1997

Survival and growth characteristics of bacteria in irradiated meat

Raveendran Jolarpettai Venugopal
Iowa State University

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Survival and growth characteristics of bacteria in irradiated meat

by

Raveendran Jolarpettai Venugopal

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Co-majors: Food Science and Technology; Microbiology
Major Professors: James S. Dickson and Alan A. Dispirito

Iowa State University
Ames, Iowa
1997

Graduate college

Graduate college

Iowa State University

This is to certify that the doctoral dissertation of

Raveendran Jolarpettai Venugopal

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

For the Co-major program

Signature was redacted for privacy.

For the Co-major program

Signature was redacted for privacy.

For the Graduate College

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 3

Chapter 4

Chapter 5

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

ABSTRACT

Several key factors wee investigated in order to improve the safety of foods and to better understand the survival of microorganisms when exposed to stress. Pork patties were irradiated at 0, 0.5, 1, and 2KGy, and stored at 0°C. The shelf life of these irradiated pork patties (aerobically packaged and vacuum packaged; fresh and temperature abused) were studied and predictive models were developed based on generation time and lag phase extension. Next, the physiological response of *Salmonella typhimurium* ATCC 14028 to media composition and pH was investigated by studying the changes in the cell membrane fatty acids, cell surface charge and ability to attach to beef tissue. Three different media, nutrient broth, tryptic soy broth and tryptic soy broth supplemented with 0.5% yeast extract and 0.1% pyruvate at pH 5.0, 6.0, 7.0, 8.0, and 9.0 were studied. Media-dependent responses of *S. typhimurium* revealed a more diverse fatty acid profile when grown in nutrient broth and qualitative differences were observed in the presence of C 18:1 and C 18:2 with lower pH. Scanning- and transmission-electron microscopy revealed morphological changes in viable cells grown at pH 5.0 and 9.0.

Quantitative estimations of microbial populations in a food product are fundamental to assessing microbial quality of that food product. In this study we have used flow cytometer to rapidly estimate numbers of fluorescently stained microorganism and to identify injured sub populations. In order to identify a suitable fluorescent stain, we evaluated rhodamine 123, carboxy fluorescein diacetate, fluorescein diacetate, propidium iodide, and a commercially available stain, *Bac*Light viability kit. *Escherichia coli, Staphyloccus aureus* and *Listeria innocua* were exposed to heat, acid, irradiation and starvation and stained with these dyes. Among the dyes studied, *Bac*Light viability kit gave positive results in certain cases and it appears that viability staining with these vital stains was dependent upon the type of stress undergone by bacteria and may not be suitable for all kind of stresses. Finally, a simple column filtration using sand particles was developed to clarify meat homogenate suitable enough to quantify bacterial population in spoiling pork patties, using flow cytometry. By this method, bacterial cells in meat suspensions were stained with *Bac*Light viability kit and were quantified rapidly.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Introduction

The microbial quality of foods has acquired increased importance and priority over the last few years. This increased importance is mainly attributed to an increased public awareness of the harmful effects of certain microorganisms and improved legislation on food quality. Further, the economic losses associated with food borne infections have attracted increasing attention in developed countries in recent years, particularly in the United States, Canada and in European countries. This impact may be greater in developing countries. Of the approximately 1,500 million global episodes of diarrhea occurring annually, resulting in 3 million deaths among children under age five (mainly in developing countries), 70% have been estimated to have been caused by biologically contaminated food (Anon, 1997). Thus, the problem of food poisoning has major social, economic, and political implications. Despite the increased awareness, the problem of spoilage and poisoning of foods by microorganism is not yet under adequate control. This problem is further compounded by the current consumers' demand for a more natural and fresh-like foods which necessitates the use of mild preservatives. The eventual implication of microbial quality of foods is safety and shelf life extension of food products.

The most active market segment is the chilled, ready-to-heat foods, typically produced in single-serving packages, designed to meet the needs of convenience and simplicity (Ohlsson, 1994). But most of these products have a short shelf life, thus limiting their geographic area of distribution. Technologies that allow for extension of shelf life are in great demand by the food industry. Apart from extended storage time, the food industry is also seeking for increased product safety. The fresh-like products including muscle foods are highly perishable, and treatments that improve the safety are very important, especially in the light of new legislation on food quality. Successful preservation techniques to manufacture food products should also be inherently safe.

Irradiation is emerging as a potential method of food preservation. It is being used to extend the shelf life of raw and processed foods in many countries world wide. Certain types

of ionizing radiation penetrate deep into the food and, through their physical effects, interact with atoms and molecules of foods and also with those of bacteria, molds, parasites and insects. This irradiation causes chemical and biological changes in microbial populations which are lethal, hence are utilized in beneficial ways for food preservation. Thus, most of the applications of food irradiation aim at the destruction of microorganisms, both food spoilage and food poisoning microorganisms, prevention of sprouting and complete destruction of insects. The net result being a safe food with extended shelf life.

Today's food industry uses several preservation techniques which include freezing, blanching, pasteurizing, canning, modified atmosphere packaging, etc. The fact that a combination of preservative factors influence the microbial stability and safety of foods has been known for many centuries (Leistner and Gorris, 1995). This development in the food industry has made it increasingly difficult for us to rely on personal evaluation regarding safety and shelf life. There is a great need to generate and compile information objectively on the survival potential and the growth rates of microorganisms under given environmental conditions. Predictive microbiology is part of this new approach to the microbiological assurance of food quality and safety. It can be used to predict the shelf life and safety of food products. It may also predict the consequences of changes in product formulation and provide food manufacturers with objective evaluation of processing operations. Predictive microbiology has emerged as a separate discipline and is gaining acceptance as a part of food microbiology.

Quantitative estimation of microbial populations in a food product are fundamental to assessing microbial quality of that food product. Conventional microbiological techniques require time-consuming and laborious preparations and procedures. These procedures become even more cumbersome with the presence of sub-lethally injured cells. These cells, although a part of the viable microbial population, usually fail to grow under conventional plating conditions. Overlooking this sub-population could present a potential health hazard. On the other hand, the usual resuscitation, pre-enrichment, selective enrichment and isolation of colonies constitute a relatively lengthy procedure and increases the time involved in assessing the microbial quality. Rapid methods attempt to facilitate a more extensive and

expedient turnover of samples. Recently, rapid methods have been developed for microbial concentration, separation and identification systems. Most of these methods are unique in that they are not limited by bacterial growth or metabolism, and are thus very rapid.

Among the different rapid methods for the detection of food borne pathogens, flow cytometry (FCM) offers a sophisticated technique for both direct quantification of microorganisms and determination of their physiological characteristics on a single-cell basis. The ability of multiparametric analysis in conjunction with fluorescent dyes allows identification of heterogeneity within populations with regard to structure and function. The speed at which FCM permits large number of cells to be analyzed is another attractive feature of this instrument. As a result, application of flow cytometry in the food industry for the detection of spoilage and pathogenic microorganisms is becoming more feasible. The improvements in fluorochromes and instrumentation is increasing the scope of application of FCM.

Understanding the physiology of food-poisoning microorganisms is central to today's preservation techniques. It is increasingly becoming clear that many non-spore-formers could adapt to a variety of stresses by becoming more resistant. As a result, many food poisoning microorganisms escape preservation techniques altogether, reaching the consumer more or less directly from contaminated foods. This is more often true for foods of animal origin. Thus, the growth of microorganisms may not be limited to the specified range of temperature, pH and water activity. The ability of food borne pathogens to adapt and tolerate stress is an important topic and is of great concern to the food industry. This requires a deeper understanding of the basic physiology of food borne microorganisms. To help prevent food poisoning the approach should be more inclusive to accommodate the entire chain of food poisoning from farm to fork.

1.2 Dissertation Organization

This dissertation has a total of seven chapters. Chapters 2 - 6 are five manuscripts arranged in five different chapters. Chapter 1 covers introduction, dissertation organization, and literature review. The first manuscript is on predictive modeling and is presented in

Chapter 2. Chapter 3 contains the second manuscript and it deals with the influence of pH on cell wall fatty acids of Salmonella. The usefulness of certain fluorescent dyes in differential staining of injured bacteria is the topic of the third manuscript and this is presented in chapter 4. Chapter 5 deals with staining of stressed bacteria with *Bac*Light viability kit and this is the fourth manuscript. The final manuscript is about the use of flow cytometer in estimating the bacterial population present in meat samples and development of suitable clarification procedures. The final chapter, Chapter 7 contains a general discussion and recommendations for future research.

1.3 Literature Review

1.3.1 Introduction

Food is part of our life and healthy food is important to our well being. Diet and health are now firmly established in our life style. However, the food habits are constantly changing and the recent trend calls for minimally processed foods. There is an emphasis on natural ingredients, convenience, and simplicity in preparing our food that results in little change in the inherent freshness of the food. At the same time, preparation should ensure the food product with adequate microbial safety. On the other hand, the food industry, with its diverse range of products, is strictly governed by food legislation, with microbiological safety being the key issue. Implementation of food legislation requires identification of microbiological hazards and faulty practices and procedures in food processing. From a shelf life perspective, substantial advances in our knowledge concerning the effects of various preservation factors on the growth and survival of both food-poisoning and food-spoilage microorganisms are required. Monitoring microbiological safety demands analytical techniques that are simple to use, cost-effective, quick and reliable. The ability of microorganisms to adapt and survive various stresses has changed the perspective of food preservation. This has made it necessary to re-examine the whole approach and formulate new procedures based on a thorough understanding of microbial physiology.

1.3.2 Predictive Microbiology

Predictive modeling of bacterial growth expanded as a research area in the early 1980's. Since 1983, predictive microbiology has attracted considerable research interest and funding, particularly in the United States of America, the United Kingdom, and also Australia (Ross and McMeekin, 1994). By definition, predictive microbiology is the term used to describe the mathematical modeling of the effects of temperature, water activity, pH and other environmental influences on the rate of microbial growth or the time to reach specified numbers of microorganisms or their metabolites (Ratkowsky *et al.* 1991). Proponents claim that such an approach will enable (Ross and McMeekin, 1994):

(1) Prediction of the consequences of product shelf life and safety and changes in product formulation, will enable the rational design of new processes and products to meet required levels of safety and shelf life;

(2) Objective evaluation of processing operations will strengthen the Hazard Analyses Critical Control Point approach;

(3) Objective evaluation of the consequences of lapses in process and storage will enable food industries to formulate meaningful remedial procedures.

1.3.2.1 History

The use of mathematical models in food microbiology is not new. However, the application of mathematical modeling techniques to the growth and survival of microorganisms in foods did not receive wide attention until the 1980's. Increased attention was given to modeling the growth of microorganisms of concern with a number of groups publishing in this area (Metaxapoulos *et al.* 1981a, 1981b; Ratkowsky *et al.* 1982). The paper by Roberts and Jarvis (1983) may be viewed as the foundation for some of the rapid developments in predictive microbiology. In that paper they challenged traditional methods of food quality and assurance testing, which they described as an "expensive and largely negative science", and advocated a more systemic and cooperative approach.

1.3.2.2 Models

Predictive microbiology is usually considered under two main headings, the kinetic model and probability model. Kinetic modeling involves the extent and rate of growth of microorganisms of concern and probability modeling involves the construction of models to predict the likelihood of some event such as spore germination or detectable amount of toxin being formed, within a given period of time. In kinetic modeling, one approach is to model growth rate to make predictions based on exponential population growth. This approach has been used by a number of groups (Broughall *et al.* 1983; Smith, 1987; Blakenship *et al.* 1988; Fu *et al.* 1991; Dickson *et al.* 1992; McMeekin *et al.* 1993). The other approach, introduced by Gibson *et al.* (1988), is a sigmoid function fitted to the observed population growth curve and the effect of environmental factors on the values of parameters of that fitted sigmoid curve are modeled. Predictions are then made by evaluating the fitted function. Models are constructed in both approaches by following the increase in numbers or biomass of the organism for a range of levels and combinations of environmental factors of interest, to generate information about lag phase duration, rate of growth, and maximum population density achieved (Ross and McMeekin, 1994).

In kinetic models the response variable is expressed in time-based units (i.e., as rate, or the time taken for a particular response). In this category, four main models are recognized. They are Belehradek or square-root model (Ratkowsky *et al.* 1982), Arrhenius-type models (Johnson and Lewis, 1946; Hultin, 1955; Sharpe and DeMichele, 1977; Schoolfield *et al.* 1981), Modified Arrhenius or Davey models (Davey 1989, 1991) and Polynomial or response surface models (Metaxopoules *et al.* 1981a, b; Einarsson and Eriksson, 1986).

Probability models aim at providing quantitative data for factors other than thermal processing in order to predict safe combinations of conditions to prevent pathogen growth and toxin formation (Genigeorgis *et al.* 1971). In this model, the use of decimal reduction as a response variable is consistent with the description of the effects of thermal processing. This model type was used for a number of other organisms and combination of environmental factors (Raevuori and Genigeorgis, 1975; Yip and Genigeorgis, 1981).

The approach to date has been to assemble a collection of growth curves for the organism growing in broth media on the assumption that cultural factors such as temperature, pH, salt etc., are the main determinants of growth of bacteria, both in model media and in foods. Predictive models have now been developed in model media for a range of pathogens, e.g., Salmonella (Gibson *et al.* 1988), *L. monocytogenes* (Buchanan and Phillips 1990; Wijtzes *et al.* 1993; Duh and Schaffner, 1993), *Aeromonas hydrophila* (Palumbo *et al.* 1991; Hudson, 1992; McClure *et al.* 1994), *Bacillus cereus* (Baker and Griffiths, 1993), *Yersinia enterocolitica* (Adams *et al.* 1991), and have been shown to give good estimates for growth in these media. Models for predicting growth of *L. monocytogenes* under conditions simulating those in dairy products have also been developed (Murphy *et al.* 1996).

The results of these works on predictive mathematical models have yielded important quantitative information on the behavior, especially the growth of pathogens in model systems. In 1994, UK-based 'Food Micromodel' was launched in a commercially available PC-based version (available from Leatherhead Food Research Association, Leatherhead, UK). In the US, 'Microbe-Model', an Internet forum for sharing information is maintained by the Microbial Food Safety Research Unit, US Department of Agriculture, Philadelphia (Knøchel and Gould, 1995).

1.3.2.3 Limitations

Predictive microbiology is still in its developing stages, currently mainly at a single or a few environmental factors in pure culture broth systems. Researchers in the field of predictive modeling are striving to develop mechanistic models for microbial growth and death based on an understanding of cell variability and physiology that could be used to extrapolate to other conditions. However, at present there are no mechanistic models that are able to describe the way in which microorganisms respond to three or four interacting factors, such as temperature, pH, water activity and preservation (Cole, 1991). Exposing bacteria to sublethal stresses may significantly alter their responses to further stresses and may induce cross-protection. All these factors cannot be taken into account. Besides, the pre-adaptation of contaminating pathogens, other factors such as dynamic developments in, for example,

water activity and pH, and the heterogeneity in foods (including composition, structure and gradients) may complicate the accuracy of predictions more than what is assumed (Robins and Wilson, 1994).

It is therefore important to recognize that it may not be possible to accurately predict the kinetics of all the interactions that may take place between bacterial contaminants and the various environmental situations that exist in a food ecosystem. However, in many cases, a number of strategies can be applied to simplify the problem, and to allow useful predictions and analyses, to be made. There are many reports (Langeveld and Cuperus, 1980; Gill 1984; Dickson *et al.* 1992) based on a range of products which indicate that predictions from models based on data generated under constant conditions can reliably predict growth under dynamic temperature conditions.

1.3.3 Food Preservation and Irradiation

Fresh meat, a highly perishable food commodity, provides an excellent substrate for bacterial colonization and proliferation. This necessitates appropriate preservation and storage conditions to prevent rapid spoilage resulting from the growth of microorganisms. Microbial spoilage is the main limiting factor in the shelf life of fresh meat. Meat processing and distribution is another limiting factor of shelf life. Fresh meat distribution is a complex system with the meat going through many processes from the time it leaves the slaughter house until it reaches the ultimate consumer. The distribution system includes retail supermarkets, hotels, restaurants and institutions (Brody, 1989), each with specific packaging system requirements. The shelf life of meat stored at room temperature is less than one day and this can be extended for a few days when stored at refrigerated temperatures. Mechanical refrigeration ($\leq 5°C$) was introduced to control spoilage and to extend the shelf life of perishable food materials. This is achieved by slowing down or preventing the production of undesirable microbial end-products by spoilage organisms, and preventing growth and/or toxin production by pathogenic organisms (Palumbo, 1986).

Mechanical refrigeration alone is not adequate to prevent spoilage. Growth of various spoilage bacteria, yeast and molds in a wide range of meat, fish, poultry and dairy products

held at 5°C have been reported (Elliott and Mutineer, 1964; Shaw and Shawn, 1968; Thomas, 1953). In addition, temperature abuse of refrigerated foods can occur during processing, distribution, retailing and even while in storage after reaching the consumers. All these limitations, made it impossible to rely on this technology, which could extend the shelf life of refrigerated perishable foods, particularly meat products. Techniques that have been studied include product formulation, irradiation and packaging.

Irradiation is emerging as a potential method of food preservation. It is being used to extend shelf life of raw and processed foods in many countries world wide. Irradiation involves exposing food material to ionizing radiation. Certain types of radiation such as X-rays and gamma rays penetrate deep into the food and through their physical effects, interact with atoms and molecules of foods and also with those of bacteria, molds, parasites and insects. This causes chemical and biological changes in them which are utilized in beneficial ways (Thakur and Singh, 1995). Treatment with ionizing radiation can result in an immediate reduction of the total contamination level (Lefebvre *et al.* 1994) and this leads to an increase in the storage life. Irradiation of food also offers the following advantages (Dempster, 1985):

- avoidance of recontamination by treatment packaged food
- low energy requirement
- diminished usage of chemical additives
- tentative elimination of pathogenic microorganisms
- conservation of the food's nutritional value.

The US Food and Drug Administration (FDA) approved the treatment of pork meat products with a minimum dose of 0.3 KGy and a maximum of 1.0 KGy of ionizing radiation to control *Trichinella spiralis* (Anon., 1985). Although doses of ionizing radiation below 1 KGy are adequate to control helminths, they are not generally considered adequate to control many food-borne pathogens or to provide significant extensions in shelf life. Government and industry have indicated interest in ionizing radiation doses in excess of 1.0 KGy but less than

10 KGy to increase shelf life and improve safety of products for consumers (Thayer *et al.* 1993).

Mattison et al. (1986) studied the effects of a 1 KGy gamma radiation dose at ≅ 0°C on microflora, sensory characteristics and fat stability of vacuum-packaged pork loin. Microbial evaluations revealed reduced numbers of mesophiles, psychrotrophs, anaerobic bacteria and staphylococci. Ehioba *et al.* (1987, 1988) characterized bacterial cultures from vacuum-packaged irradiated and non-irradiated ground pork stored upto 12 days at 5°C. They reported decreased numbers of mesophiles, psychrotrophs and anaerobes and an extension of shelf life by 2-3 days (8 to 11.5 days). Lebepe *et al.* (1990) studied the effect of a 3 KGy radiation dose on microflora and other characteristics of fresh, vacuum-packaged pork loins stored at 2°C upto 98 days. He reported a shelf life increase from 41 to 90 days by a 3 KGy radiation dose.

Like other methods of preservation, irradiation has a number of limitations. The complete sterilization of foods based on the '12D' concept (the 12D value is the time in minutes required at a specified temperature to reduce spore counts from 10^{11}/ml to 0/ml) for the destruction of spores of *Clostridium botulium* requires a radiation dose in the range of 23-58 KGy, depending upon the food and radiation conditions (Savagaon, *et al.* 1972). Another problem associated with irradiation technology is the development of undesirable sensory and chemical changes in some foods. Irradiation has been reported to cause the development of off-odors in foods even at permissible dosages of ≤ 10 KGy (Giddings and Marcotte, 1991). Thus, radiation dose that can be applied to a particular food and hence the extent of microbial kill will be limited by undesirable changes in flavor (Thakur and Singh, 1995). In order to achieve the desired shelf life for foods using permissible doses of radiation and to prevent undesirable sensory and chemical changes in irradiated foods, the process should be combined with other preservation treatments such as heat or packaging. In this way it is possible to obtain the desired results at low doses of irradiation. Thus, with proper consumer education, food irradiation should emerge as an important method of food preservation.

1.3.4 Acid Adaptation and Food borne Pathogens

Microorganisms that are responsible for a majority of outbreaks of food poisoning, include among others, organisms such as salmonella, campylobacter and enteropathogenic strains of *Escherichia coli*. *Staphylococcus aureus* and *Clostridium botulium* belong to the toxinogenic group of organisms, although infrequent, may have particularly severe consequences. One of the most fruitful research themes of recent years has been the discovery that pathogenic microorganisms are not limited to cardinal ranges of temperature, pH and a_w, but can adapt to survive at values outside of those given in textbooks (Hill *et al.* 1995). An additional realization is that microorganisms can respond to mild stresses in ways that enable them to survive more severe challenges (Gould *et al.* 1995). Organic acids are frequently used in foods to inhibit the growth and survival of undesirable organisms. In some foods organic acids are intrinsic or they may be deliberately added as preservatives or be present as a consequence of microbial fermentation processes. The most effective acidulants currently available for food preservation, act to a large extent by interfering with the homeostatic reactions that are involved in maintaining internal pH of microorganisms. It has been clearly demonstrated that the cytoplasmic pH of microorganisms is tightly regulated and that this 'pH homeostasis' is essential for continued growth and viability (Booth, 1985). A major consequence of this action is the disturbance of membrane physiology.

The ability of food borne pathogens to adapt to acidic conditions is a concern in the food industry (Brown and Booth, 1991) and there has been recent interest in how acidic external pH affects enterobacteria, because there are many sites in the environment, in food and in the animal or human body where organisms face acidity (Rowbury, 1995). Survival in acidic conditions may have clinical significance, because enteric pathogens must pass through the acidic stomach before colonizing the intestinal tract. It has been demonstrated for a number of food borne microorganisms that the exposure of microbial cells to low pH values can lead to increased tolerance or 'habituation' to acid. This is also true for other stresses that these microorganisms may encounter in foods and food processing environments (Kroll and Patchett, 1992).

1.3.4.1 Effect of Environmental Conditions on Acid Sensitivity

Acid sensitivity is influenced by a number of environmental factors. Acid sensitivity varies with culture temperature, *E.coli* and *S. enteritidis* grown at 42-45°C are more acid-resistant than cultures grown at 37°C. On the other hand, growth at 20-25°C reduces resistance (Humphrey *et al.* 1993). Cold-shock enhances acrylic acid resistance, possibly by inducing a cold-shock operon allowing enhanced repair of DNA damage caused by the weak acid (Whiting and Rowbury, 1995). No studies have been reported on the effects of irradiation on sensitivity to organic acids (Rowbury, 1995).

Induced tolerance to acid by growing the organisms at acidic pH have been reported widely. This so called acid tolerance response (ATR) involves two distinct stages: pre-acid-shock, induced at approximately pH_o 5.8 and post-acid-shock, induced at approximately pH_o 4.5. Pre-acid shock makes organisms to maintain a neutral internal pH (pH_i) at low pH_o (Foster and Hall, 1991). On the other hand, exposure to alkaline pH has been reported to induce acid sensitivity in *E. coli* (Rowbury *et al.* 1993). Certain chemical compounds such as phosphates and sodium ions have been reported to influence the acid sensitivity of organisms. Phosphates and polyphosphates inhibit induction of acid tolerance at pHo 4.5-5.8 by competitively preventing protons crossing the outer membrane and reaching the periplasmic sensor (Rowbury, 1995). Rowbury et al. (1994) reported that *E. coli* grown in LSB, which are acid-resistant, when transferred to condition where internal Na^+ rises markedly become acid-sensitive. Other factors such as growth media, growth phase and anaerobic conditions have been reported to influence the acid sensitivity of enterobacteria (Rowbury, 1995).

1.3.4.2 Physiology of Acid Adaptation

An intact plasma membrane is critical for the continued survival and successful growth of bacteria. This membrane performs vital functions such as the maintenance of the proton-motive force and the uptake of nutrients. It is also an interface between the external environment and the cellular cytoplasm, and the composition of membranes is a function of the external medium. Most important environmental parameters in the context of food spoilage and poisoning are the changes in water activity, pH and temperature (Russell *et al.*

1995). All these factors affect the structure and function of membranes. Although much physiological work has been carried out on non food-poisoning microorganisms, relatively little work has been carried out on the food-poisoning microorganism themselves (Knφchel and Gould, 1995).

The most extensively studied members in terms of acid stress responses are *Salmonella*, *Shigella* and *E. coli*. Much of the work in the area of acid habituation has been carried out in external acid-regulated genes in *S. typhimurium* and *E. coli* (Foster and Hall, 1990). Approximately 50 acid shock proteins (ASP) that are believed to prevent or repair macromolecular damage are induced during this stage (Foster and Hall, 1990). Thus, acid tolerance is governed by several physiological changes in response to the production of ASPs. Emergency pH homeostasis systems induced by mild acid function is operated by several inducible amino acid decarboxylases (Park *et al.* 1996). Another aspect, the alternative sigma factor σ^s, encoded by *rpoS*, regulates one aspect of acid tolerance (Hengge-Aronis, 1996). Ferric uptake regulator (Fur) is another regulator of acid tolerance (Hall and Foster, 1996). Other genes with demonstrable effects on acid tolerance include *PolA* and *Ada* genes involved in DNA repair, FabF involved in fatty acid synthesis, the cAMP receptor protein CRP and the Mg^{2+} -dependent proton-translocating ATPase.

However, most of the organic acids target the cell membrane for their antimicrobial action (Richards *et al.* 1995). As a result, changes could occur in the membrane composition to cope with the stress. It has been reported that changes in the composition of bacterial membranes caused by environmental factors are thought to act as adaptive responses in order to maintain stability and proper membrane function (Imhoff and Thiemann 1991). There are reports of phenotypic alterations in the amount of lipids in microbial membranes resulting from a change in growth temperature (Russell and Fukunaga, 1990). Also, influence of salt concentration on the fatty acid composition of *Ectothiorhodospira* spp., *Rhodobacter*, *Rhodopsendomonas*, and *Chromatium* has been reported (Imhoff and Thiemann, 1991). In *Salmonella typhimurium*, growth in the presence of sodium benzoate and sodium deoxycholate have been reported to induce changes in membrane lipid composition and changes in the thermal sensitivity (Tomlins *et al.* 1982). Among the environmental factors,

information about phenotypic thermoadaptation of bacterial fatty acid composition is abundant in the literature (Russell and Fukunga, 1990), while information on other environmental factors is relatively scarce.

1.3.5 Bacterial Injury and Fluorescent Probes

Techniques that are employed to prevent microbial growth and proliferation in food, usually inhibits the growth of microorganisms rather than inactivating them. These techniques deliver stresses at sublethal levels which invariably result in bacterial injury. Many of the physical and chemical treatments used in food processing have been shown to injure microorganisms associated with foods (Russell, 1984; Flowers and Ordal, 1978). Injured microorganisms are not easily detected and quantified by conventional plating media. In food microbiology, this often corresponds to a state in which cells are neither dead nor alive to form a colony when plated on a suitable solid medium. However, under favorable incubation conditions they can revert, by a process known as resuscitation, to a state of aliveness as so defined (Kaprelyants *et al.* 1996). The existence of this reversible cell injury in microorganisms has profound implications for food microbiology and public health microbiology. Detection and quantification of injured populations is of critical importance to food industry. Pathogens in this injured state are a potentially unrecognized cause of disease outbreak and may constitute a public health hazard. Thus, our ability to describe microbial populations using methods based on colony formation has serious limitations.

1.3.5.1 Fluorescent Stains

Compounds exhibiting fluorescence are called fluorophores or fluorochromes. When a fluorophore absorbs light, energy is taken up for excitation of electrons to higher states. Fluorescent stains are widely used in a variety of biological applications where the response of individual cells can be observed microscopically (Haugland, 1992). However, this approach has been exploited in only a limited number of microbiological applications (McFeters *et al.* 1995). With improvements in characterization of fluochromes, there are a number of dyes available to assess different physiological properties of the bacterial cell.

The intracellular pH and the pH gradient in a cell can potentially provide information about viability. Viable cells are able to maintain a pH-gradient when the extracellular pH is moderately decreased. To measure the intracellular pH, fluorescent probes such as carboxy-fluorescein or BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein) are well suited as they are highly pH dependent and have a fast response time (Haugland, 1992). Molenaar *et al.* (1991) described a method for measuring cytoplasmic pH in *Lactococcus lactis* and Futsaether *et al.* (1993) used BCECF to measure the intracellular pH of *Propionibacerium acnes*. However, its application to bacteria has been limited (Molenaar *et al.* 1991; Efiok and Webster, 1990).

1.3.5.2 Fluorescent Nucleic Acid Stains

A large number of fluorescent nucleic acid stains have been used in cell biology (Haugland, 1992). In microbiology, a limited number of these dyes have been used to determine total bacterial count, cell size and DNA content. The compound 4',6-diamidino-2-phenylindole (DAPI) has been used as a counter-stain in procedures where contrasting fluorochromes are used. In this way, it is possible to obtain total bacterial counts in marine samples (Porter and Feig, 1980). DNA content can be measured using the membrane permeable, UV-excitable dye Hoechst 33342 with blue fluorescence, which binds specifically to A-T base pairs (Petit *et al.* 1993). Robertson and Button (1989) characterized aquatic bacteria according to population, cell size and DNA content using DAPI and fluorescent microspheres.

1.3.5.3 Membrane Integrity

The bacterial membrane is assumed to be the primary target for several inactivation treatments applied in the food industry. Stains commonly used for determination of membrane integrity are ethidium bromide and propidium iodide (PI). Both dyes are mutagenic, positively charged and almost membrane impermeable. They can enter cells mainly via damaged membranes of stressed, injured or dead cells and inercalate into DNA

and RNA, after which fluorescence is enhanced (Ueckert *et al.* 1995). PI is better than EB and is bigger, higher charged and less membrane-permeable (Pinder *et al.* 1990). Several papers report energy-coupled EB efflux systems to be present in *E. coli* and other Gram-negative bacteria (Midgley, 1986; Jernaes and Stress, 1994). PI has been widely used and usually in combination with other fluorescent dyes (Miller and Quarles, 1990; Crissman *et al.* 1979).

Certain dyes such as calcofluor white M2R and dansyl lysine are referred to as fluorescent membrane dyes. Calcafluor white (CFW) is the disodium salt of 4,4'-bis(4 anilino-bis-diethyl amino-s-triazin-2-ylamino)-2,2'-stilbene disulphonic acid). These compounds are highly fluorescent when excited by ultra violet. light. Absorption and transport of these dyes into microorganisms has been investigated. Viable cells are able to exclude this dye whereas non-viable cells appear brightly fluorescent, although the mode of binding is unknown (Mason *et al.* 1995). Measurements of cell viability and staining with CFW have been found to correlate well with propidium iodide and fluorescein diacetate fluorescence, a correlation coefficient of 0.9886 was recorded with PI (Berglund *et al.* 1987). Dansyl lysine, a nontoxic fluorescent membrane dye, has been used for assessing heat-induced killing and thermotolerance in eukaryotic cells (Fisher *et al.* 1986). Preliminary results obtained with this stain indicated a potential use for assaying inactive bacteria (McFeters *et al.* 1995).

1.3.5.4 Membrane Potential Dyes

Fluorescent dyes as indicators of membrane potential have been in use for the past 20 years (Loew, 1993). Among the available potentiometric dyes, rhodamine 123 (Rh-123) has been extensively employed for staining bacterial cells (Yu and McFetrs, 1994; Matsuyama, 1984) and more recently by microbiologists using flow cytometry (Kaprelyants and Kell, 1992; Diaper *et al.* 1992). The gram-negative envelope is only slightly permeable to Rh-123 and a permeation procedure is used to obviate that problem (Yu and McFeters, 1994). A wide range of gram-positive and gram negative bacteria have been reported to stain efficiently with Rh 123 (Matsuyama, 1984; Diaper *et al.* 1992; Kaprelyants and Kell, 1992).

Cyanine dyes are highly fluorescent and used in cells in which a potential gradient is present (Smith, 1990). Upon hyperpolarization (increase in $\Delta\psi$) cellular fluorescence is enhanced. Different cell types appear to have different degrees of susceptibility to cyanine toxicity (Petit *et al.* 1993), which can be minimized by using low dye concentrations. Cyanine dye has been used with *E. coli* K 12, *Salmonella typhimurium* ATCC 14028, and *Staphylococcus aureus* NCTC 6571 (Mason *et al.* 1995). In another experiment, drug-induced membrane potential changes were studied using 3,3-dipentyloxacarbocyanine iodide in *Staphylococcus aureus* (Ordo'nez and Wehman, 1993). Anionic oxonol dyes are another group of membrane potential dyes used in the study of microorganisms. These dyes enter depolarized cells and bind to lipid-rich intracellular components, changing the emission spectrum and fluorescence intensity (Mason *et al.* 1994). The responses to changes in partitioning are opposite to that of cyanine or rhodamine. Mason *et al* (1995) reported oxonol dye (bis 1,3-dibutylbarbituric acid trimethine oxonol) to be superior to Rh123 and carbocyanine.

1.3.5.5 Fluorescence Based on Esterase Activity

Esterases are present in all living organisms. Generally, the polar, membrane impermeable fluorescent dyes (usually fluorescein or fluorescein derivatives) may be loaded into intact cells by incubation with the respective non-fluorescent permeable acetyl or acetoxymethyl esters. Once inside the cells, these esters (prefluorochromes) are hydrolyzed by non-specific intracellular esterases into more polar fluorescent products, which are retained by intact cells (Ueckert *et al.* 1995). Reports of Molenaar *et al.* (1991) described leakage of fluorescein derivatives from intact cells of *Lactococcus lactis* and suggested an energy-dependent extrusion mechanism. The ability of FDA to stain viable bacteria has been reported by several workers (Brunius, 1980; Bercovier *et al.* 1987). It has also been reported that once cleaved, the released fluorescein was poorly retained by viable bacterial cells and therefore has limited applications (Fry, 1990; Edwards *et al.* 1993).

1.3.6 Flow Cytometry Applications in Food Microbiology

Flow cytometry is a specialized form of fluorescence microscopy (Melamed *et al.* 1990) and it permits the examination of biological surfaces of cells when they pass through a beam of excitation light from a laser. This sensitivity and ability to analyze large numbers of particles, makes flow cytometry a sophisticated technique for research on microorganisms to determine physiological characteristics on a single-cell basis. Many of the early techniques were targeted at eukaryotes and mammalian cells in particular, and the application in microbiology is comparatively recent. The ability to conduct multiparameter studies has made flow cytometry more powerful and more versatile by allowing measurements of several characteristics of cells simultaneously (Miller and Quarles, 1990). Early work by Hutter and Eipel (1979) demonstrated the feasibility of conducting two-parameter flow cytometry. This was achieved by utilizing fluorescein isothiocyanate (FITC) and propidium iodide (PI) to measure protein and DNA simultaneously in yeast cells. Later, Van Dilla *et al* (1983) studied six species of bacteria by two parameters of fluorescence. This ability to measure specific properties of microorganisms suggests the possibility of developing methods of "fingerprinting" diverse organisms as a means of differentiating and identifying them (Miller and Quarles, 1990).

Of late, with an increase in the choice of fluorescent dyes and the availability of commercial preparations, flow cytometry is increasingly being used in identifying physiological difference in a bacterial population. Fluorogenic esters were used to detect viable *Klebsiella pneumoniae* by flow cytometry (Diaper and Edwards, 1994; Brunius, 1980). Rhodamine 123 was used to assess viability and vitality of *Micrococcus luteus* (Kaprelyants and Kell, 1992) and *Bacillus subtilis* (Diaper *et al.* 1992). In the later report, fluorescein diacetate and 3,3'-dihexyloxacarbocyanine iodide were also used. Various other dyes such as fluorescien isothiocyanate (FITC) and propidium iodide (PI) (Miller and Quarles, 1990), ChemB, Rh123 and carboxy fluorescein diacetate (Porter *et al.* 1995) and Rh 123-carbocyanine, oxonol (bis 1,3 - dibutylbarbituric acid trimethine oxonol) and calcafluor (Mason *et al.* 1995) were also used.

Flow cytometric analyses of microorganisms in food systems is complicated due to the nature of food particles. For successful analyses, the bacteria should be in a liquid medium suitable enough to be passed through FCM. Three phases can be distinguished with respect to detection of microorganisms in foods (Ueckert *et al.* 1995):

(i) Extraction of the microorganisms from the food sample

(ii) Fluorescent labeling of the target cells

(iii) Analysis of the fluorescently stained cells by FCM.

For use in FCM, the food material should be filterable and filterability should not interfere with recovery of microbial cells present in the food. This is an essential prerequisite because, food debris and/or somatic cells can make the counting of microorganisms difficult or impossible. Various mechanisms involving physically agitating samples to aid in dispersion have been tested and compared in soil and aquatic microbiology (Bakken, 1985; Yoon and Rosson, 1990) and different methods have been recommended for various sample types. Mechanical homogenization may be the best way for removing bacteria from sediments, sand grains, soil particles, and plant surfaces (Fry *et al.* 1985; Babink and Paul, 1970). For turbid sea water, Yoon and Rosson (1990) found that treatment with 10 μg of Tween 80 ml^{-1} and sonication with a half-wave step titanium horn (10W for 30s) yielded 10 times more attached bacteria than were obtained from untreated controls. In food microbiology, pretreatment and enzymatic digestions have been reported to be successful for clarification, suitable enough to be used in DEFT-technique (Jaeggi *et al.* 1989a; Jaeggi *et al.* 1989b). The enzymatic treatment in this procedure involves heating the food samples at 50°C for 15 minutes and thus this procedure may not be suitable for studying metabolically injured cells or even live cells in some cases and hence the need for better separation techniques.

Fluorescent labeling of target cells is another critical step in the use of FCM. The choice of stains should be such that the background fluorescence is eliminated completely or reduced. Thus, dyes that has an affinity for proteinaceous material may not be suitable for use in FCM. The concentration of the dye to be used becomes critical under these conditions of non-specific bindings. Finally, the food suspensions should be free of particulate materials

which would otherwise clog the equipment. The main advantages are accuracy and speed of analysis. In these respects FCM is suitable for rapid quality control of food raw materials and to check hygiene procedures. Additional applications in food industry will emerge as soon as the potential of flow cytometry has been recognized completely and techniques to specifically detect or analyze food borne microorganisms have been improved.

1.4 References

1. Adams, M. R., C. L. Little and M. C. Easter. 1991. Modeling the effect of pH, acidulant and temperature on the growth rate of *Yersinia enterocolitica*. J. Appl. Bacteriol. 71: 65-71.

2. Anonymous. 1985. Irradiation in the production, processing and handling of food: Final Rule. 21 CFR Part 179. Federal Register 50(140): 29658.

3. Anonymous, 1997. Food safety - a worldwide public health issue. WHO Report, WHO home page. http://www.who.ch:80/programmes/fnu/fos/fctshtfs.html. 02/02/97.

4. Babink, L. A., and E. A. Paul. 1970. The use of fluorescence isothio-cyanate in the determination of the bacterial biomass of grassland soil. Can J. Microbiol. 16: 57-62.

5. Baker, J. M. and M. W. Griffiths. 1993. Predictive modeling of psychrotrophic *Bacillus cereus*. J. Food Prot. 56: 684-688.

6. Bakken, L. R. 1985. Separation and purification of bacteria from soil. Appl. Environ. Microbiol. 49: 1482-1487.

7. Bearson, S., B. Bearson, and J.W. Foster. 1997. Acid stress responses in enterobacteria. FEM Microbiol. Lett. 147: 173-180.

8. Bercovier, H., M. Resnick, Kornitzer and L. Levy. 1987. Rapid method for testing drug-susceptibility of *Mycobacterium* spp. and gram-positive bacteria using rhodamine 123 and fluorescein diacetate. J. Microbiol. Methods. 7: 139-142.

9. Berglund, D. L., R. E. Taffs, and N. P. Robertson. 1987. A rapid analytical technique for flow cytometric analysis of cell viability using calcofluor white M2R. Cytometry 8: 421-426.

10. Blankenship, L. C., S. E. Craven, R. G. Leffler, and L. Custer. 1988. Growth of *Clostridium perfringens* in cooked chili during cooling. Appl. Environ. Microbiol. 54: 1104-1108.

11. Booth, I. R. 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. 49: 359-378.

12. Brody, A. L. 1989. Modified atmosphere/vacuum packaging of meat, p. 17-37. *In* A.L. Brody, Controlled/modified atmosphere/vacuum packaging of foods. Food and Nutrition Press Inc., Florida, USA.

13. Broughall, J. M., P. Anslow, and D. C. Kilsby. 1983. Hazard analysis applied to microbial growth in foods: development of mathematical models describing the effect of water activity. J. Appl. Bacteriol. 55:101-110.

14. Brown, M. H., and I. R. Booth. 1991. Acidulants and low pH, p. 22-43. *In* N.J. Russell and G.W. Gould (ed.), Food Preservative. Blackie, Glasgow, Scotland.

15. Brunius, G. 1980. Technical aspects of the use of 3',6'-diacetyl fluorescein for vital fluorescent staining of bacteria. Current Microbiology 4: 321-323.

16. Buchanan, R. L. and J. G. Phillips. 1990. Response surface model for predicting the effects of temperature, pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes*. J. Food Prot. 53: 370- 376.

17. Cole, M. 1991. Predictive modeling - Yes it is! Lett. Appl. Microbiol. 13: 218-219.

18. Crissman, H. A., D. J. Orlicky, and R. J. Kissame. 1979. Fluorescent DNA probes for flow cytometry-considerations prospects. J. Histochem. Cytochem. 27: 1652-1654.

19. Davey, K. L. 1989. A predictive model for combined temperature and water activity on microbial growth during the growth phase. J. Appl. Bacteriol. 67: 483-488.

20. Davey, K. R. 1991 Applicability of the Davey linear Arrhenius predictive model to the lag phase of microbial growth. J. Appl. Bacteriol. 70: 253-257.

21. Dempster, J. F. 1985. Radiation preservation of meat and meat products Meat Sci. 12: 61-89.

22. Diaper, J. P. and C. Edwards. 1994. The use of fluorogenic esters to detect viable bacteria by flow cytometry. J. Appl. Bacteriol. 77: 221-228.

23. Diaper, J. P., K. Tither and C. Edwards. 1992. Rapid assessment of bacterial viability by flow cytometry. Appl. Microbiol. Biotechnol. 38: 268-272.

24. Dickson, J. S., G. R. Siragusa, and J. E. Wray Jr., 1992. Predicting the growth of *Salmonella typhimurium* on beef by using the temperature function integration technique. Appl. Environ. Microbiol. 58: 3482-3487.

25. Duh, Y. H. and D. W. Schaffner. 1993. Modeling the effect of temperature on the growth rate and lag time of *Listeria innocna* and *Listeria monocytogenes*. J. Food Prot. 56: 205-210.

26. Edwards, C., J. P. Diaper, J. Porter, and R. Pickup. 1993. Applications of flow cytometry in bacterial ecology, p.121-129. *In* D. Lloyd (ed.), Flow cytometry in Microbiology, Springer Verlag, London.

27. Efiok, B. J. S., and D. A. Webster. 1990. Respiratory-driven Na^+ electrical potential in the *Bacterium vitreoscilla*. Biochemistry 29: 4734-4739.

28. Ehioba, R. M., A. A. Kraft, R. A. Molins, H. W. Walker, D.G. Olson, G. Subbaraman, and R. P. Skowronski. 1988. Identification of microbial isolates from vacuum-packaged groundpork irradiated at 1 KGy. J. Food Sci. 53: 278-279.

29. Ehioba, R. M., A. A. Kraft, R. A. Molins, H. W. Walker, D.G. Olson, G. Subbaraman, and R. P. Skowronski. 1987. Effect of low-dose (100 krad) gamma radiation on the microflora of vacuum-packaged ground pork with and without added sodium phosphates. J. Food Sci. 52: 1477-1148,1173.

30. Einarsson, H. And S. G. Eriksson. 1986. Microbial growth models for prediction of shelf life of chilled meat. In: Meat Chilling 1986. Proc. Meeting International Institute of Refrigeration Commission c2, Bristol, U.K. 10-12 September 1986. Institute International du Froid/International Institute of Refrigeration, Paris, pp. 301-305.

31. Elliott, R. P., and H. D. Michener. 1964. Factors affecting the growth of psychrotrophic microorganisms in Food - A Review Tech. Bull. No. 1320. U.S. Dept. Of Agriculture, Agricultural Research Service, Washington, D.C.

32. Fisher., G. C. Rice and G. M. Hahn. 1986. Dansyl lysine, a new probe for assaying heat-induced cell killing and thermotolerance in *in vitro* and *in vivo*. Cancer Res. 46: 5064-5068.

33. Flowers, R. S. and Z. J. Ordal. 1979. Current methods to detect stressed staphylococci. J. Food. Prot. 42: 368-369.

34. Foster, J.W., and H.K. Hall. 1990. Adaptive acidification tolerance response of *Salmonella typhimurium* . J. Bacteriol. 172: 771-778.

35. Foster, J. W. and H. K. Hall. 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. 173: 5129-5135.

36. Fry, J. C. 1990. Direct methods and biomass estimation., Methods in Microbiology 22: 41-85.

37. Fry, J. C., R. Goulder, and C. Rimes. 1985. A note on the efficiency of stomaching for the quantitative removal of epiphytic bacteria from submerged aquatic plants. J. Appl. Bacteriol. 58: 105-112.

38. Fu, B., P. S. Taoukis, and T. P. Labuza. 1991. Predictive microbiology for monitoring spoilage of dairy products with time-temperature integrators. J. Food Sci: 56: 1209-1215.

39. Futsaether, C. M., B. Kjeldstad, and A. Johnson. 1993. Measurement of the intracellular pH of *Propionibacterium acnes*: comparison between the fluorescent probe BCECF and 3'p-NMR spectroscopy. Can. J. Microbiol. 39: 180-186.

40. Genigeorgis, C., S. Martin, C. E. Franti and H. Riemann. 1971. Initiation of staphylococcal growth in laboratory media. Appl. Microbiol. 21: 934-939.

41. Gibson, A. M., N. Bratchell, and T. A. Roberts. 1988. Predicting microbial growth: growth responses of salmonellae in a laboratory medium as affected by pH, sodium chloride and storage temperature. Inter. J. Food. Microbiol. 6: 155-178.

42. Gibson, A. M., N. Bratchell, T. A. Roberts. 1988. Predicting microbial growth: growth responses of salmonellae in a laboratory medium as affected by pH, sodium chloride and storage temperature. Int. J. Food. Microbiol. 6: 155-178.

43. Giddings, G. G., and M. Marcotte. 1991. Poultry irradiation for hygiene/safety and market-life enhancement. Food Rev. Int. 7: 259-282.

44. Gill, C. O. 1984. Prevention of early spoilage of livers, p. 287-290. *In* Proc. 30th Eur. Meeting Meat Research Workers, Bristol, U.K.,.

45. Gould, G. W., T. Abee, P. E. Granum, and M. V. Jones. 1995. Physiology of food poisoning microorganism and the major problems in food poisoning control. Int. J. Food Micrbiol. 28: 121-128.

46. Hall, H.K., and J.W. Foster. 1996. The role of Fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron aquisition. J. Bacteriol. 178: 5683-5691.

4.7 Haugland, R. P. 1992. Molecular Probes, p.421. *In*: K. D. Larison(ed.),Handbook of Fluorescent Probes, Molecular Probes, Eugene, Oregon, USA.

48. Hengge-Aronis, R. 1996. Back to log phase: σs as a global regulator in the osmotic control of gene expression in *Escherichia coli*. Mol. Microbiol. 21: 887-893.

49. Hill, C., B. O'Driscoll, and I. Booth, 1995. Acid adaptation and food poisoning microorganisms. Int. J. Food Microbiol. 28: 245-254.

50. Hudson, J. A. 1992. Construction of and comparisons between response surface models for *Aeromonas hydrophila* ATCC 7966 and a food isolate under aerobic conditions. J. Food Prot. 55: 968-972.

51. Hultin, E. 1955. The influences of temperature on the rate of enzymatic processes. Acta Chem. Scand. 9: 1700-1710.

52. Humphrey, T. J., N. P. Richardson, K. M. Statton, and R. J. Rowbury. 1993. Effects of temperature shift on acid and heat tolerance in *Salmonella enteritidis* phase type and Appl. Environ. Microbiol. 59: 3120-3122.

53. Hutter, K. J. and H. E. Eipel. 1979. Simultaneous measurements of DNA and protein content of microorganisms by flow cytometry. Eur. J. Appl. Microbiol. 6: 223-231.

54. Imhoff, J. F. and B. Thiemann. 1991. Influences of salt concentration and temperature on fatty acid compositions of Ectothiorhodospira and other halophilic phototrophic purple bacteria. Arch. Microbiol. 156: 370-375.

55. Jaeggi, N. E., R. Brunner and W. Schmidt-Lorenz. 1989b. DEFT-technique in the analysis of foods. II. Agreement with the standard plate count. Acta Alimentaria. 18: 79-88.

56. Jaeggi, N. E., R. Brunner, and W. Schmidt-Lorenz. 1989a. DEFT-technique in the analysis of foods. 1. Pretreatment and enzymatic digestion. Acta Alimentaria. 18: 71-77.

57. Jernaes, M. W. and H. B. Steen. 1994. Staining of *Escherichia coli* for flow cytometry: Influx and efflux of ethidium bromide cytometry 17: 302-309.

58. Johnson, F.H., and I. Lewis.1946. The growth rate if *E. coli* in relation to temperature, quinine and coenzyme. J. Cell. Comp. Physiol. 28: 47-75.

59. Kaprelyant, A. S. and D. B. Kell. 1992. Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. J. Appl. Bacteriol. 72: 410-422.

60. Kaprelyants, A. S., G. V. Mukamolova, H. M. Davey and D. B. Kell. 1996. Quantitative analysis of the physiological heterogenity within starved cultures of *Micrococcus luteus* by flow cytometry and cell sorting. Appl. Environ. Microbiol. 62: 1311-1316.

61. Knochel, S. And G. Gould. 1995. Preservation microbiology and safety: Quo vadis? Trends in Food Sci. Technol. 6: 127-131.

62. Kroll, R. G., and R. A. Patchett., 1992. Induced acid tolerance in *Listeria monocytogenes*. Lett. Appl. Microbiol. 14: 224-227.

63. Langeveld, L.P.M. and F. Cuperus. 1980. The relation between temperature and growth rate in pasteurized milk or different types of bacteria which are important to the deterioration of that milk. Neth. Milk. Dairy J. 34: 106-125.

64. Lebepe, S., R. A. Molins, S. P. Charoem, H. Farrar IV, and R.P. Skowronski. 1990. Changes in microflora and other characteristics of vacuum-packaged pork loins irradiated at 3.0 KGy. J. Food Sci. 55: 918-924.

65. Lefebvre, N., C. Thibault, R. Charbouneau, and J. P. G. Piette. 1994. Improvement of shelf-life and wholesomeness of ground beef by irradiation - 2. Chemical analysis and sensory evaluation. Meat 36: 371-380.

66. Leistner, L., and L. G. Gorris. 1995. Food Preservation by hurdle technology. Trends in Food Sci. Technol. 6: 41-46.

67. Loew, L M. 1993. Potentiometric membrane dyes, p. 150-160. *In* W.T. Mason (ed.),Fluorescent and luminescent probes for biological activity, Academic Press, London.

68. Mason, D. J., R. Allman, J. M. Stark, and D. Lloyds. 1994. Rapid estimation of bacterial antibiotic susceptibility with flow cytometry. J. Microscopy 176: 8-16.

69. Mason, D. J., R. Lopez-Amoros, R. Allman, J. M. Stark and D. Lloyd. 1995. The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. J. Appl. Bacteriol. 78: 309-315.

70. Matsuyama. T. 1984. Staining of living bacteria with rhodamine 123. FEMS Microbiol. Lett. 21: 153-157.

71. Mattison, M. C., A. A. Kraft, D. G. Olson, H. W. Walker, R. E. Rust, and D. B. James. 1986. Effect of low dose irradiation of pork loins on the microflora, sensory characteristics and fat stability. J. Food Sci. 51: 284287.

72. McClure, P.J., M.B. Cole, and K.W. Davis. 1994. An example of the stages microbial growth: the effects of NaCl, pH and temperature on the growth of *Aeromonas hydrophila*. Int. J. Food Microbiol. 23: 359-375.

73. McFeters, G. A., F. P. Yu, B. H. Pyle, and P. S. Stewart. 1995. Physiological assessment of bacteria using fluorechromes. J. Microbiol. Meth. 21: 1-13.

74. McMeekin, T. A., J. Olley, T. Ross and D. A. Ratkowsky. 1993. Predictive microbiology: Theory and Application. Research Studies Press, Taunton, U.K.

75. Melamed, M. R., T. Lindmo and M. L. Mendelsohn. 1990. Flow cytometry and sorting. John Wiley and Sons, New York.

76. Metaxapoulos, J., C. Genigeorgis, M. J. Fanelli, C. Franti and E. Cosma 1981. Production of Italian dry Salami, I: invitation of staphyococcal growth in salami under commercial manufacturing conditions. J. Food Prot. 44: 347-352.

77. Metaxapoulous, J. C. Genigeorgis, M. J. Fanelli, C. Franti, and E. Cosma. 1981 b. Production of Italian dry salami: effect of starter culture and chemical acidulation on staphylococcal growth in salami under commercial manufacturing conditions. Appl. Environ. Microbiol. 42: 63-71.

78. Midgley, M. 1986. The phosphomium ion eflux system of *Escherichia coli*: Relationship to the ethidium efflux system and energetic studies. J. Gen. Microbiol. 132: 3187-3193.

79. Miller, J. S. and J. M. Quarles. 1990. Flow cytometric identification of microorganisms by dual staining with FITC and PI cytometry 11: 667-675.

80. Miller, J. S., and J. M. Quarles, 1990. Flow cytometric identification of microorganisms by dual staining with FITC and PI. Cytometry 11: 667-675.

81. Molenaar, D., T. Abee, and W. N. Konings. 1991. Continuous measurement of the cytoplasmic pH in Lactococcus lactis with a fluorescent pH indicator. Biochem. Biophys. Acta 1115: 75-83.

82. Murphy. P. M., M. C. Rea, and D. Harrington. 1996. Development of a predictive model for growth of *Listeria monocytogenes* in a skim milk medium and validation studies in a range of dairy products. J. Appl. Bacteriol. 80: 557-564.

83. Ohlsson, T. 1994. Minimal processing-preservation methods of the future: an overview. Trends in Food Sci. Technol. 5: 341-344.

84. Ordo'n-ez, and J. V. and N. M. Wehman. 1993. Rapid Flow Cytometric antibiotic susceptibility assay for *Staphylocioccus aureus*. Cytometry 14: 811-818.

85. Palumbo, S.A. 1986. Is refrigeration enough to restrain food borne pathogens? J. Food Prot. 49: 1003-1009.

86. Palumbo, S. A., A. C. Williams, R. L. Buchanan, and J.G. Phillips. 1991. Model for the aerobic growth of *Aerononas hydrophila* K144. J. Food Prot. 54: 429-435.

87. Park, Yong-K., B. Bearson, S.H. Bang, I.S. Bang, and J.W. Foster. 1996. Internal pH crisis, lysine decarboxylase and the acid tolerance response of *Salmonella typhimurium*. Mol. Microbiol. 20: 605-611.

88. Petit, J. M., M. Denis-Gracy, and M. H. Ratinand. 1993. Assessment of fluorochromes for cellular structure and function studies by flow cytometry. Biol. Cell 78: 1-13.

89. Pinder, A. C., P. W. Purdy, S. A. . Poulter, and D. C. Clark. 1990. Validation of flow cytometry for rapid enumeration of bacterial concentrations in pure culture. J. Appl. Bacteriol. 69: 92-100.

90. Porter, J., C.Edwards and R. W. Pickup. 1995. Rapid assessment of physiological status in *Escherichia coli* using fluorescent probes. J. Appl. Bacteriol. 79: 399-408.

91. Porter, K. G. and Y. S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25: 943-948.

92. Raevuori, M, and C. Genigerogis. 1975. Effect of pH and sodium chloride on growth of *Bacillus cereus* in laboratory media and certain foods. Appl. Microbiol. 29: 68-73.

93. Ratkowsky, D. A., J. Olley, T. A. McMeekin and, A. Ball. 1982. Relationship between temperature and growth rate of bacterial cultures. J. Bacteriol. 149: 1-5.

94. Ratkowsky, D. A., T. Ross, T. A. McMeekin and, J. Olley. 1991. Comparison of Arrhenius-type and Belehradek-type models for prediction of bacterial growth in foods. J. Appl. Bacteriol. 71: 452-459.

95. Richards, R. M. E., D. K. L. Xing, and T. P. King. 1995. Activity of *p*-aminobenzoic acid compared with other organic acids against selected bacteria. J. Appl. Bacteriol. 78: 209-215.

96. Roberts, T. A., and B. Jarvis. 1983. Predictive modeling of food safety with particular reference to Food Microbiology: Advances and Prospects. Academic Press, New York, pp 85-95.

97. Robertson, B. R. and D. K. Button. 1989. Characterizing aquatic bacteria according to population, cell size and apparent DNA content by flow cytometry. Cytometry 10: 70-76.

98. Robins, M. M., and P. D. E. Wilson. 1994. Food structure and microbial growth. Trends Food Sci. Technol. 5: 289-293.

99. Ross, T., T. A. McMeekin, 1994. Predictive Microbiology, Int. J. Food Microbiol. 23: 241-264.

100. Rowbury, R. J. 1995. An assessment of environmental factors influencing acid tolerance and sensitivity in *Escherichia coli*, *Salmonella* spp. and other enterobacteria. Lett. Appl. Microbiol. 20: 333-337.

101. Rowbury, R. J., M. Goodson, and T. J. Humphrey. 1994. Sodium chloride induces and NhaA/NhaR - independent acid sensitivity at neutral external pH in *Escherichia coli*. Appl. Environ. Microbiol. 60: 1630-1634.

102. Rowbury, R. J., M. Goodson, and T. J. Humphrey. 1993. Acid sensitivity induction (ASI) at alkaline pH in *Escherichia coli* involves two major sensitization components, induction of both being switched on by increased internal pH. Lett. Appl. Microbiol. 17: 272-275.

103. Russell, N. J., R. I. Evans, P. F. Ter Steeg, J. Hellemons, A. Verheul and T. Abee. 1995. Membranes as a target for stress adaptation. Int. J. Food Microbiol. 28: 255-261.

104. Russell, N. J., and N. Fakunaga. 1990. A comparison of thermal adaptation of membrane lipids in psychrotrophic and thermophillic bacteria. FEMS Microbiol. Rev. 75: 171-182.

105. Russell, S. D. 1984. Potential sites of damage in microorganisms exposed to chemical and physical agents, p. 1-15. *In* M. H. E. Andrews and A. D. Russell (Ed.), The Revival of Injured Microbes, Academic Press, Orlando, FL.

106. Savagaon, K. A., V. Venugopal, S. V. Kamat, U. S. Kumta, and A. Sreenivasan, 1972. J. Food Sci. 37: 148-150.

107. Schoolfield, R. M., P. J. H. Sharpe, and C. E. Magnuson. 1981. Non-linear regression of biological temperature-dependent rate models based on absolute reaction-rate theory. J. Theor. Biol. 88: 719-731.

108. Sharpe, P. J. H., and D. E. DeMichele. 1977. Reaction kinetics and poikilotherm development. J. Theor. Biol. 64: 649-670.

109. Shaw, B. G. and J. M. Shewan. 1968. Psychrotrophic spoilage bacteria of fish. J. Appl. Bacteriol. 56: 25-40.

110. Smith, J. C. 1990. Potential-senstitive molecular probes in membranes of bioenergetic relevance. Biochem. Biophys. Acta. 1016: 1-28.

111. Smith, M. G. 1987. Calculation of the expected increases of coliform organisms, *Escherichia coli* and *Salmonella typhimurium* in raw blended mutton tissue. Epidemiol. Infect. 99: 323-331.

112. Thakur, B. R. and R .K. Singh. 1995. Combination processes in food irradiation. Trends in Food Sci. Technol. 6: 7-11.

113. Thayer, D. W., G. Boyd and R. K. Jenkins. 1993. Low-dose gamma irradiation and refrigerated storage in vacuo affect microbial flora of fresh pork. J. Food Sci. 58: 717-719, 733.

114. Thomas, S. B. 1953. Psychrotrophilic microorganisms in milk and dairy products. Part II. Dairy Sci. Abstr. 20: 448-468.

115. Tomlins, R. I., T. R. Watkins, and R. J. H. Gray. 1982. Membrane lipid alterations and thermal stress in *Salmonella typhimurium* 7136. Appl. Envrion. Microbiol. 44: 1110-1117.

116. Ueckert, J., P. Breeuwer, T. Abee, P. Stephens, G. Nebe von Caron, and P. F. ter Steeg. 1995. Flow cytometry applications in physiological study and detection of food borne microorganisms. Int. J. Food Microbiol 28: 317-326.

117. Van Dilla, M., R. G. Langlois, D. Pinkel, D. Yajko and W. K. Hadlkey. 1983. Bacterial Characterization by flow cytometry. Science 220: 620-621.

118. Whiting, G. C., and R. J. Rowbury. 1995. Increased resistance of *Escherichia coli* to acrylic acid and to copper ions after cold-shock. Lett. Appl. Microbiol. 20: 240-242.

119. Witzer, T., P. J. McClure, M. H. Zwietering, and T. A. Roberts. 1993. Modeling bacterial growth of *Listeria monocytogenes* as a function of water activity, pH and temperature. Int. J. Food Microbiol. 18: 139-149.

120. Yip, B. And C. Genigeorgis. 1981. Interactions of *Clostridium perfringens* and certain curing factors in laboratory media. In: Proc. World Congr. Foodborne Infections and Intoxications, 980, Berlin. Paul Parey, Berlin [cited in Farber, 1985 (q.v.)].

121. Yoon, W.B., and R.A. Rosson. 1990. Improved method of enumeration of attached bacteria for study of fluctuation in the abundance of attached and free-living bacteria in response to dial variation in sea water turbidity. Appl. Environ. Microbiol. 56: 595-600.

122. Yu, F. P., and G. A. McFeters. 1994. Rapid in sity assessment of physiological activities in bacteria biofilms using fluorescent probes. J. Microbiol. Methods. 20: 1-10.

CHAPTER 2. DEVELOPMENT OF PREDICTIVE GROWTH RATES OF AEROBIC PSYCHROTROPHIC AND PSYCHROPHILIC BACTERIA IN LOW DOSE IRRADIATED MEATS

A paper to be submitted to the Journal of Food Microbiology

Raveendran Venugopal and James Dickson

2.1 Abstract

Frozen pork patties, thawed overnight at 0°C were packaged under vacuum and air or temperature abused by holding at 15°C for 24 hr. and then packaged under vacuum and air. Immediately after packaging, both sets were irradiated at 0, 0.5, 1, and 2 KGy. All the samples were stored at 0°C and were analyzed for bacterial counts every three days for 30 days. The populations of total aerobic bacteria, psychrotrophs and lactic acid bacteria were enumerated. Fresh pork patties under air and vacuum, receiving a dose of 0 KGy had a shelf life of 11 and 16 days, respectively, and for abused patties it was 7 days for both air and vacuum packaging. By considering our criteria for spoilage which was 10^7 cells/g, both fresh and abused (both air and vacuum) that received a dose of 2 KGy had a shelf life greater than 30 days at 0°C. Models were developed based on generation time and lag phase extension.

2.2 Introduction

The quality of meat is largely dependent on the level of microbiological contamination. The chances of microbial contamination are greater in processed meat than in fresh meat. There is general agreement that onset of microbial growth is a principal factor affecting the shelf life of a food product. Irradiation has the potential to emerge as one of today's most significant food preservation technologies. Government and industry have indicated interest in ionizing radiation doses in excess of 1.0 KGy but less than 10 KGy to increase shelf life and improve safety of products for consumers (Thayer *et al.* 1993). When considering irradiation at an optimal dose for extending the shelf life of meat products,

parameters other than the microbial reduction must be considered. These include the organoleptic properties such as color, odor, flavor and nutrient retention. Thus it is essential to know the optimum dose required for effective control of microbial growth without compromising the organoleptic properties of the food.

Some of the potential advantages of food irradiation include the elimination of pathogenic and spoilage organisms, a reduction in the waste of food resulting from premature decay, ripening, germination or sprouting (Webb and Lang, 1990). All these lead to an increase in shelf life. This process has not become widely accepted, partly because of consumer concerns which includes the possibility of masking unhygienic food by eradicating detectable contamination (Lowe and Towner, 1994). Although the FDA has approved the treatment of pork meat and products with a minimum dose of 0.3 KGy and a maximum 1.0 KGy of ionizing radiation to control *Trichinella spiralis* (Anon, 1985), this level is not sufficient to control the growth of microorganisms.

It is well known that chilled meat products are preferred by consumers over frozen meat products (Seman *et al.* 1988). The most active market segment is the chilled, ready-to-eat or ready-to-heat foods, typically produced in single serving packages, designed to meet the needs of convenience and simplicity (Ohlsson, 1994). Thus, there is a push towards more natural and more fresh-like products. All these have reinforced the need for improving the safety and shelf life of chilled foods and appropriate means for more accurate determination of its shelf life. A part of this approach to microbiological safety and food quality is called predictive microbiology. Predictive microbiology is the term used to describe the mathematical modeling of the effects of temperature, water activity, pH and other environmental influences on the rates of microbial growth or the time to reach specified numbers of microorganisms or their metabolites (Ratowsky *et al.* 1991). Predicting the growth rate during storage of packaged meat could enable food manufacturers to formulate effective control measures. Such a model could establish the optimum treatment dose required for extending the quality of food materials. Several attempts have been made towards predictive modeling of the growth of microorganisms inside, or on the surface of foods as a function of time. These models are usually analytical expressions such as the

Gompertz or the logistic curve, which exhibit the typical appearance of the bacterial growth curve (Nicolai *et al.* 1993).

In this study, predictive models of growth rates of aerobic bacteria and aerobic psychrotrophic bacteria in aerobic and vacuum packaged ground pork patties are developed. Both, fresh and temperature abused pork patties are used in this study. Models are developed for 0, 0.5, 1.0 and 2.0 KGy irradiation doses.

2.3 Materials and Methods

2.3.1 Meat Samples

Frozen pork patties were purchased from the Meat Laboratory at Iowa State University and were thawed overnight. One set of patties were packaged in vacuum bags (Curlon 861 Film, oxygen transmission rate 3-4 cc/100 in^2/24 hrs @ 22.7°C, Curwood, Oshkosh, WI) or using styrofoam trays covered with saran wrap (Cryovac SSD-310 Film; Oxygen transmission rate 9,000-11,000 cc/m^2/24 hr. @ 22.7°C; Cryovac Division of W.R. Grace and Co., Duncan, SC). Another set of patties were temperature abused by holding at 15°C for 24 hr. and then packaged either by vacuum or air as described above.

2.3.2 Irradiation

Immediately after packaging, the samples, (both fresh and temperature-abused) were irradiated using accelerated electron beams at the Linear Accelerator Facility at Iowa State University. The meat patties were irradiated to an average absorbed dose of 0, 0.5, 1.0, and 2.0 KGy. Immediately after irradiation, the samples were stored in a walk-in cooler at 2°C.

2.3.3 Microbiological Analyses

Microbiological analyses was carried every three days over a period of thirty days. Aerobic plate count (Tryptic soy agar (Difco), 35°C, 48h), lactics count (MRS agar (Difco), 35°C, 48h), and psychrotrophic count (Tryptic soy agar; 7°C for 10 days) were done on both aerobically packaged and vacuum packaged irradiated patties. Samples were platted by spread plate method.

2.3.4 Model Development

The model development was done as reported by Dickson *et al.* (1992). The estimates of bacterial populations were converted to $\log_{10}$ CFU per gram and each plating type-packaging combination was independently replicated three times. Data from each growth curve [the bacterial population at time t in CFU per gram, N(t)] were fitted to the Gompertz equation:

$$N(t) = A + \{C[e^{-e^{-B(t-M)}}]\}$$

(Buchannan, 1991; Gompertz, 1825) by use of a nonlinear regression procedure (Statistical Analysis System, 1985); *A* is the initial level of bacteria (CFU/g), *B* is the relative growth rate of bacteria at the time, in days, when the growth rate of bacteria is maximal, C is the asymptotic amount of growth that occurs as time increases indefinitely, and M is the time, in days, when the growth rate is maximal. Recent reviews have concluded that the Gompertz equation is preferred for modeling bacterial growth curves (Garthright, 1991; Zwietering *et al.* 1990). Lag and generation times were defined as the points at which the second and first derivatives, respectively, of each growth curve attained a maximum (Buchanan and Phillips, 1990; Garthright, 1991; Gibson *et al.* 1988; Palumbo *et al.* 1991). The individual lag and generation times from each replication were graphed using the irradiation dose as the x axis.

2.4 Results

Predictive models were developed for total aerobic bacteria, lactic acid bacteria, and psychrotrphic bacteria for both aerobic and vacuum packaged, fresh and temperature abused ground pork. The lag and generation times for all the three types of bacteria resulted in curves which fit the following model.

$$Y = a + b(dose)^c$$

where Y is the lag or generation time, dose is from 0 to 2 KGy, and a, b, and c are derived coefficients. The calculated values for these coefficients are presented in Tables 7 - 10. The choice of this function resulted in predicted values that closely approximated the numerical averages of the laboratory data. The shelf life and growth characteristics (generation time and end of lag phase) of aerobic bacteria, lactic acid bacteria, and psychrotrophic bacteria grown on aerobically packaged, fresh, irradiated pork patties are given in Table 1. Samples with a microbial population in excess of 10^7 cells/g were considered spoiled and this point in the storage time was considered as the shelf life of the samples. Based on this criteria, the shelf life of unirradiated aerobically packaged pork patties was 11 days and this increased to 27 days when a dose of 0.5 KGy was used. When 1.0 KGy was used, the shelf life was greater than 30 days. A similar trend was observed with generation time and end of lag phase. Both increased significantly with an increase in irradiation dose. At 0 KGy, aerobic bacteria had a generation time of 0.602 days while it was 0.458 days for lactic acid bacteria and 0.616 days for psychrotrophic bacteria. This generation time doubled for aerobic bacteria and lactic acid bacteria when the irradiation dose was increased to 0.5 KGy. The influence of this dose on psychrotrophic bacteria was relatively less. At 1 KGy, generation time for aerobic bacteria and lactic acid bacteria tripled (1.613 and 1.575 days, respectively) while it doubled (1.322 days) for psychrotrophic bacteria. The extension of lag phase showed a similar trend, increasing with an increase in irradiation dose. For unirradiated patties, the lag phase for aerobic bacteria, psychrotrophic bacteria, and lactic acid bacteria were 1.08, 1.08 and 3.83 days, respectively. The maximum lag phase extension was obtained at 2 KGy.

The growth characteristics of bacteria grown on vacuum packaged, irradiated fresh pork patties are presented in Table 2. The unirradiated samples had a shelf life of 16 days, and there were no differences at 0.5 and 1.0 KGy when compared with aerobic packaging. Irradiation did not influence the generation time of aerobic bacteria but there was a significant difference in the extension of lag phase. In the case of lactic acid bacteria, dose had a significant influence on generation time but the extension of lag phase was not affected. For aerobic psychrotrophic bacteria, irradiation dose increased the generation time and extended the lag phase significantly. Models were not developed for fresh pork patties at 2.0

KGy because of the inconsistent counts obtained. Majority of sampling points over a period of 30 days had a population that was below our detection limit (data not presented). The aerobic plate count showed a value of 3.0 logs cfu/g at the end of 30 days and the cells were below our detection limits for the first nine days.

Pork patties were temperature-abused to increase their initial microbial load and then irradiated. Table 3 shows the growth characteristics and shelf life for aerobically packaged pork patties. When the product was temperature abused, it was possible to develop models for irradiation at 2 KGy. The shelf life was a maximum of 31 days for 2.0 KGy and a minimum of 7 days at 0 KGy. The generation time of aerobic bacteria was not affected by irradiation but the extension of lag phase was affected. For lactic acid bacteria, irradiating pork patties at 1 and 2 KGy gave a significant increase in generation time of 1.03 and 1.38 days, respectively. The lag phase was also extended at higher doses. A similar trend was also seen with the growth characteristics of psychrotrophic bacteria. The generation time, 0.561 day at 0 KGy was greater than aerobic bacteria and lactic acid bacteria.

The vacuum-packaged, temperature abused patties when irradiated, showed an increase in shelf life to 19 and 33 days at 0.5 KGy and 1-2 KGy, respectively (Table 4). Here again, the generation time was unaffected by dosage with significant differences in the extension of lag phase in the case of aerobic bacteria. This trend was also observed with lactic acid bacteria. Significant differences were observed with regards to generation time and end of lag phase of psychrotrophic bacteria.

Tables 5 and 6 show the percentage kill for aerobic and vacuum packaged, abused pork patties. For both packaging, pshychrotrophic bacteria showed the maximum sensitivity. At 2.0 KGy, 70% kill was obtained under aerobic packaging and 100 % kill was obtained for vacuum packaging. At 2.0 KGy, vacuum packaged patties had a higher percent of kill than aerobic packaging.

2.5 Discussion

Consumer interest in chilled food-products and ready to eat food products are increasing. The present trend is towards more extensive open date-marking of foods which

has reinforced the need for systemic information on the expected shelf life of chilled foods and appropriate means for its determination (Pooni and Mead, 1984). There is general agreement that rate of microbial growth is the principal factor determining the shelf life of flesh foods such as red meat, poultry and fish. The shelf life of pork patties increased with an increase in the dose used. Packaging alone influenced the shelf life of irradiated pork patties; unirradiated vacuum packaged patties had a shelf life of 16 days as opposed to 11 days for aerobically packaged patties. Vacuum packaging alone is known to enhance the shelf life of perishable foods such as cuts of fresh meat (Nicolai *et al.* 1993).

It is surprising that packaging did not influence the shelf life of irradiated pork patties, although it did influence the non-irradiated ones. The main reason attributed to this could be the nature of destruction of microorganisms by irradiation. Though it has been reported that spoilage of fresh meat cuts in contact with air differs considerably from that of vacuum-packaged fresh meats (Lambert *et al.* 1991), irradiation acts by inactivating microorganisms in a non-specific way. However, it is possible that vacuum packaging may provide an anaerobic atmosphere which is conducive for repair of sublethally injured bacteria. It is already known that the presence or absence of oxygen during irradiation can have an important influence on the course of radiolysis (Diehl, 1995). Thus, it is possible that an anaerobic atmosphere devoid of oxygen could accelerate the rate of resuscitation of spoilage bacteria and reduce the shelf life. In comparison, the fresh pork patties had a better shelf life than the temperature-abused patties. This highlights the need for a low initial microbial load and it is likely that the efficacy of antimicrobial treatment will reduce with increasing microbial load.

Factors which influence the shelf life are the initial microbiological quality of the product, the storage time and temperature. The extension of shelf life of foods processed with ionizing radiation is well documented. With refrigerated meats, this extension of shelf life is usually characterized, microbiologically, by an initial reduction of microorganisms, followed by an extended lag phase and subsequent growth of the surviving population. The increase in generation time and lag phase is dose dependent, higher the dose greater is the extension of generation time and lag phase. Lactic acid bacteria were incubated aerobically

and had the maximum extension of lag phase when compared to the other two types of bacteria. In addition to the antimicrobial nature of irradiation, the slow growing nature of lactics should also be accounted. Aerobic bacteria were the most resistant group among the three types enumerated. The population of the spoiling microorganisms under aerobic condition is extremely diverse and hence the behavior may not always be the same (Nicolai *et al.* 1993). The influence of irradiation was more pronounced in extending the lag phase than on increasing the generation time. The generation time was calculated by taking the steepest part of the growth curve and it is possible that, once the cells start growth, they grow as normal cells.

The initial microbial load of the product is very critical for shelf life. Thus, temperature-abused products had a relatively shorter shelf life than the fresh products. Though there appears to be no significant differences in the growth characteristics, the reduced shelf life could be an indication of survival of a greater percentage of sublethally injured population. This conclusion is supported by Tables 5 & 6. Even at a dose of 2 KGy, 100% kill was only obtained for psychrotorphic bacteria under vacuum packaged conditions. It is possible that refrigerated incubation acted in a synergistic way to inactivate the survived microbial population. Again, packaging does not seem to influence the percentage kill and one would expect a higher percentage of kill under aerobic conditions because of the presence of oxygen in the packages while irradiation.

For accurate prediction of shelf life, it is necessary to have a reliable indication of initial numbers of spoilage organisms on the processed product (Pooni and Mead, 1984). Obtaining this information with sufficient rapidity will influence the setting of 'a' value and a quicker prediction of shelf life. While many investigators have examined the extension of shelf life by irradiation, few have reported on the relative growth rates. Most reports typically list the number of days for the population of a specific group of bacteria to reach an arbitrary level, which is then described as shelf life. As for predictive models, models have been developed for a range of food borne pathogens with respect to different cultural factors such as temperature, pH, salt, etc., (Murphy *et al.* 1996) and very few for shelf life of processed foods. Growth curves can be modeled using a variety of mathematical equations,

with the Gompertz equation being one of the more widely accepted equations. Characterization of the growth of the surviving bacteria in irradiated meat in conventional kinetic growth terms, (i.e., lag and generation times) would provide an equivalent method for comparing different radiation processes and initial product quality. In addition, these growth parameters would allow for the development of predictive equations for optimizing radiation processes for products of known microbiological quality.

2.6 References

1. Anonymous. 1985. Irradiation in the production processing and handling of food: Final Rule 21 CFR Part 179. Federal Register 50(140): 29658.

2. Buchanan, R. L. 1991. Using spreadsheet software for predictive microbiology applications. J. food Saf. 11: 123-134.

3. Buchanan, R. L., and M. L. Cygnarowicz. 1990. A mathematical approach toward defining and calculating the duration of the lag phase. Food Microbiol. 7: 237-240.

4. Buchanan, R. L., and J. G. Phillips. 1990. Response surface model for predicting the effects of temperature, pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes*. J. Food Prot.53: 370-376.

5. Dickson, J. S., G. R. Siragusa, and J. E. Wray, JR. 1992. Predicting the growth of *Salmonella typhimurium* on beef by using the temperature function integration technique. Appl. Environ. Microbiol. 58: 3482-3487.

6. Diehl, J. F. 1995. Chemical effects of ionizing radiation, p. 43-88. *In* J. F. Diehl (Ed), Safety of Irradiated Foods. Marcel Dekker, Inc., New York.

7. Fielding, L. M., P. E. Cook, and A. S. Grandison. 1994. The effects of electron beam irradiation and modified pH on the survival and recovery of *Escerichia coli*. J. Appl. Bacteriol. 76: 412-416.

8. Garthright, W. E. 1991. Refinements in the prediction of microbial growth curves. Food Microbiol. 8: 239-248.

9. Gibson, A. M., N. Bratchell, and T. A. Roberts. 1988. Predicting microbial growth: growth responses of salmonellae in a laboratory medium as affected by pH, sodium chloride and storage temperature. Int. J. food Microbiol. 6: 155-178.

10. Gompertz, B. 1825. On the nature of the function of the law of human mortality, and on a new mode of determining the value of life contingencies. Philos. Trans. R. Soc. London 115: 513-585.

11. Gould, G. W., and M. V. Jones. 1989. Combination and synergistic effects, p. 401-421. *In* G.W Gould (ed.), Mechanisms of Action of Food Preservation Procedures. Elsevier Applied Science, London.

12. Lambert, A. D., J. P. Smith, and K.L. Dodds. 1991. Shelf life and microbial safety of fresh meat - review. Food Microbiol. 8: 267-297.

13. Murphy, P. M., M. C. Rea, and D. Harrington. 1996. Development of a predictive model for growth of *Listeria monocytogenes* in a skim milk medium and validation studies in a range of dairy products. J. Appl. Bacteriol. 80: 557-564.

14. Nicolai, B. M., J. F. Van Impe, B. verlinder, T. Martens, J. Vandewalle, and J. De Baerdemaeker. 1993. Predictive modeling of surface growth of lactic acid bacteria in vacuum-packed meat. Food Microbiol. 10: 229-238.

15. Ohlsson, T. 1994. Minimal processing-preservation methods of the future: an overview. Trends Food Sci. Technol. 5: 341-344.

16. Palumbo, S. A., A. C. Williams, R. L. Buchanan, and J.G. Phillips. 1991. Model for the aerobic growth of *Aeromonas hydrophila* K144. J. Food Prot. 54: 429-435.

17. Pooni, G. S., and G. C. Mead. 1984. Prospective use of temperature function integration for predicting the shelf life of non-frozen poultry meat products. Food Microbiol. 1: 67-78.

18. Ratowsky, D. A., T. Ross, T. A. McMeekin, and J. Olley. 1991. Comparison of Arrhenius-type and Belehradek-type models for prediction of bacterial growth in foods. J. Appl. Bacteriol. 71: 452-459.

19. Rowe, T. F., and K. J. Towner. 1994. Effect of irradiation on the detection of bacterial DNA in contaminated food samples by DNA hybridization. Lett. Appl. Microbiol. 18: 171-173.

20. Statistical Analysis System. 1985. NLIN, p. 575-606. In SAS user's guide: statistics, version 5 ed. SAS Institute, Inc., Cary, N.C.

21. Suman, D.L., K.R. Drew, P.A. Clarken, and R.P. Littlejohn. 1988. Influence of packaging method and length of chilled storage on microflora, tenderness and colour stability of vension loins. Meat Sci. 22: 267-282.

22. Thayer, D.W., G. Boyd and R.K. Jenkins. 1993. Low-dose gamma irradiation and refrigerated storage *In vacuo* affect microbial flora of fresh pork. J. Food Sci. 58: 717-719,733.

23. Webb, T., and T. Lang. 1990. Food Irradiation: The Myth and the Reality, 2nd ed. Wellingborough. Thorsons.

24. Zwietering, M.H., I. Jogenburger, F.M. Rombouts, and K. van't Riet. 1990. Modeling of the bacterial growth curve. Appl. Environ. Microbiol. 56: 1875-1881.

Table 1. Growth characteristics of aerobic bacteria, lactic acid bacteria and aerobic psychrotrophic bacteria grown on aerobically packaged, irradiated fresh pork patties.

		Aerobic bacteria		Lactic acid bacteria		Psychrotrophic bacteria	
Dose (KGy)	Shelf life (Days)	Generation time (Days)	End of lag phase (Days)	Generation time (Days)	End of lag phase (Days)	Generation time (Days)	End of lag phase (Days)
0	11.0	0.602	1.08	0.458	3.83	0.616	1.083
0.5	27.0	1.194	3.00	1.057	8.08	0.715	5.75
1.0	>30.0	1.613	7.41	1.575	5.66	1.322	7.33

Values are mean of three replications
Shelf life denotes number of days for the population to reach 10^7 CFU/g by aerobic plate count

Table 2. Growth characteristics of aerobic bacteria, lactic acid bacteria and aerobic psychrotrophic bacteria grown on vacuum packaged, irradiated fresh pork patties.

		Aerobic bacteria		Lactic acid bacteria		Psychrotrophic bacteria	
Dose (KGy)	Shelf life (Days)	Generation time (Days)	End of lag phase (Days)	Generation time (Days)	End of lag phase(Days)	Generation time (Days)	End of lag phase (Days)
0	16.0	0.710	2.75	0.644	3.16	0.631	1.5
0.5	27.0	1.201	5.41	1.351	5.08	1.176	2.16
1.0	>30.0	1.254	8.75	1.946	5.58	1.522	10.0

Values are mean of three replications
Shelf life denotes number of days for the population to reach 10^7 CFU/g by aerobic plate count

Table 3. Growth characteristics of aerobic bacteria, lactic acid bacteria and aerobic psychrotrophic bacteria grown on aerobically packaged, irradiated, temperature-abused pork patties.

		Aerobic bacteria		Lactic acid bacteria		Psychrotrophic bacteria	
Dose (KGy)	Shelf life (Days)	Generation time (Days)	End of lag phase (Days)	Generation time (Days)	End of lag phase (Days)	Generation time (Days)	End of lag phase (Days)
0	7.0	0.322	1.875	0.436	0.833	0.561	1.75
0.5	17.0	0.825	0.916	0.35	5.583	0.556	3.66
1.0	28.0	1.07	4.75	1.03	7.33	1.045	10.0
2.0	31.0	0.843	10.0	1.38	9.30	1.297	7.5

Values are mean of three replications
Shelf life denotes number of days for the population to reach 10^7 CFU/g by aerobic plate count

Table 4. Growth characteristics of aerobic bacteria, lactic acid bacteria and aerobic psychrotrophic bacteria grown on vacuum packaged, irradiated temperature-abused pork patties.

		Aerobic bacteria		Lactic acid bacteria		Psychrotrophic bacteria	
Dose (KGy)	Shelf life (Days)	Generation time (Days)	End of lag phase (Days)	Generation time (Days)	End of lag phase (Days)	Generation time (Days)	End of lag phase (Days)
0	7.0	0.475	2.25	0.416	0.875	0.53	0.0
0.5	19.0	0.88	2.16	0.912	1.75	0.883	2.33
1.0	33.0	1.011	6.41	1.273	6.58	1.06	10.0
2.0	33.0	0.817	8.93	1.256	10.0	1.29	8.91

Values are mean of three replications
Shelf life denotes number of days for the population to reach 10^7 CFU/g by aerobic plate count

Table 5. Percentage kill of aerobic bacteria, lactic acid bacteria and aerobic psychrotrophic bacteria grown on aerobically packaged, irradiated temperature-abused pork patties.

Dose (KGy)	Aerobic bacteria	Lactic acid bacteria	Psychrotrophic bacteria
0	0	0	0
0.5	21	32	21
1.0	34	37	87
2.0	47	43	70

Numbers are expressed as percentage and are calculated from values that are mean of three replications

Table 6. Percentage kill of aerobic bacteria, lactic acid bacteria and aerobic psychrotrophic bacteria grown on vacuum packaged, irradiated temperature-abused pork patties.

Dose (KGy)	Aerobci bacteria	Lactic acid bacteria	Psychrotrophic bacteria
0	0	0	0
0.5	21	26	17
1.0	35	38	83
2.0	53	60	100

Numbers are expressed as percentage and are calculated from values that are mean of three replications

Table 7. Derived coefficients, a, b, and c used in the calculation of growth characteristics of aerobic bacteria, lactic acid bacteria and aerobic psychrotrphic bacteria grown on aerobically packaged, irradiated fresh pork patties.

Dose	Aerobic Plate Count			Lacitcs Count			Psychrotrophic Count		
(KGy)	a	b	c	a	b	c	a	b	c
0	4.47	0.386	4.09	3.40	0.538	2.98	4.58	0.33	4.10
0.5	2.66	0.339	4.37	2.40	0.281	3.39	3.39	0.344	3.79
1.0	2.90	0.221	3.16	2.10	0.276	3.60	3.21	0.248	3.09

Values are mean of three replication
a = start of log phase; b = slope; c = difference between the highest point and lowest point of the slope.

Table 8. Derived coefficients, a, b, and c used in the calculation of growth characteristics of aerobic bacteria, lactic acid bacteria and aerobic psychrotrphic bacteria grown on vacuum packaged, irradiated fresh pork patties.

Dose	Aerobic Plate Count			Lacitcs Count			Psychrotrophic Count		
(KGy)	a	b	c	a	b	c	a	b	c
0	4.35	0.367	3.08	3.63	0.327	3.76	3.94	0.328	3.72
0.5	3.3	0.403	3.23	2.38	0.252	3.24	3.68	0.370	3.29
1.0	2.71	0.306	3.3	2.00	0.216	3.59	2.16	0.228	3.64

Values are mean of three replication
a = start of log phase; b = slope; c = difference between the highest point and lowest point of the slope.

Table 9. Derived coefficients, a, b, and c used in the calculation of growth characteristics of aerobic bacteria, lactic acid bacteria and aerobic psychrotrphic bacteria grown on aerobically packaged, irradiated abused pork patties.

Dose	Aerobic Plate Count			Lacitcs Count			Psychrotrophic Count		
(KGy)	a	b	c	a	b	c	a	b	c
0	3.05	0.453	4.16	2.71	0.338	3.27	2.46	0.407	4.5
0.5	2.0	0.247	5.51	1.82	0.345	4.52	1.65	0.209	5.81
1.0	2.00	0.170	6.15	2.00	0.239	5.38	1.83	0.224	6.32
2.0	1.20	0.209	5.58	1.00	0.178	5.53	2.11	0.247	5.44

Values are mean of three replication
a = start of log phase; b = slope; c = difference between the highest point and lowest point of the slope.

Table 10. Derived coefficients, a, b, and c used in the calculation of growth characteristics of aerobic bacteria, lactic acid bacteria and aerobic psychrotrphic bacteria grown on vacuum packaged, irradiated abused pork patties.

Dose	Aerobic Plate Count			Lacitcs Count			Psychrotrophic Count		
(KGy)	a	b	c	a	b	c	a	b	c
0	3.924	0.355	4.24	2.20	0.407	4.70	2.97	0.377	5.14
0.5	2.85	0.305	4.25	2.49	0.32	3.84	1.65	0.221	5.78
1.0	1.22	0.287	6.31	1.81	0.25	4.77	1.00	0.247	5.67
2.0	1.00	0.299	6.43	1.00	0.234	4.92	1.20	0.305	4.97

Values are mean of three replication
a = start of log phase; b = slope; c = difference between the highest point and lowest point of the slope.

CHAPTER 3. INFLUENCE OF pH ON CELLULAR FATTY ACIDS AND CELL SURFACE CHARGE OF *Salmonella typhimurium* AND ITS ABILITY TO ATTACH TO BEEF TISSUE

A paper to be submitted to the journal of Applied and Environmental Microbiology

Raveendran Venugopal and James Dickson

3.1 Abstract

Salmonella typhimurium ATCC 14028 was grown at 37°C in nutrient broth, tryptic soy broth and tryptic soy broth supplemented with 0.5% (w/v) yeast extract and 0.1% (w/v) pyruvate at pH 5.0, 6.0, 7.0, 8.0, and 9.0. The effects of media and pH on cellular fatty acid composition, cell surface charge and ability of the bacteria to attach to beef tissue were investigated. Media-dependent responses of *S. typhimurium*, revealed a more diverse fatty acid profile when grown in nutrient broth compared with the other two media. The influence of pH was more pronounced when the organism was grown in tryptic soy broth with the supplement. Qualitative differences were observed in the presence of C 18:1 and C 18:2 with lower pH, whereas C 16:0 was the major fatty acid produced.

The net negative charge was maximum when the cells were grown at alkaline pH and in general, acid or alkaline shocked cells had a higher net negative charge. The ability of the cells to attach to beef tissue appears to be reduced with decreasing pH. Scanning- and transmission- electron microscopy revealed morphological changes in viable cells grown at pH 5.0 and 9.0

3.2 Introduction

Salmonella spp. are major food borne pathogens that are a constant threat to the food industry. In the control of Salmonella, along with other food borne pathogens many techniques such as modified atmosphere packaging, organic acid sprays, irradiation etc., are being tried. Among organic acids, acetic acid and lactic acid have been used to reduce

contamination on the surface of raw meat. The use of acetic acid in a model spray-chilling system for raw beef reduced the numbers of *Salmonella typhimurium, Listeria monocytogenes,* and *Escherichia coli* O157:H7 (Dickson, 1991). On the other hand, the antibacterial properties of high pH on *Salmonella enteritidis* on shell eggs also have been reported (Catalano and Knabel, 1994). Salmonella, being a gastrointestinal pathogen with an oral route of infection, has to pass through the highly acidic stomach. Thus, *S. typhimurium* can encounter a wide variety of environments during its life cycle. One component of the environment which will fluctuate widely is pH and this organism survives dramatic shifts in pH, both *in vitro* and *in vivo.*

The ability of food borne pathogens to adapt to acidic conditions is a concern in the food industry (Brown and Booth, 1991). There has been recent interest in how acidic external pH affects enterobacteria, because there are many sites in the environment, in food and in the animal or human body where organisms face acidity (Rowbury, 1995). Survival in acidic conditions may have clinical significance, because enteric pathogens must pass through the acidic stomach before colonizing the intestinal tract. It has been demonstrated for a number of food borne microorganisms that the exposure of microbial cells to low pH values can lead to increased tolerance or 'habituation' to acid. This is also true for other stresses that these microorganisms may encounter in foods and food processing (Kroll and Patchett 1992). Adaptive responses in S. *typhimurium* have been reported to acid (Foster and Hall, 1990), salt (Csonka, 1989), heat (Lindquist, 1986) and, hydrogen peroxide and oxygen radicals (Christman *et al.* 1985). This phenomenon has also stirred speculation that acid-habituated cells may be able to survive better in the gastric fluid and in phagocytes (Foster and Hall, 1990). Likewise, alkali habituation in *Escherichia coli* has been shown to induce increased resistance to normally lethal alkalinity, ultra.violet. light and thermal stress (Goodson and Rowbury, 1990; Humphrey *et al.* 1991), while sensitivity towards acid may increase (Rowbury *et al.* 1993).

Much of the work in this area of acid habituation has been carried out in external acid-regulated genes in *S. typhimurium* and *E. coli* (Foster and Hall, 1990). Although much physiological work has been carried out on non food-poisoning microorganisms, relatively

little work has been carried out on the food-poisoning microorganisms themselves (KnØchel and Gould, 1995). Most of the organic acids target the cell membrane for their antimicrobial action (Richards *et al.* 1995) and act to a large extent, by interfering with homeostatic reactions that are involved in maintaining internal pH of microorganisms. As a result, changes could occur in the membrane composition to cope with the stress.

Environmental variables, such as temperature, pressure, pH, and ionic strength, are likely to influence the structure and fatty acid composition of the membranes (Suutari and Laakso, 1994). Among these environmental variables, information about phenotypic thermoadaptation of bacterial fatty acid composition is abundant in the literature (Russell and Fukunaga, 1990), while information on other environmental factors is relatively scarce. There are reports of phenotypic alterations in the amount of lipids in microbial membranes resulting from a change in growth temperature (Russell and Fukunaga, 1990). Influence of salt concentration on the fatty acid composition of *Ectothiorhodospira* spp., *Rhodobacter*, *Rhodopseudomonas*, and *Chromatium* has been reported (Imhoff and Thiemann, 1991). In *S. typhimurium*, growth in the presence of sodium benzoate and sodium deoxycholate have been reported to induce changes in membrane lipid composition and changes in the thermal sensitivity (Tomlins *et al.* 1982).

The purpose of this study was to investigate the changes brought about in the fatty acid composition of *S. typhimurium* by pH and media composition. Morphological changes were studied through scanning- and transmission-electron microscopy. Further, the influence of pH stress and media composition on the ability of the bacteria to attach to meat surfaces and the changes in their cell surface charge were also studied.

3.3 Materials and Methods

3.3.1 Bacterial Strains and Growth Conditions

Salmonella typhimurium ATCC 14028 was grown and maintained in tryptic soy broth (TSB, BBL) slants, and used throughout this study. Nutrient broth (NB, Difco), TSB and TSB supplemented with 0.5% (w/v) yeast extract (Difco) and 0.1% (w/v) sodium pyruvate (referred to as TSBY) (Sigma, St. Louis, MO) were the three media used in this study.

Media were acidified using lactic acid (Sterling Chemicals, TX) to pH 5.0 or 6.0; or adjusted to pH 8.0 or 9.0 with 3M NaOH. TSB and TSBY had an initial pH of 7.3 which was brought down to 7.0 using lactic acid. The media were adjusted to respective pH before autoclaving and the change in the pH after autoclaving was within ± 0.3 units. This change was compensated for, accordingly, prior to autoclaving. The temperature of incubation was 37°C and the culture was grown under static conditions.

For adaptation studies, *S. typhimurium* was grown in NB, TSB, and TSBY adjusted to pH 5.0, 6.0, 7.0, 8.0, or 9.0 and grown until late log phase before inoculation into the respective media and pH conditions. For shock studies, the organism was grown in NB, TSB, and TSBY adjusted to pH 7.0 until late log phase and inoculated into the respective media adjusted to pH 5.0, 6.0, 8.0 or 9.0. In all cases, the initial inoculum was diluted in phosphate buffer to give a cell population of less than 100 cells. The experiments were replicated thrice.

3.3.2 Cell Dry Weight

S. typhimurium was grown in 400 ml of NB, TSB or TSBY adjusted to pH 5.0, 6.0, 7.0, 8.0 or 9.0 until late log phase and harvested by centrifugation at 5000x*g* for 20 minutes. The cell pellet was washed twice in deionized water and dried in pre weighed aluminum foil cups at 105°C for 12 hr. The difference in weight was recorded as the dry weight of cell biomass.

3.3.3 Protein Determination

Five milliliters of cells grown to late log phase was centrifuged at 5000x*g* for 20 minutes, washed twice with deionized water and suspended in 2 ml of 0.1 N NaOH. The suspension was boiled for 15 minutes in a heating block at 90°C, and total protein was determined using Lowry's method (Lowry *et al.* 1951).

3.3.4 Generation Time and End of Lag Phase

Generation time and end of lag phase were calculated as reported by Dickson *et al.* (1992).

3.3.5 Tissue Preparation and Attachment Studies

Post rigor beef tissue was obtained as bone less trim from the Meat Lab at Iowa State University. The tissue was sliced into 0.5-cm thick slices and frozen in sealed bags. The initial population of these samples were less than 25 cells/cm^2. Prior to use, the slices were cut into 2.0 x 2.0 cm^2 (sample size 2.0 x 2.0 x 0.5 cm, surface area 12 cm^2) and tempered to room temperature.

Inocula of the control (pH 7.0) and pH stressed cultures grown in all the three media were grown to late log phase and diluted to levels of approximately 10^7 cfu.ml^{-1}. Tissue samples were inoculated by immersion for 10 or 20 or 30 minutes at room temperature (Ca 23°C), allowed to drain briefly and then transferred to 99 ml bottles of phosphate buffer. Bottles were gently inverted 25 times within 15s to remove planktonic bacteria trapped in the water film on the surface, and the tissue samples removed. Bacteria remaining on the tissue after washing were considered to be attached. Washed tissue samples were homogenized in 99-ml phosphate buffer for 2 minutes in Stomacher 400 (Tekmar Inc., Cincinnati, OH), and serially diluted as necessary in phosphate buffer and enumerated on tryptic soy agar (TSB, BBL).

3.3.6 Chromatography

Electrostatic interaction chromatography (ESIC) columns (Pederson, 1980) were prepared using polystyrene columns (Bio-Rad, Richmond, CA) packed with 1 ml of a 1:1 (w/v) mixture of Dowex chloride (1 by 8) (Sigma, St. Louis, MO; capacity 1.2 meq ml^{-1}). Columns were washed with 3 ml of deionized water and 1 ml of cell suspension was adsorbed on to the column and eluted by centrifuging the columns at 200x*g* for about 20 seconds. The populations of the initial and eluted suspensions were enumerated, and the relative negative charge was expressed as the ratio of the initial population and eluted

population. Dickson and Siragusa (1994) described the use of spin columns and validation with Peterson's method.

3.3.7 Analysis of Cellular Fatty Acids

The lipid portion of the cells was extracted and analyzed by gas-chromatography as reported by Venugopal *et al.* (1994). Briefly, the cells were grown to late log phase, harvested by centrifugation at 5000x*g* for 20 minutes and washed twice with deionized water. The wet cell biomass was saponified (15% NaOH in 1:1 aqueous methonol for 30 minutes at 90°C in a heating block and acidified with methonol-HCl (75:25 v/v) for 15 minutes at the same temperature. The hydrolysates were extracted five times with 1:1 ether:hexane (v/v); and the extract was pooled, washed free of acid with deionized water, passed through granular anhydrous sodium sulphate and finally dried completely under nitrogen. The extracted lipid was redissolved in 1 ml of hexane and 1 µl was injected in a gas chromatograph (GC) equipped with a flame-ionization detector (FID) and a capillary column, SPB-1, 30m x 0.25mm ID, 0.25µm film (non polar; Supelco, PA). The instrument used was a HP 5890 Series II gas chromatograph (Hewlett-Packard) with a HP 3396A integrator. Helium was used as the carrier gas (1.03 ml/min), while the flow rates of air and hydrogen were 400 ml/min and 43 ml/min, respectively. The split ratio was 12.5. The column was held initially for 4 minutes at 150°C, then raised to 250°C at the rate of 4°C/min, and subsequently held there for 5 minutes. The injector was maintained at 250°C and the FID at 280°C. Fatty acids were identified by comparing their retention times with those of known fatty acid methyl esters, ranging in carbon length from C 11:0 to C 22:0 (Bacterial acid Methyl Esters CP™ mix, Matrya Inc., Pleasant Gap, Pennsylvania, USA).

3.3.8 Extraction of Total Cellular Lipids

Using the dry cell biomass, the lipid fraction was extracted as described above. The extracted lipid fraction was weighed to calculate the cellular lipid content.

3.3.9 Scanning and Transmission Electron Microscopy

The bacterial cells were centrifuged at 5000x*g* for 20 minutes, washed twice with deionized water and the cell pellet was made into a thick suspension in deionized water. This suspension was filtered through polyester membranes (0.4 μm, 13 mm diameter; Poretics Corporation, CA). The filters were fixed in 4% glutaraldehyde/ 3% paraformaldehyde in 0.05M cacodylate buffer, pH 7.2, overnight at 4°C. The filters were washed 3 times in the same buffer for 10 minutes, and fixed in 1% Osmium Tetroxide (OsO4) in same buffer for 1 hour at 4°C. It was washed three times in the same buffer, (10 minutes/wash) and dehydrated in ethanol series to 100% (50%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 100%, and 100%). For scanning electron microscopy, the filters were critical point dried from 100% ethanol in CO_2, mounted on brass discs and sputter coated in a Polaron E5100 Sputter Coater with platinum/palladium target (60:40). Observations were made with a JEOL JSM-35 at 20kV.

For transmission electron microscopy, dehydrated specimens were infiltrated to 100% propylene oxide as an intermediate fluid from 100% ethanol, embedded in epoxy resin and cured for 24 hrs at 60°C. Thin sections were then cut with a diamond knife on a Reichert Ultracut E ultramicrotome and placed on copper grids. Post-stained sections with uranyl acetate and lead citrate were photographed on a JEOL 1200EX scanning transmission electron microscope (STEM) at 80kV.

3.3.10 Statistical Analysis

The estimates of bacterial populations were converted to $\log_{10}$ cfu.cm^{-2} values. Statistical analysis was conducted using the General Linear Models procedure of the Statistical Analysis System (1991). Reported means are the average of three independent replications of each experiment, with the population estimates of the initial inocula used as covariates in the analysis to normalize the results between replications. Significance is expressed at 0.05%.

3.4 Results

S. typhimurium 14028 was grown to late log phase for all the assays. The overall difference between acid-adapted and acid-shocked cells was not significant and hence the data for adapted cells alone are presented for attachment studies and fatty acid analysis.

3.4.1 pH Stress on the Growth Characteristics of *S. typhimurium* 14028

The influence of pH stress on the growth characteristics of this organism was studied through biomass production, protein content, lipid content, generation time and extension of lag phase. Both pH and media composition influenced biomass production and protein content. Results indicate that the optimum pH was 7.0 and maximum biomass production was for the cells grown in TSB with the supplements (results not presented). pH had a profound influence on the dry weight and protein content of the cells. Irrespective of the media composition, the dry weight and protein content reduced significantly when the pH of the media was shifted away from pH 7.0. Growth in TSB and TSBY, acidified to pH 5.0 resulted in a four to five-fold reduction in cell biomass and the same media, at pH 9.0 also gave similar results. Media composition and pH also influenced the protein content of the cells and a similar trend was observed. The influence of pH and media composition on the microbial lipid content was studied by isolating the lipid fraction of the cells and calculating the percentage on a dry weight basis. At pH 7.0, the influence of media composition is minimum and at unfavorable pH conditions, the cells appear to increase their lipid content especially when grown in NB when compared to the other two media (Fig. 1).

The other aspect of growth characteristic studied in this work is the influence on generation time and end of lag phase (Tables 1 and 2). The generation time was shortest for cells grown in TSB but it was not significantly different from that of TSBY and NB. However, the generation time increased when the pH was shifted from 7.0 and it was significantly different from pH 5.0 and 9.0. Media composition influenced the generation time at pH 5.0 and 9.0. A similar trend was observed for acid or alkaline adapted cells with the exception that there were no significant differences between pH 7.0 and 9.0 for NB and TSB.

The end of lag phase was influenced by pH and composition of the growth media (Tables 3 and 4). Typically, the lag phase extended up to 7 to 8 h under ideal growth conditions (pH 7.0) and it increased with pH shifting to either acidic or alkaline side. Both acid or alkaline shocked and adapted cells, in all three media at pH 9.0 and 5.0 had a lag phase which were significantly different from pH 7.0. It was surprising to note that at these pHs, the lag phase was 10 hrs in TSBY which is greater when compared to the other two media. The media composition did not influence the end of lag phase when grown at pH 6.0, 7.0 and 8.0.

3.4.2 Attachment Studies

Similar attachment patterns were observed for both adapted and shocked cells, so only the results for adapted cells are presented (Tables 5-7). The influence of pH and time of incubation in the three media were investigated separately and hence the influence of media as such was not studied. The cells, when grown in NB at pH 5.0, 8.0 or 9.0, differed in their ability to attach to beef tissue. In TSB, pH 9.0 influenced the ability of cells to attach to beef tissues whereas in TSBY, pH 9.0 and 5.0, at 20 and 30 minutes, influenced cells attachment to beef tissues. Under all the three media, time was not a factor in the cells ability to attach to beef tissues because there were no significant differences between 10, 20, and 30 minutes of incubation.

3.4.3 Electrostatic Interaction Chromatography

The net negative charge on *S. typhimurium* 14028 was influenced by pH and media composition of acid or alkaline shocked and adapted cells, respectively (Tables 8 and 9). The influence of alkaline pH in NB and TBS was greater on the cell surface charge of acid or alkaline shocked cells. In TSB, pH did not influence the cell surface charge. In both, shocked and adapted conditions, the cell surface charge seems to increase with increasing pH and the changes are more pronounced when the cells where grown in NB. The influence of media composition was not seen at acidic pH, whereas at alkaline pH it was minimum.

3.4.4 Changes In Cellular Fatty Acids

The analyses of cellular fatty acids revealed a diverse profile for cells grown in NB. The fatty acid profile included C 12:0, C 14:0, C 15:0, 3-OH-C 14:0, C 16:0, C 17:0Δ, C 18:0 and C 19:0Δ among the saturated fatty acids and C 16:1, C 18:1*cis*, C 18:1*trans* and C 18:2*cis* among the unsaturated fatty acids (Table 10). Qualitative and quantitative changes were observed due to the influence of media composition and pH. C 15:0 and C 18:2*cis* were only observed in NB-grown cells. Here, the major fatty acids detected, in the order of greatest amount were C 16:0> C 16:1> C 18:1*trans*> C 17:0Δ. This order changed to C16:0> C17:0Δ> C 18:1*trans*> C 16:1 when the cells were grown in TSB and TSBY. This order of production shifted due to the influence of both pH and media composition and are presented in Figures 2-4. In the three media, the production of C 17:0Δ increased at pH 5.0. Alkaline pH had an influence on C 16:1 in TSB and TSBY because the percentage of C 16:1 was more than C 16:0. C 18:2*cis* was the only polyunsaturated fatty acid detected and it was observed in cells grown in NB.

The influence of pH also was studied through changes in the degree of saturation. The degree of saturation was studied by calculating the ratio of total unsaturated fatty acids/total saturated fatty acids (Drici-Cachon *et al.* 1996). C 18:0/C 16:0 ratio was calculated for NB alone (data not presented). This showed a separate trend for acid and alkaline pH. Similar studies were not done for the other two media because C 18:0 was absent. The degree of saturation increased with increase in pH for all acid or alkaline adapted cells when grown in NB. In TSB, there was a decrease in the degree of saturation at pH 7.0 whereas in TSBY, there was a decrease at pH 7.0 and 8.0 before showing an increase at pH 9.0. In the case of acid or alkaline shocked cells, when grown in NB and TSBY, there was a decrease in the degree of saturation at pH 9.0 (Figs. 5-7).

3.4.5 Changes In Cell Morphology

Cells grown in NB under acid shock condition at pH 5.0 showed an altered cell morphology from rods to coccal cells (Figures 8a-8c). This was not observed in the case of

acid-adapted cells grown at pH 5.0. At pH 9.0, both alkaline or acid adapted cells grown in all the three media appeared elongated and convoluted (Fig. 9).

3.5 Discussion

Acidic pH is a common stress encountered by microorganisms in a food ecosystem. In salmonella, several inducible acid tolerance response systems have been reported (Foster and Spector, 1995). Further, abrupt exposure or cells growing at neutral pH to acid conditions induce acid tolerance to extremely low pH (Foster, 1993). On the other hand, Huttanen (1975) found that *S. typhimurium* could adapt to grow at lower pH on solid media. He found that cells taken from the acidic-growth edge were more acid resistant and could grow farther into the acidic end of a fresh gradient plate. Both, acid adaptation and acid shock are known to trigger a number of physiological changes apart from inducing certain cross-protection in microbial cells. In this study, the adaptation/shock phenomenon did not influence *S. typhimurium* 14028 in its ability to attach to beef tissues. The influence on the cell surface charge and cellular fatty acids was minimum. This organism tolerated a wide range of pH from 5.0 to 9.0 and this is within the range reported in the literature (Banwart, 1989). This strain failed to grow in NB, TSB and TSBY when the pH dropped below pH 5.0. Though the media composition had a profound influence on the biomass production, it did not alter the pH range of this organism. Initially, it was thought that the presence of yeast extract and sodium pyruvate in TSBY would expand the pH range of the organism, but we failed to make any such observation. Sodium pyruvate was thought to act as a protectant in the growth media because it is usually added in the medium to recover injured cells (Leyer and Johnson, 1992).

The biomass production and protein content was reduced significantly at acidic and alkaline pH. It is obvious that the external pH affects the cell homeostasis by interfering with the internal pH and finally inhibiting the macromolecular synthesis of the cell (Cherrington *et al.* 1990). All these changes could result in reduced ATP synthesis ultimately reducing the cell yield. Reduced biomass could also suggest that the organism could adopt a fermentative mode of survival at unfavorable growth conditions. It is interesting to note the increased

lipid content of the cells when grown in NB and this strongly suggests the influence of pH and nutrient availability on the lipid content of cells and that cells, under pH stress undergo changes in their lipid profile. A 8-30% increase in total phospholipid content of four Vibrio spp. was reported when the growth temperature was reduced from 15° to 0°C (Bhakoo and Herbert, 1979). In many organisms, the cell envelope is believed to contain all of the cellular lipids (both free and conjugated) wheras free (readily extractable) lipid typically amounts for 5-10% of the dry cell mass (Wilkinson, 1988). Our results exceed this range and this could be due to different growth conditions and extraction procedure.

The influence of pH and media composition on generation time was minimal. This is because the generation time was calculated from the steepest part of the growth curve and thus the organism, after entering log phase grows at the same rate irrespective of growth conditions. The data on the end of lag phase suggest that the cells require a longer period for adaptation in order to grow at suboptimal pH conditions. Gibson *et al.* (1988) reported a lag time of 2.8 h for salmonellae in media with 2.75% (w/v) NaCl at a pH of 6.12 and 44.6 hours at a pH of 5.63 and a salt concentration of 1.30% (w/v). The media composition or the adaptation to acid or alkaline conditions does not seem to influence the end of lag phase. Thus, it is important to prevent the cells from entering log phase in order to prevent their spoilage and pathogenicity in foods where acidity is used as a preventive measure. With our information, comparison cannot be made about the survival of this enteric pathogen in the stomach, because of the difference in the pH range studied. However, the cells were viable and growing at these pH values. The initial inoculum in this study was less than 100 cells in all the treatment and this could have significantly influenced the generation time and end of lag phase.

Although the phenomenon of acid shock and adaptation of cells did not influence the ability of the bacteria to attach to beef tissues, different pH treatments, individually, when compared to neutral pH, had an influence on the ability of the bacteria to attach to beef tissues. This indicates that growth pH does have an influence on bacterial attachment and colonization of beef tissue. In other studies, *S. typhimurium* ATCC 14028 surviving starvation exhibited decreased ability to attach to beef tissue surface (Dickson and Frank,

1993). The influence of starved *S. typhimurium* to attach to eukaryotic cells (Hep 2 cells) were investigated by Galdiereo *et al.* (1994). They found that starved cells were significantly less adhesive than the control cells. In our study, the incubation time did not seem to influence the rate of attachment. This could be either due to inadequate incubation time or due to growth conditions. There was no evidence in this study that cells adapted to pH stress showed increased ability to attach to beef tissue. In the case of fresh meat, it is essential for salmonella to attach irreversibly in order to persist through decontamination and processing. It appears that environmental stress could increase the sensitivity of the cells to decontamination steps and reduce their chance of survival in a food chain.

Foster and Hall (1990) have reported that pH could cause changes in cell membranes in order to adapt to growth conditions. Similarly, starvation stress can lead to either increases or decreases in exopolysaccharide production (Lappin-Scott and Costerton, 1990) which could affect surface charge and cell attachment. Hence, it was thought that pH stress could influence the net negative charge on bacterial cell walls. The influence of pH and media were greater at alkaline pH and in general, acid or alkaline shocked cells had a higher net negative charge. The cell surface charge of the cells is important because it has been correlated to their ability to attach to beef tissues (Dickson and Koomaraie, 1989). Galdiereo *et al.* (1994) have reported that starvation reduces the surface hydrophobicity of *S. typhimurium* and become more susceptible to phagocytosis by peritoneal macrophages. They have further hypothesized that the structural alterations observed reveal hydrophilic components specific for the appropriate receptors on the surface of the macrophages.

The diversity seen in the fatty acid profile of cells grown in NB is an interesting outcome of this work. This indicates that though the composition of the media influences the fatty acid profile, its complexity is not necessarily correlated with the changes. The fatty acid profile obtained were in agreement with those compiled and reported by Wilkinson (1988). Additionally, under our growth conditions at pH 7.0, we have reported the presence of a hydroxy fatty acid, 3-OH 14:0, C 15:0 and C 18:2*cis* in NB and both C 18:1*trans* and C 18:1*cis* in all the three media. Though C 18:1 has already been reported, it is not known whether it is *cis* or *trans*. BØe and Gjerde (1980) have reported small quantities of C 13:0,

anteiso C 15:0, anteiso C 17:0, and C 20:0 in *S. typhimurium* FSK1 grown on plate count agar and extracted by a different method. Thus, we can conclude that our procedure of extraction, though different, gave good results.

S. typhimurium clearly displays a variety of changes in lipid composition as revealed by changes in fatty acid profile in response to alterations in pH and composition of media. Growth temperature is the environmental factor which has been most extensively studied for its effect on microbial lipid composition (Rose, 1989). Despite the well recognized functions of proteins, the properties of eubacterial membranes to a great part depend on their lipid composition, on the properties of their head groups and on their fatty acids, which form the hydrophobic membrane continuum of the lipid bilayer (Imhoff and Thiemann, 1991). Media composition and pH are important environmental factors that influence the growth and proliferation of food borne pathogens.

The degree of saturation increased with an increase in the pH in all three media. This is the result of an increase in the total unsaturated fatty acid content with an increase in pH of the media. The chain length of the fatty acids were not affected by the pH or the media composition. Considering pH 7.0 as the optimum, there was a separate trend in the degree of saturation for the acidic and alkaline pH. It is possible that pH has an important role in bringing about these changes in the composition of lipid profile. The common explanation for this action of weak acids is that they cross the membrane as undissociated molecules and dissociate inside the cells or vesicles, thus eliminating the transport-driving pH difference between the two sides of the membrane (Richards *et al.* 1995). Functionally, this correlation could indicate that the changes in the degree of saturation also is an important property in determining the membrane physiology of the bacteria concerned, particularly the membrane fluidity. The degree of fluidity of the membrane continuum primarily depends on the fatty acid composition (Inmhoff and Thiemann, 1991). Membrane fluidity is also important for lateral movement of membrane components essential for many important membrane functions (Cronan and Gelmann, 1975).

The influence of temperature on the membrane is well known. The chief response to a lowering of growth temperature is a raise in unsaturation of lipids, probably because of the

energetic considerations involved in relation to the amount of alteration in membrane fluidity (Russell, 1984). The outcome of our study substantiates the fact that changes in the level of saturation is correlated to the membrane fluidity. However, bacteria could also have different mechanisms to adapt to different stress conditions. Imhoff and Thiemann (1991) have reported minimum values of C 16:0 and saturated fatty acids at optimum salt concentration in *Ectothiorhodospira*. In this study, these values were found at pH 9.0.

We also studied the profile in terms of changes in the cyclopropane fatty acids (C 17:0Δ and C 19:0Δ). It has been reported that cyclopropane fatty acid formation in phospholipids appears to reflect changes in the growth conditions and control of membrane functions (Kito *et al.* 1973). The overall negative correlation between the total unsaturated fatty acids and cyclopropane fatty acids found in our study could be due to the fact that cyclopropane fatty acids are derived from mono-unsaturated fatty acid precursors by the addition of a methyl group across the double bond (Russell and Fakunaga, 1990). As for the actual function of cyclopropane fatty acids, it has been reported to behave like *trans*-unsaturated fatty acids in terms of chain packing (Cullen and Phillips, 1971). The results of our study do not support this because there was a negative correlation to the level of unsaturated fatty acids.

Acidity and alkalinity both had a different influence on the cell morphology. Acid shocked cells (pH 5.0) had the appearance of coccoid cells and were similar to starved non-viable salmonella cells (Galdiereo *et al.* 1994). It is thought that this change in morphology could be a survival mechanism. In starved cells, a decrease in size provides the cells with an increased surface to volume ratio and therefore a more efficient substrate scavanging capacity (Gonzalez *et al.* 1990). It is possible that a similar situation occurred in these cells because this change was observed only in the case of NB-grown cells. They have also reported that small cells (starved cells) have better chances to escape predation. The elongated cell morphology observed in both alkaline adapted and alkaline shocked cells is thought to be due to inhibition in cell division. It has already been reported that organic acids cause severe inhibition of DNA synthesis and cell division (Cherrington *et al.* 1990). Incubation of *E. coli* and *S. enteritidis* in the presence of formic and propionic acids resulted in larger cells,

possibly as a result of DNA inhibition, with no apparent damage to cell membrane (Thompson and Hinton, 1996).

In summary, *S. typhimurium* grows well over a wide range of pH. The difference between the acid or alkaline shocked cells versus the acid or alkaline adapted cells is minimum. Our results showing the changes in the lipid content needs further study. The changes in the cell surface charge and the ability of the organism to attach to meat tissues needs further correlation and the outcome will have practical utility. The elaborate changes in the fatty acid profile is sufficient information warranting further study in the phospholipid content and composition of this organism. This will help us in understanding the mechanism of survival of this organism under stress conditions. The changes in the cell morphology is another interesting outcome of this study. The changes in cell wall characteristics and in cell morphology have given us sufficient reason to speculate changes on the cell surface and possibly some serological changes.

3.6 References

1. Banwart, G.J. 1989. Factors that affect microbial growth in food, p. 101-163. *In* G.J. Banwart, Basic Food Microbiology (2nd ed.). Chapman and Hall, New York.

2. Block, M.C., E.C.M. Van Der Neut-Kok, L.M. Van Deenen, and J. De Gier. 1975. The effect of chain length and lipid phase transitions on the selective permeability properties of liposomes. Biochim. Biophys. Acta. 406: 187-196.

3. BØe, B., and J. Gjerde. 1980. Fatty acid patterns in the classification of some representatives of the families *Enterobacteriaceae* and *Vibrionaceae*. J. Gen. Microbiol. 116: 41-49.

4. Brown, M.H., and I.R. Booth. 1991. Acidulants and low pH, p. 22-43. *In* N.J. Russell and G.W. Gould (ed.), Food Preservative. Blackie, Glasgow, Scotland.

5. Catalano, C.R., and S.J. Knabel. 1994. Destruction of *Salmonella enteritidis* by high pH and rapid chilling during simulated commercial egg processing. J. Food Prot. 57: 592-595, 603.

6. Cherrington, C.A., M. Hinton,and I. Chopra. 1990. Effect of short-chain organic acids on macromolecular synsthesis in *Escherichia coli*. J. Appl. Bacteriol. 68: 69-74.

7. Christman, M., R. Morgan, F. Jacobson,a nd B. Ames. 1985. Positive control of a regulon for defense against oxidative stress and some heat shock proteins in *Salmonella typhimurium*. Cell 41: 753-762.

8. Cronan, J.E. Jr., E.P. Gelman. 1975. Physical properties of membrane lipids: Biological relevance and regulation. Bacteriol. Rev. 39: 232-256.

9. Csonka, L.N. 1989. Physiological and genetic response of bacteria to osmotic stress. Microbiol. Rev. 53: 121-147.

10. Dickson, J.S., G.R. Siragusa, and J.E. Wray, JR. 1992. Predicting the growth of *Salmonella typhimurium* on Beef by using the temperature function integration technique. Appl. Environ. Microbiol. 58: 3482-3487.

11. Dickson, J.S. 1991. Control of *Salmonella typhimurium, Listeria monocytogenes* and *Escherichia coli* O157:H7 on beef in a model spray chilling system. J. Food Sci. 56: 191-193.

12. Dickson, J.S., and J.F. Frank. 1993. Bacterial starvation stress and contamination of beef. Food Microbiol. 10: 215-222.

13. Dickson, J.S., and M. Koomaraie. 1989. Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. Appl. Environ. Microbiol. 55: 832-836.

14. Drici-Cachon, Z., J.F. Cavin, and C. Divies. 1996. Effect of pH and age of culture on cellular fatty acid composition of *Leuconostoc oenos*. Lett. Appl. Microbiol. 22: 331-334.

15. Foster, J.W. 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. 175: 1981-1987.

16. Foster, J.W., and H.K. Hall. 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. 172: 771-778.

17. Foster, J.W., and M.P. Spector. 1995. How *Salmonella* survive against the odds. Ann. Rev. Microbiol. 49: 145-174.

18. Galdiereo, E., G. Donnarumma, L. de Martino, A. Marcatili,G. Cipollaro de l' Ero., and A. Merone. 1994. Effect of low-nutrient sea water on morphology, chemical composition, and virulence of *Salmonella typhimurium*. Arch. Microbiol. 162: 41-47.

19. Giannella, R.A., S.A. Broitman, and N. Zamcheck. 1972. Gastric acid barrier to ingested microorganisms in man: studies in vivo and in vitro. Gut 13: 251-256.

20. Gibson, A.M., N. Bratchell, and T.A. Roberts. 1988. Predicting microbial growth: growth responses of salmonellae in a laboratory medium as affected by pH, sodium chloride and storage temperature. Int. J. Food Microbiol. 6: 155-178.

21. Gonzalez, J.M., E.B. Sherr, and B.F. Sherr. 1990. Size-selective grazing on bacteria by natural assemblages of estuarine flagellales and ciliates. Appl. Environ. Microbiol. 56: 583-589.

22. Goodson, M., and R.J. Rowbury. 1990. Habituation to alkali and increased U.V. - resistance in DNA repair-proficient and -deficient strains of *Escherichia coli*. Lett. Appl. Microbiol. 12: 258-260.

23. Herbert, R.A. and M. Bhakoo. 1979. Microbial growth at low temperatures, p. 1-16. *In* A.D. Russell and R.Fuller (ed.), Cold Tolerant Microbes in Spoilage and the Environment. Academic Press, New York.

24. Humphrey, T.J., N.P. Richardson, A.H. Gawler,a nd M.J. Allan. 1991. Heat resistance of *Salmonella enteritidis* PT4: the influence of prior exposure to alkaline conditions. Lett. Appl. Microbiol. 12: 258-260.

25. Imhoff, J.F., and B. Thiemann. 1991. Influence of salt concentration and temperature on fatty acid compositions of *Ectothiorhodospira* and other halophilic phototrophic purple bacteria. Arch. Microbiol. 156: 370-375.

26. Kito, M., M. Ishinga, S. Aibara, M. Kato and T. Hata. 1973. Relation between cyclopropane fatty acid formation and saturation of phospholipid species in *Escherichia coli* K-12. Agr. Biol. Chem. 37: 2647-2651.

27. Kn∅chel, S., and G. Gould. 1995. Preservation microbiology and safety: Quo vadis? Trends in Food Sci. Technol. 6: 127-131.

28. Lappin-Scott, M.H., and J.W. Costerton. 1990. Starvation and penetration of bacteria in soils and rocks. Experientia 46: 807-812.

29. Leyer, G.J., and E.A. Johnson. 1992. Acid adaptation promotes survival of *Salmonella* spp. in cheese. Appl. Environ. Microbiol. 58: 2075-2080.

30. Lindquist, S. 1986. The heat shock response. Ann. Rev. Biochem. 55: 1151-1191.

31. Lowry, O.H., N.J. Rosebrough. A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

32. Pederson, K. 1980. Electrostatic interaction chromatography, a method for assaying the relative surface charges on bacteria. FEMS Microbiol. Lett. 12: 365-367.

33. Richards, R.M.E., D.K.L. Xing, and T.P. King. 1995. Activity of p-aminobenzoic acid compared with other organic acids against selected bacteria. J. Appl. Bacteriol. 78: 209-215.

34. Rose, A.H. 1989. Influence of the environment on microbial lipid composition, p. 255-278. *In* C. Ratledge and S.G. Wilkinson (ed.), Microbial Lipids, vol. 2. Academic Press, New York.

35. Rowbury, R.J. 1995. An assessment of environmental factors influencing acid tolerance and sensitivity in *Escherichia coli*, *Salmonella* spp. and other enterobacteria. Lett. Appl. Microbiol. 20: 333-337.

36. Rowbury, R.J., M. Goodson, and T.J. Humphrey. 1993. Acid sensitivity induction (ASI) at alkaline pH in *Escherichia coli* involves two major sensitization components, induction of both being switched on by increased internal pH. Lett. Appl. Microbiol. 17: 272-275.

37. Russell, N.J. 1984. Mechanism of thermal adaptation in bacteria: blueprints for survival. Trends Biochem. Sci. 9: 108-112.

38. Russell, N.J., and N. Fakunaga. 1990. A comparison of thermal adaptation of membrane lipids in psychrotrphic and thermophillic bacteria. FEMS Microbiology Reviews 75: 171-182.

39. Suutari, M., and S. Laakso. 1994. Microbial fatty acids and thermal adaptation. Crit. Rev. Microbiol. 20: 285-328.

40. Thompson, J.L., and M. Hinton. 1996. Effect of short-chain fatty acids on the size of enteric bacteria. Lett. Appl. Microbiol. 22: 408-412.

41. Tomlins, R.I., T.R. Watkins, and R.J.H. Gray. 1982. Membrane lipid alterations and thermal stress in *Salmonella typhimurium* 7136. Appl. Environ. Microbiol. 44: 1110-1117.

42. Venugopal, R.J., S.C. Ingham and A.R. McCurdy. 1994. Identification of psychrotrophic *Micrococcaceae* spp. isolated from fresh beef stored under carbon dioxide or vacuum. Int. J. Food Microbiol. 23: 167-178.

43. Wilkinson, S.G. 1988. Gram-negative bacteria, p. 299-488. *In* C. Ratledge and S.G. Wilkinson (ed.), Microbial lipids, vol. 2. Academic Press, New York.

Table 1. Effect of pH stress (acid or alkaline shocked) on the generation time (hr) of *Salmonella typhimurium* grown in three different media at 37°C.

	pH				
Media	5.0	6.0	7.0	8.0	9.0
NB	$0.59^{b,1}$	$0.14^{a,4}$	$0.13^{a,5}$	$0.11^{a,6}$	$0.26^{c,7}$
TSB	$0.11^{d,2}$	$0.11^{d,4}$	$0.09^{d,5}$	$0.13^{d,6}$	$0.14^{e,8}$
TSBY	$0.17^{g,3}$	$0.11^{f,4}$	$0.11^{f,5}$	$0.13^{f,6}$	$0.45^{h,9}$

NB=Nutrient broth, TSB=Tryptic soy broth, TSBY=Tryptic soy broth supplemented with 0.5% yeast extract and 0.1% sodium pyruvate.
Values are expressed as least square mean $\log_{10}$ cfu/cm^2; average of three independent replications.
Means with different superscripts are significantly different ($P<0.05$).
Letters denote level of significance within columns.
Numbers denote level of significance within rows.

Table 2. Effect of pH stress (acid or alkaline adapted) on the generation time (hr) of *Salmonella typhimurium* grown in three different media at 37°C.

	pH				
Media	5.0	6.0	7.0	8.0	9.0
NB	$0.46^{b,1}$	$0.15^{a,4}$	$0.13^{a,5}$	$0.14^{a,6}$	$0.23^{a,8}$
TSB	$0.53^{d,2}$	$0.13^{e,4}$	$0.09^{c,5}$	$0.10^{c,6}$	$0.14^{c,9}$
TSBY	$0.14^{g,3}$	$0.11^{f,4}$	$0.11^{f,5}$	$0.08^{f,7}$	$0.21^{h,8}$

NB=Nutrient broth, TSB=Tryptic soy broth, TSBY=Tryptic soy broth supplemented with 0.5% yeast extract and 0.1% sodium pyruvate.
Values are expressed as least square mean $\log_{10}$ cfu/cm^2; average of three independent replications.
Means with different superscripts are significantly different ($P<0.05$).
Letters denote level of significance within columns.
Numbers denote level of significance within rows.

Table 3. Effect of pH stress (acid or alkaline shocked) on the end of lag phase (hr) of *Salmonella typhimurium* grown in three different media at 37°C.

	pH				
Media	5.0	6.0	7.0	8.0	9.0
NB	$8.50^{a,1}$	$8.0^{a,3}$	$8.0^{a,4}$	$7.58^{a,5}$	$9.25^{b,6}$
TSB	$8.33^{c,1}$	$7.83^{c,3}$	$7.58^{c,4}$	$7.91^{c,5}$	$7.41^{c,7}$
TSBY	$10.0^{e,2}$	$7.66^{d,3}$	$7.66^{d,4}$	$8.08^{d,5}$	$10.0^{f,6}$

NB=Nutrient broth, TSB=Tryptic soy broth, TSBY=Tryptic soy broth supplemented with 0.5% yeast extract and 0.1% sodium pyruvate.
Values are expressed as least square mean $\log_{10}$ cfu/cm^2; average of three independent replications.
Means with different superscripts are significantly different ($P<0.05$).
Letters denote level of significance within columns.
Numbers denote level of significance within rows.

Table 4. Effect of pH stress (acid or alkaline adapted) on the end of lag phase (hr) of *Salmonella typhimurium* grown in three different media at 37°C.

	pH				
Media	5.0	6.0	7.0	8.0	9.0
NB	$10.0^{b,1}$	$8.16^{a,3}$	$8.0^{a,4}$	$8.16^{a,5}$	$9.41^{c,6}$
TSB	$9.00^{e,2}$	$8.0^{d,3}$	$7.58^{d,4}$	$7.66^{d,5}$	$8.00^{d,7}$
TSBY	$8.66^{g,2}$	$7.75^{f,3}$	$7.66^{f,4}$	$7.58^{f,5}$	$10.0^{h,6}$

NB=Nutrient broth, TSB=Tryptic soy broth, TSBY=Tryptic soy broth supplemented with 0.5% yeast extract and 0.1% sodium pyruvate.
Values are expressed as least square mean $\log_{10}$ cfu/cm^2; average of three independent replications.
Means with different superscripts are significantly different ($P<0.05$).
Letters denote level of significance within columns.
Numbers denote level of significance within rows.

Table 5. Effect of pH stress (acid or alkaline shocked) on the ability of *Salmonella typhimurium* grown in Nutrient broth at 37°C to attach to beef tissue.

	pH				
Time (minutes)	5.0	6.0	7.0	8.0	9.0
10	$4.56^{a,1}$	$5.43^{b,2}$	$5.16^{b,3}$	$4.03^{c,4}$	$3.80^{d,5}$
20	$4.56^{f,1}$	$5.43^{e,2}$	$5.30^{e,3}$	$4.43^{g,4}$	$3.86^{h,5}$
30	$4.76^{j,1}$	$5.70^{i,2}$	$5.46^{i,3}$	$4.70^{k,5}$	$4.16^{l,5}$

Values are expressed as least square mean $\log_{10}$ cfu/cm^2; average of three independent replications.
Means with different superscripts are significantly different ($P<0.05$).
Letters denote level of significance within columns.
Numbers denote level of significance within rows.

Table 6. Effect of pH stress (acid or alkaline shocked) on the ability of *Salmonella typhimurium* grown in Tryptic soy broth at 37°C to attach to beef tissue.

	pH				
Time (minutes)	5.0	6.0	7.0	8.0	9.0
10	$5.70^{a,1}$	$5.30^{a,2}$	$5.70^{a,4}$	$5.40^{a,5}$	$4.10^{b,6}$
20	$5.76^{c,1}$	$5.73^{c,2}$	$5.83^{c,4}$	$5.63^{c,5}$	$4.26^{d,6}$
30	$5.80^{e,1}$	$5.93^{e,3}$	$5.96^{e,4}$	$5.60^{e,5}$	$4.16^{f,6}$

Values are expressed as least square mean $\log_{10}$ cfu/cm^2; average of three independent replications.
Means with different superscripts are significantly different ($P<0.05$).
Letters denote level of significance within columns.
Numbers denote level of significance within rows.

Table 7. Effect of pH stress (acid or alkaline shocked) on the ability of *Salmonella typhimurium* grown in Tryptic soy broth with the supplements at 37°C to attach to beef tissue.

	pH				
Time (minutes)	5.0	6.0	7.0	8.0	9.0
10	$5.70^{a,1}$	$5.60^{a,2}$	$5.80^{a,4}$	$6.03^{a,6}$	$4.96^{b,7}$
20	$5.76^{d,1}$	$5.70^{e,2}$	$6.33^{c,5}$	$6.13^{c,6}$	$4.96^{f,7}$
30	$5.80^{h,1}$	$6.13^{g,3}$	$6.40^{g,5}$	$6.13^{g,6}$	$5.26^{i,7}$

Values are expressed as least square mean $\log_{10}$ cfu/cm^2; average of three independent replications.
Means with different superscripts are significantly different ($P<0.05$).
Letters denote level of significance within columns.
Numbers denote level of significance within rows.

Table 8. Electrostatic interaction chromatography estimates of the net negative charge on *Salmonella typhimurium* cell walls as influenced by pH stress (acid or alkaline shock) in three different media at 37°C.

	pH				
Media	5.0	6.0	7.0	8.0	9.0
NB	$1.89^{a,1}$	$2.31^{a,2}$	$2.30^{a,3}$	$5.20^{b,5}$	$4.69^{c,8}$
TSB	$1.63^{d,1}$	$1.61^{d,2}$	$1.56^{d,3}$	$2.22^{d,6}$	$2.07^{d,9}$
TSBY	$1.65^{e,1}$	$1.98^{e,2}$	$1.51^{e,4}$	$3.16^{f,7}$	$2.30^{g,9}$

NB=Nutrient broth, TSB=Tryptic soy broth, TSBY=Tryptic soy broth supplemented with 0.5% yeast extract and 0.1% sodium pyruvate.
Values are expressed as least square mean $\log_{10}$ cfu/cm^2; average of three independent replications.
Means with different superscripts are significantly different ($P<0.05$).
Letters denote level of significance within columns.
Numbers denote level of significance within rows.

Table 9. Electrostatic interaction chromatography estimates of the net negative charge on *Salmonella typhimurium* cell walls as influenced by pH stress (acid or alkaline adapted) in three different media at 37°C.

	pH				
Media	5.0	6.0	7.0	8.0	9.0
NB	$2.14^{a,1}$	$2.26^{a,2}$	$2.30^{a,3}$	$3.89^{b,5}$	$10^{c,7}$
TSB	$2.17^{d,1}$	$1.82^{d,2}$	$1.56^{d,3}$	$2.78^{e,6}$	$4.18^{f,8}$
TSBY	$1.75^{g,1}$	$2.31^{h,2}$	$1.51^{g,4}$	$2.11^{g,6}$	$3.30^{i,7}$

NB=Nutrient broth, TSB=Tryptic soy broth, TSBY=Tryptic soy broth supplemented with 0.5% yeast extract and 0.1% sodium pyruvate.
Values are expressed as least square mean $\log_{10}$ cfu/cm^2; average of three independent replications.
Means with different superscripts are significantly different ($P<0.05$).
Letters denote level of significance within columns.
Numbers denote level of significance within rows.

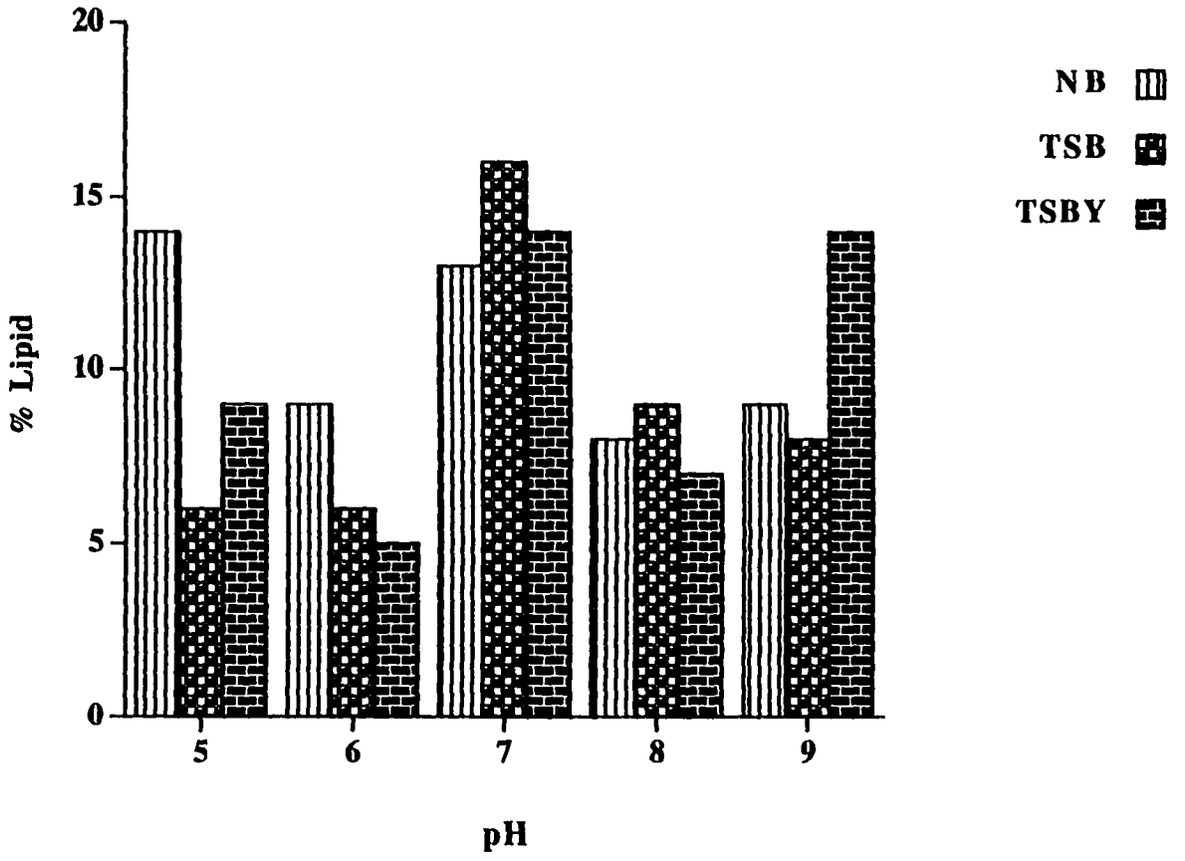

Fig. 1. Influence of pH on lipid content (% of dry weight) of acid or alkaline adapted *Salmonella typhimurium* grown in NB, TSB and TSBY at 37°C. Values are mean of three replication.

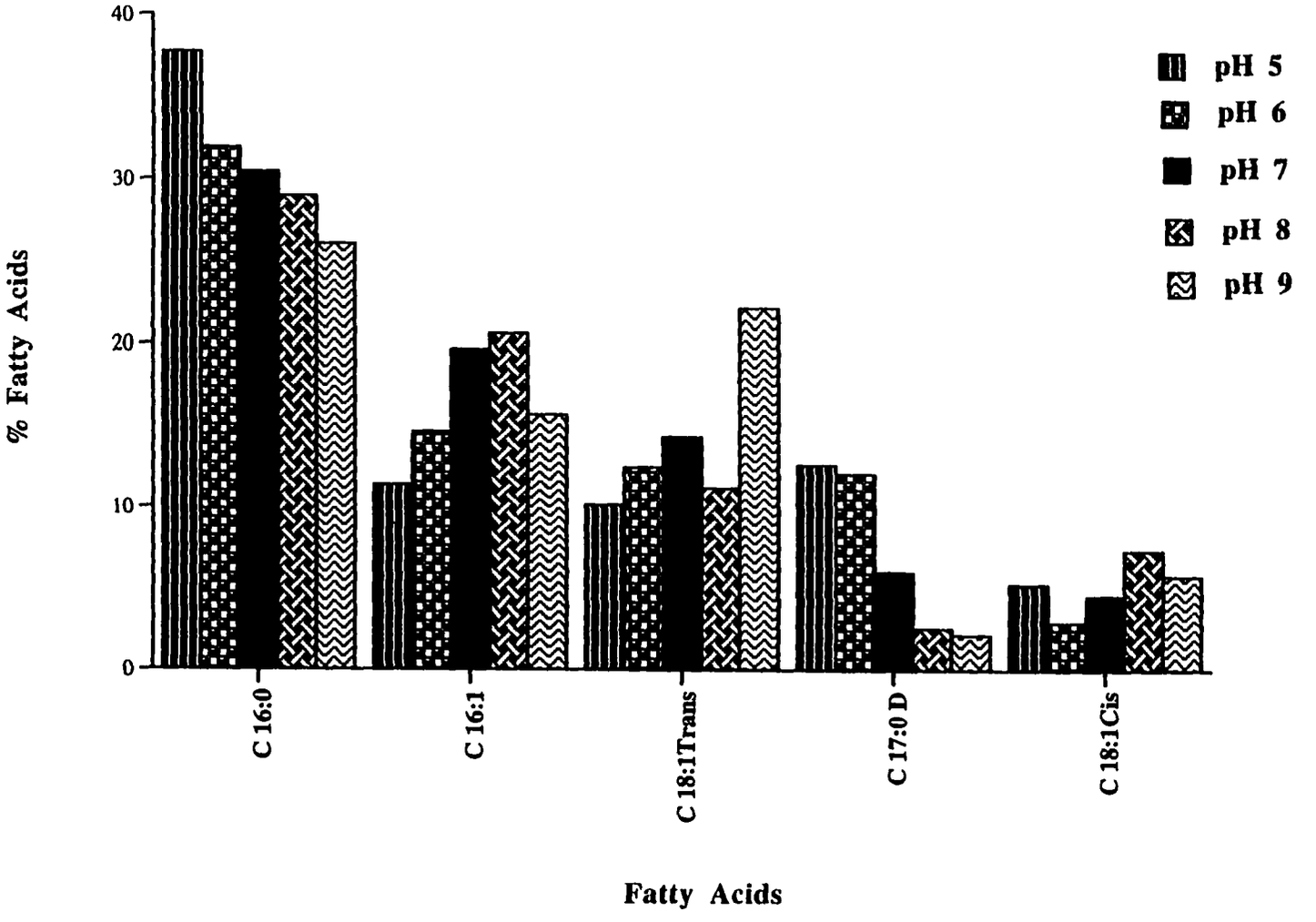

Fig. 2. Influence of pH on major fatty acids of acid or alkaline adapted *Salmonella typhimurium* grown in nutrient broth at 37°C. Values are mean of three replication.

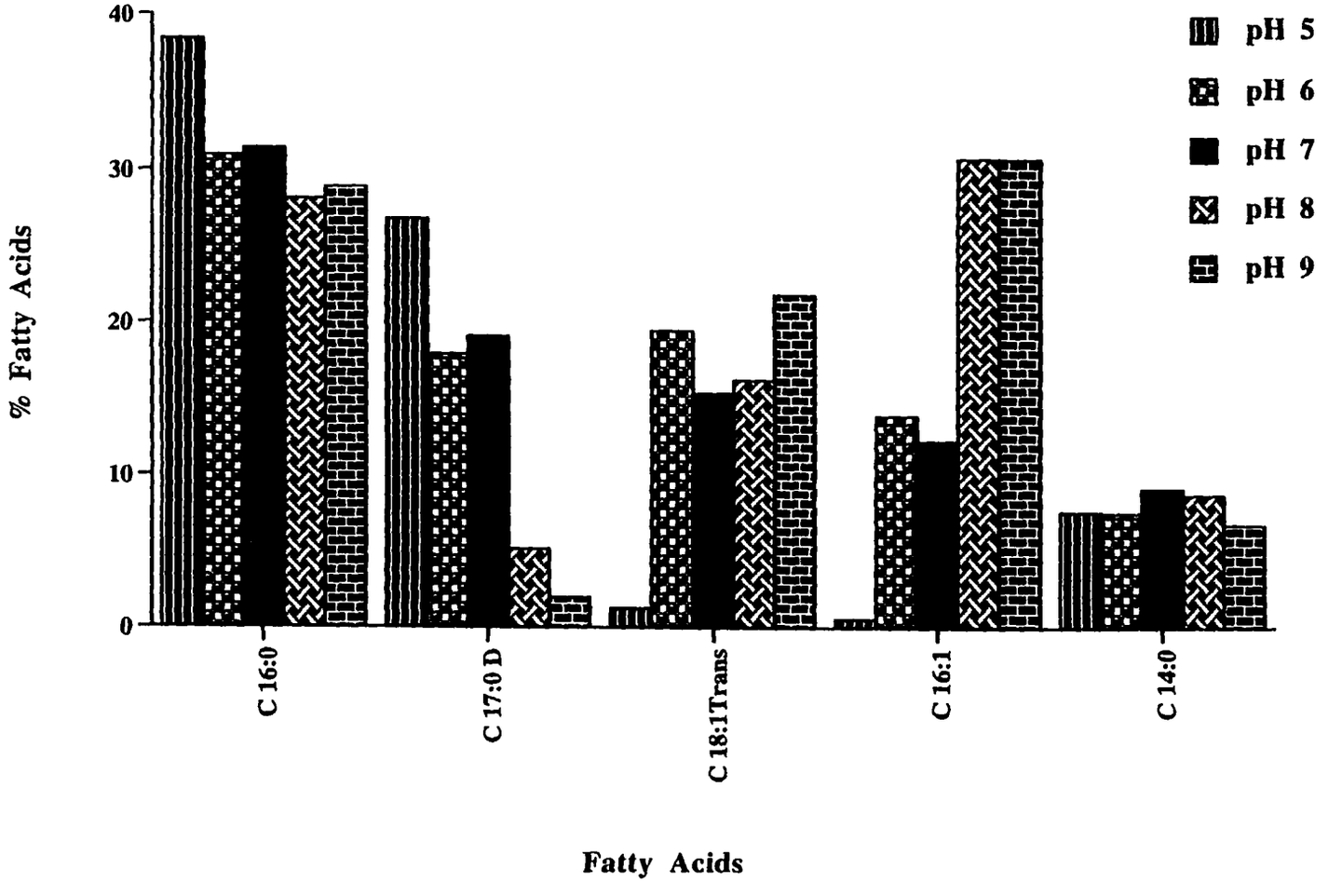

Fig. 3. Influence of pH on major fatty acids of acid or alkaline adapted *Salmonella typhimurium* grown in tryptic soy broth at 37°C. Values are mean of three replication.

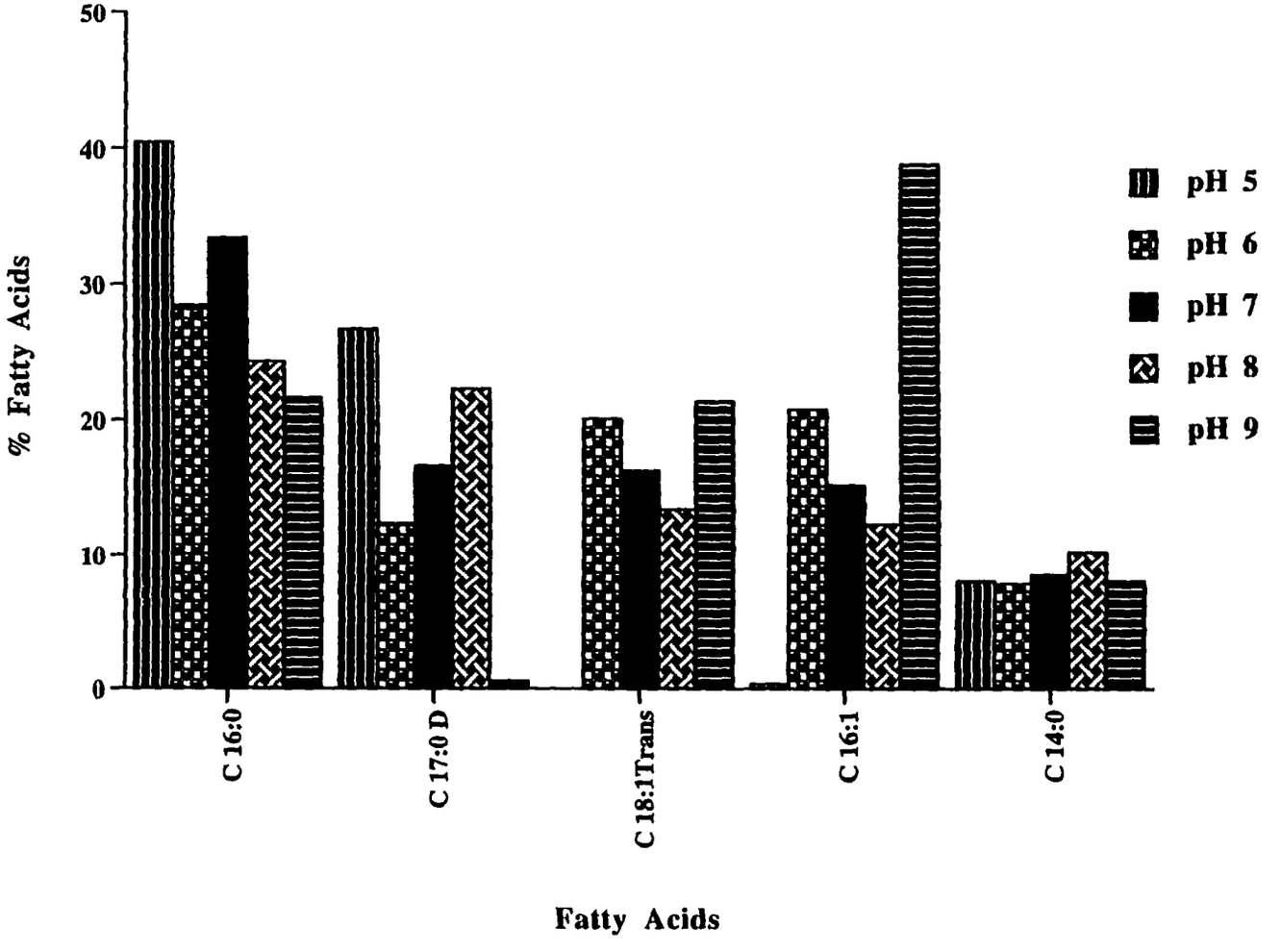

Fig. 4. Influence of pH on major fatty acids of acid or alkaline adapted *Salmonella typhimurium* grown in tryptic soy broth with the supplements at 37°C.

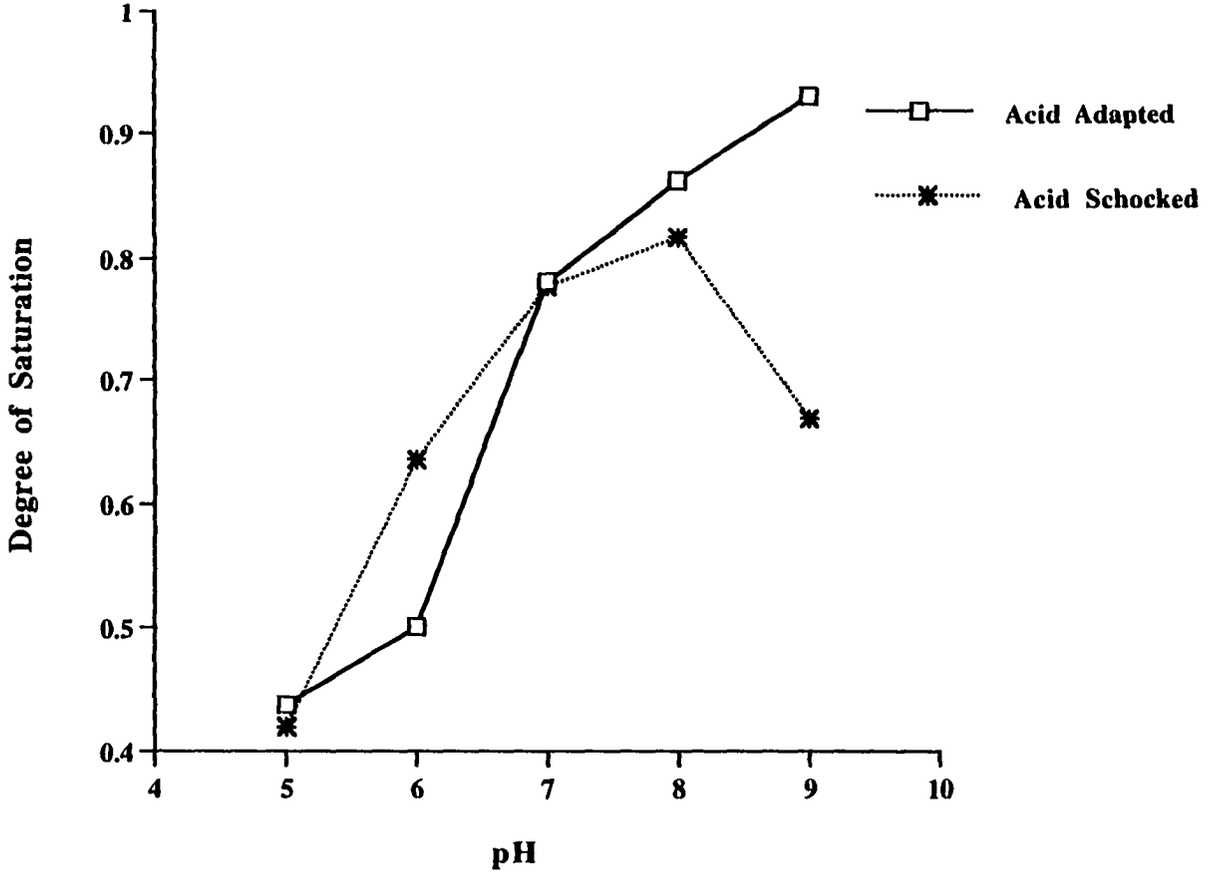

Fig. 5. Influence of pH on the degree of saturation of acid or alkaline adapted *Salmonella typhimurium* grown in Nutrient broth at 37°C. Values are mean of three replication.

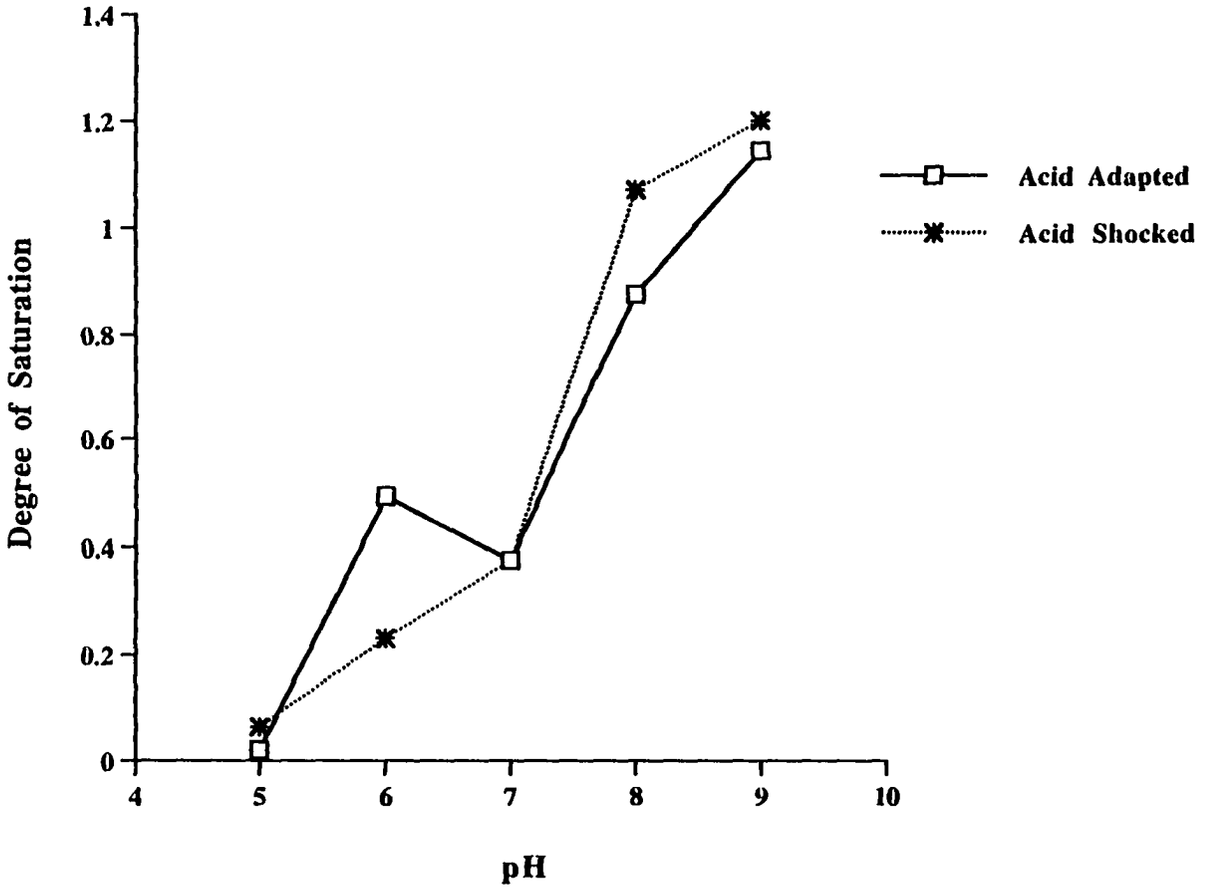

Fig. 6. Influence of pH on the degree of saturation of acid or alkaline adapted *Salmonella typhimurium* grown in Tryptic soy broth at 37°C. Values are mean of three replication.

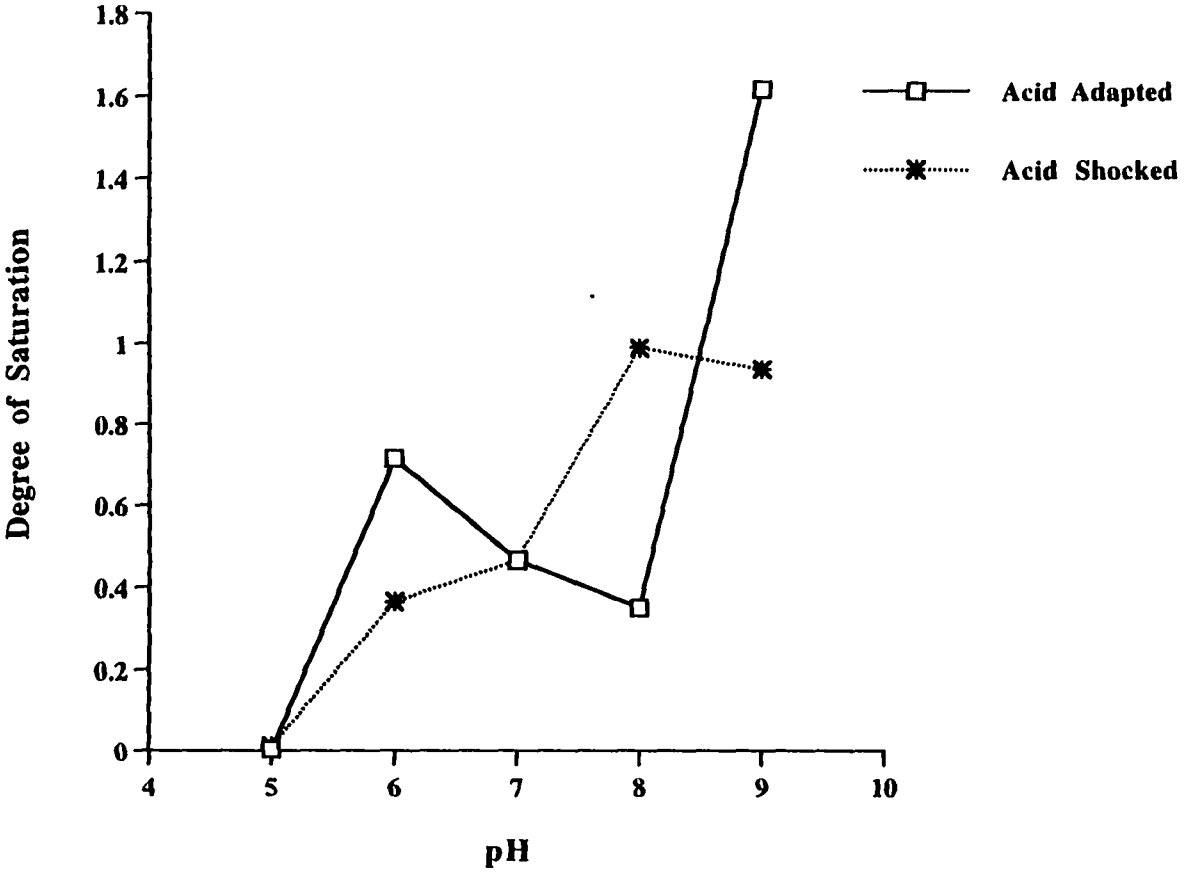

Fig. 7. Influence of pH on the degree of saturation of acid or alkaline adapted *Salmonella typhimurium* grown in Tryptic soy broth with the supplements at 37°C. Values are mean of three replication

Figure 8a. Transmission electron micrograph of *Salmonella typhimurium* grown at pH 7.0 in TSBY at 37°C. Bar indicates 500 μm.

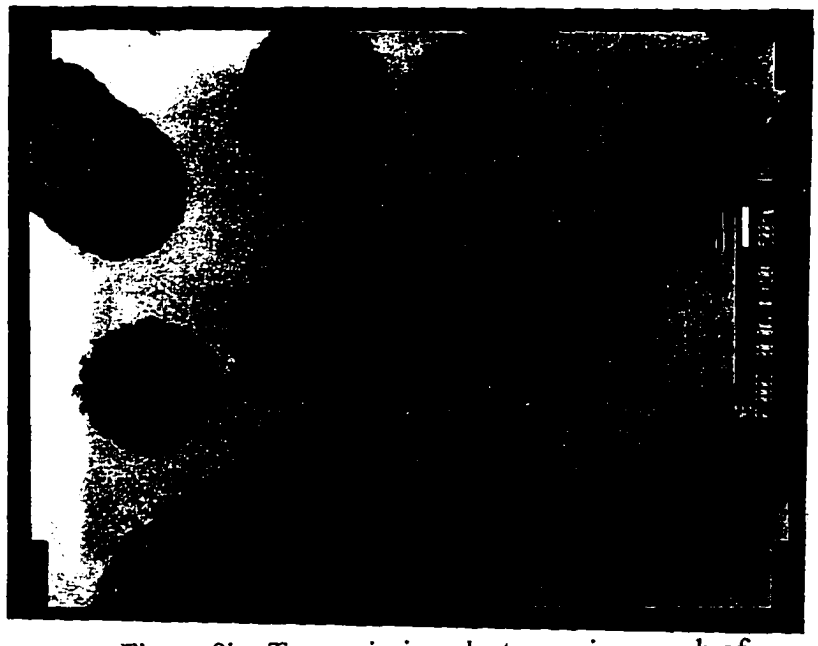

Figure 8b. Transmission electron micrograph of *Salmonella typhimurium* grown at pH 9.0 in TSBY at 37°C under alkaline shock condition. Bar indicates 500 μm.

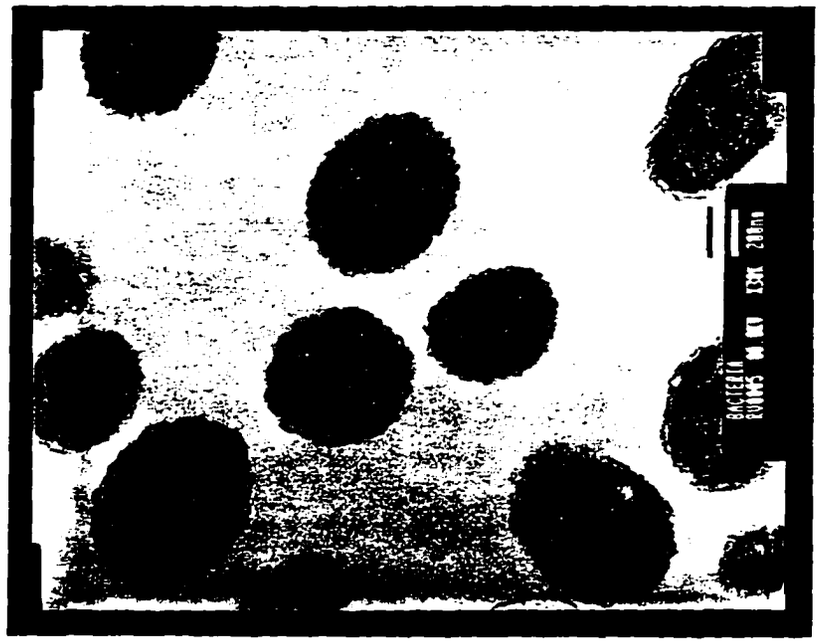

Figure 8c. Transmission electron micrograph of *Salmonella typhimurium* grown at pH 5.0 in TSBY at 37°C under acid shock condition. Bar indicates 200 μm.

Figure 9. Scanning electron micrograph of *Salmonella typhimurium* grown at pH 9.0 in TSBY at 37°C under alkaline shock condition.

CHAPTER 4. EVALUATION OF CERTAIN FLUORESCENT PROBES TO ASSESS BACTERIAL INJURY CAUSED BY HEAT, ACID, AND IRRADIATION BY USING FLOW CYTOMETRY

A paper to be submitted to the Journal of Letters in Applied Microbiology

Raveendran Venugopal, Christi Harkins and James Dickson

4.1 Abstract

Four different fluorescent stains, Rh 123, a carbocyanine dye, carboxy fluorescein diacetate and fluorescein diacetate were used as vital stains in this study. The former two are membrane potential dyes while the later two are fluoregenic esters. Propidium iodide (PI), a nucleic acid stain, was studied in combination with all the four dyes and also individually. These dyes were used to study *Escherichia coli*, *Staphylococcus aureus*, and *Listeria innocua*. The bacteria were subjected to heat, acid, and irradiation stress. Treatments were chosen in such a way to yield live, injured, and dead bacteria. The usefulness of these dyes to identify the three stages were studied using a flow cytometer (FCM). The fluorogenic esters and carbocyanine dye gave weak fluorescence that were not detected by the FCM. Rh 123 stained the cells but failed to differentiate the three stages of the bacteria used in this study. With PI, it was possible to differentiate between cells with intact membrane and those with compromised membranes.

4.2 Introduction

The use of flow cytometry (FCM) in various branches of microbiology is increasing. The attractive attributes of this technology are its speed, sensitivity, ability to make several measurements simultaneously, and its quantitative analyses of the samples. FCM is increasingly used to evaluate bacteria (Monfort and Baleux, 1992), to characterize and quantify cellular parameters such as DNA content (Boye and LØbner-Olsen, 1991), 16s RNA sequence (Wallner *et al.* 1995), antigen expression (McClelland and Pinder, 1994), β-galactosidase (Nir et al. 1990), and viability (Diaper and Edwards, 1994) in various fields of

fundamental and applied microbiology. Of particular interest to microbiology is the ability of FCM to study heterogeneity in bacterial populations.

The detection of viable bacteria is a perennial problem in microbiology (Diaper and Edwards, 1994). Conventional methods such as growth on laboratory media are time-consuming and there is now a growing body of evidence that a portion of the metabolically-active bacteria in the environment cannot be cultured (Rozak and Colwell, 1987). Alternative and more direct method to detect and enumerate viable bacteria is available through the use of FCM. Bacterial viability is measured by FCM using appropriate fluorochromes. Dyes such as ethidium bromide, acridine orange, propidium iodide and fluorescein diacetate have all been successfully used for this purpose, both in fluorescence microscopy and flow cytometry (Mason *et al.* 1995). Of these, propidium iodide and ethidium bromide are also used in determination of membrane integrity. They can enter cells mainly via damaged membranes of stressed, injured or dead cells and intercalate into DNA and RNA, whereafter fluorescence is enhanced (Ueckert *et al.* 1995).

Potentiometric dyes have also been used as indicators of bacterial cell viability, the fluorescent response of these dyes varies with the magnitude of the membrane potential (Mason *et al.* 1993). Rhodamine 123 is a membrane potential dye and its cellular uptake is mediated by a transmembrane potential which maintains a negatively charged cytoplasm (Johnson et al. 1981). A number of workers have used Rh123 to study bacterial viability (Diaper *et al.* 1992; Davey *et al.* 1993) and Mason et al. (1995) studied bacterial stress due to heat and gramicidin treatments. Rh 123 was also used in the quantitative analysis of starved cultures of *Micrococcus luteus* by flow cytometry and cell sorting (Kaprelyants *et al.* 1996).

The carbocyanine dyes are a family of membrane potential sensitive lipophilic cations. The fluorescence of cyanine dye is enhanced (about 6 fold in the case of $DiOC_6(3)$, less for other dyes) when the dye is in a hydrophobic environment (Sims et al. 1974) such as membranous structures in which it can be observed in cells. These have been used to determine membrane potential in a diversity of cells and vesicles including mouse ascites tumor cells (Hargittai *et al.* 1991), red blood cells (Sims *et al.* 1974), yeast (Pena *et al.* 1984) and bacteria (Zaritsky *et al.* 1984; Mason *et al.* 1993). Another group of liphophilic dyes are

fluorogenic esters. Fluorogenic esters such as fluorescein diacetate (FDA) are non-fluorescent derivative of fluorescein which readily diffuses through cell membranes (Diaper and Edwards, 1994). Once inside the cell it is cleaved by non-specific esterases to release fluorescein, which is polar and therefore retained inside the cells where it fluoresces brilliant green when irradiated with blue light (Chrzanowski *et al.* 1984). The ability of FDA to stain viable bacteria has been reported by several workers (Brunius, 1981; Lundgren 1981; Bercovier *et al.* 1987).

In this study, the potential of fluorescent probes to assess bacterial viability as influenced by common bacterial stress conditions is evaluated. This was combined with FCM because of the ease and precision with which use of these probes could be validated. The stress conditions were chosen in such a way to reflect the conditions experienced by bacteria in food processing.

4.3 Material and Methods

4.3.1 Bacterial Strains and Growth Conditions

The bacterial strains used in this study were *Escherichia coli* 12-1, *Staphylococcus aureus* and *Listeria innocua* ATCC 33090. *S. aureus* was obtained from the culture collection available at Department of Microbiology, Iowa State University. Growth of all cultures was in Tryptic soy broth (Difco, Detroit, MI, USA) at 37°C in a shaker at 130 rpm. For the study, overnight grown cultures were centrifuged at 8000x*g* for 10 minutes at 4°C and the cell pellet was washed twice in sterile PBS and redissolved in 1 ml of the same solution. Appropriate volume of this sample was inoculated into minimal broth to give a final concentration of 10^8 cells/ml before exposing the cells to stress conditions. The composition of minimal media used was as given by Atlas and Parks (1990) and the composition (g/l) is as follows: K_2HPO_4 - 7.0 g; KH_2PO_4 - 2.0 g; $(NH_4)_2SO_4$ - 1.0 g; glucose - 1.0 g; sodium citrate - 0.5 g; $MgSO_4.7H_2O$ - 0.1 g; pH 7.0.

4.3.2 Stress Conditions

4.3.2.1 Heat

Minimal media was preheated in a water bath at 65°C and suitable volume of the innoculum was inoculated to give a concentration of 10^8 CFU/ml. Samples were taken at 0 minutes (live), 5 minutes (intermediate) and 30 minutes (dead). The cells from all the three stages were plated on tryptic soy agar for enumeration of viable colony forming units. Also, a 1:100 dilution from minimal media was prepared in PBS for staining and subsequent FCM analyses. The temperature and time of exposure to stress was determined separately for each organism to obtain the three stages mentioned above within 30 minutes.

4.3.2.2 Acid

Minimal media were acidified with 1 ml of 85% lactic acid (Sterling Chemicals, TX) to give a final concentration of 0.5% lactic acid in the media. As mentioned above, three samples were taken at 0 minutes (live), 5 minutes (Intermediate) and 30 minutes (dead) and prepared for FCM analyses as mentioned above. For *S. aureus* alone, sampling for dead cells was done at 60 minutes. The time of exposure to stress was determined separately for each organism to obtain the three stages mentioned above within 30 minutes.

4.3.2.3 Irradiation

The bacterial suspensions (10^8 cfu/ml) in minimal broth were poured into a 50 ml flat tissue culture flask with screw caps (Costar) and filled up to the brim. Care was taken to exclude atmospheric air inside the flasks. These flasks, with the inoculated broth were irradiated using accelerated electron beams at the Linear Accelerator Facility at Iowa State University. The inoculated media were irradiated to an average absorbed dose of 0, 2, and 5 KGy. The samples were then processed for FCM analyses.

4.3.3 Fluorescent Dyes and Staining Protocol

All the fluorescent dyes used in this study were purchased from Sigma Chemicals, MO. The stains were directly added to the 1:100 dilution made in PBS. The concentration of the cells were kept constant to maintain the same dye: cells ratio for the dyes used in this study. For *E. coli* alone, the cells were suspended in 5 ml of TE buffer (10mM TRIS, 1mM EDTA, pH 8.0) before adding the stains. Rh 123 was added as described by Diaper *et al.* (1992), $DiOC_6$ by the method of Shapiro (1990), FDA by the method of Brunius (1980) and CFDA was used as described by Porter *et al.* (1995). A 13 mM solution of propidium iodide was prepared in deionized water and added to the cells to give a final concentration of 1µl/ml.

4.3.4 Flow Cytometry

Flow cytometer (Coulter EPICS XL-MCL) available in the Cell and Hybridoma Facility at Iowa State University was used in this study. The flow cytometer was equipped with a FL-1 green filter of 525 nm band pass filter (505 to 545 nm) and a FL - 4 red filter of 675 nm band pass filter (660 to 700 nm). All the samples were counted up to 2000 events. Data were interpreted based on quadrant analyses. The first quadrant, C1 is red positive, detecting the fluorescence of PI, the second quadrant, C2 is both red and green positive, detecting the fluorescence of PI and all the other stains. The third quadrant, C3 is both red and green negative and the fourth quadrant, C4 is green fluorescent, detecting the fluorescence of all the stains except PI. Cells staining with the former four stains will show up in the fourth quadrant of the histogram and the cells staining with PI will show in the first quadrant of the histogram.

4.4 Results

The population of live, intermediate, and dead cells was enumerated on TSA by aerobic plating. Live cells had a population of 10^7 cells/ml, intermediate cells had a population of 10^2 cells/ml and for the dead cells, it was below our detection limits (100 cells and more). The fluorescence of Rh 123, DiOC6, CFDA, and FDA were measured as log of

green fluorescence in the fourth quadrant whereas the fluorescence of PI was measured as log of red fluorescence in the first quadrant. Among the stains used, bacteria were successfully stained with Rh 123 and PI. Fluorescence was weak with the other three stains and hence the results were not presented here. Uptake of Rh 123 by live *E. coli*, *S. aureus*, and *L. innocua* are presented in Figs. 1b, 1c, and 1d. The staining pattern of heat treated *E. coli* is presented in Table 1. No fluorescence was measured in unstained cells as presented in Fig. 1a. The cells, when stained with Rh 123, showed maximum fluorescence in the fourth quadrant (green-positive) and when combined with PI, red-positive (quadrant 1) and red-positive, green-positive (quadrant 2) shared the observed fluorescence. No red fluorescence was observed with live cells and PI uptake increased with progressing death of cells. In all the three stages, Rh 123 fluorescence were observed. Table 2 (Fig. 3a) presents the data obtained with acid-killed *E. coli* and a similar trend was observed. Cells, dead by plate count observations, showed green fluorescence. Rh 123 stained cells showed 58.5% positive for live cells, 80.4% positive for intermediate (Fig. 2a) cells and 86.3% for dead cells. When combined with PI, the percentage of cells showing green fluorescence dropped to 35.9, 23.1 and 4.1%, respectively. Live cells did not stain with PI (Fig. 2d) while there was PI uptake with the dead cells; intermediate cells had a poor uptake of PI.

The results obtained with *S. aureus* are presented in Tables 3 & 4 (Fig. 2b, 3b and 3d) for heat and acid treatments, respectively. In the acid treated cells, 98.9% showed green fluorescence with Rh 123 and this percentage dropped 81.4% for intermediate cells and to 53.9% for dead cells. In the case of heat treated cells, there were no differences. Again, when combined with PI, Rh 123 lost its fluorescence significantly from 79.8 to 2.7% for intermediate stage and 17.7% for dead stage cells. With *L. innocua*, essentially the same trend observed with *E. coli* was recorded. Intermediate stage cells and dead cells lost green fluorescence of Rh123 when PI and Rh 123 were stained together (Fig. 2c, 3c and 4c).

Irradiated cells were stained individually with Rh 123 & PI and also with the combination of both Rh 123 & PI. The results are presented in Tables 7a, 7b, and 7c for *E. coli, S. aureus* and *L. innocua* respectively. Rh 123 stained all three stages of cells. For *E. coli* it was 57.3, 53.9 and 73.8% for live, intermediate and dead cells, respectively. This was

92.9, 94.8, and 94.4% for *S. aureus* and 76.9, 74.7 and 86.9%, respectively for *L. innocua*. Dead cells did not stain with PI. The percentage cells that were red positive were 2.0, 2.0, and 1.9% for live, intermediate and dead *E. coli* cells, respectively. This was 0.6, 0.6 and 0.8% for *S. aureus* and 5.2, 6.5 and 2.9% for *L. innocua*, respectively. In case of live *E. coli*, the green-positive region of Rh 123 reduced from 57.3 to 45.6% when Rh 123 & PI were combined as stains. This reduction was not compensated for in the red-positive region because the increase was from 0.0 to 0.7%. For intermediate cells, the reduction in the green-positive region was from 53.9 to 48.3%. On the other hand, the gain in the red region was only 1.2%. For dead *E. coli* cells, the loss in the green positive region was from 73.8 to 65.6% and gain in the red region was a mere 3.2% (Fig. 4a, 4b and 4d).

Similar calculations were made for the data presented in Tables 7b for *S. aureus*. The loss in the green fluorescence in Rh 123-stained cells when stained with PI was 7.5% and gain in red fluorescence was 3.7% for live cells. For intermediate cells, these numbers were 11.6 and 4%, respectively and for dead cells, this was 4.2 and 2.0%, respectively. For *L. innocua*, the data in Table 7c shows a better uptake of PI in the intermediate cells and dead cells. When stained with Rh 123 alone, 76.9% of live cells showed positive fluorescence for green and 1.5% for red. When stained with both Rh 123 and PI, the cells showing green fluorescence was 69.0% and those that were showing red fluorescence were 3.7%. For intermediate cells, green fluorescence reduced from 74.7 (in case of Rh 123-stained cells) to 38.4% (in the case of Rh 123 and PI-stained cells). On the other hand, the gain in red fluorescence was 27.4% for Rh 123-PI stained cells. This gain was not reflected in PI-stained cells because the percentage of cells showing red fluorescence was 6.3%. A similar trend was observed with dead *L. innocua* cells. The loss in green fluorescence from Rh 123 stained cells to Rh 123-PI stained cells was 43.8% and the gain in red fluorescence was 26.6%.

4.5 Discussion

Visualization of microbial sub-populations based on viability has enormous significance in public health microbiology and food microbiology. The stress conditions, heat, acid, and irradiation are common antimicrobial agents used in food industry to inhibit

microbial growth and proliferation. All these antimicrobial agents affect the structure and function of bacterial cells in different ways. The majority of these, at permitted doses, combat microorganisms that cause food spoilage and poisoning by inhibiting their growth rather than destroying them completely (Gould *et al.* 1995). Thus, there is scope for bacteria to survive these treatments in an injured state and go undetected through conventional plating techniques.

A diverse group of fluorochomes were used in this study to stain the cells. The rationale behind this choice was to study the staining patterns with reference to differences in bacterial injury caused by heat, acid, and irradiation. Carboxy fluorescein diacetate (CFDA) and fluorescein diacetate (FDA) depend on the esterase activity of the cells for their fluorescence. The weak fluorescence obtained in this study may be due to inefficient penetration in some types of membranes. Another reason attributed to weak fluorescence is that fluorescein produced from FDA tend to leak from cells (Soderstrom, 1979; Thomas *et al.* 1979). On the other had, CFDA, a derivative of FDA produces a fluorescent compound, 6-carboxyfluorescein (Cohen, 1984) which is better retained (Thomas *et al.* 1979) but its fluorescence intensity was not high enough to escape interference from the autofluorescence of soil particles (Tsuji *et al.* 1995). In our case, though the staining was with pure cultures, we did not obtain adequate fluorescence to be detected by FCM. Additionally, we speculate that fluorogenic esters may not be suitable for staining heat, acid or irradiation injured cells. This is because, esterase being a protein, may a take a longer time to get inactivated. Thus fluorescence obtained may not be a true indication of cell viability.

Several disadvantages have been reported for carbocyanine dyes in their use as indicators of cell membrane potential. The increased lipid solubility of $DiOC_7(3)$ (conferred by its longer alkyl side chains) could prevent it from being a good indicator of membrane potential (Mason *et al.* 1995). The weak fluorescence obtained in this study may have been due to the low concentration of the dye used. A low concentration of 100nM was used in this study as reported by Shapiro (1990). This is because carbocyanine dyes have been found to be toxic to bacteria in some cases (Shapiro, 1990; Diaper *et al.* 1992). $DiOC6_{(3)}$, when used at a concentration of 0.05.mg l^{-1} resulted in a low fluorescence yield with viable cultures

(Mason *et al.* 1995). However, Ratinaud and Revidson, (1996) successfully used $DiOC6_{(3)}$ at a concentration of 0.1 μM to measure the functional state of listeria as affected by antimicrobial peptides, mY105 extracted from *Leuconostoc mesenteroides*. Thus, it appears that concentration and non-specific binding may be two most important limitations of carbocyanine dyes.

The intensity of fluorescence obtained with Rh 123 was measurable. However, no difference in intensity was observed between the three stages of the cells in all three types of bacteria used. Intermediate cells which had a population of about 3 logs, and dead cells in which no viable cells were detected on plate count, stained as efficiently with Rh 123 as that of live cells. We suspect that non-specific energy-independent binding may be the reason for fluorescence in dead cells as reported by Mason *et al.* (1995). However, Rh 123 was successfully used with starved *Micrococcus luteus* (Kaprelyants and Kell, 1992; Keprelyants *et al.* 1996).

The mode of destruction of microbial cells due to irradiation is different from that of heat or acid. Irradiation kills microbial cells without disrupting its membrane. This may prevent instant loss of cell-membrane-potential with death. This could be an additional reason for observed fluorescence in irradiation-killed cells. On the other hand, heat and acid are widely known to disrupt bacterial membranes and the probability of losing membrane potential in bacterial cells under this situation is high. Good results were obtained with PI staining. PI stains bacterial cells with compromised membranes. Our results indicate a positive red fluorescence for acid and heat killed cells and a negative red fluorescence for irradiation-killed cells. From these results, it could be concluded that irradiation does not act by disrupting the cell membranes and thus vital stains may have little value as indicators of cell viability.

Another interesting observation is that the intermediate and dead cells stained with Rh 123 and PI showed a positive red fluorescence even though they showed a positive green fluorescence when they were stained with Rh 123 alone. Under these circumstances, injured cells are likely to have both red and green fluorescence with a heterogeneous mixture of live, injured and dead cells. Rh 123 is cationic lipophilic dye, accumulated cytosolically by the

cells with an inside negative transmembrane electrochemical potential (Mason *et al.* 1995). PI is also a positively charged molecule that can enter cells mainly via damaged membranes of stressed, injured or dead cells and intercalate into DNA and RNA (Ueckert *et al.* 1995). Thus it appears that PI fluorescence is more efficient than RH 123 in membrane compromised cells.

The results of this study indicate that the success of a particular fluorescent dye depends on the nature of bacterial injury. The observations with irradiated cells clearly substantiates this conclusion. Further, concentration of the dyes are a critical component in staining protocol. FCM is a better tool, more convenient and accurate in evaluating these dyes than with fluorescence microscopy.

4.6 References

1. Atlas, R.M. 1993. Minimal broth, Davis, p. 609. *In* L.C. Parks (ed.), Handbook of Microbiological Media, CRC Press, Boca Raton.

2. Bercovier, H., M. Resnick, D. Kornitzer, and L. Levy. 1987. Rapid method for testing drug-susceptibility of *Mycobacterium* spp. and gram-positive bacteria using Rhodamine 123 and fluorescein diacetate. J. Microbiol. Meth. 7: 139-142.

3. Boye, E., and A. Lϕbner-Olesen. 1991. Bacterial growth control studied by flow cytometry. Res. Microbiol. 142: 131-135.

4. Brunius, G. 1980. Technical aspects of the use of 3',6'-diacetyl fluorescein for vital fluorescent staining of bacteria. Curr. Microbiol. 4: 321-323.

5. Cohen, S.D. 1984. Detection of mycelium and oospores of *Phytophthora megasperma* forma specialis Glucinea by vital stains in soil. Mycologia 76: 34-39.

6. Chrzanowski, T.H., R.D. Crotty, J.G. Hubbard, and R.P. Welch. 1984. Applicability of the fluorescein diacetate method of detecting active bacteria in fresh water. Microbiol. Ecol. 10: 179-185.

7. Davey, H.M., A.S. Kaprelyants, and D.B. Kell. 1993. Flow cytometric analysis, using rhodamine 123, of *Micrococcus luteus* at low growth rate in chemostat cultures, p. 83-93. *In* D. Lloyd (ed.), Flow cytometry in Microbiology, London, Springer-Verlag.

8. Diaper, J.P., K. Tither, and C. Edwards. 1992. Rapid assessment of bacterial viability by flow cytometry. Appl. Microbiol. Biotechnol. 22: 1-5.

9. Diaper, J. P., and C. Edwards. 1994. The use of fluorogenic esters to detect viable bacteria by flow cytometry. J. Appl. Bacteriol. 77: 221-228.

10. Gould, G.W., T. Abee, P.E. Granum, and M.V. Jones. 1995. Physiology of food poisoning microorganisms and the major problems in food poisoning control. Intl. J. Food Microbiol. 28: 121-128.

11. Hargittai, P.T., S.J. Youmans, and E.M. Lieberman. 1991. Determination of the membrane potential of cultured mammalian Schwann cells and its sensitivity to potassium using a thiocarbocyanine fluorescent dye. Gila 4: 611-616.

12. Johnson, L.V., M.L. Walsh, B.J. Bockus, and L.B. Chen. 1981. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. J. Cell Biol. 88: 536-535.

13. Kaprelyant, A.S., G.V. Mukamolova, H.M. Davey, and D. B. Kell. 1996. Quantitative analysis of the physiological heterogeneity within starved cultures of *Micrococcus luteus* by flow cytometry and cells sorting. Appl. Environ. Microbiol. 62: 1311-1316.

14. Keprelyants, A.S., and D.B. Kell. 1992. Rapid assessment of bacterial viability and vitality using rhodamine 123 and flow cytometry. J. Appl. Bacteriol. 72: 410-422.

15. Lundgren, B. 1981. Fluorescein diacetate as a stain of metabolically active bacteria in soil. OIKOS 36: 17-22.

16. Mason, D.J., R.Lopez-Amoros, R. Allman, J.M. Stark, and D. Lloyd. 1995. The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. J. Appl. Bacteriol. 78: 309-315.

17. Mason, D.J., R. Allman, and D. Lloyd. 1993. Uses of membrane potential dyes with bacteria, p.69-81. *In* D. Lloyd (ed.), Flow cytometry in microbiology, Springer-Verlag, London.

18. McClelland, R.G., and A.C. Pinder. 1994. Detection of low levels of specific *Salmonella* species by fluorescent antibodies and flow cytometry. J. Appl. Bacteriol. 77: 440-447.

19. Monfort, P., and B. Baleux. 1992. Comparison of flow cytometry and epifluorescence microscopy for counting bacteria in aquatic ecosystems. Cytometry 13: 188-192.

20. Nir, R., Y. Yisraeli, R. Lamed, and E. Sahae. 1990. Flow cytometry sorting of viable bacteria and yeast according to β-galactosidase activity. Appl. Environ. Microbiol. 56: 3861-3866.

21. Pena, A., S. Uribe, J.P. Pardo,and M. Borbolla. 1984. The use of a cyanine dye in measuring membrane potential in yeast. Archives of Biochem. Biophys. 231: 217-225.

22. Porter, J., C. Edwards, and R.W. Pickup. 1995. Rapid assessment of physiological status in *Escherichia coli* using fluorescent probes. J. Appl. Bacteriol. 79: 399-408.

23. Ratinaud, M.-H., and S. Revidon. 1996. A flow cytometric method to assess functional state of the *Listeria* membrane. J. Microbiol. Meth. 25: 71-77.

24. Roszak, D.B., and R.R. Colwell. 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51: 365-379.

25. Sims, P.J., A.S. Waggoner, C.-H. Wang, and J.F. Hoffman. 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry 13: 3315-3330.

26. Shapiro, H.M. 1990. Cell membrane potential analysis. Methods Cell Biol. 33: 25-35.

27. Soderstrom, B.E. 1977. Vital staining of fungi in pure cultures and in soil with fluorescein diacetate. Soil Biol. Biochem. 9: 59-63.

28. Thomas, J.A., R.N. Buchsbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry 18: 2210-2218.

29. Tsuji, T., Y. Kawasaki, S. Takeshima, T. Sekiya, and S. Tamaka. 1995. A new fluorescence staining assay for visualizing living microorganisms in soil. Appl. Environ. Microbiol. 61: 3415-3421.

30. Ueckert, J., P. Breeuwer, T. Abee, P. Stephens, G. Nebe von Caron, and P.F. ter Steeg. 1995. Int. J. Food Microbiol. 28: 317-326.

31. Wallner, G., R. Erhart, and R. Amann. 1995. Flow cytometric analysis of activated sludge with rRNA-targeted probes. Appl. Environ. Microbiol. 6: 1859-1866.

32. Zaritsky, A., M. Kihara, and R.M. MacNab. 1981. Measurement of membrane potential in *Bacillus subtilis*: a comparison of lipophilic cations, rubidium ion, and a cyanine dye as probes. J. Mem. Biol. 63: 215-231.

Table 1. Staining pattern of live, injured and dead stages of heat treated (65°C) *E. coli* cells with rhodamine 123 and PI.

	% Live				% Intermediate				% Dead			
Stains	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**
No stain	0.0	0.0	100	0.0	0.0	0.0	99.5	0.5	1.4	0.0	97.8	0.8
Rh 123	0.0	1.2	41.4	57.3	0.0	0.0	11.9	88.1	0.0	0.1	29.4	70.5
Rh 123 + PI	0.8	3.6	39.7	55.9	66.2	26.3	4.5	3.0	90.1	0.0	9.8	0.1
PI	1.4	0.4	91.3	6.8	88.2	0.1	10.1	1.6	96.7	0.0	2.3	1.0

R+ = Red positive, R&G+= Red and Green positive, R&G-= Red and Green negative, G+= Green positive

Table 2. Staining pattern of live, injured and dead stages of acid treated *E. coli* cells with rhodamine 123 and PI.

	% Live				% Intermediate				% Dead			
Stains	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**
No stain	0.1	0.0	94.3	5.7	0.1	0.0	98.4	1.6	0.4	0.0	98.7	0.9
Rh 123	0.0	0.2	41.3	58.5	0.0	0.2	19.3	80.4	0.2	1.9	11.5	86.3
Rh 123 + PI	13.3	16.9	33.8	35.9	31.3	34.1	11.6	23.1	77.6	13.1	5.2	4.1
PI	27.8	0.0	64.6	7.5	53.3	0.0	43.9	2.8	90.4	0.0	9.3	0.3

R+ = Red positive, R&G+= Red and Green positive, R&G-= Red and Green negative, G+= Green positive

Table 3. Staining pattern of live, injured and dead stages of heat treated (65°C) *S. aureus* cells with rhodamine 123 and PI.

	% Live				% Intermediate				% Dead			
Stains	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**
No stain	0.4	0.0	96.9	2.8	0.2	0.0	99.7	0.1	0.3	0.0	99.6	0.0
Rh 123	0.0	0.6	1.1	98.3	0.0	0.0	3.1	96.9	0.0	0.0	2.0	98.0
Rh 123 + PI	2.4	17.9	1.0	78.7	14.3	16.1	6.4	63.0	50.3	36.0	5.7	8.0
PI	1.8	0.0	97.6	0.6	19.7	0.0	80.0	0.3	82.1	0.0	17.9	0.0

R+ = Red positive, R&G+= Red and Green positive, R&G-= Red and Green negative, G+= Green positive

Table 4. Staining pattern of live, injured and dead stages of acid treated *S. aureus* cells with rhodamine 123 and PI.

	% Live				% Intermediate				% Dead			
Stains	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**
No stain	0.1	0.0	99.8	0.2	3.9	0.0	96.1	0.1	6.1	0.0	93.8	0.0
Rh 123	0.0	0.5	0.6	98.9	0.0	0.0	18.6	81.4	0.0	0.0	46.1	53.9
Rh 123 + PI	2.0	17.1	1.1	79.8	12.9	41.0	23.5	22.7	23.0	8.5	50.7	17.7
PI	2.0	0.0	97.2	0.7	69.9	0.0	30.1	0.0	44.9	0.0	55.1	0.0

R+ = Red positive, R&G+= Red and Green positive, R&G-= Red and Green negative, G+= Green positive

Table 5. Staining pattern of live, injured and dead stages of heat treated (65°C) *L. innocua* cells with rhodamine 123 and PI.

	% Live				% Intermediate				% Dead			
Stains	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**
No stain	0.0	0.0	99.8	0.2	0.0	0.0	99.3	0.1	0.0	0.0	99.9	0.1
Rh 123	0.4	0.3	18.4	80.9	0.0	0.0	29.5	70.5	0.0	0.0	19.8	80.2
Rh 123 + PI	9.4	10.2	22.1	58.3	31.0	5.4	35.8	27.8	90.9	2.8	4.9	1.3
PI	4.9	0.0	92.0	3.1	25.5	0.0	74.4	0.1	97.1	0.0	2.9	0.0

R+ = Red positive, R&G+= Red and Green positive, R&G-= Red and Green negative, G+= Green positive

Table 6. Staining pattern of live, injured and dead stages of acid treated *L. innocua* cells with rhodamine 123 and PI.

	% Live				% Intermediate				% Dead			
Stains	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**
No stain	0.2	0.0	99.7	0.1	0.2	0.0	99.7	0.1	1.2	0.0	98.7	0.0
Rh 123	0.6	0.7	18.3	80.4	0.0	0.0	15.8	84.2	0.0	0.0	9.0	90.9
Rh 123 + PI	5.7	7.5	23.8	63.0	72.1	17.3	6.4	4.2	59.8	30.7	4.6	4.8
PI	3.9	0.1	92.4	3.5	92.2	0.0	7.8	0.0	94.2	0.0	5.8	0.0

R+ = Red positive, R&G+= Red and Green positive, R&G-= Red and Green negative, G+= Green positive

Table 7a. Staining pattern of live, injured and dead stages of irradiation treated *E. coli* cells with rhodamine 123 and PI.

	% Live				% Intermediate				% Dead			
Stains	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**
No stain	0.0	0.0	99.7	0.3	0.0	0.0	99.6	0.4	0.0	0.0	99.7	0.3
Rh 123	0.0	0.0	42.7	57.3	0.0	0.0	46.1	53.9	0.0	0.0	26.2	73.8
Rh 123 + PI	0.7	0.7	53.0	45.6	1.2	1.1	49.4	48.3	3.2	2.2	29.0	65.6
PI	2.0	0.0	96.4	1.6	2.0	0.0	96.4	1.6	1.9	0.0	96.2	1.9

R+ = Red positive, R&G+= Red and Green positive, R&G-= Red and Green negative, G+= Green positive

Table 7b. Staining pattern of live, injured and dead stages of irradiation treated *S. aureus* cells with rhodamine 123 and PI.

	% Live				% Intermediate				% Dead			
Stains	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**
No stain	0.0	0.0	99.9	0.1	0.0	0.0	99.9	0.1	0.0	0.0	99.9	0.1
Rh 123	0.0	0.1	7.0	92.9	0.0	0.0	5.2	94.8	0.0	0.0	5.5	94.4
Rh 123 + PI	3.7	4.8	6.1	85.4	1.2	5.0	7.9	83.2	2.0	5.1	3.0	89.9
PI	0.6	0.0	99.0	0.4	2.0	0.0	99.2	0.2	0.8	0.0	98.8	0.4

R+ = Red positive, R&G+= Red and Green positive, R&G-= Red and Green negative, G+= Green positive

Table 7c. Staining pattern of live, injured and dead stages of irradiation treated *L. innocua* cells with rhodamine 123 and PI.

	% Live				% Intermediate				% Dead			
Stains	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**	**R+**	**R&G +**	**R&G-**	**G+**
No stain	0.2	0.0	97.6	2.2	0.1	0.0	98.4	1.5	0.1	0.0	98.7	1.2
Rh 123	1.5	0.7	20.8	76.9	0.2	0.4	24.7	74.7	0.1	0.3	12.8	86.9
Rh 123 + PI	3.7	2.7	24.6	69.0	27.6	8.6	25.5	38.4	26.6	9.6	20.7	43.1
PI	5.2	0.0	94.6	0.2	6.3	0.9	92.2	0.7	2.9	0.0	97.0	0.1

R+ = Red positive, R&G+= Red and Green positive, R&G-= Red and Green negative, G+= Green positive

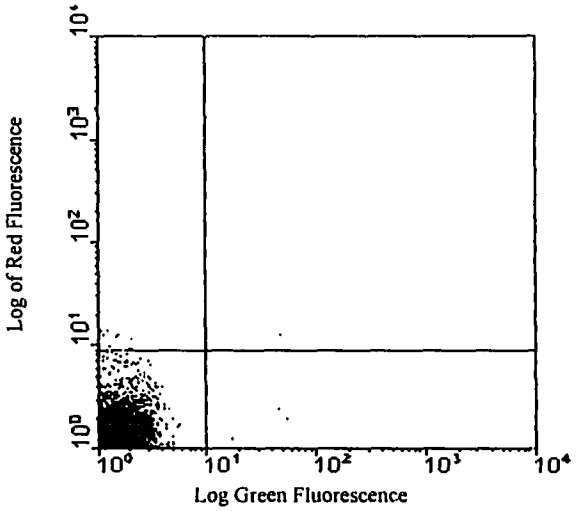

Fig. 1a. Live *s.aureus* cells without stains.

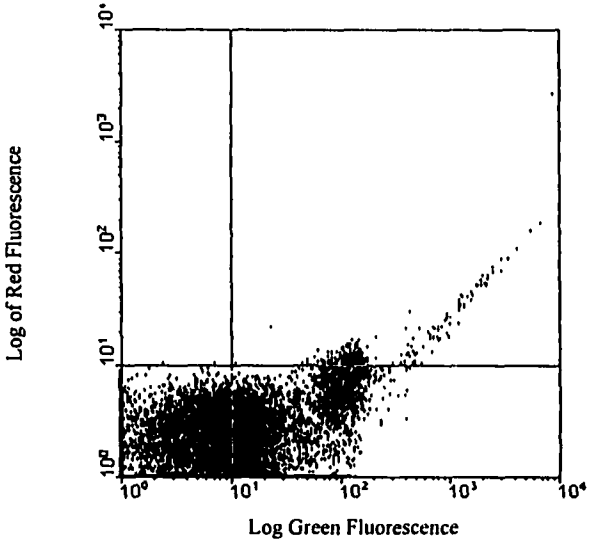

Fig. 1b. Live *E. coli* cells stained with Rh123.

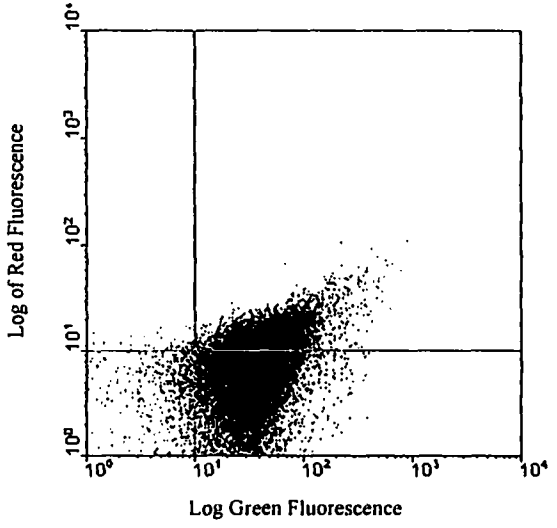

Fig. 1c. Live *s.aureus* cells stained with Rh123.

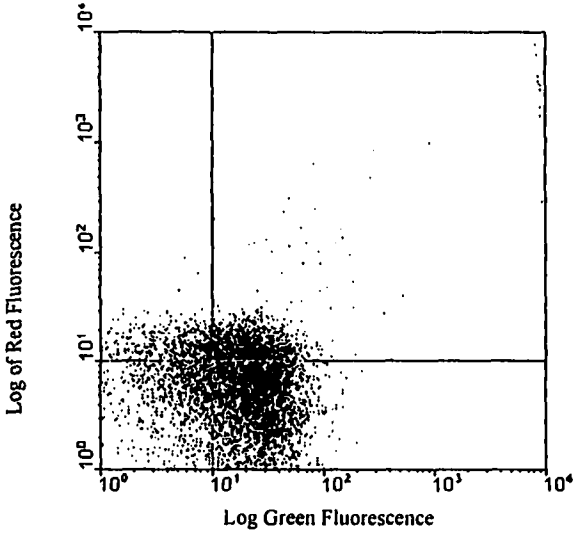

Fig. 1d. Live *L. innocua* cells stained with Rh123.

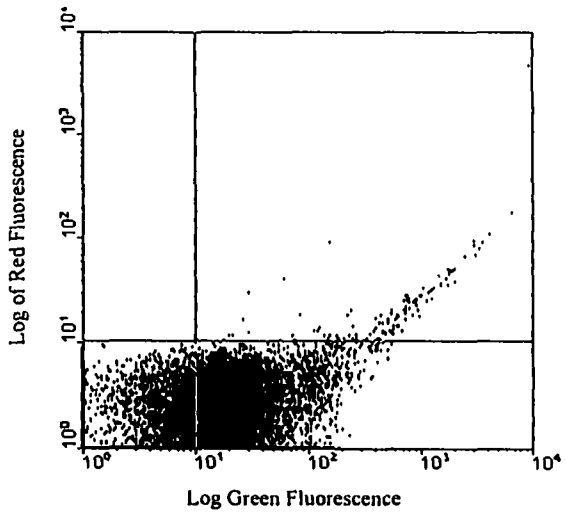

Fig. 2a. Intermediate cells of acid-treated *E. coli* stained with Rh 123 & PI.

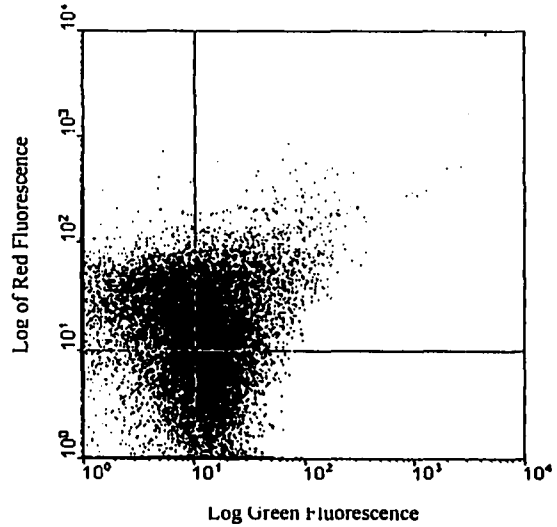

Fig. 2b. Intermediate acid-treated *S. aureus* cells stained with Rh123 and PI.

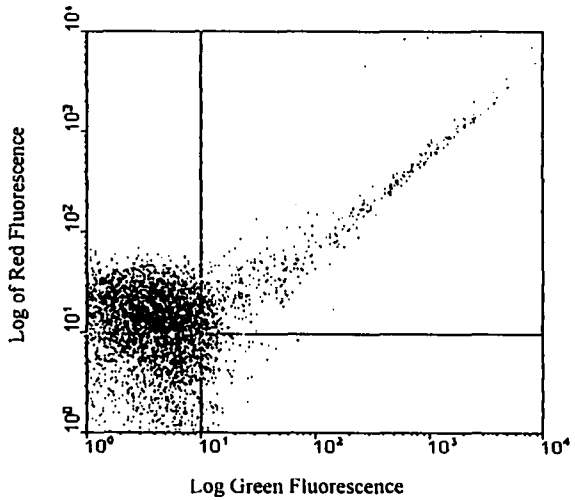

Fig. 2c. Intermediate cells of acid-treated *L. innocua* stained with Rh123 and PI.

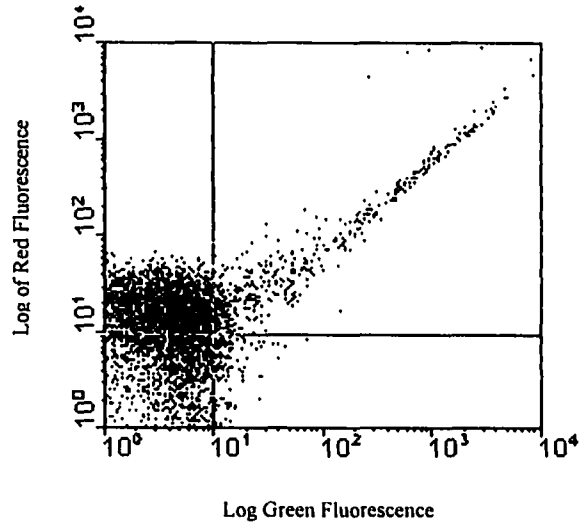

Fig. 2d. Live *E. coli* cells stained with PI.

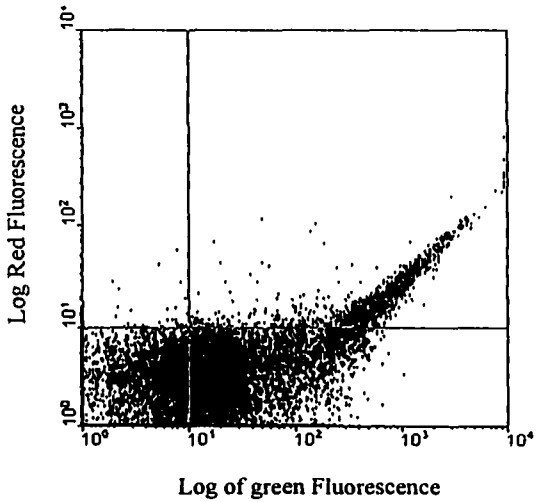

Fig. 3a. Dead cells of acid-treated *E. coli* stained with Rh 123.

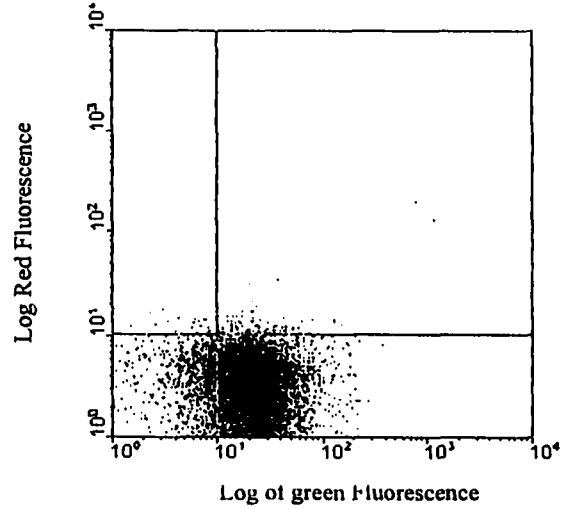

Fig. 3b. Dead cells of acid-treated *S. aureus* stained with Rh 123.

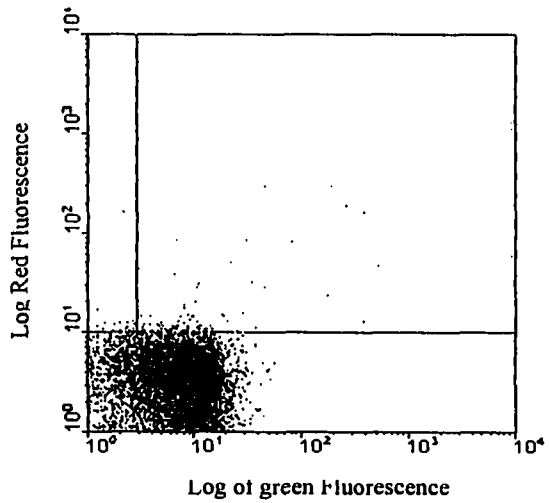

Fig. 3c. Dead cells of acid-treated *L. innocua* stained with Rh 123.

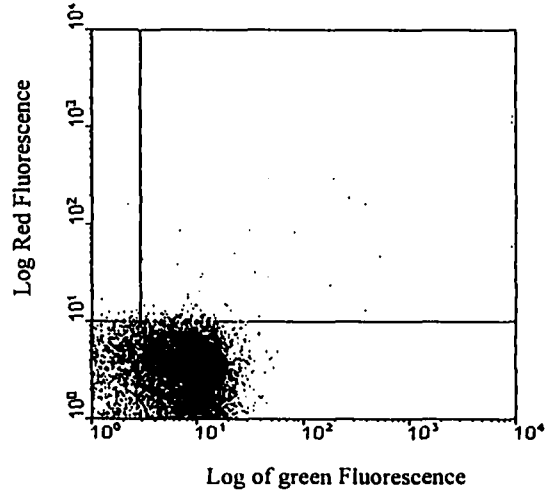

Fig. 3d. Dead cells of acid-treated *S. aureus* stained with PI.

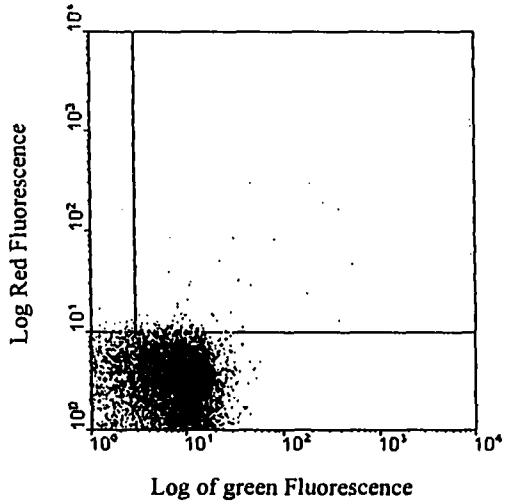

Fig. 4a. Dead cells of irradiated *E. coli* stained with Rh 123.

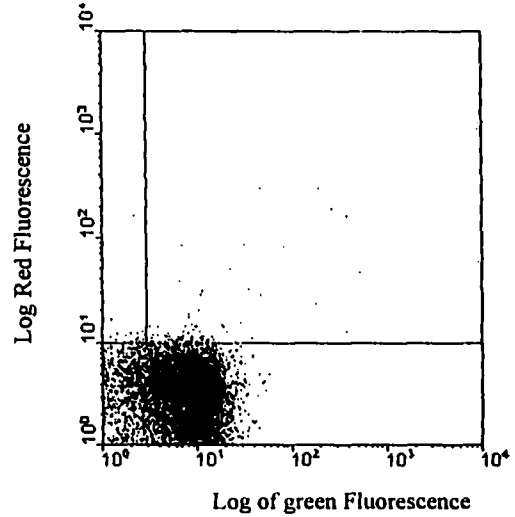

Fig. 4b. Dead cells of irradiated *S. aureus* stained with Rh 123.

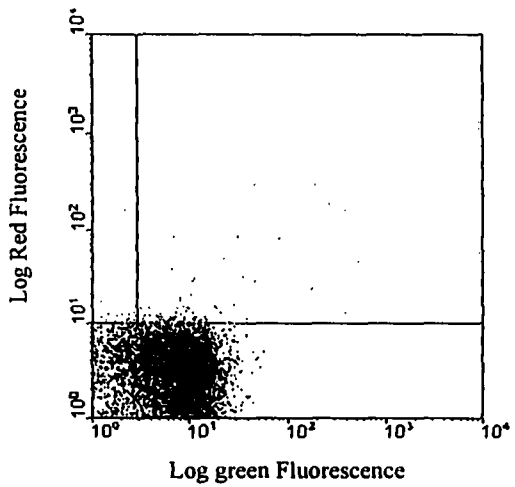

Fig. 4c. Heat-killed cells of *L. innocua* stained with Rh 123.

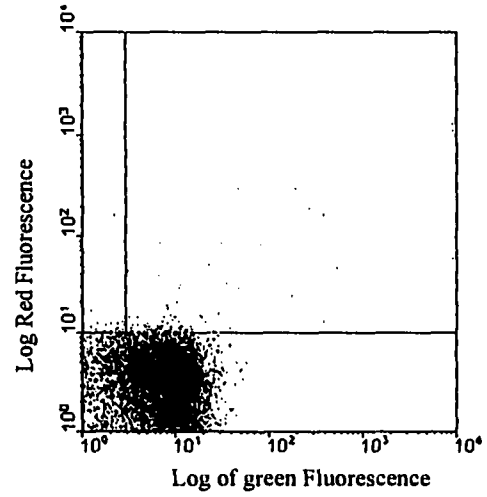

Fig. 4d. Dead cells of irradiated *L. innocua* stained with PI.

CHAPTER 5. EVALUATION OF *BAC*LIGHT VIABILITY KIT TO ASSESS AND QUANTIFY BACTERIAL INJURY AND DEATH CAUSED BY HEAT, ACID, IRRADIATION AND STARVATION

A paper to be submitted to the Journal of Applied Microbiology

Raveendran Venugopal, Christi Harkins and James Dickson

5.1 Abstract

Flow cytometry was used to rapidly asses and quantify viability of bacteria stained with *Bac*Light viability kit. *E. coli*, *S. aureus*, and *L. innocua* were exposed to heat, acid, starvation and irradiation, and stained with LIVE/DEAD stains of *Bac*Light viability kit. Live bacteria stained with LIVE/DEAD stain were quantified using flow cytometer and correlated with plate counts on TSA. Using flow cytometer, the bacteria were counted as viable, injured and dead, compared with plate counts obtained using TSA and on selective agar, VRBA was used for *E. coli*, BP for *S. aureus* and MOX agar was used for *L. innocua*.

Good correlations were obtained for live bacteria stained and counted with flow cytometer, but the discrimination of bacterial viability was not clear for heat, acid and starvation-treated cells. The stain failed to differentiate irradiated cells. Viability of stressed bacteria were also confirmed by inoculating them in enrichment broths. Our results indicate that flow cytometer is effective in rapidly quantifying bacteria in pure cultures when the population is between 10^3 to 10^7 cells/ml. However, viability staining with this vital stain is dependent upon the type of stress undergone by bacteria and may not be suitable for all kinds of stress.

5.2 Introduction

Microbiologists involved in quality control of food products are often faced with the challenge of detecting and enumerating bacteria that are sub lethally injured. Bacteria, when exposed to an inimical process, might survive, be killed or be sufficiently injured so as to be

unable to produce colonies on a medium on which undamaged organisms can grow and multiply (Russell, 1991). A variety of procedures are employed to maintain the high microbial quality of foodstuffs during processing, storage, distribution and sale. The majority of techniques that are employed to combat microorganisms that cause food poisoning and spoilage act by inhibiting or completely preventing their growth rather than by inactivating them (Gould *et al.* 1995). Bacteria injured by such treatments have been known for many years now. Allochthonous and autochthonous bacteria exposed to potentially lethal environmental conditions including nutrition restriction, ultraviolet irradiation or sublethal concentrations of antibacterial compounds undergo physiological and morphological alterations that complicate the detection and accurate enumeration of such stressed bacteria using available cultural approaches (Kjellberg *et al.* 1993; Rozak and Colwell, 1987; McFeters, 1990). Also, it has been estimated that only 20% of the bacteria within autochthonous communities are known (Wayne *et al.* 1987). As a result, the use of most established media often leads to serious underestimations of the complexity and bacterial population density of natural and engineered systems (McFeters *et al.* 1995). The potential public health significance of undetected and underestimated populations of injured organisms such as *Bacillus* or *Clostridium* spp., *Listeria*, staphylococci or salmonellas in food is immense (Russell, 1991).

Microbiological recovery and enumeration methods can influence the survival of microorganisms. Ideally, recovery techniques should allow for enumeration of all viable cells, including sublethally injured cells (Ahmed and Conner, 1995). However, to facilitate the detection of specific microorganisms, chemicals with selective properties are often added to media. Some of these agents inhibit repair of injured cells (Ray and Adams, 1984). A sublethally injured cell, when given appropriate environmental conditions, can repair damage and regain pathogenicity (McCarthy, 1991). However, injured cells are unable to multiply in the presence of selective agents commonly added to enrichment media and would not allow for the detection of injured cells (Jay, 1986). Thus, microbial cells, having undergone exposure to stresses such as heating, freezing, and exposure to acids and sanitizers, can exist in an injured state (Flanders *et al.* 1995).

The sensitivity of flow cytometry allows the discrimination of bacterial populations based on their viability and physiological activity. Flow cytometry has been used as a rapid method for testing viability of bacteria in water and soil (Page and Burns, 1991; Diaper and Edwards, 1994; Porter *et al.* 1995), investigating dormancy and starvation (Kaprelyants and Kell, 1993; Diaper and Edwards, 1994), testing susceptibility to antibiotics (Martinez *et al.* 1982; Gant et al. 1993; Mason *et al.* 1994), assessment of viability after exposure to quaternary ammonium compounds (Langsrud and Sundheim, 1996) and for studying the effect of heat, sonication and electroporation on bacterial viability (Lopez-Amoros *et al.* 1995). Fluorescent reagents are currently used in an impressive range of biological applications where the response of individual cells can be observed microscopically (McFeters *et al.* 1995). A number of fluorescent dyes have been described for flow cytometric assessment of viability of bacteria. Accumulation of some dyes is dependent on cell membrane potential as is with rhodamine 123 (Rh 123). Rh 123 has been used successfully to determine the viability of a range of laboratory, environmental and chemical strains (Lopez-Amoros *et al.* 1995; Kaprelyants and Kell, 1992). A major problem with Rh 123 is poor accumulation in some Gram-negative species (Matsuyama, 1984). Carbocyanine dyes are another group of membrane potential dyes used to assess cell viability. The disadvantage with these dyes are their toxicity to bacteria (Shapiro, 1990) and their increased lipid solubility (Mason *et al.* 1995). Fluorogenic esters such as fluorescein diacetate (FDA) are non-fluorescent derivatives of fluorescein which readily diffuses through cell membranes (Diaper and Edwards, 1994) and once inside the cell, they are cleaved by non-specific esterases to release fluorescein, which is polar and therefore retained inside the cells where it fluoresces brilliant green, when irradiated with blue light (Chrzanowski *et al.* 1984). The problem associated with FDA is that fluorescein produced tend to leak from cells (Soderstrom, 1979; Thomas *et al.* 1979). As a result of these reported disadvantages of single dyes, it is usually necessary to use at least two dyes to elucidate cell metabolic activity.

In this study, flow cytometry was used to determine and quantify live, injured and dead cells stained with *Bac*Light viability kit. *E. coli*, *S. aureus*, and *L. innocua* were exposed to heat, acid, irradiation and starvation, and cell viability were assessed at various

time points, using conventional plate counts and counts by flow cytometry. The viability of stressed cells were also assessed by resuscitation in liquid broth media.

5.3 Materials and Methods

5.3.1 Bacterial Strains and Growth Conditions

The bacterial strains used in this study were *Escherichia coli* 12-1, *Staphylococcus aureus* and *Listeria innocua* ATCC 33090. *S. aureus* and *E. coli* were obtained from the culture collection available at the Department of Microbiology, Iowa State University. Growth of all cultures was in Tryptic soy broth (TSA) (Difco) at 37°C in a shaker at 130 rpm. For the study, overnight grown cultures were centrifuged at 8000x*g* for 10 minutes at 4°C and the cell pellet was washed twice in sterile PBS and redissolved in 1 ml of the same solution. Appropriate volume of this inoculum was inoculated into minimal broth to give a final concentration of 10^8 cells/ml, before exposing the cells to stress conditions. The composition of minimal media was as described by Atlas and Parks (1990). It contained (g/l): K_2HPO_4 - 7.0 g; KH_2PO_4 - 2.0 g; $(NH_4)_2SO_4$ - 1.0 g; glucose - 1.0 g, sodium citrate - 0.5 g, $MgSO_4.7H_2O$ - 0.1 g, pH 7.0.

5.3.2 Stress Conditions

5.3.2.1 Heat

Minimal media was preheated in a water bath and a suitable volume of the inoculum was inoculated to give a concentration of 10^8 cells/ml. For *E. coli* and *L. innocua*, minimal media was preheated to 57°C and for *S. aureus* it was preheated to 58°C. Samples were taken at 0 minutes and then, once every 10 minutes over a period of 60 minutes. The cells from all the samples were diluted in 0.2% (w/v) peptone water (Oxoid) and plated on TSA or appropriate selective agar. *E. coli* were plated on violet red bile agar (VRBA), *S. aureus* were plated on Baird-Parker agar supplemented with egg yolk tellurite enrichment and *L. innocua* were plated on Modified Oxford medium (MOX). All the three selective media were purchased from Difco (Difco Laboratories,Detroit, MI). Plates were incubated at 37°C for 48 hr before the colonies were counted. The detection limit of plating scheme used was

10 and more cells. Simultaneously, for all the sampling points and for all the three cultures, a 1:100 dilution were made in phosphate buffered saline (PBS) and held on ice until they were stained and analyzed using flow cytometer (FCM)

5.3.2.2 Acid

Minimal media were acidified with 1 ml of 85% lactic acid (Sterling Chemicals, TX) to give a final concentration of 1.6% lactic acid in the media. The initial inoculum of *E. coli*. *S. aureus* and *L. innocua* were prepared as described above and inoculated into acidified minimal media. Samples were taken at 0 minutes and thereafter, once every 5 minutes over a period of 30 minutes for *E. coli* and *L. innocua* and once every 10 minutes over a period of 70 minutes for *S. aureus*. Plating conditions, media used and FCM sample preparation were as mentioned above for heat stress.

5.3.2.3 Irradiation

The bacterial suspensions, *E. coli*, *S. aureus*, and *L. innocua* (10^8 CFU/ml) in minimal broth were poured aseptically into 50 ml flat tissue culture flasks with screw caps (Costar) and filled up to the brim. Care was taken to exclude atmospheric air inside the flasks. These flasks, with the inoculated broth were irradiated using accelerated electron beams at the Linear Accelerator Facility at Iowa State University. The depth of the liquid column inside the flask available for irradiation was approximately 15 mm. The inoculated broth were irradiated to an average absorbed dose of 0, 0.5, 1.0, 2.0, and 5.0 KGy. As described above, the samples were plated and processed for FCM analyses.

5.3.2.4 Starvation

Appropriate volumes of the bacterial suspensions (*E. coli*, *S. aureus*, and *L. innocua*) were each inoculated in 0.8% (w/v) saline separately, to give a final concentration of 10^8 cells/ml. The cultures were starved at 35°C for 72 days for *E. coli* and for 48 days for *S. aureus* and *L. innocua*. The samples were plated once every 7 days on TSA and on respective selective media as mentioned above. For FCM analyses, initial samples were

diluted 1: 100 in PBS and for *E. coli* and *S. aureus*, starting with 21 days, 1:10 dilutions were used because of a drop in cell counts as estimated b plate count method. For *L. innocua*, 1: 10 dilutions were used starting 14 days and starting 21 days, no dilutions were made and the samples were taken directly, stained and analyzed in FCM.

5.3.3 Fluorescent Dye and Staining Protocol

*Bac*Light viability kit was purchased from Molecular Probes, Inc., Eugene, OR. The kit has two dyes, LIVE stain and DEAD stain. The active ingredient of DEAD stain is propidium iodide (13 mM) and the active ingredient of LIVE stain is proprietary. Live cells exclude DEAD stain and stain with LIVE stain, fluorescing green. DEAD stain, stains dead cells, replacing LIVE stain and fluorescing red. Both the reagents were added at 1μl/ml, vortexed and incubated in dark for 10 minutes at room temperature before analyzing in FCM. The analyses were done within 30 minutes after staining.

5.3.4 Quantification of Bacterial Cells Using FCM

Bacterial cells were quantified by using fluorescent beads (6 μm yellow green polystyrene latex microparticles, Polysciences, Inc., Warrington, PA). A 25 μl of a known concentration (10^5/ml) of the bead suspensions in PBS were inoculated into each sample before FCM analyses and after staining. Similar volumes of the same concentration of beads inoculated into 500 μl of PBS, served as the control. The beads showed up in a separate region on the histograms and were gated and counted along with bacterial cells, but separately. After FCM analyses, the beads in the samples were counted using a coulter counter (Coulter Electronics, Inc., Hialeah, FL). This was done by pipetting 200 μl of FCM-analyzed samples with beads, into 10 ml of PBS, and the beads were counted. This dilution factor was found suitable to calculate 10^5 beads/ml and gave a final ratio of 1:1 with bacterial cells, if the samples contained 10^5 cells/ml. The number of bacterial cells were calculated by the following formula:

$$\frac{\text{Number of cells}}{\text{Number of beads}} \times \text{Number of beads (coulter counter)} \times \text{Dilution factor} = \text{No. of cells/ml}$$

5.3.5 Flow Cytometry

Flow cytometry (Coulter EPICS XL-MCL) available in the Cell and Hybridoma Facility at Iowa State University was used in this study. The flow cytometer was equipped with a FL-1 green filter of 525 nm band pass filter (505 to 545 nm) and a FL-4 red filter of 675 nm band pass filter (660nm to 700nm). All the samples were counted upto 20000 events. Regions based on fluorescence intensity can be separated and counted. This process is usually referred to as gatting. Data were interpreted based on gatting on differentially stained cells. Cells that had a positive uptake of green dye (LIVE stain) were gatted as live cells, cells with red fluorescence (DEAD stain) were gatted as dead cells and cells both red and green fluorescence were gatted as injured cells. Each organism was gatted separately for live, injured and dead cells because of the difference in the pattern of histogram, which differed according to the genus. Once gatted for a particular organism, all the samples of that organism were analyzed under the same gatting scheme. This enabled us to study objectively, the shift in the fluorescence, from green to red.

5.3.6 Resuscitation Studies

One hundred microliters of stressed cells were inoculated in 9 ml volumes of minimal media (MB), Nutrient broth (NB) (Difco), Tryptic soy broth (TSB) (Difco), Tryptic soy broth (TSB++) supplemented with 0.5% (w/v) yeast extract (Difco) and 0.1% (w/v) sodium pyruvate (Sigma Chemical Co., St. Louis, MO),and respective selective broth (Violet red bile medium for *E. coli* and Baird-Parker medium supplemented with egg yolk tellurite enrichment for *S. aureus*). Both the selective media were formulated based on the composition of manufacturer's, with the exclusion of agar. MOX media was not used in this study. Inoculated broth media were incubated at 37°C and were observed for growth. Visible turbidity was counted as positive for growth of the organism and all the inoculated broth were incubated for 96 hours before concluding negative results and observations were made once in 6 hours.

5.3.7 Cell injury

Cell injury was calculated as % injury by the following formula as reported by Bunduki et al. (1995):

$$\% \text{ Injury} = \left[1 - \frac{\text{count of selective media}}{\text{count on non selective media}} \right] \times 100$$

5.4 Results

Healthy cells were stained with *Bac*Light viability kit and pattern of cell fluorescence were studied. Simultaneously, the counts obtained with flow cytometer was compared with conventional plate count on TSA, the results of which are presented in Table 1. The pattern of fluorescence obtained, differed with each organism studied in this work. Fig. 1a, 2a and 3a shows the type of fluorescence obtained with live *E. coli*, *S. aureus*, and *L. innocua* and it could be clearly seen that the histogram obtained differs with the type of organism. In order to compare FCM counts with conventional plate counts, five different concentrations (10^3 to 10^7 ml/l) of each of the three organisms were prepared and their cell densities were compared. Table 1 shows the comparison of plate count obtained on TSA with FCM counts and Table 2 shows the correlations obtained. *E. coli* had a correlation coefficient of 0.976, it was 0.977 for *S. aureus* and 0.973 for *L. innocua*.

5.4.1 Heat Stress

Comparisons between plate counts and FCM counts were made for heat stressed *E.coli*, *S. aureus*, and *L. innocua*, the results of which are presented in Tables 3-5. The initial population of *E. coli* was 8.6 and 8.46 on TSA and VRBA, respectively, *S. aureus* was 8.58 and 8.68 on TSA and BP, respectively, and *L. innocua* was 8.79 and 8.75 on TSA and MOX respectively. This population steadily decreased with increase in time for all three organisms. At the end of 60 minutes, *E. coli* and *S. aureus* went below the detection limit when counted on TSA and when counted on their respective selective agar, both the organisms went below detection limit by 50 min. *L. innocua*, when counted on TSA had a population of 2.17

CFU/ml at the end of 60 min wheras it went below the detection limits when counted on MOX agar, by 40 min. The percentage of injured cells was higher in case of *L. innocua* than with *E. coli* or *S. aureus*, because *L. innocua*, by 30 minutes had 72% injured cells and from 40 minutes onward the percentage of injured cells were 100%.

FCM counts for percentage live *E. coli* dropped significantly from 62% at 0 min to 0% by 30 min. This loss was compensated with an increase in percent injured cells which increased from 35% at 0 min to 82% at 30 min and fluctuated around this value after 30 minutes. The percent dead cells by FCM showed an increase from 3% at 0 min to 15% at 20 min and stabilized at 20% after 40 minutes. Figs. 1b and 1c show the histograms of heat injured and heat killed *E. coli*. FCM counts for percentage live *S. aureus* cells showed a gradual decrease with progression of time. At 0 min the percentage of cells that were live was 89% and this reduced to 14% after 60 min exposure. The percentage of injured cells increased from 11% at 0 min to 43% at 50 min and then dropped to 33% by 60 min. The percentage of dead *S. aureus* cells increased from 0% at 0 min to 53% after 60 min of exposure to heat. *L. innocua* did not respond to dual staining because even after exposing the cells to 57°C for 60 min, 87% of cells were live. The percentage injured cells did not increase during the course of exposure to heat and the percentge dead cells increased from 2% at 0 min to 10% after 60 min of exposure to heat. Figs. 2b and 2c show the staining pattern of injured and dead *S. aureus* cells respectively and Fig. 3d shows heat killed *L. innocua*.

5.4.2 Acid Stress

Comparison of counts for acid stressed cells of *E. coli*, *S. aureus*, *and L. innocua* by plate counts and FCM counts are given in Tables 6 - 8 respectively. *E. coli* cells, with an initial population of 8.70 cfu/ml and 8.50 cfu/ml on TSA and VRBS, respectively, lost culturability after 10 minutes of exposure to acidified minimal media. The percentage of injury increased from 1% to 100% within 10 min of exposure. There was a similar drop in FCM counts for live cells, a drop from 65% initially to 0% within 5 minutes of exposure. The injured cell count increased from 33% at 0 min to 94% after 5 min and dropped

gradually to 86% after 30 min exposure. The percentage of dead cells increased from 2% at 0 min to 14% at 30 min. Acid killed *E. coli* cells are shown in Fig. 1d. Comparatively *S. aureus* was resistant to acid treatment because we were able to recover about 3.07 log CFU/ml and 1.99 log CFU/ml on TSA and BP, respectively, even after a 70 min exposure to acidified minimal media. The staining pattern for acid stressed *S. aureus* are shown in Fig. 2d. The percent of injured cells as assessed by plate counts was 35% at this time. The live count using flow cytometry remained high through out the exposure period. At 0 min, the percentage of cells live was 80 and this dropped to 64% at 30 and 40 min and then increased to 80% at 70 min. The percentage of injured cells and dead cells did not show any significant shift in their populations. The sensitivity of *L. innocua* was comparable to *E. coli*. *L. innocua* lost its culturability within 20 min of exposure to acidic media. When plated on MOX agar, the number of cells went below the detection limit within 10 min, and the percentage of injured cells at this time was 100. The percentage of live count by FCM dropped from 97% at 0 min to 2% by 10 min and to 0% by 25 min. The proportion of injured cells increased from 1% at 0 min to 11% at 5 min and fluctuated around this level and the count was 13% at 30 min. The dead count on the other hand increased with a decrease in the live count. The initial count was 2% and at 30 min, it was 88%. The staining pattern of acid-injured and acid-killed *L. innocua* are shown in Figs. 3b and 3c.

5.4.3 Starvation Stress

The results of starvation stress are given in Tables 9 - 11. *E. coli* survived starvation stress remarkably well, registering a drop of 2 log cfu/ml in their population over a period of 70 days. The percent of injured cells were less than 5% throughout this period. The percentage of live count obtained by flow cytometry was 86% on day 0 and this dropped to 10% on day 7 and remained less than 23% for the next 42 days. It then increased to 40% on day 70. The injured population was 11% on day 0 and increased to 89% on day 7. After this, it fluctuated around this level over the remaining period of the study. The percent of injured cells on day 70 was 57%. There were no changes recorded in the population of dead cells and this was less than 3% over the entire period of incubation. *S. aureus* cells and *L. innocua*

cells were starved for a period of 49 days. At the end of 49 days, the population dropped from 8.12 log cfu/ml to 4.77 log cfu/ml, about 58% of the cells were recovered on both TSA and BP agar. The percentage of injured cells as estimated by plate count was less than 7% at any point of time during this period of starvation. Live counts by FCM dropped from 95% on day 7 to 2% on day 49. There were no changes in the population of injured cells. Percent dead cells increased from 4% on day 0 to 97% on day 49. *L. innocua* was more sensitive to starvation than *E. coli* and *S. aureus*. At the end of 49 days incubation, about 40% of the cells were recovered on TSA and MOX. The percent injury was 15% on day 49. The live count dropped gradually over the entire period of starvation. It dropped from 98% on day 0 to 12% on day 49. The percent injured cells increased from 2% on day 0 to 22% on day 49. Dead cells were 0% on day 0 and increased to 66% on day 49.

5.4.4 Irradiation

E. coli, *S. aureus* and *L. innocua* were irradiated at a final dose of 0, 0.5, 1.0, 2.0, and 5.0 KGy. The results are shown in Tables 12 - 14 and Figs. 4a - 4d. Irradiation did not influence the dual staining pattern of stressed cells. *E. coli* and *S. aureus* lost culturability at a dose of 2.0 KGy on both TSA and on VRBA and BP respectively, while *L. innocua* lost its culturability above 2.0 KGy. *L. innocua*, at 2.0 KGy had about 48% of injured cells by plate count estimations whereas the other two organisms had a very low percent of injured cells (2, 1, and 9% for 0, 0.5, and 1.0 KGy, respectively, for *E. coli* and 1 and 2% for 0.5 and 1.0 KGy, respectively, for *S. aureus*). The percent of live cells dropped from 72 (0 KGy) to 56% at 5.0 KGy, while the percentage of injured cells increased from 26% (0 KGy) to 42% (5.0 KGy). The percentage of dead cells were between 1 and 2% for the entire range of dosage used in this study. *S. aureus* had a similar pattern of staining and the percent of live cells was between 68 and 70% over the entire range of dosage used. The percentage of injured cells was between 22 and 24% while the percentage of dead cells was between 7 and 9%. The percentage of viable *L. innocua* cells at 5.0 KGy was 100%. Injured and dead populations were not observed and the position of the cells with respect to fluorescence did not change.

5.4.5 Resuscitation of Stressed Cells

Stressed cells of *E. coli*, *S. aureus*, and *L. innocua* from various time points were inoculated into MMB, selective media, (VRBB or BPB for *E. coli* or *S. aureus* only), NB, TSB and tryptic soy broth with supplements, 0.5% (w/v) yeast extract and 0.1% (w/v) sodium pyruvate (TSB++). The results of resuscitation of heat stressed cells of *E. coli*, *S. aureus*, and *L. innocua* are presented in Table 15. *E. coli* went below the detection limit after 60 min of exposure to heat when plated on TSA, however, when inoculated into resuscitation broths, the cells were revived and growth was visible by 24 hr in VRBB and NB, and in 18 hr in TSB and TSB++. The control cells of *E. coli* took 24 hr to grow to visible turbidity in MMB while this was in 6 h in TSB and TSB++, and 36 hr for 10 and 20 min exposed cells. MMB was unable to support heat stressed cells after exposing them for 30 min. *S. aureus* and *L. innocua* did not grow in MMB. *S. aureus* exposed to heat for 60 min failed to grow on both solid and liquid media and 50 min exposed cells grew on TSA, took 24 hr to show visible turbidity in TSB and TSB++, and failed to grow in NB and BPP. *L. innocua*, when exposed to heat for 60 min, grew when plated on TSA but failed to grow on MOX agar. When inoculated in resuscitation broth, growth was observed only in TSB and TSB++, after 36 hr when compared to 18 hr for control cells. In general, growth was observed for all three organisms, in the order of TSB++/TSB > SB/NB > MM.

The results of resuscitation of acid stressed cells are given in Table 16. MMB failed to support growth of *S. aureus* and *L. innocua* even for control cells and for *E. coli*, control cells alone grew in MBB. *E. coli* cells exposed to acidic media for more than 5 min, failed to grow in VRBB and NB wheras cells exposed to more than 10 min failed to grow in TSB and TSB++. Similar results were obtained with *L. innocua*. When plated, *E. coli* failed to grow on TSA after 15 min exposure and failed to grow on VRBA, after 10 min exposure. *L. innocua* failed to grow on TSA after 20 min exposure and on MOX, after 10 min exposure. However, 15-min exposed *L. innocua* did not revive in all four liquid broth media. *S. aureus* withstood acidic media even after 70 min exposure and cells were revived on both solid and liquid media (TSA, BPA, BPB, TSB, & TSB++). MMB failed to support the growth of *S. aureus* completely and NB failed to support growth after 40 min exposure to acidified

minimal broth. It took 60 hr to observe turbidity in BPB for 70 min exposed cells while it was 48 hr in TSB and TSB++.

Irradiated *E. coli* and *S. aureus*, at a dose of 2.0 KGy failed to grow in liquid media whereas *L. innocua* grew even after receiving a dose of 2.0 KGy, but failed to grow when the dose was increased to 5.0 KGy. Minimal broth did not support the growth of *S. aureus* and *L. innocua*. For all three organisms, growth in both solid and liquid media was in agreement with relation to dosage used.

5.5 Discussion

A combination of suitable fluorescent stains with flow cytometry could enable us to quantify and study the physiological heterogeneity within a bacterial population. Assessing the physiological state of bacterial population could provide us with valuable information about the viability of cells, which in turn has serious implications in public health microbiology including food microbiology. Flow cytometry and fluorescent dyes for rapid detection of microorganisms in foods have been developed and its use have been successfully demonstrated (Laplace-Builhe *et al.* 1993). Using *E. coli*, *S. aureus*, and *L. innocua*, we obtained good correlations with conventional plate counts and have demonstrated the feasibility of flow cytometry for assaying a variety of bacteria in cultures over the range 10^3 to 10^7/ml. The three organisms chosen, a gram-negative rod, a gram-positive cocci and a gram-positive rod, have wide differences in cell morphology and cell wall characteristics. Though not a representation of the entire microbial group, the successful staining of live cells with the *Bac*Light kit, shows the usefulness of flow cytometry and this fluorescent stain, in rapid detection and quantification of microorganisms. According to the manufacturer, the dye accumulates in both gram-positive and gram-negative species and can be used to discriminate between viable bacteria, with intact plasma membrane, and dead bacteria (Langsrud and Sundheim, 1996).

A particular disadvantage is the detection limit of this equipment. The lowest concentration used in this study was approximately one thousand cells and it has already been reported that the ideal concentration for flow cytometric quantification is 10^6 cells/ml

(Robertson and Button, 1989), although Pinder *et al.* (1990) have successfully assayed bacteria at a concentration of 10^2 cells/ml in pure cultures. We estimated the cell counts in pure culture and this detection limit could raise when quantification is attempted in a natural system. We have found the detection limit to be about 10^5 cells/ml when flow cytometry was used to quantify microbial cells in spoiling meat samples (Venugopal *et al.* 1997b). Figures 1a, 2a, and 3a shows the histograms of live bacterial cells. Each species examined had a different histogram, suggesting a characteristic pattern in the histogram, unique for that bacterial species. Similar differences were also reported in the literature (Pinder *et al.* 1990; Miller and Quarles, 1990), and they are mainly due to the differences in cell morphology and GC content of nucleic acid.

Microbial cells must have an intact plasma membrane for their continued survival and successful growth (Russel *et al.* 1995). The bacterial membrane is assumed to be the primary target for several inactivation treatments applied in the food industry. The most important of these in the context of food spoilage and poisoning are the lowering of water activity, pH and temperature, with high temperature through heating is one of the commonest methods of presering food (Gould, 1995). All of these are likely to affect the structure and function of membranes in bacterial cells. Basically, the *Bac*Light viability kit is a DNA stain . DNA was selected as the target for staining because of its ubiquitous association with living organisms and good stainability (Robertson and Button, 1989). Though membrane is also responsible for such vital functions as the maintenance of proton-motive force, membrane potential dyes such as Rh 123 and $DiOC_6$, they did not give good results with heat, acid and irradiation treated cells (Venugopal *et al.* 1997a).

*Bac*Light viability kit was also used to investigate its ability to stain and distinguish bacteria exposed to heat, acid, starvation, and irradiation, based on their viability. Bacteria were exposed to these stresses in such a way to create a sub-population of injured bacteria and we attempted to use the fluorescent stains to distinguish the population of cells into viable, injured and dead. Heat stressed *E. coli* responded favorably by staining differentially, according to its cell viability. The shift in fluorescence between the control cells and heat stressed cells clearly indicates the permeabilization of the membrane and uptake of

propidium iodide (PI). PI, a nucleic acid marker excluded by intact cells, has been used to label dead cells (MacCoubrey *et al.* 1990). However, the differentiation between injured and dead state was not clear, because majority of the cells took both the stains, and PI, the DEAD stain was unable to replace the green dye (LIVE stain) completely. According to the manufacturer, uptake of PI by dead cells should replace the LIVE stain and thus should fluoresce red. These results also could indicate the presence of an injured sub-population of cells (probably dying) because this was in agreement with our resuscitation studies. Though the cells exposed to heat for 60 min were unable to grow on solid TSA, growth was observed in liquid TSB. The fact that growth was not observed in minimal broth shows that the cells were injured and required complex, nutrient rich media for their resuscitation and growth. Heat stressed *S. aureus* lost its viability completely after 60 minutes of exposure to heat. The viability was confirmed by its inability to revive in resuscitation broth. The histogram of heat stressed *L. innocua* did not change even after the plate count showed a reduction of about 6 log cfu/ml in the population. Thus, the dye uptake is relatively poor for *S. aureus* and *L. innocua* or the degree of heat treatment is not sufficient to permeabilize the membrane, sufficient enough for PI uptake and to replace the LIVE stain.

Acid stressed *E. coli* and *L. innocua* responded remarkably to the dye uptake by a remarkable shift in fluorescence but the response of *S. aureus* was not good enough in comparison with the other two organisms. Acid treatment was more severe as indicated by the results of our plate counts. Again, with *E. coli*, the distinction between dead and injured cells was not clear and there was an overlap in the histogram. In starvation stress, all the three types responded to the dye uptake and we did observe a shift from live region to injured region but it did not move to dead region. The population declined slowly for *E. coli* while it was most rapid for *L. innocua*. The dyes did not prove useful in the case of irradiated cells. Cells irradiated at a dose of 5.0 KGy, stained with the LIVE stain. This is a relatively high dose, inactivating the microbial cells completely and there could be no survivors at this dose when compared to the D-values given for common food borne pathogens (Radomyski *et al.* 1994).

PI was excluded from live cells of all the three organisms studied though there were differences in the position of fluorescence observed. PI was not effective in providing a good discrimination between injured and dead cells for heat, acid and starvation, and completely failed to discriminate irradiated cells. According to the manufacturer, PI, the DEAD stain enters dead cells and binds to the DNA replacing the LIVE stain. Thus the affinity of DEAD stain for DNA is more than the LIVE stain.

The inability of PI to enter the dead cells completely may be due to inadequate damage caused to the cell membrane by these stresses. The molecule is bigger and more positively charged than ethidium bromide and intercalates with DNA and RNA, where fluorescence is enhanced (Ueckert *et al.* 1995). Lopez-Amoros *et al.* (1995) have reported that PI did not provide good discrimination of the sub-populations arising during starvation, indicating that important damage to the cell membrane does not occur during starvation. However, for acid and heat treatments, lesions in cellular membranes are likely to result, increasing the permeability of membranes. For *S. aureus*, lesions to the cellular membranes as a result of sublethal heat treatment have been reported. This was evidenced by the loss of 260- and 280 nm absorbing material as well as Na^+, K^+, and Mg^{++} in the heating medium (Hurst *et al.* 1974; Iandolo and Ordal, 1966). Acid killing has been claimed to result mainly from membrane damage (Rowbury, 1995), and cell membrane damage has been reported in the case of *E. coli* cells exposed to *p*-aminobenzoic acid. In this study, electron micrographs revealed a detached outer membrane and a damaged cytoplasmic membrane have been reported (Ricards *et al.* 1995). Thus, there is adequate evidence in literature for sufficient membrane damage to be caused by heat and acid. The inability of heat stressed *L. innocua* and acid stressed *S. aureus* for PI uptake remains to be studied.

All these stresses also have some form of effect on cellular DNA and RNA, and are essential for staining microbial cells with PI and LIVE stain. Decline in protein, DNA and RNA levels in starving vibrios (Hood *et al.* 1986) and *S. aureus* (Diaper and Edwards, 1994) have been reported. With *E. coli*, damage to DNA, membranes and enzymes, occurs at roughly the same H^+ concentration, and the acid-sensitivity of DNA repair-deficient strains (Sinha, 1986) suggests that damage to DNA is a critical event (Rowbury, 1995). In the case

of irradiation, microorganisms are destroyed by partial or total inactivation of the genetic material, either by its direct effect on DNA or through the production of radicals and ions that attack DNA. An exposure of 0.1 KGy results in 2.8% of the DNA being damaged whereas 0.14% of enzymes and 0.005% of amino acids are altered with the same dose (Diehl, 1990). In our study, irradiation-killed cells failed to stain with PI but they stained with the LIVE stain and it is not known whether degradation of DNA prevented PI binding and the subsequent failure to fluoresce red. Thus further studies are required on stress related DNA damage and availability of it for fluorescent staining. This is particularly important in starvation stress because in our studies, the histogram obtained with all three organisms kept reducing with an increase in starvation time, possibly due to a reduction of cell biomass or reduction in DNA content of the cell material.

The use of selective media in enumeration of stressed bacteria appears to influence the quantification. *L. innocua* was sensitive to MOX agar and this may be due to the inclusion of antibiotic supplement to the media whereas BP agar, though selective for *S. aureus* had minimum inhibitory properties and this could be due to the egg yolk supplement in the media in spite of the presence of tellurite. The selective media did not affect the culturability of starved cells while irradiation and acid treatment resulted in direct death of bacteria, without passing through injured state. It is interesting to note that irradiation destroys microorganisms completely without the possibility of leaving sublethally injured bacteria.

Using flow cytometry, the state of the bacteria can be determined within a few minutes (Langsrud and Sundheim,1996). On the other hand the lower detection limit of flow cytometry is not sensitive enough to replace conventional plate count. Thus, for enumeration of viable microorganisms, traditional plate count is still the benchmark method. Assessment of viability using dyes that depend on membrane integrity is more a function of the nature of stress and membrane permeabilization than viability of bacterial cells. In a practical situation, viability of bacterial cells could be lost with intact membranes as seen with irradiated cells. However, this situation could change with improvements in dye formulations, selective treatments of the samples and multiple staining. Our results indicate

that with fairly simple staining technique flow cytometry could be used to quantitate bacterial cells(> 1000 cells/ml) within an hour. Also, a broad assessment of viability as influenced by heat, acid, and starvation is possible using *Bac*Light viability kit.

5.6 References

1. Ahmed, N.M., and D.E. Conner. 1995. Evaluation of various media for recovery of thermally-injured *Escherichia coli* O157:H7. J. Food Prot. 58: 357-360.

2. Bunduki, M.M.-C., K.J. Flanders, and C.W. Donnelly. 1995. Metabolic and structural sites of damage in heat- and sanitizer-injured populations of *Listeria monocytogenes*. J. Food Prot. 58: 410-415.

3. Chrzanowski, T.H., .D. Crotty, J.G. Hubbard, and R.P. Welch. 1984. Applicability of the fluorescein diacetate method of detecting active bacteria in fresh water. Microbiol. Ecol. 10: 179-185.

4. Diaper, J.P., and C. Edwards. 1994. The use of fluorogenic esters to detect viable bacteria by flow cytometry. J. Appl. Bacteriol. 77: 221-228.

5. Diaper, J.P., and C. Edwards. 1994. Survival of *Staphylococcus aureus* in lake water monitored by flow cytometry. Microbiology 140: 35-42.

6. Flanders, K.J., T.J. Pritchard, and C.W. Donnelyy. 1995. Enhanced recovery of *Listeria* from dairy-plant processing environments through combined use of repair enrichment and selective enrichment/detection procedures. J. Food Prot. 58: 404-409.

7. Gant, V.A., G. Warnes, I. Phillips, and G.F. Savidge. 1993. The application of flow cytometry to the study of bacterial responses to antibiotics. J. Med. Microbiol. 39: 147-154.

8. Gould, G.W. 1995. New Methods of Food Preservation, Blackie. Glasgow and London.

9. Gould, G.W., T. Abee, P.E. Granum, and M.V. Jones. 1995. Physiology of food poisoning microorganisms and the major problems in food poisoning control. Int. J. Food Microbiol. 28: 121-128.

10. Hood, M.A., J.B. Guckert, D.C. White, and F. Deck. 1986. Effect of nutrient deprivation on lipid, carbohydrate, DNA, RNA, and protein levels in *Vibrio cholerae*. Appl. Environ. Microbiol. 52: 788-793.

11. Hurst, A., A. Hughes, and D.L. Collins-Thompson. 1974. Relationship between loss of magnesium and loss of salt tolerance after sub-lethal heating of *Staphyloccocus aureus*. Can. J. Microbiol. 20: 1153-1158.

12. Iandolo, J.J. and Z.J. Ordal. 1966. Repair of thermal injury of *Staphyloccus aureus*. J. Bacteriol. 91: 134-142.

13. Jay, J.M. 1986. Determining microorganisms and their products in foods. p. 97-127. *In* J.M. Jay (ed.), Modern Food Microbiology. Van Nostrand Reinhold, New York, N.Y.

14. Kaprelyants, A.S., and D.B. Kell. 1992. Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. J. Appl. Bacteriol. 72: 410-422.

15. Kaprelyants, A.S., and D.B. Kell. 1993. Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. Appl. Environ. Microbiol. 59: 3187-3196.

16. Kjelleberg, S., K.B.G. Flardh, T. Nystrom, and D.J.W. Moriarty. 1993. Growth limitation and starvation of bacteria, p. 289-320. *In* Aquatic Microbiology, Ford, E.T. (Ed.) , Blackwell Scientific, Oxford.

17. Langsrud, S., and G. Sundheim. 1996. Flow cytometry for rapid assessment of viability after exposure to quaternary ammonium compound. J. Appl. Bacteriol. 81: 411-418.

18. Laplace-Builhe, C., K. Hahne, Y. Tirilly, and J.L. Drocourt. 1993. Application of flow cytometry to rapid microbial analysis in food and drink industries. Biol. Cell 78: 123-128.

19. Lopez-Amoros, R., J. Comas, and J. Vives-Rego. 1995. Flow cytometric assessment of *Escherichia coli* and *Salmonella typhimurium* starvation survival in sea water using rhodamine 123, propidium iodide and oxonal. Appl. Environ. Microbiol. 61: 2521-2526.

20. MacCoubrey, I.C., P.L. Moore, and R.P. Haugland. 1990. Quantitative fluorescence measurements of cell viability (cytotoxicity) with a multi-well plate scanner. J. Cell Biol. 111: 58

21. Martinez, O.V., H.G. Gratzner, T.I. Malinin, and M. Ingram. 1982. The effect of some β-lactam antibiotics on *Escherichia coli* studied by flow cytometry. Cytometry 3: 129-133.

22. Mason, D.J., R. Allman, J.M. Stark, and D.Lloyd. 1994. Rapid estimation of bacterial antibiotic susceptibility with flow cytometry. J. Microscopy 176: 8-16.

23. Mason, D.J., R. Lopez-Amoros, R. Allman, J.M. Stark, and D. Lloyd. 1995. The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. J. Appl. Bacteriol. 78: 309-315.

24. Matsuyama, T. 1984. Staining of living bacteria with rhodamine 123. FEMS Microbiol. Lett. 21: 153-157.

25. McCarthy, S. 1991. Pathogenicity of nonstressed, heat stressed and resuscitated *Listeria monocytogenes* 1A1 cells. Appl. Environ. Microbiol. 57: 2389-2391.

26. McFeters, G.A. 1990. Enumeration, occurrence and significance of injured indicator bacteria in drinking water, p. 478-492. *In* Drinking water Microbiology: Progress and Recent Developments (McFeters, G.A. Ed.), Springer-Verlag, New York.

27. McFeters, G.A., F.P. Yu, B.H. Pyle, P.S. Stewart. 1995. Physiological assessment of bacteria using fluorochromes. J. Microbiol. Meth. 21: 1-13.

28. Miller, J.S., and J.M. Quarles.1990. Flow cytometric identification of microorganisms by dual staining with EITC and PI. Cytometry 11: 669-675.

29. Page, S., and R.G. Burns. 1991. Flow cytometry as a means of enumerating bacteria introduced into soil. Soil Biology and Biochemistry 23: 1025-1028.

30. Porter, J., J. Diaper, C. Edwards, and R. Pickup. 1995. Direct measurements of natural planktonic bacterial community viability by flow cytometry. Appl. Environ. Microbiol. 61: 2783-2786.

31. Pinder, A.C., P.W. Purdy, S.A.G. Poulter, and D.C. Clark. 1990. Validation of flow cytometry for rapid enumeration of bacterial concentrations in pure cultures. J. Appl. Bacteriol. 69: 92-100.

32. Radomyski, T., E.A. Murano, D.G. Olson, and P.S. Murano. 1994. Elimination of pathogens of significance in food by low-dose irradiation: a review. J. Food Prot. 57: 73-86.

33. Ray, B., and D.M. Adams. 1984. Repair and detection of injured microorganisms. p. 112-123. *In* M.L. Speck (ed.). Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, DC.

34. Richards, R.M.E., D.K.L. Xing, and T.P. King. 1995. Activity of *p*-aminobenzoic acid compared with other organic acids against selected bacteria. J. Appl. Bacteriol. 78: 209-215.

35. Robertson, B.R., D.K. Button. 1989. Characterizing aquatic bacteria according to population, cell size, and apparent DNA content by flow cytometry. Cytometry 10: 70-76.

36. Rowbury, R.J. 1995. An assessment of environmental factors influencing acid tolerance and sensitivity in *Escherichia coli*, *Salmonella* spp. and other enterobacteria. Lett. Appl. Microbiol. 20: 333-337.

37. Rozak, D.B., and R.R. Colwell. 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51: 365-376.

38. Russell, A. D. 1991. Injured bacteria: Occurrence and possible significance. Lett. Appl. Microbiol. 12: 3-7.

39. Russell, N.J., R.I. Evans, P.F. ter Steeg, J. Hellemons, A. Verheul, and T. Abee. 1995. Membranes as a target for stress adaptation. Int. J. Food Microbiol. 28: 255-261.

40. Shapiro, H.M. 1990. Cell membrane potential analysis. Methods Cell Biol. 33: 25-35.

41. Sinha, R.P. 1986. Toxicity of organic acids for repair-deficient strains of *Escherichia coli*. Appl. Environ. Microbiol. 51: 1364-1366.

42. Soderstrom, B.E. 1977. Vital staining of fungi in pure cultures and in soil with fluorescein diacetate. Soil Biol. Biochem. 9: 59-63.

43. Thomas, J.A., R.N. Buchsbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopy probes generated in situ. Biochemistry 18: 2210-2218.

44. Ueckert, J., P. Breeuwer, T. Abee, P. Stephens, G. Nebe von Caron, and P.F. ter Steeg. 1995. Flow cytometry applications in physiological study and detection of foodborne microorganisms. Inter. J. Food Microbiol. 28: 317-326.

44. Van Dilla, M., R.G. Langlois, D. Pinkel, D. Yajko, W.K. Hadley. 1983. Bacterial characterization by flow cytometry. Science 220: 620-621.

45. Venugopal, R.J., K. Harkins, and J.S. Dickson. 1997a. Evaluation of certain fluorescent probes to assess bacterial injury caused by heat, acid, and irradiation, using flow cytometery.

46. Venugopal, R.J., K. Harkins, and J.S. Dickson. 1997b. Clarification of meat samples using a simple column filtration and quantification of microbial cells using flow cytometry.

47. Wayne, L.G., D.J. Brenner, R.R. Colwell, P.A.D. Grimont, O. Kandler, M.I. Krichevsky, L.H. Moore, W.E.C. Moore, R.G.E. Murray, E. Stackerbrandt, M.P. Starr, and H.G. Truper. 1987. Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37: 463-464.

Table 1. Comparison of plate counts with FCM counts obtained with *E. coli*, *S. aureus*, and *L. innocua* at five different concentrations.

Concentraions	E. coli		S. aureus		L. innocua	
	Plate count (Log cfu/ml)	FCM count (Log cfu/ml)	Plate count (Log cfu/ml)	FCM count (Log cfu/ml)	Plate count (Log cfu/ml)	FCM count (Log cfu/ml)
I	7.56	7.23	7.53	6.95	7.40	7.00
II	6.70	6.27	6.42	5.71	6.42	6.10
III	5.47	5.33	5.30	4.91	5.27	5.38
IV	4.52	4.53	4.42	4.46	4.26	5.11
V	3.33	4.29	3.11	3.92	3.13	4.69

Values are mean of three replications
Concentrations denote five different levels (based on population) of inoculum used in the study

Table 2. Correlation between percentage of viable cells determined by staining with *Bac*light viability kit using FCM and colony counts on tryptic soy agar.

Organisms	RL	r
E. coli	0.711x + 1.610	0.976
S. aureus	0.673x + 1.585	0.977
L. innocua	0.523x + 2.886	0.973

RL=Regression line ((best fitted strainght line between x and y); y=FCM count, x=plate count
r=correlation coefficient

Table 3. Comparison of counts of heat stressed *E. coli* by tryptic soy agar (TSA), violet red bile agar (VRBA) and flow cytometry.

Time (Min)	TSA (Log cfu/ml)	VRBA (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.6	8.46	1.62	62 (7.60)	35 (7.33)	3 (6.37)	7.81
10	5.03	3.70	26	14 (6.33)	78 (7.28)	8 (6.27)	7.39
20	3.65	2.53	31	3 (5.43)	82 (7.17)	15 (6.43)	7.26
30	2.86	1.91	32	0 (3.15)	82 (7.12)	18 (6.46)	7.21
40	2.62	1.61	39	0 (0)	80 (7.01)	20 (6.40)	7.10
50	1.90	BDL	100	0 (0)	80 (7.02)	20 (6.40)	7.11
60	BDL	BDL	ND	0 (0)	79 (7.09)	21 (6.52)	7.20

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 4. Comparison of counts of heat stressed *S. aureus* by tryptic soy agar (TSA), Baird-Parker agar (BPA) and flow cytometry.

Time (Min)	TSA (Log cfu/ml)	BPA (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.58	8.68	-	89 (7.69)	11 (6.64)	0 (0)	7.74
10	4.54	4.32	5	79 (7.30)	15 (6.57)	6 (6.37)	7.40
20	3.08	2.39	22	59 (7.29)	32 (7.02)	9 (6.48)	7.52
30	2.56	1.96	23	42 (6.51)	38 (6.67)	19 (6.69)	7.21
40	1.97	1.45	26	36 (6.92)	41 (6.99)	22 (6.71)	7.38
50	1.39	BDL	100	32 (6.69)	43 (6.89)	25 (6.62)	7.26
60	BDL	BDL	ND	14 (6.09)	33 (6.70)	53 (6.90)	7.18

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 5. Comparison of counts of heat stressed *L. innocua* by tryptic soy agar (TSA), Modified Oxford agar (MOX) and flow cytometry.

Time (Min)	TSA (Log cfu/ml)	MOX (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.79	8.75	0	97 (8.02)	1 (5.83)	2 (6.33)	8.03
10	5.86	3.54	40	96 (7.92)	1 (6.02)	3 (6.33)	7.94
20	4.19	2.34	44	95 (7.82)	2 (6.09)	3 (6.34)	7.84
30	3.76	1.07	72	94 (7.74)	2 (6.04)	4 (6.35)	7.76
40	3.19	BDL	100	92 (7.48)	2 (5.83)	6 (6.30)	7.52
50	2.46	BDL	100	90 (7.46)	2 (5.86)	8 (6.40)	7.50
60	2.17	BDL	100	87 (7.45)	2 (5.87)	10 (6.50)	7.51

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 6. Comparison of counts of acid stressed *E. coli* by tryptic soy agar (TSA), violet red bile agar (VRBA) and flow cytometry.

Time (Min)	TSA (Log cfu/ml)	VRBA (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.70	8.50	1	65 (7.70)	33 (7.40)	2 (6.33)	7.89
5	5.28	3.25	38	0 (5.08)	94 (7.83)	6 (6.64)	7.85
10	1.57	BDL	100	0 (4.96)	91 (7.79)	9 (6.76)	7.83
15	BDL	BDL	ND	0 (4.15)	89 (7.89)	11 (6.95)	7.93
20	BDL	BDL	ND	0 (4.23)	88 (7.81)	12 (6.94)	7.87
25	BDL	BDL	ND	0 (4.14)	86 (7.82)	14 (7.02)	7.89
30	BDL	BDL	ND	0 (4.30)	86 (7.84)	14 (7.06)	7.91

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 7. Comparison of counts of acid stressed *S. aureus* by tryptic soy agar (TSA), Baird-Parker agar (BPA) and flow cytometry.

Time (Min)	TSA (Log cfu/ml)	BPA (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.46	8.46	0	80 (7.47)	18 (6.81)	2 (5.98)	7.57
10	6.68	6.40	4	70 (7.13)	26 (6.73)	4 (5.90)	7.32
20	5.03	4.84	4	66 (7.04)	30 (6.70)	4 (5.82)	7.22
30	4.61	4.52	2	64 (7.24)	31 (6.80)	5 (5.98)	7.30
40	4.30	4.29	1	64 (7.12)	29 (6.78)	7 (6.09)	7.31
50	3.97	3.33	16	73 (7.04)	20 (6.52)	8 (6.10)	7.23
60	3.30	2.55	23	76 (7.04)	18 (6.41)	6 (5.91)	7.15
70	3.07	1.99	35	80 (7.02)	10 (6.07)	10 (6.06)	7.11

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 8. Comparison of counts of acid stressed *L. innocua* by tryptic soy agar (TSA), Modified Oxford agar (MOX) and flow cytometry.

Time (Min)	TSA (Log cfu/ml)	MOX (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.75	8.39	4	97 (7.82)	1 (5.76)	2 (6.10)	7.83
5	5.10	3.84	25	25 (7.12)	11 (6.75)	63 (7.52)	7.72
10	2.38	BDL	100	2 (6.02)	10 (6.72)	88 (7.68)	7.70
15	1.18	BDL	100	1 (5.56)	9 (6.64)	90 (7.66)	7.70
20	BDL	BDL	ND	1 (5.27)	7 (6.43)	92 (7.54)	7.58
25	BDL	BDL	ND	0 (4.74)	9 (6.58)	91 (7.61)	7.65
30	BDL	BDL	ND	0 (4.81)	13 (6.74)	88 (7.61)	7.66

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 9. Comparison of counts of starved *E. coli* by tryptic soy agar (TSA), violet red bile agar (VRBA) and flow cytometry.

Time (Days)	TSA (Log cfu/ml)	VRBA (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.18	8.09	1	86 (7.42)	11 (6.53)	3 (5.98)	7.81
7	7.87	7.48	5	10 (5.87)	89 (6.81)	1 (4.96)	6.87
14	6.78	6.68	2	14 (5.89)	84 (6.67)	2 (5.07)	6.75
21	6.83	6.63	3	23 (5.98)	75 (6.50)	2 (4.84)	6.63
28	6.24	6.17	1	14 (5.52)	84 (6.29)	2 (4.56)	6.37
35	6.36	6.22	2	11 (5.57)	88 (6.47)	1 (4.48)	6.52
42	6.30	6.16	2	13 (5.18)	86 (6.00)	1 (4.05)	6.07
49	6.33	6.03	5	12 (5.23)	85 (6.21)	3 (4.11)	6.31
56	6.35	6.14	3	26 (5.54)	71 (5.98)	3 (4.59)	6.14
63	6.09	5.82	4	27 (5.19)	71 (5.64)	2 (4.16)	5.80
70	6.20	6.13	1	40 (3.73)	57 (3.90)	3 (2.53)	4.15

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 10. Comparison of counts of starved *S. aureus* by tryptic soy agar (TSA), Baird-Parker agar (BPA) and flow cytometry.

Time (Min)	TSA (Log cfu/ml)	BPA (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.12	8.08	-	86 (7.35)	10 (6.41)	4 (6.12)	7.42
7	8.09	8.12	-	95 (7.61)	0 (5.21)	5 (6.26)	7.63
14	6.27	6.37	-	75 (7.46)	4 (6.00)	21 (6.88)	7.58
21	6.11	5.90	3	28 (6.49)	5 (5.80)	67 (6.90)	7.08
28	5.82	5.39	7	15 (6.01)	5 (5.63)	80 (7.11)	7.11
35	5.36	5.10	5	10 (5.78)	4 (5.11)	86 (6.82)	6.93
42	4.93	4.80	3	2 (5.36)	1 (5.04)	97 (7.02)	7.03
49	4.77	4.56	4	2 (4.21)	1 (3.88)	97 (5.94)	5.95

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 11. Comparison of counts of starved *L innocua* by tryptic soy agar (TSA), Modified Oxford agar (MOX) and flow cytometry.

Time (Min)	TSA (Log cfu/ml)	MOX (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.40	8.36	0	98 (7.81)	2 (6.05)	0 (5.43)	7.82
7	6.52	6.34	3	97 (5.25)	0 (2.79)	3 (3.71)	4.96
14	6.15	5.90	4	85 (4.66)	3 (3.17)	12 (3.80)	4.93
21	5.87	5.82	1	40 (4.99)	15 (4.58)	45 (5.05)	5.58
28	6.14	5.91	4	36 (4.53)	16 (4.11)	48 (5.11)	5.38
35	5.33	4.91	8	20 (4.36)	18 (4.78)	62 (5.28)	5.51
42	4.69	3.85	18	13 (4.27)	18 (4.42)	69 (5.00)	5.16
49	3.86	3.27	15	12 (4.29)	22 (4.54)	66 (5.01)	5.20

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 12. Comparison of counts of irradiated *E. coli* by tryptic soy agar (TSA), violet red bile agar (VRBA) and flow cytometry.

Dose (KGy)	TSA (Log cfu/ml)	VRBA (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.44	8.27	2	72 (7.95)	26 (7.51)	1 (6.14)	8.09
0.5	5.72	5.65	1	73 (7.34)	25 (6.88)	2 (5.64)	7.48
1.0	2.53	2.29	9	67 (7.90)	32 (7.58)	1 (6.12)	8.08
2.0	BDL	BDL	ND	73 (7.91)	25 (7.44)	1 (6.00)	8.05
5.0	BDL	BDL	ND	56 (7.80)	42 (7.68)	1 (6.12)	8.04

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 13. Comparison of counts of irradiated *S. aureus* by tryptic soy agar (TSA), Baird-Parker agar (BPA) and flow cytometry.

Dose (KGy)	TSA (Log cfu/ml)	BPA (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.13	8.16	-	69 (7.63)	22 (7.13)	8 (6.72)	7.79
0.5	6.32	6.28	1	69 (6.97)	23 (6.49)	8 (6.05)	7.13
1.0	3.40	3.32	2	69 (6.29)	22 (5.81)	9 (5.39)	6.45
2.0	BDL	BDL	ND	68 (7.62)	24 (7.17)	8 (6.70)	7.79
5.0	BDL	BDL	ND	70 (7.75)	23 (7.27)	7 (6.76)	7.91

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 14. Comparison of counts of irradiated *L innocua* by tryptic soy agar (TSA), Modified Oxford agar (MOX) and flow cytometry.

Dose (KGy)	TSA (Log cfu/ml)	MOX (Log cfu/ml)	% Injured (Plate count)	FCM Count (% Live)	FCM Count (% Injured)	FCM Count (% Dead)	FCM Count (Total-Log cfu/ml)
0	8.30	8.31	-	99 (8.76)	0 (5.88)	1 (6.45)	8.76
0.5	6.01	5.54	8	100 (8.67)	0 (5.66)	0 (6.26)	8.67
1.0	4.47	4.10	8	100 (8.63)	0 (5.59)	0 (6.20)	8.64
2.0	2.51	1.30	48	100 (8.71)	0 (5.82)	0 (6.26)	8.71
5.0	BDL	BDL	ND	100 (8.67)	0 (5.60)	0 (6.10)	8.67

Numbers in parenthesis denote values in Log cfu/ml.
Values expressed are mean of three replication.
BDL - Below Detection Limit; ND - Not Determined.

Table 15. Resuscitation of heat stressed cells in five different media of varying nutrient composition.

Time (min)	Organism	Media				
		M M	S M	N B	T S B	T S B++
0	*E. coli*	24	12 (VRBA)	12	6	6
	S. aureus	NG	6 (BP)	6	6	6
	L. innocua	NG	ND	24	18	18
10	*E. coli*	36	18 (VRBA)	18	12	12
	S. aureus	NG	18 (BP)	18	18	18
	L. innocua	NG	ND	36	24	24
20	*E. coli*	36	18 (VRBA)	18	18	18
	S. aureus	NG	24 (BP)	24	18	18
	L. innocua	NG	ND	48	24	24
30	*E. coli*	NG	18 (VRBA)	18	18	18
	S. aureus	NG	24 (BP)	24	18	18
	L. innocua	NG	ND	48	24	24
40	*E. coli*	NG	18 (VRBA)	24	18	18
	S. aureus	NG	36 (BP)	36	18	18
	L. innocua	NG	ND	48	24	24
50	*E. coli*	NG	24 (VRBA)	24	18	18
	S. aureus	NG	NG (BP)	NG	24	24
	L. innocua	NG	ND	48	36	36
60	*E. coli*	NG	24 (VRBA)	24	18	18
	S. aureus	NG	NG (BP)	NG	NG	NG
	L. innocua	NG	ND	NG	36	36

Numbers denote the time (hrs) at which growth was observed.
MM - Minimal media; SM - Selective media; NB - Nutrient broth; TSB - Tryptic soy broth;
TSB++ -Tryptic soy broth with 0.5% yeast extract and 0.1% sodium pyruvate.
VRBA - Violet red bile agar; BP - Baird Parker agar.
NG - No growth; ND - Not determined.

Table 16. Resuscitation of acid stressed cells in five different media of varying nutrient composition.

Time (min)	Organism	Media				
		M M	S M	N B	T S B	T S B++
0	*E. coli*	36	18 (VRBA)	18	12	12
	L. innocua	NG	ND	48	18	18
5	*E. coli*	NG	24 (VRBA)	36	18	18
	L. innocua	NG	ND	60	24	24
10	*E. coli*	NG	NG (VRBA)	NG	36	36
	L. innocua	NG	ND	NG	36	36
15	*E. coli*	NG	NG (VRBA)	NG	NG	NG
	L. innocua	NG	ND	NG	NG	NG
20	*E. coli*	NG	NG (VRBA)	NG	NG	NG
	L. innocua	NG	ND	NG	NG	NG
25	*E. coli*	NG	NG (VRBA)	NG	NG	NG
	L. innocua	NG	ND	NG	NG	NG
30	*E. coli*	NG	NG (VRBA)	NG	NG	NG
	L. innocua	NG	ND	NG	NG	NG

Numbers denote the time (hrs) at which growth was observed.
MM - Minimal media; SM - Selective media; NB - Nutrient broth; TSB - Tryptic soy broth;
TSB++ - Tryptic soy broth with 0.5% yeast extract and 0.1% sodium pyruvate.
VRBA - Violet red bile agar; BP - Baird Parker agar.
NG - No growth; ND - Not determined.

Table 17. Resuscitation of acid stressed cells of *S. aureus* in five different media of varying nutrient compostion.

	Media				
Time (min)	**Minimal Media**	**Selective Media (Baird-Parker)**	**Nutrient Broth**	**Tryptic Soy Broth**	**Tryptic soy Broth ++**
0	NG	18	18	12	12
10	NG	24	24	18	18
20	NG	36	36	24	18
30	NG	36	36	24	24
40	NG	48	NG	36	36
50	NG	48	NG	36	36
60	NG	48	NG	36	36
70	NG	60	NG	48	48

NG - No growth
Numbers denote time(hrs) at which growth was observed
++ - supplemented with 0.5% yeast extract and 0.1% sodium pyruvate

Table 18. Resuscitation of irradiated cells in five different media of varying nutrient composition.

Dose (KGy)	Organism	Media				
		M M	S M	N B	T S B	T S B++
0	*E. coli*	18	12 (VRBA)	18	12	12
	S. aureus	NG	18 (BP)	18	12	12
	L. innocua	NG	ND	24	18	18
0.5	*E. coli*	18	18 (VRBA)	18	12	12
	S. aureus	NG	18 (BP)	18	12	12
	L. innocua	NG	ND	36	18	18
1.0	*E. coli*	48	48 (VRBA)	24	18	18
	S. aureus	NG	36 (BP)	24	18	18
	L. innocua	NG	ND	24	12	12
2.0	*E. coli*	NG	NG	NG	NG	NG
	S. aureus	NG	NG	NG	NG	NG
	L. innocua	NG	ND	72	48	48
5.0	*E. coli*	NG	NG	NG	NG	NG
	S. aureus	NG	NG	NG	NG	NG
	L. innocua	NG	NG	NG	NG	NG

Numbers denote the time (hrs) at which growth was observed.
MM - Minimal media; SM - Selective media; NB - Nutrient broth; TSB - Tryptic soy broth;
TSB++ - Tryptic soy broth with 0.5% yeast extract and 0.1% sodium pyruvate.
VRBA - Violet red bile agar; BP - Baird Parker agar.
NG - No growth; ND - Not determined.

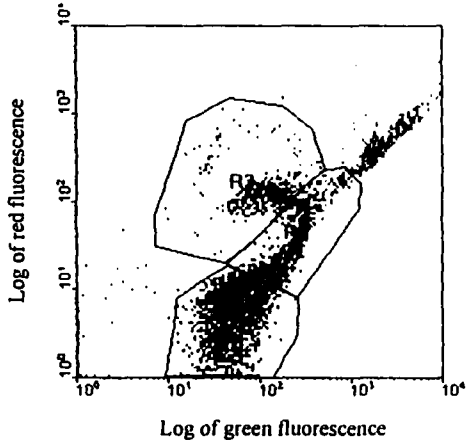

Fig. 1a. Live *E. coli* cells stained with *Bac*Light viability kit. R1=Live; R2=Injured; R3=Dead.

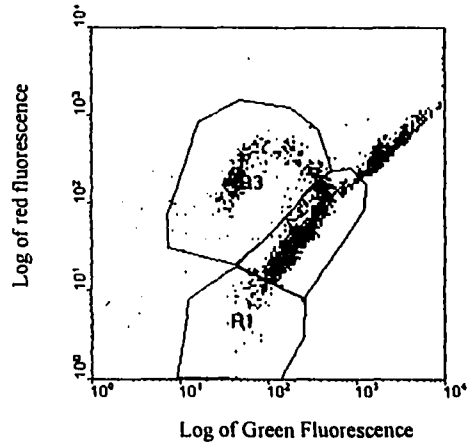

Fig. 1b. *E. coli* cells exposed to 57 C for 20 min & stained with *Bac*Light viability kit.R1=Live; R2=Injured; R3=Dead.

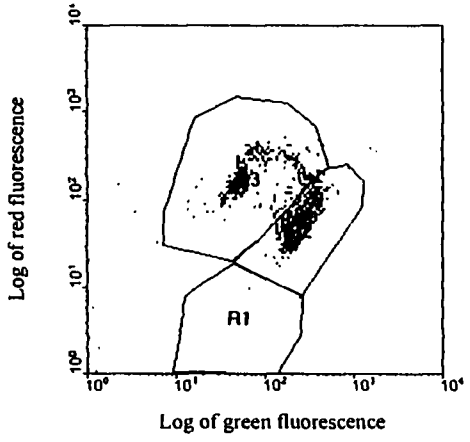

Fig. 1c. *E. coli* cells exposed to 57 C for 60 min & stained with *Bac*Light viability kit. R1=Live; R2=Injured; R3=Dead.

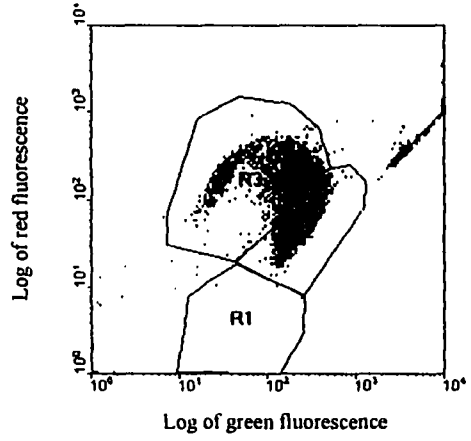

Fig. 1d. *E. coli* cells exposed to acidic meida for 30 min & stained with *Bac*Light viability kit. R1=Live; R2=Injured; R3=Dead.

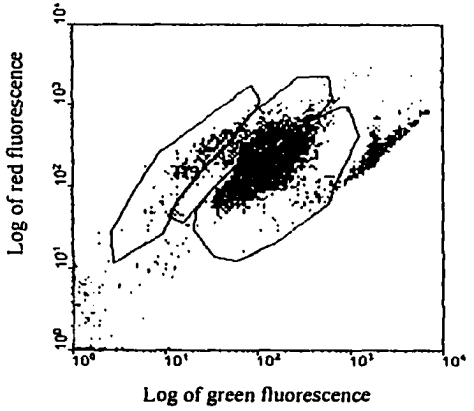

Fig. 2a. Live *S. aureus* cells stained with *Bac*Light viability kit. R1=Live; R2=Injured; R3=Dead.

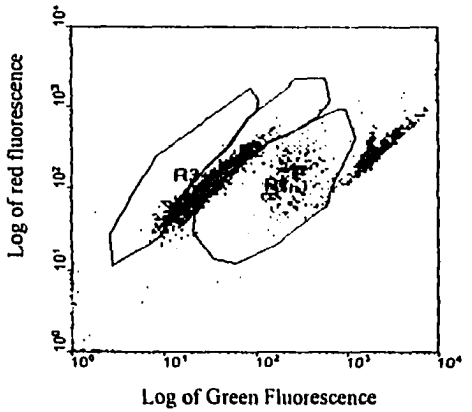

Fig. 2b. *S. aureus* cells exposed to 58 C for 30 min & stained with *Bac*Light viability kit. R1=Live; R2=Injured; R3=Dead.

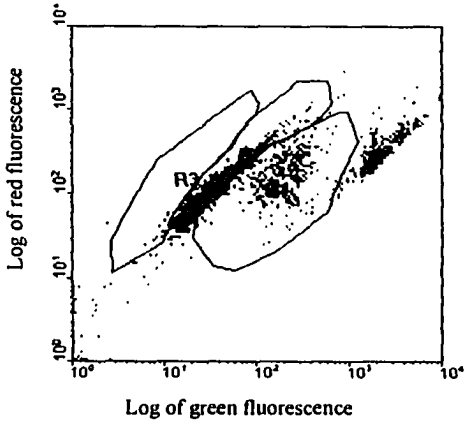

Fig. 2c. *S. aureus* cells exposed to 58 C for 60 min & stained with *Bac*Light viability kit. R1=Live; R2=Injured; R3=Dead.

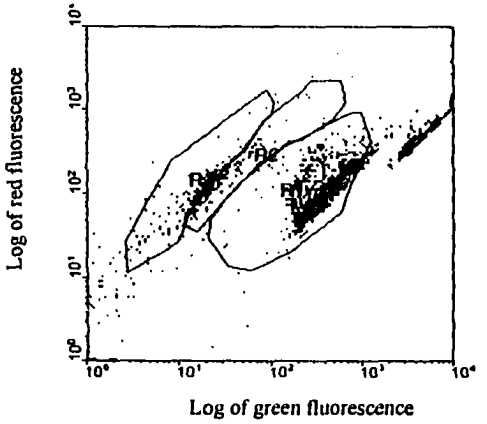

Fig. 2d. *S. aureus* cells exposed to acidic meida for 70 min & stained with *Bac*Light viability kit. R1=Live; R2=Injured; R3=Dead.

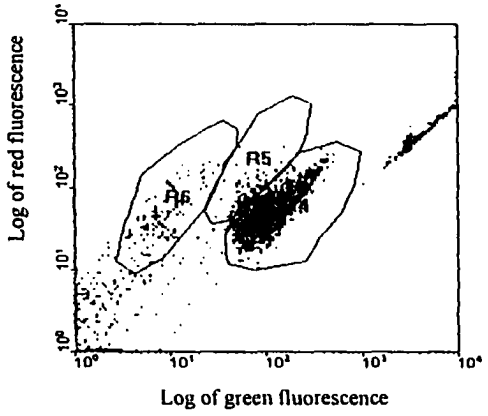

Fig. 3a. Live *L. innocua* cells stained with *Bac*Light viability kit. R4=Live; R5=Injured; R6=Dead.

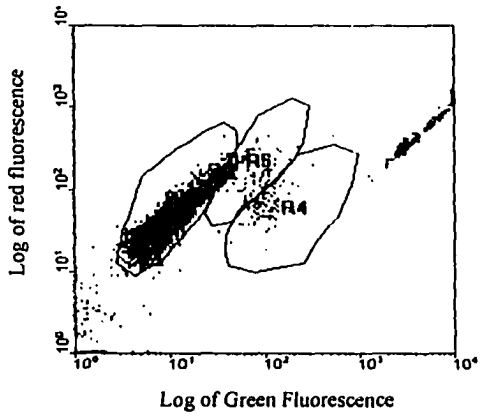

Fig. 3b. *L. innocua* cells exposed to acidic media for 10 min & stained with *Bac*Light viability kit. R4=Live; R5=Injured; R6=Dead.

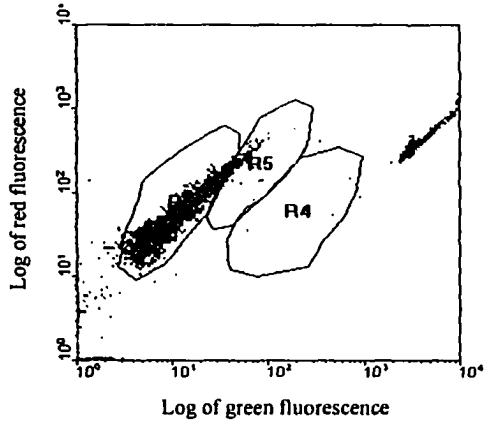

Fig. 3c. *L. innocua* cells exposed to acidic meida for 30 min & stained with *Bac*Light viability kit.R4=Live; R5=Injured; R6=Dead.

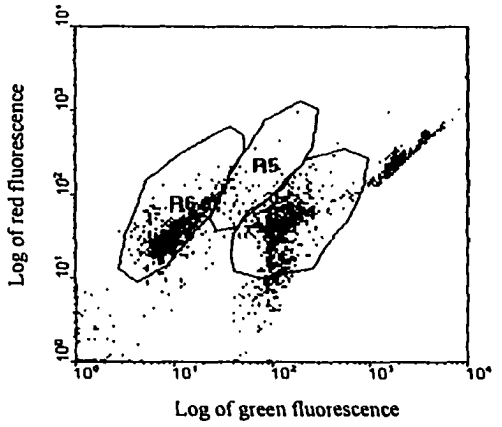

Fig. 3d. *L. innocua* cells exposed to 57 C for 60 min & stained with *Bac*Light viability kit.R4=Live; R5=Injured; R6=Dead.

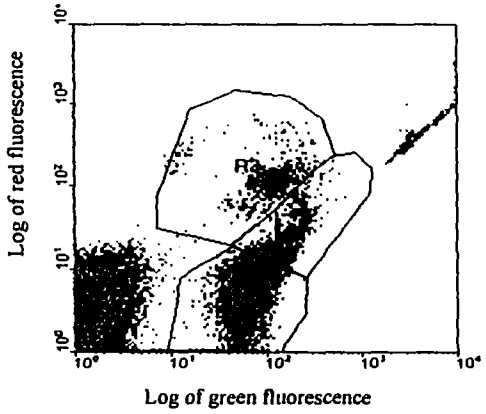

Fig. 4a. *E. coli* cells irradiated at a dose of 1.0 KGy & stained with *Bac*Light viability kit. R1=Live; R2=Injured; R3=Dead.

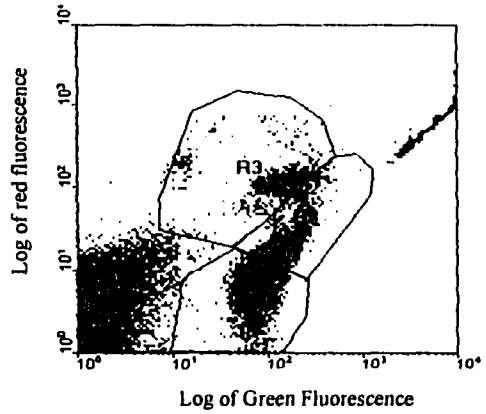

Fig. 4b. *E. coli* cells irradiated at a dose of 5.0 KGy & stained with *Bac*Light viability kit.R1=Live; R2=Injured; R3=Dead.

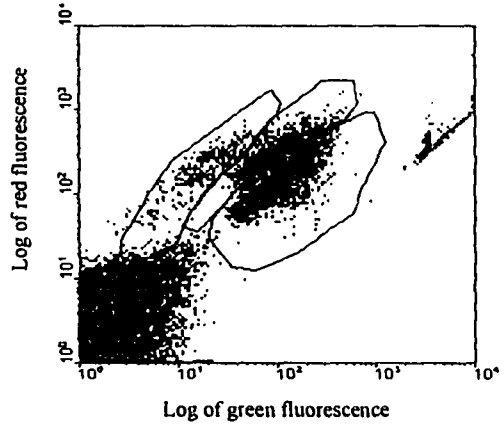

Fig. 4c. *S. aureus* cells irradiated at a dose of 5.0 KGy & stained with *Bac*Light viability kit. R1=Live; R2=Injured; R3=Dead.

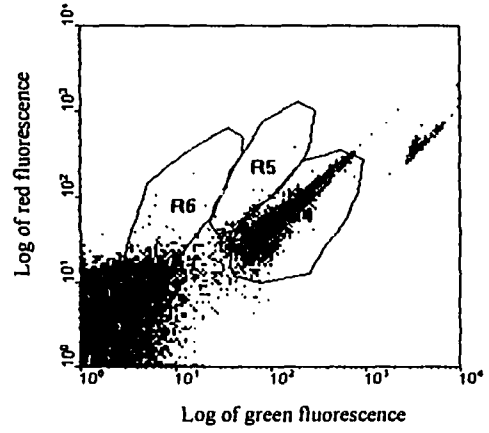

Fig. 4d. *L. innocua* cells irradiated at a dose of5.0 KGy & stained with *Bac*Light viability kit.R4=Live; R5=Injured; R6=Dead.

CHAPTER. 6. CLARIFICATION OF MEAT SUSPENSIONS USING A SIMPLE COLUMN FILTRATION AND QUANTIFICATION OF MICROBIAL CELLS USING FLOW CYTOMETRY.

A paper to be submitted to the Journal of Food Protection

Raveendran Venugopal, Christi Harkins and James Dickson

6.1 Abstract

A simple column filtration was developed to clarify meat homogenate to quantify bacterial populations in pork patties, using flow cytometry. The removal of debris from homogenized meat suspensions was achieved by passing it through different column materials such as sand particles (Sand I and II), glass beads, dowex resin, and cell debris remover (CDR). For column evaluation, total plate counts on trypticase soy agar and lactic acid bacteria count on MRS agar were done on the clarified suspensions. Degree of clarification was studied by measuring the absorbance of the clarified suspensions at 600 nm. Among the materials compared, sand II and glass beads gave more than 90% recovery of bacterial cells. The absorption spectra of the filtered suspensions were considerably less when compared to the initial sample and the best results were obtained with CDR followed by dowex resin.

The clarified suspensions were found suitable for flow cytometric analysis and bacterial cells were successfully gated on a separate region of the histograms. Quantification of cells were achieved by the use of fluorescent microspheres. *Bac*Light viability kit was used to stain the bacterial cells in meat suspensions. The bacterial populations in retail samples (ground beef) were also assayed using flow cytometer.

6.2 Introduction

Quantification of bacterial numbers is important to our understanding of the microbiological quality of food products and this is directly related to its safety and shelf life. Present day food legislations require a rapid and reliable estimate of microbial load of food products. Conventional culture methods for determining total viable counts on meat and meat products take at least 1 day to produce results (Shaw *et al.* 1987). This has aroused considerable interest in developing rapid methods in the meat industry. The main reasons for this are the advantages related to the speedy release of products, and that rapid results in monitoring critical control points offer the possibility for early corrections during production (Qvist and Jakobsen, 1985). Finally, rapid results enable the manufacturers to select raw materials to conform to their microbiological quality standards and also there is a possibility for a quick assessment of meat products following transportation.

In order to overcome the limitations of conventional plate counts, several techniques are now available to the food industry for rapid estimation of bacterial counts in foods. Among the new approaches, the advent of flow cytometry in food microbiology is most recent. Flow cytometry is a specialized form of fluorescence microscopy (Melamed *et al.* 1990) and it permits the examination of biological surfaces of cells when they pass through a bean of excitation light from a laser. This sensitivity and the ability to analyze large numbers of cells in a relatively short period of time (usually in seconds) makes flow cytometer an ideal instrument to obtain rapid microbial counts in a food system. However, visualization and quantification of microorganisms in meat samples using flow cytometer requires sample pretreatment. Often food debris and/or somatic cells can make the counting of microorganisms difficult or impossible. The suspended particles could clog the orifice and prevent the sample flow. Thus, its application is limited, among other reasons, by the filterability of food samples. In majority of cases, food samples require pre-treatment because filterability alone does not guarantee accurate microbial count. For successful analyses, bacteria should be in liquid medium suitable enough to be passed through FCM. Three phases can be distinguished with respect to detection of microorganisms in food (Ueckert *et al.* 1995):

(i) Extraction of microorganims from the food samples

(ii) Fluorescent labeling of the target cells

(iii) Analyses of fluorescently stained cells by FCM

Successful extraction of microorganisms require an efficient clarification and improved filterability of food materials. Sample pretreatment has been widely used in Direct Epifluorescent Filter Technique (DEFT), and milk is one of the foods to which DEFT has been widely applied (Fernandez-Astorga *et al.* 1995). The method described by Pettipher *et al.* (1980) is a traditional pre-treatment method used in improving the filterability of milk samples. This method involves sample incubation at 50°C for 10 minutes with Triton-X and trypsin. A similar method was also used in pretreatment of food samples that included minced meat, frozen peas, natural yoghurt and pre-cooked pasta (Jaeggi *et al.* 1989). Other pre-treatment methods are based on this method and include modifications of the enzyme concentration and/or rinses with organic solvents (Buchrieser and Kaspar, 1993; Tortorella and Gendel, 1993). In all these methods, both surfactant and the incubation period are a constant in spite of the fact that such aggressive treatments could have a negative effect on or destroy the bacterial cells present (Fernandez-Astorga *et al.* 1995). Thus, it would be advantageous to resolve these problems of clarification and filterability without the possibility of cell destruction, since FCM in conjunction with appropriate fluorochromes has the ability to discriminate bacterial cells into live, injured, and dead cells. Under the above mentioned methods, this attribute of the FCM could be compromised.

Labeling of target cells with appropriate stains is another important factor in successful application of FCM in food microbiology. Specificity of the dye is essential because of the nature of food debris and somatic cells which could either mask the fluorescence of cells and/or could interfere with data interpretation through autofluorescence. Hence, the dye should bind only to the target cells and still should not have any affinity to proteins, fat or carbohydrates in food suspensions. Finally, in the analysis of the fluorescently stained cells by FCM, the most important factor is the signal to noise ratio of labeled cells and background (Ueckert *et al.* 1995). The background noise is directly

proportional to the amount of food debris present in the sample and thus successful clarification is central to flow cytometric analyses of food suspensions.

In this study, we have focused upon the development of a clarification method which neither involves digestion of meat homogenate with proteases nor heating. The efficiency of the method is demonstrated by its suitability to be analyzed using FCM and thus obtaining microbial counts in a rapid way.

6.3 Materials and Methods

6.3.1 Meat Samples

The meat samples used in this study were either purchased from Meat Lab at Iowa State University or from a local grocery store..

6.3.2 Microbial Recovery Studies

In order to evaluate the various column materials used in this study, frozen pork patties were thawed overnight in a refrigerator at 4°C and the thawed patties were placed in sterile plastic bags (WHIRL-PAK) and incubated in the same refrigerator at the same temperature. Aerobic plate counts (APC) and lactic acid bacteria counts were done once a day for a period of 3 days including day 0 as the first sampling point. The experiment was stopped at the end of day 3 when the aerobic plate count exceeded 10^6 cells/g of meat samples.

6.3.3 Microbiological Analyses

A 1:10 dilution of meat homogenate was made by placing 11 g of ground pork in a stomacher bag and mixing it with 99 ml of 0.2% (w/v) buffered peptone water (Oxoid) and stomaching it in a stomacher (Stomacher 400 Lab blender, Mark II, Seaward Medical, London) at medium speed for 2 minutes. One milliliter of this homogenate was used for clarification studies, and aerobic (TSA, Difco Laboratories, Detroit, MI) and lactic acid counts (MRS, Difco) were done at every step of the clarification procedure and compared it with the initial plate count. The plates were incubated aerobically at 30°C and the colonies

were counted after 48 hr. The detection limit of our plating scheme was 100 cells or more, and the counts are expressed $\log_{10}$ colony forming units/g.

6.3.4 FCM Analyses of Spoiling Pork Patties

Among the five column materials evaluated, sand II (100 mesh and finer) was found suitable for clarification of meat samples. A 10ml of a 1:10 dilution (processed and prepared as mentioned previously) was clarified using sand II and the clarified samples were used in FCM to visualize and quantify bacterial cells. In this study, APC on TSA alone were done to compare with the results obtained using FCM.

6.3.5 FCM Analyses of Retail Samples

Ground beef (80% lean) was purchased in a local grocery store in the morning and the samples were transported to the laboratory on ice and clarified using sand II immediately. Both, clarification and FCM analyses were done on the purchased samples as described above.

6.3.6 Quantification of Bacterial Cells Using FCM

Bacterial cells were quantified by using fluorescent beads (6μm yellow green polystyrene latex microparticles, Polysciences, Inc., Warrington, PA). A 25 μl of a known concentration (10^5/ml) of the bead suspensions in PBS were inoculated into each sample before FCM analyses. Similar volumes of the same concentration of beads inoculated into 500 μl of PBS served as the control. The beads showed up in a separate region on the histograms and were gated and counted along with bacterial cells but separately. After FCM analyses, the beads in the samples were counted using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). This was done by pipetting 200 μl of FCM-analyzed samples into 10 ml of PBS and the beads were counted. This dilution factor was found suitable to calculate 10^5 beads/ml. The number of bacterial cells were calculated by the following formula:

Number of cells

---------- X Number of beads (Coulter Counter) X Dilution factor = No. of

Number of beads cells/ml

6.3.7 Evaluation of Column Materials

The following column materials were evaluated for their suitability in clarification of meat suspensions:

(a) Sand, white Quartz (-50 +70 mesh), referred as Sand I

(b) Glass Beads (106 μ and finer, acid washed)

(c) Dowex 1 (Ionic form: chloride-strongly basic anion exchanger)

(d) Cell Debris Remover (CDR) (Modified cellulose)

(e) Sand II (100 mesh & finer, acid washed)

Sand I, glass beads and Dowex 1 were purchased from Sigma Chemical Co., (St. Louis, MO) and CDR from Whatman, UK. Sand II was prepared in our lab by grinding Sand I in a pestle and mortar. It was sieved using a 100 mesh sieve followed by three acid washes (10% Nitric acid) and three sterile deionized water washes. It was then dried at 110°C for 12 hr.

Each of the above materials were evaluated individually in a 1 ml Bio-Spin disposable chromatography columns (Bio-Rad Laboratories, CA). The column support provided at the base of each column was removed and two filter paper discs (Whatman 41) were cut to size and were placed one above and one below the column support. The columns were then filled with the various column materials and autoclaved. A 1 ml of meat suspensions (1:10 dilution), after letting it stand for 10 minutes were loaded onto the column and filtered by pulling vacuum through the columns. Absorption of the filtered meat suspensions was measured at 600 nm using a spectrophotometer (Spectronic 601, Milton Roy). After initial filtration, the columns were washed 9 times with 1 ml of sterile deionized water. APC on TSA and lactic acid bacteria on MRS were done on each eluted 1 ml of water. For FCM analyses, 10 ml of meat suspensions (1:10 dilution), after centrifuging at 200 x*g* for 10 minutes were loaded onto 10 ml Bio-Spin disposable chromatography columns. After initial

filtration of the meat suspensions, the filtrates were concentrated by centrifugation followed by filtration through a 60 μm and a 20 μm nylon mesh (Nitex Nylon Mesh, Tetko, Inc., Briarcliff Manor, NY) before FCM analyses.

6.3.8 Clarification of Meat Suspensions for Column Material Evaluation

In order to evaluate column materials used in this study, 1 ml of a 1:10 dilution of meat suspensions were filtered through a 1 ml Bio Rad column filled with respective column materials. The clarification protocol is outlined below:

11 g of ground pork in 99 ml Buffered Peptone Water (BPW)

↓

Homogenization in a stomacher (2 min @ Medium speed)

↓

↓ ⇒⇒ **Initial (Plate Count on TSA & MRS)**

↓

Transferred 10 ml into a centrifuge tube and made to stand for 10 min

↓

↓ ⇒⇒ **After 10 min (Plate Count on TSA & MRS)**

↓

1 ml loaded onto the column with column material and vacuum-filtered

↓

Clarified meat suspension ⇒⇒ **After filtration (Plate Count on TSA & MRS)**

↓

1 ml of sterile deionized water was loaded and the column washed

↓

↓ ⇒⇒ **Wash 1 (Plate Count on TSA & MRS)**

↓

(This procedure was repeated a total of nine times ⇒⇒ **Wash 2 to Wash 9**)

6.3.9 Clarification of Meat Suspensions for Flow Cytometry

For FCM analyses, 10 ml of a 1:10 diluted meat suspension was filtered through a 10 ml Bio Rad column filled with Sand II. The clarification protocol is outlined below:

11 g of ground pork in 99 ml of BPW

↓

Homogenization in a stomacher (2 min @ Medium speed)

↓ ⇒⇒ **Plate Count A**

↓

Transferred 30 ml into a centrifuge tube and centrifuged at 200 x*g* for 10 min

↓ ⇒⇒ **Plate Count B**

↓

10 ml loaded onto a column with Sand II and filtered under vacuum

↓

↓ ⇒⇒ **Plate Count C**

↓

Clarified meat suspension was centrifuged at 12000 rpm for 10 min (the supernatant was discarded and cell pellet retained)

↓

↓

↓

Cell pellet redissolved in 1 ml of phosphate buffered saline and passed sequentially through a 60 μm and a 20 μm nylon mesh

↓

↓ ⇒⇒ **Plate Count D**

↓

FCM ⇒⇒ **Plate Count E**

6.3.10 Flow Cytometry

Flow cytometer (Coulter EPICS XL -MCL) available in the Cell and Hybridoma Facility at Iowa State University was used in this study. The flow cytometer was equipped with a FL - 1 - green filter of 525 nm BP (505 to 545 nm) and FL - 4 - red filter of 675 nm BP (660 to 700 nm). The excitation of the dye was at 488 nm. All 0.5 ml samples were counted for 30 seconds.

6.3.11 Staining Protocol

*Bac*Light Viability Kit (Molecular Probes Inc., OR, USA) was used to stain the bacterial cells. The kit consisted of two stains, LIVE stain and DEAD stain, the DEAD stain being propidium iodide and the active ingredient of the other stain is not known. Both the stains were added at a concentration of 1µl/ml each. The samples were incubated in dark for 15 min before they were analyzed by FCM.

6.3.12 Statistics

Statistical analysis was done using Microsoft Excel software available on Microsoft Office for Windows 95.

6.4 Results

6.4.1 Evaluation of Column Materials

The various column materials, Sand I & II, Glass beads, Dowex resin, and CDR used in this study were evaluated for their ability to retain suspended particulate matter in meat suspensions and at the same time allowed the passage of microorganisms. The colony counts obtained on the initial 1:10 dilution of the meat homogenate was taken as 100 % and the counts obtained during subsequent stages of clarification were compared to the initial count. The results were expressed as percent recovery in comparison to this initial count. Statistical analyses were done on the first three counts (initial, after 10 minutes, and after filtration) to observe any significant differences between these counts. For all five columns, there were no statistically significant differences among the three counts. The results obtained with sand I

(70 mesh) are presented in Table 1. After filtering through the column, the percentage of microbial cells recovered from the original sample (plated on TSA) were 98, 97, 99, and 100% for Days 0, 1, 2, and 3, respectively. On MRS agar, these numbers were 95, 107, 95, and 99% respectively. During the subsequent washes, the population of the cells went below our detection limits after wash 1 for Day 0 and, after wash 2 for Day 1. On Day 2, it was possible to enumerate cells up to wash 5 and for Day 3, the cells were enumerated up to wash 9 and wash 9 had 58% of the cells. A similar trend was observed with counts obtained on MRS agar, where on Day 3 samples, cells were enumerated up to wash 7 which had 53% of initial population.

The results of Table 2 show the percent recovery of cells in meat homogenate, filtered through glass (106 μ & finer) and as enumerated on TSA and MRS. On TSA, the percent recovery after filtration for Day 0, Day 1, Day 2, and Day 3 were 92, 89, 95, and 101%, respectively. On MRS, it was 90, 96, 95, and 98%, respectively. For Day 0, counts on TSA plates went below the detection limits after wash 2 and for Day 1 it was after wash 7 and for Day 3, cells were counted even on wash 9 where the recovery of cells was 63%. When counted on MRS, for Days 0 and 1, we were unable to count the cells after wash 1. For Day 2 the last count obtained was 55% on wash 4 and for Day 3 it was 64% on wash 7. The results obtained with Sand II (100 mesh & finer) are presented in Table 3. The recovery of cells after filtering, as enumerated by TSA showed a percentage recovery of 93, 95, 89, and 93 % for Days 0, 1, 2, and 3, respectively. For MRS, these numbers were 96, 98, 92, and 92 % for Days 0, 1, 2, and 3, respectively. We were able to enumerate cells eluted from the column up to wash 2 for Day 0 (41%), wash 3 for Day 1 (55%), wash 5 for Day 2 (47%) and wash 7 for Day 3(52%). These counts were obtained on TSA. When plated on MRS, the last count for Day 0 was obtained on wash 2 (45%), for Day 1 it was on wash 4 (52%), for Day 2 it was on wash 6 (51%) and wash 8 (40%) for Day 3.

The recovery of microorganism in meat homogenate, filtered through Dowex resin was less compared to sand and glass beads (Table 4). After filtration, aerobic plate counts showed 87, 89, 83 and 84% recovery for Days 0, 1, 2, and 3, respectively. On MRS plates, these numbers were 78, 73, 73, & 81% for Day 0, Day 1, Day 2, & Day 3 respectively. On

TSA, for Day 0, the number of cells went below the detection limit after wash 3 for Day 0, after wash 5 for Day 1, after wash 2 for Day 2 and after wash 5 for Day 3. For MRS, we were unable to enumerate cells on wash 1 for Days 0 and 1, and after wash 1 for Day 2 and after wash 2 for Day 3. The results obtained with cell debris remover are presented in Table 5. The recovery of cells was lowest among all the filter materials evaluated in this study. APC on TSA gave a 0% recovery for Days 0 and 1, for suspensions after filtration. For Day 2 there was 23% recovery and for Day 3 it was 21% recovery after filtration. For MRS, no cells were recovered after filtration for all four days.

The degree of clarification of filtered meat suspensions were quantified by studying the absorbance at 600 nm and the results are presented in Table 6. The average initial absorbance was 1.8 and this slightly reduced to about 1.1 after making the homogenate stand for 10 minutes. Among the materials evaluated , Dowex resin and CDR had the greatest reduction in absorbance (about 90%). For Sand I, the reduction was about 65%, for Sand II and glass beads, it was about 80%.

6.4.2 Quantification of Bacterial Cells in Meat Homogenate Using FCM

The results of direct quantification of bacterial cells in spoiling pork patties are given in Table 7. The initial plate count (count A) on Day 0 was 5.06 log CFU/g. The same sample, after centrifugation had a population of 4.79 (count B) and after filtration through the column, had a population of 4.51 (count C). At the final stage, i.e., after centrifugation-concentration (count D), the population was 5.64. The FCM count was 5.04. For Day 1 sample, the initial count was 5.59 and final count was 6.75, while the FCM count was 5.45. For Day 2 sample, the initial count was 6.45, and the final count was 6.82 and FCM count was 5.60. For Day 3 samples, the initial count was 6.98, final count was 7.61 and FCM count was 6.57. Figure 1 shows the comparison between the initial count and the count obtained through FCM. FCM was successfully used to visualize the bacterial cells in a different region of the histogram (R1), distinct from the fluorescence created by meat debris (Figure 2). Statistically no differences were observed between the plate counts. The correlation coefficient between Count A (initial count) and Count E (FCM count) was 0.97

The validity of column filtration method was tested using meat samples purchased from a retail outlet. The results of this study are presented in Table 8. As described in the above paragraph, a total of 5 counts were obtained on each sample and a total of 10 samples, purchased at various time intervals were used in this study. The retail samples, ground beef, had an initial population between 5.98 to 6.72 log CFU/g. Count D had a maximum population of 7.28 log CFU/g and a minimum population of 6.46. The minimum population obtained with FCM was 5.97 and the maximum was 7.28. Close to 50% of the beads (fluorescent beads used in the standardization of FCM) were lost when they were analyzed in the meat homogenate (data not shown). Hence the meat homogenate was diluted 10 times and analyzed. On these diluted samples, the bead counts were in agreement with the control samples (data not presented) and FCM counts were in agreement with the plate counts, the results of which are also present in Table 8. Comparisons between the initial plate count and FCM counts of retail samples were diluted 10 times and cells were counted by plate count and by FCM, the results of which are shown in Figure. 3b while the histograms of FCM are shown in Figure 4. Statistically no significant differences were observed and the correlation coefficient between Count A (initial count) and Count E (FCM count) was 0.84.

6.5 Discussion

The criteria adopted in evaluating column materials were the degree of clarification of meat homogenate and recovery of microbial cells after clarification. Among the column materials evaluated, Sand II and glass beads were found suitable for clarification of meat homogenate. The results obtained with Sand I was more favorable in terms of microbial recovery but the degree of clarification was less when compared to other column materials used in this study. Particle size of the column materials used is the main reason for efficient clarification. The particle size of the column material influences the retention of suspended debris in food suspensions by controlling the pore size formed in the column bed. Among the column materials used, sand I had the largest particle size (200 to 300 μ) and this may have prevented it from forming a compact bed. This observation is confirmed by the measurements obtained on the light absorption at 600 nm. Sand II and glass beads were both

equally good, the main reason attributed to this is due to finer particle size of the beads. Glass beads were 106 μ and finer according to manufacturer's information and Sand II beads were 149 μ and finer. A 100 mesh sieve will retain particles that are 150 μ and bigger (manufacturer's information). This gave a better compaction in the column which in turn resulted in a smaller pore size formed. As a result of this, a lot of suspended debris were retained by the column. Again, we were able to confirm this observation through light absorbance of the clarified meat suspension. At the same time the recovery of bacterial cells was good and agreed with the initial plate counts.

The other two column materials, Dowex resin and CDR were not suitable for use in clarification. Though both gave good results in retaining suspended debris, the microbial recovery was poor when compared to the other three materials used. Dowex resin is a strongly basic anion exchanger. It is possible to have influenced bacterial passage through the column, because bacterial cells are known to be associated with a net negative charge on their cell surface. Dowex resins are particularly used to estimate the relative negative charge associated with bacterial cell surface (Pederson, 1980; Dickson and Koomaraie, 1989). CDR, due to its extremely fine particles, gave very good results in clarification, but at the same time, retained microbial cells. As claimed by the manufacturer and as the name indicates, CDR is used to retain cell debris in the purification of cellular DNA and RNA by a different procedure. Thus, it is not surprising to note that cells are being held back by this column material. The particle size of CDR is not known and it appears to have extremely fine particles, smaller than all the other four column materials used.

In evaluation of column materials, it was also observed that the retention of cells by the column materials was population dependent. Higher the population, greater is the retention of cells. This was confirmed by eluting the column materials with sterile deionized water and enumerating the bacterial cells in the eluted water. The Day 3 samples which had the maximum population, bacterial cells were enumerated even after washing the column nine times. The degree of clarification was objectively quantified by simple measurements of light absorbance. This gave us another means of evaluating the efficiency and suitability of the column materials used in this study. Though the onset of spoilage influenced the

microbial retention by the columns, it did not influence the clarification process. Changes were not seen in the absorption spectra obtained within each column material. It was initially thought that clarification would become easier with the onset of spoilage due to increased proteolysis. The data obtained with sand and glass beads indicate the absence of any non-specific attachment of bacterial cells to the column materials, because we were able to wash the cells from the column materials when the population was low. The two column materials, Sand II and glass beads could be used for clarification without compromising on microbial counts. However, with an increase in population, it appears that a certain percentage of cells adhere to the column materials in spite of repeated washings. This may not be a concern because no significant differences were observed between the initial population and the population enumerated after clarification.

The main objective of this clarification procedure was to make the meat homogenate suitable for use in flow cytometry to quantify the bacterial population. A method for rapid assessment of microbial load is a major requirement in several areas of microbiology including public health, clinical sector and food industry. Flow cytometry measures physical or chemical characteristics of individual cells as they move in fluid stream past optical or electronic sensors (Diaper *et al.* 1992). The ability to rapidly and precisely detect, characterize and identify cells in a mixed population demonstrates its potential as a tool for the analyses of microbial populations (Shapiro, 1990) in natural environments. In this study, flow cytometry was successfully used in rapid detection of microbial cells in meat homogenate. Meat homogenate, in liquid form, as such is not suitable for analysis in FCM. The orifice that permits the flow of liquid in the FCM has an exclusion limit of particles that are 80-100 μ or greater and hence the samples should be clear of any floating debris larger than 100 μ. An additional advantage of this clarification procedure is that the viability of cells is not affected. This could pave the way for differentiating microbial populations based on their viability, directly in a naturally spoiling food.

The histograms obtained clearly showed the bacterial cells and the meat debris in separate regions making it possible to visualize bacterial cells in the sample. The appearance of bacterial cells in the histogram indicates that they are predominantly stained with the green

dye and showed up as log of green fluorescence in the histogram. According to the manufacturer, green fluorescence indicates live cells and red fluorescence indicates dead cells. The dye used is a two component dye and the composition of one alone is known, which is propidium iodide, a nucleic acid stain (dead cells are permeable to propidium iodide while live cells are impermeable). Initial plate counts were in agreement with the counts obtained by FCM and no significant differences were observed between the two counts. However, count D, the population of concentrated cells was considerably higher than both the initial plate count and the FCM count. This concentration step was mainly introduced in the clarification scheme to offset any possible loss in numbers of bacterial cells and to obtain a count comparable to the initial plate count. From the data it is obvious that some cells escaped detection by the detector. Further, about 40-50% of the inoculated fluorescent beads were lost in counting. The reason could be due to the presence of fluorescing somatic cells of muscle tissues which have the capacity to mask fluorescence of the beads and cells. This hypothesis was tested by diluting the final samples ten times and we were able to recover the beads and the counts agreed with the bead count obtained in the control samples. The plate counts were also in agreement. The fluorescence of somatic cells and fragments that escaped through the column was a major problem in the study. The stains used were both nucleic acid stains and thus bound non-specifically to both bacterial cells and the tissue fragments with DNA. Thus, improvements in fluorescent dyes could improve the efficiency of FCM counting.

Flow cytometry is being successfully used in aquatic environments (Robertson and Button, 1989; Button and Robertson, 1989; Simon *et al.* 1995). However, the outcome of this study has highlighted certain limitations of flow cytometric analyses of food samples. The ideal population to be measured by FCM is about 10^6 cells/ml (Robertson and Button, 1989). Fresh meat, processed through proper slaughtering procedure, usually is low in microbial numbers and may not be suitable for FCM analyses. The initial microbial population on fresh pork patties, though low in numbers, were quantified by plate counts but not with FCM. However, FCM could be effectively used in the analyses of processed food samples which invariably have a population of 10^6 cells/g. This was clearly demonstrated in

the analyses of retail ground beef by FCM. Another factor, important in the use of FCM is the need to keep the ratio of cells to stain concentration, a constant. It was demonstrated with DAPI (4',6-diamidino-2-phenylindole) that a significant amount of this stain was removed by the cells from the stain solution, and fluorescence became a function of biomass (Robertson and Button, 1989). In food samples, the microbial population varies widely and may or may not contain adequate numbers for successful quantification.

Pre-treatment and clarification of food homogenate are crucial steps in techniques used for direct enumeration of bacteria. Inert column materials such as sand particles and glass beads proved effective when compared with the other materials used in this study and sufficient clarification could be obtained with simple column filtration using these materials of suitable particle size. Flow cytometry offers a means to obtain direct, rapid microbial counts in a non-destructive way on clarified meat suspensions. The ability of FCM to assess microbial populations on a cell-by-cell basis provides us with a tool to probe the viability of the population. The limitations realized in this study could be overcome with improvements in fluorescent stains and with the availability of simple methods to separate prokaryotic cells from eukaryotic cells. As a tool for discriminating among bacterial cells based on viability, it should be possible to study the efficacy of anitmicrobial treatments in a rapid way. As demonstrated, the potential for rapid quantification of microbial loads in retail samples is enormous.

6.6 References

(1) Buchrieser, C., and C.W. Kaspar.1993. An improved direct viable count for the enumeration of bacteria in milk. Int. J. Food Microbiol. 20: 227-236.

(2) Button, D.K., and B.R. Robertson. 1989. Kinetics of bacterial processes in natural aquatic systems based on biomass as determined by high-resolution flow cytometry. Cytometry 10: 558-563.

(3) Diaper, J.P., K. Tither, and C. Edwards. 1992. Rapid assessment of bacterial viability by flow cytometry. Appl. Microbiol. Biotechnol. 38: 268-272.

(4) Dickson, J.S., and M. Koomaraie. 1989. Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. Appl. Environ. Microbiol. 55: 832-836.

(5) Fernandez-Astorga, A., M.J. Hijarrubia, B. Lazaro, and I. Barcina. 1995. A useful and rapid method to recover bacterial cells from milk samples for microscopic count. J. Microbiol. Meth. 24: 111-115.

(6) Jaeggi, N. E., R. Brunner, and W. Schmidt-Lorenz. 1989. DEFT-Technique in the analysis of foods. 1. Pretreatment and enzymatic digestion. Acta Alimentaria 18: 71-77.

(7) Melamed, M.R., T. Lindmo, and M.L. Mendelsohn. 1990. Flow cytometry and sorting. John Wiley and Sons, New York.

(8) Pederson, K. 1980. Electrostatic interaction chromatography, a method for assaying the relative surface charge, on bacteria. FEMS Microbiol. Lett. 12: 365-367.

(9) Pettipher, G.L., R. Mansell, C.H. Mckinnon, and C.M. Cousins. 1980. Rapid membrane filtration-epifluorescent microscopy technique for direct enumeration of bacteria in raw milk. Appl. Environ. Microbiol. 39: 423-429.

(10) Qvist, S.H., and M. Jakobsen. 1985. Application of the direct epifluorescent filter techniques as a rapid method in microbiological quality assurance in the meat industry. Int. J. Food Microbiol. 2: 139-144.

(11) Robertson, B.R., and D.. Button. 1989. Characterizing aquatic bacteria according to population, cell size, and apparent DNA content by flow cytometry. Cytometry 10: 70-76.

(13) Shapiro, H.M. 1990. Flow cytometry in laboratory microbiology: new directions. ASM News 56: 584-588.

(14) Shaw, B.G., C.D. Harding, W.H. Hudson, and L. Farr. 1987. Rapid estimation of microbial numbers on meat and poultry by the direct epifluorescent filter technique. J. Food Prot. 50: 652-657.

(15) Simon, N., N. LeBot, D. Marie, F. Partensky, and D. Vaulot. 1995. Fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. Appl. Environ. Microbiol. 61: 2506-2513.

(16) Tortorello, M.L., and S.M. Gendel. 1993. Fluorescent antibodies applied to direct epifluorescent filter technique for microscopic enumeration of *Escherichia coli* O157:H7 in milk and juice. J. Food Prot. 56: 672-677.

(17) Ueckert, J., P. Breeuwer, T. Abee, P. Stephens, G. Nebe von Caron, and P.F. ter Steeg. 1995. Flow cytometry applications in pysiological study and detection of foodborne microorganisms. Int. J. Food Microbiol. 28: 317-326.

Table 1. Percent recovery of microorganisms in meat homogenate of spoiling pork patties filtered through sand (70 mesh) columns.

	Aerobic Plate Count on TSA				Aerobic Plate Count on MRS agar			
Steps	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**
Initial	100 (3.64)	100 (3.81)	100 (4.72)	100 (5.89)	100 (2.79)	100 (2.92)	100 (4.06)	100 (4.97)
After 10	99 (3.62)	99 (3.79)	101 (4.77)	101 (5.95)	96 (2.68)	102 (2.97)	98 (3.98)	100 (4.97)
After Filt.	98 (3.56)	97 (3.71)	99 (4.68)	100 (5.92)	95 (2.66)	107 (3.13)	95 (3.87)	99 (4.93)
Wash 1	68 (2.47)	71 (2.70)	80 (3.79)	91 (5.35)	79 (2.20)	75 (2.20)	77 (3.13)	87 (4.32)
Wash 2	-	59 (2.23)	62 (2.92)	76 (4.47)	-	-	60 (2.42)	75 (3.74)
Wash 3	-	-	53 (2.50)	69 (4.08)	-	-	49 (2.00)	66 (3.30)
Wash 4	-	-	50 (2.35)	69 (4.04)	-	-	-	60 (2.99)
Wash 5	-	-	50 (2.37)	65 (3.84)	-	-	-	56 (2.77)
Wash 6	-	-	-	60 (3.49)	-	-	-	59 (2.92)
Wash 7	-	-	-	60 (3.51)	-	-	-	53 (2.64)
Wash 8	-	-	-	60 (3.51)	-	-	-	-
Wash 9	-	-	-	58 (3.44)	-	-	-	-

Values are expressed as percentage of cell count which are mean of three replication
Numbers in parenthesis denote cell count in log cfu/ml
After 10 - After 10 min standing; After Filt. - After filtration through columns

Table 2. Percent recovery of microorganisms in meat homogenate of spoiling pork patties filtered through glass beads (100 μ and finer) columns.

	Aerobic Plate Count on TSA				Aerobic Plate Count on MRS agar			
Steps	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**
Initial	100 (3.64)	100 (3.81)	100 (4.72)	100 (5.89)	100 (2.79)	100 (2.92)	100 (4.06)	100 (4.97)
After 10	100 (3.65)	99 (3.79)	101 (4.77)	101 (5.95)	96 (2.68)	102 (2.97)	98 (3.98)	100 (4.97)
After Filt.	92 (3.34)	89 (3.38)	96 (4.52)	100 (5.92)	90 (2.51)	96 (2.81)	93 (3.85)	98 (4.87)
Wash 1	76 (2.75)	79 (3.02)	88 (4.16)	94 (5.57)	81 (2.27)	78 (2.27)	81 (3.29)	90 (4.45)
Wash 2	59 (2.15)	61 (2.34)	72 (3.39)	82 (4.83)	-	-	68 (2.76)	76 (3.80)
Wash 3	-	58 (2.23)	65 (2.90)	78 (4.46)	-	-	53 (2.15)	73 (3.65)
Wash 4	-	-	61 (2.78)	72 (4.23)	-	-	55 (2.24)	70 (3.50)
Wash 5	-	-	59 (2.56)	68 (4.00)	-	-	-	65 (3.25)
Wash 6	-	-	54 (2.56)	66 (3.88)	-	-	-	63 (3.12)
Wash 7	-	-	50 (2.37)	65 (3.81)	-	-	-	64 (3.17)
Wash 8	-	-	-	65 (3.82)	-	-	-	-
Wash 9	-	-	-	63 (3.69)	-	-	-	-

Values are expressed as percentage of cell count which are mean of three replication
Numbers in parenthesis denote cell count in log cfu/ml
After 10 - After 10 min standing; After Filt. - After filtration through columns

Table 3. Percent recovery of microorganisms in meat homogenate of spoiling pork patties filtered through sand (100 mesh and finer) columns.

	Aerobic Plate Count on TSA				Aerobic Plate Count on MRS agar			
Steps	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**
Initial	100 (3.67)	100 (4.32)	100 (5.43)	100 (6.86)	100 (3.00)	100 (3.68)	100 (4.20)	100 (4.79)
After 10	100 (3.66)	98 (4.23)	97 (5.26)	100 (6.87)	100 (3.01)	98 (3.60)	97 (4.07)	100 (4.77)
After Filt.	93 (3.41)	95 (4.10)	89 (4.83)	93 (6.37)	96 (2.88)	98 (3.59)	92 (3.86)	92 (4.40)
Wash 1	63 (2.31)	90 (3.88)	61 (3.31)	90 (6.17)	46 (1.38)	86 (3.16)	92 (3.86)	80 (3.83)
Wash 2	41 (1.50)	72 (3.11)	56 (3.04)	83 (5.69)	45 (1.35)	63 (2.31)	81 (3.40)	79 (3.78)
Wash 3	-	55 (2.37)	51 (2.76)	75 (5.14)	-	50 (1.84)	66 (2.77)	70 (3.35)
Wash 4	-	-	52 (2.82)	70 (4.80)	-	52 (1.91)	60 (2.52)	64 (3.06)
Wash 5	-	-	47 (2.55)	69 (4.73)	-	-	55 (2.31)	65 (3.11)
Wash 6	-	-	-	60 (4.11)	-	-	51 (2.14)	56 (2.68)
Wash 7	-	-	-	52 (3.56)	-	-	-	42 (2.01)
Wash 8	-	-	-	-	-	-	-	40 (1.91)
Wash 9	-	-	-	-	-	-	-	-

Values are expressed as percentage of cell count which are mean of three replication
Numbers in parenthesis denote cell count in log cfu/ml
After 10 - After 10 min standing; After Filt. - After filtration through columns

Table 4. Percent recovery of microorganisms in meat homogenate of spoiling pork patties filtered through Dowex resin columns.

	Aerobic Plate Count on TSA				Aerobic Plate Count on MRS agar			
Steps	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**
Initial	100 (3.82)	100 (4.21)	100 (5.43)	100 (6.61)	100 (3.14)	100 (3.16)	100 (3.96)	100 (4.57)
After 10	101 (3.89)	101 (4.25)	102 (5.57)	99 (6.54)	101 (3.16)	101 (3.18)	102 (4.05)	99 (4.56)
After Filt.	87 (3.34)	89 (3.74)	83 (4.50)	84 (5.56)	78 (2.44)	73 (2.30)	73 (2.91)	81 (3.68)
Wash 1	68 (2.59)	63 (2.64)	55 (3.01)	67 (4.45)	-	-	52 (2.05)	61 (2.81)
Wash 2	61 (2.32)	67 (2.83)	39 (2.13)	52 (3.43)	-	-	-	44 (2.00)
Wash 3	61 (2.32)	62 (2.62)	-	48 (3.18)	-	-	-	-
Wash 4	-	57 (2.41)	-	39 (2.59)	-	-	-	-
Wash 5	-	55 (2.31)	-	32 (2.10)	-	-	-	-
Wash 6	-	-	-	-	-	-	-	-
Wash 7	-	-	-	-	-	-	-	-
Wash 8	-	-	-	-	-	-	-	-
Wash 9	-	-	-	-	-	-	-	-

Values are expressed as percentage of cell count which are mean of three replication
Numbers in parenthesis denote cell count in log cfu/ml
After 10 - After 10 min standing; After Filt. - After filtration through columns

Table 5. Percent recovery of microorganisms in meat homogenate of spoiling pork patties filtered through columns loaded with cell debris.

	Aerobic Plate Count on TSA				Aerobic Plate Count on MRS agar			
Steps	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**
Initial	100 (3.11)	100 (3.93)	100 (5.26)	100 (6.11)	100 (2.86)	100 (3.11)	100 (3.83)	100 (4.29)
After 10	102 (3.16)	101 (3.92)	98 (5.17)	99 (6.07)	102 (2.92)	100 (3.10)	98 (3.77)	99 (4.26)
After Filt.	-	-	38 (2.00)	33 (2.00)	-	-	-	-
Wash 1	-	-	-	33 (2.00)	-	-	-	-
Wash 2	-	-	-	-	-	-	-	-
Wash 3	-	-	-	-	-	-	-	-
Wash 4	-	-	-	-	-	-	-	-
Wash 5	-	-	-	-	-	-	-	-
Wash 6	-	-	-	-	-	-	-	-
Wash 7	-	-	-	-	-	-	-	-
Wash 8	-	-	-	-	-	-	-	-
Wash 9	-	-	-	-	-	-	-	-

Values are expressed as percentage of cell count which are mean of three replication
Numbers in parenthesis denote cell count in log cfu/ml
After 10 - After 10 min standing; After Filt. - After filtration through columns

Table 6. Absorbance of 1:10 dilution of meat homogenate before and after clarification using different column materials at 600 nm.

Days	Initial	After 10 min.	Sand I	Glass	Sand II	Dowex Resin	Cell Debris Remover
Day 0	1.941	1.089	0.672	0.416	0.455	0.136	0.126
Day 1	1.760	1.142	0.663	0.451	0.497	0.111	0.183
Day 2	1.798	1.142	0.726	0.488	0.404	0.263	0.211
Day 3	1.968	1.241	0.730	0.372	0.451	0.172	0.191

Table 7. Comparison of plate counts on TSA, done during the different stages of clarification of spoiling pork patties stored at 4°C, and counts obtained with FCM.

Days	Count A	Count B	Count C	Count D	Count E
Day 0	5.06	4.79	4.51	5.64	5.04
Day 1	5.59	5.41	5.29	6.75	5.45
Day 2	6.45	6.39	6.07	6.82	5.60
Day 3	7.65	7.40	7.42	8.55	6.64

Count A = Initial count; Count B= After 10 minute-standing;
Count C = After filtering through the column;
Count D = After concentrating the cells; Count E = FCM counts

Table 8. Comparison of plate counts on TSA, done during the different stages of clarification of ground beef obtained at a retail outlet, and counts obtained with FCM.

Samples	Count A	Count B	Count C	Count D	Count E	Count D (1:10)	Count E (1:10)
Sample 1	6.03	5.6	5.39	6.46	5.97	5.01	5.41
Sample 2	5.98	5.73	5.42	6.61	6.11	5.24	5.67
Sample 3	6.21	6.02	5.66	7.00	6.39	5.44	6.13
Sample 4	6.16	5.91	5.73	7.36	5.98	5.63	6.41
Sample 5	6.56	6.23	5.86	7.51	6.03	5.71	6.65
Sample 6	6.72	6.58	6.51	7.08	7.19	6.46	6.65
Sample 7	6.85	6.48	6.54	7.05	7.28	6.52	6.49
Sample 8	6.62	6.51	6.40	7.05	7.22	6.45	6.56
Sample 9	6.60	6.50	6.69	7.18	7.19	6.50	6.58
Sample 10	6.72	6.87	6.55	7.30	7.21	6.50	6.68

Count A = Initial count; Count B= After 10 minute-standing;
Count C = After filtering through the column;
Count D = After concentrating the cells; Count E = FCM counts

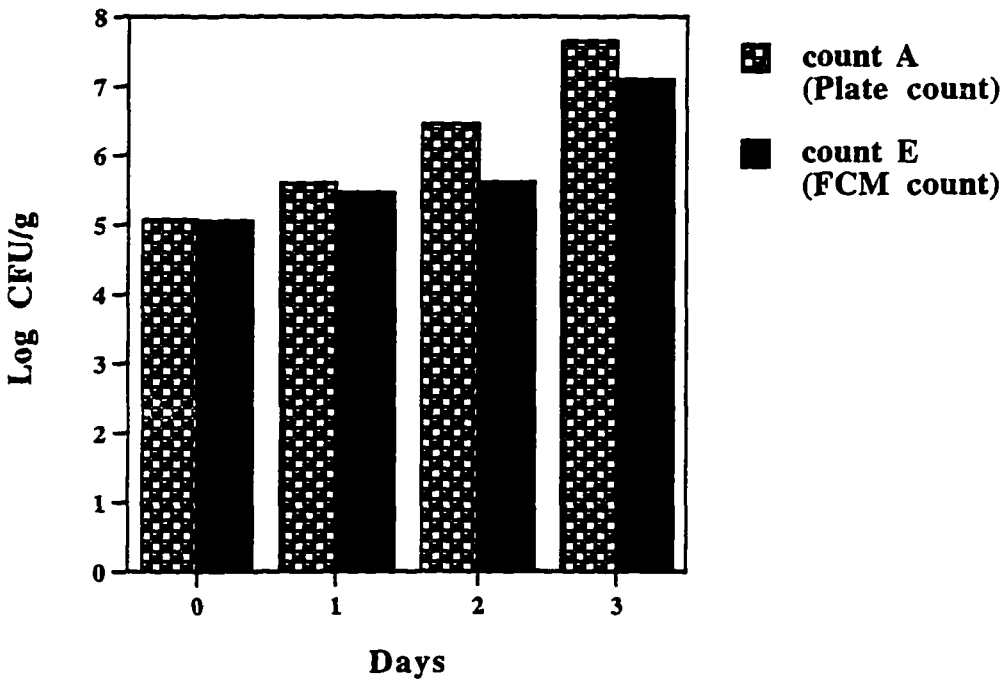

Fig. 1. Comparison of plate counts with FCM counts on spoiling pork patties stored aerobically at 4 °C. Values are mean of three replication.

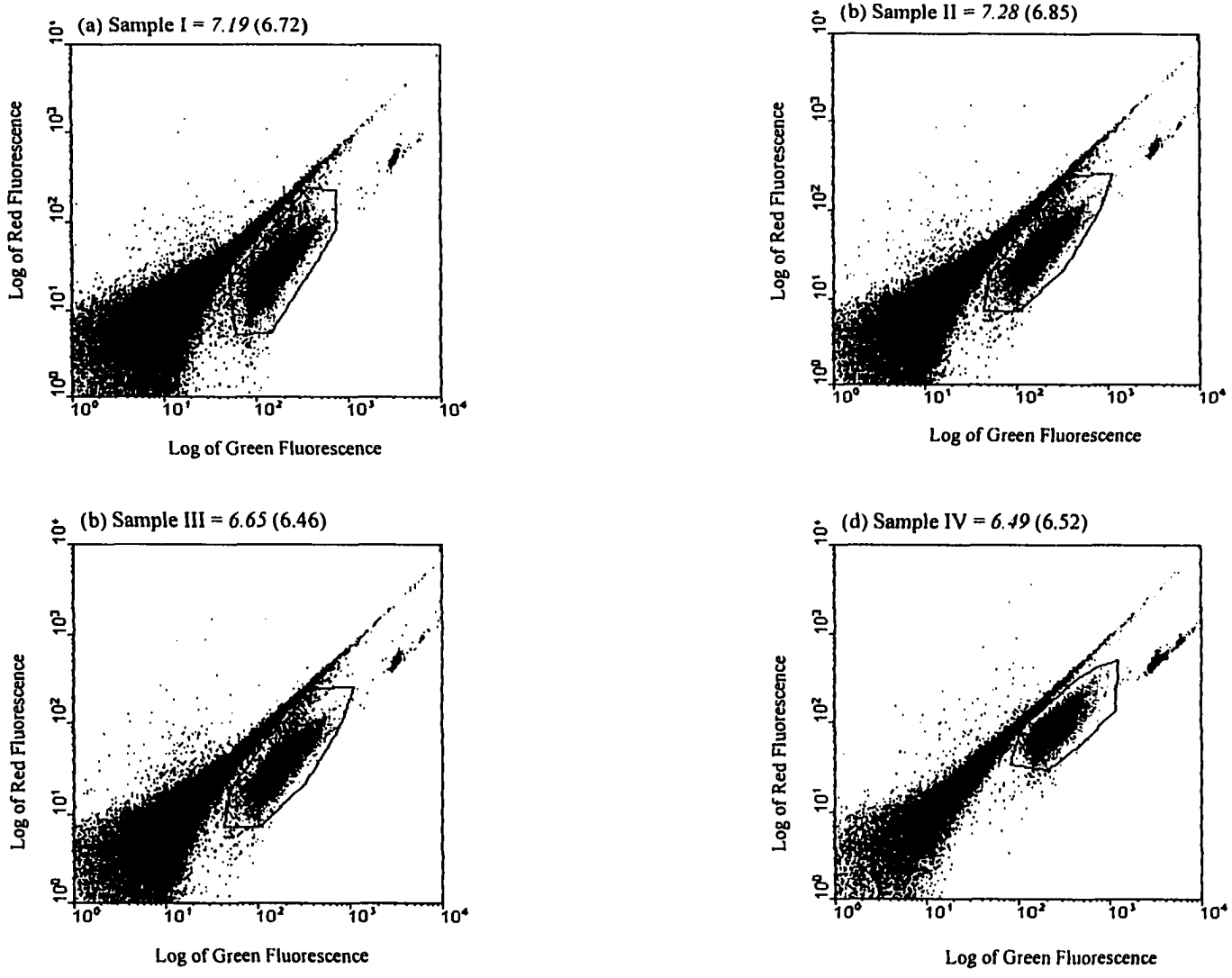

Fig. 4. FCM analyses of ground beef (80% lean) purchased from a retail outlet. Numbers in italics denote bacterial population estimates obtained by FCM and those in parenthesis denote estimates obtained by aerobic plate count. Region, R1 denotes bacterial population.

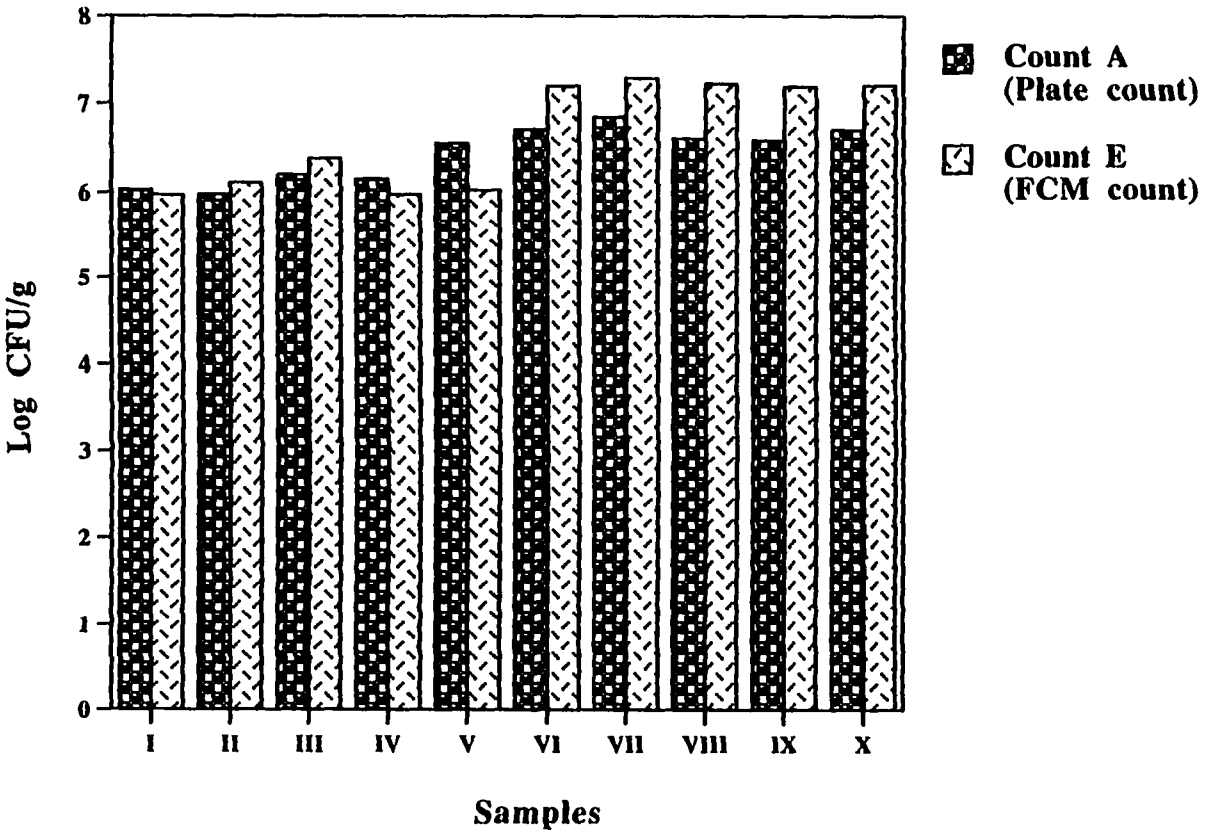

Fig. 3a. Comparison of plate counts with FCM counts on Retail samples. Values are mean of three replication.

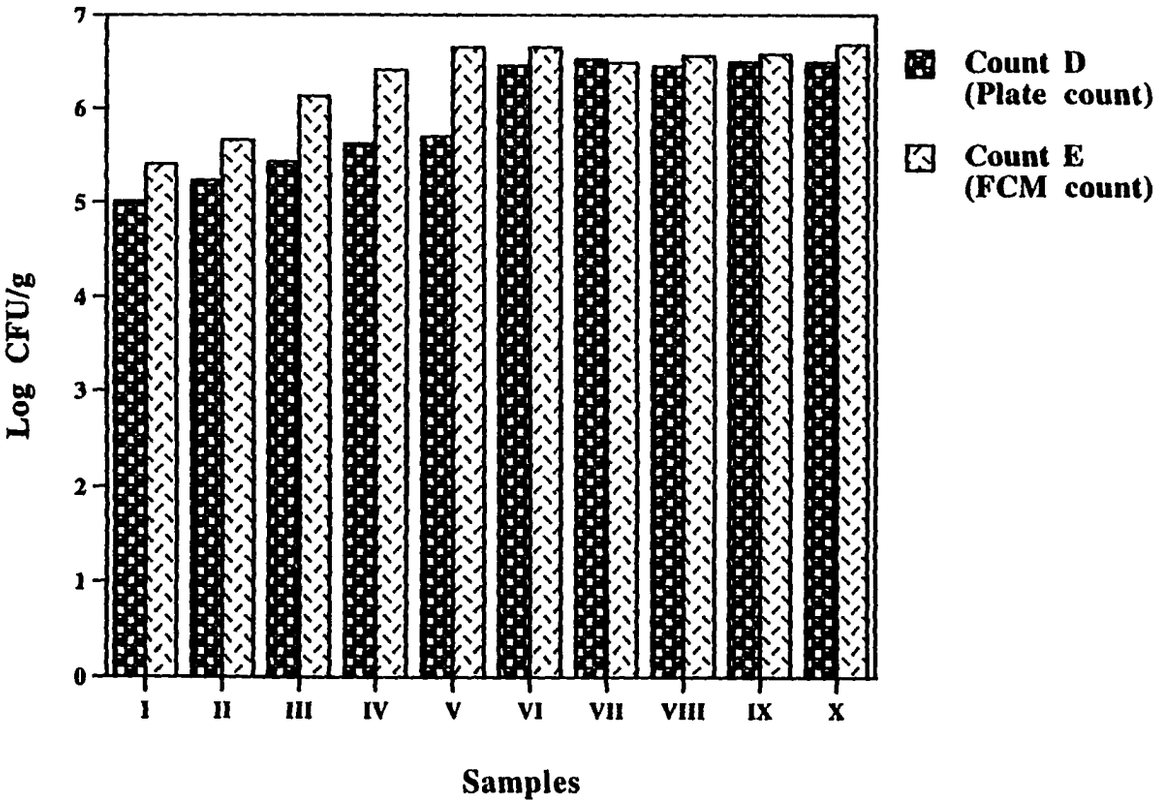

Fig. 3b. Comparison of plate counts with FCM counts on 1:10 diluted samples.

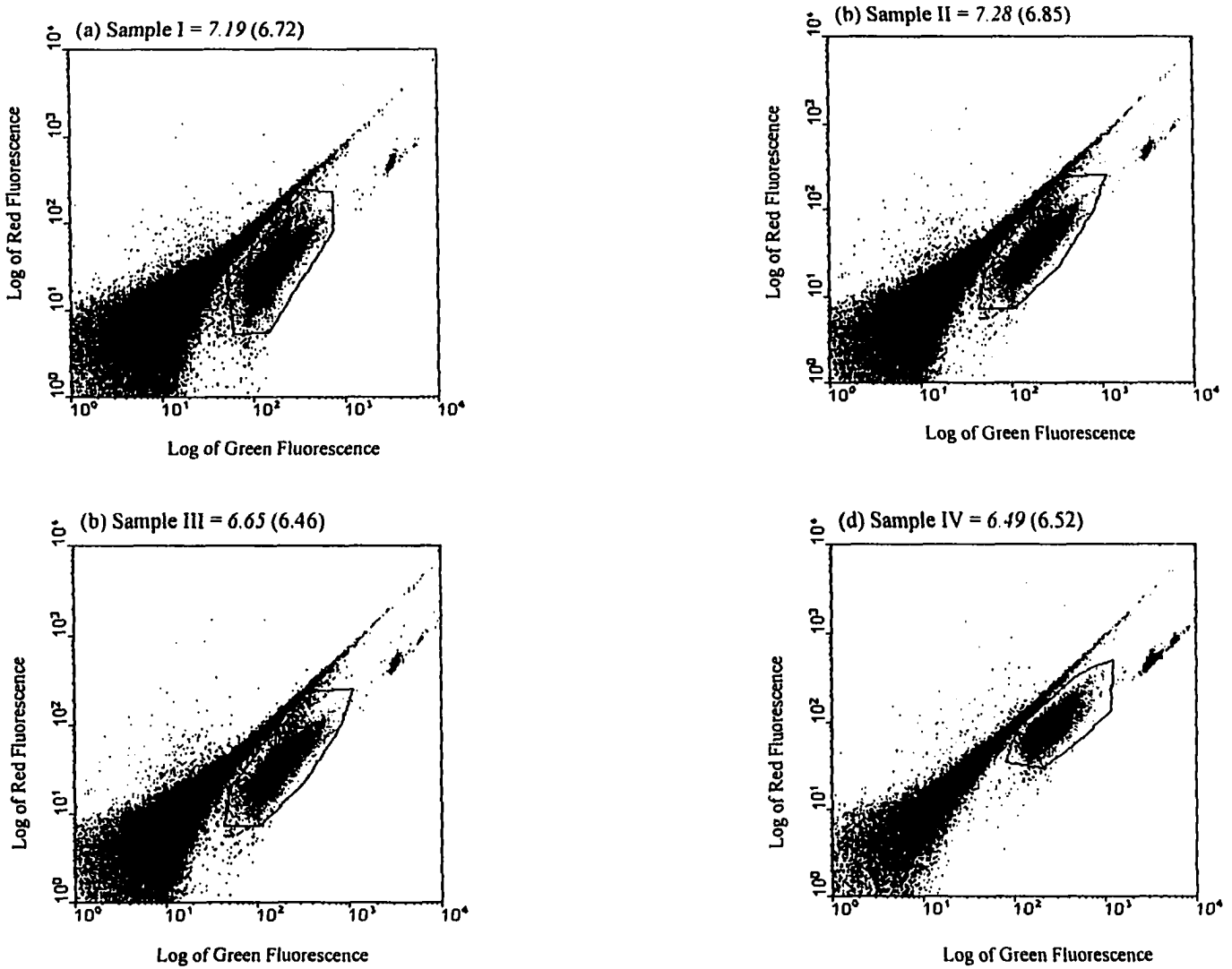

Fig. 4. FCM analyses of ground beef (80% lean) purchased from a retail outlet. Numbers in italics denote bacterial population estimates obtained by FCM and those in parenthesis denote estimates obtained by aerobic plate count. Region, R1 denotes bacterial population.

CHAPTER 7. GENERAL CONCLUSIONS

7.1 General Discussion

The central theme of this project was microbial safety of foods and in this we chose to investigate several key aspects inorder to improve our understanding about the growth and behavior of microorganisms. Documented information on the expected shelf life and appropriate means for its determination are important in today's food industry. There is general agreement that rate of microbial growth is the principal factor determining the shelf life of flesh foods such as red meat, poultry and fish. Other factors which influence the shelf life are the storage temperature and time of storage. The results obtained with fresh and temperature-abused patties have demonstrated that the initial microbial load of the product is critical for shelf life. It is likely that the efficacy of antimicrobial treatment will reduce with increasing microbial load. The shelf life of pork patties increased with an increase in the dose used and packaging did not influence the shelf life of irradiated pork patties although it did influence the non-irradiated ones. Growth curves can be modeled using a variety of mathematical equations, with the Gompertz equation being one of the more widely accepted equations.

Characterization of the growth of the surviving bacteria in irradiated meat in conventional kinetic growth terms, (i.e., lag and generation times), would provide an equivalent method for comparing different radiation processes and initial product quality. In addition, these growth parameters would allow for the development of predictive equations for optimizing radiation processes for products of known microbiological quality. Predictive models have been developed for a range of food borne pathogens with respect to different cultural factors such as temperature, pH, salt, etc., and very few for shelf life of processed foods.

Salmonella typhimurium grows well over a wide range of pH. The difference between the acid or alkaline shocked cells versus the acid or alkaline adapted cells is minimum. Our results show a clear change in the lipid content of the cells in response to changes in pH. The changes in the cell surface charge and the ability of the organism to attach to meat tissues also was influenced by changes in pH. This indicates that growth pH

has an influence on the bacterial attachment and colonization of beef tissue. Elaborate changes in the fatty acid profile as influenced by media composition and pH were observed.

A more diverse fatty acid profile of cells grown in nutrient broth when compared to tryptic soy broth and tryptic soy broth supplemented with yeast extract and pyruvic acid was also observed. This indicates that although the composition of the media influences the fatty acid profile, its complexity is not necessarily correlated with the changes. The degree of saturation increased with an increase in the pH in all three media. Considering pH 7.0 as the optimum, there was a separate trend in the degree of saturation for the acidic and alkaline pH. Acidity and alkalinity both had a different influence on cell morphology. Acid shocked cells (pH 5.0) had the appearance of coccoid cells whereas elongated cell morphology was observed with alkaline adapted and alkaline shocked cells.

A diverse group of fluorochromes were used in this study to stain cells of *E. coli*, *S. aureus* and *L. innocua*, and to differentiate them into live, injured and dead cells as influenced by heat, acid, and irradiation. The fluorescent stains used were rhodamine 123, a carbocyanine dye, carboxy fluorescein diacetate (CFDA) and fluorescein diacetate (FDA). Propidium iodide (PI) was studied in combination with all the four dyes and also individually. The usefulness of these dyes to identify the three stages were studied using a flow cytometer (FCM). The fluorogenic ester, CFDA and FDA, and the carbocyanine dye gave weak fluorescence that were not detected by the FCM. Rh 123 stained the cells but failed to differentiate the three stages of the bacteria used in this study. With PI, it was possible to differentiate between cells with intact membrane and those with compromised membranes. The results of this study indicate that the success of a particular fluorescent dye depends on the nature of bacterial injury. The observations with irradiated cells clearly substantiates this conclusion. Further, concentration of the dyes are a critical component in staining protocol. FCM is a better tool, more convenient and accurate in evaluating these dyes than with fluorescence microscopy.

Using FCM, the state of the bacteria can determined within a few minutes. Assessing the physiological state of bacterial population could provide us with valuable information about the viability of cells, which in turn has serious implications in public health

health microbiology including food microbiology. Based on the results obtained with our study on the evaluation of fluorescent dyes, *Bac*Light viability kit was used to stain and distinguish bacteria exposed to heat, acid, starvation, and irradiation, based on their viability. *E.coli*, *S.aureus*, and *L. innocua* were exposed to these stresses in such a way to create a sub population of injured bacteria and we attempted to use the fluorescent stains to distinguish the population of cells into viable, injured and dead. Good correlations were obtained for live bacteria stained and counted with FCM, but the discrimination of bacteria viability was not clear for heat, acid and starvation-treated cells. The stain failed to differentiate irradiated cells. Viability of stressed bacteria were also confirmed by inoculating them in enrichment broths. FCM is effective in rapidly quantifying bacteria in pure cultures when the population is between 10^3 to 10^7 cells/ml. Assessment of viability using dyes that depend on membrane integrity is more a function of the nature of stress and membrane permeabilization than viability of bacterial cells.

FCM was successfully used in rapid detection of microbial cells in meat homogenate. Meat homogenate, in liquid form, as such is not suitable for analysis in FCM. Clarification procedures using a simple column filled with sand particles was developed and clarified meat homogenate was analyzed using FCM. FCM offers a means to obtain direct, rapid microbial counts in a non-destructive way on clarified meat suspensions. The results indicate that FCM could be effectively used in the analyses of processed meat samples which invariably have a population of 10^6 cells/g. This was clearly demonstrated in the analyses of retail ground beef by FCM. On the other hand, the lower detection limit of flow cytometry is not sensitive enough to replace conventional plate count. Thus, for enumeration of viable microorganisms, the traditional plate count is still the benchmark method.

Pre-treatment and clarification of food homogenate are crucial steps in techniques used for direct enumeration of bacteria. Inert column materials such as sand particles and glass beads proved effective when compared with the other materials used in this study and sufficient clarification could be obtained with simple column filtration using these materials of suitable particle size. The ability of FCM to assess microbial populations on a cell-by-cell basis provides us with a tool to probe the viability of the population. The limitations realized

in this study could be overcome with improvements in fluorescent stains and with the availability of simple methods to separate prokaryotic cells from eukaryotic cells. As a tool for discriminating among bacterial cells based on viability, it should be possible to study the efficacy of anitmicrobial treatments in a rapid way. As demonstrated, the potential for rapid quantification of microbial loads in retail samples is enormous.

7.2 Recommendations for Future Research

1. Predictive microbiology has enormous potential in food microbiology and more parameters should be tried in order to generate more data. A combination of various antimicrobial conditions should be tried to improve the data base.

2. It is obvious from the results of this study that pH has a definite influence on the fatty acid content of *Salmonella typhimurium*. Further studies are required to ascertain whether such changes help the survival of the organism in the presence of suboptimal growth conditions. Supplementing fatty acid synthesis inhibitors in the growth media could be an useful strategy in this type of studies.

3. Vital fluorescent stains are useful in rapid identification of microorganisms. A greater number of dyes in conjunction with a variety of microorganisms should be studied. This will give a better understanding about the differences in microbial physiology and cell wall characteristics.

4. Clarification methods are required for rapid, accurate and direct enumeration of microorganisms in meat samples. The clarification method developed proved useful in this study and further studies are required to understand the nature of adhesion of microorganisms to column support. This will help us to develop better column materials with more specificity for removal microorganisms.

ACKNOWLEDGMENTS

I am indebted to my major professor, Dr. J.S. Dickson for his guidance and support throughout the course of this study. He is a wonderful person and an excellent teacher, it is my privilege to be known by him.

I extend my sincere thanks to my committee members, Dr. A. Dispirito, Dr. A. Pometto, Dr. D. Olson, Dr. G. Phillips and Dr. D. Hayes for agreeing to serve in this committee and for their constructive ideas in executing this work.

I wish to thank my parents for their encouragement and support to explore this part of the globe. I also wish to thank my brothers and sister for relieving me of some of my family obligations, they were my role models and inspiration in my academic pursuit.

I am thankful to Barbara and Steve Ingham for their support and permitting the use of their resources for the preparation of this dissertation. The financial support provided by the Department of Food Science and Human Nutrition through a research assistantship is gratefully acknowledged.