hamburger patties were ground in a Cuisinart food processor (Weil Co. Ltd., Mississuaga, Ontario). A 40-g sample of ground meat was weighed into a sampling bag (Whirl-Pak, Nasco, Fort Atkinson, WI) for growth of UAL26.CL97 in hamburger meat and 20 g of ground meat plus 20 mL of 50 mM phosphate buffer (pH 7.0) for growth in hamburger slurry.

A 24 h culture of UAL26.CL97 was resuspended in 0.1% peptone water after centrifugation at 8000 x g to give a cell concentration of 10^9 CFU/mL. A 10 μL portion of the resuspended cells was inoculated into 10 mL of APT broth and Cooked Meat Medium in screw capped tubes, while 40 μL was inoculated into the hamburger meat and meat slurry (containing 20 mL of 50 mM phosphate buffer). All samples were mixed to distribute the bacterial cells. The final concentration of inoculum in each of the samples was approximately 10^6 CFU/g. Samples were incubated in anaerobic jars flushed with 10% CO_2 and 90% N_2 at 10°C and 4°C for up to 10 days.

(2) Evaluation of growth

Duplicates of each inoculated sample type were stored at 10°C and 4°C, except for the broth samples which were only stored at 4°C. Samples were enumerated initially and every 2 days up to and including day 10. Approximately 1-g subsamples were weighed from the slurry and meat samples, before blending exact weights were recorded and 9 mL of 0.1% peptone water was added to the subsample and blended

in a Stomacher for 1 min. Suitable dilutions were made directly from the Cooked Meat Medium and broth samples, spotted onto APT.Cm agar, dried in a laminar flow hood (The Baker Co. Inc., Sanford, ME) and incubated anaerobically at 25°C for 48 h. Colonies were counted and the number of CFU/g was calculated.

(3) Detection of bacteriocin production in broth and cooked meat medium

Spot-on-lawn assay as described by Barefoot and Klaenhammer (1983) was used to determine if bacteriocin was being produced by UAL 26.CL97 in broth and Cooked Meat Medium on each of the sampling days. The pH of the samples was measured using a combination electrode (Fisher Scientific, Fair Lawn, NY) connected to a pH meter (Model 230 - Fisher Accumet^{R}). If the pH was below pH 6.0, it was adjusted to pH 6.5 with 1 N NaOH (Fisher). After adjustment of pH, cells were inactivated by (a) heating at 62°C for 30 min or by adding (b) an equal volume of chloroform. Following heat treatment supernatants were further treated with (a) protease from *Streptomyces griseus* (1 mg/mL, Sigma), (b) heat denatured protease (100°C for 10 min) (c) catalase from bovine liver (68 units/mL, Biopharmaceutiques).

After treatments, supernatants were centrifuged in a Hermle centrifuge (Model-Z230M, National Labnet Co., Woodbridge, NJ) at 10.400 rpm for 5 min. A 20-*u*L amount of supernatant was spotted onto APT agar plates overlayered with 10 mL of APT soft agar (0.75% agar) inoculated with an overnight culture of UAL 26 (1% inoculum) as the indicator organism. Incubation conditions have been described in 4.2.11. Inhibition was recorded.

(4) Extraction of bacteriocin from hamburger meat and hamburger slurry

Each of the samples that were used for microbiological analysis were also tested for bacteriocin production by a modification of the method used by Schoebitz (1988). Hamburger slurries were centrifuged at 10,000 x g for 15 min and the supernatant removed and placed in a sterile tube.

To extract bacteriocin from the hamburger meat, 20 mL of 50 mM phosphate buffer was added to 40 g of meat and blended in a Stomacher for 1 min. Samples were allowed to equilibrate for 24 h at 4°C, and treated in the same manner as the meat slurries. The pH of the supernatants was measured and adjusted with 1 N NaOH if the pH was below pH 6.0. Subsequent treatment of the supernatants was the same as described for the broth and Cooked Meat Medium in 4.12.3.

(5) Determining the concentration of bacteriocin produced by UAL 26.CL97

Bacteriocin activity was determined by the spot-on-lawn technique described by Barefoot and Klaenhammer (1983). A two-fold serial dilution of the heat treated supernatant was made in 50 mM phosphate buffer (pH 7.0). A 20-uL amount of the dilutions was spotted on to APT agar overlayered with soft APT agar containing UAL 26 as the indicator organism. Activity units/mL were expressed as the reciprocal of the highest dilution showing a zone of inhibition.

(6) Addition of sodium dodecyl sulphate (SDS) and Tween 80 to aid in the extraction of bacteriocin

SDS (Calbiochem) and Tween 80 (Fisher) were added separately to a concentration of 1% in hamburger meat at the time of bacteriocin extraction with 20 mL of 50 mM phosphate buffer, prior to the 24-h equilibration. Supernatants were heat treated and spotted onto indicator lawns to determine if these detergents would help dissociate the proteins and release the bacteriocin.

4.13 Inhibition of Enterococcus faecium 11576 by UAL 26.CL97 in Hamburger Meat

Cooked ground hamburger meat (40 g) was weighed into Whirl-Pak bags as described in 4.2.12.(1). *E. faecium* grown in APT broth at 35°C for 24 h and UAL 26.CL97 grown in APT.Cm broth at 25°C for 24 h were centrifuged and the pellets were resuspended in 0.1% peptone water to give concentrations of 10^8 and 10^9

CFU/mL, respectively. A 40-uL volume of each cell suspension was inoculated into the ground meat to give the following concentrations of the test organisms.

Sample	Concentration of Bacterial Inoculum	
	E. faecium	UAL 26.CL97
E. faecium	10^5 CFU/g	-
E. faecium & UAL 26.CL97	10^5 CFU/g	10^6 CFU/g

Samples were manipulated by hand to mix the bacterial inoculum into the meat. Inoculated meat samples were incubated anaerobically at 10°C and sampled initially and every 2 days for 10 days. The samples were suspended in 99 mL of tryptic soy broth (TSB) by blending for 2 min using a Colworth Stomacher. APT.Cm and KF Streptococcus agar plates were spotted with 50 uL of the dilutions for the enumeration of UAL 26.CL97 and *E. faecium*. KF agar was incubated aerobically at 35°C for 48 h and APT.Cm at 25°C for 48 h.

4.14 Inhibition of Listeria monocytogenes 15313 by UAL 26.CL97 in Hamburger Meat

The same principles as those used in 4.2.13 were used in this experiment, with the following exceptions: *L. monocytogenes* was grown in tryptic soy broth with yeast extract (TSB-YE) at 35°C for 24 h, cells were resuspended to give a concentration of 10^8 CFU/mL and inoculation of meat samples was done as follows:

Sample	Concentration of Bacterial Inoculum	
	L. monocytogenes	UAL 26.CL97
L. monocytogenes	10^5 CFU/g	-
L. monocytogenes & UAL 26.CL97	10^5 CFU/g	10^6 CFU/g

The diluent used was TSB-YE broth. *L. monocytogenes* was enumerated on PALCAM agar (refer to Table 4.3) incubated aerobically at 35°C for 48 h.

4.3 Results

4.3.1 Effect of Storage Temperature and Method of Packaging on the Microflora of Cooked Hamburger Patties

(1) Total count

The development of microflora in hamburger patties (4 patties/pound) cooked to an internal temperature of 80°C and stored for 6 weeks was influenced by the storage temperature and method of packaging. Patties in thermoform packaging (approximately 40% CO_2 with the balance N_2) and stored at 4°C had the best shelf-life, as shown in Figure 4.1. These patties reached a total count of 10^5 CFU/g at the end of six weeks of storage. Only minimal bacterial growth was detected in these samples until the end of the third week of storage, by which time 1 log cycle (10^2 to 10^3 CFU/g) increase was observed. In contrast, patties packaged in 50% air and 50% CO_2 and stored at 4°C showed an increase in the total count of 3 log cycles (10^2 to 10^5 CFU/g) after only 2 weeks of storage and reached a count of 10^7 CFU/g by the end of the sixth week.

The development of microflora in patties stored at 10°C, whether in thermoform packaging or in 50% air and 50% CO_2, was the same. Total counts increased 3 log cycles (10^2 to 10^5 CFU/g) after the first week of storage and continued to increase. After 6 weeks, the patties packaged in thermoform packaging reached a total count of 10^7 CFU/g while those packaged in 50% air and 50% CO_2 had count of 10^8 CFU/g.

(2) Coliform and Enterobacteriaceae counts

There was no growth of coliform bacteria or Enterobacteriaceae in the thermoform packaged patties stored at either 4 or 10°C throughout the 6 week experimental period. Coliform bacteria and Enterobacteriaceae grew on patties packaged in 50% air and 50% CO_2 at both temperatures (Figure 4.2).

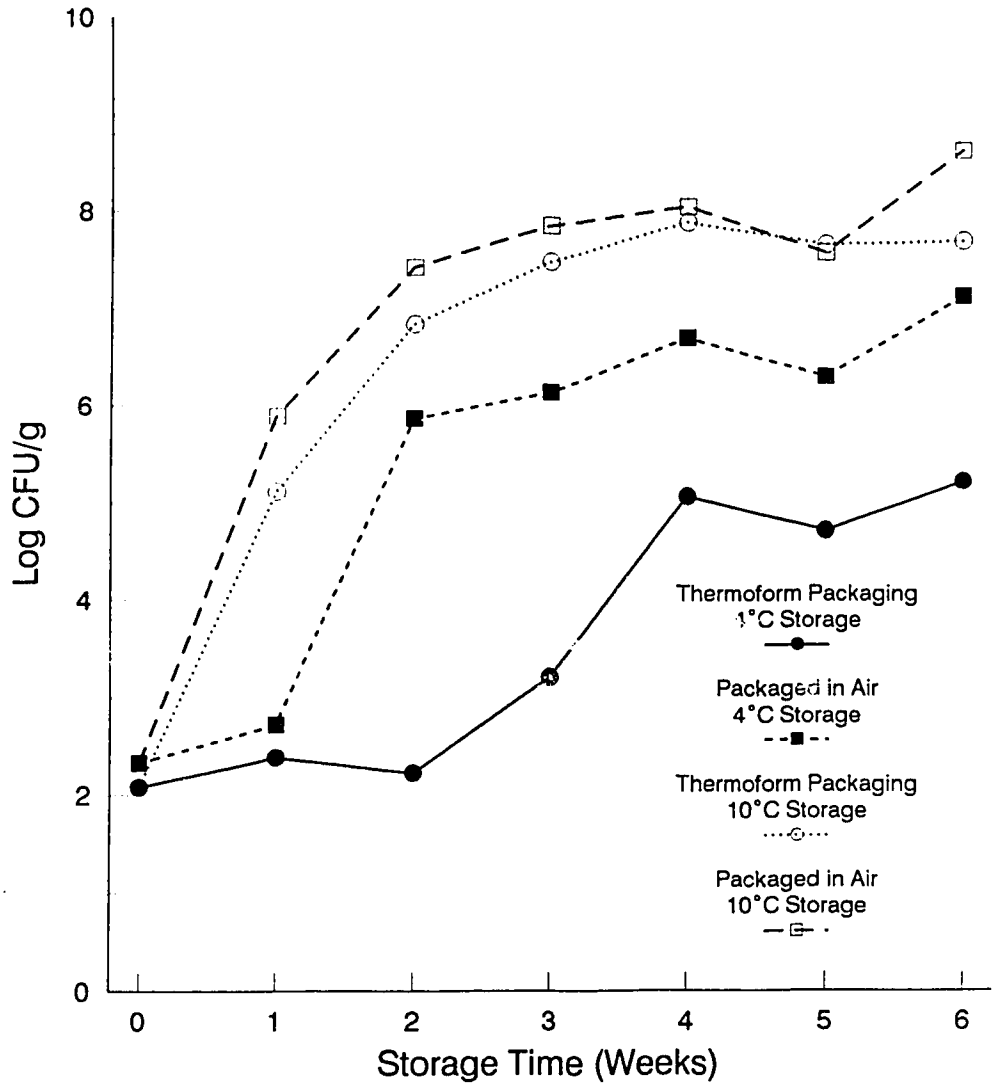


Figure 4.1. Development of Microflora in Cooked Hamburger Patties Packaged with and without Oxygen and Stored at 4 and 10°C

Thermoform packaging - approximately 40% carbon dioxide with the balance nitrogen

Air packaging - approximately 50% carbon dioxide and 50% air

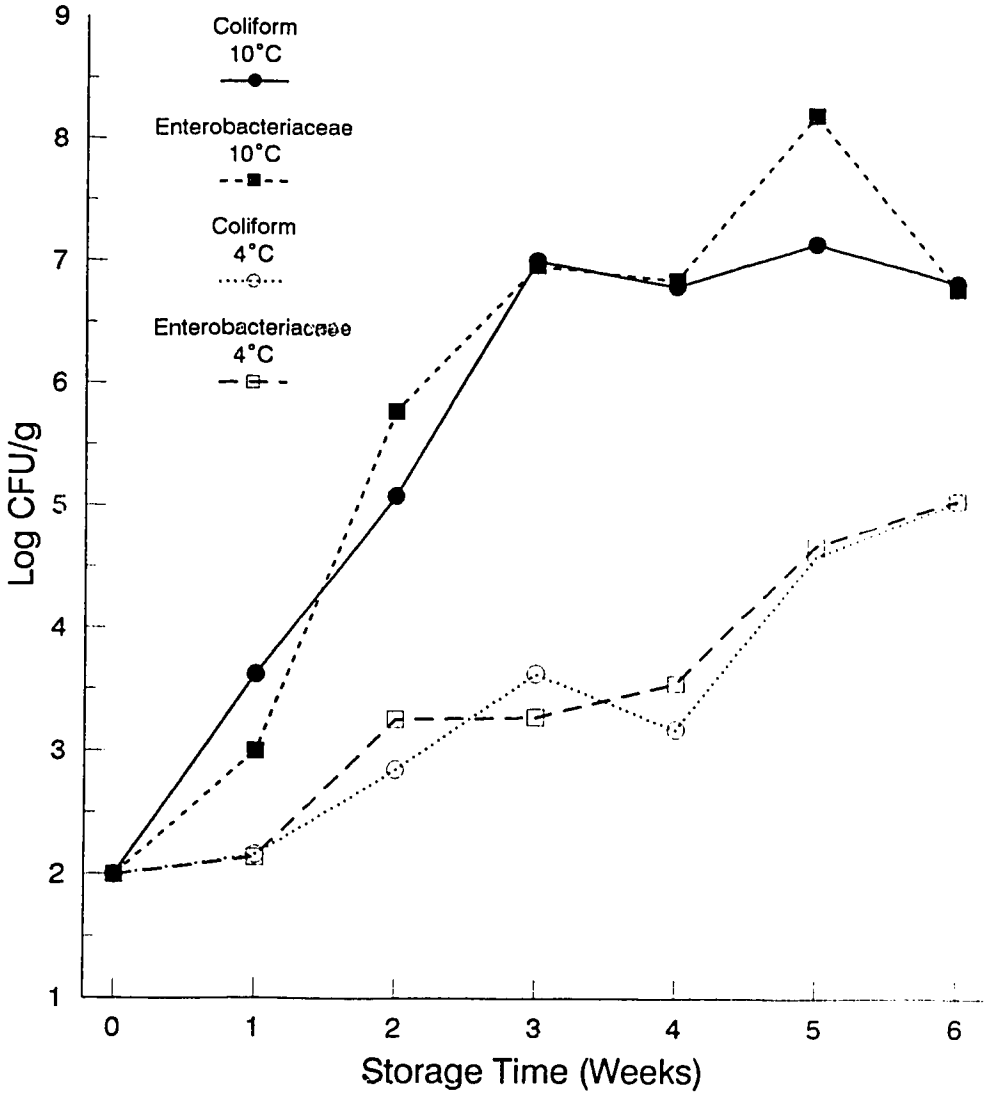


Figure 4.2. Coliform and Enterobacteriaceae Counts of Hamburger Patties Packaged in 50% Air and 50% Carbon Dioxide and Stored at 4 and 10°C

A total of ten colonies was picked from VRBG plates to confirm that they were Enterobacteriaceae. This was confirmed by oxidase test and growth in glucose salt medium. All of the colonies were oxidase negative and 9 out of 10 grew in glucose salt medium, indicating that 90% (9 out of 10) of the colonies were Enterobacteriaceae.

(3) Lactic acid bacteria

Development of lactic acid bacteria was slower in the thermoform packages than those stored in air, as shown in Figure 4.3. Storage at 4 or 10°C did not have a marked influence on the growth of the lactics. From the data obtained, growth rate of the lactics was faster when the patties were packaged in 50% air and 50% CO_2. After 6 weeks of storage, lactic counts in the packages with 50% air and 50% CO_2 reached 10^7 and 10^8 CFU/g at 4 and 10°C, respectively. In comparison, counts of 10^3 CFU/g were obtained in the thermoform packages at either temperature.

The data shown in Table 4.5 indicate that the lactic microflora was not predictable. There was variability not only in the number of patties that developed a lactic microflora but also in the numbers of lactic acid bacteria at the different storage times. The variability in lactic development was more pronounced in the thermoform packages than in packages with 50% air and 50% CO_2 mixture.

(4) Yeasts and molds

The growth of yeasts and molds in cooked hamburger patties packaged in 50% air and 50% CO_2 and in thermoform packaging is shown in Figure 4.4. Patties packaged in 50% air and 50% CO_2 and stored at 4°C developed a detectable yeast and mold microflora of 10^3 CFU/g after three weeks of storage; while samples stored at 10°C reached this stage of growth within the first week of storage.

Yeasts and molds were not detected until after the fourth week of storage in thermoform packaged hamburger patties stored at 4°C. At 10°C, yeasts and molds were detected by the end of the first week of storage, but only limited growth

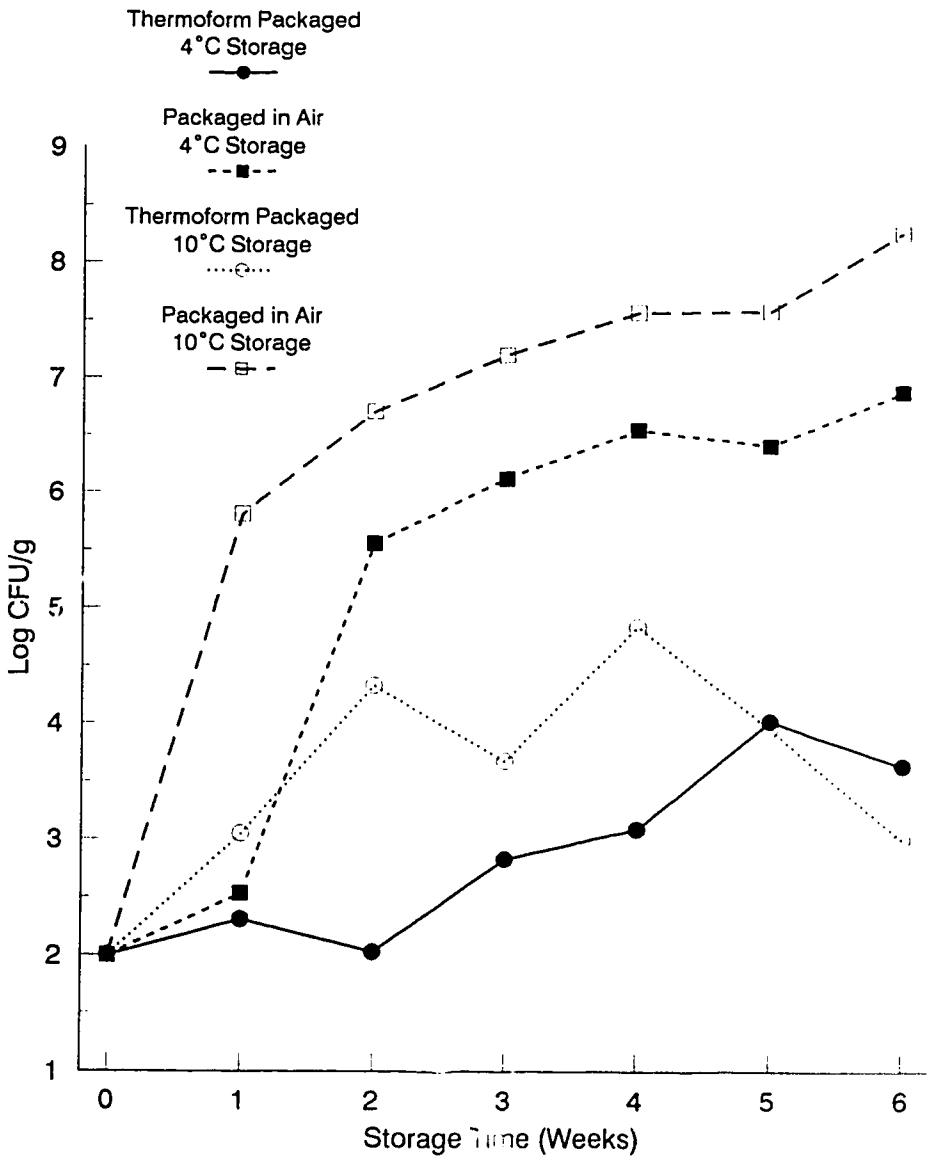


Figure 4.3. Development of Lactic Acid Bacteria in Cooked Hamburger Patties Packaged with and without Oxygen and Stored at 4 and 10°C

Thermoform packaging - approximately 40% carbon dioxide with the balance nitrogen

Air packaging - approximately 50% carbon dioxide and 50% air

Table 4.5. Variation in the Development of Presumptive Lactic Acid Bacteria in Hamburger Patties Stored at 4 and 10°C for up to 6 Weeks

Samples	TP[a] 4°C Storage		AP[b] 4°C Storage		TP 10°C Storage		AP 10°C Storage	
	Number of Samples Developing a Lactic Microflora and Counts (CFU/g) of Positive Samples							
Storage Time (Weeks)								
0	0/5	5-<10^2	0/5	5-<10^2	0/5	5-<10^2	0/5	5-<10^2
1	1/5	1- 10^3	4/5	4- 10^2	3/5	1- 10^2	5/5	2- 10^4
	4/5	4-<10^2	1/5	1-<10^2		1- 10^3		3- 10^6
						1- 10^5		
2	1/5	1- 10^2	5/5	4- 10^5	4/5	2- 10^4	5/5	3- 10^6
	4/5	4-<10^2		1- 10^6		2- 10^5		2- 10^7
					1/5	1-<10^2		
3	2/5	1- 10^2	5/5	3- 10^5	2/5	1- 10^5	5/5	5- 10^7
		1- 10^5		2- 10^6		1- 10^7		
	3/5	3-<10^2			3/5	3-<10^2		
4	2/5	2- 10^4	5/5	2- 10^5	3/5	3- 10^6	5/5	1- 10^6
	3/5	3-<10^2		1- 10^6	2/5	2-<10^2		2- 10^7
				2- 10^7				2- 10^8
5	4/5	1- 10^3	5/5	2- 10^5	2/5	1- 10^6	5/5	5- 10^7
		3- 10^4		3- 10^6		1- 10^7		
	1/5	1-<10^2			3/5	3-<10^2		
6	2/5	1- 10^5	5/5	3- 10^6	1/4	1- 10^6	5/5	1- 10^7
		1- 10^6		2- 10^7	3/4	3-<10^2		4- 10^8
	3/5	3-<10^2						

[a]Thermoform packaging (approximately 40% carbon dioxide with the balance nitrogen)

[b]Air packaging (50% air and 50% carbon dioxide)

Determined on MRS agar adjusted to pH 5.6 with 85% lactic acid.

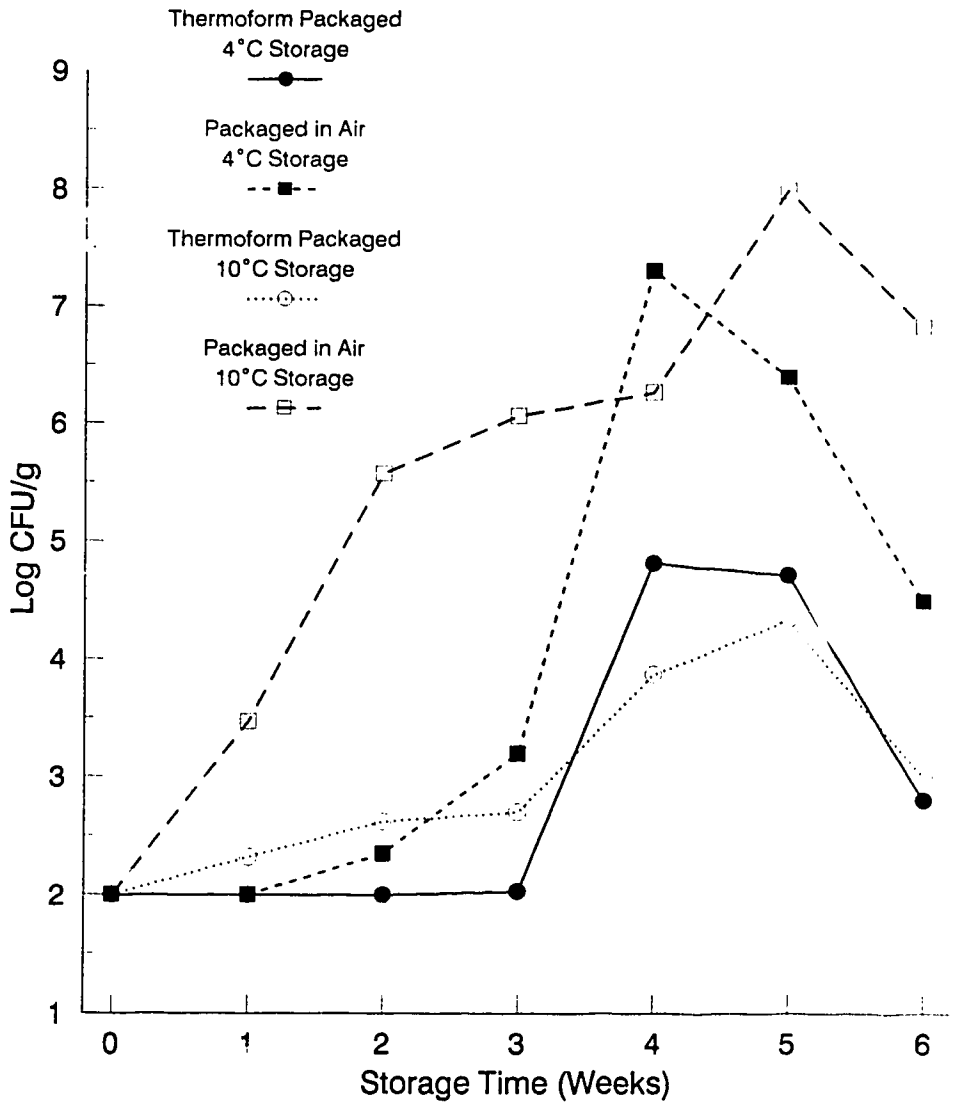


Figure 4.4. Development of Yeasts and Molds in Cooked Hamburger Patties Packaged with and without Oxygen and Stored at 4 and 10°C

Thermoform packaging - approximately 40% carbon dioxide with the balance nitrogen

Air packaging - approximately 50% carbon dioxide and 50% air

occurred until the end of 4 weeks of storage. After 6 weeks of storage at 4 and 10°C, the samples in thermoform packages supported the least amount of yeast and mold development (10^3 CFU/g); while samples packaged in 50% air and 50% CO_2 developed a yeast and mold count of 10^4 and 10^7 CFU/g at 4 and 10°C, respectively.

The data in Table 4.6 shows the variability observed within treatments in the development of yeasts and molds in the hamburger patties during 6 weeks of storage. The variability was noticeable but not to the same degree that was observed with lactic acid bacteria.

(5) Presumptive counts of Staphylococcus aureus

S. aureus grew at 10°C in patties packaged in 50% air and 50% CO_2. Growth of *S. aureus* was not significant at either 4 or 10°C in the thermoform packaged patties nor in the samples packaged in 50% air and 50% CO_2 and stored at 4°C.

(6) Anaerobic sporeformers and enterococci

The only hamburger patties to support the growth of sporeformers were those in the thermoform packages stored at 10°C. This occurred after 4 weeks of storage. Wet mount microscopy confirmed the presence of spores. The patties in the thermoform packages stored at 10°C developed a microflora that was resistant to heating at 75°C for 20 min, but were not believed to be sporeformers. It was suspected that these bacteria were *Enterococcus* species. Colonies were streaked onto KF agar plates, Gram stained, and checked for production of catalase (-), growth in BHI with 6.5% NaCl and growth at 45°C, confirming that these strains were *Enterococcus* species and not anaerobic sporeformers.

4.3.2 Characteristics of UAL 26.CL97

(1) Rate of Plasmid Loss

This experiment showed that the CL97 plasmid is stable, no loss of chloramphenicol resistance and bacteriocin production, even after 50 generations of growth in APT broth. The growth of UAL 26.CL97 in APT and APT.Cm broth was

Table 4.6. Variation in the Development of Yeasts and Molds in Hamburger Patties Stored at 4 and 10°C for up to 6 Weeks

Samples	TP[a] 4°C Storage		AP[b] 4°C Storage		TP 10°C Storage		AP 10°C Storage	
	Number of Samples Developing a Yeast and Mold Microflora and Counts (CFU/g) of Positive Samples							
Storage Time (Weeks)								
0	0/5	5-<10^2	0/5	5-<10^2	0/5	5-<10^2	0/5	5-<10^2
1	0/5	5-<10^2	0/5	5-<10^2	3/5	3-<10^2 1- 10^2 1- 10^3	5/5	4- 10^3 1- 10^4
2	0/5	5-<10^2	1/5 4/5	1-<10^2 4- 10^2	3/5 2/5	3-<10^2 2- 10^3	5/5	5- 10^4
3	1/5 4/5	1- 10^2 4-<10^2	5/5	1- 10^2 4- 10^3	3/5 2/5	3-<10^2 2- 10^3	5/5	3- 10^5 2- 10^6
4	5/5	1- 10^3 3- 10^4 1- 10^7	5/5	5- 10^7	1/5 4/5	1-<10^2 1- 10^2 1- 10^3 1- 10^5 1- 10^6	5/5	1- 10^5 3- 10^6 1- 10^7
5	5/5	1- 10^3 2- 10^4 2- 10^5	5/5	1- 10^5 4- 10^6	5/5	5- 10^4	5/5	2- 10^7 3- 10^8
6	2/5 3/5	1- 10^2 1- 10^5 3-<10^2	1/5 4/5	1-<10^2 2- 10^4 2- 10^5	5/5	5- 10^3	5/5	3- 10^6 2- 10^7

[a]Thermoform packaging (approximately 40% carbon dioxide with the balance nitrogen)

[b]Air packaging (approximately 50% air and 50% carbon dioxide)

Determined on Potato Dextrose Agar adjusted to pH 3.5 with 10% tartaric acid.

compared after 24 h. A 24-h culture (1% inoculum) would have gone through 7 generations of growth, thus subculturing every 24 h (7 times) would result in 50 generations of growth.

Growth in APT.Cm broth selects for the expression of chloramphenicol resistance (Cm^R). The results (Table 4.7) indicate that plasmid CL97 was not lost after 7 generations nor after 50 generations of growth in APT broth. This suggests that it is possible to grow UAL 26.CL97 in APT broth without the loss of chloramphenicol resistance and expression of CL97 plasmid for at least 50 generations. If the CL97 plasmid was lost, it would have been indicated by a difference in APT and APT.Cm counts, where APT.Cm count would have been lower than the APT count, since only Cm^R colonies would have been enumerated.

(2) Tolerance to NaCl

Various concentrations of NaCl (0.5, 1.0, 1.5 and 2.0 percent) were added to APT broth and Cooked Meat Medium to determine the sensitivity of UAL 26.CL97 to NaCl. After 24 h of incubation at 25°C, UAL 26.CL97 grew in APT broth and Cooked Meat Medium containing the various NaCl concentrations. This suggests that the salt content (0.3%) in the hamburger patties should not affect the growth of UAL 26.CL97.

(3) Heat resistance in APT broth

Heat resistance of UAL 26.CL97 at 60°C was determined as described in section 4.2.5. From this study it was concluded that UAL 26.CL97 is heat sensitive in broth. Figure 4.5 illustrates the death curve obtained for UAL 26.CL97 at 60°C. From the three replicates a mean D_{60} of 13.2 s was calculated. The data at the 2 min sampling interval cannot be considered as very accurate because it represents only 1 or 2 colonies on an agar plate.

Table 4.7. Enumeration of UAL 26.CL97 after 7 Generations of Growth in APT and APT.Cm Broth and after Growth in APT Broth for 50 Generations

	APT Count Log CFU/mL[a]	APT.Cm Count Log CFU/mL[a]
7 generations		
growth in APT	9.00	8.85
growth in APT.Cm	8.89	8.79
50 generations		
growth in APT	8.96	8.86

[a]Mean of 2 replicates

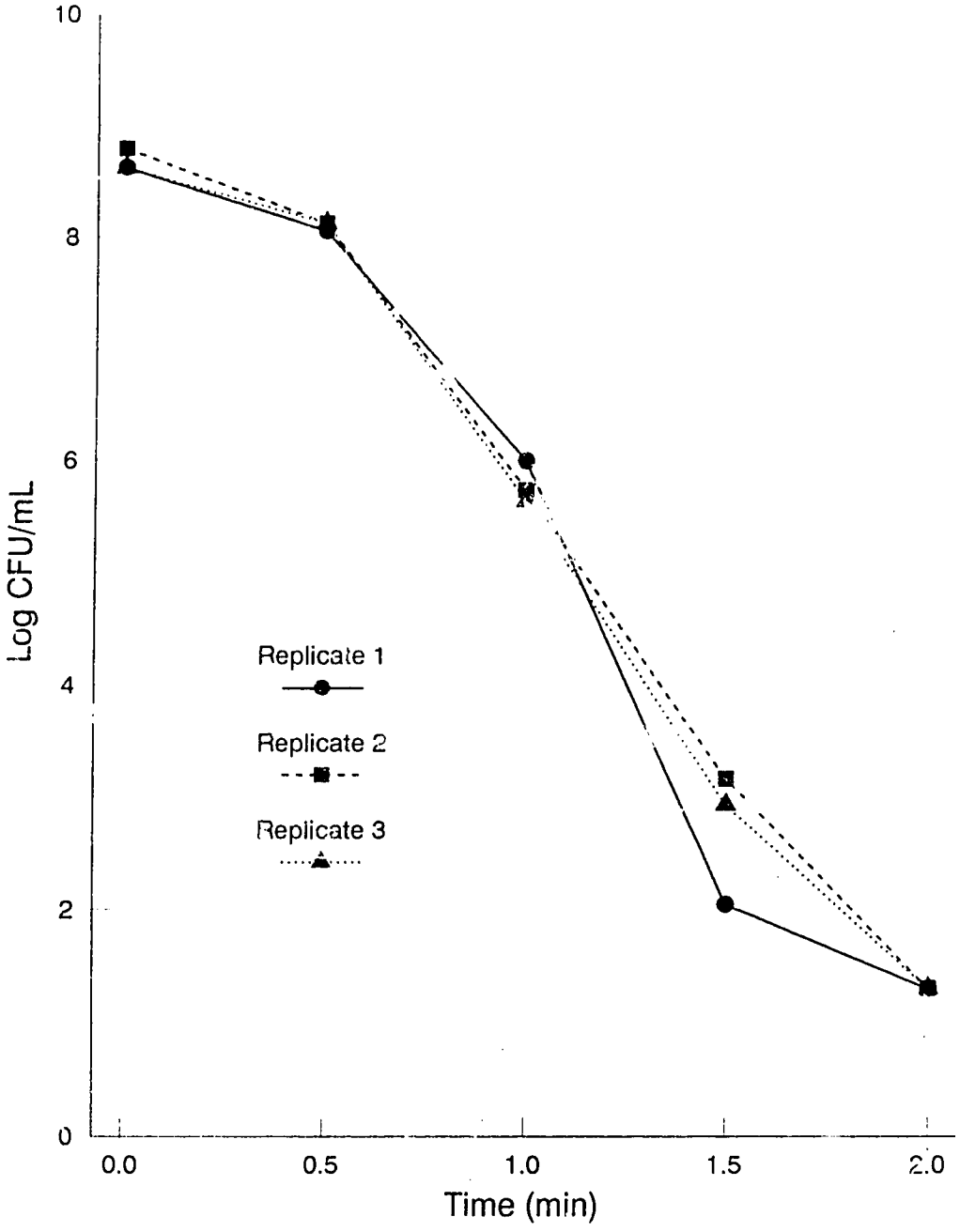


Figure 4.5. Death Curve for UAL 26.CL97 at 60°C

(4) Growth at 4°C

UAL 26.CL97 was shown to grow well in culture media at 4°C. The growth of UAL 26.CL97 in APT broth, APT.Cm broth and Cooked Meat Medium at 4°C is shown in Figure 4.6. At 4°C UAL 26.CL97 reached 10^8 CFU/mL after 4 and 6 days in Cooked Meat Medium and APT broth, respectively. Maximum population was reached after the six days of storage. Growth in APT.Cm broth was considerably slower, reaching 10^7 CFU/mL after 10 days of growth at 4°C.

4.3.3 Calibration of Thermometer and Data Logger

A Fisher partial immersion thermometer was calibrated to both an ice point reference and a steam point reference as described in section 4.2.9. The calibration of the thermometer was done on two different days. On both days the ice point readings were 0°C and the boiling point readings as follows:

Reading	Theoretical
97.3°C	97.5°C
97.8°C	98.2°C.

It was concluded that the thermometer was sufficiently accurate to be used as a reference in the calibration of the data logger (Hanzon Data Inc., Woodinville, WA).

In the calibration of the data logger, an ice point reference was taken but it did not correspond well with the thermometer reading. This was not considered to be a concern because the temperature range of interest was between 50-80°C. To insure that the data logger was reading accurately at this range of temperatures, a circulating waterbath was set at varying temperatures and the temperature readings were recorded with the data logger using the calibrated thermometer as a reference. The data in Table 4.8 illustrates that the temperature readings of the data logger correspond well with the readings of the calibrated thermometer. In addition, there was little variation between the readings recorded for the individual thermocouples.

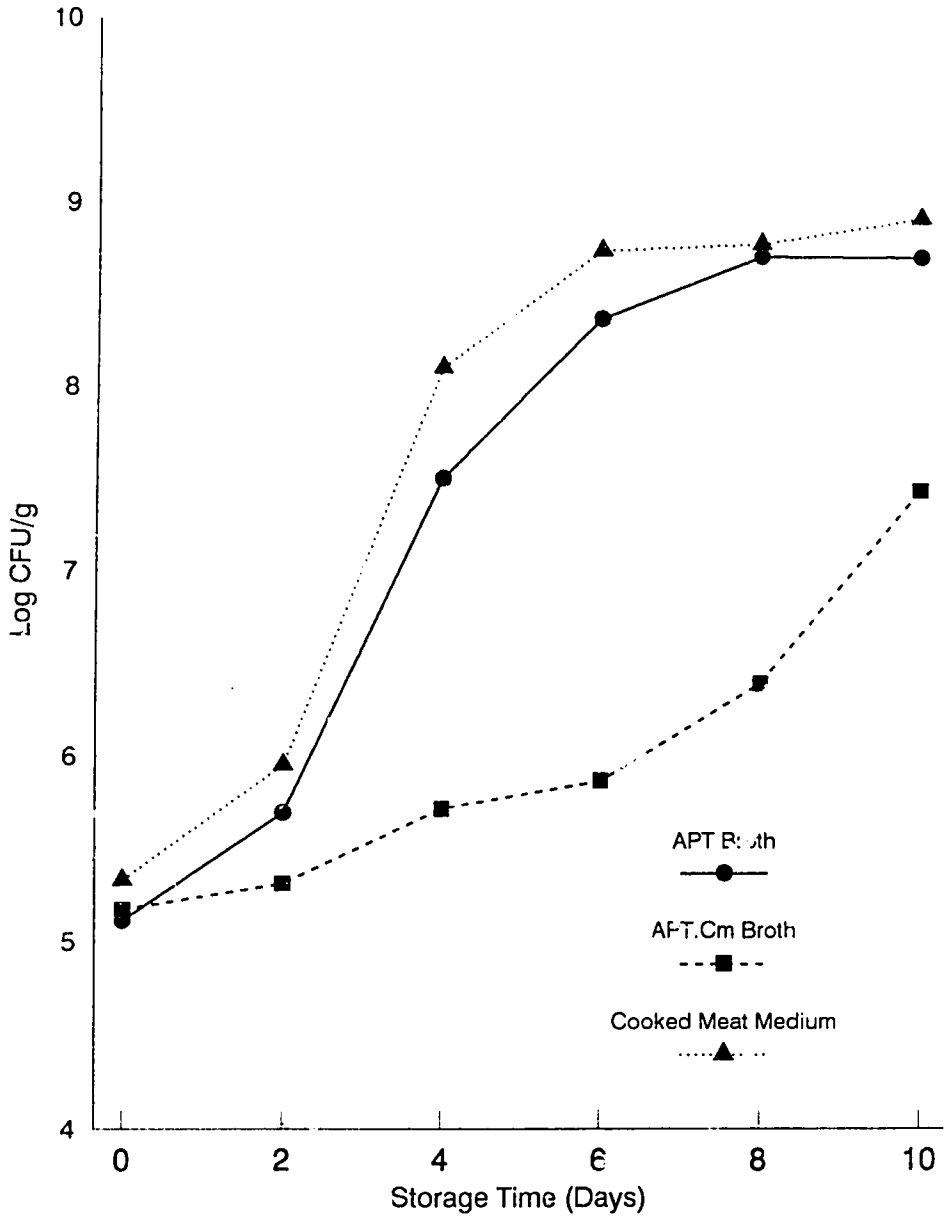


Figure 4.6. Growth of UAL 26.CL97 in Broth and Cooked Meat Medium at 4°C

Table 4.8. Data Logger Calibration Readings Over a Temperature Range of 50-85°C

Waterbath Setting (°C)	Calibrated Thermometer Reading (°C)	Data Logger Reading (°C)[a]
50.0	50.0	49.7
60.0	60.1	60.0
70.0	70.0	70.0
75.0	75.1	75.1
80.0	80.1	80.3
85.0	85.1	85.2

[a]Means of 8 thermocouple readings

Thus, the temperature readings recorded by the data logger appeared to be accurate.

4.3.4 Microbial Analysis of Raw Inoculated Patties

Reformed hamburger patties inoculated with liquid culture (10^9 - 10^{10} CFU/mL) and freeze dried culture (10^{10} CFU/mL prior to freeze drying) were analyzed to determine the actual number of UAL 26.CL97 present in frozen patties inoculated with these two types of bacterial cultures. Results of the enumeration (Table 4.9) indicate that all three sets inoculated with liquid culture had counts of approximately 10^7 CFU/g. The counts from the two sets inoculated with freeze dried culture were variable. One set had counts similar to those inoculated with the liquid culture, while the second set had counts 10-fold less. The variation was not due to differences in the growth of the culture nor in the resuspension of the cells, because both sets began with a cell concentration of 10^{10} CFU/mL. Variation could be due to differences in the number of times the culture was previously subcultured, making the bacterial cells more sensitive to the freeze drying process or to loss of cells when inoculating the hamburger patties. The data was inconclusive as to whether inoculating patties with liquid culture or with freeze dried culture result in the same concentration of cells in hamburger patties.

4.3.5 Survival of UAL 26.CL97 after Cooking Hamburger Patties to Various Internal Temperatures

(1) Inoculum size required to obtain survivors

Initially, 1 mL portions of cell suspensions containing 10^5 to 10^8 CFU/mL were seeded into hamburger patties (approx. 113 g). Patties were cooked in a commercial broiler at a temperature setting that would give an internal temperature of approximately 70-75^{o}C.

Plating of the cooked patties to determine survivors on APT.Cm agar indicated that these concentrations were not high enough to allow UAL 26.CL97 to survive.

Table 4.9. APT.Cm Counts of Frozen Raw Hamburger Patties Inoculated with Liquid and Freeze Dried Cultures

	Liquid Culture[a] Log CFU/g[c]		Freeze Dried Culture[b] Log CFU/g		
Replicate	1	2	1	2	3
Set 1	7.54	7.45	7.95	7.72	7.93
Set 2	7.60	7.57	6.11	6.49	6.28
Set 3	7.59	7.54			

[a]Containing 10^9 - 10^{10} CFU/mL

[b]Containing 10^{10} CFU/mL prior to freeze drying

[c]Mean of duplicate data

The surviving microflora was low numbers of spreader type colonies. It was concluded that a larger inoculum size was required and that temperature monitoring of individual patties was necessary, due to variation in the degree of doneness at the chosen temperature setting.

Subsequently, UAL 26.CL97 was grown in 500 mL APT.Cm broth and after centrifugation cells were resuspended in 50 and 10 mL of 0.1% peptone water giving cell concentrations of 2.2×10^{10} and 9.4×10^{10} CFU/mL, respectively. Examples of temperature and survivor data are shown in Table 4.10. Data collected indicated that UAL 26.CL97 survived internal cooking temperatures between 60-70°C. However no survivors were detected at 75°C. However, at these inoculum concentrations, and storage at 4°C, UAL 26.CL97 may resuscitate and grow in the hamburger patties. Hence a storage experiment was designed.

(2) Survival and Development after Storage for 2 Weeks at 4°C

Reformed hamburger patties inoculated with a culture of UAL 26.CL97 containing 10^{10} CFU/mL were cooked in a commercial broiler at two different temperature settings (lower setting - top setting at 1000, bottom at 1050 and higher setting - top 975, bottom 1100) with the cooking time set at 3.5 minutes. Internal temperature of each patty was measured by inserting an alcohol sterilized thermocouple into the patty as it dropped from the broiler. Patties were cooled to 4°C and then divided in half. One half of the patty was used to enumerate survivors by plating on APT.Cm agar. The other half of the patty was packaged in gas impermeable film flushed with 50% CO_2/50% N_2. Packaged patties were stored for 2 weeks at 4°C. Four sets, each on different days, were obtained and enumerated to determine if there was consistency in survival and development of UAL 26.CL97. The data are summarized in Table 4.11.

The hamburger patties were cooked at two temperature settings, but there was variation in temperatures within the temperature settings. Temperatures ranged

Table 4.10. Survival of UAL 26.CL97 in Cooked Hamburger Patties

Inoculum Size Log CFU/mL		Internal Temperature (°C)	APT.Cm Count Log CFU/g
10^{10}	Replicate		
	1	62.4	3.62
	2	65.4	3.89
	3	70.4	3.04
	4	75.2	< 2.00
	5	75.5	< 2.00
10^{11}	Replicate		
	1	60.4	3.59
	2	60.6	5.36
	3	61.6	3.32
	4	62.8	2.18
	5	67.0	2.15

Table 4.11. Survival of UAL 26.CL97 after Cooking at Two Temperature Settings and Recovery after 2 Weeks of Storage at 4°C

Temperature Setting	Top Control Setting - 1000 Bottom Control Setting - 1050		Top Control Setting - 975 Bottom Control Setting - 1100		
	Initial Count[a] Log CFU/g	2 Week Count[a] Log CFU/g		Initial Count Log CFU/g	2 Week Count Log CFU/g
Set 1	Temperature (°C)		Temperature (°C)		
Replicate					
1	67.3	< 2.00	3.60	71.1	< 2.00	< 2.00
2	69.3	2.43	4.75	73.7	< 2.00	< 2.00
3	71.1	< 2.00	< 2.00	74.0	< 2.00	< 2.00
4	71.7	< 2.00	< 2.00	75.1	< 2.00	< 2.00
5	71.9	< 2.00	< 2.00	75.4	< 2.00	< 2.00
6	74.0	< 2.00	2.20			
Set 2						
Replicate						
1	65.3	< 2.00	3.65	71.4	3.08	3.32
2	66.7	< 2.00	3.92	72.4	< 2.00	2.79
3	67.8	< 2.00	3.32	73.0	2.11	3.20
4	68.2	< 2.00	< 2.00	74.4	< 2.00	< 2.00
5	72.6	< 2.00	< 2.00	74.9	< 2.00	3.20
6	74.9	< 2.00	3.15	75.6	< 2.00	3.98

Table 4.11. (Continued)

Temperature Setting	Top Control Setting - 1000 Bottom Control Setting - 1050			Top Control Setting - 975 Bottom Control Setting - 1100		
		Initial Count[a] Log CFU/g	2 Week Count[a] Log CFU/g		Initial Count Log CFU/g	2 Week Count Log CFU/g
Set 3	Temperature (°C)			Temperature (°C)		
Replicate						
1	64.5	5.15	7.66	77.1	3.72	6.28
2	65.9	2.53	6.59	70.6	< 2.00	4.36
3	66.9	< 2.00	6.45	70.6	2.26	7.23
4	68.2	3.28	6.40	71.4	< 2.00	4.87
5	69.3	3.40	7.11	72.6	< 2.00	4.15
6	70.6	2.45	4.88	77.1	< 2.00	4.49
Set 4						
Replicate						
1	64.8	3.11	4.96	70.1	< 2.00	4.52
2	65.2	< 2.00	3.87	73.0	< 2.00	3.04
3	65.4	3.84	5.38	73.8	< 2.00	< 2.00
4	66.3	2.70	5.67	73.8	< 2.00	4.98
5	70.8	3.18	5.16	76.5	< 2.00	< 2.00

[a]Initial and 2 week counts enumerated on APT.Cm agar

from 67-71°C at the lower setting and 71-74°C at the higher setting. The data from all four sets showed that UAL 26.CL97 survived in 35% of the patties cooked to temperatures between 67-71°C and 17% of the patties cooked to temperatures between 72-74°C. After storage for 2 weeks at 4°C, 78% and 61% of the patties showed growth of UAL 26.CL97, respectively.

The higher temperature setting was more favorable in that the patties were well cooked and had a uniform brown color. Ideally this was the temperature desired. In some instances, UAL 26.CL97 did not survive heat treatment even at the lower temperatures in some patties. The 2-week storage period allowed heat injured cells to resuscitate and grow. The ability of UAL 26.CL97 to grow in some of the patties after a 2 week storage was encouraging.

(3) Survival and Development of UAL 26.CL97 During Storage

Reformed patties were cooked in the same manner as described in the previous section except that one temperature setting was used to obtain internal cooking temperatures over 70°C. Two sets of data were collected. Patties were divided into 5 sections and plated initially, after 1 week at 10°C and after 2, 4, and 6 weeks at 4°C. Data from this storage experiment are shown in Table 4.12. Similar to the results of the previous experiment, only 17% of the cooked patties showed initial survival of UAL 26.CL97. Detection of UAL 26.CL97 after 1 week at 10°C and after 2, 4 and 6 weeks at 4°C was 58%, 33%, 42% and 58%, respectively. Even after 6 weeks of storage 42% of the patty sections failed to show growth of UAL 26.CL97. Data indicate that the survival and growth of UAL 26.CL97 at temperatures above 70°C is not guaranteed.

4.3.6 Effect of Freeze Drying Bacterial Culture on Survival After Cooking

Patties inoculated with a freeze dried culture were cooked to temperatures above 70°C to determine if the survival rate would be the same as those patties inoculated with liquid culture. The two sets of data obtained showed that freeze

Table 4.12. Recovery of UAL 26.CL97 after Cooking and Storage

	APT.Cm Counts (Log CFU/g)				
	Initial Count	1 Week at 10°C	2 Weeks at 4°C	4 Weeks at 4°C	6 Weeks at 4°C
Temperature (C)					
Set 1					
71.2	< 2.00	6.08	4.80	7.81	7.36
71.2	< 2.00	4.23	4.00	6.62	5.56
71.9	2.30	5.45	< 2.00	< 2.00	< 2.00
72.9	< 2.00	< 2.00	< 2.00	< 2.00	5.79
74.6	< 2.00	3.73	3.67	6.40	7.63
77.5	< 2.00	< 2.00	< 2.00	6.23	6.26
Set 2					
73.0	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
74.2	< 2.00	3.00	< 2.00	< 2.00	5.18
74.5	2.41	5.26	2.43	7.15	6.60
75.1	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
75.1	< 2.00	2.23	< 2.00	< 2.00	< 2.00
77.8	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00

drying was detrimental to the survival and subsequent growth on storage of UAL 26.CL97. After 1 week of storage at 10°C, growth of UAL 26.CL97 was detected in 27% of patty sections. There was no growth in the majority of patties stored at 4°C.

4.3.7 Confirmation that Survivors were UAL 26.CL97

Confirmation that the survivors were UAL 26.CL97 was determined by a direct antagonism method as described by Tagg *et al.* (1976) and outlined in section 4.11. UAL 26 was the main indicator organism with UAL 8 used occasionally to compare inhibitory activity of the surviving colonies.

Approximately 87% of colonies picked from the APT plates and 100% of colonies picked from APT.Cm plates showed inhibitory activity against UAL 26. Plates overlayered with the indicator organism showed distinct zones of inhibition confirming that the colonies were UAL 26.CL97. A number of colonies that were believed not to be UAL 26.CL97 were also tested against UAL 26 to determine if these colonies had inhibitory activity against UAL 26. No zones of inhibition were observed.

Colonies were also tested against UAL 8 to determine if both bacteriocins of UAL 26.CL97 had the same level of activity. The results showed that UAL 8 was inhibited, but the zones of inhibition were considerably smaller and not all of the surviving colonies showed inhibition against UAL 8. This suggests that Bac^{8} is more active than Bac^{26}, but that both bacteriocins were being produced.

4.3.8 Relationship Between Growth Rate and Bacteriocin Production in Culture Medium and Meat

(1) Growth of UAL 26.CL97 at 4 and 10°C

Cooked Meat Medium, meat slurry and hamburger meat inoculated with UAL 26.CL97 (10^6 CFU/g) and stored at 4 and 10°C showed differences in the growth of UAL 26.CL97. Figures 4.7 and 4.8 show that the growth of UAL 26.CL97 was very rapid at 10°C, reaching 10^8 CFU/g after 2 days of storage in Cooked Meat Medium

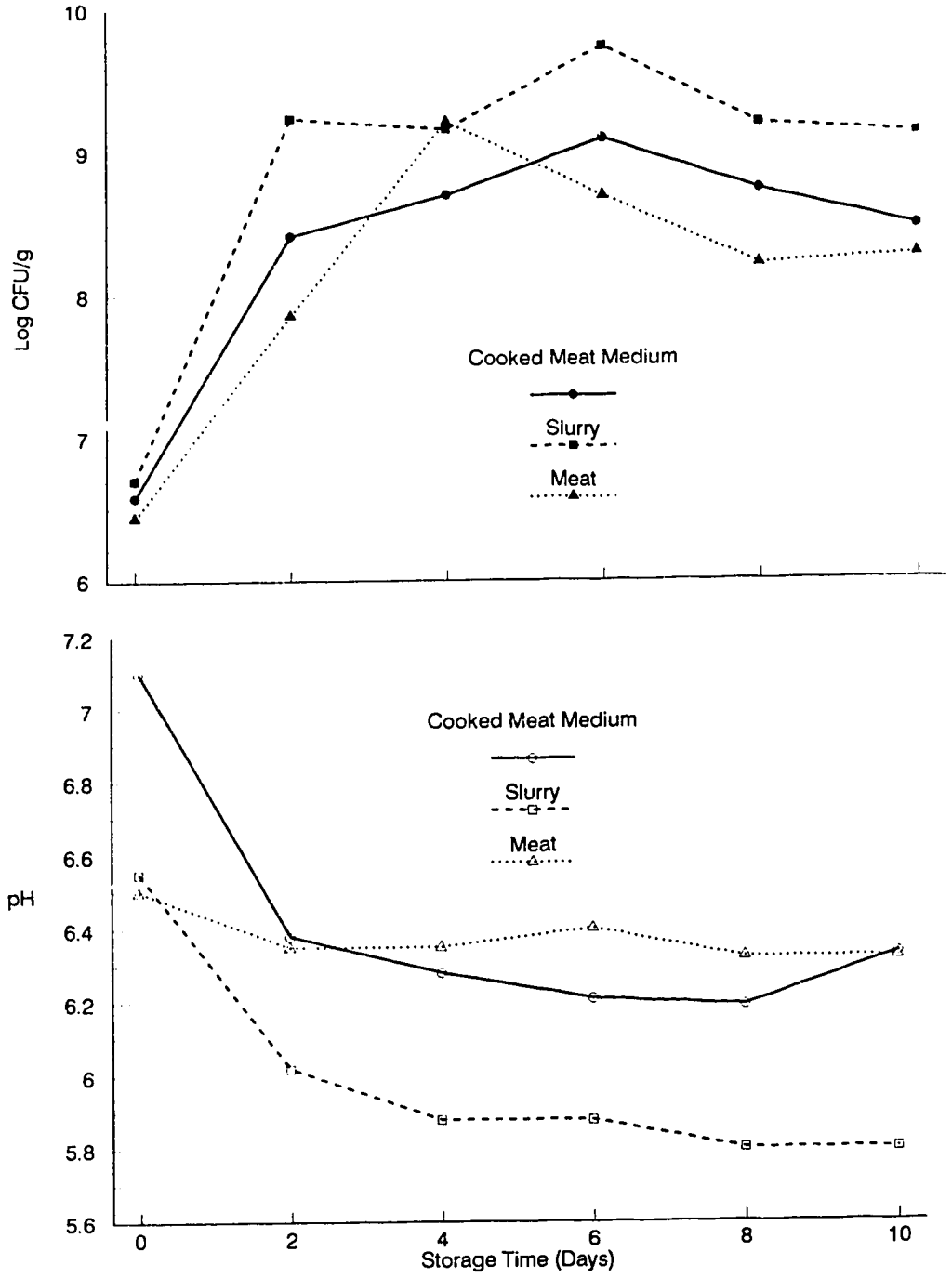


Figure 4.7. Growth of UAL 26.CL97 and pH Changes in Cooked Meat Medium and Meat at 10°C

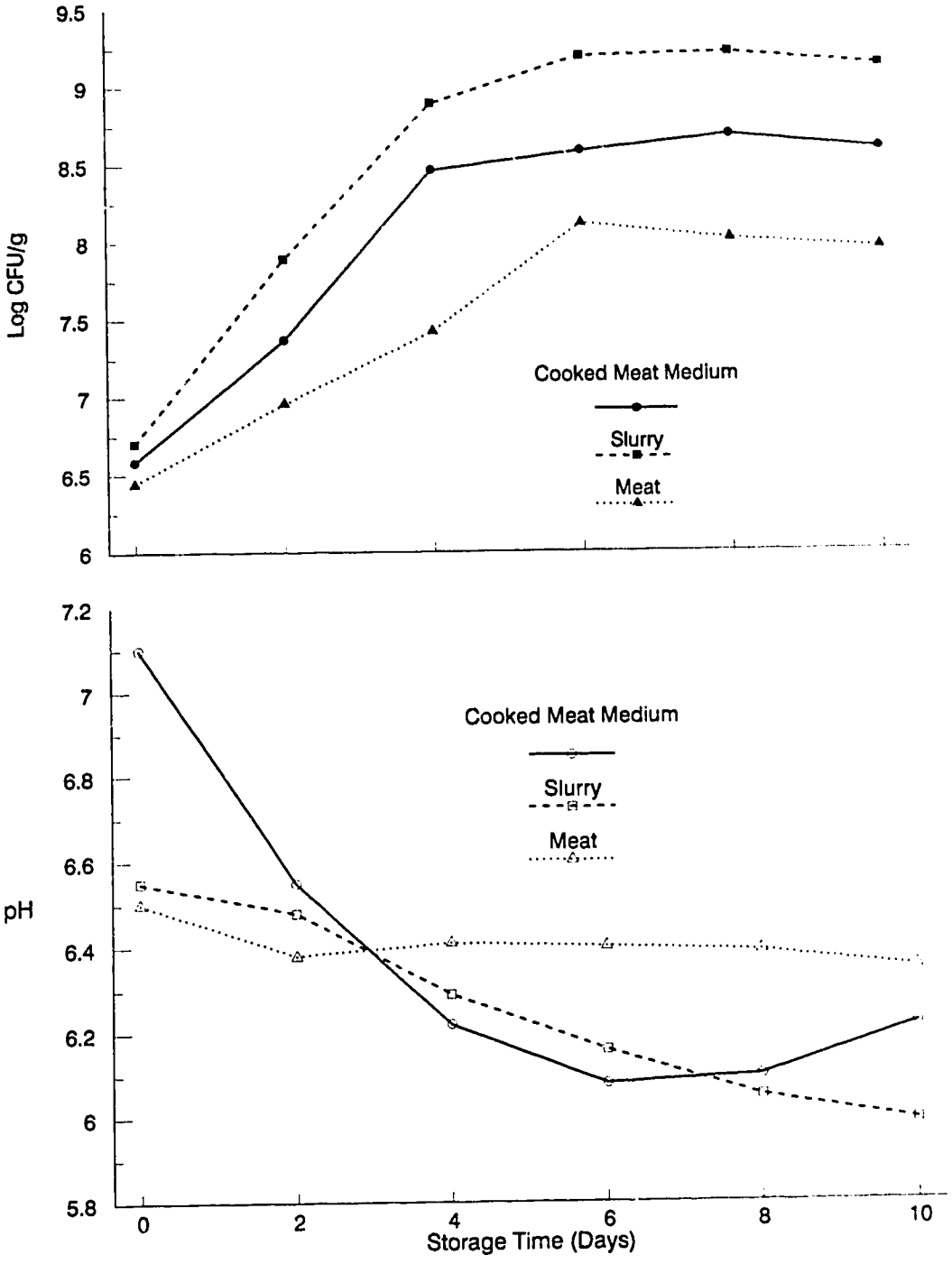


Figure 4.8. Growth of UAL 26.CL97 and pH Changes in Cooked Meat Medium and Meat at 4°C

and meat slurry and after 4 days in the hamburger meat. At 4°C growth was slower with a count of 10^8 CFU/g being reached after 4 days in the Cooked Meat Medium and meat slurry and after 6 days in the hamburger meat. Growth in the hamburger meat reached maximum population after 4 days at 10°C and after 6 days at 4°C. Rapid growth was accompanied by a rapid decrease in pH in the Cooked Meat Medium and slurry. The hamburger meat is more of a buffer than other substrates. The pH was relatively stable throughout the 10 days of storage at both storage temperatures, however the meat was suspended in buffer at pH 7.0.

(2) Production of bacteriocin by UAL 26.CL97 in culture medium and meat

UAL 26.CL97 produces bacteriocin in Cooked Meat Medium, meat slurry and hamburger meat at 4 and 10°C. Detection of bacteriocin occurred after UAL 26.CL97 reached a population of 10^8 CFU/g. Bacteriocin production was detected in Cooked Meat Medium and meat slurry after 2 days at 10°C and after 4 days at 4°C; while in hamburger meat it was detected after 4 days at 10°C and after 10 days at 4°C. Samples having pH below 6.0 were adjusted to 6.5 to ensure inhibition of the indicator strain was not due to acidity.

Production of bacteriocin was in greatest in Cooked Meat Medium with 400 AU/mL compared to 200 AU/mL in the meat slurry (Figures 4.9 and 4.10). Bacteriocin production in hamburger meat was less with 100 AU/mL and 50 AU/mL at 10 and 4°C respectively (Figure 4.11).

The addition of 1% SDS or Tween 80 to hamburger meat to aid in the extraction of the bacteriocin resulted in no apparent advantage. In samples containing Tween 80 detection of bacteriocin at 4 and 10°C was the same as those without Tween 80. Bacteriocin appeared to be inhibited by the SDS because samples containing SDS showed no inhibition of the indicator organism.

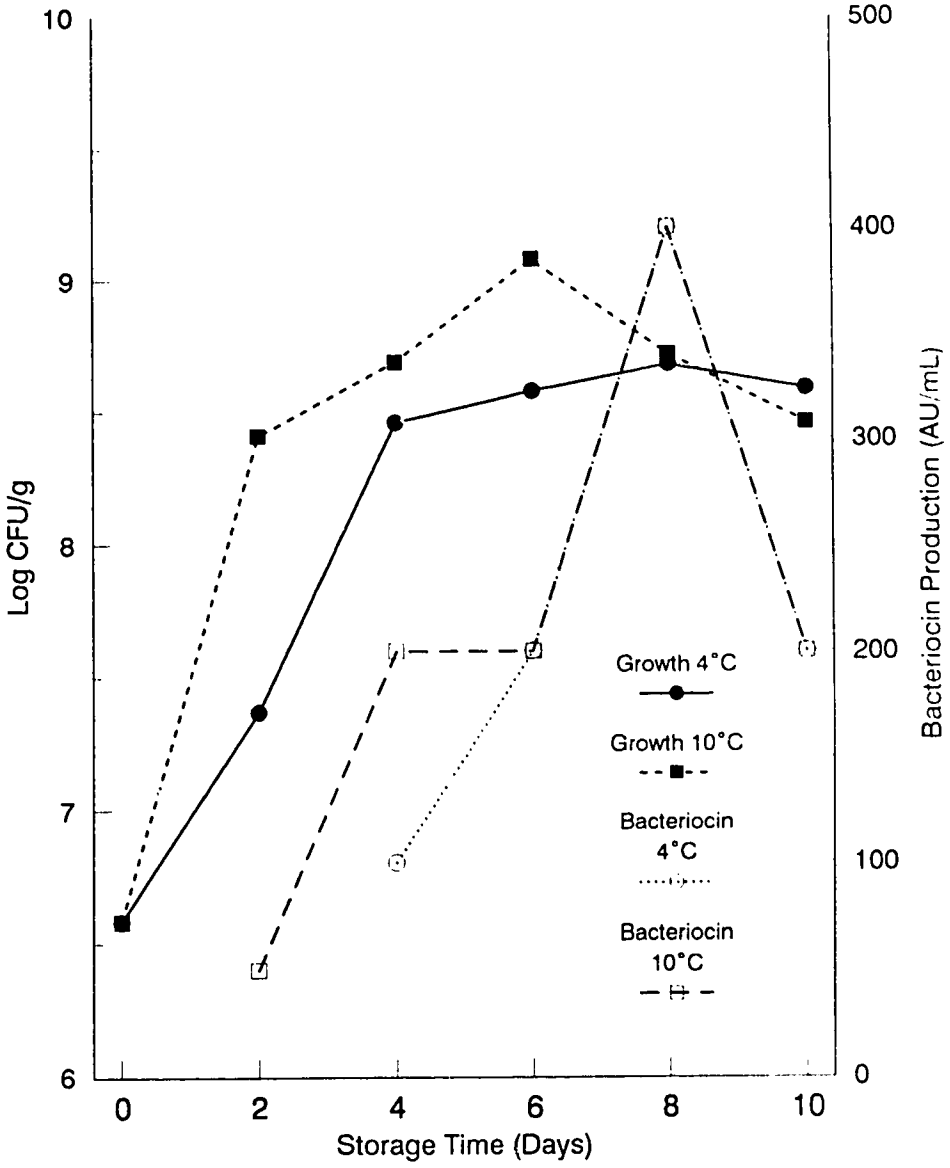


Figure 4.9. Growth of UAL 26.CL97 in Cooked Meat Medium and Bacteriocin Production at 4 and 10°C

AU/mL = reciprocal of the highest dilution showing inhibition against UAL 26

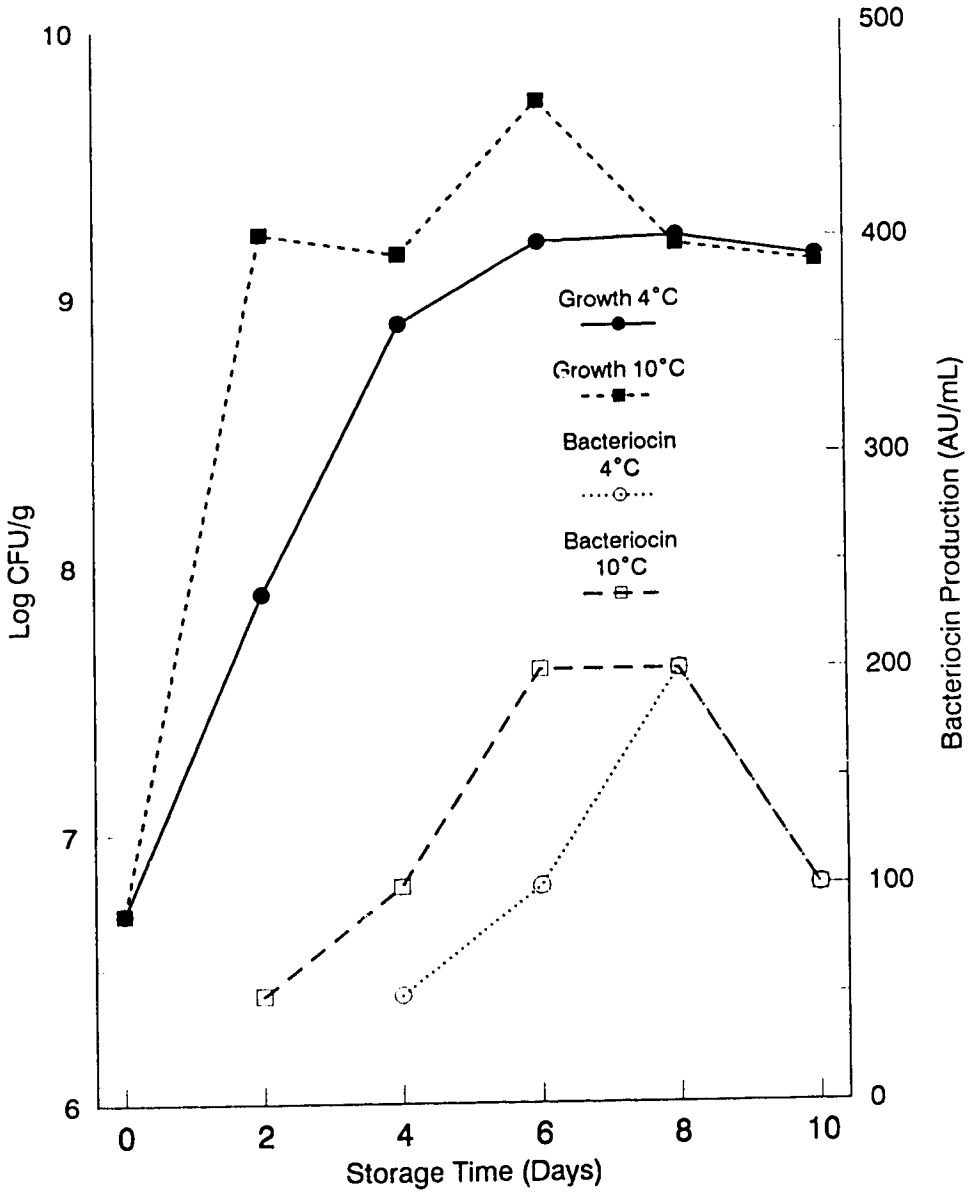


Figure 4.10. Growth of UAL 26.CL97 in Meat Slurry and Bacteriocin Production at 4 and 10°C

AU/mL = reciprocal of the highest dilution showing inhibition against UAL 26

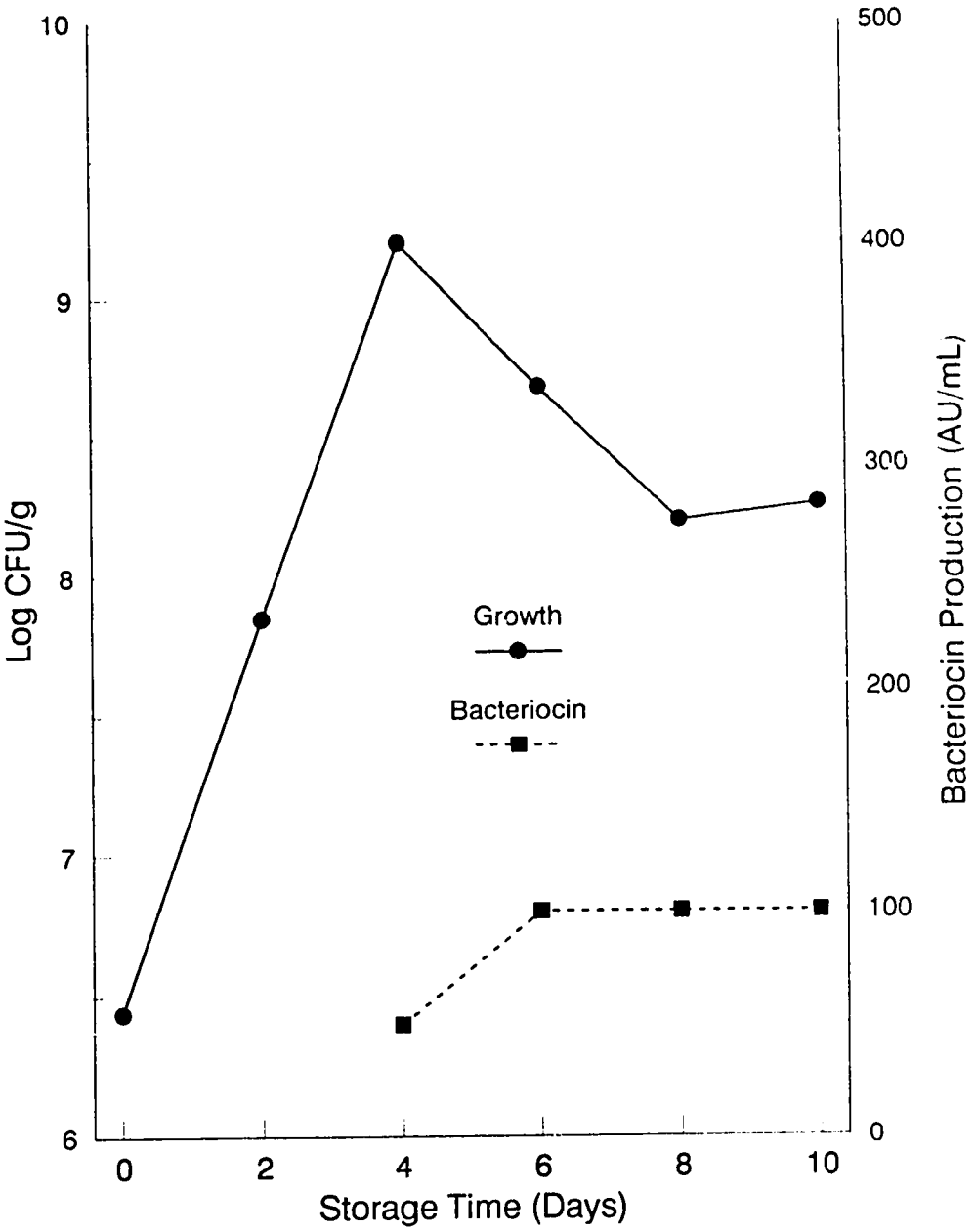


Figure 4.11. Growth of UAL 26.CI97 in Cooked Hamburger Meat and Bacteriocin Production at 10°C

AU/mL = reciprocal of the highest dilution showing inhibition against UAL 26

(3) Confirmation that inhibition was due to bacteriocin activity

After heat treatment (62°C for 30 min) active supernatants from Cooked Meat Medium, meat slurry and hamburger meat were tested for inhibition of activity by protease (1 mg/mL) and inactivation of activity by catalase (68 units/g). To reconfirm that inhibition was due to a proteinaceous substance supernatants were treated with denatured protease (1 mg/mL). Figures 4.12 to 4.14 illustrate the results obtained after 10 days of storage in each growth medium.

4.3.9 Ability of UAL 26.CL97 to Suppress the Growth of Spoilage and Pathogenic Organisms in Hamburger Meat

(1) Effectiveness of UAL 26.CL97 against Enterococcus faecium and Listeria monocytogenes

Heat treated (62°C for 30 min) supernatant of UAL 26.CL97 was spotted onto APT agar plates seeded with *E. faecium* and APT and tryptic soy agar with yeast extract (TSA-YE) seeded with *L. monocytogenes*. After incubation at 25 and 35°C, zones of inhibition were indicative that *E. faecium* and *L. monocytogenes* were sensitive to the inhibitory substances produced by UAL 26.CL97.

(2) Inhibition of Enterococcus faecium in hamburger meat

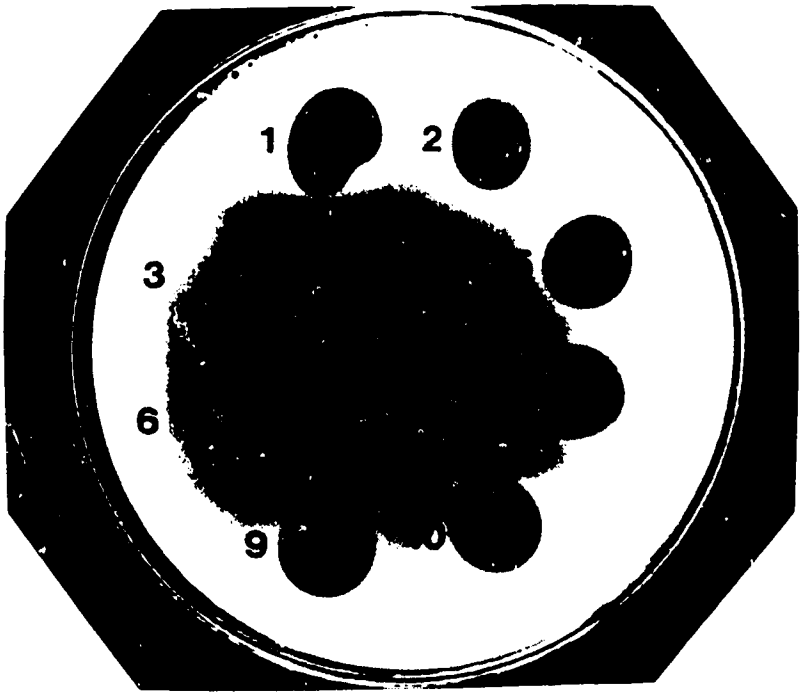
E. faecium was grown alone and in association with UAL 26.CL97 in hamburger meat for 8 days at 10°C. Figure 4.15 depicts the growth of both organisms under these conditions. UAL 26.CL97 was initially present at about 10 times higher density than *E. faecium* and was able to proliferate faster than *E. faecium*. *E. faecium* grew well at 10°C in the hamburger meat. Its growth was approximately 10-fold less when grown in association with UAL 26.CL97.

(3) Inhibition of Listeria monocytogenes in hamburger meat

Figure 4.16 shows that *L. monocytogenes* (grown alone) and UAL 26.CL97 grew to levels of 10^8 CFU/g after 8 days of storage at 10°C. Growth of *L. monocytogenes*

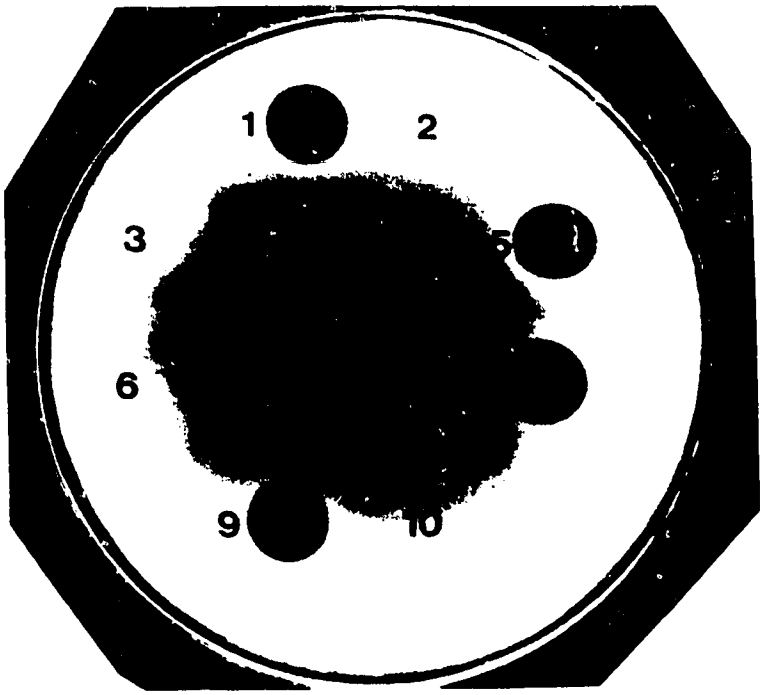
in association with UAL 26.CL97 was suppressed in that the count was less than 10^8 CFU/g after 10 days of storage.

At 4°C (Figure 4.17) there were marked differences between growth rates of UAL 26.CL97 and *L. monocytogenes*. UAL 26.CL97 proliferated well, while *L. monocytogenes* decreased in number both when grown alone and in association with UAL 26.CL97. The decline was greater when grown with UAL 26.CL97. After storage for 8 days *L. monocytogenes* started to increase in population, but still was below the initial inoculum level indicating that there was a resistant population that could grow.



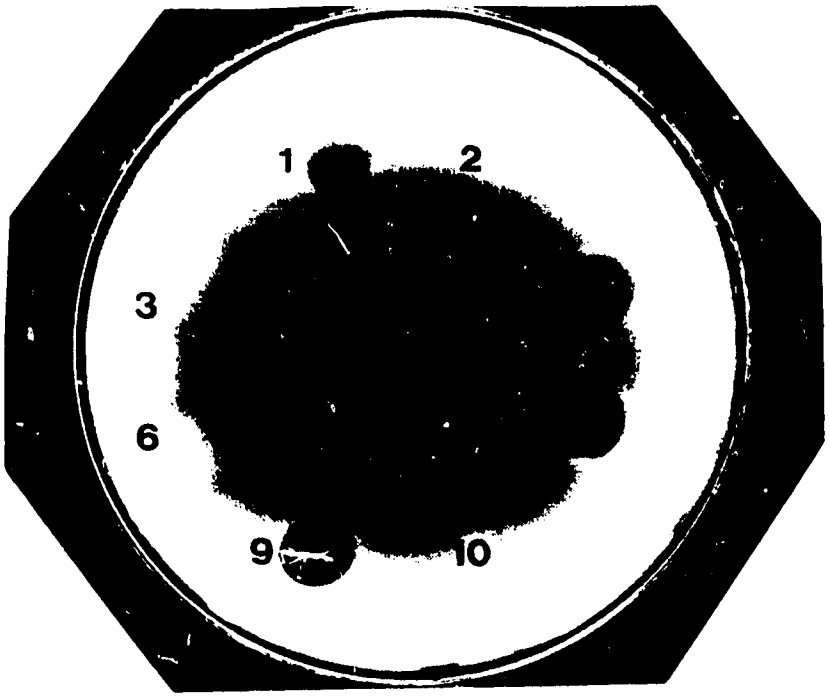
4°C	10°C
1 Heat 62°C for 30 min	6 Protease 1 mg/mL
2 Chloroform equal volume	7 Denatured Protease 1 mg/mL
3 Protease 1 mg/mL	8 Catalase 68 units/mL
4 Denatured Protease 1 mg/mL	9 Heat 62°C for 30 min
5 Catalase 68 units/mL	10 Chloroform equal volume

Figure 4.12. Inhibition of Indicator UAL 26 by Treated Supernatants of UAL 26.CL97 Grown in Cooked Meat Medium



4°C	10°C
1 Heat 62°C for 30 min	6 Protease 1 mg/mL
2 Chloroform equal volume	7 Denatured Protease 1 mg/mL
3 Protease 1 mg/mL	8 Catalase 68 units/mL
4 Denatured Protease 1 mg/mL	9 Heat 62°C for 30 min
5 Catalase 68 units/mL	10 Chloroform equal volume

Figure 4.13. Inhibition of Indicator UAL 26 by Treated Supernatants of UAL 26.CL97 Grown in Meat Slurry



4°C	10°C
1 Heat 62°C for 30 min	6 Protease 1 mg/mL
2 Chloroform equal volume	7 Denatured Protease 1 mg/mL
3 Protease 1 mg/mL	8 Catalase 68 units/mL
4 Denatured Protease 1 mg/mL	9 Heat 62°C for 30 min
5 Catalase 68 units/mL	10 Chloroform equal volume

Figure 4.14. Inhibition of Indicator UAL 26 by Treated Supernatants of UAL 26.CL97 Grown in Hamburger Meat

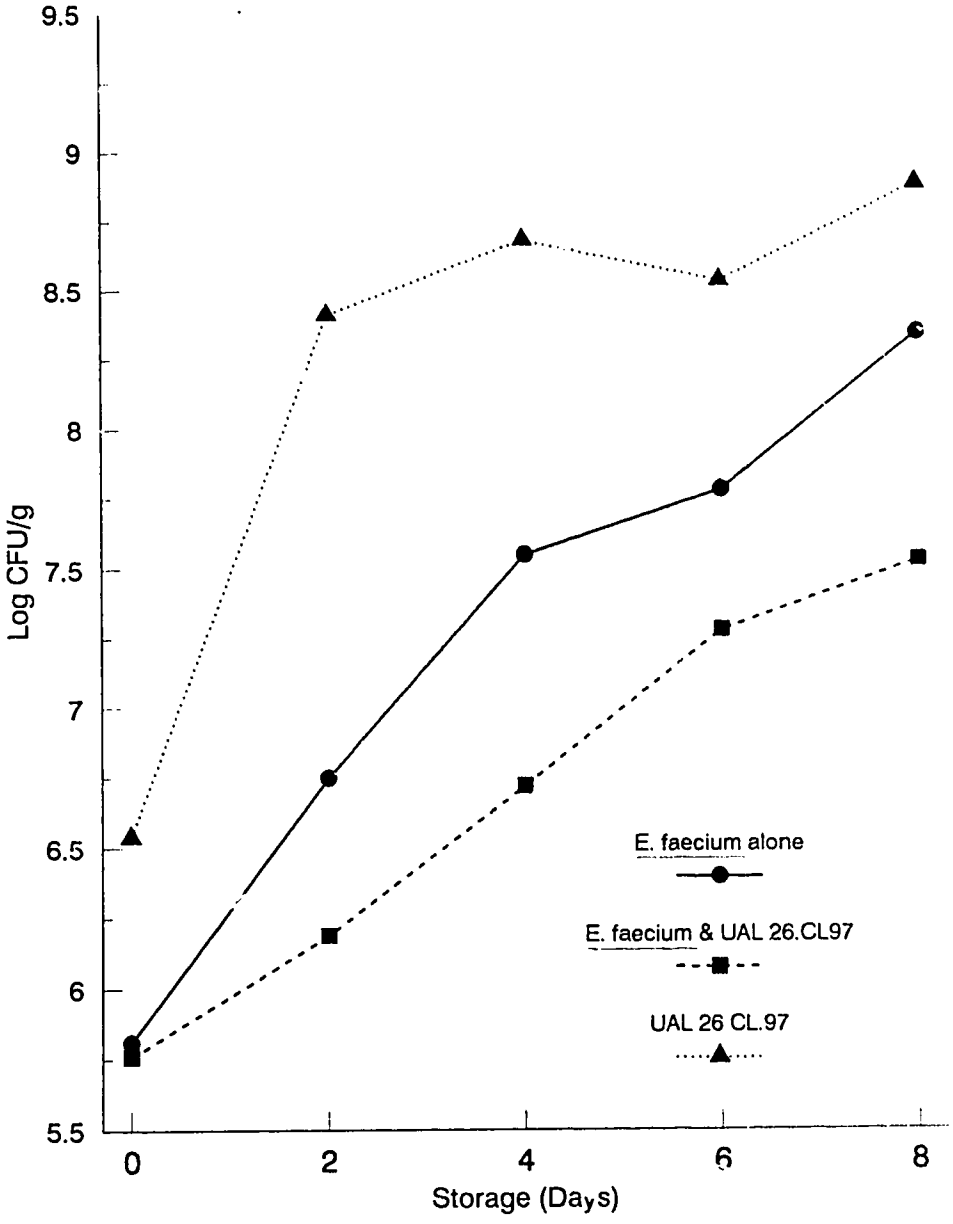


Figure 4.15. Growth of *Enterococcus faecium* 11576 in Hamburger Meat Alone and in Association with UAL 26.CL97 at 10°C

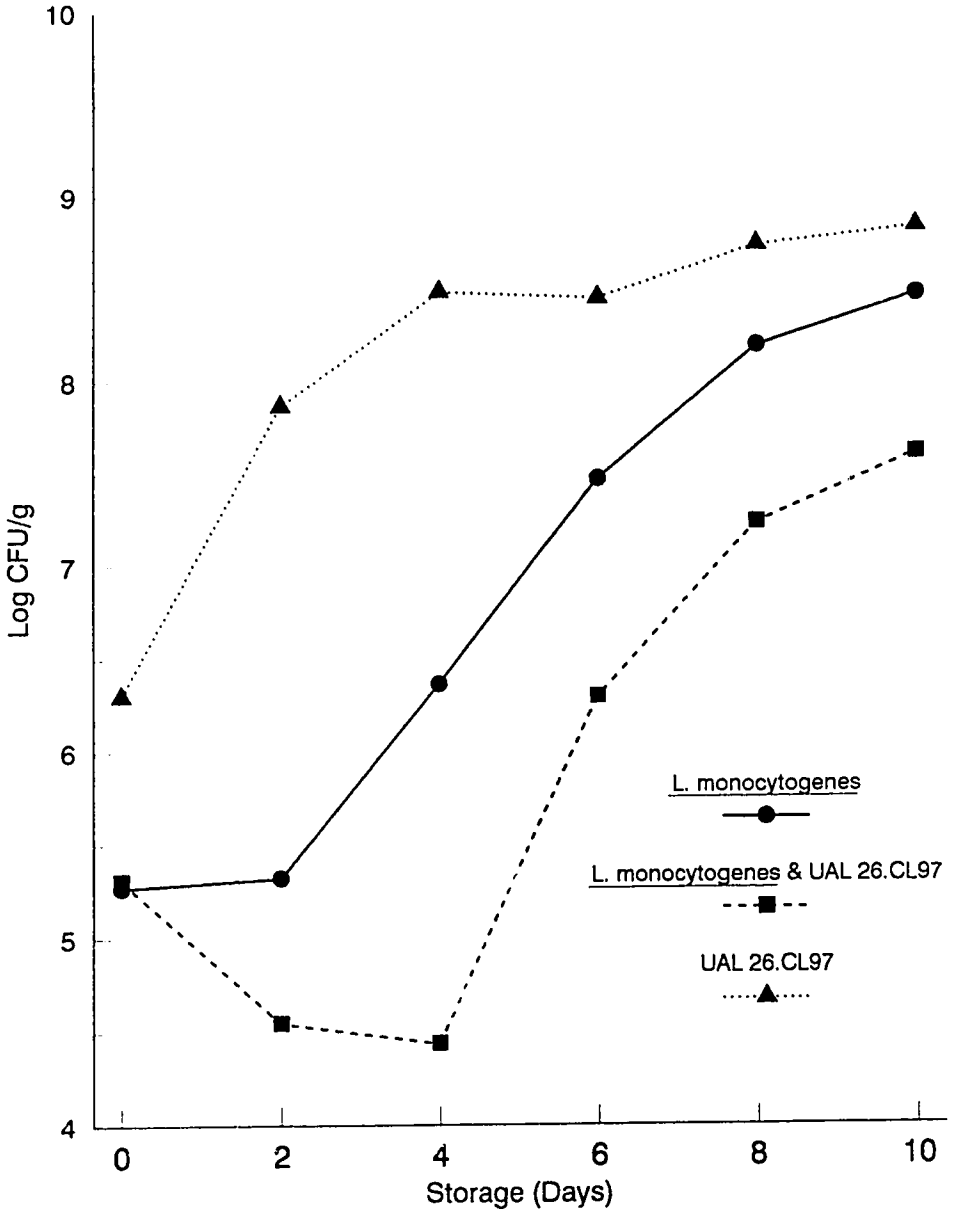


Figure 4.16. Growth of *L. monocytogenes* 15313 in Hamburger Meat Alone and in Association with UAL 26.CL97 at 10°C

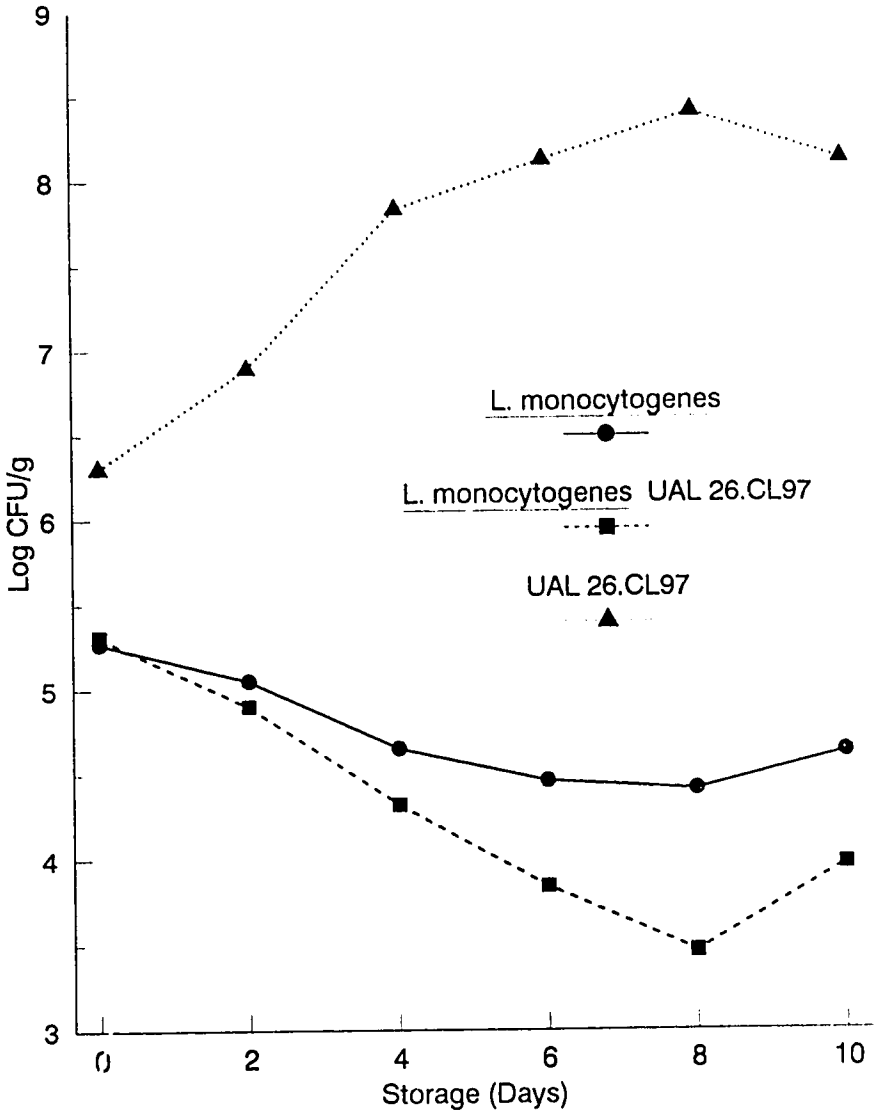


Figure 4.17. Growth of *L. monocytogenes*15313 in Hamburger Meat Alone and in Association with UAL 26.CL97 at 4°C

5. DISCUSSION

Hazard Analysis Critical Control Point (HACCP) is an effective means to anticipate microbiological hazards in food systems, to identify risks in new and existing products and to guide safe design of products (Corlett, 1989). It is a preventative system which incorporates safety into the food product and process by which it is produced. HACCP is a more effective, structured and critical approach than traditional quality control in identifying hazards, for it shifts the emphasis from final product testing to raw material and process control. It focuses on specifics of each production line for each type of product in a processing plant, interlocking an entire company for control of microbiological hazards.

HACCP concept was initially applied to low-acid canned foods. Other areas of food production that have incorporated HACCP include:

(a) frozen foods (Peterson and Gunnerson, 1974);

(b) refrigerated foods (Corlett, 1989; Bryan, 1990; Smith *et al.*, 1990);

(c) meat and poultry products (Tompkin, 1990);

(d) foodservice (Bobeng and David, 1977; Munce, 1984).

The HACCP analysis conducted at Quality Fast Foods Ltd. identified time/temperature, equipment sanitation and hygiene of employees as the critical control points that need to be controlled and monitored to ensure a safe product. A pre-operative inspection was conducted daily prior to commencement of production by a plant employee. Inspection involved visual examination of both food contact and non-food contact surfaces for adequate cleaning. Weekly sanitation checks were done on selected processing areas. It was felt that inspection by the same employee resulted in oversight. It was suggested that the responsibility for the inspection not reside with one employee and on regular occasions, a second, independent inspection should be done. The institution of regular, independent inspections

would allow for comparison of observations and a more reliable pre-operation inspection process.

The sanitation evaluation revealed some inconsistency in efficacy of sanitation achieved from day to day. Better control of yeasts and molds was required. This was due to the inadequate sanitation process and (or) the inappropriate concentration of quaternary ammonium compound (QAC) used. Limitations in using a QAC are that increased concentrations would require rinsing of work surfaces after sanitation and this compound is only effective in controlling Gram-positive organisms (Marriott, 1985; Troller, 1983). An alternate sanitizer (ie. iodophor or hypochlorite) that has a broader antimicrobial range and that is compatible with the equipment was recommended to be efficacy tested, followed by a microbial audit to assure the effectiveness of the sanitizing agent.

Microbiologically, the hamburger sandwich is an interesting product. Hamburger patties must be cooked adequately to eliminate a potential hazard from the survival of pathogenic microorganisms (ie. *E. coli*, *S. aureus*, *Salmonella*) which may be present in the raw hamburger patties. Cooking temperatures of 74°C or higher must be consistently achieved (Smith *et al.*, 1990). The cooking process at Quality Fast Foods, although based on visual appearance, seemed to be effective. Periodic temperature monitoring was recommended to ensure efficient cooking and broiler operation.

Once cooked, the patties are susceptible to time/temperature abuse, cross-contamination with raw materials, unsanitary equipment and by employees practicing poor personal hygiene. Rapid cooling of cooked hamburger patties to 4°C prior to assembly of the sandwiches and maintenance of 10°C or less during assembly is important to prevent microbial growth. Extended delays could allow growth of aerobic psychrotrophs. Improper handling of cooked patties during assembly of sandwiches could introduce pathogenic organisms into the product.

The only means by which growth of microorganisms is controlled in the hamburger sandwich is by time/temperature control and to some extent by packaging. The method of packaging used incorporates 50% air into the packages, resulting in the presence of oxygen which would allow facultative pathogens to grow if the product is temperature abused. Preservatives are not permitted as additives in hamburger meat to aid in eliminating the growth of microorganisms, so the product relies on temperature control as the "hurdle" for product safety. Proper refrigeration must be maintained not only at the processing plant but throughout distribution, retail and until consumption by the consumer.

Microbiological quality of ground beef is a reflection of the quality of meat used, level of sanitation and time/temperature control during processing and storage (Duitschaever *et al.*, 1973). Psychrotrophic organisms are the dominant spoilage organisms of refrigerated ground beef (Kirsch *et al.*, 1952; Ayres, 1960; Gill and Newton, 1977). Researchers (Duitschaever *et al.*, 1973 and 1977; Goepfert, 1976; Pivnick *et al.*, 1976; Westhoff and Feldstein, 1976) who have examined the bacteriological quality of ground beef have generally reported that it has a poor microbiological quality.

Goepfert (1976) and Pivnick *et al.* (1976) showed that aerobic plate counts varied with the incubation temperature used. Counts at 35°C were approximately 10-fold less than counts at 20-21°C. The counts at 20-21°C are considered to reflect more accurately the microbial content of raw ground beef and to provide a better estimate of the keeping quality of the product (Goepfert, 1976) because psychrotrophic microorganisms grow better at the lower incubation temperature.

Duitschaever *et al.* (1977) examined three types of ground beef products finding that the microbial quality of frozen ground beef (19% greater than 1×10^7 CFU/g) was better than fresh ground beef (44% greater than 5×10^7 CFU/g) due to microbial counts stabilizing or even decreasing during frozen storage, while fresh

counts may increase depending on temperature of storage. The study also showed that 76% of cooked hamburgers had counts of less than 10^3 CFU/g.

Our evaluation of frozen raw hamburger patties showed that the microbial quality (10^5 to 10^6 CFU/g) was good, being close to the Canadian microbial guideline of less than 10^6 CFU/g for frozen hamburger (Pivnick *et al.*, 1976). Our counts were based on a 30°C incubation temperature. Of the five code dates sampled, one code date contained several boxes of patties that had counts of 10^6 CFU/g compared with 10^5 CFU/g. This resulted in a significant difference in counts between code dates and boxes at the 95% confidence level. There was consistency between patties of the same code date.

A limited number of studies have been conducted on cooked ground beef quality and on the shelf life of this product (Mueller, 1975; Duitschaever *et al.*, 1977; McMullen and Stiles, 1989; Farber *et al.*, 1990). Our study showed that initial microbial quality of cooked hamburger patties was good, with counts of 10^2 CFU/g or less. This suggests that the cooking process used with initial loads of 10^5 -10^6 CFU/g was sufficient to reduce the microbial load. Differences in the development of microflora were observed between the hamburger patties packaged in thermoform packaging (approximately 40 % CO_2 with the balance nitrogen) and 50% CO_2 and 50% air packaging. These differences could be due to the initial lower levels of oxygen in the thermoform packages, thus reducing the growth of aerobic microorganisms.

Thermoform packaged patties stored at 4°C had the best storage life, with a total count of 10^5 CFU/g after 6 weeks of storage. The products packaged in 50% CO_2 and 50% air had counts about 100-fold higher. Patties stored at 10°C, in both types of packaging, had counts of 10^7 to 10^8 CFU/g after this storage period. This illustrates the ability of microorganisms to grow in cooked hamburger at

temperatures of refrigerator abuse which may occur during distribution or under retail conditions before consumption by the consumer.

Organisms having the potential to cause foodborne illness were not detected in the thermoform packaged patties at both storage temperatures. Packages containing air supported the growth of coliforms and Enterobacteriaceae at both temperatures and *Staphylococcus* species were detected at 10°C. Mueller (1975) and Duitschaever *et al.* (1977) did not isolate microorganisms known to cause foodborne illness from cooked hamburger.

Yeasts and molds appeared to be the major spoilage organisms in hamburger patties packaged in 50% CO_2 and 50% air. The thermoform packaged patties also supported the growth of yeasts and molds. Anaerobic sporeformers were detected in thermoform packaged patties stored at 10°C. The identity of these organisms was not determined.

Modified atmosphere packaging is advantageous in that the increased levels of CO_2 inhibit the growth of aerobic spoilage organisms and support the growth of lactic acid bacteria (Egan, 1983; Genigeorgis, 1985; Hotchkiss, 1988). In our study the packaging methods resulted in difference in the development of lactic acid bacteria. Lactic development was 10^3 to 10^4-fold greater in patties packaged in 50% CO_2 and 50% air. There was variability in the development of the lactic microflora, similar to observations made by McMullen and Stiles (1989). Variability was more pronounced in the hamburger patties packaged in thermoform packaging.

Lactic acid bacteria have the ability to dominate the microflora of modified atmosphere packaged fresh and cured meats. This is attributed to the fact that they do not require O_2 to grow, they are resistant to CO_2 and low pH and they tolerate high salt concentrations in the food (Egan, 1983). Proliferation of lactic acid bacteria in refrigerated meat products may be considered desirable because spoilage occurs long after populations have reached 10^8 CFU/g and spoilage is due

to souring rather than putrefaction. However, the development of a lactic microflora in cooked hamburger has been shown to be unpredictable (McMullen and Stiles, 1989 and this study). Lack of a competitive lactic microflora and decreased bactericidal and bacteriostatic effect of carbon dioxide due to increased temperatures could lead to microbial problems. Post-cooking contaminants, *E. coli*, *Listeria* and *S. aureus*, would be able to grow in the hamburger meat without the presence of a competitive lactic microflora. Cooking raw meat destroys the vegetative bacterial cells but spores may not be destroyed. Although extreme temperature abuse is required for *C. perfringens* to grow, *C. botulinum* may grow at 10°C (Hintlian and Hotchkiss, 1986). Under conditions of temperature abuse, pathogens will grow in almost any atmosphere - including air (Silliker and Wolfe, 1980; Hintlian and Hotchkiss, 1986).

Lactic acid bacteria isolated from meat and meat products are believed to be the best candidates for improving the microbiological safety of meats (Schillinger and Luecke, 1989). Therefore, our study considered the characteristics of *Carnobacterium piscicola* (UAL 26.CL97) to determine its suitability for inoculation into hamburger meat. Chloramphenicol resistance was incorporated in the genetic derivation of UAL 26.CL97 (Ahn, 1991) as a marker for selective growth on APT.Cm agar. The plasmid (CL97) responsible for chloramphenicol resistance and bacteriocin production was shown to be stable after 50 generations of growth. Indicating that this plasmid would not be lost if the organism was subjected to stressful conditions. Its tolerance of up to 2% sodium chloride suggests that the salt content in the hamburger meat should not affect its growth. UAL 26.CL97 grew well at 4°C in broth and Cooked Meat Medium as its parent strain UAL 26 (Burns, 1987). Therefore, its ability to grow in a meat system and produce bacteriocin at 4 and 10°C was examined. Inoculation of Cooked Meat Medium, meat slurry and hamburger meat with 10^6 CFU/g of UAL 26.CL97 resulted in good growth in all

three growth media at both temperatures. Growth in the hamburger meat was the slowest, but it still reached a maximum population of 10^8 CFU/g after 6 days of growth at 4°C. This indicated that UAL 26.CL97 could grow well in a meat system.

A rapid growth rate in the meat slurry and Cooked Meat Medium was accompanied by a rapid decrease in pH. The greatest drop in pH occurred in the meat slurry reaching pH 5.8. The hamburger meat provided the best buffering capacity with the pH being relatively stable throughout the 6 weeks of storage.

Inhibitory substance activity was detected in all three growth media confirming the ability of *Carnobacterium piscicola* to produce extracellular inhibitory substances (Ahn, 1991). Inhibition was shown to be due to a proteinaceous compound by inactivation of activity by protease and no effect on activity when treated with denatured protease. To eliminate the possibility of inhibition by hydrogen peroxide, catalase (68 units/g) was added to the heat-treated supernatant with inhibition still being produced. Therefore it was concluded that inhibition was due to the production of a bacteriocin. Heating supernatants to kill bacterial cells did not appear to have any affect on inhibitory activity as was observed by Schoebitz (1988). This could be because the hamburger meat was cooked rather than raw and meat proteases were inactivated by the cooking process, therefore they were not interfering with the bacteriocin. Treatment of supernatants with chloroform resulted in the inactivation of bacteriocin activity in the meat slurry and hamburger meat. There was no effect in Cooked Meat Medium. A possible explanation for this is could be that the bacteriocin in the meat slurry and hamburger meat was associated with a lipid component of the meat. Bacteriocin extraction with the lipid, indicates that the bacteriocin has lipophilic properties. Rammelsberg and Radler (1990) observed a similar inactivation by chloroform of a lipophilic bacteriocin, brevicin 37 produced by *Lactobacillus brevis* B 376.

UAL 26.CL97 produces two bacteriocins. One that is mediated by plasmid pCP49 (from UAL 8) and its own chromosomally mediated bacteriocin. Bacteriocin mediated by pCP49 is produced early in the growth phase, while the chromosomally mediated bacteriocin is produced in the stationary phase. (Ahn, 1990)

Bacteriocin production in Cooked Meat Medium was the most rapid and the greatest with a maximum of 400 AU/mL detected. In the meat slurry 200 AU/mL of activity was observed. In both of these media inhibitory activity was observed after 8 days of storage. After this there was a decline in the activity similar to an observation made by Schoebitz (1988) in raw ground beef. The bacterial population by this time was in the stationary phase, therefore the production of bacteriocin mediated by pCP49 could have slowed down and only chromosomally mediated bacteriocin was being produced. Inhibitory activity mediated by pCP49 appeared to be more intense than the chromosomally mediated bacteriocin as seen by greater zones of inhibition of UAL 26 than UAL 8.

Bacteriocin production in the hamburger meat followed a different pattern. The level of activity was lower, with a maximum of 100 AU/mL being produced at 10°C. There was no decline in activity after 8 days of storage. At 4°C, bacteriocin production was not observed until after 10 days of storage.

The ability of bacteriocin-producing lactic acid bacteria to inhibit pathogenic organisms is well documented (Daeschel and Klaenhammer, 1985; Carminati *et al.*, 1989; Harris *et al.*, 1989; Spelhaug and Harlander, 1989; Okereke and Montville, 1991). Schillinger and Holzapfel (1990) showed that *Carnobacterium* species have very narrow inhibitory spectra, active only against closely related organisms. However, Ahn and Stiles (1990) demonstrated that *Carnobacterium piscicola* strains were inhibitory towards *E. faecium* and *L. monocytogenes*. This was also shown in this study by the inhibition of these organisms by UAL 26.CL97. Further, to demonstrate inhibitory activity, *E. faecium* and *L. monocytogenes* were grown in

association with UAL 26.CL97 in hamburger meat. At 10°C the growth of *E. faecium* and *L. monocytogenes* in association with UAL 26.CL97 was approximately 10-fold less than when grown alone. Growth of *L. monocytogenes* at 4°C was reduced under both conditions, but there was a greater decline when grown with UAL 26.CL97. Schillinger and Luecke (1990) demonstrated that meat inoculated with *L. monocytogenes* and a bacteriocin-producing strain of *Lactobacillus sake*, the bacteriocin contributed to the inhibition of *L. monocytogenes*.

The presence of *L. monocytogenes* in meat products is a microbiological concern (Tompkin, 1990). Its ability to grow on meat at 4°C (Glass and Doyle, 1989) and its resistance in meat to cooking temperatures of up to 70°C (Boyle *et al.*, 1990) raises concern on how to control this potential pathogen. Continued work with bacteriocin-producing lactic acid bacteria in meat systems may have a potential benefit in controlling the growth of *L. monocytogenes* in meat products.

The data obtained in this study indicated that UAL 26.CL97 is capable of growing and producing bacteriocin in hamburger meat. To aid in the development of a predictable microflora, inoculation of UAL 26.CL97 into hamburger meat was investigated. Since UAL 26.CL97 was to be inoculated into hamburger patties that were to be subsequently cooked, the heat resistance of this organism was determined. From the experiment a D_{60} of 13.2 s was determined, indicating that UAL 26.CL97 died rapidly at 60°C. This was not encouraging because the hamburger patties were to be cooked to temperatures of 70-75°C.

It was assumed that meat would provide more protection to the organism than broth, hence UAL 26.CL97 was inoculated at various concentrations to find out that high concentrations were required to obtain survivors (inoculum concentrations of 10^{10} to 10^{11} CFU/mL). Two types of inocula were tried, and it was shown that liquid cultures gave better results than freeze dried cultures.

Variability in internal temperatures of the hamburger patties required that temperatures for each individual patty be taken. Temperature variations could have been due to inconsistency in patty size or variation in insertion of thermocouples into patties.

UAL 26.CL97 was shown to survive initially in 35% of hamburger patties cooked to temperatures between 67-71°C and in 17% of the patties cooked to 72-75°C. The higher temperatures are more desirable for achieving the proper cooking of the patties and insuring the destruction of potentially pathogenic organisms. Percentage of survivors was not as high as anticipated. After 2 weeks of storage at 4°C, 78% of the patties cooked at the lower temperatures and 61% of the patties cooked at the higher temperature developed a UAL 26.CL97 microflora. The two week storage allowed heat injured cells to resuscitate and grow.

Considering that two weeks of storage was not very long, the storage period was extended in a subsequent experiment. In patties that were stored at 10°C for 1 week, 58% of the patties developed a UAL 26.CL97 microflora the same percentage as those stored for 6 weeks at 4°C. The organisms surviving and developing on storage were shown to be UAL 26.CL97 by demonstrating chloramphenicol resistance and inhibition of a sensitive indicator organism (UAL 26).

From these storage experiments it was observed that survival of UAL 26.CL97 in hamburger meat was not consistent at temperatures over 70°C, therefore development on storage was inconsistent. However, the microbial growth that was occurring was that of UAL 26.CL97. The levels of growth reached after 6 weeks of storage was 10^5 - 10^7 CFU/g as compared to 10^2 - 10^6 CFU/g in the study by McMullen and Stiles (1989). Although survival of UAL 26.CL97 was not seen in all hamburger patties, in those that it did survive UAL 26.CL97 appeared to have an influence on the development of the lactic microflora.

This study applied the HACCP concept to the production of hamburger sandwiches as a systematic approach in preventing microbiological hazards. Time and temperature, identified as the most important factors, must be controlled. Cooked hamburger has the potential of being temperature abused prior to consumption by consumers. Microbial growth in this type of product is controlled by temperature control. Modified atmosphere packaging controls microbial growth to some extent but development of lactic microflora is unpredictable. Inoculation of hamburger patties with UAL 26.CL97 failed to provide a consistent predictable microflora, but it did appear to have an affect on lactic development. UAL 26.CL97 was shown to grow well, produce bacteriocin and reduce the growth of *E. faecium* and *L. monocytogenes* at 4 and 10°C in hamburger meat.

Further studies on the ability of UAL 26.CL97 to inhibit spoilage and pathogenic organisms in meat, particularly *Clostridium botulinum*, are required. Suitability of a more heat resistant organism needs further study. Through genetic engineering, genetic material responsible for bacteriocin production in UAL 26.CL97 could be transferred to a more heat resistant organism, for example *Sporolactobacillus* (Holzapfel and Botha, 1988), providing the same bacteriocin characteristics. For lactic acid bacteria to be inoculated into meat and meat products as preservatives, they must not alter the meat in any way or not impart any toxic effects onto the consumer. Further work is needed as to the practicality of using lactic acid bacteria and bacteriocins as food preservatives.

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Statistical Analysis

Appendix I

Comparison of Microbial Counts Between Code Dates

Code Date	Microbial Counts (Mean Count – CFU/g)*
X1	Y1
May 30, 1990	5.58
May 30, 1990	5.45
May 30, 1990	5.49
May 30, 1990	5.52
May 30, 1990	5.47
Jun 08, 1990	5.58
Jun 08, 1990	5.61
Jun 08, 1990	5.64
Jun 08, 1990	5.65
Jun 08, 1990	5.60
Jul 04, 1990	5.79
Jul 04, 1990	5.75
Jul 04, 1990	5.71
Jul 04, 1990	5.79
Jul 04, 1990	5.75
Aug 28, 1990	6.61
Aug 28, 1990	5.58
Aug 28, 1990	6.62
Aug 28, 1990	5.56
Aug 28, 1990	6.56
Nov 21, 1990	5.64
Nov 21, 1990	5.61
Nov 21, 1990	5.57
Nov 21, 1990	5.54
Nov 21, 1990	5.32

* Mean of 5 replicates.

One Factor ANOVA X_1: Code Date Y_1: Microbial Counts CFU/g

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	4	1.554	.388	5.758
Within groups	20	1.349	.067	p = .003
Total	24	2.903		

Model II estimate of between component variance = .08

1

One Factor ANOVA X_1: Code Date Y_1: Microbial Counts CFU/g

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
May 30, 1990	5	5.502	.051	.023
Jun 08, 1990	5	5.616	.029	.013
Jul 04, 1990	5	5.758	.033	.015
Aug 28, 1990	5	6.186	.563	.252
Nov 21, 1990	5	5.536	.127	.057

2

One Factor ANOVA X_1: Code Date Y_1: Microbiel Counts CFU/g

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
May 30, 1... vs. Jun 08, ...	-.114	.343	.12	.694
May 30, 1... vs. Jul 04, ...	-.256	.343	.607	1.558
May 30, 1... vs. Aug 28, ...	-.684	.343*	4.334*	4.164
May 30, 1... vs. Nov 21, ...	-.034	.343	.011	.207
Jun 08, 19... vs. Jul 04, ...	-.142	.343	.187	.864

3

* Significant at 95%

One Factor ANOVA X_1: Code Date Y_1: Microbial Counts CFU/g

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Jun 08, 19... vs. Aug 28,...	-.57	.343*	3.01*	3.47
Jun 08, 19... vs. Nov 21,...	.08	.343	.059	.487
Jul 04, 1990 vs. Aug 28,...	-.428	.343*	1.697	2.605
Jul 04, 1990 vs. Nov 21,...	.222	.343	.457	1.351
Aug 28, 19... vs. Nov 21,...	.65	.343*	3.914*	3.957

4

* Significant at 95%

Appendix II

Comparison of Microbial Counts Between Code Dates and Boxes

Code Date	Microbial Counts (CFU/g)				
	Box 1	Box 2	Box 3	Box 4	Box 5
X1	Y1	Y2	Y3	Y4	Y5
May 30, 1990	5.64	5.46	5.43	5.53	5.43
May 30, 1990	5.63	5.46	5.56	5.45	5.46
May 30, 1990	5.52	5.38	5.51	5.46	5.53
May 30, 1990	5.58	5.48	5.43	5.56	5.48
May 30, 1990	5.53	5.48	5.51	5.59	5.45
Jun 08, 1990	5.61	5.62	5.69	5.59	5.58
Jun 08, 1990	5.63	5.58	5.62	5.69	5.59
Jun 08, 1990	5.49	5.56	5.59	5.73	5.61
Jun 08, 1990	5.64	5.64	5.64	5.66	5.58
Jun 08, 1990	5.53	5.63	5.67	5.59	5.64
Jul 04, 1990	5.72	5.76	5.69	5.80	5.82
Jul 04, 1990	5.80	5.65	5.72	5.76	5.73
Jul 04, 1990	5.85	5.79	5.74	5.75	5.67
Jul 04, 1990	5.73	5.78	5.65	5.82	5.79
Jul 04, 1990	5.83	5.76	5.74	5.81	–
Aug 28, 1990	6.65	5.59	6.67	5.64	6.57
Aug 28, 1990	6.61	5.51	6.60	5.58	6.52
Aug 28, 1990	6.59	5.60	6.61	5.49	6.53
Aug 28, 1990	6.60	5.59	6.66	5.57	6.60
Aug 28, 1990	6.60	5.62	6.54	5.51	6.60
Nov 21, 1990	5.71	5.61	5.61	5.60	5.30
Nov 21, 1990	5.65	5.62	5.51	5.56	5.30
Nov 21, 1990	5.70	5.61	5.52	5.51	5.34
Nov 21, 1990	5.57	5.56	5.59	5.51	5.28
Nov 21, 1990	5.58	5.64	5.62	5.51	5.36

– Not determined

Anova table for a 2-factor repeated measures Anova.

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Code Date (A)	4	7.767	1.942	1060.759	.0001
subjects w. groups	19	.035	.002		
Repeated Measure (B)	4	1.259	.315	124.684	.0001
AB	16	5.49	.343	135.925	.0001
B x subjects w. groups	76	.192	.003		

1

There were no missing cells found. 1 case deleted with missing values.

The AB Incidence table

Repeated Mea...		Box 1	Box 2	Box 3	Box 4	Box 5	Totals:
Code Date	May 30, 1...	5 5.58	5 5.452	5 5.488	5 5.518	5 5.47	25 5.502
	Jun 08, 19...	5 5.58	5 5.606	5 5.642	5 5.652	5 5.6	25 5.616
	Jul 04, 19...	4 5.775	4 5.745	4 5.7	4 5.782	4 5.753	20 5.751
	Aug 28, 1...	5 6.61	5 5.582	5 6.616	5 5.558	5 6.564	25 6.186

2

Page 2 of the AB Incidence table

Repeated Mea...		Box 1	Box 2	Box 3	Box 4	Box 5	Totals:
Cod.:	Nov 21, 1...	5 5.642	5 5.608	5 5.57	5 5.538	5 5.316	25 5.535
Totals:		24 5.84	24 5.592	24 5.808	24 5.602	24 5.74	120 5.716

3

Appendix III

Comparison of Microbial Counts Between Code Dates and Patties

Code Date	Microbial Counts (CFU/g)				
X1	Patty 1 Y1	Patty 2 Y2	Patty 3 Y3	Patty 4 Y4	Patty 5 Y5
May 30, 1990	5.64	5.63	5.52	5.58	5.53
May 30, 1990	5.46	5.46	5.38	5.48	5.48
May 30, 1990	5.43	5.56	5.51	5.43	5.51
May 30, 1990	5.53	5.45	5.46	5.56	5.59
May 30, 1990	5.43	5.46	5.53	5.48	5.45
Jun 08, 1990	5.61	5.63	5.49	5.64	5.53
Jun 08, 1990	5.62	5.58	5.56	5.64	5.63
Jun 08, 1990	5.69	5.62	5.59	5.64	5.67
Jun 08, 1990	5.59	5.69	5.73	5.66	5.59
Jun 08, 1990	5.58	5.59	5.61	5.58	5.64
Jul 04, 1990	5.72	5.80	5.85	5.73	5.83
Jul 04, 1990	5.76	5.65	5.79	5.78	5.76
Jul 04, 1990	5.69	5.72	5.74	5.65	5.74
Jul 04, 1990	5.80	5.76	5.75	5.82	5.81
Jul 04, 1990	5.82	5.73	5.67	5.79	-
Aug 28, 1990	6.65	6.61	6.59	6.60	6.60
Aug 28, 1990	5.59	5.51	5.60	5.59	5.62
Aug 28, 1990	6.67	6.60	6.61	6.66	6.54
Aug 28, 1990	5.64	5.58	5.49	5.57	5.51
Aug 28, 1990	6.57	6.52	6.53	6.60	6.60
Nov 21, 1990	5.71	5.65	5.70	5.57	5.58
Nov 21, 1990	5.61	6.62	5.61	5.56	5.64
Nov 21, 1990	5.61	5.51	5.52	5.59	5.62
Nov 21, 1990	5.60	5.56	5.51	5.51	5.51
Nov 21, 1990	5.30	5.30	5.34	5.28	5.36

- Not determined

Anova table for a 2-factor repeated measures Anova.

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Code Date (A)	4	7.777	1.944	5.47	.0042
subjects w. groups	19	6.753	.355		
Repeated Measure (B)	4	.007	.002	.733	.5725
AB	16	.035	.002	.946	.5226
B x subjects w. groups	76	.176	.002		

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There were no missing cells found. 1 case deleted with missing values.

The AB Incidence table

Repeated Mea...		Patty 1	Patty 2	Patty 3	Patty 4	Patty 5	Totals:
Code Date	May 30, 1...	5 5.498	5 5.512	5 5.48	5 5.506	5 5.512	25 5.502
	Jun 08, 19...	5 5.618	5 5.622	5 5.596	5 5.632	5 5.612	25 5.616
	Jul 04, 19...	4 5.742	4 5.733	4 5.783	4 5.745	4 5.785	20 5.757
	Aug 28, 1...	5 6.224	5 6.164	5 6.164	5 6.204	5 6.174	25 6.186

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Page 2 of the AB Incidence table

Repeated Mea...		Patty 1	Patty 2	Patty 3	Patty 4	Patty 5	Totals:
Cod...	Nov 21, 1...	5 5.566	5 5.528	5 5.536	5 5.502	5 5.542	25 5.535
Totals:		24 5.729	24 5.711	24 5.709	24 5.717	24 5.723	120 5.718

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