The effect of MAP on the growth and survival of *Escherichia coli* O157:H7 and *Staphylococcus aureus* in chilled minced beef.

By

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Declaration

I declare that the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria, has not previously been submitted by myself for a degree at any other university of institution of higher education.

Theon Montaque du Preez

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Abstract

The effect of MAP on the growth and survival of *Escherichia coli* O157:H7 and *Staphylococcus aureus* in chilled minced beef.

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With modern culture moving towards convenience in terms of fresh produce, especially with meat products, science needs to constantly evolve to serve these customer needs. These needs however can sometimes be implemented too hastily without the proper assessment done for factors such as food safety. One such improvement is the use of modified atmosphere packaged minced meat. This form allows the minced meat to be kept for much longer than normal without freezing the product, providing the fast pasted consumer both the convenience of having fresh, unfrozen meat as well as an added shelf life.

MAP works by disrupting the atmosphere within these packages, retarding the growth of the spoilage micro-organisms, thus causing them to require a longer time span to reach spoilage numbers. The problem however arises that although most of these techniques are tried and trusted on the products' spoilage organisms, it does not take into consideration the effect MAP and the altered spoilage organism communities would have on a pathogen that might be present on the products.

This study thus aimed to assess the effect of both factors on *Escherichia coli* O157:H7 (phase 1) and *Staphylococcus aureus* (phase 2) in minced meat kept at 5°C as well as trying to identify the major affecting factors. The two subject organisms were each inoculated into 2 different types of MAP packs and a non-atmosphere modified PVC overlaid minced meat sample at two differing concentrations of 10^5 and 10^2 to also assess the impact of high and

low initial pathogen presence. These packs were then analysed over a time period of 16 days to track the changing minced meat environment. APC, Pseudomonads, LAB and Enterobacteriaceae counts were all investigated along with the pathogenic counts.

Apart from colony enumeration, the colour of the minced meat samples were also taken to determine the effect that these parameters have on the appearance of the product, as colour is often the first sensorial characteristic that determines the purchase of fresh meat products. pH was determined to ascertain the environmental changes occurring in the product and whether groups such as the LAB would change the environment to better suit their needs. Finally the atmospheric makeup was also measured to determine the effect of the MAP system and the change occurring in a closed system that could be attributed to the growth and respiration of the bacterial communities present.

Apart from the main aim of the study, two additional studies were performed that arose during the planning and analyses of the two primary phases. Firstly the use of a quarter versus a full plate enumeration was studied to determine its accuracy as well as possibility of use in full studies to aid enumeration and decrease time and financial input. Here a direct comparison was done between the two techniques after which they were compared and assessed in their functionality for both homogenous and heterogeneous community enumeration on selective and non-selective media.

The other secondary study focussed on the use of new technology for both the enumeration and tracking of genetically modified organisms in a variety of different environments. Here a bioluminescent imaging system was used on a genetically modified strain of *E. coli* to track its spread through minced meat, packaged either in a MAP or PVC overlaid pack, over 48 hours in an accelerated shelf life study. Enumeration of said organism was also undertaken whereby the intensity of emitted light would correspond to a defined count, enabling rapid enumeration of samples, whether overgrown or not.

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Chapter 1: Introduction

Minced meat is a form of ground or comminuted meat, usually produced from inferior sections or less noble cuts of a carcass containing a high concentration of connective tissue. Through the comminuting process, the meat gets broken down, making it easier to chew and improving the products quality and use. This breakdown of the meat has a drastic influence on the bacterial populations present due to an increased surface area and increase oxygen penetration, resulting in minced meat products often having a shorter shelf-life than its intact meat origins due to increased bacterial growth and associated spoilage linked with higher numbers (Ranken, Kill & Baker 1997). This shorter shelf-life prompted the use of novel packaging methods such as modified atmosphere packaging (MAP), to ensure an improved shelf-life.

Not only does the processing and packaging procedures affect the possible spoilage organisms, but also any pathogenic species that might be present in the product. The effect of MAP on some organisms has been studied (Eklund 1984; McMillin 2008; Ooraikul & Stiles 1991) however; the direct effect of one or a group of organisms on another and the dynamics within the microbial ecology in minced meat is yet to be evaluated.

Problem Statement

Recent years have seen an increase in the amount of pathogenic outbreaks pertaining to food, causing a spurring of consumer concern related to the safety of food products and the steps the food industry is taking in combating the prevalence. Various organisms present in a variety of food sources have contributed to these outbreaks, however some organisms such as *Staphylococcus aureus* and *Escherichia coli* seem to be involved in a majority of these (Greig & Ravel 2009).

Food borne outbreaks due to *S. aureus* has increased during the last few years with some countries such as France attributing 25.6%, or 1651 of their cases to *S. aureus* during 1999 and 2000 (LeLoir, Baron & Gautier 2003). *S. aureus* cases have also been reported in South

Africa, with the most recent outbreak being linked to *S. aureus* occurring in 2007 in an incident involving prepared meals that resulted in 38 confirmed illnesses of which 6 patients had to be admitted to hospital (NICD 2007).

A similar trend is seen in outbreaks associated with *E. coli* O157, with the outbreak either claiming human lives as seen with the Californian spinach outbreak that caused 205 confirmed illnesses as well as three deaths (FDA 2007), or having substantial financial implications on the producers or companies involved, as seen with the recall of nearly 10 million kilograms of ground beef patties in the United States in 2007 (CDC 2007). Of particular concern for this study is the incidence of these two organisms causing food borne outbreaks in beef. A recent article showed how, in the time period spanning 1988-2007, 44.2% of all the *E. coli* and 13.7% of all the *S. aureus* food borne outbreaks worldwide were associated with beef, totalling 197 outbreaks all together (Greig & Ravel 2009).

Various macroscopic reasons were given for the increased prevalence compared to previous years, including free-trade and international shipping that causes the worldwide distribution of organisms as well as providing an increased growth period, allowing these organisms to reach infective dose levels. There are however various microscopic reasons that can also be related to this increased prevalence. These are however often overlooked due to their good intentioned and beneficial actions such as increasing shelf-life or enhancing convenience.

One of these actions pertains to the principle of microbial interference whereby background micro-organisms themselves would inhibit or interfere with other micro-organisms, thus acting as direct competition for substrates and binding sites (Jay 1997). However, processing and packaging treatments, including MAP, aiming to reduce the growth of spoilage organism on food products have been some of the main perpetrator in removing the natural predominant micro-organisms and the inter micro-organism competition they provide (Enfors 1979), thereby leaving the environment more conducive for the pathogenic bacteria to proliferate.

Over the past few years, the popularity of MAP and case-ready packaging approaches has grown worldwide (Belcher 2006), with it also gaining popularity in SA, especially in the meat industry. MAP works on the principal of filling the headspace in a product's packaging, normally on a 1:1 basis according to the product, with a gas mixture that varies between

industries, with the mixture for the meat industry generally being 80% O_2/20% CO_2. This mixture creates a homeostatic environmental disturbance for the natural aerobic spoilage organisms on the food product due to the anti-microbial effect of CO_2 and the higher than normal O_2 concentration. This high oxygen type packaging also provides enough O_2 so as to retain the meat product's acceptable oxymyoglobin or red appearance throughout its shelf-life (Belcher 2006).

The product is normally packaged, sealed and priced at the processing plant to decrease back store handling. This disturbance in atmosphere aims to decrease the rate of growth seen in the spoilage organisms and in effect, increase the shelf-life of the product. It is however unclear what effect this reduction in the background spoilage bacteria would have on potential pathogenic organisms that might find their way onto the meat samples (Belcher 2006).

This study aims to determine the effect that MAP treatments, using atmospheric concentrations of a commercially used mix ($O2=60\%$/ $CO2=30\%$/ $N2=10\%$ [Mix]; allowed variation of 5% per gas) and 100% CO2, as well as the background spoilage bacteria would have on the growth occurring in both *E. coli* O157:H7 and *S. aureus* at high and low initial contamination levels.

Chapter 2: Literature Review

Escherichia coli

Introduction

First described by Theodor Escherich in 1885, *E. coli* is a rod-shaped, gram-negative, facultative anaerobic organism found within the intestinal tract of both healthy and diseased humans and animals. It forms part of the *Enterobacteriaceae* or enteric bacteria which includes other organisms such as *Salmonella* and *Enterobacter* and thus due to its consistent occupancy in the intestinal tract of humans as the predominant facultative organism, it is used as an indicator organisms for the presence of faecal contamination in both water and food (Todar 2009). It grows anaerobically through either fermentation, producing a mixture of acids and gas, or via respiration with the final electron acceptor either being NO_3, NO_2 or fumarate. This characteristic allows *E. coli* to adapt to its environment whether within the intestinal tract (anaerobic) or after excretion (anaerobic and aerobic). The different serotypes of *E. coli* are classified according to their O, H and K antigens with O referring to the somatic antigen, the H to the flagellar antigen and K to the capsule antigen, resulting in over 700 different serotypes presently known (Todar 2009).

E. coli as a diarrheal agent first attracted attention in the 1940's with an epidemic in nurseries that resulted in a mortality rate as high as 50%, while its interest in food related diseases took spotlight during the 1971 cheese outbreak in the US, at a time when medical microbiologists were busy examining *E. coli* as a possible cause of infantile diarrhoea (Brown 1982).

E. coli can be present in a variety of raw and processed foods, especially in those originating from an animal source with contamination occurring in a multitude of ways. Carcasses can normally be contaminated directly during slaughtering due to the meat coming into contact with the intestinal contents of the animal. The level of contamination however may have no relation to the counts found, as unlike in water where it is used as an index organism, meat provides significant nutrients allowing for growth (Bell & Kyriakides 2009).

Even though a large variety of *E. coli* can be isolated from food, a small percentage of these are indeed pathogenic when ingested as found by Sack *et al.* (1977) when they isolated 240 different strains of *E. coli* from food and found that only 8% (19) were toxigenic.

Clinical and epidemiological features

Food poisoning through the ingestion of *E. coli* normally involves differing incubation times and can be anything between 7-72 hours with symptoms lasting 1-7 days. These could include symptoms similar to cholera including stomach cramps and watery diarrhoea to dysentery with stool samples containing both blood and mucus. Fever is associated with a few of the pathogenic strains and in severe cases, seizures, blood clots, comas and death is also possible (Bell & Kyriakides 2009). The infective dosages for the different classes differ from 10^1, O157:H7, to 10^8, enterotoxigenic, cells which could mean dosages as low as 10 organisms per gram of food would be sufficient to cause illness, while the elderly, infants and immune-repressed would require even less. This is of special concern due to the fact that *E. coli* is found to be consistently present, in varying numbers, in common food products such as undercooked ground beef, raw milk and vegetables (Todar 2009).

E. coli strains

The strains of *E. coli* involved in food associated illness can be grouped into five major classes namely enteropathogenic *E. coli* (EPEC), entero-haemorrhagic *E. coli* (EHEC) or vero-cytotoxigenic *E. coli* (VTEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC) and diffusely adherent *E. coli* (DAEC), each containing specific characteristics relating to their pathogenicity (Bell & Kyriakides 2009).

EPEC does not normally produce enterotoxins but is found in third-world countries to be the primary cause of infantile diarrhoea. It produces an adherence factor which allows localized adherence on the intestinal cells followed by cell actin rearrangement. This rearrangement is then thought to be the reason for the diarrhoea since it can lead to cellular interference of signal transduction and inflammation, causing improper absorption and functioning of the epithelial cells (Todar 2009).

For ETEC to effectively cause illness, it requires colonization using fimbrial adhesion and amplification of numerous enterotoxins in the gastrointestinal tract, after which a broad

variety of symptoms would become apparent, ranging from minor discomfort to a cholera type disease. ETEC essentially produces two types of toxins, a heat sensitive and a heat stable enterotoxin. The heat sensitive toxin is quite similar to that of the cholera toxin in its ability to induce the formation of antibodies, while it is identical in its enzymatic function and its binding to the ganglioside receptors (Todar 2009).

The heat stable toxin (ST) is of a low molecular size, which can explain the heat tolerance, and can withstand boiling temperatures for up to 30min without losing its viability. The toxin appears in various variants within humans and animals with some variants such as ST1b and STh being found primarily in humans while others such as ST1a and STp are found in both animals and humans. The toxin acts by binding to a guanylate cyclase on the apical membrane of cells, causing an increase in cyclic GMP which leads to the secretion of electrolytes and fluids (Todar 2009).

EIEC is sometimes referred to as *Shigella* due to the high resemblance found within the pathogenic mechanisms, the clinical symptoms shown as well as the fact that there exists no identifiable taxonomical difference between *E. coli* and *Shigella* (Kaper 2005). EIEC acts by infiltrating and proliferating within the host cell, causing extensive damage which normally leads to both a fever and dysentery. However, it does not produce any enterotoxins, its pathogenicity rather being attributed to its invasive properties (Kaper 2005).

The characteristic feature that differentiates EAEC from the other classes is the aggregative manner in which it binds to the epithelial cell without causing inflammation or occupying these cells. Its manner in causing persistent diarrhoea, especially in young children, arises from its use of a heat sensitive entero-aggregative toxin as well as a hemolysin, although the exact mechanism by which these two function is still in doubt (Kaper 2005).

The final class is occupied by EHEC or VTEC, which includes the infamous *E. coli* serotype O157:H7. In 1982, an unusual gastrointestinal illness characterized by excessive, severe and bloody dysentery affected numerous people in the United States that had consumed undercooked hamburger meat. The causative agent was an atypical *Escherichia coli* serotype O157:H7 (Riley et al. 1983). This serotype was not previously recognized as a causative diarrheal agent in humans but has since then been linked as such and its strains are often primarily identified through its involvement in hemorrhagic colitis (HC), thrombotic

thrombocytopenic purpura (TTP) and the potentially fatal kidney disease, haemolytic uremic syndrome (HUS). Its virulence lies in its production of vero- or Shiga toxins (Stx). Although the toxins are almost indistinguishable, EHEC strains do not invade the host cell as enthusiastically as *Shigella* does (Tarr 1995).

Staphylococcus aureus

Introduction

Staphylococcus aureus, so called due to their clustering that has the appearance of a bunch of grapes--, *staphele* (Greek), and due to the characteristic golden colour of the colonies on agar--, *aureus* (Latin), is a gram-positive, non spore forming, catalase-positive bacterium. It is a facultative anaerobe that inhabits the nasopharyngeal regions and the intestinal tract in humans and animals as a saprophyte, living off mucal secretions and other organic substances while also existing as an ephemeral inhabitant on the skin (Ayres, Mundt & Sandine 1980).

S. aureus is sometimes used as an indicator organism, as the presence of this and other staphylococci usually points to the food being contaminated by its interaction with the food handler. This contamination can occur due to the food coming into contact with the food handler's skin or due to mucal secretions originating from the handler's mouth or nose. However this contamination may also be secondary due to the "clean" meat coming into contact with inadequately cleaned equipment such as cutting boards and knives that may have been contaminated with previous meat samples. If large amounts of staphylococci are present on these samples, it normally serves as a warning of inadequate hygiene and temperature management, allowing either high initial contamination numbers or adequate growth conditions (DOH 2002; Normanno et al. 2007).

It is generally assumed that *S. aureus* can grow at temperatures between 7 and 47.8°C, but with particular interest to cold storage of possible contaminated products, some strains have been recorded to grow at temperatures as low as 6.7°C, which falls below the temperature experienced, and often regarded as being safe, in meat display counters in supermarkets (Jay 2000). Other growth requirements include a pH between 4.0 and 9.8, with the optimum between 6.0 and 7.0, and a water activity of 0.86, for aerobic, and 0.9, for anaerobic growth.

The nutritional requirements for *S. aureus* are simple but it does however encounter some difficulty in having to compete with other bacteria that might be present on food (Jay 2000).

Although growth might occur due to the acceptable pH levels found in meat and temperature abuse cycles that might occur during storage, the ingestion of the *S. aureus* vegetative cells are not the major concern, but rather the ingestion of the enterotoxins produced by it while in the product. However, requirements for toxin production are much stricter and fall into a smaller range than that required for growth. It is generally accepted that toxin production could occur in the temperature range of 10 to 46°C, with optimum toxin production occurring between 40 and 45°C, while any anaerobic conditions would also impact on toxin production (Jay 2000).

Clinical and epidemiological features

If food poisoning occurs as a result of *S. aureus*, symptom development normally occurs within a period of 1 to 6 hours, with 4 hours being the general time frame after ingestion of the food. In contrast to other food poisoning organisms such as *E. coli* and *Salmonella* whose symptoms could stretch over days, the duration of the symptoms for *S. aureus* is normally found to be between 24 and 48 hours. These symptoms could include nausea, abdominal pain, vomiting and diarrhoea with fever however lacking in most cases, while severe cases could also include dehydration and loss of consciousness. The mortality rate is low with only bed rest, re-hydration and electrolyte replenishment often being prescribed for healthy individuals (Jay 2000).

Although rare, acute staphylococcal enterotoxicosis could prove fatal due to accompanying complications, this however often only occurs in the immune-suppressed, elderly and very young. Although the primary symptoms of vomiting and diarrhoea appear to be as the result of the action of the toxin on the abdominal viscera, the exact mechanism is not yet fully understood. (Brown 1982). As illness is caused by the ingestion of enterotoxins, stool cultures may be negative for enterotoxigenic *S. aureus* cultures and thus confirmation of food poising is often performed by investigating samples from both the leftover food and from the victim's faecal matter. Ideally if possible, isolation of enterotoxins from the food samples would also be advised (Jay 2000).

It is believed that between 25 and 50% of human beings could be carrying the organism at any instance and of that number 15 to 20% could be carrying strains that are enterotoxigenic. Thus man constitutes the primary reservoir and one of the main sources of transmission of *S. aureus* (Brown 1982).

Contamination of food, and especially meat, through *S. aureus* can occur in numerous different ways of which the most common ones are, [A.] local transfer from the outer surface to the inner surface of a carcass through various processes such as cutting and slaughter, [B.] from a foreign source such as processing equipment or as the result of a contaminated operational environment, [C.] via contamination arising from the hands or clothing of staff and [D] various transfer mediums and point of contact including aerosols, biofilms and water. Thus it is common for more than one strain to be present on the meat as contamination origins could include both animal and human sources (Jay 2000; Davies & Board 1998).

Although it is often found that a large number of meat products do become contaminated with *S. aureus*, it is generally not a concern since the lower storage temperatures and the fact that it would be destroyed through the cooking process, both play a role in minimising its effect. It was also found that in general, few outbreaks of food poisoning occurred from *S. aureus* strains isolated from raw meat products (Brown 1982). It was shown by Wieneke (1974) that only 23.2% of strains found on raw meat and raw meat products produce enterotoxins while 34.4%, 38%, 45% and 94% of strains found on cooked foods, in hospital admissions, from hospital lesions and from outbreaks pertaining to food poisoning respectively, could produce enterotoxins. Regarding enterotoxins, the one which was seen to be produced most often in food products was that of Staphylococcal enterotoxin A (SEA) followed by Staphylococcal enterotoxin D (SED), while in raw meat products the enterotoxin found to be most prevalent was SED (Jay 2000).

In terms of incidence of *S. aureus* found in raw meat and especially beef, various studies found that incidents could be anywhere between 7.5% to 83%, depending on the form of delivered meat, be it steak, minced meat or whole carcasses (Desmarchelier et al. 1999) (Sorianoa et al. 2001), with 22 outbreaks being attributed to *S. aureus* in beef between 1973 and 1987 in the United States of America (Jay 2000).

Toxin production

To date, it is recorded that *S. aureus* can produce ten different Staphylococcal enterotoxins (SE) in food, namely enterotoxin A, B, C_1, C_2, C_3, D, E, G, H and I . It was found that the type and amount of toxin produced is often dependant on the conditions or type of food as well as the geographical location (Jay 2000). These toxins are produced as a survival mechanism, implemented by *S. aureus* through the use of a global regulator, its accessory gene regulator (agr) locus, which is cell density dependant. This quantification of cells is however managed through the organism's use of quorum sensing, an inter-cellular communication system that uses the amount of a specific peptide present, to determine the amount of cells in the colony (Pragman & Schlievert 2004).

Depending on the type of toxin produced, it has been reported that counts as low as 10^4 cfu/g, for the production of Staphylococcal enterotoxin A (SEA), and 10^5 cfu/g, for the production of SEB, SED (Jay 2000) and others (DOH 2002), have been experienced in a variety of food products. In general the enterotoxins are found to be produced during all phases of growth. SEA is found to be the most prevalent toxin from *S. aureus* associated with food borne illness, which might be attributed to the fact that SEA is more often found to be produced during the logarithmic growth phase of *S. aureus*. SEB and SEC are primarily produced later in the logarithmic cycle or during the stationary phase of growth. Illness through SEA can thus occur at much lower *S. aureus* numbers compared with the rest of the toxins (Jay 2000).

Other factors that influence toxin production include pH, water activity and NaCl concentration. SEA is produced in a wider range of pH starting at around a minimum of 4.5 while the range for SEB and SEC is much narrower and centralizes at a pH of around seven. Production of SEB and SEC decreases as the NaCl concentrations increases to between 0% and 10% with only SEC still being produced above 10%. While *S. aureus* cannot grow at a water activity of less than 0.86, toxin production is halted at any water activity less than 0.96 (Jay 2000).

The large concerns associated with SE in raw food products, especially meats, are that foods that were prepared at high temperatures are generally considered safe, since the high temperatures are believed to kill any possible unwanted organism that might be present. This is true for some living organisms such as *S. aureus* that might be present on the food's surface, but can be a costly mistake when dealing with SEs due to their heat stability. In

some cases it was found that SEA could remain viable after 28min at 121°C (Anderson, Beelman & Doores 1996) while others had it down to 8min at 121°C (Denny, Tan & Bohrer 1966). SEB on the other hand remained biologically active after 16h at 60°C while SEC showed no serological reaction change after 30min of treatment at 60°C.

The general thermal stability showed by the enterotoxins can be grouped as SEC > SEB > SEA (Jay 2000). It should however be stated that the temperature-time combination needed for the inactivation of these SEs are dependent on various factors either in the food or media including pH, salt concentration and physical factors such as pieces of food product that could insulate the toxin from external heat sources. Apart from heat stability, some of the toxins also exhibit resistance towards inactivation by pepsin, gastrointestinal proteases found in the human digestive system (Balaban & Rasooly 2000).

These SE's along with other exoproteins produced by *S. aureus* such as the toxic shock syndrome toxin-1 (TSST-1) can be grouped as pyrogenic toxin super-antigens with their primary function in vivo being to inhibit an immune response from the host towards *S. aureus*. This is done by encouraging the body's production of non-specific T-lymphocytes, often leading to inflammation and fever. The SE's however also exhibit a strong emetic reaction in the subject (Dinges & Orwin 2000) which often requires a dosage of between 0.0004μg/kg (Ler, Lee & Gopalakrishnakone 2006) and 20 ng, while earlier findings pointed towards a dose of between 20-30μg of pure SEB toxin to evoke such a reaction in adults (Jay 2000).

Red meat safety and the role of background organisms

Meat environment and its microorganisms

By the time a food product reaches the consumer, the microbial load and population could well be different from that seen at its point of origin. A number of factors play a role in determining these properties including the original environment from which it was obtained, the microbiological quality of the raw product, the conditions under which processing and packaging took place and the quality of the packaging, handling and storage of the product. Although all food products, except those manufactured to be sterile, contain a certain amount of microorganisms, their numbers should be as low as possible with the intention of

increasing food safety and shelf life and decreasing any negative sensory aspects that might arise due to the growth and proliferation of the present microorganisms (Jay 2000).

Fresh meat is prone to microbiological contamination and is able to support the growth and survival of a variety of microbial species due to its rich nutrient composition, which includes proteins, carbohydrates and various non-protein organic and inorganic substances; it's slightly acidic pH of between 5.5 and 6.5; and its high water activity in the range of 0.98 to 0.99 (Samelis 2006). Even though these conditions would suit the growth of the large variety of different micro-organisms normally found on fresh meat, various studies have found that only a percentage of these ends up dominating the microbial load on the meat (Samelis 2006).

These organisms are normally determined by a variety of different intrinsic and extrinsic factors associated with the meat, its storage conditions and its processing, which in turn affect the survival, growth and competition experienced by the organisms present. The intrinsic and extrinsic factors normally include the pH, type of meat or level of processing, temperature and the prevailing atmosphere which is usually affected by the type of packaging be it overlay and un-altered or modified and hermetically sealed (Samelis 2006). The quantity, type and physical state of the organisms would also influence the final load.

The bacterial load found on red meat products are normally heterogeneous and mainly consists of mesophilic bacteria.   The source of microbial contamination of approved meat can usually be traced back to several sources of origin namely, any part of the animal; water and soil bacteria derived from its environment; contamination from infected surfaces including cutting boards and processors; or due to un-hygienic practices from meat handlers, causing the spread of resident human microbes or a pathogenic organisms, if done during illness (Davies & Board 1998).The greatest proportion however originates from the animal itself, with the sources ranging from its hide, hoofs and hair to the internal biota found in its intestinal tract and excrement. The numbers found at these sources is often of great concern to both abattoir's and meat processors alike, as incorrect handling of both the animals and carcasses could result in high contamination numbers on the meat, which would lead to ultimate shortening of storage life (Ayres, Mundt & Sandine 1980; Davies & Board 1998).

Hides are often associated with high numbers of microorganisms, and could contain counts ranging between 10^5 and 10^7 aerobic colony forming units per cm^2 (Small, Wells-Burr &

Buncic 2005). These organisms play a big role in the storage life of the meat product as can be seen with the paradoxical way beef from warmer; more tropical areas (usually exhibiting higher microbial loads on the hides) often have a longer storage life than beef from more temperate or colder areas that normally have a lower microbial load on the hide. This can be attributed to a smaller proportion of the total hide microbial load being made up out of mesophiles akin to lower temperatures (Ayres, Mundt & Sandine 1980; Davies & Board 1998). This possible high contamination load prompted the use of a variety of different measures or procedures to reduce these numbers including water sprays at high pressure, chemicals including cetylpyridinium chloride and combinations of various techniques which reduced aerobic, coliform and *Escherichia coli* O157:H7 numbers by between 0.1-5.3 $\log_{10}$ cfu/cm^2 on cattle hides and 0.1-5.2 $\log_{10}$ cfu/cm^2 on the eviscerated carcass and carcass parts (Loretz, Stephan & Zweifel 2011).

Intestinal biota acts as another large reservoir for a variety of microorganisms of which most are derived from the environment through the consumption of either food or water. This is however of no great concern as most of these organisms, excluding the enteric pathogens, are either inhibited or killed due to the unfavourable conditions, brought about by enzymes, acids, bile salts and an anaerobic environment, that thrive in the gastrointestinal system. In general the largest amounts of microbes found in the gastrointestinal tract inhabits the small intestine and colon, with 15 to 19% of an animal's faecal matter often being made up out of living and dead bacteria while bacterial numbers were recorded as high as 10^7 and 10^5 for calves and cows respectively. This would be of major concern in the event of faulty evisceration which would cause the contamination of the internal sterile meat of the carcass (Ayres, Mundt & Sandine 1980; Jay 2000).

Bacterial counts where investigated on meat carcasses pre and post processing and found that after slaughter, the carcass surface may contain micro-organisms in the range of 10^2-10^4 cfu/cm^2 (Robertson 1993). By the time the consumer would purchase a retail cut, the bacterial load could be as high as 1.5 x 10^7 cfu/g for Enterobacteriaceae, 2.42 x 10^5 cfu/g for *S. aureus* and 1 x 10^6 cfu/g for *E. coli* (Nel et al. 2004). When these figures are compared with the guidelines set by the Department of Health (South Africa) pertaining to raw meat, the levels of less than 10 cfu/g for *E. coli* with the highest level at less than 1000 cfu/g for *Listeria monocytogenes,* a common meat contaminant that can grow below 3.3°C (DOH

2002), indicates the exceeding extent of contamination occurring after slaughter as well as the possible risk associated with meat products.

To ensure that meat does not decrease in quality and has an optimum shelf-life pertaining to microbial spoilage, the meat carcasses normally undergo a rapid decrease in temperature to either 10°C or lower for sale product or to sub 0°C if they are to go into storage. This storage preservation procedure works two-fold by firstly, inhibiting the growth of the anaerobic, mesophilic bacteria that may cause putrification or meat discoloration and secondly by inhibiting possible pathogens that might be present on the meat since none of the pathogenic organism associated with meat can grow below 3.3°C (Robertson 1993).

The storage temperature of the meat does not stop aerobic growth from occurring on the surface of the meat but rather retards it, with the spoilage aerobes being dominated by *Pseudomonas* spp., the anaerobes are dominated by *Lactobacillus* spp. and the facultative anaerobes by Enterobacteriaceae species, due to their ability to outgrow other rival species that may be present (Nel et al. 2004). A clear indication of the presence of *Pseudomonas* comes from the elevated pH and the off odour of ammonia occurring due to the breakdown of amino acids, in the absence of glucose, giving a clear indication of the meat's risk factor (Robertson 1993).

Factors affecting microbial growth in red meat

Growth of microbes on meat gets affected by two main groups of factors, namely intrinsic and extrinsic factors. Intrinsic factors are characteristics innate to the meat including pH, water availability, nutrient concentration and availability, redox potential and structure which among others affects diffusion rates (Jay 2000). Most organisms grow best at pH values of around 7.0, or within a range of 6.6-7.5 thereof. Out of all the microorganisms, bacteria are the most particular about pH, with this being ever more the case with pathogenic bacteria. With the pH of the meats, and especially minced beef, normally being within the range of 5.1-6.2, it is a predisposed food for any type of microorganism, not just bacteria. This pH range does however limit some bacterial species as a pH of 5.1 would range in the minimum growth range of most bacteria, owing to the correlation of certain bacterial species such as *Salmonella* and *Escherichia* with minimal growth ranges below a pH of 5 to that of meat products (Jay 2000).

The pH of the meat is determined by the glycogen levels found in muscle tissue at slaughter. Glycogen reserves are converted to lactic acid once blood circulation has ceased which causes a drop in the tissue pH. The final pH of the meat is thus an indication of both the glycogen levels at slaughter and the state of the animal pre-slaughter, as any rigorous activity before slaughter that might lead to fatigue would have expended vast amount of glycogen and have formed lactic acid in the process. Numerous studies have found that when the pH of two groups of animals, one rested and one fatigued group, were compared, the pH of the tissue of the fatigued group was much higher owning to the fact that less lactic acid was formed due to the depleted glycogen levels (Ranken 2000).

An unfavourable pH generally affects the microbial cell in two primary ways. It would either affect the functionality of the cell's enzymes, causing them to be unproductive or to switch off, or by affecting the nutrient transfer into the cell. When affected by adverse pH conditions, the cell generally has to correct the internal pH equilibrium to stop the internal system from shutting down as some enzymes are extremely sensitive to differences in pH. In an acidic environment, this would normally involve either the expulsion or exclusion of H^+ ions. With regard to the nutrient transfer, a shift in pH could lead to the denaturing of membrane and transport enzymes while it would also influence the type of compounds that would be able to enter and exit the microbial cell as a result of ionization (Jay 2000).

The oxidation reduction or redox potential of food also plays a prominent role in affecting the growth of microorganisms as some of them display varying levels of sensitivity towards this potential. This potential can be seen as the ease with which a substrate gains or losses electrons. In general, the highest potential is normally a result of the atmosphere around the food and in this regard this subject will be discussed in a later section (Jay 2000).

Structure is one of the critical intrinsic factors that differentiates and induces a significant difference, both in keeping quality and microbial growth, between minced meat and other whole cuts. Normally the natural structure and often the structure covering the muscle tissue including the peri-, epi- and endomysium, provides good protection against the entry and/or damage caused by foreign organisms, however a breakdown of this structure often leads to a greater rate of spoilage in food (Jay 2000). Apart from the barrier properties, a broken down

structure often also causes the leakage of nutrients and moisture which provides any spoilage organism with a rich growing broth. Water and nutrient availability are self-explanatory.

The extrinsic factors of meat are normally the environment conditions in which the meat is held including temperature, relative humidity and the gaseous environment surrounding the meat. These extrinsic factors are often the main focus area for meat producers regarding the meats quality and storage life, since these are often the easiest factors to manipulate advantageously without significantly affecting the quality (Jay 2000).

The usage of temperature can be seen at any retailer or butcher by the way meat is often kept at temperatures below 5°C. This is due to the fact that all organisms have both an optimum and a growth range temperature, with the majority of spoilage organisms found on meat exhibiting an optimum growth temperature equal to that of body temperature or around 37°C. By lowering this temperature, the growth rates of these organisms are decreased, giving meat an extended shelf life (Ranken 2000). Relative humidity often has a direct correlation with water activity whereby if a product that has a low water activity was placed in a room with a comparably high relative humidity, the product would start absorbing moisture from the atmosphere and in this regard increase its water activity (Jay 2000). Gaseous environments will be discussed in the section regarding modified atmosphere packaging.

Mince meat

Minced beef, ground beef or mince meat, as it is more commonly known in South Africa, is a form of comminuted red meat that varies in functionality from the making of hamburger patties, to the production of beef sausages in a variety of forms to the simplest function of consuming it in its comminuted form as mince. The need for the mincing however occurs due to a high incidence of connective tissue in certain cuts, resulting in these cuts being chewy and less appetizing to the consumer. Through the mincing process, these cuts are shredded or broken down, resulting in the connective tissue being reduced in size and making the meat easier to chew (Ranken, Kill & Baker 1997).

This process however also has a significant effect on the microbes that might be present on the meat and it has been consistently reported that ground beef has a much higher incidence of microorganisms than intact meat portions such as steaks. This is due to various different

factors, as stated below, that all contributions to this increased microbial load (Jay 2000). Minced meat manufactured for commercial consumption normally consists of meat trimmings obtained from various different meat cuts originating from various different processing areas and to this regard, these trimmings would normally have been handled more excessively than whole cuts. Due to the larger variety and numbers of different pieces of meat being used in the production of minced meat, only one piece of highly contaminated meat is often needed to ensure the contamination of an entire processing batch. The mincing of the meat also leads to a smaller particle size and an increased surface area and accessibility of nutrients, which leads to increased microbial growth (Ranken 2000).

This breakdown also benefits the growth of aerobic bacteria, the normal spoilage organisms, as greater volumes of oxygen can be exchanged. To allow the meat to be comminuted, a variety of grinding equipment and cutting knives are used which increases the processing surface the meat comes into contact with. As this equipment is difficult to clean, it is generally experienced that they are cleaned less often which allows for the build-up of microorganisms that can then be passed along to all process batches (Jay 2000).

Microbial growth on the surface of intact or quartered carcasses is also restricted due to the enveloping coverage of both fat and connective tissue that is found both sub-dermal and covering the internal viscera of the carcass. This is due both to the fat and connective tissue being a poor supply of nutrients for the microbes (Ayres, Mundt & Sandine 1980).

Spoilage associated with storage of fresh meat.

To ensure maximum keeping quality, fresh meat is stored under chilled conditions often in vacuum sealed bags, controlled atmosphere rooms, in O_2 permeable wrapping or in its natural state with no atmosphere modification. However, these conditions merely retard the growth and metabolic activities of the spoilage organisms and upon extended storage conditions, meat often develop off odours and a shiny, slimy appearance (Ranken 2000).

These odours are often sweetly rancid with a hint of ester compounds and are frequently compared with the smells of butter and caramel. The aroma is often a precursor to the appearance of small translucent colonies on the surface of the meat which acts as an indication of the developing spoilage. In minced meat, the off odour is always apparent with

microbial populations exceeding 10^6 cfu/cm^3. These colonies are often what form the slimy appearance as they grow, develop a creamy colour and unite into a single layer on the surface of the meat (Gill 1996).

Spoilage and the rate of spoilage of chilled stored meat are often not only affected by the level or concentration of the initial contamination but also by the makeup of the microbial spoilage community (Gill 1996). An initial decline in bacterial numbers is often observed on meat that is stored between 0 and 5°C, with often only the psychroduric spoilage organisms persevering.

Ayres (1960) showed how two samples of minced beef, exhibiting approximately similar initial bacterial loads, experienced a dissimilar transition into spoilage and the production of slime and off odours. The two samples, one prepared from frozen trimmings and the other from fresh pieces of meat, were microbiologically examined and their microbial composition was found to consist of 78% and 18% of the spoilage organisms *Pseudomonas* respectively. Upon storage, the meat sample obtained from the frozen trimmings spoiled within three days while the sample prepared from fresh meat took six days to spoil. However, once spoilage was confirmed, through the appearance of slime, for both packs on the respective days, the microbial load's composition comprised more than 98% typical spoilage organisms (Ayres, Mundt & Sandine 1980).

Under certain conditions, the putrid by-products formed by organisms such as pseudomonads and enterobacteria might even be evident sooner than the expected 10^6 cfu/cm^2 level if their numbers reach 10^4 cfu/cm^2 in a biota composition of 10^7 cfu/cm^2 owning to the spent glucose in the meat (Gill 1996).

Main bacterial species associated with meat

Pseudomonas spp.

Bacteria in the genus *Pseudomonas* are gram-negative rods, typically originating from soil and water sources, with *Pseudomonas* forming the largest bacterial genus associated with both fresh foods and the spoilage thereof. *Pseudomonas* can be found on a variety of different food sources including vegetables, meat, poultry and seafood and due to the high number of strains that are psychotropic, they populate the largest group associated with the spoilage of refrigerated food products (Jay 2000). Phylogenetically the genus can be

clustered into five different groups with the first group (Group I), forming the most important group associated with the spoilage of meat. Some organisms included in this group are *P. fluorescens, P. putida, P. lundensis* and *P. fragi* (Samelis 2006).

When meat is kept at lowered storage temperatures, the microbial biota present would normally be dominated by the pseudomonads. This could be attributed to several distinct growth advantages utilized by the pseudomonads namely, their advantageous growth at temperatures between 2 and 15°C; a generation time that is often 30% shorter than other biota species present on the meat; their high affinity for oxygen, a relative insensitivity towards the presence and growth of most of the spoilage bacteria found on meat and their ability to utilize the amino acids in meat as an energy source if the glucose gets depleted (Ayres, Mundt & Sandine 1980; Jay 2000; Samelis 2006).

Lactic acid bacteria

When meat is often examined for the presence of lactic acid bacteria (LAB), the dominant groups that are normally associated with either vacuum packed or modified atmosphere packaging (MAP) meat are the *Lactobacillus, Leuconostoc* and *Carnobacterium*. Other genera that would also often make up the mix include *Weisella, Enterococcus* and *Pediococcus*. The spoilage of vacuum and MAP packed fresh meats by LAB occurs as a result of various organic acids being produced from the endogenous glucose, inevitably lowering the pH. These organic acids, of which the majority is made up out of lactic acid, results in a typical fermenting type spoilage occurring, that is accompanied by off odours of both an acidic and volatile fatty acid nature. Other spoilage effects include the production of gas, slime formation and discoloration of the meat upon opening due to the formation of H_2O_2 (Samelis 2006).

Certain LAB, such as *Lactobacillus sakei*, can also cause spoilage of meat through the production of H_2S which would then lead to both the formation of odours and black spots on the meat, as a result of its interaction with the iron molecules of the myoglobin. Even when all these spoilage factors are taken into consideration, the spoilage caused by LAB is often much less profound than that caused by the gram-negative organisms that are present on meat (Samelis 2006).

The most advisable precaution to LAB spoilage is the use of 100% CO_2 in packaging, but this however leads to other issues, not least of which is the discoloration of the meats due to the need of the myoglobin for the presence of oxygen. Although not effecting LAB as severely as a 100% CO_2 atmosphere, vacuum packaging and MAP packaging containing oxygen still results in a weaker proteolytic activity seen in LAB (Samelis 2006).

Lactobacillus sakei and *Leuconostoc gelidum,* are often dominant on meat, noting a possible special attribute or adaptation against other LAB and bacteria. Although certain general attributes such as the *in situ* production of bacteriocins, H_2O_2 and other antimicrobial compounds; psychrotrophy; glucose fermentation and anaerobiosis could lead to an advantage over other bacteria, the advantage is thought to be more likely as a result of these species' higher adaptability to meat and a lower specificity when regarding growth conditions (Samelis 2006). An example of this comes in the form of *L. sakei*, being able to hydrolyze arginine and grow in environments containing minute amounts of glucose. As arginine is freely available in meat, low amounts of glucose would not have the same impact on its growth when compared to other LAB (Samelis 2006).

Enterobacteriaceae

The family Enterobacteriaceae includes some of the most common bacteria and bacterial groups that are tested for in the food industry including salmonellae, total coliform and faecal coliform bacteria, of which *E. coli* forms part of (NCBI 2010). There is often a direct correlation between the amount of these bacteria present on the food and their prevalence in the environment, indicating that an absence of these organisms is often not possible and that their detectable numbers only need be compared with prior knowledge to establish tolerable parameters (Ayres, Mundt & Sandine 1980; NCBI 2010).

Salmonellae are spread greatly amongst all animals, wild and domesticated, in nature and can often use other carriers such as wind, dust, water and insects to spread from one area to another, making the process of controlling contamination difficult and limited. Low levels of salmonellae on meat often do not pose any risk as long as temperature control is maintained. However, heat treatment does render salmonellae defunct with any reappearance indicating human negligence (Bell & Kyriakides 2009).

When regarding the microbiology of food, it becomes crucial to differentiate between faecal and non-faecal coliform bacteria as their origin, characteristics and form of transmission differ greatly. Non-faecal coliforms normally contaminate food as part of the normal aerial microbiota or via surface contamination from packaging materials or personnel. Due to this type of contamination, it is difficult to establish limits as numbers can constantly vary. Most members grow optimally in the low mesophilic temperature range while some species grow at temperatures above 43.3°C (Ayres, Mundt & Sandine 1980).

Faecal coliforms originate from the intestinal track of animals and humans with contamination and spread on meat normally occurring during slaughter and dressing of the carcass. *E. coli* is normally regarded as an indicator organism of faecal contamination in food and thus the food's history and future needs to be taken into consideration when assessing safety and quality as the destruction of *E. coli* can be obtained through mild heat treatments (Bell & Kyriakides 2009; NCBI 2010).

Aerobic bacterial plate count (APC)

The standard aerobic bacterial plate count is often used to determine the overall number of micro-organisms in the food product and is used as an indication of the general conditions of the raw products and any processing, handling and storage steps linked to the product. Any competitive effect, such as competition for nutrients and growth space, brought about onto an artificially introduced bacterium by APC, could be seen as a sum of the whole of all the organisms found on the meat. Here certain properties or aspects might work synergistically or competitively leading to an overall effect that could be associated with the APC.

The effect of spoilage organisms on pathogens

Competitive interactions

Interference between micro-organisms was first observed in 1852 by Mosse when he treated human furunculosis, a condition resulting in boils caused by *S. aureus*, with yeasts. The general idea of this interference pertains to the likelihood of the proliferation of low numbers of pathogens when compared with their presence in either low or higher numbers of harmless microbes (Jay 2000). Jay (1997) showed that the numbers of *E. coli* O157:H7 cells were reduced by 4.28 log cycles/g on fresh beef stored at between 3-5°C when the background

aerobic microbes increased on the samples from $10^{5.97}$cells/g to 10^{9}cells/g, while no viable *E. coli* cells were detected in a 1:10 dilution after 11 days in the presence of $10^{8.93}$ aerobic cells per gram. It was thus argued that high levels of inherent non-pathogenic micro-organisms present in food, the body or on the surface of an animal might be able to out-compete any foreign or natural pathogenic species and in turn provide a protective effect (Jay 1996).

S. aureus was shown to be inhibited by various bacteria, namely *Proteus vulgaris, Achromobacter* spp. and *Pseudomonas* spp., found naturally in a variety of food sources (Troller & Frazier 1963). As the number of inhibiting organisms increased, the log count of *S. aureus* increased less dramatically in the broth over the same time frame. Of importance though, was that not only the amount but also the type of bacteria affected the inhibition of *S. aureus*. The most significant individual inhibition was seen with *Pseudomonas* sp. CS-1 which resulted in a decrease in *S. aureus* plate count numbers from 8.4 x 10^{8} to 9 x 10^{5} per ml over an incubation period of 24 hours. These inhibitory effects however are dependent on environmental and medium factors with different temperature incubations and amounts of solutes having a definite effect on *S. aureus*. These factors also affect the inhibiting bacteria with some of them showing optimal inhibition with different environmental variables (Troller & Frazier 1963)

Enabling a better growth environment for pathogens

Recent outbreaks and the possibility of illness or death associated with various pathogenic micro-organisms connected with food, has prompted an enhanced effort to try and eradicate or limit these pathogens from consumed food products (CDC 2010). In general, most slaughtering and processing sites employ various different antimicrobial treatments including the use of hot water sprays, cleaning chemicals and the use of irradiation or ultrasonic to try and combat these pathogens (Nissen, Maugesten & Lea 2001). These treatments, albeit effective to an extent, could also in turn prepare the environment for the proliferation of the pathogen by the removal of background organisms (Nissen, Maugesten & Lea 2001) or select for stronger, more resilient strains which could lead to a greater danger and spread of these adapted organisms (Stopforth et al. 2007).

However, not all pathogens get affected in the same manner by the competitive actions of innate microorganisms. Nissen *et al.* (2001) compared the effect of the background organisms on three different pathogens namely *E. coli* O157:H7, *Yersinia enterocolitica* and *Salmonella* Enteritidis. In terms of background organisms, three different meat types namely chicken, pork and beef were either decontaminated by being placed into a decontamination rig, resulting in a decrease in the natural background organisms, or left as is prior to being inoculated. The three pathogenic inoculums were as follows; *Salmonella* Enteritidis on the chicken, *Yersinia enterocolitica* on the pork and *E. coli* O157:H7 on the beef (Nissen, Maugesten & Lea 2001).

For the chicken and pork samples, no significant difference was observed in the growth of the pathogenic species between the decontaminated and the non-treated samples, indicating no substantial effect that could be attributed to the natural background organisms. An entirely different scenario was however observed with the *E. coli* O157:H7 inoculums. It was found that on the decontaminated beef, the growth rate and numbers for high inoculums were significantly higher by the fifth day of storage while higher growth rates were also found with low inoculums in vacuum packaged beef. The conclusion of the study found that although decontamination of meats could be beneficial in certain circumstances, this could also lead to higher pathogen contamination of the meat (Nissen, Maugesten & Lea 2001).

Mechanisms of interference

On comparing the different interference methods or mechanisms employed by microorganisms, several were noted as possible main factors. The first is that of the production or excretion of certain inhibitory or bactericidal substances by the innate or introduced organisms that could either adversely affect or kill any pathogen present. This mode of action can most clearly be observed with lactic acid bacteria or with any fermented food product. Apart from a decreasing pH, lactic acid bacteria can also produce substances such as H_2O_2, diacetyl and acetoin which has been shown to be inhibitory with regards to other bacteria (Jay 1996).

Another main mode of action could be in the competition for oxygen, nutrients and space, which could include viable attachment or adhesion sites on the surface. The effect of space was observed when a virulent strain of *S. aureus* was prevented from attaching to the nasal mucosa of infants due to the previous colonization of the nasal passage by less virulent strains that were introduced into the nose by swabbing. Competitive exclusion was also noted for the prevention of colonization by *Salmonella* and *Campylobacter* of the digestive system of hatchling chickens (Jay 1996).

The ability of micro-organisms to alter their environment to the extent that it becomes undesirable to other micro-organisms is another form of exclusion practised by some species. Although similar to the excretion of certain substances, this could also include the alteration of the gaseous atmosphere or a drop or increase in the environmental pH (Jay 1996). The concept of inhibition through micro-organisms does however still require a lot of research to determine both the unique mixtures of bacteria needed to provide such protection in different food sources as well as the numbers in which they need to be present to inhibit possible pathogens proliferation.

MAP and its effects through varying levels of CO_2 and O_2 on meat

Introduction

To be more competitive in an ever changing market, many packaging companies are looking at various ways to stay ahead of fluctuating consumer and retail demand. These driving forces or trends can be summed up into two major contributing factors. The first factor is a

result of many supermarket chains extending their shopping hours to meet consumer demand, with some even being open 24 hours a day (Belcher 2006). This can however result in either a retailer being out of stock of a specific type of meat due to it only pre-packing a definite amount of meat per day, or to combat such an incident, the retailer would have to increase its labour force to allow 24 hour meat preparation ability, thus increasing costs. The second factor pertains to the modern consumer's need for both fresh and convenient meat products, which often has an implication on the safety of the product. Although much work is being done to ensure adequate hygiene and the elimination of pathogens at meat processing plants, this is often found to be inefficient or of no interest as some of the contamination can occur during transport, processing or packaging (Belcher 2006).

In recent years, the trend has shifted from back of house processing and packaging to the production of a case-ready product at the meat processing source. The prevalence of case-ready products was put at 60% of all the meat products in the US in 2004, with red meat having the smallest impact at 23%. With red meat however, this was seen as an improvement since this constituted a rise of 9% from 2002. Of this 23%, ground beef made up the majority of the conversion coming in at 66% of the red meat's proportion, this in part being due to heightened safety concerns regarding ground meat as well as the desired oxymyoglobin state for whole meat cuts (Belcher 2006). Case-ready products derive much of their success from sealing the product at their primary location, inhibiting possible contamination down the logistical line, as well as the use of modified atmospheres within the product.

History

Modified atmosphere packaging (MAP) and controlled-atmosphere packaging (CAP) are preservation methods that employ the use of gasses such as CO_2 and N_2 to replace the atmospheric environment around a food product, so as to cause a homeostatic disturbance for possible aerobic spoilage organisms that might be on the food's surface (McMillin 2008). This was first observed in 1922 by Brown (Ooraikul & Stiles 1991) during a study that was prompted by the noticed effects that varying concentrations of O_2 and CO_2 had on fruit rotting fungi's germination and growth. Brown noticed that CO_2 concentrations of 10% and higher at temperatures lower than 10°C had an effect of retarding both the growth and germination of these fungi.

These results inspired further studies by Moran (1930) and Haines (1933) which indicated, that concentrations as low as 4% could retard mould growth in meat and that bacteria required double the time to reach a designated number if held in a concentration of 10% CO_2 compared to air at 0°C, respectively (Ooraikul & Stiles 1991). Even though the exact mechanism of function was not understood, it started to be used in prolonging the shelf-life of fresh and minimally processed food sources and was first employed practically in the 1930's when fresh meat in the form of intact refrigerated beef carcasses were shipped from Australia to the United Kingdom in a container containing heightened CO_2 levels (Dixon & Kell 1989).

Although its use has now been proven, the adoption of the technology and its implementation progressed slowly due to an already established refrigeration and transportation systems and the minimally regulated use of preservatives. The technology only started re-surfacing in the midst of harsher regulations and higher energy costs which forced manufacturers to re-think their conventional techniques in terms of shelf-life extension (Ooraikul & Stiles 1991).

Function

Although MAP and CAP could at times be used inter-changeably, the more general term referring to products that are supplied to vendors and customers is that of MAP and will further be referred to as such. CAP is employed more in the area of ripening of fruits and vegetables and their preservation against spoilage and normally occurs in containers much larger than those used for consumer distribution (McMillin 2008).

MAP creates a homeostatic disturbance in two ways. Firstly, by replacing the standard atmosphere that contains enough oxygen for aerobic spoilage organisms to grow and cause spoilage, it causes the environment to only allow for the growth of micro-aerophilic or anaerobic organisms, thus cancelling out any spoilage organism that might have been on the outer coating or skin of the produce or animal. Secondly it employs the anti-microbial properties associated with the higher partial pressure of the CO_2 in the container, if a technique other that vacuum packaging is used (Ooraikul & Stiles 1991; McMillin 2008; Tarafa et al. 2010).

Initially it was thought that the increased CO_2's anti-microbial action was due to a decrease of pH in the environment by forming carbonic acid, thus having the same effect as a weak

acid on the spoilage organisms. This idea was however discarded due to the unlikely possibility that carbonic acid would have such a drastic effect in an organism's environment (Eklund 1984). It is thought the increased CO_2's anti-microbial action was due to it diffusing into the cytoplasm and membrane of the microbial cell, unbalancing the cell and causing a decrease of pH in the inner cell environment by forming carbonic acid, thus having the same effect as a weak acid on the spoilage organisms (Tarafa et al. 2010).

Tan & Gill (1982), however theorized that CO_2 could have other potential methods by which it would act to cause inhibition of microbial growth; namely that CO_2 could inhibit both decarboxylating and other susceptible enzymes within the cell and that CO_2 and through it altering the properties of membrane of the organisms, would inhibit the functions of the membrane and thus retard or inhibit growth. The notion of CO_2's effect on interfering with trans-membrane uptake was later discarded as growth did not seem to be effected in the same drastic way as the uptake of certain amino acids such as serine and phenylalanine (Eklund 1984).

CO_2's effect on the intercellular environment was later assumed to be its major anti-microbial effect with the carbon dioxide diffusing into the cytoplasm and membrane of the cell, unbalancing the cell and causing a decrease of pH in the inner cell environment by forming carbonic acid (Tarafa et al. 2010). This along with the effect that carbon dioxide has on disrupting the cell membrane, affecting the transport of CO_2 and expulsion of the carbonic acid from the cell, has been shown to affect the growth and survival of certain organisms (McIntyre & McNeil 1997).

With regard to proteins and enzymes, CO_2 is thought to affect these compounds by changing their physio-chemical characteristics in such a way as to disrupt their function through the change in their shape or solubility. This would then cause the precipitation or dilution of certain enzymes, taking them out of their base of functionality. The addition of CO_2 could also disrupt an enzyme's, for instance decarboxylase's function by causing a CO_2 equilibrium dilemma in the cell, resulting in the enzyme being non-functional due to feedback inhibition rather than a disruptive change (Dixon & Kell 1989). This causes retarded growth due to its effect on both the energetics and metabolism of the cell by placing the cell into a futile cycle of energy consumption involving phosphoenolpyruvate and oxaloacetate (Teixeira de Mattos et al. 1984).

Although the exact mechanism by which CO_2 acts as an anti-microbial substance is still unclear, its effect on bacterial growth is significant. It was found that when comparing the growth of bacteria on pork, the samples placed in the presence of high amounts of CO_2 required a time period 7 times longer to reach an aerobic count of $5 \times 10^6/cm^2$ than those samples placed in atmospheric air (Enfors 1979), giving some indication of the inhibitory effect that CO_2 could exhibit on aerobic spoilage bacteria.

Packaging

When retailers and producers opt for the use of MAP meat and more specifically case-ready MAP meat, there are certain criteria in terms of both the packaging and the contents that they deem necessary to be met. These criteria involve the quality of the meat, the appearance of the entire package and the reduction of labour at the retailing end to reduce costs. Extension of the shelf life of the product is foremost on the list of both producers and retailers as sufficient keeping time is needed due to distribution of the packs occurring from a centralized packaging facility (Belcher 2006).

To ensure that such a scenario is viable, impeccable management needs to be exercised on temperature control, during packaging and transport, as well as hygiene of workers and equipment, prior to and during packaging. This requirement is usually met with the implementation of a HACCP (Hazard Analysis Critical Control Procedures) system and its accompanying SOP's (Standard Operating Procedures) as well as through good manufacturing principles (GMP).

Display of the product comes next, with the meat product preferably needing to be displayed in its bright red or oxymyoglobin state. The mixture of used gasses thus needs to provide both the added benefits of MAP as well as the maintenance in colour of a fresh product. This is of great concern as to the points discussed in the colour of meat and the consumer's perception relating to it in the following chapter (Belcher 2006).

Apart from ensuring the uniform hygienic quality of the meat through the air tight sealing of the pack and the colour of the product through the correct gas mixtures, the centralized packaging facility also takes responsibility for the labelling and pricing of each pack. This

ensures that the correct price to weight ratio is enforced as well as reducing the turn-around time between packaging and store display. Less handling of the packs also provides for a more attractive packaging and reduces the risk or breaking the hermetically sealed pack (Belcher 2006).

Importance of the colour of meat

Introduction

Most consumers base their meat purchases and to an extent all their foodstuff purchases on two groups of cues, namely intrinsic and extrinsic. Intrinsic factors, as pertaining to meat, includes colour, fat content and cut while extrinsic factors are price, origin, place of purchase and any extra information pertaining to the animal prior to slaughtering. Thus for most consumers and in terms of marketing by the butcher or supermarket, the initial sensory trigger that is of highest importance and most influential when buying meat is that of appearance and colour (Grunert, Bredahl & Brunsø 2004). Meat that may seem unappealing or differing from the norm, may often deny the other two sensory attributes, namely texture and taste, from being involved in the quality evaluation process. In a recent study, appearance and colour consistently ranked within the top five factors influencing the consumers' interpretation or perception of quality when considering the purchase of beef in countries such as Germany and the United Kingdom (Grunert, Bredahl & Brunsø 2004). It can thus be seen that these two factors are of high concern to meat producers and sellers, which through various methods, they would aim to utilize to their advantage.

What affects colour?

The colour of meat can be affected by numerous independent variables including the age of the slaughtered animal; handling pre- and post-slaughter; and the display and storage conditions. The colour in the meat (**Error! Reference source not found.**) is as a result of two ferrous heam protein pigments, myoglobin and to a lesser extent hemoglobin. Myoglobin's innate colour as found in meat is purple, which in the presence of oxygen or during oxygenation, can change to oxyheamoglobin which is bright red in colour. The meat can however revert back to a purple colour in the absence of oxygen as can be seen with vacuum-packed samples. Raw meat can also have a third colour often described as grey-brown which is the result of either oxidation where the ferrous compound in the myoglobin

gets changed to its ferric form or through the deoxygenation occurring within the meat (MacDougall 1977; Jakobsen & Bertelsen 2002; Miller 2002).

Deoxygenation   Oxidation

Oxymyoglobin ⟵   Myoglobin ⟶ Metmyoglobin
        ⟶

Oxygenation

Bright red   Dark, purplish red   Brown

Figure 1: Diagrammatical representation of the processing affecting the colour of red meat. (Jakobsen & Bertelsen 2000)

The extent of oxidation and deoxygenation is largely determined by the storage conditions of the meat and the species and variety of meat. Low oxygen pressures that cause a low level of oxygen penetration of the meat, temperatures where a higher temperature hinders diffusion of the oxygen and increases the oxidation reaction, high pH levels, residual enzyme activity that utilizes oxygen and an extended storage period all increases the extent and rate of browning (MacDougall 1977; Jakobsen & Bertelsen 2000). Out of these various factors, the parameters that were found to be most influential in the rate of oxidation was that of temperature and storage time (Jakobsen & Bertelsen 2000) while the easiest two to control in an attempt to improve shelf-life and appearance, are those of temperature and oxygen pressure. This can often be seen in the presentation of the meat in butchers and supermarkets where the meat may be displayed in an open showcase, in an oxygen permeable film or packaging, or sealed in high oxygen modified atmospheres, all at temperatures less than 5°C (MacDougall 1977).

Temperature also has other effects in that at lower temperatures, the red or oxymyoglobin form is more unwavering due to the rate of oxidation, affecting and changing the pigment to metmyoglobin, being diminished. Oxygen is also more soluble and consumed at decreased volumes by metabolic reactions at lower temperatures which cause greater penetration by the oxygen, in turn resulting in redder meat. Length of storage at chilled temperature was also shown by Feldhusen *et al* (1993) to have an effect on the rate of colour change with lightness, redness and yellowness all showing a significant increase of between 3 and 4 units over 5 days at 5°C, with diminishing increases with periods longer than 5 days (James & James 2009).

Colour determination

Although appearance as a whole can be a subjective evaluation, keeping in mind that both the type of meat cut (steak, minced, etc.), as well as the texture (smooth, grainy, etc.) might affect the appearance subjectively as a whole, it is possible to determine and compare the colour of the different types of meat. This can be done using any of a variety of instrumentation to determine the colour spectrum, using either the Commission Internationale d'Eclairage (CIE) colour terms for luminous reflectance (Y), chromaticity co-ordinates (y and x), the dominant wavelength λ_d and the purity percentage P_e; or using the Hunter colour space expressions that determines lightness (L), green to red hue (a) and blue to yellow hue (b).

Using the values gained from the latter colour space, it is possible to also determine the saturation (S) of the meat tested by using the equation $S = (a^2 + b^2)^{1/2}$. Meat saturation can be grouped into four primary groups with the saturation value being assigned to one of these. A saturation value higher than 20 is deemed bright red, 18 as a dull red, 14 as a distinctly brown colour and less than 12 is deemed to be perceived as brown to grey-brown (MacDougall 1977). Thus a retailer would opt to have all his presented meat cuts appear to have a saturation value of above 18 as to illicit an appealing response from the customer.

Bioluminescence and the role it can play in detection of microbes in meat

What is bioluminescence?

Bioluminescence is the process whereby an enzyme catalysed reaction in a living organism produces visible light *in vivo* that would normally act in a molecular reporter function. This process is achieved through the production of the enzyme luciferase that acts as a catalyst in the oxidative conversion of D-luciferin to oxyluciferin, resulting in the emission of light (Zinn *et al*. 2008). Organisms such as the North American firefly (*Photinus pyralis*), the click beetle (*Pyrophorus plagiophthalamus*) and the jelly fish (*Aequorea*) all employ bioluminescence, with the luciferase from the firefly however being both the first luciferase to be cloned and the most widely used bioluminescence reporter in research. The popular use of luciferase resulted due to the its ideal gene expression marking capabilities including the lack of post-translational modifications and its *in vivo* half-life of 2-3 hours (Sadikot & Blackwell 2005; Zinn *et al*. 2008).

During bioluminescence, luciferase requires adenosine triphosphate (ATP), magnesium and a benzothiazoyl-thiazole luciferin to produce photons over a wide emission range of 530-640 nm, with peak emissions often being around 562 nm. Aligned with the optical characteristics of biological tissue, this allows the detection of the emitted photons through several centimetres of biological mass with a stable photon emission of up to 60 minutes (Sadikot & Blackwell 2005).

Use of *in vivo* bioluminescence

Detection of emitted photons during bioluminescence is realized through the use of a charge-coupled device (CCD), essentially a light sensitive camera, with the subject of detection needing to be placed in a dark chamber (Zinn *et al*. 2008). One such device is the Xenogen IVIS Lumina II that can be used for both fluorescent and bioluminescent imaging. Exposure times range from 1 second to 10 minutes, depending on the experiment, with light intensity normally being overlaid over a black and white image and expressed through a pseudo-colour scaling system with red indicating the highest and blue indicating the lowest light intensity. The acquired light intensity can either be interpreted as photons or counts with additional features often accompanying the system through the provided software applications (Zinn *et al*. 2008).

Research incorporating in vivo bioluminescence can modestly be placed into either routine applications, including gene therapy and infection detection, or specialised applications, including protein-protein interaction and signal transduction. The general difference between the two types of applications occurs with the routine applications normally opting to construct the control promoter in an always active or "on" state. The consequence of this is a constant emission of photons without the need for a trigger or the promoter to be activated, allowing constant tracing of the source as would be preferred when detecting bacteria (Zinn et al. 2008).

Objectives and Hypotheses

Hypotheses

Staphylococcus aureus' growth rate will increase more rapidly in a 100% CO_2 (100%) atmosphere compared with an atmosphere of O_2=60%/ CO_2=30%/ N_2=10%. This can be due to a decreased number of natural background aerobic spoilage organisms in the 100% CO_2 (Jay, 1997) in addition to the various resulting anti-microbial properties attributed to the higher partial pressure of CO_2 (Dixon & Kell 1989) (Teixeira de Mattos *et al*. 1984).

The higher initial inoculation levels (10^5) of *S. aureus* will result in higher final numbers when compared with low inoculation levels (10^2) due to a colonization of the meat that will restrict the growth of background organisms due to their comparative lower numbers (Jay 1997).

E. coli O157:H7's growth will be more rapid in a O_2=60%/ CO_2=30%/ N_2=10% atmosphere compared with an atmosphere of 100% CO_2, due to the detrimental effect 100% CO_2 has on the specific growth rate of *E. coli*, since its optimal growth range lies between 2-10% CO_2 (Lacoursiere et al. 1986).

High inoculation levels (10^5) of *E. coli* will result in higher final numbers when compared with low inoculation levels (10^2) due to faster colonization of the meat, restricting the background organisms' influence on its growth due to their comparative lower numbers (Vold et al. 2000)

Due to the stress applied to *E. coli* - Xen 14 by the MAP environment, less spread over the meat surface with lower numbers will be observed when compared to the PVC overlay packs (Barrera et al. 2007)

Objectives

To determine the effect of MAP: O_2=60%/ CO_2=30%/ N_2=10% and CO_2 (100%); and storage time on the growth of high and low inoculation levels of *S. aureus* on minced beef.

To determine the effect of MAP: O_2=60%/ CO_2=30%/ N_2=10% and CO_2 (100%); and storage time on the growth of high and low inoculation levels of *E. coli* O157:H7 on minced beef.

To determine the relationship, in terms of growth and interactions, between the naturally present background organisms and the introduced pathogens, *S. aureus* and *E. coli* O157:H7, on minced beef.

To determine the effect brought about by different packaging types, MAP and PVC overlay, on the spread of *Escherichia coli* - Xen 14 in a minced beef sample during an accelerated shelf life of 48h.

Chapter 3: Comparison study between the use of Quarter and Full Plate methods for microbiological enumeration

Abstract

Microbiological research is often burdened by the constraints of budget and time, with a large proportion of both going towards medium production for enumeration. The quarter plating technique allows for a reduction of >50% on consumables and enumeration of an 8-fold dilutions on two plates. A comparative study between quarter plating and normal spread plating was done to establish a correlation between the two, with the results pointing towards a standard over-estimation of close to a log. This allows for inter-changeable use between the two plating methods as long as the over-estimation is kept under consideration.

Introduction

Enumeration of microorganisms in samples ranging from swabs to food products can be done in primarily two ways, the traditional enumeration methods of counting colony forming units (CFU) on an agar plate or either by the use of ever improving rapid enumeration techniques such as competitive Polymerase Chain Reactions (cPCR) (Choi & Hong 2003). Although rapid techniques would be preferred due to their, as their name refers, rapid acquisition of results, labs that still perform conventional research and routine analyses and smaller, less advanced testing labs as a result of the increased cost factor associated with rapid techniques, often opt towards the traditional enumeration methods.

This said however, does not mean that the traditional methods are *per se* inexpensive. Basic components such as agars and Petri dishes can account for a large part of a budget, while extras such as supplements, that are often essential for enumeration of certain microorganisms, are often sold in small quantities at a costly price. All these factors often influence a researcher's budget to the point that only the essential parts of an experiment can be performed. When a microbiological study is conducted to determine the colony forming

units (CFU) of a specific group or species of organism on food, the test sample is prepared in various dilutions and inoculated onto or within the media, depending on the procedure and type of organism. The main focus of this technique study however is with the use of the spread plate method.

An alternative to the conventional spread plate method is what is generally regarded as the quarter-plate method (Buys et al. 2000; Dainty et al. 1979), which as the name suggests, utilises only a quarter of the plate per dilution (Figure 1). This method thus allows a researcher to do a range of 8 dilutions on only two plates, resulting in less media being used and less time being spent on preparation of said media.
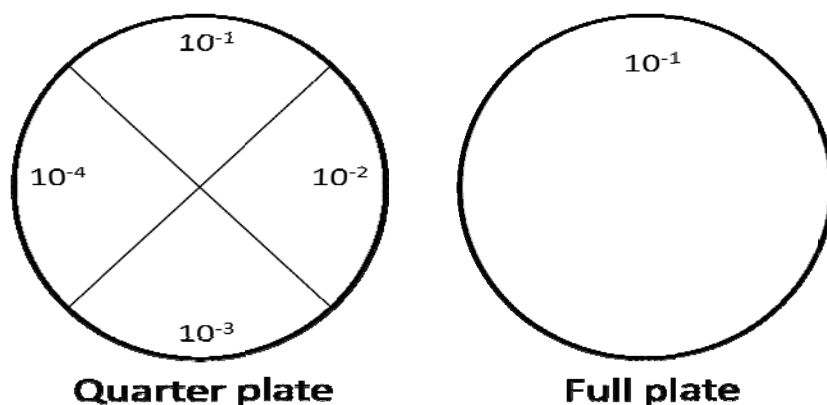
Figure 2: Graphical comparison between a quarter and full plate

It needs to be noted however that the detection level of the quarter plate method is >100 CFU which suggests that a conventional spread plate for the 10^{-1} and 10^{-2} dilutions, needs to accompany any analyses with the Quarter-plate method to ensure that counts smaller than a 100 CFU isn't noted as 0 (Buys et al. 2000; Dainty et al. 1979). When using two different colony forming count techniques, it often needs to be highlighted that numerous factors or errors can also affect these techniques and their counts in entirely different ways. These errors can be broadly summated into three groups mainly dilution and distribution; incubation; and counting errors (Jarvis 2008).

Distribution and dilution errors are associated with the aliquot sampling and transferring, with the major factor here being the volume of aliquot. The first arises with the acquisition of the aliquot, where a distribution error involving the organisms in the prepared sample could result in an unrepresentative sample being taken due to the decreased volume. This could

result both in an under or over estimation of the average, depending on where the aliquot was taken within the container. The second, and probably the largest factor to influence the counts, is the accuracy of the actual transferred amount. This can be affected by both the accuracy of the pipette being used as well as the amount of fluid remaining on the outside of the tip. As the precision is increased with the decrease of the volume of the aliquot, a 1µl error in either pipette accuracy or in the presence of additional aliquot on the tip, would have a 1% influence on a 0.1ml aliquot, but a 7% influence on a 15µl aliquot (Jarvis 2008).

Incubation errors can arise due to aspects brought on by both the plates and the organisms during incubation. To adequately grow, some organisms need their incubation temperature to be within a certain range, with a specific point being marked as their optimum growth temperature. In an incubator, the temperature profile associated with Petri dishes will vary, depending on their numbers and their position. More Petri dishes, higher and tightly packed, would have a different temperature profile when compared with a less and more dispersed group, with the possibility of "cold" pockets even forming within the incubator without convection fans (Jarvis 2008).

Another aspect is that of oxygen consumption, which again is affected by the amount of plates, and to that extent, the amount of organisms growing on those plates. The gas profile would then be variable throughout the incubator, which may have adverse effects on strict aerobes (Jarvis 2008).

The final group of errors is associated with colony counting accuracy. In the absence of an automated colony counter, an increase in plates and occurrences of colonies on the plates have been shown to affect the efficiency of plate counting, often resulting in only estimate counts being derived (ICMSF 1978). A larger number of colonies present on a plate may also coalescence over time, which would cause fewer colonies being counted than should have been reported (Hedges, Shannon & Hobbs 1978), affecting the final CFU. It also needs to be noted that batch variation and supplier specifications could also have an influence on selectivity outcomes of media.

Due to these restrictions and the possibility of using the two suggested methods inter-changeably, the methods needed to provide similar or proportionally similar results if tested under controlled laboratory conditions. Thus the purpose of this study was to determine the

correlation between enumeration using the conventional spread plate technique and the quarter plate method.

Materials and Methods

Quarter Plate Method

Preparation of the quarter plate occurs similar to the conventional spread plate method of preparation. After preparation and setting of the media the plate gets divided either virtually with a permanent marker on the bottom of the plate or physically through use of sterile dividers to indicate the borders of the respective dilutions. A sixty-sixtieth of a millilitre (15µl) volume from the respective dilution series of the sample is spread plated. The CFU's for the quarter plates are determined as follows:

Equation 1: Quarter plate formula used to convert the quarter plate counts into colony forming unit counts.

$$CFU = \frac{(n \times 66 \times (m + d) \times c)}{(1.1 \times m)}$$	**CFU** – Colony Forming Units, given as CFU/g of sample **n** – Count obtained from either one or two consecutive dilutions **66** – factor used to multiply 15µl to achieve ±1ml **m** – mass of the sample **d** – volume of diluent used to macerate **c** – lowest dilution series used for counting (dilution with the most colonies) **1.1** – used when two consecutive dilutions were counted (1 is used when only one dilution count is used)

This equation in its use refers to the counts obtained from two consecutive dilutions or a single dilution, if consecutive numbers were not obtained (n); the factor required to multiply and compare the 15µl aliquot with an aliquot of 1ml (66); the mass of the sample (m); the volume of diluent used to macerate the sample (d); the lowest dilution series used for counting the colonies (c); and a factor of 1.1 used when two consecutive dilutions or 1 when a single dilution was used to count the CFU's.

Preparation of inoculums and comparative plates

Pure cultures of both *Escherichia coli O157:H7* (ATCC 35150) and *Staphylococcus aureus* (NCTC 10654, Health Protection Agency Culture Collections, London, UK) were grown in a solution of Tryptone Soy Broth (Biolab, Wadeville, RSA) for 3.5 hours and 12 hours respectively at 37°C. The variable incubation times were chosen as to ascertain whether the numbers could have been impacted by the phase of growth.

A dilution series of the suspensions were prepared using 0.1% buffered peptone water (BPW) (Oxoid, Basingstoke, UK) after which 0.1ml and 15µl aliquots of the dilutions were plated onto their corresponding respective nutrient agar plates as well as Baird-Parker agar (Baird-Parker 1962; Baird-Parker 1963) for the *S. aureus* culture. *E. coli O157:H7* plates were incubated at 37°C for 24 hours in and *S. aureus* plates at 37°C for 48 hours. Thus a non-selective and a selective media were used to determine the CFUs of grown, pure cultured *S. aureus* using the two different methods.

A mixed culture analysis was also done whereby a 10g minced beef sample from a modified atmosphere packaged retail pack was placed along with 90ml 0.1% sterile BPW in a stomacher for 2min. A serial dilution was then performed in a similar manner to that mentioned above and plated onto Nutrient agar (Oxoid), CFC supplemented Pseudomonas agar (Oxoid) and MRS agar (Oxoid). Both the Nutrient and Pseudomonas plates were incubated at 25°C for 72 hours while the MRS plates were incubated at 30°C for 72 hours.

Statistical analyses

The averages as well as the standard deviations (SD) concerning the CFU counts were determined using Excel 2007 (Microsoft Corporation), with the differences in average CFU also determined by implementing Excel 2007.

Results

Compared with the conventional spread plate, the quarter plate consistently over-estimated the CFU counts regardless of the organism or media used for comparison (Table 1).

Table 1: Colony forming units (CFU) per ml count averages, standard deviation and the difference obtained during the comparison of the normal and the quarter plate methods on nutrient agar (n=3) with pure cultures.

Organism	Normal plate		Quarter plate	
	Avg. Log CFU/ml	±SD	Avg. Log CFU/ml	±SD
E. coli O157:H7	7.84	0.13	8.80	0.13
S. aureus	8.79	0.09	9.76	0.14
Average	**8.32**		**9.27**	
CFU Difference			**0.96**	**0.08**

The counts done on the nutrient agar plates with both the pure cultures of *E. coli* O157:H7 and *S. aureus*, resulted in an average difference between the two methods being 0.959±0.08 log in favour of the quarter plates, which indicates that the quarter plate method over-estimates the CFU counts, when compared to the spread plate method being used as the standard, during an experiment to the magnitude of 0.959 log (Table 1). When comparing the CFU averages that were obtained for *S. aureus* from the Baird-Parker agar, the average difference was found to be 0.844±0.12 log (Table 2), again in favour of the quarter plate. Finally the mixed culture meat sample analysis done with the aerobic plate counts, pseudomonads counts and the Lactic acid bacteria counts fluctuated in their overall difference between 0.855-0.965 with the average and standard deviation being 0.927±0.063 log (Table 3).

Table 2: Colony forming units (CFU) per ml count averages, standard deviation and the difference obtained during the comparison of the normal and the quarter plate methods on Baird-Parker agar (n=5) with a pure culture.

Organism	Normal plate		Quarter plate	
	Avg. Log CFU/ml	±SD	Avg. Log CFU/ml	±SD
S. aureus	8.89	0.05	9.74	0.08
CFU Difference			**0.84**	**0.12**

Table 3: Colony forming units (CFU) per ml count averages, standard deviation and the difference obtained during the comparison of the normal and the quarter plate methods for Aerobic plate count (n=3), Pseudomonas spp. (n=5) and Lactic acid bacteria (n=8) from a mixed cultured meat sample.

Organisms	Normal plate		Quarter plate	
	Avg. Log CFU/ml	±SD	Avg. Log CFU/ml	±SD
APC Day 4	7.86	0.08	8.71	0.01
Difference			**0.86**	**0.07**
Pseudomonads Day 1	3.86	0.04	4.97	0.09
Pseudomonads Day 4	7.74	0.11	8.55	0.06
Difference			**0.96**	**0.16**
LAB Day 1	3.37	0.33	4.36	0.23
LAB Day 4	4.51	0.10	5.41	0.10
Difference			**0.97**	**0.07**
Total CFU Difference			**0.93**	**0.06**

Discussion

This study attempted to determine the validity of using the quarter plate method in general lab and routine analyses work as both an alternative and an aide to that of conventional

spread plating on a variety of media sources. It was consistently found that the quarter plate technique over-estimated the total CFU counts regardless of media type or culture type. This log difference effect ranged from a difference of 0.84±0.12 to 0.97±0.07 between the different media and cultures. This discrepancy has a significant influence for both research and industry that may use both these two techniques, which in this light, would have resulted in uneven comparisons.

The repeated experiment on the different agar types gave slightly deviating averages which might indicate that the type of media, and in this regards the type of organism it differentiates towards as pertaining to its growth, may also have an influence on this dissimilarity. It is of the authors' opinions that although a study to determine a correlation between agar specificity and the average difference could be conducted, such a study would not be conclusive to use as a benchmark for conversion of obtained data. This is due to the fact that all the possible factors, as stated above, could also be considered to be influential on the results of both the quarter and full plate methods.

Thus it should be stressed that the values obtained within this study should not be regarded as an absolute and that if the quarter plate method were to be used as a substitute to the conventional full plate method, an initial test study should be conducted before hand. This would create the necessary benchmark for the researcher, equipment and media used as to obtain accurate results that could then be compared with results obtained from normal plating procedures. For internal comparison however, no benchmarking would be required.

Quarter plating is a comparable enumeration method in quality and precision to more conventional methods, although it should be noted that it cannot be used in direct comparison. Although it does require a 10^{-1} and 10^{-2} conventional spread plate to be done in conjunction if detection below 100 CFUs is required, it can stand on its own as an alternative method where a complete dilution series is required in the midst of financial and time constraints.

Chapter 4: Effect of modified atmospheres and natural background spoilage organisms on the growth of inoculated *E. coli* O157:H7 in minced beef.

Abstract

The aim of this study was to determine the effect of differing atmospheric packaging conditions and the presence of innate bacterial populations, determined by aerobic plate, *Pseudomonas* spp., lactic acid bacteria and Enterobacteriaceae counts, on high and low levels of 10^2 and 10^5 of *E. coli* O157:H7 inoculated onto minced beef. It was seen that although both storage days and the type of atmospheric packaging accounted for a large percentage of the variance found within the growth of *E. coli* O157:H7, only the inoculation level was significant (p-value <= 0.05).

From the innate bacterial groups tested for, the Enterobacteriaceae family seemed to have the largest effect on the growth of *E. coli* O157:H7. It must be emphasized however that in all cases tested, none of the factors resulted in the elimination of *E. coli* O157:H7 from the minced beef. In most cases the growth was either hindered or numbers decreased slightly. Therefore, it is evident from the research that if *E. coli* O157:H7 is found in the food, there is a risk of presence upon consumption.

Introduction

Ruminants, such as cattle and sheep, are believed to be the main source of *E. coli* O157:H7, with cattle however believed to be the major contributor due to the large numbers of food borne infections being attributed to bovine products. The growth and survival of *E. coli* O157:H7 on raw minced beef is affected by various different factors including temperature, presence and influence of competitive microbiota and the packaged gaseous environment (Barrera et al. 2007). Although the general assumption is that *E. coli* O157:H7 would not be able to multiply at temperatures below 7°C and thus one of the reason why retailers keep their meat products chilled below 5°C, certain opportunities for growth in the form of temperature abuse or temperature fluctuations could arise to provide an opportunity for

growth of the pathogen. Similarly a change in the gaseous environment, intended to reduce spoilage and increase shelf life, could also serve as an opportunity for a facultative anaerobe such as *E. coli* 0157:H7, to outperform normally dominant aerobic spoilage bacteria such as *Pseudomonas* species. These opportunities for growth however would also serve the same function for most of the microbiota present on the beef with both temperature and environmental atmosphere variations affecting growth (Barrera et al. 2007).

So although extensive studies have been done on the effects of both temperature and modified atmosphere packaging on pathogens, the effect of competitive microbiota, has not been sufficiently studied. In this regard, this study investigates the importance of understanding the influence that the naturally present spoilage microbiota has on the growth and survival of *E. coli* 0157:H7 in varying low and high levels in minced beef intended for retail consumption within 3 packaging types consisting of 100% CO_2 gas, commercial gas mixture ($O_2=60\%/ CO_2=30\%/ N_2=10\%$) and no atmosphere modification in the form of PVC overlay packaging.

Materials and Methods

Acquisition of samples

Packaged samples of 500g lean beef mince, produced from 90, 95 and 98% visual lean beef trimmings to ensure correct fat percentage of $10\pm1\%$ fat, were obtained from a fresh meat processor, Johannesburg, RSA on three different occasions, packaged and transported at <5°C to Department Food Science, University of Pretoria, Pretoria, RSA.   100% CO_2 and commercially used MAP mix ($O_2=60\%/ CO_2=30\%/ N_2=10\%$ [Mix]; allowed variation of 5% per gas) packs were prepared and packaged at location.

Before gas flushing and sealing, two ethanol sterilized rectangular polystyrene splitters, measuring nearly the inner dimensions of the pack, were placed along the breadth of the packs and mince blocks to effectively split the block into 3 segments of roughly equal size. This allowed the gaseous environment to be unrestricted in its diffusion but also allowed an effective and definitive separation of three mince blocks in each pack.

Control PVC packs were repacked from additional acquired Mix packs in the experimental meat factory in the department. The mix packs were sterilely cut open and a 100g of mince

meat was transferred onto sterilised food grade polystyrene trays and PVC overlay wrapped in the meat processing unit of the Food Science Department.

Preparation of the inoculums

A single colony of *E. coli* O157:H7 (ATCC 35150) was taken from a Sorbitol MacConkey agar (Oxoid, Southampton, UK) plate supplemented with Cefixime Tellurite (Oxoid), inoculated into a prepared Tryptone Soy Broth (Biolab, Midrand, RSA) solution and incubated at 37°C for 18-24 hours to ensure that the culture had entered the stationary phase in its growth cycle. Using the MacFarland standards (McFarland 1907; PML microbiologicals 2001) approach to obtain a dilution of 10^8, two inoculation dilutions of 10^5 and 10^2 were prepared using a 0.1% (w/v) buffered peptone solution (Oxoid) as diluents, along with a control solution containing only a 0.1% buffered peptone solution (Oxoid).

To ensure the accuracy of the MacFarland standard adjusted dilutions, each inoculation dilution that was used to inoculate the minced beef with, was plated out onto Nutrient agar and incubated for 24 hours at 37°C, to allow optimum growth of the *E. coli* O157:H7, after which each dilutions' CFUs were counted and recorded.

Inoculation of samples

One third of the mince block in the CO_2 packs (Figure 3) was only inoculated with sterile buffered peptone water (BPW) and served as control, another 1/3 of the mince block was inoculated with a 10^2 dilution of *E. coli* O157:H7 and the remaining 1/3 of the mince block was inoculated with a 10^5 dilution. Sequence of inoculation was randomized for each pack. The inoculation procedure was repeated for the mix packs.
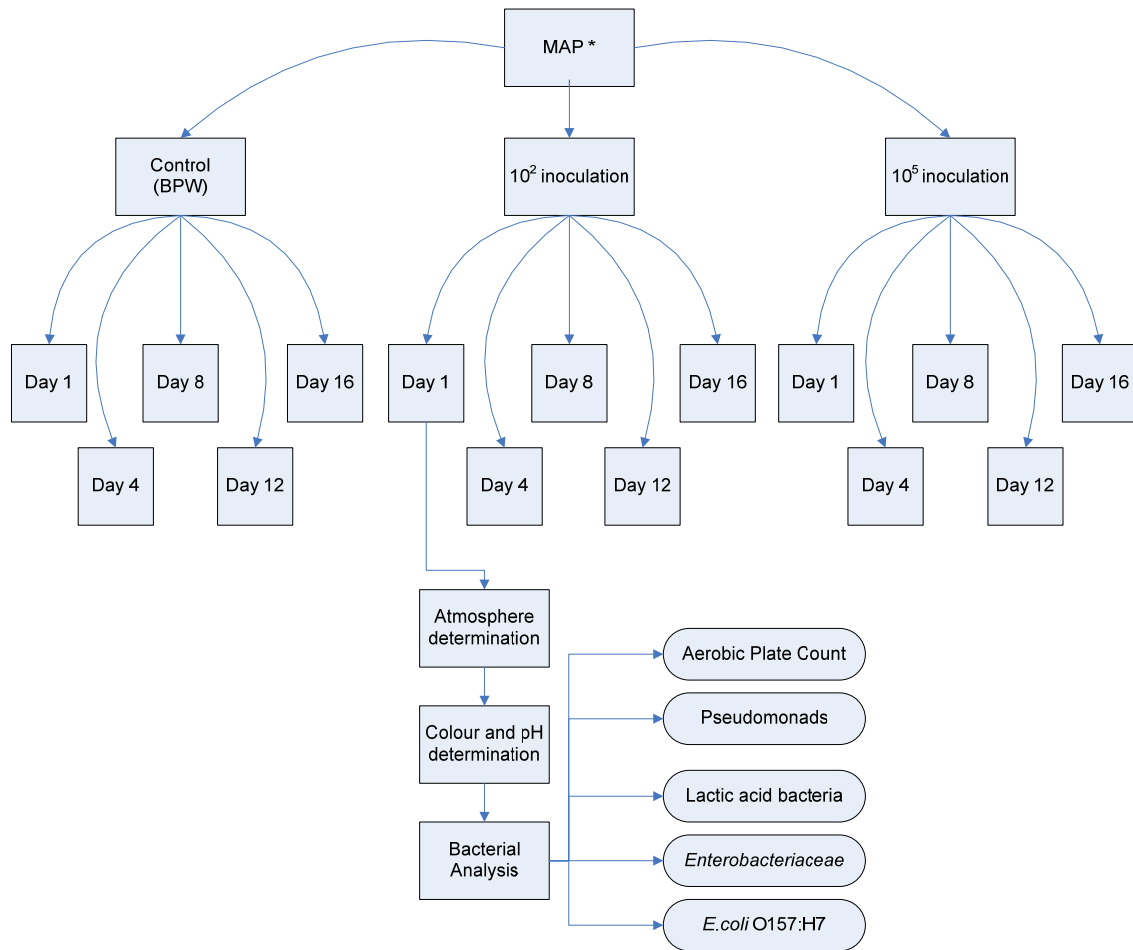
Figure 3: Flow diagram of the process of atmosphere, colour, pH and bacterial numbers determination in minced beef under modified atmosphere packaging. (* MAP interchanges for both the commercial mix [O_2=60%/ CO_2=30%/ N_2=10%] and the 100% CO_2 packs) (BPW – 0.1% Buffered Peptone water)

As the PVC packs were repackaged on location, each respective testing day had three separate PVC packs assigned, inoculated either with sterile BPW or with one of the possible *E. coli* O157:H7 dilutions series (Figure 4). This provided three independent PVC packs.
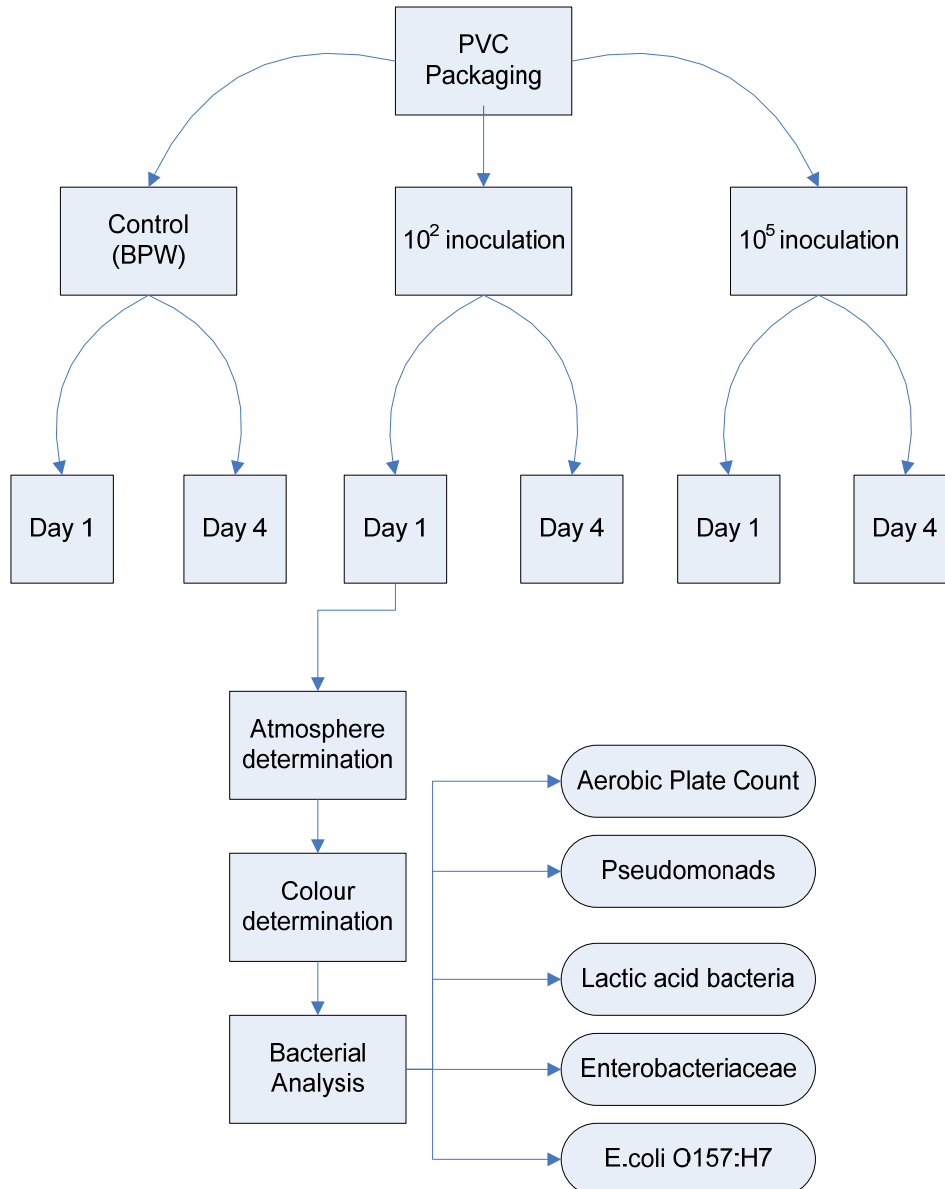
Figure 4: Flow diagram of the process of atmosphere, colour, pH and bacterial numbers determination in minced beef packaged in PVC overlay packs. (BPW – 0.1% Buffered Peptone water)

The inoculation of the packs with the three different dilutions had to occur in such a way as to ensure that the gaseous environment within the packs was not significantly modified during the procedure by means of leakage of the gasses through the inoculation site. This was accomplished using sterile 10-ml syringes and sterile disposable 0.90x38mm needles to penetrate the packs and introduce the respective dilutions onto the meat's surface. The area around the needle insertion point in the pack was swabbed with 100% ethanol prior to inoculation.   The needle was then inserted through the side of the minced meat pack and 1ml

of the specific dilution was introduced onto the surface of the minced beef at a definitive point, which was then marked to aid with later sampling. Gas leakage was minimized through the use of sticky tape that was placed over the needle insertion point immediately after the needle was removed, following inoculation.

Storage of inoculated packs

A total of 16 inoculated packs per repetition were prepared for storage. These included five packs for the five test intervals within 16 days, every 4 days, respectively for both of the MAP packs and six packs for the PVC control which included three different inoculation packs per day, both for day 1 and 4.

The inoculated packs were stored refrigerated at $5\pm2°C$ to ensure that the commercial conditions for storage and display of the packs, as in their respective stores, prevailed. Upon maturation of storage time, a 100% CO_2, commercial Mix and the three differently inoculated PVC packs were removed from storage and sampled. Microbiological analyses were performed on all three inoculations sites in the commercial Mix and 100% CO_2 packs for days 1, 4, 8, 12 and 16 while the analyses on the inoculations sites for the PVC overlay packs were only performed for days 1 and 4.

Gas, pH and Colour analyses after storage

The percentage O_2, CO_2 and balance, the remainder of gas, was measured (Systech Instruments Ltd, Oxon, UK) through an analysis of an atmosphere sample taken from each of the MAP packs. The meat's colour was determined using a Chroma meter CR-400 (Konica Minolta Sensing Inc., Osaka, Japan). The Hunter Lab values (L*, a* and b*) were then used to determine the psychological colour saturation value $S = (a^2 + b^2)^{1/2}$ which provides a measure for the red colour intensity (MacDougall 1977). pH for all the samples was determined (Testo AG, Lenzkirch, Germany) at the centre of the meat block straight after the stomacher sample was taken.

Bacterial analyses of the inoculation sites after storage

All samples were spread plated using either the normal full or the quarter plate techniques. Samples, 10g, from each respective treatment on the indicated days, were stomached for two

minutes with 90ml of 0.1% (w/v) sterile buffered peptone water. The suspensions were serially diluted and plated onto Nutrient Agar (Oxoid), supplemented *Pseudomonas* agar (Oxoid), MRS Agar (Oxoid), supplemented Sorbitol MacConkey Agar (Oxoid) and Violet Red Bile Glucose Agar (Oxoid). Aerobic plate count (APC) was determined with Nutrient Agar using quarter plate (Chapter 3: Comparison study between the use of Quarter and Full Plate methods for microbiological enumeration).

Samples were plated out in duplicate with the plates being incubated for 72 hours at 25°C before enumeration. Enumeration of *Pseudomonas* spp. was accomplished using a *Pseudomonas* agar base supplemented with a *Pseudomonas* CFC (Centrimide, Fucidin, Cephalosporin) selective agar supplement, SR0103 (Oxoid). Plates were incubated at 25°C for 72 hours. MRS agar was used to enumerate the lactic acid bacteria and was incubated at 30°C for 72 hours after which enumeration occurred.

To enable the enumeration of *E. coli* O157:H7, Sorbitol MacConkey agar, a selective media for *E. coli* O157:H7 was reconstituted with the addition of Cefixime-Tellurite, a selective supplement for *E. coli* O157:H7, according to the procedure set out by Oxoid CM0813 and SR0172. These plates were incubated for 24 hours at 37°C. No diagnostic confirmations were performed and as such all counts should be deemed presumptive *E. coli* O157:H7. Enterobacteriaceae was incubated and enumerated using Violet Red Bile Glucose agar (VRBGA) that was incubated for 24 hours at 37°C (CM0485, Oxoid).

The analysis for APC, *Pseudomonas*, lactic acid bacteria and Enterobacteriaceae all contributed towards the determination of the natural background organisms present in the samples.

Statistical analyses

Analysis of Variance (ANOVA) and a Multiple Regression (GenStat® 2009) using a stepwise addition were performed. ANOVA was used to determine whether the main effects, inoculation number; MAP and storage had a statistically significant effect ($P<0.01$) on the growth and survival of the test organism, *E. coli* O157:H7, APC, *Pseudomonas* spp., LAB and Enterobacteriaceae.

The ANOVA was constructed in two different manners since the PVC packaging analyses only ran for days 1 and 4 while the other two packaging samples ran the entire 16 days. Thus the first analysis was done without taking the PVC packaging values into consideration, allowing an ANOVA test to be determined over the entire 16 days of the experiment. The second analysis included the PVC packaging values, but this however resulted in only the values on days 1 and 4 from the other two packaging sets being incorporated into the analysis. Using the ANOVA values, percentage accounted variation or the amount of change brought about in the counts for a specific bacterial population by a specific factor, was also determined to identify the greatest affecting forces. This is indicated by %SS.

Multiple Regressions using a stepwise addition process was employed whereby the possible predictor variables, including the independent variables such as MAP and the dependant variables such as the obtained bacterial counts, were added to the analysis periodically. In determining the effect of the natural background organisms on *E. coli* O157:H7, the stepwise regression analysis was split into two analyses, determined by their different factors. The first one (primary analysis) evaluates the influence of the background organisms per specific packaging type, excluding the other independent factors namely storage days and inoculation type. The second (secondary analysis) focuses on the effects that both the dependent and independent factors have in combination on *E. coli* O157:H7 in the specific packaging type. The multiple regression was conducted to try and establish a relationship between the various factors that would best describe the observed numbers and growth of *E. coli* O157:H7 and in doing so formulate a simple regression equation that would account for the largest impact factors (StatSoft 2009).

Preparation of inoculums, inoculation and storage of samples for use with the IVIS system

Spread experiment

Two sample trials consisting of 6 packs (Figure 5) each were prepared for the experiment, consisting either of a MAP packaged minced beef sample or a PVC overlay packed minced sample, which did not have any environmental modification. All the minced meat samples were acquired from a local producer and packer.

A genetically modified strain of *Escherichia coli* - Xen 14, possessing a stable copy of the *Photorhabdus luminescens LUX* operon on its bacterial chromosome, derived from the parental strain *E. coli* WS2572 (Weihenstephan Culture Collection, Munich Technical University, Freising-Weihenstephan, Germany) was grown in a buffered peptone broth containing 30µg/ml Kanamycin.   The added Kanamycin acted as a selective agent as the modified *E. coli* strain also possessed Kanamycin resistance (Kadurugamuwa et al. 2003).

Each minced meat pack was inoculated with 0.5ml of the broth containing the modified *E. coli* strain in the concentration of 10^8 CFU/ml, similarly to what was done in Chapter 4 (Inoculation of samples). The volume of 0.5ml was chosen as higher volumes resulted in the inoculants flowing off the minced beef samples and collecting in the package' drip tray. 0.5ml also allowed for a high enough number of *E. coli* bacteria to be present to enable clear fluorescent imaging.

Both sets of packs were stored at 30°C over a period of 48 hours with interval image analysis being done at hour 0, 4, 8, 12, 24, 36 and 48. Images were taken at an exposure of 60s were possible, except where it resulted in over-exposure in which case exposure was reduced to 10s.
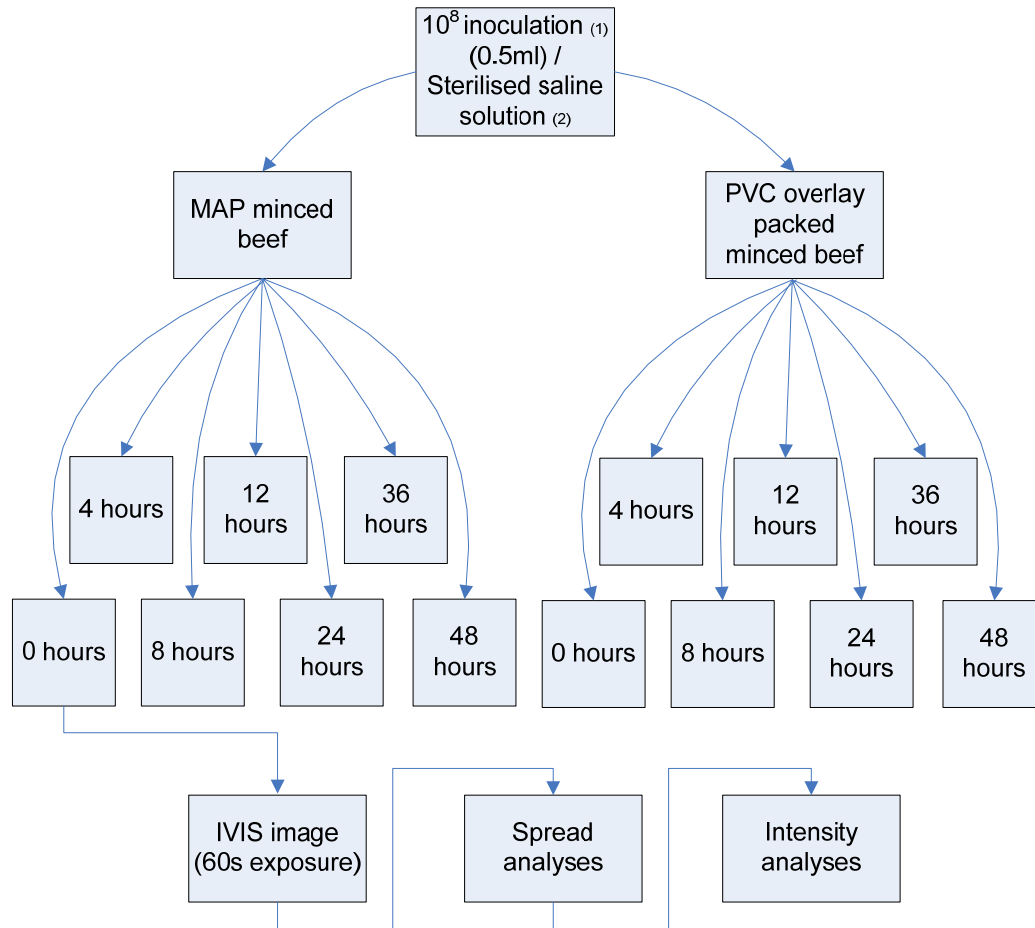
Figure 5: Flow diagram of the spread experiment over the two types of packaging with IVIS images taken hourly for spread and intensity analysis

Intensity curve – experiment

The same modified *E. coli* strain as mentioned above was grown and used with the spread experiment (Figure 6). Samples of minced beef were aseptically placed into sterile Petri dishes while a series of Nutrient Agar plates (Oxoid), each containing 30µg/ml Kanamycin, was also prepared.

```
               ┌──────────────────┐
               │  10¹ - 10⁸       │
               │  inoculation     │
               │  (0.5ml)         │
               │  E. coli         │
               └──────────────────┘
          ┌─────────────┴─────────────┐
┌──────────────────┐         ┌──────────────────┐
│  Minced beef in  │         │  Nutrient agar   │
│  petri dish      │         │  (30mg Kanamycin)│
└──────────────────┘         └──────────────────┘
          └─────────────┬─────────────┘
               ┌──────────────────┐
               │  IVIS image      │
               │  (60s exposure)  │
               └──────────────────┘
                        │
               ┌──────────────────┐
               │  Intensity       │
               │  analyses        │
               └──────────────────┘
```
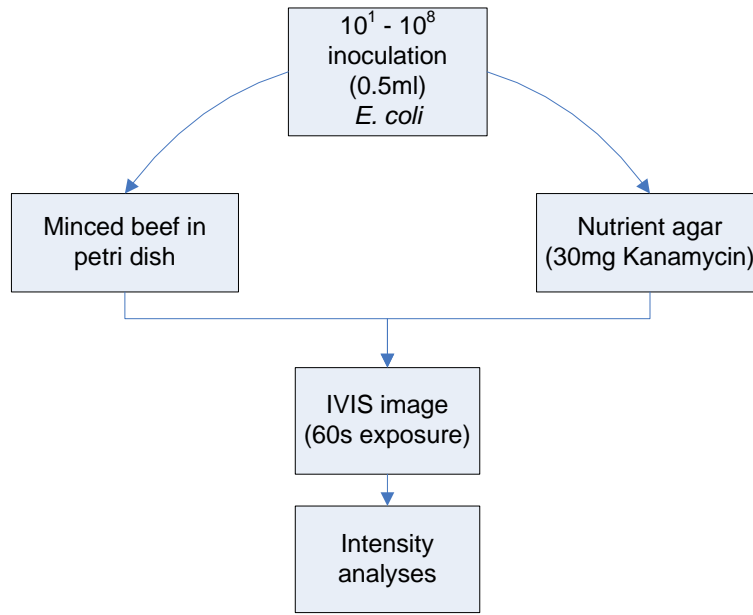
Figure 6: Flow diagram for the intensity-curve experiment where an intensity to count correlation was sought from minced meat and nutrient agar.

A dilution series of 10^{-1} to 10^{-7} of the modified *E. coli* strain was prepared and inoculated onto both the minced meat Petri dishes and the Nutrient Agar plates. This was done with four replicates per plate type.

Analysis

During the analysis of the image data from the samples, a region of interest (ROI) was specified and overlay the image to serve as a border for detection of photons. In the spread experiment, the ROI changed in size, depending on the migration of the *E. coli* strain through the minced meat pack. During the intensity curve experiment, the ROI remained constant since the outline of the Petri dishes were used as the edge. The total, average and max counts as determined through the IVIS system were recorded for both experiments.

Results

Bacterial quality of fresh minced beef meat under MAP and PVC overlay packaging

Primary bacterial analyses indicated that the initial APC counts for all three packaging methods averaged between 5.10 and 5.48 $\log_{10}$ CFU/g with a mean and standard deviation of 5.20±0.11 $\log_{10}$ CFU/g while the *Pseudomonas* and LAB counts had a mean and standard deviation of 4.41±0.09 $\log_{10}$ CFU/g and 3.90±0.07 $\log_{10}$ CFU/g respectively. The

Enterobacteriaceae counts varied the most from all the innate species at a maximum initial difference of 0.67 $\log_{10}$ CFU/g seen between the packaging types and a mean and standard deviation of 3.17±0.20 $\log_{10}$ CFU/g.

The initial *E. coli* O157:H7 numbers analysed at the different concentration sites of inoculation, as expected, were not as close in number as was seen with all the innate microbes that were analyzed. What was interesting though was the initial high numbers seen at the control site and the lower than expected numbers, when compared with the inoculation fluid, seen at the 10^5 inoculation site. For the control, 10^2 and 10^5 inoculation sites, irrespective of packaging type, the respective mean and standard deviation was 2.20±0.14 $\log_{10}$, 2.46±0.24 $\log_{10}$ and 2.98±0.11 $\log_{10}$.

As can be noticed (Figure 7), the APC numbers for both the 100% CO_2 and the commercial Mix packs were on par, when comparing enumerated counts, with each other at initial analysis while later analysis showed a greater difference in the enumerated counts arising as storage days proceeded, as can clearly be seen on day 16, with the 100% CO_2 pack indicating lower total aerobic plate counts.

Additionally (Figure 8) the same pattern can be observed as to that of Figure 7, with the exception of the PVC overlay packs already indicating a substantially higher count compared to the other packaging types at day four. On the analyses including all the APC data, storage days was the only factor that had an effect (p = 0.013) while also accounting for 33.7% of the variation in the growth.

Figure 7: Aerobic plate counts, $\log_{10}$ CFU/g, per inoculation level of *E. coli* O157:H7 for the 100% CO_2 and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)
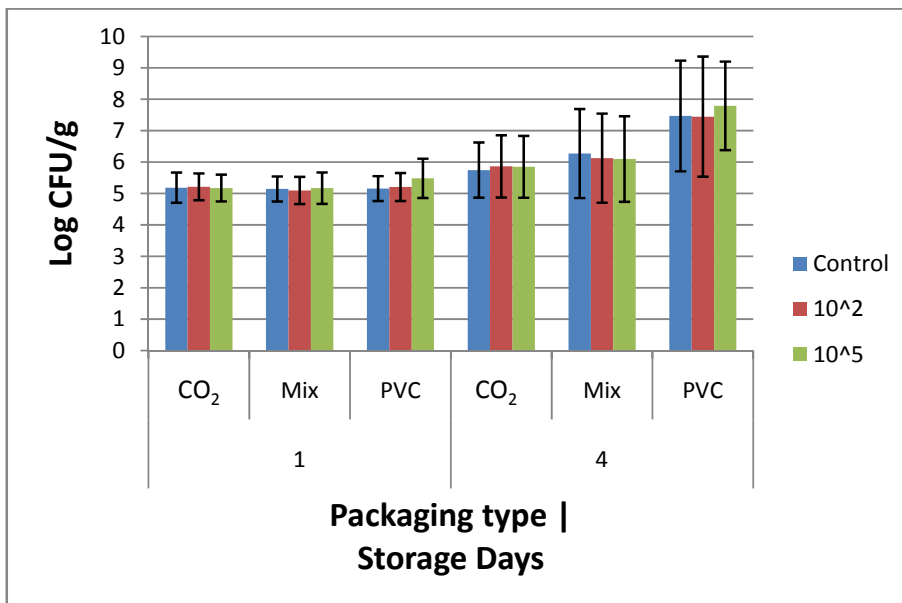
Figure 8: Aerobic plate counts, $\log_{10}$ CFU/g, per inoculation level of *E. coli* O157:H7 for the 100% CO_2, commercial mix and PVC overlay packs over 4 storage days. (Temp. 5°C, n=3)

For the analyses spanning 16 days (Figure 9), the highest percentage accounted variation (20%) in the Enterobacteriaceae counts were observed from the storage days. When the

analyses was ran over the 4 days, including the PVC pack data, both storage days (p = 0.032) and inoculation levels (p = 0.002) was found to be significant, accounting for 32.8% and 2.6% of the variation respectively.
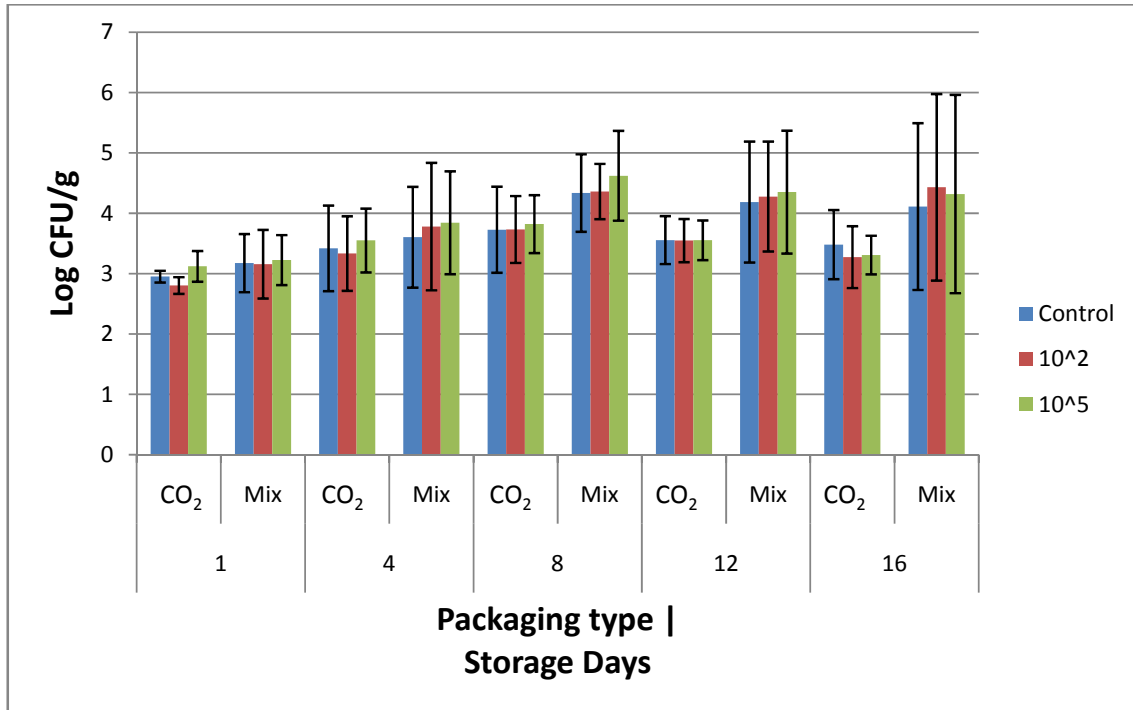
Figure 9: Enterobacteriaceae counts, $\log_{10}$ CFU/g, per inoculation level of *E. coli* O157:H7 for the 100% CO$_2$ and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)

Comparing the growth of the *Pseudomonas* sp. between the two different modified atmosphere packaging types (Figure 10), it was found that the largest factor affecting these organisms was the gaseous atmosphere (p = 0.018) accounting for 20.4% of the accounted variation in the counts. Storage days and inoculation levels were only found to be significant (p = 0.23; p=0.008 respectively) when the counts from the PVC overlay packs were also considered in the 4 day storage analysis and elucidated to 22.1% and 0.1% of the accounted variation.
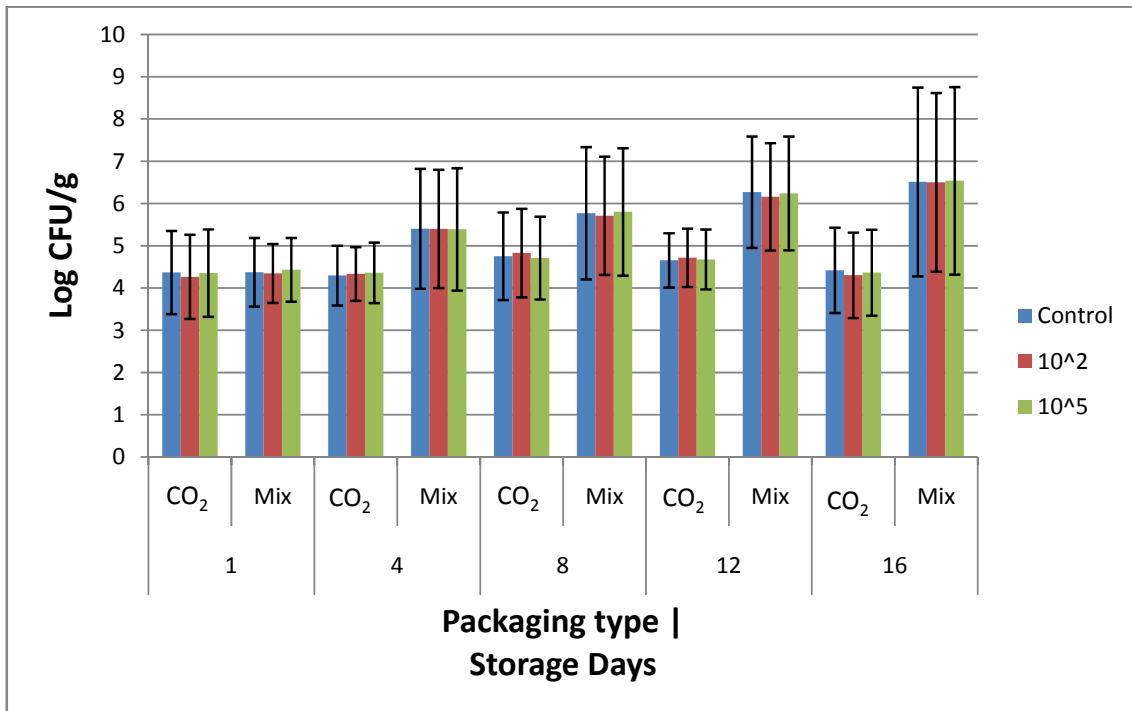
Figure 10: Pseudomonad counts, $\log_{10}$ CFU/g, per inoculation level of *E. coli* O157:H7 for the 100% CO_2 and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)
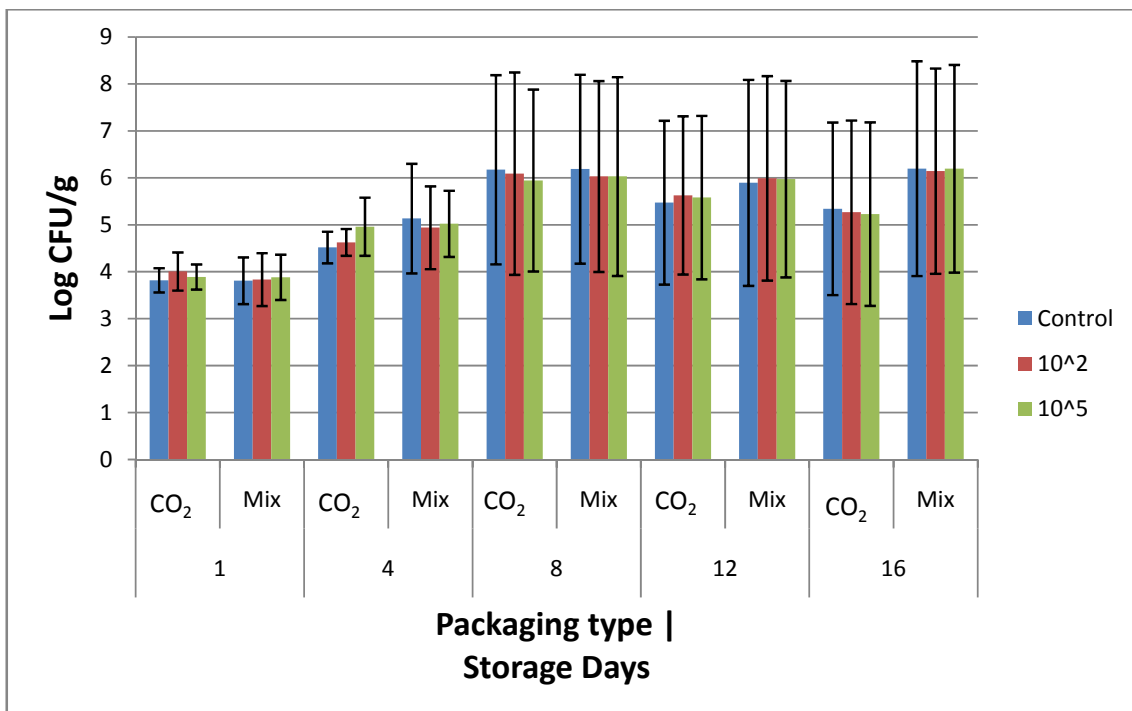
Figure 11: LAB counts, $\log_{10}$ CFU/g, per inoculation level of *E. coli* O157:H7 for the 100% CO_2 and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)

When the LAB values were compared between the two MAP packs (Figure 11), none of the tested factors were found to have a significant influence on the counts obtained. When the values of all three packs were however compared over the 4 days, storage days were found to be significant (p = 0.004) and influential (%SS = 53.2%) in affecting the obtained counts.

Influence of the independent variables on the growth and survival of *E. coli* 0157:H7

An ANOVA (Table 4) on all the values for *E. coli* O157:H7 (Figure 12), excluding those obtained from the PVC overlay packs, over the 16 days of storage, indicated that both the inoculation level (p < 0.001) and the combined effect of storage days and inoculation level (p = 0.025) was significant, with storage days nearly showing statistical significance (p = 0.052). The two effects however were, although significant, found to only account for a small percentage of the accounted variation at 4.2% and 1.5% respectively.

If the PVC data (Figure 13) was included in the analyses (Table 5) for storage days 1 and 4, again only inoculation level and a combination with storage days was found to be significant (p < 0.001; p = 0.045 respectively) with a low accounted variation of 9.7% and 2.0%.

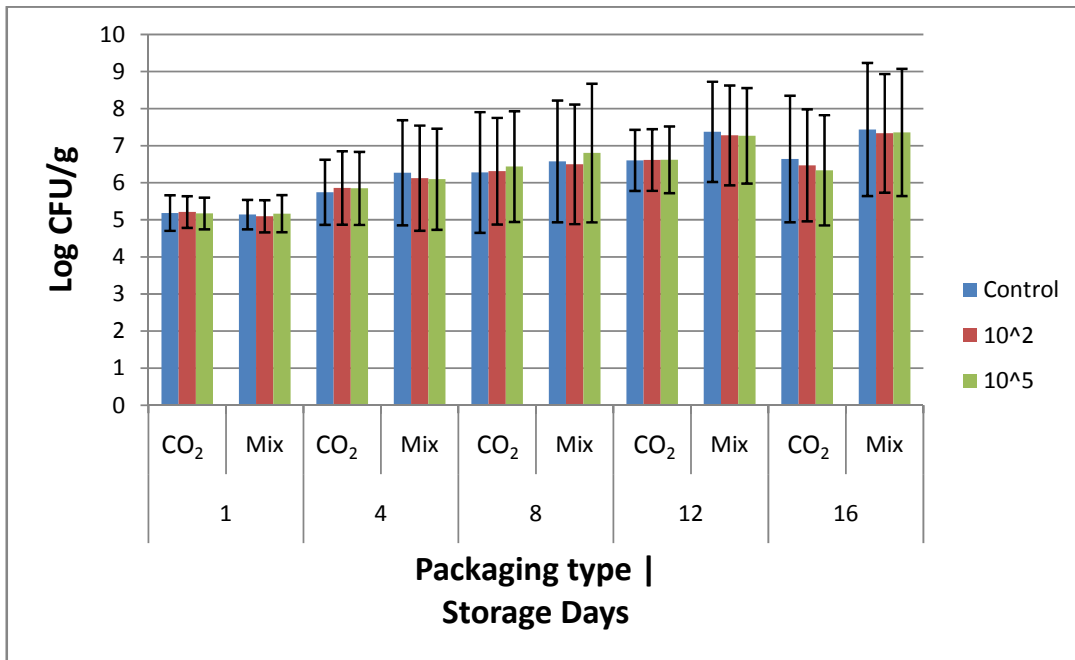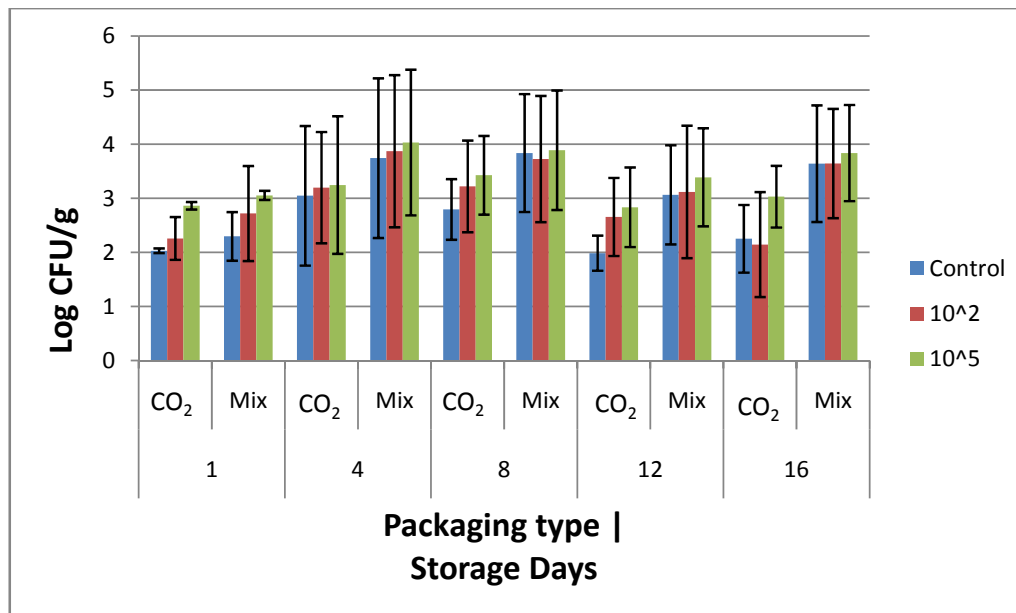**Figure 12:** *E. coli* **O157:H7 counts, $\log_{10}$ CFU/g, per inoculation level of *E. coli* O157:H7 for the 100% CO_2 and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)**
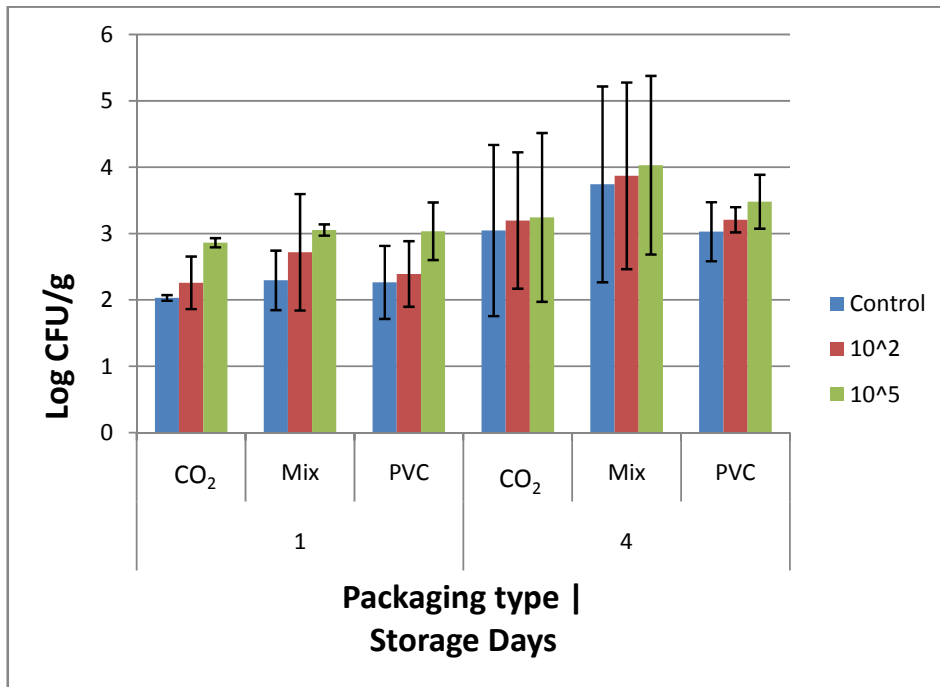
Figure 13: *E. coli* O157:H7 counts, $\log_{10}$ CFU/g, per inoculation level of *E. coli* O157:H7 for the 100% CO_2, commercial mix and PVC overlay packs over 4 storage days. (Temp. 5°C, n=3)

Table 4: P-values and %SS of split-plot ANOVA (inoculation as sub-plot) for *E. coli* O157:H7 counts, excluding the PVC overlay pack's counts, over the 16 days of storage. %SS added to indicate accounted variation.

Treatment	Degrees of Freedom	P-value	%SS
Day	4	0.293	47.92
Gas	1	0.052	38.66
Inoculation	2	<.001	11.27
Day.Gas	4	0.932	7.28
Day.Inoculation	8	0.025	3.93
Gas.Inoculation	2	0.107	0.90
Day.Gas.Inoculation	8	0.241	2.09

Table 5: P-values and %SS of split-plot ANOVA (inoculation as sub-plot) for *E. coli* O157:H7 counts, including the PVC overlay pack's counts, over the 4 days of storage. %SS added to indicate accounted variation.

Treatment	Degrees of Freedom	P-value	%SS
Day	1	0.069	36.83
Gas	2	0.594	8.96
Inoculation	2	<.001	9.75
Day.Gas	2	0.865	2.36
Day.Inoculation	2	0.045	1.95
Gas.Inoculation	4	0.904	0.25
Day.Gas.Inoculation	4	0.857	0.32

Influence of the microbial community singularly and in conjunction with the independent variables on the growth and survival of *E. coli* 0157:H7

The first statistical analysis for *E. coli* O157:H7 (Table 6) was where all independent factors were excluded for the purpose of determining which of the four bacterial species that were tested for, has the greatest impact on the growth of *E. coli* O157:H7. The largest single bacterial effect was seen from the Enterobacteriaceae group which accounted for 29.7% of the variation in *E. coli* O157:H7. Overall, if all the bacterial species tested for were combined, they accounted for 57.2% of *E. coli* O157:H7's variation.

Table 6: Stepwise regression in response to the adjusted *E. coli* O157:H7 counts, excluding the effects of storage days, inoculation type and type of packaging.

Step	Variable entered	Adjusted r^2	SER	Change P-value	Regression P-value
1	Enterobacteriaceae	0.297	0.763	<0.001	<0.001
2	APC	0.494	0.648	<0.001	<0.001
3	Pseudomonads	0.528	0.625	0.008	<0.001
4	LAB	0.572	0.596	0.002	<0.001

The second statistical analysis (Table 7) involved the effects brought about by all the bacterial species and the independent factors with the exclusion of the packaging type factor. As with the previous table, the four bacterial species were found to be most influential and occupied steps 1 to 4 of the stepwise regression. The combination of storage days*inoculation

level and Pseudomonads*storage days*inoculation level filled in steps five and six, with step six however being seen as non-significant.

Table 7: Stepwise regression in response to the adjusted *E. coli* O157:H7 counts, including the effects of storage days and inoculation type and excluding type of packaging.

Step	Variable entered	Adjusted r^2	SER	Change P-value	Regression P-value
1	Enterobacteriaceae	0.297	0.763	<0.001	<0.001
2	APC	0.494	0.648	<0.001	<0.001
3	Pseudomonads	0.528	0.625	0.008	<0.001
4	LAB	0.572	0.596	0.002	<0.001
5	Day.Inoculation	0.602	0.574	0.007	<0.001
6	Pseudomonads.Day.Inoculation	0.614	0.566	0.061	<0.001

The first packaging type to be statistically analysed was that of the 100% CO_2. When a regression was drawn up to determine the effects of only the background organisms in the CO_2 pack (Table 8), it was found that after three steps involving *Pseudomonas*, APC and Enterobacteriaceae that the accounted variation on *E. coli* O157:H7 was 57.2%. If however, a fourth step was included in the regression by adding LAB, the variation would increase to 60.3% with the regression still being significant but the addition of the fourth step however being insignificant.

Table 8: Stepwise regression in response to the adjusted *E. coli* O157:H7 counts in the 100 % CO2 packs, excluding the effects of storage days and inoculation type.

Step	Variable entered	Adjusted r^2	SER	Change P-value	Regression P-value
1	Pseudomonads	0.065	0.785	0.059	0.059
2	APC	0.295	0.682	<0.001	<0.001
3	Enterobacteriaceae	0.572	0.531	<0.001	<0.001
4	LAB	0.603	0.511	0.054	<0.001

When the independent factors were included (table not shown), significant combinations of *Pseudomonas*, APC and LAB with the inoculation level was seen to account in three steps for 35.2% of the variation in *E. coli* O157:H7.

The primary statistical analysis (Table 9) with the commercial mix packs had Enterobacteriaceae, APC and *Pseudomonas* sp. accounting within three steps for 60.5% of *E.*

coli O157:H7's variation, with all the steps being significant. The secondary analysis saw exactly the same regression being drawn, regardless of the additional independent factors included.

Table 9: Stepwise regression in response to the adjusted *E. coli* O157:H7 counts in the commercial mix packs, excluding the effects of storage days and inoculation type.

Step	Variable entered	Adjusted r^2	SER	Change P-value	Regression P-value
1	Enterobacteriaceae	0.396	0.743	<0.001	<0.001
2	APC	0.481	0.688	0.012	<0.001
3	Pseudomonads	0.605	0.600	0.001	<0.001

When the primary statistical analysis (table not shown) was done on the PVC overlay packs, three steps were added to the regression without a single one being significant. LAB, APC and Enterobacteriaceae accounted for 35% of the variation of *E. coli* O157:H7 in this non-significant regression.

The secondary (Table 10) analysis saw a similar trend with only three of the five steps adding a significant change to the regression analysis, but unlike the primary analysis the secondary analysis did culminate in a significant regression. Steps one and two saw a combination of storage days*inoculation levels and Enterobacteriaceae*storage days*inoculation levels respectively while steps 3 to 5 comprised of LAB, APC and Enterobacteriaceae. The regression accounted for 88.8% of the variation seen within *E. coli* O157:H7.

Table 10: Stepwise regression in response to the adjusted *E. coli* O157:H7 counts for the PVC overlay packs, including the effects of storage days and inoculation type.

Step	Variable entered	Adjusted r^2	SER	Change P-value	Regression P-value
1	Day.Inoculation	0.389	0.434	0.018	0.018
2	Enterobacteriaceae.Day.Inoculation	0.524	0.383	0.082	0.014
3	LAB	0.618	0.343	0.112	0.013
4	APC	0.758	0.273	0.049	0.006
5	Enterobacteriaceae	0.888	0.186	0.023	0.001

Colour analysis

Over the four days of storage (Table 11, Figure 14), it was found that storage days (p = 0.001), packaging type (p = 0.003) and a combination of storage days and inoculation levels

(p = 0.032) affected the colour saturation determination of the minced meat. Inoculation levels on its own did not seem to have any significant effect on the meats colour perception. The two largest influences to the saturation of the meats of the four days were seen to be storage days and packaging type which accounted for 33.6% and 38.1% of the variation.
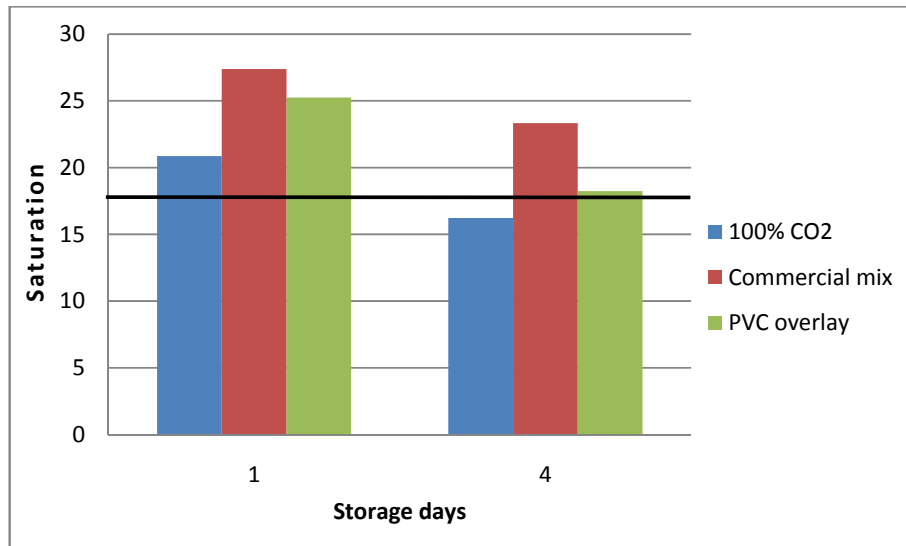
Figure 14: Saturation numbers for both MAP packs and the PVC overlay packs over the 4 days of storage.

(Above line at saturation level 18 depicts the desired level (MacDougall 1977))

Table 11: P-values and %SS of split-plot ANOVA (inoculation as sub-plot) for colour saturation, including the PVC overlay pack's counts, over the 4 days of storage. %SS added to indicate accounted variation.

Treatment	Degrees of Freedom	P-value	%SS
Day	1	0.001	33.63545
Gas	2	0.003	38.0906
Inoculation	2	0.894	0.014768
Day.Inoculation	2	0.032	0.521974

As can be seen (Figure 15), the commercial mix packs maintained their above required saturation up until day 4 after which a steady decline occurred up to date eight. For the 100% CO_2 pack, by the first day of analysis it can be seen that its value, compared to the commercial pack, was much lower but still above the required value. A decrease then

occurred from day one up until day four, after which the values slowly increased up until day 12 when again it was measured and indicated a value above that of the required minimum before decreasing to below 18 at day 16.
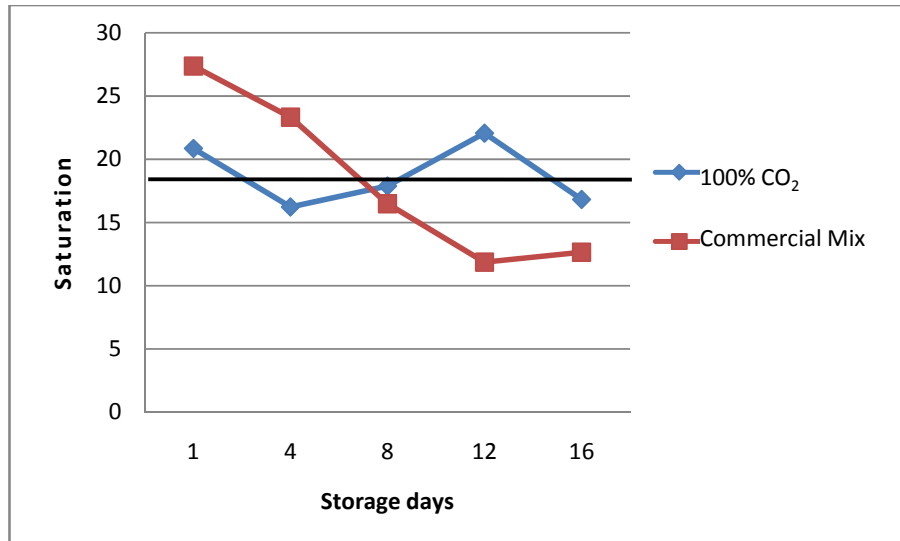
Figure 15: Saturation numbers for both MAP packs over the 16 days of storage. (Above line at 18 depicts desired level (MacDougall 1977))

When the statistical colour analysis was calculated over the 16 days of storage (Table 12) using only the two modified atmosphere packaging types, it was found that only storage days (p = 0.046) and a combination of storage days and packaging type (p = 0.04) significantly influenced the colour determination. These two factors also accounted for 27.3% and 28.6% respectively of the saturation accounted variation determined.

Table 12: P-values and %SS of split-plot ANOVA (inoculation as sub-plot) for colour saturation, excluding the PVC pack's counts, over the 16 days of storage. %SS added to indicate accounted variation.

Treatment	Degrees of Freedom	P-value	%SS
Day	4	0.046	27.31425
Gas	1	0.819	0.123047
Inoculation	2	0.696	0.007886
Day.Gas	4	0.04	28.60992

As can be noticed from (Figure 16), the O_2 percentage in the commercial mix packs saw a significant and systematic decrease over the 16 days of storage, while the CO_2 percentage saw an increase over the same period. The O_2 and CO_2 levels in the 100% CO_2 pack however stayed fairly constant throughout storage.
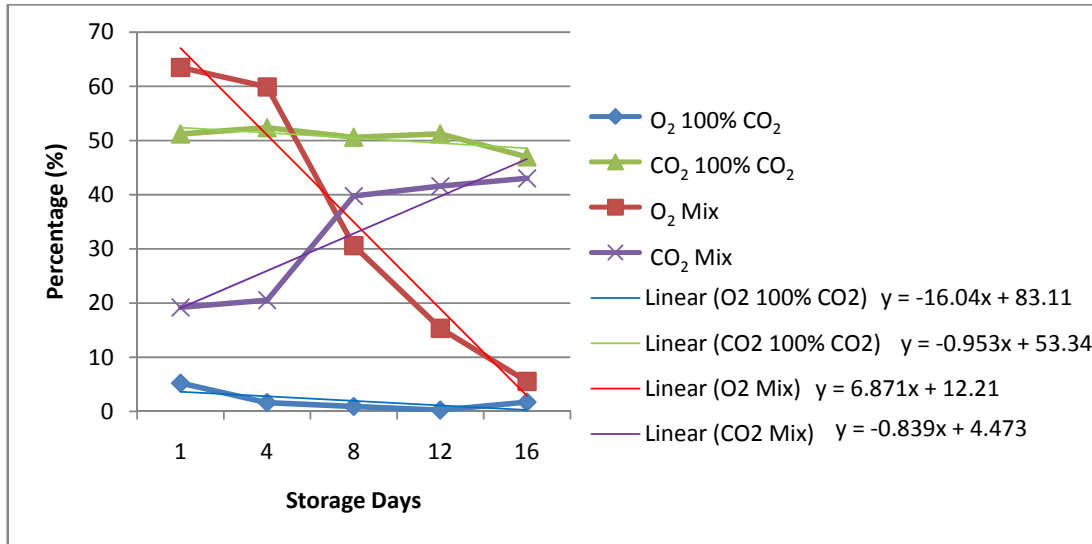
Figure 16: Percentage O_2 and CO_2 within the 100% CO_2 and commercial mix packs over the 16 days of storage.

pH determination

The pH values for the different packs of minced meat remained fairly constant with the average difference between the different packaging types not differing by more than a pH of 0.12 while the average difference between inoculation levels were no greater than a pH of 0.02. These observations were backed by the statistical analysis done of the packaging types as neither inoculation levels nor packaging types were seen to be significantly influential. Statistical analysis did however point towards a highly significant difference between storage days ($p < 0.001$) with the two modified atmosphere packaging types when being compared over the 16 days of storage.

Table 13: Mean, standard deviation (SD), max and min pH values for the different packs and inoculation sites over their storage periods.

		Inoculation site		
		Control	10^2	10^5
CO_2	**Mean**	5.61	5.61	5.62
	SD	0.21	0.20	0.21
	Max	5.96	5.95	5.88
	Min	5.28	5.24	5.26
Mix	**Mean**	5.58	5.59	5.60
	SD	0.19	0.19	0.18
	Max	5.85	5.88	5.86
	Min	5.23	5.24	5.30
PVC	**Mean**	5.69	5.71	5.69
	SD	0.09	0.10	0.11
	Max	5.79	5.83	5.88
	Min	5.56	5.58	5.56

Bioluminescence

In the intensity curve study, there was no single uni-directional correlation that could be applied throughout the entire dilution series to enable a direct comparison between fluorescence and actual plate counts. Sectional uni-directional correlations between certain dilutions were however observed, that could be correlated to their corresponding plate counts to enable count determination using the IVIS system. Since the counts determined by the IVIS system did not correlate with the counts obtained with plated samples, all the counts provided by the IVIS software was regarded as proposed counts, not actual counts.

There was a proportional increase in proposed total counts (PTC) (Figure 17) between dilutions 10^{-7} to 10^{-5}, an even plateau from 10^{-5} to 10^{-4} and a decrease between dilutions 10^{-4} to 10^{-2}. The proposed average counts (PAC) saw a similar trend to that of the PTC, with the PAC however being much lower with a maximum of 3.6 $\log_{10}$ compared to the 8 $\log_{10}$ found

with the PTC. The proposed max counts (PMC) portrayed a general decrease over the dilution series but was not proportional between dilutions
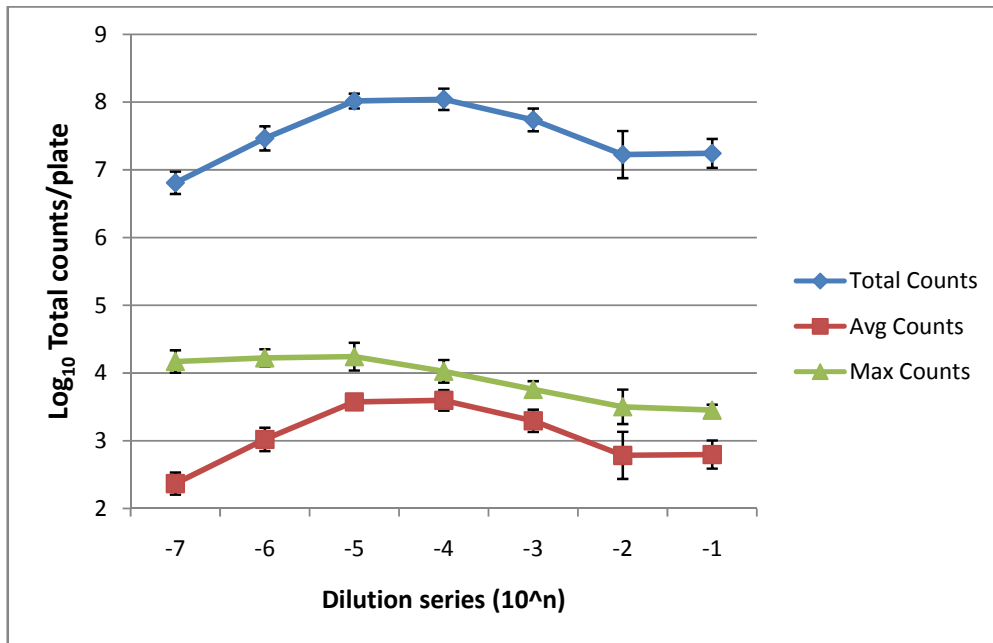
Figure 17: Intensity curve of the proposed averaged total, average and maximum counts per inoculation dilution in the constant ROI on nutrient media (n=4)

An un-proportional decrease in PTC, PAC and PMC (Figure 18) occurred on the minced samples, with detection limited to the 10^{-3} solution at 60s exposure. All three series portrayed quite similar graphs with only the PMC deviating slightly with an increase at dilution 10^{-3}, essentially ending higher than the 10^{-2} dilution.
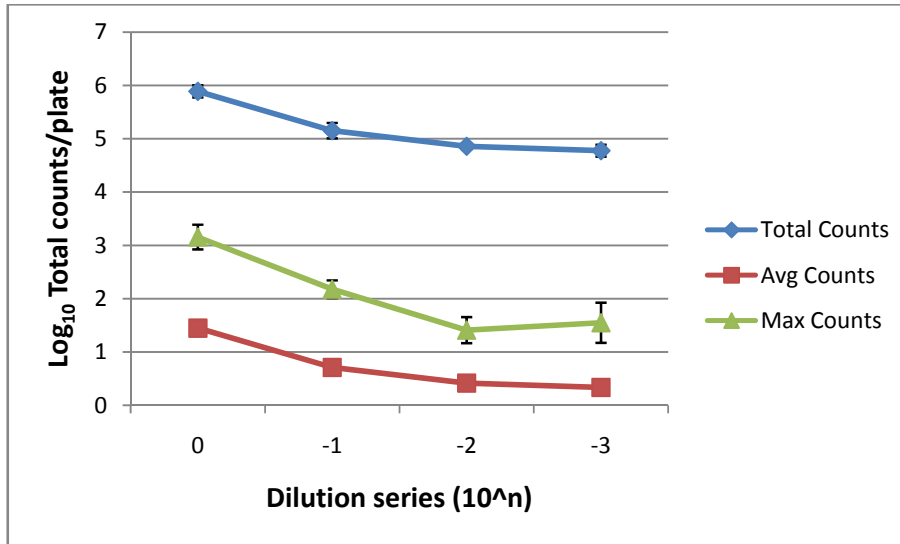
Figure 18: Intensity curve of the proposed averaged total counts per inoculation dilution in the constant ROI on minced meat (n=4)

The PTC (Figure 19) in the MAP minced meat packs saw an increase from the initial count of 5 $\log_{10}$ between the hours of 0 and 8, then remained constant around 8 $\log_{10}$ between hours 8 and 12 before finally entering a gradual decrease cycle to levels of 3.5 $\log_{10}$ at hour 48, thus ending below the initial recorded count. The PMC and PAC followed a similar trend as that of the PTC with the counts generally being 2.5 $\log_{10}$ and 3.5 $\log_{10}$ less at a specific point when compared with those of the PTC.
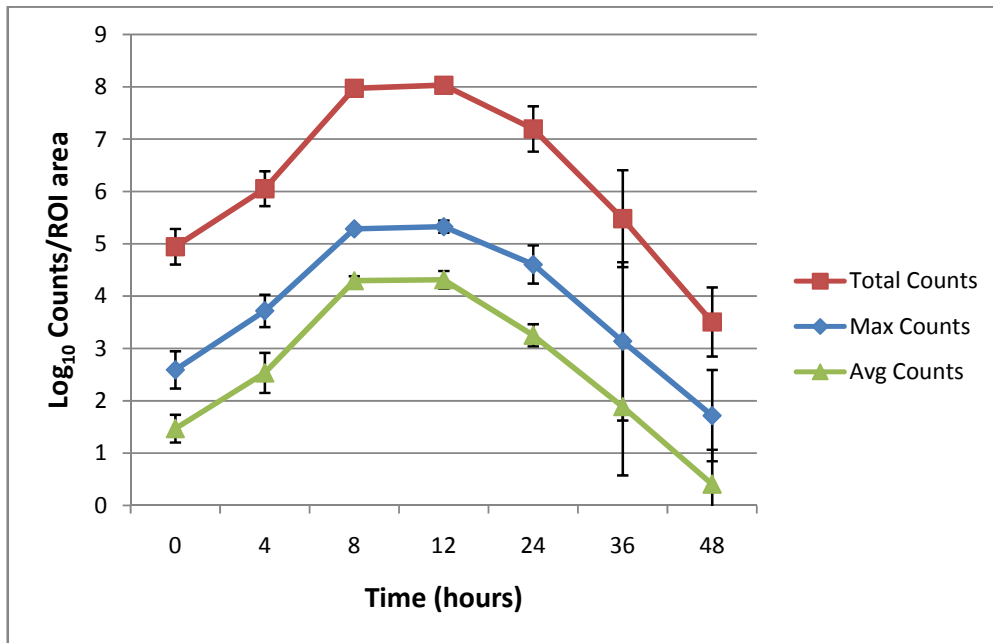
Figure 19: Averaged total, average and max counts obtained in the ROI area per hour analysis for the MAP packaged minced meat (n=6)

The PTC (Figure 20) in the PVC overlay packed minced meat packs saw an increase from the initial count of around 5 $\log_{10}$ between the hours of 0 and 8, then remained constant around 8 $\log_{10}$ between hours 8 and 12 before gradually decreasing cycle to levels of 6.5 $\log_{10}$ at hour 48, ending above the initial recorded count. The PAC followed a similar trend to that of the PTC with the counts generally being 3.5 $\log_{10}$ less at a specific point when compared with those of the PTC. The PMC showed a similar graph to that of the PTC with the exception of the data point at hour 36 being the highest point in the series.
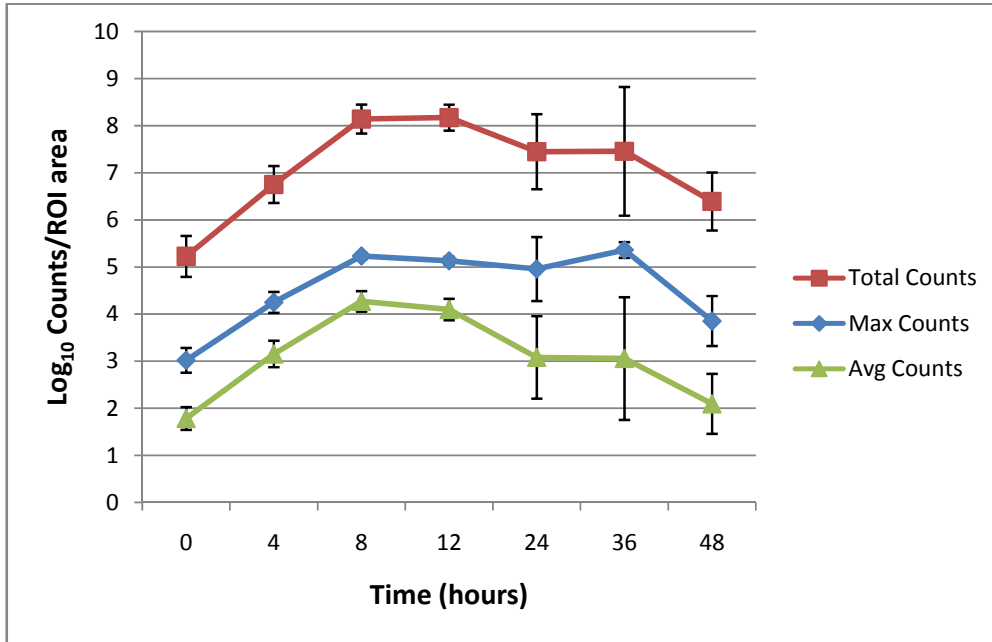
Figure 20: Averaged total, average and max counts obtained in the ROI area per hour analysis for the PVC overlay packaged minced meat (n=6)

The average ROI area between the MAP and PVC overlay packs (Figure 21) at the different intervals differed substantially from one another, with time specific intervals differing between $0.4cm^2$ and $64cm^2$. Peak ROI areas for the MAP packs were reached by hour 24 while the PVC packs reached peak ROI area by hour 48.
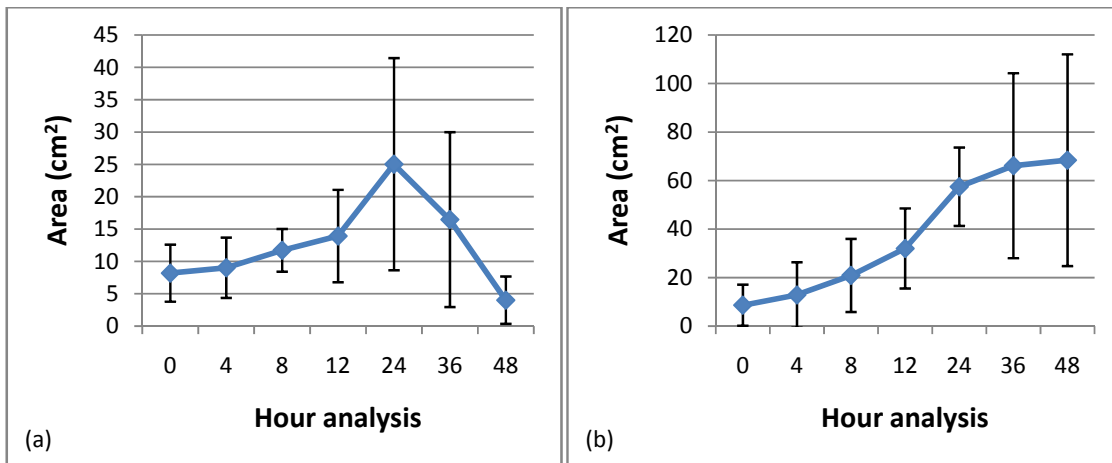
Figure 21: Average ROI area per (a) MAP and (b) PVC overlay packed minced meat over the different hours of analysis

Discussion

The effect of the innate background organisms on *E. coli* O157:H7

Even though two of the independent factors, storage days and *E. coli* O157:H7 inoculation levels, were found to have a substantial effect on the *E. coli* O157:H7 numbers, when the attributed variation in the numbers were considered, the inoculation levels and modified atmosphere packaging only elucidated to 4.5% and 15.4% of the accounted variation respectively. This still left quite a substantial portion of the accounted variation, unaccounted for in factors that were either seen to be non-significant, as is the case with storage days that accounted for 19.1% of the variation but was found to be insignificant or factors that fell outside the realm of the three independent factors tested for.

The dependent factor that was hypothesised to affect the growth of *E. coli* O157:H7 was that of the existence and growth of the natural background spoilage organisms. The stepwise regression analysis that excluded all of the independent factors, estimated the influence of the four background organism groups at 57.2%, which means that the natural background organisms found in minced beef could account for more than half of the influence or factors affecting the growth of *E. coli* O157:H7.

Even when adding the independent factors of storage days and inoculation level the background organisms still occupied the first four steps of the regression, again accounting for 57.2% of the variation, with a significantly valid inclusion of the two independent factors in combination pushing up that value to 60.2%. This indicated that the natural background spoilage organisms present on minced beef does in fact have a direct influence on the growth and survival of *E. coli* O157:H7. This supports Prendergast and his colleagues (2009) where they found that *E. coli* O157:H7 exhibited differing final numbers when inoculated onto either irradiated or non-irradiated beef surfaces.

The authors found that the natural occurring levels of background organisms resulted in significant reductions and inhibition of growth not only for *E. coli* O157:H7, but for all *E. coli* serotypes tested. It needs to be noted however that the authors also harboured the idea that natural background organisms might not only be detrimental to the growth and survival of *E. coli* O157:H7 but that it might actually aid in its growth and survival as well (Prendergast et al. 2009). This was brought about by the theory of metabiosis, whereby the

microbiota present on the surface of the meat might actually produce metabolites that would be favourable to the growth of *E. coli* O157:H7 (Gram et al. 2002).

A similar trend was noticed by Vold *et al*. (2000) when an artificially introduced *E. coli* O157:H7 inoculation of 10^3 was inhibited by various different groups of background organisms found inherently on meat. In most instances the background biota restricted the growth of *E. coli* O157:H7, resulting in maximum concentrations being reached much later when compared with meat containing no background biota. It was however noticed that no *E. coli* O157:H7 deaths were observed as a direct result of the background organisms, but that they merely acted as a suppressant of growth (Vold et al. 2000).

In similar fashion to what was seen by Vold *et al*. (2000), the dominating species in the low oxygen environment was seen to be the LAB while in the higher oxygen packaging, the *Pseudomonas* sp. emerged as the dominating species. This however came as a surprise when comparing these results with that of the stepwise regression, as this analysis indicated that the species that accounted for the greatest amount of growth influence on *E. coli* O157:H7 was that of Enterobacteriaceae. Since *E. coli* O157:H7 falls within the family of Enterobacteriaceae, one reason why the Enterobacteriaceae species might have the biggest influence could be that of nutritional and growth requirements or their adhesion dynamics being similar. Thus these species could be in direct competition with one another whenever present in communal unsustainable numbers on the surface of minced beef.

Initial levels of tested organisms on minced beef

Initial mean APC counts of 5.2 $\log_{10}$ CFU/g seem to correlate with that found in literature indicating counts in the vicinity of 4.7 $\log_{10}$ CFU/g for either coarse or finely ground beef (Eisel, Linton & Muriana 1997).

The initial presumptive *E. coli* O157:H7 counts of 10^2 found on the control samples were quite high when compared to the standard of $< 10^1$ set out in the South African regulations regarding un-cooked fresh meat (DOH 2002) and did not quite correlate in magnitude to anything found in literature. In general it was seen to be higher by one log cycle when compared to a study by Scanga *et al*. (2000), where ground beef from fresh trimmings examined 1 to 2 days after grounding indicated total *E. coli* counts at 1.12±0.34 $\log_{10}$ CFU/g.

When regarding the prevalence of positively identified *E. coli* species in raw beef products, incidences as high as 16.8% and 15.5% has been confirmed in countries such as the USA and Sweden (Rhoades, Duffy & Koutsoumanis 2009). Concerning *E. coli* O157:H7, although no serological analysis was done on the detected *E. coli* O157:H7 colonies obtained from the meat packs, it's presence is not unlikely as inspections by the US Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) obtained positive results during raw ground beef testing between 1994 and 2006 that detected *E. coli* O157:H7 percentage positive levels between 0.0% and 0.86% (Koohmaraie et al. 2007). Similar results were also found in other countries with prevalence as high as 3.8% and 2.8% associated with minced beef products in Botswana and Ireland respectively (Rhoades, Duffy & Koutsoumanis 2009).

Low detection limits of *E. coli* with high inoculations

Regarding the low initial values obtained for *E. coli* O157:H7 with the 10^5 inoculation level, ranging at just below 3 $\log_{10}$ CFU/g, this might be attributed to the heterogeneous growth parameters that exists between individual cells within a specific bacterial strain. As the environmental parameters differed between the inoculums' culturing growth vile and the meat packs, in both temperature and nutritional availability, the injection of the inoculums onto the meat surface might have resulted in environmental shock to certain individual cells which resulted in their death (Koutsoumanis & Sofos 2005). As illustrated by Koutsoumanis and Sofos (2005), a normal distribution of heterogeneous groups of cells in a high inoculation would result in a much higher variation and heterogeneous diversity for possible growth, but the exclusion of a single group by specific environmental factors could also have a major impact on the overall numbers.

Effect of the independent variables on the background organisms

The independent factors including storage days, *E. coli* O157:H7 inoculation level and packaging type, that were incorporated did not have any statistically significant effect on the APC, LAB or Enterobacteriaceae numbers over 16 days of storage, which also correlated with that found in literature (Nissen, Sørheim & Dainty 1996). This reiterates the importance of proper bacterial quality regarding the manufacturing of minced beef. The fact that the type of packaging almost had a significant effect on the numbers of Enterobacteriaceae indicates the effect that the modified atmosphere has on Enterobacteriaceae as was found to be similar

to that in literature when comparing storage using high and low concentrations of carbon dioxide (Nissen, Sørheim & Dainty 1996).

The most significant independent factor that influenced the growth of *Pseudomonas* was that of the modified atmosphere which was seen to be highly significant. As stated by Geysen *et al.* (2005) and Masson *et al.* (2002) and as can be seen here, an increased concentration of either oxygen or carbon dioxide has a significant influence on the growth of *Pseudomonas*. It also appears as if a higher carbon dioxide and lower oxygen concentration has a greater effect on the growth of *Pseudomonas* than that of a high oxygen and carbon dioxide mixture as can be seen in the final numbers obtained for both the 100% CO_2 and commercial Mix packs (Figure 10).

Nissen et al. (1996) showed that the mere presence of a higher than normal atmosphere of carbon dioxide significantly affected *Pseudomonas* numbers, with the concentration also being of importance with meat stored above the 2°C. They saw that higher concentrations had a greater suppressing effect at higher temperatures, which would explain the difference seen between the commercial mix and a 100% carbon dioxide packs.

The effect of the independent variables on *E. coli* O157:H7

The significant effect that different inoculation levels had on the *E. coli* O157:H7 counts, was seen to correspond with data found in literature where differing initial inoculations resulted in a varying final counts (Skandamis et al. 2007).   It was deduced that in general, a population consisting of a higher initial level of an organism would be less affected by the various environmental forces present in its surroundings than would lower levels. This effect could be explained due to varying heterogeneous growth limitations found between individual cells within a population as well as the reactions by the different groups towards varying environmental stresses. If these different heterogeneous groups are spread within a population in terms of the normal distribution, it can be expected that if the overall inoculation is higher, larger groups of these heterogeneous cells would exist thus increasing the growth seen within these populations. To this regard, environmental stresses that would otherwise have a damning effect on a homogeneous population would not be as disastrous in such a heterogeneous population (Koutsoumanis & Sofos 2005).

The influence of the type of packaging on the growth of *E. coli* O157:H7 was observable ,but was not found to be statistically significant, and was seen to be similar to that found in literature (Barrera et al. 2007) where elevated levels of CO_2 has a greater effect when compared with elevated levels of oxygen.

Colour changes in MAP minced meat

Concerning the colour of the commercial mix packs, the saturation value was held above the minimum level just prior to storage day number eight, which is as expected as most commercially packed minced beef packs are regarded to have a shelf life of seven days. The gradual decrease in saturation numbers seen with the commercial packs (Figure 15) are comparable with the drop in the oxygen concentration (Figure 16) found within the packs during the progression of the storage period.

The saturation numbers for the 100% carbon dioxide packs was initially much lower (Figure 15) than those seen with the commercial mix packs and fell below the minimum saturation number much quicker than was seen with the commercial mix packs. However an interesting phenomenon occurred within the 100% CO_2 packs whereby, after the fourth day of storage, the saturation numbers picked up and was measured above the minimum of 18 on day 12. This might be due to the formation of carboxymyoglobin, whereby a carbon monoxide molecule can bind to the deoxymyoglobin molecule at position six resulting in a bright red colour.

Research has shown that the formation of carboxymyoglobin can occur with much more ease and frequency in the presence of deoxymyoglobin than in the presence of either oxymyoglobin or metmyoglobin (Mancini & Hunt 2005). Although all the fundamental reactions are not entirely understood, carbon monoxide will slowly dissociate from the myoglobin molecule when the atmospheric concentration of carbon monoxide decreases, explaining the final saturation value obtained for the 100% CO_2 pack.

Jeremiah and Gibson (2001) showed that storage time has a detrimental influence on the colour of meat as was experienced in this study and could be due to de-oxygenation or oxidation of the myoglobin resulting in a discoloration and loss of the bright red colour in the meat (Mancini & Hunt 2005). The type of atmosphere packaging did not seem to have a

significant influence, however when it was compared alongside that of storage days it was also found to be statistically significant. This indicates that the concentrations of oxygen and carbon dioxide within a sealed meat pack does indeed have an effect on the meat colour, which in this instance would be related to either a lower ratio of oxygenation due to lower volumes of oxygen present or a higher rate of oxidation due to increased levels of carbon dioxide (Mancini & Hunt 2005; Martínez et al. 2005).

When considering the influence that the different bacterial populations might have on the colour or saturation value, a trend was sought to indicate such an effect. For the commercial mix packs, the most obvious trend was drawn between that of saturation and percentage O_2, as both showed a similar decreasing relationship. This can however be brought back to being the influence of the various bacterial populations as this decrease in both saturation and O_2 percentage was in an inverse relation to the increase in the APC counts.

The effect on the meat colour could also be attributed to the change brought about by the bacterial populations, in the physiological environment of the meat, through the production of amino acids, amines, and other compounds that can react with the heam pigments or through the lowering of the meat pH (Illinois 2011). Whether directly influential through the production of compounds or indirectly through the altering of the environmental conditions, meat colour seems to degrade in terms of visual acceptance much quicker in the presence of larger numbers of bacteria.

Accelerated shelf-life: Intensity Curve

The irregular pattern and un-proportional increases and decreases witnessed with the intensity curve on the nutrient agar, indicates that the IVIS system cannot be used for the enumeration of bacteria from a dilution series. This irregularity might be due to the higher bacterial counts on the higher dilutions acting against one another in that although the plate might be over-grown, the colonies might not be viable due to a lack of space and nutrients. Thus an accurate count could only be obtained from plates that are not over grown and where the inter-bacterial impact on the enumeration plate is not substantial enough.

Although a gradual decrease in the PTCs with the minced meat Petri dish samples were observed, this decrease was un-correlated to the dilution series and thus it could not serve in the process of enumeration of a specific meat sample.

Accelerated shelf-life: MAP packs

A steep decline in numbers were observed after hour 12 which might indicate the accumulation of by-product build-up or this could be due to the decreasing levels of O_2 or increasing concentration of CO_2, as was seen with the MAP pack in the above mentioned experiment. During hours 12 and 24, the largest difference between the PTC and PMC and the largest ROI were observed which could indicate the formation of sustainable colonies after which spread occurred within the product, limiting the localisation of *E. coli*. The lowest numbers and ROI was observed at hour 48 which, when viewing the images and comparing the data, could indicate increased stress from the environment as well as the elimination of the explorer colonies by the intrinsic bacteria, while the oldest colonised sites still remain, albeit exhibiting a lower fluorescence.

An alternative reason for the substantial increase in ROI during hours 12 and 24 could be the heightened presence and volumes of meat water at the bottom of the container. Although a drip tray was inserted into the container, the microbial breakdown of the muscular tissue over time was substantial enough to saturate the drip tray, allowing "free" water to migrate throughout the pack.

Accelerated shelf-life: PVC packs

During hours 12 and 24, the most substantial increase in ROI was observed, as with the MAP samples, and this could again be contributed to the increase in "free" meat water. This increased volume of meat water could also be responsible for the high average and standard deviation experienced after hour 24. Less substantial decreases in PTC, PMC and PAC were observed after hour 12 when compared to the MAP packs. This most likely point towards the influence an atmosphere of high CO_2/ low O_2 concentrations have on *E. coli*, limiting sustainable numbers. The environmental stresses most likely affecting the numbers in the PVC packs was the influence of other innate bacterial populations and the build-up of waste products and a decrease in nutrients at the respective locations.

Accelerated shelf-life: Migration

Out of the various stresses that could be experienced by bacteria in different environments including atmospheric, chemical and colonisation stresses, the type of stress often dictates the migration of bacterial colonies. As was seen with the MAP packs, the proposed highest stress experienced there was that of atmospheric stress whereby there was a decrease in O_2 concentrations while a simultaneous increase in CO_2 concentrations occurred. Due to the nature of atmospheric stress being equal everywhere within an enclosed environment, this would serve as a migration limiting stress since a change in location would not lessen the stress experienced.

In contrast a stress such as the accumulation of waste and by-products or space and colonisation stress is a localised stress that can often be overcome by migrating away from the location. This would serve as a migration enhancing stress, enhancing outward migration, and could result in a quicker spread by a bacterial species.

Conclusion

The type of packaging and the initial starting number population of *E. coli* O157:H7 both efficiently influences the growth and survival of *E. coli* O157:H7 on minced beef while the storage period did not significantly influence either growth or survival. Innate background spoilage organisms found on meat have a significant effect on the growth and maximum population numbers of *E. coli* O157:H7 in minced beef (Prendergast et al. 2009), regardless of the type of modified atmosphere packaging used, as no significant difference brought about by the innate biota could be observed within the two different packaging types when storage days and inoculation levels were disregarded.

It needs to be reiterated however that regardless of the factors directly influencing the growth and survival of *E. coli* O157:H7, none resulted in a substantial decrease as to result in food safety numbers concerning *E. coli* O157:H7 being reached.

Chapter 5: The effect of MAP, storage time and spoilage bacteria in minced beef on the growth of *Staphylococcus aureus*

Abstract

In this study the effect that both MAP and the inherent spoilage organisms found on minced beef has on *S. aureus* was investigated with variable initial *S. aureus* levels. It was found, in contradiction, that the growth of *S. aureus* in meat is largely independent on the presence of other bacterial species. LAB was indicated to have the largest effect on the growth and survival of *S. aureus* of all the bacterial groups enumerated. It was also shown that high initial levels of *S. aureus* are often more resilient to corrective or preventative action in minced beef and as such, the most important aspect to consider in meat contaminated with *S. aureus*, would be the initial levels present on the meat.

Introduction

The shelf-life of fresh meat products are of a great financial concern and as such industry has been seeking various means such as MAP (Ooraikul & Stiles 1991) to try and enhance this shelf-life. Although the effect of MAP on spoilage organism and pathogens has been extensively studied, the indirect effect of the spoilage bacteria on pathogens is still lacking. This is of considerable concern as the prevalence of human pathogens such *S. aureus* in red meat has been widely noted (Shale et al. 2005).

S. aureus is a facultative anaerobic bacterium that inhabits the nasopharyngeal regions and intestinal tract of both humans and animals. It exists as a saprophyte on the mucal secretions and other organic substances of its host and can also exist for short periods of time on the skin of its hosts (Ayres, Mundt & Sandine 1980). Due to these habitation factors, the presence of *S. aureus* on food is often used as an indicator organism for the contamination of food through the interaction of the food with the food handlers. This interaction can come about both through direct contacts with the handler's skin or through mucal secretions from the handler findings its way onto the food (Accoa et al. 2003).

The risk in the consumption of food containing *S. aureus*, lies not in the presence of the vegetative cells, but in the presence of the various enterotoxins that can be produced by a variety of *S. aureus* species, with the exception of Methicillin resistant *S. aureus* (MRSA). Risk is heightened further due to certain of the enterotoxins, including SEA (Staphylococcal enterotoxin A) and SEB, illustrating heat stability up to 121°C (Anderson, Beelman & Doores 1996).

Growth of *S. aureus* can occur at temperatures between 6.7°C and 47.8°C, a pH between 4.0 and 9.8, with the optimum between 6.0 and 7.0, and a water activity of 0.86, for aerobic, and 0.9, for anaerobic growth. Toxin production however has stricter production requirements with a temperature range of 10°C to 46°C and optimum toxin production occurring between 40°C and 45°C. Depending on the type of toxin to be produced, a population of between 10^4-10^5 CFU/g is often required before toxin production is initiated (Jay 2000). Apart from a higher water activity being required for *S. aureus* when grown in anaerobic environments, different MAP methods do not have a significant effect on the growth of *S. aureus*, although

differing levels of CO_2 have been shown to provide varying levels of inhibition (Kimura, Yoshiyama & Fujii 1999; Saucier, Gendron & Gariepy 2000).

Although the nutritional requirements for growth of *S. aureus* are simple, it does however encounter some difficulty in having to compete with other bacteria that might be present on food (Jay 2000). This deficiency could however be nullified in a gaseous environment not suitable for the normal aerobic spoilage organisms of meat, due to *S. aureus*' being facultative anaerobic. Thus the aim of this study was to determine the effect that packaging in differing modified atmospheres, 100% CO_2 and commercial mix (O_2=60%/ CO_2=30%/ N_2=10%), and the presence and growth of the natural spoilage organisms on minced meat, would have on the growth and survival of *S. aureus*.

Materials and Methods

 The experimental setup involved two separate experimental flows. The primary experimental flow (Figure 22) involved the inoculation of two modified atmosphere packaged minced meat samples, 100 % CO_2 and commercial mix (O_2=60%/ CO_2=30%/ N_2=10%), with either a 0.1% buffered peptone water solution, to act as a control; a 10^2 or 10^5 concentration of *S. aureus*. These samples were then stored and analysed over a 16 day period.

The secondary experimental flow (Figure 23) involved the inoculation of a PVC overlaid packaged minced meat samples with either a 0.1% buffered peptone water solution, to act as a control; a 10^2 or 10^5 concentration of *S. aureus*. These samples were then stored and analysed over a 4 day period.
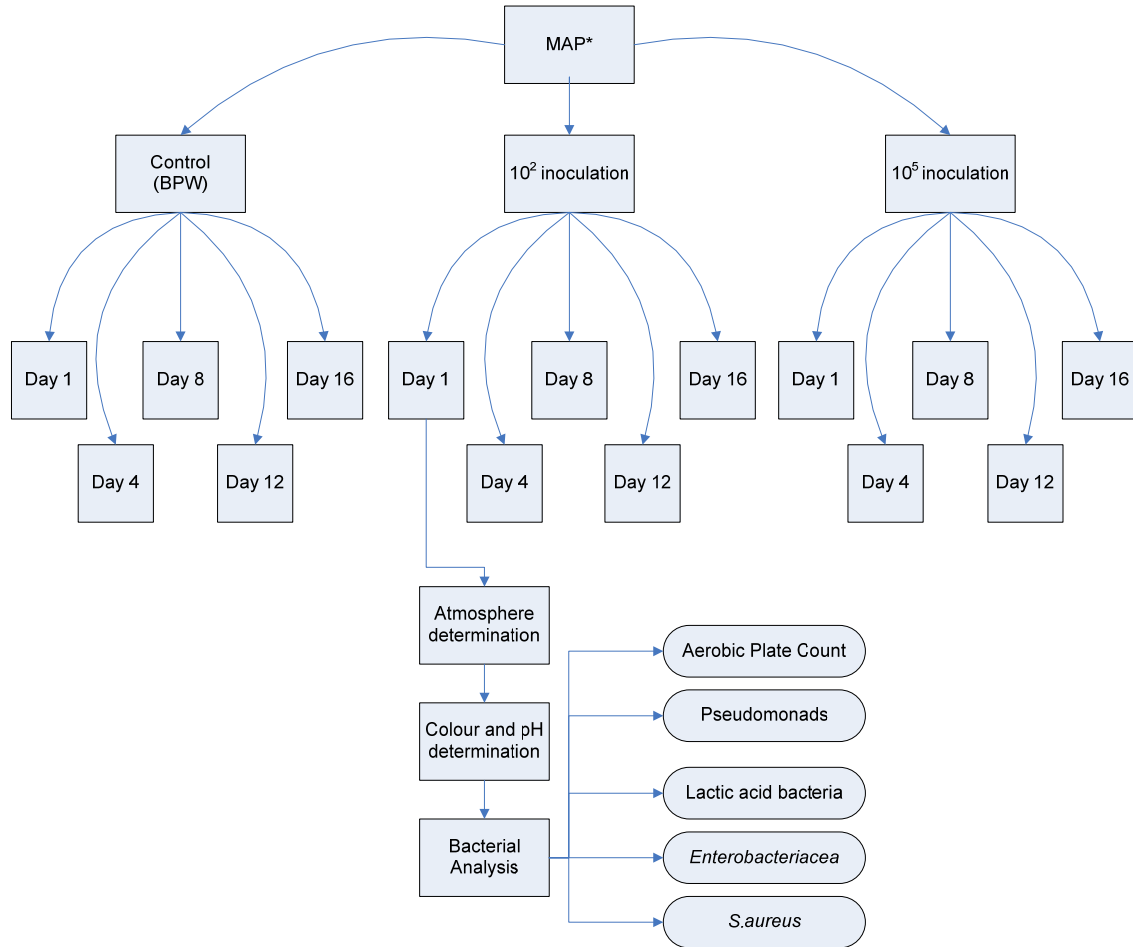
Figure 22: Flow diagram of the process of atmosphere, colour, pH and bacterial numbers determination in minced beef under modified atmosphere packaging. (* MAP interchanges for both the commercial mix [O_2=60%/ CO_2=30%/ N_2=10%] and the 100% CO_2 packs) (BPW – 0.1% Buffered Peptone water)
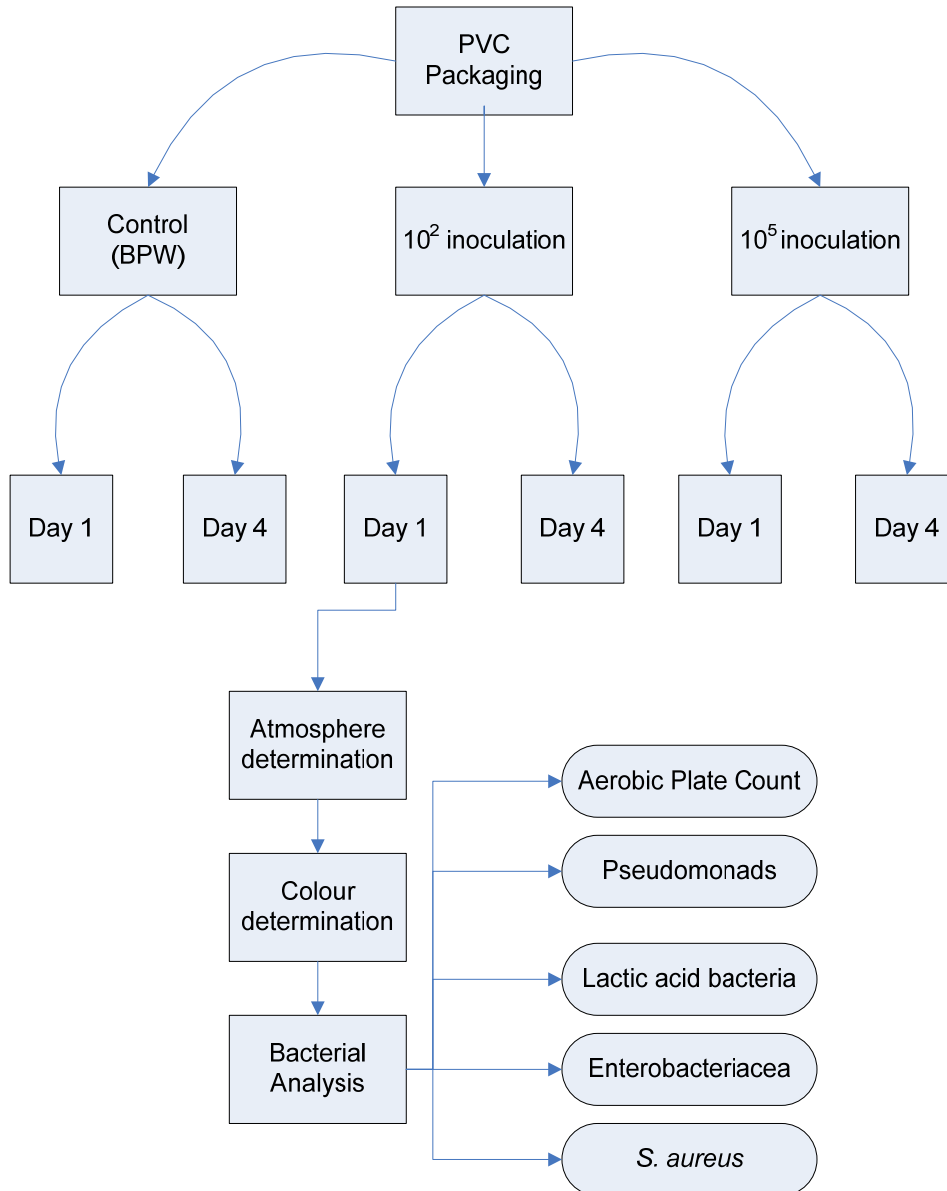
Figure 23: Flow diagram of the process of atmosphere, colour, pH and bacterial numbers determination in minced beef packaged in PVC overlay packs. (BPW – 0.1% Buffered Peptone water)

Refer to the methodology found in Chapter 4: Effect of modified atmospheres and natural background spoilage organisms on the growth of inoculated *E. coli* O157:H7 in minced beef., with the exception of inoculated organism changing from *E. coli* O157:H7 in Chapter 4 to *S. aureus* in Chapter 5. The following changes can be noted.

Preparation of the inoculums

A single colony of *S. aureus* (NCTC 10654) was taken off a Baird-Parker agar base (Oxoid, Southampton, UK) plate supplemented with an Egg Yolk Tellurite emulsion (Oxoid), inoculated into a prepared Tryptone Soy Broth (Biolab, Midrand, RSA) solution and incubated at 37°C for 18-24 hours to ensure that the culture had entered the stationary phase in its growth cycle. Further detection of *S. aureus* was done using the same supplemented agar medium. For confirmation of enumerated *S. aureus* colonies, a latex slide agglutination test was used (Oxoid, DR0850)

Statistical analyses

Analysis of Variance (ANOVA) and a Multiple Regression (GenStat® 2009) using a stepwise addition were performed. ANOVA was used to determine whether the main effects, inoculation number; MAP and storage had a statistically significant effect ($P<0.01$) on the growth and survival of the test organism, *S. aureus*, as well as the APC, *Pseudomonas* spp., LAB & Enterobacteriaceae.

The ANOVA was constructed in two different manners since the two modified atmosphere packaged samples ran the entire 16 days (Figure 22), while the PVC packaging analyses only ran for days 1 and 4 (Figure 23). Thus the first analysis was done without taking the PVC packaging values into consideration, allowing an ANOVA test to be determined over the entire 16 days of the experiment. The second analysis included the PVC packaging values, but this however resulted in only the values on days 1 and 4 from the other two packaging sets being incorporated into the analysis. Using the ANOVA values, percentage accounted variation or the amount of change brought about in the counts for a specific bacterial population by a specific factor, was also determined to identify the greatest affecting forces. This is indicated by %SS.

Multiple Regressions using a stepwise addition process was employed whereby the possible predictor variables, including the independent variables such as MAP and the dependant variables such as the obtained bacterial counts, were added to the analysis periodically. In determining the effect of the natural background organisms on *S. aureus*, the stepwise regression analysis was split into two analyses, determined by their different factors. The first one (primary analysis) looks at the influence of the background organisms per specific

packaging type, excluding the other independent factors namely storage days and inoculation type. The second (secondary analysis) focuses on the effects that both the dependent and independent factors have in combination on *S. aureus* in the specific packaging type. The multiple regressions was done to try and establish a relationship between the various factors that would best describe the observed numbers and growth of *S. aureus* and in doing so formulate a simple regression equation that would account for the largest impact factors (StatSoft 2009).

Results

Bacterial quality of fresh minced beef meat under MAP and PVC overlay packaging

APC on the first day of testing did not vary significantly between the inoculations within the packaging types, but did vary between the packaging types, with counts of 4.97 ± 0.03 $\log_{10}$ CFU/g, 5.49 ± 0.01 $\log_{10}$ CFU/g and 5.91 ± 0.05 $\log_{10}$ CFU/g being recorded for the 100% CO_2, the commercial mix and the PVC overlay packs respectively (Figure 24, Figure 25).

A clear difference in the APC between the 100% CO_2 and the commercial mix pack was however noted during the progression of days (Figure 24), with the commercial mix pack recording an average APC of $\log_{10}10.15\pm0.03$ CFU/g by day 16. This difference was also statistically noted, with differences seen between storage days (P <0.001) and the type of packaging (P <0.001) over the 16 days of storage. No significant difference could be observed for the APC between the different inoculation levels.

Similarly both storage days (P = 0.003) and packaging type (P = 0.038) was also seen to be significant over the four days of storage with the PVC (Figure 25) overlay packs included. In both instances, storage days were seen as the largest influence on the APC obtained.

Figure 24: Aerobic plate counts, $\log_{10}$ CFU/g, per inoculation level of *S. aureus* for the 100% CO_2 and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)
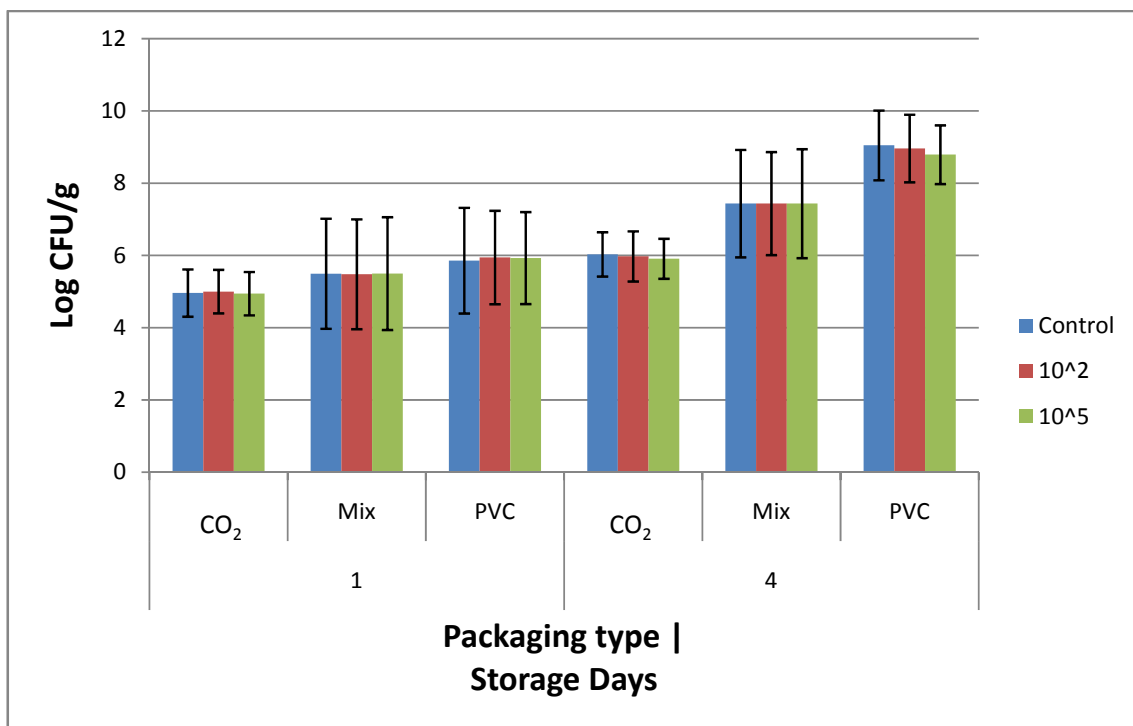
Figure 25: Aerobic plate counts, $\log_{10}$ CFU/g, per inoculation level of *S. aureus* for the 100% CO_2, commercial mix and PVC overlay packs over 4 storage days. (Temp. 5°C, n=3)

Concerning the Enterobacteriaceae counts, a clear count increase was observed in the commercial mix packs while the 100% CO_2 packs all but remained constant over the 16 days of storage (Figure 26). This was also observed with both storage days ($P < 0.001$) and the type of packaging ($P < 0.001$) being seen to have a significant impact. Unlike the APC, type of packaging had the greatest influence on variation seen within the Enterobacteriaceae counts. Again no difference was observed that could be attributed to the different inoculations.

A similar trend was observed when regarding the three different packs over the four storage days with storage days ($P < 0.001$) and type of packaging ($P < 0.001$) again being seen to be significant. With this trend however storage days were seen to have the greatest influence on variation (Data not shown).
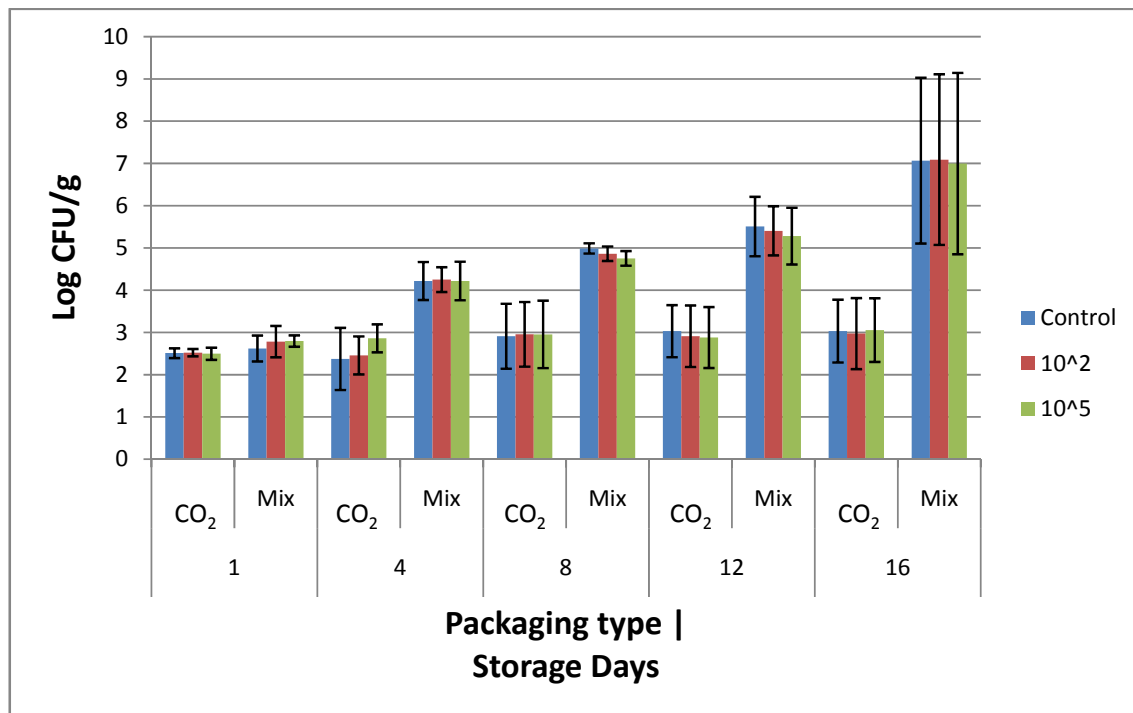
Figure 26: Enterobacteriaceae counts, $\log_{10}$ CFU/g, per inoculation level of *S. aureus* for the 100% CO_2 and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)

When the counts of the Pseudomonad species were observed (Figure 27), a similar trend to that of Enterobacteriaceae was noted. Initial counts between the two different packaging types was not substantially different, however a considerable increase curve was again noted for the Pseudomonad counts in the commercial mix pack while the counts in the 100% CO_2 packs remained virtually unchanged. The type of packaging ($P < 0.001$) accounted for almost

half of the variation seen within the Pseudomonad counts. Storage days however was also seen to be significant (P = 0.018), but did not contribute to the variation in such a large extent as was seen with the type of packaging. No difference in Pseudomonad counts were noted between the different inoculation levels.
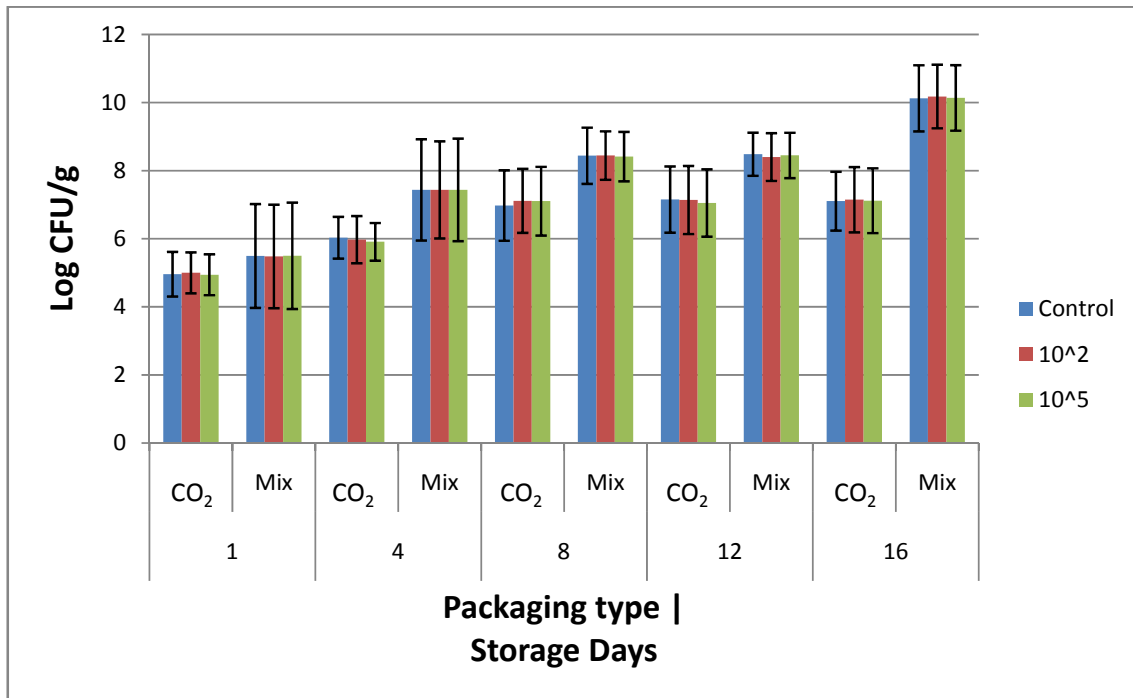

Figure 27: Pseudomonad counts, $\log_{10}$ CFU/g, per inoculation level of *S. aureus* for the 100% CO_2 and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)

In the analysis including the PVC overlay packs, both storage days (P = 0.017) and packaging type (P = 0.021) was again seen to be significant, with the PVC packs indicating the largest growth in Pseudomonas numbers. Initial average levels of LAB (Figure 28) in the 100% CO_2 and the commercial mix packs, 24 hours after packaging, did not differ substantially and were both recorded between 3.5 and 4 $\log_{10}$ CFU/g. After 4 days however, a clear difference in the counts were observed with the commercial mix identifying higher levels of LAB, continuing throughout the 16 days of storage.

The greatest difference was observed on day 16, with the commercial mix pack indicating a higher average count, with an average difference with the 100% CO_2 packs at almost 2 $\log_{10}$ CFU/g. Storage days indicated statistical significance (P < 0.001), accounting for 52.2% of

the variation while the type of packaging also proved significant (P = 0.027), but accounted for much less variation (9.31%) in the detected numbers.
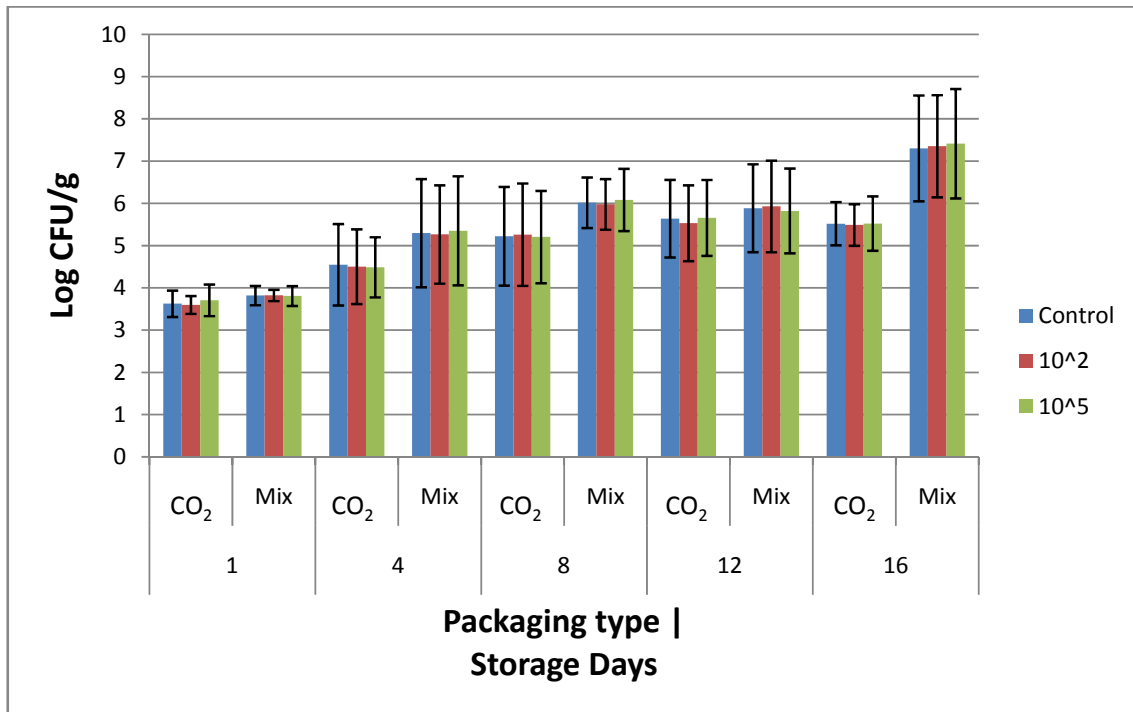
Figure 28: LAB counts, $\log_{10}$ CFU/g, per inoculation level of *S. aureus* for the 100% CO_2 and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)

Similarly, storage days also had an effect (P < 0.001) when taking the PVC counts into consideration for the 4 storage days and accounted for a similar variation (51.5%) when compared to the 16 days analysis.

Influence of the independent variables on the growth and survival of *S. aureus*

A clear distinction, in detected CFU/g for *S. aureus* (Figure 29) was observed between the 10^2 and 10^5 inoculation sites throughout the 16 days of storage. The 10^5 inoculation consistently averaged higher in CFU/g counts throughout the 16 days and differed with the 10^2 counts by at least $\log_{10}$ 1 CFU/g. The 10^5 inoculation however never reach the 10^5 level, with the highest CFU/g of $\log_{10}$ 3.1 being recorded on day 1 in the 100% CO_2 pack, after which a steady decline was observed. The 10^5 inoculation in the commercial mix packs remained steady throughout the 16 days of storage at around $\log_{10}$ 2.6 CFU/g.

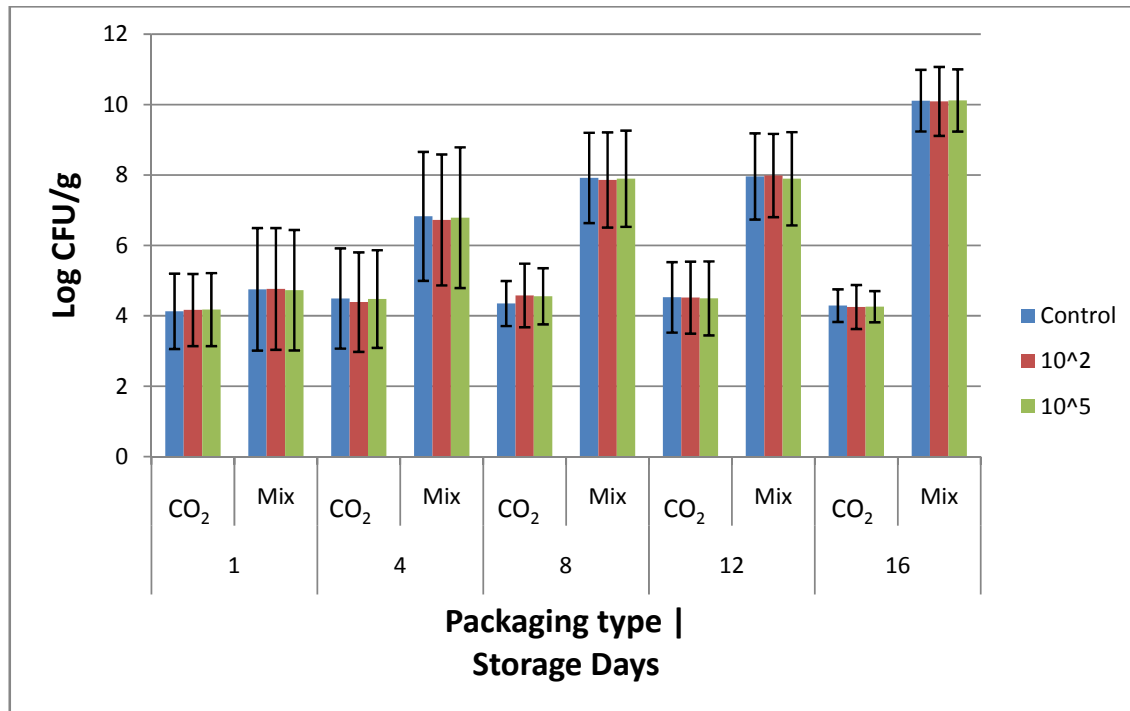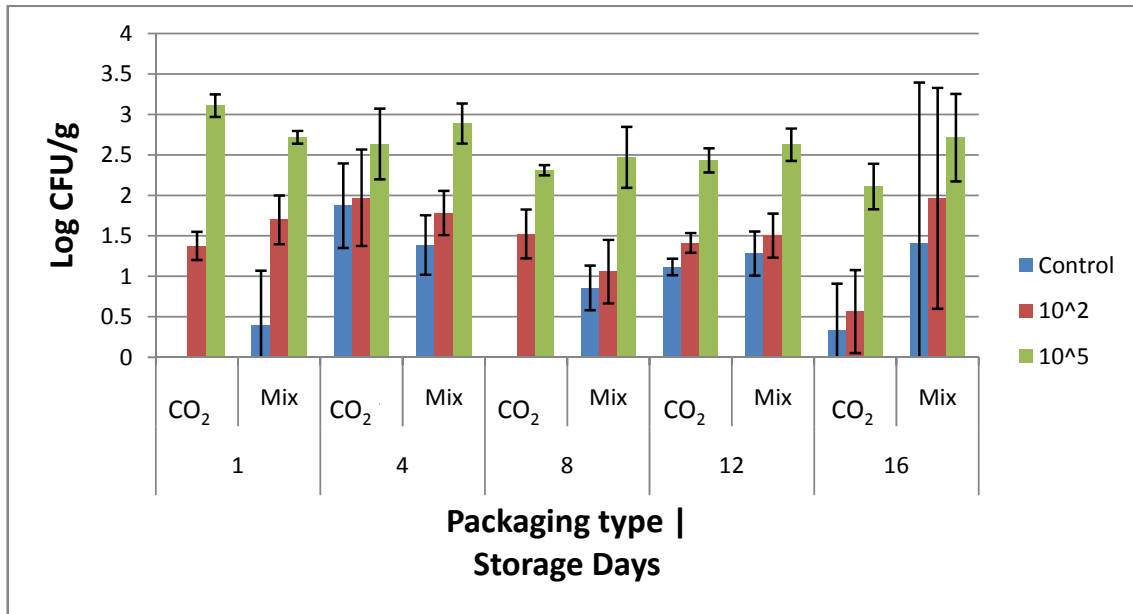**Figure 29:** *S. aureus* counts, $\log_{10}$ CFU/g, per inoculation level of *S. aureus* for the 100% CO_2 and commercial mix packs over 16 storage days. (Temp. 5°C, n=3)

For the 10^2 inoculation in the 100% CO_2 pack (Figure 30), a rise was seen until day 4 after which a steady decline resulted in a recorded average of *S. aureus* at $\log_{10}$ 0.5 CFU/g while the 10^2 inoculation held steady at between $\log_{10}$ 1.5-2 CFU/g in the commercial mix pack, with the exception of a recorded average count on day 8 close to $\log_{10}$ 1 CFU/g.

The control samples in the 100% CO_2 packs (Figure 29) displayed a high-low detection pattern whereby on alternating days, opposite levels compared to the previous observations were recorded. On day 1 and 8, no colonies of *S. aureus* were detected while an average high of $\log_{10}$ 1.8 CFU/g was recorded for day 4 in the 100% CO_2 packs.

In the commercial mix packs (Figure 30), an initially low, $\log_{10}$ 0.4 CFU/g, count was observed for day 1 followed by a sharp rise by day 4, $\log_{10}$ 1.4 CFU/g, after which the counts stabilised around $\log_{10}$ 1 CFU/g. A significant standard deviation, $\log_{10}$ 2 CFU/g, was however noted in the control sample for the observed colonies on day 16.

Storage days (P = 0.023), inoculation levels (P < 0.001) and the combination of the two (P < 0.001) were all found to be significant, with the inoculation level accounting for the largest variation (60.1%) affecting observed colony counts.
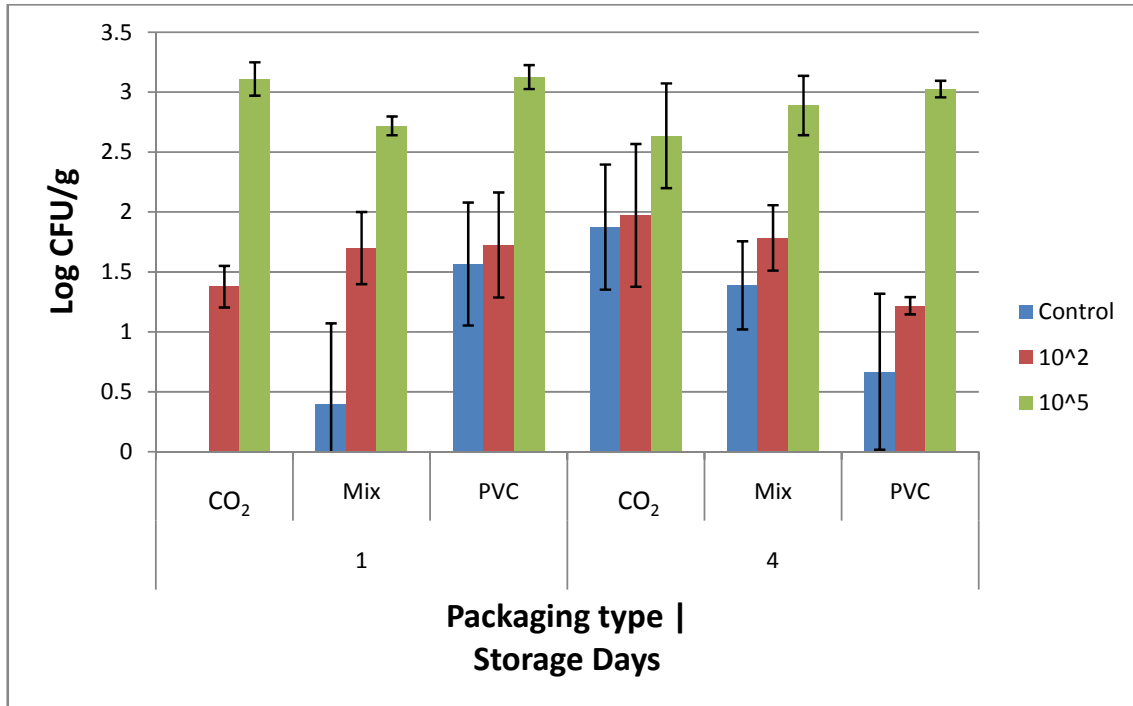
Figure 30: *S. aureus* counts, $\log_{10}$ CFU/g, per inoculation level of *S. aureus* for the 100% CO_2, commercial mix and PVC overlay packs over 4 storage days. (Temp. 5°C, n=3)

With the PVC overlay packs (Figure not shown), average counts of $\log_{10}$ 3 CFU/g were observed for the 10^5 inoculations while a decrease in average colony counts for both the control and 10^2 inoculations occurred between days 1 and 4. The level of inoculation (P < 0.001) and combinations of storage days*packaging (P = 0.002), storage days*inoculation level (P = 0.01) and storage days*packaging*inoculation level (P < 0.001) all were found to be statistically significant with inoculation level again accounting for the largest count variation (69.8%).

Influence of the microbial community singularly and in conjunction with the independent variables on the growth and survival of *E. coli* 0157:H7

By analyzing the effect on the growth and survival of *S. aureus* by only regarding the other bacterial populations present (Table 14), it was found that at most, only 30% of *S. aureus*'

growth variation could be accounted for in a non-significant regression. Once the independent factors were included (Table 15) however, the inoculation level accounted in the first step for 61.5% of the growth variation of *S. aureus* while a four step regression could account for 70.5% of the variation. The most significant step involving a dependant factor, Enterobacteriaceae*Day, appeared as the third step and accounted for 4% variation.

Table 14: Stepwise regression in response to the adjusted *S. aureus* counts, excluding the effects of storage days, inoculation type and type of packaging.

Step	Variable entered	Adjusted r^2	SER	Change P	Regression P
1	Enterobacteriaceae	0.01	0.945	0.189	0.189
2	LAB	0.3	0.934	0.067	0.079

Table 15: Stepwise regression in response to the adjusted *S. aureus* counts, including the effects of storage days and inoculation type and excluding type of packaging.

Step	Variable entered	Adjusted r^2	SER	Change P	Regression P
1	Inoculation	0.615	0.588	<0.001	<0.001
2	Day.Inoculation	0.638	0.570	0.007	<0.001
3	Enterobacteriaceae.Day	0.678	0.538	<0.001	<0.001
4	APC.Day	0.705	0.515	0.002	<0.001
5	Enterobacteriaceae.Inoculation	0.706	0.514	0.219	<0.001

When doing a regression analyses on only the 100% CO_2 packs (Table 16), a similar trend was seen with inoculation level again accounting for the largest variation, 59.7%, in the first step. The addition of significant steps up to step 4 resulted in a correlated variation of 68.6% while the addition of non-significant steps, resulted in a correlation of 75% in a still significant regression. Again the effects of the dependant factors were minimal.

In the commercial mix packs, the bacterial colonies on their own did not owe to substantial correlation (Table 17) while the Enterobacteriaceae group in the mixed analyses (Table 18) displayed accountability for a fair amount of the variability in the *S. aureus* counts. Significant changes in the added steps were observed until step 4 with the accounted variation amounting to 78.5% while a significant regression accounting for 80.4% of the variation was achieved in 7 steps.

Table 16: Stepwise regression in response to the adjusted *S. aureus* counts for the 100% CO₂ packs, including the effects of storage days and inoculation type.

Step	Variable entered	Adjusted r^2	SER	Change P	Regression P
1	Inoculation	0.597	0.624	<0.001	<0.001
2	Day.Inoculation	0.648	0.583	0.010	<0.001
3	Enterobacteriaceae	0.650	0.582	0.025	<0.001
4	LAB	0.686	0.551	0.021	<0.001
5	Pseudomonads.Day	0.704	0.535	0.073	<0.001
6	LAB.Inoculation	0.710	0.529	0.179	<0.001
7	Enterobacteriaceae. Day.Inoculation	0.750	0.492	0.012	<0.001

Table 17: Stepwise regression in response to the adjusted *S. aureus* counts in the commercial mix packs, excluding the effects of storage days and inoculation type.

Step	Variable entered	Adjusted r^2	SER	Change P	Regression P
1	Enterobacteriaceae	0.051	0.864	0.080	0.080
2	LAB	0.067	0.856	0.201	0.097

Table 18: Stepwise regression in response to the adjusted *S. aureus* counts for the commercial mix packs, including the effects of storage days and inoculation type.

Step	Variable entered	Adjusted r^2	SER	Change P	Regression P
1	Inoculation	0.594	0.565	<0.001	<0.001
2	Enterobacteriaceae	0.673	0.507	0.002	<0.001
3	Day	0.745	0.448	0.001	<0.001
4	LAB.Inoculation	0.785	0.411	0.007	<0.001
5	Enterobacteriaceae.Day	0.789	0.407	0.206	<0.001
6	Enterobacteriaceae.Inoculation	0.797	0.400	0.131	<0.001
7	Day.Inoculation	0.804	0.393	0.140	<0.001

A regression analyses on the PVC overlay packs including both the dependant and independent factors (Table 19), could account for 84.7% of the variation in 2 steps, with inoculation level, 77.6%, and APC contributing.

Table 19: Stepwise regression in response to the adjusted *S. aureus* counts for the PVC overlay packs, including the effects of storage days and inoculation type.

Step	Variable entered	Adjusted r^2	SER	Change P	Regression P
1	Inoculation	0.776	0.466	0.018	<0.001
2	APC	0.847	0.385	0.011	<0.001
3	APC.Inoculation	0.856	0.374	0.187	<0.001

Colour analysis

As expected (Figure 31), both the commercial mix and the PVC overlay packs scored a saturation value above 18 upon testing during storage day 1, while the 100% CO_2 pack was below. These positions however changed around by day 4 with only the 100% CO_2 packs maintaining a saturation value above 18.
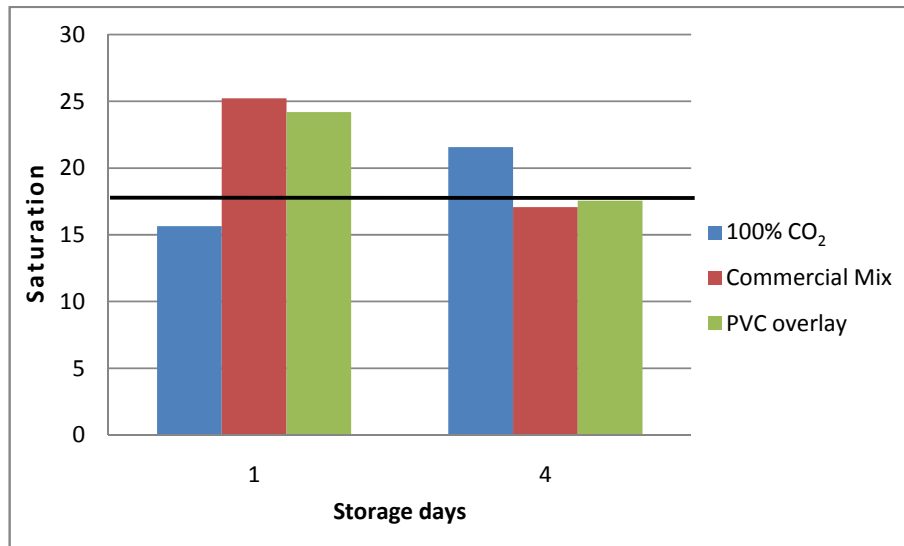
Figure 31: Saturation numbers for both MAP packs and the PVC overlay packs over the 4 days of storage.
(Above line at 18 depicts desired level (MacDougall 1977))

With four days of packaging (Table 20), only the combination of storage days*packaging type (P = 0.009) was found to influence the colour of the minced beef. It also accounted for the largest portion of variation (44%) in colour.

Table 20: Ps and %SS of split-plot ANOVA (inoculation as sub-plot) for colour saturation, including the PVC pack's counts, over the 4 days of storage. %SS added to indicate accounted variation.

Treatment	Degrees of Freedom	P	%SS
Day	1	0.103	9.59
Gas	2	0.421	5.73
Inoculation	2	0.398	0.15
Day.Gas	2	0.009	43.95
Day.Gas.Inoculation	4	0.014	1.26

Over 16 days of storage (Figure 32), the 100% CO_2 and the commercial mix packs reacted in an opposite fashion as the commercial pack only recorded a saturation value above 18 on the first day of storage while the 100% CO_2 pack recorded saturation values above 18 from storage day 4 to 16. The decrease in saturation values for the commercial mix pack was more even and eventually reached a plateau while the saturation value for the 100% CO_2 pack was erratic.
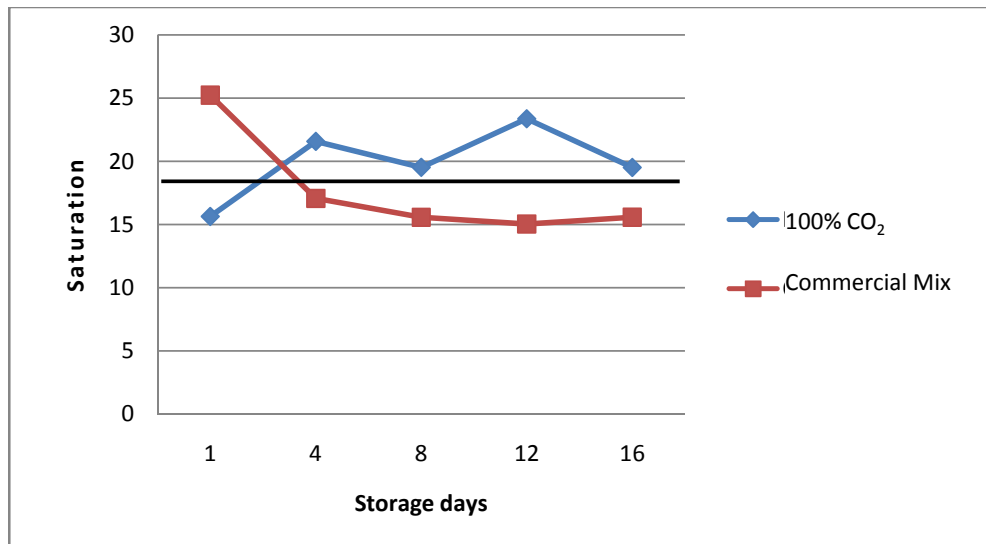
Figure 32: Saturation numbers for both MAP packs over the 16 days of storage. (Above line at 18 depicts desired level (MacDougall 1977))

As was seen with the analysis over four days of storage, a 16 day storage analysis (Table 21) also resulted in the combination of storage days*packaging type being statistically significant while also forming the largest single part for the accounted variation (41.8%).

Table 21: Ps and %SS of split-plot ANOVA (inoculation as sub-plot) for colour saturation, excluding the PVC pack's counts, over the 16 days of storage. %SS added to indicate accounted variation.

Treatment	Degrees of Freedom	P	%SS
Day	4	0.638	5.56
Gas	1	0.126	5.50

Inoculation	2	0.332	0.13
Day.Gas	4	0.007	41.83
Day.Inoculation	8	0.048	1.04

CO_2 and O_2 levels (Figure 33) in the 100% CO_2 packs remained fairly constant over the 16 days of storage, with a slight dip only notice in the CO_2 levels on day 8. The CO_2 and O_2 levels for the commercial mix pack however behaved rather unpredictable with the O_2 levels dipping on even sampling days and rising on un-even days while the CO_2 levels saw a steady increase up to day 12, after which it took a drastic drop to return to storage day 1 levels.
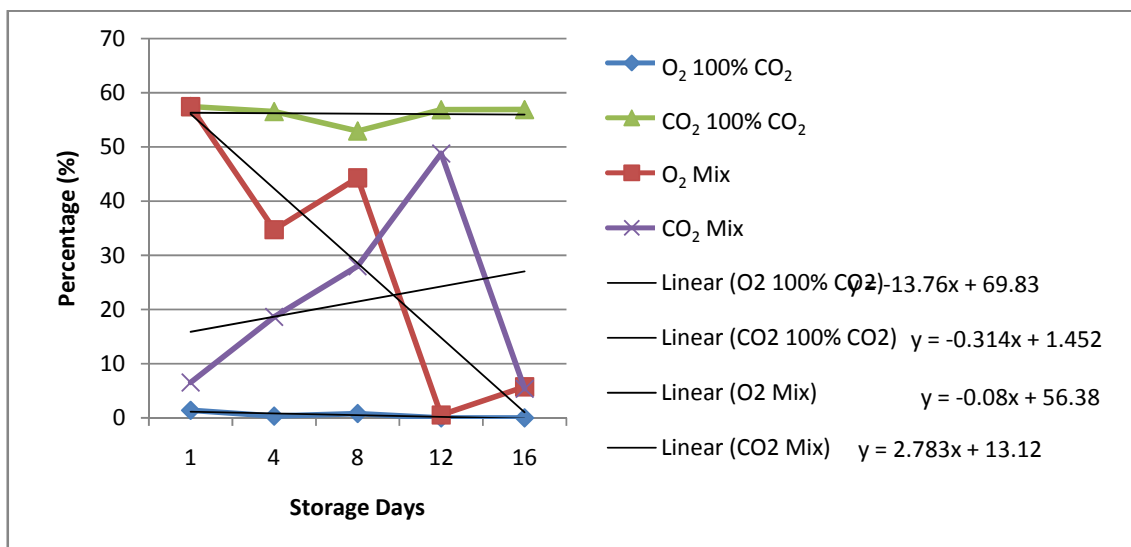
Figure 33: Percentage O_2 and CO_2 within the 100% CO_2 and commercial mix packs over the 16 days of storage.

pH determination

Significant differences in pH (Table 22) were observed when regarding both storage days (P < 0.001) and packaging type (P < 0.001) over the 16 days of storage. The commercial mix packs also had a substantially higher standard deviation and maximum pH when compared with both of the other packaging types. With the PVC overlay packs included in a variance analysis, only the packaging type was found to be significant (P = 0.025).

Table 22: Mean, standard deviation (SD), max and min pH values for the different packs and inoculation sites over their storage periods

		Inoculation site		
		Control	10^2	10^5
CO_2	Mean	5.53	5.53	5.52
	SD	0.19	0.19	0.20
	Max	5.78	5.80	5.76
	Min	5.21	5.22	5.19
Mix	Mean	5.78	5.78	5.77
	SD	0.42	0.43	0.39
	Max	6.95	6.92	6.76
	Min	5.35	5.33	5.35
PVC	Mean	5.79	5.84	5.75
	SD	0.08	0.21	0.06
	Max	5.93	6.26	5.81
	Min	5.67	5.69	5.68

Discussion

The effect of the background organisms on *S. aureus*

The effect of the dependent variables, the naturally present spoilage bacteria, on *S. aureus* was seen to be low when compared with the independent variables used in the experiments. Out of the four spoilage bacteria enumerated, only the Enterobacteriaceae and LAB was seen to contribute to the regression line drawn against *S. aureus* with them only contributing towards a 30% correlation. Neither organism was found to be significant in the correlation while the standard error was also high, with the whole correlation in the end also not being significant.

This indicates an almost entire independence, dependant on certain factors, of *S. aureus'* growth and survival relating to these enumerated spoilage bacteria, which contradict the literature, where it is stated that *S. aureus* does not compete well with other bacteria. The

deviation here might be caused by both the environment in which it is being tested as well as the independent factors that influence is it. This idea is supported by Charlier *et al.* (2009) who found that the environment, in terms of the effects of acidification, played a significant role in its effect on *S. aureus*. Here it was also found that the difference in initial levels also had a great influence on the way *S. aureus* acted in its environment.

Out of the two bacteria that form part of the stepwise regression analysis on *S. aureus*, the LAB appeared the most influential in the regression, even though it was not included at the first step. Much research has been done in terms of the effect LAB has on the inhibition of *S. aureus* in fermented foods (Charlier et al. 2009). Effects ranging from acidification of the environment to the production of bacteriocins and H_2O_2, have all been linked to this inhibitory action. However as the type of packaging was not found to be significant even though the 100% CO_2 packs had a slightly more acidic environment and the crude product was not a fermented one, the effect of the LAB could most likely not be attributed to the acidification of the environment

Since the other two enumerated groups, APC and Pseudomonas, only contributed to the regression in combination with the independent factors of storage days and inoculation level, the effect of the natural spoilage organisms could most likely only be attributed to an interaction or competition involving growth area and nutrients.

The effect of the independent variables on *S. aureus*

Out of the three independent factors tested against *S. aureus*, the level of inoculation had the greatest effect on the growth of *S. aureus* resulting in up to 70% of the accounted variation in the enumerated colonies. This was expected as initial higher levels of an organism should, if un-impeded by its own presence and growth and applied within the same dominating environment, result in higher enumerated values through the progression of days, when compared with lower initial values (Rho & Schaffner 2007). This effect however seemed to be of greater benefit for the higher level of inoculation in environments containing external stresses, 100% CO_2, than for lower initial levels and could be attributed to an increased rate of reproduction when comparing the exponential growth rate or the lower level of homogeneity (Koutsoumanis & Sofos 2005).

With the 10^2 inoculation concentration, initial levels were close to the applied concentration in testing after 24 hours, but only reached equivalent levels in the 100% CO_2 pack by day 4 while this only occurred in the commercial mix pack by day 16. This might be the result of the atmospheric stresses applied to the inherent spoilage bacteria, of which Pseudomonads are the dominating species and most oxygen sensitive, in the 100% CO_2 packs (Geysen et al. 2005; Masson et al. 2002), which decreased their growth, allowing faster colonisation by *S. aureus* of the meat surface.

The decrease after day 4 corresponds to a decrease in enumerated colonies experienced to large extent by all the other groups enumerated, with the exception of the LAB. The opposite was seen in the commercial mix packs whereby the decrease up to day 8 followed by the increase in *S. aureus* numbers, coincide with the increasing levels of CO_2 and decreasing levels of O_2 within the packs that would have affected the other species such as Pseudomonas a lot more than it would have affected *S. aureus* and its facultative aerobic characteristics.

With the 10^5 level of inoculation, in the commercial mix packs this level of inoculation seemed to have acted as a buffer towards the stresses experienced within the pack and can be related back to the work done by Koutsoumanis and Sofos (2005). In the 100% CO_2 packs, the steady decrease observed over the storage days could be due to the stresses affecting all the enumerated groups in the pack as well as the formation of dominance by the LAB and the effect they have on *S. aureus* (Charlier et al. 2009)

Although a slightly lower pH was recorded for the 100% CO_2 packs and the inhibitory effects of acidification on *S. aureus* have been described (Charlier et al. 2009), this had a slight effect, if at all, on the growth of *S. aureus* as the pH did not go lower that the minimal pH growth ranges of 4.6 and due to the packaging type not attribute much in the way of the accounted variation even though different pH values were obtained for the different packaging types..

An interesting observation was that in the PVC overlay packs, the general trend followed was that regardless of enumerated group, the counts obtained by day 4 was always higher than those obtained at day 1. This however did not ring true for the 10^2 inoculation levels of *S. aureus*, where an actual decrease in numbers was observed. This is most likely due to the

competitive effect that the other inherent bacteria could have on *S. aureus* through their numbers and their stability due to their presence, prior to inoculation.

Effect of the independent variables on the background organisms

The enumerated APC were mostly affected by the type of packaging as well as the days of storage, which was expected and coincided with similar findings (Nissen, Sørheim & Dainty 1996). In the commercial mix packs, APC was observed to increase constantly over the days, while in the 100% CO_2 packs, growth increased up to day 8 after which a plateau was reached which might be due to the restrictions conveyed by the high CO_2 and low O_2 levels in the packs.

For the Enterobacteriaceae and Pseudomonads, both were significantly affected by the type of packaging and the storage days with the steady growth observed in the commercial mix packs owning to the availability of oxygen as well as the low levels of CO_2 (Nissen, Sørheim & Dainty 1996).

The LAB was the least affected by the packaging type, with the commercial mix packs however showing a faster growth and higher final numbers. This could indicate that although LAB are not un-accustomed to high CO_2/low O_2 environments, a threshold of ideal ratios does exist that would in turn affect their growth. This warrants further investigation

Colour changes in MAP minced meat

By far the most puzzling and interesting aspect regarding the colour or saturation values of the minced beef where due to the fact that for the 100% CO_2 packs, all but the first days readings had values above the threshold value of 18 (Figure 32), which indicates consumer accepted colour (MacDougall 1977), while the exact opposite was observed for the commercial mix packs. The mere fact that the commercial mix packs contained a higher level of oxygen than the 100% CO_2 packs resulted in the commercial mix packs having more value above the 18 score threshold (Jakobsen & Bertelsen 2000; Miller 2002; MacDougall 1977).

The saturation readings over the different days also prompted interesting questions as to the role and level of influence of the O_2 and CO_2 concentrations in determining the colour of the

minced meat, as the reading for the commercial mix pack were stable while the readings of the 100% CO_2 pack was sporadic. As expected, these readings had no correlation to the CO_2 and O_2 readings obtained from the packs (Figure 33) as here the levels for O_2 and CO_2 remained relatively constant in the 100% CO_2 packs while fluctuations were observed in the commercial mix packs.

The results for the 100% CO_2 packs might be explained through the formation of carboxymyoglobin which would result in bright red colours being produced in meat (Mancini & Hunt 2005) through the binding of carbon monoxide to the deoxymyoglobin. This is the most feasible reason due to the high levels of CO_2 and low levels of O_2 detected in the 100% CO_2 packs.

The low saturation numbers obtained for the commercial mix packs might be as a result of the bacteria within the meat. APC levels were higher on a day to day basis in the commercial mix packs when compared with the 100% CO_2 packs, with one of the most substantial jumps in enumerated levels being recorded between day 1 and 4 (Figure 25). Their high numbers and increased growth might have resulted in them acquiring the oxygen and leaving low levels to bind to the haemoglobin in the meat.

This is substantiated when comparing the data with similar results obtained in the Chapter 4: Effect of modified atmospheres and natural background spoilage organisms on the growth of inoculated *E. coli* O157:H7 in minced beef. Here the saturation value for the commercial mix packs dropped belong 18 at the point that the APC counts reached 7 $\log_{10}$ CFU/g, while this same APC level was reached by day four in the enumerated APC counts in this study involving *S. aureus*. This hypothesis represents an interesting possibility that the colour of red meat is not only affected by the atmosphere in which it finds itself, but also by the level of organisms present (Buys et al. 2000).

Conclusion

S. aureus showed a high level of independence from other inherent bacterial groups in minced beef when regarding its growth and survival. The greatest effects were seen arising from the independent factors such as initial level in the meat and the type of storage and

packaging. Keeping this in mind, the single most important factor when considering the effects and the possible counter actions against *S. aureus*, is that nothing is a substitute for the good manufacturing practises of limiting the levels of *S. aureus* on the meat, regardless of packaging or other preventative measures that might be put in place.

This said, *S. aureus* never reached the level of 10^5 that is often cited in literature as the required level for the production of enterotoxins in either of the MAP packs prior to their spoilage that would in most cases prevent human consumption. This indicates that the stresses applied in the process of MAP, whether independent of the inherent spoilage organisms or not, are sufficient to not consider *S. aureus* a potential risk in MAP minced beef.

Apart from the safety issue of buying the contaminated meat, the packaging that suppressed the growth of *S. aureus* the most also had the best colour saturation out of the three packs tested. This would have drawn the costumer to purchase the "safest" mince meat pack unknowingly, with them basing their purchase solely on visual appearance. This also shows that the saturation level would often be unacceptable before the meat would be deemed un-safe, providing customers with a measurable scale related to food safety.

Chapter 6: General Discussion, conclusions and recommendations

Due to mince being composed of separate meat cuts that are comminuted and mixed, a large variability could exist between batches. Ideally packs from similar batches would have been preferred for the two separate pathogens, to enable a direct correlation to be drawn as well as allowing a direct comparison to be done. This however was not possible due to the large volumes of meat that was used as well as the need for sampling to occur within a specific time frame, which would not have allowed for the enumeration of two pathogens as well as their surrounding inherent meat bacteria.

Although the pathogen insertion technique was confirmed to not have a significant effect on the overall atmosphere, the possibility of errors during the insertion is always present. This insertion technique is also not the preferred one, as the predetermined volume of inoculants might not be proportional to the sample taken due to migration of the inoculants through the meat block into the drip sheet or the spread being non-uniform.

The ideal situation would have been to inoculate an entire block of meat prior to mincing with a volume proportional to the weight. This would have ensured that on average each gram of meat product contained the same amount of inoculated pathogen as well as removing the need for point inoculation and modified atmosphere pack puncturing. This was however not possible due to the need to involve the industry directly to ensure that the samples obtained were as close to what is being done on a daily basis as possible.

When comparing the use between normal full plate plating and quarter plate plating, the quarter plate method was the preferred one due to the decreased time spent in preparing media as half the media is required compared to the full plate method. Handling time was also decreased as four dilutions could be done through the handling of only a single plate while plating out an entire dilution series of 8 dilutions would only involve the handling of 4 plates. In terms of incubation of the dilution plates, the quarter plate method decreased the amount of space needed for incubation, with the decreased number of plates within a closed environment also allowing better air circulation and equal heat distribution.

Regarding the atmosphere analysis, it would have been preferable if the analyser could distinguish the composition of the remaining gas, after the detection of oxygen and carbon dioxide. Although this would not have influenced the study's results, it would have been interesting to compare the atmospheric breakdowns between the different packaging types and storage days.

During this study it was seen that the effect of the inherent background bacteria on the growth and survival of pathogen, was dependant on the specific pathogen observed. Neither *E. coli* 0157:H7 nor *S. aureus* was affected in the same way or by the same organisms across the board. *S. aureus* seemed to be minimally affected by the inherent bacteria while up to 60% of the variation in growth for *E. coli* 0157:H7 was attributed to the inherent bacteria. The bacterial group that was found to be most influential was that of the Enterobacteriaceae. The Enterobacteriaceae was seen to be the most influential to affect the growth and survival of both test pathogens in the commercial mix packaging. It was also the most influential towards *E. coli* 0157:H7's growth in the 100% CO_2 packaging and in the absence of the effects of the independent factors.

In the author's opinion, this finding could be due to the nature of the Enterobacteriaceae group in that it includes certain known pathogens as well as non-pathogenic varieties of certain organisms, including *Salmonella* and *E. coli*. Due to their similar nature; the presence of high counts of Enterobacteriaceae could result in some growth parameters being in over demand due theses high counts, resulting in direct competition between them and the test pathogens. Other bacterial groups of interest were the LAB, most likely owing its effect to the bactericidal substances and other metabolites it produces and APC which would most likely be due to the high numbers of organisms, competing for the same limited resources. Although there was competitive interaction seen, this in no way advocates the addition of certain species of bacteria as higher numbers would lead to a decreased shelf-life.

Of all the inherent meat bacteria enumerated, MAP seems to have the biggest effect on the *Pseudomonas* spp. compared to the effects noticed for LAB, APC and Enterobacteriaceae. This is of special concern to the meat industry as Pseudomonas is the main meat spoilage bacteria on fresh meat. Across the board, the 100% CO_2 seemed to have the largest detrimental effect on all the inherent meat bacteria with numbers in these packs being significantly lower than for the other two packaging methods. The PVC overlay packs,

containing no form of atmosphere modification, had the largest advantageous effect on the inherent meat bacteria with enumerated figures obtained by day 4 of storage in the PVC packs, often only being obtained at day 12 or 16 in the MAP systems.

As stated, the high CO_2 levels were found to have a detrimental effect on the growth and survival of the inherent meat bacteria, but high CO_2 levels alone did not give a uniform effect. It was found that apart from the high CO_2 levels playing a role in affecting the inherent meat bacteria that the remaining mix within the packaged atmosphere also contributed to this effect.

The two most prominent atmosphere combinations that were observed was a high CO_2/low O_2 and a high CO_2/high O_2 gas mixture. Out of these two, it was seen that the combination of a high CO_2/low O_2 had the largest detrimental effect on the inherent meat bacteria, often resulting in lower enumerated numbers. This however is of concern to the meat industry as a high CO_2/low O_2 combination would result in the meat products not displaying the bright red colour often associated with fresh meat. This can however be circumvented through the use of CO, carbon monoxide, that has been show to produce the bright red colour in meat as well as retarding bacterial growth. South African legislation does however not allow the use of CO and the public perception that precedes it would often not be positive.

The effect of the independent factors on the inherent meat bacteria

When taking into consideration the effect on the two pathogens, *E. coli* 0157:H7 and *S. aureus*, brought about by the independent factors; packaging type, inoculation level and storage days; the single largest correlation that was observed was in the inoculation level. For both organisms it was found that when the inoculation level was high, thus a high initial number, it always corresponded to a higher final number, indicating that any stresses or shelf-life improvement measures implemented post-processing would in most cases only keep pathogens at an initial level. This finding stresses the importance of good manufacturing principles (GMP) to ensure that whatever is not desired in the final product at consumption, should not be in the product before packaging.

The effects brought about by storage days and type of packaging was seen to be dependent on the organism. Here certain internal factors of the organism need to be considered including

its make-up of energy production through a final electron accepter and its resilience to external stresses, to determine the ideal type of packaging to be used. It was however noted in the packaging type that for both organisms, the larger the factor of CO2 to O2, the more suppressive to growth and survival of the pathogen the packaging method was.

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