BACTERIAL ATTACHMENT TO MEAT SURFACES

A thesis presented in partial fulfilment of the requirements for the degree of

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ABSTRACT

The aim of this study was to optimise the hygienic efficiency of slaughter and dressing operations. Three strategic approaches, namely reducing and removing or killing the bacteria attached to meat surfaces, were considered. The second option of removal was selected for development, as current technology inevitably results in bacterial contamination, while killing bacteria on meat surfaces requires drastic treatments that may adversely affect quality parameters.

The initial attachment mechanism between bacteria and the carcass surface (reversible attachment) was studied using the collagen film model system. Bacterial attachment to the collagen model was compared with attachment to cut beef muscle and uncut beef muscle using viable count procedure. Scanning electron microscopy and direct microscopic count procedure using an epifluorescence microscope was also developed using both collagen films mounted on microscope slides and collagen coated microscope slides. The collagen film viable count system was the method selected to model bacterial attachment to meat because of ease and consistency of quantification.

There was no positive correlation between attachment and many bacterial cell surface factors such as charge, hydrophobicity, protein and polysaccharide surface molecules. Different eluents were used to identify the principal component interfering with single attachment mechanisms on electrostatic interaction and hydrophobic interaction chromatographic columns and on collagen film. Three components interfering with the isolated attachment mechanisms were identified. They were Tween, sodium chloride (NaCl) and mannose. Further column studies indicated that cell surface proteins play a more important role in cell surface negative charge and hydrophobicity than do surface polysaccharides.

A wash solution was formulated using the components Tween, NaCl and mannose to reverse what were believed to be the major attachment mechanisms. Further trials

with Tween, NaCl and mannose and increasing their concentrations and the application of increased vigorous rinsing also proved ineffective for washing the cells from meat surfaces. This result also supports the hypothesis that bacterial attachment to meat surface is very complex and multifactorial. Elution studies using 10 % Tri sodium orthophosphate pH 12.0 killed the cells rather than removing them and further work will be directed towards the killing.

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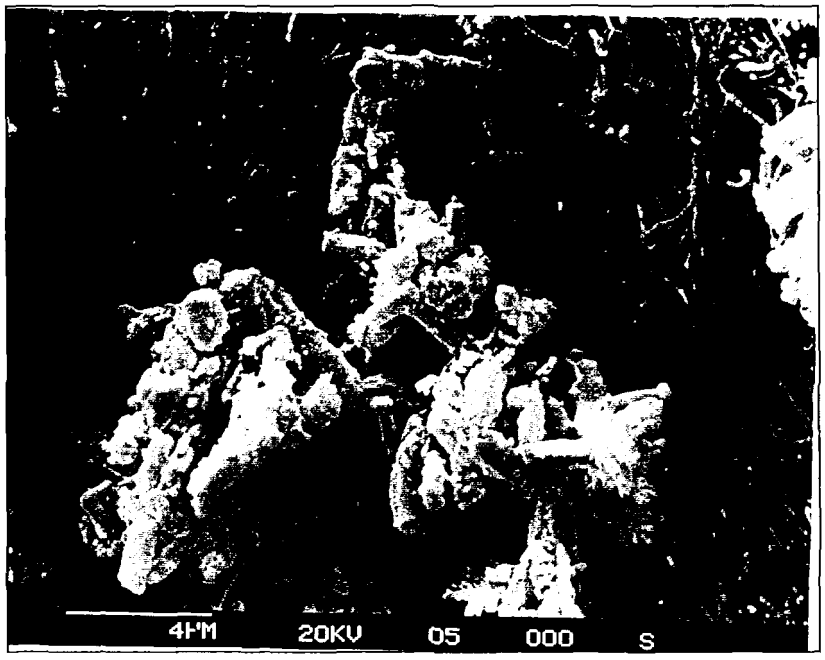

Scanning electron micrograph of ***E. coli*** **E6** attaching to cut beef muscle surface. The cells are in clumps and appear to be preferentially colonising the muscle fibres Magnification = 7000 x.

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LIST OF PRESENTATIONS

This work has been featured in the following presentations at scientific conferences:

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ABBREVIATIONS

APC	-	Aerobic plate count
Ab-DEFT	-	Antibody- direct epifluorescent filter technique
BSA	-	Bovine serum albumin
CF	-	Collagen Film
CCS	-	Collagen coated slide
CPC	-	Cetyl pyridinium chloride
DF	-	Diluent fluid
DEFT	-	Direct epifluorescent filter technique
EIC	-	Electrostatic Interaction chromatography
ETEC	-	Entero toxigenic *Escherichia coli*
FCU	-	Femoris culna ulnaris
FISH	-	Fluorescent in situ hybridisation
GMP	-	Good manufacturing practices
HIC	-	Hydrophobic Interaction chromatography
HCGR	-	High concentration gentle rinse
HCVR	-	High concentration vigorous rinse
LCGR	-	Low concentration gentle rinse
LCVR	-	Low concentration vigorous rinse
LPS	-	Lipo polysaccharides
MRGR	-	Multiple rinse gentle rinse

MRVR	-	Multiple rinse vigorous rinse
MIRINZ	-	Meat Industrial Research Institute
MATH	-	Microbial adhesion to hydrocarbons
NaCl	-	Sodium chloride (salt)
PW	-	Prewetting
PB	-	Phosphate buffer
PBS	-	Phosphate buffered saline
REA	-	Restriction endonuclease analysis
SEM	-	Scanning electron microscopy
SRGR	-	Single rinse gentle rinse
SRVR	-	Single rinse gentle rinse
STD-DF	-	Standard diluent fluid
TSM	-	Tween, Salt (NaCl) and Mannose
TEM	-	Transmission Electron Microscopy
TVC	-	Total Viable Count
TSP	-	Trisodium ortho phosphate

DEFINITIONS

ADHESION: Adhesion is a physicochemical process of interaction between molecules that are situated in the outermost layer of the inert surface and of the microorganisms, and molecules of the surrounding fluid. This term is used when the process is permanent and irreversible.

ATTACHMENT: Attachment is defined as a physicochemical process of interaction between molecules that are situated in the outermost layer of the inert surface and of the microorganisms, and molecules of the surrounding fluid. This term is used when the process is temporary and reversible.

CARCASS: is the body of any slaughtered animal or bird often, but not always, after bleeding and dressing.

CUT BEEF MUSCLE: Meat which has only muscle and is free from subcutaneous fat and fascia.

EVISCERATION: is the removal of the viscera from a carcass.

FRESH MEAT: is meat that has not been treated in any way other than refrigeration, with or without preservative packaging to maintain its fitness for human consumption.

INITIAL MICROFLORA: is the association of microorganisms present on an eviscerated carcass after skin removal (if appropriate) but prior to washing, grading, chilling and further processing.

MEAT: is the edible part (musculature and edible offal) of an animal or bird slaughtered for human consumption.

MICROBIAL CONTAMINATION: refers to microorganisms directly or indirectly transferred onto a carcass or edible offal, hence contaminating microflora means those microorganisms present as a consequence of such transmission.

RIGOR: The postmortem changes that occur during conversion of muscle to meat resulting in stiffening of a body after death.

SPOILAGE: describes changes that render meat objectionable to consumers: hence spoilage microflora describes an association of microorganisms whose development on meat renders that meat objectionable to consumers.

SPOILAGE POTENTIAL: is a measure of the propensity of microorganisms to render meat objectionable to consumers through the production of offensive metabolic by products.

SLAUGHTER: is the killing of an animal or bird for human consumption generally but not necessarily performed within premises (abattoir) that are approved and registered for that purpose.

UNCUT BEEF MUSCLE: Meat which is completely surrounded by fascia which is a membranous connective tissue covering.

Chapter 1
BACTERIAL ATTACHMENT TO MEAT SURFACES

This thesis forms part of a programme of research, centred at AgResearch, MIRINZ Centre, Hamilton, New Zealand, conducted in association with Massey University, Palmerston North, studying bacterial attachment and its control with respect to meat surfaces. Bacterial attachment to many surfaces has been studied in a wide range of environments for many years. Bacterial attachment to surfaces may be beneficial in the case of waste water treatment and in fermentation technology. However, attachment of bacteria to food and food contact surfaces is considered to pose a food safety or food spoilage problem.

The mechanism by which bacteria attach to surfaces is far from completely understood. Many models of attachment to different surfaces have been described. These models can all be simplified into three main stages – initial (reversible) attachment, growth and detachment. Adhesion involves a complex interaction between a substrate and the microorganisms. This process is influenced by the surrounding environment and is followed by biofilm growth, which is usually associated with the production of extracellular polysaccharide material that may bond the biofilm colony together, forming a more secure attachment to the substrate. Biofilm growth usually leads to shedding of cells and pieces of the film, resulting in contamination of the product and processing environment.

Although biofilms have been studied in many natural environments, it is only since the early 1980s that biofilms have been recognised as a concern in food processing environments. Bacterial adherence to meat was recognised as a food related biofilm problem with the potential to accelerate spoilage onset and compromise food safety.

Meat surfaces were found to be prone to colonisation by a wide variety of microorganisms present in the processing environment.

The attachment of bacteria to meat surfaces was recognised as the first step in microbial contamination of freshly slaughtered carcasses. Colonisation of meat plants with bacteria potentially affects the quality of carcasses which are likely to contact meat plant surfaces. The mechanisms by which bacteria initially attach to meat surfaces are poorly understood and so are difficult to control, with meat plants relying on routine washing procedures to reduce microbial contamination. However, once microbial cells have attached, they may be difficult or impossible to remove and have been shown to be resistant to the washing treatments commonly practised in New Zealand meat plants.

Chapter 2
LITERATURE REVIEW

2.1 SCOPE OF LITERATURE REVIEW

- The literature covered in this review is categorised into four major areas of relevant research:
- Sources of carcass contamination and associated concerns;
- Bacterial attachment to meat surfaces;
- Cell surface properties of bacteria; and
- Decontamination methods to control initial flora and the requirements for further research in this field.

2.2 SOURCES OF CONTAMINATION

The New Zealand meat industry produces a wide range of fresh meat and meat products which are perishable and subject to spoilage by a broad range of microorganisms, some of which may be pathogenic (Milner, 1996). The presence of the latter microorganisms on meat can result in major disease outbreaks. The major sources of microbial contamination of meat carcasses are the animal being slaughtered, the process workers and the processing environment (Anon, 1991; Bauman, 1995; Tompkin, 1994).

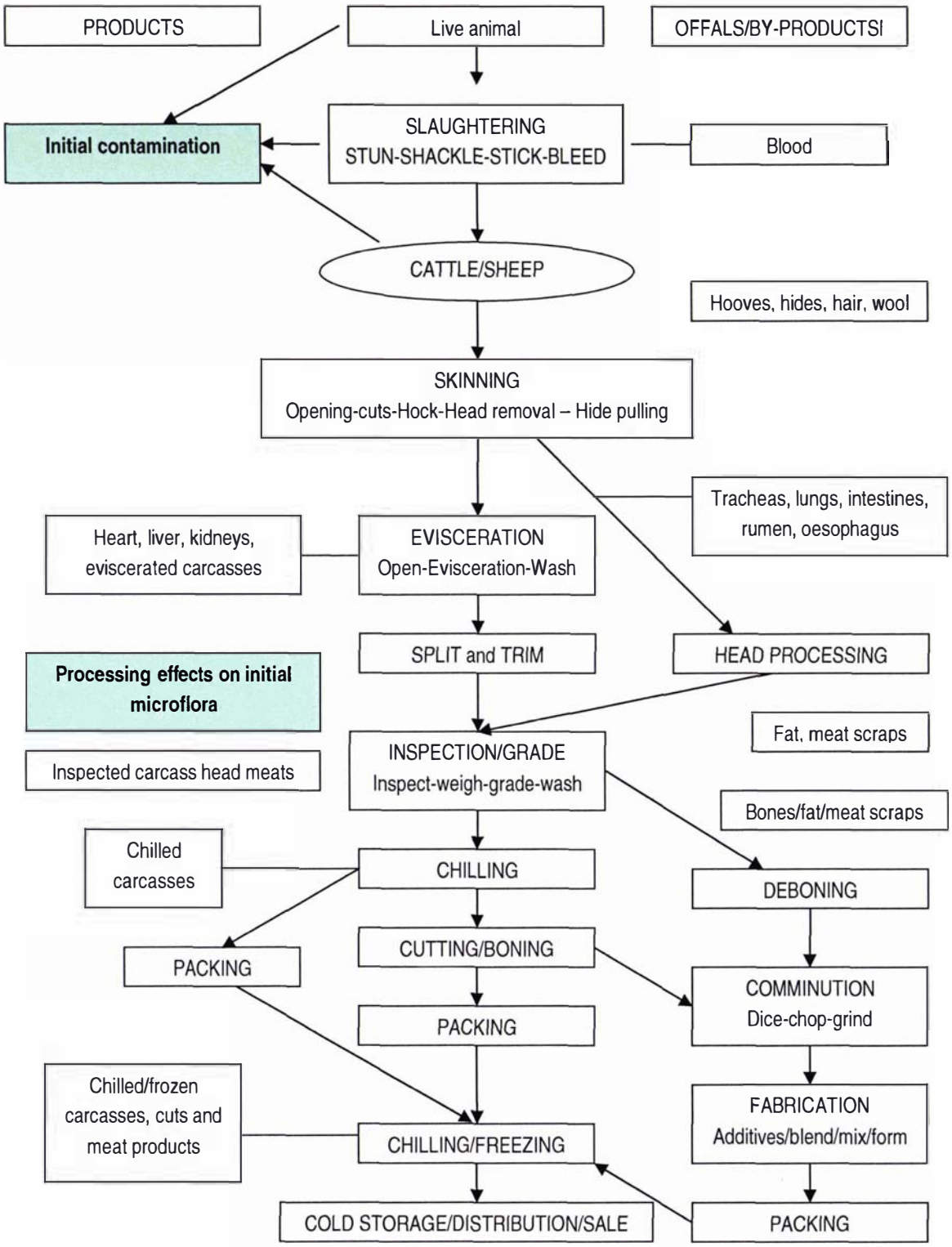
2.2.1 Animals as a source of contamination

At the time of slaughter, internal muscle tissues from healthy, hygienically killed animals are thought to be either sterile or to contain very few bacteria (Gill et al., 1979; Mackey & Derrick, 1979). In contrast, on skinning, carcass surfaces are exposed to varying degrees of contamination from animal sources, including external body surfaces, skin, fleece, feet and hooves and the gastrointestinal and respiratory tracts.

2.2.2. Hygienic consequences of "Skin-Off" meat processing:

Meat processing "skin-off" is the usual form of processing for cattle and sheep. The objective of the process is to obtain a skinned carcass with minimal transfer of microbial contamination to the carcass surface during slaughter and dressing. "Skin-on" processing is typically used in pork and poultry processing.

2.2.2.1 Flow chart 1: Generic process flow diagram for "skin-off" meat animal processing



Flow Chart 1: Generic process flow diagram for chilled, frozen raw whole meat carcasses, cuts and meat product (Bell, 1996). Published with permission from the author and Leatherhead Publishers.

2.2.2.2 Stock presentation

Carcass contacts with the hide, hooves, feet and hair introduce 30% of the microflora typically found on a dressed carcass (Empey & Scott, 1939). Transportation of slaughter stock especially, over long distances, usually results in gross faecal contamination of the animal exterior. Furthermore, transportation stress potentiates the excretion of pathogenic organisms, particularly *Salmonella*, by symptomless carriers Grau et al., (1968) resulting in dispersion of this pathogen within a transport unit.

2.2.2.3 Cleaning the live animals

Wet and dry preslaughter cleaning of stock to remove soil and faecal material, respectively, through washing or shearing is intended to reduce visible carcass contamination and to minimise the microbial load transferred from the animal to the carcass surface. Cold water pre-slaughter spray washing reduces 50% of the microflora present on hides (Empey & Scot, 1939). This reduction represents only a small improvement (Roberts, 1980). Unfortunately washing results in the addition of moisture, which subsequently helps the bacteria to proliferate on the hide during the postwash, preslaughter period. Contrary to hygienic expectations, preslaughter crutching (removal of wool and excrement from ovine hindquarters) increases subsequent carcass microbial contamination of both wet and dry stock (Biss & Hathaway 1995; Roberts, 1980).

2.2.2.4 Slaughter

Evidence shows that bacteria can be introduced into deep tissues through the use of contaminated slaughter equipment (Mackey & Derrick, 1979). Hygienic slaughter practice therefore recommends procedures to minimise the introduction of microbial contaminants during slaughter, in particular the use of 82°C water to pasteurise slaughter instruments (Bell, 1997).

2.2.2.5 Carcass dressing

Immediately after slaughter, the edible tissue is normally sterile. Microbial contamination of these tissues, however inevitably occurs during carcass dressing and butchering. Skinning introduces many microorganisms onto carcasses as a result of carcass hide or carcass fleece contact (Gill & Penney, 1979; Macay & Derrick, 1979). The gastrointestinal tract contains a high microbial load and is a significant source of contamination in respect of enteric pathogens. Modern evisceration procedures minimise such contamination by securing both ends of the intestinal tract and avoiding its perforation (Grau, 1979; Nottingham et al., 1974). The key to hygienic slaughter, dressing and handling practices lies in reducing or eliminating the opportunity for contact between the skinned carcass and contaminated body parts, process workers, processing surfaces and instruments.

2.2.2.6 Washing

A cold potable water carcass wash is widely practised following the completion of dressing (Bell, 1997). Spray washing is the most common. The washing may be followed by decontamination interventions such as organic acids, chlorine and so on. to remove, or at least reduce, microbial contamination. Such decontamination processes may extend the product storage life through a general reduction in product bioload or may resolve microbiological safety problems by targeting the pathogens concerned. This topic of decontamination is discussed more fully in section 2.5.

2.2.2.7 Carcass cooling/chilling

Carcass cooling (freezing or chilling) represents a change in the method of microbiological control from minimising contamination to reducing microbial growth. Delay in further processing leads to the growth of psychrotolerant organisms. However, cooling of carcasses to temperatures below 7° C has become a feature of most meat hygiene regulations.

2.3 CONCERNS ASSOCIATED WITH INITIAL MICROFLORA OF CARCASS IN MEAT PLANTS

The microorganisms present on dressed eviscerated carcasses before washing can be considered as the true initial microflora. The initial microflora associated with "skin-off" meat processing is composed of microorganisms transferred onto the naked carcasses during slaughter and dressing. The initial contaminating microflora of the carcass surface are increasingly implicated as a significant source of contamination found on raw meat and meat products (Doyle, 1989; Varnam et al., 1991).

Gram positive cocci dominate the initial microflora of skinned carcasses (Stringer et al., 1969). However, the initial microflora of the carcass are very diverse and may contain the following groups: *Acinetobacter, Aeromonas, Coryneforms, Enterobacteriaceae, Flavobacterium, Moraxella* and *Pseudomonas* in addition to *Micrococcus* and *Staphylococcus* species. Lactic acid bacteria, *Brochothrix thermosphacta* and *Bacillus* species are found less often and in low numbers. However, yeast and molds are rarely reported.

Such a saprophyte-dominated microflora may also include pathogens introduced by direct or indirect faecal contamination from the processing environment. They are present in generally low numbers except under unusual circumstances. During chilled storage, pathogenic microorganisms derived from the intestinal tract will not usually contribute significantly to spoilage, but may represent a safety hazard (Al-MohiZea et al., 1994).

Approximately 90% of the initial bacterial load of the dressed carcass is thought to be aerobic and mesophilic (Newton et al., 1978; West et al., 1972). These mesophilic bacteria are unable to grow at refrigeration temperatures and so will not contribute to spoilage.

The presence of psychrotolerant microorganisms is of concern because not only can they directly potentiate product spoilage, but also they may pose a secondary contamination hazard if they become established in a chilled processing environment

through inadequate or ineffective sanitation. In the following section the problems posed by both pathogens and spoilage microorganisms are discussed in some detail.

2.3.1 Pathogens

Pathogenic microorganisms, particularly those of animal origin, can be expected to be found on occasion in the saprophyte-dominated initial microflora and this is of public health significance. Their numbers are usually low except in unusual circumstances. With the exception of skin-associated *Staphylococcus aureus* and *Yersinia enterocolitica*, most of the major food poisoning organisms (*Salmonella* spp, *Clostridium perfringens*, pathogenic *Escherichia coli* and *Campylobacter jejuni*) are associated with direct or indirect faecal contamination of carcasses. Other pathogens such as *Listeria monocytogenes* may be animal associated but can also be found in the processing environment. Sometimes their growth and survival following cooking procedures may lead to major food-borne outbreaks of disease. The microbiological characteristics of meat-borne pathogens causing food poisoning are briefly outlined in Table 2.1.

Table 2.1. Food poisoning microorganisms associated with chilled and frozen raw meat.

Microorganisms	Temperature	Mode of pathogencity
Aeromonas hydrophila	Psychrotolerant	Infection
Bacillus cereus (a)	Psychrotolerant	Intoxication
Bacillus cereus (b)	Mesophilic	Intoxication
Campylobacter spp	Mesophilic	Infection
Clostridium botulinum	Mesophilic	Intoxication
Clostridium perfringens	Mesophilic	Intoxication
Escherichia coli	Mesophilic	Infection
Listeria monocytogenes	Psychrotolerant	Infection
Salmonella spp	Mesophilic	Infection
Staphylococcus aureus	Mesophilic	Intoxication
Yersinia enterocolitica	Psychrotolerant	Infection

2.3.2 Spoilage flora and types of spoilage

Prolonged chilling and storage of raw meat products may lead to spoilage problems as a result of the growth of elements of the initial contaminating microflora. The microorganisms most frequently associated with the spoilage of chilled raw meats are shown in Table 2.2. Which of these bacteria develop to cause spoilage is determined by a complex interaction between the initial microflora, the substrate (meat) parameters, particularly pH, Aw and glucose content; and the environmental variables of temperature and gas composition. In frozen storage the temperature is reduced below that conducive to microbial growth. While frozen storage prevents microbial spoilage, however, frozen meat is subject to chemical and physical spoilage such as oxidative rancidity and freezer burn.

Table 2.2. Genera of bacteria found on raw meat and poultry and their association with spoilage development.

Bacteria	Spoilage potential
Gram Positive	
Bacillus	Low
Brochothrix	High
Clostridium	High
Lactic acid bacteria	Low
Gram Negative	
Acinetobacter group	Low
Enterobacteriaceae	Low
Pseudomonas	High
Shewanella	High

The following discussion concentrates on microbial spoilage, using temperature as the primary means of classifying spoilage; other factors affecting the rate of spoilage

onset, and gaseous environment (packing) are regarded as secondary criteria for microbial spoilage.

2.3.2.1 Warm temperature spoilage

Holding meat at warm temperatures (above 15°C) is not generally advocated because of the potential it affords for pathogen growth. Exceptions are the special case of the hot meat, or wet market meat distribution system operating in many undeveloped parts of the world where animals are slaughtered and the meat is sold and consumed within a matter of hours (Bell, 1996).

Aerobic

At temperatures above 20°C pseudomonads do not have a growth rate advantage over competing psychrotolerant or mesophilic bacteria present on meat. However, it is not until temperatures reach about 30°C, the maximum for the growth of many psychrotolerant pseudomonads, that mesophilic strains of *Acinetobacter* and *Enterobacteriaceae* become the dominant spoilage microflora (Gill & Newton, 1980).

Anaerobic

A mixed spoilage microflora develops under anaerobic conditions, including psychrotolerant and mesophilic strains, some of which may be pathogenic. Strains of *Enterobacteriaceae* dominate the spoilage microflora developing at room temperature. Raising the temperature to 30°C will allow *Clostridium perfringens*, if present in the initial microflora, to bring about spoilage (Gill & Newton, 1980). Similarly, the slow cooling of carcasses can allow the growth of *Clostridia* in deep tissue, resulting in the spoilage condition known as "bone taint". In the temperature range of abusive chilled storage, 5 to 15°C, pack distension (blown pack spoilage) resulting from the production of large volumes of gas within the pack is associated with the growth of psychrotolerant *Enterobacteriaceae.*

2.3.2.2 Chill-temperature spoilage

The optimum temperature for chilled storage of meat is the lowest temperature that can be maintained without freezing the product.

Aerobic

Under aerobic chilled storage, *Pseudomonas* spp will predominate in the developing spoilage microflora, Ayres (1960), that will usually also contain significant numbers of *Acinetobacter* and *Moraxella* strains. Rapid spoilage results from the unrestricted growth of the *Pseudomonas* spoilage consortium. Off-odours become evident when numbers reach 10^8cells/cm^2 and surface slime is visible as numbers approach 10^9 cells/cm^2.

Anaerobic

Vacuum packaging: Vacuum packaging, in which an oxygen-deficient environment is created around the product by evacuation, is the most widely used preservative packaging system (Bell, 1996). By changing the gaseous environment from aerobic to anoxic, an ecological shift is effected that sees the strictly aerobic high spoilage potential *Pseudomonas* microflora replaced by low spoilage potential lactic acid bacteria. Spoilage potential is a measure of the propensity of microorganisms to render meat objectionable to consumers through the production of offensive metabolic by-products. With spoilage microflora dominated by lactic acid bacteria, spoilage does not become evident until several weeks after maximum numbers of 10^7 to 10^8cells/cm^2 have been reached (Bell, 1996). Lactic acid bacteria are, however, usually present as an undetectably small fraction of the initial contaminating microflora, so it is possible for early spoilage to be caused by slower growing higher spoilage potential species present in the initial microflora in relatively large numbers. Facultative microorganisms that are particularly troublesome in this regard are *Shewanella putrefaciens, Brochothrix thermosphacta* and the psychrotolerant *Enterobacteriaceae.* All these organisms have a propensity to become established in processing environments where sanitation procedures are ineffective. Such pre-packaging contamination can produce serious, if intermittent, early spoilage problems.

Of increasing concern with vacuum- packed meat produced during the winter months in temperate regions is a type of "blown pack" spoilage resulting from the growth of psychrotolerant and psychrophilic *Clostridia* (Broda et al., 1996).

Carbon dioxide packaging: The preservative effect of anaerobic conditions in conventional vacuum packaging is enhanced by elevated concentrations of carbon dioxide, typically 100% carbon dioxide, in the pack atmosphere. Chilled storage life can be extended by 50 to 100% over that achieved by vacuum packaging. The differential inhibitory effect of carbon dioxide and lag phase extension due to chilling retards the growth of the psychrotolerant pathogens such as *A. hydrophila, L. monocytogenes and Y. enterocolitica.*

2.3.2.3 Freezing temperature spoilage

At temperatures, below –12 °C, at which frozen meat is normally held, microbial growth is effectively prevented. However, at temperatures only marginally higher, fungal growth has been reported (Lowry & Gill, 1984).

To summarise, the keeping quality of meat depends directly on the initial contaminating microflora, subsequent processing and extrinsic environmental conditions that were applied to the meat surface. New Zealand is a major exporter of red meat in the global meat market and requires meat processors to ship fresh meat over long distances. Many advanced preservative and packaging technologies have been developed. However, success relies on storage and shipment temperatures being maintained as close as possible to the freezing point of meat and high hygienic quality of carcasses.

Thus, preventing initial contamination of the carcass is currently receiving increased commercial interest due to the trends towards producing products with longer shelf life, stricter hygiene requirements for international trade and the increasing awareness of the problems posed by *Listeria, Salmonella and E. coli* O157:H7. Recognition of these problems has focused research towards the need for a better understanding of bacterial attachment to meat surfaces.

2.4 BACTERIAL ATTACHMENT TO MEAT SURFACES

Bacterial attachment to meat surfaces is similar to bacterial attachment to many surfaces and occurs in two stages, namely: reversible attachment and irreversible attachment (Firstenberg-Eden, 1978). Marshall et al. (1971) reviewed bacterial attachment to meat surfaces and concluded that physical forces were involved in the initial reversible attachment, whereas subsequent irreversible attachment required the formation of a cementing matrix composed of extracellular polysaccharides. Fletcher & Floodgate (1973) demonstrated the role of acidic polysaccharides in the cementing layer involved in the adhesion of bacteria to marine surfaces. An intermediate stage between reversible and irreversible attachment of marine bacteria to mammalian cells involving pili and flagella has also been demonstrated. The formation of hydrogen and ionic bonds is involved and this is followed by the excretion of extracellular polysaccharide (Rogers, 1979).

The classical theories of adhesion suggest two to five stages in the attachment process, involving van der Waals attractive forces, electrostatic interactions, hydrophobic and later more active adhesion through the production of extracellular polymers by the bacteria (Oliveria, 1992). Marshall et al. (1971) divided the adhesion process into two distinct, and now well recognised phases – reversible and irreversible adhesion – on the basis of the force required to remove the cells from the surface. Busscher & WeerKamp (1987) proposed a three-step model, adding an extra initial stage where the cells are prevented from direct contact with a surface because of van der Waals and electrostatic forces which are eventually overcome by stronger attractive forces. This stage is often referred to as the "DLVO theory" (developed independently by two research teams, Derjaguin & Landau (1941); Verwey & Overbeek (1948) to explain the stability of colloids). The "DLVO theory" stresses the influence of electrostatic interactions in the adherence of particles, including microorganisms, to solid surfaces (van Loosdrecht et al., 1989). Characklis & Cooksey (1983) also expanded the two-stage model to include: cell transport to a wetted surface, adsorption of a conditioning film, and adhesion of microbial cell, reactions within the biofilm and, finally, detachment of the biofilm.

The physical description of bacterial attachment to surfaces can be conveniently divided into three phases (Fletcher et al., 1980).

Bacterial deposition on the collector surface: Fletcher (1980) reported that in the initial attachment process, van der Waals forces, free energies and bridging interactions play a vital role. The meat surface is negatively charged as is the bacterial surface. In this situation there is an overall tendency for mutual repulsion by the two surfaces, but this is opposed by forces, known as *van der Waals attraction energies*. Bacterial cell appendages facilitate the close contact of cell and surface by reducing overall electrostatic repulsion. Flagella and pili can increase the cells' surface free energy. This energy may be strong enough to overcome, at least locally, the double layer repulsion forces. However this type of attachment or association between cell and collector surface is reversible and the bacteria can be removed by shear forces associated with the washing process.

Bacterial consolidation on the collector surface: Extracellular polymers are produced by the bacteria on the surface that provide a means of bridging the gap between the cell and the surface, thereby effectively anchoring the bacteria to the surface (irreversible) attachment (Allison & Sutherland, 1998; Oliveria, 1992).

Growth on and colonisation by bacteria of the collector surface: Within hours of the initial association of a bacterium with a surface, an extracellular polymeric matrix is produced (Firstenberg – Eden et al., 1979). This matrix is often referred to as the *glycocalyx*. The extracellular polymer has adhesive properties and reinforces the adhesive bond between the collector surface and bacterium; making the latter more difficult to remove that is it becomes irreversibly attached (Firstenberg –Eden et al., 1979).

As a result of the growth of adherent bacteria, surface colonisation starts with the formation of microcolonies of bacteria bound together by a dense mass of glycocalyx (Delaquis et al., 1992). With time, these colonies eventually fuse together to form a continuous slime layer on the collector surface, which in the case of meat spoilage

occurs at a cell concentration of approximately 10^9cfu/cm^2. Meat in this condition would be in an advanced state of spoilage.

2.4.1 Basic modes of association of bacteria to meat surfaces

Piette and Barriga (1994) described three ways in which bacteria can reach a newly exposed muscle surface: (1) the surrounding environment; including the air and process water (2) contaminated surfaces, including equipment and (3) through cross contamination by direct contact with other carcasses.

Waterborne contamination is well researched in poultry processing (Lillard, 1986a; Notermans & Kampelmacher, 1975; McMeekin & Thomas, 1978; Thomas & McMeekin, 1980; Thomas & McMeekin, 1981a; Thomas & McMeekin, 1981b). Immersion chilling introduces planktonic bacteria (free floating in the cooling water) onto the carcass surface water film (Lillard, 1986a; Notermans & Kampelmacher, 1975). The bacteria are generally reversibly attached at this stage and can be removed by rinsing. However, it has been reported (McMeekin & Thomas, 1978; Thomas & McMeekin, 1981b; Thomas & McMeekin, 1984) that a proportion of surface-associated bacteria become physically entrapped in crevices and channels formed in chicken skin and on skinless pieces of chicken breast muscle as a result of such immersion. Therefore, prolonged contact with large amounts of water is necessary to cause the structural surface modifications that facilitate entrapment. Entrapment occurs in the three-dimensional network of collagen mesh fibres created through hydration of muscle fascia (Thomas & McMeekin, 1981a).

When observed by transmission or scanning electron microscopy the bacteria are in direct contact with the meat surface (Thomas & McMeekin, 1981a). Lillard (1986a) demonstrated that with time there is a change in the distribution of surface associated bacteria between the thin surface water film, water pockets filling the crevices, and the meat surface itself, with a fraction of free floating and entrapped bacteria being transferred to the meat surface, to which they become attached. The surface microtopography, with deep crevices, and channels presents the problem of bacterial

retention and trapping on the surface features being confused with surface attachment (Thomas & McMeekin, 1981).

2.4.2 Attachment of bacteria to meat surfaces

Microbial attachment to meat surfaces has been reviewed several times over the past twenty years (Benedict, 1988; Firstenberg-Eden, 1978; Frank, 2001; Notermans & Kampelmacher, 1983). The process of attachment is influenced by various factors which include pH, temperature, time, cell concentration, type of meat surface, type of bacterial strain, culturing methods, surface structures and surface properties such as charge and hydrophobicity (Butler et al., 1979; Chung et al., 1989; Dickson 1991; Notermans & Kampelmacher, 1975; McMeekin & Thomas, 1978). Attachment of bacteria to the meat surface occurs rapidly in the first minute of contact between bacterial suspensions and meat tissue (Bouttier et al., 1997; Butler et al., 1979). These observations indicate that initial attachment is due to physiochemical properties of bacteria and the contact surface. Piette & Idziak (1992) confirmed this by demonstrating that inactivation of cells had no effect on their attachment to tendon. The question is "Do dead cells adhere as well as live ones?" However Flint et al. (1997) and Parkar et al. (2001) demonstrated that dead cells adhere as well as do live ones. In contrast, Dickson & MacNeil (1991) observed that attachment of *L. monocytogenes* continued to increase after 120 minutes' which may be related to a growth effect during immersion with viable bacteria suspended in phosphate buffer suspension, however, the same nonviable bacteria in a sterilised manure showed no further increase after 10 minutes' and this might be due to the blocking of bacterial attachment sites by the components present in the manure.

Attachment to meat surfaces increased with increase in cell concentration in the suspending media (Butler et al., 1979; Chung et al., 1989; Dickson, 1991; Notermans & Kampelmacher, 1975). This indicates that maximal numbers saturate the attachment sites and low numbers are too light to occupy all the attachment sites. Dickson (1991) observed that *Salmonella* grown at 25°C attached to fat in higher numbers than bacteria grown at 37°C or at 37°C followed by refrigeration at 5°C, whereas attachment to lean tissue was unaffected by growth temperature. Although

the effects observed in this study were relatively small, they indicate that different mechanisms are active in bacterial attachment to different tissue types. Fat tissue has more lipid content whereas lean muscle has more protein therefore the attachment reaction occurs to both protein and lipid biomolecular tissue surfaces.

Depending on the experimental conditions, increases in temperature or pH were found to have a positive, negative or neutral effect on bacterial attachment to meat (Butler et al., 1979; Delaquis & McCurdy, 1990; Lillard, 1988; Notermans & Kampelmacher, 1974). Duffy & Sheridan (1999) demonstrated that the pH and culture procedures had significant effects on attachment of meat spoilage flora to polycarbonate membranes. The bacteria seem to adhere at both high and low temperatures and across a wide range of pH. No generalised conclusion can be made regarding the influence of temperature or pH on the attachment of bacteria to meat.

The extent of bacterial attachment is influenced by the composition of the suspending fluid, especially by the presence or otherwise of various cations, proteins or glucosaminoglycans (Lillard, 1988; Piette & Idziak, 1991a, 1992; Thomas & McMeekin, 1981b). Such effects can be very pronounced. For example, various proteins reduce the adhesion of *Lactobacillus* to collagen casings by more than one log cycle and the adhesion to beef tendon by more than three log cycles (Piette & Idziak, 1991a).

The effect of suspending fluid is, however, complex and-on occasion-contradictory. For example, sodium chloride completely inhibits the attachment of *Salmonella* species to chicken muscle fascia (Thomas & McMeekin, 1981b), but slightly increases the adhesion of *Pseudomonas fluorescens* to beef tendon (Piette & Idziak, 1992).

Attachment of many different types of bacteria can occur to meat surfaces. The majority of the bacteria tested demonstrated potential for attachment; attaching bacteria include A*cinetobacter, Bacillus, Brochothrix, Campylobacter, Clostridium, Enterobacter, Erwinia, Escherchia, Klebsiella, Lactobacillus, Listeria, Micrococcus, Moraxella, Proteus, Pseudomonas, Salmonella, Serratia, Staphylococcus and*

Streptococcus. There are, however a few exceptions. Certain strains of *Escherichia coli, Campylobacter jejuni, Staphylococcus, Proteus mirablis, Pseudomonas* and *Micrococcus* did not attach to chicken muscle fascia under conditions which permitted attachment of various other organisms (Campbell et al., 1987). *B. thermosphacta* did not show attachment to beef muscle pieces (Ockerman et al., 1992).

It is not known if all these organisms would be capable of attachment under different conditions. The relative ability of bacteria to attach to meat surfaces is not exclusively related to the Gram stain reaction, cell morphology, genera or species. However, there are some trends worth noting. In most studies *Pseudomonas* spp attach in high numbers (Butler et al. 1979; Farber & Idziak 1984; Kim & Slavik 1994), whereas lactobacilli attach poorly, demonstrating that different bacterial species have different abilities to attach to meat. Chung et al. (1989) reported no competitive interactions between several strains of spoilage and pathogenic bacteria during attachment to beef lean or fat tissue. The different bacteria neither enhanced nor interfered with the attachment of the other bacteria.

Benedict (1988) asked two interesting questions in his review on attachment and cell surface carbohydrates. Do pathogens bind more firmly than nonpathogens? Can initial attachment be blocked? Both pathogens and non pathogens tend to bind firmly to the surfaces whereas, with meat research, no attempts have been made so far to block the initial adhesion.

Attachment models sausage casings, collagen casings, and skins of lamb and pork have been used to imitate meat surfaces to minimise the variables and to provide simple models instead of using the complex natural meat surface (Butler *et al.*, 1979; Piette & Postec, 1993; Walls et al., 1993). Attachment of bacteria to these model systems was comparable with those reported for bacterial attachment to meat surfaces.

Different types of meat tissue allow attachment at different levels. Firstenberg-Eden et al. (1978), found that chicken breast fascia allows the greatest attachment, with

decreasing attachment observed in descending order for cut chicken muscle, cut beef muscle, cow teats and beef fascia. In contrast, the studies on the relative attachment to lean and adipose tissue indicate that bacteria attach equally well to both adipose and muscle tissue (Benedict et al., 1991; Cabedo et al., 1997; Chung et al., 1989; Dickson & Frank, 1993; Dickson & Macneil, 1991; Piette & Idziak, 1989). However, Bouttier et al. (1997); Dickson (1991) observed a statistically significantly greater attachment of bacteria to muscle as compared to fat. As the difference was less than 0.5 log units its biological significance remains questionable. Benedict et al. (1991) and Schwach & Zottola (1982) noted that adherence of bacterial cells to beef tissue was probably a result of physical attachment of the cells to the tissue and entrapment of the cells by beef tissue fibrils. The interplay of these two mechanisms may well explain the differences in attachment observed by different researchers employing different conditions.

2.4.3 Bacterial penetration of muscle after attachment

Muscle fibres undergo radial shrinkage and pull away from the surrounding endomysia when rigor develops. The resulting gaps provide a route for bacterial penetration (Gill et al., 1984). Cell motility and proteolysis enhance penetration especially in the presence of excess water, which may enlarge these gaps. Gill et al. (1984) concluded that the fluid in gaps between muscle fibres in chicken meat presents little barrier to the passage of motile bacteria. They and others also found that the presence of proteolytic bacteria could speed up the penetration of non-proteolytic types (Gill & Penny, 1977, 1982; Thomas et al., 1987b). Proteolysis aids penetration but is not a requirement (Thomas et al., 1987b). The ability of bacteria to penetrate meat tissues has significant implications for the effectiveness of a decontamination process, as these cells will be protected from the effect of sanitising chemicals. Woody et al. (2000) showed high pH treatments were ineffective in killing *E. coli* O157:H7 cells, which have penetrated deep into beef muscle tissue. Therefore this indicates that deep penetration reduces the effectiveness of decontamination procedures.

2.4.4 Specific bacterial structures

Bacterial surface structures are involved in mediating attachment. Such bacterial structures include flagella, fimbriae or pili and extracellular polymers.

The involvement of flagella, the organelles of locomotion, in adhesion to surfaces has been reported by many workers (Bouttier et al., 1997; Meadows, 1971; Notermans & Kampelmacher, 1974; Piette & Idziak, 1991b). The cumulative evidence suggests that these surface structures have a definitive role in attachment of bacteria to surfaces. However, Lillard (1989) could find no such role for either fimbriae or flagella.

The possible involvement of motility in attachment has been thought to be that of aiding bacteria to reach the surface (Alder, 1966). Farber & Idziak (1984) reported that motile cells attached better to chicken muscle fascia and beef than did non-motile cells. In contrast, Lillard (1985) concluded that nonflagellated cells attach more readily to poultry skin than do flagellated cells.

Intrinsic properties of the bacterial cell may also affect attachment to tissue surfaces. DeGraft-Hanson & Heath (1990) reported that treatment of *Pseudomonas* cells with D-mannose reduces cell motility, indicating an effect on flagella, but did not reduce attachment to poultry skin. While this work supports the observation of Lillard (1985) research into the effect of motility or flagella remains equivocal.

Similarly, fimbriae seem to have no critical role in the attachment of *Salmonella*. Typhimurium to chicken skin (Lillard, 1986 b). In contrast, the fact that mannose decreases the attachment of fimbriated *E. coli* to the teats of cows suggested a possible interaction between mannose sensitive type 1 fimbriae of this organism and the meat surface (Notermans & Kampelmacher, 1983). Most tissue culture studies show that mannose has specificity towards *E. coli* and helps in the washing of attached cells from the surface (Sharon et al., 2002). Ofek et al. (1977) showed evidence for pili mediated attachment in a specific and nonspecific manner, which suggests pili have a significant role in attachment. Ward & Berkeley (1980) reported the role of extracellular polymers and capsular material in bacterial adhesion.

2.4.5 Meat surface characteristics

Surface characteristics of the meat play a significant role in attachment. Studies with *Salmonella, E. coli* O157:H7 and *P. fluorescens* indicate that bacteria preferentially attach to connective tissue, with collagen fibres being the preferred bacterial attachment sites. Benedict et al. (1991); Fratemico et al. (1996); Piette & Idziak (1992) and Walls et al. (1993) reported that bacteria will almost completely cover a tendon surface indicating that any specific binding is either with collagen or proteoglycan molecules. Piette & Idziak (1992) demonstrated that *E. coli* and Enteropathogenic *Yersinia* have outer membrane proteins that mediate specific binding to collagen and fibronectin. The adhesion of *E. coli* to the pig intestine showed the involvement of Type I Pili which, in turn, suggested the presence of specific receptors on the epithelial surface (Dugid et al., 1966). Beachy & Simpson (1980), presented clear evidence that specific binding sites are involved in attachment and that *Streptococcus* LTA (Lipoteichoic acid) interacts with specific binding sites present on epithelial cell surfaces. Parry & Craig (1984), Ljungh et al. (1991) and Schulze – Koops et al. (1992, 1993) stated that Lipoteichoic acid of cell wall mediates adherence to fibronectin, a mechanism that could apply to many Gram positive bacteria (Courtney et al., 1983). *S. aureus* also has specific binding sites for collagen and fibronectin (Speziale et al., 1986).

Evidence for different binding mechanisms for Gram positive and Gram negative bacteria was provided by Vercellotti et al. (1985) who found that Gram positive bacteria will aggregate with extracellular matrix proteins (fibronectin, laminin and type IV collagen).

Lectin proteins produced by plant cell surfaces have specific receptors for bacterial polysaccharides which are involved in attachment (Dazzo, 1980). Although lectins may be involved in specific binding mechanisms, mannose-a lectin binding agent, only slightly inhibited attachment of *Salmonella* to poultry meat (Benedict et al., 1991).

2.4.6 Methodological considerations

The difference between strongly attached cells and loosely attached cells has been expressed in terms of the strength of attachment or S – value (Firsternberg-Eden, 1978). These terminologies have since been slightly modified by Butler et al. (1979); Dickson & Koohmaraie (1989); Farber & Idziak (1984) who all consider that bacterial populations associated with tissue surface are in fact attached cells.

In most previous research the bacterial populations were determined by conventional culture techniques, that is plate counts. However, several researchers have used electron microscopy for both qualitative and quantitative studies. Thomas & McMeekin (1981a) attempted to quantify numbers of attached cells on chicken surfaces by scanning electron microscopy (SEM), and reported that the presence of low levels of salt in the inoculating menstrua (0.9% W/V) prevented the attachment of *Salmonella* to chicken skin. However, when Lillard (1988) repeated the experiments of Thomas & McMeekin on chicken surfaces using both SEM and conventional culture techniques, she could not confirm the earlier results. The bacterial cells in saline inoculum apparently attached in the crevices in the tissue itself where they were not visible by SEM.

Researchers of bacterial attachment to poultry Lillard (1986), Thomas & McMeekin (1981) and beef tissue surfaces (Butler et al. 1979; Dickson, 1988) have used liquid attachment medium as a method of inoculation. This is a suitable method for inoculation for poultry because it simulates conditions commonly found in the chilling process with poultry processing (Lillard, 1989) which is a significant source of contamination. Since most contamination of beef carcasses results from solid or semisolid material, the use of a liquid inoculation system may not be representative of contamination under beef processing conditions. Bacterial contamination of beef tissue by inoculated manure has been studied. Dickson & Koohmaraie (1989) explained the observation that fewer bacteria deposited on the surface over time from the manure than from the liquid inoculation system compared with a natural inoculum by saying that this could be an effect of bacterial surface binding sites being blocked by the components in the manure slurry. These differences suggest

that an appropriate inoculating system must be used relevant to the animal species being studied. The transfer of bacteria between beef tissue surfaces has also been documented (Dickson, 1990), with the numbers of bacteria transferred decreasing as the length of time the initial inoculum was allowed to adsorb onto the base tissue increased. This observation indicates that the strength of attachment increases with time after initial (i.e. reversible) contact has taken place.

2.4.7 Attachment of bacteria to other food surfaces

Liao & Sapres (2000) reported that bacteria attached more firmly to injured fruit tissue when compared to skin. Sanitiser treatments were partially successful but were considered as a major problem in the subsequent production of juice. Takeuchi *et al.* (2000) demonstrated that different species attach differently to lettuce leaf structures and this was confirmed by confocal scanning laser microscope studies.

Vatsos et al. (2001) observed that different isolates of the fish pathogen *Flavobacterium psychrophylum* exhibit the same attachment ability to unfertilized eggs of rainbow trout as they do to the fish surface. Increased surface hydrophobicity of the cells and greater ability to attach to egg surfaces was found to be more pronounced with fresh cultures when compared to that of old cultures.

Bacterial attachment to meat surfaces appears to be very complex and multifactorial as meat surfaces present different tissue types interacting with complicated cell wall components of bacterial surfaces. Numerous variables require careful consideration while studying adhesion to meat surfaces. These variables include growth and culturing procedures of bacteria and surface associated liquid films and so on.

2.4.8 Methods for studying attachment of bacteria

In the meat plant, bacterial enumeration by traditional dilution and plating procedures by swabbing and excision were used to detect bacterial attachment to meat surfaces. The samples are collected in a clean stomacher bag and processed further to determine the counts. The total viable count (TVC) developed is perhaps the most convincing and widely used method of detecting viable cells, irrespective of

culturabilty. Standard plate count cannot be used to enumerate viable but nonculturable organisms, therefore alternative methods have been developed to enumerate these bacteria based on direct counting, the most common of which are epifluorescence microscopy and acridine orange staining (Daley & Hobbie, 1975). Problems occur as the cells may be difficult to remove and methods relying on the culture of bacteria will fail to detect viable but nonculturable forms which may later threaten product quality (Wong & Cerf, 1995). In this situation there is a need for rapid detection methods to provide meat processors with a quick assessment of the microbiological quality of carcasses. Techniques that show promise for detection of bacteria on meat surface include microscopy, ATP measurement, flow cytometer, immuno fluorescent labelling methods, impedance measurement and molecular detection methods (Polymerase chain reaction, DNA or gene probes – fluorescent hybridization). Meat industry should focus on new reliable rapid methods in order to maintain the quality and shelf life of meat.

Microscopic Methods

Microscopy is widely used for visualising bacterial attachment, growth and biofilm formation in food processing systems. Many researchers used different microscopes for biofilm observation which include electron microscopy, scanning electron microscopy, low voltage field emission SEM, variable pressure SEM, cryo SEM, environmental SEM, TEM, light microscopy, phase contrast microscopy, confocal scanning electron microscopy and epifluorescence microscopy. However, epifluorescence microscopy is a powerful tool to use in quantifying microbial biofilms. It has been used by many investigators to study biofilm activity in either a simulated or an actual food processing plant. This system utilises the property of fluorescent molecules to visualize cells, either directly, as in fluorescent dyes, such as acridine orange, or indirectly when coupled in specific tags, such as fluorescent antibodies. These fluorochromes emit light of a given wavelength when excited by incident light of shorter wavelength. Filters are used to eliminate light of wavelengths different from that being emitted by the fluorochrome to give specific and sensitive detection of the specimen (Rawlins, 1992). Holah et al. (1989); Pettipher (1986); Pettipher et al. (1992,1995); Stanley et al. (1983); Wirtanen & Mattila-Sandholm (1993) and Yu et al. (1993) used epifluorescent microscopy to

study the attachment of bacteria to the beef and stainless steel surfaces. In epifluorescent microscopy the specificity is determined by the types of chemical staining reagents used for the visualisation of the cell. The DEFT uses acridine orange, a stain that binds to nucleic acids (Hobbie et al., 1977; Kepner & Pratt 1944). Holah et al. (1988) and Yu et al. (1993) described a procedure comparing direct epifluorescent microscopy and viable count for determining numbers of cells in a biofilm on stainless steel coupons and data were statistically analysed and gave comparable results. However, there are other methods to quantify the attached cells. These include those of Restaino et al. (1996); Rodrigues & Kroll (1990); Tortorello & Stewart (1994) estimated the attachment of *E. coli* O157:H7 and *Salmonella* in beef in a long-term survival study by application of the antibody–direct epifluorescent filter technique (Ab-DEFT). The Ab-DEFT is an example of how microscopy can be utlilised, despite traditional misconceptions, as a rapid screening test for detection of food pathogens.

Non Visual methods

There are a number of methods available for the determination of number of viable cells in a microcolony or a biofilm. The usual procedure involves obtaining a sample, diluting the sample in a sterile diluent, adding the diluted sample to a Petri dish, adding a nutrient medium, incubating at an appropriate temperature for a selected time interval, and then counting the samples that are formed. Several of the procedures are described in some detail in the *Compendium of Methods for the Microbiological Examination of Foods* (Vanderzant & Splittoessar, 1992).

Impedence microbiology is a rapid alternative method for the enumeration of total bacteria, evaluation of biofilms, coliforms, yeasts and molds. It offers advantages over traditional plating methods and eliminates the need for serial dilution of highly contaminated samples (Dhaliwal et al., 1992; Giese, 1995; Silley & Forsythe 1996). The automatic real time measurement of impedence makes it possible to test the effects of biocides, preservatives and other antimicrobial agents in the development of new food, pharmaceutical or cosmetic products. Impedence measurements offer today's microbiologist a tool for use in development of new products and a way to automate product and raw material testing.

The content of nucleoside phosphate AT P in bacterial cells is fairly constant and can be measured relatively easily. The amount of ATP present in a sample can be determined by a bioluminescent reaction between luciferin and the enzyme luciferase. If ATP is present in excess, the maximum intensity of the light given off is proportional to the amount of ATP present in the sample (Giese, 1995). Since food samples will also contain ATP, this procedure cannot be used to evaluate food samples or other materials containing biological matter for numbers, but it can be used to evaluate the food contact surfaces to determine how much organic material remains on the surface. This could then be related to the potential for microbial biofilms to be present on the surface. Nowadays, the best use of this procedure is to determine if cleaning and sanitization have been done correctly. Many food processing companies are using this methodology to determine the efficacy of their cleaning and sanitising practices. It has become a very useful tool. If calibrated properly it should be possible to use such a system to identify potential biofilm problem spots in a food system.

Flow cytometry

Flow cytometer as a tool for food microbiology offers the advantage of rapid and objective sample analysis. Analysis of 20-50x 10^4 individual bacteria can be accomplished in 1-2 minutes. The ability to collect in real time and store multi parameter data for subsequent reanalysis and the ability to sort bacteria of interest from mixed populations are unique features of this technology. Flowcytometry enumeration of *E. coli* O157:H7 in nonselective beef enrichment cultures was equivalent to Ab-DEFT, especially at higher concentrations of O157:H7. The major drawbacks are the background fluorescence counts seen with the flow cytometer but not with Ab-DEFT, which limit sensitivity, and a shortage of specific antibody probes for important bacterial pathogens. Other areas in food microbiology where the flow cytometer may be particularly useful are in the analysis and sorting of subpopulations of bacteria from complex mixtures and in the development of automated systems for quality control (McClelland & Pinder, 1994a, 1994b; Tortorella & Stewart, 1994).

Molecular methods

Molecular methods are becoming popular because of their role in detecting pathogens on the surface using automated PCR machines. Polymerase chain reaction using species specific primers were also used by food microbiologists to identify different isolates (Klijn et al., 1992; Taylor, 1992). PCR amplification of DNA encoding 16SrRNA for partial sequence analysis can be used to provide further evidence for the classification of an isolate as a new species (Klijn et al., 1991). Random amplified polymorphic DNA (RAPD) analysis was also used to screen rapidly for the similarities between different isolates (Williams et al., 1990).

To determine the difference in isolates in their DNA composition, restriction endonuclease analysis (REA) and ribotyping (restriction patterns of DNA encoding rRNA) were used (Grimont & Grimont, 1991). Using REA, differences between isolates are determined visually by the pattern of fragments of DNA, following digestion with restriction enzymes, separated in agarose gel. Ribotyping involves transferring these fragments of DNA to a membrane and probing with labeled rRNA. This results in fewer bands being identified and allows easier interpretation of results. Fluorescent in situ hybridisation (FISH) using oligonucleotides conjugated to a fluorescent dye enables the direct identification of single cells by fluorescence microscopy (Amann et al., 1990). The technique is useful for identifying bacteria in biofilms (Manz et al., 1993). DNA probe-based methods have been established as powerful tools for use in microbiological examination of foods. Where such methods were once considered esoteric and experimental they are now routinely used in laboratories. Probe based methods have proven to be especially useful in determination of pathogenic bacteria in foods, particularly in a situation where a high degree of specificity is required. An excellent review is available summarising the field of nucleic acid hybridisation and DNA probe technology (Hill & Keasler, 1991). However, efforts at further improvement of probe methods are aimed at making these methods faster, more sensitive and more convenient for the user. Manual formats are evolving to automated alternatives and nucleic acid amplification techniques are being coupled with detection chemistries and introduced to the food microbiologists as test systems. The major challenge will be making these amplified

methods user friendly and at the same time, economical enough for routine use in quality control applications.

To successfully study biofilms in food systems requires the use of a number of these procedures. The ideal system for carrying out quantification studies on biofilms in terms of an ecological approach should include use of viable count or epifluorescent microscopic procedures and molecular detection methods can be used to detect specific or new isolates in biofilms. There are many challenges awaiting those of us interested in biofilm formation in food systems. We have only scratched the surface as to what is to be learned about the microcolonies that inhabit the nooks and crannies of our food processing systems. To resolve these potential problems will require creativity and perseverance, but eventually problems with biofilms will be a problem of the past. We have to overcome some of the traditional thinking in food microbiology and develop concepts and ideas consistent with what is now known and what can be learned with the new techniques available.

2.5 SURFACE PROPERTIES OF BACTERIA AND MEAT

2.5.1 Cell surface properties of bacteria

Bacterial attachment to the meat surface is a complex process and it involves physicochemical parameters such as surface charge and hydrophobicity of cells and surfaces (Fletcher & Loeb, 1979; van Loosdrecht et al., 1987 a, b). In addition to charge and hydrophobicity, ionic interaction, surface tension and the composition of the liquid phase surrounding the meat have a major influence on attachment. Consequently, it is very important to focus on cell surface properties of bacteria to understand the mechanisms by which bacteria attach to meat surfaces.

Busscher & Weerkamp (1987) reviewed the role of specific and nonspecific interactions in bacterial adhesion to solid surfaces. Mechanisms of attachment involve two types of interactions: (1) Specific and (2) Nonspecific. Three distinct interaction regions are involved in bacterial attachment to solid substrata.

At separation distance >50nm only van der Waals forces operate. Nonspecific macroscopic cell surface properties play the dominating role in this stage of adhesion.

At separation distances between 10 and 20 nm electrostatic repulsion is active and results in reversible secondary minimum adhesion.

At separation distances <1.5nm- that is, at extremely small separation distances, specific interaction can take place. The organism must be capable of extruding adhesion probes with available hydrophobic groups, dehydrate the surfaces, thereby, facilitating direct contact. The capability to effect specific interactions is highly strain dependent.

2.5.1.1 Nonspecific interactions

Attachment to surfaces is mediated by physicochemical parameters of the bacterial cell surface that are important in the initial role of adhesion (Castellanos, 1997; Van Loosdrecht et al., 1987). In general, bacteria and meat surfaces carry a net negative charge at neutral pH and so they repel each other. However, most meat-associated bacteria are weakly hydrophobic and their initial adhesion to meat may be hydrophobic in nature and therefore are believed to vary with the electric charges on both the bacterial cells and meat surfaces (Bouttier et al., 1997; Dickson & Koohmaraie, 1989 ; Notermans & Kampelmacher, 1975).

Bouttier et al. (1997) and Lillard (1989) concluded that electrostatic interactions were not involved in initial attachment to meat surfaces. In contrast, Dickson & Koohmaraie (1989) reported that the relative negative charge on the bacterial cell wall correlated with attachment to beef tissue, although the correlation was greater with lean rather than fat tissue. In support of this view it was found that changing the surface charge of the meat with an electrical current affected bacterial attachment (Dickson & Crouse 1989). van Loosdrecht et al. (1987) found both surface charge and hydrophobicity influence adhesion and also found that as electrokinetic potential increases hydrophobicity decreases. The literature indicates an apparent discrepancy

over the role of electrostatic interactions in attachment to meat surfaces and this needs further research.

Electrostatic interactions are dependent on the ionic strength and pH of the surrounding medium (Bellon-Fontaine et al., 1996; Li & McLandsborough, 1999; Oliveira, 1992; Piette & Idziak, 1992). Adhesion of *P. fluorescens* increases slightly as the ionic strength of the solution increases (to <100mM) (Piette & Idziak, 1992). Piette & Idziak (1992) interpreted the earlier reports of Jones et al. (1981), Marshall et al. (1971); Orstavik (1977) and Stanley (1983) as indicating that adhesion increases with increased concentration of divalent or monovalent cations, which supports their own observations of the direct relationship between adhesion and ionic strength. Ions in solution reduce the thickness of the electrical diffuse double layer on each surface, which allows negatively charged cells to approach the negatively charged meat tissue more closely.

Different buffer strengths influence the distribution of charges on the surface. In the microbial adhesion to solvents test, *Streptococcus themophilus B* (STB) and *Leuconostoc mesenteroides NCDO 523 (LM 523)* display maximum affinity for acidic solvents and low affinity for basic solvents. This indicates that bacteria are strong electron donors and electron acceptors which might have a role in the interfacial region during the process of adhesion, (Bellon-Fontaine et al., 1996). Other attractive forces (i.e., van der Waals forces or surface appendages) can overcome the force of gross electrostatic repulsion and strengthen the attachment, thereby supporting local electrostatic attraction.

Electrostatic and hydrophobic forces may play a greater role in increasing the strength of the adhesive bond rather than increasing the number of attached cells (van Pelt et al., 1985). No correlation was established between electrostatic interactions and adhesion rate constants in the work of (Vanhaecke et al., 1990).

The practical importance of these electrostatic interactions is not truly known but, according to the electrokinetic theory of adhesion, they are expected to substantially affect bacterial adhesion (Piette & Idziak, 1990). The general lack of correlation

between cell surface characteristics and adhesion to meat surfaces might indicate that electrokinetic adhesion models when studied in isolation are inappropriate to describe bacterial adhesion to meat.

The cell surface property of hydrophobicity plays an initial role in adhesion (Castellanos, 1997; Dickson, 1989; van Loosdrecht et al., 1987). Interesting vibration studies of CCl_4 / water and hydrocarbon/ H_2O show that hydrogen bonding between adjacent water molecules is weak compared to the generally accepted models of water next to fluid hydrophobic surfaces where hydrogen bonding is strong. These results have important implications for understanding water adjacent to hydrophobic surfaces and the penetration of water into hydrophobic phases (Scatena, 2001).

In general there is no difference in attachment of a variety of bacteria to lean or fatty beef tissues, and that attachment was found to be poorly, or not correlated at all, with the bacteria cell hydrophobicity (Benito et al., 1996; Bouttier et al., 1997; Dickson & Koohmaraie, 1989; Piette & Idziak, 1991a). Studies with strains of Lactic acid bacteria showed positive correlation between hydrophobicity and strength of attachment (Marin et al., 1997). The discrepancy between the ability of organisms to adhere and their hydrophobicity probably indicates that other factors are involved in adhesion. No evidence has been found that the hydrophobicity of meat surfaces is critical in adhesion. The significance of hydrophobic interactions is unclear, as cells with either hydrophilic or hydrophobic surfaces will adhere to meat tissue (Bouttier, 1997).

The presence and chain length of my*colic* acids of bacteria were shown to be related to cell surface hydrophobicity (Bendinger et al., 1993). Del Re (2000) showed a relationship between adh + and adh – cells' ability to autoagglutinate and cell surface hydrophobicity measured by MATH test and adhesion. A parabolic relationship was established between contact angle measurement and adhesion rate constants to stainless steel (Vanhaecke et al., 1990). In another study, Bos et al. (2000) showed that bacteria do not have strong preferences for adhesion to hydrophobic and hydrophilic surfaces and concluded that substratum hydrophobicity is a major determinant of bacterial retention rather than adhesion.

Hydrophobic interactions, their role in adhesion and the significance of microbial cell surface hydrophobicity were reviewed by Duncan-Hewitt (1990), Rosenberg & Kjelleberg (1986) and Rosenberg & Doyle (1990) and these reviews explain the role of hydrophobicity in bacterial adhesion more elaborately.

As with any other physicochemical process, adhesion is subject to the laws of thermodynamics. The surface of the substratum is considered, as temporarily invariant and that of bacterial cells as a function of their physiological state. The physiological state of bacterial cells impacts on thermodynamics and adhesion (Grasso et al., 1996). Bacterial adherence correlated well with thermodynamic predictions and these data were used to determine the surface tension of the bacterial species (Absolom, 1983). The adhesion of a bacterium to a meat surface in a liquid phase will occur only if the event results in a decrease in the total free energy of the bacterium liquid surface system. The thermodynamic theory of adhesion implies that hydrophobicity of both the bacterium and the surface play a major role in adhesion. In effect, adhesion is expected to increase with increasing hydrophobicity of bacteria and on surfaces when the liquid phase is water or saline as is the case in most adhesion studies.

In practice, however, there is no general agreement between the predictions of the thermodynamic theory and experimental results. The general lack of correlation between cell surface characteristics and adhesion to meat surfaces might indicate that the thermodynamic adhesion models are inappropriate to describe bacterial adhesion to meat.

Surface active compounds tend to interact with interfaces and play a role in adhesion and desorption from the surfaces. Neu (1996) reviewed surface active compounds and concluded that surface activity plays a significant role in adhesion but more importantly desorption from interfaces. The general characteristics of surfactants are their concentration at interfaces, which lowers interfacial surface tension, and their ability to facilitate micelle formation. Rosenberg (1986) reviewed developments in microbial surfactant research. He emphasised that 0.1 to 1.0% of a polymeric bio-emulsifier or biodispersant is enough to saturate the surface of the dispersed material

resulting in micelle formation. Even a small quantity of surfactant can dramatically alter the surface properties, such as surface charge and hydrophobicity of bacteria. The effects on adhesion and desorption are discussed by Rosenberg, (1986). Whitekettle (1991) reported that 90% of microbial adhesion could be inhibited by use of non-ionic surfactants. Biosurfactants produced by *Lactobacillus* species reduced the initial deposition rate of *Enterococcus* by 70% and the number of adhering *Enterococci* after 4 hours of interaction was reduced by an average of 77% (Velraeds etal., 1996). Farghaly (2002) demonstrated that the surfactant "Spraycult^{TM}" is capable of dissolving biofilms covering meat processing equipment. Cacioglu (2000) demonstrated that pre-spraying beef surfaces with Tween 80 reduces the bacterial adhesion by lowering the surface tension and thereby interfering with hydrophobicity.

Piette & Idziak (1992) demonstrated that electrokinetic, hydrophobic and thermodynamic models were inappropriate to study attachment. This may be because

- The models are too crude to address the complexity of the bacterium/meat system.
- Comparing the adhesion of a pool of organisms in order to infer mechanisms of adhesion is flawed.
- The organisms selected might have very different adhesion mechanisms, which would make the whole attempt futile.

Chemical modification of the cell surface had been proposed as an alternative method to investigate the role of hydrophobic and electrostatic interaction in adhesion. This presents some disadvantages like changing the surface charge to affect hydrophobicity. This may explain the poor correlations obtained between attachment and surface characteristics. In contrast, Jones et al. (1996) suggested that standardising the buffer system would help to establish a relationship between cell surface properties and attachment.

Wilson et al. (2001) reviewed the methods for measuring cell surface charge. An & Freidman (1997) reviewed the laboratory methods used in studies on bacterial

adhesion. Methods for measuring hydrophobicity of microorganisms were reviewed by Mozes & Rouxhet, (1987). Jones *et al.* (1996) discuss the drawbacks of methods involved in measuring bacterial adhesion. These reviews about the difficulties associated with correlating each cell surface property with adhesion of bacteria to surfaces.

In the microbial adhesion to hydrocarbon (MATH) test Rosenberg et al. (1984) which measures cell surface hydrophobicity, the choice of organic phase is important as some organic phases destroy the surfaces of certain bacteria, giving a false indication of hydrophobicity. The implication of the MATH test for evaluating cell surface hydrophobicity state is that maximum removal rates of bacteria are achieved by a given hydrocarbon at pH values where electrostatic repulsion is absent. Such measurements can be considered as a meaningful measure of microbial hydrophobicity (van der Mei et al., 1995). However, Geertsema-Doornbusch *et al.* (1993) consider that the MATH test does not measure hydrophobicity, but rather the interplay of charge and hydrophobic properties.

Measurement of each property can be achieved by different methods. Results obtained by different methods are often poorly comparable. Each method has its own merits and demerits. Establishing a meaningful relationship of surface properties and adhesion is a difficult problem faced by most researchers. Hence ranking them in a particular high to low order appears to facilitate comparison of the hydrophobicity of various isolates.

2.5.1.2 Specific interactions

There is growing evidence that some bacteria attach to meat through specific interactions involving mutual recognition, then binding of ligands at the cell surface and attachment sites on the meat.

The fact that *Salmonella* species appear to attach preferentially to connective tissue fibres suggests a specific interaction with collagen or the glycoprotein substance around it (Benedict et al., 1991). Furthermore, the adhesion of *S.* Typhimurium and *S.* Singapore to swollen collagen fibres of chicken muscle fascia is reduced by

hyaluronan, a major component of the glucosaminoglycan matrix surrounding the collagen fibers (Sanderson et al., 1991). The association between hyaluronan and the cell surface appears to be specific and involve at least one complete monomer subunit of hyaluronan. The cell surface moiety has not been positively identified, but it might be one or several of five surface exposed proteins that interact with hyaluronan *in vitro*. Similarly, the adhesion of *S.* Typhimurium and of *Lactobacillus* species to collagen casings is reduced by preparations of solublised collagen (Barriga & Piette, unpublished results; Walls et al., 1993 b). The *Lactobacillus* species appear to possess a common 30 k Da surface protein that binds collagen specifically (Aleljung et al., 1991).

Proteins and polysaccharides play a major role in attachment of bacteria to surfaces. Coconnier et al. (1992) demonstrated that protein-mediated adhesion is very important in adhesion of *Lactobacillus acidophilus* to human enterocytes and mucus secreting cell lines in tissue culture and also indicated that polysaccharide-mediated attachment is partially involved in the process of adhesion. Hood & Zottola (1989) studied the adherence of *L. acidophilus* to human intestinal cells *in vitro* and revealed that polysaccharides mediated adhesion to surfaces.

Sarem et al. (1996) observed that trypsin and periodate treatment reduced attachment, emphasising the importance of both surface proteins and carbohydrate-mediated attachment of *Lactobacillus* strains to Caco 2 and Int-7407 intestinal cell lines. Tuomola et al. (2000) studied the effects of pepsin and trypsin treatments on the adhesion of *Lactobacillus* strains to intestinal glycoproteins, emphasising that bacterial protein structures are important in adhesion. Flint et al. (1997) investigated the cell surface properties of thermophilic streptococci on attachment to stainless steel, and found that protein-mediated attachment is very important in the early stages, while polysaccharide also played a part in attachment. Santiago et al. (1999) demonstrated that a 104 k Da surface protein designated "*Listeria* adhesion protein" (LAP) is responsible for the adhesion of *L. monocytogenes* to intestinal cells. This opinion is supported by Pandiripally et al. (1999) who demonstrated that a mutant strain AAMU572, which lacks the 104 k Da protein, exhibited lower levels of adhesion to human enterocytes such as Caco-2 cells than did wild type cells.

Expression of LAP protein was different at different growth temperatures and different growth phases. However, enhanced expression did not result in increased adhesion, suggesting that possibly a few LAP molecules are sufficient to initiate adhesion. This study signals the role of cell surface proteins and polysaccharides in adhesion of bacteria to surfaces. No studies have been conducted to explore the role proteins and polysaccharides play in the attachment of bacteria to meat surfaces.

Microbial blocking agents (MBAs) provide a unique microbial hurdle in a food processing cascade. They inhibit the expression of specific colonisation factors on the microbial surface that promote tissue attachment and compete for tissue binding sites on the epithelial surface to block or detach microorganisms. Because of this two fold effect the microorganism is repelled from the surface. Since the blocking activity is a molecular phenomenon related to structure and function, the activation of functional sites and a specific target delivery mechanism are critical for the efficacy of an MBA system (Naidu, 2000). Blocking of surface receptors was discussed by Fujiwara et al. (2001). Mannose –adherence of certain *E. coli* strains to various epithelial cell types has been shown to be mediated by common *type 1 pili* or *fimbriae* and can be inhibited by D-mannose (Dugid & Gillies 1957; Ofek et al. (1977); Salit & Gotschich (1977). Benedict et al. (1993) and Walls et al. (1991) demonstrated inhibition of adhesion with mannose with meat surfaces. Lammler et al. (1988) demonstrated that serum albumin - protein partially blocks the adherence of other bacteria. streptococci possess multiple adhesins mediating attachment (Hasty *et al.,* 1992). The role of proteins in adhesion was demonstrated by Cowart et al., (1989); Caccava et al., (1999); Pandiripally et al., (1999); Falkow et al., (1992) and Westerlund et al., (1993). Mannose is used as anti-adhesive sugar for fighting infectious diseases (Sharon & Ofek, 2002). Cell surface bound carbohydrate binding proteins (animal lectins) also act as attachment points for microorganisms carrying the appropriate sugars at their surfaces (Sheriff et al., 1994). Dominguez (1991) demonstrated adhesion between Listerial surface protein A and host cell heparin sulphate proteoglycans. Cell surface lectins (Sharon & Lis, 1989) are also involved in cell to cell attachment and/or signalling.

2.5.1.3 Factors influencing physicochemical properties

Peng et al. (2001) found adhesion increased markedly as cells entered different growth phases from the end of log to late stationary phase. The surface characteristics were measured and it was found that vegetative cells of *B. cereus* tend to be more hydrophobic at late stationary phase, followed by those from stationary and log phase. Vegetative cells prepared from the stationary and log phase had the highest and lowest surface charge values respectively. Various food processing surfaces present the risk of growth of *L. monocytogenes.* If the surface is wet and not maintained in a sanitary condition, attachment is enhanced (Blackman, 1996). van Loosdrecht et al. (1987) also found cell surface properties were influenced by growth conditions. At high growth rates, bacteria tended to be more hydrophobic and attached more readily. Growth conditions also influenced the cell surface properties of *L. monocytogenes* and its attachment to stainless steel (Briandet, 1999).

The genetic profiles of *Lactobacillus* strains correlated well with the physicochemical properties of lactobacilli and their ability to adhere to uroepithelial cells and hexadexane (Reid et al., 1999). Initial adhesion to substratum, coaggregation between microbial pairs and co adhesion between planktonic and sessile bacteria was reviewed by (Bos, 1999). The meat research shows lack of understanding of the role of coaggregation and coadhesion of microbes in attachment to meat surfaces.

On the whole, bacterial attachment to any surface is affected by cell surface charge as a function of ionic interaction and pH; hydrophobicity affected by surfactants involved in lowering surface tension properties of the air-water interface; reduction of surface free energy leading to the prediction of thermodynamic nature of interfacial properties; blocking of attachment sites with proteins or polysaccharides; removal of cell surface protein and polysaccharide biomolecules apparently offering a way to perturb bacterial associations with surfaces. Cell surface properties and adhesion mechanisms are poorly understood in terms of meat surfaces. However fundamental understanding of bacterial cell surface interactions in terms of meat surface may offer a way to optimise cell removal from the meat surface.

Comprehensive understanding of these major attachment mechanisms will be pivotal to improving the currently practised washing process in the meat plant. Therefore, understanding the role of cell surface properties in adhesion to meat surfaces in terms of single attachment mechanisms should enable blocking or reversal of the initial attachment of bacteria to meat surfaces. Most of the previous research has failed to correlate adhesion with cell surface properties.

2.5.2 Meat surface properties

Surface roughness of the substratum is important in adhesion to stainless steel, though cell surface roughness showed no correlation to attachment (Jones, 1999). Detachment of adhering *Streptococcus* occurred from salivary mediated-adhesion to teeth because cohesive failure of the substratum conditioning film leads to easy wipe off surfaces (Busscher et al., 1995). Formation of such a film is not reported in meat surfaces. The roles of meat surface roughness, substratum topography and surface properties have not been investigated. Understanding the substratum properties may lead to a better understanding of adhesive interactions.

2.6 DECONTAMINATION TECHNOLOGY

Fresh carcasses experience a series of contamination episodes during slaughter and processing to the dressed condition. The bacterial contamination of beef carcasses differs between processing plants and is influenced by plant design, speed of slaughter and skill of operators. Additionally, contamination varies with season of the year, type of animal slaughtered, anatomical carcass site and stage in the dressing process (Sofos et al., 1999).

In addition to minimising contamination through hygienic slaughter, dressing and handling practices, contamination, can be reduced by antimicrobial interventions that decontaminate the carcass. Such measures, by reducing the bioload on the surface of meat, contribute to the keeping quality of stored chilled products.

According to Smith (1999) of the Center for Red Meat Safety "Meat-borne pathogens are a genuine problem with which the red meat industries deal. Each year, more than

5 million cases of illness and 4000 deaths can be attributed to food-borne pathogens in meat and poultry products. The microflora of carcasses is raising safety concerns and the 1993 outbreaks caused by bacteria such as *E. coli* O157:H7 and *L. monocytogenes* increased consumer awareness and has resulted in intensified efforts to reduce the contamination of carcasses."

Carcass washing or cleaning are highly recommended as processes for separating out contaminants from raw materials. Physically removing bacteria remaining on carcass surfaces by washing with water and subsequent sanitising has been shown to be a practical and effective means of improving the microbiological quality of fresh meat (Dickson & Anderson 1992; Hardin et al., 1995).The objective of cleaning is to remove contaminants especially those that constitute a health hazard and to control microbiological loads that impair product quality (Brennan et al., 1990). Wet cleaning methods include rinsing, dipping or soaking, fluming, flotation, ultrasonic cleaning, filtration and settling.

Brennan et al. (1990) indicated that an acceptable washing process must satisfy the following requirements:

- Separation efficiency of the process must be as high as possible.
- It should be consistent with minimum wastage of good material.
- The contaminant must be removed completely after separation to avoid recontamination of the cleaned food.
- The process and equipment should be designed to limit recontamination of cleaned food by, for example, airborne dust or by contaminated washing water from previous batches.
- The cleaning process must leave the cleaned surface in an acceptable condition and product damage must be avoided.
- Volumes and strength of liquid effluents must be kept to a minimum and disposed of effectively.

Carcass washing and decontamination procedures used in the slaughter industry are limited mainly to the use of cold potable water. A typical meat plant cleaning

programme consists of cold potable water used to remove visible and faecal contamination, followed by a decontamination step such as organic acid and chlorine washes. If microbial contamination is reduced with such treatments, the reduction is not sufficiently large to extend the storage life of chilled carcasses (Sheridan, 1982).

In the last few decades, various processes have been developed to decontaminate carcass surfaces or minimise the bacteria on the surface and are now widely practised by the slaughter industry. Carcass decontamination methods employed by meat plants include both physical and chemical methods. The purpose of the review in this section is to summarise the principal features of each class of physical and chemical decontaminant.

2.6.1 Postslaughter physical treatments

2.6.1.1 Trimming

Trimming is the most widely practised method of decontaminating carcasses and is an approved method for removing faecal and ingesta contamination (Gill et al., 1996).

2.6.1.2 Cold water wash

Cold water washing is of low efficacy in removing bacterial contamination, but by raising water pressures, bacterial removal efficacy can be increased. Many researchers have investigated pressure as a variable in spray washing (Bracket et al. 1994; Crouse et al., 1988; Gorman et al. 1995a; Kotula et al. 1974) investigated the effect of spray wash pressures of 2,412 Kpa and 4,134 kpa and duration of washing on the natural microflora in beef carcasses. They found high pressure sprays to be effective in reducing Enterobacteriaceae counts. The application of pressure presents some disadvantages, including physical damage to the meat surface and bacteria being forced into the meat tissues (De Zuniga et al., 1991).

2.6.1.3 Hot water sprays

Hot water sprays are effective in reducing bacterial contamination. From a hygiene point of view, the higher the temperature the greater the microbial reduction.

Temperature, as a spray wash variable, has been included in most spray washing studies. Many researchers have shown temperature to be directly related to microbial removal (Barkate et al., 1993; Gorman et al., 1995 a; Kelley et al., 1981 Raegan et al., 1996). Temperatures above 70°C have been shown to reduce general bacterial populations, including *E. coli* and *Salmonella* on beef or lamb carcasses, (Barkate *et al.*,1993; Davey & Smith, 1989; Dorsa et al., 1996; Dorsa et al., 1997; Gorman et al., 1995 b; Reagan et al., 1996). Concerns with the use of hot water are discoloration and surface damage. Barkate et al. (1993) found that discoloration disappeared after cooling for 24 hours. Dorsa et al. (1996) observed visual appearance recovery after a 12-hour period.

2.6.1.4 Ionising radiation and Electron accelerators

Low dose ionising radiation of 2-3 (kgy) was effective in the elimination of *E. coli* O157:H7, *C. jejuni* and *Salmonella* spp from red meats and poultry. There is, however, considerable consumer reaction against the irradiation of food in many parts of the world (Solar, 1995). In time, consumer demand for safe food will outweigh this fear of radiation technologies.

Electron accelerators, which require no isotope, are used in France to decontaminate raw chicken portions. The main disadvantage of this approach is that electrons with the required energy level (up to 10 MeV) do not penetrate more than 1-2 cm, so large pieces of meat or whole poultry carcasses cannot be treated (Hinton & Corry, 1980).

2.6.1.5 Other postslaughter physical treatments

Many postslaughter treatments are widely practised to maintain microbiologically cleaner carcasses. Various physical principles have been implemented to reduce the bacterial numbers from meat surfaces. The physical methods employed to reduce

carcass contamination presents both advantages and disadvantages. Of all the physical decontamination procedures steam treatment appears to be the most effective whereas other methods fail to maintain the carcass quality and appearance. Some of the postslaughter physical treatments to reduce carcass contamination are highlighted below:

Table 2.3. A selection of physical treatments to reduce carcass contamination

Water, steam vacuuming systems	(Donnelly, 1995; Smith, 1996)
Steam	(Morgan et al., 1996)
Steam at atmospheric pressure	(Vogel & Silliker, 1972)
Steam under vacuum	(Klose & Bayne, 1970)
Ultrasound	(Banwart, 1989)
Electromagnetic radiations:	
Microwaves	(Fung & Cunningham, 1980)
Visible light	(Banks et al., 1985)
Laser energy	(Takahashi et al., 1975)
Infrared rays	(Brennan et al., 1990)
Ultraviolet radiation (UV)	(Haines & Smith, 1993)
Electricity, high voltage pulsed electric field	(Zhang et al., 1994)
Oscillating magnetic field pulsed pressure	(Mertens & Knor, 1992)
Air ions	(Gysin, 1986)
Freeze thaw cycling	(Olson et al., 1981)

2.6.2 Postslaughter chemical treatments

Many chemical methods have been employed to reduce the bacterial contamination present on carcasses. Chemicals are generally assessed with respect to their efficacy and practical suitability for use as decontaminating sprays or dips.

2.6.2.1 Organic Acids

The most frequently used chemical decontaminants are solutions (1-3%) of organic acids such as acetic and lactic acids, which reduce the number of bacteria on carcass surfaces (Smulders et al., 1986; Sofos et al., 1999). Organic acids can be used as dips or sprays.

The effects of commercially acceptable organic acids at different concentrations, temperatures and in mixtures on meat microflora have been studied. Such organic acids are most useful as warm rinses 50-55°C applied before chilling, especially in combination with preceding treatment using hot water or steam (Gorman et al., 1997; Hardin et al., 1995). Quartey et al. (1980) reported that 2% formic and 1% formic acids plus 1% acetic acid were most effective against microorganisms from meat samples, destroying 84 and 73% respectively.

Dickson (1998) showed that osmotic stress or desiccation in combination with 2% acetic acid is more effective than acid alone in reducing numbers of *S.* Typhimurium or *L. monocytogenes* on beef tissue. The use of saline acetic acid produced the greatest reduction, reducing numbers of *S.* Typhimurium an additional 1.5-2.0 log compared with acetic acid alone. Physical desiccation was effective in reducing the numbers of *Enterobacteriaceae* on inoculated carcasses prior to acid treatment and was seen as the more acceptable process in practice.

Nan et al. (1991) showed that a mixture of acetic acid and chitosan was more effective in inhibiting the growth of bacteria on pork stored at temperatures of 10 and 20°C than acetic acid treatment alone. Organic acids have been found to exert a more marked antimicrobial effect at higher concentrations. Higher temperatures also enhanced the antimicrobial effect.

Carpenter (1972) showed that acetic acid solution was more effective than pH 2.5 or 3.0. Treatments were most effective at the maximum concentration and temperature studied, namely: 3% solution at 70°C. Maximum reductions were of the order of 1.5 log cfu/gram for total aerobic *Enterobacteriaceae* and *S.* Typhimurium (< 1.0 log for

E. coli). Treatments with 2% lactic acid applied at a meat surface temperature of 37 °C were recommended as the optimal decontamination treatment to retain acceptable appearance and minimise treatment time. However, Brackett et al. (1994) judged acetic, citric and lactic acid to be ineffective against *E. coli* O157:H7 on beef.

The action of either warm (25°C) or hot (55°C) applications of organic acids did not differ appreciably. The reduction in population differed by < 0.3 log cfu/g immediately after treatment and <0.5 log cfu/g after 13 days' incubation compared to untreated controls. These reported reductions are significantly lower than those reported by Cutter & Siragusa, (1994). One reason for this contradiction may be the substantially lower acid concentration used by Brackett et al. (1994), 1.5% compared to the maximum 5% levels used by other researchers. However, Brackett et al. (1994) explained the differences between their work and previous studies by citing differences in methodology for enumerating *E. coli* O157:H7 and treatment factors such as spray pressure.

Cutter & Siragusa (1994) showed that acid concentration, tissue type and bacterial strain were more significant than the type of acid used to treat beef carcasses. The highest concentration studied, 5%, proved the most effective in reducing populations of *E. coli* O157:H7 *or P. fluorescens*. Bacterial reductions were consistently greater on adipose than on lean tissue. Strains of *E. coli* O157:H7 were more resistant to acid treatment than *P. fluorescens*; and *E. coli* 43895 was the most resistant *E. coli* O157:H7 strain tested.

Cutter et al. (1997) observed when using 2% acetic acid that while initial bacterial level, and to a lesser extent tissue type and menstruum, had an effect on bacterial reductions from beef surfaces, temperature did not. In challenge studies, Dickson (1992) showed that a 2% acetic acid solution reduced the level of *S.* Typhimurium on the surface of lean and fat beef tissue by between 0.5 and 0.8 log cfu/gram. The effectiveness of the treatment was independent of the level of initial contamination. The bactericidal effect was influenced by the amount of organic material on fat but not on lean beef tissue. Acid treatment sublethally injured about 65% of the population of both fat and lean tissue.

Dorsa et al. (1997b) conducted laboratory studies to determine the effect of water, Trisodium ortho phosphate, acetic acid or lactic acid washes on both pathogenic and nonpathogenic bacteria on beef carcass tissue handled and stored under normal industrial practices. Organic acid and TSP treatments were equally effective for controlling the growth of *E. coli* O157:H7 and *Clostridium. sporogenes*, but TSP was less effective in reducing the aerobic plate count (APC), *L. innocua* or Lactic acid bacteria counts.

Not all studies have found organic acids to be effective. Sanitising treatment on pork carcasses was shown to have limited effectiveness in terms of long term storage (Fu et al., 1994). In other words, growth of survivors during storage rapidly compensated for the reductions in numbers caused by the acid treatment.

Among the organic acids, acetic and lactic acids are the most widely used and are the most effective. Potential concerns associated with the use of organic acids include selection of acid-resistant bacteria that may accelerate rates of product spoilage, increase undesirable effects on product appearance and speed equipment corrosion (Gill, 1998). Collagen is soluble in acetic acid so perhaps there is a possibility of acetic acid dissolving collagen at the meat surface and thereby, threatening both product appearance and its keeping quality.

2.6.2.2 Chlorine

Chlorine compounds for example, sodium hypochlorite and chlorine dioxide gases, are widely used in the food industry. They are antimicrobial due to their oxidising effects. Patterson (1968) studied the hygienic effects of chlorinating water and concluded that chlorine at 10 ppm in wash water would reduce bacterial contamination and increase the general hygiene status of products. Spraying beef carcass surfaces with hyochlorite solution significantly reduced counts of aerobic mesophiles and faecal coliforms two days after treatment in comparison with water spraying (Titus et al., 1978). By contrast, Cutter & Dorsa (1995) found ClO_2 to be ineffective in reducing faecal contamination on samples of beef. Addition of chlorine to water washes provides little to no additional advantage over washing with water

alone. However, Skelley et al. (1995) showed that spray washing pork carcasses with 200 ppm NaOCl solution reduced the bacterial count and weight loss during chilling. Spray washing of beef forequarters with chlorinated water at 200 ppm reduced total aerobic bacterial count up to 4 logs. Spray washing of carcasses with hot chlorinated water 80°C 450 ppm substantially reduced bacterial contamination (Kelly et al., 1982; Kotula et al., 1974). Chlorine is not approved for use in red meat abattoirs in New Zealand but its use reduces bacterial counts (Kenney et al., 1995).

2.6.2.3 Polyphosphates

Polyphosphates have high pH and detergent properties and can be used as antimicrobial agents. TSP works by removing a thin layer of fat from the carcass surface and in the process the microorganisms attached to the surface are removed (Giese, 1992). The action of TSP is first to detach bacteria from the skin surface and then to cause rupture of the bacterial cell membrane. Ruptured cells are not protected and succumb to the ionic strength and high pH of the medium. Leakage of constituents from the bacterial cells following the use of other polyphosphates has also been observed (Lee et al., 1994).

There are conflicting reports on the sensitivities of Gram positive and Gram negative bacteria to polyphosphates (Lee et al., 1994). But TSP has been reported to be most active against Gram negative bacteria such as *Salmonella* spp, *Campylobacter* and *Pseudomonas* spp (Cory & Mead, 1996). Lee et al. (1994) carried out experiments on the inhibition of *S. aureus* by a number of food grade polyphosphates. Growth was totally inhibited by the polyphosphates. They speculated that efficacy would be higher in cooked meat and in fresh poultry than in fresh meat because the phosphatases present in fresh meat reduce the antibacterial effects of the polyphosphates.

Kim & Slavik (1994) pre-inoculated samples of beef or fascia with 9 log cfu/ml of *E. coli* O157:H7 or *S.* Typhimurium before immersion in a 10% TSP solution at 10°C for 15 seconds. The TSP solution *was* better in removing *E. coli* O157:H7 than *S.* Typhimurium and more effective in removing both species from fat surfaces than

fascia. TSP was found to be less effective than lactic or acetic acid for controlling aerobic plate count, *L. innocua* or lactic acid bacteria, (Dorsa et al., 1997 b). Reductions in the bacterial populations of 1-1.5 log were obtained in laboratory trials by Dickson et al. (1994) on lean beef tissue contaminated with Gram negative pathogens *S.* Typhimurium and *E. coli* O157:H7. Smaller reductions, however, were obtained with *L. monocytogenes.* Samples were treated by immersion in 8%, 10%, 12% TSP solutions at 25°C, 40°C, 55°C with contact times for up to 3 minutes. Higher reductions were obtained as the solution temperature and concentration increased. Greater reductions were observed on adipose tissue, with maximum reduction of 2-2.5 log cfu/gram in *S.* Typhimurium and *E. coli* O157:H7 and 1-1.5 log cfu/gram in *L. monocytogenes.* Trials using TSP to sanitise beef carcass surface tissue inoculated with *E. coli* ATCC 25922 showed reductions comparable with those observed in the laboratory for *E. coli* O157:H7 on lean tissue. Again, greater reductions were observed on adipose tissue. The greater efficiency was attributed to the contribution of the washing stage of the system in physically removing some of the bacterial populations.

2.6.2.4 Antibiotics

Antibiotics for food preservation have been restricted for reasons that were reviewed by Sofos & Busta (1992). Briefly, the restriction of antibiotic use is associated with the widespread development of antibiotic resistance.

2.6.2.5 Disinfectants

Commercial disinfectants such as glutaraldehyde (Bailey et al. 1977; Mast & Macneil 1978; Thompson et al. 1977); Iodophore (Spencer *et al.* 1968) and Cetyl pyridinium chloride (CPC) (Kim & Slavik, 1996) were assessed as meat sanitisers and were also successfully used to decontaminate produce as either washes or additions to immersion chilling operations. Cutter et al. (1996 b) showed that use of Carnatrol ™ and Timsen ™ was more effective than water washes for reducing bacterial populations associated with faecal contamination on beef tissue. Research by Cutter et al. (2000) showed that spray washing of beef fat with a CPC solution reduced inoculum levels (10^5 to 10^6 cfu/gram) *of E. coli* O157:H7 and *S.*

Typhimurium to virtually undetectable levels. A similar study by Ransom *et al.* (2001) produced the same conclusions. Residual CPC levels following treatment were considered excessive for human consumption and these chemicals have yet to receive federal approval for use.

2.6.2.6 Lysozyme

Lysozyme treatments are effective in reducing the attachment of Gram positive and Gram negative (Banwart 1989; Chatzopoulou, 1991; Sofos & Busta 1992) on surfaces. Lysozyme treatments combined with osmotic shock treatments have been shown to be effective against bacteria contaminating lamb muscle (Chatzopoulou, 1991). Lysozyme is currently expensive and so lysozyme-based decontamination would be uneconomical. However, lysozyme has recently been produced in industrial quantities and the price is likely to fall significantly.

2.6.2.7 Salts

Potassium chloride in unbuffered rinse solution increases bacterial detachment from meat surfaces, (Appl & Marshall 1984). A concentration of 0.1 M KCl removed 50% of attached bacteria. NaCl solution, on the other hand, was found to have a negligible effect on meat-borne bacteria (Dickson, 1988).

2.6.2.8 Hydrogen Peroxide

Hydrogen peroxide is an oxidising agent used as an antimicrobial agent. H_20_2 is effective in reducing bacteria in poultry chiller water (Lillard & Thomson, 1983) and on poultry carcasses (Izat et al., 1989; Lillard & Thomson, 1983; Mulder et al., 1987). However, the results of this treatment are bleached and bloated carcasses which would be unacceptable for a fresh or frozen product.

2.6.2.9 Ozone

Ozone, a powerful oxidising agent, is a naturally occurring gas. It has been used to extend the refrigerated storage life of meat and found to be effective in controlling

bacteria (Greer and Jones, 1989). Ozone also increased weight loss and trim losses and darkened the lean muscle colour.

2.6.2.10 Sorbates

Sorbic (2, 4 –hexadienoic) acid is a straight chain trans- unsaturated fatty acid. Sorbic acid itself is more soluble in lipid materials than in water. Its solubility in water is only 0.16% w/v at 20° C, but this increases with temperature and in buffered solutions with pH values above 4.4. Its pKa value is 4.76. Dips containing potassium sorbate with other organic acid salts at high pH were found to be effective for the extension of shelf life of unchilled beef samples at 30°C and 20°C up to 44h and 68 h (Kondaiah et al., 1985). The salts of sorbates inhibit the growth of organisms but they do not kill them. Their major effect is on yeasts and moulds. Robach and Ivey (1978) and Cunningham (1981) reported that potassium sorbate significantly inhibits the growth of *Salmonella* on poultry carcasses. At 5°C all treatments had a significant inhibitory effect on *E. coli* and *S. aureus, E. faecalis* and *C. perfringens* inoculated into unchilled fresh beef samples in comparison with untreated control.

2.6.2.11 Summary of postslaughter physical and chemical treatments

Many physical washing treatments are currently used to remove bacteria from meat surfaces. However, these treatments present the difficulty of driving the bacterial cells deep into the surfaces upon application, which creates a threat to the keeping quality and appearance of meat.

A very diverse range of chemicals, including organic acids, inorganic acids, chlorine, hydrogen peroxide and triphosphates, is being assessed in respect of their efficacy and practical suitability for use as decontaminating sprays or dips. Such antimicrobial treatments and strategic interventions, are actively being researched. Among the organic acids, acetic and lactic are the most widely used as well as being the most effective. As with the other antimicrobials, concentration and temperature of application influence their effectiveness. Sensory considerations severely limit the concentration of organic acids that can be applied, as these acids impart distinct

odours and flavours, as well as causing surface discoloration of meat. These changes can make the meat unacceptable to consumers, e.g., vinegar flavoured lamb in the Middle East.

The use of antimicrobial sprays and dips appears likely to become widely accepted within the meat and poultry industries as a cost-effective method of meeting ever more stringent microbiological standards or commercial requirements. Ideally, antimicrobial sprays must be non-toxic and should not affect: sensory quality, organoleptic quality, or cause discoloration and should be cheap and simple to apply. Selective antimicrobial activity, however, raises safety concerns that suppression of the spoilage microflora will provide an opportunity for unrestricted growth of any pathogens present.

Recently new methods have been developed to decontaminate carcass surfaces using Lactoferritin Naidu (2000); Spraycult^{TM} biofilm dissolving agent (Farghaly, 2000); CPC (Cutter et al., 2000; Ransom et al., 2001); peroxyacetic acid (Ransom et al., 2001); acidified sodium chlorite (Castillo et al., 1999b) and bactericidal agents (Ammor et al., 2004); microbiologically produced products such as the bacteriocin nisin (Chung *et al.* 1989 b) and biosurfactants have been reported to have activity against bacteria of concern to meat plants, with biosurfactants having anti adhesive properties. These developments indicate the importance that an understanding of microbial attachment has in the effective control of the bioload on carcass surfaces.

Decontamination procedures practised today clearly signal that meat researchers have failed to understand the fundamental adhesive interactions taking place between bacteria, meat surfaces and the environment. Focusing research in this area will aid the development of novel decontamination technologies that interfere with the major attachment mechanisms and will aid in optimising existing decontamination procedures.

2.6.3 Resistance to chemicals and heat tolerance

Adhesion to, and detachment of, bacteria from food contact surfaces have been reviewed by Bower et al. (1996) who outlined developments in biofilm research.

Bower & Daeschel (1999) suggest that the resistant nature of microorganisms present in biofilms on the food contact surfaces to the application of chemical and physical methods is an increasing problem in the assurance of food safety. The increased heat tolerance of *S.* Typhimurium *DT 104* isolates attached to muscle tissue was reported by Humpherey et al. (1997) and related to possible outbreaks of food poisoning involving cooked meat products. Development of resistance mechanisms by bacteria to the physical and chemical agents is an alarming issue for the maintainenance of food safety.

2.7 SUMMARY

The meat industry is conscious that bacterial contamination of meat and processing equipment is a major problem and seeks to investigate its control in a cost-effective manner. To date, in New Zealand, control of this problem has been by good hygienic practice. However, understanding the mechanics of initial bacterial attachment to meat surfaces may lead to new decontamination strategies.

Several researchers have addressed the problems of bacterial attachment to meat surfaces, which appears to involve a complex multifactorial mechanism. The physicochemical properties of the bacterial cell surface appear to be fundamental to the attachment process, but previous research has failed to adequately establish the relationship between surface properties and attachment to meat surfaces.

Many aspects of the process of attachment to meat surfaces remain unexplored. These include the role that bacterial surface proteins, polysaccharides, charge and hydrophobicity play in attachment and blocking of attachment sites and their interaction with the substratum.

Previous research has shown that numerous postslaughter physical and chemical treatments are widely used to reduce bacterial contamination. However, whether these treatments are effective by killing or removal of cells remains a matter of conjecture. The public health implications in terms of meat surfaces or any food surface- is important as molecular testing becomes more widely applied to tracing bacterial contamination.

Sequential decontamination treatments are attracting attention as a method of application because of possible synergistic or additive effects to remove bacteria from surfaces. This approach is commonly referred to as the *multiple hurdle effect* (Leistner, 1995). For example, Delmore et al. (1998) reported laboratory reductions in *E. coli* counts on beef fat when preevisceration washing was followed by acetic acid solution rinsing, warm water washing and a final washing with an acetic acid solution. This approach seems to be an effective decontamination technology for use in beef plants.

To summarise, previous research has not intensively studied the fundamental process of bacterial attachment to meat surfaces, which should be the theoretical basis for the development of procedures for the physical removal of microorganisms contaminating meat surfaces. In addition, better understanding of adhesive interactions and mechanisms which will lead to interventions against these interactions in order to remove attached bacterial cells from the surface must represent a vital step in improving the currently practised washing procedures.

Thus the objectives of the present research are to:

- Develop an understanding by which bacteria attach to meat surfaces.
- Understand the underlying theoretical principles of attachment to surfaces by exploring the role of cell surface properties.
- Introduce various interventions acting on major attachment mechanisms to remove attached cells from meat surfaces.
- Develop wash solutions to reverse the initial attachment of bacteria to meat surfaces.

Chapter 3
STRATEGIC EXPERIMENTAL APPROACH MATERIALS AND METHODS

New Zealand is a major exporter of red meat in the world market. Globalisation of the world's meat market has required meat processors to ship fresh meat over long distances. Many efficient preservative packaging technologies have been developed. Their successful application relies heavily on two factors: (1) storage and shipment temperatures being maintained as close as possible to the freezing point of meat (about $-2^{o}C$) and (2) exceptional hygienic quality of meat carcasses and cuts derived therefrom.

In order to meet the requirement for microbiologically cleaner carcasses, efforts have been made to develop new technologies and processes that will minimise the contamination of meat by bacteria during slaughter, dressing and subsequent handling. These efforts will be less empirical and all the more successful as the nature of the association between the contaminating bacteria and meat surfaces is better understood. The Literature Review presented in the previous chapter evaluated the current knowledge of the association between bacteria and meat surfaces.

It is increasingly evident that attachment of bacteria to meat surfaces is a complex phenomenon, which involves several distinct mechanisms, particularly electrostatic and hydrogen bonding, hydrophobic attraction and specific attraction between coordinating complexes, which in turn are controlled by many different factors. It is obvious that the slaughter technologies used today and subsequent handling of carcasses will affect meat surface characteristics such as microtopography and state of hydration. These characteristics, in turn, greatly influence the distribution of surface associated bacteria between free floating, entrapped and adherent bacteria. Adhesion itself is complex and probably always concurrently involves, to various extents, several physicochemical parameters, such as the surface charge and hydrophobicity of both the bacterial cell and

the meat surface. Also the surface tension, ionic content and the organic composition of the fluid phase surrounding the meat play a critical role in bacterial adhesion. Finally some bacteria probably interact with particular components of the meat surface through ligand-receptor specific interactions. Faced with these facts, it seems most unlikely that a single treatment applicable in industrial settings will be found that will efficiently decontaminate carcasses by removing the surface associated bacteria. Therefore, expanding understanding of the mechanisms of initial attachment and factors involved in the contamination of carcasses will contribute to the development of new treatments to reduce initial contamination and to remove or kill a substantial portion of the surface associated flora. The research objectives embodied in this thesis are presented in four self-contained chapters where:

Chapter 4 describes the development and validation of simple model systems for studying attachment to surfaces and the application of electrostatic interaction and hydrophobic interaction columns for studying attachment mechanisms and other physical factors controlling attachment.

Chapter 5 concerns the cell surface properties of bacteria, which include surface charge, hydrophobicity, protein and polysaccharide surface molecules. In this chapter, blocking of potential attachment sites is also investigated.

Chapter 6 explores the mechanisms of attachment interacting between bacteria and model and meat surfaces. This approach allows the principal components interfering with single attachment mechanisms (hydrophobic, ionic, protein and polysaccharide mediated attachment) to be identified.

Chapter 7 focuses on the design, formulation and testing of a wash solution to interfere with the principal attachment mechanisms. The solution could be applied to meat surfaces and achieve decontamination by either removing or killing the attached bacteria.

An overall evaluation of the whole research programme is presented as **Chapter 8**.

3.1 SOURCES OF ISOLATES

The cultures used in this study originated either from meat surfaces or reference culture collection. The meat spoilage and pathogenic bacteria used in this study are listed in Table 3.1 and were used for this study on microbial attachment to meat surfaces and collagen films.

Table 3.1. Sources of Isolates

Reference strains	Strain number	Source	Culture media and growth conditions
Brochothrix thermosphacta	NZRCCATCC 11509	Fresh pork sausage	25°C, 48 hours in BHI
E. coli O157:H7 *(+VT)*	NZRCC ATCC 35150	Human faeces	37°C, 16hrs in BHI
E. coli O157:H7 *(-VT)*	NZRM 3614	Not Known	37°C, 16 hrs in BHI
Enterobacter aerogenes	NZRCC ATCC 13048	Sputum	37°C, 16 hrs in BHI
Escherichia coli 916	NZRCC ATCC 25922	Clinical isolate	37°C,16 hrs in BHI
Wild E. coli E6	MIRINZ	Meat	37°C, 16 hrs in BHI
Lactobacillus viridescens	NZRCC ATCC 12706	Cured meat products	25°C CO_2, 16 hrs in BHI
Listeria innocua	NZRCC ATCC 19119	Sheep	37°C, 16 hrs in BHI
Listeria monocytogenes	NZRCC ATCC 35152	Guineapig/mesentric lymphnode	37°C, 16 hrs in BHI
Pseudomonas aeruginosa	NZRCC ATCC 25668	Clinical isolate	37°C, 16 hrs in BHI
Salmonella menston	NZRM 383	Not Known	37°C, 16 hrs in BHI
Staphylococcus aureus	NZRCC ATCC 25923	Clinical isolate	37°C, 16 hrs in BHI

3.2 CULTURE METHODS AND MEDIA

Preparation of Bacterial Suspension for Attachment Assays: Cell cultures were grown on Brain Heart Infusion (BHI) agar slopes at 37°C for 18 hours and the cells were washed from the slopes using 10 ml of dilution fluid (0.1% peptone, 0.85% NaCl). The cell suspensions were centrifuged at 1000 'g' at 4°C for 10 minutes. The supernatants were decanted and cell pellets re-suspended in 10ml of dilution fluid. This wash-centrifugation – re-suspension cycle was repeated three times leading to a final suspension in dilution fluid which contained approximately 10^9 cells/ml.

The same cell washing step was carried out using a phosphate buffered saline system (PBS) (5.0 g of NaCl, 7.0 g of Na_2HPO4 and 3.0 g of KH_2PO4 in 1000 ml of deionised water, pH 7.0). The meat industry uses dilution fluid for current industry practice and cell surface studies were done by using PBS. This study employed standard dilution fluid and a PBS system for studying attachment and cell surface properties in order to compare our work with that of previous researchers.

Preparation of stained cell suspension: *E. coli* E6 was grown in 10 ml of BHI at 37 °C for 18 hours and 10 ml of 0.001% of Acridine orange was then added to the broth culture and allowed to react for three minutes. The stained cell suspension was then centrifuged and washed as described above. The pellet was resuspended in diluent fluid to contain approximately 10^9 cfu/ml. The stained cell suspension was prepared in similar fashion for all the experiments.

Preparation of bacterial suspension for column assays: Cell cultures were prepared as previously described and resuspended in PBS.

3.3 DEVELOPMENT OF ATTACHMENT ASSAY

Procedure

Preparation of attachment bath: Bacterial suspensions for the attachment bath (sterile 500 ml beakers) were prepared from the final wash suspensions as follows: 2.5 ml of cell suspension was transferred into 250 ml of dilution fluid in the attachment bath giving a concentration of approximately 10^7 cells/ml.

Preparation of rinse bath: Rinse baths consisted of 100 ml of dilution fluid in sterile 500 ml beakers.

Preparation of medium for cell recovery: 100 ml of dilution fluid was added to each of the 18 stomacher bags.

All materials used in the attachment experiments were equilibrated at 20°C. The concentration of *E. coli* E6 cells in the attachment baths was determined by plating duplicate 0.1 ml volumes of ten-fold dilutions (10^{-3} to 10^{-5}) onto plate count agar half plates with developing colonies enumerated after 48 hours' incubation at 30°C. Background bacterial contamination on the cut beef muscles was determined in duplicate by placing each of the two samples into stomacher bags, stomaching for two minutes at the high setting and plating 0.1 ml volumes of appropriate dilutions onto plate count agar. The pH and age postmortem of each meat sample as well as a visual estimate of fascia and fat cover were recorded. The pHs of the attachment, rinse and recovery media were measured using a glass electrode.

Attachment experiments were conducted using the following protocol: Sixteen 48 cm^2 cut meat samples (4 x 4 x 1 cm) were placed into attachment bath containing an approximately 10^7 cfu/ml of a suspension of *E. coli* E6 in dilution fluid and incubated at 20°C. After 0.5, 5, 10, 15, 20, 30, 40 and 50 minutes two meat samples were individually removed from the attachment bath with alcohol-sterilised forceps. The meat pieces were each gently swirled five times in a rinsing bath and transferred to individual stomacher bags containing dilution fluid (see medium for cell recovery). Ten-fold dilutions of stomacher homogenates were prepared in the range of (10^{-1} to 10^{-3}) and 0.1 ml volumes of appropriate dilutions were plated in duplicate onto APC (aerobic plate count). After incubation at 30°C for 48 hours, colonies were counted and results were expressed as cfu/cm^2. The process was repeated by slightly changing the diluent fluid to PBS to study the attachment of bacteria in a buffer system.

3.3.1 Definition of attached cells

This experiment uses the procedure in section 3.3 with slight modification. The samples used collagen film, which is explained below. The attachment of *E. coli* E6 was studied

for the samples removed after five minutes from the immersion bath followed by a single rinse, or up to six rinses, before stomaching.

Preparation of attachment bath: As described in section 3.3.

Preparation of rinse bath: Rinse baths consisted of 100ml of diluent fluid in sterile 500 ml beakers. Experiment I utilised a single rinse bath. Subsequent experiments, numbered II to VI, used equivalent numbers of baths up to six baths.

Preparation of medium for attached cell recovery: The rinse steps described above were followed by a single stomaching in 100 ml of diluent fluid.

Attachment experiments were conducted using the following protocol: Two 4 x 4 cm pieces of collagen film samples 64 cm^2 were placed into separate attachment baths containing a suspension of approximately 10^7 cfu/ml of *E. coli* E6 in dilution fluid and incubated at 20°C. After 5 minutes' immersion, the two collagen films were removed with alcohol-sterilised forceps. The pairs of collagen films were swirled five times in a rinsing bath and then each film sample was transferred into an individual stomacher bag containing dilution fluid (see medium for cell recovery). Colonies were enumerated as described in section 3.3. The process was repeated, using increasing numbers of rinses as described above.

3.3.2 Selection of optimal cell concentration for the studies on attachment

This experiment used the procedure in section 3.3 with slight modification. The samples used were collagen film. The attachment of *E. coli* E6 was studied after immersion for five minutes in the attachment bath.

Preparation of attachment baths: Bacterial suspensions for the standard attachment bath were prepared from the final wash suspensions (refer section 3.2) as follows: 2.5 ml of cell suspension was transferred to 2.5ml, 25 ml and 250 ml of diluent fluid in the attachment bath giving a concentration of approximately 10^9,10^8,10^7 cells/ml. Attachment baths with lower cell concentrations were prepared in diluent fluid as approximately ten-fold serial dilutions of the initial bath i.e 10^6, 10^5, 10^4,10^3, 10^2 ,10^1

cells/ml by transferring 20 ml volumes of cell suspensions into 230 ml of diluent fluid contained in each of six 250 ml beakers.

Preparation of rinse bath: As described previously in section 3.3.

Preparation of medium for cell recovery: As described previously in section 3.3.

Attachment experiments were conducted using the following protocol: Pairs of 32 cm^2 collagen film samples were placed into each of the attachment baths containing approximately10^9, 10^8, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 cfu/ml of a suspension of *E. coli* E6 in dilution fluid and incubated at 20°C. After 5 minutes the pairs of collagen films were individually removed from each cell concentration attachment bath using alcohol-sterilised forceps. Collagen films were each swirled five times in a rinsing bath and transferred into individual stomacher bags containing dilution fluid (see medium for cell recovery). Colonies were enumerated as described in section 3.3.

3.4 SAMPLES USED IN ATTACHMENT ASSAY

3.4.1 Cut beef muscle

Hot boned vacuum packaged beef striploin (*Longissimus dorsi*) muscle was obtained from an export meat plant. The meat was trimmed to remove fascia.

Pre rigor meat samples: Trimmed samples were used directly in the experiments before rigor was complete. Pre-rigor state is a natural physical state of muscle immediately after slaughter and persists for some time before rigor is complete. The samples were cut with a knife sterilised by wiping the blade with 70% alcohol between cuts, into 4 cm x 4 cm x 1 cm samples i.e., with a 48 cm^2 surface area. The level of background contamination on these aseptically prepared beef samples was low compared to the numbers of cells attaching on placement into the test suspensions.

Postrigor meat samples: Samples were also placed in a chiller operating at 4°C for 24 hours for rigor to occur. Before being cut for use in experiments, these trimmed striploins were equilibrated at room temperature.

3.4.2 Uncut beef muscle

Hot boned fore shin conical muscle covered with fascia of the *Femoris capna ulnaris* muscle (FCU) was obtained from an export meat plant.

Prerigor meat samples: Refer previous section 3.4.1 for the explanation of prerigor meat samples. The samples were cut aseptically with a knife sterilised by wiping the blade with 70% alcohol to a size approximately 200 cm^2. The level of background contamination on these aseptically prepared beef samples was low compared with the numbers of cells attaching on placement into the test suspensions as assessed by plating appropriate dilutions in plate count agar and Mc Conkey plates. Following the rinse step the exposed cut muscle surface was trimmed off using a sterile knife before stomaching. Introduction of trimming of muscles following the rinse step is to exclude the measurement of attachment of bacteria to the exposed cut muscle surface.

Post rigor meat samples: Refer to section 3.4.1 for the explanation of postrigor meat samples and also refer to the previous paragraph for the steps involved in preparation of samples for this experiment.

Sample (Femoris Capri Ulnaris) Dissection

1. 10 carcasses (left/right sides) were selected immediately after slaughter. Carcasses were then railed into a chiller for (FCU) removal.

2. Alcohol wipes were used to sterilise equipment (knives, hook) before an incision was made from the inner ulna area down to the carpus (front hock joint).

3. The breakdown hook was inserted into the muscle (FCU) by the ulna area, this would ensure that an adequate length could be obtained, allowing the muscle to be dissected further to prevent cross-contamination.

4. By cutting across the muscle (FCU) behind the hook with a sterile knife at the same time as pulling upwards with the breakdown hook the muscle could be freed with minimal knife work, except at the hock area. A knife was used to cut the tendon by sliding along the foreshin bone. Enough tendons remained attached to the muscle so

that trimming could be conducted if needed. Samples were then placed into numbered bags.

3.4.3 Collagen film

Collagen films were obtained from Devro Corporation Bathurst, NSW, Australia. This Devro –Teepak is a clear edible food grade film which appears transparent with a slightly textured surface. This film is 0.25 mm thick, has a pliable texture, and is hydrophilic and nonwater soluble. The film contains 70% collagen, 20% humectant and 10% water. The film is stable when stored below 25°C in a dry cool place. These collagen films are prepared from cattle hide. The hide is split to remove the outer leather layer and the inner flesh and fat layer, leaving the protein-rich collagen centre. The collagen is purified, washed and then ground into a protein gel. The protein gel is then hygienically conditioned by a method exclusive to Devro; a continuous uniform protein film is formed from the conditioned gel. The collagen films are cut into a convenient length and marketed by the manufacturers in roll form.

For this study Devro–Teepak Collagen film was cut into 4 cm x 4 cm pieces (i.e., with a surface area of 32 cm^2) with scissors which were wiped with 70% alcohol between pieces.

3.4.4 Preparation of collagen film on slides

Clean new microscopic glass slides were used. Devro – Teepak Collagen film was cut to the size of glass microscopic slides. Two drops of super glue (Epoxy Resin) were placed on each glass slide and spread evenly over the surface. Films were placed onto the glued surface of the glass slide taking care to avoid wrinkles and distortion of the collagen film. Slides with mounted collagen film were allowed to dry for half an hour at room temperature.

3.4.5 Preparation of collagen coated slides

0.001 grams of soluble Collagen type III (Sigma) was added to 100ml of 0.1M acetic acid and allowed to dissolve at room temperature. Dissolution required approximately two hours. Clean new glass slides were soaked in 96% ethanol overnight and flamed before use. 0.1 – 0.2 ml of the acetic acid solution of Type III collagen was placed on

the centre of the slide and a spreader was used to distribute the solution evenly over the slide surface. The coated slides were conditioned overnight in an incubator operating at 42°C.

3.5 EPIFLUORESCENT MICROSCOPY

3.5.1 Collagen films as model for attachment of *E. coli* E6 to meat using epifluorescence microscopy – Qualitative study

Reagent Preparation

Stock Solution: A stock solution of Acridine orange was prepared for all the epifluorescent microscopic experiments by dissolving 0.1 gram of acridine orange (Cal Biochem San Diego, California) in 100 ml of distilled water. From this stock solution a staining solution of 0.001% of Acridine orange was prepared in distilled water and then transferred into an amber bottle and stored in the dark.

Preparation of cell suspension and stained cell suspension: For all the epifluorescent microscopic experiments refer section 3.2.

Method 1 Pre-stained cells on collagen film slides

A 0.1ml volume of pre-stain cell suspension was placed on the centre of the collagen film mounted on glass microscopic slide and spread evenly on the surface. The staining step was omitted from this procedure. Care was taken to avoid disturbing the cell suspension, which was allowed to react for three minutes. Then the stained area was flooded gently with distilled water. Washed slides were then allowed to dry naturally for two hours. Stained cells were observed through a 10x eyepiece in combination with 10x, 20x, 40x, 50x 0il iris and 100x oil immersion objectives of an epifluorescent microscope (Olympus MFS 493,Tokyo, Japan.) with a filter range WIB at 470 nm at phase III.

Method 2 Post-stained cells on collagen film slides

A 0.1ml volume of washed cell suspension was placed on the centre of the collagen film mounted on a glass microscopic slide. Then 0.2 ml of 0.001 % Acridine orange was gently added to the cell suspension and allowed to react for three minutes. Care was taken to avoid disturbing the cell suspension. The stained area of collagen film was then

flooded gently with distilled water. Washed slides were then allowed to dry naturally for two hours. The cells were observed as explained above.

3.5.2 Type III Collagen coated slides as a model for attachment of *E. coli* E6 to meat using epifluorescence microscopy – Qualitative study

Method 1 Pre-stained cells on collagen coated slides

The conditioned collagen coated slides were taken and 0.1 ml of the washed pre-stained cell suspension was placed on the centre of the collagen coated slide and spread evenly on the surface. The staining step is omitted in this procedure. Care was taken to avoid disturbing the cell suspension and it was allowed to react for three minutes. Then the slide was flooded gently with distilled water. Washed slides were then allowed to dry naturally for two hours. Stained cells were observed with an epifluorescence microscope as before.

Method 2 Poststained cells on collagen coated slides

The conditioned collagen coated slides were taken and 0.1 ml of the washed cell suspension was placed on the centre of the collagen coated slide and spread evenly on the surface. 0.2 ml of 0.001 % Acridine orange was then gently flooded onto the dispersed cell suspension and left to react for three minutes. The stained area of the collagen coated slides was then gently flooded with distilled water. Washed slides were then allowed natural drying for two hours. Stained cells were observed with an epifluorescence microscope as before.

3.5.3 Quantitative assessments collagen films as a model for attachment of *E. coli* E6 to meat using epifluorescence microscopy and compared with viable count

Method 1 Prestained cells on collagen film slides

Attachment experiments were conducted using the following protocol: Ten collagen film slide samples were placed into an attachment bath containing approximately 10^7 cfu/ml of an Acridine orange pre-stained suspension of *E. coli* E6 in dilution fluid and incubated at 20°C. After 0.5, 5, 10, 15 and 20 minutes two collagen film slides were individually removed from the attachment bath with alcohol-sterilised forceps. Collagen films were each swirled five times in a rinsing bath. The pre-stained Acridine orange

collagen film slides were taken out from the rinse bath and allowed natural drying for two hours. Stained cells were observed through a 10x eyepiece in combination with 10x, 20x, 40x, 50x oil iris &100x oil immersion objectives of a epifluorescence microscope (Olympus MFS 493,Tokyo, Japan.) with a filter range WIB at 470 nm at phase III. For all the quantitative methods cells were counted using the formula:

Number of cells/cm^2 = Number of cells/Number of fields x Microscopic area x 100

Method 2 Poststained cells on collagen film slides

The unstained collagen film mounted on glass microscope slides were taken out from the rinse bath and then 0.2 ml of 0.001 % Acridine orange was gently applied to the collagen film and allowed to react for three minutes. Care was taken to avoid disturbing the attached cells. The stained area was then flooded gently with distilled water and allowed to dry naturally for two hours. Stained cells were counted by epifluorescence microscopy as before.

3.5.4 Quantitative assessments - Type III Collagen coated slides as a model for attachment of *E. coli* E6 to meat using epifluorescence microscopy and compared with viable count

Method 1 Prestained cells on collagen coated slides

With prestained Acridine orange cells, slides were taken out from the rinse bath and allowed to dry naturally for two hours at room temperature, then counted using epifluorescence microscopy as before.

Method 2 Poststained cells on collagen coated slides

With unstained cells, the collagen coated slides were taken from the rinse bath and 0.2ml 0.001 % Acridine orange was then gently flooded onto the collagen surface and allowed to react for three minutes. The stained area was then gently washed by flooding with distilled water and then allowed to dry naturally for two hours before counting by epifluorescence microscopy as before.

3.6 SCANNING ELECTRON MICROSCOPY (SEM)

3.6.1 Surface study of cut beef muscle, uncut beef muscle and collagen film: SEM Investigations

The experiments under this section, used fresh meat samples (refer section 3.4.1, 3.4.2 & 3.4.3.). SEM samples of all the three materials, of appropriate size, were fixed in the TEM 1 degree fixative consisting of 3% (w/v) glutaraldehyde (BDH), 2% formaldehyde solution (BDH) in 0.1 M phosphate buffer (PB) (0.1 M KH_2PO_4 (BDH); 0.1 M Na_2HPO_4 at pH 7.2 for one to two days at room temperature and then washed three times with 0.1 m PB at pH 7.2. The preparations were then stained with 1% osmium tetroxide in PB and allowed to stand for two to three hours at room temperature. After staining, samples were again washed three times with buffer. Samples were then dehydrated in a graded acetone (BDH) water series to two changes of 100 % acetone. The samples were then critical point dried, using liquid carbon dioxide as the critical point fluid, in a Polaron critical point drier. The preparations were mounted on aluminium stubs using conductive silver paint or double-sided tape, gold sputter coated using a Polaron E5 100 cold stage sputter coater and examined in a Cambridge 250 Mk III Scanning Electron Microscope.

3.6.2 Attachment of *E. coli* E6 to uncut beef muscle, cut beef muscle and collagen Film: SEM Investigations

A slight modification of the attachment protocol described in section 3.3.was used. Each set of samples was removed from the immersion bath after the 10^{th} minute. The samples were then processed using the procedure detailed previously in section 3.6.1.

3.7 TRANSMISSION ELECTRON MICROSCOPY (TEM)

To visualise features of the trypsin, lysozyme and periodate treated cell surfaces (*S. aureus; B. thermosphacta and E. coli* E6) were examined by TEM. Sections of resin embedded preparations were prepared as follows: an 18-hour culture of cells was centrifuged at 1000 g for 10 minutes and resuspended in deionised water. The cells, controls and those treated as described in sections 3.12.1 and 3.12.2 were fixed at ambient temperature for 2-3 hours in a primary fixative solution consisting of 3 % (w/v)

glutaraldehyde (BDH), 2 % formaldehyde solution (BDH) in 0.1 M PB at pH 7.2. The cells were washed with three changes of PB and exposed to a secondary fixative solution consisting of 1-% osmium tetroxide (Pro Tech, Queensland, Australia) in 0.1 M PB, pH 7.2 at ambient temperature for 30 minutes. After two washes in PB, the cells were dehydrated using acetone (BDH) and embedded in Procure 812 epoxy resin (Pro tech). Thin (90 nm) sections were cut using a microtome, stained with saturated uranyl acetate (BDH) in 50 % ethanol followed by lead citrate (BDH) and examined using a Philips 201c (Eindhoven, The Netherlands)TEM.

To observe cell surface structures that might be responsible for adhesion, whole control and treated cells were negatively stained. One drop of culture was placed on a 200 mesh Formvar-coated copper grid, followed by a drop of 1% phosphotungstic acid (Hopkins & Williams, London, UK), air dried and viewed in the Philips 201 C (TEM).

3.8 WHOLE CELL CHROMATOGRAPHY

3.8.1 Electrostatic interaction chromatography (EIC)

The relative surface charge of each strain was tested by separation through anionic (Dowex AG 18* 8, 100-200 mesh) and cationic (Dowex AG 50W *8, 100-200 mesh) exchange resins. The method was based on that of Pedersen, (1980). Ion exchange columns were prepared as follows. Pasteur pipettes were plugged with glass wool and filled with 0.5 gram of ion exchange resin suspended in PBS and then eluted with a further 3ml of PBS. The final cell suspension (section 3.2) in PBS was adjusted to OD 1.0-1.2 at 540nm. Each column was loaded with 1ml of suspension and eluted with 3ml of PBS. The absorbance was measured at 540nm, and the percentage of bacteria bound to the resin was calculated from the absorbance of a one-quarter dilution of the bacterial suspension (A_o) and the absorbance of the sample eluted from the column (A_1) using the following formula $[(A_o-A_i)/A_o]$ x100. The mean value of five measurements was calculated and used to determine the relative charges based on chromatographic data and bacterial attachment.

3.8.2 Hydrophobic Interaction Chromatography (HIC)

Pasteur pipettes were plugged with glass wool and filled with 0.6 ml of phenyl sepharose (CL-4B Pharmacia Sweden) resin suspended in PBS pH 7.0 and were eluted with 3ml of PBS. The final concentration of washed cell suspension in PBS was adjusted to OD 1.0-1.2 at 540nm. Each column was loaded with 1ml of bacterial cell suspension and eluted with 3ml of PBS. The absorbance of the eluted suspension was measured at 540nm and the percentage of bacteria bound to the resin was calculated from the absorbance of a one quarter dilution of the bacterial suspension (A_o) and the absorbance of the sample eluted from the column (A_1) using the following formula $[(A_o-A_i)/A_o] *100$. The mean value of five measurements was calculated to determine the correlation between cell hydrophobicity and bacterial attachment.

3.8.3 Adhesion studies

The ability of different isolates and differently treated samples to adhere to meat surfaces was determined using the protocol described previously – (Section 3.3). Correlation of attachment with cell surface charge and hydrophobicity used a standard diluent fluid suspending system and a standardised buffer system using PBS at pH 7.0. All the studies on attachment were performed in duplicate. The cell surface charge and hydrophobic measurements were correlated with the attachment of cells to the collagen film model surface using Microsoft Excel.

3.9 EIC AND HIC COLUMN MODELS TO IDENTIFY COMPONENTS INTERFERING WITH SINGLE ATTACHMENT MECHANISMS

3.9.1 Influence of cell concentration on retention on the resins

Preliminary investigations were done to determine the optimal cell optical density with which to load resin columns. Tests were conducted on EIC (anionic and cationic resins) and HIC columns using *E. coli* E6. *E. coli* E6 cells were grown in BHI broth over night and washed in PBS pH 7.0 (as set out in section 3.2). Various cell concentrations of washed solutions were prepared by adjusting optical density to 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0. at 540 nm using a spectrophotometer. The cell concentrations

of different optical density were run through EIC and HIC columns (sections 3.8.1 and 3.8.2) and the percentage retention of each suspension was calculated and compared and optimal optical densities were chosen for further study. This test procedure employed PBS pH 7.0 as an eluent.

3.9.2 Spectrophotometric calibration of various eluents interfering with optical density reading

Tests were conducted to determine if the use of various eluents would interfere with the optical density reading of the cell suspensions by comparing the optical density reading of cell suspension in PBS pH 7.0 as an eluent. 3ml of each of the eluents were added to separate 1ml aliquots of washed cell suspension (adjusted to O.D 1.0 to 1.2) of Gram positive, Gram negative and Gram variable bacteria. The optical density of each mixture was recorded at 540 nm using a spectrophotometer.

3.9.3 Effect of various eluents interfering with ionic and hydrophobic attachment

EIC and HIC tests were employed to identify the various components interfering with the single attachment mechanism's charge and cell surface hydrophobicity, using the method described previously (sections 3.8.1 and 3.8.2) with slight modification. Several eluent components, 0.85%; 1.5 %; 2.0% and 3.0% NaCl; 0.1% Tween 80; 0.1% mannose; 0.1% peptone; PBS pH 7.0; PBS pH 5.0; PBS pH 9.0; 10% TSP pH 12.0; PB and distilled water were trialled. The attachment of bacteria to the resins using other eluents were compared to that achieved using PBS. The use of different eluents was expected to give different results, as the attached cells experience environment conditions that differ from those under which attachment occurred.

S. aureus 916 was identified as a bacterium possessing high positive, negative charge and hydrophobicity when compared to all the other isolates used in the study of the cell surface properties of bacteria. Consequently this bacterium was selected as the organism of choice for this study on the identification of the components interfering with single attachment mechanisms (charge and hydrophobicity). These studies were extended to include selection of agents interfering with attachment mechanisms applied to Gram

negative *E. coli* E6 and Gram variable bacteria *B. thermosphacta*. The *E. coli* E6 isolate was also used in the study of attachment, rinse and pre-wetting effects.

3.10 ESTIMATION OF ZETA POTENTIAL

The surface charge of 13 isolates was expressed as the Zeta potential measured in a Malvern Zetasizer IV (Malvern Instruments Ltd, UK) (Denyer et al., 1993). The operating principle of the Zetasizer is discussed briefly below: The measurement of bacterial cell electrophoretic mobility and Zeta potential can be made by the technique of quasi elastic laser light scattering. This involves the application of a known electric field across the suspension of the microbial sample contained in a cylindrical sample cell. The sample is illuminated with a laser light source (15 mW helium – neon laser) which is split into two beams of equal intensity. The split beams are made to cross at a stationary level (zero electro osmotic flow) in the sample cell which forms an ellipsoid measuring volume. In this region a pattern of interference fringes is formed as a result of the coherence of the two laser beams. The spacing of these light and dark bands is an exact function of the beam crossing angle and laser frequency particles moving through the fringe system will scatter light with a frequency different from that of the incident beam as a result of the Doppler effect. To determine the charge and velocity of the particles under investigation, one of the laser beams is modulated by a frequency of 250 hertz. This causes the interference fringes to drift in a direction parallel to the particle motion. Charged particles will move either with the fringe system or against it depending on the polarity of the applied electric field. Synchronisation of the modulating frequency and the field polarity with the particle movement then provides information about the sign and the magnitude of the particle surface charge. The Malvern Zetasizer II (Malvern Instruments U.K) is a system which determines the Doppler shift frequency by generating a correlation function of the scattered light intensity and converting this to a frequency spectrum via a fourier transform. In order to prevent contamination of the equipment, formaldehyde (0.4% vol/vol) was added to the culture prior to the centrifugation. After being washed twice samples were prepared for analysis by suspending them in 50 $mmol^{-1}$ PBS pH 7.0 and the cell concentration was adjusted to optical density 1.2 at 600nm using a Spectronic 20 spectrophotometer. The

mean value of five measurements was calculated and used for studying the correlation of Zeta-potential with attachment.

3.11 MICROBIAL ADHESION TO HYDROCARBON TEST - (MATH)

The cell surface hydrophobicity was determined using the MATH test (Rosenberg et al., 1980). An overnight culture was centrifuged at 1000 g for 10 minutes and the cells resuspended in sterile distilled water to an optical density at 600 nm of 1.0 -1.2. Samples of each suspension (3 ml) were added to 3 ml n-hexane and mixed on a vortex mixer at ambient temperature for two minutes. After settling at ambient temperature for 20 minutes, the absorbance of the aqueous phase was measured at 600nm. The percentage hydrophobicity was determined from the absorbance of the initial bacterial suspension Ai and the absorbance of the aqueous phase after mixing with hexane Af using the formula % H = Ai-Af /Ai*100. The mean value of five measurements was calculated and used to determine the correlation between cell hydrophobicity and bacterial attachment.

3.12 TREATMENT OF BACTERIAL CELL SUSPENSIONS WITH PROTEIN AND POLYSACCHARIDES DISRUPTING AGENTS

3.12.1 Removal of cell surface protein

To investigate the effect bacterial cell surface proteins have on adhesion, a trypsin treatment was used to remove cell surface proteins. Cultures were centrifuged at 1000 'g' for 10 minutes and the cells were resuspended in a 0.1 % trypsin solution incubated at (37°C for 60 minutes) and 1.0% trypsin solution incubated at (37°C for 24 hrs). After treatment, cells were centrifuged twice (1000 'g' for 10 minutes) and resuspended in sterile PBS pH 7.0. The procedures outlined in sections 3.3 & 3.4.1 were then followed to study adhesion of the treated samples.

3.12.2 Removal of cell surface polysaccharides

To investigate the effect on adhesion of removing cell surface polysaccharides, two chemical treatments were used to remove cell surface polysaccharides. Cultures were centrifuged 1000 'g' for 10 minutes and the cells were resuspended in one of the following solutions: 50 millimolar sodium meta periodate (22 °C for 1 hour & 22°C for 24); 0.1% and 1.0% lysozyme (37°C for 60 minutes). After treatment, cells were centrifuged twice (1000 'g' for 10 minutes) and resuspended in sterile PB pH 7.0. The procedures outlined in section 3.3 and 3.4.1 were then followed to study the adhesion of the treated bacterial cells.

3.12.3 Removal of cell surface proteins and polysaccharides; Comparison of optical density of treated and untreated cells

Preliminary studies were conducted to investigate differences in optical density before and after treatment (trypsin, periodate and lysozyme). 20 ml of washed cell suspension (*E. coli* E6; *B. thermosphacta* and *S. aureus*) were prepared (section 3.2) and the optical density was adjusted to 1.0 to 1.2 at 540 nm using a spectrophotometer. 10 ml of each of the adjusted cell suspensions were taken and treated with trypsin, as described in section 3.12.1 and 3.12.2. After treatment and washing, the optical densities were compared with those of the untreated cell suspensions. The same procedure was used for the other treatments, periodate and lysozyme respectively, to compare the optical density before and after treatment. Significant differences in optical densities after treatment of cells were considered to be evidence of cell damage.

3.12.4 Effect of various eluents interfering with ionic and hydrophobic attachment after removal of cell surface proteins and surface polysaccharides

The EIC and HIC test procedures described in sections 3.8.1 and 3.8.2 were employed to identify the various components interfering with single attachment mechanisms, charge and cell surface hydrophobicity, of treated (trypsin, periodate and lysozyme) and untreated cells. 1.5 % NaCl; 0.1% Tween; PBS pH 7.0 were used as eluents, selected as

a result of the previous work, which showed them to be the most effective eluents. The organisms chosen for this study were *E. coli* E6; *B. thermosphacta* and *S. aureus*.

3.13 QUALITATIVE ANALYSIS OF SUPERNATANT AFTER REMOVAL OF CELL SURFACE PROTEINS - PIERCE BCA ASSAY PROCEDURE

The protein concentrations in the supernatant after trypsin treatments were determined using the Pierce BCA Assay procedure. The Pierce BCA protein assay employs a detergent compatible formulation based on bi-cinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. The method combines the well known reduction of Cu^{2+} to Cu^{+1} by protein in alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation Cu^{+1} using a unique Bicinchoninic acid reagent. The purple coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. A 25 µl aliquot of each standard or unknown sample replicate was pipetted into individual wells of micro plates. 200 µl of the working reagent was added to each sample and mixed thoroughly by shaking on a plate shaker for 30 seconds. The plate was covered with a glass lid and incubated at 37 ° C for 30 minutes. After incubation, the plate was cooled to room temperature. The absorbance at, or near, 562 nm of each well was then measured using a plate reader. Calibration curves were prepared against standard bovine serum albumin (BSA) solutions. The results were expressed as BSA mg/ml.

3.14 QUALITATIVE ANALYSIS OF SUPERNATANT AFTER REMOVAL OF CELL SURFACE POLYSACCHARIDES – NEUTRAL GLYCOSE ESTIMATION

The polysaccharide concentration present in the supernatant after periodates and lysozyme treatment was determined using the phenyl sulphuric acid method. A 50 – 200 µl sample of the supernatant (any concentration above 0.5 mg/ml) was taken and 0.5 ml of 5% aqueous phenol and 2.5 ml of concentrated sulphuric acid were added. The mixture was cooled and the absorbance at 490 nm was measured (Dubois et al., 1956). When sugar is present, the solution turns yellow / brown and sugar can be quantitatively

estimated by reading the absorbance in a UV Spectrophotometer at 490 nm and comparing the results with a calibration. Calibration curves were prepared against standard lipoglycan solutions. The results expressed as lipoglycan mg/ml.

3.15 DETACHMENT STUDIES

The *E. coli* E6 meat indicator organism isolated at MIRINZ was used in all decontamination studies. Meat samples were prepared as described in section 3.4.1. For detachment studies only the rinse bath step was modified, which is described below. All the other steps were previously described as attachment assay in section 3.3. The following section presents the design of various rinse treatments trialled on meat surfaces.

3.15.1 Effect of single rinse treatments on attachment of *E. coli* E6 to cut beef muscle

The 0.1% Tween, 0.1%mannose and 1.5%; 2.0% and 3.0% NaCl; and solutions were applied as single rinse treatments.

3.15.2 Effect of combination rinse treatments on attachment of *E. coli* E6 to cut beef muscle

The 0.1% Tween, 1.5% NaCl and 0.1% mannose solutions were combined and applied as a single rinse treatment.

3.15.3 Effect of sequential rinse treatments on attachment of *E. coli* E6 to cut beef muscle

The 0.1% Tween, 1.5% NaCl and 0.1% mannose (six different combinations identified as TSM, TMS, STM, SMT, MTS and MST) were applied as sequential rinse treatments. The attachment assay described in section 3.3 was modified in this step, with three rinse baths used, following immersion in the attachment bath. The sequential rinse treatments studies were carried out using factorially designed experiments. The significance of differences between treatments were assessed by analysis of variance which was performed by Genstat version 6.1.0.20 (Lawes Agricultural Trust, supplied by VSN

International Ltd, Oxford, UK. Between treatments, selected group means were compared in pairs using Student's T test which was carried out by Microsoft Excel 2002 (Microsoft Corporation). The mean value of quadruplicate measurements was calculated and used for studying the rinse effect of the principal components in removing the bacteria from meat surfaces. The attachment was measured following the removal of samples from the sequential rinse baths after 5 minutes' and 20 minutes' immersion in the attachment bath.

3.15.4 Effect of sequential rinse treatments on attachment of *E. coli* E6 to cut beef muscle using high concentrations and vigorous rinsing of selected chemicals

After 5 minute' immersion in the attachment bath the Tween, NaCl and mannose solutions (six different combinations TSM, TMS, STM, SMT, MTS and MST) were applied as sequential rinse treatments with high component concentrations (5% of each component) and vigorous shaking (by maintaining the range of hand movement, swirling vs. back and forth etc.) of respective rinse baths for (1 minute) were applied in the rinsing step.

3.15.5 Effect of single prewetting treatments on attachment of *E. coli* E6 to cut beef muscle

This experiment investigated the prewetting of surfaces for 5 minutes by immersion in 250 ml of 0.1% Tween, 1.5%; 2.0% and 3.0% NaCl, 0.1% mannose, diluent fluid and 0.1% peptone before immersion in the attachment bath.

3.15.6 Effect of 10% TSP pH 12.0 treatments on attachment of *E. coli* E6 to cut beef muscle

10% TSP pH 12 was applied as a single rinse treatment following immersion in the attachment bath for 0 to 50 minutes.

3.16 ACCURACY AND REPRODUCIBILITY

Throughout this study, care was taken to ensure accurate and reproducible results. All tests were carried out in duplicate and, where appropriate, quantitative data were reported with standard deviations. The pH meter used for pH determinations was calibrated on a regular basis. Errors for incubation temperatures were +/- 0.5 ° C and for pH +/- 0.1.

3.17 STATISTICAL ANALYSIS

Stringent statistical analysis was used throughout this study.

Chapter 4
DEVELOPMENT OF MODEL SYSTEM

4.1 INTRODUCTION

Meat surfaces are structurally and chemically complex in nature. Slaughter operations, processing environments and processing procedures introduce spoilage and pathogenic bacteria onto carcass surfaces. Attachment of bacteria to meat surfaces is a complex phenomenon mediated by multiple attachment mechanisms which include electrostatic interactions, hydrophobic interactions, and more specific interaction involving surface protein and polysaccharide molecules (Benedict et al., 1991; Bouttier et al., 1994; Flint et al., 1997).

Meat is composed primarily of muscle tissue plus variable quantities of adipose and connective tissues as well as smaller amounts of epithelial and nervous tissue. Although all connective tissue types are present in the carcass, adipose tissue, bone and cartilage, connective tissue proper which envelopes muscle fibres and bundles and finally muscles themselves predominate. Muscle, fat and bone constitute the gross components of carcasses. Muscle tissues contain 65 to 80% of water; proteins (contractile, structural, cytoskeletal, sarcoplasmic and stromal proteins (which latter group includes collagen and other insoluble proteins); lipids, non-protein nitrogenous substances, carbohydrates; non nitrogenous substances and inorganic constituents (Judge et al., 1988).

In structural terms collagen is a major component of muscle. The sarcolemma (outer cell membrane) of individual muscle fibres is surrounded by the endomysium, a delicate connective tissue covering. This endomysium is composed of fine collagen fibres which are continuous with those in the perimysium, surrounding muscle bundles, and in the epimysium surrounding the whole muscles. Therefore even a cut muscle surface will present collagen molecules to attaching bacteria (Figure 4.1). Consequently, collagen film, which is derived from natural collagen, may provide a simple model system with which to study bacterial attachment to meat.

The validity of using a collagen film as a model for a meat surface could be demonstrated by comparing the influence of contact time on the bacterial attachment to the two surfaces. Assuming that bacteria attach to collagen film in similar manner as to meat surfaces, then the use of collagen films will provide a cost-effective model with which to investigate attachment and decontamination mechanisms. Many collagen surfaces like collagen casings, artificial sausage casings, and tendons, skin of lamb, pork and beef have been used to study bacterial attachment to meat surfaces (Butler et al., 1979; Walls et al., 1993). Development of a simple collagen film model would be a useful tool for investigation of bacterial attachment to meat surfaces.

The use of collagen film and collagen coated slides as a model system may allow direct microscopic assessment of bacterial attachment in addition to viable count assessments. This preliminary study concerned the development of a model system that would allow quantification of the total number of bacteria attached to the surface. Qualitative studies using SEM showed the deposition of bacteria on the collagen model system. Microscopic count techniques require collagen film to be mounted on a microscopic slide or collagen to be coated onto slides. These films may then be exposed to suspensions of bacteria in diluent fluid for various times followed by removal of unattached cells by washing. Bacterial cells remaining attached to the mounted collagen film or collagen coated slides after washing can then be observed using epifluorescence microscopy. For counting by epifluorescent microscopy, bacterial cells must be stained with a fluorescent stain before they can be visualised. The attachment of bacteria to the collagen film appeared as a reasonable and convenient model system.

Firstenberg Eden et al. (1978) have commented that the interpretation of attachment studies may be influenced by many variables, such as the number of loosely attached cells present in the surface associated liquid film. These variables need to be investigated.

It is increasingly evident that bacterial attachment to, or interaction with, meat surfaces is a complex multimechanism association (Benedict et al., 1991; Piette & Idziak, 1991). Attachment of bacteria to the surfaces probably always involves, to various extents, several physicochemical parameters, such as surface charge and hydrophobicity of both

the bacterial cell and of the meat surface. Also the surface tension, ionic content and the organic composition of the fluid phase surrounding the meat play a critical role in bacterial adhesion. Some bacteria probably interact with particular components of the meat surface through ligand receptor-specific interactions. There is a need to understand these complex attachment mechanisms by separating them in terms of single attachment mechanisms using simple practical systems.

The use of real meat to study attachment makes the interpretation of results very difficult. This study aimed to develop a simple and convenient model with which to study bacterial attachment to meat surfaces.

4.2 PROCEDURES

- To develop a collagen model system and to compare with regard to the attachment of *E. coli* E6 to the model surface, cut beef and uncut beef muscle (Figure 4.2). Refer section 3.3; 3.4.1; 3.4.2 & 3.4.3.
- To define an attached cell (refer section 3.3.1)
- Select the optimal cell concentration for attachment studies(refer section 3.3.2)
- To use SEM to compare bacterial attachment to meat and model surfaces (refer section 3.6.1&3.6.2)
- To validate the collagen film model system for qualitative and quantitative assessments using epifluorescence microscopy of bacteria attached to mounted collagen films on microscopic slides and collagen coated slides (Figure 4.3). Refer section (3.5.1; 3.5.2; 3.5.3; 3.5.4; 3.4.4 & 3.4.5).
- To compare the attachment of spoilage and pathogenic bacteria to model surfaces using standard diluent fluid and PBS system (refer section 3.1; 3.2; 3.3 & 3.4.3).

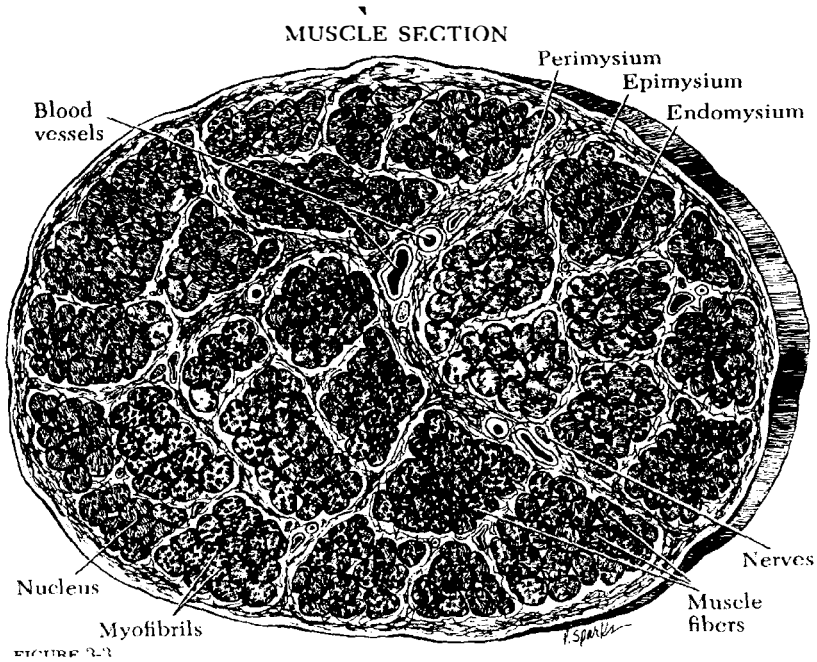


Figure 4.1. Drawing of a skeletal muscle in cross section showing muscle fibres, bundle arrangement, pervading connective tissues and blood vessels (Reference - *Principles of meat science*, Forest et al., 1975, Page no - 28)

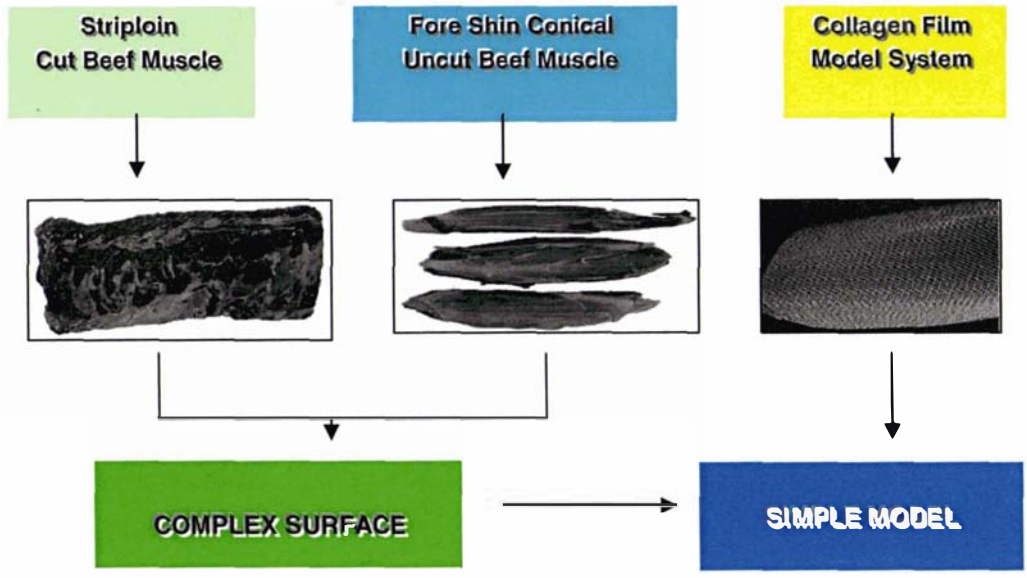


Figure 4.2. Proposed experimental design to develop a collagen film model system: Attachment of bacteria to cut beef, uncut beef muscle and collagen film model were compared using viable count procedure.

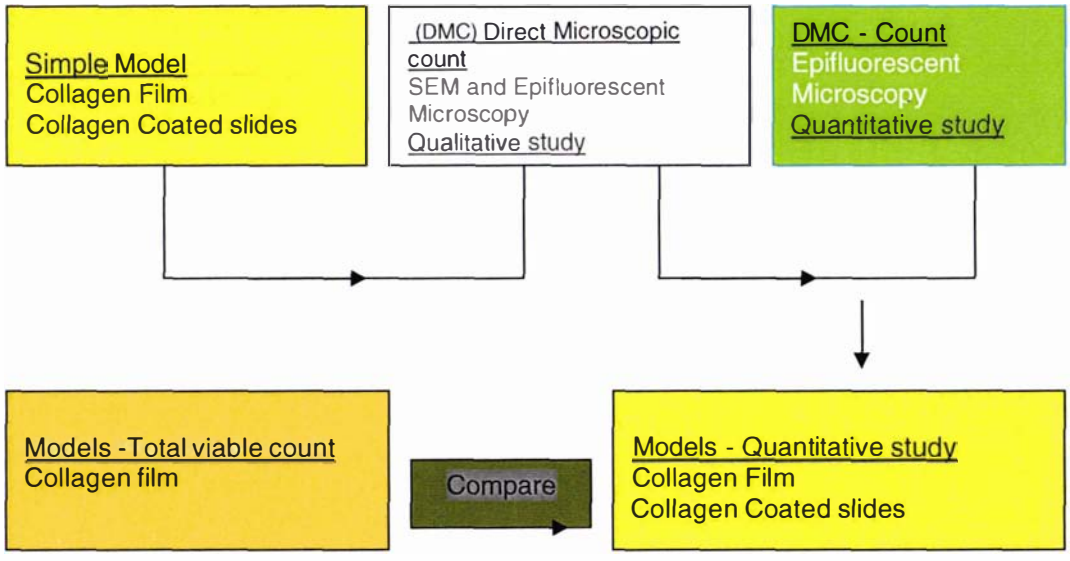


Figure 4.3. Proposed experimental design to develop a collagen film and collagen coated slide model system using qualitative (SEM and Epifluorescence Microscopy) and quantitative microscopic procedures (Epifluorescence Microscopy): Attachment of bacteria to collagen film model system using microscopy is confirmed by comparing the attachment of bacteria to model and meat surfaces using viable count procedures.

4.3 RESULTS

4.3.1 Attachment Protocol

The basic attachment protocol was trialled and the results are variously reported in subsequent sections.

Note: *The 10- second result has been arbitrarily placed at 20 seconds to separate this result from line 0.* ***Fig 4.4; 4.5; 4.6; 4.15; 5.9; 6.13;6.14; 7.2; 7.3; 7.6 & 7.7****. The time scale shows O, 5, 10, 15 and 50 immersion for figures (****4.6; 5.9; 6.13; 6.14; 7.2; 7.3 & 7.6*** *)* ; 0,5,10,15 and 20 for (Figure ***4.15***) *microscopic count* and *0 to 50 minutes for (Figure* **4.4)** *cut beef muscle and* **7.7** *(TSP treatments).*

4.3.2 Attachment of *E. coli* E6 to cut beef muscle

The meat was fresh and in prerigor state when it was placed into the attachment bath. No fascia was evident on any of the meat samples which all appeared to be 100% visually lean (i.e., had no subcutaneous fat), and had a mean pH of 5.4. The attachment, rinse and diluent media all had a pH of 7.0. Before immersion the natural contamination on cut beef surfaces was 1 log cfu/cm^2. The influence of time on the attachment of *E. coli* E6 cells to prerigor cut beef surfaces is shown in Fig 4.4. An attachment of approximately 4.5 log cfu/cm^2 occurred within seconds on immersion into a cell suspension containing approximately 10^7 cfu/ml and did not increase significantly with time. A similar pattern of results was observed with postrigor (which was 24 hrs postmortem when placed in an attachment bath), cut beef muscle with an attachment of 5 log cfu/cm^2.

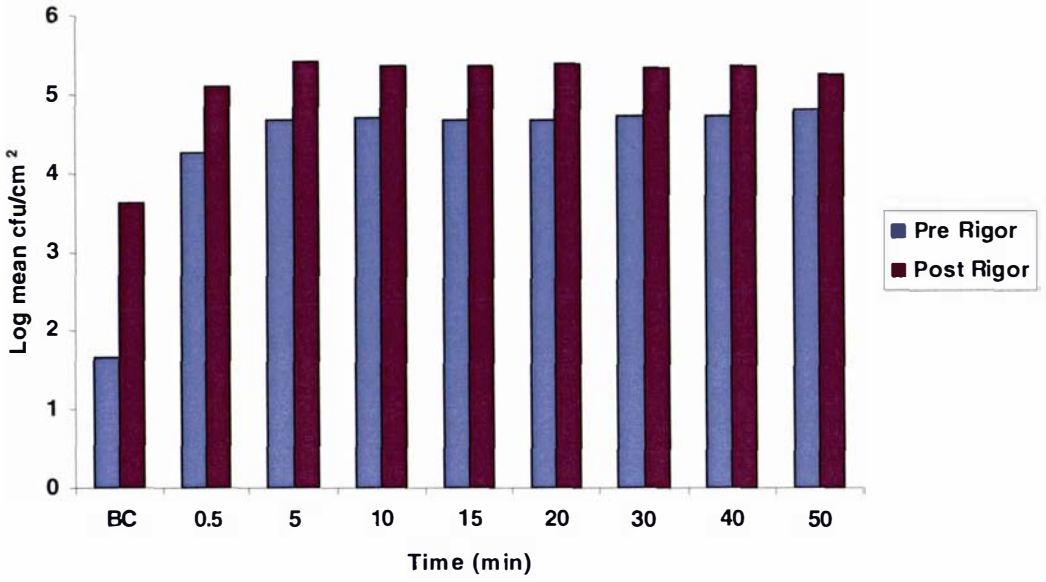


Figure 4.4. Influence of time on the attachment of ***E. coli*** **E6** to cut beef muscle surfaces (pre and postrigor) immersed in a cell suspension of 10^7 cfu/ml in dilution fluid (0.1% peptone and 0.85% NaCl). BC- Background contamination

4.3.3 Attachment of *E. coli* E6 to uncut beef muscle

The uncut beef fore shin conical muscle was fresh and in prerigor state when it was placed in the attachment bath. 95 % fascia and 1 to 5 % of muscle and other connective tissue layers were evident on the meat samples, which had a mean pH of 6.5 to 7.0. The attachment, rinse and diluent media all had a pH of 7.0. Before immersion the natural contamination on uncut beef surfaces was 1 log cfu/cm^2. The influence of time on the attachment of *E. coli* E6 cells to prerigor uncut beef surfaces are shown in Fig 4.5. An attachment of approximately 5 log cfu/cm^2 occurred within seconds of immersion in a cell suspension containing approximately 10^7 cfu/ml and did not increase significantly with time. A similar pattern of results was observed with post-rigor (which was approximately 24 hours postmortem) uncut beef muscle with an attachment of 5 log cfu/cm^2. The extent of attachment was quite comparable with the counts obtained using APC and McConkey agar medium.

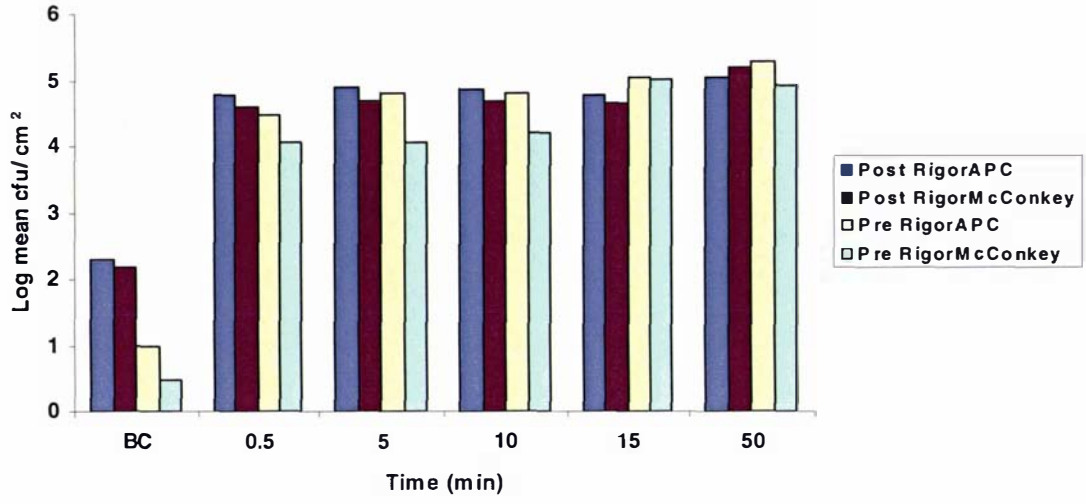


Figure 4.5. Influence of time on the attachment of ***E. coli*** **E6** to uncut beef muscle surfaces (pre and postrigor plated in APC and McConkey agar) immersed in a cell suspension of 10^7 cfu/ml in dilution fluid (0.1% peptone and 0.85% NaCl). BC-Background Contamination

4.3.4 Attachment of *E. coli* E6 to collagen film model

The pH of the collagen films was 3.88 and the attachment, rinse and diluent media all had a pH of 7.0. Before immersion, no natural contaminating microflora were detected. The influence of time on the attachment of *E. coli* E6 cells to the films is shown in Fig. 4.6. An attachment of approximately 5 log cfu/cm^2 occurred within seconds of immersion into a cell suspension containing approximately 10^7 cfu/ml and did not increase significantly with time. Therefore the attachment of *E. coli* E6 to collagen film model system is quantitatively comparable to that of attachment of the same bacteria to cut beef and uncut beef muscle. These results suggest that collagen film is a reasonable model system with which to investigate bacterial attachment to meat.

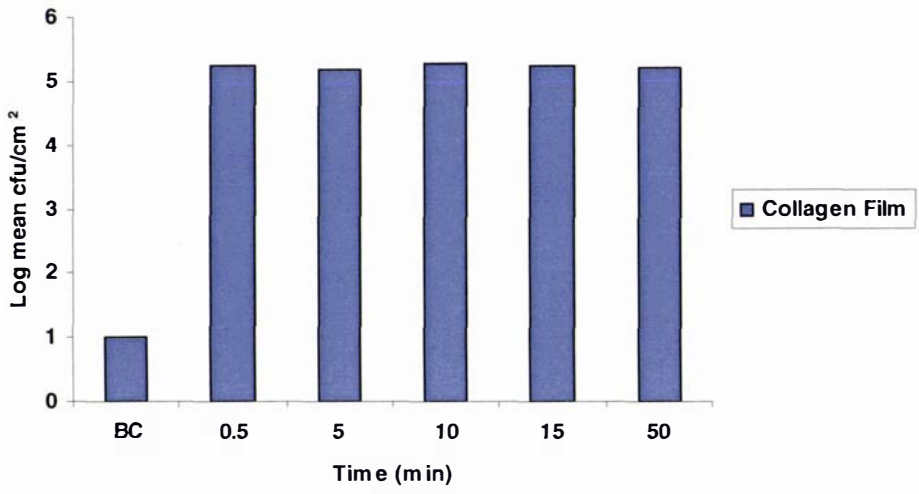


Figure 4.6. Influence of time on the attachment of ***E. coli*** **E6** to collagen films (model surface) immersed in cell suspensions of 10^7 cfu/ml in dilution fluid (0.1% peptone and 0.85% NaCl).BC- Background contamination

4.3.5. Definition of an attached cell

The influence of multiple rinses on the extent of attachment of *E. coli* E6 cells to collagen film model surfaces after five minutes' immersion is shown in Fig 4.7. Before immersion, the natural contamination on the model surface was 1 log cfu/cm^2. An attachment of approximately 5 log cfu/cm^2 occurred on immersion into a cell suspension containing approximately 10^7 cfu/ ml followed by one to six rinses. As evident from the figure, the statistical analysis also showed no significant differences $p>0.05$ in the number of cells counted on the surface after one to six rinses, which indicates that the microbial count on the surface remained constant and suggests that the bacteria are attached, rather than held in a liquid film. Therefore *attached cells* are defined as the cells that are recovered by stomaching followed by a single prerinse. Multiple rinsing does not remove greater numbers of unattached cells associated with liquid film than a single rinse.

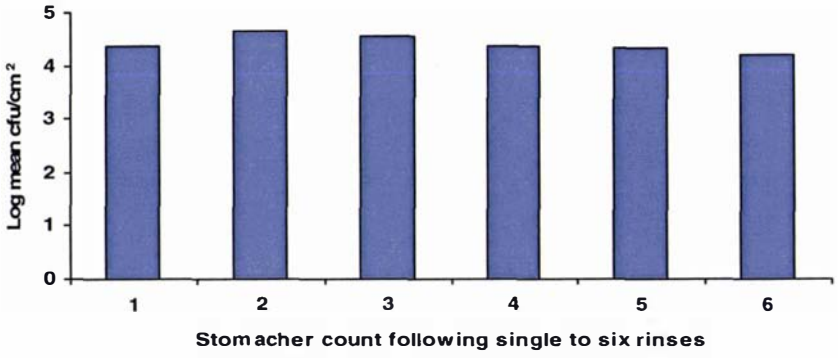


Figure 4.7. Influence of multiple rinsing on the attachment of ***E. coli*** **E6** to collagen films immersed in cell suspension of 10^7 cfu/ml in dilution fluid (0.1% peptone and 0.85% NaCl). *Rinses-1-6 (single to multiple rinsing followed by stomaching)

4.3.6 Selection of optimal cell concentration for attachment studies

The influence of cell concentration on the attachment of *E. coli* E6 to collagen films was determined by immersion of films in suspensions varying from 10^1 to 10^9 cfu/ml *E. coli* in dilution fluid. A linear relationship existed between cell concentration and attachment of *E. coli* E6 to the collagen film in Fig 4.8. The consistent attachment of 5 logcfu/cm^2 was observed when 10^7cfu/ml was used in the attachment bath.

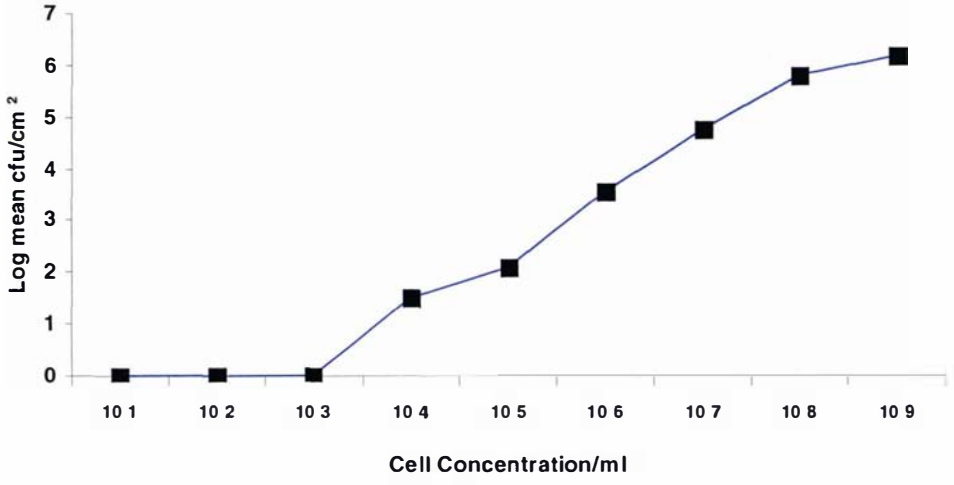


Figure 4.8. Influence of cell concentration on the attachment of ***E. coli*** **E6** to collagen films immersed in varying cell suspensions in dilution fluid.

4.3.7 SEM of cut beef, uncut beef muscle and collagen film surfaces

The scanning electron micrograph (Fig. 4.9) shows the structural complexity of cut beef muscle surface which appears rough with bundles of interwoven, muscle fibres, irregular surface, damaged muscle components, particulate material or debris, tears or depressions and a nonuniform surface with crevices.

The scanning electron micrograph (Fig. 4.10) shows the structural complexity of uncut beef muscle (fascia) which appears rough with torn membrane or connective tissues, irregular surface, damaged components of connective tissue, particulate material or debris, tears or depressions and a nonuniform surface.

The scanning electron micrograph (Fig. 4.11) shows the surface structure of the collagen film model system which appears as a smooth, uniform sheet overlying a filamentous matrix which contains small tears. The surface is relatively flat without any major depressions or crevices.

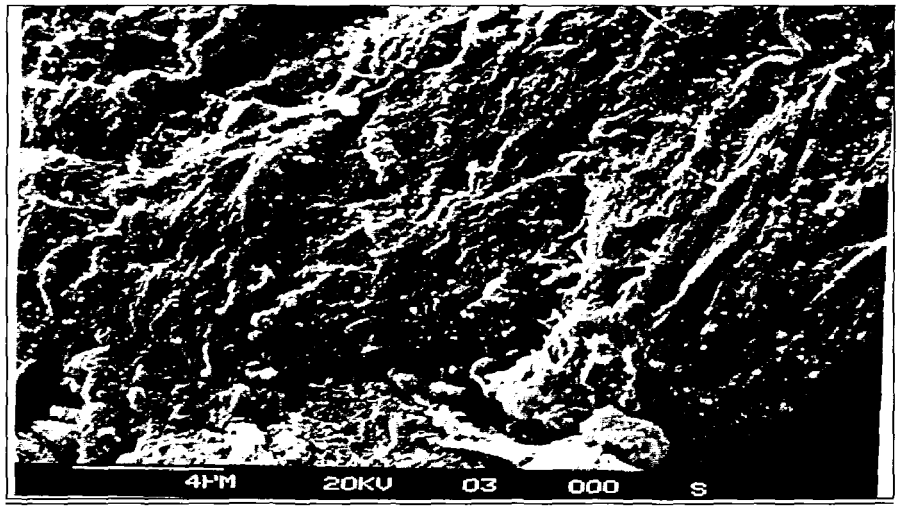


Figure 4.9. SEM of control surface of cut beef muscle – 5000 x

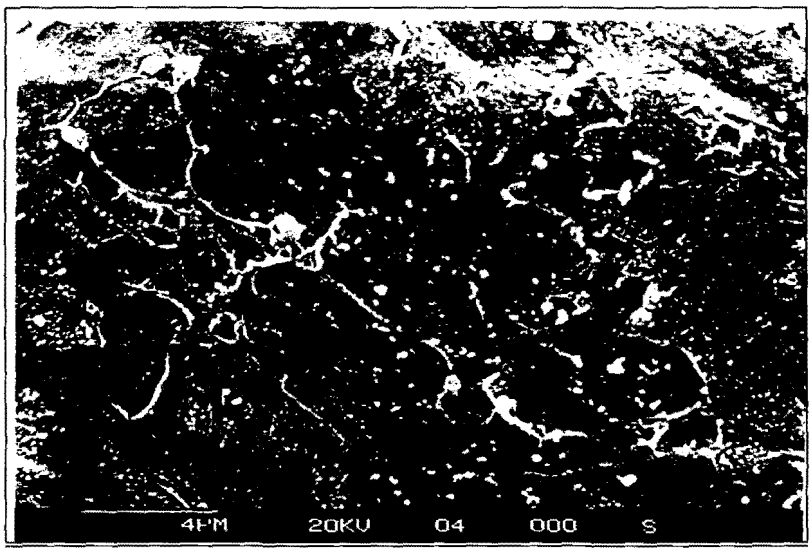


Figure 4.10. SEM of control surface of uncut beef muscle – Fascia – 5000 x

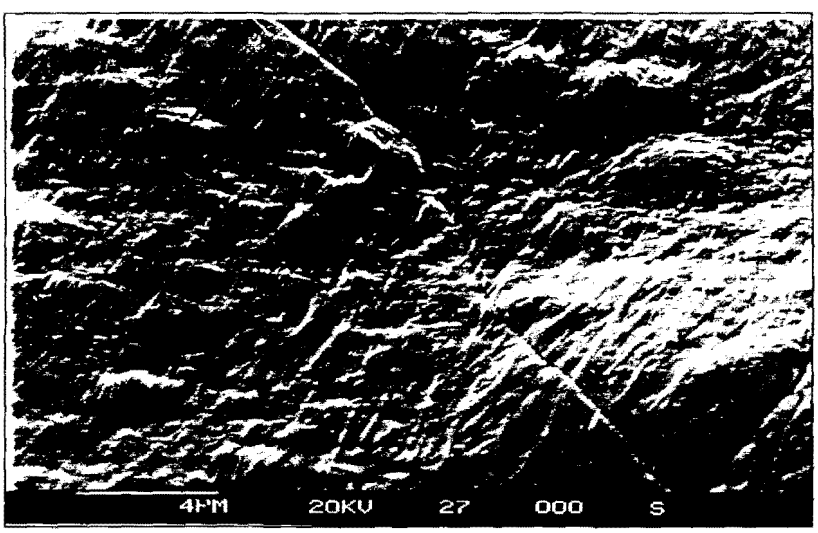


Figure 4.11. SEM of surface of collagen film model – 5000 x

4.3.8 SEM of attachment of *E. coli* E6 to cut beef, uncut beef and collagen film

The scanning electron micrograph in Fig 4.12 shows *E. coli* E6 associated with the surface of cut beef muscle. The micrograph also shows the coaggregation and coadhesion between bacteria. The sample illustrated had been immersed for 10 minutes in the rinse bath.

Fig 4.13 shows *E. coli* E6 associated with the surface of uncut beef muscle. The sample illustrated had been immersed for 10 minutes in the rinse bath.

The scanning electron micrograph in Fig 4.14 shows *E. coli* E6 associated with the surface of collagen film model. Coaggregation and coadhesion between bacteria are also evident in this micrograph. The sample illustrated had been immersed for 10 minutes in the rinse bath.

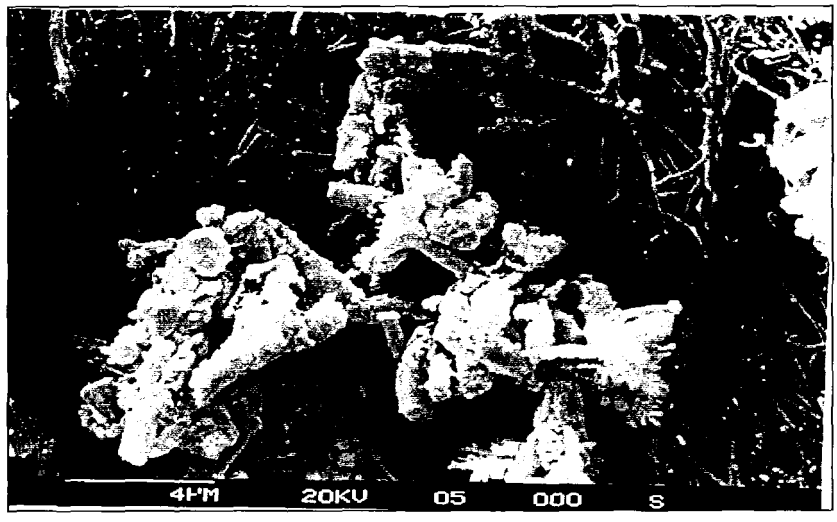


Figure 4.12. SEM of ***E. coli* E6** deposited on cut beef muscle -6500 x

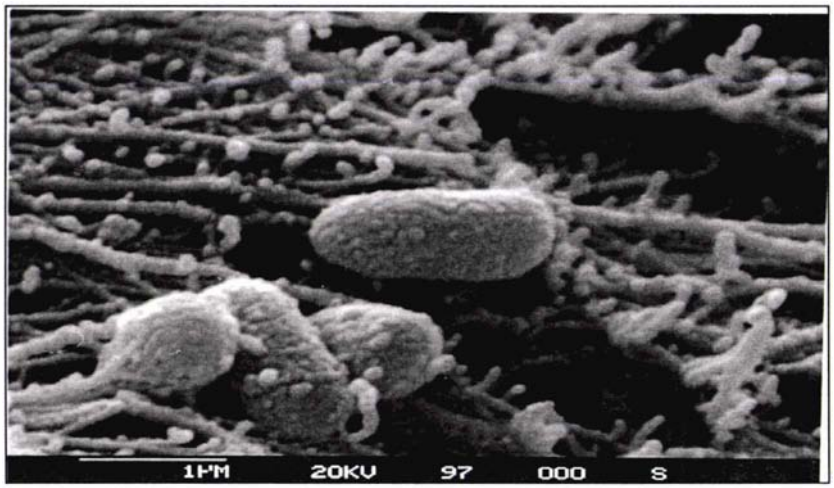


Figure 4.13. SEM of ***E. coli*** **E6** deposited on uncut beef muscle fascia -20000 x

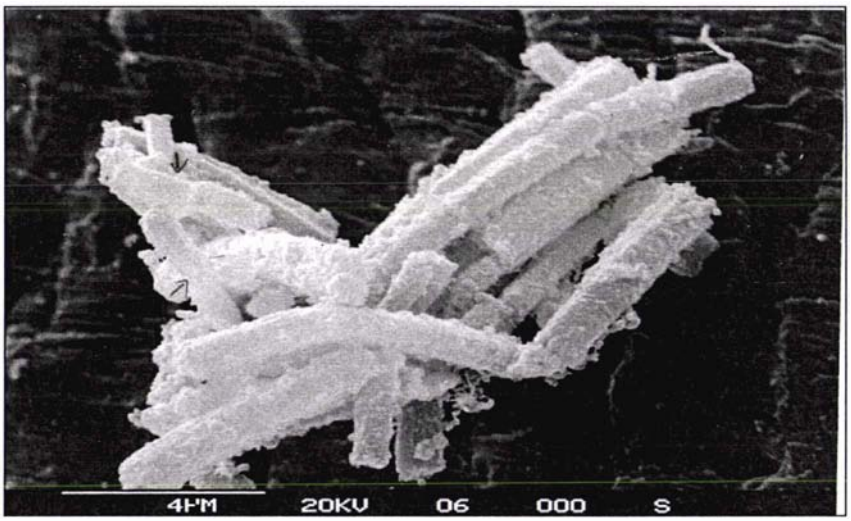


Figure 4.14. SEM of ***E. coli*** **E6** deposited on a collagen film model system - 7000 x

4.3.9 Qualitative studies – collagen film – microscopic procedure development and confirmation of model

Epifluorescence microscopy confirms that *E. coli* E6 cells are present on the collagen film using the pre- and poststaining procedures. The Acridine orange stained attached

cells fluoresce as green and orange coccobacilli. Photographs are not shown as they were out of focus.

4.3.10 Qualitative Studies – Type III collagen coated slide – microscopic procedure development and confirmation of model

The collagen coated slide model system demonstrated the Acridine orange stained attached cells fluorescing as green and orange coccobacilli (photographs are not shown as they were out of focus).

4.3.11 Collagen film, Type III collagen coated slides – Development of microscopic procedures for quantitative assessments and compared with viable count

The influence of time on the extent of attachment of prestained and poststained *E. coli* E6 cells to collagen film mounted on slides and is shown in Fig 4.16. Attachment of approximately 4 to 4.5 log cfu/cm^2 occurs on prestained cells and poststained cells upon immersion into a cell suspension containing approximately 10^7 cfu/ml compared to a viable count of approximately log 5 cfu/cm^2. Similarly the influence of time on the extent of attachment of prestained and poststained *E. coli* E6 cells to collagen coated slide surfaces is also shown in Fig. 4.15. Attachment of approximately 4.5 to 5 log cfu/cm^2 occurs with pre and poststained cells upon immersion of a collagen coated slide into a cell suspension containing approximately 10^7 cfu/ml compared to viable count which is log 5 cfu/cm^2. These results show that collagen coated slides appear to be a good microscopic model for studying bacterial attachment to model surfaces. Microscopic methods proved less satisfactory for studying attachment to meat surfaces. Therefore, viable count assessment using the standard protocol remains the method of choice for studying attachment to meat surfaces as well as to model surfaces.

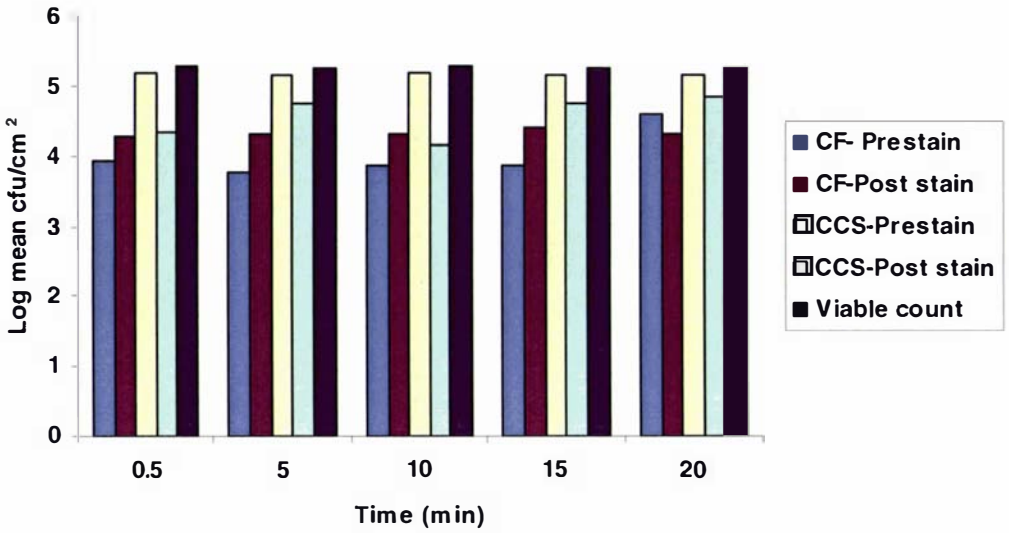


Figure 4.15. Comparison of viable count and microscopic method - Attachment of ***E. coli* E6** to collagen films and collagen coated slides immersed in cell suspension containing 10^7 cfu/ml. CF- Collagen film; CCC- Collagen coated slide.

4.3.12 Attachment of different bacteria to collagen film model

Most of the bacteria tested attached well to the collagen film model system immersed in diluent fluid and PBS system (Fig 4.16 a & b). These include *E. coli* E6*; B. thermosphacta; E. coli* O157*:* H7 *(VT^+); E. coli* O157*:* H7 *(VT^-); E. aerogenes; E. coli* 916*; L. viridescens; L. innocua; L. monocytogenes; P. aeruginosa; S.* Menston *and S. aureus.* Of all the bacteria tested, *E. coli* E6 attached best to the model system; few *P. aeruginosa* attached to the model surface. The attachment of *E. coli* E6*; S.* Menston*; S. aureus; L. monocytogenes; B. thermosphacta; L. innocua and P. aeruginosa* to collagen films immersed in PBS showed the comparable attachment to that of diluent fluid system. *E. coli* E6 and *S.* Menston showed maximum attachment and *B. thermosphacta* and *P. aeruginosa* showed poorest attachment. Seven isolates were used to study the attachment of bacteria to the model system immersed in PBS.

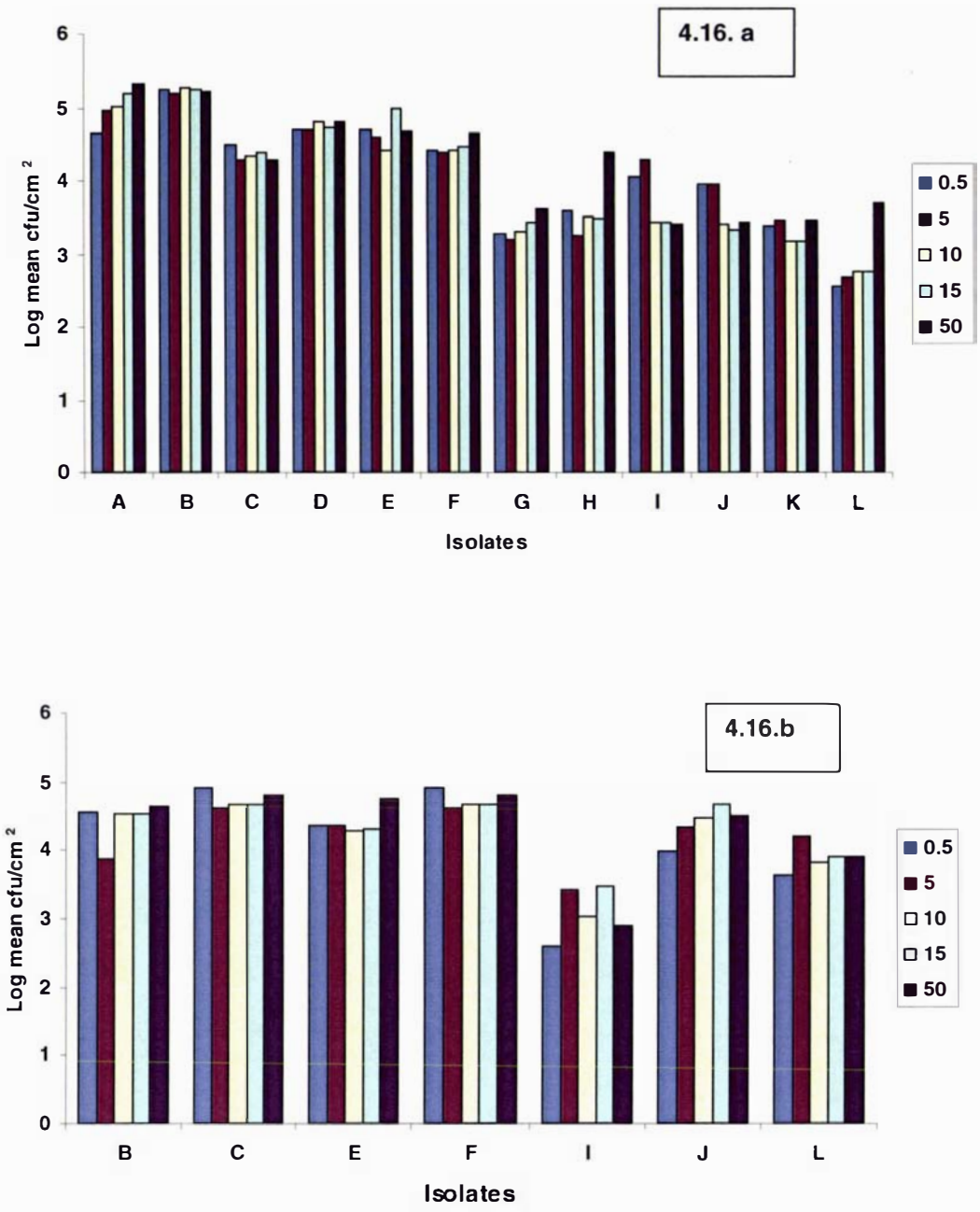


Figure 4.16 a & 4.16 b. Attachment of different bacteria to collagen films immersed in dilution fluid and PBS containing a cell suspension of10^7 cfu/ml. Gram-negative and Gram –positive strains (**A**) *E. coli* 916; (**B**) *E. coli* E6 ;(**C**) *S.* Menston; (**D**) *E. coli* O157:H7 VT-; (**E**) *S. aureus* ;(**F**) *L. monocytogenes;* (**G**) *E. aerogenes*; (**H**) *E. coli* O157:H7 VT +; (**I**) *B. thermosphacta* ;(**J**) L. *innocua*; (**K**) L. *viridiscens* and (**L**) *P. aeruginosa*.

4.4 DISCUSSION

This chapter described the development of a collagen film model system to help in understanding the process of bacterial attachment in relation to meat surfaces.

A convenient collagen film model system was developed and validated using both viable count and direct microscopic procedures. The attachment of bacteria to the model system was comparable to the attachment of bacteria to cut beef muscle and uncut beef muscle. Further studies using SEM confirmed the complexity of the meat surfaces, but suggested deposition of bacteria to the three surfaces. Many variables interfere with the measurement of attachment, such as the loosely attached cells in the surface associated liquid film, cell concentration and the situation is complicated by the variable degree of attachment of different bacterial strains. The process of attachment is influenced by various factors which include pH, temperature, time, cell concentration, type of meat surface, type of bacterial strain, culturing methods, surface structures and surface properties such as charge and hydrophobicity (Butler et al., 1979; Chung et al., 1989; Dickson, 1991; McMeekin & Thomas, 1978; Notermans & Kampelmacher, 1975).

Development of collagen film model system by comparing attachment to cut beef and uncut beef muscle using viable count:

In developing an immersion protocol to study the attachment of *E. coli* E6 to post rigor cut beef muscle, meat with normal ultimate pH was used i.e., rigor was complete. The absence of both fascia and subcutaneous fat from this meat ensured that the attachment of *E. coli* E6 was to a cut lean meat surface. The rinse process was used to remove unattached cells present in the residual surface film of attachment suspension. Shear forces removal of attached cells was minimised in the rinse procedure by employing a gentle swirl technique. The similarity in pH of the attachment and rinse media favoured the removal of unattached cells present in the surface liquid film, rather than promoting release of attached cells through pH related surface character changes. The degree of attachment was approximately 5 log cfu/cm^2 on immersion in a cell suspension containing approximately 10^7 cfu/ml. Attachment to cut lean muscle surfaces appeared to occur very quickly (Fig 4.4), with little, if any, additional attachment occurring with

continued immersion. Therefore long immersion times are not required to achieve maximum attachment. Consequently, in future studies shorter immersion times can be used without compromising attachment levels. Similar attachment patterns were observed with the attachment of *E. coli* E6 to pre-rigor cut beef muscle surfaces. This observation has an important implication for beef processing: that attachment of bacteria to meat surfaces occurs very rapidly. It is therefore important to minimise contact between bacteria and meat surfaces because the microorganisms will quickly become unremovable.

With postrigor and pre-rigor uncut beef muscle, the attachment of *E. coli* E6 was to both fascia and subcutaneous fat, rather than to the underlying meat tissue. Attachment occurred at approximately 5 log cfu/cm^2 on immersion in a cell suspension containing approximately 10^7 cfu/ml (Fig 4.5). As with cut surfaces, attachment occurred very quickly with little, if any, additional attachment occurring with continued immersion. The small difference in numbers enumerated using pre rigor meat in McConkey agar and APC suggests that level of background contamination was low. The attachment of *E. coli* E6 to pre-rigor cut beef muscle was found to be similar. Therefore it appears that bacterial attachment to cut, fascia and adipose tissues is similar and is not influenced by the rigor state of the meat.

The similarity in attachment of *E. coli* E6 to collagen film and meat indicates that commercial collagen film is a reasonable model system with which to study bacterial attachment to meat surfaces. Additionally, attachment kinetics (Fig 4.6) and reaction to rinse treatments are similar.

The results of the present study and those of (Bouttier et al. 1997; Butler et al. 1979) show that the attachment of bacteria to the meat and model surface occurs rapidly in the first minute of contact between bacterial suspensions and meat tissue. These observations indicate that initial attachment is due to physiochemical interactions between the bacteria and the contact surface. Firstenberg-Eden et al. (1978) found that different types of meat tissue allow attachment at different levels: chicken breast fascia allows the greatest attachment with decreasing attachment observed in descending order for cut chicken muscle, cut beef muscle, cow teats and beef fascia. In contrast, the

studies on the relative attachment to lean and adipose tissue indicate that bacteria attach equally well to both adipose and muscle tissue (Benedict et al., 1991; Cabedo et al., 1997; Chung et al., 1989; Dickson & Macneil, 1991; Dickson & Frank, 1993; Piette & Idziak, 1989) which are in agreement with the present studies. The differences in attachment observed by different researchers may be due to employment of different attachment conditions.

Artificial collagen films are composed principally of collagen but, unlike collagen in connective tissue associated with muscle, have been purified and denatured. These changes may lead to a surface that lacks some of the collagen associated attachment molecules found in natural collagen upon purification of collagen. This might result in removal of cementing substances (glucosaminoglycans) that have been suggested as providing specific attachment opportunities (Sanderson et al., 1991). Bacterial clumping or association with other bacterial cells is a problem with attachment studies. It is also difficult to know that each colony represents the progeny of a single organism when using culture methods. However, stomaching is believed to break up the clumping of cells or the attachment of bacteria to other cells when culture methods are used to count the bacteria. With microscopic procedures (SEM and Epifluorescence microscopy) clumping of bacterial cells and artefacts would be a major problem when compared to the viable count method. Use of a collagen model surface for studies on attachment of bacteria to meat surfaces introduces fewer variables and is inexpensive. Nevertheless, the collagen model system offers advantages over meat in that the surface is virtually sterile, uniform, and simplifies attachment interactions to those between a single bacterial surface type and a single meat protein. This collagen film system was therefore chosen as a model with which to study the influence of a range of variables on bacterial attachment. Other collagen attachment models, such as sausage casings, collagen casings, and skins of lamb and pork have been used to imitate meat surfaces to minimise the variables and to provide simple models for the complex natural meat surfaces (Butler et al., 1979; Piette & Postec, 1993; Walls et al., 1993). The extent of attachment of bacteria to these model systems was comparable with that reported for bacterial attachment to meat surfaces.

SEM investigation of control meat, model surfaces and attachment of bacteria to meat and model surfaces

SEM study on attachment of *Pseudomonas fragi* to sarcoplasm depleted cut beef muscle was made by (Yada et al., 1982). Attachment of bacteria to model and meat surfaces was also examined in the present study by SEM. Electron micrographs of cut beef muscle show that the muscle is composed of individual muscle cells that are grouped together into bundles or fasciculi. The muscle bundles are enveloped in collagen fibres, which appear rough and folded. The surface is neither relatively flat nor uniform. These fibres are composed of collagen (Swatland, 1978). The greater magnification and resolution of electron microscopy reveal collagen fibrils ranging from 20 to 100nm. Collagen fibrils typically have diameters which are multiples of 8 nm and this may reflect the manner in which they grow radially (Parry & Craig, 1981).

Scanning electron micrographs of the fascia surface of beef muscle revealed the rough appearance of the surface, with exposed connective tissue. This observation confirms that fascia or connective tissue proper surfaces are composed of striated, non-fibrous basement membrane and filamentous collagen which appears as an irregular torn surface at lower magnifications in Figure 4.8. They are interwoven and at the higher magnifications can be seen to be arranged in bundles lying parallel to each other. Connective tissues are distributed throughout the body in sheaths that surround structures such as tendons and muscles. The skin or hide is attached to the body by connective tissue. Connective tissue envelops muscles, muscle bundles and muscle and is known as connective tissue proper. Connective tissues are made up of collagen which is an elongated protein which forms extremely strong microscopic fibrils. Collagen fibrils may be bound together to form microscopic and macroscopic structures in meat known as *gristle.*

Scanning electron micrographs of commercial collagen film show it to be smooth and uniform, which will reduce the non-specific entrapment of cells within fibres (Benedict et al., 1991). It also shows commercial collagen film to be a thin, flat continuous sheet with few tears or holes in the surface. Unlike meat, collagen films are sterile, smooth and were generally free from particulate material. Lillard (1986) observed that

mechanical entrapment of cells occurs between muscle fibres or within the connective tissue matrix. As a result, trapped cells often could not be visualised easily using SEM. Entrapment of cells therefore reduces the accuracy of enumeration of attached bacteria using SEM. The use of a relatively flat and uniform collagen film surface totally avoids the problem of cell entrapment. Therefore collagen films seem to be more advantageous for studies on attachment to meat tissue than muscle tissue itself as it eliminates the complication of entrapment of cells at the surface. A surface that is simple and smooth in nature introduces fewer variables and allows study on the interaction of bacterial cells with single major meat protein.

SEM showed that *E. coli* E6 deposited on cut beef muscle after a 10-minute immersion in the attachment bath followed by a diluent fluid rinse. The rough and folded surface holds the clumps of bacteria which may indicate that they were retained on the surface due to the problems associated with the sample preparation procedure using SEM.

SEM images showed that *E. coli* E6 deposited to connective tissue fibres or fascia, which is made up of collagen, following a 10 minute immersion of beef muscle in the attachment bath. Specific adhesion of *S.* Typhimurium to a network of collagen fibres on the muscle fascia has been reported (Thomas & McMeekin, 1986). Very few cells were observed with SEM, probably reflecting the relatively low level of attachment 5 log cfu/cm^2 produced by the standard immersion attachment procedure. Problems associated with the technique would have prevented the observation of larger numbers of bacteria attached to fascia using SEM.

SEM showed *E. coli* E6 stranded on the collagen film model system after 10 minutes of immersion in the attachment bath.

Scanning electron micrographs of meat tissue and of purified collagen suggested that bacteria attached primarily to collagen fibres (Benedict et al., 1991; Fratamico et al., 1996; McMeekin et al., 1984). Several studies have indicated that connective tissue fibres such as collagen may play a major role in bacterial attachment to meat tissue (Benedict et al., 1991; Kim et al., 1994; McMeekin et al., 1984; Piette & Idziak, 1989; Walls et al., 1993).

The present SEM study showed the bacterial deposition but not necessarily attached to all three surfaces. It is well known that SEM specimens prepared by chemical fixation and dehydration contain artefacts which may lead to misinterpretation (Gilmour et al., 1993). Similarly the clumping of cells probably resulted from the surface tension forces during dehydration. It is now believed that SEM is not an appropriate method to quantify the number of bacteria attached because of the many technical problems associated with sample preparation; mechanical entrapment of cells, which occurs between muscle fibres, or within the connective tissue matrix and finally the complexity of the surface. In addition, it is unfortunate that attachment for all the three surfaces was not visualised at the same magnification and also appear to be a different shape which could be due to the problems associated with sample preparation. The SEM study was not intended to compare the numbers with standard viable count assessment; it was used to confirm the association of the bacteria to all the three surfaces. It is believed that, in this situation, viable count assessment using the standard immersion procedure gains its importance as a means of quantifying the attached bacteria. By taking all these parameters into account, collagen film is confirmed as a near ideal surface for studying bacterial attachment to meat surfaces using either viable count or microscopic procedures. However, fascia and muscle surfaces are clearly more complex, therefore the collagen film model must be expected on occasion to give attachment reactions that differ from those of red meat surfaces.

Confirmation of model system using qualitative and quantitative assessments by developing microscopic procedure using epifluorescence microscopy; comparison of quantitative assessments using microscopic procedure with viable count

In this study, considerable time and effort were devoted to the development of a model system to observe the bacteria attached to collagen film using epifluorescence microscopy. After extensive experimentation for qualitative assessments, the two methods (pre and poststain) trialled produced reproducible results, though a clear preference in the staining methodology was not immediately obvious. In the present study, slight changes in the microtopography were noticed. It is believed that the use of a water resistant silicone RTV adhesive sealant in place of "super glue" prevented the

total distortion and changes in microtopography of collagen film resulting from water uptake on immersion. The surface of collagen film is relatively flat, which reduces the possibility of bacterial entrapment. However with meat, cells may become trapped in crevices and consequently would not be enumerated by direct microscopic observation. Therefore the model system would give higher microscopic counts than a meat surface experiencing a comparable bacterial contact. However, the swelling property of collagen film upon immersion may also prevent the observation of some bacteria.

The problem of change in the microtopography associated with the collagen film is totally avoided by the use of collagen coated slides. The surface of collagen coated slides is flat which precludes the entrapment of bacterial cells which would produce microscopic counts lower than those for meat surfaces experiencing comparable bacterial contact. The observation of bacterial cells attached to collagen film or collagen coated slides unfortunately does not distinguish between specific (binding molecules or attachment receptors) or nonspecific binding (physical forces - charge, hydrophobicity and vander Waals) of the bacterial cells to the collagen surface. However, collagen coated slides appear to be a convenient model with which to study the attachment and detachment mechanisms. The preference in model system for microscopic counts will be apparent when quantitative assessments are conducted.

Quantitative studies on attachment of *E. coli* E6 to mounted pre and poststained collagen film slides indicated that the attachment of *E. coli* E6 was to a collagen. The rinse process removes unattached cells present in the residual surface film of the attachment suspension. As discussed previously, the similarity in pH of the attachment and rinse media favours the removal of unattached cells present in the surface liquid film rather than release of attached cells resulting from pH related surface character changes. Attachment to mounted collagen film appears to occur very quickly (Fig 4.15), with little, if any, additional attachment occurring after continued immersion. Preference was given to the prestain procedure, as the post stain procedure washes the cells that are present on the surface and makes microscopic examination considerably more difficult. A 1 log variation in attachment of *E. coli* E6 using pre-stain and post stain methods occurred when compared with that measured by the viable count procedure. Presumably acridine orange treatments particularly prestain affected the

viability and/or changed the surface properties of the cells leading to apparently less surface associated cells following immersion. The variation in numbers may be due to several difficulties associated with the counting of bacteria using epifluorescence microscopy. The difficulties experienced include: depth of focus problems, as the swelling property of collagen film is such that it prevents the observation of all cells in a field under the microscope at a single point of focus; differentiating cells and artefacts; changes in the microtopography; staining of the background; partial distortion of the film; washing and blotting of the cells . Such problems should be prevented by the use of collagen coated slides.

Attachment of *E. coli* E6 to pre and poststained collagen coated slides indicate that the attachment of *E. coli* E6 was to a collagen dominated surface. Attachment to collagen coated slides appears to occur very quickly (Fig 4.15), with little, if any, additional attachment occurring after continued immersion. The problem of swelling in the collagen film model is totally avoided by the use of collagen coated slides because the collagen coating gel is already fully hydrated. Preference can be given to both the pre and poststain methods. However, pre staining of cells for use with coated slides is preferred, as the post-attachment staining procedure washes the cells that are present on the surface. The number of *E. coli* E6 enumerated by microscopy on Type III collagen coated slides is comparable to that of the numbers recovered using the viable count procedure. Therefore collagen coated slides appear as the better model for studying the mechanisms of bacterial attachment to carcass surfaces, affording comparability between the viable count procedure and microscopic procedures.

It is believed that both prestain and poststain could present a problem in terms of quantitative attachment studies. Presumably prestaining with acridine orange kills the cells and might also have impact on changing the surface property of the cells. With post acridine orange staining, repeated washing would influence the actual count. Holah et al. (1989), Stanley et al. (1983), Pettipher (1986), Pettipher et al. (1992, 1995), Wirtanen & Mattila-Sandholm (1993) and Yu et al. (1993) used epifluorescent microscopy to study the attachment of bacteria to the beef and stainless steel surfaces. In epifluorescence microscopy the specificity is determined by the types of chemical staining reagents used for the visualization of the cell. The DEFT uses acridine orange,

a stain that binds to nucleic acids (Hobbie et al., 1977; Kepner & Pratt, 1944). Yu et al. (1993) and Holah et al. (1988) described a procedure comparing direct epifluorescence microscopy and viable count for determining numbers of cells in a biofilm on stainless steel coupons and data were statistically analysed and gave comparable results. These results are in agreement with present studies using the collagen film and collagen coated slide model systems. Researchers have also used other methods to quantify the attached cells. These include application of the antibody –direct epifluorescent filter technique (Ab-DEFT) (Restaino et al., 1996; Rodrigues & Kroll, 1990; Tortorello and Stewart, 1994) who estimated the attachment of *E. coli* O157:H7 and *Salmonella* in beef in a long term survival study. The Ab-DEFT is an example of how microscopy can be utlilised, despite the traditional misconceptions, as a rapid screening test for detection of food pathogens. These problems could be minimised by focusing on several other alternative methods to study the attachment of bacteria using microscopy. Fluorescent in situ hybridisation (FISH) using oligonucleotides conjugated to a fluorescent dye enables the direct identification of single cells by fluorescence microscopy (Amann et al., 1990). The technique is useful for identifying bacteria in biofilms (Manz et al., 1993).

However, because of its simplicity, ease and consistency of quantification, the collagen film viable count system remains the preferred method with which to model bacterial attachment to meat. Viable count assessment holds priority because it can be used to study attachment on both meat and model surfaces and the clumping problem observed with microscopic procedures is also minimised by using this method as the stomaching step is expected to break up the clumps. The microscopic method is suitable only to enumerate the attached numbers using the model surface and must be expected to present technical difficulties when used for enumeration with a complex meat surface.

Definition of an attached cell

The term "attached cells" is used variously in the international literature to describe the interactions of microorganisms and surfaces. The differentiation of cells associated with the surface liquid film that is, loosely attached cells, and firmly attached cells is still under debate. Thomas & McMeekin (1981a) reported that with chicken muscle fascia the microtopography was significantly altered by immersion in aqueous solutions,

increasing the amount of water held at the surface. It was concluded that since bacteria are retained within the liquid film, attachment levels are related to the amount of water held by the meat tissue. They hypothesised that bacterial retention may be a function of the amount of water loosely bound by the connective tissue, with increasing amounts of water being bound with time. In the present study, it is presumed that cells present in the surface associated liquid film are fully accounted for as being recovered in the rinse liquid. However, boundary layer effect could mean that a single rinse does not remove all liquid film cells. Multiple rinses are not required because there is little or no statistically significant difference between attached cell numbers recovered after one to six rinses. This experimental evidence clearly shows that attached cells are different from the surface associated cells in the liquid film. Therefore *attached cells* are defined as the *cells that are recovered by stomaching following a single rinse.* Notermans & Kampelmacher (1975) showed that a part of the bacterial flora of the broiler skin is present in the surrounding water film and can be removed easily by rinsing for three minutes in running water. The extent of attachment with or without the removal of the water film was about the same; however, removal of portion of more firmly attached cells to the skin was more restricted. After immersion in inoculated attachment medium, the samples were allowed to drain and then were rinsed by immersion in sterile diluent fluid. Under these conditions, attachment to the samples probably was restricted to the more firmly attached bacteria. Differences in the rinsing procedures caused little difference in attachment. Notermans & Kampelmacher (1975) also showed that the skin proper and the water film affected bacterial counts obtained with different procedures. The microflora of the skin could be evaluated more effectively by a skin maceration procedure than by a skin rinse method. The reports by Notermans & Kampelmacher (1974; 1975) and van Schothorst et al. (1976) provide a better understanding of the discrepancies in bacteriological counts and types when different sampling and testing procedures are used.

Influence of cell concentration on attachment of bacteria

In the present study on inoculum concentration, it was shown that a linear relationship existed between cell concentration and the number of attached cells. With low inoculum numbers less attachment was observed, but at high inoculum levels the attachment

reaches a maximum. A consistent attachment of 5 log cfu/cm^2 was obtained by using the 10^7 cfu/ml. This concentration was chosen for further studies on attachment to meat surfaces. In studies on meat surfaces Dickson (1991) observed that an increase in inoculum level resulted in a proportionate increase in the number of attached cells of *S.* Typhimurium and *L. monocytogenes.* Similarly, attachment of *S.* Typhimurium to sausage casings increased linearly. Attachment to meat surfaces increased with increase in cell concentration in the suspending media (Butler et al., 1979; Chung et al., 1989; Dickson, 1991; McMeekin & Thomas, 1978; Notermans & Kampelmacher, 1975). Fratamico et al. (1996) showed that the number of attached organisms depended on the concentration of bacteria in the liquid inoculum.

Attachment of different bacteria to model surfaces

All the bacteria investigated in the present study of attachment to the collagen film model system using dilution fluid and the PBS system showed different attachment. These include Gram positive bacteria, Gram negative bacteria, Gram variable bacteria, spoilage and pathogenic bacteria. With the dilution fluid system of all the bacteria tested *E. coli* E6 and *E. coli* 916 attached best to the surface and *P. aeruginosa* showed the poorest attachment which could be due to a lack of major attachment molecules on the model surface. *E. coli* E6 was chosen for further studies on bacterial attachment to meat and model surfaces. The extent of attachment using the PBS system showed comparable attachment with slight variation. *E. coli* E6 and *S.* Menston showed greatest attachment and *B. thermosphacta* and *P. aeruginosa* showed poorest attachment which could be due to the lack of specific binding molecules on the model surface. This might suggest that *P. aeruginosa* and *B. thermosphacta* attaches on meat to something other than collagen. No significant difference in the extent of attachment of bacteria to the model system was noticed by changing the cell suspending fluids. However, the present study employed these two different systems to obtain a positive correlation with the cell surface properties of bacteria and to compare the present study with work done by previous researchers.

Delaquis & McCurdy (1990) observed the colonisation of beef muscle surfaces by *P. fluorescens* and *P. fragi.* Attachment of many different types of bacteria can occur to

meat surfaces. The majority of the bacteria tested demonstrated that potential for attachment; attaching bacteria include *Acinetobacter, Bacillus, Brochothrix, Campylobacter, Clostridium, Enterobacter, Erwinia, Escherichia, Klebsiella, Lactobacillus, Listeria, Micrococcus, Moraxella, Proteus, Pseudomonas, Salmonella, Serratia, Staphylococcus* and *Streptococcus.* There are, however, a few exceptions. Certain strains of *E. coli, C. jejuni, Staphylococcus, P. mirabilis, Pseudomonas* and *Micrococcus* did not attach to chicken muscle fascia under conditions which permitted attachment of various other organisms (Campbell et al., 1987). *B. thermosphacta* did not show attachment to beef muscle pieces (Ockerman et al., 1992). However, in the present study *B. thermosphacta* attached well to the model system. The relative ability of bacteria to attach to meat surfaces is not exclusively related to the Gram stain reaction, cell morphology, genera or species. However, there are some trends worth noting. In most studies *Pseudomonas* spp attach in high numbers (Butler et al. 1979; Farber & Idziak 1984; Kim & Slavik 1994), whereas lactobacilli attach poorly.

4.5 CONCLUSION

The initial (reversible) attachment mechanism between bacteria and the carcass surface was studied using a collagen film model system. The model system was used to develop a reasonable methodology to define "attached cells" and to differentiate them from cells that are present in the surface associated liquid film. The model collagen film system with a viable count procedure was used to simulate the attachment of bacteria to the meat surface. Bacterial attachment to the collagen model was compared with attachment to cut and uncut beef muscle. These studies confirmed that the model system reasonably imitated bacterial attachment to both cut and uncut surfaces. SEM studies confirmed that bacteria deposited on muscle, fascia and collagen model surfaces owing to technical problems.

A direct microscopic count procedure using epifluorescence microscopy was also developed, using collagen film mounted on microscope slides and collagen coated slides. The direct microscopic counts from these two model systems were compared with data obtained with the viable count procedure. The collagen coated slide was the better microscopic model for studying attachment of bacteria to carcass surfaces,

allowing comparison between the viable count and microscopic procedures. However, because of its simplicity and consistency of quantification, the collagen film viable count system was the method selected to model bacterial attachment to meat. *E. coli* E6 at an optimal cell concentration of 10^7 cfu/ml was chosen for further studies.

Chapter 5
CELL SURFACE PROPERTIES OF BACTERIA

5.1 INTRODUCTION

The attachment of bacteria to meat tissues or any solid surface is a complex phenomenon. Many theories have been put forward to account for adhesion, these include physicochemical properties of surfaces (charge and hydrophobicity); thermodynamic properties based on interfacial free energies and specific molecular interactions between the surface of the bacterial cell and biological tissues. Therefore, attachment of bacteria to meat surfaces most probably involves multiple interactions, which at present are far from fully understood.

It is generally accepted that bacterial attachment occurs as a two-stage process: Reversible and irreversible attachment (Marshall et al., 1971). Initially, a portion of the total population forms a reversible association with the attachment surface. The second stage, irreversible attachment, occurs as the bacteria form a more permanent physical and chemical attachment. Reversible and irreversible attachment correspond to the terms "loosely" and "strongly" attached bacteria used by Firstenberg Eden et al. (1978) with respect to meat surfaces.

The classical theories of adhesion suggest two to five stages in the attachment process, involving van der Waals attractive forces, electrostatic interactions, hydrophobic and (later) more active adhesion through the production of extracellular polymers by the bacteria (Oliveria, 1992). Marshall et al. (1971) divided the adhesion process into two distinct, and now well recognised phases – reversible and irreversible adhesion – on the basis of the force required to remove the cells from the surface. Busscher & Weerkamp (1987) proposed a three-step model, adding an extra initial stage where the cells are prevented from direct contact with a surface because of vander Waals and electrostatic forces that are eventually overcome by stronger attractive forces. This process is often referred to as the "DLVO theory" (developed independently by two research teams, Derjaguin & Landau, (1941) and Verwey &

Overbeek, (1948), to explain the stability of colloids. The "DLVO theory" stresses the influence of electrostatic interactions in the adherence of particles, including microorganisms, to solid surfaces (van Loosdrecht et al., 1989). Characklis & Cooksey (1983) also expanded the two-stage model to include: cell transport to a wetted surface, adsorption of a conditioning film, adhesion of microbial cell reactions within the biofilm and, finally, detachment of the biofilm. Whether all these stages are involved in the initial attachment process is not known. For example, it is still unclear whether all microorganisms preferentially adhere to a conditioning film (Zottola & Sasahara, 1994).

The whole process of bacterial attachment to meat surfaces is poorly understood. It is generally agreed that the adhesion of microorganisms to surfaces is dependent on interactions between the bacterial species, the substrate and its surrounding environment. A better understanding of these factors will help in the systematic design of methods to control initial reversible attachment through the prevention of adhesion or by enhancing the removal of bacteria should they become attached (Neu, 1996).

It has become increasingly apparent that little is known about the factors involved in the adhesion of bacteria to meat surfaces in the meat processing environment. Studies to date have shown that bacterial attachment is influenced by cell surface charge (Bayer & Sloyer, 1990; Bouttier et al., 1997; Dickson & Koohmaraie, 1989; and Fletcher & Loeb, 1979). As an imbalance of charges exists at a solid liquid interface, all surfaces possess a characteristic surface charge. The Zeta potential at the plane of shear of the moving particle and surface charge can be calculated from the particle's electrophoretic mobility (Abramson et al., 1942). The following have all been shown to be associated with attachment. Hydrophobicity (Dahlback et al., 1981; Gerson & Scheer, 1980; Marshall, 1976; van Loosdrecht et al., 1987 a, b; Rosenberg & Kjelleberg, 1986); surface energy (Busscher et al., 1984); cell surface structures including extracellular polysaccharides (Fletcher & Floodgate, 1973); cell surface proteins (Flint *et al.*, 1997; Parkar *et al.*, 2000) and flagella (Fletcher & Loeb, 1979) are all associated with attachment. There is disagreement, however, over the role of surface structures, since non-fimbriated and non-flagellated cells have been reported

to attach more rapidly than non- motile strains (Butler *et al.*, 1979; Farber & Idziak 1984). The actual role of flagella in attachment is most probably dependent on the specific strain of a bacterium as well as the growth conditions it has experienced. The presence of bacterially produced components such as external appendages and extracellular polymers may also have an influence on the adhesion of some species (Oliveria, 1992). The composition of cell surface structures involved in attachment, both bacterial and those of the meat surface, are also important in establishing attachment (Beachy, 1981). Environmental factors such as temperature, pH and culturing method may also contribute to, and/or modify attachment (Firstenberg-Eden et al., 1978).

In the present study some of the properties of the cell surface of pathogenic bacteria and bacteria causing meat spoilage (hydrophobicity, charge, polysaccharide and protein) were examined and the role of these properties in the attachment of bacteria to collagen film model surfaces determined.

5.2 PROCEDURES

- To determine the relative surface charge of each isolate using (a) anionic (Dowex AG 1 x 8 100-200) mesh and (b) cationic (Dowex AG 50 W x 8 100-200 mesh) exchange resins (Bio rad laboratories Ltd, Glenfield, Auckland, New Zealand) and (c) the zeta potentials of all the isolates using ZetaSizer electrophoretic mobility measurements. The attachment of all the isolates, in standard diluent fluid and (PBS) suspending systems, was related to bacterial cell surface charge measurements (refer section 3.8.1 & 3.10)

- To determine cell surface hydrophobicity using MATH and HIC of all the isolates. The attachment of all the isolates in standard diluent fluid and PBS suspending systems was related to cell surface hydrophobicity measurements (refer 3.11 & 3.8.2)

- To determine the influence of bacterial protein and polysaccharide removal on the adhesion of *E. coli* E6 to cut beef muscle (refer 3.3; 3.12.1; 3.12.2 & 3.8.3)

5.3 RESULTS

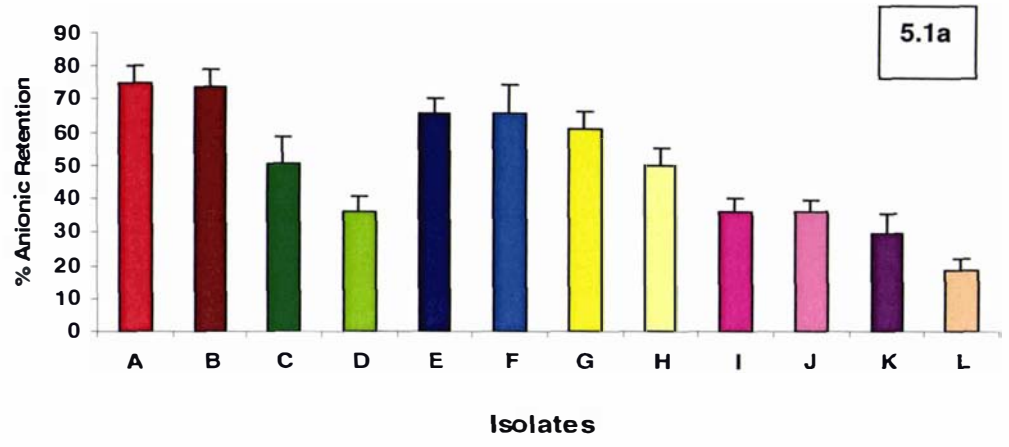
5.3.1 Surface charge measurements

5.3.1.1 The effect of cell surface charge on adhesion using EIC and its relationship to attachment

All the 12 isolates indicated by greater charge interaction retention on the anionic than on the cationic column (Fig 5.1 a & b). The net positive charged cells retained on the column ranged from 5 to 68 % and the percentage with a net negative charge ranged between 20-70 %.

For *S. aureus,* similar numbers of cells were retained on both anionic and cationic exchange resins, presumably due to a balance of positive and negative charges on the cell surface. Similarly *E. aerogenes* showed positive and negative charges almost in balance. There was no difference in the charges on killed *E. coli* E6 cells and live *E. coli* E6 cells. There appears to be a discernable relationship between the Gram stain reaction of bacteria and their retention on EIC.

Gram positives gave 35 to 70 % negative and 20 to 68 % positive interaction charge retention. Gram variable *B. thermosphacta* possesses 65 % negative charge and 30 % positive charge interaction distribution. Gram negatives possess 18 to 65 % negative and 4 to 50 % positive interaction charge retention.



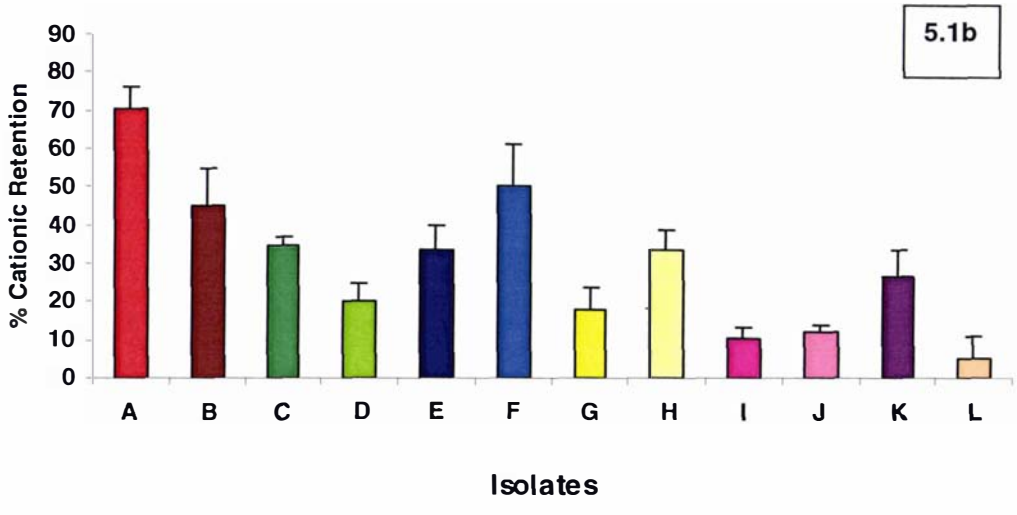


Figure 5.1. a & b Proportion of negatively, positively charged cells retained on anionic and cationic exchanger of all the 12 isolates, eluted using PBS (pH 7.0) *S. aureus* (**A**); *L. viridescens* (**B**); *L. monocytogenes* (**C**); *L. innocua* (**D**); *B. thermosphacta* (**E**); *P. aerouginosa* (**F**); *E. coli* 916 (**G**); *E. coli* O157:H7 (verotoxin negative) (**H**); *S.* Menston (**I**); *E. coli* E6 (**J**); *E. aerogenes* (**K**); *E. coli* O157:H7 (verotoxin positive) (**L**). Error bars represent the standard deviation from the mean of five replicates.

There was no relationship between the negative or positive charge interaction retention of the cells of the test bacteria and the numbers of those cells adhering to meat surfaces using diluent fluid and PBS suspending systems (Fig 5.2 a,b,c and d).

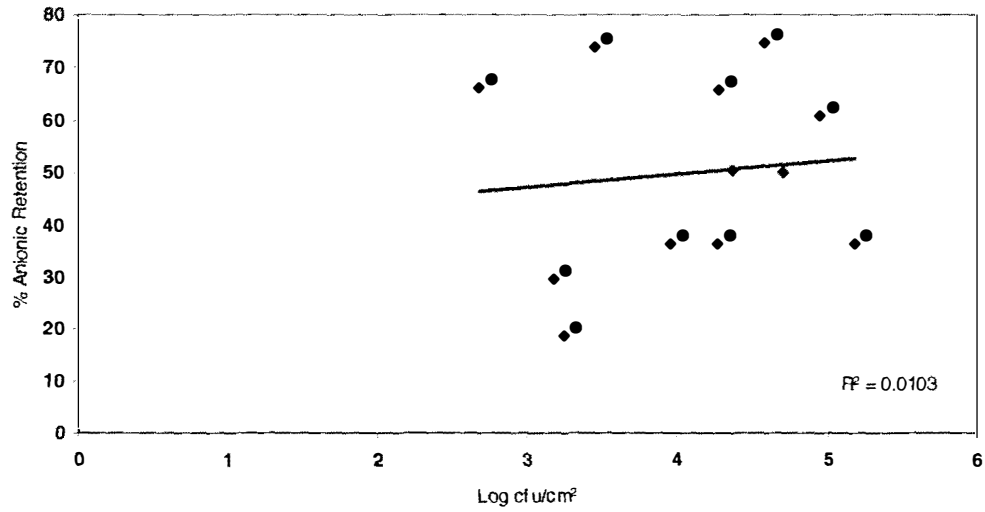


Figure 5.2a. Relationship between cell surface negative charge and attachment to model system of twelve isolates using diluent fluid system.

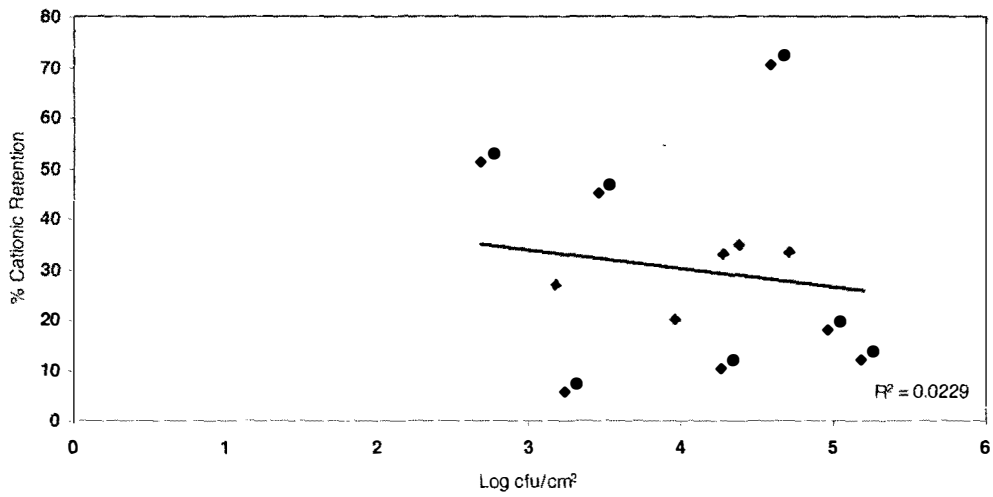


Figure 5.2b. Relationship between cell surface positive charge and attachment to model system of twelve isolates using diluent fluid system.

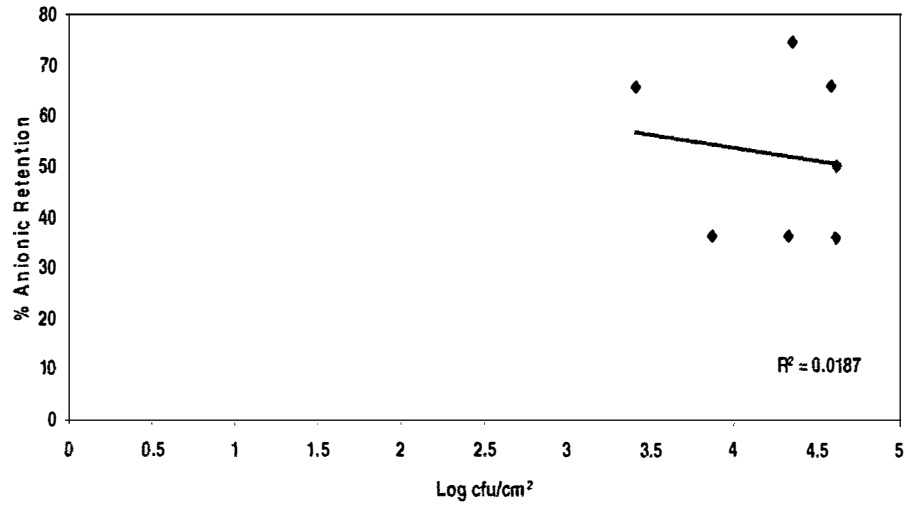


Figure 5.2c. Relationship between cell surface negative charge and attachment to model system of seven isolates using PBS suspending system.

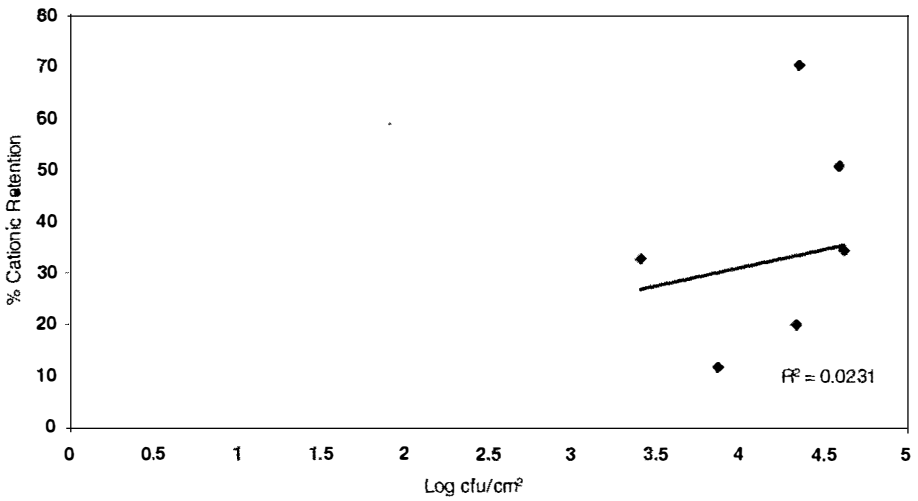


Figure 5.2d. Relationship between cell surface positive charge and attachment to model system of seven isolates using PBS suspending system.

5.3.1.2 Estimation of zeta potential using zetasizer and its relationship to attachment

The Zeta potential of the isolates in PB ranged from (- 4mv to -30mv) (See Fig 5.3). All the strains showed low negative Zeta potential at pH 7.0. There was no difference in Zeta potentials between live *E. coli* E6 and killed *E. coli* E6 cells. Zeta potentials of Gram positive microorganisms ranged from (-14 to -20mv), Gram variable (-30mv) and Gram negatives (-4 to -25 mv).

Bacterial cell surface charge varied greatly depending on the method of measurement i.e., the EIC and Zetasizer measurement for the test strains were not in the same sequence (Fig 5.1 and Fig 5.3). Although relative surface charges and Zeta potentials as determined by each method cannot be directly compared, ranking the bacteria from most charged to least charged for Gram positives and Gram negative strains is a useful means of comparison Table 5.2. Despite the fact that each method of determination resulted in a completely different ranking, Gram positive strains appeared to be more charged when compared to Gram negative strains.

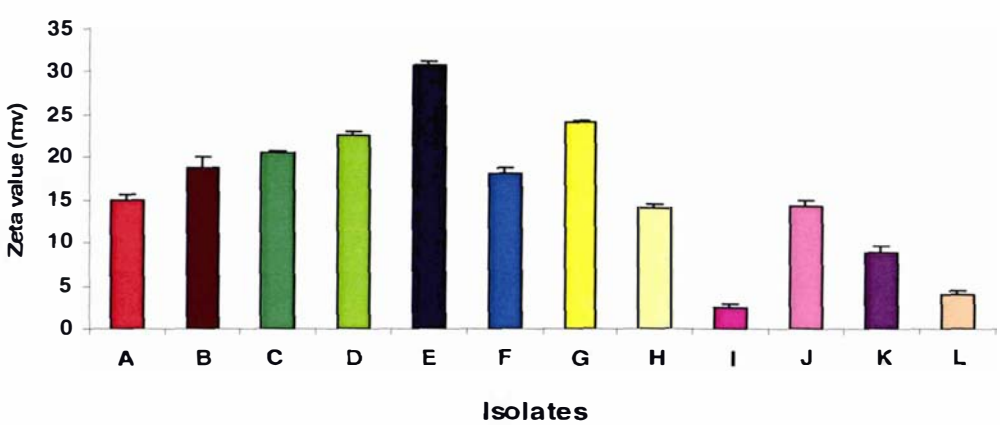


Figure 5.3. Zeta potential measurements of 12 isolates *S. aureus* (**A**); *L. viridescens* (**B**); *L. monocytogenes* (**C**); *L. innocua* (**D**); *B. thermosphacta* (**E**); *P. aerouginosa* (**F**); *E. coli* 916 (**G**); *E. coli* O157:H7 (verotoxin negative) (**H**); *S.* Menston (**I**); *E. coli* E6 (**J**); *E. aerogenes* (**K**); *E. coli* O157:H7 (verotoxin positive) (**L**). Error bars represent the standard deviation from the mean of five replicates.

Table 5.1. **Comparison of cell surface charge measurements using EIC and Zeta potential methods**

Isolates	EIC Measurements		Zeta Potential +/-SD
	Positive charge	Negative Charge	
S. aureus	70.42 +/- 5.77	74.86 +/-5.38	-14.9 +/-0.8
L. viridescens	45.11 +/- 9.82	73.88 +/-5.32	-18.8 +/-1.2
L. monocytogenes	34.54 +/- 2.36	50.50 +/-7.95	-22.5 +/-0.3
L. innocua	20.11 +/- 4.64	36.27 +/-4.41	-20.5 +/-0.4
B. thermosphacta	33.02 +/- 6.34	65.85 +/-4.07	-30.8 +/-0.4
P. aeruginosa	51.08 +/-10.77	66.16 +/-8.83	-18.1 +/-0.7
E. coli 916	17.85 +/- 5.58	60.89 +/-5.06	-24.1 +/-0.3
E. coli O157:H7 (VT-)	33.21 +/- 5.50	49.89 +/-5.23	-14 +/-0.4
S. menston	10.18 +/- 3.16	36.24 +/-3.76	-2.4 +/-0.4
Wild E. coli E6	11.89 +/-2.06	36.26 +/-3.22	-14.3 +/-0.5
E. aerogenes	26.67 +/- 7.04	29.53 +/-6.14	-8.9 +/-0.7
E. coli O157:H7 (VT+)	5.55 +/- 5.46	18.74 +/-3.39	-4 +/- 0.8

There was no relationship between the Zeta potential and the number of cells adhering to meat surfaces using diluent fluid (pH 7.0) and phosphate buffer (pH 7.0) suspending system (Fig 5.4a and 5.4 b).

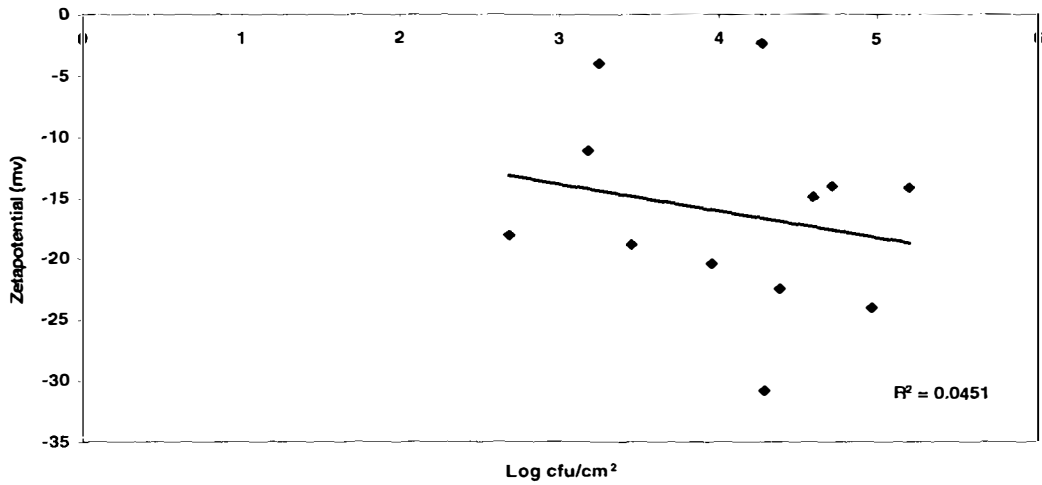


Figure 5.4a. Relationship between cell surface Zeta potentials and attachment to model system of twelve isolates using diluent fluid system.

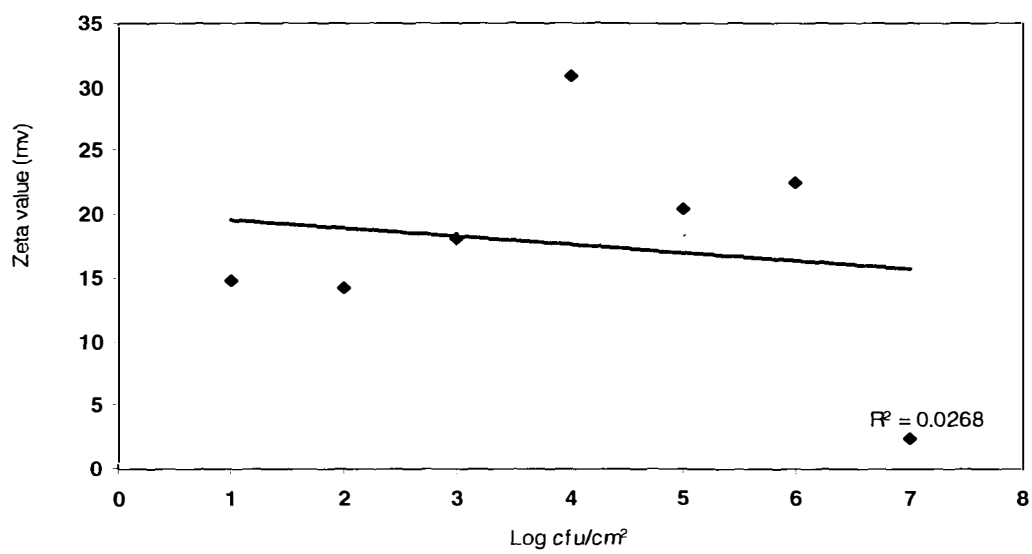


Figure 5.4b. Relationship between cell surface Zeta potentials and attachment to model system of seven isolates using PBS suspending system.

5.3.2 Hydrophobic measurements

5.3.2.1 Determination of cell surface hydrophobicity using HIC and its relationship to attachment

The percentage of cells retained by HIC ranged from 20 to 48 % for Gram positives and 4 to 28 % for Gram negatives; Gram variable shows 25 % (Fig 5.5). All the meat spoilage and pathogenic bacteria tend to be moderately hydrophobic to highly hydrophilic cells. With HIC measurements Gram positives show more hydrophobicity than Gram negatives.

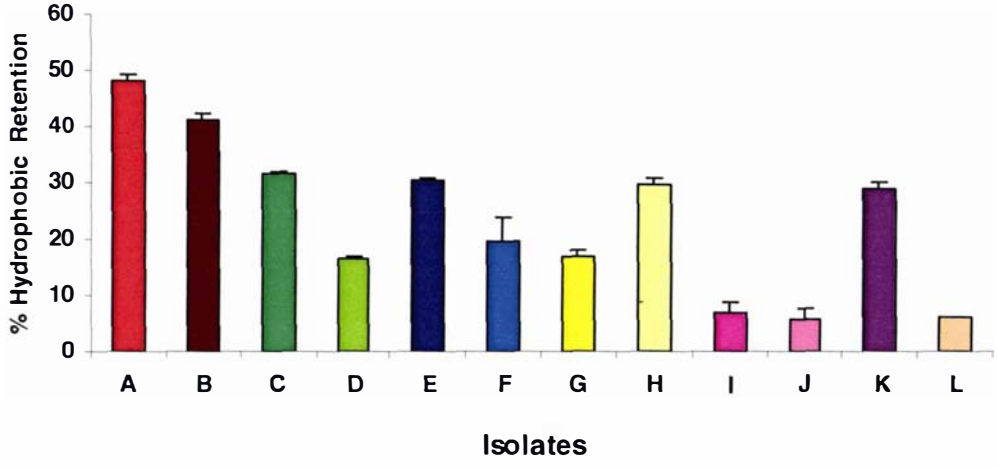


Figure 5.5. Proportion of hydrophobic cells retained on hydrophobic resin of all the 12 isolates, eluted using PBS (pH 7.0) *S. aureus* (**A**); *L. viridescens* (**B**); *L. monocytogenes* (**C**); *L. innocua* (**D**); *B. thermosphacta* (**E**); *P. aerouginosa* (**F**); *E. coli* 916 (**G**); *E. coli* O157:H7 (verotoxin negative) (**H**); *S.* Menston (**I**); *E. coli* E6 (**J**); *E. aerogenes* (**K**); *E. coli* O157:H7 (verotoxin positive) (**L**). Error bars represent the standard deviation from the mean of five replicates.

No relationship could be established between degree of hydrophobicity and the adhesion of cells to meat surfaces using both diluent fluid and PBS suspending system (Fig 5.6a and b).

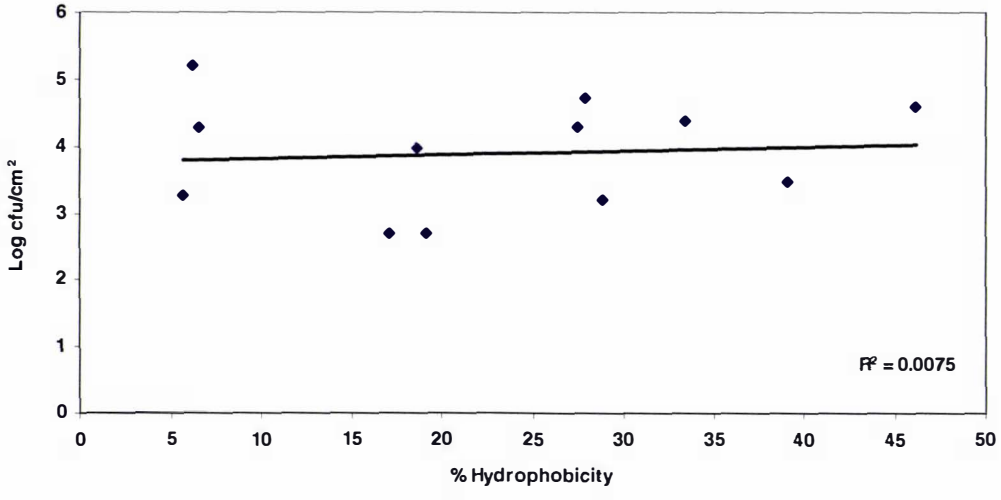


Figure 5.6a. Relationship between HIC cell surface hydrophobicity and attachment to model system of twelve isolates using diluent fluid system.

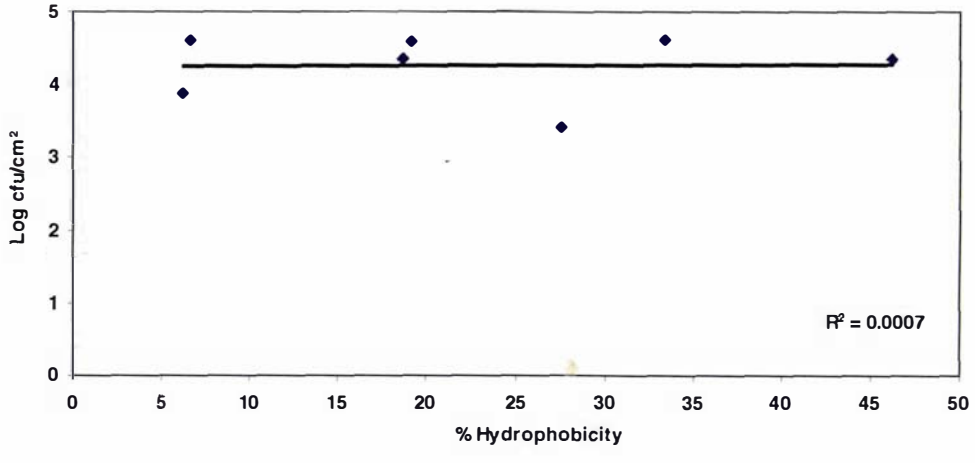


Figure 5.6b. Relationship between HIC cell surface hydrophobicity and attachment to model system of seven isolates using PBS suspending system.

5.3.2.2 Determination of cell surface hydrophobicity - (MATH test) and relationship with attachment

The percentage of hydrophobic cells assessed by the MATH test ranged from 2 to 80% for Gram positives and 0 to 10 % for Gram negatives and Gram variable

showed 5 % (Fig 5.7). Gram positives appeared to be more hydrophobic than Gram negatives.

Bacterial hydrophobicity within strains and species (Table 5.2) varied slightly depending on the method of measurement (HIC versus MATH). Although relative hydrophobicities as determined by each method cannot be directly compared, ranking the bacteria from the most hydrophobic to least hydrophobic is a useful means of comparison. Each method of determination resulted in a completely different ranking (Table 5.2), Nevertheless, Gram positives appear to be generally more hydrophobic than Gram negatives, which are typically hydrophilic.

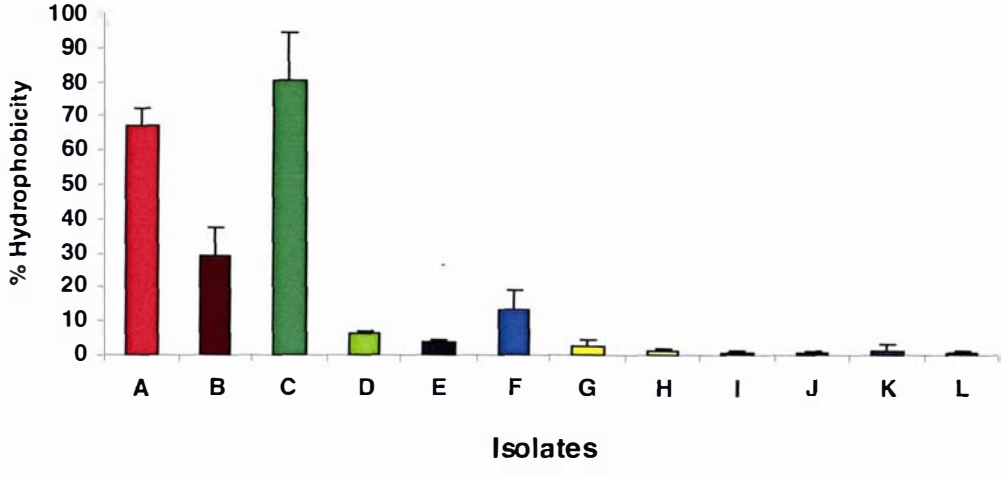


Figure 5.7. Percentage hydrophobic measurements of 12 isolates using the MATH test *S. aureus* (**A**); *L. viridescens* (**B**); *L. monocytogenes* (**C**); *L. innocua* (**D**); *B. thermosphacta* (**E**); *P. aerouginosa* (**F**); *E. coli* 916 (**G**); *E. coli* O157:H7 (verotoxin negative) (**H**); *S.* Menston (**I**); *E. coli* E6 (**J**); *E. aerogenes* (**K**); *E. coli* O157:H7 (verotoxin positive) (**L**). Error bars represent the standard deviation from the mean of five replicates.

Table 5.2. **Comparison of cell surface hydrophobicity using HIC and MATH methods**

Isolates	HIC	MATH
	% Hydrophobicity	% Hydrophobicity
S. aureus	46.23 +/- 2.1	66.8 +/- 1.0
L. viridescens	39.1 +/- 2.4	29.26 +/- 8.2
L. monocytogenes	33.41 +/- 2.2	80.33 +/- 1.9
L. innocua	18.7 +/- 2	6.6 +/- 0.3
B. thermosphacta	27.54 +/- 3.1	3.63 +/-1.1
P. aeruginosa	19.2 +/- 0.9	13.56 +/- 5.2
E. coli 916	17.12 +/- 1.4	2.8 +/- 1.4
E. coli O157:H7 (VT-)	5.76 +/- 0.9	0.7 +/- 0.5
S. menston	6.6 +/- 1.9	-0.44 +/- 0.2
Wild E. coli E6	6.2 +/- 0.6	-0.33 +/- 0.4
E. aerogenes	28.83 +/- 1.3	1.27 +/- 1.6
E. coli O157:H7 (VT+)	5.76 +/- 0.9	0.7 +/- 0.5

No relationship could be established between degree of hydrophobicity and the adhesion of cells to meat surfaces using both diluent fluid and PBS suspending system (Fig 5.8a and b).

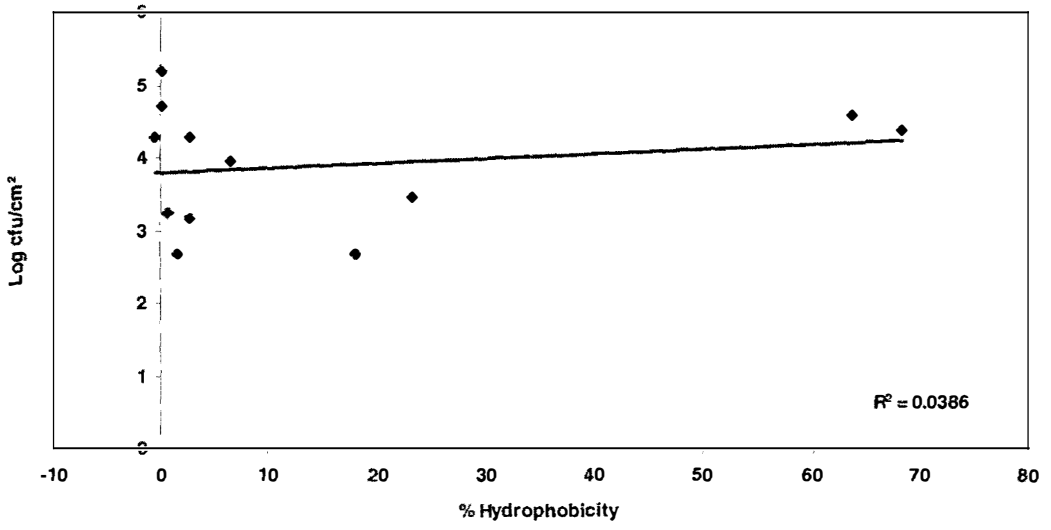


Figure 5.8a. Relationship between MATH hydrophobicity measurements of twelve isolates and attachment of isolates to model system using the diluent fluid system.

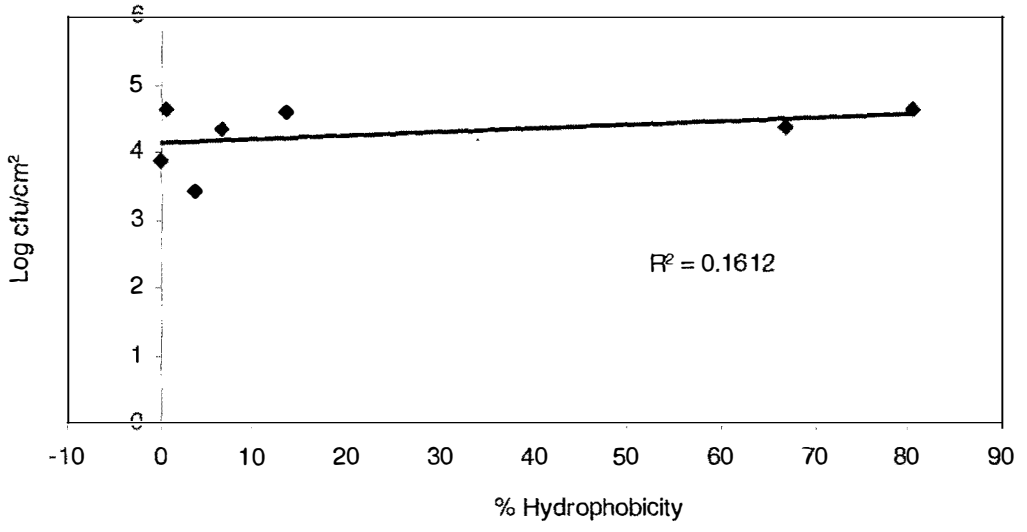


Figure 5.8b. Relationship between MATH hydrophobicity measurements of seven isolates and attachment to model surfaces using the PBS suspending system.

5.3.3 Effect of removal of cell surface proteins and polysaccharides components of cell walls on adhesion of *E. coli* E6

An attachment of approximately 5 log cfu/cm^2 occurred within seconds following immersion into a cell suspension containing approximately 10^7cfu/ml for 0.1% and 1.0% trypsin; 0.1% lysozyme treated cells and untreated cells did not increase significantly with time (Figure 5.9). The attachment, rinse and diluent media all had a pH of 7.0. Before immersion the natural contamination on cut beef surfaces was 10^1cfu/cm^2. Since periodate (50 millimolar) and lysozyme (1.0%) treatment killed the cells, the attachment to meat surfaces could not be established using viable count assessments. The fact that neither the trypsin nor lysozyme treatments reduced or increased attachment may be interpreted as showing that attachment can be affected by both cell surface proteins and polysaccharides. An alternative interpretation is that neither cell surface polysaccharides nor proteins play a significant role in the attachment of bacteria to meat.

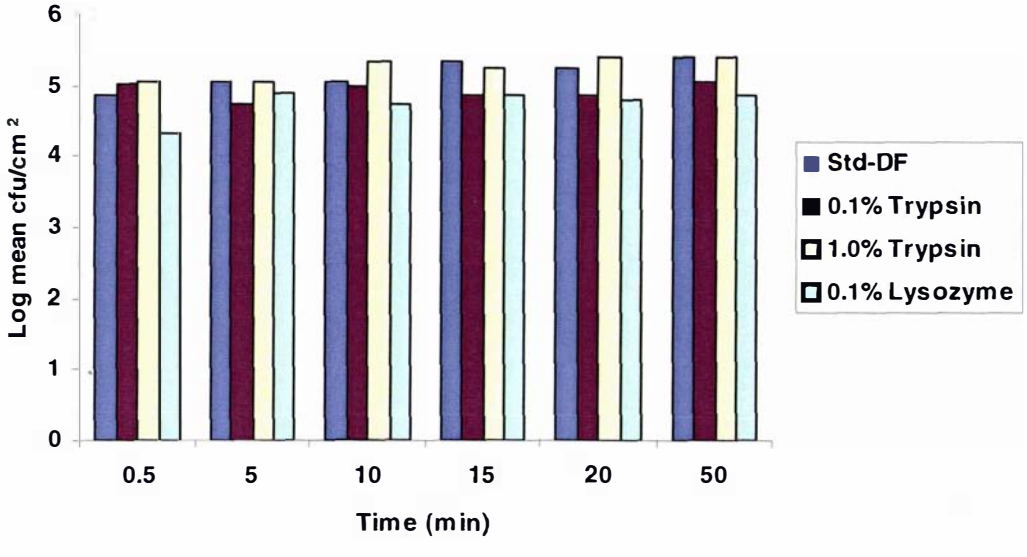


Figure 5.9. Attachment of ***E. coli*** **E6** to cut beef muscle following treatment to remove surface protein and polysaccharides from the bacteria.

5.4 DISCUSSION

Bacterial cell surface properties affecting the adhesion of meat spoilage and pathogenic bacteria were studied in order to learn more about the attachment of bacteria that persist on meat surfaces.

Cell surface charge, hydrophobicity, protein and polysaccharide removal were investigated to determine their role in the adhesion of spoilage and pathogenic bacteria to meat surfaces. These factors have been reported to be involved in the adhesion of bacteria to surfaces with the importance of factors varying from species to species (Bouttier et al., 1997; Doyle et al., 1990; Dickson & Koohmaraie, 1991; Flint et al., 1997; Hood & Zottola, 1995; Marshall et al., 1971; Neu, 1992; Parkar et al., 2001). In this study, no relationship was shown between adhesion and any of these four factors considered in isolation. This suggests that either these factors are unimportant, or that the attachment process to meat surfaces is complex and multifactorial.

There are numerous methods available to characterise the cell surface hydrophobicity and cell surface charge of microorganisms. In this study the techniques EIC and Zeta potential measurements were used to determine cell surface charge; MATH and HIC were used to measure hydrophobicity. In addition there is some debate concerning the correlation of results between individual methods. To allow meaningful comparisons, the buffer system employed in each test was standardised using PBS pH 7.0. A batch approach with interaction chromatography would eliminate any problems of retaining cell aggregates by blocking the voids. Therefore improvements can be made in future work by use of batch experiments.

Twelve isolates were used in this study. The isolates examined exhibited primarily medium to high cell surface hydrophobicity and cell surface charge. The current study employed two methods to determine the surface charge of meat isolates. They were (1) EIC, giving the general estimate of positive and negative charges present on the cell surface and (2) The Zeta sizer, which measures zeta potentials or electrophoretic mobility of the bacterial cell surface.

The two methods indicated a range of high and low positive and negative charges and lower zeta potentials (indicating less electrostatic interactions between surfaces) occurred for all the isolates used in this study. Zeta potentials were measured using formalin treated isolates. This was thought necessary to prevent contamination of the equipment which was kindly made available by the Fonterra Research Centre, Palmerston North, New Zealand. A comparison of Zeta potential measurements of killed and live *E.coli* E6 cells showed that formalin treatment had very little effect. The methods used to determine cell surface charge showed much variation between isolates. However, both the methods are measuring the same amount of charge distributed on the cell surface. With the EIC the positive and negative charge retentions are measured separately while Zeta potentials measure the net effective charge. Therefore Zeta potential in millivolts is approximately equivalent to difference between negative charge and positive charge measured on ionic interaction column or a net effective charge. In general bacteria that contaminate meat exhibit a surface with both negative and positive charges which result in low negative Zeta potentials in other words, the strains had more negative than positive charges on the cell surface. But there was no correlation between negative charge, positive charges or Zeta potentials and attachment to meat in standardised diluent fluid and PBS suspending systems.

Although it was not possible to demonstrate a relationship between the net cell negative charge and adhesion, nevertheless surface charge may still influence the adhesion process. The predominantly negative charge on the cell surface is likely to cause repulsion of bacteria from negatively charged surfaces. This repulsion is overcome by stronger attractive forces (e.g. van der Waals forces or hydrophobic interaction) to produce adhesion. Based on electrical double layer theory, the positive charges present on the cells would be more likely to adhere. The present studies are in agreement with Flint et al. (1997) who demonstrated there was no relationship between the surface charge and adhesion to stainless steel surfaces. On the contrary, the studies of Dickson & Koohmaraie (1989) show that bacterial attachment to any surface is related to charges on both the cells and surfaces. A positive correlation can be demonstrated by using various pH and ionic strength solutions to confound or

enhance the charge related ionic attachment process (Bouttier et al., 1994; Li & Mclandsborough, 1999).

Hydrophobic interactions are also thought to play a role in attachment (Benito et al., 1997; Busscher et al., 1987; Dickson & Koohmaraie, 1989; Rosenberg & Doyle, 1990; Stenstrom, 1989; van der Mei et al., 1987; van Loosdrecht et al., 1987). Two methods were employed to determine the hydrophobicity of the surface of meat isolates: HIC (Phenyl sepharose) and MATH test. The two methods determine the binding of hydrophobic groups on the bacteria to those on hydrophobic surfaces. The two methods indicated that the surfaces of meat isolates ranged from moderately hydrophobic to highly hydrophilic.

Comparison between methods introduces considerable variation in the hydrophobicity of meat isolates, but overall conclusions are consistent. In the MATH test Rosenberg et al. (1984) which measures cell surface hydrophobicity, the choice of organic phase is important, as some organic phases destroy the surfaces of certain bacteria giving a false indication of hydrophobicity. The overall conclusion concerning the two methods is that there is good agreement as to the hydrophilic and hydrophobic bacteria. These results are in agreement with the conclusion arrived at by (Mozes & Rouxhet 1987). The Implication of the MATH test for evaluating cell surface hydrophobicity state is that maximum removal rates of bacteria are achieved by a given hydrocarbon at pH values where electrostatic repulsion is absent. Such measurements can be considered as a meaningful measure of microbial hydrophobicity (van der Mei et al., 1995). However Geertsema-Doornbusch et al. (1993) consider that the MATH test does not measure hydrophobicity, but rather the interplay of charge and hydrophobic properties. Measurement of each property can be achieved by different methods; however results obtained by different methods are often poorly comparable. Each method has its own merits and demerits. Establishing a meaningful relationship of surface properties and adhesion is a difficult problem faced by most researchers. Jones et al. (1996) discuss the drawbacks of methods involved in measuring bacterial adhesion and also review about the difficulties associated with correlating each cell surface property with adhesion to surfaces and

also recommend that the buffer system should remain constant throughout to achieve consistency of results.

Hence ranking them in a particular high to low order appears to facilitate comparison of the hydrophobicity of various isolates. In the present trials the hydrophobicity of all the meat isolates studied differed; there was no obvious positive relationship between hydrophobicity and absolute attachment of cells to the model meat surface. This might be attributed to the heterogeneous nature of microbial surfaces.

The results of the present study were similar to those of Boutteir et al. (1993) and Piette & Idziak (1997) who demonstrated that hydrophobicity did not affect the adhesion of bacteria to meat surfaces. In contrast, Benito et al. (1997) demonstrated positive correlation of cell surface hydrophobicity and attachment strength of meat spoilage and pathogenic bacteria. A positive correlation found by this group of researchers may be due to the application of an entirely different system to correlate properties with attachment. It is believed that hydrophobicity plays an important role in surface attachment of bacteria. Since the process of attachment is complex and multifactorial it is essential to understand attachment in terms of single attachment mechanisms and it is necessary to identify the components interfering with or overriding hydrophobic concurrent attachment mechanisms rather than trying to establish a relationship between gross surface properties and attachment. Jones et al. (1996) reported that positive correlations can be obtained between cell surface properties and adhesion by standardising the buffer system. The present study with standardised PBS as the suspending medium failed to produce a positive correlation between cell surface properties and adhesion.

The present study used *S. aureus* in further experiments because it gives high retention in all the assay methods and therefore is more readily investigated. Generally, Gram positives appear to be highly charged and hydrophobic, with few exceptions, which may be due to presence of teichoic acid on the cell wall when compared with Gram negatives, which tend to demonstrate less charge and hydrophobicity irrespective of the methods used. However, all the isolates used in the present study exhibit a wide range of charge and hydrophobicity.

Several attempts were made to determine bacterial cell surface hydrophobicity using contact angle measurements with the goniometer and photographic methods. As the water droplet persisted for seconds and soaked into the bacterial lawns rapidly on films, measurements could not be determined using a manual goniometer. Most of the meat spoilage bacteria used in this study were highly hydrophilic strains, based on tests conducted using MATH and HIC. Therefore it is presumed that the difficulty in assessing the hydophobicity using contact angle measurements was due to the highly hydrophilic nature of the isolates used in this study. Time and financial constraints prevented continuation of work on this area. Failure to obtain the contact angle measurements did not allow the prediction of surface tension and thermodynamic aspects of bacterial adhesion to meat surfaces.

Extracellular polymers of bacterial origin have frequently been reported to aid the attachment of bacteria to surfaces in aquatic environments (Fletcher, 1980; Salas & Geesey, 1989; Wardell et al., 1980) in the oral cavities (Rogers et al., (2001) and stainless steel (Lewis et al., 1989). In contrast, the presence of extracellular polymers has been reported also to inhibit attachment under certain circumstances (Kogure *et al.*, 1982; Pringle *et al.*, 1986; van Houte, 1983). Extracellular polysaccharides (EPS) are believed to be more important in the adhesion of some bacteria to surfaces and have been termed *adhesive polymers* (Neu, 1992). Marshall et al. (1971) suggested that EPS plays a role in both the initial adhesion and the irreversible adhesion. Some authors consider that EPS can promote a preconditioning or viscosity change of the surface, making adhesion more favourable (Oliveira et al., 1994). Herald & Zottola (1989) showed that compounds that bind to or disrupt carbohydrates, such as sodium meta-periodate, cetavlon and concanavalin A, all decreased the adhesion of *Pseudomonas fragi* to stainless steel. However, Allison & Sutherland (1987) queried the extent to which the EPS are involved in the initial adhesion.

In dairy plants extracellular polysaccharides (EPS) play only a small part in the initial adhesion of cells of thermoresistant streptococci (Flint et al., 1997). No such study has been conducted in relation to meat surfaces. The first trials of the present study quantified the influence of time on adhesion following treatment of *E. coli* E6

cells with chemicals that disrupt the surface polysaccharides, but no reduction in adhesion to meat surfaces occurred with mild concentration lysozyme treatments. However, higher concentration killed the cells. Therefore, either cell surface polysaccharides have a negligible effect on adhesion, or the concentrations of chemical treatments used were too low to cause a significant effect. Milder concentrations of lysozyme and periodate were selected in an attempt to maintain the viability of cells during treatments. However, periodate treatment of 0.1 and 1.0% concentration killed all the cells and attachment to meat surfaces could not be detected using viable count procedures.

Treatments used were lysozyme, which dissolves bacterial cell wall mucopolysaccharides by hydrolysing the beta (1-4) linkages between N–acetyl-amino-2 deoxy D glucose residues (Windholz, 1983) and sodium meta-periodate, which oxidises adjacent hydroxyl groups of component monosaccharides (Gopal & Reilly, 1995).

Many researchers have pointed to the role of proteins associated with the adhesion process in terms of adhesion proteins. Lorca et al. (2002) demonstrated that lactobacilli expressed cell surface proteins which mediate the binding of immobilized collagen and fibronectin, indicating that protein-protein interactions mediate adhesion to extracellular matrix proteins. Lammers et al. (1999) demonstrated that fibronectin proteins of *S. aureus* were essential for adhesion to occur on bovine mammary gland cells. Cheung et al. (1989) demonstrated that staphylococcal surface proteins mediate the adherence of S. *aureus* to fibres *in vitro.* Tuomola et al. (2000) showed that cell surface proteins are important in the adhesion of lactobacilli to human intestinal mucus glycoproteins.

In biological systems, proteins on the bacterial cell surface mediate adherent interactions with host tissues. For example, site-specific colonisation by oral streptococci results from interplay between the host receptor and bacterial adhesion protein expression (Jenkinson, 1994). The adhesion of *Azospirillum brasilense* to glass and polystyrene surfaces indicated a correlation between the protein concentration at the cell surface and adhesion density (Dufrene et al., 1996). The

importance of proteins in the adhesion of cells to meat substrates is less well documented. In the present investigation, the effect of protein degrading treatments on the adhesion of *E. coli* E6 to meat surfaces was determined. The removal of cell surface proteins from the bacteria using trypsin did not lead to an observed reduction in attachment numbers. The action of trypsin involves removal of dipeptide units of polypeptide chains of protein molecules. This suggested that though proteins are the major component involved in the initial phase of adhesion, the treatments used may be not effective in reducing attachment. The reasons for the failure of the treatment to reduce attachment may be that trypsin removed some molecules and unmasked others, restoring the attachment potential. Trypsin removes a fraction of molecules irrespective of the concentration used and the cells attach to the surface using other binding molecules (i.e., other cell surface molecules like peptidoglycan or lipo mannan) that may contribute to attachment. The present observations are contrary to those of Flint et al. (1997) and Parkar et al. (2001) who demonstrated that cell surface proteins of thermophilic streptococci and thermophilic bacilli are involved in adhesion to stainless steel. However, in contrast to dairy plant surfaces, the meat surface is a complex biological surface presenting different attachment modes, sites and mechanisms for bacterial adhesion processes. That the bacterial cell surface protein is not the only factor contributing to the process of attachment to meat clearly signals the possible involvement of other attachment mechanisms.

From an industrial point of view, these treatments cannot be applied to meat surfaces as attachment inhibitors, since the bacteria are already present on the freshly slaughtered meat surface as an inevitable consequence of the process by which slaughter stock are turned into meat. Since meat is a food commodity, regulations on the application of chemicals are strictly enforced. Moreover, the slaughter and washing process in the meat plant are rapid and the fresh meat reaches the chiller within as little as 20 minutes after slaughter. With hot boning procedures, the fresh meat is immediately subjected to packaging procedures to minimise contamination problems. Consequently, proteolytic treatments may not be effective in reducing adhesion with meat surfaces and cannot be recommended as an intervention.

For future research on adhesion mechanisms, there are several different types of enzymes available other than trypsin or lysozyme, which might be used to study further the adhesive interactions between bacterial cell and meat surfaces.

In order to see any change in the cell surface following treatment with trypsin, lysozyme and periodate, sections of cells were examined by TEM. No bacterial structures such as pili were seen (unfortunately, the experimental work did not produce any result). The wet mount motility test showed that the organisms were motile, which indicates E. *coli* E6 has pili and other appendages on cell surface.

The *E. coli* E6 cells remain as a whole cell without any damage occurring to the surface. No morphological changes were observed with trypsin and periodate treated cells (Fig 6.7 A-D). This clearly indicates that no reduction in adhesion to meat surfaces occurred upon treating with trypsin, which implies that the continued attachment was because no significant surface modifications occurred. TEM observations after treatments with 0.1% lysozyme showed lysis (Fig 6.7 D), which was presumed to be only partial, as the cells were capable of adhering to the surface without presenting any biologically significant reduction. Application of 1% lysozyme caused complete lysis of the cells of *E. coli* E6 and thus no attachment to a model system was observed. Previous studies on lysozyme treatment of Gram negative bacteria have shown that Gram negatives are more resistant to lysozyme because of the presence of a "thick" outer membrane (Chatsapozalu et al., 1991). The present TEM study demonstrates that *E. coli* E6 Gram negative bacteria *are* sensitive to lysozyme, where partial and complete lysis of cells is believed to be due to the presence of unusually sensitive permeable outer membrane, which is not usually a target site for the lysozyme. The variation in result might be due to the source of lysozyme, or possible contamination with other enzymes. However, more work in this area might provide new informations for future food microbiologists.

It therefore appears that following removal of a fraction of the cell surface protein or polysaccharide, bacteria are still capable of attaching to the surface using other attachment modes, and partially lysed cells can adhere to the meat surface. While treatments removed a fraction of surface proteins and polysaccharides, the exposure

of deeper layers may restore attachment potential. Targeting the polysaccharide component and releasing the intracellular components of the cells may be one of the ways to reverse the attachment or prevent whole cells from attaching by blocking sites with cell fragments. However, lysozyme cannot be recommended because of the problems associated with the practical application of wash treatments on meat surfaces, which were discussed in previous paragraphs.

It is generally accepted that physico-chemical properties of both the substrate and bacterial surfaces play some role in the adhesion of bacteria (Carpentier & Cerf, 1993). The physico-chemical properties include electrostatic interactions, van der Waals attractive forces, hydrophobicity and other steric forces and surface molecules like polysaccharides and proteins. The importance of each property varies depending on the type of organism, its stage of growth, the nature of the environment and the composition of the substrate. In addition, variations in the different test procedures used (e.g., for the cell surface hydrophobicity and cell surface charge), make comparisons between the results from different laboratories difficult. In summary no single cell surface feature was shown to be the primary cause of bacterial attachment to meat surfaces. It is hypothesised that attachment to meat surfaces is mediated by multiple mechanisms acting simultaneously, making the process of desorption difficult. The current knowledge suggests that the potential for microorganisms to adhere cannot be predicted from the physicochemical properties of either the substrate or microbial surfaces (Carpentier & Cerf, 1993). None of the single target treatments appears to correlate positively or negatively with attachment. It has been suggested that interfering simultaneously with the multiple initial attachment mechanisms will contribute to the detachment of bacteria from the surfaces (Busscher et al., 1995). Targeting the combined inter-surface relationships indicates the direction for reversing bacterial attachment by altering the surface related characters.

5.5 CONCLUSION

The current study on cell surface properties of bacteria and attachment showed that no simple relationship existed between individual physicochemical properties and the process of attachment. Even cell surface proteins and polysaccharides did not apparently individually contribute much to attachment to meat surfaces. Attachment to meat surfaces is, therefore, believed to be mediated by more complex physical and chemical interactions between the cell and substratum. Targeting the multiple attachment mechanisms simultaneously may offer a way to perturb the initial attachment, thereby reducing the adhesion.

Chapter 6
MODEL SURFACE INTERACTIONS

6.1 INTRODUCTION

The interaction between a microbial surface and any substratum results from numerous types of adhesive reactions mediated by multiple attachment mechanisms. Without an understanding of individual contributing mechanisms, bacterial attachment to meat surfaces may be too complex to understand; factors that may be involved include electrostatic interaction (Dickson & Koohmarie, 1989); cell surface hydrophobicity (Benito et al., 1997); cell surface molecules like surface proteins (Flint et al.,1997; McNab & Jenkinson, 1998; Schneewind *et al.*, 1995); surface polysaccharides Fletcher and Corpe et al. (1976); Floodgate (1973); Geesey *et al.* (1977) and Sutherland (1980); surface structures which additionally also possess specific receptors to promote ligand receptor binding reactions (Benedict et al., 1991; Ofek et al., 1977; Vercelloti et al., 1985); environmental conditions such as electrolyte concentrations, fluid dynamics (Mittleman et al., 1990); bulk phase biotic and abiotic constituents (Marshall, 1988); the physicochemical characteristics of the substratum (Absolom et al., 1983; Fletcher, 1992). Several, or all, of these mechanisms may act simultaneously to mediate adhesion or to complicate the reversibility of the process of bacterial adhesion which was considered in the previous chapter. Cheng et al. (2000) demonstrated that the bacterial attachment process involves a sequence of different types of chemical entities and biopolymers, taking part firstly in the initial attachment and secondly in biofilm development.

Busscher et al. (1995) and Bos et al. (1999) hypothesised that the initial microbial adhesion was important in the overall process of biofilm formation, as they realised that the bond between initial adhering organisms and a surface must be broken if detachment rather than adhesion is to be promoted. The adhesive interactions of microbial cells and other substrates can be perturbed by various interventions such as changing the pH, ionic

strength or wettabilty; use of blocking agents, surface protein and polysaccharide removal agents, hydrogen and hydroxyl ions. These interventions are expected to interfere with the charge-charge and hydrophobic attractions, blocking attachment sites, adhesive proteins and polysaccharides, and reducing surface free energy, and so on. This may facilitate desorption of bacteria by altering the surface characters of the microbial cell, substratum and associated environmental conditions (Absolom et al.,1983; Bellonfontaine et al., 1996; Busscher et al., 1995; Grasso et al., 2001; Flint et al.,1997; Piette & Idziak, 1991; Parkar et al., 2001; Stewart, 2000; Whitekettle, 1991). Studies of attachment mechanisms with meat surfaces include those by (Bouttier et al., 1994; Li & McLandsborough, 1999; Piette & Idziak, 1991). These workers clearly show that our understanding of attachment to meat surfaces is poor. A study of the fundamental process of bacterial attachment to meat surfaces should be undertaken to form the theoretical basis for the development of procedures for the physical removal of microorganisms contaminating meat surfaces (James & James, 1997; Pordesimo et al., 2002). An understanding of the interaction of bacteria with the surface water layer of carcasses and the mechanisms of bacterial attachment will be pivotal if carcass washing treatments are to be made more effective in bacterial removal or inactivation (Dickson & Siragusa, 1994).

The objective of this chapter was to examine some of the single attachment mechanisms using EIC and HIC column models (Fig 6.1 a). This study also aimed to identify the principal components, such as pH, interfering with these single attachment mechanisms (Fig 6.1b and 6.1c) with these single attachment mechanisms. Such a fundamental understanding may allow the development of procedures for enhanced removal of bacteria attached to the surfaces.

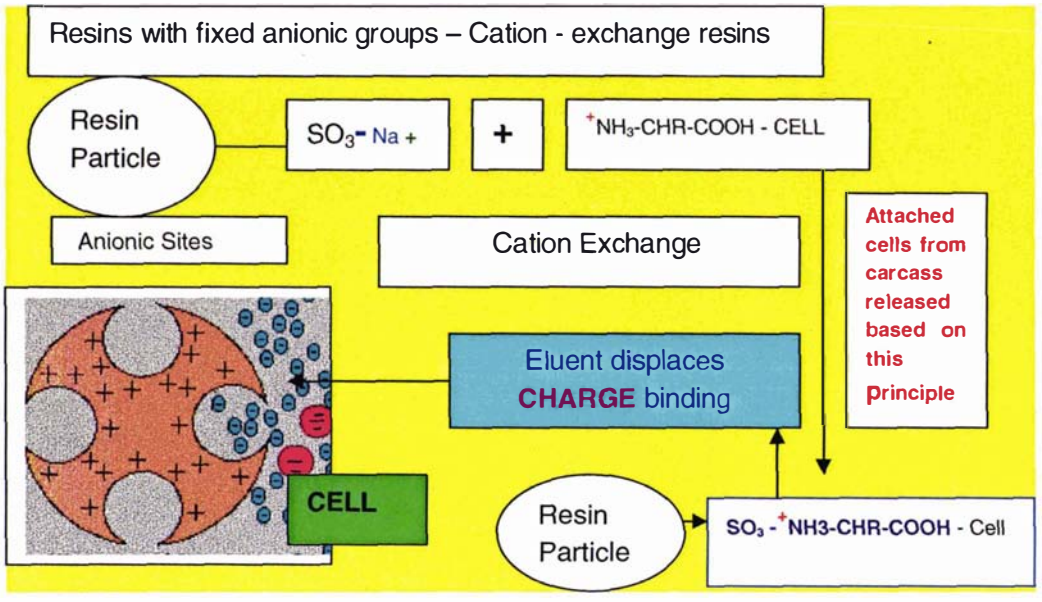


Figure 6.1a. Resin cell interaction: example which shows binding of a positive charge amino acid group of cell surfaces to a cation exchange resin by displacing Na+counter ions; and the use of eluents to displace the charge binding by weakening the electrostatic interaction to favour greater removal of cells from columns.

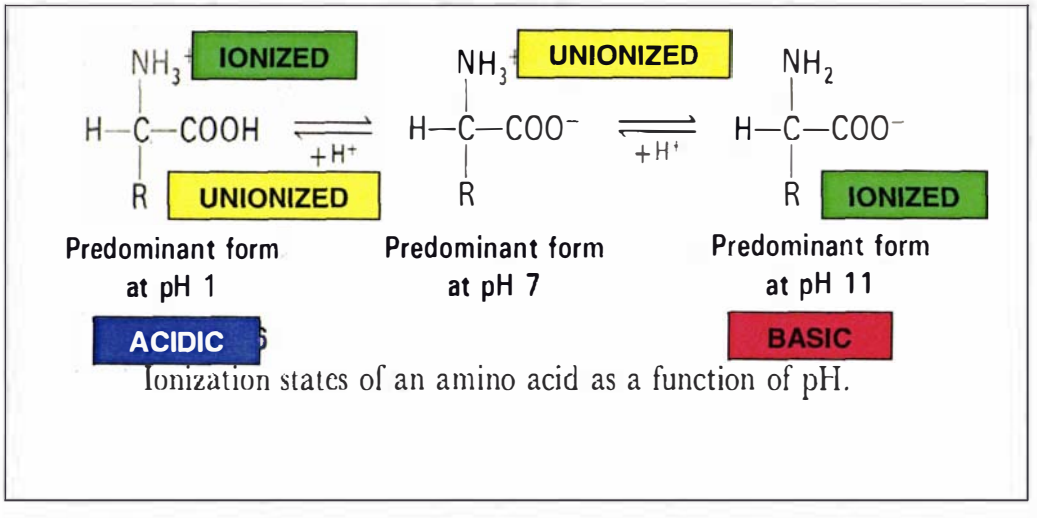


Figure 6.1b. Ionization states of amino acid (Ref: *Biochemistry* by Stryer, 2nd edition) and modified slightly.

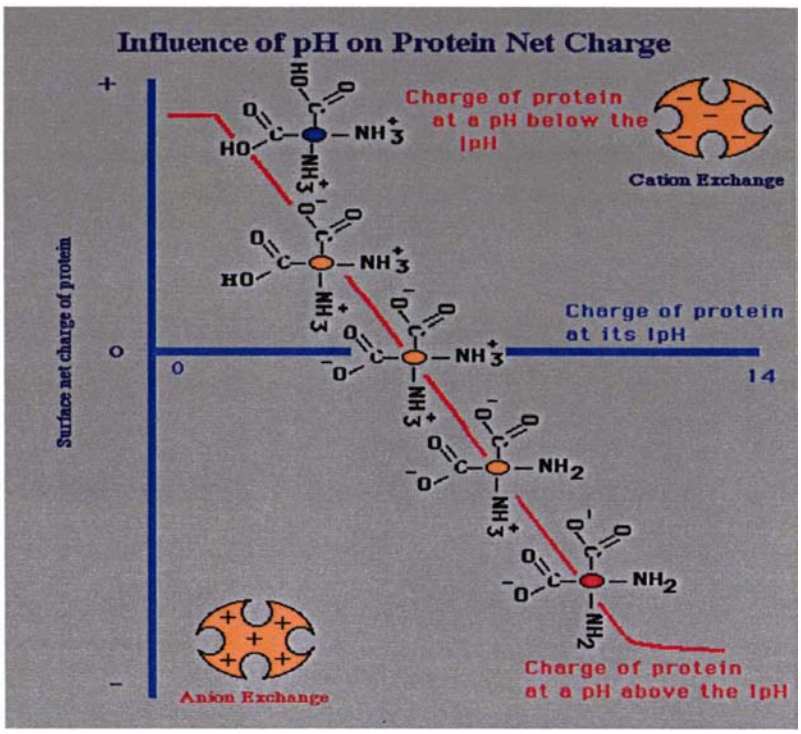


Figure 6.1c. Charge of protein components of meat and microbial surface undergo changes as pH of the suspending medium changes. Ref: (website http://ntri.tamuk.edu/hplc/selectivity.gif) Published with the permission from John C. Perez, Director of the Natural Toxin Research Center, Department of Biology, Texas.

6.2 PROCEDURES

1. To study the effect of various eluents interfering with single attachment mechanisms using *S. aureus* (refer section 3.9).
   (a) To select optimal cell concentrations to load on the resin columns (refer section 3.9.1).
   (b) To validate spectroscopically and observe the effects of interference by the eluents in attachment to resin columns (refer section 3.9.2).
   (c) To identify the components interfering with anionic attachment (refer section 3.9.3).

(d) To identify the components interfering with cationic attachment (refer section 3.9.3).

(e) To identify the components interfering with hydrophobic attachment (refer section 3.9.3).

2. To study the effect of removal of cell surface protein and polysaccharides on surface charge and hydrophobicity (refer section 3.12.1 &3.12.2).

(a) To validate spectroscopically the effect of protein and polysaccharide removal on the attachment of cells (refer section 3.12.3).

(b) To analyse qualitatively and quantitatively the supernatant of treated (Trypsin; Lysozyme and Periodate) and untreated cells (refer section 3.13 & 3.14).

(c) To conduct TEM investigations on three isolates to observe the effect of removal of cell surface proteins and polysaccharide molecules (refer section 3.7).

(d) To test the viability of treated and untreated cells (refer section 3.12.3)

(e) To identify the components interfering with charge (ionic and electrostatic) and hydrophobicity following the removal of cell surface proteins (refer sections 3.12.1; 3.12.4; 3.8.1& 3.8.2).

(f) To identify the components which interfere with charge and hydrophobicity following the removal of cell surface polysaccharides (refer sections 3.12.2; 3.12.4; 3.8.1&3.8.2).

3. To use the collagen film model system to investigate ionic and hydrophobic rinse treatments interfering with the attachment of *E. coli* E6 (refer sections 3.3& 3.4.3).

4. To use the collagen film model system to investigate the blocking of attachment sites for *E. coli* E6 with carbohydrate, proteins, NaCl and Tween (refer sections 3.15.5; 3.3; 3.4.3).

6.3 RESULTS

6.3.1 Isolates of this study

Isolates used in this study included *S. aureus; E. coli* E6 and *B. thermosphacta.*

6.3.2 Selection of optimal sample (cell concentration) optical density to apply on resin columns

The mean percentage retention obtained for *E. coli* E6 for O.D 0 to 2.0 with anionic, cationic and hydrophobic resins is shown in Fig 6.2. With optical densities too low (0.2 and 0.6) the retention was high indicating that maximum retention sites were present because the cell densities were too light to indicate the change. With high optical densities, the bacterial numbers were too dense and made it difficult to detect the changes when most of the retention sites were saturated. Cell concentrations of O.D 1.0 to 1.2 were chosen for further study as this density did not result in saturation of all the retention sites on anionic, cationic and hydrophobic columns.

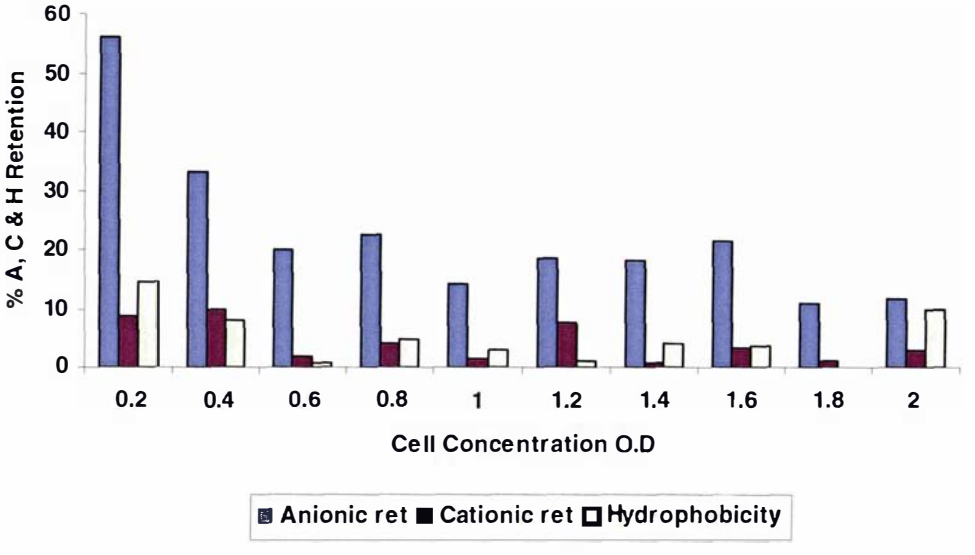


Figure 6.2. Selection of optimal cell density (optical density) based on percentage retention of ***E. coli*** **E6** on anionic, cationic and hydrophobic columns.

6.3.3 Determination of optical density of cell suspensions interacting with various eluents using a spectrophotometer

No differences in mean optical densities of Gram positive *L. monocytogenes,* Gram negative *E. coli* E6 and Gram variable *B. thermosphacta* bacteria suspended in various eluents were observed compared to optical density of cells suspended in phosphate buffered saline as an eluent (Fig 6.3). Slight differences in optical density between Gram positive and Gram negative bacteria were noticed. However, these results suggest that the different eluents do not influence optical density readings, therefore difference occur as a result of differing numbers of bacteria eluted compared to the PBS control.

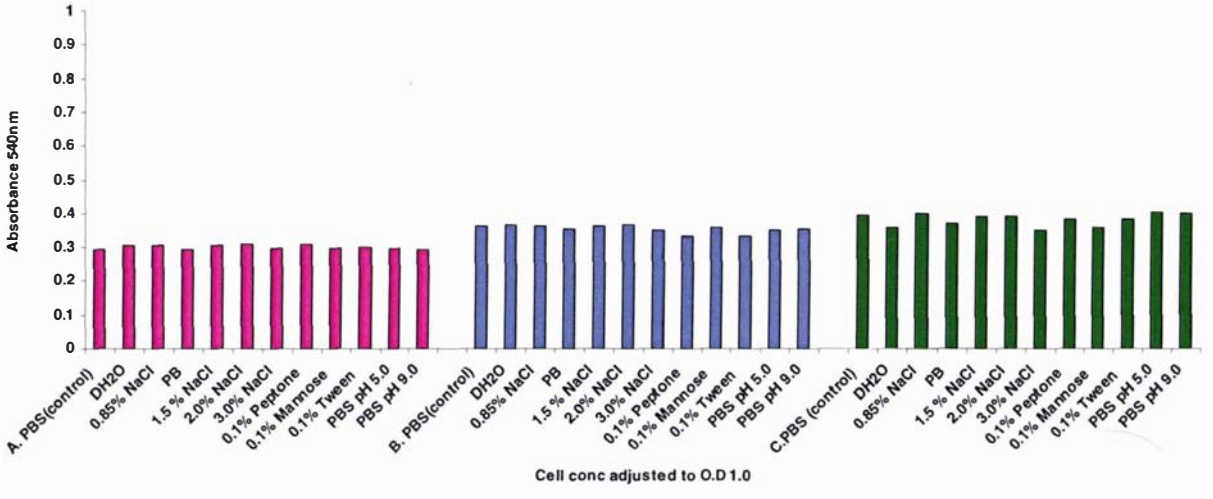


Figure 6.3. Comparison of optical densities of Gram positive, Gram variable and Gram negative cells with PBS pH 7.0 and various other eluents using spectrophotometer. (**A**) *L. monocytogenes*; (**B**). *B. thermosphacta*; (**C**). *E. coli* E6.

6.3.4 Effect of various eluents in eluting *S. aureus* cells from EIC column

6.3.4.1 Effect of various eluents in eluting *S. aureus* from anionic column

The surface of *S. aureus* is both negatively and positively charged; therefore the cells are expected to bind to a positively charged group on the anionic exchanger. Fig 6.4 presents elution profiles of *S. aureus* cells that were applied to anionic exchange columns. The cells bound efficiently to the column in PBS at pH 7.0 and were eluted most efficiently with NaCl concentrations of 1.5% as the percentage of anionic retention decreased as negative charge decreased. The percentage of anionic retention slightly increased as the negative charge slightly increased at NaCl concentrations of 0.85%, 3.0%, 2.0%, PB, pH 9.0, and pH 5.0 of PBS, distilled water, 0.1% peptone, 0.1% Tween, 10% TSP pH 12.0 and 0.1% mannose used as eluents. It appears that increased effective negative charge results from both increased ionic strength and hydrogen ion concentration (falling pH). All the eluents used showed moderate interference with the electrostatic binding on anionic columns, with 1.5 % NaCl demonstrating lowest retention. The retention of cells on anionic columns is associated with a net negative charge as well as its distribution.

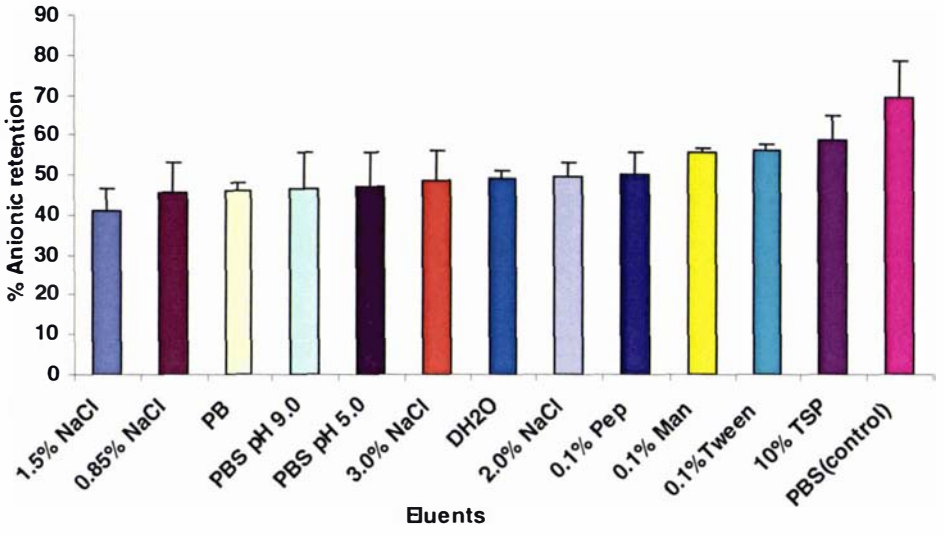


Figure 6.4. Effect of various eluents on retention of ***S. aureus*** by anionic exchange resin. The error bar represents the standard deviation from the mean of five replicates.

6.3.4.2 Effect of various eluents in eluting *S. aureus* cells from cationic exchangers – EIC column

The surface of *S. aureus* carries both positive and negative charges and the cells are expected to bind to a negatively charged carrier on cationic exchangers. Fig 6.5 presents elution profiles of *S. aureus* cells which were applied to the cation exchange columns. The cells bound efficiently to the cationic column in PBS pH 7.0 and were eluted with peptone and Tween at concentrations of 0.1% where retention on the cationic column decreased as the positive charge decreased. Retention tended to increase slightly as the positive charge slightly increased with all the other eluents (Fig 6.5). All the eluents used caused interference with the electrostatic binding on cationic columns, with 0.1% peptone demonstrating the lowest retention. Retention of cells on the cationic column is associated with a net positive charge as well as with its distribution.

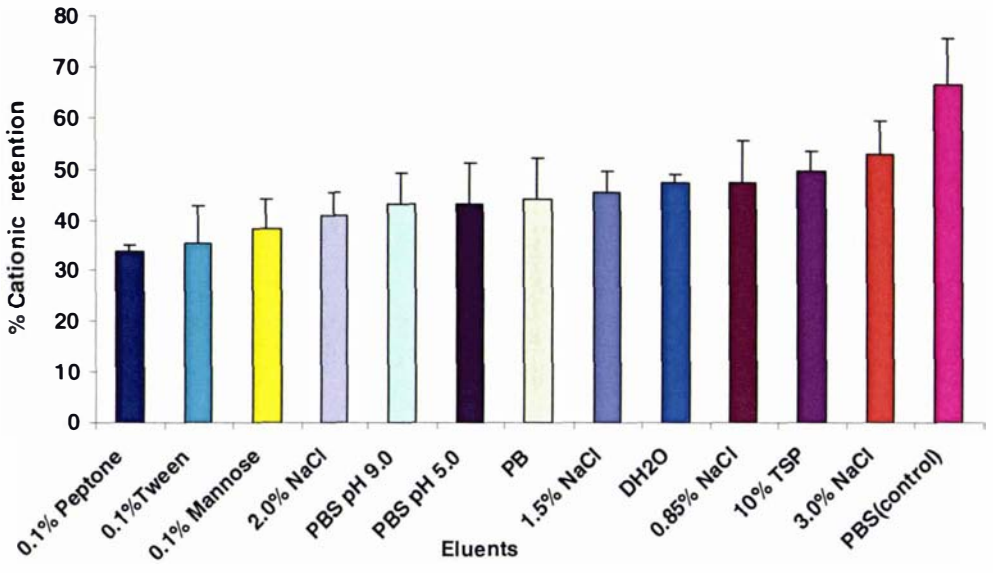


Figure 6.5. Effect of various eluents on retention of ***S. aureus*** by cation exchange resin. The error bar represents the standard deviation from the mean of five replicates.

6.3.4.3 Effect of various eluents in eluting S. aureus cells from hydrophobic column – HIC Column

The surface of *S. aureus* is moderately hydrophobic and the cells were expected to bind to an alkyl and aryl group of the hydrophobic column. Fig 6.6 presents elution profiles of *S. aureus* cells which were applied to HIC. The cells bound efficiently to the hydrophobic column in PBS pH 7.0 and when eluted with Tween concentrations of 0.1% the maximum decrease in the retention on hydrophobic column was observed as hydrophobicity decreased and hydrophilicity increased. Hydrophobic retention increased as hydrophobicity of cells increased, with PB, NaCl concentrations of 0.85% and 1.5%, 0.1% peptone, distilled water and 0.1% mannose. The hydrophobic binding to the hydrophobic column was enhanced above that observed with the PBS standard with 2.0% and 3% NaCl; pH 5.0 and pH 9.0 of phosphate buffered saline and 10% TSP pH 12.0.

The observed increase in attachment (decreased elution) associated with higher ionic strength eluents is compatible with the manufacturer's statement that ionic strength will increase the attachment environment to hydrophobic columns. Of all the eluents used, 0.1% Tween showed the highest interference with the hydrophobic binding to hydrophobic columns.

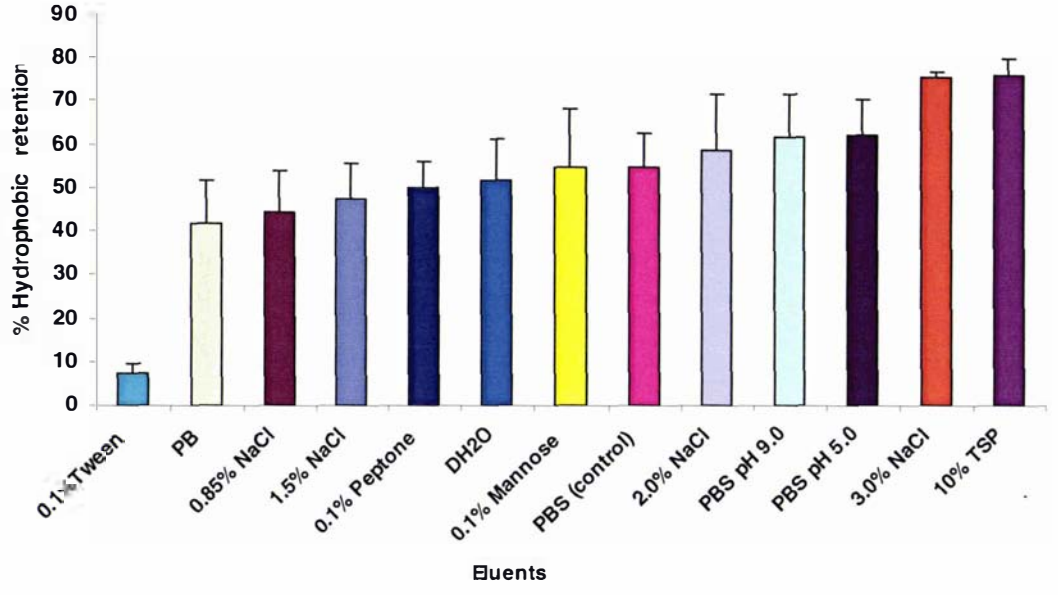


Figure 6.6. Effect of various eluents on retention of ***S. aureus*** by phenyl sepharose hydrophobic resin. The error bar represents the standard deviation from the mean of five replicates.

6.3.5 Comparison of optical density of treated cells and untreated cells

The influence of cell surface treatments on the optical density of *E. coli* E6, *S. aureus* and *B. thermosphacta* suspesions is shown in Table 6.1: There is no significant difference in the optical density of treated and untreated cells except for lysozyme treatment on *E. coli* E6 and *B. thermosphacta.*

Table 6.1. Mean optical density of *E. coli* E6, *S. aureus* and *B. thermosphacta* after treatment with Trypsin, Lysozyme and Periodate

Isolates	Control mean O.D.*	Trypsin mean O.D.	Lysozyme mean O.D.	Periodate mean O.D.
***E. coli* E6**				
Before treatment	1.338	1.329	1.352	1.355
After treatment		1.221	0.49	1.329
B. thermosphacta				
Before treatment	1.12	1.041	1.045	1.008
After treatment		1.004	0.15	1.039
S. aureus				
Before treatment	1.018	1.122	1.077	1.09
After treatment		1.056	1.0	1.025

* Optical Density

6.3.6 Qualitative estimation of protein concentration in supernatant after treatment of cells

The qualitative analysis of protein concentration of supernatants of treated *E. coli* E6, *S. aureus* and *B. thermosphacta* suspensions are shown in Table 6.2: In each case, the trypsin treatment resulted in a significant increase in the supernatant protein concentration. Since the cells were not lysed, this in turn suggests the removal of at least some of the surface proteins which produced purple colour.

Table 6.2. Determination of proteins estimated qualitatively in supernatants of treated and untreated cells

Organism	Protein removal	Qualitative analysis
***E. coli* E6**	Control	No colour
	0.1% Trypsin	Purple
B. thermosphacta	Control	No colour
	0.1% Trypsin	Purple
S. aureus	Control	No colour
	0.1% Trypsin	Purple
Reagent Control	Reagent + Trypsin (less than 2.5 nanaogram)	No colour

6.3.7 Qualitative estimation of polysaccharide concentration in supernatant after treatment of cells

The qualitative analysis of polysaccharide concentrations in supernatants of treated *E. coli* E6, *S. aureus* and *B. thermosphacta* suspension are shown in Table 6.3. With all the test organisms polysaccharide removal resulted in a significant release of polysaccharides and produced light brown colour reaction.

Table 6.3. Determination of polysaccharides estimated qualitatively in supernatants of treated and untreated cells

Organism	Treatment-Polysaccharide Removal	Qualitative analysis
***E. coli* E6**	Control	No colour
	0.1% Periodate	Light brown
	0.1% Lysozyme	Light brown
B. thermosphacta	Control	No colour
	0.1% Periodate	Light brown
	0.1% Lysozyme	Light brown
S. aureus	Control	No colour
	0.1% Periodate	Light brown
	0.1% Lysozyme	Light brown
Reagent Control	Reagent + Lysozyme	No colour

6.3.8 TEM studies of *E.coli* E6 after removal of cell surface proteins and polysaccharides and compared with untreated cell

Figure 6.7A-D, are electron micrographs of *E. coli* E6; Untreated control and trypsin, periodate and lysozyme treated cells, respectively. Cells remained intact except in the case of lysozyme, treatment where cell lysis occurred.

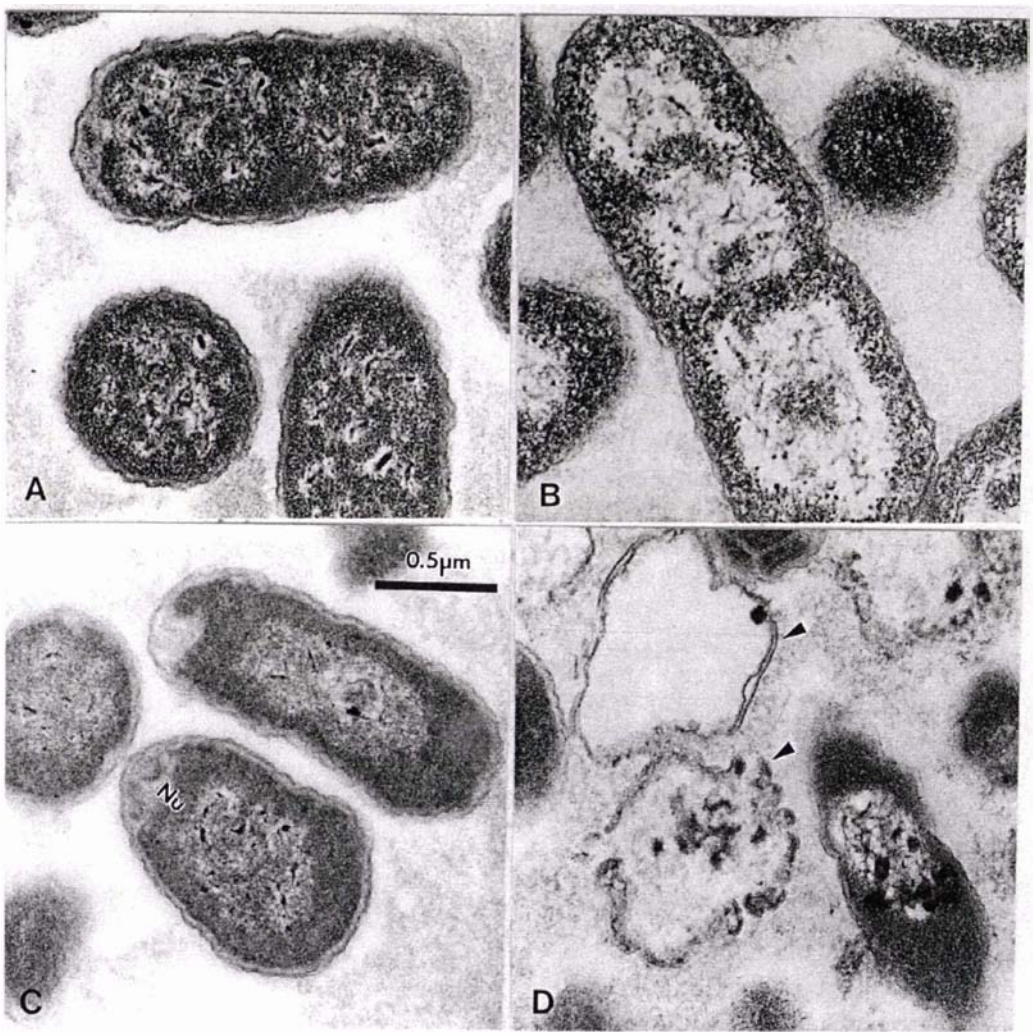


Figure 6.7A-D. Comparison of untreated and treated cells of ***E. coli* E6.** A. Control; B. Trypsin treated cell; C. Periodate treated cell; D. Lysozyme treated cell. Arrows indicate lysed cells.

6.3.9 TEM studies of *B. thermosphacta* after removal of cell surface proteins and polysaccharides and compared with untreated cells

Figure 6.8 A-D, are electron micrographs of *B. thermosphacta*; Untreated control and trypsin, periodate and lysozyme treated cells, respectively. Cells remained intact except in the case of lysozyme treatment, where cell lysis occurred.

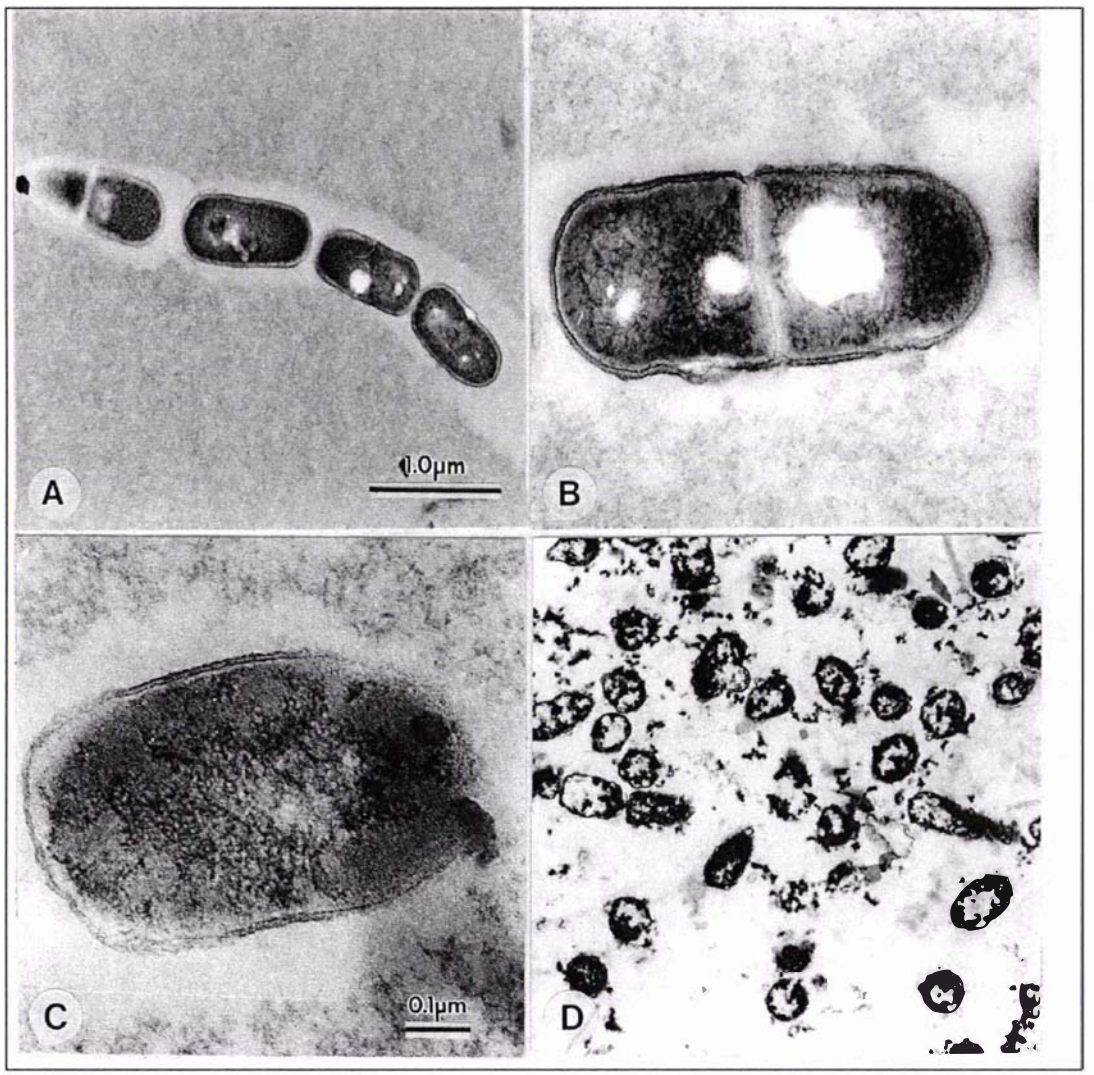


Figure 6.8 A-D. Comparison of untreated and treated cells of ***B. thermosphacta.*** A. Control; B. Trypsin treated cell; C. Periodate treated cell; D. Lysozyme treated cell.

6.3.10 TEM studies of *S. aureus* after removal of cell surface proteins and polysaccharides - untreated and treated cells

Figure 6.19 A-D, are electron micrographs of S. *aureus*; untreated control and trypsin, periodate and lysozyme treated cells, respectively. Cells remain intact following all three surface treatments.

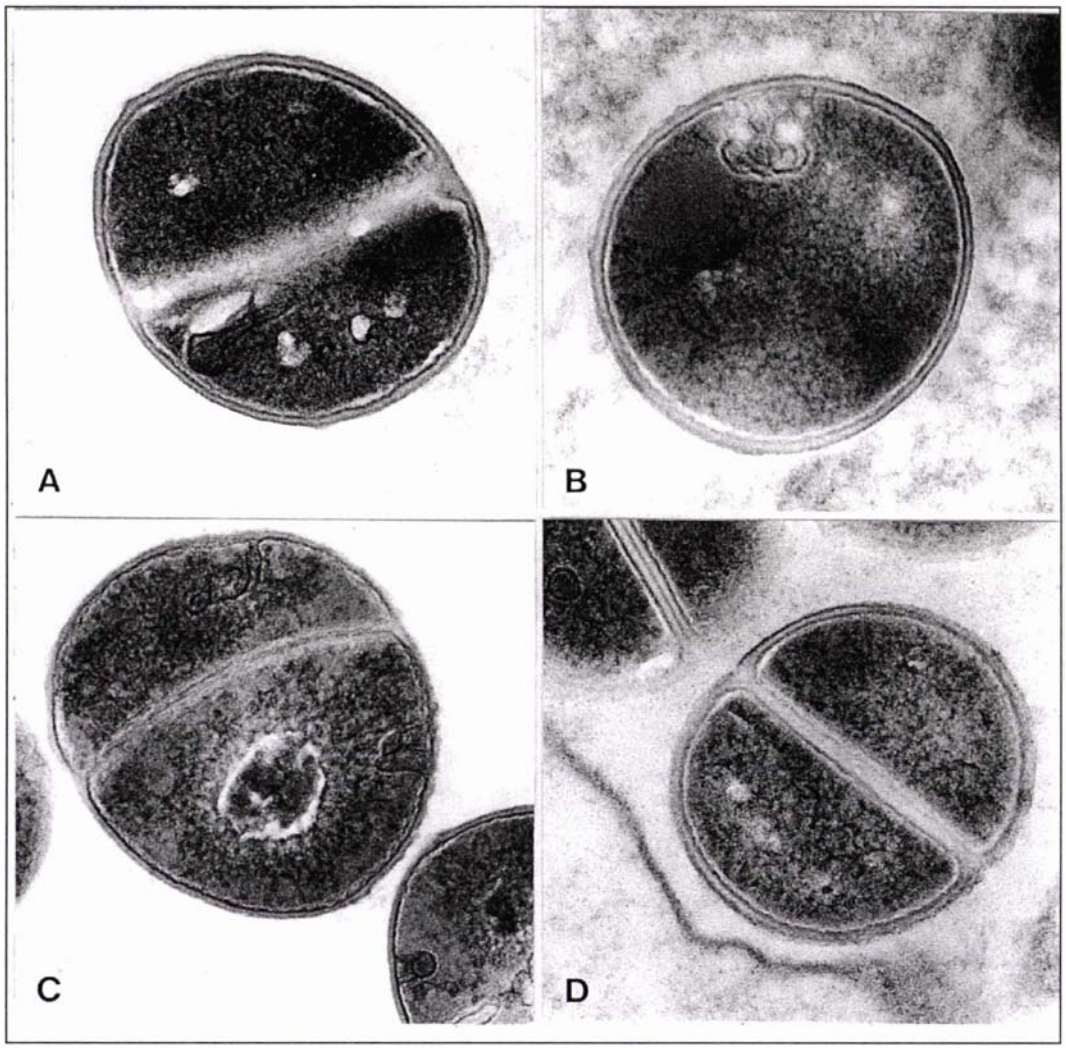


Figure 6.9 A-D. Comparison of untreated and treated cells of ***S.aureus.*** A. Control; B. Trypsin treated cell; C. Periodate treated cell; D. Lysozyme treated cell.

6.3.11 Effect of various eluents eluting bacteria from EIC and HIC after removal of cell surface proteins and polysaccharides

6.3.11.1 *E. coli* E6

The percentage retention following the removal of some surface proteins and polysaccharides of *E. coli* E6 slightly increased with the increased negative and positive charge and hydrophobicity of treated cells when compared with untreated cells (Fig 6.10). Salt as an eluent interferes with ionic attachment and hydrophilicity for both trypsin and periodate treated cells and untreated cells.

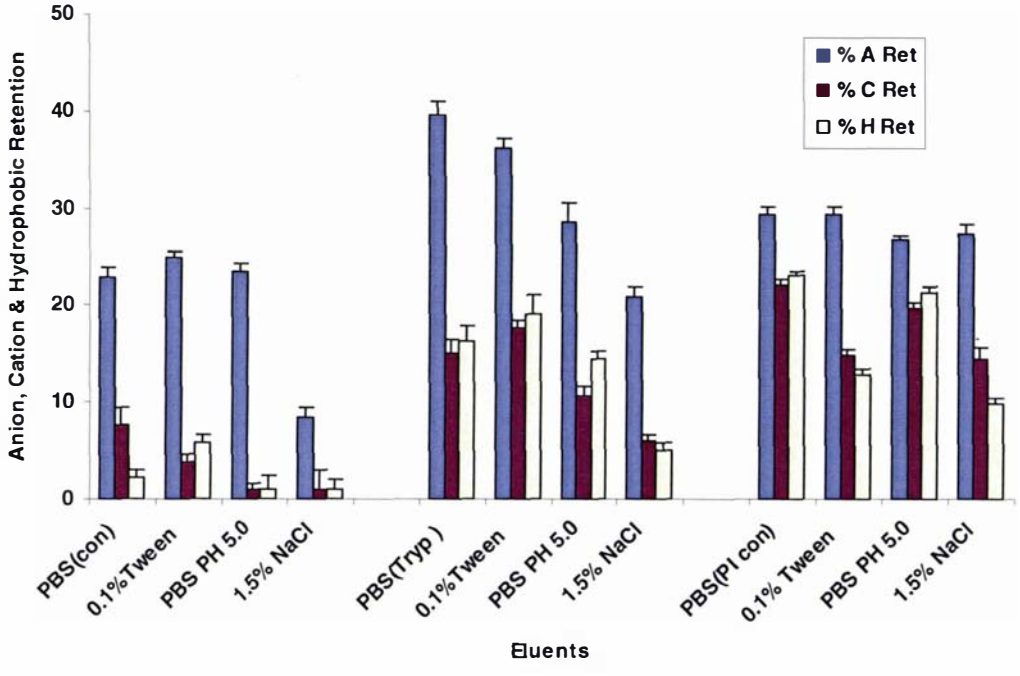


Figure 6.10. Effect of various eluents eluting ***E. coli*** **E6** from anionic, cationic and phenyl sepharose hydrophobic resin after Trypsin and Periodate treatments compared with untreated cells. *A- Anionic Retention; *C- Cationic Retention; *H – Hydrophobicity.

6.3.11.2. *B. thermosphacta*

The removal of surface proteins from the surface of *B. thermosphacta* increased the percentage retention with the distribution of negative charge and hydrophobicity whereas a slight decrease in the distribution of positive charges was observed when compared with untreated cells (Fig 6.11). The removal of cell surface polysaccharide molecules of *B. thermosphacta* slightly increased the percentage retention of negative charge. Neither an increase nor a decrease in positive charge was observed and a slight decrease in hydrophobicity was demonstrated when compared with untreated cells. The effect of eluents showed no statistically significant difference ($p>0.05$) with untreated cells. These results suggest that trypsin treated cells increased the charge and hydrophobicity when compared to those of periodate treated and untreated cells of Gram variable bacteria. Untreated *B. thermosphacta* eluted using eluents with 1.5% NaCl, surprisingly, produced no elution effect. However few independent replicates were examined; the results suggested that 1.5% NaCl interfered with anionic attachment and Tween and NaCl interfered with cationic and hydrophobic attachment. With trypsin treated cells Tween now interfered with anionic retention, pH 5.0 with cationic and 1.5 NaCl % with hydrophilicity by reducing percentage retention on EIC and HIC columns respectively. While the major effect of Tween relates to its surface active properties it also produces a minor ionic effect. With periodate treated cells, 1.5% NaCl and 0.1%Tween 80 decreased anionic retention and 0.1% Tween 80 decreased cationic and hydrophobic retention.

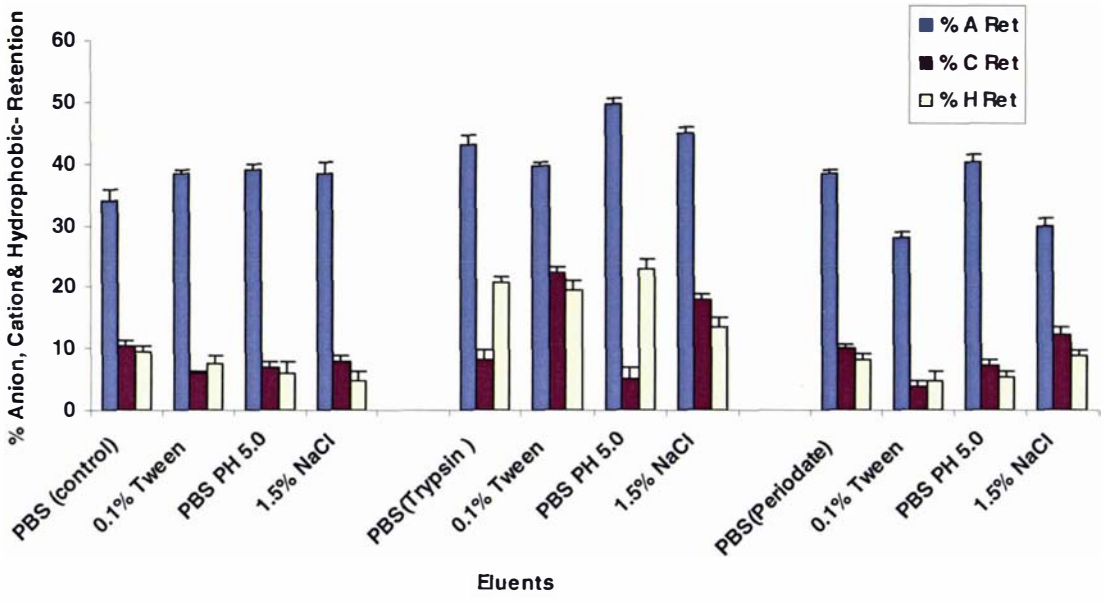


Figure 6.11. Effect of various eluents eluting ***B. thermosphacta*** from anionic, cationic and phenyl sepharose hydrophobic resin upon trypsin and periodate treatments compared with untreated cells. *A- Anionic Retention; *C- Cationic Retention; *H – Hydrophobicity.

6.3.11.3 S. *aureus*

The removal of surface proteins from the surface of *S. aureus* neither increased nor decreased anionic retention; however, the percentage retention decreased with positive charge and hydrophobicity (Fig 6.12). The removal of polysaccharides using periodate resulted in no reduction in anionic, cationic or hydrophobic retention. Removal of polysaccharide using lysozyme reduced the percentage anionic, cationic and hydrophobic retention. For control the eluents pH 5.0 and 1.5% NaCl interfered with anionic and cationic retention and Tween reduced the hydrophobic retention. Trypsin treated cells 1.5 % NaCl reduced anionic and Tween reduced hydrophobic retention. For periodate treated cells 1.5% NaCl interfered with anionic retention, pH 5.0 reduced cationic retention and Tween reduced hydrophobic retention. With lysozyme treated cells pH 5.0 reduced anionic, cationic retention and Tween reduced hydrophobic retention. Both

protein and polysaccharide removed cells showed either increase or decrease in percentage retention when compared with untreated cells.

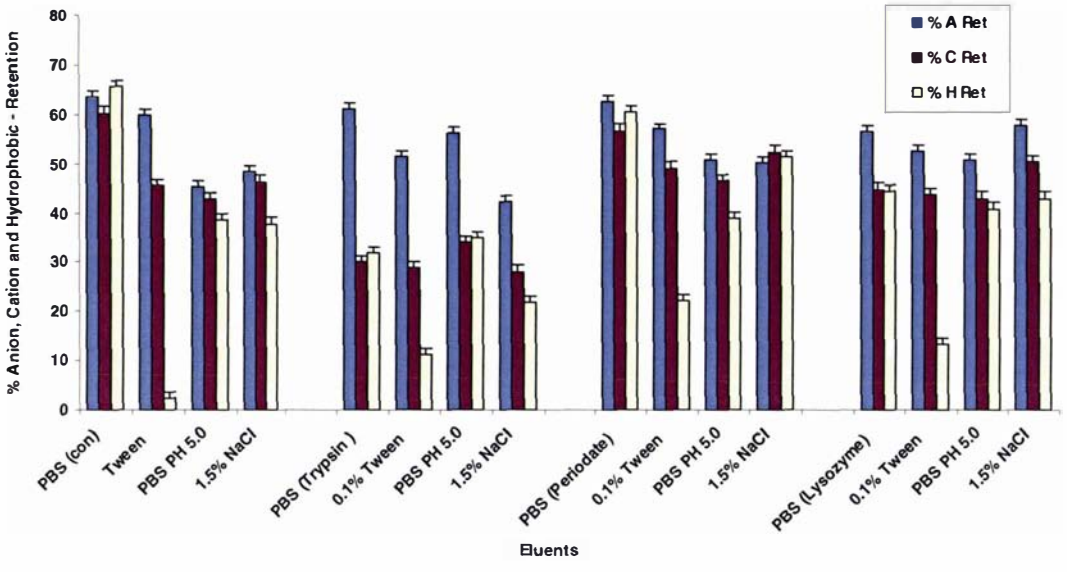


Figure 6.12. Comparison of effects of various eluents eluting *S. aureus* from anionic, cationic and phenyl sepharose hydrophobic resin after Trypsin and Periodate treatments compared with untreated cells. A- Anionic Retention; *C- Cationic Retention; *H – Hydrophobicity; *Con – Untreated cells.

6.3.12 Effect of various rinse treatments on attachment of *E. coli* E6 to the collagen film model system

Collagen films of pH 4.0 were placed into the attachment bath. The attachment, rinse and diluent media in the standard experiments all had a pH of 7.0. The pH of twelve different rinses (0.85% and 1.5 % NaCl; distilled water; 0.1 M and 0.01M PBS; 0.1 % peptone; 0.1% mannose; 0.1% Tween; diluent fluid rinse (0.85% NaCl, 0.1% peptone in distilled water); except for an additional 0.1% peptone rinse which was pH 5.3. Before immersion no natural contaminating microflora were detected on the collagen film. The influence of different rinse media and time on the attachment of *E. coli* E6 cells to collagen films in comparison with the standard diluent fluid is shown in Fig 6.13. An attachment of

approximately 5 log cfu/cm^2 occurred on immersion into a cell suspension containing approximately 10^7 cfu/ml in diluent fluid followed by a diluent fluid rinse i.e the standard procedure. The various rinse treatments, in particular NaCl and PB, produced a significant reduction in attachment numbers after 50 minutes immersion compared with the standard diluent fluid rinse. A log reduction occurred with distilled water; 0.1%mannose rinse and 0.1% peptone pH 7.0 rinses, 1.5 % NaCl and 0.1% Tween and two log reduction was obtained with 0.1% peptone (pH 5.0), 0.85 % NaCl, PBS of 0.1 M and 0. 01 M as rinse media.

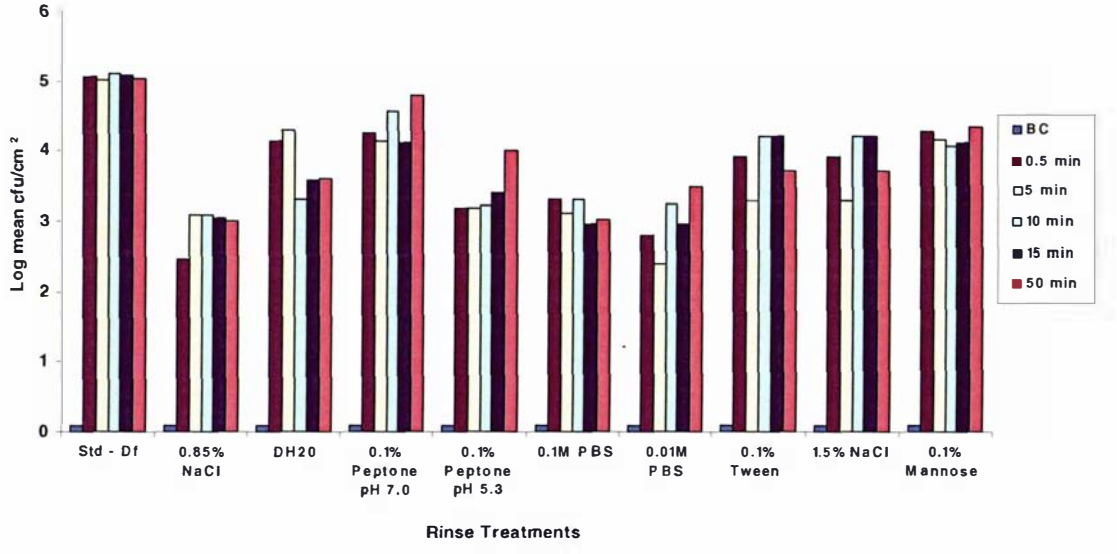


Figure 6.13. Influence of rinses on the residual attachment of ***E. coli*** **E6** to collagen films. Std-Df – standard diluent fluid system; BC- Background or initial contamination.

6.3.13 Effect of various prewetting treatments on attachment of *E. coli* E6 to collagen film model system

Collagen films of pH 3.88 were placed into the attachment bath. The attachment, rinse and diluent media of the standard experiment all had a pH of 7.0. The six prewetting

baths were: 1.5 % NaCl, 1.0% Tween 7.0, 0.1% mannose, 0.1 % peptone and diluent fluid and all were at pH 7.0. Collagen films were prewetted for 5 minutes. Before immersion no natural contaminating microflora was detected on the collagen film. The influence of different prewetting media and time on the extent of attachment of *E. coli* E6 cells to collagen films compared with the standard diluent fluid is shown in Fig 6.14. An attachment of approximately 5 log cfu/cm^2 occurred on immersion into a cell suspension containing approximately 10^7 cfu/ml in diluent fluid followed by a diluent fluid rinse (i.e., the standard procedure). All prewetting treatments produced a reduction in attachment of *E. coli* E6 to collagen, even after 20 minutes' immersion compared with the standard diluent fluid rinse. 1.0% Tween and 0.1% mannose demonstrated significant 2 log reduction, while 1.5 % NaCl, 0.1 % peptone and diluent fluid showed a log reduction.

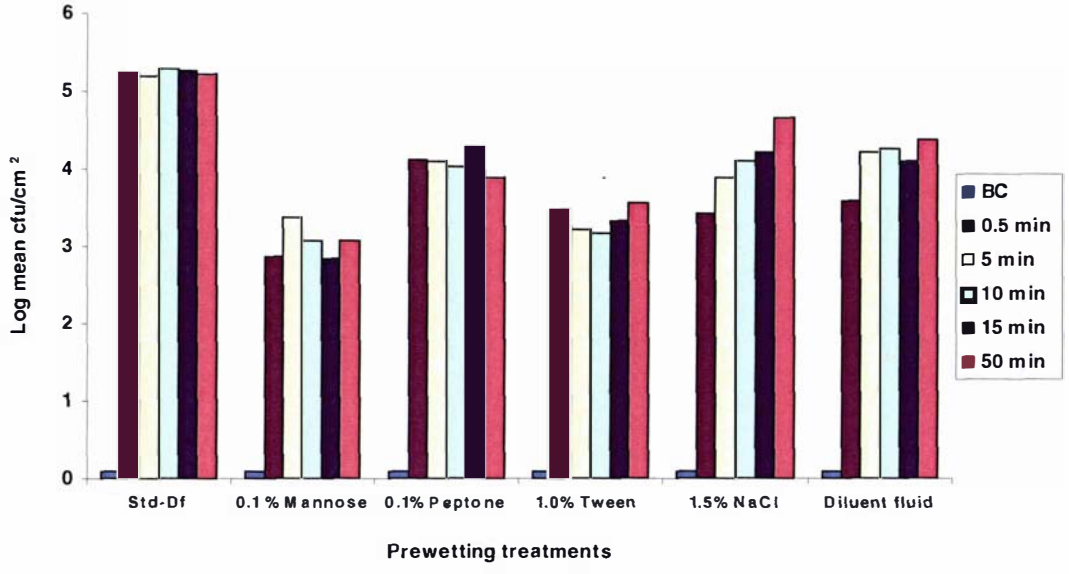


Figure 6.14. Influence of prewetting on the attachment of ***E. coli* E6** to collagen films immersed in a 10^7 cfu/ml cell suspension in dilution fluid. *Std -DF- Dilution Fluid; BC – Intial background contamination

6.4 DISCUSSION

In this chapter various adhesive interactions between the microbial cell surface and different model surfaces were investigated using various agents that were believed to interfere with the major attachment mechanisms.

EIC and HIC are useful microbial separation techniques. The column separates microbes on the basis of their relative hydrophobicity and charge distribution. Ionic and hydrophobic alkyl and aryl groups are covalently attached to the supports. Bacteria that have been bound to the resin through hydrophobic and ionic interactions are variously eluted according to the strength of this interaction. Elution is promoted by alterations in hydrophobicity, ionic strength, pH or temperature of the eluting buffer. The attachment of bacteria to anionic and cationic exchange resins has been demonstrated by several workers Hattorri & Furusaka, 1959; Zvanginstev, 1962). Kato et al. (2002) demonstrated the separation of proteins by hydrophobic columns at low salt concentration. Advantage may be taken of this behaviour to study the correlation between charge, hydrophobicity and attachment of bacteria to the collagen films and to meat surfaces. However, it is important to note that a correlation between these parameters does not necessarily indicate that this is the basis of an attachment mechanism.

The use of ion exchange and hydrophobic resins in the study of adhesion offers several practical advantages. The most important is the elimination of simultaneous multiple attachment or dispersion reactions (Daniels, 1972). By the use of these resin columns, it is possible to isolate the influence of net charge of the surface molecules and hydrophobicity on attachment of cells to surfaces. The assessment of attachment of bacteria to the resins was based on the use of PBS eluent. As with the use of rinses of the same pH as attachment bath liquids, elution removes bacteria associated with films around the resin beads and does not release those attached to those beads. The use of different eluents may give different results as attached cells under changed attachment conditions may be removed in addition to those in the liquid film associated with the bead surface.

Development of simple column models using EIC and HIC and manipulation of the parameters of their use permit separations of the microbial species by selective adsorption or selective elution of the cells. These studies show that ionic and hydrophobic binding between protein, cell and resin beads was weakened by changes in pH, ionic strength and lowering surface tension, thereby releasing the attached proteins and cells from the column. It is possible to take advantage of this behaviour in formulating protocols to remove bacteria from meat surfaces. The use of whole cell chromatography presents the problems of cells' aggregation, and blocking the voids would interfere with the final results. Vortexing the cells thoroughly would eliminate the problems of inadequate flow through. As an alternative method a batch approach with interaction chromatography can also be used in future studies. In future work, improvements need to be made by employing batch methods. While using buffers like PBS of high pH 9.0 and low pH 5.0 the range should be within the useful range of buffer pH (5.8 - 8.0). Exceeding the limits of useful range of buffers would further increase more HCl and NaOH concentrations in the eluents which might lead to erroneous results. These areas need considerable attention in future work.

EIC and HIC were used as models to elucidate the single attachment mechanisms. Bacterial interactions with surfaces are mediated by a complex of attachment mechanisms. These include surface charge, hydrophobicity and interactions involving surface proteins and polysaccharide molecules (Benito et al., 1995; Bouttier et al., 1994; Dickson & Koohmaraie, 1989; Li & McLandsborough, 1999). All these factors acting together contribute to the establishment of an inter-surface relationship commonly referred to as *attachment*. Experimentally, individual elements of bacterial initial reversible attachment to meat surfaces can be studied using interaction chromatography columns. Single mechanism attachment columns allow study of isolated attachment mechanisms such as the role of charge and hydrophobicity. EIC and HIC are two such techniques that can be used to model single bacterial attachment mechanisms. The strength of hydrophobic and ionic interactions can be examined by comparing the elution

of bacteria by different eluents. Different substances have different degrees of interaction with attachment (retention) of bacteria by the ion exchanger and hydrophobic resins due to differences in their charges, charge densities, distribution of charge on their surfaces and hydrophobicity. These interactions can be modulated by varying conditions such as surface tension, ionic strength and pH of the attachment environment. The differential attachment of bacteria to the resins can be compared, using PBS pH 7.0 as the standard eluent. By the use of other eluents, different elution rates result as attached cells, in addition to those in the bead surface associated film, are removed under different environmental conditions. Elution of attached bacteria would be promoted by alterations in the hydrophobicity, ionic strength, pH and by the presence of wetting agents in the eluent fluid if these factors weaken the hydrophobic and electrostatic interactions between the molecules bridging the bacterium and substratum.

Preliminary investigations were conducted to determine the optimal cell concentration for the experiments. A cell suspension with an optical density between 1.0 to 1.2 was chosen as providing the optimal number of bacteria. Whereas at lower optical densities not all adhesion sites would be occupied, at higher optical densities all adhesion sites would be saturated.

Similarly, concerns arose about possible changes in the optical density caused by the eluents. However, none of the eluents affected the optical density of Gram positive (*L. monocytogenes*), Gram negative (*E. coli* E6) or Gram variable bacteria (*B. thermosphacta*). However, the optical density of Gram negatives appears to be slightly higher when compared to Gram positives of the same cell concentration. It is believed that this may reflect the presence of extracellular polysaccharides (EPS), which are frequently associated with Gram negative bacteria.

A key finding using EIC and HIC columns was that *S. aureus* possessed both high positive and negative charge as well as high hydrophobicity. This bacterium is therefore very useful for investigating the processes of electrostatic and hydrophobic adhesion using the cell resin model system and so was used preferentially for these purposes.

Cell surface negative charge is an important physicochemical parameter acting between meat and bacterial surfaces (Dickson, 1991). As both bacterial and meat surfaces possess high net negative charge, repulsion occurs. The present studies showed that in addition to negative charge, microbial cell surfaces possess lower positive charge. Despite, or because of this, bacteria nevertheless become attracted, and later attached to meat surfaces (Notermans & Kampelmacher, 1975).

Daniels (1972), Hydon (1961) and Hogg (1976) reported on the effects on elution of different pH, NaCl and adhesive interactions between anionic exchange resins and microbial cells. For the present study in order to understand the electrostatic interactions, simple EIC resins were used to investigate anionic and cationic attachment of the microbial cells. Different ionic strengths and pH influenced anionic attachment. The use of various eluents suggested that ions like Na^+, Cl^-, PO_4^-, H^+, OH^-, protein fragments or amino acids, hydrogen , hydroxyl and alcohol groups of mannose interfered with anionic attachment of *S. aureus* cells in the resin adsorption system. The degree of retention of cells was reduced by changing the components of the eluting buffer, which weakened the electrostatic binding between the respective resin chemical group and the bacterial cell surface. It is presumed that the ionic component of the eluting buffer disrupts the bonding of the resin surface positive group that is bound to the negative charge of the bacterial cell. Bound groups displaced from the resin are more likely to be a negatively charged group, with a negatively charged group from the eluent mediating charge repulsion. The elution of attached bacteria from the resin system is promoted by alterations in ionic strength, pH, specific ions and by the presence of wetting agents in the eluent fluid changing the attachment environment, which in turn changes surface-related characters. The present studies showed that, of all the eluents used, NaCl removed more attached bacteria as the Na^+ Cl^- ions of salt effectively confound electrostatic attachment (Fig. 6.1a).

The bacterial cell surface also posseses positively charged areas. Both bacterial and meat surfaces possess high negative charge on the surface, when compared to positive charge,

and therefore have a net negative charge. However, the smaller positive charge also has a role in mediating adherence. Daniels (1972), Hydon (1961) and Hogg (1976) studied the role of eluents and interactions of positive charge on the cell surface with cationic exchange resins. The strength of the ionic adhesive interactions influencing cationic attachment was studied using various eluents. This suggested that various ions like Na^+, Cl^-, $PO4^-$, H^+, OH^-, hydrogen, hydroxyl and alcohol groups of mannose and digested protein fragments or amino acids interfered with the cationic attachment in the cell resin adsorption system. The percentage cationic retention was reduced by changing the components of the eluting buffer which weaken the electrostatic binding of negatively charged chemical groups of respective resins and bacterial cell surface positive charges. It is presumed that the ionic component of the eluting buffer disrupts the positive group on the bacterial cells that is bound to the negative group of the resin, replacing it with a positively charged ion. This then mediates charge-charge repulsion, thereby enhancing the removal of cells from the resin. The removal of bacterial cells was variable and was based on the strength of the eluent and its ability to displace and replace. Thus, elution of attached bacteria from the cationic resin system was promoted by alterations to the ionic strength, pH, ionic environment and by the presence of wetting agents in the eluent fluid that change the surface-related characters. Peptone plays a role in confounding electrostatic attachment (e.g., cationic attachment) and it is presumed that either partially digested peptide groups- or the positively charged or protonated amino group-displace the negatively charged group of the stationary phase making them more positive (Fig 6.1 b and 6.1.c). Based on their iso electric points, peptone eluents tend to carry a net negative charge which confounds cationic attachment. Sodium chloride solution has a net neutral charge, but nevertheless its ionic species interfere with cationic attachment and with anionic attachment (Fig. 6.1a). The above findings of this study clearly indicate that salt and pH interfere with the major electrostatic and hydrophobic attachment mechanisms.

These findings are in agreement with Daniels (1972), Hydon (1961) and Hogg (1976) who reported on the adhesive interactions between ion exchange resins and microbial

cells. On adsorption of *E. coli* and *S. aureus* to the resin system, NaCl interfered with ionic attachment and released more cells from the column. They also described differences in recovery rate of *E. coli* E6 and *S. aureus* upon elution. The present findings confirmed that differences in the release of different bacterial cells from the column upon elution are related to inherent cell surface properties of each bacterium. *E. coli* E6 is less charged and highly hydrophilic compared with *S. aureus,* which is highly charged and moderately hydrophobic. In addition, Jarvis et al. (1977) showed that NaCl dependent release of *S. aureus* from anion exchange varied with the pH. Ou & Marquis (1970) reported the adsorption of *S. aureus* to strongly acidic cation exchanger. They also reported that pH and the presence of NaCl in the suspension had a very marked effect upon cationic attachment.

Several workers have demonstrated the role of salt, pH and surfactants in hydrophobicity (Ahimou et al., 2000; Antipova et al., 2000; Cserhatti et al., 1999; Forgacs et al., 2001; Li & Mclandsborough, 1999; Otta, 1999; Yu et al., 2000). Cell surface hydrophobicity is an important physicochemical parameter acting between meat and bacterial surfaces. Both the bacterial and meat surfaces express both hydrophobic and hydrophilic properties (Benito et al., 1997; Bouttier et al., 1994; Li & Mclandsborough, 1999). The strength of hydrophobic interactions influencing hydrophobic attachment were studied using various eluents, suggesting that various ions like Na^+, Cl^-, $PO4^-$, H^+, OH^-, and CO=NH group, hydrogen, hydroxyl and alcohol groups of mannose and wetting agents (Tween) interfere with hydrophobic attachment in the cell resin adsorption system. Retention was reduced by changing the components of the eluting buffer which weakened the hydrophobic binding of the respective resin's chemical group and bacterial cell surface. It is presumed that the surface active components of the eluting buffer displace the hydrophobic groups that are bound to the resin and the bacteria. Therefore elution of attached bacteria from the resin system is promoted by alterations in the ionic strength, pH, ions and the presence of wetting agents in the eluent fluid by changing the surface related characters. Tween 80 interferes with hydrophobic retention, as wetting agents interfere with the binding of cells to hydrophobic resins, thereby promoting the elution of attached *S.*

aureus from the resin system. The results from the present study are in agreement with those of Ahimou et al. (1997) who showed that the bio-surfactants, Surfactin and Inturin A, reduced the hydrophobicity for hydrophobic strains.

Many researchers have reported the role of cell surface proteins and polysaccharides in adhesion (Allison & Sutherland, 1987; Flint et al., 1997; Fuller et al., 1974; Parkar et al., 2001; Sharon, 1993). It has been shown by Zvaginstev & Gusev, (1971) that cell surface charge may be due to the presence of surface molecules like teichoic acid, phosphate groups, carboxyl and other anionic groups of both Gram positive and Gram negative species of bacteria. A better understanding of the more specific surface role of proteins and polysaccharides in distribution and magnitude of charge and hydrophobicity and to identification of the various components interfering with the attachment mechanisms were sought. Therefore, adhesive interactions were investigated using various eluents, and chemically modified cells following removal of surface proteins and polysaccharides using trypsin, periodate and lysozyme. Qu & Marquis (1970) studied the effect of bacterial adsorption and recovery from columns following the removal of surface proteins using a Pronase a (nonspecific peptidase) enzyme preparation. The adsorption and elution of cells were affected by pH, and PB interfered with the charge binding. The use of various chemicals, temperatures and enzymatic treatments changed the cell surface charge and hydrophobicity (Castellanos et al., 1997). Therefore, these parameters were investigated using the Gram positive *S. aureus* (strongly charged and moderately hydrophobic); Gram negative *E. coli* E6 (slightly negatively charged and less positively charged and highly hydrophilic) and *B. thermosphacta* (moderately negatively charged and less positively charged and slightly hydrophilic).

Preliminary investigations demonstrated that modification of the cell surface by long, high temperature treatments with trypsin, periodate and lysozyme interfered with the optical density reading. The treatment times were consequently reduced to one hour and trials were again designed to establish the relationship between optical density and the modified chemical cell surface. The studies demonstrated that treatments did not interfere

with the optical density reading for any of the isolates used in the study, except for lysozyme, which altered the optical density reading of *E. coli* E6 and *B. thermosphacta* suspensions, but not that of *S. aureus*. These differences in O.D. suggest that the rupture of cells or leaking of intracellular constituents occurs upon lysozyme treatment.

The above studies were augmented by phase contrast microscopy and TEM investigation of the cell surface. Both techniques showed that treatments using lysozyme lysed the cells of *E. coli* E6 and *B. thermosphacta* because of the presence of unusually sensitive permeable outer membrane which is not usually a target site for the lysozyme. The variations in result might be due to the source of the lysozyme or possible contamination with other enzymes. The resistance of *S. aureus* to lysozyme may be due to the presence of a high level of teichoic acid on the cell wall surface. These results indicated that lysozyme treatment cannot be used for these studies except for *S. aureus*. Trypsin and periodate treatment did not alter the optical density and no rupture of cells was evident in electron micrographs for *E. coli* E6; *B. thermosphacta and S. aureus*. Based on this result, the organisms chosen for further investigations were *E. coli* E6, *S. aureus and B. thermosphacta*. The treatments selected were trypsin and periodate for all three isolates and lysozyme treatment was applied only to *S. aureus*. Chemically modified cells were also tested for the influence of various eluents. Optical density readings demonstrated that none of the eluents interfered with the optical density reading of treated and untreated cells. However, long exposure times caused the cells to rupture. Therefore shorter treatment times of 1 hour at 37°C were selected.

The qualitative analysis of supernatant showed that the presence of proteins and polysaccharides produced purple and light brown colour upon trypsin, periodate and lysozyme treatments. These tests confirmed that treatments are effective (refer to Tables 6.2 and 6.3). Alternative methods like sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS page) and western blot can also be used to detect the proteins released to determine the effectiveness of trypsin treatments.

Chemical modification of Gram negative bacteria *E. coli* E6 with trypsin and periodate treatments increased the magnitude of negative charge, positive charge and hydrophobicity, possibly because of the exposure of more ionogenic amino and sugar groups associated with deeper layers of the cell surface. NaCl as an eluent interferes with ionic and hydrophobic attachment as sodium and chloride ions weaken electrostatic interactions of both treated and untreated cells. Tween appears to play no role in reducing hydrophobic attachment of *E. coli* E6 probably because of the highly hydrophilic nature of this bacterium (Fig 6.10). However, the study result is in agreement with that of Ahimou et al. (1997) who showed that the biosurfactants: Surfactin and Inturin A increased the hydrophobicity of hydrophilic strains, and also with that of (Ou & Marquis, 1970) who demonstrated that charge binding was as a function of the pH and ionic strength of the buffer system used. In addition, protein removal increases the anionic retention more than polysaccharide removal, whereas polysaccharide removal increases cationic and hydrophobic retention more than does protein removal. Therefore, while both proteins and polysaccharides contribute to anionic, cationic and hydrophobic retention, polysaccharides are more important for negative charge and hydrophilicity and proteins are more important for positive charge and hydrophobicity.

Surface modification of Gram variable *B. thermosphacta* with trypsin and periodate treatments neither increased nor decreased the magnitude of negative, positive charges but increased hydrophobicity for trypsin treated cells (Fig 6.11). These results suggest that surface properties of this bacterium are slightly different from those of *E. coli*. NaCl, PB at pH 5.0 and Tween as eluents slightly interfere with ionic and hydrophobic attachment as sodium ions, chloride ions and hydrogen ions and the surface active nature of the Tween weaken the electrostatic and hydrophobic interaction of the cell resin system of both treated and untreated cells. Untreated and periodate treated cells removed fewer cells from the anionic column than did trypsin treated cells. Untreated cells removed fewer cells from cationic column than did periodate and trypsin treated cells. Untreated cells and periodate treated cells removed fewer cells from the hydrophobic column than did trypsin treated cells. The results indicate that the effect of eluents on

untreated cells, protein and polysaccharide removed cells were not statistically significant ($p>0.05$) in removing the attached cells effectively from the columns. The resistance to eluents exhibited by these bacteria may be due to difference in the surface chemistry of the cell wall molecules. However, protein removal increased the anionic retention more than did polysaccharide removal, whereas polysaccharide removal decreases the hydrophobic retention more than did the protein removal. Therefore it appears that proteins increase negative charge and hydrophobicity and polysaccharide removal reduce the negative charge and hydrophobicity. However, polysaccharides appeared to be more important for negative charge and hydrophilicity and proteins are important in positive charge and hydrophobicity. Beveridge & Graham (1991) and Ellwood et al. (1972), reported that cell wall polysaccharides are highly negatively charged and hydrophilic in nature. Schneewind et al. (1995) demonstrated that cell wall anchored proteins posses hydrophobic residues that span the cytoplasmic membrane and also several positive charge residues. The proteins have hydrophobic domain and provide anchorage to the outer face of cytoplasmic membrane (Mazmanian et al., 2000 and 1999; Ton –That et al., 1997; Ton-That & Schneewind, 1999).

Chemical modification of Gram positive *S. aureus* with trypsin and periodate treatments neither increased nor decreased the magnitude of surface negative charge, but positive charge and hydrophobicity were decreased for lysozyme treated cells and no effect was observed with periodate treatments. These results again suggest that the surface properties of this bacterium are very different from those of either *E. coli* or *B. thermosphacta.* Alternatively, the treatment may not have any effect on cell surface negative charge, and the decrease in positive charge and hydrophobicity may be due to ionogenic amino, sugar and hydrophobic binding groups in the freshly exposed layers of the cell surface. NaCl, PB at pH 5.0 and Tween as eluents interfere with ionic and hydrophobic binding of both treated and untreated cells. The removal of cell surface proteins appeared to have more effect on decreasing the charge and hydrophobicity compared with the cells treated to remove surface polysaccharides. These results suggest that for a Gram positive bacterium whose surface is highly charged and is moderately hydrophobic the magnitude of charge and hydrophobicity varies with both protein and

polysaccharide removal from the cell surface. Protein removal increased the anionic retention more than did polysaccharide removal; whereas lysozyme treated polysaccharide removal increased cationic and hydrophobic retention more than did protein removal (compared between protein removal and polysaccharide removal not with control). Therefore, while both proteins and polysaccharides contribute to anionic, cationic and hydrophobic retention, again polysaccharides are more important for negative charge and proteins are important in positive charge and hydrophobicity.

Generally, binding and elution from resin columns was affected for all the three isolates following the removal of surface protein and polysaccharides. The extent of these effects varied with the bacterium. This observation points to the diversity in the adhesion mechanisms of different groups of bacteria. It was apparent that the magnitude of net negative charge or the percentage of positive charges are strongly influenced by cell surface molecules. Retention on anionic exchange resin increases if negative charge increases or positive charge decreases. Similarly retention on HIC increases with an increase in hydrophobicity or a decrease in hydrophilicity. Both proteins and polysaccharides contribute to the charge and hydrophobic characteristics of the cell and affect attachment to a surface.

Protein and polysaccharide removal treatments tended to increase the charge and hydrophobicity of cells. However, cell surface proteins are more important in determining the magnitude of surface charge than the cell surface polysaccharides. With Gram positives, surface protein removal treatments decreased the positive charge and hydrophobicity, indicating the importance of proteins in determining these surface properties. With Gram negatives the extracellular polysaccharides are more important than protein as periodate treatments reduce the charge and hydrophobic properties of the cells. Gram negatives, on the other hand, possess highly hydrophilic polysaccharide outer membranes therefore it is these molecules which contribute most to observed cell surface properties.

The present study clearly demonstrates that the surface protein and polysaccharide molecules contribute to charge and hydrophobic attachment. In addition, the use of eluents with different pH, NaCl concentration or surface activity had a role in the release of attached cells due to their interference with electrostatic binding and hydrophobicity. However, removal of the surface proteins or polysaccharides produced no dramatic change in recovery of cells, as elution from the columns was the same as that of untreated cells. It is believed that directly targeting the major electrostatic and hydrophobic interactions by introducing various interventions between these two mechanisms would be a wise choice to promote detachment.

However, the present study is in agreement with that of Ou & Marquis (1970) and indicates that cell surface proteins are involved in the adsorption of *S. aureus* to resins. They showed that treatment with pronase reduced the percentage retention on anionic exchange resin. Pronase treatments of the adsorbed organisms partly released cells as a function of pH and buffer which interfered with mechanisms of adsorption. A similar effect was found with weak cation exchange resin. At pH 5.5 in 0.05 M phosphate citrate buffer the adsorption of pronase treated cells of *S. aureus* to the columns indicates that at pH 5.5 the surface proteins tend to possess more positive charge and $PO4^{2-}$ mediating charge repulsion. This study is in agreement with the present findings and indicates that removal of cell surface protein influences charge and also eluent pH and PB interfered with electrostatic attachment. Many studies have been conducted in order to determine the role of carbohydrates in adhesion. Numerous surface carbohydrates on various forms of animal cells provide attachment points for bacterial lectins. Fuller et al. (1975) observed pronounced inhibition of adhesion by periodate treatments, which suggested the involvement of carbohydrate. The present is the first study in which EIC and HIC columns have been used, following the removal of cell surface carbohydrates, to identify the components interfering with attachment mechanisms. There is little information in the literature concerning the removal of cell surface polysaccharides and subsequent attachment to resins. However, the present study confirms the findings by Castellanos et al. (1997) that enzymatic treatment affects the distribution of surface charge.

Similarly, this is also the first study conducted to provide an understanding the role of hydrophobicity following the removal of cell surface protein and polysaccharides using the HIC column model. Some of the findings of the previous research coincide with those of the current work. These include the research of Smyth et al. (1978) who separated cells based on hydrophobicity and used the technique to detect surface proteins of enterotoxigenic *E. coli* (ETEC) strains using HIC. They also showed that the lipopolysaccharide on the surface of ETEC strains and other Gram negative organisms provide the organisms a hydrophilic character and contribute to the net surface charge. Surface capsular material is usually found to be of a carbohydrate nature and will probably enhance the hydrophilic character of the cell. High NaCl concentration causes an increase in hydrophobic interaction which promotes bacterial aggregation. The bacterial cell surface contains hydrophilic as well as hydrophobic sites and the relative importance of each is partly dependent on the surrounding medium and the attachment substrate. The former consist mainly of charged groups such as carboxyl, phosphate, amino or guanidyl groups and the noncharged hydroxyl groups. The hydrophobic sites, on the other hand, consist of lipids and lipopolysaccharides. Cunlifee (1999) demonstrated that hydrophilic uncharged surfaces showed a greater resistance to cell attachment than charged hydrophobic surfaces. The present study agreed with the findings of Castellanos et al. (1997) that the use of various chemicals, temperatures and enzymatic treatments changed the cell surface hydrophobicity.

The column studies described above clearly indicated the role of major attachment mechanisms and the agents that may interfere with these mechanisms. However, these interfering components were also investigated using the simple collagen film model system with the intention of discovering rinse components that might aid in the removal of cells from the collagen film surface.

Attachment in the collagen film model system appears to occur very quickly, generally with little, if any, additional attachment occurring after five minutes' immersions. These results are in agreement with those of many other attachment studies in other systems

(e.g., *Streptococcus thermophilus* to stainless steel [Flint et al., 1997]). Shear force effect rinsing was done in such a manner to minimise shear force removal of attached cells. Therefore changes in cell removal can be attributed to differing conditions experienced at the contact surface. In the present work, an attachment of 3 to 4 log cfu/cm^2 occurred on immersion in a cell suspension containing 10^7 cfu/ml followed by rinsing with the trial media (Tween, salt, water, PB of different strengths, peptone and mannose). Cells treated to remove protein and polysaccharide and rinsed with diluent fluid showed a 0.5 log to 1 log reduction in attachment compared to untreated cells rinsed with the diluent. These reductions suggest the possible involvement of interference of the components of various rinses (e.g., proteins and polysaccharides in attachment mechanisms to a collagen film model system when compared to a standard diluent fluid system). The maximal attachment 5 log cfu/cm^2 of collagen film was obtained using standard diluent fluid system. The rinse process using the same medium as that used in the attachment bath removes unattached cells present in the residual surface liquid film from the attachment suspension. The use of rinse media that differ from that used in the attachment bath may result in the release of some of the attached cells, in addition to removing unattached cells present in the residual surface film. Release is likely to occur as a result of changes in the physicochemical bonds between the cell surface and the model surface produced by the different rinse media. When used separately, the components of the diluent fluid distilled water, 0.85% saline and 0.1% peptone were more effective in releasing attached cells than when combined as diluent fluid. Note that the 0.85% NaCl and the distilled water were adjusted to pH 7.0 (i.e., the same as complete diluent fluid). The pH of 0.1% peptone was 5.33. At a pH 5.3 rather than 7.0 the proteins in 0.1% peptone would be less negatively charged as they approach iso-electric point (Fig 6.1b and 6.1c). Therefore it would appear that at neutral pH the more negatively charged protein components of complete dilution fluid interfere with the ability of the 0.85% NaCl to release attached cells. It appears that pH dependent protein mediated attachment at pH 7.0 greatly exceeds the loss of ionic attachment caused by the presence of charged ions (Figs. 6.1a and 6.1d). The Tween rinse provides an indication of hydrophobic attachment by decreasing interfacial surface tension properties. The effectiveness of the mannose rinse suggests a

potential role in a wash process for blocking attachment sites of *E. coli* E6. Ionic rinse solutions such as 0.85% and 1.5% NaCl and changes in PB strengths are likely to confound electrostatic attachment. The observed reduction was a by a factor of approximately 100. The effect of various rinse treatments suggests that all the rinse treatments reduced the attachment and also indicated the involvement of ionic and hydrophobic attachment mechanisms and also cell surface protein molecules upon interaction with a single meat protein model system. These results with a single meat protein surface point to treatments for enhanced removal of cells from meat surfaces.

The practical feasibility of using the different treatments was considered in all these studies on the removal of bacteria from the collagen model system. The application of enzymes to remove the attached cells from the carcass surface appears to be impractical in the meat industry as carcasses cannot be treated for a prolonged time with enzymes. Feasible options to treat the meat surfaces include rinses, preferably at neutral pH (as above), and may include blocking agents such as mannose. For a meat model system, a neutral attachment pH appears appropriate because the pH of freshly slaughtered meat is close to neutral but gradually decreases with the onset of rigor mortis. Changing rinse media would be comparable to changing wash treatments during carcass dressing. The extent of attachment to the collagen model system may be validated by comparing the effect of the same rinse treatments on bacterial attachment to cut beef muscle.

The results from the present studies are in agreement with the findings of Castro-Rosas & Escartin, (2002) who reported that attachment of *Vibrio cholerae* O1 to a chitinous surface was affected by NaCl concentrations of 1.00 to 1.5% NaCl. Hood & Winter (1996) and Huq et al. (1984) also reported that NaCl plays a role in detachment of bacteria from surfaces. Various strengths of the buffer interfered with ionic attachment (Bellon Fontaine et al., 1996); mannose as a rinse agent affects the attachment of *Salmonella* to meat surfaces (Benedict et al., 1991; Grants, 2002); removal of cell surface proteins affects attachment (Flint et al., 1997; Parkar et al., 2001). However, when cell surface polysaccharides are considered in relation to adhesion, Allison & Sutherland

(1987) and Fuller et al. (1975) observed pronounced inhibition of adhesion by periodate treatments, which suggested the involvement of carbohydrate; but in contrast, our studies showed that enzymes known to attack carbohydrate substrates were inactive in reducing adhesion. The reason for the lack of effect on adhesion in the present study may have been the use of mild conditions or low concentration of enzyme.

Surfactants play a role in reducing surface tension and therefore alter the inherent hydrophobic nature of the cell surface. Polysorbates (e.g., Tween) are hydrophilic non ionic surfactants. They are molecules that do not dissociate in solution and are therefore compatible with both anionic and cationic compounds. The addition of surface active agents reduces the surface tension and improves the ability of the water to wet a surface (Edelmeyer, 1982). Surfaces that appear smooth to the naked eye may, in fact, be very rough when viewed under a microscope. Without the use of surface active agents to reduce surface tension, water will tend to settle as a drop over the cavities and will not penetrate into them. The water can penetrate only if the surface tension is reduced. Noda (1986) determined hydrophobicity on bacterial surfaces by using nonionic surfactants. Farghaly et al. (2001) used Spraycult^{TM}, a surface active compound, to disintegrate biofilms from the surface of meat processing equipment. The present study is in broad agreement with these findings (i.e., Tween 80 removed attached cells by interfering with hydrophobic attachment by lowering the surface tension).

Prewetting studies with various agents using the collagen film model system identified mannose as a principal component interfering with attachment, either blocking attachment sites or reducing the total surface free energy in the interfacial area of the bacteria-surface environment. Mannose binds competitively to the bacteria lectins, occupying sites that would normally bind host cell mannose receptors, which prevents attachment; without bacterial attachment, infection is prevented (Beuth et al., 1994). Most tissue culture studies have used mannose as a blocking agent to enhance the removal of bacterial cells from the intestinal cell wall (Cox et al., 1990; Michaela et al., 1980; Nishikawa et al., 1991; Nequin et al., 2002; Oyofo et al., 1989; Ofek et al., 1977).

Blocking of surface receptors was discussed by Fujiwara et al., (2001). Mannose – adherence of certain *E. coli* strains to various epithelial cell types has been shown to be mediated by common type 1 pili or fimbriae and can be inhibited by D-mannose (Dugid & Gillies, 1957; Ofek et al., 1977; Salit & Gotschich, 1977). Benedict et al. (1993), deGraft-Hanson & Heath (1990) and Walls *et al.* (1991) demonstrated inhibition of adhesion with mannose with meat and poultry surfaces. Mannose is used as anti-adhesive sugar for fighting infectious diseases (Sharon & Ofek, 2002; Zafriri *et al.*, 1984). In the present study, the interaction was specific between collagen and mannose. Therefore, it appears that mannose blocks the specific bacterial binding sites on the collagen which could be due to possible mechanisms whereby mannose may block collagen or other mannose receptors on the collagen film material which may contain other residual beef hide components like collagen and humectant. Bacterial binding to collagen type I molecules was inhibited by a composition of D-galactose/D-Mannose/N-acetyl muraminic acid (Stepinska & Trafny 1995). In experiments where mannose was used to prewash the collagen, any *E. coli* E6 that attached were likely to be bound to the mannose blocked components of the model surface. This was believed to be the explanation for the ease with which the diluent rinses removed bacteria from the mannose prewetted surfaces compared with the control, non-treated surfaces.

Several workers have examined the role of mannose in bacterial attachment. Dickson (2000) attempted unsuccessfully to block the mannose receptors on *E. coli* pili with mannose containing solutions in the expectation that this would interfere with the initial stages of bacterial attachment to animal tissue surfaces and facilitate the removal and destruction of contaminating bacteria on meat surfaces. Many of the genera of the Enterobacteriaceae *Escherichia, Shigella, Salmonella* and *Vibrio* possess external cell structures termed *common pili* or *type I fimbriae* (Eisenstein, 1988). These are composed of 127 to 21 KDa repeating units with minor proteins inserted periodically near the tip. These proteins are termed *adhesins* and these adhesins function to bind D mannose residues on eukaryotic cells. Preliminary experiments (Grant & Dickson, 2000) indicated that mannose-treated meat supported 2.5 to 3 times fewer bacteria when compared with

meat that was not treated with mannose, and confirmed that mannose treatments were of little use in reducing numbers of bacteria attaching to pork tissue. However, Benedict *et al.* (1991) demonstrated that a mannose rinse reduced the attachment of *Salmonella* to beef tissues. Similarly a reduction of high numbers of *E. coli* E6 occurred when prewetting the surfaces with Tween. Tween interferes with the interfacial surface tension properties of the liquid, thereby influencing the hydrophobicity of the cell and the attachment surfaces resulting in the removal of bacteria from the single meat protein collagen system. Calicioglu et al. (2000) demonstrated that pre-spraying of carcasses with Tween 20 (5%) prior to treatment with lactic acid and sodium benzoate resulted in significantly larger reductions of *E. coli* O157:H7 compared with control carcasses. In addition, the present prewetting studies with peptone and diluent fluid show a slight reduction, demonstrating the potential role of proteins as blocking agents or in reducing the surface free energy. Therefore this study suggests that the goal of removal of attached cells may be attained after further examination of the interactions between proteins and carbohydrates or proteins with other proteins, interference with surface free energy and surface tension and blocking of attachment sites.

The present studies using mannose prewetting were in agreement with Fujiwara et al. (2001) who discussed blocking of surface receptors. Both carbohydrates and proteins are involved in bacterial adhesion. These include numerous surface carbohydrates on various forms of animal cells which provide attachment points for bacterial lectins. Streptococci possess multiple adhesins mediating attachment (Hasty et al., 1992). The present studies on prewetting collagen with mannose and Tween confirm that mannose has a role in blocking attachment sites to produce pronounced reduction in adhesion.

Similarly, cell surface protein involvement in adhesion was demonstrated by Caccava et al. (1999); Cowart et al. (1990); Pandiripally et al. (1999); Westerlund et al. (1993). Cell surface-bound carbohydrate binding proteins (animal lectins) also act as attachment points for microorganisms carrying the appropriate sugars at their surfaces (Sheriff et al., 1994). Dominguez (1991) demonstrated adhesion between Listerial surface protein A and

Host cell heparan sulphate proteoglycans. Cell surface lectins (Sharon & Lis, 1989) are also involved in cell-to-cell attachment and/or signalling. Lammler et al. (1988) demonstrated that serum albumin protein partially blocks the adherence of other bacteria. Piette & Idziak (1993) showed proteins to be blockers reducing the adhesion of lactobacilli to collagen casings. In the present protein prewetting study results, also coincided with these findings that peptone blocked attachment of *E. coli* E6 to the collagen film model system.

Microbial blocking agents (MBAs) provide a unique microbial hurdle in a food processing cascade. They inhibit the action of specific colonisation factors on the microbial surface that promote tissue attachment and compete for tissue binding sites on the epithelial surface to block or detach microorganisms. Because of this two-fold effect the microorganism is repelled from the surface. Since the blocking activity is a molecular phenomenon related to structure and function, the activation of functional sites and a specific target delivery mechanism are critical for the efficacy of an MBA system (Naidu, 2000).

The studies on bacterial interactions using EIC, HIC and collagen film model systems emphasised that electrostatic repulsion is dependent on both pH and ionic strength; hydrophobicity is related to surface tension and wettabilty; bio molecules - carbohydrates and proteins - attached to the substratum block attachment sites or reduce the surface free energy; the unsatisfied bonding capacity of the surface thereby reduces attachment. Cell surface molecules are accessory proteins associated with pili, cell wall, outer membrane and lectin polysaccharides (protein polysaccharide or polysaccharide protein partners). These molecules ultimately interfere with the distribution of charge and hydrophobicity, which appear to be primary and important attachment mechanisms. Perturbing these diverse adhesive interactions helped to identify three principal components interfering with single attachment mechanisms. They were salt interfering with ionic attachment, Tween interfering with hydrophobic attachment, and mannose playing a role in blocking the attachment sites on the collagen surface or reducing the surface free energy.

6.5 CONCLUSION

The study of interactions of microbial cells with ion exchange resins and hydrophobic resins (simple practical systems) demonstrated numerous adhesive interactions and identified the principal components interfering with those attachment mechanisms. In addition the collagen film model system subjected to various rinse and prewetting environments on attachment of *E. coli* E6 highlighted the fact that a single attachment mechanism could be blocked by mannose.

The collagen surface proved to be a good model, allowing examination of interaction with a single major meat protein. The eluent, rinse and prewetting studies showed that attached cells can be released from the surface if surface-related characters are changed by modifying the eluting, rinse and prewetting parameters.

The three most effective agents were: salt interfering with ionic attachment; Tween interfering with hydrophobic attachment and mannose blocking the attachment sites or reducing the surface free energy. In addition, this study has shown that removal of cell surface protein molecules alters the charge and hydrophobic characteristics of cell surfaces more than does removal of cell surface polysaccharides. Thus proteins are probably more important in determining surface characteristics associated with attachment.

The selected agents, Tween, salt and mannose, might be applied to meat surfaces as an enhanced wash process either singly, in combination, or as sequential treatments in order to remove attached bacteria from the meat surface.

Chapter 7
APPLICATION OF DECONTAMINATION TECHNOLOGY TO MEAT SURFACES

7.1 INTRODUCTION

The New Zealand slaughter industry uses good manufacturing practices (GMPs) when processing beef carcasses and as a result the major portion of the carcass surface is relatively clean during this process (Fig 7.1). However, hygienic interventions in the process alone do not necessarily lead to safe products. Owing to the constant flow of bacteria into the processing plant and the unavoidable occurrence of contamination, all carcasses have microbial flora associated with them (Dickson & Anderson, 1992).

This unavoidable contamination of carcasses occurs despite GMP, and thus necessitates the use of effective antimicrobial intervention strategies to improve the microbiological status of carcasses. This is one of the greatest challenges in meat hygiene practice (Anon, 1993). In addition, the control of contamination and product hygiene are of the utmost concern from both a public health and a commercial point of view. Currently, a substantial reduction in microbial contamination of red meat is achieved by employing various decontamination procedures. These methods, which include physical, chemical and biological techniques, are practised widely to remove the surface-associated bacteria from meat. These processes are discussed in Chapter 3 and have recently been reviewed by (James & James, 1997).

However, the mechanisms of attachment of microorganisms to the meat surface remain poorly understood. Previous decontamination research was hampered by a gap in our understanding of the underlying attachment mechanisms, which may identify alternative decontamination strategies or aid the optimum application of existing methods (James & James, 1991). Pordesimo *et al.* (2002) explained that

understanding the fundamental mechanisms will be the theoretical basis for the development of procedures for the removal of attached cells from the surface.

Meat research has failed to consider the attachment mechanisms in the development of procedures for removal of bacteria from the surfaces (James, 1997 and Pordesimo et al., 2001). The current research focused on understanding the fundamental attachment mechanisms between meat and bacterial surfaces, which has been considered as a theoretical basis for the development of procedures favouring the removal of bacteria from the surfaces. However, an understanding of the interaction of bacteria with the surface water layer of carcasses and the mechanisms of bacterial attachment will be a pivotal step if carcass washing treatments are to be made more effective in bacterial removal or inactivation (Dickson & Siragusa, 1994).

In terms of cost, corrosive impact on plants and environmental friendliness, potable water sprays remain the preferred option in New Zealand where treatments must be acceptable to all markets serviced by an export- oriented industry (Bell, 1997).

Following the elucidation of attachment mechanisms using the collagen and column models, described in the previous chapter, the next step was to apply wash solutions to remove bacteria attached to meat surfaces. In chapter 6, Tween, NaCl and mannose were selected as the agents most effectively interfering with the single attachment mechanisms by modifying hydrophobicity, charge and blocking attachment sites respectively. Delmore et al. (2002) tried various methods of application: single, combinations, sequential and prewetting with these chemicals to enhance the removal of bacteria from the meat surfaces. The objective of the final phase of the present work was to improve the existing decontamination technology for removing, reducing or killing bacteria attached to meat surfaces.

Figure 7.1. Beef carcass in meat plants

7.2 PROCEDURES

- To remove attached *E. coli* E6 cells from meat surfaces by application of selected components as single rinse treatments (refer section 3.15.1).
- To remove attached *E. coli* E6 cells from meat surfaces by application of selected components as combination rinse treatments (refer section 3.15.2)
- To remove attached *E. coli* E6 cells from meat surfaces by application of selected components as sequential rinse treatments using factorial design experiments. (refer section 3.15.3)
- To remove attached *E. coli* E6 cells by application of selected components at high concentrations and high vigorous rinsing as sequential rinse treatments from meat surfaces (refer section 3.15.4)
- To evaluate the application of selected components as single prewetting treatments to prevent *E. coli* E6 attaching to meat surfaces (refer section 3.15.5).
- To evaluate the effect of 10% TSP pH 12.0 as a single rinse treatment on the attachment of *E. coli* E6 cells attaching meat surfaces (refer section 3.15.6).

7.3 RESULTS

7.3.1 Effect of single rinse treatments on attachment of *E. coli* E6 to cut beef muscle

The mean age postmortem of cut beef muscles was approximately 36 hours when they were placed in the attachment bath. No fascia was evident on any of the meat samples, which all appeared to be 95% visual lean (i.e., 5.0% visual intramuscular fat (marbled meat) and had a pH of 5.4. The attachment and recovery media had a pH of 7.0 as did all the 8 rinse baths. The seven rinses were 1.5%; 2.0% and 3.0% NaCl; 0.1% Tween; 0.1% mannose; diluent fluid rinse for standard diluent fluid. Before immersion the natural contamination on cut beef surfaces had a mean value of 2 log cfu/cm^2. An attachment of approximately 5 log cfu/cm^2 occurred on immersion in the

cell suspension containing approximately 10^7 cfu/ml when the standard diluent fluid rinse was used. The influence of different rinse suspensions with time on the extent of attachment of *E. coli* E6 cells to cut beef muscle tissue compared to those with the standard diluent fluid as rinse medium is shown in Figure 7.2. No significant difference in reducing attachment of *E. coli* E6 occurred upon using single rinse treatments with NaCl, Tween and mannose as rinse components when compared to the standard diluent fluid system.

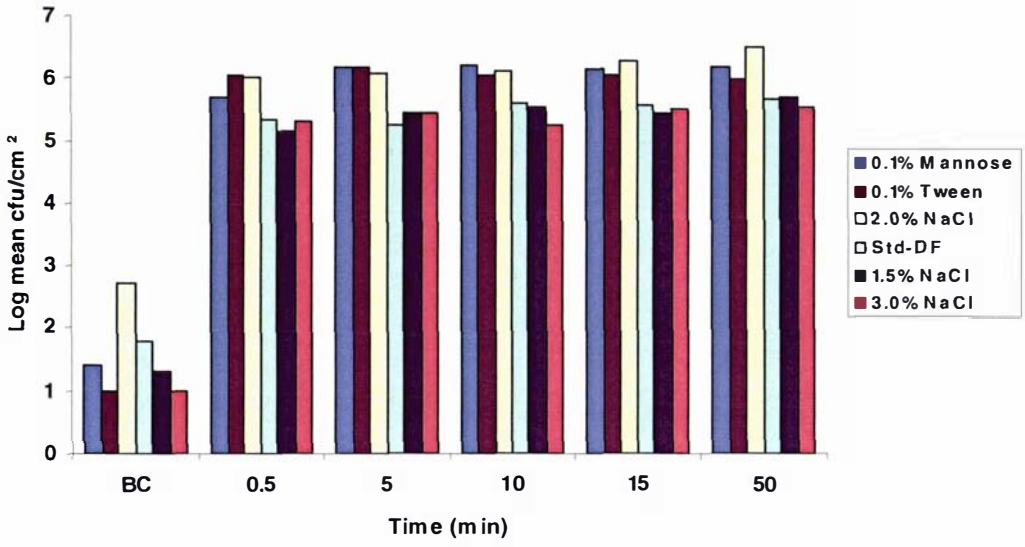


Figure 7.2. Effect of single rinse treatments using 0.1%Tween, NaCl (1.5%; 2.0% and 3.0%) and 0.1%mannose on attachment of ***E. coli*** **E6** to cut beef muscle and compared to standard diluent fluid system. BC- Background contamination.

7.3.2 Effect of combination rinse treatments on attachment of *E. coli* E6 to cut beef muscle

The influence of the three component rinse suspensions with time on the attachment of *E. coli* E6 cells to cut beef muscle tissue compared to those with the standard diluent fluid as rinse medium is shown in Figure 7.3. The numbers attached after 20 minutes' immersions were: 5 log cfu/cm^2 for the combined rinse treatment. No significant reduction in attachment of *E. coli* E6 occurred upon using combined rinse

treatments with NaCl, Tween and mannose as rinse components when compared to standard diluent fluid system.

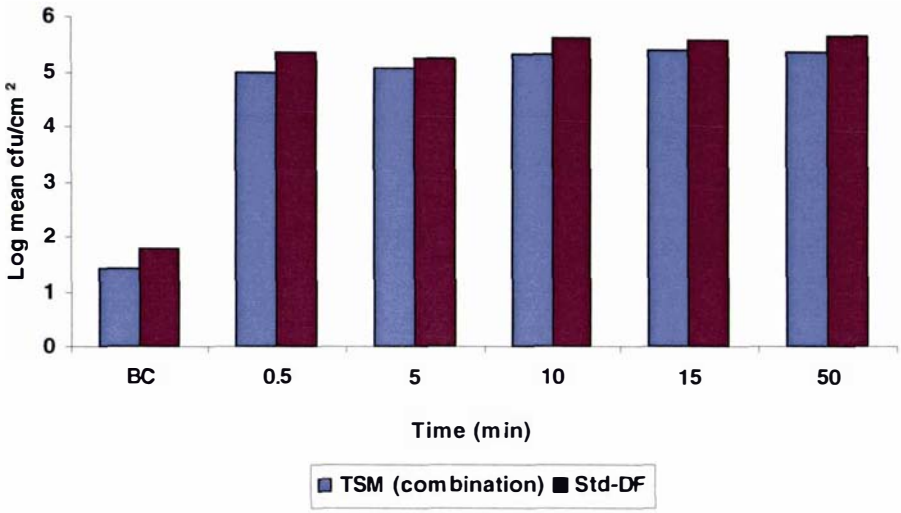


Figure 7.3. Effect of combination rinse treatments using (TSM)-0.1%Tween, 1.5% NaCl and 0.1%mannose on attachment of ***E. coli*** **E6** to cut beef muscle compared with standard diluent fluid system. BC- Background contamination.

7.3.3 Effect of sequential rinse treatments on attachment of *E. coli* E6 to cut beef muscle

The influence of different sequential rinse suspensions in various combinations with time on the attachment of *E. coli* E6 cells to cut beef muscle tissue, compared with those with the standard diluent fluid as rinse medium, is shown in Figure 7.4. The numbers attached after 5 and 20 minutes' immersion were 5 log cfu/cm^2 for all the seven sequential combinations. No significant difference in reducing attachment of *E. coli* E6 occurred when sequential treatments with diluent fluid, NaCl, Tween and mannose were used. As is evident from the figure, the statistical analysis using analysis of variance showed no significant differences between various combinations of rinse treatments trialled for two different attachment bath immersion times and the standard system ($P>0.05$). No difference in detachment occurred for 5 minute and 20 minute immersion with any of the combinations used. The standard single diluent

fluid system wash appeared to be the best compared with the more complex sequential treatments evaluated.

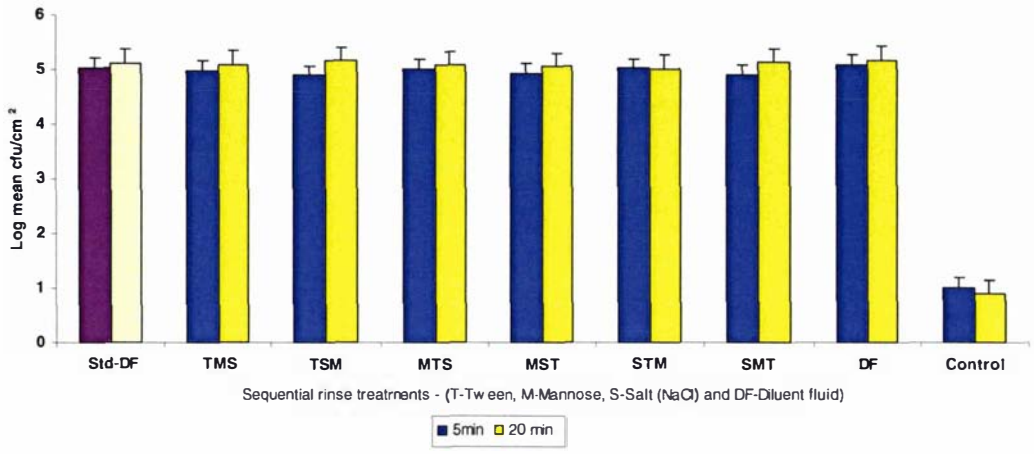


Figure 7.4. Effect of sequential rinse treatments using 0.1%Tween, Salt (1.5%NaCl), 0.1%mannose and diluent fluid on attachment of ***E. coli* E6** to cut beef muscle compared with standard diluent fluid system. Control- Background contamination.

7.3.4 Effect of (TSM) sequential rinse treatments on attachment of *E. coli* E6 to cut beef muscle with high concentration and high vigorous shaking

The influence of different sequential rinse suspensions of TSM combinations and diluent fluid of high and low concentrations with gentle and vigorous rinse treatments with time on the extent of attachment of *E. coli* E6 cells to cut beef muscle tissue compared to those with the standard diluent fluid as rinse medium is shown in Figure 7.5. The numbers retained after 5 minutes' immersion for all the TSM combinations (high and low concentrations and gentle and vigorous rinse treatments) were 5 log cfu/cm^2. No significant difference in attachment of *E. coli* E6 occurred upon using sequential rinse treatments with TSM combinations at high concentration or with vigorous rinses when compared to the standard diluent fluid system. The standard single diluent fluid system appears to be best compared to the

more complex treatments. Attachment was not influenced by increasing the concentration of TSM or vigorous shaking exerted during rinsing.

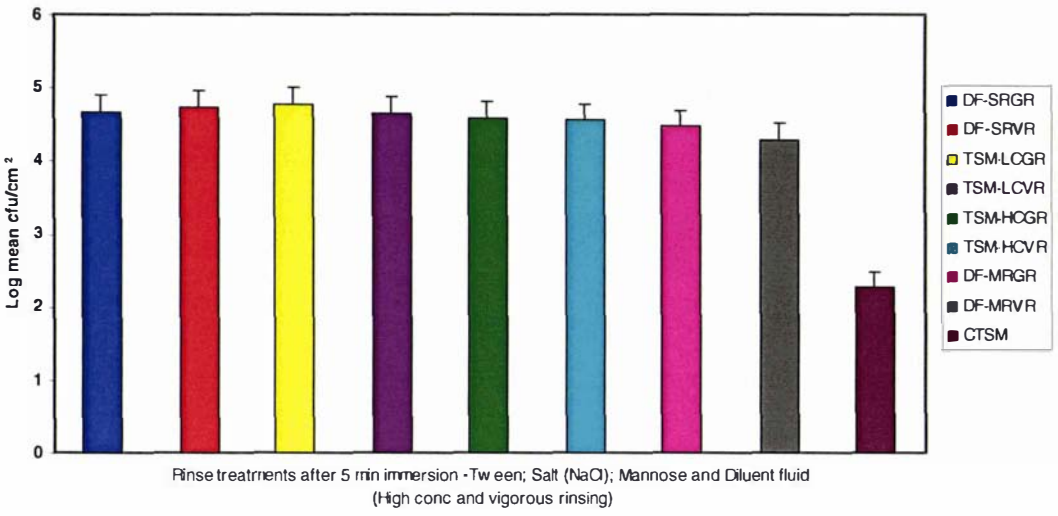


Figure 7.5. Effect of sequential rinse treatments using high concentration and vigorous rinse treatments with 5%Tween, Salt (5%NaCl) and 5%mannose on attachment of *E. coli* E6 to cut beef muscle compared to standard diluent fluid system. SRGR- Single rinse gentle rinse (std DF system); LCGR- Low concentration gentle rinse; LCVR- Low concentration vigorous rinse; HCGR- High concentration gentle rinse; HCVR- High concentration vigorous rinse; SRVR- Single rinse vigorous rinse'; MRGR- Multiple rinse gentle rinse; MRVR- Multiple rinse vigorous rinse; DF- Diluent fluid rinse; CTSM (Control) - Background contamination.

7.3.5 Effect of prewetting treatments on attachment of *E. coli* E6 to cut beef muscle

The influence of different prewetting suspensions and immersion time on the extent of attachment of *E. coli* E6 cells to cut beef muscle tissue compared with those using the standard diluent fluid as the rinse medium is shown in Figure 7.6. The numbers retained after 20 minutes' immersion were: 5 log cfu/cm^2 for 1.5%; 2.0% and 3.0% NaCl and diluent fluid and 6 log cfu/cm^2 for 0.1% Tween and 0.1% mannose. No significant reduction in attachment of *E. coli* E6 to meat surfaces occurred after using prewetting treatments with NaCl, Tween and mannose as wetting fluids when compared to the standard diluent fluid system.

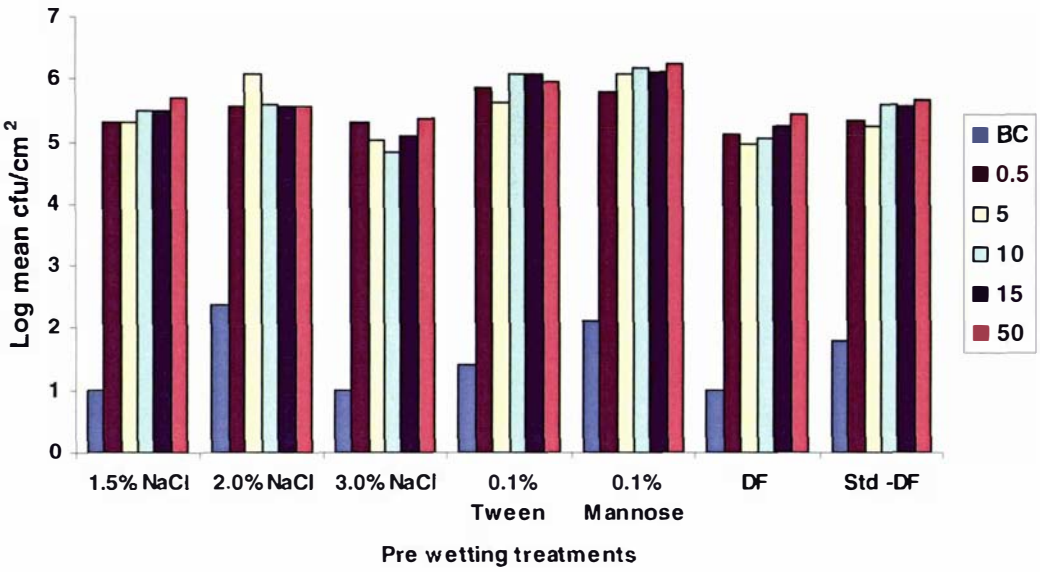


Figure 7.6. Effect of prewetting treatments with NaCl (1.5%; 2.0% and 3.0%); 0.1%Tween; 0.1% mannose and DF-diluent fluid compared with standard diluent fluid on attachment of ***E. coli*** **E6** to cut beef muscle. BC - Initial background contamination.

7.3.6 Effect of 10% TSP pH 12.0 on attachment of *E. coli* E6 to cut beef muscle

The influence of 10% TSP pH 12.0 rinse suspensions and immersion time on the attachment of *E. coli* E6 cells to cut beef muscle tissue compared to those with the standard diluent fluid as rinse medium is shown in Figure 7.7. The number of cells retained after 50 minutes' immersion were 5 log cfu/cm^2. No significant reduction in the attachment of *E. coli* E6 occurred with the use of a rinse treatment containing 10% TSP pH 12 in conjunction with a gentle rinse. However, no viable cells could be detected in the rinse solution. This suggests that harsh chemical treatments such as 10% TSP pH 12.0 kill cells when the contact time is sufficiently long. Therefore any reduction in bacterial numbers associated with 10% TSP pH 12.0 is most probably due to cell death rather than cell removal.

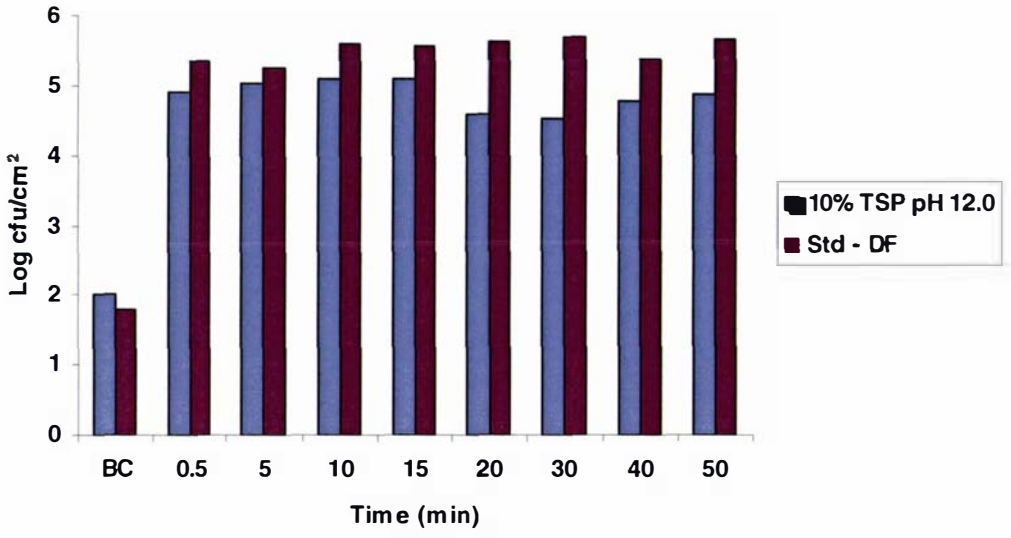


Figure 7.7. Effect of 10% TSP pH 12.0 on attachment of ***E. coli*** **E6** to cut beef muscle compared to standard diluent fluid system.

7.4 DISCUSSION

The work reported in this chapter investigated the efficiency of decontamination treatments designed from an understanding of the attachment mechanisms acting between model systems and bacterial cell surfaces. Using the information on single attachment mechanisms gained in the previous experiments, rinse techniques were designed and tested. The components Tween, NaCl and mannose were selected for their interference with the major attachment mechanisms-hydrophobicity, electrostatic interactions and blocking of attachment sites or reducing surface free energy.

E. coli E6 was selected for this study because *E. coli,* an indicator of faecal contamination, is used as a measure of process hygiene and indirectly to assess the safety of food (Gill, 1991). Moreover these bacteria attached well to meat surfaces as compared with all the other isolates used in this study. The decontamination trials were expected to produce better removal of bacteria from meat surfaces than the standard system, as these were designed to theoretically maximise attachment.

Post rigor cut beef muscles were used in all the experiments because of the difficulties associated with collection and dissection of pre rigor fascia samples from the meat plant. Since post rigor cut beef muscle showed an attachment similar to that of pre rigor fascia, the former could be used in these experiments without compromising the interpretation of the results.

Another important parameter which was considered seriously was the use of mild chemical treatments with short exposure times. Since meat is a food, drastic treatments would be highly likely to affect flavour, texture and colour. Very mild treatments, therefore, were preferred for the removal of microbial cells from the meat surface. A decontamination treatment of choice must have no adverse effect on product appearance or on any other sensory attribute, must pose no unacceptable risks to humans, the product or the environment, and must not add significantly to processing costs. As it is essential to prevent bacteria from becoming irreversibly attached to carcasses or tissues, treatments should be applied as soon as possible after surfaces are exposed and may require combinations of interventions (James, 2002).

It is well established and accepted that washing meat with water can effectively remove macrophysical contaminants such as soil, hairs and other debris. However, its effect on bacterial numbers is marginal. It is through an understanding of the adhesive interactions between meat surfaces and bacterial cells that improvement to washing technology will be most readily achieved (Dickson & Siragusa, 1994).

Current meat plant practice utilizes cold water for washing and there is evidence that cold water treatment has little effect on microbial numbers. Siragusa (1995) has stated that it is not possible to reduce the level of microbial contamination on a carcass to zero using a simple washing system. Meat carcasses typically carry between 1 and 4 log cfu/cm^2 (Bell, 1997). To achieve any significant improvement in the microbiological condition of such products requires a 4 log unit reduction in total bacterial numbers. To date, no adequate method of achieving this has been found without affecting the sensory quality of meat. Furthermore, no treatment as yet can be relied upon to eliminate all pathogens. However, Belk et al. (2001) have demonstrated that decontamination technologies are more effective when used in

combination, or sequentially as multiple hurdle systems to improve regulatory compliance and enhance product safety.

Taking all the above parameters into account, along with the mechanistic understanding of adhesive interactions between model and bacterial surfaces, a wash solution was sought that might interfere with the major attachment mechanisms namely: charge, hydrophobicity and blocking of attachment sites, in order to enhance removal of attached cells and thereby improve the hygienic status of carcasses. The principal components interfering in isolation with the presumptive major attachment mechanisms were Tween, NaCl and mannose. These agents were applied to meat surfaces using single, combined, prewetting and sequential rinse treatments.

Some of the decontamination procedures reported in the literature are complicated or require major capital investment. In the present study we have tried to focus on decontamination technologies that are simple and cheap to apply in the commercial setting.

Attachment of *E. coli* E6 to cut beef muscle rinsed using Tween, salt and mannose appears to occur very quickly with little, if any, additional attachment occurring beyond 5 minutes' immersion. An attachment of approximately 5 log cfu/cm^2 occurred on immersion in a cell suspension containing 10^7 cfu/ml when standard diluent fluid or other rinsing solutions were used. This level of attachment was consistently recorded (Figures 7.2 to 7.6). The components interfering with major individual attachment mechanisms (Tween, NaCl and mannose) appeared to be ineffective in reducing bacterial retention on meat, as no statistically significant reduction in attachment was observed using single, prewetting, combination and sequential rinse treatments. In addition, both the application of these chemicals at (5 times the original test rate) and increasing the rinsing forces failed to produce a reduction in retention. These results suggest that once the bacterium is attached to the meat surface removal treatments become ineffective, indicating that attachment to the meat surface is strong and mediated by multifactorial attachment mechanisms. Therefore, removing cells from a meat surface will be difficult once the bacteria have attached. Thus washing processes that target the major attachment mechanisms are

ineffective. The current research focussed on removal of already attached cells from meat surfaces. This is perhaps not surprising since the meat presents attachment surfaces every bit as complex as those of the attaching bacteria.

Li & Mclandsborough (1999) and Piette & Idziak (1991) demonstrated that pH, ionic strength and different concentrations of Tween interfered with the ionic and hydrophobic attachment of *E. coli* O157:H7 to beef tissue. Calicioglu et al. (2000) demonstrated that pre-spraying of carcasses with Tween 20 (5%) prior to treatment with lactic acid and sodium benzoate resulted in significantly larger reductions of *E. coli* O157:H7 compared with control carcasses. Dickson (1988) demonstrated that there was no significant difference in the detachment of bacteria from lean tissue with NaCl, the reductions being less than 1 log cfu/cm^2.

Several workers have examined the role of mannose in bacterial attachment. Dickson (2000) attempted unsuccessfully to block the mannose receptors on *E. coli* pili with mannose containing solutions in the expectation that this would interfere with the initial stages of bacterial attachment to animal tissue surfaces and facilitate the removal and destruction of contaminating bacteria on meat surfaces. Many of the genera of the Enterobacteriaceae *Escherichia, Shigella, Salmonella* and *Vibrio* possess external cell structures termed *common pili* or *type I fimbriae* (Eisenstein, 1988). These are composed of 127 to 21 KDa repeating units with minor proteins inserted periodically near the tip. These proteins are termed *adhesins* and these adhesins function to bind D mannose residues on eukaryotic cells. In preliminary experiments, Grant & Dickson (2000) indicated that mannose-treated meat supported 2.5 to 3 times fewer bacteria when compared with meat that was not treated with mannose, and confirmed that mannose treatments were of little use in reducing numbers of bacteria attaching to pork tissue. However, Benedict et al. (1991) demonstrated that a mannose rinse reduced the attachment of *Salmonella* to beef tissues.

As removal treatments appear to be ineffective, the use of 10% TSP at pH 12, was tried and this too produced no reduction in retention of *E. coli* E6 to cut beef muscle. This particular chemical is widely recognised for its killing effect on bacteria (Cutter

& Siragusa, 1994). The fact that no reduction in attached cells was observed in the present study may be due to short immersion time in the rinse solution. A significant drop in rinse count was observed, clearly indicating that with time TSP killed all the cells. Future studies can be focused on killing cells present on the surface by exposing them to 10% TSP pH 12.0 for longer immersion times to obtain greater effectiveness. TSP has been used to treat meat as well as being widely used in the poultry industry. Capita et al. (2000) demonstrated that treatments with 8 to 10% of TSP do not alter the organoleptic properties of poultry meat. Cutter & Betancourt (2000) demonstrated 3.8 log reductions of *Salmonella* and *E. coli* O157:H7 strains attached to beef tissue surfaces using 10% TSP treatments.

Comparable reduction levels were not obtained with meat surfaces to those obtained with the collagen film model system. This may be because of the simplicity of the model system and suggests that attachment to cut beef surfaces must be influenced by additional factors that are not present in a simple collagen model. These factors could include the presence of serum proteins, fat and muscle collagen associated N – acetyl glucosamine polymers (Sanderson et al., 1995). However, the use of collagen film still appears to afford a reasonable, if simplified, representation of the attachment of bacteria occurring at cut beef surfaces. Some uncertainties were associated with the model system in simulating the detachment of cells. However, the collagen film model, because of its simplicity showed that adhesive interactions can be disturbed when compared to that of the complex meat surface.

Greater reductions in microbial populations have been reported when decontaminating treatments were used on meat in combination than when they were applied individually (Castillo et al., 1998; Dorsa et al., 1996a; Delmore et al., 1998; Hardin et al., 1995; Phebus et al., 1997). Many reports support the concept of using sequential decontamination (hurdle technology) processes in beef packing plants as a means of improving the microbiological quality (Bacon et al., 2000). The results of the present study do not agree with those of Bacon et al. (2000) who examined single or separate rinse systems.

Understanding the fundamental adhesive mechanisms and, through their frustration, attempting to remove attached cells and thereby improving the washing efficiency has clearly failed; the components identified as interfering with single attachment mechanisms between microbial cells and meat surfaces did not yield improved rinse solutions. In addition, the present study demonstrated no significant differences between individual, combinations, prewetting and sequential treatments. This study confirms that bacterial attachment and removal from the meat surface is a complex and multifactorial process. The killing of attached cells or clean practice in slaughter plant may prove better options for producing microbiologically cleaner carcasses (Ammor et al., 2004).

7.5 CONCLUSIONS

Bacterial detachment from meat surfaces was studied with respect to the basic attachment mechanisms acting between the cell and substratum. The major components interfering with presumed attachment mechanisms by altering charge or hydrophobicity or blocking of attachment sites were selected and applied as a wash technology to meat surfaces. Various treatment regimes were evaluated in order to remove attached bacteria from meat surfaces. These included single, combination and sequential rinse treatments, higher chemical concentration and high vigorous shaking and prewetting treatments applied to the meat surfaces prior to exposure to the cell suspension. It must be concluded that the process of bacterial detachment is the reverse of the complex attachment process. Therefore, improving the efficiency of washing treatments by interfering with the attachment mechanisms remains an unrealised dream. Future studies therefore will focus on the killing of attached cells.

Chapter 8
OVERALL CONCLUSIONS

The aim of this study was to optimise the hygienic efficiency of the carcass washing procedure in slaughter and dressing operations. Three strategic approaches, namely reducing, removing or killing the bacteria attached to meat surfaces, were considered. The second option of removal was selected for development, as current technology inevitably results in bacterial contamination, whereas killing bacteria on meat surfaces normally requires drastic treatments that may adversely affect quality parameters of the meat.

The initial attachment mechanism between bacteria and the carcass surface (reversible attachment) was studied using a collagen film model system. The model system was used to develop a reasonable methodology to define "attached cells" and to differentiate them from cells that are present in the surface-associated liquid film. The model collagen film system employed a viable count procedure to determine the numbers of attached cells. Bacterial attachment to the collagen model was compared with attachment to cut beef muscle and uncut beef muscle. These studies confirmed that the model system reasonably imitated bacterial attachment to meat, in terms of the numbers of bacteria attaching to both cut and uncut surfaces.

A direct microscopic count procedure using epifluorescence microscopy was also developed, using both collagen films mounted on microscope slides and collagen coated microscope slides. The direct microscopic counts obtained with these two model systems were compared with those obtained with the viable count procedure. The collagen coated slide was the better microscopic model for studying bacterial attachment mechanisms to carcass surfaces, allowing comparison between the viable count procedure and microscopic procedures. However, because of ease and consistency of quantification, the collagen film viable count system was the method selected to model bacterial attachment to meat. Further studies using scanning

electron microscopy also confirmed that bacteria were deposited on meat and collagen film surfaces due to the technical difficulties associated with sample processing.

There was no positive correlation between attachment and many single bacterial cell surface factors such as charge, hydrophobicity, protein and polysaccharide surface molecules. *S. aureus* was selected as a test species, as it possesses both high surface charge and hydrophobicity, to explain the attachment mechanisms of all other 11 isolates used in this study.

The process of attachment was thus found to be mediated simultaneously by multiple attachment mechanisms. However, a comprehensive understanding of the fundamental process of bacterial attachment to meat surfaces remains an elusive theoretical basis for the development of procedures for the physical removal of microorganisms contaminating meat surfaces.

Adherence of bacteria to meat surfaces was studied experimentally by examining single attachment mechanisms acting in isolation. Different eluents were used to identify the principal components interfering with single attachment mechanisms by using EIC and HIC columns and with attachment to collagen film. Three components interfering with the isolated attachment mechanisms were identified. These were Tween, which interferes with hydrophobic attachment, NaCl which interferes with ionic attachment and mannose, which blocks specific attachment sites. Further column studies indicated that cell surface proteins play a more important role in cell surface negative charge and hydrophobicity than do cell surface polysaccharides. Results of the model study indicated that attachment is a multifactorial process and that interference with the mechanism's association with surface charge, hydrophobicity, proteins and polysaccharides could all theoretically contribute to enhanced removal by elution.

A wash solution was formulated using the components Tween, NaCl and mannose to reverse what were believed to be the major attachment mechanisms. Factorial trials were undertaken using these three components to remove the bacterial cells attached

to meat surfaces. These trials confirmed that bacterial attachment to meat surfaces could not be easily reversed by sequential or simultaneous treatments with Tween, NaCl or mannose solution. Further trials with increasing concentrations of these agents and the application of increased shear forces also proved ineffective in enhancing the washing of cells from meat surfaces. Further elution studies using 10% TSP pH 12.0 showed that this agent killed the attached cells rather than removing them.

This is the first comprehensive study made to understand the fundamentals of the complex attachment mechanisms of bacteria to meat surfaces. The approach was to introduce various interventions and a novel feature was the identification of some components interfering with these attachment mechanisms. The information was used to design a classical wash solution formulation to remove the bacteria from the meat surface.

This whole study supports the hypothesis that bacterial attachment to meat surface is very complex and multifactorial. This study strongly emphasises that removal of bacteria from the meat surface through washing is difficult, even when the cells have only recently attached. If future work is to produce an effective wash solution, it will be necessary to target simultaneously the multiple mechanisms that appear to be involved in the initial attachment or killing of cells.

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