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Quantitative measurements of factors influencing bacterial attachment to meat surfaces

by

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Statement of co-authorship

This thesis includes work, which has been published or submitted for publication in a peer-reviewed journal. More details for each chapter are described in the section of "Publications and conference presentation relevant to the thesis". Proportionate co-author contributions were as follows:

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Abstract

Pathogenic microbial contamination of meat can lead to foodborne illness following consumption of contaminated products. In abattoirs, contamination of carcass surfaces normally occurs when microorganisms are transferred from the outer surface of the carcass to sterile underlying tissues during slaughter and subsequent post-slaughter processes. Bacterial attachment to surfaces is a complex phenomenon that is influenced by many factors including the physicochemical properties of the bacterial cell and meat tissues, interacting surface structures and other factors.

The complexity and the heterogeneous nature of meat tissue, as well as physical entrapment within tissue fibres, have made it difficult to describe the specific tissue structures involved in the attachment process. A more systematic approach can be achieved by studying binding interactions between bacteria and specific structures of meat tissue.

Beef carcass prepared for commercial trade mainly consists of muscular and fatty tissue, bones and connective tissues. Approximately 49 to 68% of carcass weight consists of the muscular tissue whereas connective tissues are ubiquitous on the carcass surface, located at the fascia (between the skin and the skeletal tissues) and also within the muscle. The connective tissues are mainly composed of extracellular matrix (ECM) proteins.

This study aimed to determine the mechanisms of bacterial attachment to specific meat surface structures, namely the ECM proteins and muscle cells, by a range of enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella* spp. strains. The influence of physicochemical and environmental factors on attachment to meat structures was also investigated.

Attachment properties of the *E. coli* and *Salmonella* strains to four major ECM proteins (collagen I, fibronectin, collagen IV and laminin) were measured by a microtiter plate assay using crystal violet

staining, and by epifluorescence microscopy. The effect of temperature (4, 25 and 37°C) and protein concentration were also determined. A wide variation in attachment to ECM proteins among strains was observed. In general, *E. coli* strains had a higher binding capacity to ECM proteins compared to *Salmonella* strains. Bacterial attachment was also found to be selective based on the anatomical location of the ECM proteins. Specifically, a higher proportion of strains attached to basement proteins (laminin and collagen IV) than to interstitial proteins (collagen I and fibronectin).

Protein concentration had a minor effect on bacterial attachment to ECM proteins; however attachment was significantly influenced by temperature. Highest attachment levels occurred at 4°C for collagen I and at 25°C for the other three ECM proteins. A strong positive correlation was found between the results of both the crystal violet and epifluorescence methods ($r \geq 0.905$, $p < 0.05$) indicating that the former method is useful to study bacterial attachment to ECM proteins, especially in determining the attachment properties of high binding strains.

Based on these results, a subset of strains representing 'high-', 'intermediate-' and 'low-' binding were chosen for further investigation. The influence of pH and salt (NaCl, KCl and $CaCl_2$) on attachment to ECM proteins was assessed *in vitro*. pH, within the range of 5 to 9, had no effect on attachment to ECM proteins, whereas the effect of salt type and concentration (0.1 – 5%) varied depending on strain-ECM protein combination. The effects of three chemical rinses commonly used in commercial abattoirs (2% acetic acid, 2% lactic acid and 10% trisodium phosphate (TSP)) on the attachment were also investigated. Rinses containing TSP were the most effective, producing >95% reduction in attachment to all ECM proteins. Acetic and lactic acids also markedly reduced bacterial attachment to ECM proteins, but at a lower level than TSP.

In addition to ECM proteins, bacterial attachment to animal muscle cells was measured. Specifically, attachment of *E. coli* and *Salmonella* strains to primary bovine muscle cells and a cultured muscle cell line, C_2C_{12}, at 10 and 37°C was measured by plate-count assay. As shown for ECM proteins, attachment to muscle cells was strain dependant, with temperature being a

significant factor. The attachment properties of the two muscle cell types differed significantly, indicating that C_2C_{12} cells are not suitable as surrogates for bovine muscle cells.

Finally, the specificity of interactions between bacterial cells and ECM proteins was studied. In general, addition of certain soluble ECM proteins had significant inhibitory or enhanced effects on binding interactions, depending on the type of ECM protein. For example, while soluble collagen IV inhibited attachment of *E. coli* M23Sr to laminin, basement membrane proteins increased attachment of *E. coli* EC614 and H10407 to the interstitial ECM proteins. However, soluble fibronectin did not affect binding interactions. These results could serve as the basis for future studies of potential synthetic analogs to inhibit bacterial attachment to meat surfaces.

Through this study, there is greater understanding of bacterial attachment to specific meat tissues. The knowledge obtained from this study may be beneficial in developing new and more targeted intervention systems for carcass decontamination, potentially reducing carcass contamination, product spoilage and health risk associated with meat.

Publications and conference presentation relevant to the thesis

The work presented in this thesis has so far resulted in the following peer reviewed publications and conference proceedings.

Publications

- **Chapter 2**. Zulfakar, S.S., White, J.D., Ross, T., and Tamplin, M.L., 2012. Bacterial attachment to immobilized extracellular matrix proteins *in vitro*. *International Journal of Food Microbiology* 157:210-217. (date of submission: 23rd December 2011 . Accepted on: 8th May 2012)
- **Chapter 3**. Zulfakar, S.S., White, J.D., Ross, T., and Tamplin, M.L., 2013. Effect of pH, salt and chemical rinses on bacterial attachment to extracellular matrix proteins. *Food Microbiology* 34:369-375. (date of submission: 3rd September 2012. Accepted on: 12th January 2013)
- **Chapter 4**. Zulfakar, S.S., White, J.D., Ross, T., and Tamplin, M.L., 2013. Cultured C_2C_{12} cell lines as a model for assessment of bacterial attachment to bovine primary muscle cells. *Meat Science.* 94:215-219. (date of submission: 15th October 2012. Accepted on: 26 January 3013)
- **Chapter 5**. Zulfakar, S.S., White, J.D., Ross, T., and Tamplin, M.L., Influence of soluble extracellular matrix proteins on bacterial attachment to immobilized extracellular matrix proteins. (*Manuscript being prepared for publication)*

Conference Proceedings

Oral Presentation:

- Zulfakar, SS, White, JD, Ross, T and Tamplin, ML, 'Effect of pH salt and chemical rinse on bacterial attachment to meat extracellular matrix proteins', Oral Presentation to the 23rd International ICFMH Symposium, FoodMicro 2012, 3-7 September 2012, The Grand Cevahir, Istanbul, Turkey (2012).

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Poster Presentation:

- Zulfakar, SS, White, JD, Ross, T and Tamplin, ML, 'Bacterial attachment to Muscle Extracellular Matrix Proteins', Proceedings of the 14th Australian Food Microbiology Conference 2nd IAFP Asia Pacific Symposium on Food Safety: Securing Global Food Safety Conference, 26-28 September 2011, The Sebel Albert Park, Melbourne, Victoria (2011).

- Zulfakar, SS, White, JD, Ross, T and Tamplin, ML, 'Bacterial Attachment to Muscle Extracellular Matrix Proteins', Proceedings of 2011 Australian Society for Microbiology (ASM) Annual Scientific Meeting, 4-8 July 2011, Hotel Grand Chancellor, Hobart, Tasmania (2011).

Chapter 1

Literature review and study aims

Introduction

Food contaminated with pathogenic microorganisms can cause a range of diseases including acute illnesses (e.g. gastroenteritis, hepatitis A), and long term sequelae (e.g. haemolytic uraemic syndrome, irritable bowel syndrome). According to the Australian National Gastroenteritis Survey (Hall and Kirk, 2005), there are approximately 17.2 million cases of gastroenteritis in Australia each year with 5.4 million (32%) that are estimated to originate from contaminated food. Common pathogens responsible for foodborne gastroenteritis were pathogenic *Escherichia coli*, norovirus, *Campylobacter spp*. and non-typhoidal *Salmonella*.

These events significantly impact Australia's economy, productivity and lifestyle through loss of food products, loss of work time, increases in medical and health care costs and also the governance of public health actions such as food surveillance, outbreak investigations and maintaining food safety systems (Dalton and March, 1998; Hall and Kirk, 2005). Extensive media coverage of outbreaks of contaminated foods and resultant recalls markedly affects the buying habits of the consumer and results in decreases in the market for such products (Abelson et al., 2006). The total cost of foodborne illness in Australia is estimated at $1,249 million per annum for circa 2000, with foodborne gastroenteritis accounting for approximately $811 million (Abelson et al., 2006).

Pathogenic microbial contamination of food surfaces is significant, because microorganisms transmitted through food products cause human illness. Meats and meat products are contaminated with a wide variety of organisms that vary with processing and storage conditions (Benedict, 1988; Mackey and Roberts, 1993). Fresh muscle tissue is a highly favourable environment for microbial growth and is subject to rapid spoilage. The water activity (a_w) of fresh muscle tissue is high and it also contains readily available glycogen, peptides, and amino acids, as well as metal ions and soluble phosphorus. These factors contribute to the suitability of muscle tissue as a substrate to support microbial growth (Jackson et al., 2001).

Contaminating microbiota of muscle food varies as a result of the resident microorganisms in and on the live animal; environmental sources such as vegetation, water, and soil; ingredients used in meat products; workers' hands; and contact surfaces in processing facilities (Sofos et al., 1999). Following contamination, microorganisms attach to food surfaces and can spoil food products (Benedict, 1988; Sofos, 2008). Microbial spoilage of meat becomes evident when the number of the spoilage bacteria at the surface reach and exceed 10^7 CFU/cm^2 (Lawrie, 1998).

Sources of carcass contamination in abattoirs

Slaughter processes as sources of contamination

Microbial contamination of carcass surfaces during slaughter and post-slaughter procedures is inevitable. In live animals including cattle, microorganisms usually are present on the crotch, brisket and hind hocks region, and also in the gastrointestinal tract (Gill and McGinnis, 2003). In contrast, bacteria are normally absent in internal tissues, other than the gastrointestinal tract, due to immunological and non-immunological defence mechanisms (Davies and Board, 1998; Huffman, 2002).

The population of microorganisms that contaminate meat is influenced by intrinsic microbiota of the animals and environmental conditions (Jackson et al., 2001). Contamination initially occurs when pathogenic and spoilage microorganisms are transferred from the outer surface of the carcass to internal tissues during different slaughter processes. The primary source of carcass contamination by microorganisms is the hide, which may be contaminated with faeces, soil and filth (Antic et al., 2010). A positive relationship between hide cleanliness and carcass contamination has been reported (McEvoy et al., 2000). Common microorganisms on the hide include skin microbiota such as

Staphylococcus, *Micrococcus*, and *Pseudomonas* species, yeast and moulds, as well as enteric species found in faecal material and soil.

The hide is removed at the intersection of the skin and fat/subcutaneous muscles, leaving all fat and subcutaneous muscles intact and attached to the carcass (Belk and Scanga, 2004). Hide removal normally starts at the hock and butt area, followed by cutting down the midline to remove the hide from the flank, brisket and forelegs, manually with knives, before completely removing it from the carcass by an upward-pulling automatic hide puller (Bolton et al., 2001). During this event, microorganisms present on the hide will be carried onto the now-exposed underlying tissue with the initial cut.

It has been reported that the sites that correspond to the skin opening cuts and/or hide-meat contact during hide removal, presents higher microbial contamination of carcass meat (Antic et al., 2010; Bell, 1997). Microorganisms are also transferred through direct contact with the hide or indirectly through contact with workers' hands or equipment used, and also via aerosols and dust generated from the hide during removal process. Thus the hide cleanliness and the worker's skill are two important factors that contribute to the degree of carcass contamination (McEvoy et al., 2000).

Another potential source of contamination is the animal's gastrointestinal tract. Contamination occurs if the intestinal tract is pierced or if faecal material contacts the carcass surface from the rectum during the removal of abdominal contents (Lawrie, 1998). Such contamination can result in cross-contamination to other carcasses and other working surfaces, leading to additional contamination during carcass processing (Davies and Board, 1998; Gill et al., 2001).

During the evisceration process, the risks of microbial contamination increase as microorganisms on the contaminated meat surface are transferred to internal tissues and also by cross-contamination via hands and cutting equipment (Nørrung and Buncic, 2008). In addition, the water used for cleaning and sanitising floors, instruments and containers also serve as the sources of contamination (Lawrie, 1998).

Pathogenic bacteria associated with carcass contamination

A large variety of pathogenic microorganisms are commonly associated with carcass contamination. These include *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella* spp., *E. coli*, *Campylobacter* spp., *Listeria monocytogenes* and *Yersinia enterolitica* (Marshall and Bal'a, 2001). Pathogenic *E. coli* such as *E. coli* O157:H7 and *Salmonella* spp. are the most frequently associated with fresh meat products safety concerns and product recalls (Sofos, 2008).

Escherichia coli

Escherichia coli is a member of the family Enterobacteriaceae; a Gram-negative, facultatively anaerobic, straight rod, typically 1.1-1.5 × 2-6 µm in dimension. Some *E. coli* are motile by peritrichous flagella while others are not. *Escherichia coli* are normal flora of the lower intestine of warm-blooded vertebrates. Serotyping of *E. coli* is based on three fundamental antigens; O, K and H. O antigen groups are differentiated by structural diversity of the polysaccharide part of the outer membrane of lipopolysaccharide. K antigens are a polysaccharide, capsular or micro-capsular antigen, whereas H antigens are associated with the flagella of motile *E. coli* strains. Fimbrial antigens have also been used for further differentiation of fimbriated strains (Desmarchelier and Fegan, 2003).

Shiga toxin-producing *E. coli* (STEC) are a group of diarrheagenic *E. coli* that produce one or more types of Shiga toxins. They can cause haemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), but not all STEC strains have this capability. The STEC strains that cause HC are referred to as enterohaemorrhagic *E. coli* (EHEC). Predominant serogroups associated with this group include O157, O111, O26, O45, O91, O121 and O145. Enterohaemorrhagic strains colonize

primarily the human large intestine and are capable of producing attaching and effacing (A/E) lesions on epithelial cells (Torres et al., 2005). Other than severe HC, EHEC infections can be fatal since infection may lead to the development of acute kidney failure due to haemolytic uremic syndrome(HUS) especially in young children and the elderly (Desmarchelier and Fegan, 2003). Transmission to humans may occur through contaminated food, water, environment or person-to-person contact.

The intestinal tract of ruminants especially cattle has been implicated as the primary reservoir of EHEC strains (Desmarchelier and Fegan, 2003). Weighted prevalence of STEC in bovine faeces found in abattoirs was reported to range from 0% to 57%. The prevalence on hides of cattle pre-slaughter is generally much higher, with a weighted mean of 44% (range from 7.3-76%) (Rhoades et al., 2009).

Salmonella

Similar to *E. coli*, *Salmonella* are a member of the Enterobacteriaceae family and are Gram-negative, facultatively anaerobic non-spore forming rods of 0.7-1.5 × 2-5 μm in dimension. *Salmonella* are predominantly motile via peritrichous flagella and most form type-1 fimbriae. *Salmonella* are found in a wide variety of warm-blooded and cold-blooded animal intestinal tracts. Usually, animals colonised with *Salmonella* showed no sign of illness. Serotyping is based on O and H antigens. Phage and molecular-typing methods are also used to subtype more common serovars (Jay et al., 2003). All *Salmonella* serovars are considered potential pathogens to humans (Groisman and Ochman, 1997).

Salmonella shedding in faeces can contaminate soil, agricultural pastures, water bodies and subsequently be the source of contamination for other animals. *Salmonella* infections, or salmonelloses, are zoonotic with food animals and animal-derived food products as the main source

of human infections (Groisman and Ochman, 1997; Wick, 2004). Infections normally occur by ingestion of food contaminated with high concentrations of the organism. Children, elderly and immune-compromised persons are more susceptible to infection and severe symptoms. *Salmonella* attach to small intestine epithelial cells via type-1 or type-3 fimbriae and non-fimbriate haemagglutinins. The pathogen also produces a heat-labile enterotoxin; most strains also produce heat-labile cytotoxins (Jay et al., 2003).

Although poultry has been reported as the most important reservoir for *Salmonella* in human infections, red meats have also often been implicated in human salmonellosis (Jay et al., 2003; Mrema et al., 2006). The weighted mean prevalence of *Salmonella* on pre-slaughter cattle hides is reported to be as high as 60% (Rhoades et al., 2009), and *Salmonella* can be transferred to the carcass surface during dehiding and evisceration processes.

Mechanisms of bacterial attachment to surfaces

Attachment is the essential first step in surface contamination (Selgas et al., 1993), however it is not a simple phenomenon due to the complexity of the interacting surfaces (both substrata and microbial cell), and is influenced by various factors (Goulter et al., 2009; Selgas et al., 1993). Properties of attachment for each microorganism differ among surface types, species and even experimental methods used to study the process (An and Friedman, 1997).

The binding of bacterial cells to food surfaces can pose a risk to product safety and quality. Cells may attach to food contact surfaces and processing equipment, potentially leading to further contamination of other food products. Strongly attached bacterial cells can be difficult to remove, thereby interfering with decontamination processes (Benedict et al., 1991; Fratamico et al., 1996; Tamblyn and Conner, 1997). Therefore, appropriate and thorough intervention treatments are necessary to inhibit, remove or kill attached bacterial cells efficiently but without markedly affecting

food quality. Development of effective food safety and quality intervention systems can only be achieved by understanding the specific mechanisms that mediate the bacterial attachment process.

Attachment is generally described as a two-stage process: initial reversible attachment and subsequent irreversible attachment. This idea has been accepted by a majority of researchers (An and Friedman, 1998; Frank, 2001; Garrett et al., 2008; Ofek et al., 2003; Selgas et al., 1993; Shoaf-Sweeney and Hutkins, 2009). The term 'reversible' and 'irreversible' also correspond to the terms 'loosely' and 'strongly' attached bacteria used by Firstenberg-Eden et al. (1978).

Reversible processes

Reversible attachment occurs when bacteria become trapped in a surface water film on the surface (Selgas et al., 1993). Initially, microorganisms approach the contact surface, either by flagella that help in bacterial motility or by being transported to the surface through external physical forces such as fluid flow (Benedict, 1988; Frank, 2001; Garrett et al., 2008). Pathogenic bacteria and adhesive substrata on most biological and inert surfaces are negatively charged (Frank, 2001; Ofek et al., 2003), repelling bacteria from the surface.

In order to attach, a bacterial cell must first overcome repulsive forces as it approaches the surface, regardless of the mechanisms used (Ofek et al., 2003; Shoaf-Sweeney and Hutkins, 2009). Therefore, energy is involved (An and Friedman, 1998; Ofek et al., 2003). The forces that allow the bacterial cell to overcome the energy barrier includes van der Waals interactions, coulombic forces, hydrophobic interactions and eventually complementary interactions (Frank, 2001; Shoaf-Sweeney and Hutkins, 2009).

When the bacterial cell is within a range of 100-10 nm from the surface, van der Waals attractions and coulombic forces play a role in closing the gap (within 5 nm) between the two

interacting surfaces (Busscher and Weerkamp, 1987; Hermansson, 1999). During the event, maximum repulsion occurs in that region (10-100 nm) between the overlapping electrical double layers due to the net negative charges of the opposing surface (Garrett et al., 2008).

Because the repulsive forces of the electrical double layer increase in proportion to the diameter of the approaching particles, bacterial physical appendages with a small diameter, such as flagella, fimbriae and pili, may aid in overcoming the barrier (Ofek et al., 2003). Initiation of the second stage of attachment (irreversible attachment) results from these structures acting as a bridge to reduce the distance between the cell and the substratum (Weiner et al., 1995). At a closer distance, hydrophobic interactions mediated by the hydrophobic moieties on the bacterial surface and the host cell surface take place. This leads to formation of loose, weak, and reversible attachment.

Irreversible processes

Successful completion of the first step is requisite before the attachment process proceeds to the second stage, irreversible attachment. In time, reversibly attached bacterial cells form stronger permanent bonds with the surface via specific reactions between structures on the bacterial cell and specific complementary receptors on the surface or by producing extracellular binding polymers (Firstenberg-Eden et al., 1978; Frank, 2001). These structures may pre-exist on the bacterial cell surface before attachment occurs or their production is induced following attachment (Fletcher and Floodgate, 1973; Ofek et al., 2003). Irreversible attachment of bacteria to meat tissues may also occur via physical entrapment of cells within the muscle fibres, making it difficult to remove them (Frank, 2001).

Factors that influence bacterial attachment

Physicochemical properties

Bacterial attachment to surfaces has been characterized by physicochemical properties such as surface charge and hydrophobicity of the bacterial cell and the substratum (Hassan and Frank, 2003; Jana et al., 2000; Katsikogianni and Missirlis, 2004; Rivas et al., 2007) that involves a combination of both hydrophobicity and electrostatic interactions (Geertsema-Doornbusch et al., 1993).

Cell surface hydrophobicity

Hydrophobicity is the term that refers to hydrophobic properties of a cell surface. It generally describes the bacterial cell preferences to interact with cells or molecules with similar hydrophobicity, rather than water molecules (Geertsema-Doornbusch et al., 1993; Goulter et al., 2009). Some of the common methods used to measure hydrophobicity include bacterial adherence to hydrocarbons (BATH), hydrophobic interaction chromatography (HIC), bacterial adhesion to nitrocellulose-filters (NCF), contact angle measurements (CAM) and salt aggregation test (SAT) (An and Friedman, 1997; Rivas et al., 2005).

Cell surface hydrophobicity varies among bacterial species, strains and serotype (Bouttier et al., 1997; Chia et al., 2008; Goulter et al., 2009; Sinde and Carballo, 2000). Hydrophobicity also varies depending on growth conditions and growth stage of bacterial cells, and can also differ among methods used to measure the surface hydrophobicity (Benito et al., 1997; Goulter et al., 2009; Rivas et al., 2005). The presence of residues and surface structures such as polysaccharides in the cell envelope can also influence bacterial cell hydrophobicity in attachment to the meat surface (Boonaert and Rouxhet, 2000; Geertsema-Doornbusch et al., 1993).

The significance of bacterial cell's hydrophobicity in attachment to meat surface is unclear. Studies of lactic acid bacteria (Marin et al., 1997) on beef tissues showed a correlation between cell surface hydrophobicity and strength of attachment, but not for *E. coli* and *Salmonella* strains (Bouttier et al., 1997; Li and McLandsborough, 1999; Rivas et al., 2006). However, other authors whose studies included *E. coli* and *Salmonella* along with other bacterial species, found a correlation between cell hydrophobicity and strength of attachment to either lean or adipose beef tissue (Benito et al., 1997; Dickson and Koohmaraie, 1989). This correlation may be the result of averaging the bulk behaviour of the large numbers of bacterial cells used in these studies, which could overlook the key interactions occurring at a nano-scale level. The discrepancy of the role of cell hydrophobicity could also be attributed to the heterogenous nature of the meat tissue where both hydrophobic and hydrophilic groups may be present on the meat surface (Bouttier et al., 1997).

Cell surface charge

Surface charge is the overall net charge on a specific surface (Goulter et al., 2009; van Loosdrecht et al., 1987). It is related to a bacteria cell's hydrophobic properties as a high cell surface charge is normally accompanied by low hydrophobicity characteristics (An and Friedman, 1998). The bacterial cell surface charge is mostly influenced by protein carboxyl groups in the S-layer or by surface polysaccharides (Boonaert and Rouxhet, 2000). The majority of bacterial cells are negatively charged and the degree of this charge varies among species and strains (Goulter et al., 2009). Lillard (1985) found that the excess presence of carboxyl and phosphate groups in the bacterial cell wall is the primary cause of a negative charge. The presence of certain bacterial surface structures such as flagella, which are rich in protein associated COOH/COO- groups, is also a factor that determines bacterial surface charge (Briandet et al., 1999; Hassan and Frank, 2004).

Cell surface charge can be quantified by measuring the electrokinetic (zeta) potential (Rivas et al., 2005; van Loosdrecht et al., 1987). Electrophoretic mobility is the moving velocity of bacterial cells

suspended in a given solution under the influence of an electric field (Bouttier et al., 1997). Through measurement of electrophoretic mobility, the zeta potential value can be calculated (Li and McLandsborough, 1999). Furthermore, the zeta potential relates to the involvement of electrostatic interactions in the attachment process (van Loosdrecht et al., 1987).

By attracting ions of opposite charge in the medium, the cell surface charge forms an electrostatic double layer. Naturally, cells attach to surfaces of opposite charges, however if the electrostatic double layers are formed on each interacting surface, electrostatic interactions between like charges can occur (van Loosdrecht et al., 1987). Both cell surface charge and electrostatic interactions are influenced by ionic strength, cation concentration and the pH of surrounding medium (Bouttier et al., 1994; Bouttier et al., 1997; Piette and Idziak, 1992).

Direct correlation between cell surface charge and attachment have been observed by Dickson and Koohmaraie (1989) but not by other authors (Bouttier et al., 1997; Rivas et al., 2006). However, although Li and Mclandsborough (1999) did not observe a correlation between cell surface charge and attachment to beef tissues, their results showed that electrostatic interactions may be involved; agreeing with the findings of Piette and Idziak (1992). Li and Mclandsborough (1999) suggest that this observation may be attributed to the strain's reaction to different pH levels and ionic strengths of the test medium.

Bacterial surface structures involved in attachment

The bacterial cell envelope is the outermost structure of the Gram-negative cell and as a result, has a major influence on the attachment of bacteria to surfaces. This layer consists of surface structures, such as flagella, fimbriae or exopolysaccharides (EPS) that may mediate attachment to target surfaces by altering the physicochemical properties of the cell, thus helping to overcome electrostatic forces between interacting surfaces or via interactions between specific receptors (Ofek

et al., 2003; van Loosdrecht et al., 1987). Therefore, identification of bacterial structure(s) with adhesive properties provides a better understanding of bacterial attachment processes.

Generally, bacterial structures with adhesive activities are referred to as adhesins (An and Friedman, 1998). Numerous studies have identified adhesive properties of pathogenic bacteria that mediate attachment to epithelial cells (Shoaf-Sweeney and Hutkins, 2009). Although much is known about adhesive structures on pathogenic bacteria, more information is needed about their role in attachment of bacterial pathogens to beef tissue.

Flagella

Flagella are long, hair-like filaments with a helical shape that extend from the cytoplasmic membrane through the cell wall (Moens and Vanderleyden, 1996). A bacterial cell can have a single flagellum or multiple flagella. This structure produces a propulsive force that affords cell motility (Fernández and Berenguer, 2000). Flagella are considered virulence factors and potential adhesins, as they initiate cell-surface contact (Houdt and Michiels, 2005).

Flagellar-mediated motility provides active transportation of bacterial cells to surfaces, thus increasing the probability to initiate interaction with the target substratum (Firstenberg-Eden et al., 1978; Pratt and Kolter, 1999). Butler et al. (1979) reported that motile Gram-negative cells have a higher attachment rate to meat tissues than non-motile Gram-positive cells. Treatment of bacterial cells with anti-flagellin-antiserum also resulted in lower association with host cells as compared to untreated bacteria (Moens and Vanderleyden, 1996). It was also reported that the attachment rate of *S.* Choleraesuis to beef tissues was reduced after treatment with specific anti-flagella serum and mechanical removal of the flagella (Bouttier et al., 1997).

However, other studies on the importance of flagella in the attachment process do not support the results above. Prigent-Combaret et al. (2000) found that flagellar-mediated motility was not essential for initiating attachment nor for biofilm development. Studies on poultry showed that

motility of microorganisms did not influence attachment capabilities (McMeekin and Thomas, 1978) and the attachment rates of non-flagellated cells were not different from flagellated cells (Lillard, 1985). Studies on the attachment of *E. coli* strains to beef tissues showed a similar level of attachment between flagellated and non-flagellated strains, indicating that this structure did not play a major role in this process (Fratamico et al., 1996; Rivas et al., 2006).

Piette and Idziak (1991) who studied the role of flagella in the attachment of *Pseudomonas fluorescens* to beef tendon slices concluded that flagella indirectly mediate attachment by increasing the number of cells that reach the tendon surface and make the first contact with the target surface. However, attachment is reversible. The authors also concluded that although flagella contributed to attachment, their presence is not crucial to the process, as they observed de-flagellated cells were also able to attach to tendon in large numbers.

Fimbriae/pili

Fimbriae and pili are filamentous appendages anchored to the outer membrane of the bacterial surface particularly Gram-negative bacteria (An and Friedman, 1998; Frank, 2001; Madigan et al., 2009). These appendages are heteropolymers, built by repeating protein subunits called fimbrins or pilins with diameters ranging from 2 to 8 nm and between 0.2 and 2 µm in length, arranged peritrichously around the cells (Fernández and Berenguer, 2000; Klemm and Krogfelt, 1994). Fimbriae are considered a virulence factor in Gram-negative bacteria such as *E. coli* and *Salmonella* spp., in that cells with fimbriae are more infectious (An and Friedman, 1998).

Fimbriae are associated with host tissue adhesion properties of pathogenic strains. The most common adhesins found in fimbriae are type-1 fimbriae, P-fimbriae, and type-IV fimbriae (Fernández and Berenguer, 2000; Ofek et al., 2003). Type-1 and P-fimbriae are long rod-shaped rigid adhesive structures measuring approximately 7 nm in diameter with various lengths (Houdt and Michiels,

2005). P-fimbriae are composite structures composed of PapA subunits with a distinct fibrillar tip. The tip fibrillum is composed of PapE, PapF, PapK and the PapG adhesin. In contrast, type-1 fimbriae are primarily composed of the FimA subunit but with shorter fibrillar tip adhesins than those of P-fimbriae. The type-I fibrillar tip consists of FimF, FimG and the FimH adhesins. The type-IV fimbriae are 4 to 6 nm in diameter, more flexible and often form bundles (Ofek et al., 2003; Ørskov and Ørskov, 1990).

Aside from adhesin tips, fimbriae also mediate attachment by fimbriae-dependent surface hydrophobicity. Bacterial cell hydrophobicity was reported to be dependent on the types of fimbriae present (An and Friedman, 1998). Fimbriated bacteria are often more hydrophobic due to the presence of amino acids in proteins that contain numerous non-polar side chains, making the structure more hydrophobic (Frank, 2001).

Curli fimbriae

Curli are a type of fimbriae with a thin, wiry and highly aggregated surface. They appears as a 4-12 nm protein fibres but frequently appear as a capsule under the electron microscope (Ofek et al., 2003). Curli are expressed by many strains of *E. coli* including enterohemorrhagic (EHEC) and enterotoxigenic (ETEC) strains and also by certain *Salmonella* strains (Houdt and Michiels, 2005). Curli are heteropolymeric filamentous appendages, which are composed of a major subunit, CsgA, a 15-kDa protein and a minor subunit, CsgB, that may be distributed along the length of the curli fibre (Houdt and Michiels, 2005; Ofek et al., 2003).

Expression of curli on bacterial cells is influenced by environmental factors. Expression of curli on *E. coli* normally occurs during the stationary phase of growth, at low temperature (22-26°C), on low osmolarity medium but not at the optimum growth temperature (35-37°C)(Olsén et al., 1993a). Curli

may also be expressed by *Salmonella* and *E. coli* serotypes under stressful environmental conditions as a survival mechanism (Prigent-Combaret et al., 2000).

Curli expression is associated with attachment to cultured cell lines (Barnhart and Chapman, 2006), extracellular matrix proteins (Westerlund and Korhonen, 1993), abiotic surfaces (Pawar et al., 2005) and also in the development of biofilm on surfaces (Ryu et al., 2004). It was reported that curli production did not affect the initial attachment to stainless steel coupons (Ryu and Beuchat, 2005; Ryu et al., 2004) and the attachment to lettuce surfaces (Boyer et al., 2007). However, under appropriate conditions, curli-expressing EHEC strains were observed to attach more efficiently to beef tissues than the noncurli-expressing variants (Chen et al., 2007). However, the difference between attachments of both types of strains was minor. A similar observation was found for attachment of *E. coli* O157:H7 to Teflon and glass surfaces (Goulter-Thorsen et al., 2011).

Extracellular polysaccharides (EPS)

Extracellular polysaccharides (EPS) are substances secreted by bacterial cells (Bazaka et al., 2011). They can either be present as unbound "slime", which freely dissociates from the cell, or as cell-bound capsular polysaccharides anchored to the outermost surface of the cell and forming a distinct layer that encloses the cell (An and Friedman, 1998; Frank, 2001). Polysaccharides mainly consist of repeating monosaccharide units joined by glycosidic linkages (Bazaka et al., 2011). The production of these structures is highly influenced by environmental factors such as temperature, growth phase, nutrients, pH and macromolecule concentration (Junkins and Doyle, 1992; Ryu and Beuchat, 2005; Weiner et al., 1995)

The main function of EPS is as a physical protective barrier in response to environmental stresses; this structure had also been reported to play a role in bacterial adhesion and colonization (Weiner et al., 1995). Components of unbound EPS can be secreted by bacterial cells as a conditioning film on

target surfaces (Bazaka et al., 2011). Capsular polysaccharides can either be adhesive or anti-adhesive, as a capsular material can mask surface adhesins. When present, a hydrophillic capsule can mask bacterial cell hydrophobic components thus inhibiting bacterial attachment to hydrophobic surfaces (Ofek et al., 2003). In their studies, Hassan and Frank (2004) found that *E. coli* O157:H7 grown in media to enhance production of capsule had a greater ability to attach to apple and lettuce surfaces as compared to cells grown in media that did not support capsule production.

Meat surface structures involved in attachment

Carcass anatomy

After removal of the head, feet, hide, adhering fat tissues and internal organs, the beef carcass mainly consists of muscular and fatty tissue, bones and residues such as connective tissues. Approximately 49 to 68% of carcass weight represents muscular tissue (Kempster, 1993). Muscle consists of several levels of structure (Fig. 1) (Renou et al., 2003). The whole muscle body is surrounded by a sheet of connective tissue referred to as the epimysium (Valin et al., 1992). Below the epimysium is the perimysium that contains large blood vessels and nerves. The perimysium penetrates into the muscle, dividing the muscle body into primary and secondary muscle fibre bundles composed of variable numbers of individual muscle fibres. Perimysium components vary between muscle type, age, species and region within muscle (Taylor, 2004). Each of the individual muscle fibres is surrounded by fine collagenous endomyseal connective tissue known as the endomysium. Different from the variable perimysium, the endomysium composition does not differ in all muscle types or between species (Lawrie, 1998). Also, unlike both perimysium and epimysium that can be seen by the naked eye, endomysium can only be observed microscopically (Greaser, 1997). The collagenous fibres of the endomysium are linked to the muscle cell membrane through the basement membrane (Lawrie, 1998).

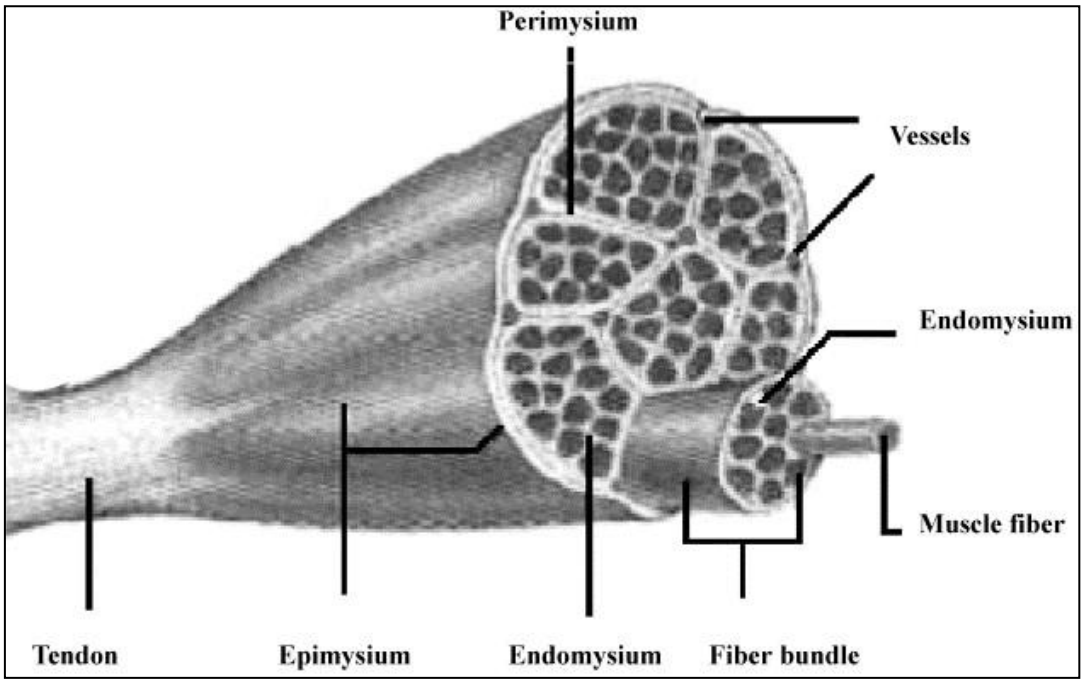

Fig. 1. Diagram of skeletal muscle with surrounding connective tissues. Image reproduced from Renou et al. (2003).

Muscle fibre

Muscle fibres are the core structure of the muscle body that consists of hundreds of myofibrils with multinucleated cells that contract. This structure develops from the fusion of mono-nucleated myoblasts that withdraw from the normal cell cycle, and subsequently differentiate into multinucleated myofibres during myogenesis (Swatland, 2004). Each of the individual muscle fibres are surrounded by a plasma membrane, the sarcolemma, located underneath the endomysium. The fibres are narrow, 10-100 µm in diameter and may reach 34 cm in length. Myofibrils are composed of repeating contractile units known as sarcomeres (Gregorio and Antin, 2000). The sarcomere is composed of two set of filaments that overlap within the sarcomere, giving rise to a pattern of alternating dark (A-band) and light (I-band) bands when viewed by light microscopy, hence, the term 'striated, skeletal muscle' (Calkins and Kilinger, 1993).

Studies on bacterial attachment to whole pieces of lean muscle tissue have been reported (Benito et al., 1997; Bouttier et al., 1997; Cabedo et al., 1997; Li and McLandsborough, 1999; Marin et al., 1997). However these studies were inconclusive in describing the specific meat structure(s) involved in the attachment process, since whole pieces of meat, comprised of muscle and fat fibres and intramuscular connective tissues were used.

Connective tissue

Connective tissue is a smaller component in most muscle types comprising approximately 1-4% of muscle dry weight (Taylor, 2004). Although it is only a minor component, its presence is ubiquitous and its role is important in tissue organization, fibre adhesion, force transmission and also as protective cover of the muscle body (Davies, 2004; Taylor, 2004). In farm animals, connective tissues are located at the fascia (between the skin and the skeletal tissues) and also within muscular tissues. The proportion of connective tissue and muscle fibre determine the relative toughness of meat and varies between muscles. The connective tissue structure is highly stable post-mortem and only changes slightly after several weeks (Greaser, 1997).

Connective tissue consists of fixed cells (fibroblasts and fat cells), free cells (macrophages and mast cells) and protein fibres that are all embedded in the extracellular matrix (ECM). Fibroblasts produce both the fibrous and the extracellular matrices of connective tissue. The protein fibres in the connective tissue mainly consist of collagen and elastin. Elastin fibres, although associated with the walls of blood vessels, are generally minor constituents of the connective tissue of muscle (Taylor, 2004).

Collagen is the major protein component (10-30mg/g of wet muscle weight) of connective tissues (Bailey and Light, 1989). Currently, 28 different types of collagen have been identified with each type varying in distribution in connective tissue layers (Miner, 2010). Collagen molecules (Fig. 2)

(approximately 300 nm long and 1.5 nm in diameter) are comprised of three polypeptide chains (also known as α-chains) wound into triple helical trimers with Gly-X-Y amino acid triple repeats (Alberts et al., 1994; Canty and Kadler, 2002). Collagen I, a fibril-forming collagen type, is the most abundant protein (approximately 90% of total collagen) in the skeletal muscle, dominating in the epimysium layer but can also be found in the perimysial and endomysial layers of connective tissue (Bailey and Light, 1989; Gelse et al., 2003). Collagen IV is mainly found in the endomysium/basement membrane of the ECM. In contrast with the fibril-forming collagen I, type IV collagen assembles into a meshwork type arrangement in the basement membrane (Greaser, 1997).

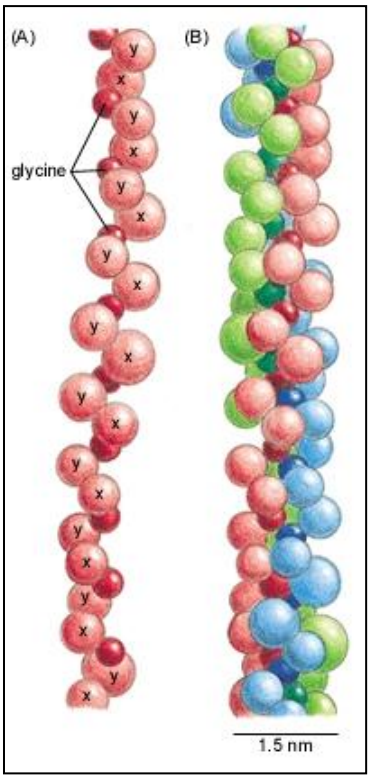

Fig 2. Diagram of collagen molecule structure. (A) A single collagen polypeptide chain composed of a series of triplet Gly-X-Y amino acids. (B) A triple stranded helical collagen structure composed of three polypeptide chains (represented in three different colours). Image reproduced from Alberts et al. (1994).

Besides collagen, intramuscular connective tissues consist of proteoglycans and glycoproteins. Proteoglycans, which include compounds such as hyaluronic acid and heparin sulphate, are large molecules in which a protein core is covalently linked to glycosaminoglycan chains. Glycoproteins anchor cells to basement membranes and other types of collagen. They consist of large protein polysaccharide molecules such as fibronectin and laminin that are found in the ECM basement membrane (Greaser, 1997).

Fibronectin is a disulfide-linked, dimeric glycoprotein (approximately 440 kDa) (Fig. 3) found in the endomysium and perimyisum layers of connective tissues (Alberts et al., 1994; Greaser, 1997). It is composed of three distinct repeating polypeptide modules in each fibronectin subunit (Xu and Mosher, 2011). Fibronectin plays a major role in cell-to-cell adhesion through interaction with cell surface receptors (intergrins) and in organizing the components of the ECM by binding with other extracellular matrix proteins such as collagen and laminin (Ito et al., 2003; Miner, 2010).

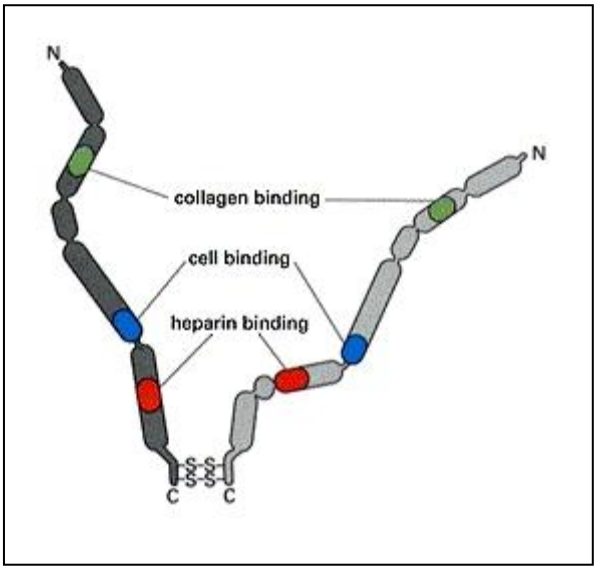

Fig. 3. Schematic model of a fibronectin dimer structure. Image reproduced from Alberts et al. (1994)

Laminin is a large protein (approximately 900 kDA) made of three different polypeptide chains, covalently linked by disulfide bonds, forming a cruciform- or T-shaped, four armed structure (Fig.4) (Alberts et al., 1994; Beck et al., 1990; Durbeej, 2010; Sasaki et al., 2004). It is the prominent glycoprotein in the basement membrane. Similar to fibronectin, laminin possesses distinct domains with properties for cell-to-cell adhesion, and also interaction with other ECM proteins including fibronectin, collagen IV and also to itself (Martin and Timpl, 1987).

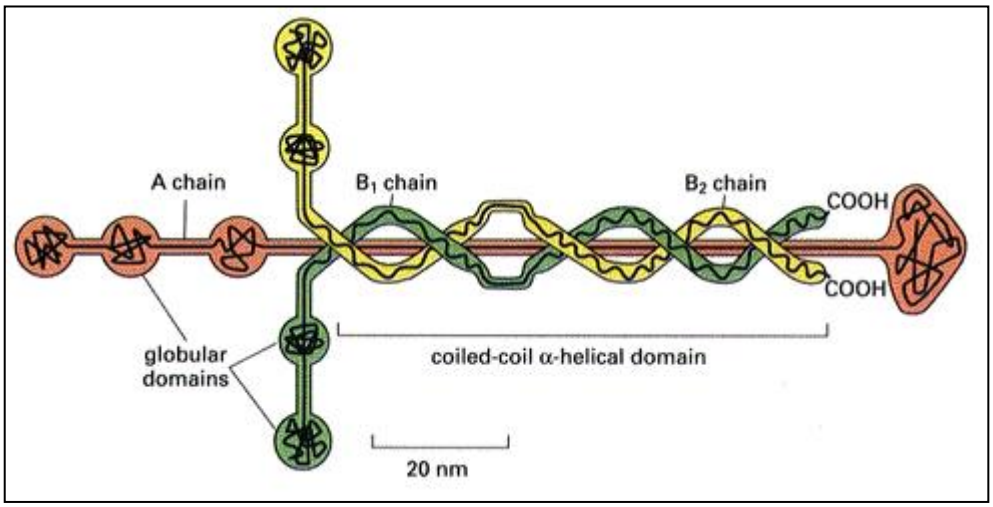

Fig 4. Laminin molecule structure. Image reproduced from Alberts et al. (1994).

Previous studies show that bacterial species such as *Salmonella, E. coli* O157:H7 and *Pseudomonas fluorescens* attach preferentially to connective tissues, such as collagen fibres (Frank, 2001). Also, *Salmonella* was reported to attach firmly to collagen fibrils of poultry muscle (McMeekin and Thomas, 1978; Sanderson et al., 1991). Microscopic examination of attachment to purified bovine collagen I and beef lean tissues show that *E. coli* and *Salmonella* isolates attach preferentially to collagen fibres and to endomysial reticulin fibrils that are composed of collagen I that surrounds muscle fibres (Benedict et al., 1991; Fratamico et al., 1996). This observation was also supported by

findings of Medina (2001) that show selective attachment of *E. coli* O157:H7 to veal breast collagen fibrils on skeletal muscle.

Various *E. coli* and *Salmonella* isolates were found to bind specifically to other ECM proteins, including fibronectin, collagen I, collagen IV and laminin via specific bacterial surface structures (Henderson et al., 2011; Ljungh et al., 1991; Ofek et al., 2003; Patti et al., 1994; Speziale et al., 1982; Visai et al., 1990; Westerlund and Korhonen, 1993). However, the studies on bacterial attachment to ECM proteins other than collagen I primarily focused on the human infection process. Studies of bacterial attachment to ECM proteins relevant to meat slaughter and processing are limited to reports by Medina and co-workers. However, only one *E. coli* O157:H7 and *Salmonella* Typhimurium as well as limited factor that influence attachment were tested in these studies (Medina, 2001; Medina, 2002; Medina, 2004; Medina and Fratamico, 1998).

Adipose tissue

Fat, or adipose tissue, is distributed in the beef carcass at three anatomically distinct locations; a subcutaneous depot on both sides of the superfacial fascia, the cutaneous muscle layer and the intermuscular depot (Davies, 2004). Adipose tissue is composed of adipocytes that are derived from mesenchymal cells. The adipocytes develop after proliferation and differentiation of adipoblasts which cannot initially be distinguished from other fibrogenic cells (Valin et al., 1992).

Bacterial strains were shown to attach to adipose tissue at similar rates as lean meat tissues (Cabedo et al., 1997; Dickson, 1991; Dickson and Koohmaraie, 1989). However, in another study, *Salmonella* Choleraesuis attached to beef adipose tissue in lower numbers than on lean meat (Bouttier et al., 1997).

Other factors

Temperature

Bacterial attachment to surfaces is influenced by temperature (Frank, 2001; Notermans and Kamplemacher, 1974; Selgas et al., 1993). Previous related studies were done either by varying the ambient experimental temperature during attachment or by testing the effect of different temperatures used to grow the inoculum.

Dickson (1991) determined the effect of culturing bacteria (*S*. Typhimurium and *L. monocytogenes*) at 23 and 37°C on attachment to beef tissues. The author observed that attachment of these bacterial strains to lean beef tissue was not affected by growth temperature, whereas cells grown at 23°C attached to adipose tissues in higher numbers than bacteria grown at 37°C. A storage temperature of 4°C was reported to have no effect on the strength of attached *E. coli* O157:H7 cells to lean beef tissues (Cabedo et al., 1997).

Growth temperature has been reported to influence the attachment of bacterial cells on various surfaces other than meat (Bonaventura et al., 2008; Dourou et al., 2011; Ells and Hansen, 2006; Norwood and Gilmour, 2001; Pompermayer and Gaylarde, 2000). The effect of temperature on bacterial attachment has been associated with production of bacterial surface structures. For example, it has been reported that expression of bacterial structures such as curli and EPS that may be involved in the attachment process are highly influenced by temperature (Junkins and Doyle, 1992; Olsén et al., 1993a; Ryu and Beuchat, 2005).

Ionic strength

The ionic strength of the surrounding medium affects the electrostatic interaction between surfaces (Bouttier et al., 1997; Piette and Idziak, 1992). However, there are different views on the

importance of electrostatic interactions in bacterial attachment to meat surfaces (Bouttier et al., 1994; Chung et al., 1989; Li and McLandsborough, 1999; Rivas et al., 2006).

An increase in the ion concentration of the attachment medium can reduce the thickness of electrostatic double layers formed by the bacterial cell and the target substratum surface charge (Frank, 2001). Consequently, the effect of surface charge may be masked. This enables the negatively charged bacterium to overcome the electrostatic repulsion forces, reduces the gap between the cell and the substratum, thus increasing the opportunities to initiate attachment (Hermansson, 1999). *Pseudomonas fluorescens* shows greater attachment to beef tendon tissue with increasing ionic strength of a potassium phosphate buffer and monovalent cation solutions, indicating the role of electrostatic interactions in attachment (Piette and Idziak, 1992). However, at higher concentrations of ionic strength (> 100mM), attachment of *P. fluorescens* and *E. coli* to beef lean tissue is inversely proportional to ionic strength (Li and McLandsborough, 1999; Piette and Idziak, 1992). It was suggested that at these high concentrations of salt, bacterial cells and meat tissue surface structures may be altered or denatured and therefore influence attachment. However, studies on other STEC *E. coli* (Rivas et al., 2006) and on *S*. Cholerasuis found that attachment to beef lean and adipose tissue are similar at different suspending medium ionic strength (Bouttier et al., 1997).

pH

pH has a variable effect on properties of bacterial attachment to whole meat tissues (Bouttier et al., 1994; Li and McLandsborough, 1999; Notermans and Kamplemacher, 1974; Selgas et al., 1993). It has been reported that attachment of *P. fragi* and *B. thermosphacta* to beef lean tissues is not affected by pH within the range of 5.8-7.2 (Bouttier et al., 1994). However, in a separate study that tested a wider range of pH, *E. coli* O157:H7 strongly bound to lean meat tissues at acidic (pH 4) or alkaline (pH 10) conditions as compared to a neutral pH (Li and McLandsborough, 1999). pH also

affects the bacterial cell surface charge, hydrophobicity and production of EPS that could affect cell attachment properties (Bouttier et al., 1994; Katsikogianni and Missirlis, 2004; Oliveira et al., 1994).

Bacterial attachment to receptors on mammalian cells

Lectin-carbohydrate

This type of interaction involves binding of lectins on bacterial surfaces to specific complementary carbohydrate receptors such as the glycolipids, glycoproteins and proteoglycans on a host cell surface (Sharon and Ofek, 2000; Shoaf-Sweeney and Hutkins, 2009). Various studies have identified lectins on bacterial cells such as *E. coli*, *Salmonella* spp., *Pseudomonas aeruginosa* and *Helicobactor pylori* that bind to specific carbohydrates on epithelial cells (Sharon and Ofek, 2000). In Gram-negative cells, bacterial surface lectins are frequently in the form of fimbriae (or pili) or as parts of fimbriae or as other types of outer membrane components, often made-up of hundreds of protein subunits that bind host oligosaccharides (Ofek et al., 2003; Shoaf-Sweeney and Hutkins, 2009).

Bacterial surface lectins may be found in the peptidoglycan matrix or may protrude through the cell wall from the cytoplasmic membrane in gram-positive bacteria (Ofek et al., 2003). Some known types are type 1 fimbriae that bind α-mannosyl groups in glycoproteins, P-fimbriae of uropathogenic *E. coli* that bind glycolipids containing α-D-galactopyranosyl-(1-4)-β-D-galactopyranoside (Galα(1-4)Galβ) moieties and the S–fimbriae of *E. coli* that bind NeuNAc α(2-3)Galβ. Type 1 mannose-specific fimbriae that contain the FimH adhesin have a single binding site for α-linked mannose derivatives, and are the most studied *E. coli* lectin. FimH adhesin is present and functional at the fimbrial tip and also along the fimbrial shaft at roughly 100-150 nm intervals (Pieters, 2011).

Bacterial cells express either various types of lectins (fimbrial adhesins) or different alleles of the same lectins with different carbohydrate receptor specificities or affinities. The expression of bacterial surface lectins is dependent on growth conditions (Ohlsen et al., 2009). Reduced mannose-binding activity was observed for *E. coli* K12 and *E. coli* 7343 strains grown to exponential phase, compared to stationary-phase strains showing reduced attachment to macrophages (Bar-shavit et al., 1980).

Bacterial surface lectins are considered virulence factors due to their ability to mediate attachment to host tissues. For example, type-1 fimbriae of urinary tract *E. coli* strains promote bacterial persistence thus increasing its virulence (Sharon and Ofek, 2000).

Bacterial attachment to host tissue mediated by bacterial surface lectins is very specific. The binding specificity can be determined by observing inhibition of attachment to target substratum with either simple or complex carbohydrates. The specificity of bacterial lectins is usually classified by both primary and fine sugar specificity. Primary sugar specificity refers to the simplest carbohydrate structure that inhibits lectin-mediated adhesion, whereas the fine sugar specificity defines the specific oligosaccharide structure of the most potent carbohydrate inhibitor (Ofek et al., 2003). Soluble carbohydrates recognized by the bacterial lectins block adhesion of the bacteria to host cells and can also displace attached bacteria from host cells. High concentrations of mannose have been shown to prevent the adhesion of type-1 fimbriated *E. coli* to epithelial and phagocytic cells (Bar-shavit et al., 1980; Pieters, 2011). Studies about the effect of carbohydrate on bacterial attachment to meat surfaces, especially beef, are very limited. Attachment of *Salmonella* spp. to beef tissues is not mediated by carbohydrates commonly found on epithelial cells. Bouttier et al. (1997) observed no effect on the attachment of *S.* Choleraesuis to lean meat tissue when different carbohydrates (D-mannose, D-mannosamine, methyl α-D-mannopyranoside, D-galactose, D-arabinose, D-glucosamine or D-galactosamine) were added into attachment medium. However,

there is little or no information on the effect of carbohydrates on attachment of *E. coli* cells to beef carcass surfaces.

Protein-protein

Protein-protein interactions involve recognition between a bacterial surface protein and complementary protein(s) on the host surface (Ofek et al., 2003). These types of interactions are usually associated with exposed ECM proteins and proteoglycans present on the surface of host tissues. Bacterial proteins that recognize and attach to one or more host ECM protein ligand, including fibronectin, laminin, collagen and elastin, are referred to as MSCRAMMs (microbial surface components recognizing adhesive matrix compounds) (Shoaf-Sweeney and Hutkins, 2009). Similar to bacterial lectins, MSCRAMMs are bacterial virulence factors due to an ability to mediate attachment to host tissue.

A bacterial cell can express several MSCRAMMs that recognize the same ECM protein ligand, whereas some individual MSCRAMM attach to more than one ECM protein. Interactions between a MSCRAMM with its ECM protein ligand are usually of high affinity and very specific (Ofek et al., 2003; Patti et al., 1994).

Bacterial adhesins such as type-1 fimbriae are an example of a MSCRAMM. Although this structure is classified as a bacterial lectin, studies have shown that type-1 fimbriae bound to fibronectin and attachment are not affected by periodate treatment of the ECM protein. This suggests that it is a protein-protein interaction and may not involve the carbohydrate component of fibronectin. This suggests that fimbrial-associated adhesins may serve a dual function in carbohydrate and protein ligand recognition (Patti et al., 1994).

Other examples of MSCRAMM are the Dr fimbriae of *E. coli* that attach to the amino-terminal triple helical 7S domain of collagen IV (Westerlund and Korhonen, 1993), and the ShdA autotransporter protein of *S.* Typhimurium that displays specific attachment to fibronectin (Henderson et al., 2011). P-fimbriae of *E. coli* possess two distinct adhesive proteins, one of which attaches to glycoconjugates and the other to ECM proteins that attach to the amino- and carboxy-terminal fragments of the fibronectin molecule (Henderson et al., 2011; Westerlund and Korhonen, 1993).

Studies on protein-protein interactions in the context of meat safety are limited to studies by Medina and co-workers (Medina, 2002; Medina, 2004; Medina and Fratamico, 1998). These authors examined interactions of *E. coli* O157:H7 and *S.* Typhimurium with ECM proteins, (collagen, laminin and fibronectin) and glysaminoglycans (hyaluronic acid and chondroitin sulphate), using a surface resonance biosensor (BioCore). Although this provided useful information, only two bacterial strains were used and the influence of abattoir-relevant conditions on the attachment process was not included in their studies. It would be beneficial if the experimental design was extended to factors relevant to meat slaughter and processing, such as temperature and pH that may influence bacterial interactions with ECM protein ligands on the carcass surface.

Problem statement

Published studies describe mechanisms and factors that mediate bacterial attachment to meat surfaces; however they were limited to whole pieces of meat, either lean or fat tissue (Cabedo et al., 1997; Chen et al., 2007; Marin et al., 1997; Rivas et al., 2006; Warriner et al., 2001). The heterogeneous nature of whole meat tissue (Bouttier et al., 1997; Cabedo et al., 1997) however, precludes understanding specific processes involved with bacterial attachment to carcass tissue structures. Such information could lead to more effective carcass microbial interventions.

Little is known about attachment of diverse enterohemorrhagic *E. coli* (EHEC) and *Salmonella* strains to meat surfaces under conditions relevant to abattoir environments. Although the scientific literature contains a large number of reports about *E. coli* O157:H7 due to its impact on public health in the USA, UK and Japan (Desmarchelier and Fegan, 2003), there is inadequate information about other *E. coli* serotypes that cause EHEC infections in Australia (Anonymous, 2003; Sumner et al. 2005). Specifically, Australian epidemiological data report non-O157 EHEC serotypes that include O26, O111 and O4:H5 (Anonymous, 2003; Bettelheim, 2003; Sumner et al., 2005).

Even less is known about the attachment properties of *Salmonella* spp. that are associated with more cases of human infections than *E. coli*. For example, in 2006, 8331 cases of *Salmonella* infection were reported by OzFoodNEt compared to only 73 cases of Shiga toxin-producing *E. coli* (Anonymous, 2006).

As of yet, there has not been a single comprehensive study examining attachment properties of *E. coli* and *Salmonella* strains relevant to Australia on different types of beef tissues.

Aims of this study

By understanding the mechanisms and factors that mediate bacterial attachment to meat surfaces, novel interventions could be designed to reduce or prevent microbial contamination of raw beef products. Such knowledge will increase the safety and quality of products destined for human consumption. Therefore the aims of this project are the following:

GENERAL AIMS:

- To define the properties of bacterial attachment to meat surface structures by a range of EHEC and *Salmonella* spp. bacterial strains

SPECIFIC AIMS:

- To determine the properties of bacterial attachment to specific meat structures, namely ECM proteins (Chapter 2) and muscle cells (Chapter 4).
- To measure the influence of physicochemical and environmental factors on the attachment of EHEC and *Salmonella* sp*p* strains to meat structures (Chapter 3).
- To characterize the sites that mediates bacterial attachment to ECM proteins (Chapter 5).

Chapter 2

Bacterial attachment to immobilized extracellular matrix proteins in vitro

Abstract

Meat surfaces are contaminated with bacteria during slaughter and processing. Understanding bacterial attachment properties to specific structures of meat could result in more targeted interventions to improve its safety and quality. However, the influence of temperatures relevant to abattoir environments on bacterial attachment to specific meat structures is not known. In this study, the effect of temperature and protein concentration on attachment of 10 *Escherichia coli* and seven *Salmonella* strains to extracellular matrix (ECM) proteins (collagen I, fibronectin, collagen IV and laminin) was measured using crystal violet stain and epifluorescence microscopy assays. By crystal violet assay, only five of 17 strains showed significant attachment to any ECM protein and only one strain attached to all proteins. Strains that attached at all tested temperatures (4, 25, 37°C) were *E. coli* M23Sr and M23 (collagen I); *E. coli* M23Sr (fibronectin); *E. coli* M23Sr, O157:H12 and M23, (collagen IV); and *E. coli* M23Sr, O157:H12, O78:K80:H1, O26:H11 and M23 (laminin). A higher proportion of strains attached to basement membrane proteins (laminin and collagen IV) than to interstitial proteins (collagen I and fibronectin). Highest attachment levels occurred at 4°C for collagen I and at 25°C for the other three proteins. Generally, the attachment levels of *Salmonella* strains to all ECM proteins were lower than for *E. coli*. No significant attachment effect was found for ECM concentration for collagen I, fibronectin and collagen IV, but was for higher laminin concentrations. A strong positive correlation was found between results of both the crystal violet and epifluorescent methods ($r \geq 0.905$, $p < 0.05$). This study demonstrated that attachment properties to ECM proteins displayed distinct variation among strains, that temperature highly influenced attachment and that protein concentration had a minor effect.

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Introduction

Microbial contamination of carcass surfaces normally occurs during slaughter and post-slaughter processing. The initial cut of hides during the dehiding process is the immediate source of contamination, while subsequent post-slaughter procedures such as evisceration, trimming and washing contributes to further transfer and redistribution of microorganisms (Bell, 1997; Gill and McGinnis, 2003; Jackson et al., 2001). Besides direct introduction from contaminated sites, microorganisms can be transferred via worker's hands, equipment, aerosols and dust generated from the hide (Jackson et al., 2001). An understanding of bacterial attachment may lead to more effective carcass interventions since the strength of attachment to meat surfaces has been shown to interfere with physical and chemical removal processes (Benedict et al., 1991; Fratamico et al., 1996; Tamblyn and Conner, 1997).

Although studies have described the effect of temperature on bacterial attachment, they have been limited to either a single temperature or to temperatures that do not fully encompass those in an abattoir environment. Examples of single temperature studies include the role of bacterial physicochemical properties, and bacterial and target cell surface structures that mediate binding (An and Friedman, 1998; Benito et al., 1997; Dickson and Koohmaraie, 1989; Ryu and Beuchat, 2005). Dickson (1991) determined the effect of culturing bacteria at 23 and 37°C on subsequent attachment at 25°C. Cabedo et al. (1997) investigated the effect of 4°C storage temperature on the strength of attachment. Thus, no single study has systematically addressed bacterial attachment to beef surface proteins at temperatures (i.e. 4 to 37°C) that encompass abattoir environments, which may critically affect adherence to the carcass surface.

The majority of published studies have used pieces of meat, either lean or fat tissue, in experimental systems (Cabedo et al., 1997; Chen et al., 2007; Marin et al., 1997; Rivas et al., 2006; Warriner et al., 2001). However, due to the complex and heterogeneous properties of whole meat

tissues, results of these studies cannot describe attachment to specific tissue structures. Consequently, a more mechanistic understanding of attachment is required.

Bovine connective tissues are located at the fascia, between the skin and skeletal structure, and also within muscle. Although connective tissues are a minor component of most muscle types, their presence is ubiquitous on the carcass surface. The connective tissues are composed of a mixture of various extracellular matrix (ECM) proteins and complex polysaccharides. Dominant ECMs include collagen I, fibronectin, collagen IV, and laminin (Bailey and Light, 1989; Taylor, 2004).

Studies have shown that bacterial species such as *Salmonella* and *E. coli* O157:H7 attach preferentially to connective tissues, such as collagen fibres (Frank, 2001). In addition, Sanderson et al. (1991) reported that *Salmonella* strains attach to collagen in poultry fascia, especially to glycosaminoglycans that surround the collagen fibrils after extended immersion in water. In studies of purified bovine collagen I and beef tissues using scanning electron microscopy, *E. coli* O157:H7 were shown to attach primarily to collagen fibres and to endomysial reticulin fibrils which are composed of collagen I surrounding muscle fibres (Fratamico et al., 1996; Medina, 2001). However, such studies of connective tissue structures involved a single temperature (20-25°C), and may not explain attachment processes entirely relevant to an abattoir environment.

Studies of bacterial attachment to ECM proteins have primarily focused on temperatures relevant to human infections and not abattoirs (Baloda, 1988; Doig and Trust, 1993; Kuusela et al., 1985; Ofek et al., 2003; Olsén et al., 1993b; Patti, 1994). For example, investigators studied the attachment of various human pathogens to ECM proteins exposed during tissue injury at 37°C (Stępińska and Trafny, 1995; Visai et al., 1990). In studies relevant to food production, limited to investigations by Medina and co-workers (Medina, 2001; Medina, 2002; Medina, 2004; Medina and Fratamico, 1998), a single temperature was tested, as well as only one *E. coli* O157:H7 and *Salmonella* Typhimurium strain.

Understanding bacterial attachment to meats should reflect the variety of enterohaemorrhagic (EHEC) *E. coli* and *Salmonella* strains that contaminate abattoir environments. According to Australian surveillance data, *E. coli* O157 is not the only serogroup causing EHEC infections in Australia (OzFoodNet Working Group, 2003; Sumner et al., 2005). Rather, human illness is commonly attributed to non-O157 EHEC serotypes that include O111 and O26 (OzFoodNet Working Group, 2009). Even less is known about the attachment characteristics of *Salmonella* spp. which are associated with more cases of human infection than *E. coli.* For example, in 2008, 8310 cases of *Salmonella* infection were reported by OzFoodNet compared to only 106 cases of Shiga toxin-producing *E. coli* (OzFoodNet Working Group, 2009).

Designing interventions that prevent or reduce bacterial attachment to beef products may be attained by understanding the mechanisms involved in interactions between bacteria and meats under conditions that are relevant to abattoir environments. Currently, there is no information about the influence of different temperatures commonly found in abattoirs on the attachment of bacteria to specific connective tissues of carcasses, such as ECM proteins. This study reports attachment properties of 10 *E. coli* and seven *Salmonella* isolates to four types of ECM proteins at 4, 25 and 37°C, as well as the effect of ECM protein concentration.

Materials and methods

Bacterial strains and culture conditions

Seventeen bacterial strains consisting of *E. coli* (10 strains) and *Salmonella* (seven strains) were used in the study (Table 1). Strains were obtained from the University of Tasmania Food Safety Centre collection, along with strains generously provided by Dr Narelle Fegan (CSIRO Food and Nutritional Sciences, Werribee, Australia) and Dr. R. Robins-Browne (Department of Microbiology

and Immunology, University of Melbourne, Melbourne, Australia). Stock cultures were kept in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, England) and stored at -80°C. Working cultures were maintained on nutrient agar (NA; Oxoid (CM0003), Basingstoke, UK) and stored at 4°C for not more than one month. For experiments, bacteria were grown overnight in tryptone soy broth (TSB; Oxoid (CM0129), Basingstoke, UK) for 18-20 h at 37°C without shaking. Cell suspensions were prepared by centrifuging the TSB culture at 3210 × *g* for 5 min. Cells were washed twice in Dulbecco A phosphate buffered saline, pH 7.3 (PBS; Oxoid (BR0014), Basingstoke, UK) before resuspending in PBS to achieve approximately 10^9 CFU/ml. One hundred microliters of appropriately diluted cell suspension were plated on tryptone soy agar (TSA; Oxoid (CM0131), Basingstoke, UK) in duplicate to confirm the inoculum level. Plates were incubated at 37°C for 24 h before colonies were counted.

Preparation of extracellular matrix protein coated surface

Extracellular matrix (ECM) proteins consisting of collagen I (calf skin collagen, Sigma, USA), fibronectin (bovine plasma fibronectin, Sigma, USA), collagen IV (from Engelbreth-Holm-Swarm murine sarcoma; BD Biosciences, Bedford, USA) and laminin (from Engelbreth-Holm-Swarm murine sarcoma; Sigma, Israel) were prepared according to the manufacturer's instructions. Two hundred and fifty microliters of protein solution (collagen I and fibronectin = 50, 100, 200 µg/ml; collagen IV = 50, 100 µg/ml; laminin = 25, 50 µg/ml) (Medina, 2001; Medina and Fratamico, 1998) were coated onto 96-wells tissue culture plates (Greiner Bio-one, Germany) and incubated at 37°C for 1 h. Excess fluid was removed from coated wells and plates were kept at 4°C until experimentation. Coated surfaces were rinsed with 200 µl PBS and air-dried at room temperature for 10 min prior to experimentation. Bovine serum albumin (BSA) was not used to saturate the ECM coated wells in this study as separate trials demonstrated that strains attached to BSA, potentially masking the

differential effects of ECM proteins (see Fig. A.1: Appendix A). However even with the addition of BSA, similar trends in strain attachment to ECM proteins were observed.

Measurement of bacterial attachment using crystal violet staining

Bacterial attachment to ECM proteins was measured using modifications of the method reported by Štyriak et al. (1999). The modifications included the incubation temperatures, the usage of ethanol to release the stain bound to the bacteria and the wavelength used to measure the absorbance. Briefly, 100 µl of bacterial suspension and 100 µl of PBS (control wells) were added to protein-coated wells. After incubation for 2 h at 4, 25 and 37°C, bacterial suspensions were removed by pipette and plates were washed three times with 200 µl PBS to remove loosely attached cells. Attached cells were heat-fixed at 55°C for 20 min and were then stained with 95 µl of filtered 1% crystal violet for 45 min at 25°C. Excessive crystal violet stain was removed by rinsing the wells five times with sterile distilled water and then air-dried for 30 min at room temperature. Next, 100 µl of 95% ethanol were added to test wells for 15 min to destain the wells. Attached cells were quantified by measuring absorbance values using a microplate reader (Bio-rad® Benchmark microplate reader, USA) at 595nm. Experiments were performed in three replicate wells and the average values calculated. Initially, some experiments were repeated with biological replicates for selected strains and conditions, but no significant differences were observed. Accordingly, further biological replication was not conducted for all treatments. The readings obtained from samples using the crystal violet method were corrected by subtracting the average absorbance from control wells.

Table 1. List of experimental bacterial strains.

Bacterial species	Serotype	Strain abbreviation	Source	Notes
Escherichia coli	M23Sr (with streptomycin resistance), OR:H-	EC01	unknown	Non-pathogenic
Escherichia coli	O157:H7 Sakai	EC02	clinical isolate from Sakai outbreak	EHEC strain
Escherichia coli	O157:H7	EC03	beef cattle hide, EC623[a]	EHEC strain
Escherichia coli	O157:H-	EC04	calf feces, EC516 [a]	EHEC strain
Escherichia coli	O157:H12	EC05	beef cattle feces, EC614 [a]	Non-Shiga toxin producing strain
Escherichia coli	O26:H11	EC06	dairy cattle feces, EC473 [a]	EHEC strain
Escherichia coli	O111:H-	EC07	calf feces, EC132 [a]	EHEC strain
Escherichia coli	O127:H6	EC08	E2348/69 [b]	EPEC strain
Escherichia coli	O78:K80:H11	EC09	H10407 [b]	ETEC strain
Escherichia coli	M23 (parent strain), OR:H-	EC10	unknown	Non-pathogenic
Salmonella enterica	Typhimurium 135	ST135	beef cattle feces, Sal691[a]	
Salmonella enterica	Typhimurium 9	ST9	cattle feces, Sal1013[a]	
Salmonella enterica	Infantis	SI	cattle feces, Sal1023[a]	
Salmonella enterica	Anatum	SA	sheep feces, Sal1729a[a]	
Salmonella enterica	Chester	SC	sheep feces, Sal1613a[a]	
Salmonella enterica	Mississippi	SM	sewage	
Salmonella enterica	Sofia	SS	food (poultry product)	

[a] Strain received from Dr. Narelle Fegan, CSIRO Food and Nutritional Sciences, Werribee, Australia

[b]Strain received from Dr. R. Robins-Browne and Kristy Azzopardi/Stojcevski, Department of Microbiology and Immunology, University of Melbourne, Melbourne

Microscopic measurement of bacterial attachment to ECM proteins

Six strains were tested for each type of protein (Collagen I: *E. coli* M23Sr, O157:H7 Sakai, O157:H7, M23, *S*. Typhimurium 135 & S. Anatum; Fibronectin: *E. coli* M23Sr, O157:H7, O157:H12, O127:H6, *S*. Anatum & *S*. Mississippi; Collagen IV: *E. coli* M23Sr, O157:H-, O157:H12, O26:H11, M23 & *S*. Infantis; Laminin: *E. coli* M23Sr, O157:H7, O157:H12, O26:H11, O127:H6 & O78:K80:H11). Strains were chosen based on "high", "medium" and "low/negative" binding observed for individual ECM proteins in the crystal violet assay described above. Two hundred and fifty microliters of protein solution were coated on 8-well chamber slides (Permanox®, Nunc® Lab-Tek®, USA) according to procedures described above. Next, 100 µl of bacterial suspension were added to coated wells and incubated at 25°C for 2 h. The wells were subsequently washed three times with 300 µl PBS to remove loosely attached cells and then fixed with heat at 55°C for 20 min. The slides were kept overnight at 4°C and air-dried for 15 min prior to staining. The assay was repeated with two biological replicates for each strain and protein.

Fixed slides were stained with 0.02% acridine orange (ProSciTech, Australia) for 3 min in the dark (Duffy and Sheridan, 1998). Slides were washed with deionized water three times to remove excessive stain and then air-dried at room temperature for 15 min. The attached cells were imaged using a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Ten random fields per slide were observed under 100x oil immersion objective. Each image was analysed using ImageJ v.1.4 software (US National Institutes of Health, http://rsb.nih.gov/ij/) and the percentage of area covered by fluorescing cells calculated (total area covered with attached cells / field of view surface area).

Lower detection limit for the crystal violet assay

The lower detection limit for the crystal violet assay was determined by plotting data from both the crystal violet and epifluorescence microscopy methods for all proteins tested (see Fig. A.2: Appendix A). The line of best-fit was determined, including upper and lower 95% confidence limits. The lower detection limit of the crystal violet assay was determined by using the measurement error value of the multiplate reader and the 95% confidence interval in log CFU. To determine the measurement error of the instrument, log mean error was calculated from all the negative OD values obtained from the crystal violet stain. The calculated lower sensitivity limit was -1.63 log OD. The 95% confidence interval for the detection limit for log CFU was calculated using the equation of the trendline for both lower (y = 0.8875x – 8.2744) and upper (y = 0.8875x- 6.8483) 95% confidence limit. The estimation of the lower detection limit for the crystal violet assay was between log 5.8 and 7.4 CFU/cm^2 with the average value of log 6.6 ± 0.8 CFU/cm^2.

Statistical analysis

Statistical analysis of the effects of temperature on attachment to ECM proteins was done by pooling data for all protein concentrations tested. The same was done for the analysis of concentration-dependent effects by pooling all data for temperatures tested. Data were analysed using one-way analysis of variance (ANOVA), post-hoc comparison using the Least Significant Difference (LSD) test and Pearson product moment correlation coefficient. All statistical tests were performed using the PASW® statistic 18.0 (SPSS Inc.). Results were considered significant when $p < 0.05$, unless otherwise stated.

Results

Bacterial attachment to extracellular matrix proteins measured by crystal violet staining

Only five strains showed significant attachment to ECM proteins measured by crystal violet assay (Table 2). Other strains produced absorbance values that were equal to or less than the mean absorbance values of the negative control wells for most conditions tested (data not shown).

Results from this study showed wide variation in attachment to ECM proteins among the strains tested. In general, *E. coli* strains had higher binding capacity to ECM proteins than *Salmonella* strains. Attachment to ECM proteins also varied noticeably among different strains within the same species. There was no significant difference in attachment of O157 and non-O157 (pathogenic) *E. coli* strains.

Overall analysis of the effect of protein concentration on bacterial attachment showed no significant influence except for laminin where levels of attached cells were greater at the highest concentration of laminin tested (50 μg/ml).

Collagen I

Of the 10 *E. coli* and seven *Salmonella* strains tested, only two *E. coli* strains; *E. coli* M23Sr and M23 showed significant levels of attachment to collagen I. However, the attachment of M23 was not significant at 37°C. Both strains displayed greater attachment at 4°C than other temperatures. Attachment of M23Sr was significantly higher than M23 at 25 and 37°C, but not at 4°C where no significant difference was observed.

Table 2. Effect of temperature and protein concentration on bacterial attachment to ECM proteins assessed by crystal violet staining method. Bacteria were incubated for 2 h in wells at selected temperatures and the mean absorbance measured at 595 nm.

ECM protein	Strains[1]	Protein concentration (µg/ml)	Mean (SEM) absorbance values[2,3]		
			Temperature		
			4°C	25°C	37°C
Collagen I	EC01^{A}	50	0.262 (0.004)^{Ab}	0.160 (0.093)^{Bb}	0.144 (0.083)^{Bb}
		100	0.429 (0.009)^{Aa}	0.252 (0.146)^{Bb}	0.294 (0.170)^{Ba}
		200	0.253 (0.024)^{Ab}	0.226 (0.130)^{Bb}	0.244 (0.141)^{Bb}
	EC10^{B}	50	0.224 (0.012)^{Aa}	0.134 (0.078)^{Ba}	0.017 (0.010)^{Ca}
		100	0.460 (0.043)^{Aa}	0.070 (0.040)^{Ba}	0.032 (0.018)^{Ca}
		200	0.459 (0.041)^{Aa}	0.074 (0.043)^{Ba}	0.012 (0.007)^{Ca}
Fibronectin	EC01	50	0.016 (0.009)^{Ba}	0.092 (0.053)^{Aa}	0.046 (0.027)^{Ba}
		100	-	0.175 (0.101)^{Aa}	0.043 (0.025)^{Ba}
		200	0.071 (0.041)^{Ba}	0.187 (0.108)^{Aa}	0.049 (0.028)^{Ba}
Collagen IV	EC01^{A}	50	0.062 (0.036)^{Ba}	0.097 (0.056)^{Aa}	0.104 (0.060)^{Aa}
		100	0.067 (0.039)^{Ba}	0.137 (0.079)^{Aa}	0.160 (0.092)^{Aa}
	EC05^{B}	50	0.122 (0.070)^{Ba}	0.101 (0.059)^{Aa}	0.014 (0.008)^{Ca}
		100	0.118 (0.068)^{Ba}	0.147 (0.085)^{Aa}	-
	EC10^{C}	50	0.014 (0.008)^{Ba}	0.042 (0.024)^{Aa}	0.041 (0.024)^{Aa}
		100	0.008 (0.005)^{Ba}	0.032 (0.018)^{Aa}	0.053 (0.030)^{Aa}
Laminin	EC01^{A}	25	0.380 (0.219)^{Aa}	0.403 (0.233)^{Aa}	0.117 (0.068)^{Ba}
		50	0.418 (0.242)^{Aa}	0.294 (0.170)^{Aa}	0.190 (0.110)^{Ba}
	EC05^{B}	25	0.152 (0.088)^{Aa}	0.279 (0.161)^{Aa}	0.136 (0.079)^{Ba}
		50	0.438 (0.253)^{Aa}	0.362 (0.209)^{Aa}	0.071 (0.041)^{Ba}
	EC06^{D}	25	0.107 (0.062)^{Aa}	0.087 (0.050)^{Ba}	-
		50	0.293 (0.169)^{Aa}	0.148 (0.085)^{Ba}	-
	EC09^{C}	25	0.140 (0.081)^{Ab}	0.131 (0.075)^{ABb}	0.068 (0.039)^{Bb}
		50	0.242 (0.140)^{Aa}	0.165 (0.095)^{ABa}	0.148 (0.085)^{Ba}
	EC10^{E}	25	0.030 (0.018)^{Ab}	0.029 (0.017)^{Ab}	0.019 (0.011)^{Ab}
		50	0.060 (0.034)^{Aa}	0.038 (0.022)^{Aa}	0.050 (0.029)^{Aa}

Strains nomenclature is detailed in Table 1.

[1] Letters indicate differences among strains within the same ECM protein ($p< 0.05$).

[2] Capital letters for the same protein concentration indicate differences among temperatures within the same ECM protein and the same strain ($p< 0.05$).

[3] Small letters indicate differences between protein concentrations within the same ECM protein and the same temperature ($p< 0.05$).

- Indicates the absorbance values are equal or less than the control absorbance value

Fibronectin

Only *E. coli* M23Sr significantly attached to fibronectin at all temperatures tested. The highest level of attachment was observed at 25°C, with progressively lower attachment at 37 and 4°C. Some *Salmonella* strains showed low attachment levels (absorbance values ≤ 0.05) at 25°C (*S.* Anatum, *S.* Typhimurium 135 and *S.* Mississippi) and at 37°C (*S.* Typhimurium 9 and *S.* Infantis) but not at all protein concentrations. *Escherichia coli* O111:H- also attached to fibronectin at 37°C (absorbance values ≤ 0.053), but not at the lowest protein concentration tested (50 µg/ml).

Collagen IV

Only *E. coli* M23Sr, *E. coli* O157:H12 and *E. coli* M23 significantly attached to collagen IV at all temperatures and attachment was highest at 25°C. *Escherichia coli* M23Sr attached significantly less to collagen IV at 4°C which contrasted with *E. coli* O157:H12 where attachment was significantly lower at 37°C. *Escherichia coli* O26:H11 and *S.* Chester only significantly attached to 100 µg/ml collagen IV at 4°C (EC06 = 0.128 ± 0.074; SC = 0.033 ± 0.019), whereas at 25°C, *E. coli* O127:H6 and four *Salmonella* strains (*S.* Typhimurium 135, *S.* Typhimurium 9, *S.* Infantis and *S.* Anatum) displayed significant attachment (absorbance values ≤ 0.05).

Laminin

Bacterial strains that significantly attached to laminin were *E. coli* M23Sr, *E. coli* O157:H12, *E. coli* O78:K80:H11, and *E. coli* O26:H11. Overall, attachment was greater at 25°C; however the difference was not significant when compared to 4°C. The lowest level of attachment occurred at 37°C. For *E. coli* O26:H11, attachment was only found at 4°C and 25°C. *Escherichia coli* M23 attached significantly to laminin at all temperatures but only at the highest protein concentration (50 µg/ml).

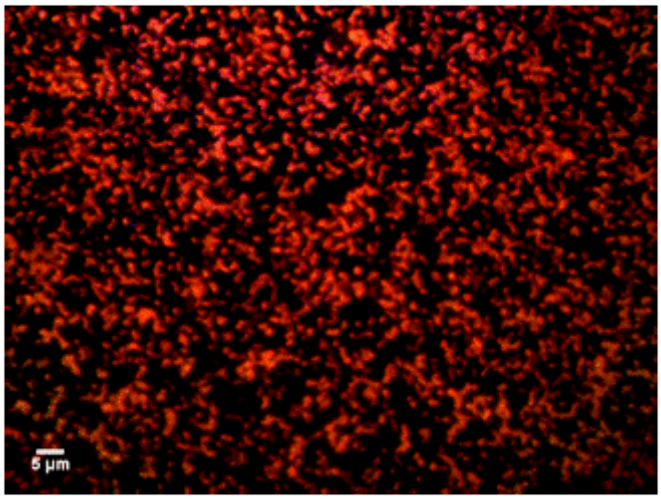
At 25°C, *S.* Typhimurium 135*, S.* Typhimurium 9, *S.* Infantis, *S*. Anatum, *S*. Chester and *S*. Sofia also attached to laminin (absorbance values < 0.1). Overall analysis on the effect of protein concentration on attachment was shown to be significant for this protein.

Microscopic assay of bacterial attachment to extracellular matrix proteins

Results of the microscopic assays showed that most of the bacterial cells, especially high binding strains, attached to ECM protein-coated surfaces in clumps and this interfered with enumeration. Representative images of high-binder strains are shown in Fig. 1. Therefore, levels of attached cells (Fig. 2) were measured by calculating the percentage area covered with cells, as described in the Materials and Methods section.

Bacterial attachment to ECM protein-coated surfaces showed a strong positive correlation with results by the crystal violet staining method for each type of protein (Collagen I: $r^2 = 0.882$, $p < 0.01$; Fibronectin: $r^2 = 0.906$, $p < 0.01$; Collagen IV: $r^2 = 0.887$, $p < 0.01$; Laminin: $r^2 = 0.819$, $p < 0.05$). Non-binding strains, as deduced by the crystal violet staining, did attach to ECM proteins as revealed by the microscopic assay (Fig. 3) but at levels slightly lower (average value = log 6.56 ± 0.37 CFU/cm^2) than what can be reliably detected by the crystal violet staining method (lower detection limit was log 6.6 ± 0.8 CFU/cm^2). The attachment levels of these non-binding strains were significantly lower than the high-binding strains observed by crystal violet assay (log 7.63 ± 0.18 CFU/cm^2).

A



B

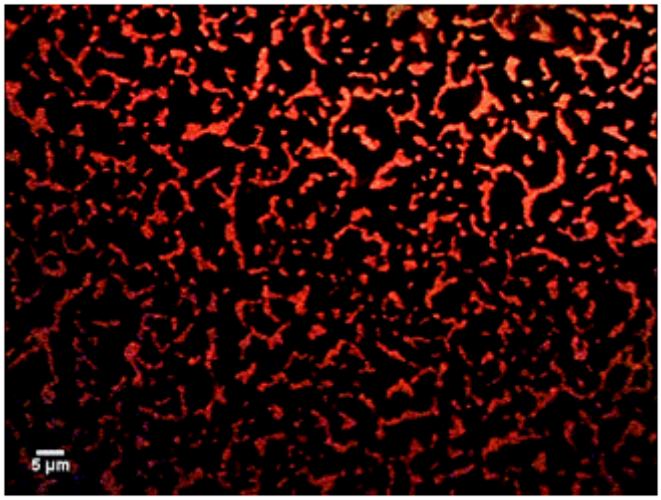
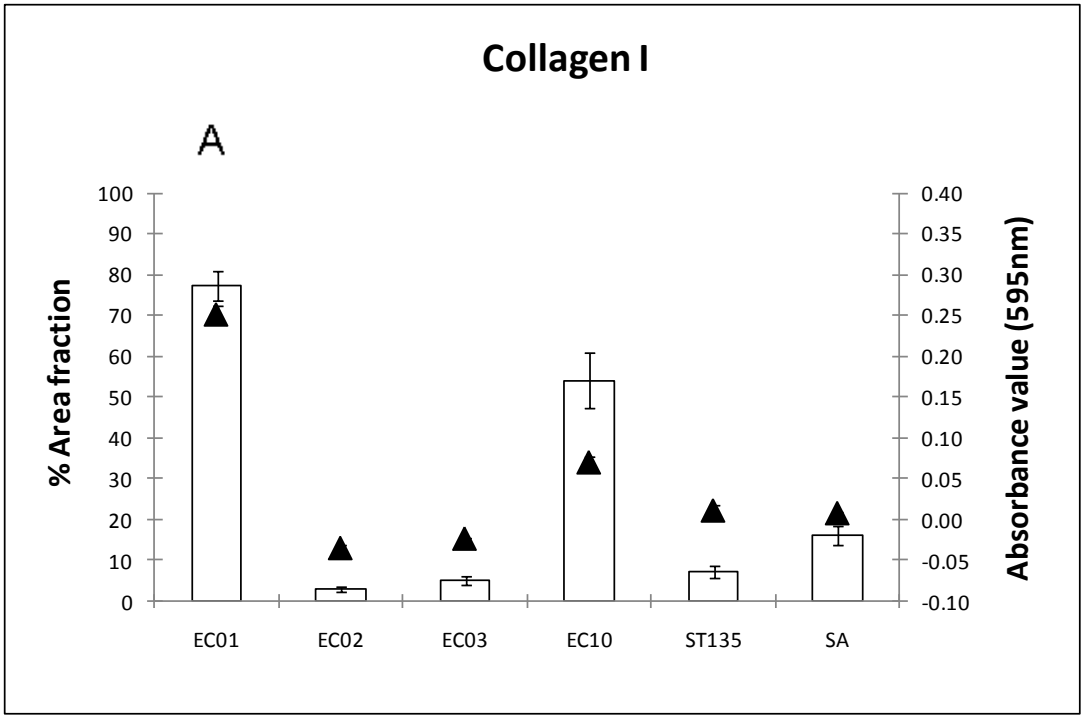
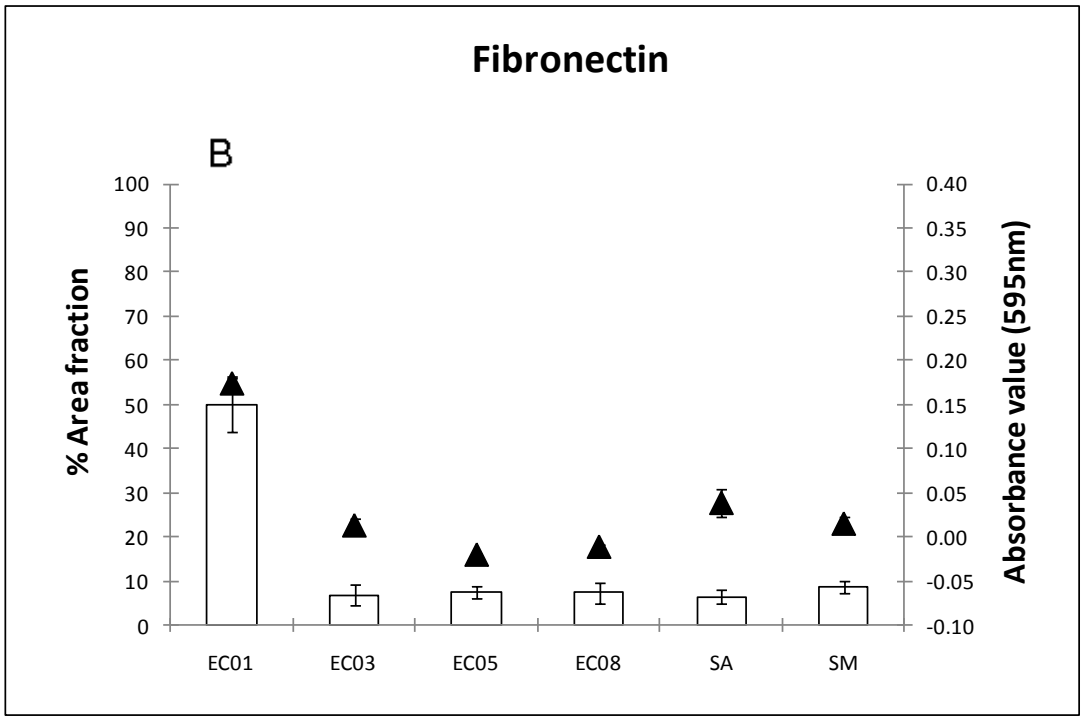


Fig. 1: Micrographs of bacterial attachment to ECM coated surface (incubated for 2 h at 25°C) that represent as high binder strains based on crystal violet stain assay. (A) *E. coli* M23 attached to collagen I (B) *E. coli* O157:H12 attached on laminin. Images were observed under oil-immersion objective.


Collagen I
A
% Area fraction
Absorbance value (595nm)
100
90
80
70
60
50
40
30
20
10
0
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
EC01
EC02
EC03
EC10
ST135
SA


Fibronectin
B
% Area fraction
Absorbance value (595nm)
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90
80
70
60
50
40
30
20
10
0
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0.35
0.30
0.25
0.20
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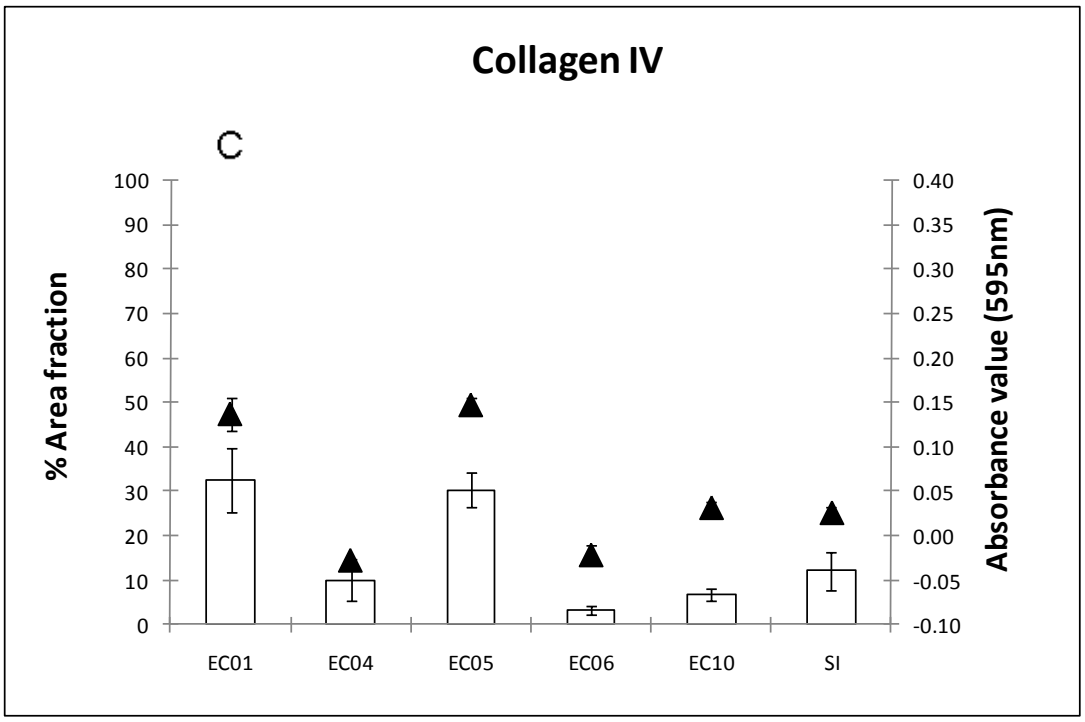
EC01
EC03
EC05
EC08
SA
SM



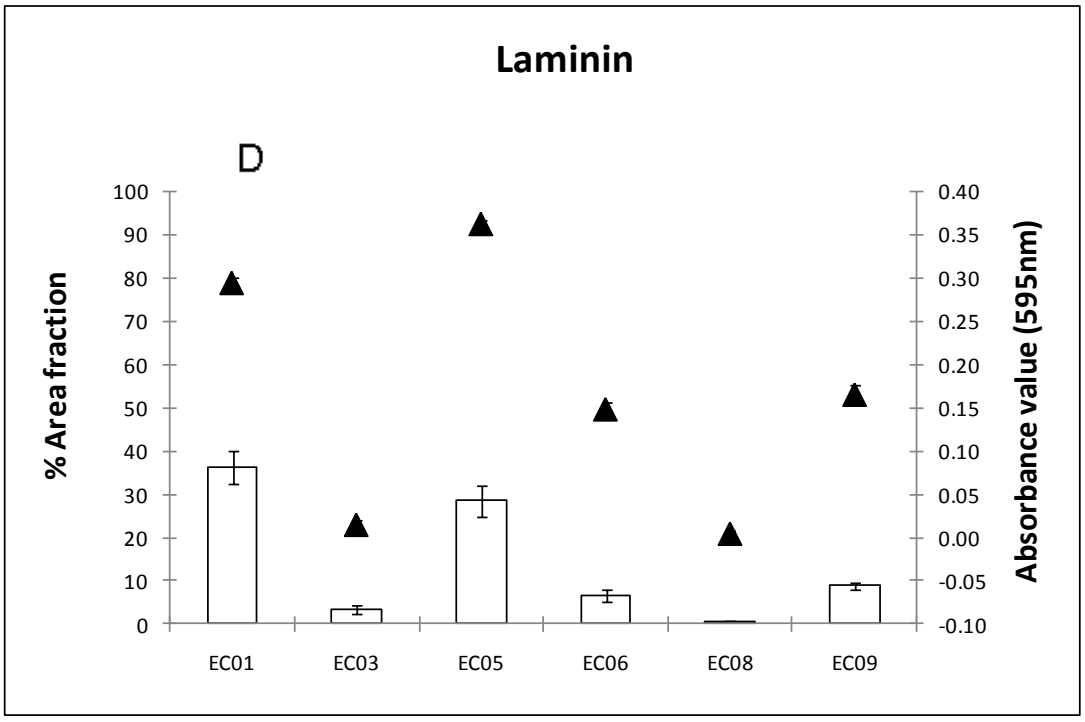
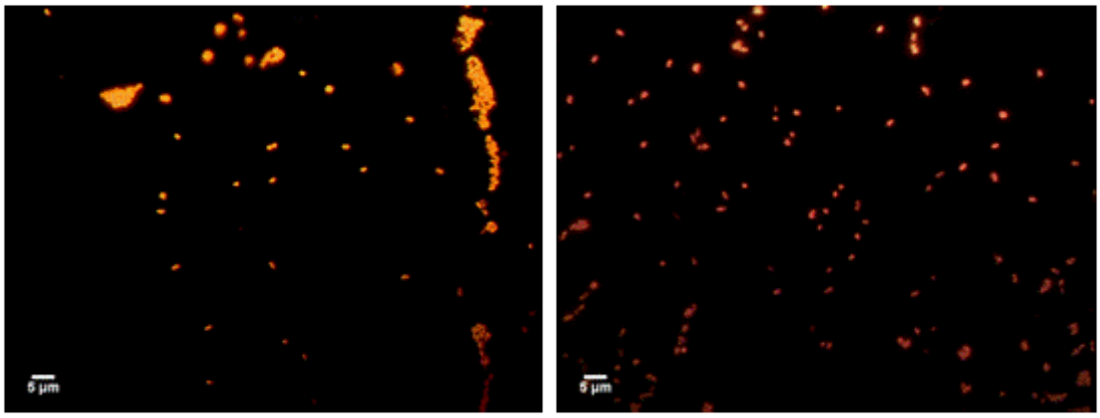


Fig. 2: Comparison between bacterial attachment to ECM proteins (A) collagen I (B) fibronectin (C) collagen IV (D) laminin at 25°C for 2 hours, measured by epifluorescence microscopy (□) and crystal violet staining methods (▲). Strains nomenclature is detailed in Table 1.

A B



C D

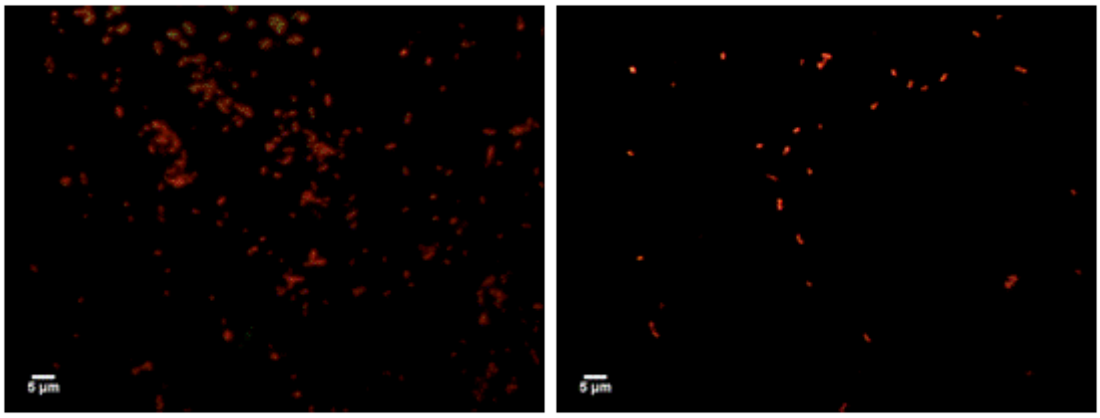


Fig. 3: Micrographs of bacterial attachment to ECM coated surface (incubated for 2 h at 25°C) that represent as negative binders based on crystal violet stain assay. (A) *E. coli* O157:H7 Sakai (EC02) on collagen I (B) *E. coli* O157:H7 (EC03) on fibronectin (C) *Salmonella* Infantis on collagen IV (D) *E. coli* O127:H6 (EC08) on laminin. Images were observed under oil-immersion objective.

Discussion

ECM proteins are abundant, protein sheets that are widely distributed throughout the carcass. They can generally be categorized into (i) interstitial proteins that include collagen I that is mainly located in the epimysium and perimysium, and (ii) basement membrane proteins that consist of

collagen IV and laminin (Beck and Gruber, 1995). It is more difficult to specifically categorize the location of fibronectin as it is found widely in the basement membrane and also in the interstitial matrices including the perimysium.

Our observations showed that bacterial attachment was selective based on the anatomical location of ECM proteins. More strains showed higher attachment to basement membrane proteins (laminin and collagen IV) than interstitial proteins (collagen I and fibronectin). Laminin supported attachment of more strains as compared to other ECM proteins, which is in concordance with previous reports (Horie et al., 2002; Speziale et al., 1982; Virkola et al., 1993). However, Ljungh et al. (1991) reported that *E. coli* NG7C attached weakly to laminin whereas *S*. Typhimurium was reported to attach at significantly higher levels to collagen I than laminin (Medina, 2004). Although the basement membrane is located at the microscopic level of intramuscular connective tissue (Bailey and Light, 1989), this protein layer can be easily exposed to the outer environment through tissue injury (Patti, 1994), such as during carcass dehiding and evisceration.

The wide variation of attachment levels among strains is consistent with previous studies (Fröman et al., 1984; Speziale et al., 1982) which showed that only selected *E. coli* strains attached to fibronectin and/or laminin. These observations also indicate that attachment likely involves specific receptors that may not be present on all strains (Ofek et al., 2003). It was found that none of the O157:H7 strains used in this study showed significantly high levels of attachment to any ECM proteins above control levels and, thus, were identified as low binding strains as compared to non-O157:H7 isolates. However, these observations could not be compared to the findings by Medina and co-workers who showed that *E. coli* O157:H7 binds to purified collagen and laminin, as their study used only one strain (Medina, 2001; Medina and Fratamico, 1998). It was also observed that in some conditions, *E. coli* M23Sr showed significantly higher attachment levels than its parent strain, *E. coli* M23. However, it is unclear whether the streptomycin resistant characteristics plays any role

in *E. coli* M23Sr attachment abilities and further investigations are needed to explain these differences.

Our study demonstrated that attachment to ECM proteins was markedly influenced by temperature. Although the initial cut during the dehiding process has been identified as the primary source of contamination (Bell, 1997) where the carcass temperature is approximately 37°C, our results have shown that the level of attachment to ECM proteins at this temperature is relatively low. The highest attachment observed occurred at 25°C for all proteins tested, except for collagen I where attachment was highest at 4°C. This is especially relevant as the range of average temperature of the carcass muscle surface before chilling is 25-29°C (Jericho et al., 1998). Introduction of bacterial contamination before chilling could lead to greater attachment to exposed meat tissues. Findings from the same study also showed that the carcass surface temperature ranged from 3 to 17°C after five hours of chilling.

The higher level of bacterial attachment to collagen type I at 4°C, a temperature normally used for abattoir storage (Crowley et al., 2009) should also be considered as this is the most abundant protein component of connective tissues, mainly found in the epimysium of skeletal muscle of the carcass (Bailey and Light, 1989). These data indicate that temperature has a major effect in determining the preferential sites and the level of attachment of bacterial cells to specific structures of the meat tissues. To our knowledge, this is the first report of the effect of abattoir-relevant temperatures on bacterial attachment to ECM proteins.

Attachment can be a cellular strategy to survive in the environment (Frank, 2001), in which bacterial cells express adhesive structures that mediate attachment to the host. Production of bacterial adhesins such as curli and exopolysaccharide (EPS) is highly influenced by temperature (Olsén et al., 1993a). Production of curli has been associated with bacterial attachment to surfaces (Chen et al., 2007; Olsén et al., 1993a) whereas there is an uncertainty in the role of EPS in initial attachment, as it may function as a mediator of attachment or inhibit the process (Weiner et al.,

1995). High production of curli in *E. coli* has been observed at room temperature (22-26°C) which may explain the higher attachment to ECM proteins (fibronectin, collagen IV and laminin) at 25°C observed in this study.

Although previous authors (An and Friedman, 1997; Fletcher and Floodgate, 1973) have suggested that centrifugation and washing of cells might remove certain bacterial structures, this step is still a common procedure used in preparing cell suspensions, including recent attachment studies (Dourou et al., 2011; Goulter-Thorsen et al., 2011; Rivas et al., 2006). The present study did not specifically investigate the potential influence of these structures on attachment to ECM proteins. Bacteria were centrifuged at a relatively low centrifugal force (3210 ×*g*), and yet results showed a significant variation in attachment properties among strains. Further studies are warranted to determine the specific bacterial structure(s) responsible for strain variation.

Our findings show that ECM protein concentration did not have a major effect on bacterial attachment, except for laminin where attachment was greater at the highest concentration tested. This contrasts with previous studies reporting concentration-dependent bacterial binding to collagen I and fibronectin (Fröman et al., 1984; Vercellotti et al., 1985; Visai et al., 1990). This may be due to different forms of proteins used in this study, in that protein-coated surfaces were used instead of matrix proteins in solutions. It has been suggested that differences in ECM protein forms, either in solution or immobilized on a surface, could explain differences in bacteria-protein interactions (Henderson et al., 2011; Vercellotti et al., 1985). However, the use of immobilized ECM proteins was chosen for this study as it has been used before as an *in vitro* model that reflects the *in vivo* eukaryotic cell systems in skeletal muscle studies (White et al., 2001).

Strains not observed to bind by the crystal violet method did actually attach to ECM proteins when measured by epifluoresence microscopy, but at a relatively lower level than that detected by crystal violet. The greater sensitivity of epifluorescence microscopy accounts for these differences. Yet, although less sensitive, the crystal violet method is useful for detecting high-binding

strains and it is less laborious and directly correlates with results by epifluoresence microscopy. This is in agreement with findings by Djordjevic et al. (2002) who also observed a positive correlation between crystal violet assay and microscopic analysis in biofilm studies of *Listeria monocytogenes* at 32°C.

From this study, we conclude that *E. coli* and *Salmonella* strains display different attachment properties to various ECM proteins and that attachment is highly influenced by temperature. As this *in vitro* study focused on specific isolated structures of meat tissue, the attachment properties of bacterial cells to actual meat or carcass surfaces could differ compared to *in vitro* results. Nevertheless, the particular findings on the effect of temperature from this study are especially relevant as it is one of the important control parameters in an abattoir and may influence the likelihood of bacterial attachment throughout post-slaughter processes. We propose that understanding bacterial attachment processes to ECM proteins could help in the design of more targeted interventions that prevent or disrupt interactions between bacterial cells and meat surface binding sites, thus reducing carcass contamination, product spoilage and health risk associated with meat.

Chapter 3

Effect of pH, salt and chemical rinses on bacterial attachment to extracellular matrix proteins

Abstract

Microbial contamination of carcass surfaces occurs during slaughter and post-slaughter processing steps, therefore interventions are needed to enhance meat safety and quality. Although many studies have been done at the macro-level, little is known about specific processes that influence bacterial attachment to carcass surfaces, particularly the role of extracellular matrix (ECM) proteins. In the present study, an *in vitro* assay to assess the effect of pH and salt (NaCl, KCl and $CaCl_2$) on attachment of *E. coli* and *Salmonella* isolates to dominant ECM proteins: collagen I, fibronectin, collagen IV and laminin was conducted. Also, the effects of three chemical rinses commonly used in abattoirs (2% acetic acid, 2% lactic acid and 10% trisodium phosphate [TSP]) were tested. Within a pH range of 5 to 9, there was no significant effect on attachment to ECM proteins, whereas the effect of salt type and concentration varied depending on combination of strain and ECM protein. A concentration-dependant effect was observed with NaCl and KCl (0.1-0.85%) on attachment of *E. coli* M23Sr, but only to collagen I. One-tenth percent $CaCl_2$ produced the highest level of attachment to ECM proteins for *E. coli* M23Sr and EC614. In contrast, higher concentrations of $CaCl_2$ increased attachment of *E. coli* EC473 to collagen IV. Rinses containing TSP produced >95% reduction in attachment to all ECM proteins. These observations will assist in the design of targeted interventions to prevent or disrupt contamination of meat surfaces, thus improving meat safety and quality.

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Introduction

Microbial carcass contamination occurs during the slaughter of beef cattle. Both pathogenic and spoilage microorganisms can be transferred from the outer surface of the carcass, via fomites and washings, to sterile underlying tissues during dehiding, evisceration, fabrication and other processing steps (Gill and McGinnis, 2003; Jackson et al., 2001). The complexity and the heterogeneous nature of meat tissue (Bouttier et al., 1997; Cabedo et al., 1997) as well as physical entrapment within tissue fibres (Frank, 2001), has meant that previous studies on whole pieces of meat tissues are inconclusive in describing the specific tissue structures involved in attachment (Cabedo et al., 1997; Chen et al., 2007; Marin et al., 1997; Rivas et al., 2006; Warriner et al., 2001). A more systematic approach can be achieved by studying binding interactions between bacteria and specific structures of meat, such as connective tissues that are primarily composed of extracellular matrix (ECM) proteins.

Published studies on bacterial attachment to ECM proteins have primarily focused on human infections (Doig and Trust, 1993; Ofek et al., 2003; Patti et al., 1994). Studies relevant to meat slaughter and processing are limited but include reports by Medina and co-workers (2001; Medina, 2004; Medina and Fratamico, 1998) and Zulfakar et al. (2012)(see Chapter 2). Previously, a distinct variation was found in the attachment of *E. coli* and *Salmonella* spp. to ECM proteins and that attachment was highly influenced by temperatures relevant to abattoir environments (Zulfakar et al., 2012) (see Chapter 2). However, information on the potential effects of other conditions relevant to meat processing on attachment to ECM proteins has not yet been reported.

Physical forces such as electrostatic interactions are generally thought to be involved in attachment (An and Friedman, 1998; Selgas et al., 1993; van Loosdrecht et al., 1987). These interactions can be influenced by pH, ionic strength and cation concentration of the surrounding medium during attachment (Frank, 2001; Li and McLandsborough, 1999; Piette and Idziak, 1992). Such factors have a direct relationship with the physicochemical properties of the bacterial cell and

the target substratum surfaces, which in turn relates to the electrical diffuse double layer (parallel layers of surface charge) between both interacting surfaces (Busscher and Weerkamp, 1987; van Loosdrecht et al., 1987). pH has also been shown to affect production of bacterial exopolysaccharides (EPS) (Oliveira et al., 1994; Ryu and Beuchat, 2004) that can influence attachment (Patti et al., 1994; Ryu et al., 2004; Weiner et al., 1995). Information on the effect of these factors on bacterial attachment to ECM proteins would provide a better understanding of mechanisms involved this process.

Techniques for carcass decontamination have been developed and reviewed (Bolder, 1997; Cutter et al., 1997; Dorsa et al., 1997; Mani-López et al., 2012; Sofos et al., 1999). Decontamination procedures that reduce or eliminate pathogenic and spoilage microorganisms (Huffman, 2002) include rinses with chemicals such as organic acids and trisodium phosphate (TSP) (Dorsa et al., 1997; Kim and Slavik, 1994; Morris et al., 1997; Stopforth et al., 2003). Organic acids and TSP are excellent antimicrobials and have been classified as "Generally Recognized as Safe" by the United States Food and Drug Administration (USFDA) for applications on beef and poultry carcasses (USDA-FSIS, 2012).

The effectiveness of chemical rinses in reducing bacterial attachment to whole meat tissues has been reported. Organic acids (acetic and lactic acid) and TSP reduce contamination levels typically more than 2 log CFU/cm^2 *E. coli* O157:H7 on beef tissues (Dorsa et al., 1997; Fratamico et al., 1996). Kim and Slavik (1994) reported reduction in bacterial attachment following TSP treatment of beef surfaces, including both surface- and strain-dependent effects. For example, treatments were more effective in removing bacteria from fat surfaces than fascia. However, the effect of chemical rinses on bacterial attachment to ECM proteins has not been studied, and therefore limits understanding about specific processes mediating decontamination.

By understanding factors that influence interactions between bacterial cells and meat ECM proteins, novel decontamination treatments could be designed. This study reports the effect of pH

and salt on the attachment of *E. coli* and *Salmonella* isolates to collagen I, fibronectin, collagen IV and laminin, and the effects of several commercial chemical rinses.

Materials and methods

Bacterial strains and culture conditions

Seven bacterial strains (*E. coli* M23Sr, *E. coli* O157:H7 Sakai*, E. coli* EC614, *E. coli* EC473, *E. coli* H10407, Sal1729a and Sal691) were used (Table 1). Strains were obtained from the Food Safety Centre collection, along with strains generously provided by Dr Narelle Fegan (CSIRO Food and Nutritional Sciences, Werribee, Australia) and Dr. R. Robins-Browne (Department of Microbiology and Immunology, University of Melbourne, Melbourne, Australia). Stock cultures were kept in microbiological storage beads, (Protect Bacterial Preservers, Technical Service Consultants Limited, Heywood, England) and stored at -80°C. Preparation of cultures and ECM protein-coated surfaces has been described (Zulfakar et al., 2012) (see Chapter 2). Briefly, working cultures were maintained on nutrient agar (NA; Oxoid CM0003, Basingstoke, UK) and stored at 4°C for less than than one month. Cultures were grown overnight in tryptone soy broth (TSB; Oxoid CM0129, Basingstoke, UK) for 18-20 h at 37°C without shaking. Washed cell suspensions were prepared by centrifuging the TSB culture at 3,210 x *g* for 5 min. Cells were washed twice in Dulbecco A phosphate buffered saline, pH 7.3 (PBS; Oxoid BR0014, Basingstoke, UK) before resuspending in PBS to achieve approximately 10^9 CFU/ml. One hundred microliters of diluted cell suspension were plated in duplicate on tryptone soy agar (TSA; Oxoid CM0131, Basingstoke, UK) to measure the inoculum level. Plates were incubated at 37°C for 24 h and colonies counted.

Table 1: List of bacterial strains tested.

Strain ID	Bacterial species	Serotype	Source
M23Sr	*Escherichia coli*	OR:H- (with streptomycin resistance)	unknown
O157:H7 Sakai	*Escherichia coli*	O157:H7 Sakai	clinical isolate from Sakai outbreak
EC614	*Escherichia coli*	O157:H12[a]	beef cattle feces
EC473	*Escherichia coli*	O26:H11[a]	dairy cattle feces
H10407	*Escherichia coli*	O78:K80:H11 [b]	clinical isolate
Sal691	*Salmonella enterica*	Typhimurium 135[a]	beef cattle feces
Sal1729a	*Salmonella enterica*	Anatum[a]	sheep feces

[a]Strain received from Dr. Narelle Fegan, CSIRO Food and Nutritional Sciences, Werribee, Australia

[b]Strain received from Dr. R. Robins-Browne and Kristy Azzopardi/Stojcevski, Department of Microbiology and Immunology, University of Melbourne, Melbourne

Preparation of extracellular matrix protein coated surface

The ECM proteins investigated were collagen I (calf skin collagen; Sigma, USA), fibronectin (bovine plasma fibronectin, Sigma; USA), collagen IV (Engelbreth-Holm-Swarm murine sarcoma; BD Biosciences, Bedford, USA) and laminin (Engelbreth-Holm-Swarm murine sarcoma; Sigma, Israel), and were prepared according to the manufacturer's instructions. Two hundred and fifty microliters of protein solution (collagen I and fibronectin = 100 µg/ml; collagen IV = 100 µg/ml; laminin = 50 µg/ml) were coated onto 96-well tissue culture plates (Greiner Bio-one, Germany) and incubated at 37°C for 1 h. Excess fluid was removed from wells and the plates stored at 4°C. Coated surfaces were rinsed with 200 µl PBS and air-dried at room temperature for 10 min prior to experimentation.

Effect of pH

Washed bacterial cell pellets were resuspended in PBS adjusted to pH 5, 6, 7, 8 and 9 with 1.2 M HCl or 1.7 M NaOH. One hundred microliters of bacterial suspension or 100 µl PBS (control wells) were added to each protein-coated well. After incubation for 2 h at 25°C, bacterial suspensions were removed by pipette and plates rinsed three times with 200 µl PBS to remove loosely attached cells. Bacterial attachment to ECM proteins was measured using a crystal violet staining method as reported by Zulfakar et al. (2012) (see Chapter 2). Briefly, attached cells were fixed at 55°C for 20 min and then stained with 95 µl of filtered 1% crystal violet for 45 min at 25°C. Excess crystal violet stain was removed by rinsing wells five times with sterile distilled water. Wells were then air-dried for 30 min at room temperature and were destained by adding 100 µl of 95% ethanol to wells for 15 min. Attached cells were quantified by measuring absorbance with a microplate reader (Bio-Rad® Benchmark microplate reader, USA) at 595 nm. Average absorbance values were calculated. The minimum detection limit was determined in our previous study, and estimated to be 6.6 ± 0.8 log

CFU/cm^2 (Zulfakar et al., 2012) (see Chapter 2). Measurements of bacterial attachment to non-coated wells (plastic only) were conducted in separate preliminary trials and showed no difference in absorbance values compared to non-inoculated controls.

Effect of salt

Based on results of our previous study (Zulfakar et al., 2012) (see Chapter 2), three *E. coli* strains displaying significant attachment to ECM proteins in PBS (M23Sr, EC614 and EC473) were tested. Although no *Salmonella* strains showed significant binding in PBS to ECM proteins, one strain (Sal1729a) was included for comparison with *E. coli* strains. Bacterial cell suspensions were prepared as described above. Washed bacterial cell pellets were resuspended in three different salt solutions (NaCl, KCl and $CaCl_2$) prepared in Milli-Q water (Merck, Millipore, USA) at five concentrations: 0.1, 0.5, 0.85, 2, 3 and 5%. For 0% salt, Milli-Q water was used to resuspend bacterial cell pellets. One hundred microlitres of bacterial suspension or 100 µl of Milli-Q water (control wells) were added to each protein-coated well. After incubation for 2 h at 25°C, bacterial suspensions were removed by pipette and wells rinsed three times with 200 µl Milli-Q water to remove loosely and unattached cells. Measurement of attached cells was performed by crystal violet assay as described above.

Effect of chemical rinses

Six strains were used to test the effect of chemical rinses on bacterial attachment to ECM proteins. *Escherichia coli* O157:H7 Sakai strain was not included since no significant attachment to ECM proteins in PBS was observed in preliminary trials. Chemical rinses tested were 2% acetic acid (pH 2.37; Scharlau, Spain), 2% lactic acid (pH 2.19; Sigma-Aldrich, Australia) and 10% trisodium

phosphate (pH 12.49; Sigma-Aldrich, Australia); solutions were prepared in Milli-Q water. Concentrations tested were based on previous reports (Cutter and Rivera-Betancourt, 2000; Fratamico et al., 1996; USDA-FSIS, 2012). PBS was used as the control treatment. For the attachment assays, washed bacterial cell pellets were resuspended in PBS (pH 7). One hundred milliliters of bacterial suspension or 100 µl of PBS (control wells) was added to each ECM-coated well. After incubation for 2 h at 25°C, bacterial suspensions were removed by pipette and wells rinsed four times, for a duration of 2 min per rinse. The first two rinses were done with the chemical treatments, followed by two final rinses with PBS (pH 7). Table 2 shows the order of first and second rinses. The level of attached bacteria remaining after the treatments was assessed by crystal violet assay, as described above.

Statistical analysis

All experiments were performed in triplicate and each experiment repeated twice. Results (absorbance at 595 nm) for all experiments were presented as mean ± standard deviation (SD). Absorbance values were corrected by subtracting the average absorbance value from the corresponding uninoculated control wells. Data were analysed using one-way analysis of means (ANOVA), followed by Least Significant Difference (LSD) test. All statistical tests were performed using IBM SPSS Statistics 19 (SPSS Inc.). Results were considered significant at $p < 0.05$.

Table 2: First and second rinse treatments to test the effect of chemical rinse on bacterial attachment to ECM proteins.

Chemical rinse abbreviations	First rinse	Second rinse
PBS-PBS[a]	PBS	PBS
AA-AA [b]	2% acetic acid	2% acetic acid
LA-LA [c]	2% lactic acid	2% lactic acid
TSP-TSP [d]	10% trisodium phosphate	10% trisodium phosphate
AA-LA	2% acetic acid	2% lactic acid
AA-TSP	2% acetic acid	10% trisodium phosphate
LA-TSP	2% lactic acid	10% trisodium phosphate

[a]Phosphate buffered saline

[b]Acetic acid

[c]Lactic acid

[d]Trisodium phosphate

Results

Effect of pH

Overall, pH within a range of 5 to 9 did not significantly influence attachment of the seven strains to ECM proteins ($p > 0.05$). Sal1729a showed significantly lower attachment to fibronectin and collagen IV at pH 6, however the difference was very low (absorbance value ≤ 0.02) and close to the minimal detection limit of the assay (see Table 1: Appendix B).

Effect of salt

None of the strains showed significant attachment to ECM proteins in Milli-Q water (0% salt concentration. For collagen I and fibronectin, salt affected the attachment of *E. coli* M23Sr only.

Increasing concentrations of NaCl and KCl (0.1-0.85%) resulted in higher levels of attachment of M23Sr to collagen I (Fig. 1), whereas 2-5% NaCl and KCl markedly reduced attachment compared to 0.85% (NaCl = 87.1% ± 4.5; KCl = 72.3% ± 3.2). In contrast, NaCl and KCl did not show a concentration-dependent effect on M23Sr attachment to fibronectin (Fig. 2). Attachment to fibronectin was markedly lower at 5% NaCl and KCl.

A concentration of 0.1% $CaCl_2$ produced the highest level of *E. coli* M23Sr attachment to both collagen I and fibronectin for this salt type, with a significant decrease at higher $CaCl_2$ concentrations (0.5-5%). The reduction in attachment to fibronectin was greater (94.3% ± 1.8) compared to collagen I (50.8% ± 3.9).

Attachment of three *E. coli* strains (M23Sr, EC614 and EC473) to collagen IV and laminin were significantly affected by salt type and concentration (Fig. 3 and 4). The effect of salt on attachment of M23Sr to collagen IV and laminin displayed a similar pattern as fibronectin (Fig. 3A and 4A), as was the effect on attachment of EC614 to collagen IV. $CaCl_2$ however, produced a concentration-dependent effect (Fig. 3B). $CaCl_2$ significantly increased attachment of EC473 compared to other salts (Fig. 3C).

For EC614, the effect of salt on attachment to laminin was similar to that observed for collagen IV, except for a sharp reduction at ≥ 0.85% $CaCl_2$ (Fig. 4B). $CaCl_2$ significantly increased attachment of EC473 to laminin only when compared with KCl (Fig. 4C). At the concentrations tested, the salts had no significant effect on attachment of Sal1729a to any ECM proteins.

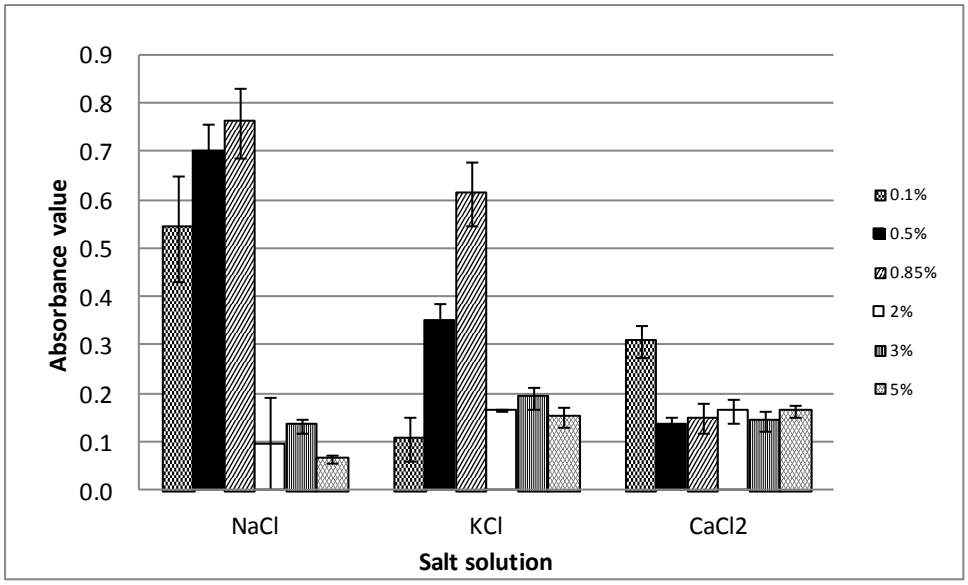


Fig. 1: Mean absorbance values at 595 nm for the effects of salt on initial attachment of *E. coli* M23Sr to collagen I. Error bars are the standard deviation.

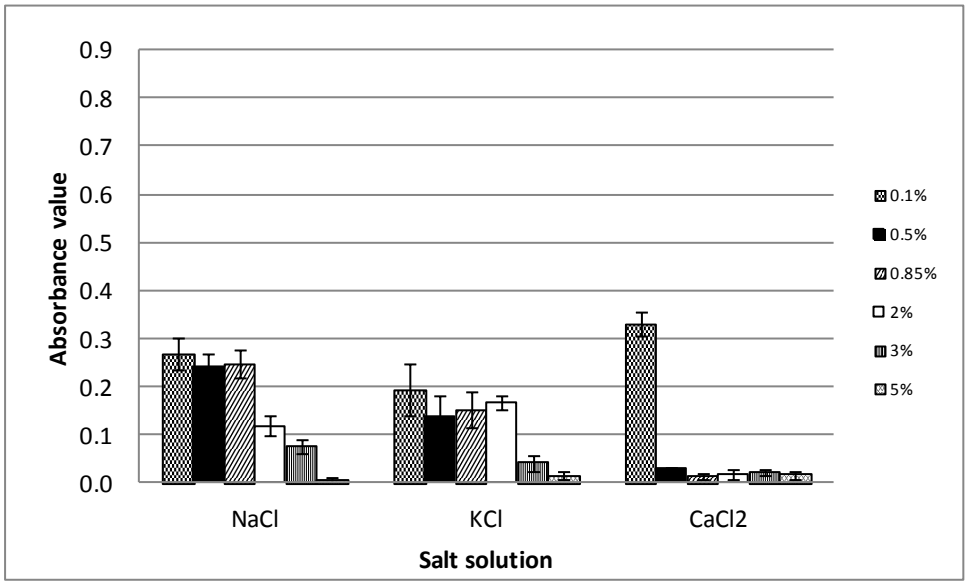
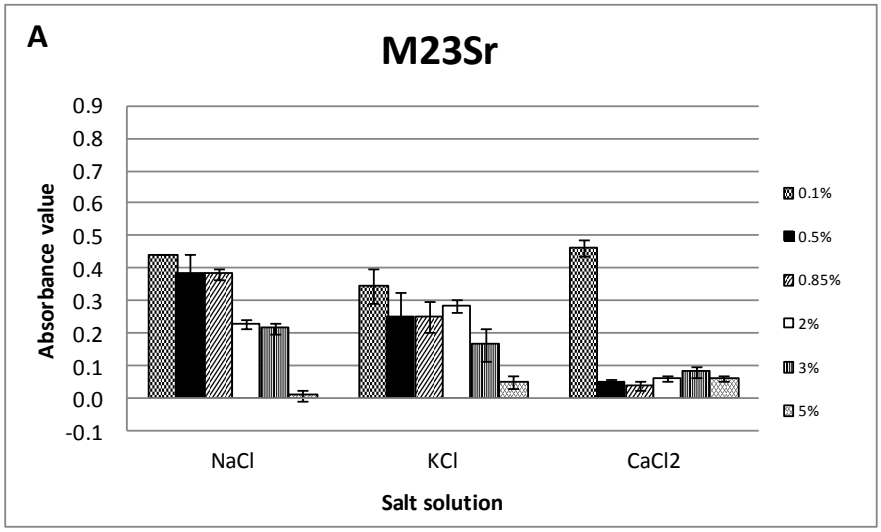
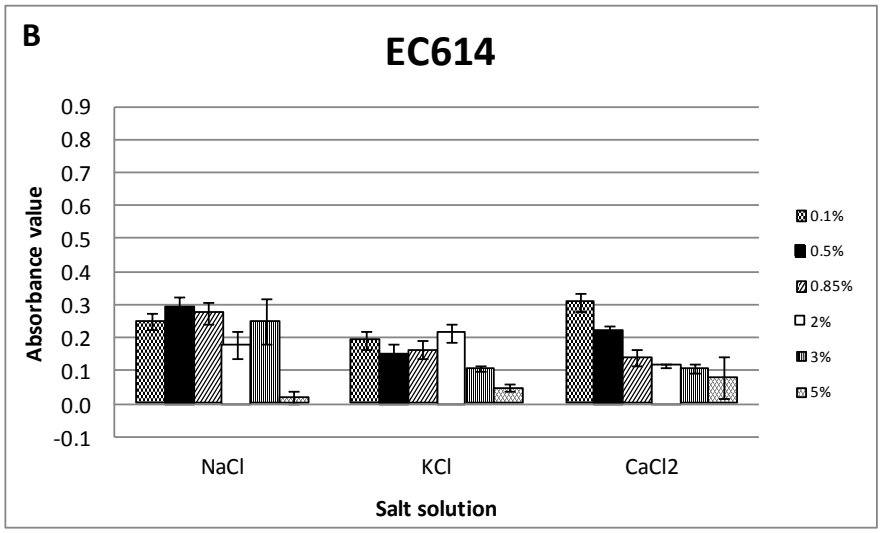


Fig. 2: Mean absorbance values at 595 nm for the effects of salt on initial attachment of *E. coli* M23Sr to fibronectin. Error bars are the standard deviation.





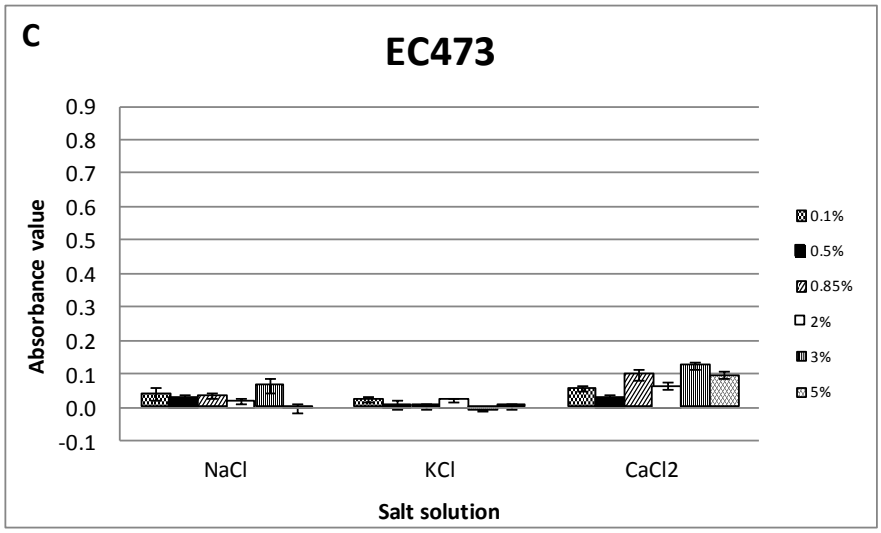


Fig. 3: Mean absorbance values at 595 nm for the effects of salt on initial attachment to collagen IV. (A) M23Sr (B) EC614 (C) EC473. Error bars are the standard deviation.

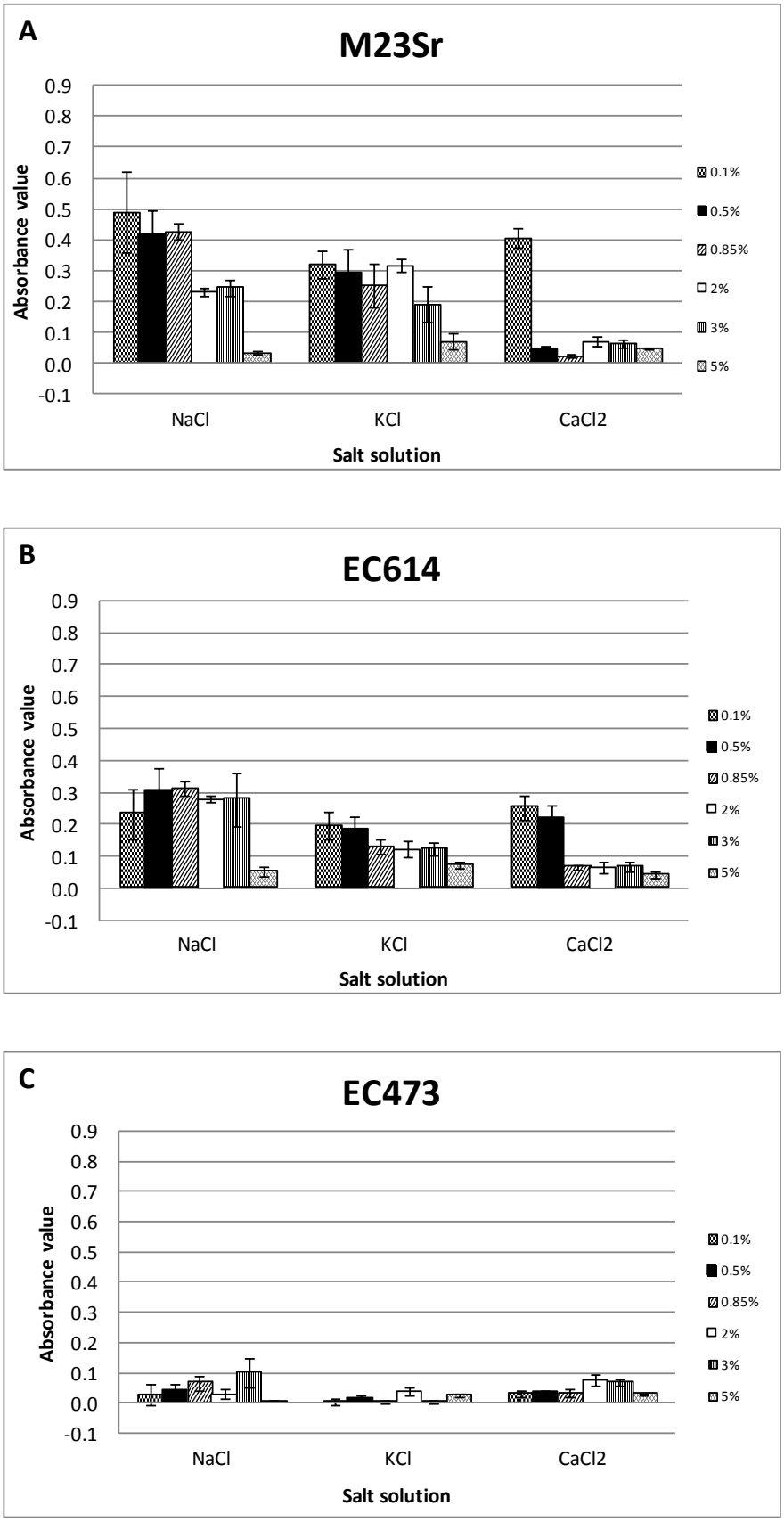


Fig. 4: Mean absorbance values at 595 nm for the effects of salt on initial attachment to laminin. (A) M23Sr (B) EC614 (C) EC473. Error bars are the standard deviation.

Effect of chemical rinses

Results for the effects of chemical rinses on attachment to collagen I and fibronectin are shown only for *E. coli* M23Sr (Fig. 5), which was the only strain that displayed significant attachment to these proteins. Rinse combinations containing TSP (TSP-TSP, AA-TSP and LA-TSP) produced >95% reduction in attachment of M23Sr to collagen I. In contrast, organic acid rinses had no significant effect. For attachment to fibronectin, all six rinse combinations reduced binding of M23Sr, although rinses containing TSP produced the greatest effect (97% ± 2.4 reduction) compared to organic acids (43.5% ± 2). Similar reductions were observed among the three rinses containing TSP, as well as among the three rinses that contained only organic acids.

Unlike collagen I and fibronectin, strains other than *E. coli* M23Sr attached more strongly to collagen IV, allowing the effects of chemical rinses to be measured for a larger number of strains (Fig. 6). Results for *Salmonella* strains were not included in the figure due to low binding to collagen IV. TSP-containing rinses produced the greatest reduction in attachment (96.6% ± 3.7). Organic acids markedly reduced (59.6% ± 18.7) attachment of M23Sr, EC614 and H10407, but were less effective for EC473 (18.8% ± 12.6). The effectiveness of the three organic acid rinses was not, however, significantly different. Results of chemical rinses on attachment to laminin produced a similar pattern as that observed for collagen IV (Fig. 7). However, organic acids had little effect on M23Sr (18.7% ± 1.2 reductions).

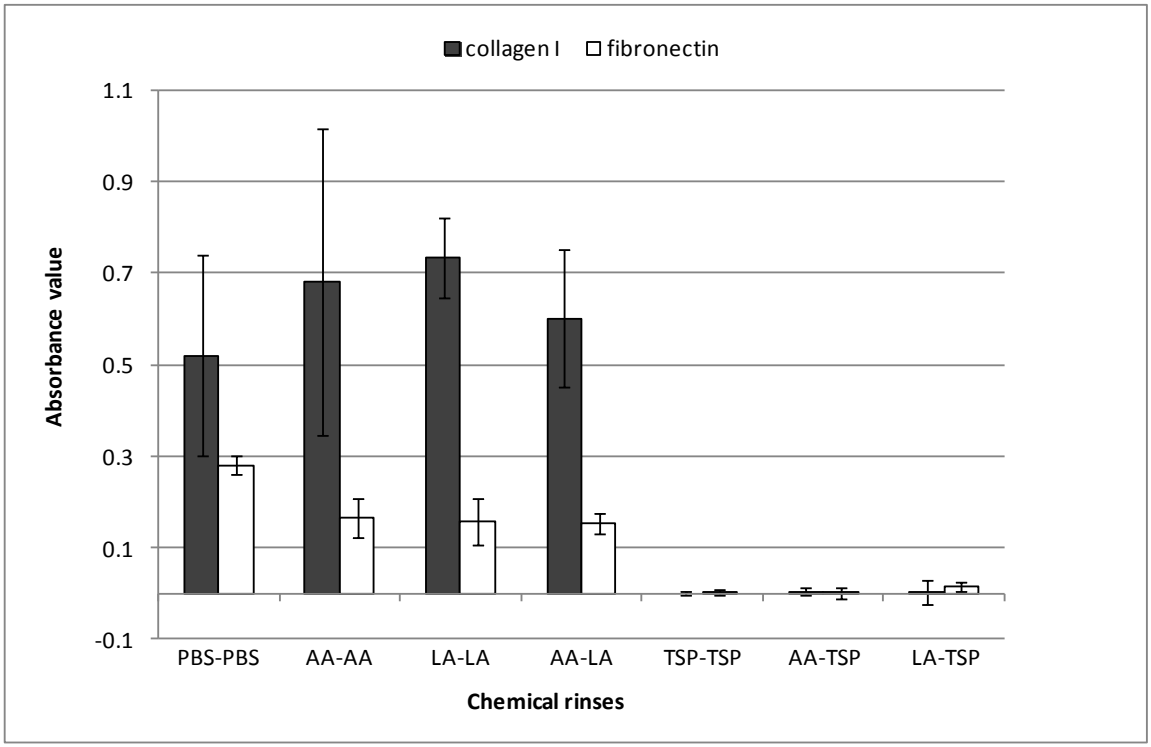


Fig. 5: Mean absorbance values at 595 nm for the effect of chemical rinses on *E. coli* M23Sr attachment to collagen type I and fibronectin. Error bars are the standard deviation.

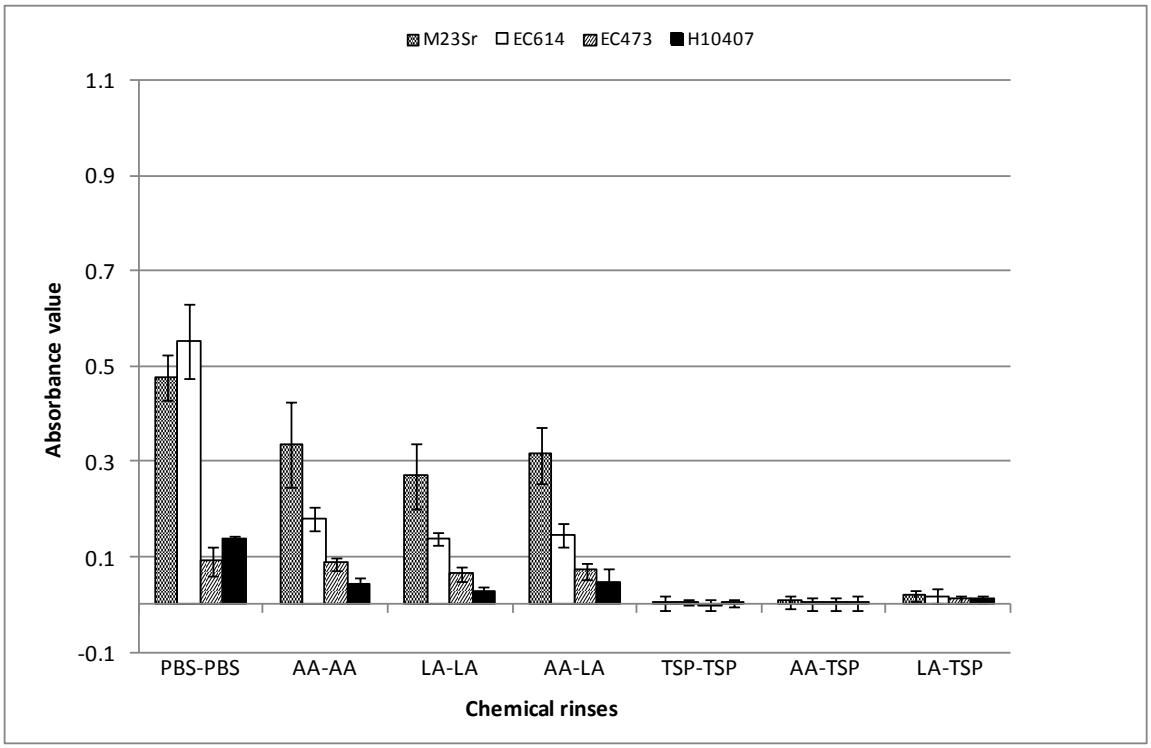


Fig. 6: Mean absorbance values at 595 nm for the effect of chemical rinses on bacterial attachment to collagen IV. Error bars are the standard deviation.

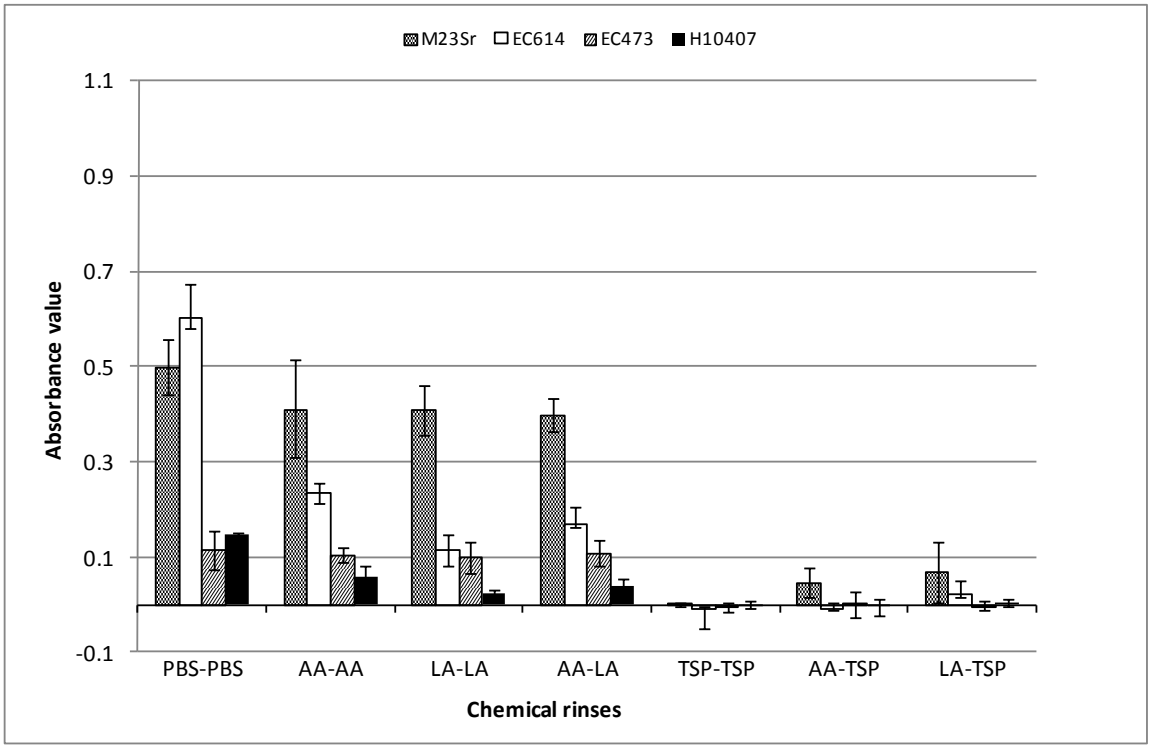


Fig. 7: Mean absorbance values at 595 nm for the effect of chemical rinses on bacterial attachment to laminin. Error bars are the standard deviation.

Discussion

Published studies have reported bacterial strain specificity in attachment to ECM proteins (Ljungh et al., 1991; Westerlund and Korhonen, 1993). Similarly, it was reported that bacterial attachment to ECM proteins varied among strains of *E. coli* and *Salmonella*, indicating that attachment likely involves interactions between specific surface receptors that are not present on all strains (Zulfakar et al., 2012) (see Chapter 2). In addition, it was also shown in the same study, that attachment was related to the anatomical location of ECM proteins, in that more strains attached to basement membrane (collagen IV and laminin) compared to interstitial proteins (see Chapter 2).

The strains used in the present study represent high- (M23Sr and EC614), intermediate- (H10407 and EC473) and low- (O157:H7 Sakai, Sal691 and Sal1729a)-binding strains, based on previous findings (Zulfakar et al., 2012). Although these included pathogenic and non-pathogenic strains, attachment did not correlate with pathogenicity.

Within the pH range tested, there was no influence on bacterial attachment to ECM proteins. When compared to published studies using whole pieces of meat, our finding contrasts that of Li and McLandsborough (1999) who observed that *E. coli* O157:H7 bound more strongly to lean beef tissues under acidic (pH 4) or alkaline (pH 10) conditions, compared to neutral pH. However, a study of different bacterial species, *P. fragi* and *B. thermosphacta*, showed that attachment to lean beef tissue was not affected by pH over a range of 5.8-7.2 (Bouttier et al., 1994).

In the current study, the pH range tested was limited to pH 5-9, a range that causes little stress to *E. coli* and *Salmonella* strains (Bearson et al., 1997; Foster, 1995; Padan et al., 2005). It is also known that bacterial cells are able to adapt to small environmental changes in pH (Garrett et al., 2008), and with the 2 h incubation period, this may have allowed bacterial cells to adapt to the changes. Within the pH range tested, there is a possibility that the conformation of ECM proteins

was altered, but our observations indicate that if such changes occurred, they did not affect the level of bacterial attachment.

There are different views on the importance of electrostatic interactions on attachment to meat surfaces (Bouttier et al., 1994; Dickson and Koohmaraie, 1989; Li and McLandsborough, 1999; Rivas et al., 2006). In this study, the effects of salt type and concentration, as a function of cation concentration, varied with bacterial strain and type of ECM protein.

A concentration-dependent (0.1 to 0.85%; equivalent to 13-145 mM) increase in attachment of *E. coli* M23Sr was found for NaCl and KCl. These findings are similar to those of Piette and Idziak (1992) who observed that the attachment of *Pseudomonas fluorescens* to tendon tissue increased over a range of 0.1-100 mM monovalent cation. It has been suggested that increased ion concentration reduces the thickness of the electrical diffuse double-layer and thereby masks surface charge (Bouttier et al., 1997; Hermansson, 1999). This enables the negatively charged bacterium to overcome electrostatic repulsion forces and move closer to the target surface, thus increasing the opportunities to initiate attachment through the action of attractive van der Waals forces or via specific surface receptors (Frank, 2001).

Piette and Idziak (1992) also observed a sharp decrease in attachment at higher ionic strengths and cation concentration that is similar to our findings for ECM proteins at concentrations ≥ 2% NaCl and KCl (equivalent to ≥ 250 mM). This effect of high ionic strength was also observed for *E. coli* O157:H7 attachment to beef muscle by Li and McLandsborough (1999), who suggested that the outer wall layers of the bacterial cell and structures involved in the attachment process may be altered or denatured at high concentrations of salt. However in the current study, the concentration-dependant effect was only observed for one strain, *E. coli* M23Sr indicating a strain-dependant response. This effect also was only observed for collagen I, suggesting that the mechanisms involved in attachment to this protein differ to those of the other ECM proteins, possibly due to differences in ECM protein structure.

With the divalent cation Ca^{2+}, maximum attachment to ECM proteins occurred at 0.1% (9 mM) but decreased at higher Ca^{2+} concentrations for M23Sr and EC614. Treatment with $CaCl_2$ increased attachment of EC473 to collagen IV and laminin, although it was still lower than attachment levels observed for other strains, emphasising the strain-dependant responses. In this regard, the effects of salt on attachment of the various strains studied were proportional to inherent attachment levels, i.e. strains that displayed "high", "intermediate" and "low" binding patterns under physiological conditions (i.e. PBS), displayed similar relative levels of attachment regardless of experimental treatment.

The two-step rinse procedure used in this study was based on combinations of chemical rinses applied to cattle hides and beef carcasses under laboratory conditions (Loretz et al., 2011). This study shows that all chemical rinses reduced bacterial attachment to ECM proteins. The effect of organic acid washes can result from the penetration of non-disassociated acids into the cytoplasm and subsequent protonation resulting in acidification of the cytoplasm and cell damage (Huffman, 2002).

In this study, it was found that treatments that included TSP were most effective in reducing bacterial cell attachment to ECM proteins, consistent with other reports that used whole piece of beef tissue (Cutter and Rivera-Betancourt, 2000; Fratamico et al., 1996). The effect of TSP inactivation/removal has been mostly attributed to high alkaline pH (pH 12.5) and sequestration of metal ions targeted at the bacterial cell wall, thus damaging the cell membrane structure (Capita et al., 2002). Electron microscopic studies of TSP-treated cells showed distortion and collapse of membrane continuity, suggesting that cell surface structures involved in attachment may be affected (Sampathkumar et al., 2003).

It should also be noted that these results were obtained under controlled experimental conditions and may differ from those obtained in commercial practice. The surfaces on which the

bacteria are deposited, the physiological condition of organisms, their disposition in the meat menstruum and the method of application of chemical treatments likely varies in commercial versus experimental settings (Gill, 2009). The efficacy of these treatments may be reduced if bacteria on the carcass surface are protected, e.g. bacterial cells that are hidden underneath flaps of tissues or in small cuts under the surface of the carcass (Acuff, 2005).

In conclusion, bacterial attachment to ECM proteins can be influenced by salt type and concentration; however pH has a less important role, at least over the range of pH 5 - 9. Manipulation of these factors would be challenging, yet this knowledge may be beneficial in developing new and better intervention systems for carcass decontamination. It was also concluded that chemical rinses commonly used in the meat industry, especially TSP, will likely reduce attachment to ECM proteins.

Chapter 4

Cultured C_2C_{12} cell lines as a model for assessment of bacterial attachment to bovine primary muscle cells

Abstract

The mechanisms of bacterial attachment to meat tissues need to be understood to enhance meat safety interventions. However, little is known about attachment of foodborne pathogens to meat muscle cells. In this study, we measured attachment of six *E. coli* and two *Salmonella* strains to primary bovine muscle cells and a cultured muscle cell line, C_2C_{12}, including the effect of temperature. Bacterial strains that showed significant attachment (p<0.05) to both muscle cell types as compared to controls were reported. At 37°C, all but one strain (EC623) attached to C_2C_{12} cells, whereas only five of eight strains (M23Sr, H10407, EC473, Sal1729a and Sal691) attached to primary cells. At 10°C, two strains (H10407 and EC473) attached to C_2C_{12} cells, compared to four strains (M23Sr, EC614, H10407 and Sal1729a) of primary cells. Comparing all strains at both temperatures, EC614 displayed the highest CFU per C_2C_{12} cell (4.60±2.02 CFU/muscle cell at 37°C), whereas greater numbers of M23Sr attached per primary cell (51.88±39.43 CFU/muscle cell at 37°C). This study indicates that primary bovine muscle cells may provide a more relevant model system to study bacterial attachment to beef carcasses compared to cell lines such as C_2C_{12}.

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Introduction

Microbial contamination of meat can lead to foodborne illness following consumption of contaminated product. During processing steps in an abattoir, spoilage and pathogenic microorganisms contaminate and attach to product surfaces that are otherwise sterile (Jackson et al., 2001). Carcasses prepared for meat trade primarily consist of muscular, fatty and connective tissues (Calkins and Kilinger, 1993) with complex and heterogeneous properties (Frank, 2001; Selgas et al., 1993). Consequently, a thorough understanding of bacterial attachment to beef products must consider the specific structures of meat tissues. Such knowledge may lead to novel carcass interventions that prevent and/or disrupt bacteria-carcass interactions.

It was previously reported that there exists a wide strain variation in the extent of attachment of *E. coli* and *Salmonella* spp. to muscle extracellular matrix (ECM) proteins, the major components of meat connective tissue (Zulfakar et al., 2012) (see Chapter 2). Temperature was shown to have a significant effect on bacterial attachment, moreover, strains showed preferential attachment to basement membrane ECM proteins compared to interstitial ECM proteins. This is relevant to abattoir environments where temperature ranges from 3 to 39°C (Crowley et al., 2009; Jericho et al., 1998).

While this information provided valuable insight about ECM proteins, there are few reports about attachment of *E. coli* and *Salmonella* spp. to muscle cells. Cultured intestinal cell lines such as Caco-2, HT-29 and others have been used extensively *in vitro* to understand bacterial attachment and invasion of human intestinal epithelial cells (Blay et al., 2004; Liu et al., 2010; Mellor et al., 2009). However, similar approaches have not been reported for bovine skeletal muscle cells. These approaches could be adapted to the muscle cell system to obtain information that would provide a more holistic perspective of bacterial interactions with the carcass surface and insight into potential intervention strategies for carcass decontamination.

Skeletal muscle tissue is composed of highly differentiated mono-nucleated cells. Differentiated myoblasts would subsequently fuse to form tube-like structures with multiple nuclei akin to muscle fibres (Swatland, 2004). The muscle cells can be derived directly from embryonic and adult muscle tissues, or from continuous myogenic cell lines (Neville et al., 1997).

The establishment of primary muscle cells involves labour-intensive preparations that include disassociation of tissues, isolation of mono-nucleated cells and maintenance in the tissue culture systems (Allen, 1987). Alternatively, muscle cells can be prepared from myogenic cell lines, such as the C_2C_{12} mouse cell line, which can be induced to form multinucleated myotubes (Blau et al., 1985; Yaffe and Saxel, 1977). This cell line is frequently used in muscle studies due to easier cultivation, maintenance and consistency compared to primary cultures.

As shown for ECM proteins (Zulfakar et al., 2012) (see Chapter 2), temperature may also be an important factor influencing bacterial attachment to muscle cells. For example, *Salmonella* Typhimurium grown at 37°C attached in higher numbers to whole pieces of lean tissue, whereas cells grown at 23°C attached preferentially to adipose tissue (Dickson, 1991). Temperature has also been shown to affect the production of bacterial surface structures that may promote or inhibit the attachment process (Chen et al., 2007; Junkins and Doyle, 1992).

This report describes the effect of temperature on attachment of *E. coli* and *Salmonella* to primary bovine muscle cells, and explores the potential of the C_2C_{12} mouse cell line as a surrogate for primary bovine muscle cells.

Materials and methods

Bacterial strains and culture

Six *E. coli* strains (M23Sr, EC614, EC473, H10407, EC623, E2348/69) and two *Salmonella* strains (Sal1729a and Sal691) were used (Table 1). Stock cultures were kept in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, England) and stored at -80°C. Preparations of cultures was as previously described (Zulfakar et al., 2012) (see Chapter 2). Briefly, working cultures were maintained on nutrient agar (NA; Oxoid CM0003, Basingstoke, UK) and stored at 4°C for no more than one month. Bacteria were grown overnight in tryptone soy broth (TSB; Oxoid CM0129, Basingstoke, UK) for 18-20 h at 37°C without shaking. Cell suspensions were prepared by centrifuging the TSB culture at 3,210 x *g* for 5 min, and cell pellets washed twice in Dulbecco A phosphate buffered saline, pH 7.3 (PBS; Oxoid BR0014, Basingstoke, UK) before resuspending in Dulbecco's modified minimal essential medium (DMEM; 1X high glucose) containing 25 mM $_{D}$-glucose, 164mM $_{L}$-glutamine and 110mM sodium pyruvate (Gibco, Life Technologies, USA) to achieve approximately 1.5 x 10^8 CFU/ml. One hundred microliters of diluted cell suspension were plated onto tryptone soy agar (TSA; Oxoid CM0131, Basingstoke, UK), in duplicate, to determine the cell concentration. Plates were incubated at 37°C for 24 h and colonies enumerated.

C_2C_{12} cell culture

C_2C_{12} (ATCC CRL-1772) cells were maintained at 37°C in a humidified 5% CO_2/ 95% air atmosphere, in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Australia) and an antibiotic solution of 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies, California USA). The culture medium was changed on alternate days until cells reached ~70% confluence, then split using 0.05% trypsin:EDTA (Gibco, Life Technologies, California, USA) for

10 min at 37°C. An equal volume of culture medium containing 10% FBS was used to stop trypsin activity. Detached cells were centrifuged at 300 x *g* for 10 min, resuspended in culture medium and then enumerated in a Neubauer counting chamber before seeding at a concentration of 2.5 x 10^4 cells/well in 24-well tissue culture plates (Greiner Bio-one, Frickenhausen, Germany) containing 2 ml growth medium/well. Cells were grown to ~90% confluence and the medium replaced with "fusion medium" consisting of DMEM supplemented with 2% heat inactivated horse serum (HS; Life Technologies, Australia) and antibiotic solution to induce differentiation and fusion (Blau et al., 1985; Katagiri et al., 1995). When >50% myotubes were formed, the culture medium was replaced with antibiotic-free fusion medium and the attachment assay was performed the following day.

Table 1. Bacterial isolates.

Strain ID	Bacterial species	Serotype
M23Sr	*Escherichia coli*	OR:H- (with streptomycin resistance)
EC614	*Escherichia coli*	O157:H12 [a]
H10407	*Escherichia coli*	O78:K80:H11 [b]
EC473	*Escherichia coli*	O26:H11 [a]
EC623	*Escherichia coli*	O157:H7 [a]
E2348/69	*Escherichia coli*	O127:H6 [b]
Sal1729a	*Salmonella enterica*	Anatum[a]
Sal691	*Salmonella enterica*	Typhimurium 135[a]

[a]Strain received from Dr. Narelle Fegan, CSIRO Food and Nutritional Sciences, Werribee, Australia.
[b]Strain received from Dr. R. Robins-Browne and Kristy Azzopardi/Stojcevski, Department of Microbiology and Immunology, University of Melbourne, Melbourne.

Establishment of bovine primary muscle culture

Muscle samples were excised from the neck of freshly slaughtered beef carcasses that were destined for sale at a commercial abattoir. The muscle samples then were transported back to the laboratory in ice-cold Hank's balanced salt solution (HBSS; no calcium and magnesium, Gibco, Life Technologies, USA) supplemented with 2.5 ug/ml fungizone (Gibco, Life Technologies, USA) and an antibiotic solution as described earlier. Excess connective tissue was trimmed and the muscle thoroughly minced. The minced tissues were subjected to enzymatic digestion with 250 U/ml collagenase (Worthington, New Jersey, USA) and 50 U/ml dispase (Worthington) at 37°C on a magnetic stirrer for 1 h. The suspension was then centrifuged at 300 x *g* for 10 min, the cell pellet resuspended in HBSS and centrifuged again as previously described. The cell pellet was resuspended in 0.05% trypsin:EDTA solution and incubated at 37°C for 15 min. An equal volume of DMEM supplemented with 10% FBS was added to stop trypsin digestion. The cell suspension was centrifuged as previously and the resulting pellet resuspended in growth media consisting of Ham's F10 nutrient mix (Gibco, Life Technologies) supplemented with 20% FBS, 2.5 ug/ml fungizone and antibiotic solution, as for C_2C_{12} cells. The digestion mixture was coarse-filtered to remove tissue debris and seeded into a T25 tissue culture flask (Greiner Bio-one, Frickenhausen, Germany). The cells were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere (Lavulo et al., 2008). After 24 h, the medium was replenished with fresh growth medium without fungizone. The culture medium was changed on alternate days until cells reached ~70% confluence, and then, they were sub-cultured. At the second passage, primary cells were split as described earlier and counted in a Neubauer counting chamber. The cells were seeded in tissue culture plates, grown to ~90% confluence and differentiated as previously described. Similar to C_2C_{12} cells, the attachment assay was performed in antibiotic-free fusion medium on the following day after >50% myotubes were formed.

Attachment assay

Bacterial attachment to muscle cells assay was determined based on the method used on epithelial cells with slight modifications (Mellor et al., 2009). In brief, prior to the attachment assay, muscle cells were washed once with HBSS to remove residual culture medium. Aliquots of 1 ml of the bacterial suspension were added to tissue culture wells and incubated at either 10°C or 37°C for 20 min. The wells were rinsed four times with 2 ml HBSS to remove unattached cells. Attached muscle and bacterial cells were detached from well surfaces by adding 250 µl of 0.05% trypsin:EDTA per well and incubating at 37°C for 10 min. The trypsin reaction was stopped using an equal volume of DMEM with 10% FBS, and then, the cell suspension was transferred into 1.5 ml reaction tubes (Greiner Bio-one, Frickenhausen, Germany). Following detachment, 100 µl of the cell suspension was 10-fold serially diluted before plating on TSA in duplicate. Plates were incubated at 37°C for 24 h. The colonies were counted and recorded as total bacterial cells recovered per well. Detached muscle cells were enumerated using a Neubauer counting chamber and the ratio of total bacterial cells attached to a muscle cell was calculated. The attachment assay was carried-out as described above, performed in triplicate and repeated on three separate days. Experiments with primary cultures were repeated with muscle cells derived from three different beef carcasses on separate days.

For control wells, antibiotic-free fusion medium was added into the wells and incubated at 37°C in a humidified 5% CO_2/95% air atmosphere two days prior to the attachment assay to simulate the conditions of plates containing muscle cells. The number of non-specific attached bacteria in control wells (no muscle cells) was used to estimate the non-specific bacterial attachment to the lateral sides of the well and the value was subtracted from test wells.

Statistical analysis

Results for all experiments were presented as mean ± standard deviation (SD). Student's *t*-test was used to assess the levels of bacterial cell attachment to the muscle cells compared to the controls. When a significant difference was found, the ratios of attached bacteria per muscle cells were compared by one-way ANOVA, followed by LSD test. Pearson product moment correlation coefficient was used to test the correlation of bacterial attachment ratio per muscle cells between C_2C_{12} and primary bovine muscle cells. All statistical tests were performed using IBM SPSS Statistics 19 (SPSS Inc.). Results were considered significant at $p < 0.05$.

Results

Bacterial attachment to C_2C_{12} cells

At 37°C, five of six *E. coli* strains (except EC623), and both *Salmonella* strains attached significantly to C_2C_{12} cells compared to the controls (Table 2). The levels of attachment to the C_2C_{12} cells differed significantly among strains at this temperature. However at 10°C, only two *E. coli* strains (H10407 and EC473), and no *Salmonella* strains, attached to C_2C_{12} cells and the levels of attachment were not different between these strains ($p > 0.05$). Also, no significant difference was observed for attachment between both H10407 and EC473 strains when attachment was measured at 37°C and 10°C.

For the subset of strains that attached to C_2C_{12} cells at either temperature, EC614 displayed the highest attachment rate at 37°C (4.60 ± 2.02 CFU/C_2C_{12} cell) ($p < 0.05$). No significant difference in attachment rate was observed among the other strains where values ranged from 0.20 to 2.22 CFU/C_2C_{12} cell).

Bacterial attachment to bovine primary muscle cells

Fewer *E. coli* strains attached to bovine primary muscle compared to C_2C_{12} cells at 37°C. Specifically, three *E. coli* strains (M23Sr, H10407 and EC473) and both *Salmonella* strains attached to bovine primary muscle cells (Table 3). In contrast, at 10°C more strains attached to bovine primary muscle cells than to C_2C_{12} cells. These included three *E. coli* strains (M23Sr, EC614 and H10407) and one *Salmonella* strain (Sal1729a).

Significant differences in attachment levels were observed among strains at 10°C but not at 37°C. Attachment levels were similar ($p > 0.05$) at 37°C and 10°C for strains that attached to primary muscle cells at both temperatures. For the subset of strains that attached to cells at either temperature, M23Sr displayed the highest attachment rate (51.88 ± 39.43 CFU/muscle cell) to primary bovine cells at 37°C, although there was no significant difference when compared to other strains at both temperatures ($p > 0.05$).

The attachment rate of Sal1729a and EC473 at 37°C was significantly higher for primary muscle than C_2C_{12} cells, whereas no significant difference was found for other strains. Correlation tests showed there was no significant relationship between bacterial attachment rate for C_2C_{12} and primary bovine muscle cells at both temperatures ($r^2 < 0.1$, $p > 0.05$).

Table 2: Bacterial attachment to C_2C_{12} muscle cells and number of bacteria attached per C_2C_{12} cell at 37°C and 10°C (mean ± SD).

Isolates	37°C		10°C	
	Log CFU bacteria attached to C_2C_{12} culture	Number of bacteria attached per C_2C_{12} cell	Log CFU bacteria attached to C_2C_{12} culture	Number of bacteria attached per C_2C_{12} cell
M23Sr	5.70 ± 0.32^{b}*	1.22 ± 1.22^{b}	NS	ND
EC614	6.36 ± 0.12^{a}	4.60 ± 2.02^{a}	NS	ND
H10407	5.69 ± 0.35^{b}	1.28 ± 1.39^{b}	5.75 ± 0.16^{a}	0.72 ± 1.16^{a}
EC473	5.67 ± 0.21^{b}	1.51 ± 0.88^{b}	5.53 ± 0.15^{a}	0.36 ± 0.57^{a}
EC623	NS	ND	NS	ND
E2348/69	5.14 ± 0.18^{c}	0.23 ± 0.08^{b}	NS	ND
Sal1729a	6.04 ± 0.24^{ab}	2.21 ± 1.20^{b}	NS	ND
Sal691	5.89 ± 0.30^{ab}	1.70 ± 0.94^{b}	NS	ND

NS- no significant attachment to C_2C_{12} cells compared to controls

ND- not determined

* Different letters indicate significant difference between strains within the same column ($p < 0.05$).

Table 3: Bacterial attachment to primary bovine muscle cells and number of bacteria attached per primary cell at 37°C and 10°C (mean ± SD).

Isolates	37°C		10°C	
	Log CFU bacteria attached to primary cell culture	Number of bacteria attached per primary cell	Log CFU bacteria attached to primary cell culture	Number of bacteria attached per primary cell
M23Sr	5.63 ± 0.24[a]*	51.88 ± 39.43[a]	5.83 ± 0.24[a]	13.17 ± 6.44[a]
EC614	NS	ND	5.71 ± 0.25[a]	22.86 ± 20.23[a]
H10407	5.28 ± 0.10[a]	11.49 ± 8.64[a]	5.53 ± 0.26[ab]	12.18 ± 8.69[a]
EC473	5.38 ± 0.04[a]	15.84 ± 4.62[a]	NS	ND
EC623	NS	ND	NS	ND
E2348/69	NS	ND	NS	ND
Sal1729a	5.24 ± 0.12[a]	11.29 ± 1.03[a]	5.11 ± 0.24[b]	15.46 ± 9.87[a]
Sal691	5.47 ± 0.27[a]	44.19 ± 60.32[a]	NS	ND

NS- no significant attachment to C_2C_{12} cells compared to controls

ND- not determined

* Different letters indicate significant difference between strains within the same column ($p < 0.05$)

Discussion

Numerous bacterial attachment studies have incorporated the use of epithelial cell lines such as Caco-2 and HT-29 to investigate mechanisms of attachment in the human intestinal system (Blay et al., 2004; Liu et al., 2010; Mellor et al., 2009). In this study, a similar approach was taken by using bovine skeletal muscle cells to understand bacterial interactions with muscle cells, one of the major components of a meat carcass. Skeletal muscle consists of multinucleated striated myofibres bound by connective tissue. This structure develops from the fusion of mono-nucleated myoblasts into multinucleated myotubes during myogenesis (Swatland, 2004).

H10407 was the only strain to attach to both C_2C_{12} and primary bovine cells at both temperatures. In contrast, EC623 did not attach to either muscle cell type under any condition. This is in agreement with the previous study that showed EC623 was a low binding strain with ECM proteins, while H10407 was an intermediate binding strain (Zulfakar et al., 2012) (see Chapter 2). In contrast, both ECM low-binding *Salmonella* strains attached to C_2C_{12} and primary bovine cells, although not under all test conditions. This indicates that some mechanisms involved in binding to ECM proteins and muscle cells are likely different.

Temperature has been shown to influence attachment of bacteria cells to bovine tissues (Cabedo et al., 1997; Selgas et al., 1993). In the previous study (Zulfakar et al., 2012) (see Chapter 2), multiple temperatures that are relevant to beef abattoirs were examined. In contrast, other published studies have either used a single experimental temperature (Benito et al., 1997; Cabedo et al., 1997) or reported the effects of bacterial cultivation temperature on attachment (Dickson, 1991). As shown previously, *E. coli* and *Salmonella* spp. showed lower attachment to ECM proteins at 37°C compared to other temperatures (Zulfakar et al., 2012) (see Chapter 2). In contrast, the present study shows that more strains and higher levels of attachment to muscle cells occur at 37 versus

10°C. Therefore, this further supports the hypothesis that different mechanisms are involved in bacterial attachment to ECM proteins and muscle cells.

There are reports of variation in bacterial attachment properties to different cell lines (Liu et al., 2010; Mellor et al., 2009). This agrees with findings in this study where more *E. coli* strains attached to the murine C_2C_{12} cell line than to primary bovine muscle cells. This indicates that C_2C_{12} cell surfaces may express receptors that mediate attachment for a larger range of strains. Although more strains attached to C_2C_{12} cells, the mean number of bacteria attached per muscle cell was higher for primary bovine cells. However, the difference in attachment rate between muscle cell types was only significant for Sal1729a and EC473, which was due to higher variation among replicates. This phenomenon has been reported in other assays that involved two independent biological systems (Mellor et al., 2009).

The establishment of primary muscle cell cultures is more labour-intensive, however these cultures more closely reflect *in vivo* properties compared to cell lines (Allen, 1987). Besides the differences due to mammalian species variation stated above, primary bovine cells were observed to be much slower than C_2C_{12} cells at producing myotubes required to conduct attachment assays. As the physiological function of myotubes *in vitro* is influenced by fusion time (Montgomery et al., 2006), the difference in fusion process duration may also contribute to differences in bacterial attachment observed in this study.

From this study, it was concluded that bacterial attachment to different types of muscle cells was strain- and temperature-dependant. Attachment rates differed between the two muscle cell types, indicating that C_2C_{12} cells are not optimal as surrogates for bovine primary muscle cells to understand attachment of bacterial pathogens to beef carcass surfaces.

Chapter 5

Influence of soluble extracellular matrix proteins on bacterial attachment to immobilized extracellular matrix proteins

Abstract

During the removal of animal hides, microorganisms are easily transferred to exposed connective tissues located between the skin and skeletal muscle tissues. These tissues are also exposed to external environments through tissue injury during fabrication and evisceration of the carcass. Connective tissue is composed of extracellular matrix (ECM) proteins that include collagen I, fibronectin, collagen IV and laminin. A possible carcass intervention is disruption of the bacterial adhesin-ECM receptor interaction via addition of soluble ECM proteins (sECM). In this study, the effect of sECM proteins on interactions between *E. coli* and immobilized ECM (iECM) proteins were characterised towards defining strategies to inhibit bacterial attachment to meat surfaces. Interaction patterns were quantified using a second-order polynomial model. Soluble basement membrane proteins (collagen IV, laminin) increased attachment of *E. coli* EC614 and H10407 to interstitial iECM proteins (collagen I, fibronectin), while soluble collagen IV and collagen I inhibited attachment of *E. coli* M23Sr to laminin and collagen IV, respectively. Soluble fibronectin had no influence on binding. These observations indicate that the addition of sECM proteins, in itself, may not be effective as an intervention strategy; however the knowledge obtained could serve as the basis for future studies of new synthetic analogs that could inhibit bacterial attachment to meat surfaces.

(Manuscript being prepared for publication)

Introduction

Microbial contamination of animal carcasses can lead to foodborne illness. In abattoirs, the initial source of carcass contamination is the hide, which may be contaminated with faeces, soil and filth (Antic et al., 2010). During slaughter and post-slaughter processes, microorganisms can be transferred from the hide onto the sterile underlying tissue; this begins with the initial cut of the carcass (Jackson et al., 2001). Subsequent sources of contamination include the gastrointestinal tract, workers' hands, equipment and other contact surfaces (Lawrie, 1998; Nørrung and Buncic, 2008).

Beef carcasses prepared for commercial trade mainly consist of muscular and fatty tissue, bones and connective tissues. Although connective tissue is a minor component, its presence is ubiquitous on the carcass surface, as it is located at the fascia (between the skin and skeletal muscle) and within muscle tissues (Davies, 2004; Taylor, 2004). During hide removal, microorganisms present on the hide can be easily transferred to exposed connective tissues that act as a protective cover for the muscle body (Valin et al., 1992).

Connective tissues are composed of a mixture of various extracellular matrix (ECM) proteins and complex polysaccharides. Dominant ECMs include collagen I, fibronectin, collagen IV and laminin (Bailey and Light, 1989; Taylor, 2004) that are widely distributed throughout the carcass. They can generally be categorized into: (1) interstitial proteins that include collagen type I (mainly located in the epimysium and perimysium) and fibronectin (located in the perimysium), and (2) basement membrane proteins made of collagen type IV and laminin (Chagnot et al., 2012). Fibronectin can also be located in the basement membrane (Beck et al., 1990).

Studies show that bacterial species including *E. coli* and *Salmonella* attach preferentially to ECM proteins (Frank, 2001; Medina, 2004; Medina and Fratamico, 1998; Zulfakar et al., 2012). It was previously found that wide strain variation in attachment of *E. coli* and *Salmonella* spp. to ECM proteins, and that attachment was selective based on the anatomical location (Zulfakar et al.,

2012)(see Chapter 2). Specifically, more strains attached to basement proteins (laminin and collagen IV) than to interstitial proteins (collagen I and fibronectin). Although the basement membrane is of microscopic size located in the intramuscular connective tissue (Bailey and Light, 1989), this protein layer can be easily exposed to the outer environment through tissue injury (Patti et al., 1994), such as that which occurs during fabrication and evisceration of the carcass.

Intervention treatments involving sprays and washes, many of which also involve physical removal of pathogens, are often applied to minimise food safety risks. Strongly attached bacterial cells are more difficult to remove, thereby reducing the effectiveness of decontamination processes (Fratamico et al., 1996; Tamblyn and Conner, 1997). Therefore, strategies are necessary to inhibit attachment, or remove or kill attached bacterial cells, without markedly affecting meat quality. Development of such intervention systems requires an understanding of the specific mechanisms that mediate the attachment process.

There is substantial evidence that bacteria-ECM interactions are based on specific molecular recognition (Boland et al., 2000; Hussain et al., 2001; Ljungh et al., 1991; Schwarz-Linek et al., 2004; Westerlund and Korhonen, 1993). As such, it may be possible to prevent initial adherence and subsequent contamination of the carcass surface by disrupting the bacterial adhesin-ECM receptors, via application of soluble inhibiting receptors (Shoaf-Sweeney and Hutkins, 2009). This study reports the effect of soluble ECM proteins on interactions between *E. coli* and immobilized ECM proteins.

Materials and methods

Bacterial strains and culture

Three *E. coli* strains (M23Sr, EC614 and H10407) were used to assess inhibition of attachment to immobilized ECM proteins (Table 1) by soluble ECM proteins. The strains were selected based upon

'high' (M23Sr and EC614) and 'intermediate'- (H10407) binding to various immobilized ECM proteins (Zulfakar et al., 2012)(see Chapter 2). Stock cultures were kept in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, England) and stored at -80°C. Preparation of cultures was as previously described (Zulfakar et al., 2012). Briefly, working cultures were maintained on nutrient agar (NA; Oxoid CM0003, Basingstoke, UK) and stored at 4°C for no more than one month. Bacteria were grown overnight in tryptone soy broth (TSB; Oxoid CM0129, Basingstoke, UK) for 18-20 h at 37°C without shaking. Cell suspensions were prepared by centrifuging the TSB culture at 3,210 x *g* for 5 min. Cells were washed twice and resuspended in Dulbecco A phosphate buffered saline, pH 7.3 (PBS; Oxoid BR0014, Basingstoke, UK) to achieve approximately 1.0 x 10^8 CFU/ml. One hundred microliters of appropriately diluted cell suspension were plated on tryptone soy agar (TSA; Oxoid CM0131, Basingstoke, UK) in duplicate, to measure the inoculum level. Plates were incubated at 37°C for 24 h and colonies enumerated.

Table 1. List of bacterial strains tested.

Strain ID	Bacterial species	Serotype
M23Sr	*Escherichia coli*	OR:H- (with streptomycin resistance)
EC614	*Escherichia coli*	O157:H12[a]
H10407	*Escherichia coli*	O78:K80:H11[b]

[a] Strain received from Dr. Narelle Fegan, CSIRO Food and Nutritional Sciences, Werribee, Australia

[b]Strain received from Dr. R. Robins-Browne and Kristy Azzopardi/Stojcevski, Department of Microbiology and Immunology, University of Melbourne, Melbourne

Preparation of extracellular matrix protein-coated surface

Extracellular matrix proteins; collagen I (calf skin collagen; Sigma, USA), fibronectin (bovine plasma fibronectin, Sigma; USA), collagen IV (Engelbreth-Holm-Swarm murine sarcoma; BD Biosciences, Bedford, USA) and laminin (Engelbreth-Holm-Swarm murine sarcoma; Sigma, Israel)

were prepared as previously described in Chapter 2 (Zulfakar et al., 2012). Briefly, 250 µl of protein solution (100 µg/ml) were coated onto 96-well tissue culture plates (Greiner Bio-one, Germany) and incubated at 37°C for 1 h. Excess fluid was removed from wells and plates stored at 4°C. Coated surfaces were rinsed with 200 µl PBS and air-dried at room temperature for 10 min prior to experimentation.

Attachment assay

One hundred microliters of bacterial suspension or 100 µl PBS (control wells) were added together with 100 µl of soluble ECM (sECM) protein (0, 50, 100 and 150 µg/ml) to each well coated with immobilized ECM (iECM) protein. For 0 µg/ml, PBS was added in place of sECM proteins. After incubation for 2 h at 25°C, bacterial suspensions were removed by pipette and plates rinsed three times with 200 µl PBS to remove loosely attached cells. Bacterial attachment to ECM-coated wells was measured using a crystal violet stain as reported by Zulfakar et. al (2012)(see Chapter 2). Briefly, attached cells were fixed at 55°C for 20 min and then stained with 95 µl of filtered 1% crystal violet for 45 min at 25°C. Excess crystal violet stain was removed by rinsing wells five times with sterile distilled water. Wells were then air-dried for 30 min at room temperature and destained by adding 100 µl of 95% ethanol to wells for 15 min. Attached cells were quantified by measuring absorbance with a microplate reader (Bio-Rad® Benchmark microplate reader, USA) at 595 nm. All experiments were performed in triplicate and each experiment was repeated three times. Results (absorbance at 595 nm) for all experiments were presented as mean optical density (OD) ± standard deviation (SD). Absorbance values were corrected by subtracting the average absorbance value from the corresponding control wells that did not contain bacteria.

Data analysis

One-way analysis of variance (ANOVA), followed by Least Significant Difference (LSD) test was used to assess the effects of adding sECM proteins at various concentrations on the attachment to iECM proteins. All statistical tests were performed using IBM SPSS Statistics 19 (SPSS Inc.). Results were considered significant at $p < 0.05$. When significant differences were found, data were fitted into a second order polynomial model.

$$y = Ax^2 + Bx^2 + C \qquad \text{(Eqn. 1)}$$

$y = OD_{595}$ value

x = ECM protein concentration

A, B and C are parameters to be estimated from the data.

The "yield of attachment" (max OD_{595} – min OD_{595}) was used to quantify and differentiate the extent of attachment due to addition of sECM proteins to the binding interactions.

A "stability factor" (max OD_{595} – fitted OD_{595} of last data point of the data for each trail)/yield) was also defined because in some trials attachment initially increased with increasing sECM, but decreased at high sECM level. Thus, the stability factor signifies the extent to which the max OD_{595} represents an asymptote, rather than a peak value. Values closer to 0 indicate the interactions were stable and reached a plateau.

Results

The effect of sECM proteins on *E. coli* attachment to iECM proteins varied with strains and ECM protein combination (Table 2). Results showed that sECM proteins increased, inhibited or showed no effect on *E. coli* attachment. Notably, soluble fibronectin did not affect attachment of any *E. coli* strain to iECM proteins (data not shown). Also notably, in most cases sECM enhanced binding or had no effect on binding.

sECM-strain-iECM interaction patterns in Table 2 were modelled using a simple second-order polynomial to quantify the interactive effect of sECM on *E. coli* binding to iECM (Table 3). Figure 1 is representative of the effects observed in the case of enhancement of attachment and shows the fit of the model to data.

Attachment to collagen I

Soluble collagen I did not affect attachment of any strain to immobilized collagen I. However, soluble collagen IV significantly increased attachment of EC614 and H10407 to collagen I, although levels of attachment were relatively low (OD_{595} < 0.1) (Table 2).

In contrast, soluble laminin produced a marked effect on EC614 and H10407. Similar to collagen IV, laminin had a relatively low (OD_{595} < 0.1) but significant enhancement effect of H10407 attachment, compared to EC614 and M23Sr. However, increasing concentrations of laminin significantly increased attachment of EC614 to collagen I, although there was little difference between 100 and 150 µg/ml (Table 2). Data fitted to Eqn. 1 suggested no additional binding of EC614 (max OD_{595} = 0.346) at concentrations of soluble laminin over 115.4 µg/ml (Table 3).

Table 2. Effect of solubilized ECM protein concentration (0, 50, 100, 150 µg/ml) on the attachment of *E. coli* M23Sr, EC614 and H10407 to immobilized ECM proteins. Optical density was measured at 595 nm.

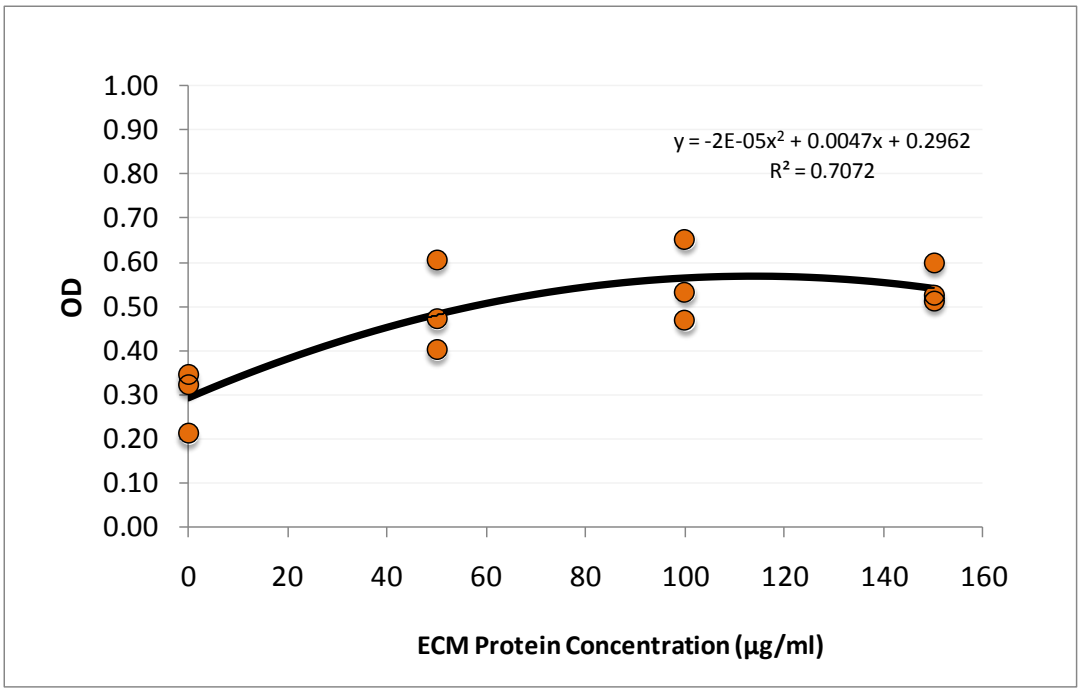
Immobilized ECM protein[1]	Soluble ECM protein	Strain	Mean OD ± SD[2]			
			Soluble protein concentration (µg/ml)[3]			
			0	50	100	150
Collagen I	Collagen IV	EC614	0.001±0.014[b4]	0.058±0.020[ab]	0.079±0.015[a]	0.091±0.045[a]
		H10407	0.004±0.008[c]	0.021±0.010[b]	0.022±0.003[b]	0.047±0.002[a]
	Laminin	M23Sr	0.274±0.017[b]	0.594±0.060[a]	0.562±0.033[a]	0.593±0.044[a]
		EC614	0.003±0.006[c]	0.267±0.023[b]	0.309±0.008[a]	0.327±0.020[a]
		H10407	0.004±0.008[c]	0.039±0.009[b]	0.062±0.018[ab]	0.084±0.029[a]
Fibronectin	Collagen IV	EC614	0.004±0.022[b]	0.202±0.033[a]	0.181±0.038[a]	0.182±0.045[a]
		H10407	0.000±0.015[c]	0.031±0.009[b]	0.055±0.022[ab]	0.076±0.009[a]
	Laminin	M23Sr	0.292±0.072[b]	0.491±0.104[ab]	0.550±0.093[a]	0.545±0.047[a]
		EC614	0.001±0.006[b]	0.416±0.082[a]	0.479±0.036[a]	0.530±0.089[a]
		H10407	0.000±0.015[b]	0.120±0.047[a]	0.165±0.043[a]	0.168±0.046[a]
Collagen IV	Collagen I	M23Sr	0.732±0.073[a]	0.745±0.117[a]	0.660±0.096[ab]	0.479±0.088[b]
Laminin	Collagen IV	M23Sr	0.547±0.024[a]	0.307±0.056[b]	0.211±0.023[c]	0.201±0.029[c]
	Laminin	M23Sr	0.500±0.007[b]	0.663±0.075[a]	0.706±0.041[a]	0.625±0.076[ab]

[1]ECM proteins (100 µg/ml) were coated on 96-well tissue culture plates.

[2]Only results that showed a significant an inhibiting/promoting effect by solubilized ECM proteins on attachment to immobilized ECM proteins are listed (p<0.05).

[3]Concentration of added solubilized ECM protein.

[4]Different letters indicate significant differences in mean OD among soluble protein concentrations for a specific strain.



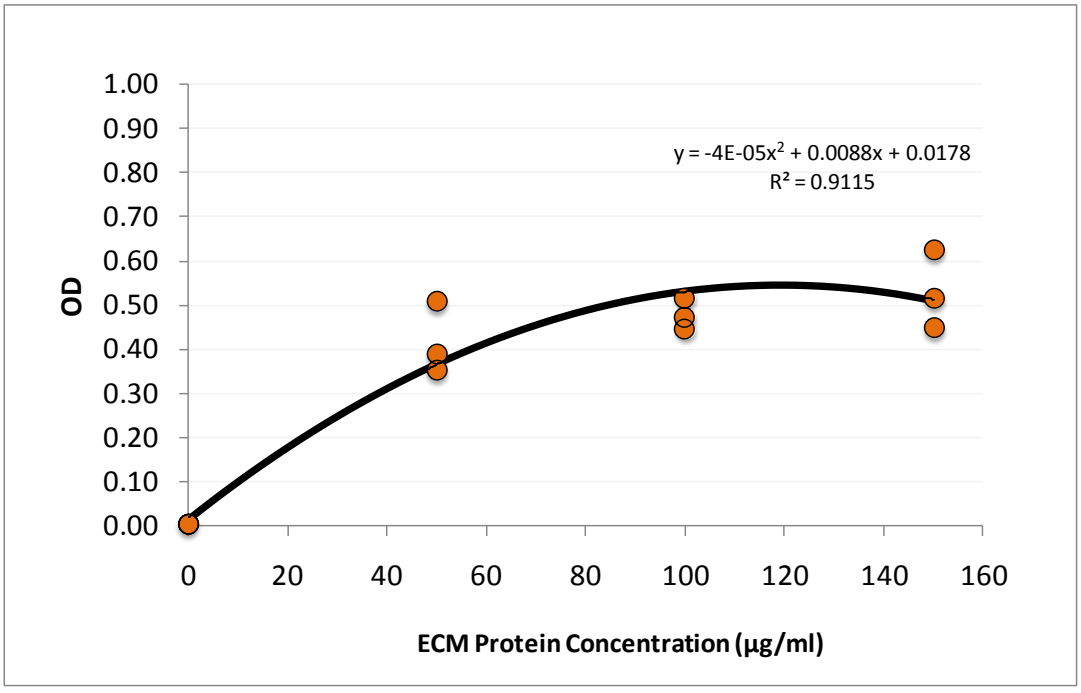


Fig. 1: Representative figures of sECM-strain-iECM interaction patterns as modelled using a simple second-order polynomial to quantify the interactive effect of soluble laminin on (A) M23Sr and (B) EC614 binding to immobilized fibronectin. Data points are average OD_{595} from three independent experiments.

Soluble laminin also significantly increased ($p < 0.05$) attachment of M23Sr to collagen I at 50 µg/ml (Table 2). However, there was no further increase in attachment at higher laminin concentrations. Although the maximum level of attachment for M23Sr was higher (max OD_{595} = 0.627; $p < 0.05$) than for EC614, there was no difference ($p > 0.05$) in the yield of attachment between these two strains (Table 3).

Attachment to fibronectin

Similar to collagen I, the addition of soluble laminin significantly increased attachment of all strains to fibronectin. Attachment increased for EC614 and H10407 ($p < 0.05$) only by addition of soluble collagen IV (Table 2). Other sECM proteins did not affect attachment for any strain ($p < 0.05$).

Soluble laminin had a significantly smaller effect (yield = 0.274) on attachment of M23Sr to fibronectin than observed for attachment to collagen I (yield = 0.337) (Table 3); the magnitude was smaller compared to EC614 (yield = 0.533). H10407 produced the lowest yield among the strains ($p < 0.05$). No increase in attachment was observed with increasing concentrations of laminin for any strain ($p > 0.05$).

The effect of soluble collagen IV on EC614 attachment to fibronectin was significantly smaller (yield = 0.209) than for laminin (Table 3). For H10407, although attachment increased with addition of collagen IV, all OD levels were > 0.1 (Table 2).

Attachment to collagen IV

The addition of soluble collagen I showed a significant inhibitory effect on attachment of M23Sr to immobilized collagen IV, but was only observed at the highest concentration (150 µg/ml)

where attachment was significantly lower than without addition of collagen I (Table 2). Attachment of EC614 and H10407 to collagen IV was not affected by any sECM proteins.

Attachment to laminin

The addition of soluble collagen IV and laminin significantly affected attachment of M23Sr to immobilized laminin, while other sECM proteins had no effect. As observed with immobilized collagen IV, EC614 and H10407 were not affected by any sECM protein.

Interestingly, soluble collagen IV produced a significant concentration-dependent reduction of M23Sr attachment to laminin. However, no difference in effect was observed between 100 and 150 μg/ml (Table 2). The inhibitory effect was significantly greater (yield = -0.353) than that of soluble collagen I on attachment of M23Sr to collagen IV (yield = 0.023) (Table 3).

Table 3. Quantitative description on the effect of sECM proteins on attachment of *E. coli* M23Sr, EC614 and H10407 to iECM proteins. Data from Table 2 were fitted into a second order polynomial model.

iECM protein	sECM protein	strain	maximum concentration[1]	maximum OD[2]	yield[3]	stability factor[4]	R^2 value
Collagen I	Collagen IV	EC614	128.849	0.090	0.092	0.027	0.705
	Laminin	M23Sr	106.338	0.627	0.337	0.169	0.828
		EC614	115.421	0.346	0.331	0.090	0.962
		H10407	248.175	0.096	0.090	0.156	0.794
Fibronectin	Collagen IV	EC614	101.050	0.218	0.209	0.235	0.794
	Laminin	M23Sr	112.762	0.568	0.274	0.109	0.707
		EC614	119.144	0.546	0.533	0.067	0.915
		H10407	121.485	0.176	0.178	0.055	0.812
Collagen IV	Collagen I	M23Sr	33.882	0.751	0.023	11.745	0.653
Laminin	Collagen IV	M23Sr	126.941	0.189	-0.353	0.033	0.957
	Laminin	M23Sr	91.118	0.569	0.226	0.418	0.748

[1]Maximum sECM protein concentration to saturate bacterial attachment to iECM protein.

[2]Maximum attachment level (OD_{595}) achieved by a bacterial strain when maximum sECM protein concentration added.

[3]Yield of bacterial attachment as an effect of sECM protein.

[4]Measurement of the stability of the fitted curve. Values closer to 0 indicate the interactions were stable and reached a plateau.

M23Sr attachment to laminin was significantly increased with soluble laminin. No difference was observed between 50 and 100 µg/ml soluble laminin ($p > 0.05$). However, at 150 µg/ml, a small reduction in attachment was observed. However the OD was not significantly different compared to attachment at other concentrations.

Discussion

Zulfakar et al (2012) reported that bacterial attachment to ECM proteins is related to the anatomical location of ECM proteins, in that more strains attach to basement membrane versus interstitial ECM proteins and that attachment varied among strains, even within the same species. These observations are consistent with earlier reports of strain specificity in attachment to iECM proteins (Fröman et al., 1984; Ljungh et al., 1991; Visai et al., 1990). Also, these observations indicate that attachment involves interactions between specific surface receptors that are not present on all strains (Ofek et al., 2003). Although the present study showed a lack of interaction between strains and iECM proteins, attachment might occur at levels below the sensitivity limit of the crystal violet assay (Zulfakar et al., 2012).

The strains used in the present study were selected based on results of previous studies (Zulfakar et al., 2012) (see Chapter 2) and included 'high' (M23Sr and EC614) and 'intermediate'-(H10407) binding strains. Relatively high levels of M23Sr attached to all ECM proteins, whereas EC614 and H10407 displayed high and intermediate attachment levels, respectively, to only basement membrane ECM proteins. This is similar as what was observed in the current study as shown by the assays in the absence of sECM proteins.

sECM proteins enhanced, inhibited or had no effect on attachment of *E. coli* strains to iECM proteins. An interesting observation was that EC614 and H10407 shared a similar attachment profile

compared to M23Sr. Specifically, EC614 and H10407 only attached to basement membrane proteins, whereas M23Sr bound to all four ECM proteins. Based on these observations, a schematic model (Figs. 2 and 3) was produced to illustrate possible interactions among sECM, bacteria and iECM proteins.

In the present and previous study (Zulfakar et al., 2012) (see Chapter 2), EC614 and H10407 only attached to iECM basement membrane proteins (laminin and collagen IV), and not to iECM interstitial proteins (collagen I and fibronectin). The addition of sECM interstitial proteins did not affect attachment to iECM proteins (Fig.2). As such, this may indicate that attachment between basement membrane and interstitial proteins involves distinct bacterial cell surface components that differ among bacteria, as reported by others (Martin and Timpl, 1987; Ofek et al., 2003; Saarela et al., 1996).

In contrast, sECM basement membrane proteins, especially laminin, increased attachment of EC614 and H10407 to immobilized collagen I and fibronectin (Fig. 2A and 2B). These effects agree with findings by Medina and Fratamico (1998) who observed synergistic binding effects of binary combinations of laminin and collagen I with *E. coli* O157:H7. It has also been reported that ECM protein molecules have an intrinsic property to self-associate and form ordered assemblies with other ECM proteins (Durbeej, 2010; Engel and Chiquet, 2011; Henderson et al., 2011; Martin and Timpl, 1987). Therefore, it can be hypothesized that soluble laminin and collagen IV may attach to iECM interstitial proteins, acting as a bridge and providing sites that mediate attachment of EC614 and H10407.

However, the presence of soluble collagen IV and laminin did not enhance attachment of EC614 and H10407 to iECM basement membrane proteins (collagen IV and laminin) (Fig. 2C and 2D). Although not tested in this study, this observation may indicate that receptors for basement membrane proteins on these strains are limited.

M23Sr has high affinity binding sites for all four iECM proteins tested (Fig. 3). Similar to EC614 and H10407, soluble laminin enhances attachment of M23Sr to iECM interstitial proteins (Fig 3A and Fig. 3B). Moreover, unlike EC614 and H10407, soluble laminin also enhances M23Sr attachment to laminin (Fig, 3D), indicating there may be more M23Sr binding sites for laminin than on the other bacterial strains tested.

Soluble fibronectin did not affect attachment of *E. coli* strains to any iECM protein. This observation was not surprising for EC614 and H10407 (Fig. 2), as these strains were previously shown, via the crystal violet assay, not to attach to fibronectin (Zulfakar et al., 2012). However, for M23Sr that displayed high attachment to immobilized fibronectin, the absence of an effect for soluble fibronectin (Fig. 3) could indicate that M23Sr does not bind to soluble fibronectin. This agrees with the study by Westerlund and Korhonen (1993) who reported some *E. coli* strains only bind to immobilized, but not soluble fibronectin.

It is possible that fibronectin binds to iECM proteins at different sites compared to M23Sr and therefore, addition of soluble fibronectin does not inhibit M23Sr attachment to the iECM protein tested. In this regard, another study indicates that *E. coli* binding sites for collagens and fibronectin are located on different structures on *E. coli* NG7C (Ljungh et al., 1991). Another possible explanation is that soluble fibronectin attaches to immobilized fibronectin that then undergoes conformational changes, thus altering binding site specificity for M23Sr.

The aim of this study was to determine if sECM proteins could inhibit bacterial-iECM protein interactions, as a means to reduce carcass contamination. However, these studies show that most bacterial-iECM interactions are enhanced by sECM proteins. Reductions were only observed when soluble collagen I and collagen IV were tested with immobilized collagen IV and to laminin, respectively. However, this effect was only observed for M23Sr. It can be hypothesized that both soluble ECM proteins share similar attachment sites with M23Sr to the respective iECM proteins and thus, block attachment of bacteria.

From this study, it is concluded that sECM proteins are not suitable intervention strategies, however they may aid in future research to explore effects of synthetic analogs to block ECM binding sites and reduce carcass contamination.

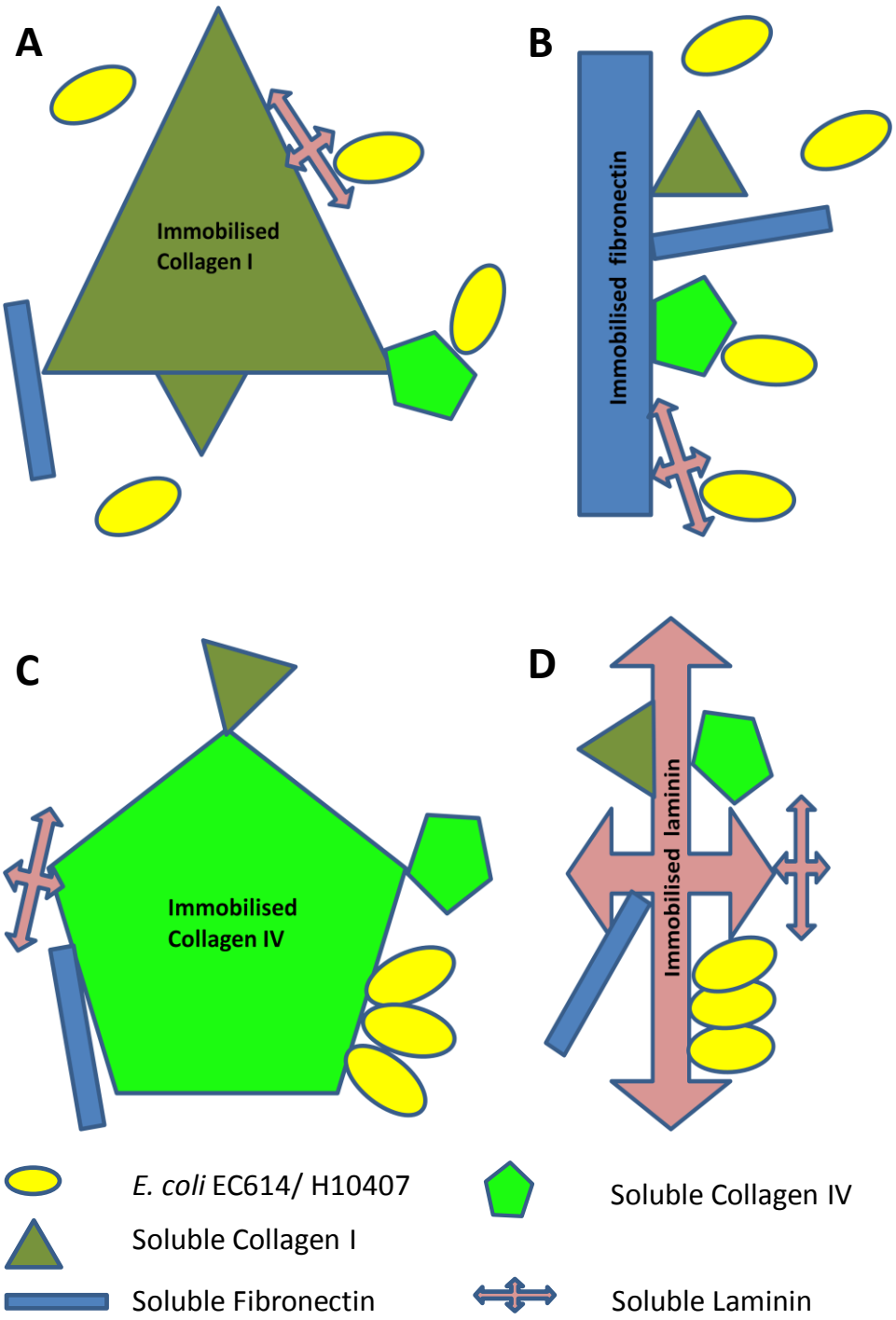


Fig. 2: Potential interactions of EC614 and H10407 with immobilized and soluble ECM proteins. EC614 and H10407 only attaches to immobilized basement membrane proteins; collagen IV (C) and laminin (D). It was hypothesized that presence of soluble basement proteins acts as a bridge and providing sites that mediates attachment to immobilized interstitial proteins; collagen I (A) and fibronectin (B). However, addition of soluble basement proteins did not enhanced attachment to their immobilized form.

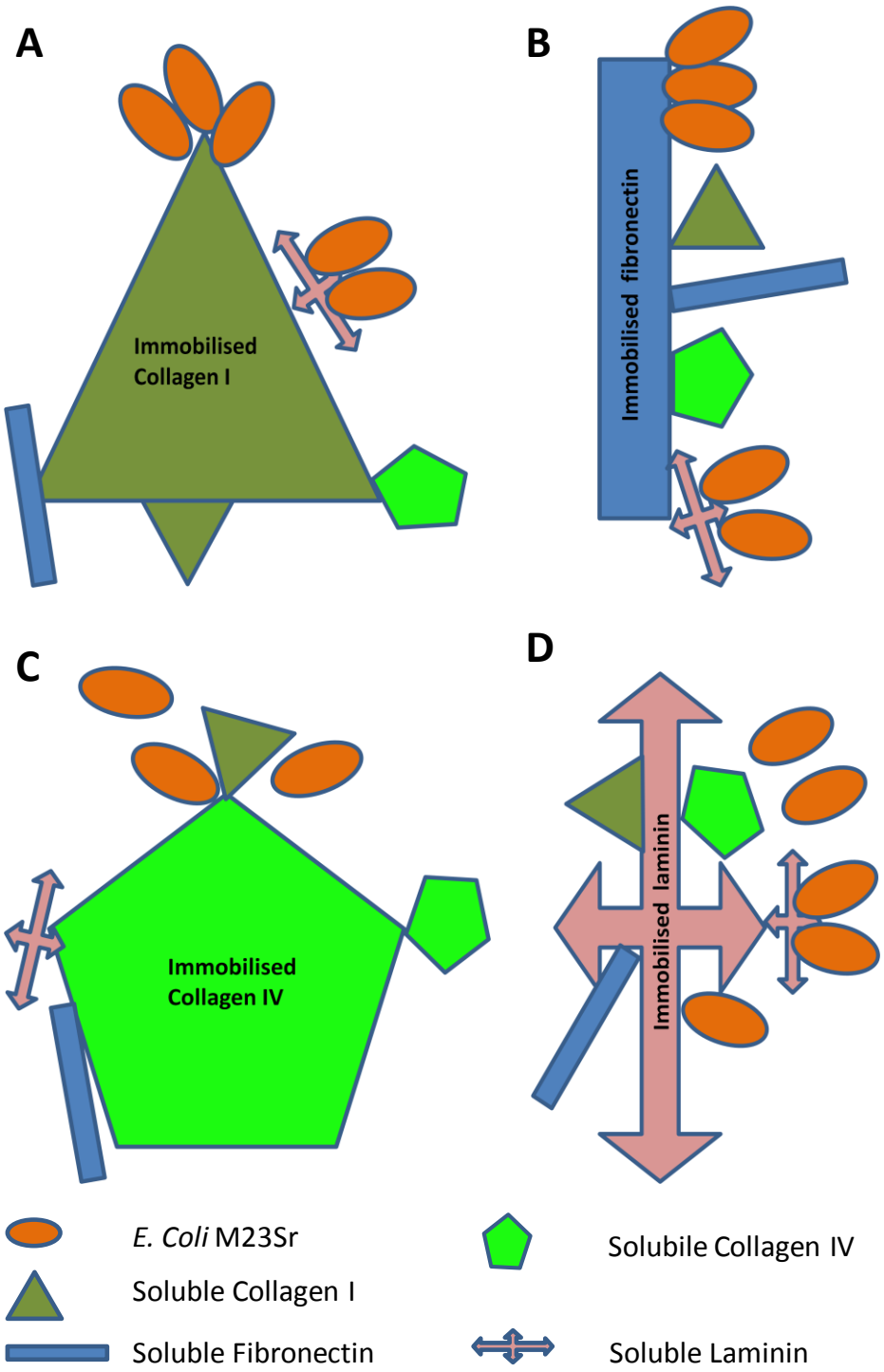


Fig. 3: Potential interactions of M23Sr with immobilized and soluble ECM proteins. M23Sr attaches to all immobilized ECM (iECM) proteins. Presence of soluble laminin enhances the attachment to immobilized collagen I (A), fibronectin (B) and laminin (D) by acting as bridge and more attachment sites. However, it was hypothesized that presence of soluble collagen I in interactions with immobilized collagen IV (C) and soluble collagen IV in interactions with immobilized laminin (D) blocks M23Sr attachment to the respective iECM proteins.

Chapter 6

General discussions

Introduction

Carcass contamination occurs during various processes in abattoirs, including dehiding, evisceration, carcass washing and fabrication. Bacterial attachment to carcases plays an important role in the spoilage and safety of meat.

The majority of published reports on factors that mediate bacterial attachment to meat surfaces used whole pieces of meat (Chen et al., 2007; Rivas et al., 2006). However, this approach does not provide insight into the specific and complex processes involved in bacterial attachment to heterogeneous meat tissue (Goulter et al., 2009; Selgas et al., 1993).

The primary objective of this thesis was to investigate the attachment of two pathogenic bacterial species, *E. coli* and *Salmonella,* to specific meat tissue structures, namely the extracellular matrix (ECM) proteins and muscle cells, and how this process is influenced by intrinsic and extrinsic factors that are relevant to the abattoir environment.

Major findings and contribution of this thesis

In the first part of this study (Chapter 2), the attachment of *E. coli* and *Salmonella* strains to four major ECM proteins (collagen I, fibronectin, collagen IV and laminin) was investigated, including the effects of temperature. Attachment varied among species and within strains of the same species, illustrating patterns of 'high-' and 'low-' binding activity, and also suggesting that receptor sites differed among strains. Attachment was not associated with strain pathogenicity, but interestingly showed a preference based on the anatomical location (basement membrane versus interstitial) of ECM protein. This indicates that attachment may involve specific receptors on the bacterial cell and meat tissues, including specific ECM proteins.

Temperature played a significant role in bacterial attachment to ECM proteins. The highest levels of bacterial attachment to ECM proteins occurred at 4°C for collagen I, and at 25°C for other ECM proteins. Bacterial attachment to ECM proteins was notably low at 37°C. This suggests that temperature may have a significant, but differential influence on preferred binding sites, and the levels of attachment to specific ECM proteins. This also implies that interventions should target carcass contamination events before the chilling process, where the average temperature of carcass muscle surface is 25-29°C (Jericho et al., 1998). These results provide fundamental knowledge about bacterial attachment to ECM proteins, and support future studies to identify receptors that mediate attachment. Based on the results from this study, a subset of strains representing 'high-', 'intermediate-' and 'low-' binding properties to ECM proteins was chosen for further investigations in this thesis.

The second phase of study (Chapter 3) tested the effects of additional conditions relevant to meat processing, namely pH and salt. pH had no effect on attachment to ECM proteins within a range of pH 5 to 9. This observation may have resulted from a limited range of pH that may, in consequence, not have induced bacterial stress (Foster, 1995; Small et al., 1994), a factor known to increase attachment. In addition, the relatively long incubation period (2 h), may have allowed the bacterial cells to adapt to changes in pH. This result also implies that if pH was intended to be manipulated for carcass decontamination treatments, a larger range of pH may be needed for better treatment efficacy.

The effect of salt concentration (0.1–5%) varied depending on strain-ECM protein combination, which could be related to surface charge interactions between the bacterial cell and ECM protein. Across the strains, the effect of salt, if observed, influenced strain attachment proportionally to levels observed without treatment. For example, strains that displayed 'high', 'intermediate' and 'low' attachment patterns in PBS, showed similar proportional levels of attachment under experimental treatments. Variation in effects among strains indicates that

manipulation of these factors would be difficult to implement as interventions in commercial processes.

The effectiveness of chemical rinses to remove surface contamination has been reported (Cutter et al., 1997; Dorsa et al., 1997; Kim and Slavik, 1994). However, the effect of rinses in reducing bacteria attached to ECM proteins has not been reported. Three rinses: acetic acid, lactic acid and trisodium phosphate (TSP), were tested and the results reported in Chapter 3. Interventions that included TSP were most effective (97.3% ± 3.9 reduction) in reducing numbers of bacteria attached to ECM proteins, as compared to organic acid rinses (41.2% ± 28.5 reduction). TSP is known to generally target the bacterial cell wall and its effect is largely attributed to its high alkaline pH (Mendonca et al., 1994) and sequestration of metal ions (Capita et al., 2002). As it is unclear whether the ECM proteins were also affected, this research could be extended to determine if TSP has an effect on ECM protein structure as well.

The third phase of study investigated bacterial attachment to bovine skeletal muscle cells, providing a broader perspective of bacterial interactions with carcass surface structures (Chapter 4). Attachment to primary bovine muscle cells and a cultured muscle cell line (C_2C_{12}), employed as a potential surrogate for primary cells was investigated, including the effect of temperature. Similar to immobilized ECM proteins, bacterial attachment to muscle cells was strain-dependant and significantly influenced by temperature. However, unlike ECM proteins, there were more strains that attached at higher levels at 37°C compared to lower temperature (10°C), suggesting that mechanisms involved in attachment to muscle cells and ECM proteins are likely different. Also, attachment of strains to the two muscle cell types differed significantly. This indicates that both types of muscle cells may not express similar surface receptors, and that C_2C_{12} cells are not suitable as a model system to understand bacterial attachment to beef carcass surfaces.

The fourth and final phase of this thesis explored the potential of soluble ECM (sECM) proteins to inhibit bacterial attachment to meat surfaces, by characterizing the effect of sECM proteins on the

interactions between bacterial cells and immobilized ECM (iECM) proteins (Chapter 5). These experiments focused on three strains (*E. coli* M23Sr, EC614 and H10407) that showed 'high' and 'intermediate' attachment levels to interstitial and basement membrane proteins (based on results presented in Chapter 2).

The schematic model (Chapter 5, Figs. 2 and 3) illustrates possible interactions among sECM, bacteria and iECM proteins. Soluble fibronectin had no effect on attachment of *E. coli* to any iECM protein. In general, sECM proteins mostly enhanced bacterial attachment to iECM proteins. An interesting observation was that soluble basement membrane proteins (collagen IV and laminin) significantly increased EC614 and H10407 attachment to immobilized interstitial proteins (collagen I and fibronectin). These strains were shown previously in earlier chapters to only attach to soluble basement membrane and not to immobilized interstitial proteins. A plausible explanation is that soluble laminin and collagen IV attach to immobilized collagen I and fibronectin, acting as a bridge between bacteria and immobilized interstitial proteins, providing sites that mediate attachment.

The affinity of EC614 and H10407 only for basement membrane proteins was also evidenced by the absence of an effect when soluble interstitial proteins (collagen I and fibronectin) were added to any of the interactions in this part of study including to immobilized basement membrane proteins. This may indicate that distinct bacteria–ECM receptor recognition was involved in the attachment to basement membrane and interstitial proteins. However, the attachment sites for basement membrane proteins on these strains might be limited, as shown when addition of soluble basement membrane proteins did not enhance attachment to immobilized ECM proteins.

Unlike EC614 and H10407, M23Sr possessed high affinity for all four iECM proteins. Soluble laminin also enhanced M23Sr attachment to iECM interstitial proteins and also to immobilized laminin. As such, M23Sr may have a higher number of binding sites for laminin than EC614 and H10407.

Of all iECM-bacteria-sECM permutations tested, inhibition of attachment only occured with addition of soluble collagen IV and collagen I, specifically with immobilized laminin and collagen IV, respectively. Furthermore, this effect was only observed for M23Sr. These results indicate that sECM proteins are likely not suitable as an intervention method to minimise carcass contamination by pathogenic bacteria. Nevertheless, these findings may be beneficial for future development of new carcass intervention systems, using synthetic analogs to effectively block bacteria-ECM proteins interactions.

Future directions

Further research can build on the findings of this research to provide a fuller understanding of mechanisms that mediate bacteria-carcass interactions. Such studies include:

- *Characterizing specific bacterial surface structures/molecules that mediate attachment*

Variations among strains that attached to ECM proteins (Chapter 2) and muscle cells (Chapter 4) may result from differences in expression of bacterial surface adhesins. This may contribute to selective attachment by certain strains and explain higher affinity to basement membrane proteins. Potential adhesive surface structure(s) to investigate include fimbriae, curli and extracellular polysaccharide (EPS). This would also include comparative studies to determine the expression of such adhesive structures on 'high-' and 'low'-binding strains and whether there is a correlation between expression and affinity for attachment to ECM or muscle cells. Evaluation of adhesion by mutants of high affinity strains, defective in specific surface structures/proteins, may provide useful insights. *Escherichia coli* M23Sr may be a good model organism, since it displayed the highest level of

attachment and was the only strain that attached to all ECM proteins tested in this thesis. The significance of M23Sr streptomycin resistance, if any, to attachment could also be investigated.

- *Characterization of ECM proteins and muscle cells structures/surface receptors that mediate bacterial attachment*

Similar to bacterial cells, specific surface receptors that interact with bacterial adhesins are present on the meat surface. Characterizing surface receptors on interstitial and basement ECM proteins, and on muscle cells, would provide a more detailed understanding of attachment mechanisms. Investigation would include identification of specific structures/molecules that interact with bacterial adhesins and characterization of different surface receptors on interstitial and basement membrane proteins which contributes to the preferential attachment to the latter ECM proteins.

- *Studies of other carcass surface structures*

Different mechanisms might be involved in bacterial attachment to ECM proteins versus muscle cells (Chapter 4). It would be beneficial to extend this study to investigate the potential role of other meat tissue structures, such as adipose tissues and muscle cell proteins, namely myosin and actin. This would provide a broader understanding of the attachment process. Also, investigations could include studies of meat tissue structures and mechanisms that could result in physical entrapment of bacterial cells to the carcass surface. Confocal scanning laser microscopy (CSLM) and transmission electron microscopy (TEM) would be useful to visualize and investigate the mechanisms of this effect.

- *Studies of other potential substances to be used as intervention systems for carcass decontamination*

In Chapter 3, trisodium phosphate (TSP) was reported to be the most effective treatment in reducing bacterial cells attached to ECM proteins. However, it should be noted that this treatment, due to its high pH level, may affect the product quality and pose a hazard to food workers and to the environment if not properly managed. Investigations would include identifying new treatments such as using substances derived from plant extracts with similar or better efficacy.

Conclusion

In conclusion, this thesis provides new insights into bacterial attachment to carcass surface structures and environmental factors that influence attachment. Based on these findings, bacterial attachment to the meat surface is strain- and tissue structure- dependent. Although attachment efficacy and specificity varied due to these factors, preferential attachment based on anatomical location of the meat tissue structures, was observed. The findings also suggest that design and implementation of carcass intervention systems should include temperature, as it significantly influences the bacterial attachment process. Such knowledge will be beneficial in designing more targeted interventions to prevent and/or disrupt interactions between bacterial cells and the meat surface, thus increasing the safety and quality of meat products for human consumption.

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Appendix A

Bacterial attachment to immobilized extracellular matrix proteins *in vitro* (Chapter 2)

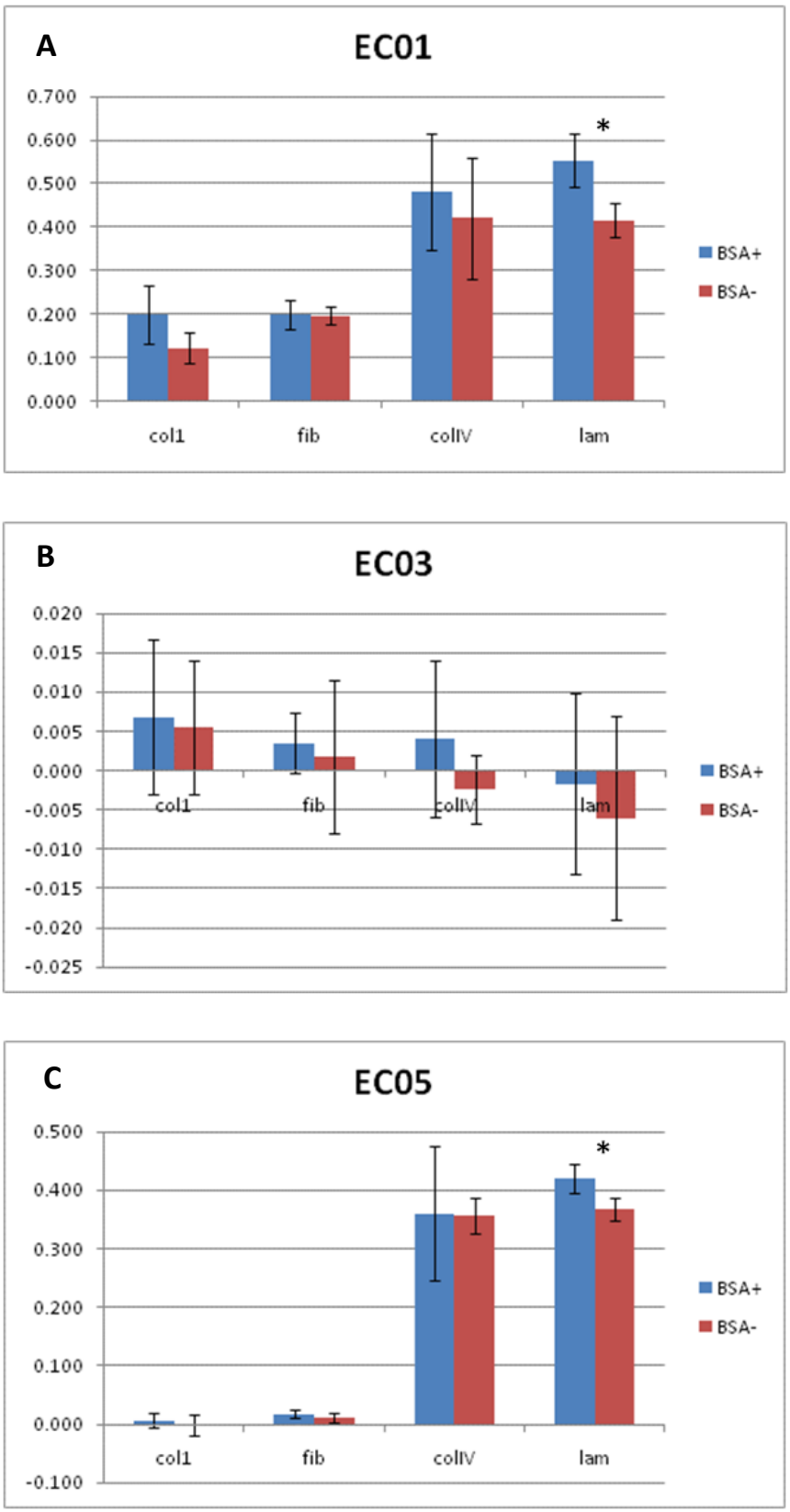

Fig. A.1 - Comparison of bacterial attachment to BSA-saturated wells (BSA+) and non-saturated wells (BSA-). A) EC01 -*E. coli* M23Sr B) EC03 – *E. coli* O157:H7 C) EC05- *E. coli* O157:H12. (*) significant difference at $p<0.05$ by student T-test.

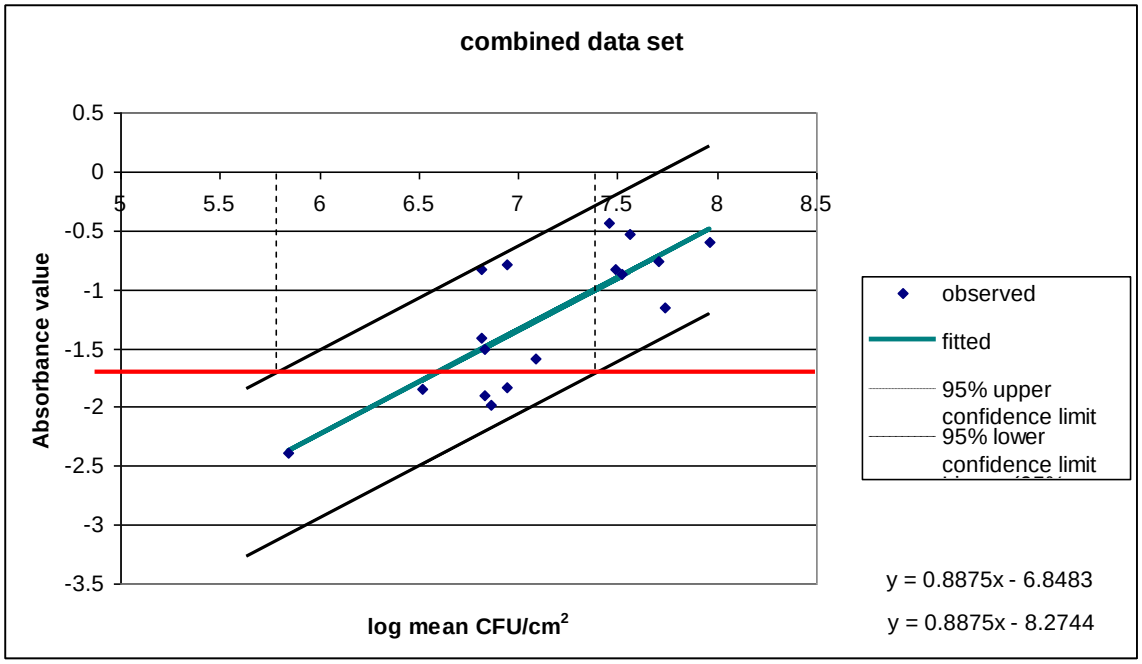

Fig. A.2: The lower detection limit for the crystal violet assay. The figure above shows the line of best fit (**—)** through the combined data set for all proteins with the upper and lower 95% confidence interval on the fitted lines. 95% lower confidence limit equation y = 0.8875x – 8.2744. 95% upper confidence limit y = 0.8875x- 6.8483. The lower detection limit range for the crystal violet assay is log 5.8 – 7.4 CFU/cm^2.

Appendix B

Effect of pH, salt and chemical rinses on bacterial attachment to extracellular matrix proteins (Chapter 3)

Table 1: Effect of pH on the bacterial initial attachment to ECM proteins.

ECM Protein	Strains	Mean absorbance values ± SD				
		pH 5	pH 6	pH 7	pH 8	pH 9
*Collagen I	M23Sr	0.762 ± 0.232 ^{A}	0.541 ± 0.244 ^{A}	0.647 ± 0.157 ^{A}	0.710 ± 0.416 ^{A}	0.737 ± 0.256 ^{A}
Fibronectin	M23Sr	0.331 ± 0.054 ^{A}	0.279 ± 0.068 ^{A}	0.314 ± 0.049 ^{A}	0.212 ± 0.106 ^{A}	0.291 ± 0.020 ^{A}
	EC614	0.036 ± 0.012 ^{A}	0.018 ± 0.011^{A}	0.036 ± 0.006 ^{A}	0.035 ± 0.033 ^{A}	0.033 ± 0.013 ^{A}
	H10407	0.015 ± 0.005 ^{A}	0.011 ± 0.005 ^{A}	0.019 ± 0.004 ^{A}	0.006 ± 0.011 ^{A}	0.014 ± 0.013 ^{A}
	Sal1729a	0.013 ± 0.009 ^{AB}	0.005 ± 0.006 ^{B}	0.023 ± 0.005 ^{A}	0.014 ± 0.012 ^{AB}	0.019 ± 0.008 ^{AB}
Collagen IV	M23Sr	0.546 ± 0.038 ^{A}	0.487 ± 0.091 ^{A}	0.505 ± 0.081 ^{A}	0.412 ± 0.056 ^{A}	0.515 ± 0.06 ^{A}
	EC614	0.444 ± 0.09 ^{A}	0.385 ± 0.050 ^{A}	0.440 ± 0.660 ^{A}	0.449 ± 0.056 ^{A}	0.449 ± 0.053 ^{A}
	EC473	0.067 ± 0.021 ^{A}	0.079 ± 0.017^{A}	0.073 ± 0.036 ^{A}	0.053 ± 0.014 ^{A}	0.056 ± 0.014 ^{A}
	H10407	0.093 ± 0.020 ^{A}	0.069 ± 0.019 ^{A}	0.073 ± 0.027 ^{A}	0.073 ± 0.024 ^{A}	0.100 ± 0.012 ^{A}
	Sal1729a	0.002 ± 0.017 ^{A}	-	0.01 ± 0.004 ^{A}	0.021 ± 0.027 ^{A}	0.013 ± 0.007 ^{A}
Laminin	M23Sr	0.529 ± 0.051 ^{A}	0.484 ± 0.078)^{A}	0.514 ± 0.056 ^{A}	0.444 ± 0.083 ^{A}	0.509 ± 0.048 ^{A}
	EC614	0.447 ± 0.032 ^{A}	0.430 ± 0.023)^{A}	0.450 ± 0.017 ^{A}	0.485 ± 0.050 ^{A}	0.445 ± 0.050 ^{A}
	EC473	0.083 ± 0.036 ^{A}	0.105 ± 0.022 ^{A}	0.084 ± 0.056 ^{A}	0.074 ± 0.034 ^{A}	0.075 ± 0.043 ^{A}
	H10407	0.081 ± 0.03 ^{A}	0.085 ± 0.025 ^{A}	0.085 ± 0.037 ^{A}	0.097 ± 0.024)^{A}	0.115 ± 0.042 ^{A}
	Sal1729a	0.005 ± 0.002 ^{A}	0.003 ± 0.011 ^{A}	0.014 ± 0.010 ^{A}	0.013 ± 0.020 ^{A}	0.012 ± 0.006 ^{A}

*Only E. coli M23Sr showed significant attachment to Collagen I as compared to control

Means with different capital letters across the columns indicate significant differences between pH levels for that bacterial strain ($p< 0.05$)

- Indicates the absorbance values are equal or less than control's absorbance values.