Bacterial Community Analysis of Meat Industry Conveyor Belts

by

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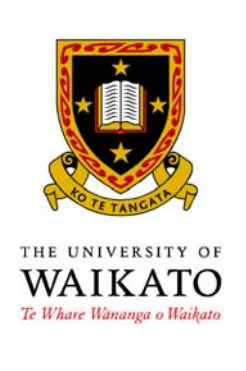

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Abstract

At the commencement of this study, some sensitive overseas markets were rejecting chilled vacuum-packed New Zealand lamb due to higher than expected total viable counts, and counts of Enterobacteriaceae, a family of bacteria used to indicate sanitary condition.

Of the many factors that influence the bacterial composition of chilled lamb in the overseas marketplace, the meat producer can only exert significant control over:

- Hygiene, ensuring the bacterial viable count on the meat prior to packaging is as low as possible, and comprised of as few species as possible that are capable of anaerobic growth at chilled meat temperatures.
- Maintaining the pH of the meat within acceptable limits, by careful animal selection and minimal pre-slaughter stress.
- Refrigeration temperatures, through rigorous maintenance of the cold-chain.
- The type of preservative packaging used, which is often limited by regulation in the marketplace.

Initial work established that the bacterial microbiota present on the meat contact surfaces in the butchering facilities at some premises, in particular conveyor belting, was excessive and comprised of species that contributed to the high counts on the meat reported above.

As a means of improving the hygiene of this process, this study investigated the hypothesis that some species of bacteria were able to form biofilms on the conveyor belt contact surfaces, becoming reservoirs for cross-contamination. This hypothesis was ***not*** been proven by this work; the results showing that biofilms were not present and that adequate hygiene of these surfaces instead depends on the ability to remove all meat-based residues from them at the completion of each

day's processing.

For premises operating interlocking belts from one manufacturer (Intralox®), a clean-in-place system is now available that is able to achieve this. Premises operating conventional disinfectant and water sanitisation of either continuous or interlocking belts must ensure that meat residue is completely removed before disinfection.

The majority of New Zealand meat industry premises can now demonstrate that their hygienic processes in this area are under control.

The microbiota of conveyor belting in this study was found to consist of bacteria from five taxonomic groups; the Flavobacteriaceae, the Actinomycetales, the Bacillus/Clostridium group, and the α- and γ- branches of the Proteobacteria. The genera present on belts from premises whose hygiene was found to be in control did not contain species known to cause food-borne disease or spoilage of vacuum packaged meats.

The bacterial viable count remains the most effective method available at this time for monitoring conveyor belt hygiene. Attempts to develop a monitoring system based on microscopy of an in-situ sampling device were unsuccessful due to an inability to penetrate the meat residue matrix. Denaturing Gradient Gel Electrophoresis (DGGE) may offer an alternative for rapid investigation of diversity, but further work is required before this can be validated for routine use.

Acknowledgements

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Chapter 1: Introduction

A significant proportion of New Zealand's export trade in chilled sheep-meat is subject to customer-imposed microbiological limits that, being based on the fresh meat supplied in the customer's own country, can offer very little margin for error for in terms of storage temperature or time. These limits have been justified by customer groups on the basis of empirical observations that exceeding these limits resulted in poor storage quality (Bell & LeRoux, 2002). Having containers of meat downgraded (from premium priced chilled meat to frozen) as a consequence of microbiological test results is costly, both to the manufacturer and New Zealand as a whole, from immediate loss of revenue and long term loss of credibility. Significant downgrades of chilled vacuum-packaged lamb began to emerge in 2001. Research to further improve the microbial status of this product was therefore considered a high priority.

The quantity and identity of bacteria isolated from meat in the marketplace is determined by many contributing factors. This review provides an overview of meat processing in New Zealand, the current knowledge concerning the microbiology of exported fresh meats, the technologies used in their preservation, the role of cross-contamination and biofilm development, and the results of the initial investigation into this matter.

Exporting of sheep-meat from New Zealand

The meat industry is a significant contributor to the New Zealand economy, which yielded $5.3 billion of earned export revenue, and $1.05 billion of domestic sales, in the year to April 2005 (MIA, 2007). Meat production in New Zealand is focussed primarily on sheep meat (lamb and mutton), and beef. Smaller amounts of veal, venison and goat meat are produced for export, whilst production of poultry and pork meats are almost completely restricted to domestic markets. As

this thesis is concerned primarily with improving the storage quality of chilled sheep-meat, this review will be restricted to this class of meat and its markets.

Total sheep meat and co-products exported in 2004 were worth $2.7 billion. Of this, exports to the European Union (EU) were worth $1.55 billion, with 181,882 tonnes product weight shipped, representing 64% of sheep meat exports by value and 50% by volume (MWNZ, 2005). The North American market was the second largest for sheep meat export, worth $382 million.

The export value of sheep-meat increased steadily up to 2004, then stabilised (**Figure 1**). A large proportion of that increase can be attributed to the increasing volume of chilled lamb cuts exported (dark blue), which have a significantly higher value than frozen meat. In 2006, the total value of chilled sheep-meat exported was approximately $588million (Ryan, 2006).

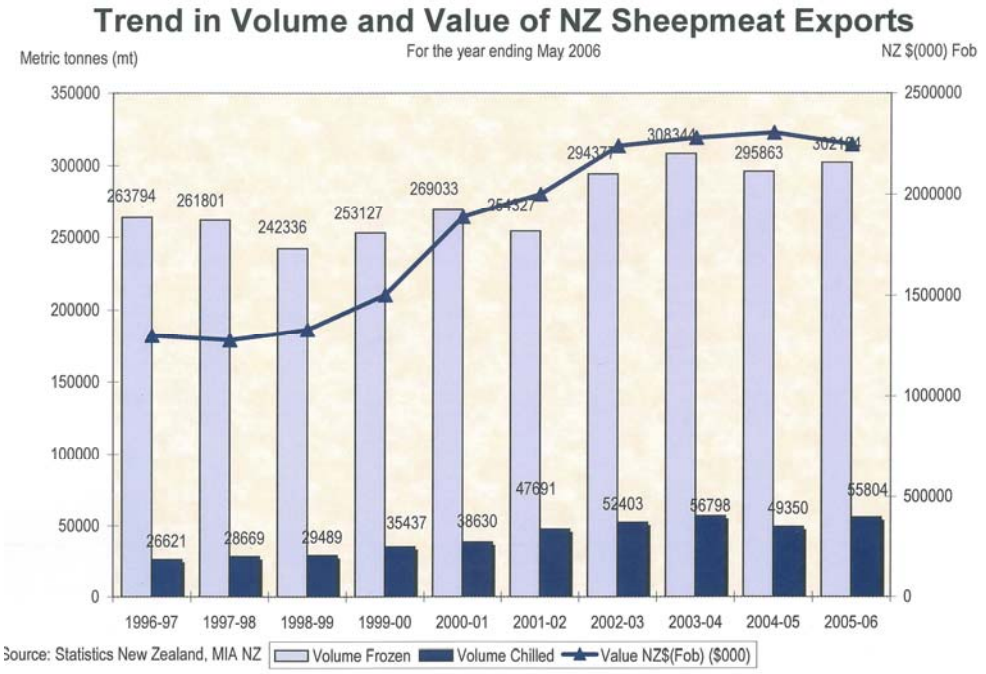


Figure 1 – Trend in volume and value of NZ sheep-meat exports (MIA, 2007). Reproduced with permission

The higher value chilled cuts do, however, place a high dependence on storage technologies (principally refrigeration to -1.5°C and packaging systems) to reach

overseas markets without significant proliferation of pathogenic or spoilage organisms. Whilst packaging in controlled CO_2/N_2 atmospheres was developed to achieve this (Gill, 2004), the technology has found only partial acceptance due to increased production costs and the requirement for additional preparation in the marketplace. In contrast, vacuum packaged meat is comparable to fresh meat fabricated in the importing country, and is therefore much more widely accepted, despite limitations on shelf-life and dependence on precise refrigeration.

Meat processing

A New Zealand export sheep and lamb processing facility is a high throughput operation designed to convert live animal tissue into meat in the most cost-effective manner. Additional revenue is derived from saleable by-products such as the fleece, and rendered meat and bone meal. The small size of sheep (relative to cattle and pigs) has however presented logistical problems in commercial slaughter operations. The effort required to produce meat using traditional methods is disproportionately high (80 man-hours per 10000Kg, compared to 22 for pork and beef), and so New Zealand has invested heavily in developing slaughter and dressing technology to reduce labour costs, maintain stringent hygiene and improve the quality of the meat and pelt (Devine & Gilbert, 2004).

Meat production commences with transport of the animals to the facility, where they are stored in “lairage” pens, and unlike other countries, washed rigorously, either by swimming or high velocity jets, prior to slaughter. The animals are pre-stunned prior to slaughter by spanning the head with metal tongs and passing an electrical current through the brain inducing an epileptic seizure. This “head only” stun does not, however, stop the heart and so actual slaughter is achieved by severing the throat prior to recovery, cutting the carotid artery, jugular veins,

trachea, oesophagus and various nerves, and the carcass hung so that blood drains from the severed vessels in a manner consistent with religious (halal) slaughter (Grandin, 2001).

Virtually all NZ sheep facilities de-pelt the carcass in the "inverted" position, hung from the forelegs with the hide removed from shoulder to hind-leg. Careful knife work opens the pelt forming a "Y" across both forelegs to join the slaughter cut. The fleece is then pulled back with the aid of a number of hand tools, and finally removed using a mechanical puller. The head is removed by cutting across the larynx and cutting through the occipital joint, which is automated at some plants. This is followed by evisceration and offal handling; the abdominal cavity is opened by cutting behind the brisket and the contents removed, the liver is cut from the diaphragm, and the contents of the thoracic cavity extracted by trimming the aorta. At this point the carcass and associated viscera remain together for inspection by veterinary trained officers. On completion, offal meats are rapidly cooled and packed, whilst muscle meats are further conditioned to achieve optimum tenderness (Devine & Gilbert, 2004; Pérez-Chabella & Legarreta, 2001).

Muscle does not terminate all functions at the time of death. Glycolysis continues, but oxidative phosphorylation cannot (due to the lack of an oxygen transport system), therefore the pyruvate generated is converted to lactic acid, which accumulates in the muscle. This persists until the majority of glycogen stores are also depleted, and the pH of the muscle falls from 7.0 to around 5.5, achieving a final "*ultimate pH*" when the process of rigor mortis (Latin – "stiffness of death") has completed (Greaser, 2001). This stage of chemical change is known as *conditioning* and is influenced by muscle type, temperature, time, cutting and hanging of the carcass, typically taking around 16 hours for lamb (Honikel, 2004b).

Rigor mortis itself is a consequence of the formation of permanent cross-linkages between the muscle filaments, due to loss of regenerated ATP as glycolysis ceases (Aberle et al., 2001). The process is gradual, as not all possible cross bridges form at the same time. The overall shortening resulting from rigor is therefore small (10-15%) and the resulting toughness can be reversed during chilled storage (aging) through the action of endogenous proteolytic enzymes (e.g. calpain) present in the meat.

If the carcass temperature falls below 10°C before rigor is complete, control of muscle contraction is lost and the muscles are reduced by as much as 50% in length, which is irreversibly tough. This is a serious defect in meat tenderness known as *cold shortening* (Honikel, 2004a). A similar problem, *heat shortening*, can arise when the pH falls below 6.0 as a result of glycolysis, whilst the carcass temperature remains above 25°C. Heat shortening typically occurs gradually, and late in glycolysis - during the conditioning phase.

Appropriate chilling of meat carcasses is therefore critical for eating quality. For optimum tenderness, heat must be removed from carcasses to achieve an internal temperature of below 20°C as soon as possible, but the temperature must not fall below 12°C until rigor mortis is complete (Ockerman & Basu, 2004). Use of these conditions has however been criticised, as holding meat at temperatures of 15-16°C for several hours may result in growth of pathogenic and spoilage causing organisms; generally, a reduction to below 7°C is required to prevent proliferation of pathogenic bacteria (Lovatt *et al.*, 2006). The maintenance of separate conditioning floors at meat processing facilities is also expensive. Electrical stimulation has now largely replaced the process of conditioning and aging (Pearson, 1986).

Electrical stimulation involves passing electrical current through the body or

carcass of freshly slaughtered animals (Hwang *et al.*, 2003). The current causes muscle to contract, increasing the rate of glycolysis and thereby reducing pH by around 0.6 pH units at 37°C and 0.18 pH units at 15°C. Following the initial reduction in pH, glycolysis is accelerated dependent on temperature, and consequently rigor mortis develops early. The combination of these effects is that muscles enter rigor mortis before the temperature falls to values that would produce cold shortening and toughening, and optimal carcass hygiene can be maintained (Devine *et al.*, 2004).

After electrical stimulation, carcasses need to be held on a marshalling floor for only 60 minutes before rapid chilling to below 7°C (usually to between 2-6 °C). Drying of the carcass (evaporative cooling from the refrigeration airflow) in combination with the reducing temperatures prevents microbial growth. The lower temperature also provides a firmer carcass so that clean cuts and tidy appearance are achieved in subsequent butchering (Bell, 1993). When this has been achieved (typically overnight) the carcasses are moved to a cutting room (also known as a boning room or further processing room). Such rooms operate at below 10°C, and often as low as 6°C, to ensure optimum quality. The carcasses are then cut and trimmed, by band-saw and knife, into particular cuts specified by the customer. These cuts are then bagged, placed in cartons, and back under refrigeration inside 30 minutes. For chilled meat, cartoned product is held until temperature has equilibrated to -1.5°C, then the cartons are strapped and loaded into refrigerated shipping containers. This temperature is maintained during shipping, until receipt in the marketplace, where the cartons and bags are opened, and the meat cut into retail portions and repackaged for sale to the consumer. Diligent meat producers log temperatures throughout this process to assure product control.

Chilled meat microbiology

Chilled meat from New Zealand is displayed in remote markets alongside domestic and other imported product, necessitating extended shelf lives that will, at the minimum, allow the product to reach the marketplace and remain on retail sale for several days. ***shelf-life*** has been defined as *the period during which (under recommended storage conditions), the product remains safe, whilst retaining desired sensory, chemical, physical and microbiological characteristics* (IFST, 1993). To successfully achieve this, the meat must (Bell, 1990):

- Retain optimum sensory qualities - an attractive fresh appearance, tender, juicy and flavourful following cooking.
- Retard microbial spoilage and inhibit the proliferation of pathogenic micro-organisms, resulting in acceptable bacterial counts.
- Minimise water loss by evaporation or exudates (as meat is sold by weight).

These objectives have largely been met through the development of refrigeration and packaging technologies.

Microbial ecology of meat

Microbial ecology has been defined as "the study of the interaction between the chemical, physical and structural attributes of a niche and the composition of its specific microbial population (Mossel & Struijk, 1992).The microbial ecology of meat is rarely viewed from this holistic aspect, but rather in terms of risk to health from pathogenic organisms and toxins, or risk to quality from microbial spoilage. The majority of this work has focussed on bacteria.

Whilst moulds and psychrotolerant[1] yeasts are capable of growing on meat aerobically, causing black, white, blue-green and whisker spots, these are unable to

[1] Optimum range for growth 20-40°; can grow at 0°C

compete with bacteria on fresh meat due to low growth rates and are therefore associated with salted, dried or frozen product. Their growth is completely inhibited under anoxic conditions (Marshall & Bal'a, 2001).

Food mediated viral disease is largely restricted to Hepatitis A and E, Norovirus, Rotavirus, and most recently, avian influenza in poultry meat (Anon, 2004; Cromeans *et al.*, 1997; Doyle & Erickson, 2006; Goens & Perdue, 2004). Little evidence exists for intrinsic transmission via red meat (as opposed to poultry and shellfish) but rather an association with food handlers, therefore control has focussed on the health and hygiene of workers. The role that viruses, particularly bacteriophage, may play in complex microbial interactions on meat, for example the transfer of antibiotic resistance via food, has yet to be established. Bacteriophage have been identified as potential agents for control of pathogenic and spoilage bacteria in foods, including meat (Hudson *et al.*, 2006).

Until recently, Archaea were considered to be confined to specialised environments at the extremes of temperature, salinity and pH, and so few attempts have been made to identify them in food processing environments (Aravalli *et al.*, 1998). One study has described the presence of the methanogens *Methanogenium*, *Methanobacterium* and *Methanosarcina* in vegetables, meat, fish and cheese, however these were considered chance contaminants as none of the foods tested showed the presence of methanogen species isolated from the human digestive tract (Brusa *et al.*, 1998; Duncan *et al.*, 2007).

In the healthy animal, tissues that ultimately become meat (muscle, fat and edible offal) are usually sterile (Gill, 1979). Animal carcasses and meat cuts are easily contaminated with bacteria during the slaughter, dressing, cutting and packing process (Koutsoumanis & Sofos, 2004). If not properly handled, processed and preserved, meat will support the growth of these organisms thereby creating a

significant health risk. Sources of contamination include faeces, ingesta, hide, lymph nodes and intestines of the animals themselves, and air, water, soil, processing equipment, utensils and personnel from the abattoir environment. After dressing, NZ lamb carcasses typically carry between 10^2 and 10^4 bacteria/cm^2 (Lovatt et al., 2006). The initial flora is very diverse, although it is predominantly mesophilic [2], with *Micrococci*, *Staphylococci*, *Bacillus*, Coryneforms, Enterobacteriaceae, *Flavobacteria*, Pseudomonads, lactic acid bacteria (LAB) and *Brocothrix* likely to be present (Dainty & Mackey, 1992).

Despite ideal conditions existing on meat for the growth of many bacterial species, not all bacteria present initially multiply during storage. Meats invariably develop a characteristic flora whose composition can largely be predicted from a knowledge of temperature, meat pH and gaseous atmosphere. It has been established that background organisms are antagonistic to pathogenic bacteria in anerobically packed chilled meats, a process known as microbial interference (Jay, 1996). Further, it has been shown that a succession of different species leads to the eventual dominant microbiota on the final product (Jones, 2004). This selection depends on the combined effect of extrinsic, intrinsic and processing factors affecting survival, growth and competition of bacteria on meat, and these can be selectively targeted by preservation technologies. Typically, the ultimate microbiota of chilled vacuum packed lamb will be dominated by lactic acid bacteria (LAB), particularly *Carnobacterium*, *Lactobacillus* and *Leuconostoc*.

Virtually all bacteria characterised as capable of growth on red meat under chilled vacuum packed or controlled atmosphere packed conditions are aerobic/facultatively anaerobic (MIRINZ, 2007b). The exceptions are the

[2] Temperature range for growth 8-46°C

psychrophilic[3]/psychrotolerant *Clostridia* spp., i.e. non-proteolytic *Clostridium botulinum* and *Clostridia* implicated in "blown pack" spoilage (Broda *et al.*, 1996).

Meat Safety

Safety is a primary concern of the consumer when purchasing meat. Customers regard "use by" dates as a guaranteed date of safety. Therefore, the manufacturer must be certain the shelf-life recommendation is correct (Becker, 2002; Eburne & Prentice, 1994).

Consumer safety issues associated with red meats are not restricted to microbiological hazards. Chemical hazards include presence of pesticides, hormones, antibiotics (the latter two being used to enhance animal growth in some countries), and chemicals from production and processing. Physical hazards are typically foreign bodies such as glass, metal and plastic. Steps to eliminate these hazards must be considered as part of the manufacturer's HACCP programme but do not impact significantly on shelf-life (Fung *et al.*, 2001).

The catalogue of pathogenic bacteria that have been found on red meat is extensive. However, only some of these bacteria are implicated in food-poisoning disease, notably *Salmonellae*, *Escherichia coli* O157:H7, *Campylobacter*, *Clostridium botulinum*, *Aeromonas hydrophila*, *Yersinia enterocolitica* in pork, and *Listeria monocytogenes* in processed meats (Fung *et al*., 2001). All of these pathogens are considered adulterants in high risk "ready-to-eat" foods (that require no additional cooking).

Despite the fact that raw, fresh meat will be subjected to cooking as a final hygienic control step prior to consumption, many countries have now adopted a "zero tolerance" approach to the presence of some pathogenic micro-organisms. In the USA, a well-publicised outbreak associated with undercooked hamburgers

[3] Optimim temperature for growth below 15°C

(501 recorded cases of food poisoning, 151 hospitalisations, 45 cases of haemolytic uraemic disease (HUD) and 4 deaths), lead to the causative organism *Escherichia coli* O157:H7 being declared an adulterant of meat. A large-scale surveillance programme was established to monitor all beef destined for the US burger market, including most of New Zealand export beef (FSIS, 2004; Fung et al., 2001; NZFSA, 2006a). A more intensive programme to effect control of Salmonellosis in Sweden and Denmark, included "top-down" eradication of infected flocks of poultry and a continual test programme in pork (Wegener *et al.*, 2003).

The hygienic objectives of post-slaughter carcass and product management are, therefore, to first minimise contamination. As total eradication is impractical, the second objective is to restrict subsequent proliferation (Bell *et al.*, 1998; Borch *et al.*, 1996). This second objective is of paramount importance when considering technologies that extend shelf-life.

Most of the meat-borne pathogens described above (*Salmonellae*, *Escherichia coli* O157:H7 and *Campylobacter*) are mesophilic. Whilst there is significant risk of these organisms proliferating (with associated hazard to health) at ambient temperatures in the "wet market" scenario, they will not multiply during chilled or frozen storage. Refrigeration therefore provides a simple technology to safely extend shelf-life (Bell, 2001). Whilst non-proteolytic *Clostridium botulinum*, *Aeromonas hydrophila*, *Yersinia enterocolitica* and *Listeria monocytogenes* can grow at lower temperatures (*Cl. botulinum* cannot produce toxin below 2-3 °C, minimum growth temperature for the remainder is 0-4°C), growth can be limited by low substrate pH and storage atmosphere. Generally, the minimum temperature for growth increases as CO_2 concentration increases and pH falls, thus storage life can be extended by incorporating CO_2 into the packaged product.

Microbial spoilage

Meat has been described as the most perishable of all important foods. The chemical composition of meat (approximate - water 71-76%, protein 20-22%, lipid 3-8%, carbohydrate ~1.2%, & soluble non-protein substances ~2.3%) provides an ideal substrate for microbial growth (Samelis, 2006). The muscles of healthy animals are essentially sterile but become contaminated at slaughter; avoiding microbial contamination is practically impossible. Consequently, the shelf life of unpreserved meat, such as is sold in the warm "wet markets" that dominate meat trading in Asia (Ho, 2004) is very short, measurable in hours, compared to days for unpreserved refrigerated meat (**Figure 2**).

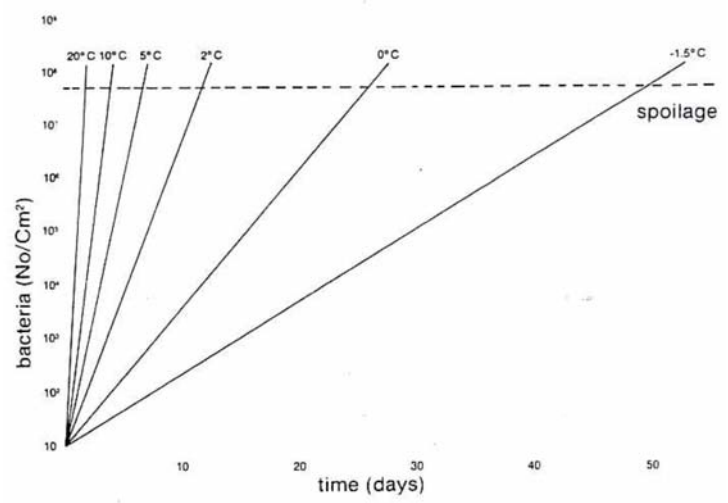


Figure 2 – Effect of storage temperature on time to spoilage (Bell, 1993).

Meat is generally considered spoiled when one or a combination of undesirable organoleptic (sensory) changes make them unacceptable to the consumer (Ellis & Goodacre, 2006). These changes can be purely visible (e.g. discolouration, surface colonies), malodorous (e.g. sulphurous smell) or due to slime formation ("sticky" texture). Whilst endogenous enzymatic activity post-mortem contributes to biochemical changes during storage, it is generally accepted that detectable organoleptic spoilage of meat is a result of the decomposition and subsequent metabolite formation caused by the growth and catabolic activity of

microorganisms.

Refrigeration effectively prevents growth of mesophilic organisms. Consequently the spoilage microbiota developing on chilled meat are mainly psychrotrophic and psychrophilic bacteria and rarely, fungii. These organisms originate mainly from soil, water and vegetation. Growth at chill temperatures is however slow, the most rapid recorded being 1 generation per 8 hours aerobically for *Pseudomonas* at 2°C. These organisms contaminate the meat either by transfer from the hide or fleece, or from work surfaces during processing (Bell, 2001). Only a small number of such bacteria regularly grow on meat at chill temperatures. These include lactic acid bacteria (LAB), *Acinetibacter*, *Moraxella*, some species of *Pseudomonas*, some members of the family Enterobacteriaceae, *Brocothrix thermosphacta*, *Shewanella putrifaciens*, and some species of *Clostridia*. The presence of these organisms, their contribution to the total bacterial count, and the total initial numbers of bacteria on the surface of the meat after processing is completed all influence the product shelf-life (**Figure 3**). Thus, good hygienic manufacturing practice is important.

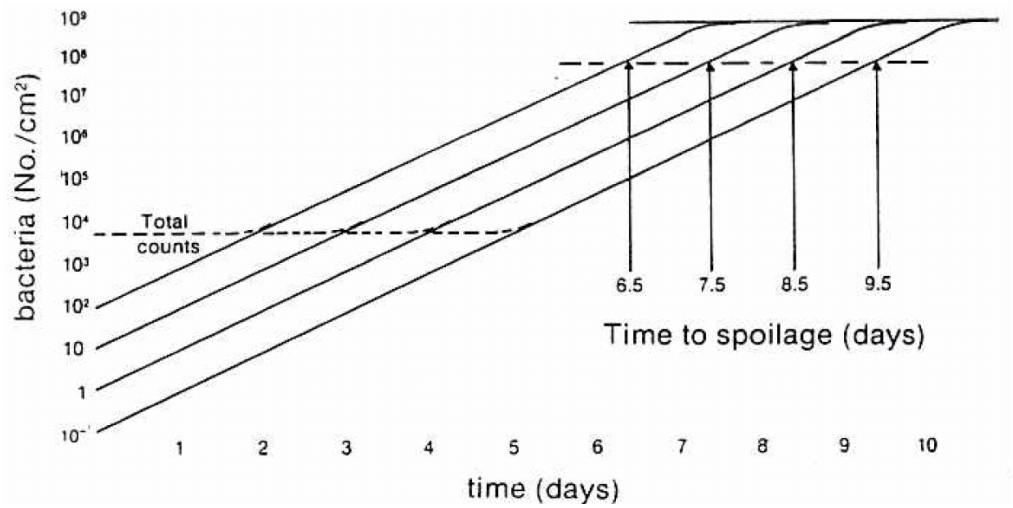


Figure 3 – Effect of the initial total bacterial count on time to spoilage for meat of normal composition, refrigerated aerobically, using a bacterial generation time of 8 hours (Bell, 1993).

The pH of the meat influences which bacteria will grow. Enterobacteria,

Brocothrix thermosphacta and *Shewanella putrifaciens* cannot multiply in low pH (<5.8-6.0) environments, whilst LAB and *Pseudomonas* can (Dainty & Mackey, 1992). LAB habitually dominate the microbiota of anaerobically stored meats, due to their tolerance of low pH, and some strains can cause offensive ("cheesy") taste and odour in very high (>10^9cfu/g) numbers. The majority of spoilage bacteria, where present, are able to co-exist with the LAB, and may utilise their metabolic products (Gram *et al.*, 2002).

Meat spoilage bacteria utilise low-molecular weight, soluble components of muscle tissue for their growth, particularly glucose and amino-acids (Bell, 2001). Glucose is the preferred substrate, and amino-acids are not significantly degraded (with accompanying release of offensive spoilage amines) so long as it is available. The glucose concentration in meat of pH 5.5-5.7 is approximately 500 μg/g. At these concentrations spoilage occurs when bacterial numbers reach $10^8 – 10^9$ cm^{-2}. In contrast, meat of high ultimate pH (>6.0) is low in glucose and spoilage occurs when bacterial numbers approach 10^6 cm^{-2}.

Oxygen is necessary for strict aerobic bacteria (notably *Pseudomonas*) so removing oxygen from the environment restricts growth. However facultative anaerobes (capable of aerobic and anaerobic metabolism, e.g. *Shewanella putrefaciens*) can still grow and spoil meats if pH and temperature allow. Carbon dioxide inhibits the growth of many bacteria. *Pseudomonas sp.* are particularly sensitive to CO_2, whilst LAB are largely unaffected. In anaerobic systems, the inhibitory effect on facultative anaerobes increases progressively, with maximum effect being achieved with pure CO_2 (Bell, 2001).

Water activity (a_w) is a measure of free water in a product, i.e. not chemically or physically bound (Young *et al.*, 2001a). Pathogenic and spoilage micro-organisms found on meat typically require an a_w for growth of >0.98 (ICFMH, 1998).

Desiccation of carcass surfaces has been observed during chilling, as the surface a_w equilibrates to the relative humidity of the surrounding environment, which results in inhibition of bacterial growth (North, M., unpublished data). Whilst this may be an important factor in preserving carcasses for local consumption, the effect does not persist in packaged product.

Quality considerations

Measures developed to preserve fresh meat for sale in overseas markets are not restricted to the control of pathogenic and spoilage micro-organisms. The acceptability of fresh red meat is largely judged on the appearance of the exposed muscle tissue, which is primarily determined by the state of the muscle pigment myoglobin (Gill, 1996). In the absence of oxygen, this pigment exists as deoxymyoglobin, which is dull-purple in colour. On exposure to air, the pigment is oxygenated to form oxymyoglobin, which imparts the bright red colour consumers find attractive in meat. Both oxy- and deoxy-myoglobin react further with oxygen to form the oxidised state metmyoglobin, which has the dull-brown colour consumers associate with deterioration of quality. Whilst oxygenation of myoglobin is rapid and reversible, oxidation to metmyoglobin is stable and will persist once formed.

Maintaining meat at as low a temperature as possible without freezing slows the reactions associated with pigment oxidation thus extending shelf-life. Oxidation of deoxymyoglobin occurs more rapidly than oxymyoglobin. Therefore, exposing the meat to high concentrations of oxygen decreases deterioration and extends shelf-life. Alternatively, oxygen is excluded from the meat altogether, thus preventing any oxidation until arrival at market, when the meat must be re-exposed to oxygen to “bloom” – i.e. oxygenate to the red colour of fresh meat.

Deterioration of other constituents (e.g. lipids, proteins) in meat is also largely a

consequence of oxidation (Aalhus & Dugan, 2004). Lipid peroxidation occurs when unsaturated fatty acids react with oxygen from the storage environment to produce aldehydes, ketones and short-chain fatty acids. These compounds produce rancid flavours and odours. Refrigerated, whole, raw meat is largely resistant to lipid peroxidation, particularly when packaged to exclude oxygen. Oxidation of proteins may also occur, usually through localised reaction with transition metal ions or lipid peroxidation products. Changes in muscle protein functionality when the meat is exposed to oxidising environments include modification of gel forming ability, meat binding ability, emulsification capacity, solubility, viscosity and water-holding capacity.

Refrigeration

Most authors agree refrigeration is the most important technology for extending meat storage life. Low temperatures control the growth of spoilage organisms, and arrest the proliferation of pathogenic organisms. Refrigeration also retards the onset of oxidative deterioration. Of the established methods for extending the shelf life of meat (e.g. drying, curing, smoking, heat process, irradiation, fermentation, canning and packaging) refrigeration has the key benefit of keeping the meat product almost unchanged and (if performed correctly) almost indistinguishable from the original fresh product (Lovatt, 2004).

Refrigeration is applied to almost all types of meat product other than those preserved by drying, salting and canning. To ensure meat is supplied safely and to the highest organoleptic quality, a chain of refrigeration processes must be carefully followed, from the initial chilling of the freshly slaughtered carcass through to the storage of the chilled retail portion in the home (James & James, 2004).

Effective temperature control during transportation is becoming increasingly

important as the shelf-life of meat is extended (James & James, 2004). To achieve a shelf-life for lamb of 60 days vacuum packaged, a temperature of -1 ± 0.5 ° C must be maintained during transportation to the importing country and until product reaches the secondary cutting facility, thereby avoiding bacterial growth *and* freezing. In countries where product is destined for local consumption it may be chill-stored unwrapped, for up to 2 weeks, to allow aging to occur before cutting and packing in case-ready form. The majority of incidents of bacterial spoilage of NZ chilled meat reported to the MIRINZ Centre during the past 5 years have resulted from some form of breakdown to this chill cycle (LeRoux *et al.*, 2007).

Packaging methods

The basic function of packaging is often overlooked (Bell, 2001). It protects product from the environment, including variables such as temperature, moisture, humidity, oxygen, airborne particles, light, and biological contamination. Packaging also acts as a facility for marketing and labelling.

Preservative packages are used routinely for exporting raw meat from New Zealand. These are characterised by an ability to extend product life by modifying or restricting bacterial growth, creating and maintaining in-pack conditions that differ from the ambient environment (Bell, 2001). Such packaging typically regulates permeation (a barrier property) of gases such as oxygen, carbon dioxide and nitrogen. This regulation, in association with refrigeration, minimises changes in meat colour, appearance and oxidation and discourages bacterial growth (Kropf, 2004b).

This study is focussed on *vacuum packaged* meat. With this type of packaging, product is placed into a low permeability bag or pouch that is evacuated then heat sealed. This extends the storage life of meat **(Table 1)** by excluding oxygen,

impeded oxidative processes and prevents growth of aerobic bacteria (Kropf, 2004c). The meat does however develop the purple colour of deoxymyoglobin which most consumers find unattractive. To prepare the pack for retail it must be opened to allow the meat to "bloom" (through oxygenation of deoxy- to oxy-myoglobin) to the bright red colour preferred by consumers. During this time the meat may be further dissected into display cuts and re-packaged. Vacuum packaging is widely used for display of sliced processed meats, where colour is stabilised by nitrate curing then protected by anoxia.

The other types of packaging available are *Modified atmosphere packaging* (MAP), where the gaseous environment around the product is modified before the package is heat sealed, and *Controlled atmosphere packaging* (CAP) a type of MAP using totally impermeable packaging film (Kropf, 2004a), which means the gaseous environment around the product can be maintained at a specified composition (e.g. CO_2/N_2).

Table 1 – Storage and retail display life of hygienically produced vacuum- and CO_2- packed meat stored at -1 °C and displayed at 4 °C (Bell, 2001). Product life is defined by failing to satisfy market need.

	Assured product-life (days)			
Packaging system	Beef	Lamb	Pork	Chicken
Transport/storage packaging				
Vacuum	84	60	28	25
Carbon dioxide	126	90	63	70

Cross-contamination from contact surfaces

Although the initial contamination of a carcass is primarily derived from its own fleece and intestine, subsequent contamination from knives, aprons, machinery (e.g. saws, skinners), cutting boards and conveyor belts is well documented (Young *et al.*, 2001b). Boning operations are therefore considered a prime source of

contamination that leads to spoilage after chill storage, due to the number of cuts made, human contact, and contact with multiple surfaces. It has also been shown that cross-contamination with pathogens can occur between successive cuts passing over a surface when the first carcass is contaminated excessively (Mills & Clemens, 2003).

Conveyor belts are common to all types of food manufacture, from primary processing of meat to higher risk operations (i.e. with no subsequent heating) like shellfish production to ready to eat meals. Many types of systems exist, with a range of materials used for construction. In New Zealand, conveyor systems prepared for the meat industry are typically custom-fabricated by an engineering company to the meat producer's specifications, based around commercially available belt materials, which are either continuous Teflon® coated composite or modular interlocking plastic (Dickinson, 2006). A typical Interlocking belt arrangement is shown in **Figure 4**:

Figure 4 – Interlocking conveyor system

Cleaning and disinfecting conveyor belts presents difficulties, particularly with mesh-based or interlocking systems which contain internal hinge areas. Belts are therefore prone to build-up of microbiological contamination throughout the

production process. The source of these organisms is not just the soil remaining on the surface providing the nutrient source but also the surrounding environment and production operatives (Rahkio & Korkeala, 1996). Two time periods are considered critical for the assessment of belt cleaning efficacy; the inter-production period, where microbial growth should be absent or minimal (Zottola & Sasahara, 1994), and late during the production day, where pathogen detections have been reported when soiling from production reaches maximum levels (Rivera-Betancourt *et al.*, 2004).

Cleaning processes in meat production are governed by product change over, where it is necessary to remove all traces of previous production (e.g. transferring processing from veal to sheep-meat), or by the frequency of breaks (typically every 2-3 hours, depending on room operating temperature). Where possible, cleaning with hot, high pressure water during breaks in production will decrease overall soiling of the belts and therefore the microbiological contamination of product (Phelps, 2006), however this adds to production costs and may not be practicable in areas with restrictions on water consumption or effluent discharge.

The primary sanitizers in use in the meat processing environments are quaternary ammonium compounds ("Quats") and chlorine-based foams. These are usually applied during a "deep clean" process, often during the night shift, as they require time to function correctly. Quaternary ammonium compounds are used due their ability to leave a residual film after disinfection, however this is minimised as sanitizers must be rinsed off to prevent contact with meat in accordance with EU regulations. Chlorine is alternated with quaternary sanitizers to reduce the potential for resistance to form to a single agent (Higgins, 2003).

The potential role of biofilms

Bacterial phenotypes change when they attach to and colonise surfaces, producing a community referred to as a *biofilm*, which is characterised by a decreased susceptibility to disinfectant and increased production of extracellular polymeric substances (EPS) (Midelet & Carpentier, 2002). To the food hygienist, the definition of a biofilm has been proposed as "a microbial community, adhering to a surface and frequently embedded in an extracellular matrix" (Carpentier & Serf, 1999). This definition is preferred due to the observation that increased resistance to antimicrobial substances (of primary importance to good hygienic practice) is acquired within a few hours of adhesion, before detectable levels of EPS are formed.

The initial adhesion of bacteria (see **Figure 5**) to the food processing surface is believed to be a random event, probably influenced by hydrophobic and electrostatic forces, and propinquity (nearness).

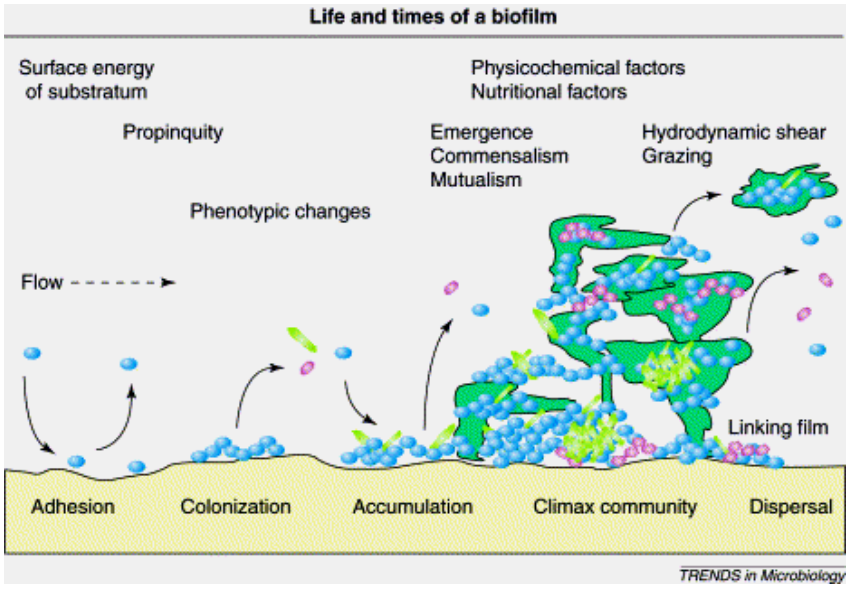


Figure 5 Stages of biofilm development. (Jenkinson & Lappin-Scott, 2001)

Adhesion may be facilitated by formation of a conditioning layer, usually protein (meat processing is a high protein, high fat environment) denatured by high or low extremes of pH or high contact surface temperature (Deibel & Schoeni, 2003). The

adhered bacteria multiply, colonising the surface, and by doing so provide for further cell adhesion and accumulation to generate a linking film from which forms the "climax community"; from this, viable cells are dispersed into the meat processing environment, most probably by "hydrodynamic shear" (Jenkinson & Lappin-Scott, 2001).

It has been noted (Carpentier & Serf, 1999) that biofilms found on common food producing surfaces frequently form discrete micro-colonies rather than continuous films. In addition, transcriptional changes occur following cell attachment and surface growth, attributed to chemotaxis (quorum sensing). In *E.* coli, it was reported that 38% of transcription was affected (Prigent-Combaret *et al.*, 1999).

Surface sanitization is difficult where biofilms are present. A multi-point cleaning and disinfecting strategy must be developed, typically involving the processes of: mechanical activity (not currently a part of meat industry cleaning practice), choice of appropriate formulation and concentration of sanitizer, exposure time, and temperature (Deibel & Schoeni, 2003). Penetration into the locking zones of interlocked belting is not possible without total dismantling of the belt, which is impractical for routine operations, although this can be scheduled over weekends or closed periods.

Antimicrobial agents can be incorporated into the plastic belt segments to inhibit to growth of bacteria. Silver-based compounds use an ion exchange mechanism that slowly releases silver ions, which interact with the bonding sites on the bacterial surface to prevent reproduction (Simpson, 2003).

Preceding work

The first contact from industry related to product exceeding the limit (10^3 cfu/g) for the family Enterobacteriaceae, which is enumerated as indicator organisms of sanitary significance. Further investigation (leRoux *et al.*, 2001) revealed that *Serratia liquifaciens*, a psychrotolerant species of this family, was responsible, with growth facilitated by minor temperature abuse during load-out and transportation. Enterobacteriaceae have since been criticised as sanitary indicator organisms on chilled meats due to the presence of psychrotolerant species (Struijk & Mossel, 2005), however their detection remains of concern as these species are also implicated in meat spoilage (Marshall & Bal'a, 2001).

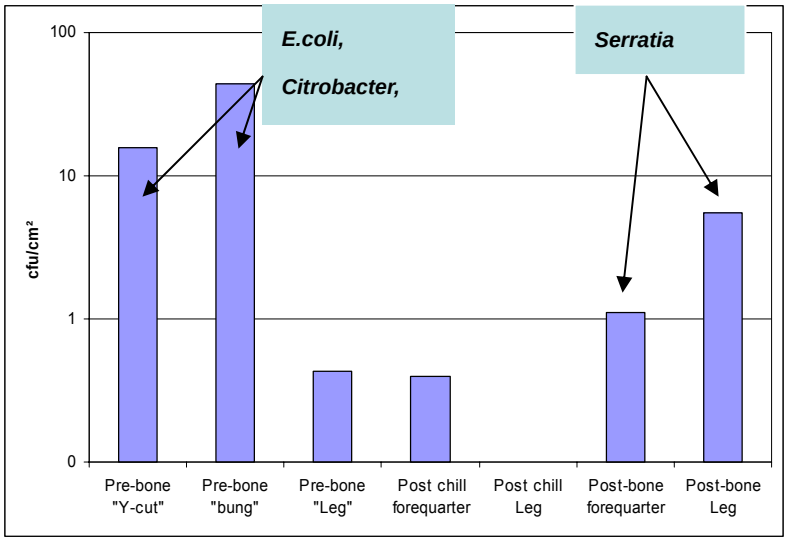
Further downgrades occurred in 2002 due to product exceeding Total Viable Count (TVC – limit 10^5 cfu/g). Investigations into this event suggested causality was more general and a revised sampling plan and alternate limits were proposed (Bell & LeRoux, 2002) pending research into reducing the growth of psychrotolerant and psychrophilic species generally on chilled vacuum-packed lamb.

Following this initial finding that Enterobacteriaceae were implicated in the downgrades, the following investigations were performed (Mills, 2006):

A longitudinal study was undertaken to determine the source(s) of these organisms. It was found that the species of Enterobacteriaceae found on meat entering boning rooms were predominantly mesophiles, whilst those on the meat immediately prior to vacuum packing were predominantly psychrotolerant (figure 6), and included *Serratia liquifaciens*.

Further investigation revealed the bearing races in the belt supporting rollers, and the hinge regions of Intralox® conveyor belting, to be reservoirs of this organism in this facility. Pulsed field gel electrophoresis (PFGE) typing of *Serratia liquifaciens* isolates from the initial downgraded meat and its manufacturer

produced identical fingerprints (**Figure 7**) between an isolate from a conveyor belt and the meat sample.



Key to sites:

Pre-bone – carcass on completion of slaughter & dressing.

Y-cut – line across both fore-legs and neck where the fleece is first cut from the carcass.

Bung – area surrounding the (eviscerated) anal cavity of the carcass.

Leg – area including hind-leg and rump from one side of the

Figure 6 - Distribution of Enterobacteriaceae in a meat production facility

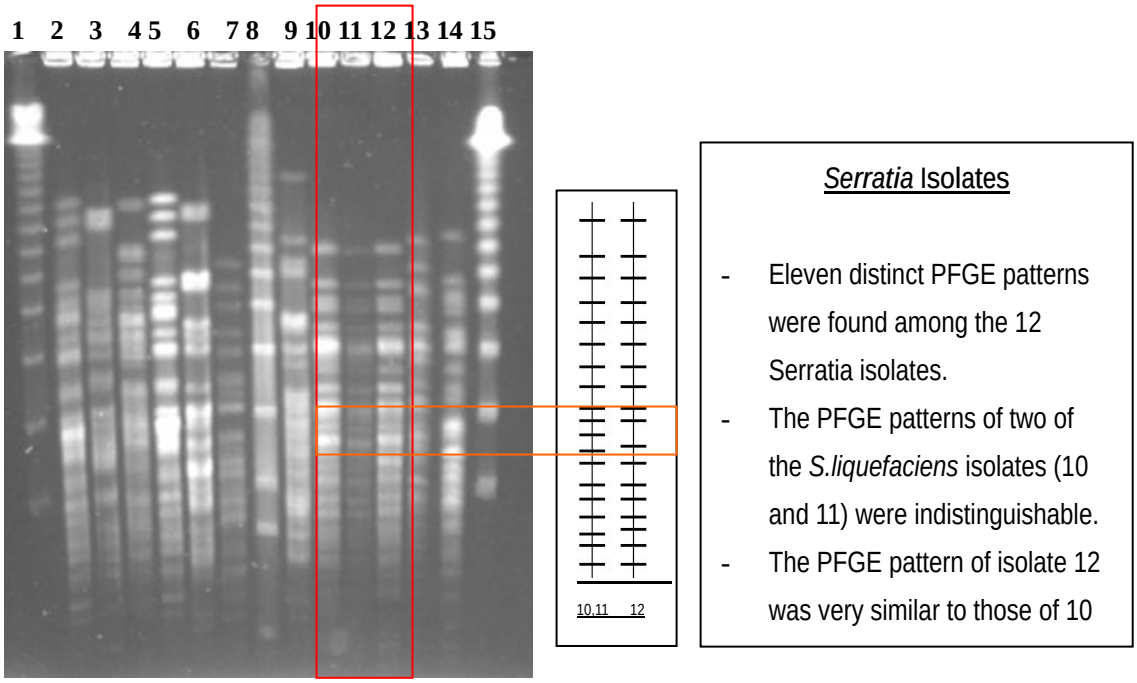


Figure 7 – PFGE analysis of *Serratia* sp. isolates

Studies conducted at two other processing facilities in 2002/3 produced similar results, with spoilage Enterobacteriaceae (predominantly *S. liquifaciens*) associated with conveyor belt surfaces (one intralox®, one continuous Teflon®), rollers, guides and under-surfaces. A third facility, however, utilizing small rooms

with very short continuous Teflon® conveyor belt runs, contained a microbiota dominated by aquatic species, particularly *Pseudomonas* sp. It was therefore concluded that plant construction and cleaning regimes could influence the microbiota found in a particular room, which offered the potential for remedial action.

The investigation into high TVC product resulted in the determination of a number of contributing factors, which needed to be addressed in total by the production companies to achieve the reduction required to reduce risk of rejection to an acceptable level. Firstly, it was determined that high initial counts on the carcasses could be attributed to excessive washing of woolly fleece (blue & yellow bars, **Figure 8**) pre-slaughter resulting in higher counts throughout the processing of the meat . Seasonal differences were also noted during this study.

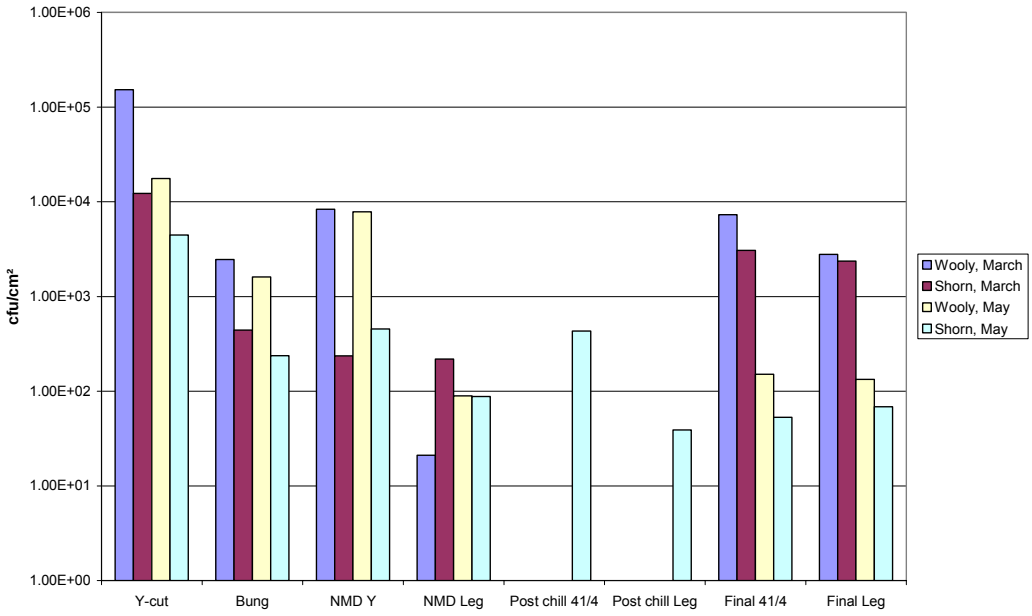


Figure 8 - Influence of fleece length

Secondly, it was determined that a high proportion of sheep-meat is of high pH (only 70% of leg meats produced measurements of less than pH 5.8, and the majority of shoulder meats exceeded pH 6.2), which not only explains the shorter shelf-life of sheep-meat compared to beef (**Figure 9**), but also how psychrotolerant

Enterobacteriaceae spp. were able to proliferate.

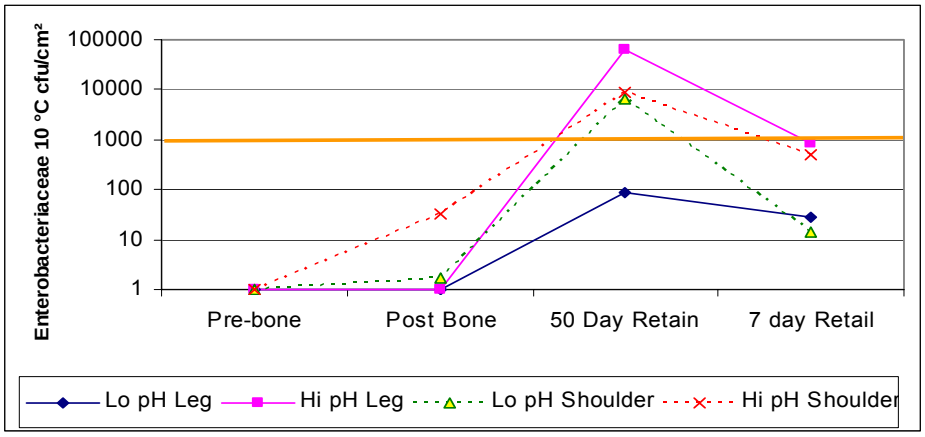


Figure 9 - Influence of pH: (longissimus) - Lo < 5.8, Hi >5.8, on the proliferation of Enterobacteriaceae

Primary importance was attached to maintenance of the cold chain, particularly assuring load-out temperatures of below 0°C, verifying performance of different phases of the chain, and keeping transportation times to a minimum.

Finally, it was postulated that the 5-10°C environment of the boning rooms made for ideal reservoirs of psychrotolerant and psychrophilic bacteria, which were adapted to growth under chilled meat conditions, and represented the primary source of transmission for these organisms onto meat. A study to determine the bacterial community on the Intralox® belts in a typical boning room, using culture-dependent and culture-independent 16S rDNA sequence analysis, revealed a predominance of *Pseudomonas* spp., and a previously uncultured (from meat environments) genus, *Sphingomonas*. Lesser numbers of *Serratia* sp., *Alcaligenes* sp., and *Microbacterium* sp., were identified from the culture-dependent library only (Brightwell *et al.*, 2006). These organisms are all considered psychrotolerant, being predominantly of soil origin (Balows *et al.*, 1992).

This study

Based on this knowledge of meat processing and data obtained from the initial work, a ***hypothesis*** was proposed that biofilms were being formed on the conveyor belting, that resisted cleaning and disinfection, and facilitated ongoing product contamination with psychrotolerant bacteria. Although the quantitative count of these bacteria on meat may remain small, early dominance of bacterial succession in vacuum pack results in their rapidly attaining unacceptable counts in the marketplace, and contributes to poor shelf-life performance.

To test this hypothesis, this study:

- Determined the comparative bacterial ecology of conveyor belting at four different facilities, evaluating different belt types (conventional continuous Teflon® and Intralox®), and cleaning regimes.
- Examined the belt materials microscopically to assess the role of biofilm formation and cleaning efficacy in-situ.
- Determined the ability of bacteria isolated from a participating facility to form biofilms on belt materials in a laboratory model.
- Determined the efficacy of silver-based antimicrobial belt materials in resisting biofilm development and reducing cross-contamination.

Chapter 2 – Materials and Methods

Sample collection

Three North Island and one South Island lamb cutting facility participated in this study. Two facilities operated only Intralox® acetyl-material conveyor systems, both with manufacturer recommended "clean-in-place"[4] sanitizers. One of these facilities (premise "A") was selected for detailed examination of segment microscopy and bacterial diversity of Intralox® systems, changing out segments for testing, inserting sterile acetyl, polyethylene and Alphasan® (silver antimicrobial) segments for removal and testing after 6 weeks, and inserting the custom in-situ testing device (refer page 30 for details). The second facility (Premise "C") was swab-tested in place and samples were investigated for bacterial diversity only.

The two remaining facilities operated a combination of both Teflon surfaced continuous belts and Intralox® acetyl-material conveyors (acetyl is the preferred Intralox® supplied material used by the NZ meat industry, due to its greater resistance to damage by bone fragments). These facilities relied upon conventional disinfectant and water sanitization. Again, one facility ("D") had Intralox® segments removed, and continuous Teflon® surfaces excised for microscopic and bacterial diversity testing, while the final facility ("B") was sampled in-place for bacterial diversity only. A separate set of samples was collected from premise "A" and washed under high pressure water for 10 seconds to simulate the clean-in-place system. A summary of all samples collected is presented in Appendix 1. While bacteria were enumerated by culture from all samples collected,

[4] Clean-in-place (CIP) is defined as the cleaning of complete items of plant with little or no manual involvement on the part of the operator. This involves the jetting or spraying of surfaces through the plant under conditions of increased turbulence or flow velocity (Bremer *et al.*, 2006).

fiscal constraints required that representative samples only be fully investigated for bacterial diversity. These are indicated in the final column of the table.

Samples for bacterial diversity studies were collected by swabs. Swab sampling is a common non-destructive method for sampling surfaces. This method was selected as it could be readily applied to sampling "in-situ", but has the limitation of removing only a portion (approximately 20%) of the total flora present on the surface, and therefore is considered to be an "indicator" of surface hygiene (ICMSF, 1980). Two different swab procedures were utilised (MIRINZ, 2007c):

- Wet/dry technique. The target area to be sampled was first measured. A sterile cotton-tipped swab was moistened in Maximum Recovery Diluent (MRD, Difco, Detroit, USA) and then one side was rubbed up and down, then across, moistening the entire measured area. The swab was turned over and the process repeated. A dry sterile swab was then rubbed over the moist area in the same manner. The swab ends were broken off (below the contaminated handling area) into a sterile polycarbonate universal bottle (Labserv, Auckland, NZ) containing 4-5 glass beads (BDH, Poole, UK). This method was applied at premises A, B and D. Where individual Intralox® segments were swabbed, the material was first returned to the laboratory and samples were obtained from the following areas (**Figure 10**):

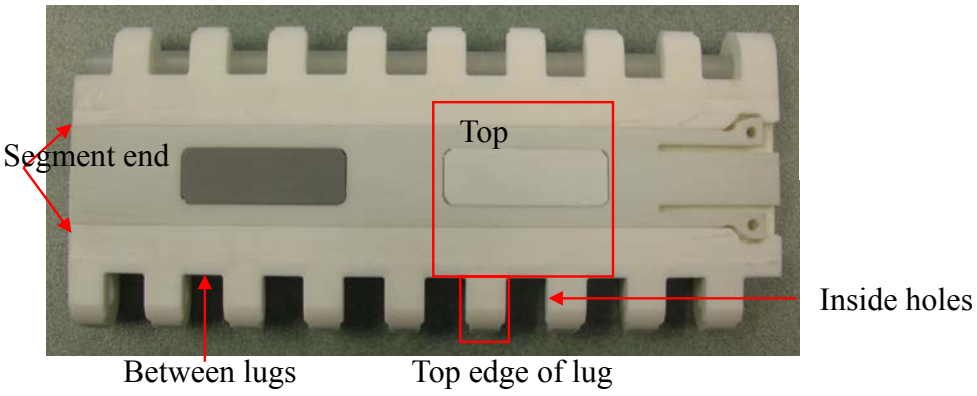


Figure 10 – Intralox® segment sampling sites

- Sponge swab. The target area to be sampled was first measured. A sterile sponge "Enviroswab" (Biolab, Auckland NZ) was rubbed up and down, then across, covering the entire measured area. The swab was turned over and the process repeated. The swab was returned to its plastic cover prior to transport to the laboratory. This method was applied to premises C, and the initial sampling at premises A. It has been shown that carcass sampling techniques can be varied without compromising the recovery of bacteria, and that swab and sponge methods are generally comparable (Gill & Jones, 1998, 2000).

Intralox® belt segments were removed by releasing and withdrawing the locking pins from the opposite side of the belt, and extracting the segment using a previously disinfected vinyl gloved hand. The segment is placed and sealed into a sterile "whirlpak" bag (Nasco, Fort Atkinson WI, USA) for transport to the laboratory.

Samples for microscopy from continuous Teflon surfaced belts were excised using a scalpel from the belt edges. The resulting fragments were transferred to sterile universal bottles using sterile forceps.

An ***in-situ sampling device*** was constructed from an unused belt edge Intralox® segment. A locking tray mechanism was incorporated into the upper surface, which allowed test material to be moved in and out using an unlocking tool (**Figure 11**). The system was installed at premise A. The test materials were changed out at the same time as the test segments and viewed microscopically.

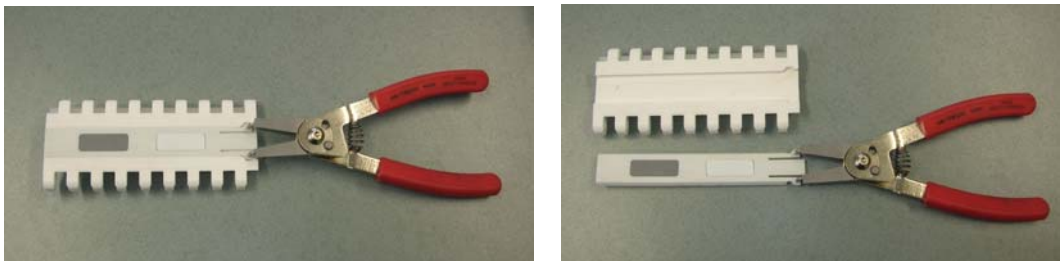


Figure 11 – in-situ sampling device

All samples were transferred from the meat premise to the laboratory on ice and testing was commenced within 24 hours of collection.

Sample preparation

Sample suspensions were made from swabs by vortexing (Ika MS-1, Sdn Bld, Malasia), and from sponges by stomaching (Seward, Worthing, UK) in 20mL phosphate buffered 0.9% saline pH 6.9 (Lorne Laboratories, Reading, UK). 10 mL of each suspension was set aside for DNA extraction for culture-independent testing (pages 33, 35-42). 1:10, 1:100 and 1:1000 dilutions were made in MRD of each sample suspension to be inoculated for culture-dependent enumeration.

Following swab sampling, Intralox® segments to be examined microscopically were held in a vice inside sterile bags, and un-swabbed portions were cut out using sterilised saw blades, such that the exposed contact surfaces remained undamaged.

Excised Intralox® fragments, and continuous Teflon® fragments obtained during sampling at the premises were either:

- Mounted on slides for direct live/dead staining.
- Submersed in 4% paraformaldehyde fixative for DAPI staining and FISH probing.
- Submersed in 2.5% gluteraldehyde/0.1N cacodylate buffer fixative (Univeristy of Waikato) for scanning electron microscopy.

Live/dead staining occurred immediately following sample preparation. Fixed specimens were processed in order based on sample stability.

Culture strategy

100 µL of each initial sample suspension, 1:10, 1:100 and 1:1000 dilutions, were pipetted onto the surface of the following agar plate media, spread across the surface of each plate according to standard methods (MIRINZ, 2007a) and incubated as indicated:

- *Columbia sheep blood agar* 35°C 48 hours is designed to isolate the majority of bacteria from animal sources (Atlas & Bartha, 1981).
- *Columbia CNA agar* 35°C 48 hours is selective for Gram-positive organisms from animal sources *(*Ellner *et al., 1966)*.
- *Chromocult coliform agar* 35°C 48 hours is designed to identify Gram-negative organisms of the coliform group (i.e. most members of the family Enterobacteriaceae) (Gonzalez *et al.*, 2003).
- *PTYG agar* 30°C 72 hours is a mineral supplemented medium designed to cultivate mesophilic and psychrotolerant heterotrophs (Balkwill & Ghiorse, 1985). It was added following a failure to cultivate *Sphingomonas* spp. identified in previous clone library studies (Brightwell *et al.*, 2006).
- *R_2A medium* 25°C 7 days is a low nutrient, mineral supplemented medium originally designed to recover stressed bacteria from potable water (Reasoner & Geldreich, 1979). The medium was included to isolate stressed heterotrophic bacteria.
- *10% TSA* 5°C 14 days is intended to isolate psychrophilic and psychrotolerant heterotrophic and oligotrophic bacteria (Elliott & Des Jardin, 1999).

On completion of incubation, viable bacterial counts were obtained for each plate using standard methods (MIRINZ, 2007a).

Ribosomal DNA libraries

Representative colonies from the above plates were selected for 16S sequencing on the basis of colony and cultural appearance (Donachie *et al.*, 2004; McCraig *et al.*, 2001) based on classical methods (Collins & Lyne, 1984). Isolates were picked and plated for purity using the same medium and incubation times as when first isolated. Pure colonies were then picked and suspended in PBS pH 6.9 for DNA extraction. A representative colony was also stored in case of problems with sequencing – initially on slopes of the same medium from which they were isolated, but later suspended in TSB-glycerol and frozen at -80°C.

DNA extraction

DNA was extracted from both culture isolates and initial sample suspensions using the Hi-Pure PCR template preparation kit (Roche, Mannheim, Germany). A modification was made to the manufacturer's instructions due to the resistance to lysozyme of Clostridial spores responsible for "blown pack" spoilage (Broda, 2007; Broda *et al.*, 1998): following washing and re-suspending the cells in 200 µL PBS pH 6.9, 5 µL lysozyme (10 mg.mL in 10mM Tris/HCl pH 8.0) was added and the mixture incubated at 37 °C for 60 minutes (as opposed to 15 per manufacturer's instructions). Whilst DNA extraction from direct sample suspensions[5] was subject to inhibitor removal, this was found to be unnecessary for culture isolates. The manufacturer's recommended wash stages were followed for all extractions. Eluted DNA was stored at -20°C from culture isolates (unless 16S PCR followed immediately), and -80°C from sample suspensions.

[5] DNA extraction from 14/2/06 sampling at premise "A" was compromised due to use of methodology from the Roche website rather than the kit insert, which prescribed an incorrect binding buffer. The author has advised Roche of their mistake, which has been corrected.

16S rDNA amplification for culture isolates

Amplification was carried out using the primers pA 5'-AGAGTTTGATCCTGGCTCAG-3' (8-28 E. coli numbering system) and pH* 5'-AAGGAGGTGATCCAGCCGCA-3' (1542-1522) (Devereux & Willis, 1995). PCR reactions were performed in 50 µL reaction mixtures containing 1x reaction buffer (Invitrogen), 1.5mM $MgCl_2$, 0.2µM of each primer (Invitrogen), 200 µM dNTP's (Roche), 1 unit of Platinum Taq polymerase (Invitrogen) and 2 µL of sample DNA template. PCR was carried out with a MJ Research PT-100 or Techne Genius thermal cycler using the conditions: 93°C for 3 minutes; 92°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes for 30 cycles; with a final extension at 72°C for 3 minutes followed by holding at 4°C. 10 µL of amplified product from each isolate was examined by gel electrophoresis in 1.5% agarose (BHD, Poole, UK) visualised with ethidium bromide by UV transillumination to verify the presence of PCR products of approximately 1500bp in size.

Restriction Fragment Length Polymorphism (RFLP)

The comparison of RFLP "fingerprints" of amplified 16S rDNA is an established method of determining taxonomic relatedness between isolates (Broda *et al.*, 2000; Heyndrickx *et al.*, 1996; Pukall *et al.*, 1998; Swaminathan & Barrett, 1995). The technique was therefore carried out on each PCR-amplified DNA product to prevent redundant sequencing and nearest-neighbour database searching. PCR products were digested separately with endonucleases *Msp*I and *Hae*III (Roche) in 20 µL reaction volumes containing 10 µL of PCR product, 2 µL reaction buffer x10, and 5 units of endonuclease in sterile ultrapure water (Invitrogen). The reaction mixtures were incubated for 1 hour at 37°C and the digestion products were separated by gel electrophoresis in a 2.5% agarose gel, visualised with ethidium bromide by UV transillumination. Isolates were compared visually for

matching fingerprints and only one example was sequenced per gel. Inter-gel comparisons were only performed where products had been generated during the same PCR reaction and digested at the same time.

Sequence analysis of PCR product from culture isolates

16S rRNA PCR product selected for sequencing was purified by the QIAquick PCR purification kit (Qiagen, Hilden, Netherlands) using the spin protocol in accordance with manufacturer's instructions. Sequencing was performed by the Waikato DNA Sequencing Facility using a MegaBASE capillary sequencer. Approximately 500 - 650 nucleotides were sequenced from the 5'- end using the forward primer pA described previously. Inspection and editing of the elecropherograms was performed using Chromas software (Technelysium, Australia). Identification of nearest-neighbour sequences was carried out with the GenBank Basic Logical Alignment Search Tool (BLAST) (Altschul *et al.*, 1997). A representative sample was scanned using the CHECK_CHIMERA software (Maidak *et al.*, 1997) for potential chimeric sequences. For the purposes of this study sequences sharing ≥ 97% identity from a sequence of >400 nucleotides were considered to belong to the same operational taxonomic unit (Donachie et al., 2004) and the nearest-neighbour identity was recorded. For sequences with less than 97% identity, or based on fewer nucleotides, the identity has been reported in the results section as being indicative only.

Denaturing Gradient Gel Electrophoresis (DGGE)

Primer sets appropriate for investigating bacterial communities on meat have recently been developed, with initial optimization and validation studies completed (Pulford *et al.*, 2006). Two primer sets, DGGE-C and DGGE-D were selected based on the results of these and other studies (Jones *et al.*, 2006; Penney

et al., 2006). The PCR conditions from these previous studies were applied (**Tables 2 & 3**):

Table 2. Reaction mixture used for amplifying amplicons **C** and **D** by PCR.		
PCR	**C**	**D**
X10 Taq Platinum buffer (Invitrogen)	5 µl	5 µl
2mM dNTP (Roche)	5 µl	5 µl
50mM $MgCl_2$ (Invitrogen)	2 µl	1 µl
10pM/µL Forward primer	5 µl	5 µl
10pM/µL Reverse primer	5 µl	5 µl
Platinum Taq (Invitrogen)	0.25 µl	0.25 µl
Ultrapure water (Invitrogen)	22.75 µl	23.75 µl
DNA sample (~10 ng/µL)	5 µl	5 µl

PCR cycling for amplicon **C** and **D** was performed on a MJ PTC100 thermal cycler using the following conditions;

PCR-**C** [94ºC 2min, (94ºC 30s, 45ºC 60s, 72ºC 45s x28 cycles), 72ºC 3min, +4ºC];

PCR-**D** [94ºC 2min, (94ºC 30s, 55ºC 60s, 72ºC 45s x28 cycles), 72ºC 3min, +4ºC].

Table 3. Primer sequences designed to amplify amplicons of the bacterial 16S gene by PCR. All CLAMP primers contain a 5' GC domain (underlined) necessary for performing the DGGE analysis. Nucleotides marked N were synthesized in equal proportions of A, C, G or T.	
C Forward	5'- GGA TTA GAT ACC CNG GTA-3'
C CLAMP	5'- <u>GCG GGC GGC GCG GGG CGC GGG CAG GGC GGC GGG GGC GGG C</u>CG CTC GTT NCG GGA CT -3'
D Forward	5'- GAG TTT GAT CAT GGC TCA -3'
D CLAMP	5'- <u>GCG GGC GGC GCG GGG CGC GGC CAG GGC GGC GGG GGC GGG C</u>CT GCC TCC CGT AGG AGT -3'

DGGE was performed on the PCR products generated using the commercial DCode™ system (Bio-Rad Life Sciences, Hercules CA, USA). Gels were assembled using components from the DCode Electrophoresis Reagent Kit for DGGE (Bio-Rad 170-9170) according to manufacturer's instructions.

PCR-**C** and –**D** amplicons were analysed on Tris/ Acetic acid/ EDTA pH 8.3 (TAE) buffered 7.5% acrylamide, 0-70% formamide/urea gradient gels. 20µl of PCR product was mixed with 5µl of sample loading buffer (glycerol based x5 sample buffer with bromophenol blue and xylene cyanol) and loaded onto the above gels, pre-warmed to 60ºC. Electrophoresis was performed at 180 volts (100mA) for 200 minutes using circulating buffer maintained at 60ºC.

Gels were stained in TAE buffer containing 1:10000 SYBR Gold (Invitrogen) for 10 minutes, and DNA was visualised using a Safe Imager™ (Invitrogen). Bands were excised from the gel using a clean scalpel blade, the gel slice was placed into an Eppendorf tube and the DNA eluted into 0.5ml of sterile ultrapure water (Invitrogen) at 4ºC overnight.

1µl of eluted DNA was re-amplified in 49µl of PCR reaction mixture as described above. This PCR amplification utilised primers without the GC clamp, over 28 cycles. DNA amplification was confirmed by 2% agarose electrophoresis, visualised with ethidium bromide by UV transillumination. Products were purified from this agarose gel using the Qiaquick Gel Extraction kit (Qiagen) according to manufacturer's instructions. Purified DNA was sequenced (Waikato DNA Sequencing Facility) and generated data was analysed using the BLAST search tool to identify the nearest neighbour of the sequence (Altschul et al., 1997).

Cloning of community 16S rDNA

16S rDNA clone libraries were prepared from three representative samples to cross-reference the results of DGGE, according to previously established protocols (Brightwell et al., 2006). 16S rRNA genes were amplified from total community DNA in three separate PCR reactions using the 16S primers, pA 5'-AGAGTTTGATCCTGGCTCAG-3' and pH* 5'-AAGGAGGTGATCCAGCCGCA-3' (Devereux & Willis, 1995). PCR

reactions were performed in 50 µl of reaction mixture containing 1 x *Pfu* Reaction buffer (Invitrogen), 1 µM of each primer (Invitrogen), 200µM of dNTPs (Roche), 1.2U of *Pfu* Polymerase (Promega, Madison WI, USA**)** and 5 µl of total community DNA template. PCR cycling was carried out in a MJ Research PT100 thermal cycler under the following conditions; 94ºC for 3 minutes, proceeded by 26 cycles of 94ºC for 1 minute, 55 ºC for 1 minute and 72ºC for 3 minutes with a final extension at 72 ºC for 8 minutes. 16S rDNA PCR products (1500bp) were verified by elecrophoresis on 1.5% agarose visualised by UV transillumination of ethidium bromide, and purified using QIAquick gel extraction kit (Qiagen) as per manufacturer's instructions. The final product was again visualised on a 1.5% agarose gel to check for a single clean band of approximately 1500bp.

A 16S rRNA clone library was generated from the three independent PCR amplification reactions using the Zero Blunt TOPO Cloning kit (Invitrogen) as per manufacture's protocol. Clones were grown overnight in Luria-Bertani media (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCL, pH 7.0) at 37ºC. PCR amplification of 16S rRNA genes from clones was carried out directly without isolation of plasmid DNA using M13 primers included in the cloning kit. PCR reactions were performed in a 100µl reaction mixture containing 1X Roche reaction buffer, 1µM of each primer, 200µM dNTP's (Roche), 2.5U of *Taq* polymerase (Roche) and 5µl of overnight clone culture. PCR was carried out in an MJ Research PT100 thermal cycler using the following conditions; 93ºC for 3 min; 92 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 2 min for 30 cycles; with a final extension of 72 ºC for 3 min. PCR products were electrophoresed in 1.5% agarose gel to confirm the presence of a single PCR product of approximately 1500bp.

To identify unique 16S rDNA types from each clone library PCR products generated from clones with M13 primers were digested with the restriction

enzymes *Msp*I and *Hae*III (Roche) in 20 µl reaction volumes containing 10 µl of PCR product, 2 µl incubation buffer, 5 units of restriction enzyme, in sterile ultrapure water. Digests were incubated for 1 hr at 37ºC. Restricted 16S rDNA fragments were analysed by gel electrophoresis in a 2.5% w/v agarose (BDH) gel run in 0.5x TBE buffer. A 1Kb+ DNA ladder (Invitrogen) was used as a size marker. Resulting bands were visualised with ethidium bromide by UV transillumination. The clones were grouped according to their RFLP pattern and an RFLP group was defined as one that had identical patterns with both restriction enzymes.

Note - the use of M13 universal primers to amplify inserts directly from clones without plasmid purification meant that each sequence type could be represented by two RFLP patterns due to two possible alternative orientations of each inserted DNA sequence in the cloning vector.

PCR products from RFLP type representatives were purified using the QIAquick PCR Purification Kit (Qiagen) as per manufacturer's protocol, prior to sequencing of approximately 600 nucleotides at the 5' end of the PCR product using the primer, M13 forward. Inspection and editing of electropherograms was performed using the Chromas software program (Technelysium, Australia), including editing out of the leader sequence (TGAATTGTAATACGACTCACTATAGGGCGAATTGAA TTTAGCGGCCGCGAATTCGCCCTT) derived from the cloning vector.

Sequence comparisons were carried out using GenBank Basic Logical Alignment Search Tool (BLAST) programme (Altschul et al., 1997). Chimeric sequences were identified by the CHECK_CHIMERA program (Maidak et al., 1997). 16S rDNA sequences identified as chimeric were omitted from further analysis. Clones with more than 97% identity with database sequences were assigned to that phylotype.

Microscopic examination

Initial scanning of fluorescent preparations was performed using an Olympus BX-60 epifluorescence microscope (Olympus, Tokyo, Japan) fitted with a mercury vapour UV source and filter cubes WU (excitation 330-385nm/Deichroic mirror 400nm/barrier 420nm), WIB (460-490/505/515) and WG (510-550/570/590). Where the presence of discernable biofilm structures was suspected, samples were to be examined in greater detail using the confocal scanning laser microscope (CSLM) facility at Waikato University.

Scanning electron microscopy was performed at the Electron Microscopy suite at Waikato University using the Hitachi S-4100 Field Emission Scanning Electron Microscope.

Live/dead staining was performed using the *Bac*Light bacterial viability kit (Molecular Probes, Eugene, USA). This kit utilises two dyes, the vital stain SYTO9 (green) which labels the majority of cells within a population, and the nucleic acid stain propidium iodide (red) which penetrates only bacteria with damaged membranes and reduces SYTO9 fluorescence when both are present. Bacteria with intact membranes (live) therefore stain green, whilst bacteria with damaged membranes (dead) stain red.

The stain was prepared by adding 3µL of each stain (as supplied) per 1 ml of deionised water. The excised fragments[6] were mounted onto a glass slide, and the meat contact surfaces flooded with the stain. The slides were then incubated in a moist chamber in the dark at room temperature for 15 minutes. The stain was washed off with deionised water and a drop of mounting oil applied, followed by a coverslip. The specimens were then examined by epifluorescence microscopy

[6] Time constraints limited the number of samples that could be processed. Processing was therefore restricted to samples from the hinge region of Intralox® belts, and edges of continuous belts.

using the WIB filter cube.

DAPI staining and FISH were performed on samples fixed in paraformaldehyde. The fixative was a 4% solution in phosphate buffered saline (PBS) pH 7.2. The excised sample fragments were submerged in the fixative for 1-3 hours at 4 °C, then washed in PBS and submerged in 1:1 PBS/absolute ethanol. The material was then dehydrated by successive passages through 50, 80 and 98% ethanol, prior to air drying and mounting onto microscope slides, which could then be stored indefinitely at room temperature (Amann, 1995). The process of fixation with paraformaldehyde and ethanol renders the cell wall permeable to small oligonucleotide probes which then hybridise specifically to ribosomal RNA.

DAPI staining was carried out using a 5µg/mL solution of 4',6-diamidino-2-phenylindole (Sigma, St Louis, USA), spotted onto the marked area on the specimen. This was incubated for 5 minutes in a wet chamber in the dark, then washed off and allowed to dry (Loge *et al.*, 1999). DAPI stained bacteria were visualised by epifluorescence microscopy using the WU filter cube.

Whole cell hybridization was carried out for EUB338 and anti-sense NonEUB as follows (Amann, 1995):

Hybridisation buffer was prepared comprising 0.9M sodium chloride, 0.01% sodium dodecyl sulphate (SDS), 20 mM Tris/HCl and 20% formamide, at pH 7.2. A wet chamber was prepared using a 50 ml centrifuge tube (Corning, New York, USA), containing a slip of No.1 filter paper (Whatman, Maidstone, UK) saturated with 0.9% sodium chloride. The chamber was equilibrated to 46.5°C for 5 minutes, then the sample slide was inserted and the slide/chamber incubated at 46.5°C for 30 minutes. 8µL hybridisation buffer was placed onto two marked areas on the sample surface, and the slide/sample again incubated for 20 minutes. 1µL of 25ng/µL of the appropriate FITC labelled probe (see table 2) was then added

(EUB338 to one site, anti-sense control to the other), and the slide/chamber incubated for 2 hours. On completion, the slide was removed, washed in pre-warmed hybridisation buffer, placed in a 50mL centrifuge tube containing pre-warmed hybridisation buffer, sealed and incubated at 46.5°C for 20 minutes. The slide was finally dipped in deionised water, dried and the sample area mounted in glycerol/PBS mountant pH 8.5 (Citifluor, Canterbury, UK). The specimen was viewed by epifluorescence microscopy using the WIB filter cube.

Probes and references to hybridisation conditions for testing positive samples are described in **Table 4**.

Table 4 - FISH oligonucleotide probes

Target	Probe	Reference
Bacteria - 16S EUB 338	5'-FITC-GCT GCC TCC CGT AGG AGT-3'	Amann et al. Micro Revs 59(1):143-169
Bacteria - Control NOT - EUB 338	5'-FITC-ACT CCT ACG GGA GGC AGC-3'	Amann et al. Micro Revs 59(1):143-169
α-Proteobacteria "ALF1b"	5'- FITC-CGTTCG(C/T)TCTGAGCCAG -3'	Manz et al. Syst. Appl. Micro. 15 (1992) 593-600
β-Proteobacteria "BET42a"	5'- FITC-GCCTTCCCACTTCGTTT -3'	Manz et al. Syst. Appl. Micro. 15 (1992) 593-600
γ-Proteobacteria "GAM42a"	5'- FITC-GCCTTCCCACATCGTTT-3'	Manz et al. Syst. Appl. Micro. 15 (1992) 593-600
Enterobactereaceae "ENT1"	5'-FITC-CUCGCGAGAGCAAGCGG-3' positions 1273 - 1289 on *E.coli* 16S rRNA	Loge et al. Water Environment Research (1999_ 71 (1) 75 - 83
Enterobactereaceae "Probe D"	5'-FITC-TGCTCTCGCGAGGTCGCTTCTCTT-3' positions 1251 - 1274 on *E.coli* 16S rRNA	Ootsubo et al. J. Appl. Micro 95 (2003) 1182-1190 J. Appl. Micro 93 (2002) 60-68
Pseudomonas spp.	5'-FITC-GAT CCG GAC TAC GAT CGG TTT -3'	Gunasekera et al. J. Appl. Micro. 94 (2003) 936-945
Sphingomonas SPH120	5'-Alexa fluor 556-GGGCAGATTCCCACGCGT-3'	Eilers et al. Appl. Env. Micro 66 (2000) 3044-3051

Scanning electron microscopy

Samples fixed in gluteraldehyde/cacodylate buffer were first dehydrated in an ethanol series. The samples were then dried using a critical point drier (Polaron, Watford, UK) using the conditions 40°C at 1200PSI; this replaced the ethanol with liquid CO_2, which was then vented to leave the dry sample. This was subsequently mounted and sputter coated using a platinum target (Hitachi E-1030, Tokyo, Japan).

Prepared samples were then examined using a Hitachi S-4100 Field Emission Scanning Electron Microscope located at the University of Waikato.

Modelling biofilm growth under laboratory conditions

Five species of bacteria isolated from boning room belt cultures were checked for purity on PTYG agar, then passaged though Brain Heart Infusion broth (BHI, Difco, Detroit, USA) for 24 hours at 30°C. 25 µL of each culture was then inoculated into respective 12 mL centrifuge tubes (Corning) containing 5 mL of BHI (4 per isolate). A 100mm rod sample of each belt material (acetyl, polyethylene and alphasan®), plus a control of stainless steel (**Figure12**), was added to a culture tube for each isolate (**Figure 13**). The tubes were incubated at 10°C for 21 days, then the rods were removed, stained by *Bac*Light (refer page 40) at the meniscus and half-way points of the culture medium, and examined microscopically by epifluorescence.

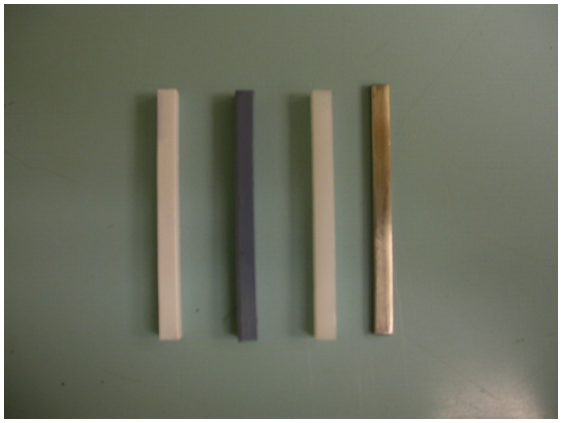


Figure 12 – rods of test material

Figure 13 – complete system, rod, culture tube and medium

Chapter 3 - Results

Culture – dependent molecular microbial ecology

Total viable counts were performed using multiple culture media on all samples collected. A complete record of quantitative data is presented in ***Appendix 2***. In total, 681 isolates were selected by colonial morphology type for identification by 16S rDNA sequencing, of which 6 (0.8%) failed to be recovered in pure culture. It was observed that *no organism dominated the microbiota of any culture sample*. A further 16 (2.4%) did not produce viable quantities of DNA following 16S PCR, the majority of which (12) were subsequently identified microscopically as yeasts. A typical gel electrophoresis of 16S PCR product is shown in **Figure 14**:

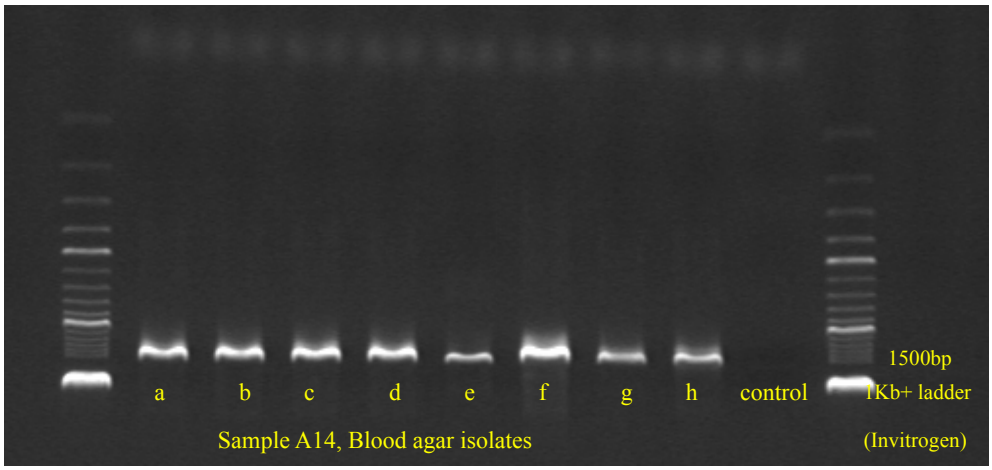


Figure 14 – Agarose gel electrophoresis of 16S PCR product, stained with ethidium bromide. The product expected is approximately 1500bp.

RFLP profiles were obtained for all isolates, from which isolates of identical pattern on the same gel were grouped together and sequencing performed only on a typical example (**Figure 15**). This procedure was credited with preventing 248 (36.4%) of samples being sequenced unnecessarily. After four 16S PCR/RFLP/sequencing runs were completed, similar patterns from all gels were placed side-by-side and the resulting sequences compared. It was concluded that RFLP was able to successfully screen isolates for sequencing from this sample type.

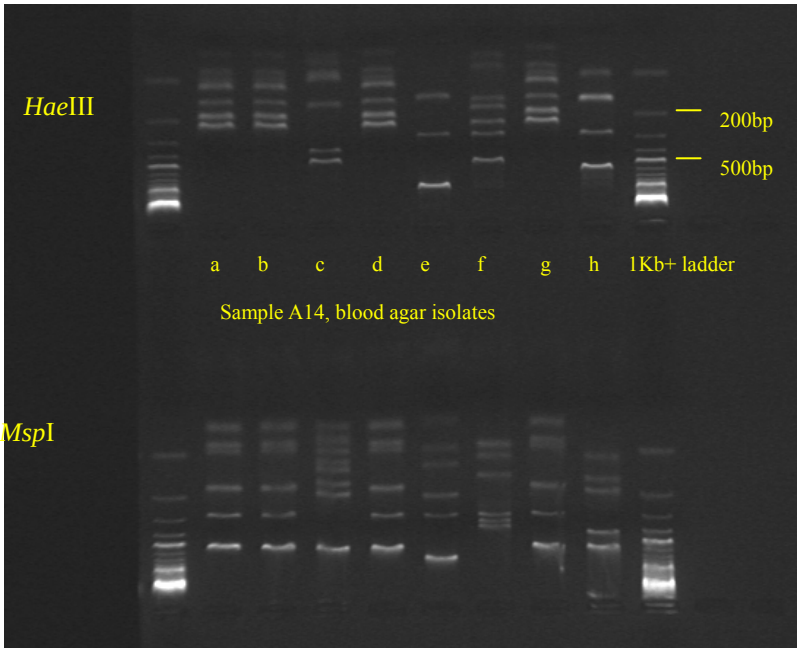


Figure 15 – RFLP of isolates from sample A14. Isolates a, b, d & g are indistinguishable.

On obtaining sequences, 17 isolates (2.5% of all tested, 3.9% of samples referred for sequencing) produced no probable bacterial identity by BLAST. Of these latter, 11 were assigned a presumptive identity based on RFLP similarity to gel images obtained from samples run on different days.

The following results (**Figures 16-20, Tables 5-9**) have been selected to answer critical questions surrounding the ecology of conveyor belts. The quantitative viable counts are illustrated in histograms, and individual isolates from all culture sources have been combined into a single table for each sample. A breakdown of isolates identified from different culture media is presented in ***Appendices 3 – 7***. One sample, ***A15***, is included in all results presented, as a point of reference.

Due to this logical examination of the results, this record does not include every isolate identified.

Note –BLAST searches for these isolates predominantly produced significant similarity results at the *genus* level. All isolates have been therefore reported as genus only in these results.

- ***What sample is most representative for monitoring Intralox® belt hygiene?***

  (Figure 16 & Table 5):

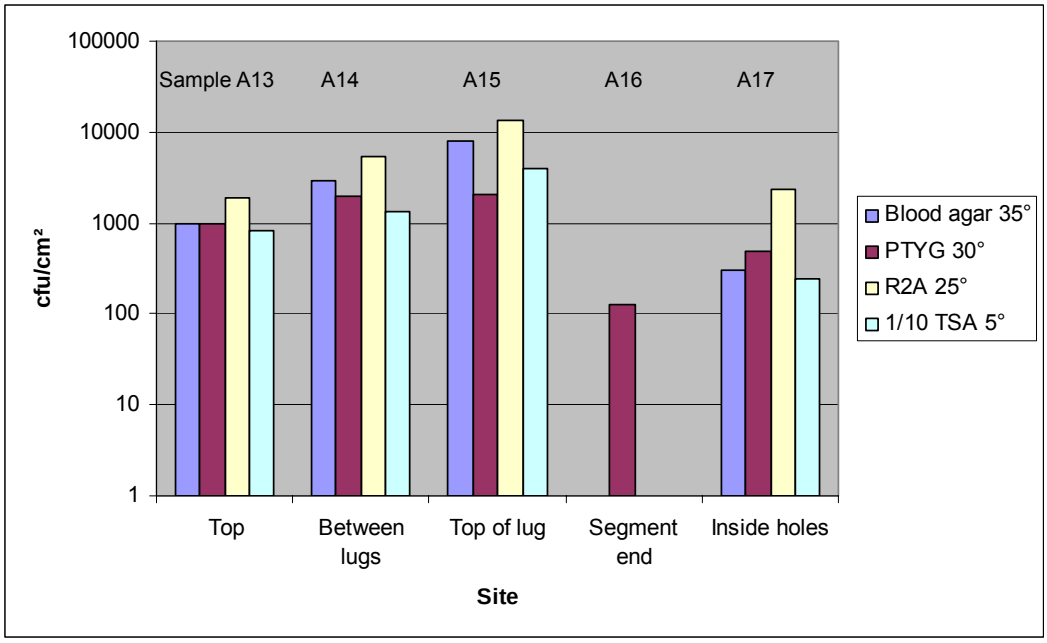


Figure 16 – Comparative viable counts on four media of sampling sites on an acetyl Intralox® segment

Table 5 - variation within a single intralox belt segment (from A samples, 8/5/06)

Sample Site	A13 Top	A14 between lugs	A15 top of lug	A17 Inside holes
Isolate	*Acinetobacter* *Arthrobacter* *Brevibacterium* *Brevundimonas* *Corynebacterium* *Cryseobacterium* *Dermacoccus* *Devosia* *Dietzia* *Flavobacterium* *Janibacter* *Jeotgalicoccus* *Kocuria* *Microbacterium* *Micrococcus* *Mycobacterium* *Stenotrophomonas*	*Agrobacterium* *Brevibacterium* *Brevundimonas* *Corynebacterium* *Haloanella* *Microbacterium* *Moraxella* *Pseudomonas* *Rhizobium* *Sphingomonas*	*Acinetobacter* *Brevundimonas* *Microbacterium* *Sphingomonas* *Stenotrophomonas*	*Brevundimonas* *Haloanella* *Microbacterium* *Mycobacterium* *Sphingomonas*

Isolates reported in ***black*** show a similarity with their nearest neighbour of >97%
Isolates reported in ***blue*** show a similarity with their nearest neighbour of <97%

Although greater diversity was evident in the "top surface" samples (A13), the results for "between" and "top" of the hinge lugs (A14 and A15) showed higher rates of contamination and more consistency across all materials (refer appendix 3). Samples from the hinge region were therefore considered to be more

representative of the actual conveyor belt ecology and contain fewer transient species. Both "top" and "between' hinge samples have been reported in this study, however the "top of hinge" sample is more applicable to routine monitoring as no deconstruction of the belt is required.

- ***How does belt hygiene vary with time?*** **(Figure 17 & Table 6):**

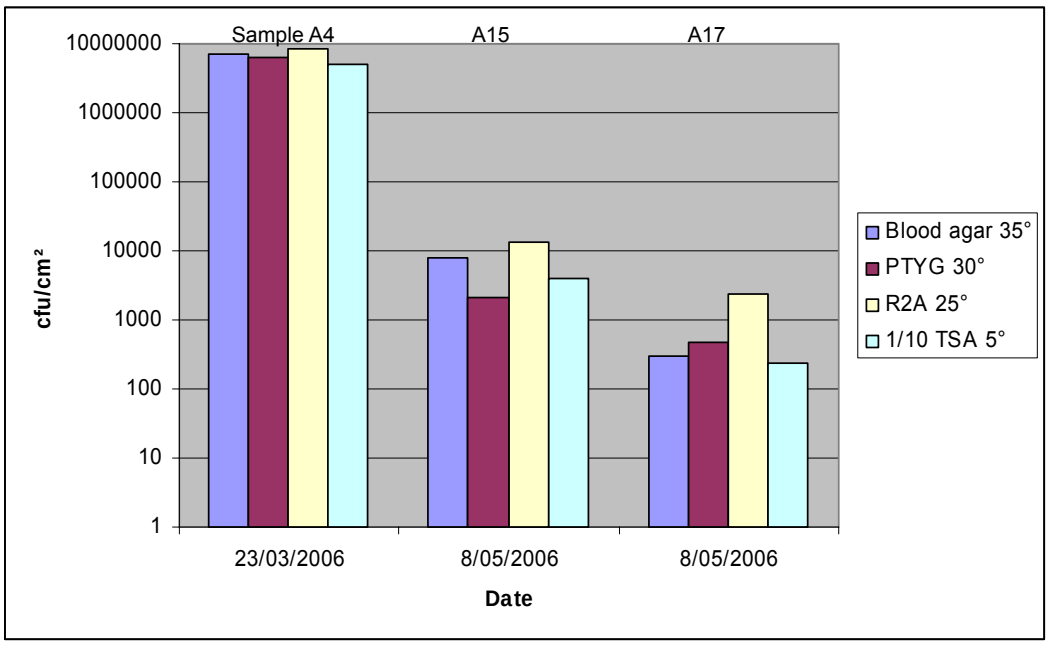


Figure 17 – Counts from the same conveyor belt over two months

Table 6 - variation between samples from the same Intralox belt over time

Date	23/03/2006	8/05/2006	
Sample	**A4**	**A15**	**A17**
Type	Inside holes	top of lug	Inside holes
Isolate	*Acidivorax/ Pseudomonas* *Acinetobacter* *Brevundimonas* *Chryseobacterium* *Clavibacter* *Microbacterium* *Moraxella* *Sphingomonas* *Stenotrophomonas*	*Acinetobacter* *Brevundimonas* *Microbacterium* *Sphingomonas* *Stenotrophomonas*	*Brevundimonas* *Haloanella* *Microbacterium* *Mycobacterium* *Sphingomonas*

BLAST results: Black = similarity >97%; Blue = similarity <97%

In the case of plant "A", a marked reduction in both viable count and bacterial diversity was noted between the two sampling events. The reasons for this are explored further in the discussion.

***How effective are different Intralox® materials in resisting bacterial proliferation?* (Figure 18 & Table 7):**

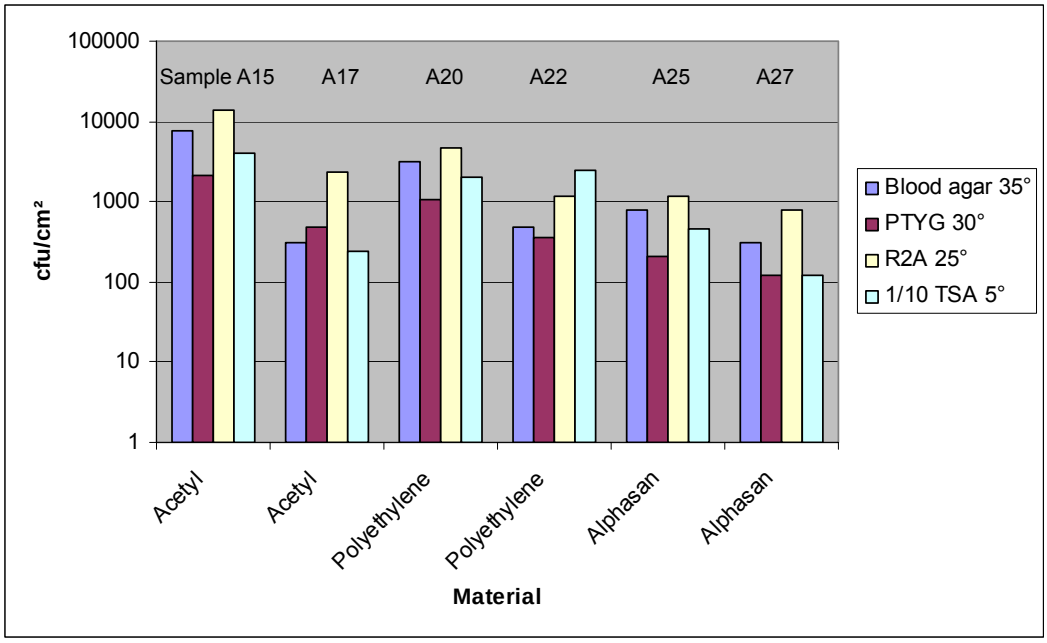


Figure 18 – Comparison of three Intralox belt materials. Alphasan is polyethylene based containing a silver-derived antimicrobial.

Table 7 - variation between different types of Intralox belt materials

Sample	A15	A17	A20	A22	A25	A27
Site	top of lug	Inside holes	top of lug	Inside holes	top of lug	Inside holes
Material	**Acetyl**		**Polyethylene**		**Alphasan**	
Isolate	*Acinetobacter* *Brevundimonas* *Microbacterium* *Sphingomonas* *Stenotrophomonas*	*Brevundimonas* *Haloanella* *Microbacterium* *Mycobacterium* *Sphingomonas*	*Acinetobacter* *Agrobacterium* *Brevundimonas* *Chryseobacterium* *Haloanella* *Luteococcus* *Microbacterium* *Pseudomonas* *Psychrobacter* *Sphingomonas* *Stenotrophomonas*	*Acidivorax* *Arthrobacter* *Brevundimonas* *Microbacterium* *Micrococcus* *Rathayibacter* *Rhodococcus* *Sphingomonas* *Stenotrophomonas*	*Acinetobacter* *Chryseobacterium* *Microbacterium* *Moraxella* *Mycobacterium* *Paracoccus* *Rhodococcus* *Serratia* *Stenotrophomonas*	*Actinobacterium* *Agrobacterium* *Brevundimonas* *Microbacterium* *Sphingomonas*

BLAST results: Black = similarity >97%; Blue = similarity <97%

No marked variations in bacterial viable counts were noted between the three materials tested. Acetyl material typically showed less bacterial diversity per site than the other materials.

- ***How effective is hot water washing alone in retarding bacterial growth?***

  (Figure 19 & Table 8):

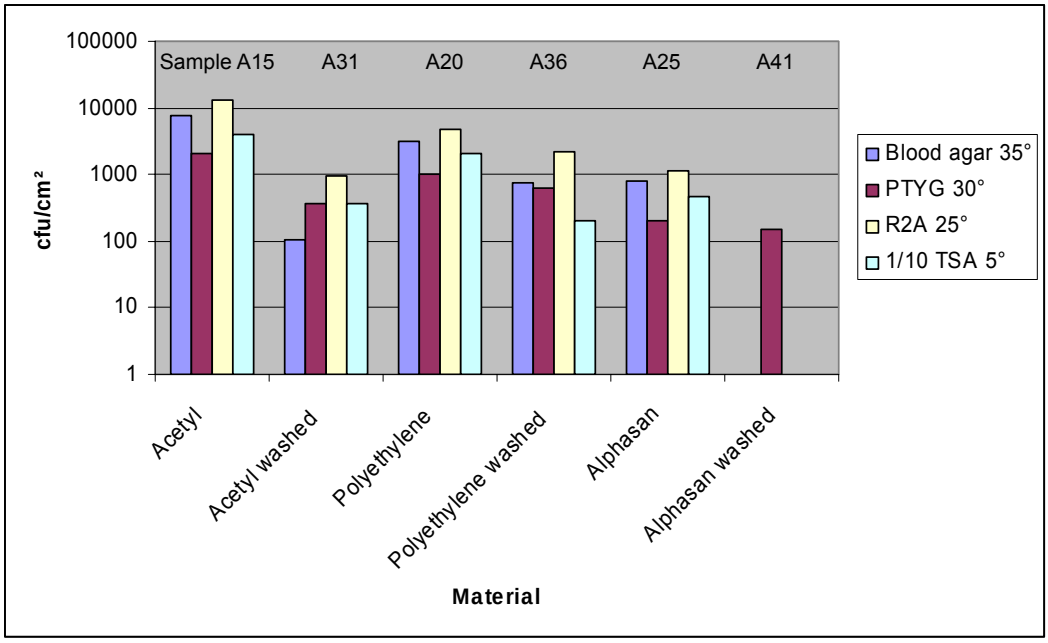


Figure 19 – Effect of hot water washing on three working Intralox belt samples (top of lug)

Table 8 - evaluation of the effect of hot water washing on samples from the top edge of the hinge lug

Sample	**A15**	**A31**	**A20**	**A36**	**A25**	**A41**
Treatment	Acetyl	Acetyl washed	Polyethylene	Poylethylene washed	Alphasan	Alphasan washed
Isolate	*Acinetobacter* *Brevundimonas* *Microbacterium* *Sphingomonas* *Stenotrophomonas*	*Brevundimonas* *Microbacterium* *Sphingomonas* *Spirosoma* *Staphylococcus*	*Acinetobacter* *Agrobacterium* *Brevundimonas* *Chryseobacterium* *Haloanella* *Luteococcus* *Microbacterium* *Pseudomonas* *Psychrobacter* *Sphingomonas* *Stenotrophomonas*	*Acinetobacter* *Haloanella* *Microbacterium* *Pseudomonas* *Psychrobacter*	*Acinetobacter* *Chryseobacterium* *Microbacterium* *Moraxella* *Mycobacterium* *Paracoccus* *Rhodococcus* *Serratia* *Stenotrophomonas*	*Microbacterium*

BLAST results: Black = similarity >97%; Blue = similarity <97%

Hot water washing typically reduced the bacterial load on acetyl material by a factor of 10. The effect was less marked on polyethylene. On Alphasan®, marked reductions in both viable counts and diversity were observed across all samples.

- *What differences in bacterial ecology are seen on conveyor belts between premises and types of construction?* **(Figure 20 & Table 9):**

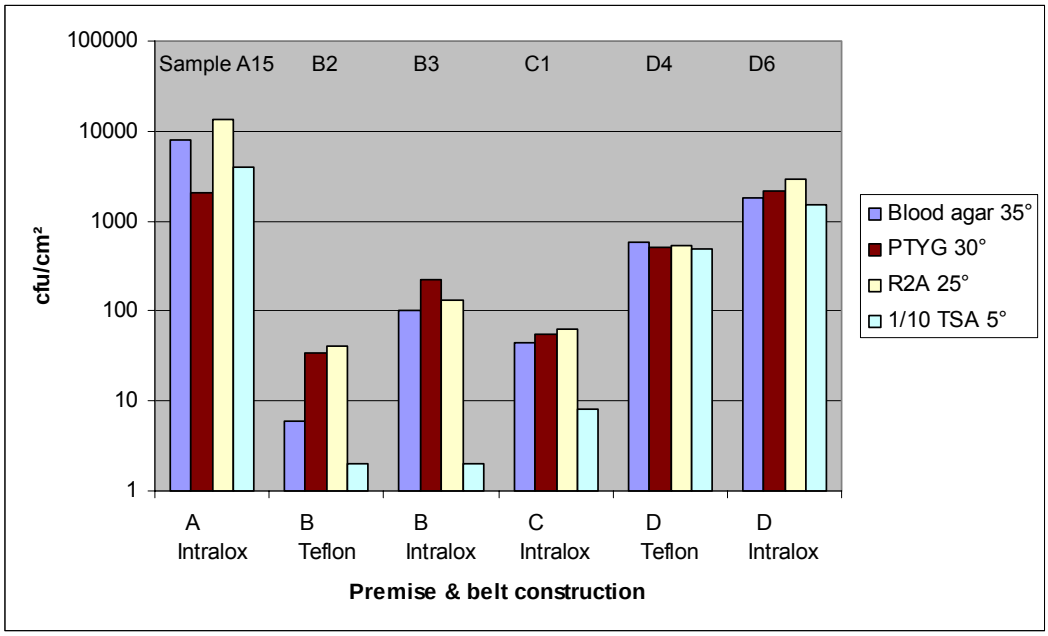


Figure 20 – Differences in viable counts observed between premises

Table 9 - variation between premises and belt construction

Sample	A15	B2	B3	C1	D4	D6
Date	8/05/2006	15/08/2006	15/08/2006	21/08/2006	4/09/2006	4/09/2006
Type	Intralox, top edge of lug	Continuous Teflon, contact	Intralox, contact surface	Intralox, Hinge	Continuous Teflon, contact surface & edge	Intralox, divider segment
Isolates	*Acinetobacter* *Brevundimonas* *Microbacterium* *Sphingomonas* *Stenotrophomonas*	*Actinobacterium* *Corynebacterium* *Dietzia* *Kaistella* *Kocuria* *Luteococcus* *Microbacterium* *Micrococcus* *Moraxella* *Psychrobacter* *Staphylococcus* *Streptococcus*	*Acinetobacter* *Bacillus* *Corynebacterium* *Deinococcus* *Janibacter* *Jeotgalicoccus* *Kaistella* *Kocuria* *Macrococcus* *Microbacterium* *Micrococcus* *Staphylococcus* *Streptococcus*	*Acinetobacter* *Arthrobacter* *Kocuria* *Pseudochrobacterium* *Pseudomonas* *Stenotrophomonas*	*Acinetobacter* *Aerococcus* *Brevibacterium* *Chryseobacterium* *Pseudomonas* *Rhodococcus* *Serratia* *Staphylococcus* *Vagococcus*	*Acidovorax* *Acinetobacter* *Aeromonas* *Agrobacterium* *Brevundimonas* *Chryseobacterium* *Comamonas* *Corynebacterium* *Flavobacterium* *Phyllobacterium* *Pseudomonas* *Staphylococcus* *Stenotrophomonas* *Watersiella*

BLAST results: Black = similarity >97%; Blue = similarity <97%

Differences are apparent between both viable counts and bacterial diversity both between and within premises. For Teflon belts, the belt edge was found to have higher counts than the main contact surface. The loads moving across these belts are largely similar in terms of quantity of product and bacterial load on that product, as measured by National Microbiological Database (NZFSA, 2006b). Some premises have therefore been able to achieve greater hygiene through sanitisation than others. This will be explored in greater detail in the discussion.

Culture-independent molecular ecology

Denaturing Gradient Gel Electrophoresis (DGGE)

The DNA product obtained from the first round of PCR amplification with DGGE-C and DGGE-D primers could not be visualised by agarose gel electrophoresis. This was not unexpected, with similar results having been reported previously and attributed to the small numbers of bacteria present (Pulford *et al.*, 2006). This DNA product was therefore used as the template for a second round of amplification (increasing to 35 cycles from the original 28) with the same primers. The resulting DNA product, whilst now visible, remained minimal, therefore experiments were conducted to optimise the reaction by varying the magnesium chloride ($MgC1_2$) concentration. **Figure 21** demonstrates the optimum concentration for DGGE-C:

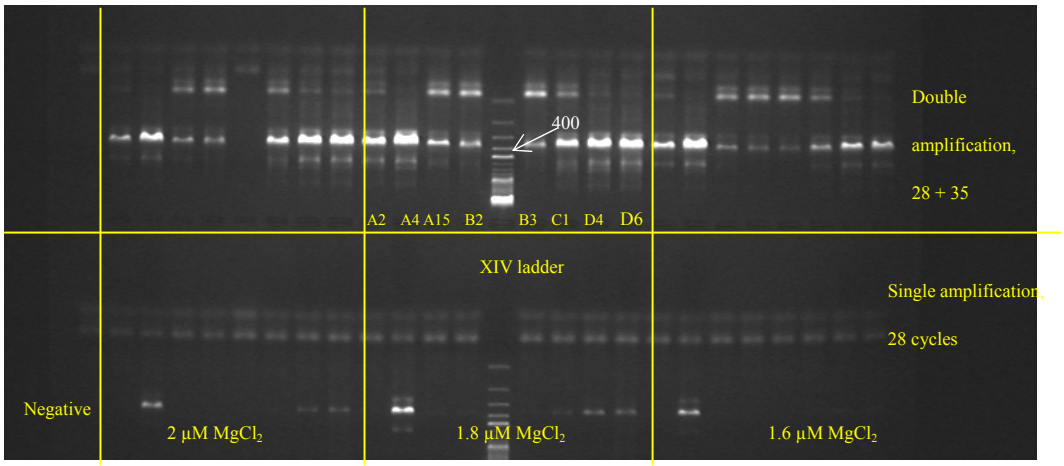


Figure 21 - 2% agarose gel electrophoresis of Fragment C PCR product. The target molecule is approximately 360bp in length.

Although additional bands were noted, the optimum product was nevertheless considered to require double amplification with a 1.8µM concentration of $MgC1_2$. This revised protocol was applied to all other samples tested by DGGE-C. A similar experiment was carried out for DGGE-D, resulting in the following revised reaction mixtures **(Table 10)**:

Table 10. Reaction mixture used for amplifying amplicons **C** and **D** by PCR (Revised 3/10/06).		
PCR	**C**	**D**
X10 Taq Platinum buffer (Invitrogen)	5 µl	5 µl
2mM dNTP (Roche)	5 µl	5 µl
50mM $MgCl_2$ (Invitrogen)	1.8 µl	1.2 µl
10pM/µL Forward primer	5 µl	5 µl
10pM/µL Reverse primer	5 µl	5 µl
Platinum Taq (Invitrogen)	0.25 µl	0.25 µl
Ultrapure water (Invitrogen)	22.95 µl	23.55 µl
DNA sample	5 µl	5 µl

The PCR products were then separated by DGGE. The example shown (Figure 22) is DGGE of Fragment C, which also includes a series of control organisms isolated during the culture library studies (**Table 11**):

Table 11. List of control isolates			
P1	*Bacillus* Sp.	P5	*Brevundimonas* sp.
P2	*Microbacterium oxydans*	P6	*Stenotrophomonas* sp.
P3	*Acinitobacter* sp.	P7	*Pseudomonas* sp.
P4	*Sphingomonas* sp.	P8	*Micrococcus* sp.

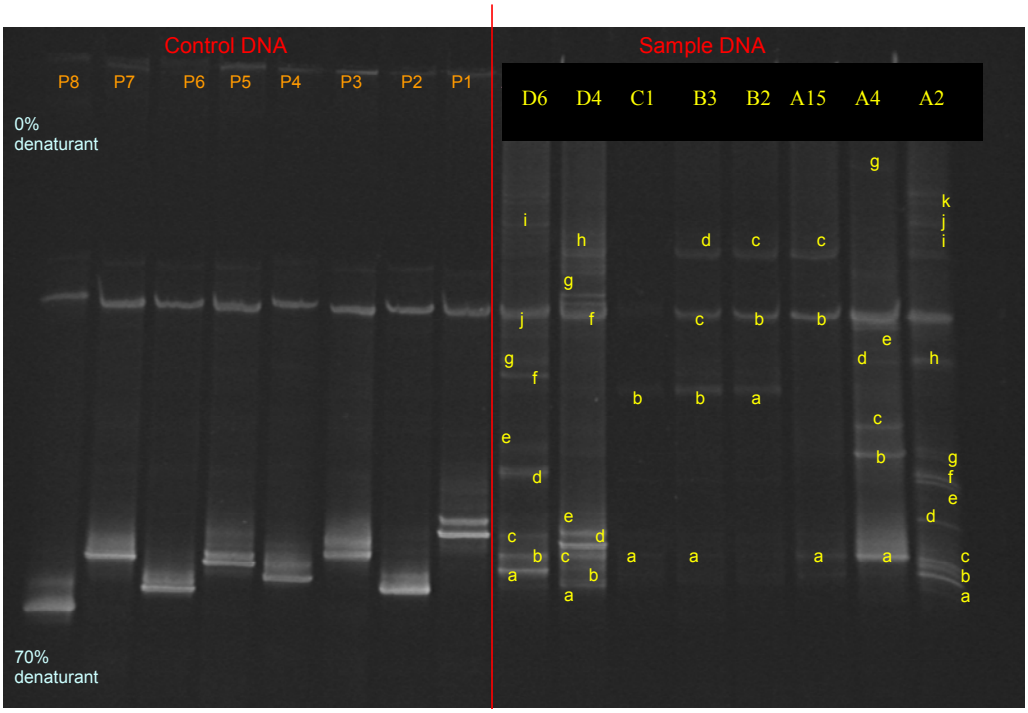


Figure 22 - Acrylamide DGGE gel of Fragment C, SYBR Gold stain (bands excised shown in yellow). *Note*: SYBR Gold was used to achieve more intense staining and safe excision of bands under blue light. This image was obtained using a UV transilluminator, and therefore not all bands are visible.

The excised DGGE-C fragments had the closest sequence similarity with the following bacteria (**Table 12**):

Table 12. ***DGGE-C*** Nearest neighbour results from BLAST search

	A2	A4	A13	A14
a	*Pseudomonas* 98%	*Acinitobacter* 98%	*Pseudomonas* 98%	*Acinetobacter* 95%
b	*Pseudomonas* 98%	*Acinitobacter* 99%	*Psychrobacter* 99%	*Acinetobacter* 97%
c	*Pseudomonas* 96%	*Acinitobacter* 99%	*Pseudomonas* 98%	*Acinetobacter* 97%
d	failed to sequence	*Acinitobacter* 99%	*Pseudomonas* 97%	*Acinetobacter* 98%
e	failed to sequence	*Acinitobacter* 98%	*Pseudomonas* 97%	
f	*Pseudomonas* 94%	*Paracoccus* 90% *Citrobacter* 90%	*Pseudomonas* 96%	
g	*Pseudomonas* 96%	*Acinitobacter* 90%		
h	*Pseudomonas* 97%			
i	failed to sequence			
j	*Pseudomonas* 94%			
k	*Pseudomonas* 95%			
	A15	**A16**	**A17**	**A20**
a	*Acinetobacter* 99%	*Acinetobacter* 97%	*Acinetobacter* 97%	*Pseudomonas* 99%
b	*Acinetobacter* 100%	*Acinetobacter* 96%	*Acinetobacter* 99%	*Pseudomonas* 100%
c	*Acinetobacter* 98%		*Acinetobacter* 97%	*Pseudomonas* 99%
d	*Acinetobacter* 98%			*Moraxella* 99%
e	*Acinetobacter* 98%			*Pseudomonas* 99%
f				*Pseudomonas* 98%
g				*Pseudomonas* 98%
	A25	**B2**	**B3**	C1
a	*Acinetobacter* 98%	*Mycoplasma ovis* 97%	*Acinetobacter* 97%	*Acinitobacter* 97%
b	*Acinetobacter* 98%	failed to sequence	*Mycoplasma ovis* 99%	*Mycoplasma ovis* 98%
c	*Acinetobacter* 97%		failed to sequence	
	D4	**D6**		
a	*Pseudomonas* 100%	*Pseudomonas* 97%		
b	*Aerococcus* 96%	*Pseudomonas* 94%		
c	*Staphylococcus* 93%	failed to sequence		
d	*Acinitobacter* 90%	*Pseudomonas* 100%		
e	failed to sequence	*Pseudomonas* 98%		
	Carnobacterium 96%	*Pseudomonas/* *Acinitobacter* 94%		
g	*Staphylococcus* 96%	*Pseudomonas* 95%		
h		*Psychrobacter* 100%		
i		*Pseudomonas* 95%		

Black - >97% similarity by BLAST
Blue - <97% similarity by BLAST

The first attempts to sequence DNA amplified from excised fragments of DGGE-D (using the forward primer) were largely unsuccessful. Sequencing was then repeated using the reverse primer, from which the majority of nearest-neighbour similarities were obtained. The combined results for DGGE-D are reported in **Table 13**:

Table 13. ***DGGE-D*** Nearest neighbour results from BLAST ***** = sequenced using reverse primer

	A2	A4	A15	B2
a	No sequence	*Exiguobacterium* 99%	*Rhodococcus* 98%	*Corynebacterium* 91% *
b	*Exiguobacterium* 97%	*Exiguobacterium* 97%	No sequence	*Actinobacterium* 96% *
c	Uncultured γ-proteobacteria	*Exiguobacterium* 95%	No sequence	*Microbacterium* 94%
d	*Chryseobacterium/ Flavobacterium* 97%	*Exiguobacterium* 98%	*Exiguobacterium* 98%	*Paenibacillus* 93% *
e	*Chryseobacterium* 95%	*Exiguobacterium* 96%	*Exiguobacterium* 95% *	Uncultured bacterial clone 98%
f		*Chryseobacterium* 94%	*Exiguobacterium* 94% *	*Exiguobacterium* 96%
h			*Exiguobacterium* 98% *	
h			*Exiguobacterium* 92% *	
	B3	**C1**	**D4**	**D6**
a	*Planomicrobium/ Planococcus* 95%	*Planococcus* (unclultured) 99% *	*Aerococcus* 99% *	*Pseudomonas* 99%
b	*Planomicrobium* 93% *	*Planomicrobium/ Planococcus* 97%	*Aerococcus* 98% *	*Microbacterium* 95%/ *Arthrobacter* 94%
c	*Propionibacterium 98%* *	*Planomicrobium/ Planococcus* 91% *	*Aerococcus* 94% *	*Carnobacterium* 100%
d	*Actinobacterium* 93% *	*Planococcus* 93 % *	*Aerococcus* 92%	*Carnobacterium 100%*
e	*Micrococcus* 97% *	*Planomicrobium* 94% *	*Aerococcus 97%*	*Carnobacterium100%*
f		*Kocuria* 91% *		
g		*		

Black - >97% similarity by BLAST Blue - <97% similarity by BLAST

Upon investigating the difficulties in sequencing the DGGE-D amplicons, it was determined that, although attempting to target the same nucleotides, the forward primer designed by Pulford et al., (2006) did not correspond exactly with the sequence targeted by pA. The comparison between the nucleotides is:

- pA (8-27F)[7] AGAGTTTGATCCTGGCTCAG
- DGGE-D forward (9-26F)[7] GAGTTTGATCATGGCTCA

It was ascertained by probing the Ribosome Database Project (RDP) (Maidak *et al.*, 1997) that the DGGE-D primer used was aligned with a smaller group (< 10%) of bacteria than that targeted by pA. It was therefore determined to discontinue work with DGGE-D until this was resolved (see Future Work, page 76).

16S rDNA clone libraries

16S rDNA clone libraries were prepared from DNA extracted from samples A4, A15 and D4. Sample A15 required two rounds of amplification for DNA product to be visualised by agarose electrophoresis. Sample A4 was investigated first, from which 34 clones were generated, all containing an insert of the correct size. Due to

[7] Priming site indicated by *E. coli* 16S numbering.

the low number of clones produced, the ligation phase was extended to 30 minutes when cloning samples A15 and D4. Amplified cloned 16S rDNA was then analysed by RFLP to prevent redundant sequencing (**Figure 23**). Note the RFLP patterns produced may differ from those obtained by culture due to the presence of plasmid nucleotides between the insert and M13 priming site.

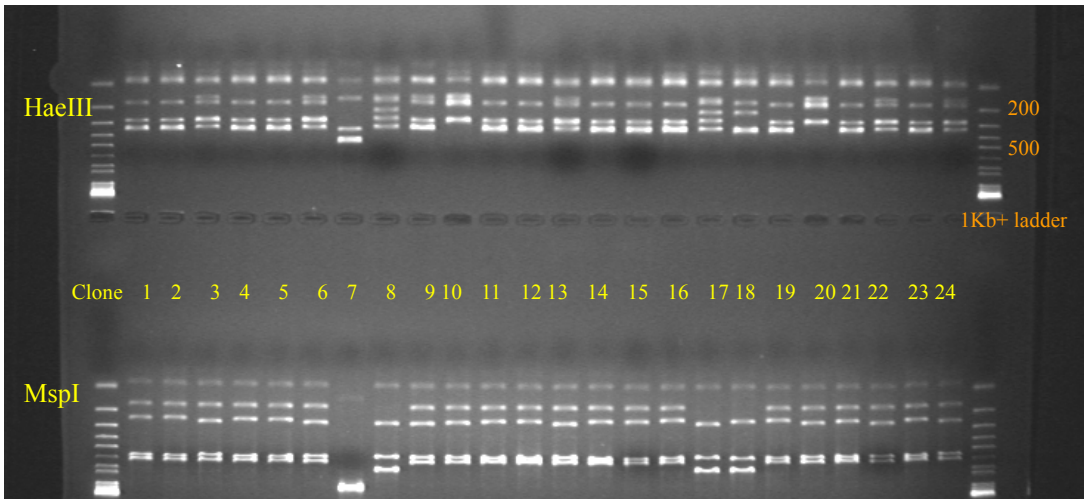


Figure 23 – RFLP of 16S rDNA clones 1-24 from sample A4. Sequence similarity is shown below.
Clones 1, 2, 4, 5, 11, 12, 14, 15, 16, 19, 21, 23 and 24 are indistinguishable
Clones 3, 6, 9, 13 and 22 are indistinguishable
Clones 8 and 17 are indistinguishable

The results of rDNA sequence analysis from representative clones of all samples are presented in **Table 14**:

Table 14 – 16S rDNA, Results of BLAST search

A4		A15		D4	
Nearest neighbour	**%[1]**	**Nearest neighbour**	**%**	**Nearest neighbour**	**%**
Acinetobacter (clone 1)[2]	55	*Escherichia coli*	42	*Acinetobacter*	18
Acinetobacter (clone 3)	18	*Escherichia coli*	28	*Aerococcus*	18
Acinetobacter (clone 8)	9	*Acinetobacter*	15	*Acinetobacter*	14
Acinetobacter (clone 10)	6	*Hafnia alvei*	3	*Staphylococcus*	6
Acinetobacter/ Alvinella (clone 18)	6	*Acinetobacter*	3	*Staphylococcus*	6
Acinetobacter (not shown)	3	*Pseudomonas*	3	*Staphylococcus*	5
Chimeric (clone 7)	3	*Acinetobacter*	3	*Serratia*	3
		failed to sequence	3	*Serratia*	1
				Acinetobacter	2
				Pectobacterium	1
				failed to sequence	26

[1] Indicates the proportion of total clones identified with this RFLP pattern
[2] Clones indicated are from RFLP shown in figure 18
Black - >97% similarity by BLAST Blue - <97% similarity by BLAST

In-situ Microscopy

Fluorescent stains & probes

The examination of stained and FISH probed preparations by epifluorescence microscopy was largely unsuccessful. This was attributed to:

- A relatively small number of bacteria being present.
- High levels of meat residue (protein and fat) in the unwashed matrix.
- Unexpected background fluorescence in Teflon®-based fragments.

The following micrographs are typical of all samples examined (*note* that the Nikon E4500 camera was not cooled, making exposures difficult to set):

***Live/Dead BacLight*® (Figures 24-27):**

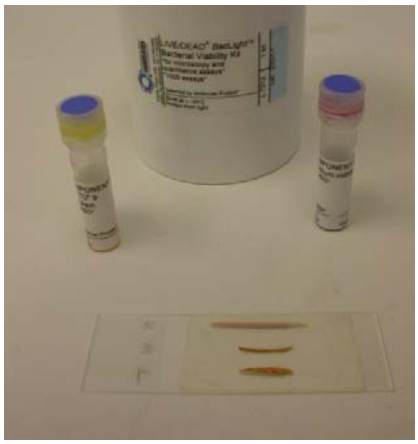


Figure 24 – stained Teflon fragments ready for examination

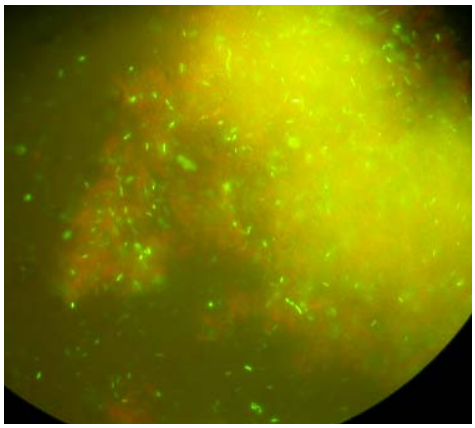


Figure 25 – control 1000x, *Pseudomonas* sp., 21 day culture in Brain-heart infusion

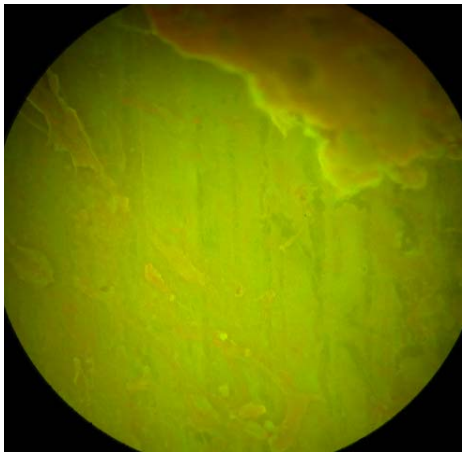


Figure 26 – Acetyl segment, 100x

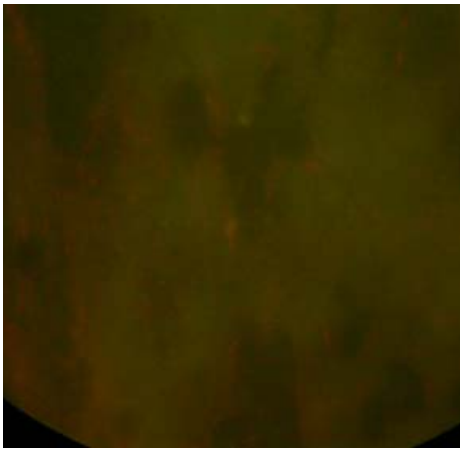


Figure 27 – Acetyl segment, 1000x

***DAPI* (Figures 28 – 31):**

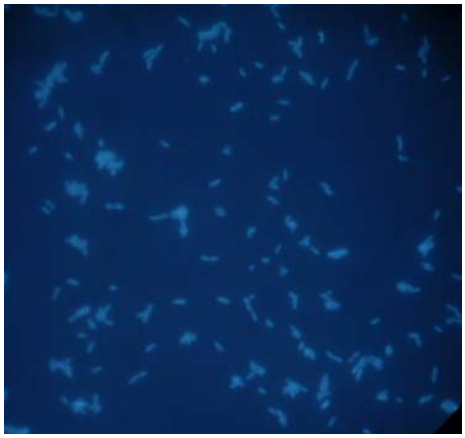


Figure 28 – control x1000, *E. coli*

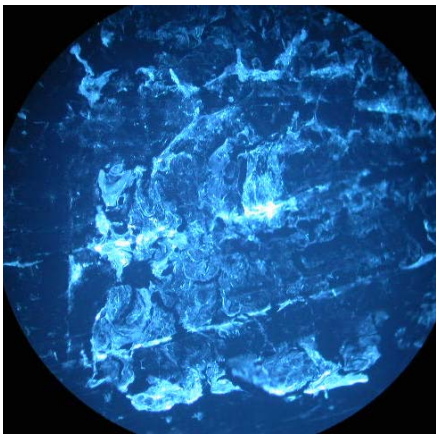


Figure 29, Acetyl belt, 100x

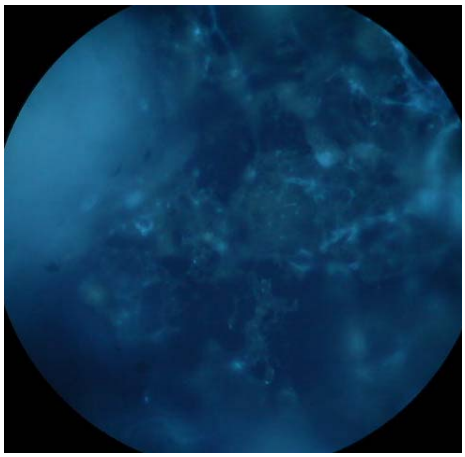


Figure 30 – Acetyl belt, 1000x

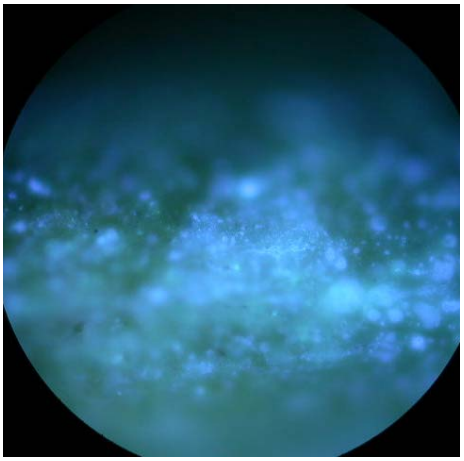


Figure 31 – Teflon belt, 1000x

***FISH* (Figures 32-37):**

Figure 32 – Alphasan insert prepared for examination

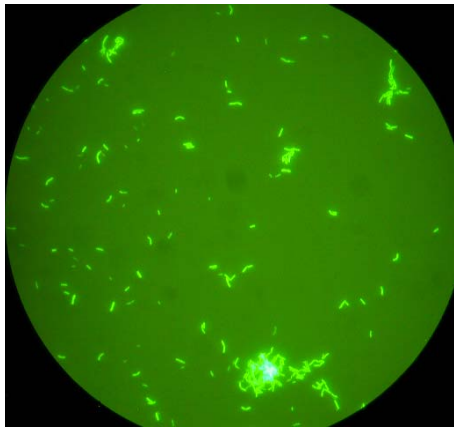


Figure 33 – control 400x, *E. coli*, EUB 338

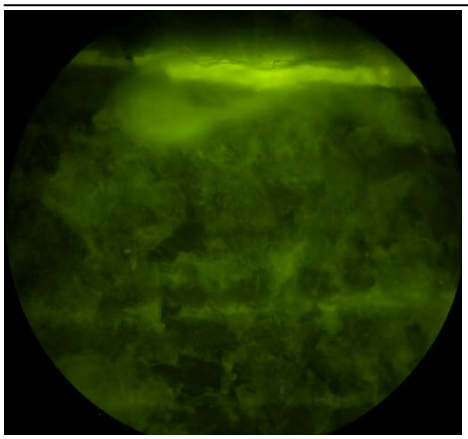


Figure 34 – Acetyl belt 400x, EUB 338

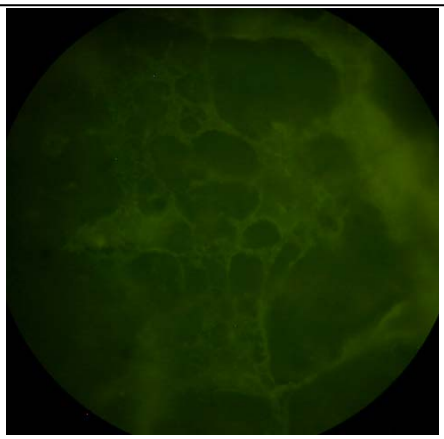


Figure 35 – Acetyl belt 1000x, EUB 338

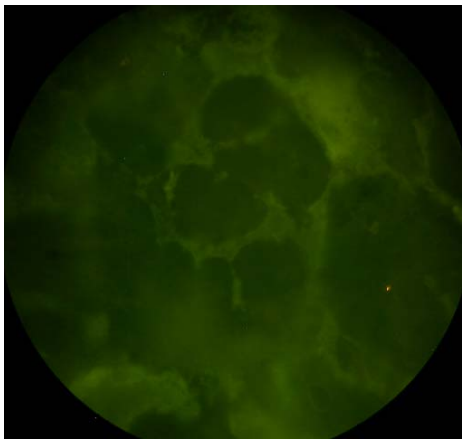


Figure 36 – Acetyl belt 1000x, NON EUB

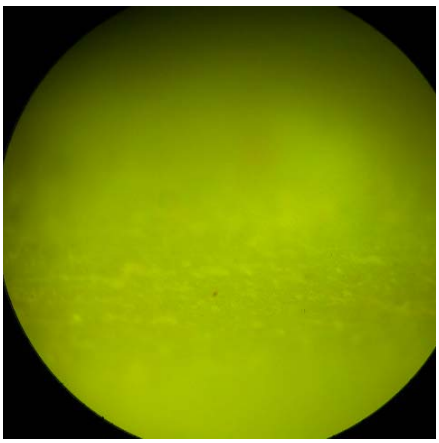


Figure 37 – Teflon belt 400x, EUB 338

Scanning electron microscopy

Scanning electron microscopy proved to be a much more successful tool than epifluorescence, however as long preparation and scanning times were required, the technique was limited to a small number of samples (the instrument was also off-line for long periods). Fixation, drying and coating proceeded routinely. It was however necessary to lower the microscope operating voltage from 20KV to 5 KV for the Intralox® specimens, due to burning under the electron beam, which affected the clarity of images available. Teflon samples were imaged successfully at 20KV. Representative images are shown in **Figures 38-43**:

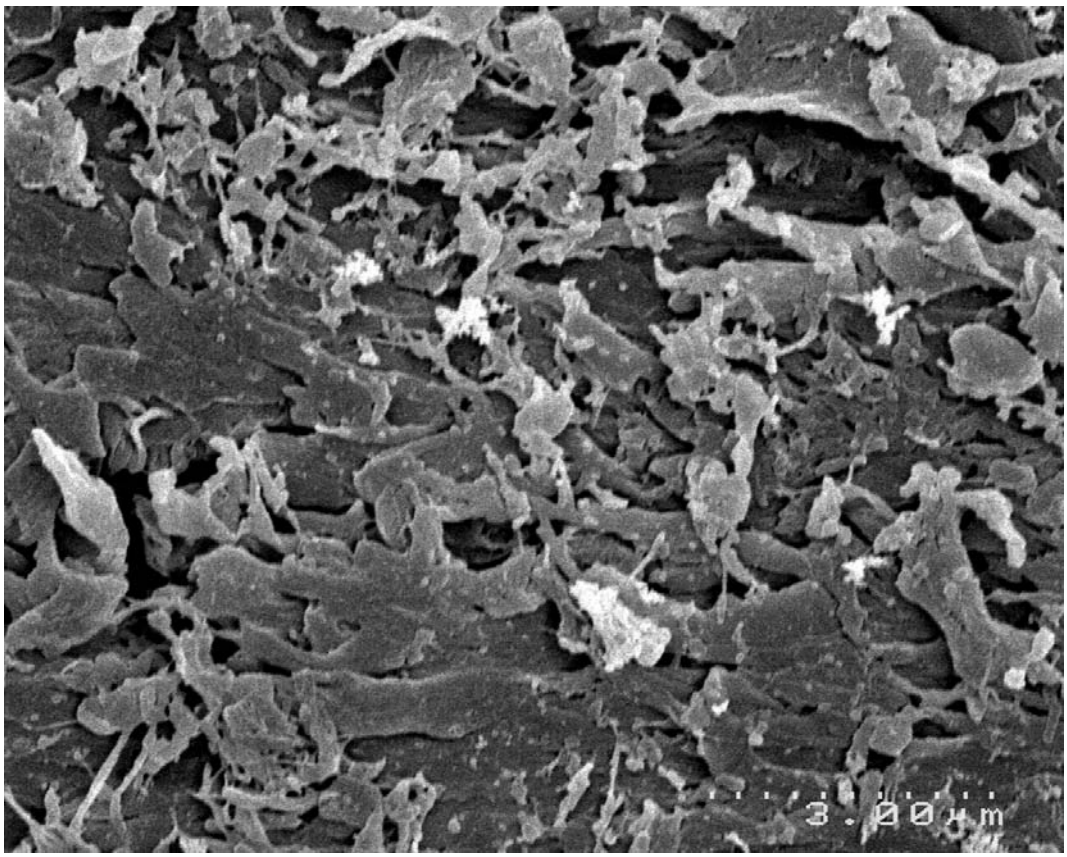


Figure 38 – Acetyl belting sampled at the close of the third working shift of the day. The matrix is formed from deposits of meat proteins and fat.

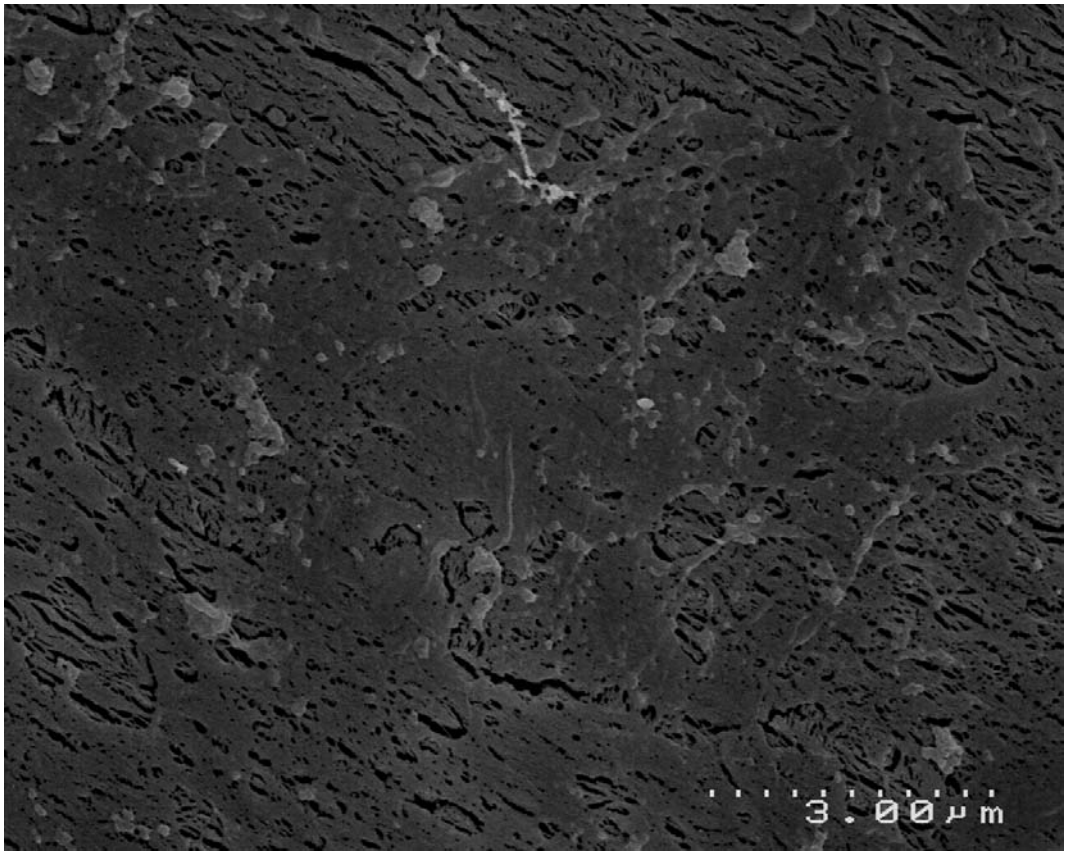


Figure 39 – The above material following 10 seconds of hot water washing at 60°C. No bacteria were visible after 30 minutes of scanning. Both micrographs are from inside a belt hinge (A17).

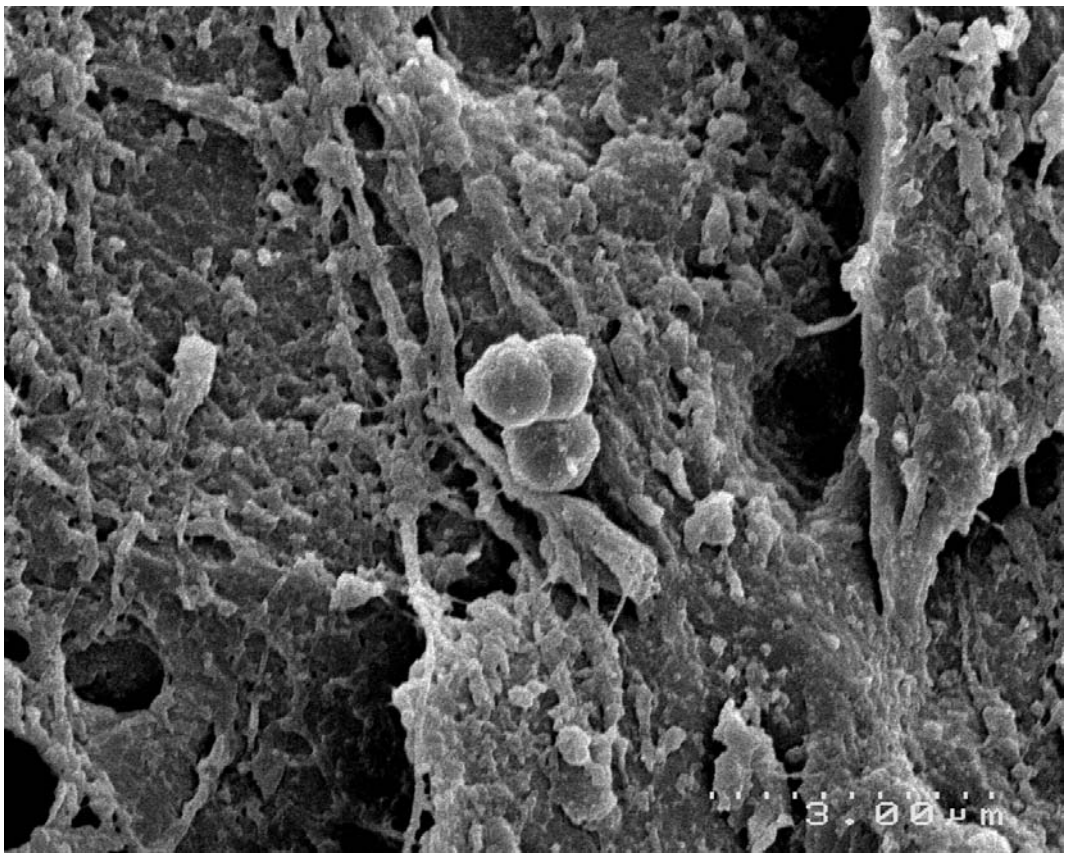


Figure 40 – Bacteria associated with the protein/fat matrix. Polyethylene Intralox® sample A20.

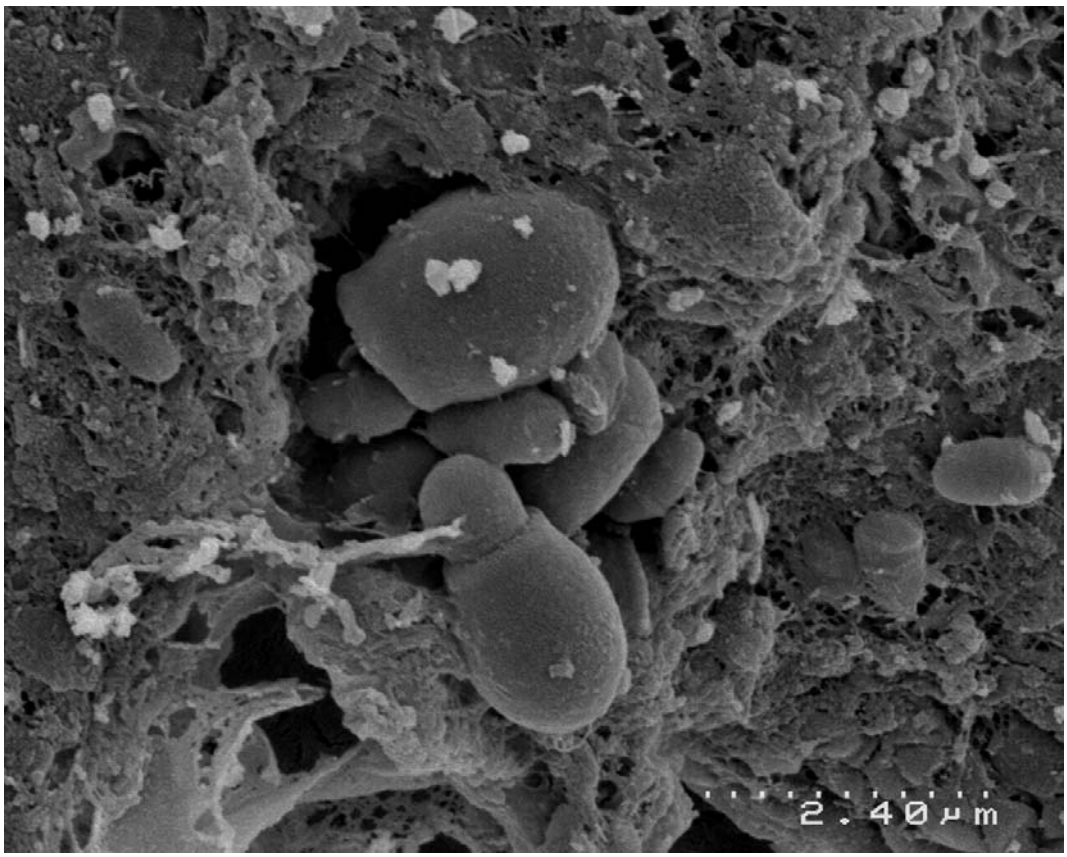


Figure 41 - Acetyl Intralox® belt D6. This was part of a small area containing numerous similar groupings of bacteria. No organisms were visible in the remainder of the sample

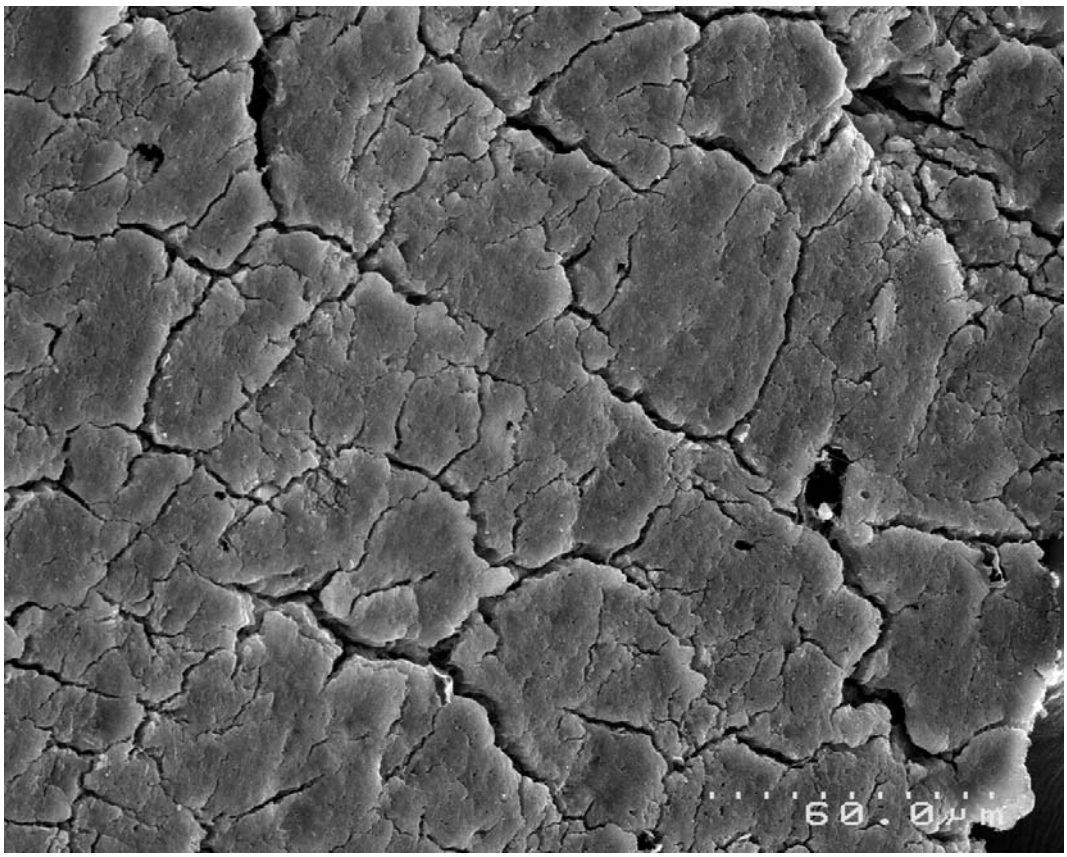


Figure 42 – Teflon belt surface (D3).

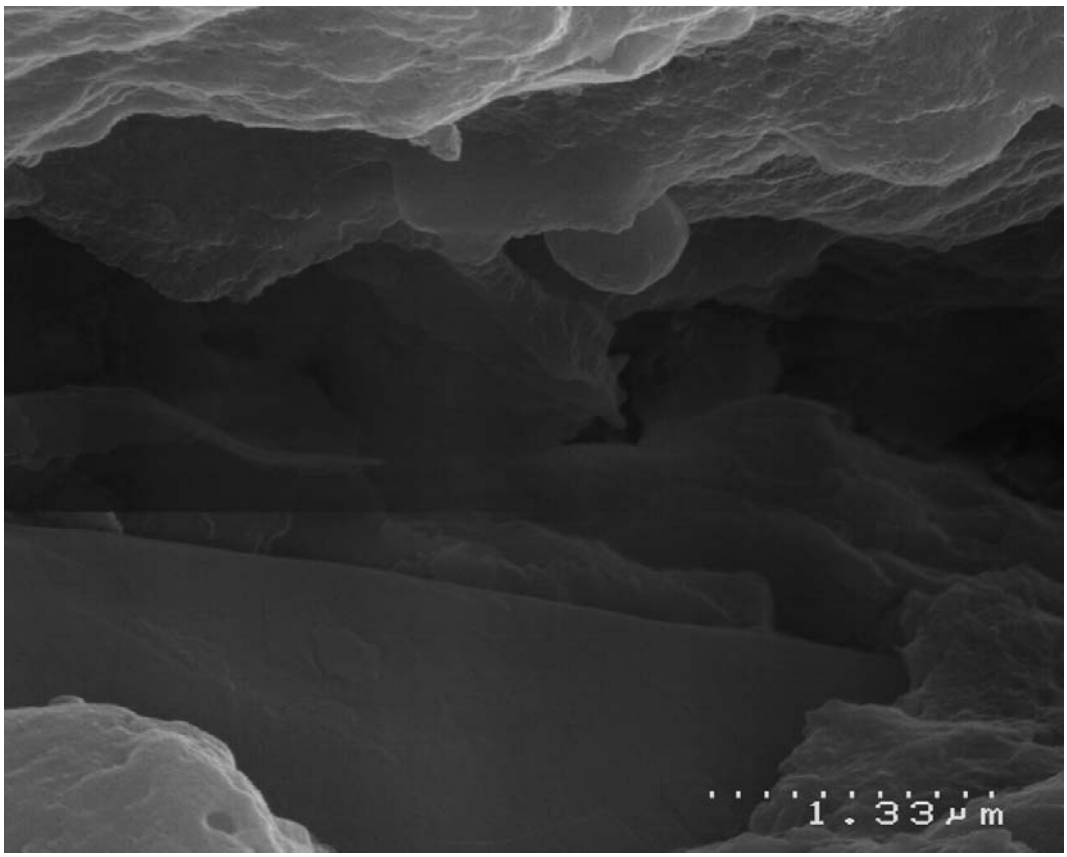


Figure 43 – A coccoid organism inside a crevice in the above belt.

Assessing the results of microscopic examination of the belts overall, it is clear that **no** aggregates of bacteria could be visualised adhering to belt material from any sample using any method. There is therefore no evidence that biofilms develop on the belts. Some evidence however exists from SEM images that bacteria are associated with the meat residue matrix, and are removed under properly conducted hygienic sanitization.

Biofilm growth under laboratory conditions

The five organisms tested were all isolated from premise A, and were of the following genera:

- *Pseudomonas*
- *Acinetobacter*
- *Sphingomonas*
- *Microbacterium*
- *Stenotrophomonas*

Stainless steel rods were included as a positive control. All test organisms formed strongly attached aggregates (**Figure 44**) except *Microbacterium*, which formed an aggregate at the meniscus but this was easily washed away.

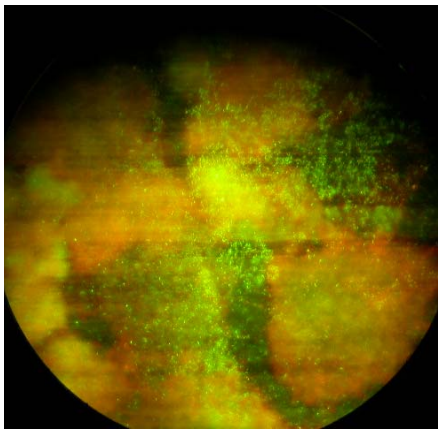


Figure 44 – Pseudomonas attached to stainless steel, BacLight 400x

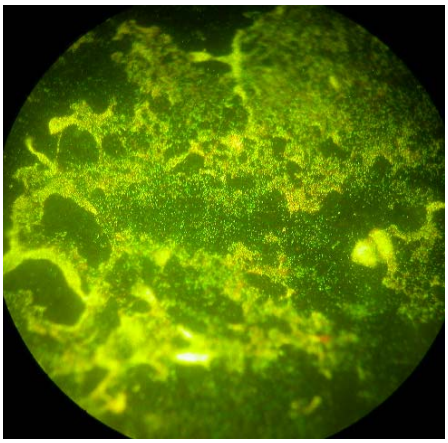


Figure 45 – Pseudomonas on the surface of Alphasan belting. BacLight 400x

For the Intralox® materials however, whilst the bacteria again formed aggregates on the surface of the material (particularly at the meniscus), all test organisms were easily removed by gently running 100µL of deionised water across the surface, leaving small numbers of planktonic cells only visible by microscopy (**Figure 45**). It was therefore deduced the bacteria were not able to strongly attach to these materials.

Quality assurance

Standard quality assurance protocols were observed throughout this study. Culture media and diluents were prepared and controlled, pipettes and incubators were calibrated, and culture method processes were monitored, as described by MIRINZ (2007d). Negative samples were processed by all methods from all sample events. Samples "spiked" with normal meat bacteria were evaluated at the start of the study to ensure PCR reactions were not inhibited by the sample matrix. All PCR reactions contained negative controls to ensure the reactions were free of contamination. Positive controls for DGGE were prepared from organisms isolated and sequenced from culture-dependent studies of the same samples. Sequences from closely related RFLP patterns were compared across the study to ensure the technique was successfully discriminating between genera.

The results of these controls were mostly unremarkable, with method processes remaining in control throughout the study; the only exception being the presence of "double-banding" in DGGE gels, which is discussed further on page 71.

Chapter 4 - Discussion

The basic questions to be answered by any study into microbial ecology are ‘who is there?’ and ‘what are they doing?’ (Dubilier, 2007). Whilst contemporary methods cannot yet provide a complete answer to these questions, a comparative approach utilising culture-dependent and culture-independent ribosomal DNA libraries, combined with in-situ microscopic observation, does permit a comparison between the microbiota occupying different habitats and the ability to assess the impact of stimuli such as antimicrobial agents (Bent *et al.*, 2007).

To gain a comprehensive representation of bacterial diversity, a combination of culture –dependent and –independent methods were used (Suzuki *et al.*, 1997). Due to known biases with culture-independent methods (e.g. preferential DNA extraction, PCR primer efficiency), two methods, DGGE and 16S clone libraries, were cross-referenced to improve the estimation of diversity.

Culture-dependent studies

The bacterial ecosystems present on meat contact surfaces are known to contain heterotrophic and oligotrophic organisms of human, animal and environmental origin. In meat boning rooms, selective ecological characteristics such as chemical cleaning regimes, low temperatures (6 – 10 °C) and periods of high and low nutrient and water availability also influence the diversity of bacteria involved (Newton *et al.*, 1978).

The nutritional requirements to maximise the recovery of culturable bacteria are unlikely to be met by a single culture medium, and biases exist even between media designed to cultivate the same bacterial groups (Balestra & Misaghi, 1997; Tabacchioni *et al.*, 2000). A variety of media with different nutritional qualities were therefore applied in preparation of the culture libraries. Provision was made

to cultivate both mesophiles of animal and human (worker) origin, psychrophiles of environmental origin, and pychrotolerant species derived from either source, as all are known to contribute through a succession process to the final microbiota of chilled meat (Jones, 2004).

Considering the performance of each culture medium in turn, the number of samples for which a medium successfully cultivated species not found on other culture plates was enumerated:

- Organisms were cultivated on blood agar only from 24 samples out of 28. These included *Microbacterium* sp., *Micrococcus* sp., *Moraxella* sp., *Corynebacterium* sp., *Staphylococcus* sp., *Sphingomonas* sp., and *Brevundimomas* sp.
- Organisms were cultivated on CNA blood agar only from 8 samples out of 18. These included *Microbacterium* sp., *Micrococcus* sp., *Sphingomonas* sp., *Streptococcus* sp., and *Arthrobacter* sp.
- Organisms were cultivated on CHROMagar Coliform only from 5 samples out of 14. These included *Aeromonas* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Sphingomonas* sp., and the only known human enteric pathogen isolated during this study, *Shigella sonnei*.
- Organisms were cultivated on PTYG agar only from 15 samples out of 28. These included *Carnobacterium* sp., *Kocuria* sp., *Agrobacterium* sp., *Stenotrophomonas* sp., *Sphingomonas* sp., *Pseudomonas* sp., and two non-pathogenic species of *Mycobacterium* sp.
- Organisms were cultivated on R_2A agar only from 21 samples out of 28. These included *Rhodococcus* sp., *Kocuria* sp., *Moraxella* sp., *Rhizobium* sp., *Sphingomonas* sp., and a non-pathogenic species of *Mycobacterium* sp.

- Organisms were cultivated on 1/10 TSA agar only from 15 samples out of 25. These included *Acidovorax* sp., *Psychrobacter* sp., *Rhizobium* sp., *Kaistella* sp., *Flavobacterium* sp., *Pseudomonas* sp., and a non-pathogenic species of *Mycobacterium* sp.

Although some of these examples could be a result of a replicate distribution affect resulting from low viable counts, this data overall confirms that multiple culture media were essential in determining the range of diversity, and that no one medium was found to be redundant by this study.

The culture library work was used to consider five basic questions, the first being to locate a representative sample site on Intralox® conveyor belts. The "top of hinge" site selected has the advantage that it can be collected without dismantling the belt, yet still provides a good indication of overall hygiene.

Question two studied the variation in belt hygiene over time, and illustrates a very important difficulty when working with any industry. In ***figure 17***, viable counts overall were observed to have been reduced by a factor of 1000 within the period investigated, with an equivalent impact on bacterial diversity. An even more marked effect was observed at premise "C", which was the location of the preceding study reported by Brightwell et al., (2006). Similarly, the dominance of members of the family Enterobacteriaceae (notably *Serratia* sp.) at three out of four premises investigated in 2003 (Mills, 2006) was no longer present, and *Serratia* sp was not recovered at all from three of four premises tested here (two of which were common to both studies). Finally, Serratia sp. that were isolated from premise "D" were most closely related to *S. proteamaculans*, a species associated with diseases of insects, rather than *S. liquifaciens* (a recognised meat spoilage organism) that was identified during the previous studies.

In order to gain access to meat premises and associated research funds, regular

updates of results-to-date must be provided to the industry, and in this case the premises upgraded (sometimes significantly) their general hygiene as a result. This is a variation on a well described (and sometimes criticised) confounding variable, known as the "Hawthorne" or "placebo" effect, where people behave or perform differently if they know they have been singled out for special study (Parsons, 1974). The consequence of this factor to the work described in this thesis was a change of focus to consider the effectiveness of these steps by comparing implementation at four different premises.

The next question considered was whether different Intralox® materials offered any benefits in terms of reduced bacterial numbers, or a microbiota containing fewer spoilage bacteria. No such benefits were observed; although Alphasan® belt material achieved a reduced count and diversity following cleaning, the effectiveness during meat processing was negligible, thus there was no justification for the additional cost to the premise.

In question four, the effectiveness of hot water was evaluated as a physical hygiene measure. Water at 60°C was applied under pressure for 10 seconds, effectively mimicking the action of a "clean in place" system. A small reduction in viable count was observed between pre- and post- wash samples for acetyl and polyethylene, but a marked reduction was noted for Alphasan®. Little difference in bacterial diversity was observed for acetyl, whilst a partial reduction in species was noted for polyethylene. For Alphasan®, no bacterial species could be detected post washing except *Microbacterium* sp.

The final question investigated the differences in bacterial ecology between premises and belt construction. Due to the different hygiene strategies in place, the belt at premise "C" was necessarily sampled in a "clean" condition, whilst the belts at the other facilities were sampled "working". The belt at premise "A" (Intralox®

acetyl, washed by clean-in-place followed by disinfectant) was in use for 10 hours prior to sampling, which may explain the higher viable count. This belt did, however, demonstrate the least diversity for a "hinge" sample, although containing isolates common to most samples from premise "A".

The two belts at premise "B", although of different construction (one continuous Teflon, one Intralox®), nevertheless displayed very similar viable counts and bacterial diversity. The marginally higher counts recorded for the Intralox® belt (B4) may reflect the fact that, at that time, this belt was sanitised by conventional disinfectant and water (identical to the Teflon belt) and not by clean-in-place.

Premises "C" and "D" differed from the others in that *Microbacterium* sp. did not feature commonly in the list of isolates; instead, *Pseudomonas* sp. were frequently present. This may be in consequence to the preferred use of quaternary ammonium compound ("Quat") disinfectants at these premises, to which this genus shows intrinsic resistance. Premise "C" nevertheless demonstrated excellent viable count results, and a small diversity due to a well optimised clean-in-place regime.

The belts at premise "D" were found to harbour significant numbers of *Serratia* sp. in 2003 (Mills, 2006), and this genus was still present on the continuous Teflon belt, albeit in smaller numbers and accompanied by a diverse microbiota. Although adopting a similar hygiene regime to premise "B", these belts had a higher bacterial viable count, and a diverse microbiota.

Based on discussions with the companies concerned, it was ascertained that premises A, B and C were not experiencing rejection in the marketplace during the course of this study (premise D did not process chilled meat on the day of sampling). Data from belts A, B and C were therefore considered to represent a premise "in control". Based on this data, a tentative standard for hygiene control can be proposed as a bacterial viable count of less than 1000 cfu/cm^2 and the

absence of Enterobacteriaceae. Statistical work will be required on additional samples before this standard can be formally adopted.

Culture-independent studies

The rationale behind including culture-independent methods in this study was two-fold. Firstly, culture-dependent methods frequently under-estimate the total bacterial diversity (Donachie *et al*., 2004), and in this study, the culture work undertaken was incubated aerobically only. Culture-independent methods were therefore included not only to provide a greater representation of the diversity, but also to expose bacteria that may have been overlooked previously.

Only one species, *Mycoplasma ovis* (a respiratory pathogen of sheep) was discovered by DGGE but not by the culture libaries.

Escherichia coli and *Hafnia alvei* were discovered using 16S rDNA clones but not by culture, which is unexpected as these species should have been readily cultivatable. *Pectobacterium* was identified by clone library alone. A clone was produced showing the same probability towards both *Alvinella* and *Acinetobacter*, however this was presumptively linked to *Acinetobacter*, as *Alvinella* are known to exist only in deep aquatic environments.

Secondly, as conventional culture methods are extremely time consuming, a system such as DGGE that can review the bacterial diversity within many samples after just a few days offers many advantages. This study was therefore also undertaken to validate DGGE for this purpose, with a secondary confirmation using 16S rDNA clone libraries.

DGGE is a technique for separating individual species from total community DNA (Watanabe *et al.*, 2001), and is based on the fact that the two strands of DNA separate, or melt, when heat or a chemical denaturant is applied. The melting

temperature is influenced by the hydrogen bonds formed between complimentary base-pairs (i.e. GC rich regions melt at higher temperatures than AT rich) and the attraction between neighboring bases on the same strand. Two DNA molecules that differ by as little as a single nucleotide within a low melting domain will nevertheless have different melting temperatures. When separated by electrophoresis through an increasing gradient of chemical denaturant, the molecule is retarded at the concentration where these strands dissociate. Complete strand separation is prevented by artificially creating a high melting domain – typically this is achieved by PCR amplifying 16S DNA using a "GC clamped" primer with a 5' tail comprising a 30-50 nucleotide GC rich sequence.

Members of bacterial communities are then visualized as bands that migrate to different positions on a gel, each band representing a different DNA sequence. Identification of bacterial species can then be determined by excising bands from the gel, amplifying the DNA and sequencing.

Whilst this method functioned satisfactorily, the results appeared biased towards different strains representing one or two genera, rather than revealing a similar diversity to that determined by culture.

Examples from Fragment C - D1 (*Pseudomonas* sp.) and Fragment C - D3 (*Acinitobacter* sp.) were studied further *in-silico* by pairwise BLAST alignment (Altschul *et al.*, 1997) and aside from one pair (D1a & D1b, separated by a single deletion) the sequences derived from each band were significantly (<98% identity) different from each other, suggesting micro-variation artifacts (Kisand & Wikner, 2003). These may have occurred by natural variability (i.e. a set of strains within a species), or artificial introduction from studying an un-cultured part of the community by PCR amplification. It was noted such bands may follow each other

in a random manner, regardless of phylogenetic relatedness, as this is not strictly related to melting temperature (Tm).

Potential causes of inaccurate phylogenetic information from PCR-DGGE are the biases introduced during DNA extraction and artifacts caused by PCR amplification of mixed templates, which can result in preferential amplification of some sequences, chimeric amplification products, and erroneous nucleotides. In one study, up to 14% of amplicons from a single clone carried aberrations (Speksnijder *et al.*, 2001). The double amplification protocol necessary to prepare adequate product involves some 63 PCR cycles, followed by a further 28 post electrophoresis, thus the potential for such errors is magnified.

Double-banding was observed, most noticeably in the control DNA lanes (P1-P8) on the Fragment C gels. Bands cut, eluted and sequenced from these control samples nevertheless showed >98% similarity to the genus identified from the cultured organism. Artefactual banding has been reported previously (Janse *et al.*, 2004) and is of concern as it can lead to an overestimation of sequence diversity.

An explanation for the formation of double bands has not been proven, but it has been hypothesized that secondary product is formed during each PCR cycle due to prematurely halted elongation, possibly by obstruction of the enzyme by secondary structures.

A high number of sequences were obtained that showed low similarity to neighboring species. This may be a consequence of introduced artifacts as described above, identification of a sub-set of strains with low representation on the NCBI database, or be due to the necessity for the sequence length to be relatively small (Muyzer & Smalla, 1998).

A small number of bands failed to produce identifiable sequences. This could represent ssDNA remaining from the PCR reaction, or 16S rDNA from more than

one bacterial species.

Strategies to resolve these issues are considered in "*Future work*".

The 16S rDNA clone libraries produced a similar dominance of *Acinetobacter* to that seen by DGGE for sample A4, which if a result of method bias may point towards DNA extraction as a possible cause. The clone libraries revealed more diversity than was found by DGGE for sample A15, and a different diversity for sample D4.

At the current level of development therefore, DGGE cannot yet be considered validated as a means of rapidly establishing bacterial diversity for samples from this environment.

Bacterial diversity

Of the organisms found in this study, only *Shigella sonnei* and some strains of *Escherichia coli* are associated with food-borne human disease (Madigan *et al.*, 2000). Whilst Enterobacteria, *Carnobacterium*, *Acinetobacter*, *Moraxella*, and *Pseudomonas* are frequently isolated from the surface of chilled meats (MIRINZ, 2007b), only *Hafnia alvei* (identified from one clone library only) is associated with the anaerobic spoilage of vacuum packaged chilled meats (Gill, 2004). *Carnobacterium* and *Pseudomonas* do however have the potential to spoil meat aerobically (Ercolini *et al.*, 2006), and their presence is therefore of considerable importance to the domestic market.

The remainder of the bacteria present are largely as predicted by Newton et al., (1978), and are derived from animal and environmental origin. Whilst some have infrequently been reported as opportunistic pathogens in humans, the risk via meat is negligible (Madigan et al., 2000).

The majority of the genera identified by this study are located in five distinct taxonomic categories (NCBI, 2005). These are:

CFB/Green sulphur bacteria
- CFB group
  - Bacteroidetes
    - Flavobacteria
      - Flavobacteriaceae (taxonomic category 1 identified)
        - ***Chryseobacterium***
        - ***Flavobacterium***
        - ***Haloanella***

Firmicutes
- Actinobacteria
  - Actinobacteridae
    - Actinomycetales (taxonomic catergory 2 identified)
      - Corynebacterinaeae
        - Corynebacteriaceae
          - ***Corynebacterium***
        - Dietziaceae
          - ***Dietzia***
        - Mycobacteriaceae
          - ***Mycobacterium***
        - Nocardiaceae
          - ***Rhodacoccus***
      - Micrococcineae
        - Microbacteriaceae
          - ***Microbacterium***
        - Dermacoccaceae
          - ***Dermacoccus***
        - Intrasporangiaceae
          - ***Janibacter***
        - Micrococcaceae
          - ***Arthrobacter***
          - ***Kocuria***
          - ***Micrococcus***
- Bacillus/ Clostridium Group
  - Bacilli
    - Bacillales (taxonomic category 3 identified)
      - Bacillaceae

- ***Bacillus***
- ***Exiguobacterium***
- Paenibacillacaeae
- Lactobacillales
- Aerococcaceae
- ***Aerococcus***
- Carnobacteriaceae
- ***Carnobacterium***
- Streptococcaceae
- ***Streptococcus***
- Staphylococcaceae
- ***Staphylococcus***

Proteobacteria

- α subdivision (taxonomic category 4 identified)
- Caulobacter group
- ***Brevundimonas***
- Methylobacterium group
- Rhizobiaceae
- ***Agrobacterium***
- ***Rhizobium***
- Rhodabacter group
- Sphingamonadaceae
- ***Sphingomonas***
- γ subdivision (taxonomic category 5 identified)
- Enterobacteriaceae group
- Enterobacteriaceae
- ***Escherichia*** (found by clone library only)
- ***Hafnia*** (found by clone library only)
- ***Serratia***
- ***Shigella***
- Pseudomonaceae/Moraxellaceae group
- Moraxellaceae
- ***Acinetobacter***
- ***Moraxella***
- ***Psychrobacter***
- Pseudomoadaceae
- ***Pseudomonas***
- Xanthomonadales
- Xanthomonas group
- ***Stenotrophomonas***

Microscopy

Microscopy is considered the only technique whereby bacteria are able to be studied at the single cell level *in-situ* (Palmer Jr. & Sternberg, 1999), thereby allowing for some estimation of the ecological question "what are they doing?". DAPI staining, and scanning electron microscopy (SEM) are non-specific, whilst live/dead staining determines the viability of bacteria in the sample, and FISH allows for discrimination at the phylogenetic level (Madigan *et al.*, 2000)

The fluorescent stains and in-situ hybridisation probes used were unable to effectively visualise bacteria in the numbers present during this study, particularly when surrounded by a protein/ fat matrix as found when the samples were in a "working" condition.

Unfortunately, this precluded the use of the in-situ sampling device, which was intended as a rapid monitor of hygiene using both direct viable counting based on the BacLight methodology and proportional diversity based in FISH.

It was, however possible to view biofilm growth under laboratory conditions using the BacLight system. This was used to determine that pseudomonas biofilms developed readily on stainless steel (effectively the control as this property is well established), but only easily detachable aggregates were formed on the Intralox® belt materials.

Scanning electron microscopy has the advantage of producing a 3-dimensional image of the sample, as the electrons bounce off the surface to provide a 3-D structure. The main disadvantage of the method is that samples needed to be dried and coated with electron dense stains which may have distorted any communities present and produced staining artefacts.

This method produced some very important results. Firstly, the surface matrix was able to be visualised, including the meat residue matrix and bacteria associated

with it. It was then possible to visualise the same surface after washing with 60°C water, showing the removal of that matrix leaving only the belt surface with no bacteria visible at all for the Intralox® materials. This strongly supports the effectiveness of the clean-in-place washing methods. A Teflon belt studied post sanitisation showed only one organism visible in a surface fissure, again after 30 minutes of examination.

No evidence of biofilm formation (i.e. adhesion and aggregation of bacteria) was seen in any of the scanning electron micrographs viewed, which effectively means ***the null-hypothesis has been proved*** for this study.

Conclusions

The preferred locations to obtain samples to monitor conveyor belt hygiene are across the hinge area for Intralox® belts, and a 100 cm^2 area covering the top surface and edge of a continuous belt.

Based on the results from premises "B" and "C" (premises that have few if any rejections in the marketplace), a provisional standard for hygiene control has been recommended as a bacterial viable count of less than 1000 cfu/cm^2 and the absence of Enterobacteriaceae.

Antimicrobial belt materials were not cost-effective in terms of added hygienic value. Biofilms were not detected in-situ on any of the belt materials tested, and did not form strongly attached sessile forms under laboratory conditions on these materials. The primary concern for all meat processors should therefore be to remove all traces of meat residue during their sanitisation processes.

The microbiota of conveyor belting in this study was found to consist of bacteria

from five taxonomic groups; the Flavobacteriaceae, the Actynomycetales, the Bacillus/Clostridium group, and the α- and γ- branches of the Proteobacteria. No dominant species was found on any sample using culture methods. *Acinetobacter* and *Pseudomonas* sp. dominated DGGE and 16S rDNA clone library studies. The genera present on belts from premises whose hygiene was found to be in control (i.e. A, B and C) did not contain species known to cause disease or spoilage of vacuum packaged meats.

The bacterial viable count remains the most effective method available at this time for monitoring conveyor belt hygiene. Attempts to develop a monitoring system based on microscopy of an in-situ sampling device were unsuccessful due to an inability to penetrate the meat residue matrix. Denaturing Gradient Gel Electrophoresis (DGGE) may offer an alternative for rapid investigation of diversity, but further work is required before this can be validated for routine use.

Future work

The processes required to control hygiene on meat conveyor belts are now largely understood. To complete the hygienic programme in boning rooms some attention must now be given to the stainless steel surfaces, which are known to harbour biofilms.

To provide a more useful tool, further development is required to the methodology of DGGE. Some of the avenues to be explored are to revisit biases introduced by DNA extraction, introducing an alternate primer to DGGE-D forward, using smaller denaturing gradients, evaluating the use of high fidelity polymerases, further optimising the PCR reactions, using a "nested" PCR to amplify low concentrations of DNA, cloning extracts from bands to determine whether more than one species is present, and finally, using mung-bean nuclease to degrade ssDNA to produce clearer gels.

Appendices 1-7

Appendix 1 - Summary of samples collected

Date	Sample			Viable count	Culture library	DGGE	Clone	Microscopy
14/02/2006	Acetyl	A1	Top of hinge	X				
		A2	Top of hinge	X		X		
		A3	Top of hinge	X				
23/03/2006	Acetyl	A4	Inside holes	X	X	X	X	
		A5	between lugs	X				
		A6	Top	X				
		A7	top edge of lug	X				
	Acetyl washed	A8	Inside holes	X				
		A9	between lugs	X				
		A10	Top	X				
		A11	top edge of lug	X				
8/05/2006	Acetyl	A13	Top	X	X	X		
		A14	between lugs	X	X	X		
		A15	top edge of lug	X	X	X	X	X
		A16	segment end	X	X	X		
		A17	Inside holes	X	X	X		X
	Polyethylene	A18	Top	X				
		A19	between lugs	X				
		A20	top edge of lug	X	X	X		
		A21	segment end	X				
		A22	Inside holes	X	X			X
	Alphasan	A23	Top	X				
		A24	between lugs	X				
		A25	top edge of lug	X	X	X		
		A26	segment end	X				
		A27	Inside holes	X	X			X
	Acetyl washed	A28	Top	X				
		A29	Inside holes	X				X
		A30	between lugs	X				
		A31	top edge of lug	X	X			X
		A32	segment end	X				
	Polyethylene washed	A33	Top	X				
		A34	Inside holes	X				
		A35	between lugs	X				
		A36	top edge of lug	X	X			
		A37	segment end	X				
	Alphasan washed	A38	Top	X				
		A39	Inside holes	X				
		A40	between lugs	X				
		A41	top edge of lug	X	X			
		A42	segment end	X				
15/08/2006	Cont Teflon	B1	Top surface	X	X			
		B2	Top surface	X	X	X		
	Acetyl Intralox	B3	Top surface	X	X	X		
		B8	Top of hinge	X	X			
21/08/2006	Acetyl Intralox	C1	Top of hinge	X	X	X		
		C2	Top of hinge	X				
		C3	Top of hinge	X				
4/09/2006	Cont Teflon	D1	Main, top	X	X			
		D2	Main, top+edge	X	X			
		D3	Main, side edge	X	X			X
		D4	Pack, top+edge	X	X	X	X	
	Acetyl Intralox	D5	hinge	X	X			
		D6	divider segment	X	X	X		X

Appendix 2 - Viable counts on all media

Date	Sample			Area cm²	Blood agar 35° cfu/cm²	CNA 35° cfu/cm²	Coliform 35° cfu/cm²	PTYG 30° cfu/cm²	R_2A 25° cfu/cm²	1/10 TSA 5° cfu/cm²
14/02/2006	Acetyl	A1	Top of hinge	120	1800		45	4300	3700	2800
		A2	Top of hinge	120	3000		25	5200	10300	3800
		A3	Top of hinge	120	3300		45	4300	4500	2200
23/03/2006	Acetyl	A4	Inside holes	3.3	6970000	>200000	1750000	6300000	8420000	5090000
		A5	between lugs	2.94	320000	135000	93000	313000	381000	388000
		A6	Top	16	3625	1775	290	5000	5750	2000
		A7	top edge of lug	1.28	1950000	>200000	119000	3125000	3360000	1560000
	Acetyl washed	A8	Inside holes	3.3	<60	<60	<60	60	<60	<60
		A9	between lugs	2.94	<70	<70	70	<70	<70	<70
		A10	Top	16	15	<15	<15	<15	<15	<15
		A11	top edge of lug	1.28	<155	<155	<155	<155	<155	<155
8/05/2006	Acetyl	A13	Top	16	990	375	15	975	1900	810
		A14	between lugs	2.94	2900	820	135	2000	5400	1360
		A15	top edge of lug	3.84	7800	4010	100	2100	13500	3960
		A16	segment end	1.6	<125	<125	<125	125	<125	<125
		A17	Inside holes	3.3	300	60	<60	485	2350	240
	Polyethylene	A18	Top	16	1250	425	<15	1430	1610	1010
		A19	between lugs	2.94	1230	680	<70	950	4760	1290
		A20	top edge of lug	3.84	3080	1300	315	1040	4740	2030
		A21	segment end	1.6	875	1000	250	1380	4250	625
		A22	Inside holes	3.3	485	120	<60	365	1150	2420
	Alphasan	A23	Top	16	690	400	<15	660	875	110
		A24	between lugs	2.94	750	270	<70	200	1910	340
		A25	top edge of lug	3.84	780	310	<50	210	1150	470
		A26	segment end	1.6	12800	2750	1380	7500	17300	12500
		A27	Inside holes	3.3	300	<60	<60	120	790	120
	Acetyl washed	A28	Top	16	25			<15	100	<15
		A29	Inside holes	3.3	1030			120	545	120
		A30	between lugs	2.94	1630			1700	4690	950
		A31	top edge of lug	3.84	100			365	940	365
		A32	segment end	1.6	<125			1130	125	<15
	Polyethylene washed	A33	Top	16	25			25	50	<15
		A34	Inside holes	3.3	485			1330	1880	180
		A35	between lugs	2.94	475			1840	820	475
		A36	top edge of lug	3.84	730			625	2190	210
		A37	segment end	1.6	500			750	875	500
	Alphasan washed	A38	Top	16	<15			25	<15	15
		A39	Inside holes	3.3	<15			425	<15	<15
		A40	between lugs	2.94	<70			340	<70	<70
		A41	top edge of lug	3.84	<50			155	<50	<50
		A42	segment end	1.6	<125			1130	125	<125
15/08/2006	Cont Teflon	B1	Top surface	100	28	10	<2	26	32	<2
		B2	Top surface	100	6	12	<2	34	40	2
	Acetyl Intralox	B3	Top surface	100	100	50	2	220	134	2
		B8	Top of hinge	15	133	40	13	173	253	53
21/08/2006	Acetyl Intralox	C1	Top of hinge	120	45	<2	10	55	63	8
		C2	Top of hinge	120	32	<2	2	50	53	12
		C3	Top of hinge	120	35	<2	5	52	85	8
4/09/2006	Cont Teflon	D1	Main, top	100	<2	<2	<2	<2	2	2
		D2	Main, top+edge	110	11	<2	7	2	25	2
		D3	Main, side edge	15	2930	147	227	3870	16700	4270
		D4	Pack, top+edge	110	580	98	36	510	545	490
	Acetyl Intralox	D5	hinge	90	560	7	31	1020	870	530
		D6	divider segment	180	1780	244	144	2110	2900	1520

Less than (<) values indicate the limit of detection for the sample type. Calculated values have been rounded to 3 significant figures.

Appendix 3 - variation within a single intralox belt segment

	Sample	Blood agar 35°C	CNA blood agar 35 °C	CHROMagar Coliform	PTYG 30 °C	R2A 25 °C	1/10 TSA 5°C
Acetyl 8/5/06	A13 Top	*Kocuria* *Psychrobacter* *Dietzia* *Acinetobacter* *Microbacterium* *Corynebacterium* *Psychrobacter/Moraxella* *Acinetobacter*	*Brevundimonas* *Microbacterium oxydans ®* *Corynebacterium* *Micrococcus indicus* *Microbacterium oxydans ®* *Corynebacterium* *Brevundimonas ®* *Corynebacterium mycetoides*	*Agrobacterium larrymoorei*	*Acinetobacter* *Dietzia* *Psychrobacter* *(Janibacter)* *Microbacterium oxydans* *Kocuria rhizophila* *Flavobacterium johnsonii*	*Microbacterium* *Cryseobacterium* *Acinetobacter johnsonii* *Mycobacterium frederiksbergense* *Brevibacterium* *Devosia* *Dermacoccus* *Microbacterium ®*	*Microbacterium* *Arthrobacter* *Stenotrophomonas* *Stenotrophomonas maltophilia (2)* *Jeotgalicoccus psychrophilus* *Microbacterium* *Arthrobacter*
	A14 between lugs	*Microbacterium* *Microbacterium ®* *Corynebacterium* *Microbacterium ®* *Flavobacteriaceae* *Sphingomonas* *Microbacterium ®* *Brevundimonas*	*Microbacterium* *Microbacterium ®* *Brevundimonas nasdae*	*Pseudomonas*	*Microbacterium oxydans* *Moraxella osloensis* *Agrobacterium larrymoorii* *Agrobacterium larrymoorii*	*Sphingomonas panni* *Microbacterium ®* *Brevibacterium* *Rhizobium* *Rhizobium/ Thiobacillus*	*Microbacterium ®* *Microbacterium*
	A15 top edge of lug	*Microbacterium* *Microbacterium* *Acinetobacter* *Sphingomonas*	*Microbacterium oxydans* *Microbacterium oxydans ®* *Microbacterium oxydans ®* *Microbacterium oxydans ®*	*Stenotrophomonas maltophilia* *Stenotrophomonas spp.*	*Microbacterium oxydans* *Acinetobacter* *Stenotrophomonas maltophilia* *Stenotrophomonas*	*Microbacterium oxydans* *Brevundimonas*	*Microbacterium oxydans ®* *Microbacterium oxydans ®* *Brevundimonas nasdae* *Stenotrophomonas maltophilia*
	A16 segment end				*Mold*		
	A17 Inside holes	*Microbacterium* *Microbacterium* *Sphingomonas* *Microbacterium*	*Microbacterium oxydans ®*		*Sphingomonas* *Microbacterium oxydans* *Microbacterium oxydans*	*Microbacterium oxydans* *Sphingomonas panni* *Haloanella gallinarum/ Candidatus* *Sphingomonas* *Mycobacterium frederiksbergense* *Mycobacterium*	*Microbacterium* *Brevundimonas nasdae* *Microbacterium ®*

Isolates reported in ***black*** show a similarity with their nearest neighbour of >97%
Isolates reported in ***blue*** show a similarity with their nearest neighbour of <97%
Isolates marked ® have been identified by RFLP only

Appendix 4 - variation between samples from the same Intralox belt over time

	Sample		Blood agar 35°C	CNA blood agar 35 °C	CHROMagar Coliform	PTYG 30 °C	R2A 25 °C	1/10 TSA 5°C
23/03/2006	A4	Inside holes	*Exiguobacterium* *Acinetobacter* *Stenotrophomonas maltophilia* *Chryseobacterium* *Microbacterium* *Microbacterium ®* *Acinetobacter ®* *Brevundimonas* *Acinetobacter*		*Stenotrophomonas* *Acinetobacter ® (3)* *Brevundimonas* *Brevundimonas ®* *Acinetobacter* *Sphingomonas*	*Acidivorax/Pseudomonas* *Chryseobacterium* *Microbacterium* *Brevundimonas* *Brevundimonas*	*Acinetobacter ® (4)* *Moraxella osloensis* *Chryseobacterium* *Chryseobacterium* *Acinetobacter*	*Chryseobacterium* *Clavibacter michiganensis* *Acinetobacter* *Acinetobacter ® (2)*
8/05/2006	A15	top edge of lug	*Microbacterium* *Microbacterium* *Acinetobacter* *Sphingomonas*	*Microbacterium oxydans* *Microbacterium oxydans ®* *Microbacterium oxydans ®* *Microbacterium oxydans ®*	*Stenotrophomonas maltophilia* *Stenotrophomonas spp.*	*Microbacterium oxydans* *Acinetobacter* *Stenotrophomonas maltophilia* *Stenotrophomonas*	*Microbacterium oxydans* *Brevundimonas*	*Microbacterium oxydans ®* *Microbacterium oxydans ®* *Brevundimonas nasdae* *Stenotrophomonas maltophilia*
	A17	Inside holes	*Microbacterium* *Microbacterium* *Sphingomonas* *Microbacterium*	*Microbacterium oxydans ®*		*Sphingomonas* *Microbacterium oxydans* *Microbacterium oxydans*	*Microbacterium oxydans* *Sphingomonas panni* *Haloanella gallinarum/ Candidatus Sphingomonas* *Mycobacterium frederiksbergense* *Mycobacterium*	*Microbacterium* *Brevundimonas nasdae* *Microbacterium ®*

Appendix 5 - variation between different types of Intralox belt materials

Material	Sample		Blood agar 35°C	CNA blood agar 35 °C	CHROMagar Coliform	PTYG 30 °C	R2A 25 °C	1/10 TSA 5°C
Acetyl	A15	top edge of lug	*Microbacterium* *Microbacterium* *Acinetobacter* *Sphingomonas*	*Microbacterium oxydans* *Microbacterium oxydans ®* *Microbacterium oxydans ®* *Microbacterium oxydans ®*	*Stenotrophomonas maltophilia* *Stenotrophomonas spp.*	*Microbacterium oxydans* *Acinetobacter* *Stenotrophomonas maltophilia* *Stenotrophomonas*	*Microbacterium oxydans* *Brevundimonas*	*Microbacterium oxydans ®* *Microbacterium oxydans ®* *Brevundimonas nasdae* *Stenotrophomonas maltophilia*
	A17	Inside holes	*Microbacterium* *Microbacterium* *Sphingomonas* *Microbacterium*	*Microbacterium oxydans ®*		*Sphingomonas* *Microbacterium oxydans* *Microbacterium oxydans*	*Microbacterium oxydans* *Sphingomonas panni* *Haloanella gallinarum/ Candidatus* *Sphingomonas* *Mycobacterium frederiksbergense* *Mycobacterium*	*Microbacterium* *Brevundimonas nasdae* *Microbacterium ®*
Polyethylene	A20	top edge of lug	*Microbacterium oxydans* *Psychrobacter* *(Mixed)* *Brevundimonas* *Acinetobacter johnsonii* *Brevundimonas* *Haloanella gallinarum* *Stenotrophomonas maltophilia*	*Brevundimonas sp.* *Sphingomonas intermedia* *Brevundimonas ballata* *Microbacterium oxydans ®*	*Stenotrophomonas* *Pseudomonas* *Acinetobacter* *Stenotrophomonas maltophilia* *Agrobacterium tumifaciens*	*Psychrobacter*	*Brevundimonas nasdae* *Agrobacterium tumefaciens* *Microbacterium ®* *Pseudomonas ® (2)* *Stenotrophomonas* *Brevundimonas nasdae ® (2)*	*Chryseobacterium* *Brevundimonas mediterranea* *Brevundimonas ballata ®* *Luteococcus japonicus*
	A22	Inside holes	*Microbacterium* *Micrococcus luteus* *Microbacterium oxydans* *Microbacterium oxydans*	*Arthrobacter* *Microbacterium ®*		*Rhodococcus* *Stenotrophomonas maltophilia* *Microbacterium oxydans* *Sphingomonas*	*Microbacterium ®* *Microbacterium ®* *Rhodococcus erythropolis* *Mycobacterium sp* *Microbacterium ®* *Brevundimonas nasdae ®*	*Rathayibacter* *Microbacterium ®* *Acidivorax*
Alphasan	A25	top edge of lug	*(Serratia liquifaciens)* *Moraxella osloensis* *Stenotrophomonas maltophilia* *Microbacterium oxydans*	*Microbacterium oxydans*		*Rhodococcus erythropolis* *Mycobacterium habria*	*Rhodococcus erythropolis* *Acinetobacter johnsonii* *Microbacterium oxydans ®* *Paracoccus yeeii*	*Chryseobacterium* *Microbacterium oxydans ®* *Microbacterium oxydans ®*
	A27	Inside holes	*Sphingomonas echinoides* *Brevundimonas* *Brevundimonas* *Microbacterium oxydans*			*Microbacterium oxydans* *Agrobacterium tumefaciens*	*Microbacterium ®* *Microbacterium ®* *Microbacterium ®* *Actinobacterium*	*Microbacterium ®* *Microbacterium ®*

Appendix 6 - evaluation of the effect of hot water washing

Treatment	Sample		Blood agar 35°C	CNA blood agar 35 °C	CHROMagar Coliform	PTYG 30 °C	R2A 25 °C	1/10 TSA 5°C
Acetyl	A15	top edge of lug	*Microbacterium* *Microbacterium* *Acinetobacter* *Sphingomonas*	*Microbacterium oxydans* *Microbacterium oxydans ®* *Microbacterium oxydans ®* *Microbacterium oxydans ®*	*Stenotrophomonas maltophilia* *Stenotrophomonas spp.*	*Microbacterium oxydans* *Acinetobacter* *Stenotrophomonas maltophilia* *Stenotrophomonas*	*Microbacterium oxydans* *Brevundimonas*	*Microbacterium oxydans ®* *Microbacterium oxydans ®* *Brevundimonas nasdae* *Stenotrophomonas maltophilia*
Polyethylene	A20	top edge of lug	*Microbacterium oxydans* *Psychrobacter* *Brevundimonas* *Acinetobacter johnsonii* *Brevundimonas* *Haloanella gallinarum* *Stenotrophomonas maltophilia*	*Brevundimonas sp.* *Sphingomonas intermedia* *Brevundimonas ballata* *Microbacterium oxydans ®*	*Stenotrophomonas* *Pseudomonas* *Acinetobacter* *Stenotrophomonas maltophilia* *Agrobacterium tumifaciens*	*Psychrobacter* *????* *????*	*Brevundimonas nasdae* *Agrobacterium tumefaciens* *Microbacterium ®* *Pseudomonas ® (2)* *Stenotrophomonas* *Brevundimonas nasdae ® (2)*	*Chryseobacterium* *Brevundimonas mediterranea* *Brevundimonas ballata ®* *Luteococcus japonicus*
Alphasan	A25	top edge of lug	*Serratia liquifaciens* *Moraxella osloensis* *Stenotrophomonas maltophilia* *Microbacterium oxydans*	*Microbacterium oxydans*		*Rhodococcus erythropolis* *Mycobacterium habria*	*Rhodococcus erythropolis* *Acinetobacter johnsonii* *Microbacterium oxydans ®* *Paracoccus yeeii*	*Chryseobacterium* *Microbacterium oxydans ®* *Microbacterium oxydans ®*
Acetyl washed	A31	top edge of lug	*Staphylococcus pasteuri* *Brevundimonas*			*Microbacterium sp ®* *Microbacterium sp ®*	*Sphingomonas* *Sphingomonas mucosissima* *Spirosoma*	*Microbacterium sp. ®*
Poylethylene washed	A36	top edge of lug	*Psychrobacter* *Acinetobacter* *Haloanella gallinarum/ Candidatus* *Haloanella gallinarum/ Candidatus*			*Haloanella gallinarum* *Psychrobacter*	*Acinetobacter* *Microbacterium ®* *Haloanella gallinarum* *Kocuria carnophila* *Mycobacterium fredericksbergense*	*Pseudomonas fluorescens*
Alphasan washed	A41	top edge of lug				*Microbacterium oxydans*		

Appendix 7 - variation between premises and belt construction

Date	Premise	Sample	Blood agar 35°C	CNA blood agar 35 °C	CHROMagar Coliform	PTYG 30 °C	R2A 25 °C	1/10 TSA 5°C
	A	15 Intralox, top edge of lug	*Microbacterium* *Microbacterium* *Acinetobacter* *Sphingomonas*	*Microbacterium oxydans* *Microbacterium oxydans ®* *Microbacterium oxydans ®* *Microbacterium oxydans ®*	*Stenotrophomonas maltophilia* *Stenotrophomonas spp.*	*Microbacterium oxydans* *Acinetobacter* *Stenotrophomonas maltophilia* *Stenotrophomonas*	*Microbacterium oxydans* *Brevundimonas*	*Microbacterium oxydans ®* *Microbacterium oxydans ®* *Brevundimonas nasdae* *Stenotrophomonas maltophilia*
15/08/2006	B	1 Continuous Teflon, contact surface	*Microbacterium lacticam* *Actinobacterium* *Actinobacterium ®* *Moraxella osloensis* *Staphylococcus*	*Microbacterium ®* *Streptococcus mitis* *Dermatophilus* *Corynebacterium ®*		*Corynebacterium ®* *Micrococcus ® (2)* *Kaistella flava* *Kocuria rhizophila* *Luteococcus japonicus* *Kocuria carnophila (2)*	*Kocuria rhizophila* *Kocuria* *Corynebacterium mycetoides*	*Dietzia* *Kocuria rhizophila ®* *Kaistella flava* *Psychrobacter*
		2 Continuous Teflon, contact surface	*Staphylococcus ®* *Kocuria ®*	*Corynebacterium ® (2)* *Micrococcus* *Micrococcus*		*Corynebacterium ®* *Micrococcus ® (2)* *Kocuria rhizophila(2)* *Brachybacterium* *Micrococcus* *Kocuria carnophila ®*	*Kocuria rhizophila ®* *Kocuria* *Moraxella osloensis* *Moraxella*	*Kaistella flava* *Kocuria ®* *Kocuria rhizophila ®* *Psychrobacter*
		3 Intralox, contact surface	*Staphylococcus* *Corynebacterium mycetoides* *Corynebacterium mycetoides ®* (2) *Kocuria* *Kocuria ®* (2) *Microbacterium ®* *Micrococcus* *Kaistella flava*	*Corynebacterium bovis* *Micrococcus ®* *Corynebacterium ®* *Janibacter anophelis* *Corynebacterium ®*	*Kocuria ®*	*Corynebacterium ® (2)* *Deinococcus radiodurans* *Staphylococcus auricularis* *Micrococcus ®* (4) *Corynebacterium camporealensis* *Acinetobacter lwoffii* *Dietzia* *Streptococcus sanguinis* *Corynebacterium xerosis*	*Kocuria* *Micrococcus* (2) *Corynebacterium mycetoides* *Acinetobacter* *Bacillus macroides*	*Kocuria ®* (2) *Micrococcus ®* *Deinococcus* *Macrococcus caseolyticus* *Jeotgalicoccus psychrophilus*
		8 Intralox, hinge	*Micrococcus ® (2)* *Corynebacterium mycetoides(3)* Actinomycetaceae *Brachybacterium*	*Staphylococcus pasteuri* *Micrococcus* *Corynebacterium*	*Staphylococcus pasteuri*	*Micrococcus* *Staphylococcus* *Microbacterium* *Micrococcus ®* *Kocuria ®*	*Kocuria ®* *Micrococcus* *Micrococcus ®* *Micrococcus ®*	*Arthrobacter* *Pseudoclavibacter* *Stenotrophomonas* *Kocuria ®*
21/08/2006	C	1 Intralox, Hinge	*Acinetobacter* *Stenotrophomonas* *Kocuria* *Pseudomonas* *Pseudomonas ®* *Kocuria rhizophila*		*Pseudomonas* *Acinetobacter ®* *Pseudomonas*	*Pseudomonas ®* *Kocuria rhizophila ®* *Kocuria* *Pseudomonas fluorescens*	*Pseudomonas fluorescens ®* *Kocuria* *Kocuria rhizophila ®* *Arthrobacter* *Pseudochrobacterium kiredjanae*	*Pseudomonas fluorescens* *Pseudomonas fluorescens ®* *Pseudomonas*
4/09/2006	D	4 Continuous Teflon, contact surface & belt edge	*Acinetobacter* *Vagococcus carnophilus* *Serratia proteamaculans* *Aerococcus viridans* *Staphylococcus equorum* *Staphylococcus equorum ® (2)* *Pseudomonas*	*Vagococcus* *Aerococcus viridans ®* *Staphylococcus equorum* *Brevibacterium sanguinis*	*Acinetobacter* *Serratia proteamaculans ® (3)* *Aerococcus viridans ® (2)* *Staphylococcus equorum* *Acinetobacter ®*	*Acinetobacter* *Staphylococcus saprophyticus* *Serratia proteamaculans ® (2)* *Chryseobacterium* *Staphylococcus equorum*	*Acinetobacter* *Acinetobacter ® (2)* *Serratia proteamaculans* *Rhodococcus fasciens*	*Acinetobacter ® (2)* *Serratia proteamaculans ®* *Aerococcus viridans* *Pseudomonas fluorescens* *Staphylococcus equorum*
		6 Intralox, divider segment	*Pseudomonas* *Pseudomonas ® (2)* *Cryseobacterium* *Agrobacterium tumefaciens* *Staphylococcus pasteuri* *Dermacoccus*	*Pseudomonas fluorescens* *Brevundimonas bullata* *Watersiella falsenii* *Staphylococcus equorum ®* *Brevundimonas bullata ®* *Corynebacterium xerosis* *Staphylococcus equorum ®* *Staphylococcus parauberis*	*Aeromonas molluscorum* *Aeromonas hydrophila* *Chryseobacterium indologenes* *Pseudomonas fluorescens* *Pseudomonas sp.* *Staphylococcus pasteuri* *Shigella sonnei* *Aeromonas* *Pseudomonas*	*Stenotrophomonas maltophilia (2)* *Chryseobacterium* *Comamonas* *Pseudomonas* *Acinetobacter* *Pseudomonas* *Acidovorax (3)* *Phyllobacterium myrsinacearum (2)* *Pseudomonas fluorescens* *Pseudomonas (2)* *Staphylococcus pasteuri ®*	*Myroides* *Staphylococcus pasteuri* *Agrobacterium tumefaciens* *Pseudomonas ®*	*Flavobacterium* *Flavobacterium* *Pseudomonas ®* *Brevundimonas bullata* *Flavobacterium* *Chryseobacterium* *Stenotrophomonas maltophilia* *Phyllobacterium myrsinacearum* *Chryseobacterium ®* *Chryseobacterium*

The isolate reported in red is the only enteric pathogen identified during the study

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