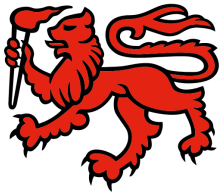

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Oxidants as an antimicrobial intervention on *Escherichia coli* during carcase chilling

by

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Bachelor of Science with Honours

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

University of Tasmania

June 2021

Declaration of Originality

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Acknowledgements

I would like to express my gratitude to the following people for their contributions to this project and to whom I am most grateful. My academic supervisors: Prof Tom Ross, Prof John Bowman, Dr Mellefont, Dr Kocharunchitt.

This work was supported by Meat and Livestock Australia Ltd., North Sydney, Australia, with special thanks to Ian Jensen, Long Huynh and John Sumner. I would sincerely like to thank the staff of the local abattoir for their help with sampling and packaging, in particular Blaze Barker and Phil Robinson.

The microbiology team at Tasmanian Institute of Agriculture, Lauri Parkinson, Zac Block, Pei Pei Zhang, Akikhun Nahar, Kaniz Mohsina, Tai Gardner, Tom McMeekin and Ross Corkrey are also gratefully acknowledged in the outcomes of this research.

Also, a special thanks to my friends and family, in particular my PhD companions: Kevin Feng, Ali Al Nassari, Tuflikha Putri, Ibrahim Abujabhah, Wossen Megenshia, and Colin Steedman.

List of Abbreviations

ABARE	Australian Bureau of Agricultural and Resource Economics and Sciences
ABI	ALIGAL Blue Ice
ahpF	Alkyl hydroperoxide reductase subunit F gene
ANOVA	Analysis of Variance
ASC	Acidified Sodium Chlorite
a_w	Water Activity
BHI	Brain Heart infusion Broth
cDNA	complementary DNA
CDC	Centre for Disease Control and Prevention
CFU	Colony Forming Units
ClO_2	Chlorine dioxide
CSIRO	Commonwealth Scientific and Industrial Research Organisation
cysK	Cysteine synthase K gene
DAWR	Department of Agriculture and Water Resources
Dps	DNA-binding Protein during Starvation gene
E-beam	Electron beam
EC	*E. coli* Strain
EMB	Eosin Methylene Blue Levine
EO	Electrolysed Water
EHEC	Enterohemorrhagic *E. coli*
FDA	Food and Drug Administration
FSANZ	Food Standards Australia and New Zealand
GLM	General Linear Model
GT	Generation Time
GRAS	Generally Regarded As Safe

grxA	Glutaredoxin A gene
HUS	Haemolytic Uremic Syndrome
H_2O_2	Hydrogen Peroxide
ibpA	Small heat shock protein IbpA gene
katG	Catalase-peroxidase gene
LAB	Lactic Acid Bacteria
USDA	United States Department of Agriculture
FSIS	Food Safety and Inspection Service
MAP	Modified Atmosphere Packaging
MLA	Meat and Livestock Australia
OD	Optical Density
PAA	Peryoxacetic acid
PAW	Plasma activated water
PFU	Phage Forming Units
PPM	Parts Per Million
RMAC	Red Meat Advisory Council
SAS	Statistical Analysis System
SDS	Sodium Dodecyl Sulfate
STEC	Shiga-Toxin producing *Escherichia coli*
TIA	Tasmanian Institute of Agriculture
TVC	Total Viable Counts
2D-LC-MS/MS	Two-dimensional liquid chromatography-tandem mass spectrometry
UV	Ultra Violet Light
VP	Vacuum Packed
yaaA	Peroxide stress resistance protein YaaA gene
yeeD	Putative sulfur carrier protein YeeD gene

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Abstract

The red meat industry is a multi-billion-dollar business for Australia and many other countries around the world. Ensuring meat quality and safety is paramount to the survival and economic success of the industry. Interventions used for hygiene and safety start from on-farm animal husbandry practices, to physical and chemical applications during processing, right up until consumption with the use of effective packaging and storage technologies. Processing-based interventions including knife trimming, organic acid washes, steam vacuums and UV light have been shown to improve the microbial shelf-life and safety of fresh meat. However, no single intervention can ensure complete safety of red meat.

Pathogenic *Escherichia coli*, especially the O157 serotype, have been linked to serval large food-borne outbreaks associated with consumption of bovine origin foods. This is despite control measures, referred to here as 'antimicrobial interventions', already in use during carcase processing. Currently, no single method is completely effective in controlling these pathogens.

Zero tolerance policies for pathogenic *E. coli* serotypes require Australian exporters to have confidence the meat products have minimal risk of *E. coli* and other pathogen contamination. Thus, there is the opportunity and desire for additional novel antimicrobial interventions within the processing system that can reliably achieve high quality, safe meat for export.

As carcase chilling is an existing step in the processing chain of meat, it would be an advantage to couple this process with an additional intervention that would enhance the inactivation of contaminant bacteria, especially pathogenic *E. coli* and *Salmonella* spp. In implementing an additional practise during chilling in plants already set up for spray chilling or spray washing of some sort, this approach should be relatively inexpensive and easy to put in place. In

deciding on any new intervention, consideration needs to be taken for the specific industry environment, ability to implement additional infrastructure, cost effectiveness for site specific issues and the types of products produced.

This study investigated the potential for oxidants as an additional intervention strategy when applied during carcase chilling. Initially broth-based studies were done to test King et al. (2016) hypothesis of increased susceptibility of gram-negative bacterial pathogens to oxidants during typical carcase chilling conditions. An oxidant (hydrogen peroxide at 75 ppm or chlorine dioxide at 7.5 ppm), at a non-lethal level under optimal (growth-permitting) conditions, was applied during dynamic changes in growth kinetics of *E. coli* O157:H7 Sakai, induced by abrupt downshifts in temperature and water (35°C a_w 0.993 to 14°C a_w 0.967). The addition of hydrogen peroxide at 1.5, 7.5, and 22.5 h after the downshift caused a reduction in viable bacterial counts (>3 log reduction) only at the 1.5 h time point. Similarly, chlorine dioxide when added to cultures under chilling conditions caused inactivation of *E. coli* O157:H7 Sakai though at all time points. Comparisons were also made with other *E. coli* and *Salmonella enterica* strains. All additions of chlorine dioxide at the early stages of the spray chilling conditions induced faster inactivation rates in culture-based trials. The results highlighted the potential application of oxidants under chilling conditions and further studies on meat tissue were warranted.

To determine further the potential utility of oxidants during carcase chilling, a series of laboratory-scale studies were undertaken to evaluate the efficacy of chlorine dioxide (ClO_2) or peroxyacetic acid (PAA) in eliminating *E. coli* O157:H7 Sakai on beef meat during the simulated process of spray chilling (4 sec every 15 min for 36 cycles) or when applied continuously prior to the spray chilling process (144 sec). In all cases, the effect of the oxidant was most evident on fat surfaces, rather than on lean surfaces. ClO_2 at 15 ppm, a non-lethal level under optimal growth conditions, when applied during spray chilling, caused higher

reductions in *E. coli* O157:H7 Sakai numbers (~3 log reduction) than when applied before the same spray chilling (~1 log reduction). This reinforces the increased susceptibility of *E. coli* O157:H7 Sakai to oxidative stress during spray chilling. In subsequent studies, both ClO_2 and PAA at levels of 20 and 200 ppm, respectively, produced more pronounced lethal effects on *E. coli*, achieving ≥4 log reduction at the end of chilling. These results indicate the potential for further developing an oxidant-based application during spray chilling as an antimicrobial intervention, to minimise the problems associated with enteric pathogens on beef meat.

The addition of an oxidant, in the early stages of chilling, warranted further studies on carcase chilling in commercial settings. Therefore, an in-plant trial was conducted to evaluate and compare the performance of two different oxidants (ClO_2 and PAA), as antimicrobial interventions for *E. coli* on beef. These trials involved the use of beef carcase sides and a chiller allowing for an industrial spray regime. Natural populations of *E. coli* were too low and infrequent to assess the effect of the oxidants. Therefore, a cocktail of non-pathogenic *E. coli* strains was deliberately applied to meat surfaces. Both oxidants reduced the total microbial count and *E. coli* significantly, although a greater effect was seen on the *E. coli* populations. A 1 - 3 log CFU/cm^2 reduction was found on the carcase sides depending on the site sampled. Sites located at the top of the suspended carcase, such as the hind leg and flank-brisket had the highest log reductions, most likely due to greater exposure to the spray. The results showed adding an oxidant to a spray chilling regime is an attractive potential antimicrobial intervention for industry, with little to no structural changes in the plant.

Shelf-life studies were also conducted following the in-plant trials with non-inoculated vacuum-packed (VP) striploins. VP striploins were stored at -1°C for up to 194 days and were assessed periodically for pH, viable count, and sensory attributes (colour, odour, and visual preference). No detrimental effects were detected for either oxidant treatment. However, some striploins had significant ($P < 0.5$) odour differences between PAA treated and non-treated

samples. There were also significant differences in visual preferences observed by sensory panellists for PAA treatments. Expansion of the sensory panel accompanied by training would make to make the results more reliable, because much variation was observed in the results of the small, untrained, panel used in this study. Also, different cuts and higher grades of meat would assist in the evaluation of the treatments from a sensory perspective.

Overall, PAA was found to be a more effective antimicrobial intervention compared to ClO_2 during chilling, with greater log reductions of enteric pathogens, greater compatibility with existing industry practices, and more positive outcomes from sensorial analysis. The current studies indicate that adding an oxidant to spray chilling systems in abattoirs can increase consumer safety and realise economic benefits for the industry.

Chapter 1

Literature review - Current and emerging interventions for red meat processing

Abstract

The red meat industry is a multi-billion-dollar global business for Australia and many other countries around the world. Ensuring quality and safety of these products is paramount to the survival and profitability of the industry. Therefore, investigations into current and emerging interventions being used or trialled in the processing of red meat, from farm to packaging, are discussed here. Interventions range from on farm animal husbandry practices, to physical and chemical applications, right up until consumption via package and storage technology. Such interventions including knife trimming, organic acid washes, steam vacuums, and UV light, have shown to improve the microbial shelf-life and safety of fresh meat. However, no single intervention can ensure the required quality and safety of red meat. A series of interventions already in use are shown to decrease the risk of contamination and therefore disease, though outbreaks still occur from meat products. This includes potentially very serious illnesses from *E. coli* O157:H7 as well as other pathogenic serotypes of *E. coli*. Chilling along with subsequent sanitation interventions provides an opportunity to apply hurdle stresses on meat surface contaminating bacteria, particularly pathogenic species. Implementing an additional sanitation step during chilling in abattoirs that are already equipped for spray chilling or spray washing of some sort should be relatively inexpensive and easy to put in place. In deciding on any new interventions, however, consideration needs to be taken for the specific industry environment, ability to implement additional infrastructure, cost effectiveness for site specific issues, worker safety, the types of products produced, and downstream effects on product quality and consumer acceptability. Ultimately the improvement of antimicrobial interventions

within the meat processing chain will benefit all, not only economically but to the health and safety of consumers.

1.1. Introduction

Australia's red meat industry is a large and profitable global business. Therefore, ensuring the quality and safety of these products is critical for the success of the industry for both domestic and international markets. Despite extensive research and technological developments in the food process industry, food-borne illness remains a global concern. In red meat processing, contamination of animal carcases during slaughter and subsequent processing include faeces, hide, oil, water, air, intestinal contents, lymph nodes, processing equipment, and from human contact (Bell 1997). The types of microorganisms and extent of contamination present on the final product are influenced by sanitation procedures, hygienic practices, application of food safety interventions, type and extent of product handling and processing, and the conditions of storage and distribution (Bell 1997). Current interventions are in place to reduce the risk of contamination of final products. Processing plants implement many antimicrobial interventions throughout the processing chain. However, no one form of intervention is totally effective at eliminating pathogenic bacteria. Cattle hides are continuously contaminated with faeces and thus also potentially contaminated pathogenic *E. coli*. Other organisms of concern to meat processors throughout the red meat supply chain (particularly during packaging and retail) include spoilage microorganisms and pathogens, such as *Salmonella enterica*, *Listeria monocytogenes* and *Clostridium perfringens*. All of these may be found in the faeces and on the hides of cattle presented for slaughter as well as being continually present in aerosols (Beuchat 2002; Rahkio et al. 1997; Reid et al. 2002; Ross 1999; Strydom et al. 1995) and can be transferred to the carcase during processing, particularly through hide removal and evisceration (Bell 1997).

The muscle itself is sterile, though in the process of hide removal contamination occurs. This contamination is a big problem for the meat industry and if not sufficiently controlled inevitably diminishes the quality and safety of the meat product. There are strict regulations in place for bacterial numbers and pathogenic detection in batches of meat, in particular with meat products undergoing export to other countries (Webber et al. 2012). Therefore, extensive interventions are put in place to minimise contamination during processing.

The aim of this review is to investigate the current and emerging interventions being used or trialled in the processing of red meat, from the farm gate to the packaging stage. Antimicrobial interventions are reviewed in how they are applied in pre-processing, processing, and post-processing stages. The goal is to identify truly and feasible effective antimicrobial interventions for the meat industry, with a particular focus on the decontamination of pathogenic *E. coli* strains.

1.1.2. *E. coli* O157 prevalence and why it is a problem

Global testing of processed beef products have shown prevalence rates of O157 to be as high as 54.2% while non-O157 occur 62.5% of samples for pathogenic *E. coli* surveyed (Hussein 2007; Hussein et al. 2005). Up to a third of meat *E. coli* isolates have the potential to cause disease. In comparing foods, Rantsiou et al. (2012) found a higher prevalence of these isolates in meat as well as in dairy, especially in fermented products. Virulent *E. coli* strains have been found in up to 42% of dairy products and 70% of meat products in certain countries (Rantsiou et al. 2012). These prevalence rates are alarming and detail the potential source of outbreaks linked to food, in particular meat and dairy products.

The first reported *E. coli* O157:H7 associated outbreak was in 1982 associated with contaminated beef mince, and from there an increasing trend in the number of outbreaks has occurred, possibly due to increasing popularity of ready to eat and highly processed foods

(Presser et al. 1998; Rangel et al. 2005). However, improved diagnostic, surveillance and incident management technology also contributes to the increased knowledge of serotypes and prevalence rates (Marder et al. 2018; Valilis et al. 2018). The outbreaks have been more notable in the US (Rangel et al. 2005; Tuttle et al. 1999). In late 1992 to early 1993, a large outbreak of *E. coli* O157:H7 infections occurred in the USA and were associated with eating ground beef patties at one fast-food chain (Tuttle et al. 1999). The source was found to be from contaminated cattle, this contamination was spread by combined batches of meat, to form an infectious dose of *E. coli* O157:H7 with less than 700 organisms (Tuttle et al. 1999). In the United States the Centre for Disease Control (CDC) has found up to 55% of all foodborne outbreaks to be caused by *E. coli*, with the majority link to raw beef products (Robertson et al. 2016). The Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) issued a zero tolerance policy after the 1992-93 outbreak, and more recently they have increased the serotype detection to include O26, O45, O103, O111, O121, and O145 pathogenic serotypes (Wang et al. 2012). The increase in serotype detection is a result of the increase prevalence of non-O157 strains and the increased incidence of pathogenicity (Schlackow et al. 2012; Valilis et al. 2018). *E. coli* are a normal gastrointestinal microorganism of humans and mammals. They assist in the essential breakdown of foods in the intestines, allowing a greater uptake of nutrients for their host. Although most strains of *E. coli* are not harmful, some strains possess characteristics including virulence factors that enable them to cause several forms of gastrointestinal disease (Todd et al. 2001). Pathogenic *E. coli*, especially the O157 strains have been linked to foodborne outbreaks associated with undercooked meat and meat products (Armstrong et al. 1996; Smith et al. 2014). *E. coli* O157:H7 is a subgroup of enterohemorrhagic *E. coli* (EHEC) which produces Shiga and hemolysin capable of enterocyte effacement. EHEC strains can cause severe gastroenteritis and potentially more serious conditions, in particular Haemolytic Uremic Syndrome (HUS) in

susceptible people (Phillips 1999). This pathogen is often present on hides, in faeces, the rumen and derived carcases (Elder et al. 2000; Hancock et al. 2001; Van Donkersgoed et al. 1999). Therefore, the potential presence of *E. coli* O157 on carcases can lead to a very serious threat to the meat industry and its consumers.

Cattle are a major reservoir for *E. coli* O157:H7, *E. coli* including O157:H7 strains are natural gastrointestinal microbiota of cattle. This pathogenic E. coli variety is not generally pathogenic to adult cattle (Armstrong et al. 1996), though may cause diarrhoea and other symptoms in young calves. Several studies have found the prevalence of *E. coli* O157:H7 to be much higher in summer months of the year (Barkocy-Gallagher et al. 2003; Khaitsa et al. 2003; Van Donkersgoed et al. 1999). This increase is detected in the faeces and subsequently also on the hides of cattle (Barkocy-Gallagher et al. 2003). Studies have also detected greater number of *E. coli* O157:H7 on the hides than in the faeces, implying a build-up of the pathogenic bacteria over time. Barkocy-Gallagher et al. (2003) also noted this increase in O157 during the summer months, though continually noticed numbers on hides to be high from spring through to autumn. However, a study by Elder et al. (2000) found the opposite with relatively low hide prevalence for EHEC O157 compared with faeces. Another study by Khaitsa et al. (2003) grouped cattle in pens to follow the shedding pattern of O157 over the summer period. They found the results varied dramatically between pens and cattle, though a distinct pattem emerged. Over a 19 week period Khaitsa et al. (2003) found after an initial low period of eight weeks, a high period of shedding occurred for 7 weeks. Heightened amounts of cells residing on the hides of cattle, for potentially a long period, highlights that hides can spread contamination to meat carcases during abattoir processing.

Bonardi et al. (2001) also found similar results to that of Khaitsa et al. (2003) with 12% of isolates off carcases being EHEC strains, with most contamination occurring on the legs. EHEC O157 subtyping showed that a strain originally present on one animal, may spread to many

others by direct contact, especially when cattle coming from different farms are kept together in abattoir pens before slaughtering (Bonardi et al. 2001). Therefore, this may explain why larger abattoirs have more cross-contamination compared to small more manual processing plants (Bonardi et al. 2001).

Beef carcases are initially sterile and during processing, hide removal and evisceration, contamination occurs from either the skin, aerosols, intestinal material (auto-contamination) or from knives, machinery, and other carcases (cross-contamination). Bacon et al. (2000) found hide counts to be 5.5-7.5 log CFU/cm^2 for *E. coli* prior to skinning with the counts reducing to 2.6-5.3 log CFU/cm^2 on the carcase. Thus, this shows skinning as a major source of contamination, particularly on the rump area (Gill 1996). Elder et al. (2000) found EHEC incidence increased on the posterior region of the carcases at the peak shedding time. In particular, the rump area had the highest level of contamination. In the abattoir a series of decontamination processes occur. Gill (1996) found two such steps, trimming and washing, did not successfully decontaminate the neck and rump areas. The rump they found was still contaminated even after extensive trimming. Other sources of significant contamination have been found from dropped carcases during de-hiding (Brown et al. 2013), as well as worker's gloves and conveyor belts (Youssef et al. 2013). Multiple interventions are used to decontaminate fallen carcases or they are condemned (Brown et al. 2013).

Studies have shown the reduction of total bacteria and *E. coli* counts from the initial hide until chilling, through a series of decontamination steps provides an additive reduction of contaminating bacterial numbers (Bacon et al. 2000). Thus, a series of decontamination steps throughout the processing chain works best at decreasing *E. coli* counts overall, with individual interventions being insufficient for practical decontamination.

1.2. Pre harvest strategies

Minimising the risk of spreading *E. coli* in the abattoir environment starts at the farm. The cattle are withheld food and in some case feed specific diets are used to minimise pathogenic *E. coli* in the gut, or to minimise the faecal waste produced from the cattle in transport and holding prior to processing (Thormar 2012). Other techniques to minimise *E. coli* contamination include the washing of the animals prior to processing. Research into various acid washes have achieve greater than 3 log unit reductions of pathogenic *E. coli*, including lactic (Yoder et al. 2012), caprylic, resorcylic (Baskaran et al. 2013) and hypobromous acid (Schmidt et al. 2012) solutions.

1.2.1 Feed supplements

Natural feed supplements are now being favoured over past traditional process such as antibiotic use. The use of antibiotics was a common practise in maintaining the health and productivity of livestock in the past and was continued for many years, until the realisation that many pathogenic bacteria, particularly foodborne pathogens were acquiring antibiotic resistance (Swartz 2002; Threlfall et al. 2003). Antibiotic use or over use has now been banned or negatively perceived by the general public in favour of more natural feed supplements (Thormar 2012). Natural feed supplements include probiotics, prebiotics, bacteriocins, organic acids, enzymes, bioactive phytochemicals, antimicrobial peptides, lipids, and bacteriophages with several products being commercially available and patented (Cheng et al. 2014; Johnson et al. 2008; Thormar 2012).

Several studies have considered the effects of different feed ingredients and diet on the shedding of pathogens by livestock, but the results are often conflicting. The change in diet and management practices could actually increase shedding of pathogens, due to the

gastrointestinal stress and potential pH change that can result (Greer et al. 1997; Markowiak et al. 2018). Other trials have used an extract from the brown seaweed *Ascophyllum nodosum* as a feed additive to promote stress tolerance, and when given 14 days prior to slaughter this product has shown potential to decrease the prevalence of *E. coli* in faeces and on hides (Barham 2001). In addition, other studies have investigated the application of chitosan micro-particles as a feed supplement and found this potentially may reduce the numbers of *E. coli* O157:H7 being shed (Browne 2007).

There is also a significant amount of research into the feeding of probiotics, bacteria, and other microbes with the purpose of providing health benefits when consumed, to livestock to competitively exclude the pathogens. Some organisms have also shown promise in reducing the incidence of *E. coli* O157:H7 in calves (Markowiak et al. 2018; Zhao et al 1998), while natural products from some non-pathogenic *E. coli*, such as 'colicins', seem to have some inhibitory effects on *E. coli* O157:H7 (Schamberger et al. 2004). On the other hand, sodium chlorate, given by mouth to cattle, sheep and pigs has been shown to reduce *S.* Typhimurium and *E. coli* O157:H7 within intestinal contents (Anderson et al 2001), and work is underway to see if this can be used in the field.

1.2.2. Vaccinations

Vaccines are under substantial research with trials into the production of a vaccine against *E. coli* O157:H7 for cattle. A two-dose vaccination regime effectively reduced *E. coli* O157:H7 faecal shedding and hide contamination (Thomson et al. 2009). The vaccines are available in Canada and the USA (Matthews et al. 2013). The USDA granted approval in 2009 for a conditional licence to Epitopix to sell a vaccine. Cull et al. (2012) examined the efficacy of an *E. coli* O157:H7 vaccine. They found that the level of *E. coli* O157:H7 positively decreased in

cattle (from 31.7% down to 17.4%) as did the incidence of high shedding cattle (from 3.5% down to 0.95%).

1.2.3. Hygienic practices

The hygiene of water troughs have been shown to support *E. coli* O157, and be a source of colonisation of previously 'clean' animals, so control of pathogen populations in the water could be a possible means of reducing the incidence (LeJeune et al. 2001). Chlorine would appear to be the treatment of choice, but some strains of *E. coli* are particularly resistant to chlorine, and animal water troughs often contain large amounts of organic material, which would inactivate the chlorine (Zhao et al. 2006).

Cattle faeces are reported to contain an aerobic plate count of 6-7 log CFU/g (Salter et al. 2000), and an adult bovine can void up to 25.5 kg of faeces and 12-22 litres of urine in 24 hours (McGrath et al. 1969). Therefore, if there is insufficient bedding or drainage in animal accommodation, or poor design or maintenance, faecal soiling of the hide can occur. A link between hide microbial contamination and subsequent carcase contamination has been reported (Zhang et al. 2010). It has been suggested that if hide contamination can be controlled, the contamination of carcases can be controlled. Several studies have also concluded that the holding environment is more significant than the originating feed lot or farm, with *E. coli* O157:H7 contamination on cattle hides (Deng et al. 1998; Guo et al. 2011; Hiramatsu et al. 2005). Conversely, others concluded that hides were more likely to be contaminated with *E. coli* O157 at the feedlot than at the abattoir, although they found high prevalence of this organism in holding pens at the abattoir (47% of samples taken from the pen rails and 42% of samples taken from the pen floor) (Fegan et al. 2009). Australian abattoirs often reject animals that are delivered exceptionally dirty or separate them for further treatment before they are processed at the end of the day prior to cleaning to prevent contamination.

1.2.4. De-hairing

De-hairing of cattle hides is another pre-processing intervention used by some abattoirs. It is considered an effective intervention to reduce the incidence of hide-to-carcase contamination with pathogens. Nou et al. (2003) showed the effectiveness of this hide intervention in beef processing to significantly reduce carcase contamination by *E. coli* O157:H7.

1.3. Processing strategies

Carcases are sterile until processing where hide removal, evisceration and anything that contacts the carcases can cause contamination. Several interventions are currently in use to control carcase contamination within a single processing plant. Techniques in place range from trimming contaminated sites on the carcase to acid wash steps. During processing the decontamination of implements and equipment is also undertaken. After processing the carcases are chilled to maintain quality, which also causes a potential decrease in microorganisms. However, no single intervention can provide 100% assurance of safe meat. This indicates the need for developing new or improving existing interventions that are effective in controlling or reducing the pathogens on carcases.

1.3.1. Carcase trimming

Trimming has previously been reported to be effective in reducing the aerobic plate count (by 3 log CFU/cm^2) and *E. coli* O157:H7 (by 3.1-4.4 log CFU/cm^2) on carcases (Akyol 2018; Neidhardt et al. 1990; Visvalingam et al. 2018). However, when trimming was used in conjunction with washing, higher counts were reported compared to trimming alone. A explanation for this may be cross-contamination during the washing process (Visvalingam et al. 2018). Gill et al. (1996) also found no conclusive evidence that trimming and washing was

an effective decontamination process although others have with aerobic counts alone (Reagan et al. 1996). Therefore, it is thought the effectiveness of trimming and washing depends on many influences such as the operator, the extent of visible contamination compared with non-visible contamination, and the temperature, angle and pressure of the wash waters used in each of these studies.

Trimming is a manually intensive process, requiring skilled personnel and careful disposal. It is also a potential cost to the industry with not only subsequent loss of meat with damage to certain cuts resulting in a lower quality product and therefore saleable price. Although there are many negatives, visible removal is still considered the easiest way to prevent potential contamination (Gill 2004).

1.3.2. Hot water

Hot water is a common decontamination step in abattoirs with its ease of use and setup though its continual use can be relatively expensive over time. The major concern with its practical use is its effect on the sensory quality of the meat, such as colour, texture and odour. Gill et al. (1999) trialled hot water treatment (85°C) to one half of a beef carcase, were they found a 2 log reduction in *E. coli* (15 and 10 sec) with a further small decrease after chilling. The hot water treatment of pasteurising the meat for 10 seconds proved not to be detrimental to the overall meat grade. Bosilevac et al. (2006) compared a lower temperature of 74°C and lactic acid. Hot water was considered significantly more effective with an 81% reduction in *E. coli* O157:H7.

Reductions in bacteria were obtained when surfaces were treated with water >80°C for 10 s, though no benefits at increasing temperatures over 85°C for longer periods (Davey et al. 1989; Gill et al. 1999; Smith et al. 1978). If water temperatures were reduced below 80°C, the treatment times were increased to reduce bacterial numbers (Smith 1992). The reduction in

time was found to reduce costs of the treatment and any sensory effects without a loss to decontamination efficacy (Gill et al. 1999). Gill et al. (2000) confirms treatment time of 11 s should be set to obtain the maximum reductions in coliform and *E. coli* numbers.

In other comparison studies, spray treatments with hot water have been shown to be the most effective, resulting in higher pathogen reductions than other antimicrobial interventions such as various acid washes (Sala et al. 2012; Yang et al. 2012). Although hot water has proven to be a good decontamination intervention, it is not 100% effective at eliminating pathogens from all over carcases, therefore an additional or substitution step is still required.

1.3.3. Lactic acid

Washing with lactic acid solutions is often used as an additional treatment after water washing occurs to the carcase. It is often used in conjunction with other treatments such as hot water and other compounds. The benefit of lactic acid is that it is an approved organic acid for food processing and consumption, and is relatively cheap to procure and utilise, particularly for abattoirs that already possess wash cabinets (Chen et al. 2012). The hazards of using lactic acid are low with it being classified only as a low irritant, although it can cause corrosion to equipment over time, accelerated meat spoilage in some instances, and possibly induces acid resistance in microbial populations (Chen et al. 2012).

The concentrations of lactic acid investigated range from 1-5% (vol/vol). In processing plants, the usual concentration used is 1-2% (v/v). Several studies have shown that the increasing concentration of lactic acid had a greater log reduction for both pathogenic *E. coli* and non-pathogenic *E. coli* (Castillo et al. 2001; Gill et al. 2004; Youssef et al. 2013), and a similar reduction between pathogenic and non-pathogenic *E. coli* (Akyol 2018; Youssef et al. 2013).

Hamby et al. (1987) applied the concept of repeated treatment of 1.0% lactic acid as a spray on beef carcases over 28 days storage. The total counts of bacteria were found to be significantly

lower overtime. Pittman et al. (2012) decided to use two lactic acid treatments on beef sub-primal cuts. Both treatments were successful in decreasing the non-pathogenic and *E. coli* O157. The initial treatment reduced *E. coli* O157 by 1.6 log units and the secondary wash by 2.8 log units. These results suggest multiple treatments of even the same treatment, as in these cases of lactic acid, can be beneficial during storage and packaging.

Lactic acid solutions are usually applied in the form of a rinse spray for economical and efficiency reasons, another way is to use it as a dip rinse of carcases. Wolf et al. (2012) compared the dip and spray two techniques. They found that the dip approach actually resulted in the largest decrease in numbers of pathogenic *E. coli,* including O157, and *Salmonella*. The log reduction for the lactic acid dips were between 0.5 to 1.41 log. The negative aspects of the dip treatments are the amount of solution required, decontamination of the waste and the potential risk of cross-contamination. Samelis et al. (2002) found *E. coli* O157:H7 survived in decontamination fluid at low temperatures, mimicking abattoir processing temperatures. In lactic acid washes *E. coli* survived for more than 2 days, and in acetic acid washes for 7 days, with better survival rates at 4˚C than 10˚C. *E. coli* survived, though did not grow in water washings.

Kalchayanand et al. (2012) found lactic acid to be effective in reducing various pathogenic strains including *E. coli* O157:H7 though found it was not as effective as hot water treatments. In contrast, Castillo et al. (1998) observed similar, >4 log unit reduction when either lactic acid spray or hot water was applied. Whereas water wash and trimming treatments were found to decrease the overall faecal contamination, they also spread pathogens on the carcases. Hot water and lactic acid treatments were the best combinations found to decontaminate the carcases by Castillo et al. (1999a), though Yoder et al. (2012) confirmed that lactic acid spraying could also achieve this with a 3.5-6.4 log reduction of *E. coli* O157 on beef carcases.

Many studies have investigated the effect of lactic acid on meat carcases or cuts, the reductions for *E. coli,* pathogenic and non-pathogenic, range from 0 to >6 log (Samelis et al. 2002; Yoder et al. 2012). The differing results may be due to other factors such as application rates, at which stage the treatment was applied, the temperature of the environment and the acid solution. The nature of interventions previous to the acid washes also influences the physiology of the *E. coli* cells and can result in acid habituation an increased acid survival (Du et al. 2019). Youssef et al. (2012) found that the type of tissue exposed to the lactic acid had a larger influence than the lactic acid concentration. They found a greater reduction of *E. coli* on the membrane of beef rather than on fat or cut muscle. Castillo et al. (2001) observed greater reductions with hot lactic acid on chilled beef carcases, while King et al. (2005) found better results with an application at the initial chilling stage; Pittman et al. (2012) on the other hand found a greater reduction of *E. coli* numbers occurred after a repeated treatment of lactic acid.

1.3.4. Peroxyacetic acid

Peroxyacetic acid (PAA) or peracetic acid is used for sanitizing specific fresh food products with concentration of up to 80 ppm in wash water, while 220 ppm is allowed by the United States (FDA 2018) on raw meat products and at non-specified levels in Australia (FSANZ 2016). The results of PAA are varied at different concentrations and for different fresh food types (Vandekinderen et al. 2009). Abadias et al. (2011) found that with apples levels as low as 20 ppm reduced *E. coli* O157 over 4 log CFU/g. Mohan et al. (2012) and Gill et al. (2004) both found PAA at 0.02% effective at lowering total counts and *E. coli* counts on beef, though not as effective as other acid washes, PAA has no detrimental sensory effects and the meat maintained suitable redness. PAA actually showed potential to maintain or improve odour sensory qualities at high levels, i.e. 200 ppm, and at the same time significantly reduced *E. coli* and *S. typhimurium* on beef trimmings (Quilo et al. 2009).

1.3.5. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a commonly used chemical in various industries and is easily available. H_2O_2 is a highly reactive oxidant that penetrates bacterial cells and can inhibit growth of *E. coli* and cause inactivation (Smirnova et al. 1997). It is been used to reduce bacterial numbers of a variety of fresh food products with relative success, although varying concentrations are required (Olaimat et al. 2012). The Food Standards Australian New Zealand, Standard 1.3.3, allows its use as a general food processing aid at a maximum of 5mg/kg residual on all food types. The FDA allows 8 mg per capita of H_2O_2. Generally, it is used for washing, bleaching, peeling, as a control for Lactic Acid Bacteria (LAB), to maintain pH, and for general water treatment. The dairy industry in Australia has recently been approved to use H_2O_2 to control LAB in dairy products to the 5mg/kg level in food. In fresh produce concentrations as low as 0.5% are used to lower bacterial populations in mushrooms (Sapers et al. 1998), and 1% has reportedly reduced *E. coli* O157 1.8-3.5 log on whole apples though not on cantaloupe, suggesting a surface effect (Sapers et al. 2003). The concentration for poultry carcases has been seen at 1.2% able to reduce 80% of *E. coli* contamination, though adverse effects of bleaching and bloating of the carcase were observed (Lillard et al. 1983). On beef carcases a usage of 3% resulted in a 2.7 log reduction of model pathogenic bacteria (Bell et al. 1997). At this concentration of H_2O_2 some level of meat lightening was seen, though only a slight change of pH occurred. In the same study by Bell et al. (Salter et al. 2000) 3% H_2O_2 in combination with acetic acid produced an even greater reduction of *E. coli* up to 4 log CFU/cm^2. The combined use of acetic acid and H_2O_2 did affect the pH and left a residual level of 1.4 ppm of H_2O_2 on the carcases not seen with H_2O_2 alone. Therefore, when using H_2O_2 consideration into the pH of the food type is an important factor, on not only the sensory qualities, though also on residual levels of H_2O_2 (Salter et al. 2000). At near neutral pH H_2O_2 degrades to water and oxygen. ther

than pH, H_2O_2 may also lose its effectiveness as a decontaminate when it reacts with different surfaces, such as soil and metals (Sapers et al. 2003).

1.3.6. Chlorine derivatives

Sodium chlorite ($NaClO_2$), chlorine dioxide **(**ClO_2) and derivatives are commonly used to produce chlorite ions (ClO^-), which are toxic to bacteria cells, *E. coli* and *Salmonella*. Most trials have applied these chemicals in carcases or other fresh produce washing. Although some studies are looking into the idea of feeding cattle, sheep and pigs sodium chlorate ($NaClO_3$) in order to reduce intestinal populations of *E. coli* and *Salmonella* (Anderson, et al. 2000; Taylor et al. 2015). In the factory environment the benefits of chlorine dioxide, the by-product of acidified sodium chlorite (ASC), is that it is non-corrosive and is also relatively inexpensive to set up in most plants, and it leaves no residual chlorine or detrimental sensory effect on the fresh produce. Rajkovic et al. (2009) studied *E. coli* O157:H7 and found no resistance with the repeat use of chlorine dioxide as a processing treatment. However, for the use of chlorine dioxide the mixture of acid and sodium chlorite has to be made up prior to use, and allowed the recommended time to activate, usually 30 minutes depending on the product (Keskinen et al. 2009). Acids used to activate sodium chlorite are phosphoric acid, citric acid, lactic acid or any 'generally regarded as safe' (GRAS) acid (FSANZ 2016). In a comparison between phosphoric acid and citric acid, Castillo et al. (1999b) found citric acid to be more effective in assisting in lowering the counts of *E. coli,* extending the reduction by ~0.5 log.

Australian and NZ food standards and the FDA indicate the maximum amount of chlorine dioxide that can be used is between 500-1200 ppm, depending on the method used, dip or spray (USDA 2013). The Australian processing aids, Standards 1.33 clause 12, states that sodium chlorite as a wash on all foods is allowed, with maximum residue levels to be no more than of

1 mg/kg (available chlorine) in the final food product, in alignment with other chlorine based agents (FSANZ 2003).

Non-pathogenic *E. coli* was shown to be effectively reduced on meat products by as much as >8 log CFU/cm^2 using 20 ppm sodium chlorite, according to Elano et al. (2010), whereas for more virulent strains, such as *E. coli* O157:H7, levels of up to 1200 mg/L were found to be more successful (Castillo et al. 1999b). Castillo et al. (1999b) found >4 log reductions on beef carcases using high levels of acidified sodium chlorite after a prewash reduction of around 2 log units. Similarly, on inoculated beef cubes Lim et al. (2004) also found a reduction of *E. coli* O157:H7 by 2.5 log and that ASC alone was the most effective wash, rather than in combination with 0.5% cetylpyridinium chloride or 0.1% potassium sorbate. Some sensory effects, mainly lightening of the beef surface, were noticed as the pH decreased for ASC treatments. ASC pH has to be specific to the product of use to prevent any loss of sensory quality (Lim et al. 2004).

Higher levels of chlorine dioxide are found to be more effective than lower concentrations according to Yoder et al. (2012) and Bosilevac et al. (2004) though lower levels are best for retaining the quality and shelf life of ground beef. In comparing chlorine dioxide with a more traditional approach of a hypochlorite wash on seafood, Andrews et al. (2002) found a greater log reduction of total bacteria counts with increased concentration up to 40 mg/L than aqueous chlorine at slightly higher levels, with no residual chlorine.

Acidified sodium chlorite has been shown to be a successful wash for fresh produce, meat and vegetables. Inatsu et al. (2005) found *E. coli* O157:H7 was reduced by up to 3 log units before sensory quality of Chinese cabbage leaves were affected. Ruiz-Cruz et al. (2007) found *E. coli* O157:H7 to be reduced by 2.5 log units on carrots. Gonzalez et al. (2004) also found ASC to reduce pathogens over 5 log units at 1000 ppm on carrots, a greater reduction than chlorine, citric acid and water. Andrews et al. (2002) and Castillo et al. (1999b) both found chlorine

dioxide or acidified sodium chlorite sprays after a pre-water wash were most effective. Alonso-Hernando et al. (2013) found ASC worked best at 4°C rather than high temperatures. In contrast Kalchayanand, Norasak et al. (2012) found acidified sodium chlorite less effective than hot water, lactic acid and peroxyacetic acid. Anderson et al. (2000) found sodium chlorate reduced *E. coli* O157:H7 5 log with concentration of 5 mM at pH 6.8, although the results are pH dependant. Keskinen et al. (2009) found with the ASC treatment the remaining bacteria were associated with biofilms or damaged tissue.

1.3.7. Ozone

Ozone has been tested for its oxidative effects on meat carcases and cuts. The results of studies have not seen a dramatic effect on reducing microbial counts with *E. coli* contamination. Studies by Cardenas et al. (2011) and Rahman et al. (2013) have shown up to a 1 log reduction in counts with relatively low concentrations of 5 ppm for carcases and 72 ppm for meat cuts. Ozone has two types of applications in the form of gas or as ozone water. In a pre-processing step of washing cattle hides with ozone water promising results have been seen. Studies by Bosilevac, J. M. et al. (2005) have shown ozone water reduces total and *Enterobacteriaceae* counts by 2.1 and 3.4 log CFU/100 cm^2, respectively. The prevalence of *E. coli* O157 on hides was also seen to reduce from 89% to 31% following treatment with ozone water, similar results to that of electrolysed water. Ozone and EO waters could be beneficial if used to decontaminate hides, significantly reducing pathogen loads on beef hides, and thereby reducing pathogens on beef carcases (Bosilevac et al. 2005). However, some studies indicate high cost and harmful effect of the gas makes it an undesirable intervention. Others justify the cost as being cheaper compared to high temperature treatments and the harmful gas as manageable, short lived issue with no storage of hazardous material being required (Pandiselvam et al. 2019).

1.3.8. Electrolysed water

Electrolysed water, which is created by electrolysis of NaCl, forming a hydrochlorous acid (HClO) solution (neutralised to around pH 6 for maximum efficacy), has shown great potential with reducing *E. coli* O157:H7 in broth and water systems with up to 8 log reductions (Kim et al. 2000; Park et al. 2004). Whereas when applied to the carcases it has been shown to have a lesser effect with reductions in *E. coli* O157:H7 up to 2 log and in combination with calcium lactate a reduction of up to 3 log (Rahman et al. 2013).

1.3.9. Plasma activated water

Plasma activated water is made up of nitrate, nitrite, and hydrogen peroxide species. At acidic pH nitrate and nitric oxide are present and act as powerful oxidants. Naitali et al. (2010) showed that the efficiency of PAW was diminished at neutral or buffered pH. Their results showed PAW was more efficient in reducing Hafnia (5 log) than the nitrogen species and hydrogen peroxide alone (Naitali et al. 2010).

1.3.10. Natural compounds

Natural antimicrobials such as the compound nisin, produced from lactic acid bacteria such as *Lactococcus lactis*, has been applied to meat and meat products owing to its specific antimicrobial qualities (De Alba et al. 2013). Nisin at 3% has been shown to have reductions of *E. coli* O157 of up to 4 log units in combination with 500 MPa high pressure (De Alba et al. 2013). Overall nisin was an effective antimicrobial on meat products with maintaining pH, though had detrimental sensory effects on the product. By comparison, other compounds such as lactate and acetate-lactate had better results with maintaining sensory attributes and reducing microbial counts (Cárdenas et al. 2013). Other compounds such as SDS (sodium dodecyl

sulfate) and levulinic acid (4-oxopentanoate) alone have very limited effect on *E. coli* O157:H7, although when combined as a treatment they were highly effective at 30 and 60 min of incubation. A solution containing 0.5% levulinic acid with 0.05% SDS results in up to 7 log reduction of *E. coli* O157 (Zhao et al. 2009). In treating vegetables 3% levulinic acid plus 1% SDS applied for 20 s reduced both *Salmonella* and *E. coli* O157:H7 by up to 6.7 log CFU/g (Zhao et al. 2009).

Other naturally occurring and novel, generally recognized as safe, compounds have been found to be effective at reducing *E. coli* O157:H7. They consist of caprylic acid (octanoic acid) and β-resorcylic acid (2,4-dihydroxybenzoate) on hides by 3 to 4 log CFU/cm^2 (Baskaran et al. 2013), acetoacetic acid and β-phenylethylamine with 50% to 90% on beef pieces (Lynnes et al. 2014) and the plant extract from olives with up to 7 log (Rounds et al. 2013).

1.3.11. Bacteriophage

Bacteriophages are considered a natural antimicrobial and have the potential to decrease pathogens on meat and meat products. Treatment with specific phage allows the control of likely pathogens. Phage are approved by US FDA and they are used for treating farm animals or animal products such as carcases, meats and vegetables (Sillankorva et al. 2012). The use of phage can also be biocontrol agents in foods, and biosanitizers of food contact surfaces (Sillankorva et al. 2012). Phage have specific interactions so have a limited efficiency to bacterial population and hence require a cocktail of multiple types. The use of cocktails also helps prevent rapid resistance and subsequent loss of efficiency over time (Brockhurst et al. 2017; Pires et al. 2016). In trials phage FAHEc1 at >10^7 PFU/ml was found to result in a 4 log reduction of *E. coli* O157:H7 at 5 °C in broth (Hudson et al. 2013). When replicating conditions of hot boning and conventional carcase cooling, inactivation of *E. coli* O157:H7 of

approximately 2 log with phage levels at 3.2×107 PFU/4 cm^2 on meat pieces (Hudson et al. 2013).

1.3.12. Steam pasteurisation

Steam pasteurisation is a fast, cost effective method, which is suitable for treating most meat products (Chen et al. 2012). The use of steam has advantages over the use of hot water due to the potential energy released when steam condenses, thus achieving a more rapid rise in the surface temperature of the meat. Steam pasteurisation was approved for use in the USA in 1996 (Signorini et al. 2018) where the standard recommends a temperature of 90°C or greater to be reached at the meat surface for 5-15 seconds. This is then followed by rapid cooling, usually immediately prior to the holding cooler. Commercial systems are available and in wide use in the USA and Australia, however the use of steam pasteurisation is not widely used in the EU. Studies on whole carcases have shown steam pasteurisation to be an effective technology. Nutsch et al. (1997) observed reductions in aerobic plate counts of around 1.5 log CFU/cm^2 and the reduction of coliforms to below detectable levels, following a 6-8 seconds treatment. Phebus et al. (1997) using a similar system obtained similar results and showed that the reduction was uniform over the surface of the carcase. More recently, Milios et al. (2011) trialled a steam pasteurisation system in a slaughterhouse in Greece and observed reductions of 0.72 log CFU/cm^2 and 0.95 log CFU/cm^2 in total viable count and *Enterobacteriaceae* on lamb carcases, respectively.

Combining two treatments, such as steam condensation on meat surfaces and hot water immersion, particularly chlorinated hot water, has been shown to effectively decrease the bacterial load on lamb (Chien et al. 2017). Dlusskaya et al. (2011) also found evidence that the use of thermal pathogen intervention treatments in commercial slaughterhouses may select for heat resistant strains of *E. coli*. However, they suggest that the use of steam pasteurisation in

combination with other treatments, such as acid washes, may help to prevent this. Smulders et al. (2011) also found that combining steam condensation (at sub-atmospheric pressures) with a hot water spray, achieved reductions of 3-4 log units in the levels of inoculated *Pseudomonas fragi* and *Yersinia enterocolitica* populations on pork skin. They recommend the use of steam condensation at sub-atmospheric pressures to minimise discolouration of the meat.

Steam pasteurisation for even a short (<15 seconds) duration results in initial surface greying of carcases, but after 24 hours chilling, the meat returns to an acceptable colour (Van Ba et al. 2018). A system of rapid cycling of steam under pressure and vacuum cooling has been designed which can give a 1.9 to 2.5 log reduction in *Listeria* numbers on beef after treatment for 48 milliseconds at 121°C (Brashears et al. 2003; Koutsoumanis et al. 2004). Steam has also been used on processed meat products; flash steam heating under pressure followed by cooling by evaporation can give up to 4 log reductions in microbial populations with a 30-40 seconds steam treatment time, without severely affecting colour or weight of beef frankfurter sausages (De Vaux et al. 2002).

1.3.14. Steam vacuum

Steam vacuum is a potential added application to steam alone. It not only loosens and kills bacteria, but also the applied vacuum physically removes contaminants. The effectiveness of steam vacuuming depends on employee diligence of application and the ability to visualise contamination. This means it is impractical as a standalone intervention as microbial contamination is not always visible (Gill 2009). Therefore, it is more of physical contamination intervention. The technique has previously shown to reduce aerobic plate count by 3 log, total coliform count by 4.0 log and *E. coli* count by 4.0 log on artificially inoculated beef short plates (Dorsa et al. 1996). Similarly, other researchers have found aerobic plate counts and total coliform counts to be reduced by up to 2.2 log (Kochevar et al. 1997). Some bleaching of the

carcase surface has been noticed, though it is found not to be a permanent discolouration. Further trials have also shown steam vacuum to be very effective at reducing the number of *E. coli* O157:H7 on beef (Dorsa et al. 1996). Steam vacuum has gained wide acceptance by the US industry as an effective tool for spot treatment on the slaughter floor prior to final inspection and chilling (Huffman 2002).

1.3.15. Irradiation

Irradiation as a decontamination step has not been approved for use on fresh foods in Europe and has a limited approval use in Australia for herbs and spices. Electron beam (E-beam) irradiation has been tested as an antimicrobial intervention on beef carcases by Arthur et al. (2005) and on beef cuts by Kundu et al. (2014) with success. They have both found around a 4 log reduction in *E. coli* O157:H7 strains. Both used 1-kGy dose of E-beam radiation to reduce *E. coli* O157:H7. Penetrations of up to 10% were found not to have any detrimental sensory effects on the end product ground beef (Arthur et al. 2005). Therefore, this treatment appears an efficient antimicrobial step during chilling.

1.3.16. Ultra-violet light

Ultra-violet light is another type of irradiation that has been assessed for its antimicrobial potential. It is commonly used for decontamination of surfaces, air, water and directly on foods. The advantages are it can be applied to raw, fresh, and minimally processed foods. Ultra-violet light causes permanent cross-links in the microbial DNA, preventing the cell from carrying out its normal functions (Sastry et al. 2000). The efficiency depends on the intensity, length of exposure, temperature, pH, relative humidity, bacterial clumping, surface texture and degree of initial contamination (Guerrero-Beltr et al. 2004; Huang et al. 1982; Oteiza et al. 2010;

Sommer et al. 2000; Wood et al. 2015). Successful use has been reported against *E. coli*, *Salmonella*, *L. monocytogenes* and *P. aeruginosa* (Abshire et al. 1981; Djenane et al. 2001; Wallner-Pendleton et al. 1994). Kim et al. (2008) found UVA irradiation did not significantly reduce *E. coli* alone, though in combination with silver ions up to 4 log reduction was detected. Other studies also report a synergistic effect being more significant in bacterial reductions the UV alone (Gayán et al. 2013; Sommers et al. 2009; Webb et al. 1978).

Alternatively, Pulsed UV light consists of short flashes of an intense broadband spectrum (100-1100 nm), it is considered safer and more effective than conventional UV treatments with a more multi-target process (Gomez-Lopez et al. 2007; Keklik et al. 2014). Pulsed UV-light has been used to inactivate *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes*, with reductions of 1-2.4 log CFU/ml and no detrimental effect to the product quality (Ganan et al. 2013; Ozer et al. 2006; Paskeviciute et al. 2011). Photosensitisation is another light-based technology, which may be useful. It involves the use of a photoactive compound selectively taken up by bacterial cells and when exposed to visible light and oxygen can cause cell death, though more research is required in this area (Bloise et al. 2017; Buchovec 2018; Luksienė et al. 2009).

1.4. Post processing strategies

1.4.1. Chilling

Chilling the carcases at the end of processing has long been recognised as a way of maintaining quality and increasing storage time (Gill 1986). It is a common process used in abattoirs around the world and in Australia. The chilling of carcases is known to generally have a small decreasing effect on bacteria, reductions of 2 log CFU and over, have been seen by Bacon et al. (2000), Tittor et al. (2011), and Youssef et al. (2013). Studies have compared the effects of

dry vs wet chilling over time to see the differences in bacterial counts. Dry chilling and aging have shown good reductions in bacteria in particular *E. coli* O157:H7. Tittor et al. (2011) found a 4.76 log CFU/cm^2 reduction in *E. coli* O157:H7 after dry chilling aged at 28 d, compared to 2.21 log reductions for wet aged samples at the same age. They also found similar results for *Salmonella*, better reduction on fat surfaces and over time, specifically against *E. coli* O157 (Tittor et al. 2011). One down-side to the chilling of dry carcases over time is a dramatic loss of weight to the carcase. This decrease in weight causes a substantial drop in carcase value, were price is derived per kilogram in most cases (Tittor et al. 2011). A more preferable method being used and under increasing investigation is the spray chilling of carcases. Kinsella et al. (2006) found no significant difference between spray chilling and chilling alone. Spray chilling in large abattoirs is effective at reducing contamination and prevents some carcase shrinkage, although at some sites such as the neck and rump it was less effective. Another technique investigated by Mohan et al. (2012) was chilling beef trimmings with rapid CO_2 chilling. Although this method was not successful in reducing bacterial counts more than 0.2 log CFU, it did however improve meat colour.

1.4.2. Modified atmosphere packaging

Modified atmosphere packaging (MAP), in particular VP, is a popular method for storing meat cuts to retain their quality and prolong spoilage (Sun et al. 2012). There is a lot of promise in studies like Smulders et al. (2013) and Wanda et al. (2013) were active packing can be combined with MAP to provide a greater reduction of microorganisms on the meat over time. These two independent studies looked into the potential effect lactic acid impregnated polyamide film can have on the microbial community of meat samples. Smulders et al. (2013) found meat samples contaminated with *E. coli* O157 reduced 2 log over the 14 days storage with levels of lactic acid below the minimum inhibitory level (0.09%v/v). Active packaging

has the potential to not only decrease pathogens on meat over time, though also slow the rate of spoilage.

The antibacterial activity of chitosan packaging also allows it to be used to increase shelf life and improve the microbial safety of meat and meat products (Baranenko et al. 2013). In studies with pathogenic *Escherichia coli* O157 in chicken juice the presence of chitosan-arginine significantly reduced their numbers and metabolic activity (Lahmer et al. 2012). High concentrations also cause a greater reduction in numbers of the pathogen in a dose-dependent manner with greater inhibition seen at higher concentration. In addition, the chitosan packaging has also shown to prevent growth of food spoilage bacteria, reduced odour and prevented pathogen re-growth for up to 72 h (Lahmer et al. 2012). Similar results were seen by (Baranenko et al. 2013) with an edible chitosan coating, were total viable count of microorganisms were reduced and no flavour characterises were negatively affected. They found a composition of 2% solution of chitosan, organic acids and gelatin had the strongest bacteriostatic effect for meat, and that the application of vacuum and protective coatings provided the strongest suppressing effect on microflora in all samples (Baranenko et al. 2013).

1.4.3. Dry ice

Dry ice is used by meat and poultry processors for temperature reduction during processing and for temperature maintenance during transportation (Fratamico et al. 2012). ALIGAL Blue Ice (ABI), which combines the antimicrobial effect of ozone (O_3) along with the high cooling capacity of dry ice has been trialled against different microorganisms, including *E. coli*, *Campylobacter jejuni*, Salmonella, and Listeria (Fratamico et al. 2012). ABI showed significant reductions of aerosol contamination with up to 5 log units inactivation for *E. coli* and *Listeria*; with *C. jejuni* 1.3 log units inactivation on chicken breast, increasing to 3.9 log units on contact surfaces, and 2 log units in liquid media. In adding ozone to the already used

dry ice, ABI could be an effective sanitiser for meat and meat products to reduce microbial growth during storage and transportation (Fratamico et al. 2012).

1.5. How to go further with antimicrobial interventions

Interventions including knife trimming, organic acid washes, steam vacuums, and UV light have been shown to improve the microbial shelf life and safety of the fresh meat (Greig et al. 2012; Pittman et al. 2012; Yoder et al. 2012). However, no single intervention can achieve a totally safe product as well as a combination of decontamination steps. This indicates why there is a wide range of research into this problem, the need for developing new or improving existing interventions that are effective in controlling or reducing the pathogens on carcases.

Implementing antimicrobial interventions at all stages from pre-processing to commercially ready packaging for consumer purchasing is important. A series of interventions causing multiple hurdles for bacteria, targeting pathogenic species ability to survive and potentially increase in numbers. As well as these interventions, 'process control points' are critical in maintaining safe conditions for fresh food production. However, apart from education and labelling there is no control over consumers handling and safety precautions, therefore guaranteeing safe products are delivered to the consumer is imperative.

The chilling stage is known to prevent growth and potentially reduce bacterial numbers, including *E. coli* O157:H7. As chilling is already a stage in the processing chain of meat, it would be advantageous to make this as an additional stress to any contaminating bacteria, particularly pathogenic species. Therefore, implementing an additional practise during chilling, in plants already setup for spray chilling or spray washing of some sort, seems a feasible option. The infrastructure to accomplish these methods should be relatively inexpensive and easy to put in place in already existing abattoirs. An overall focus on combining multiple effective interventions to increase the overall outcome of a safe product for consumers should take into

consideration the industry environment, implementation of addition infrastructure, cost effect for site specific issues, and types of products produced. Previous studies have detected a physiological weakness during the initial phase of chilling to oxidants in particular (King et al. 2014). Oxidants such as chlorine dioxide and peroxyacetic acid have shown promising results alone. Promising reductions have been seen in the literature with chlorine dioxide reductions of up to 4.5 log and peroxyacetic acid of up to 4 log CFU/cm^2.

1.6. Conclusion and thesis direction

This current research investigates further treatments that can be applied at this last processing step, chilling, prior to boning and packaging. Oxidants have the potential to efficiently decontaminate meat carcases. Chlorine dioxide and peroxyacetic acid are potential candidates for this study. This project is looking into the effects of these potential antimicrobial interventions within the abattoir environment and at the chilling stage. The chilling stage is known to not just prevent growth though in many cases to reduce bacterial numbers including *E. coli* O157:H7. As chilling is already a stage in the processing chain of meat carcases it would be advantageous to take this as an additional stress on any contaminating bacteria particularly pathogenic species. The aim of this project is to target the already stressed bacteria, targeting *E. coli* O157:H7 Sakai, when it is most vulnerable to oxidants during the chilling cycle. The microbial shelf-life and safety of the fresh meat will be assessed through the following chapters.

Following on from this review, Chapter 2 will cover the initial testing of the hypothesis, *E. coli* becoming sensitive to oxidants during the initial chilling stage. The experimental chapter details the growth response of *E. coli* O157:H7 Sakai, and to a lesser extent Salmonella species, under chilling conditions in situ broth models, first abrupt stress related to chilling (downshift in temperature and water activity) and then temperature simulation of commercial spray

chilling systems. The next chapter of the thesis, Chapter 3, expanse this concept by exploring the efficacy of an oxidant on the complex meat surface, still within a laboratory setting.

Chapter 4 takes previous experimental knowledge, from Chapters 2 and 3, and practically implements the use of an oxidant as an antimicrobial spray during chilling at an export abattoir. In these trials peroxyacetic acid and chlorine dioxide are tested as potential effective and acceptable antimicrobial for meat processing. The final experimental chapter (Chapter 5) follows the storage of striploins that underwent the oxidant antimicrobial spray chilling until end of shelf-life, to determine any detrimental effects from the oxidant treatment. The outcome of this thesis and overall relevance to industry will be discussed in the final concluding Chapter 6.

Chapter 2

Potential use of oxidants to eliminate enteric pathogens under conditions relevant to carcase chilling

Abstract

Pathogenic *Escherichia coli,* especially the O157 serotype have been linked to large outbreaks associated with consumption of foods of bovine origin. This is despite control measures, referred here as 'antimicrobial interventions', are already in use during carcase processing. To address this, our previous studies on the physiological stress of pathogenic *E. coli* suggested a potential window of susceptibility to oxidative damage during exposure to conditions present on carcase surface during cold air chilling. The present study investigated the potential for oxidants as an effective intervention strategy when applied during the carcase chilling processing. An oxidant at a non-lethal level (hydrogen peroxide at 75 ppm or chlorine dioxide at 7.5 ppm) was applied during dynamic changes in growth kinetics of *E. coli* O157:H7 Sakai induced by abrupt downshifts in temperature and water (35°C a_w 0.993 to 14°C a_w 0.967). The addition of hydrogen peroxide at 1.5, 7.5, and 22.5 h after the downshift only caused a drop in viable count (>3 log reduction) at the 1.5 h time point unlike chlorine dioxide, which caused inactivation at all time points. Comparisons were also made with the addition of chlorine dioxide between other enteric pathogens at early stages of the chilling process, and between exponential and stationary phase cells. *S. enterica* servar Typhimurium and Oranienburg were also found to be inactivated by chlorine dioxide though at slower rate than *E. coli* O157:H7 Sakai. It was evident that the addition of either oxidant after the abrupt downshift has an impact on the ability of bacterial cells to recover from chilling stress. The results highlight the potential application of oxidants to reliably eliminate or control enteric pathogens during carcase chilling.

2.1. Introduction

Escherichia coli are part of the normal microbiota in the gastrointestinal tract of cattle and most strains are not harmful. Certain strains possess virulence factors that enable them to cause gastrointestinal disease and are pathogenic to humans (Todd et al. 2001). Pathogenic *E. coli*, especially the O157 strains, have been linked to a large number of outbreaks associated with undercooked meat and meat products, particularly in Europe and the United States (Mohamed et al. 2018). Several interventions are currently in use in the meat processing industry to minimise *E. coli* contamination (Signorini et al. 2018), ranging from hide-on decontamination to spray-wash methods for whole carcases (Van Ba et al. 2018), although none of 100% effective.

There is more focus on *E. coli* when investigating the effects of potential antimicrobials. However, it is suggested that other pathogenic strains should also be trialled, in particular known hardy and pathogenic serotypes (Böhnlein et al. 2016). In this study we used pathogenic and non-pathogenic *E. coli* cocktails to encompass strain variation and two strains of *Salmonella enterica* to broaden the knowledge base for enteric pathogens. Salmonella as a meat borne pathogen is often over looked, though studies have shown it to be a potential issue for the industry (Akyol 2018).

E. coli cells are vulnerable during carcase chilling (Kocharunchitt 2012), with the process causing a 1-2 log reduction in bacterial counts (Bacon et al. 2000; Tittor et al. 2011; Youssef et al. 2013). Under conventional chilling conditions cold air is blown over the surface of the carcase and drying of the surfaces occurs. This drying relates to an equivalent water activity of 0.967 and occurs at a temperature of 7˚C or below, as required by Australian Standard 4696 (Browne 2007) within 24 hours of processing. This practice intends to reduce the risk of pathogen growth (Ross 1999). Spray chilling is now adopted by larger processing plants to reduce surface drying and overall weight loss, hence saving money (Kinsella et al. 2006; Tittor

et al. 2011). During spray chilling, intermittent and brief water sprays over the carcase are followed by a drying period prior to boning. This process was thought to cause less osmotic stress on bacterial cells due to the reduced effect of water activity compare to conventional chilling, though current technologies and systems now seem to have overcome this problem (Greer et al. 1988; Kinsella et al. 2006).

Bacterial cells can survive a range of water activity, with *E. coli* being found to withstand desiccation within dry foods, specifically at cold temperatures (Beuchat et al. 2002; Chen et al. 2004). The adaptation of *E. coli* to cold temperature has been reported to increase its ability to survive desiccation, disinfectants and even oxidants (Berry et al. 2010; Beuchat 2002). Therefore, *E. coli* adaptation to intermittent chilling may in fact enhance their ability to survive low water activity. There are various reports on the effect of spray chilling on bacterial counts and meat quality compared to conventional chilling. Some studies have reported an increase in bacterial counts (Hippe et al. 1991), though many reports have published no detrimental effect (Greer et al. 1997; Hamby et al. 1987; Kinsella et al. 2006; Strydom et al. 1995; Wiklund et al. 2010). The variation seen between studies has been attributed to the various spray regimes including cycles, time and quantity of water sprayed (Strydom et al. 1995). The end state of the carcase is thought to be the most influential factor on microbial counts and the subsequent shelf life, with differences reported between wet and dry carcases prior to boning and packaging (Strydom et al. 1995). This indicates the importance of lowering the water activity at the end of chilling and that the water activity during initial chilling is not as influential. This is supported by our previous studies of cold temperature inducing a greater stress response than water activity during the initial chilling process (King et al. 2014; Kocharunchitt 2012).

Our previous study demonstrated that exposure of *E. coli* O157:H7 Sakai to combined cold and water activity stresses (from 35˚C at a_w 0.993 to 14˚C at a_w 0.967) showed a complex pattern of population changes including 'loss of culturability and recovery' phases (King et al. 2016).

More interestingly, subsequent transcriptomic and proteomic studies using cDNA microarray and 2D-LC-MS/MS analyses revealed that *E. coli* exhibited a 'window of cell susceptibility' during those phases. Of particular note was the reduced abundance of several proteins (e.g. hydroperoxidaseI-KatG, Dps, GrxA, CysK, IbpA, YaaA, AhpF, SufABC, YeeD) associated with defence mechanisms against oxidative stress in *E. coli* (King et al. 2014). This has led to the hypothesis that *E. coli* might become more sensitive to oxidants when exposed to abrupt cold and water activity stress. To test the above hypothesis, the present study aimed to evaluate the effects of an oxidant on *E. coli* when subjected to combined cold and water activity stresses relevant to carcase chilling.

2.2. Materials and Methods

2.2.1. Overview

This study initially seeks to replicate the complex growth response of *E. coli* O157:H7 Sakai reported by King et al. (2016) under abrupt stress conditions related to chilling, specifically a drop in water activity (a_w 0.967) and temperature (14ºC). Subsequently the addition of an oxidant, either H_2O_2 or ClO_2, was explored to determine the effect on cells already stressed with a combination of temperature and water activity reduction. The response of *E. coli* cells to the oxidants was then compared further with other strains, pathogenic and non-pathogenic, and other enteric species such as two *S. enterica* strains.

2.2.1. Bacterial strains and inoculum preparation

E. coli O157:H7 Sakai (Allen et al. 1987; Fisher et al. 2009), obtained from Carlton Gyles (University of Guelph, Canada) was used in all studies, the same strain also used by King et al. (2016). In addition, ten other strains of non-pathogenic *E. coli* (EC1604, EC1605, EC1606, EC1607, EC1608) and pathogenic *E. coli* (O111:H-, O157:H-, O157:H12, O26:H11, and O157:H7), and two strains of *Salmonella* spp. (*S.enterica* Typhimurium and *S. enterica* Oranienburg) were included in this study. All strains were either obtained from the culture collection of the Tasmanian Institute of Agriculture, University of Tasmania (Hobart, Australia) or the CSIRO Agriculture and Food (North Ryde, Australia).

All isolates previously maintained at -80°C were cultured by streaking onto Brain Heart Infusion (BHI) agar and broth (Amyl Media, Australia AM11) and incubating at 37°C for 24 h. A single colony was inoculated into BHI broth and incubated at 37°C for 24 h. The culture was then stored at 4°C and used as a 'stock' culture within two weeks.

Prior to any experiments, the stock culture was diluted $1:10^4$ in 25 ml BHI broth. This 'primary' culture was incubated at 35°C in a shaking water-bath (Ratek Instruments Pty Ltd, VIC Australia) and its growth monitored turbidimetrically at 600 nm (Spectronic 20, Milton Roy Co, USA). After achieving OD of 0.1 ± 0.01 at 600 nm (i.e., mid-exponential phase of growth), the primary culture was diluted $1:10^2$ in fresh 25 ml BHI broth to prepare a 'secondary' culture. This culture was incubated at 35°C with agitation until it reached the mid-exponential growth phase (OD600 of 0.1 ± 0.01) or the early stationary phase (18 h). The cultures were then subjected to the simulated process of conventional chilling, or spray chilling with or without application of an oxidant.

2.2.2. Bacterial cocktail inoculum preparation

Prior to any experiments, each of the stock cultures was diluted 1:10^4 in a 10 ml of BHI broth. This broth culture was incubated without shaking at 35°C for 20 h to prepare stationary phase cells (~10^9 CFU/ml). A cocktail of pathogenic or non-pathogenic *E. coli* cultures was then prepared by combining an equal 5 ml volume of each stationary phase culture of its respective species. The mixtures were diluted with 0.1 ml bacteriological peptone (LP0037, Oxoid Ltd., England) to give an inoculum containing approximately 10^7 CFU/ml.

2.2.3. Preparation of salt-concentrated broth.

A BHI broth at a_w 0.760 was prepared as described by King et al. (2016). This highly concentrated salt broth was then used to adjust the water activity of the test broths to simulate the conditions as occurs during conventional chilling of carcases. Water activities of the broths were confirmed with an Aqualab CX2 dew point instrument (Decagon Devices, Pullman, USA).

2.2.4. Preparation of oxidants

Two different oxidants, H_2O_2 and ClO_2 were tested in this study. Fresh solutions were prepared prior to each experiment. H_2O_2 was purchased from Sigma-Aldrich, Australia (H1009) and used as a stock solution. For ClO_2, a stock solution was prepared from 4% stabilised chlorine dioxide (ZyDox®, ZyChem Technologies Pty Ltd., Australia), according to manufacturer's instructions. Specifically, stabilised ClO_2 was mixed with 54.6% citric acid solution (Sigma-Aldrich, C1909). The mixture was left for 30 min at room temperature in the absence of light, allowing the reaction to occur and generate 1,000 ppm ClO_2. An appropriate volume of the

stock solutions was then added to the cultures to achieve the desired concentration (Section. 2.6).

2.2.5. Exposure of bacteria to abrupt cold and osmotic stress in broth

A secondary culture (OD_{600} of 0.1) of *E. coli* O157:H7 Sakai at 35°C prepared as described in S. 2.2, had a calculated volume of saturated salt BHI broth (Section 2.3) at 14°C, added to achieve a water activity of 0.967. The saturated salt broth was made according to BHI manufactures instructions, though the addition of sodium chloride was added at 24.73% w/w before autoclaving. The salt amended *E. coli* culture was immediately transferred from 35°C to a 14°C shaking water bath.

2.2.6. Exposure of bacteria to simulated spray chilling temperature fluctuations

E. coli O157:H7 Sakai was grown to exponential phase and stationary phase separately before being treated (Section 2.2). For cocktails of non-pathogenic and pathogenic cultures, stationary phase cultures only were prepared prior to treatment (Section 2.2). An aliquot of the cultures (10 ml) was removed at 1.5, 7.5, and 22.5 h after the downshift exposure to the spray chilling condition, and was combined with an appropriate volume of 1,000 ppm ClO_2 to achieve the final concentration of 7.5, 10 or 15 ppm. It should be noted that these levels of ClO_2 were chosen based on preliminary studies, in which the level had no detrimental effects on *E. coli* O157:H7 Sakai under optimal growth conditions, at relevant physiological states used (Appendix A1).

All cultures were subjected to dynamic changes in temperature as occur during spray chilling. A profile describing changes in temperature during spray chilling was obtained from the work of Mellefont et al. (2015) and Ross (1999). This profile was based on the average surface temperature of carcases monitored throughout overnight chilling with 22°C spray water. However, the carcase was estimated to spend anywhere from 20-30 min on the dressing floor and an additional 6 min in the chiller prior to the start of temperature logging (Mellefont et al. 2015). The temperature profile was, therefore, extrapolated back 36 min to 35°C to obtain a full profile. Figure 2.3 illustrates the temperature profile used in this study to examine the growth response of bacteria under conditions relevant to spray chilling. This temperature profile was programmed into a temperature gradient water-bath to replicate the drop in temperatures of carcases from processing to end of chilling (35°C to 7°C).

2.2.7. Microbiological analysis

All experiments were performed in duplicate flasks, and the results reported as the average of the replicates. Population changes of test organisms when subjected to the chilling conditions and addition of an oxidant were determined by a surface plating method. Specifically, a 0.1 ml aliquot was taken periodically from the test cultures and serially diluted in 0.1% peptone water (Oxoid™ LP0037) containing 0.85% sodium chloride (Merck 106404). Aliquots of appropriate dilutions were plated onto BHI agar, using an automated spiral plater (Spiral Biotech Autoplate 2000). All plates were then incubated at 37°C for 24 h before colonies were counted.

2.2.8. Data analysis

Growth and/or survival responses were determined by plotting viable numbers ($\log_{10}$ CFU/ml^{-1}) against time (h). Linear regression analysis of the growth curve data was used to estimate generation time and inactivation rates. A straight line was fitted to points that appeared to represent the growth or inactivation phase. Typically, this region included ≥4–10 points of viable count data. From the inactivation plots a line of best fit was achieved and linear regression done to calculate growth and inactivation rates using Microsoft Excel. General Linear Model (GLM) of SAS version 9.4 was used to calculate analyse of variance (ANOVA). A P value of < 0.01 was the significant level adopted, when mean generation time and inactivation rates were compared.

2.3. Results

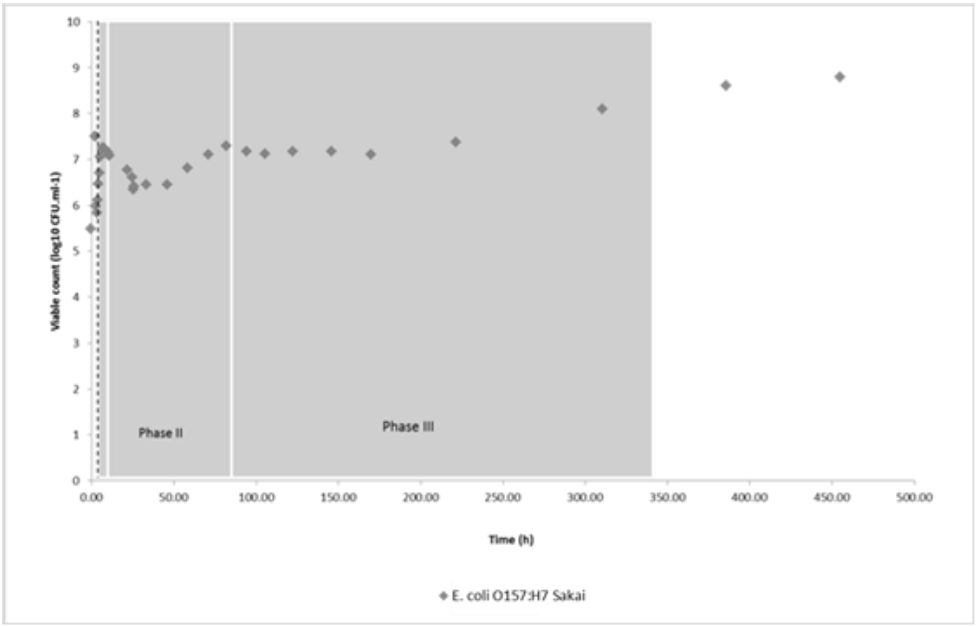
2.3.1. Growth response of exponential *E. coli* O157:H7 after combined shock

In parallel with experiments to evaluate the potential effects of an oxidant on *E. coli* O157:H7 Sakai during exposure to combined cold and water activity stresses as occur during air chilling of carcases (King et al. 2016), the growth response of exponential *E. coli* O157:H7 Sakai to those stresses were studied. This was to determine if the microbial growth responses observed previously by King et al. (2016) was replicated in our experiments, and to test the hypothesis that *E. coli* becomes more susceptible to oxidative damage.

The pattern of *E. coli* O157:H7 Sakai growth behaviour in response to temperature and water activity changes could be divided into three phases (Phases I, II and III; Figure 1) and were similar to that reported by King et al. (2016). Specifically, the initial period after the abrupt

shift, Phase I, is a sharp decrease in cell numbers followed by a rapid increase (GT = 0.35 ± 0.003 h). However, the cell numbers do not return to that of the initial cell population. 10 h post shock, Phase II has a gradual decrease followed by an increase in bacterial numbers (GT = 12.64 ± 1.91 h). Finally, Phase III starts with a 'lag-like' period of no net change in cell numbers, followed by an increase in numbers (GT = 21 ± 3.61 h). Analysis of growth kinetics revealed that the generation time for Phase I was significantly shorter than that of both Phase II and III ($P < 0.01$). However, Phase II and III were not found to be significantly different ($P > 0.01$).

a)



b)

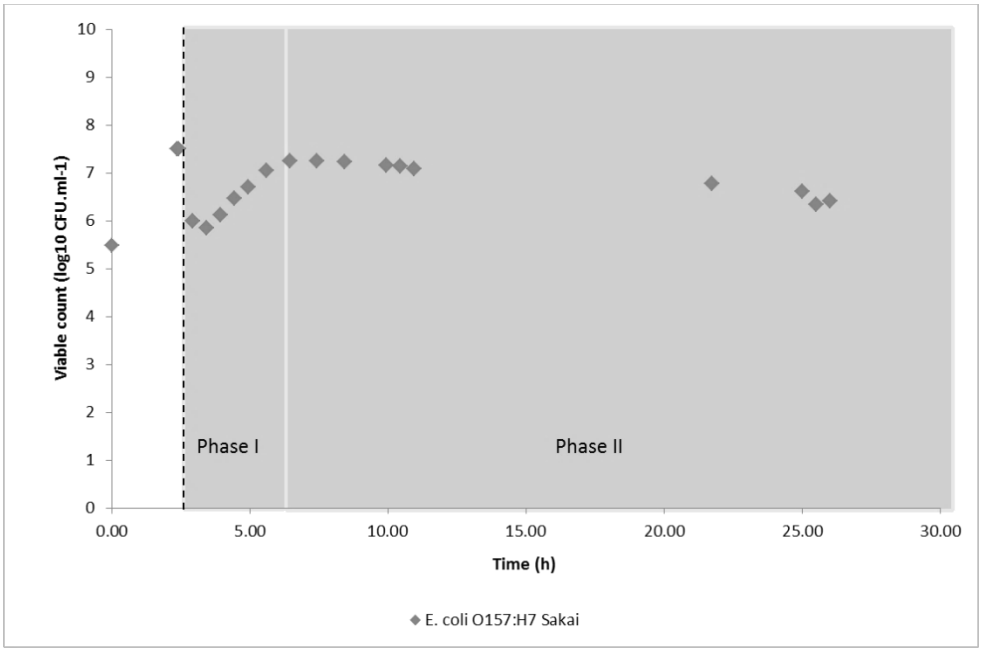


Figure 2.1a & b. Growth response of *E. coli* O157:H7 Sakai to simultaneous downshift in temperature and water activity from 35°C a_w 0.993 to 14°C a_w 0.967 (shown as dotted line). a) details the 3 growth phases; b) details the downshift and Phase I-II.

2.3.2 Potential effects of an oxidant on *E. coli* O157:H7 Sakai during exposure to combined cold and water activity stresses

Upon addition of H_2O_2 (at 75 ppm) to the cultures at 1, 7.5 and 22.5 h during exposure to combined cold and water activity stresses, *E. coli* O157:H7 Sakai exhibited differences in population kinetics compared to the control (Figure 2.2a & b). The addition of hydrogen peroxide at 1 h correlates to the point of lowest viable count (Phase I), at 7.5 h the cells had recovered in numbers at the border of Phase I and II, and at 22.5 h, well into Phase II, the first 'exponential-like' growth stage (Figure 2.2b). Although inactivation was not seen with the addition of 75 ppm, a different response was observed at the various H_2O_2 addition times after the abrupt shock. The addition of H_2O_2 at 1 h, within Phase I, significantly affected population kinetics compared to 7.5 and 22.5 h, resulting in a greater decrease in viable cell numbers and a faster generation time to recover the population to stationary phase ($P < 0.05$, Figure 2.2). The addition of H_2O_2 at both 7.5 and 22.5 h had little effect on growth pattern, with the growth response closely following that of the control (Figure 2.2). However, the population numbers of both later additions (7.5 and 22.5 h) were lower than the control. Interestingly, the 1 h addition of H_2O_2 also followed a similar growth pattern though at further reduced numbers than the other treatment times. The results highlight the ability of *E. coli* O157:H7 Sakai cells to overcome oxidative stress at all treatment times, and furthermore it suggests *E. coli* cells are more susceptible to oxidants during Phase I (Kocharunchitt 2012).

In contrast, addition of ClO_2 at 75 ppm to *E. coli* O157:H7 Sakai cultures at all time points caused a decrease in cell numbers that was maintained throughout the period of monitoring (Figure 2.3). This resulted in ≥5 log CFU/ml reduction for all treatments. However, it was observed that the rates of *E. coli* O157:H7 Sakai inactivation were significantly different ($P < 0.05$) when ClO_2 was applied at different time points. *E. coli* O157:H7 Sakai inactivation

occurred at slower rates (i.e., 0.115, 0.113 and 0.102 CFU/ml/h) when ClO_2 was applied at later time points (i.e., at 1, 7.5 and 22.5 h after the imposition of the stresses).

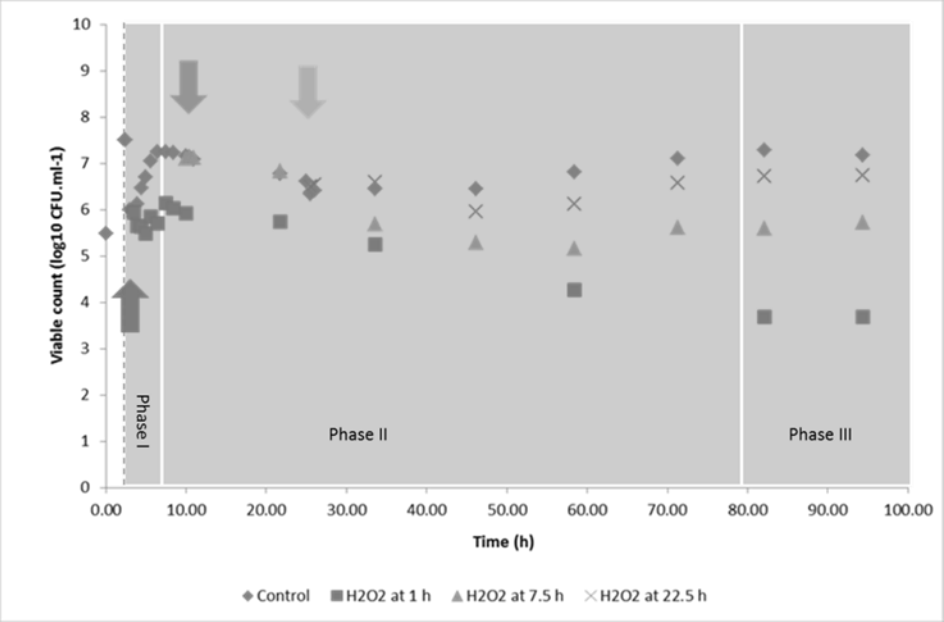


Figure 2.2a. Growth response of *E. coli* O157:H7 Sakai to simultaneous downshift in temperature and water activity from 35°C a_w 0.993 to 14°C a_w 0.967 with the addition of 75 ppm hydrogen peroxide, over 100 h. The vertical dotted line indicates the downshift. The control (◆) has no addition of hydrogen peroxide. Hydrogen peroxide is added at 1 h (■), 7.5 h (▲) and 22.5 h (X) after the downshift in temperature and water activity. The addition of hydrogen peroxide is shown by an arrow in the corresponding grey scale. The cellular response phases I, II and III are shown in shaded columns.

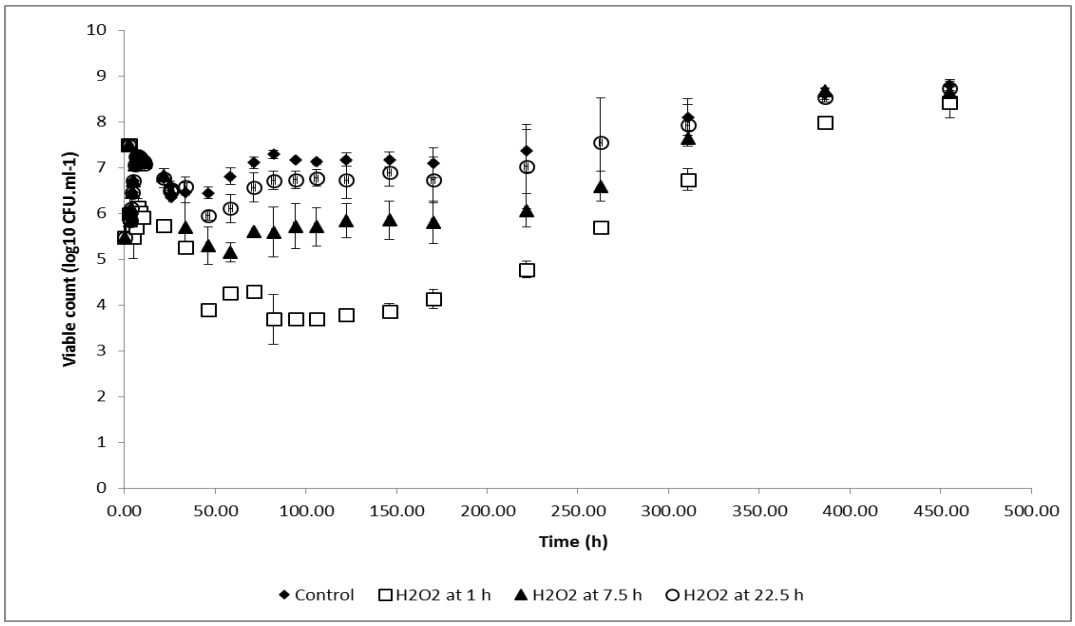


Figure 2.2b. Growth response of *E. coli* O157:H7 Sakai to simultaneous downshift in temperature and water activity from 35°C a_w 0.993 to 14°C a_w 0.967 with the addition of 75 ppm hydrogen peroxide, until stationary phase. The control (◆) has no addition of hydrogen peroxide. Hydrogen peroxide is added at 1 h (□), 7.5 h (▲) and 22.5 h (❍) after the downshift in temperature and water activity.

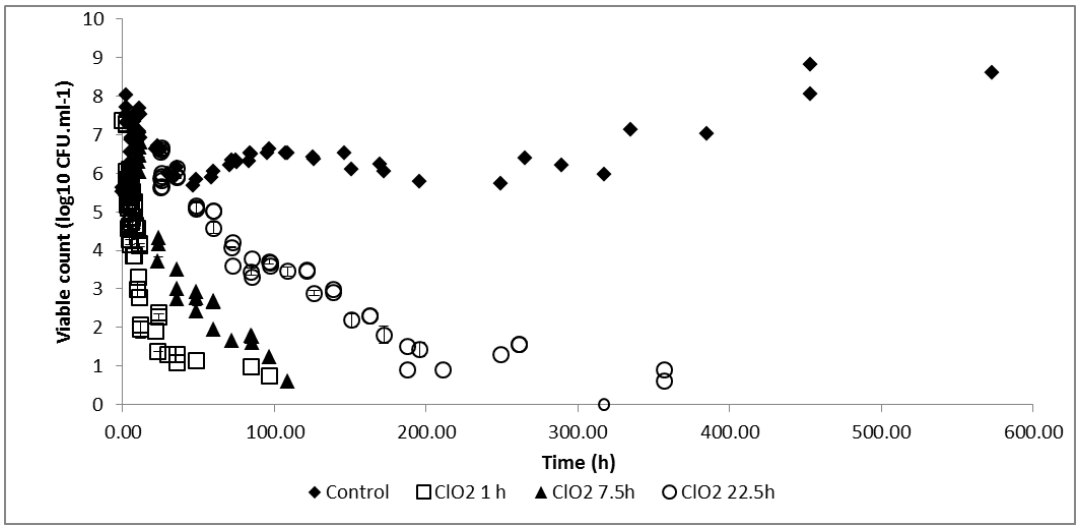


Figure 2.3. Growth response of *E. coli* O157:H7 Sakai to simultaneous downshift in temperature and water activity from 35°C a_w 0.993 to 14°C a_w 0.967 with the addition of 75 ppm chlorine dioxide. The control (◆) has no addition of chlorine dioxide. Chlorine dioxide is added at 1 h (□), 7.5 h (▲), and 22.5 h (○) after the downshift in temperature and water activity.

2.3.3. Growth response of *E. coli* O157:H7 Sakai under simulated spray chilling

To mimic temperatures encountered during spray chilling conditions, *E. coli* O157:H7 Sakai cultures were exposed to a biphasic drop in temperature. Specifically, a rapid reduction occurs from to 35°C to 20°C within the first few hours and is followed by a slower reduction to 7°C within 7 h (Figure 2.4). Temperatures thereafter remains static, and as expected *E. coli* O157:H7 Sakai exhibits exponential growth initially, until the simulated spray chilling temperature dropped below 10°C (Figure 2.4. control). At these temperatures, conditions are not supportive of cell growth, and thus numbers plateaued around 6 log CFU/ml for the remainder of incubation. The same growth pattern was seen in both exponential and stationary

phase *E. coli* O157:H7 Sakai cells, pathogenic and non-pathogenic *E. coli* cocktails, and for both Salmonella strains, Oranienburg and Typhimurium (Appendix A2).

2.3.4. Potential application of an oxidant during simulated spray chilling

The response of exponential and stationary phase cultures of *E. coli* O157:H7 Sakai, two *E. coli* cocktails, pathogenic and non-pathogenic, and two Salmonella strains under spray chilling conditions and subjected to ClO_2 treatment are detailed in Table 1. The viable count data revealed that all challenge organisms commenced growth almost immediately after imposition of the chilling conditions (data not shown). This is consistent with the growth response of exponential phase cultures of *E. coli* O157:H7 Sakai under the same conditions tested. However, it was apparent that the growth rate (0.17 CFU/ml/h) of stationary phase cells of *E. coli* O157:H7 Sakai observed was much slower than that (1.35 CFU/ml/h) of exponentially growing cells of the same organism when subjected to the same conditions tested. As the temperature declined below growth limits of 8°C, the population of both stationary and exponential phase cultures plateaued, and population levels remained consistent for the duration of the 72 h experiment. A similar response was seen with the *E. coli* cocktails and Salmonella strains tested.

Table 2.1. Inactivation rates of challenge organisms during exposure to changing temperatures as occur during the spray chilling process of carcases.

Challenge organisms	Physiological State of cells	Application of ClO_2		Inactivation rates (log10 CFU/ml/h)
		Concentration (ppm)	Time (h)	
E. coli O157:H7 Sakai	Exponential	75	1	0.115
			2	0.113
			6	0.102
			24	0.091
E. coli O157:H7 Sakai	Stationary	100	1.5	0.079
			6	0.064
			24	0.053
Non-pathogenic *E. coli* cocktail	Stationary	150	1.5	0.021
			6	0.016
			24	0.009
Pathogenic *E. coli* cocktail	Stationary	150	1.5	0.029
			6	0.019
			24	0.009
S. Oranienburg	Stationary	150	1.5	0.042
			6	0.032
S. Typhimurium	Stationary	150	1.5	0.062
			6	0.059

Stationary phase *E. coli* O157:H7 Sakai had consistently slower inactivation rates with application of the oxidant during the chilling process, in comparison to exponential phase

cultures, 0.037 ± 0.001 CFU/ml/h difference at every application time. However, the rates of inactivation were still faster the earlier the application of ClO_2, with 0.79, 0.064 and 0.053 log CFU/ml/h obtained at 1, 6 and 24 h after chilling. A faster inactivation rate for early application was seen for both *E. coli* cocktails, non-pathogenic and pathogenic, and both Salmonella strains (Table 2.1.). All stationary phase cultures displayed inactivation curves comparable to *E. coli* O157:H7 Sakai, as shown in Figure 2.4 (and Appendix A2). *E. coli* O157:H7 Sakai was the most effected by ClO_2 with the highest inactivation rate (0.115 CFU/ml/h,) followed by S. Typhimurium (0.062 CFU/ml/h). Both *E. coli* cocktails had very similar rates, though the pathogenic population was higher for the first 2 application times. Although species and strain variation were observed, the same pattern was seen with faster inactivation occurred at every initial (1-1.5 h) application trialled.

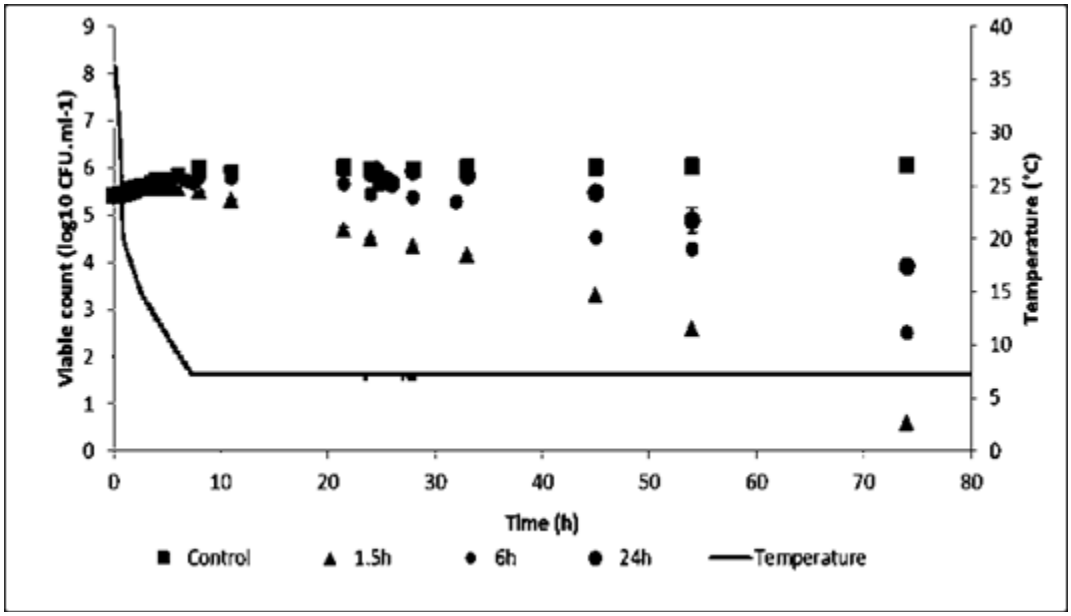


Figure 2.4. Stationary-phase cultures of *E. coli* O157:H7 Sakai during exposure to dynamic changes in temperature as occurs during spray chilling and when 100 ppm ClO_2 was applied at different time points.

2.4. Discussion

Bacteria such as *E. coli* have been extensively studied for their physiological responses to environmental stresses including cold temperature and low water activity (Beuchat et al. 2002; Ross et al. 2003; Salter et al. 2000; Thieringer et al. 1998). It has been reported that these bacteria can induce an adaptive response that involves expression changes of genes and proteins. This response allows bacteria to protect their vital processes and restore cellular homeostasis (King et al. 2014; Kocharunchitt et al. 2014). In keeping with this, our previous study (King et al. 2016) demonstrated that the response of *E. coli* O157:H7 Sakai to temperature and water activity changes relevant to carcase chilling in cold air, involved a highly complex regulatory network including changes in expression/abundance of different groups of genes and proteins. This regulatory network was mainly mediated through a transient induction of the RpoE-controlled envelope stress response and activation of the master stress regulator RpoS and the Rcs system-controlled colanic acid biosynthesis. However, that study identified a potential 'window of cell susceptibility' during the responses to the stresses. Of particular note was the down-regulation of proteins associated with defence mechanisms against oxidative damage (King et al. 2016). This has led to the hypothesis that *E. coli* potentially become more sensitive to oxidative stress under such conditions. To test this hypothesis, the present study was conducted to evaluate the potential for using an oxidant to control or eliminate *E. coli* under the conditions relevant to carcase chilling.

2.4.1. Growth response of *E. coli* O157:H7 Sakai under combined cold and low water activity

To test the hypothesis of King et al. (2016) of *E. coli* O157:H7 Sakai becoming more susceptible to oxidants under combined cold and water activity we first replicated their

experiment. The complex growth response described by King et al. (2016), as having 3 main growth phases was observed in our study. Phase I is considered a loss of culturability in response to the combined shock and subsequent recovery of cell numbers, Phase II appearing as a 'lag-like' phase and the III being growth comparable to 'normal exponential growth'.

2.4.2. Susceptibility of *E. coli* O157:H7 Sakai to oxidative stress under combined stress

Therefore, having replicated the complex growth response to the combined stress, we could further test the degree of susceptibility of *E. coli* O157:H7 Sakai to oxidative damage at different times during exposure to cold and low water activity. Appling 75 ppm H_2O_2 at different time points within the complex growth response period resulted in a different response at each time added. The addition during the first phase after the combined stress had a greater effect on *E. coli* O157:H7 Sakai cells ability to recovery from the cold and water activity stress. Although *E. coli* O157:H7 Sakai eventually recovered and reached stationary phase after 400 h for all application times, viable counts were affected under these stressful conditions. Addition of the oxidant H_2O_2 earlier rather than later in the chilling process has the potential to affect *E. coli* O157:H7 Sakai cells ability to compensate and recover at the same rate, showing an increased susceptibility at his stage.

ClO_2 even at relatively low levels of 7.5 ppm was effective at inactivating *E. coli* O157:H7 Sakai, when exposed to the temperature and water activity conditions relevant to spray chilling. Application of this oxidant in the earlier stages of chilling conditions resulted in the most rapid reduction in *E. coli* O157:H7 Sakai numbers. It was observed, however, that although reduction in numbers occurred when ClO_2 was applied at later time points, the rates of *E. coli* O157:H7 Sakai inactivation were slower. This supports the proposal from earlier work that *E. coli* has a

more pronounced period of susceptibility to oxidant stress, although they gradually recover over time, even though the stressful conditions are maintained. This contrasts with application of H_2O_2 at the same level, in which growth rather than inactivation of *E. coli* O157:H7 Sakai was observed in all cases after a period of adaptation (Figure 2.2).

E. coli O157:H7 Sakai cells were more sensitive to the oxidants tested after combined cold temperature and osmotic shock within the first phase of growth, and progressively become less sensitive when the oxidant is applied at later stages in the chilling regime. For H_2O_2, all cells recover to 'normal' growth patterns regardless of time of oxidant application. In the case of ClO_2, the effect is a slower inactivation rate. These results show that the population response is dependent on the type of oxidant used, and as noted, the timing is critical for maximum inactivation rates.

Although H_2O_2 has a higher redox potential, i.e. ability to acquire electrons and therefore cause oxidation, ClO_2 had a greater effect on the exponential cells used in this study. The differences in response to the oxidants could be attributed to their different modes of action. ClO_2 is known to be more effective at oxidizing proteins and enzyme activating inhibitors and not amino acids, unlike H_2O_2 (McDonnell et al. 2001; Young 2016). In contrast, *E. coli* can tolerate H_2O_2 due to catalase (e.g. KatG) activity (Smirnova et al. 1997). The difference in inactivation between oxidants would be affected by gene expression linked to stress responses, which further agrees with our previous study by King et al. (2016). Our preliminary results show two pathogens relevant to beef carcases, *E. coli* and Salmonella, can cope with these low levels of the oxidants under optimal growing conditions. However, with the added stress of cold shock causing expressed oxidant sensitivity, the cells can no longer retain growth or survival mechanisms depending on the oxidant.

Many studies show the ability of *E. coli* to survive at low temperature (4°C), even with lower water activities (0.4-0.6) (Hiramatsu et al. 2005). *E. coli* O157:H7 has shown enhanced survival in dry foods with a wide range of water activity and pH, particularly at refrigeration temperature, and that lower water activity allows for greater tolerance to chilling (Deng et al. 1998). Therefore, timing is critical before cells can overcome cold with their adaptive stress responses.

Both Salmonella strains showed a similar complex pattern of population kinetics when subjected to abrupt downshifts in temperature and water activity (Appendix A3). Comparison of the inactivation rates with *E. coli* revealed that although similar, the responses of *E. coli* resulted in a greater population change (Table 2.1). The data indicated that the effects of combined cold and osmotic stresses were less pronounced on Salmonella than on *E. coli*. *S.* Typhimurium rates of inactivation were found to be slower than *S.* Oranienburg, indicating that although application of 150 ppm ClO_2 was effective, there is strain variation. Our results indicate the potential intervention developed for *E. coli* during carcase spray chilling could also be used, and as effective against Salmonella spp.

2.4.3. Growth response of *E. coli* O157:H7 Sakai under simulated spray chilling

During spray chilling the temperature drops below the 'normal physiological range' for *E. coli* within 4 h of processing and below growth limits within 7 h (Mellefont et al. 2015; Neidhardt et al. 1990). Mimicking these spray chilling conditions, i.e. temperature drop from animal core temperature of 37°C to 7°C, as regulations require, causes *E. coli* O157:H7 Sakai to cease exponential growth as the temperature reaches 7°C. The observed *E. coli* O157:H7 Sakai growth responses in our studies was as expected, with exponential growth initially until the simulated spray chilling conditions temperature dropped below 10°C and stabilised at 7°C. At

these temperatures, the conditions are not supportive of cell growth. This further demonstrates the sensitivity of *E.coli* O157:H7 Sakai under chilling stress and complies with the physiological response seen in growth studies by Presser et al. (1998) .

2.4.4. Effects of an oxidant on *E. coli* O157:H7 Sakai under simulated spray chilling process

The previous trial on *E. coli* response under dynamic conditions during air chilling was trialled to see if it could also be used to eliminate or control *E. coli* on carcases during spray chilling. In contrast to the observed complex growth response during the chilling conditions, both exponential and stationary phase cells of *E. coli and* Salmonella showed a typical growth curve (exponential growth phase followed by a plateau) when subjected to the spray chilling conditions. This implies that *E. coli* potentially present on carcases might grow at a faster rate and reach higher levels under spray chilling than that of air chilling, and that water activity stress (or desiccation) as a result of the air chilling process has a significant impact on the growth of *E. coli.* Consistent with this, Kocharunchitt et al. (2012) demonstrated that water activity stress has a greater effect on the observable physiology of *E. coli* than cold stress. However, our experiments show the effectiveness of ClO_2 under spray chilling conditions, with *E. coli* and *Salmonella* strains undergoing 2-6 log reduction depending on application time and length of chilling (Figure 2.4, Appendix A2). The results highlight that it is the cold temperature that makes *E. coli* more susceptible to oxidants during the initial response to the stress before adaptive processes can occur. These results agree with others showing the promise of ClO_2 as an antimicrobial (Ndjomgoue-Yossa et al. 2015), and increased efficiency of the added stress of cold storage on *E. coli* O157 inactivation (Visvalingam et al. 2018).

2.5. Conclusion

The potential outcomes of this project are a novel and relatively easily implemented intervention applied after initial slaughter processing before packaging. It is novel because of its targeted timing for maximum efficiency not because of the type of oxidant. ClO_2 is well known for its efficiency with minimal negative effects compared to others, though also due to its long-lasting effect prior to packaging. Rather than implementing an intervention on the slaughter floor were cross contamination is bound to continue happening throughout the process. However, timing is critical for maximum efficiency of inactivation and this works on a variety of species, strains and serotypes, in particular targeting *E. coli* O157. The addition of ClO_2 to stationary phase cells, during spray chilling conditions with no water activity stress shown in this study, details how potentially effective it could be as an application option in already established abattoirs.

Temperature is the major contributing factor to oxidant susceptibility under these conditions and therefore would make an appropriate candidate for use within spray chilling systems at abattoirs. However, further studies are needed to test the effect is not inhibited by physical and chemical properties of meat surfaces, in particular iron rich lean sections and in contrast insoluble adipose tissue before the process can be deemed practical as an antimicrobial intervention. Much research has investigated usage of antimicrobials at the end of the meat production process or during the slaughter process (Chien et al. 2017; Fisher et al. 2016) but there has been limited research done on applying interventions during chilling or storage before packing. This research highlights the potential to enhance chilling as an intervention by adding a more detrimental effect that can achieve inactivation of *E. coli* cells.

Chapter 3

Potential applications of oxidant spray chilling on beef

Abstract

Shiga-toxin producing *Escherichia coli* (STEC) are a major food safety concern for the meat industry. Although many interventions have been implemented, no methods are completely effective in controlling pathogens during carcase processing. Previous research has suggested that STEC, in particular *E. coli* O157:H7 Sakai, might become more susceptible to oxidative damage when exposed to carcase chilling (King et al. 2016); broth studies in Chapter 2 confirmed this hypothesis. The present study aimed to further test this phenomenon on beef surfaces. The antimicrobial effects of oxidants, chlorine dioxide (ClO_2) and peroxyacetic acid (PAA), on beef striploins were assessed during the simulated process of spray chilling (4 sec every 15 min for 36 cycles). Preliminary trials of ClO_2 treatment on striploins at 10–50 ppm, under growth conditions (12.5 and 20°C), has no effect on *E. coli* O157:H7 Sakai at either temperature. Treating the *E. coli* O157:H7 Sakai inoculated striploins before chilling also showed no major effect (~1 log reduction), when temperatures are 37°C followed by the chilling regime of dropping below 7°C. In current trails of ClO_2 and PAA spray chilling at 20 and 200 ppm, respectively, during chilling achieved >4 log reduction at 72 h. However, the effects of the oxidants were only observed on fat surfaces and not lean surfaces. These experiments further confirmed the increased susceptibility of *E. coli* O157:H7 Sakai to oxidative stress during spray chilling. However, some limitations apply with lean tissue. These results indicate that further development of an oxidant application during spray chilling, as an antimicrobial intervention, is required to minimise the problems that effect economical and safety outcomes.

3.1. Introduction

Enteric pathogens such as Shiga-toxin producing *Escherichia coli* (STEC) represent a major food safety issues to the beef industry. *E. coli* O157:H7 have been linked to illnesses associated with the consumption of bovine products (Elder et al. 2000; Kalchayanand et al. 2015). Therefore, the U.S. Department of Agriculture-Food Safety and Inspection Service declared STEC O157:H7 and six serogroups of STEC (O26, O45, O103, O111, O121, and O145) as adulterants in beef (USDA-FSIS, 2012)

E. coli during slaughter and processing causes potential contamination of meat. Therefore, preventing this contamination on the final product are undertaken in a variety of antimicrobial interventions depending on the abattoir. Antimicrobial interventions range from knife trimming, hot water washes, to steam vacuuming. Such interventions have been demonstrated to successfully reduce carcase contamination of up to 3 log (Bacon et al. 2000; Chen et al. 2012; Wheeler et al. 2014). However, food-borne illnesses and product recalls caused by enteric pathogens still occur (Soon et al. 2011; Wheeler et al. 2014). This indicates the need for developing novel antimicrobial interventions that are reliable and effective in eliminating these pathogens on carcases.

Previously, both growth kinetics and molecular response characteristics of *E. coli* O157:H7 Sakai when subjected to temperature and water activity changes, as occur during carcase chilling, were investigated (King et al. 2016; Kocharunchitt et al. 2012). The exposure of *E. coli* O157:H7 Sakai to such stresses caused a complex pattern of growth responses including 'loss of culturability and recovery' phases. Particularly, the abundance of several proteins (AhpCF, KatG, and Tpx) in *E. coli* O157:H7 Sakai transiently decreased, a consequence of chilling stress. These findings led to the hypothesis that *E. coli* O157:H7 Sakai potentially become more sensitive to oxidative stress after exposure to chilling conditions (King et al. 2016). The hypothesis was confirmed in Chapter 2, which demonstrates the use of two different

oxidants, hydrogen peroxide and chlorine dioxide, at non-lethal levels. The results showed the oxidants, and specifically ClO_2, were very effective against *E. coli* O157:H7 Sakai in the broth-based model (≥5 log reduction with 7.5ppm ClO_2 within 24 h of application). Further investigations into the effect of oxidants are detailed in the present study. The hypothesis of King et al. (2016) was further tested here on beef meat with the intent to develop oxidants as an antimicrobial intervention. The antimicrobial effect of an oxidant (ClO_2 or PAA) on *E. coli* inoculated beef was evaluated prior and during simulated spray chilling conditions.

3.2. Methods

3.2.1. Preparation of *E. coli* inoculum

E. coli O157:H7 Sakai (Allen et al. 1987; Fisher et al. 2009) obtained from Carlton Gyles (University of Guelph, Canada) was used in preliminary studies as it is the same strain used by King et al. (2016). Also, five non-pathogen strains of *E. coli* (EC1604, EC1605, EC1606, EC1607, and EC1608), obtained from CSIRO Food and Nutritional Sciences Australia, were used in this study. All of the CSIRO *E. coli* strains are found to contain no known virulence markers for pathogenic *E. coli* but otherwise have growth characteristics very similar to various known isolates of *E. coli* O157:H7 (McPhail 2011; Small 2010).

E. coli strains are stored frozen (-80°C) on beads. Prior to the experiment strains are streaked onto brain-heart infusion (BHI) agar (AM11, AMYL, Australia) and incubating at 37°C for 18-24 h. An isolated colony from each culture was inoculated into 10 ml of BHI broth and incubated at 37°C for 20 h. The broth cultures were then diluted 1:10^4 in 10 ml of BHI broth and incubated further at 37°C for 20 h. This culture was used as stationary phase cells (~ 10^9 CFU/ml). A culture of *E. coli* O157:H7 Sakai or a cocktail of non-pathogenic *E. coli* was prepared by combining 1 ml volume of each stationary phase culture. The mixtures were

diluted with 200 ml of 0.1 ml bacteriological peptone (LP0037, Oxoid Ltd., England) to give an inoculum containing approximately 10^7 CFU/ml and used to inoculate the beef striploins.

3.2.2. Preparation of an oxidising agent

Zydox® chlorine dioxide (Zychem Technologies Pty Ltd) and peroxyacetic acid (ECOLAB Australia Pty Ltd) were the two oxidants trialled. The working solution of both oxidants were prepared in tanks and diluted with an appropriate volume of potable water to achieve the test concentration (ClO_2 at 10, 15, 20, 25 and 50 ppm or PAA at 150, 200 ppm), according to the manufacturer's instructions. ClO_2 was activated with 54.6 % citric acid (Sigma-Aldrich, C1909).

3.2.3. Preparation and treatment of inoculated meat

Beef striploins were collected from a Tasmanian export abattoir (Australia). The strip loins were VP and stored at -1°C. Prior to use each striploin was warmed to 37°C over a period of 18 h to simulate normal animal temperatures before chilling. The striploin was halved in the middle of the longest length, to make two squares. Each half striploin was then suspended by stainless steel hooks and inoculated on both fat and lean surfaces with the bacterial inoculum (Section 3.2.1) using a sterile paintbrush. The striploin was then left for 20 mins at room temperature, to allow bacterial attachment. The striploins were then subjected to either treatment (Section 3.2.4).

3.2.4. Replicating the chilling cycle on meat

A temperature-gradient incubator (KB 240, Binder GmbH, Germany) was adapted to simulate carcase chilling conditions. The incubator was modified with irrigation tubing and mist spray nozzles (MicroJet^{TM} Mist Spray, Kilkenny, SA). The incubator was programmed to simulate a temperature profile as occurs during the chilling process of carcases. This profile was obtained from the work of McPhail (2011) in which the average surface temperature of carcases was monitored throughout chilling with 22˚C spray water. However, carcases were estimated to spend anywhere from 20-30 min on the dressing floor and an additional 6 min in the chiller prior to the start of temperature logging (Mellefont et al. 2005). Therefore, an extra 36 min before spray chilling was added to the temperature profile. The spray was delivered from 4 spray nozzles, 2 per sample and delivered either deionized water or treatment solution at a flow rate of 4 ml/sec/nozzle. Prior to any applications of spray chilling, each meat sample was suspended from a stainless-steel rack inside the incubator between two nozzles, 10 cm from each nozzle.

3.2.5. Application of an oxidant during spray chilling

The inoculated meat samples were subjected to spray application during exposure to conditions simulating carcase chilling. Two meat samples were sprayed with water, ClO_2 (15 or 20 ppm) or PAA (150 or 200 ppm) for a 4 sec spray cycle every 15 min for 36 cycles during the initial stage of the chilling process and remained in the chiller (at 7°C) for a total of 72 h.

3.2.6. Surface temperature and pH measurement

Throughout the trials, temperature histories of meat pieces were collected using a DS1921G Thermochron i-button temperature logger (iButtonLink, WI, USA). The pH of both fat and lean surfaces of the meat samples was measured using an Orion 250A meter (Orion, USA) equipped with a surface probe (EPFLGN-121223, TPS Pty Ltd., Australia). The pH measurements were made before commencing the chilling process, and at 24 and 72 h of chilling.

3.2.6. Microbiological analysis

Samples were taken on both the lean and fat sides of the meat before the chilling process, at 24, 50 and 72 h after commencing chilling. A 4 x 4 cm template was used to mark out the area for sponging. A SpeciSponge whirl-pak (VW11216-790) was filled with 25 ml of 0.1% peptone, the sponge squeezed out then wiped over the marked area 5 times in one direction then 5 times in another direction. The sponge was placed back in the whirl-pak and massage for 30 sec before the samples were diluted further in 0.1% peptone to the desired level. The appropriate dilutions were plated on onto Eosin Methylene Blue Levine (EMB) for *E. coli.* All plates were incubated at 37°C for 24 h before presumptive colonies were counted. The counts are expressed as log CFU/cm^2, and their average and standard deviation calculated.

3.2.7. Data analysis

Microbiological counts were transformed into log data before statistical analysis. Log reduction values were calculated by subtracting the log counts after treatment from the log count before treatment. Least-squares means of the log counts and log reduction values were then calculated

and analysed by general linear model procedure of SAS version 9.4. The analysis of treatment effects was considered significantly different when $P < 0.05$.

3.3. Results

3.3.1. Preliminary trials of ClO_2 on *E. coli* growth

E. coli O157:H7 Sakai survival on striploin pieces treated with ClO_2 was trialled initially to confirm any future effect was only due to chilling sensitivity on meat and not concentration (Figure 3.1). Concentrations of 0, 10, 15, 20, 25, or 50 ppm at both 20°C and 12.5°C were tested. However, no detrimental growth effects were detected under all concentrations of ClO_2 trialled.

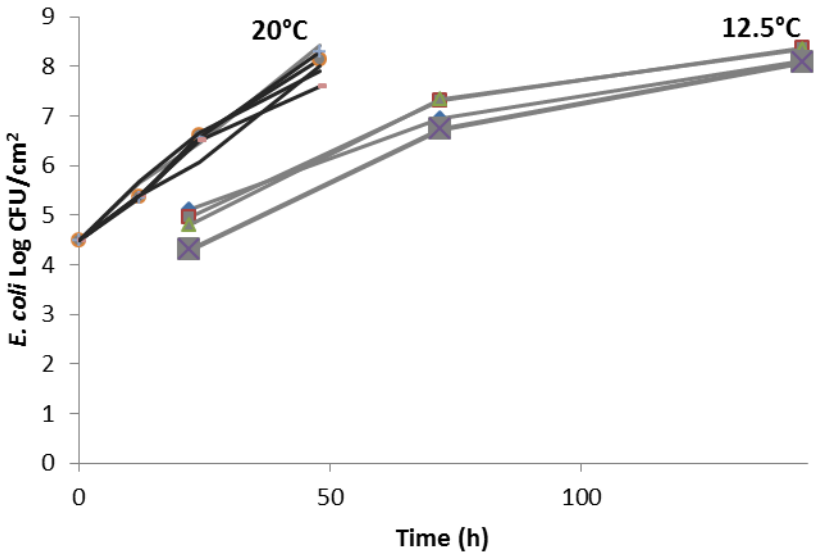


Figure 3.1. *E. coli* O157:H7 Sakai survival on striploin pieces treated with ClO_2 at 0 (◆), 10, 15 (■), 20 (●), 25 (▲), or 50 (■) ppm at both 20°C and 12.5°C.

3.3.1 Effects of stimulated spray chilling on *E. coli* O157:H7 Sakai and the addition of ClO_2 prior

E. coli O157:H7 Sakai on meat during the simulated process of spray chilling with water is shown in Figure 3.2, and a comparison to treatments, ClO_2 prior to chilling. The data revealed a significant decrease in *E. coli* O157:H7 Sakai numbers initially during chilling and from 4.5 h the count remained similar. These observations indicate that the process of spray chilling caused an overall reduction of both *E. coli* O157:H7 Sakai within the initial chilling stages during the temperature decrease from 37˚C to 10˚C. *E. coli* O157:H7 Sakai was, however, found to decrease by 1.02 ± 0.37 log CFU/cm^2 after spray chilling for 24 h.

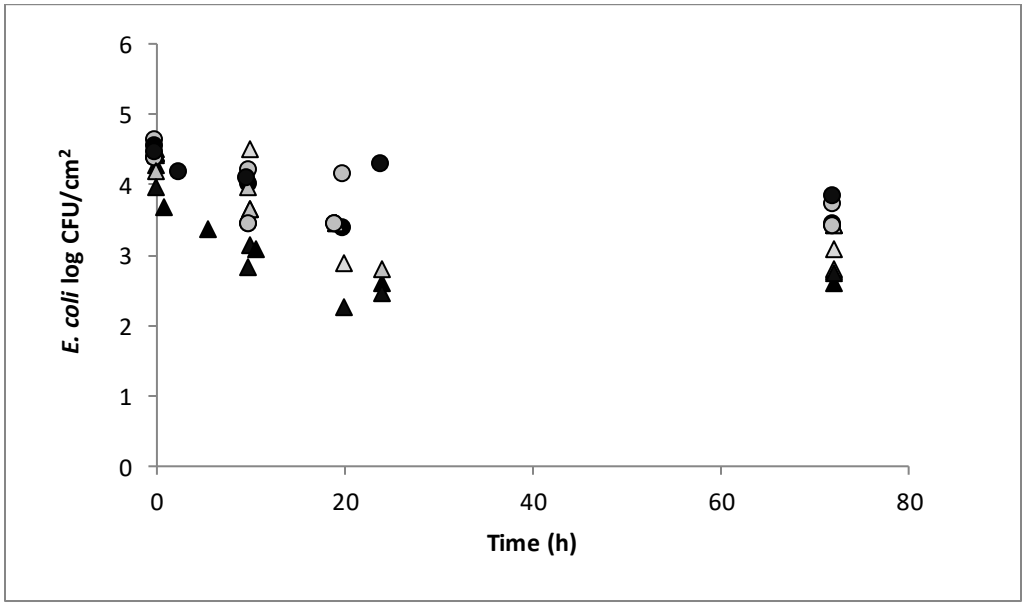


Figure 3.2. *E. coli* O157:H7 Sakai population changes after 15 ppm ClO_2 treated and non-treated, on the fat (▲and ▲, respectively) and lean (● and ●, respectively) surfaces prior to spray chilling conditions.

3.3.2 Effects of stimulated oxidant spray chilling on *E. coli*

The effect of ClO_2 at 15 and 20 ppm when applied during spray chilling (sprayed for 4 sec every 15 min for 36 cycles, 144 sec) are detailed in Figure 3.3. It was evident in all cases that the application of ClO_2 significantly reduced the numbers of *E. coli* on meat when compared to the control (water alone). ClO_2 at 15 ppm reduced *E. coli* by 3.10 ± 0.26 log CFU/cm^2 and > 4 log CFU/cm^2 for 20 ppm, after 24 h of chilling. However, this effect was only observed on fat surfaces. Thereafter, ClO_2 did not have any further effects on *E. coli* numbers.

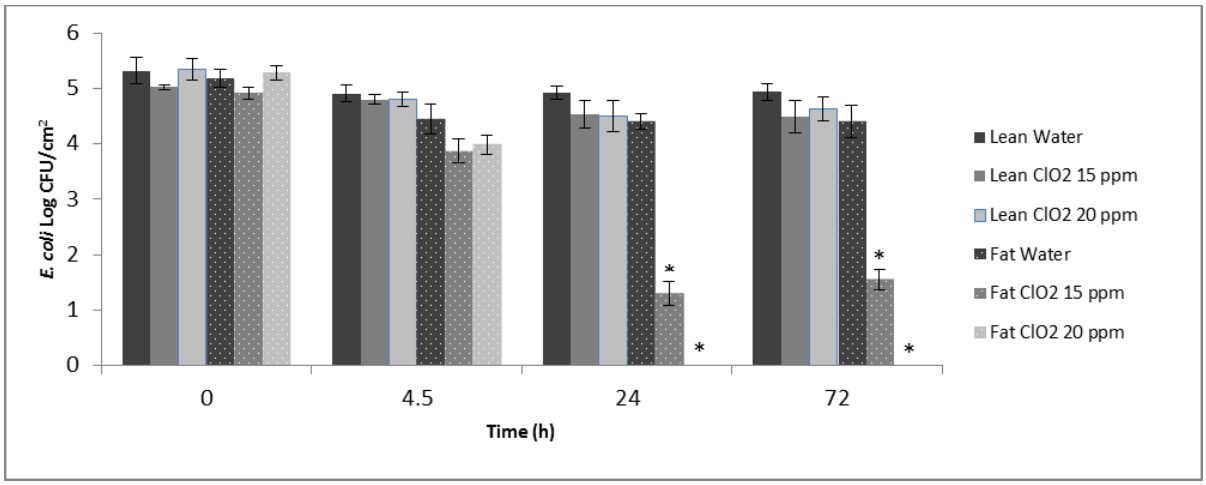


Figure 3.3. Striploin lean and fat side counts of *E. coli* cocktail log CFU/cm^2, during simulated spray chilling with water, ClO_2 15 ppm, or ClO_2 20 ppm. * Denotes significant difference to water treatment at the corresponding time.

To further evaluate the antimicrobial application of an oxidant during spray chilling, PAA was trialled at 150 and 200 ppm. The addition of PAA during spray chilling also significantly reduced the numbers of *E. coli* on meat compared to spray chilling with water alone (Figure 3.4). However, similarly to ClO_2 the effects were only significantly observed on fat surfaces.

This resulted in a >4 log reduction in *E. coli* numbers with 200 ppm and 2.68 ± 0.32 for 150 ppm, when compared to the control samples. PAA also had a similar response on *E. coli* with significantly lowering the counts at 24 and 72 h. However, PAA at 200 ppm had a greater effect than 150 ppm with count below detection limit (<0.78 CFU/cm^2) at the last time point 72 h. Therefore, showing the antimicrobial effect took longer to have maximum efficiency, unlike ClO_2 at 24 h.

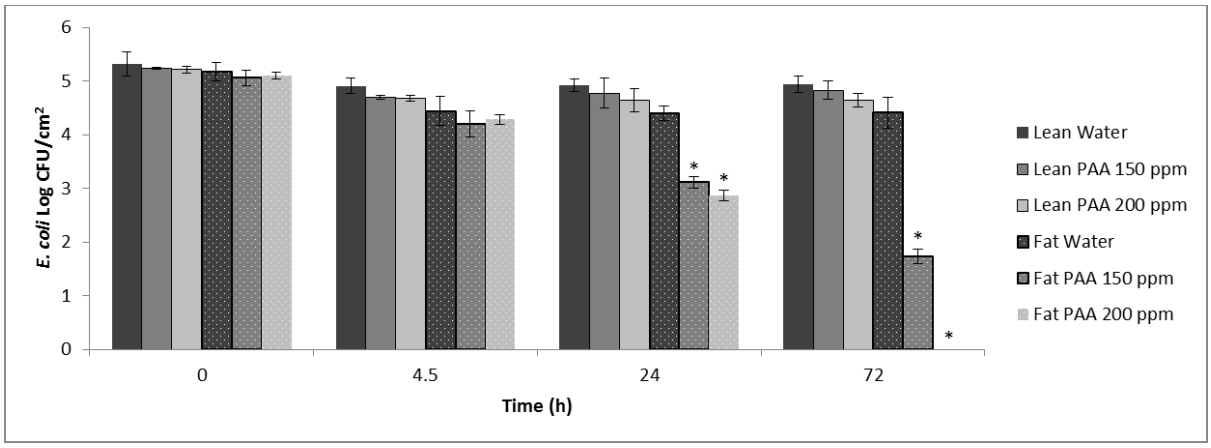


Figure 3.4. Striploin lean and fat side counts of *E. coli* cocktail log CFU/cm^2, during simulated spray chilling with water, PAA 150 ppm, or PAA 200 ppm. * Denotes significant difference to water treatment at the corresponding time.

3.4. Discussion

Previous studies, detailed in chapter 2, of *E. coli* O157:H7 Sakai response to carcase chilling confirmed the hypothesis that *E. coli* potentially becomes more sensitive to oxidative stress. Chapter 2 shows the effects of oxidants, hydrogen peroxide and ClO_2, at a non-lethal level on

E. coli in broth-based systems. Therefore, in the current study further evaluation of the antimicrobial effects of ClO_2 and PAA on beef striploins during spray chilling was conducted.

The effect of ClO_2 on *E. coli* O157:H7 Sakai at different concentrations (0-50ppm) at 20°C and 12.5°C was tested and no detrimental effect to growth was observed. Therefore, the concentration of ClO_2 use in this study was not considered lethal to *E. coli O157:H7* Sakai, highlighting the importance of chilling for inactivation to occur. Further preliminary tests were undertaken to see the effect of chilling alone and ClO_2 spray prior to chilling conditions. In comparing the non-treated and prior treatment no significant difference was found, however *E. coli* levels on fat surface were reduced over chilling ~1 log CFU/cm^2. Consistent with these results, previous studies demonstrated that both *E. coli* and *Salmonella* numbers were reduced by up to 2 log units on carcases after spray chilling (Bacon et al. 2000; Dickson 1991; Gill et al. 2003; Smirnova et al. 1997). Such conditions were not expected to be lethal to both organisms (Lanciotti et al. 2001; Presser et al. 1998). These reductions are also consistent with the results in Chapter 2 in which *E. coli* and *Salmonella* exhibited a growth response in broth when exposed to similar chilling conditions. Furthermore, once chilling conditions lowered to 7°C or below bacterial numbers are predicted to not growth and the counts were noted to remain stable.

During spray chilling ClO_2 was more effective at lower concentrations than PAA. The maximum effect of ClO_2 was also observed within 24 h, while the maximum effect for PAA was within 48 h. The highest concentrations of ClO_2 and PAA (20 ppm and 200 ppm respectively) caused the greatest reduction in number of *E. coli* as would be expected and has been reported in previous studies (Cutter et al. 1994).

ClO_2 and PAA spray chilling at 20 and 200 ppm, respectively, during chilling achieved >4 log reduction at 72 h. However, the effects of the oxidants were only observed on fat surfaces and

not lean. This confirms the increased susceptibility of *E. coli* to oxidative stress during spray chilling, however, some restrictions are observed with lean tissue.

Difference is species ability to survive and grow on fat surfaces compared to lean have also been observed. The differences may potentially be due to the composition, moisture and bacterial attachment to various tissue structure (Grau 1983). Some studies suggest bacteria survival on fat is affected by increased drying on the surface compared to lean tissue, this did not appear to be the case in the current study, although water activity was not measured throughout chilling.

It has been noted that attachment of bacterial cells to beef tissue is probably a physical attachment of the cells to the tissue (Schwach et al. 1982). The uptake of water has also thought to contribute to swelling of meat fibres and result in further entrapment of the cells (Benedict et al. 1990; Thomas et al. 1984). Pearce et al. (2008) inoculated samples on all meat tissue (fat, lean and cut) had similar counts of *E. coli* O157:H7 initially and on untreated samples over time, whereas antimicrobial treatments of a dairy extract caused the greatest loss on fat compared to the lean and no significant difference for cut samples.

Other research into antimicrobial sprays has also found greater reductions of *E. coli* on adipose tissue compared to lean (Cutter et al. 1994; Dickson et al. 1994; Marshall et al. 2005). Dickson (1992) attributed the differences in tissue to be due to pH and moisture content with lean 75% and fat 20%. Higher pH has been found to effected bacteria's survival on meat, though there was no great difference in pH noted in this study (Niebuhr et al. 2003).

Dickson et al. (1994) also found carcase surface results were comparable to laboratory trial, in which the surface of the carcass comprises of fat and connective tissue rather than lean. Therefore, the results observed in the current study warrant further investigation with oxidant sprays on carcase surfaces.

3.5. Conclusion

The present study demonstrated that an application of an oxidant, either ClO_2 or PAA, was effective at lowering *E. coli* on red meat. Such effects were more pronounced when applied during spray chilling than when applied before chilling. This highlights the potential of an oxidant-based application during spray chilling to be developed and evaluated further as an antimicrobial intervention for beef carcases in commercial settings.

Chapter 4

Commercial trials of oxidant spray chilling on beef carcases.

Abstract

E. coli contamination is a concern when exporting beef, with the potential for major economic and reputation loss. Tight safety regulations increase the need for high quality, safer meat to be available for export overseas. This highlights the need for a reliable antimicrobial intervention during meat processing. *E. coli* cells are already in a vulnerable state during chilling, and previous studies have shown O157 serotype strains are sensitive to oxidants during initial chilling. Therefore, we compared two different oxidants, chlorine dioxide and peroxyacetic acid, as a practical antimicrobial in an industry setting. The in-plant trials involved the use of beef carcase sides and a chiller undergoing an industrial spray regime. Natural populations of *E. coli* were found to be too low and infrequent to see the effect of the oxidants. Therefore, a cocktail of *E. coli* strains was applied. Both oxidants reduced the total microbial count and *E. coli* significantly, though more effect was seen on the *E. coli* populations. A 1-3 log CFU/cm^2 reduction was found on the carcase sides depending on the site sampled. Hind leg and flank-brisket sites had the highest log reductions, most likely due to greater exposure to the spray. The results show adding an oxidant to a spray chilling regime provides a potentially attractive *E. coli* intervention during carcase processing that requires little to no structural changes in the abattoir. However, the oxidants effect on a natural *E. coli* population may behave differently. Therefore, more investigations are warranted with an oxidant during spray chilling, at a larger scale and with natural populations. Although no adverse effects to the quality of the meat was seen, further studies will be required to examine any negative effects on quality and shelf-life.

4.1. Introduction

Australia exports beef to over 100 countries in Europe, Asia, Middle East and the Americas. The beef export market is worth $13.3 billion to the Australian meat industry and 1% of the gross domestic product, with Australia being the world's third largest exporter of beef (MLA 2018). Australia's largest export markets are Japan and the US, followed by Korea (DAWR 2019). Overseas regulations, particularly in the US, have strengthened with testing beef products. A more stringent requirement for the presence of pathogenic *E. coli* has recently been applied, in their zero tolerance policy O26, O45, O103, O111, O121, and O145 serotypes have been included in addition to O157 strains (Almanza 2011). If any of these serotypes are detected on meat, the meat must be discarded. Thus, any contamination has the potential for major economic and reputational loss for the Australian export market. Therefore, there is an increasing need to produce high quality with very low risk for export markets.

E. coli are part of the normal microbiota in the gastrointestinal tract of warm-blood animals including cattle. Although many strains of *E. coli* are not harmful, some strains possess virulence factors that enable them to cause several forms of gastrointestinal disease (Todd et al. 2001). Pathogenic *E. coli*, especially the O157 serotype strains have been linked to a large number of outbreaks associated with undercooked meat and meat products (Armstrong et al. 1996). Outbreaks have been more notable in the US, resulting in the US implementing a zero-tolerance policy for *E. coli* in red meat (Gansheroff et al. 2000; Tuttle et al. 1999).

At slaughter and processing the carcass can become contaminated from several points along the processing chain, from de-hiding to the removal of the gastrointestinal tract. During these processes particular sites on the carcase are more likely to be contaminated. It is well established that the hind leg, bung, neck, and cutting lines are more likely to be sites of

contamination. Therefore, in this study we focused on these areas to see the effects of our decontamination step, by changes in total viable bacterial count and *E. coli* numbers.

Several interventions are currently in use, from de-hairing to carcase washing, although none are 100% effective. Current technologies use hot water, organic acids, and pressurized washes. These wash steps are usually implemented throughout the processing chain to prevent further cross contamination. In this study we investigate an additional antimicrobial step during chilling to take advantage of the existing stress caused by chilling (Huffman 2002). This step would be a final hurdle for pathogenic bacteria before boning and packing occurs.

Regulations require beef carcase sides to be cooled by forced air refrigeration to lower their temperatures to a deep tissue temperature of 7°C within 24 h (Gill et al. 1991). Larger abattoirs now use spray chilling as a more efficient rapid cooling system. Spray chilling is the intermittent spraying of carcases with water during the early stages of the cooling of hot sides. The spray chilling systems also reduce evaporative weight loss or shrinking due to carcases drying and thus can increase yield. Food Safety and Inspection Service regulations require that the chilled carcase weight no more than the hot carcase, therefore spray chilling must be limited to prevent carcases from absorbing moisture (Prado et al. 2010). Spray chilled carcases typically retain at least 1% more of their initial weight than conventionally chilled carcases (Savell et al. 2005), although this varies with the duration of the spray cycle. Conventionally chilled carcases inhibit microbial growth by reduction in surface moisture which occurs during the chilling process (Dickson 1991). The addition of an antimicrobial oxidant to the spray may combat this potential problem during spray chilling. However, many studies find microbial growth does not occur significantly during spray chilling (Dickson 1991; Greer et al. 1997).

The focus of the experiments here was to exploit *E. coli* cells already in a vulnerable state during chilling (Kocharunchitt 2012). The chilling process itself has shown to cause a 1-2 log

reduction in bacterial counts (Bacon et al. 2000; Tittor et al. 2011; Youssef et al. 2013). Prior experiments (see thesis chapters 2 and 3) have shown how *E. coli*, and specifically O157 serotype strains, are sensitive to oxidants, chlorine dioxide (ClO_2) and peroxyacetic acid (PAA), during initial chilling in broth culture and on meat pieces. Therefore, in the current study we compare the two different oxidants within the abattoir environment on carcases.

4.2. Methods

4.2.1 Overview

The antimicrobial intervention trials were undertaken at an international export processing plant in Tasmania, Australia. These trials involved the use of beef carcase sides straight from the slaughter floor. The commercial chillers were programmed to simulate an industrial spray application over a weekend period.

4.2.2 Determination of oxidant efficiency on natural microbial population

Four different sites (hind leg, flank, fore leg and neck) of eight carcase sides were used to determine the effect of the treatment. The carcases were either subjected to the normal process of spray chilling (potable water), or to the application of an oxidising agent (ClO_2 at 35 ppm or PAA at 200 ppm) during spray chilling.

4.2.3 Determination of oxidant efficiency on inoculated carcases

4.2.3.1 Preparation of *E. coli* inoculum

Five strains of *E. coli* (EC1604, EC1605, EC1606, EC1607, and EC1608), obtained from CSIRO Food and Nutritional Sciences Australia, were used in this study. All of these *E. coli* strains are found to contain no known virulence markers for pathogenic *E. coli* but otherwise have growth characteristics very similar to various known isolates of *E. coli* O157:H7 (Small, 2010). *E. coli* strains were grown for 20 h at 37°C in brain heart infusion (BHI) broth to obtain stationary-phase cultures. Equal volumes (1 ml) of the cultures were then transferred to 500 ml of 0.1% bacteriological peptone to give an inoculum containing approximately 6-7 log CFU/ml.

4.2.3.2 Preparation of an oxidising agent

Zydox® chlorine dioxide (Zychem Technologies Pty Ltd) and peroxyacetic acid (ECOLAB Australia Pty Ltd) were the two oxidants trialled. The working solution of both oxidants were prepared in 1,000 L tanks and diluted with an appropriate volume of potable water to achieve the test concentration (ClO_2 at 35 ppm or PAA at 200 ppm), according to the manufacturer's instructions.

4.2.3.3 Preparation and treatment of inoculated test carcases

Four different sites (hind leg, flank, fore leg and neck) of three carcase sides were inoculated with the *E. coli* cocktail described above (Section 4.2.3.1). The *E. coli* cocktail was painted onto the four areas of each carcase and left to dry for 20 min. Inoculated carcases were then either subjected to the normal process of spray chilling (potable water), or to the application of an oxidising agent (ClO_2 at 35ppm or PAA at 200 ppm) during spray chilling.

4.2.3.4 Spray chiller settings

The chillers were programmed to spray in cycles as for a regular weekend chilling regime (62 h). This regime consists of 45 s sprays at 10 min intervals for the first 13 sprays, followed by 30 s sprays every 15 min for the remaining 52 sprays. The temperature cycle and fan settings were programmed to blast chill until meat surface temperature reached 5°C, at which the temperature remained constant over the weekend, prior to boning.

4.2.6 Microbiological analysis

Samples were taken with Whirlpak™ sponges by swabbing a 10 cm × 10 cm area. Each site was swabbed before the chilling process, at 24, 50 and 62 h after commencing chilling. Rehydrated Whirlpak sponges were used with 25 ml of 0.1% peptone. An aliquot of the swabbed samples was serially diluted in 0.1% peptone and plated on *E. coli* count Petrifilm™ (3M) and Total Aerobic Petrifilm™ (3M), according to the manufacturer's instructions. All Petrifilm were incubated at 37°C for 48 h before being counted. The counts are expressed as log CFU/cm^2, and their average and standard deviation calculated. The effectiveness of the proposed intervention in eliminating *E. coli* on carcases was determined by the differences in log units between untreated carcases and carcases treated with an oxidant.

4.2.7 Data analysis

Microbiological counts were transformed into log data before statistical analysis. Log reduction values were calculated by subtracting the log counts after treatment from the log count before treatment. Least-squares means of the log counts and log reduction values were then calculated

and analysed by general linear model procedure of SAS version 9.4. The analysis of treatment effects was considered significantly different when $P < 0.05$.

4.3. Results

4.3.1 Effectiveness of the proposed intervention on natural microbial population

The effectiveness of both oxidants, ClO_2 and PAA, were compared to the control, chlorinated potable water, by the difference in counts. The initial trials of natural populations showed a 1 ± 0.5 log reduction overall, with both oxidant treatments, for total viable count (Figure 4.1). A dip in total viable counts after 12-24 h of chilling was seen, followed by a total count increase at the end of weekend chilling (62 h). However, the log reduction was only significant for PAA treatment (Table 4.1). The natural population of *E. coli* was low and sparsely located on the carcases. *E. coli* was found only 4 times out of 32 control samples, and on none of the treated samples (64 samples), using the *E. coli* petrifilm method (Section 4.2.6).

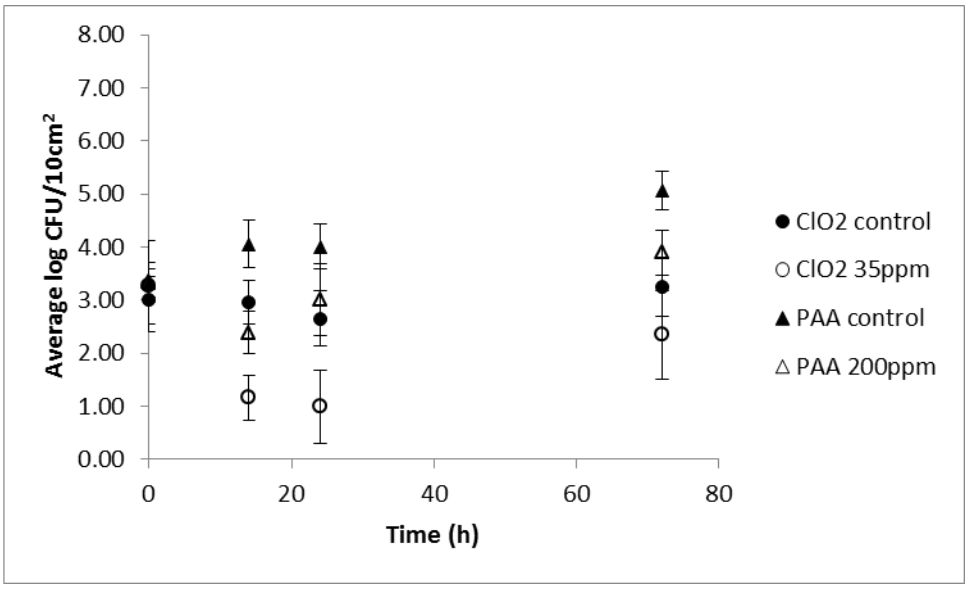


Figure 4.1. Average Total Viable counts from eight carcases through-out the chilling process, with treatments of 200 ppm PAA (△) or 35 ppm ClO_2 (❍), and non-treated samples (controls) for PAA (▲) and ClO_2 (●).

4.3.2 Effectiveness of the proposed intervention on Inoculated population

4.3.2.1 Overall effect on microbial reductions

Table 4.1. Average microbial log CFU/cm^2 reductions after treatment with either PAA or ClO_2, for both un-inoculated and inoculated carcasses, at the end of chilling.

Treatment	Carcase region	Average Log reduction CFU/cm^2			
		Un-inoculated		Inoculated	
		TVC	Ec	TVC	Ec
PAA	Hind leg	1.18*	-	2.98*	2.87*
	Flank/Brisket			2.54*	2.37*
	Fore leg			2.61*	1.61*
	Neck			1.28*	1.27*
ClO_2	Hind leg	0.90	-	2.64*	2.79*
	Flank/Brisket			2.54*	2.49*
	Fore leg			1.03*	1.27*
	Neck			0.69	0.75

*Denotes a significant reduction between the control and treatment ($P < 0.05$)

The un-inoculated carcase sample sites were all combined into a single composite sample for plating, to increase the observable counts (Table 4.1). However, composite samples still did not reveal frequent or useful numbers of *E. coli*. Therefore, further experiments were undertaken with inoculating carcases with a cocktail of non-pathogenic *E. coli* strains, so inactivation responses could be accurately observed and hence the effect of the treatments. The outcomes indicated inoculating the carcases with a cocktail of *E. coli* strains achieved valuable numbers to compare treatments. Table 4.1 compares the log reductions, the difference between the control (water) and treated (PAA or ClO_2) carcases, for each site samples. The trend, in general, is a log reduction decreasing as the samples go down the length of the carcase. For PAA trials the TVC average log reduction is from 2.98 CFU/cm^2 at the hind leg to 1.28 CFU/cm^2 at the neck. *E. coli* reductions have a similar though slightly lower count from 2.87

CFU/cm^2 at the hind leg, to 1.27 CFU/cm^2 at the neck. Similarly, for ClO_2 trials the TVC average log reduction is from 2.64 CFU/cm^2 at the hind leg, to 0.64 CFU/cm^2 at the neck. *E. coli* reductions are also similar though slightly lower count from 2.79 CFU/cm^2 at the hind leg, to 0.75 CFU/cm^2 at the neck.

4.3.2.2 Site effect of the antimicrobial

There was no significant difference between the control and treatment carcases at any site for the *E. coli* counts at the beginning of the trials, for either PAA (Figure 4.2) or ClO_2 (Figure 4.3). This is expected due to the same inoculation level being applied to all sites, in order to test the effect. The PAA trial, Figure 4.2, details the comparison in counts between the control and treatment at each site (hind leg, flank/brisket, fore leg and neck), over the 62 h weekend chilling period. The *E. coli* counts were found to be lower at the higher regions of the carcase, although for the PAA treatments all sites were significantly different at each time point except for the initial time point prior to any treatment (Figure 4.2, a-l = $P < 0.05$).

The ClO_2 trial also showed the same trend with lower counts at the higher sites of the carcase (Figure 4.3). However, ClO_2 only caused a significant degree of inactivation between the control and treatment samples for the hind leg, flank/brisket, and fore leg sites, though not the hind leg site at 50 h or the neck at any time point. Overall, for PAA and ClO_2 trials there was no significant site difference found between the control samples over the chilling period ($P < 0.05$).

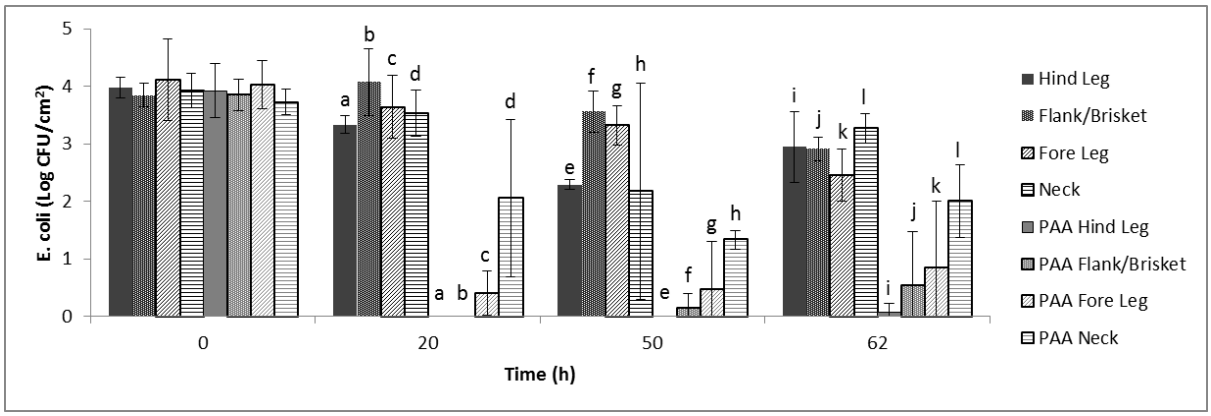


Figure 4.2. *E. coli* average log CFU/cm^2 on inoculate carcases, at four sites throughout chilling, for three control carcases (water only) and three treatment carcases (200 ppm PAA). The sample sites consisted of hind leg, flank/brisket, fore Leg, and neck regions. Significant differences between control and PAA treatment are denoted with the same letter for each corresponding pair (a-l = $P < 0.05$).

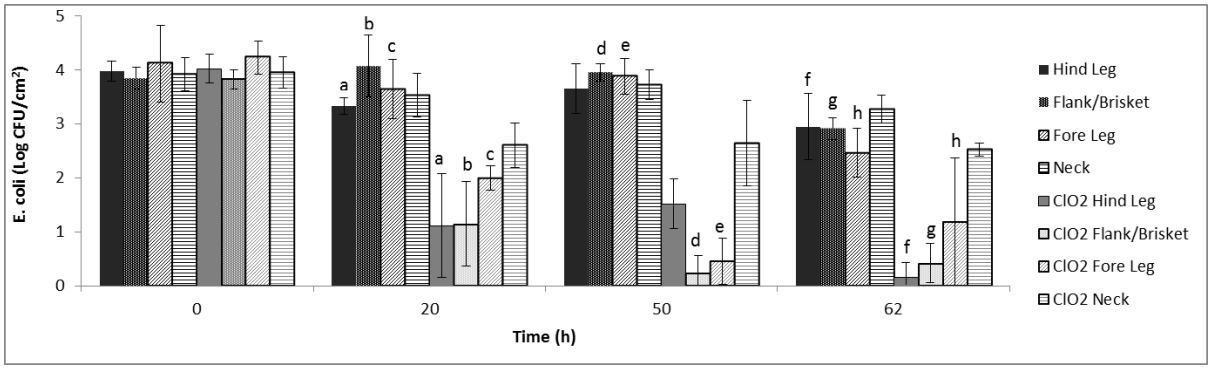


Figure 4.3. *E. coli* average log CFU/cm^2 on inoculated carcases, at four different sites throughout chilling, for three control carcases (water only) and three treatment carcases (35 ppm ClO_2). The sample sites consisted of hind leg, flank/brisket, fore leg, and neck regions. Significant differences between control and ClO_2 treatment are denoted with the same letter for each corresponding pair (a-h = $P < 0.05$).

4.4. Discussion

This study was intended to test the feasibility and efficacy of oxidants under processing conditions. The oxidants PAA and ClO_2 were both trialled in-plant under 'normal' weekend processing regimes and in comparison, to standalone water spray regimes during chilling. During chilling the number of total bacteria was lower around the 24 h time frame before increasing to original or higher numbers as initial counts at the beginning of chilling. This trend has been report by many studies with large variation between different abattoirs (Gill et al. 1997; Murray et al. 2001). The cycles of spray chilling vary between abattoirs due to different meat commodities being processed, spray nozzle configuration, spray pattern and water cycle, fat coverage, variable water sources, temperature of water and pre-chill treatment are all noted reasons for large site-to-site large variations. Carcase condition prior to chilling may also have an important effect on the spray chilling intervention. Effects such as washing, excess trimming and drainage may interact with or dilute the antimicrobial spray (Gill 2009).

At the abattoir where the experiments were performed low numbers of *E. coli* led to trials relying on artificially inoculated carcases. Artificially inoculated carcases were used to determine the specific effect of the oxidants on strains that behaved similarly to O157 serotype strains. Gill et al. (1997) found with *E. coli* inoculated meat after around 24 hrs of chilling there was a <0.5-2 Log CFU/cm^2 reduction of *E. coli* (Gill et al. 1997). This data may not be representative of the numbers after 62 h since the inactivation process could be continuing to occur through the duration of the trial.

Higher counts were consistently seen for treated carcases at the lower positions on the carcase, in particular the neck, which represents the lowest point. In this current study a washing effect was not seen, as all control sample sites had similar counts throughout the spray chilling process. Instead, we found higher counts on the neck compared to other sites of the treated

carcases, implying a lack of treatment efficiency at lower points, most likely due to sprays directed from above the carcase not reaching the lower regions. The results agree with the findings from Castillo et al. (1999b) who observed greater log reductions on hind and flank regions.

Contrary to other studies where water washes alone can yield a 1-2 log reduction (Castillo et al. 1999b), the spray chilling performed here was not found to cause any significant cell removal or inactivation. The counts increased after the weekend chilling regime (62 h). The volume and spraying process, applied as a fine mist, compared to droplets, may account for the variation between other studies (Castillo et al. 1999b; Signorini et al. 2018). Although some bleaching from water run off was seen in the current study, at lower regions of the carcases, this effect was minor and did not influence the microbial count. In previous laboratory simulation trials, a potential for a >5 log reduction, with PAA and ClO_2, on beef striploins during spray chilling was observed (Chapter 3). However, the greatest results were only seen on the fat surfaces, this agrees with other studies (Dickson 1988; Nattress et al. 2001). Higher fat coverage could be an important part in increasing the efficiency of the antimicrobial treatment during chilling. Reductions were achieved for total bacteria on fat tissue, which is important because much of the exposed surfaces of beef carcases are covered with fat. This finding could possibly lead to less trimming becoming a safer and more effective way of eliminated or decreasing pathogen counts on beef carcasses with the added potential economic gain of retaining more product for sale.

Commercial studies in the poultry industry have already shown PAA and ClO_2 are an effective decontamination wash for *Salmonella* and *Campylobacter*. The effectiveness has been seen at low levels (2 ppm) and therefore is now approved for use in commercial systems (Bauermeister et al. 2008; Berrang et al. 2011).

Studies into PAA have found positive characteristics of this oxidant over others, such as its stability, non-corrosive, economical and also being already widely excepted in the food industry (Costa et al. 2015). PAA was also found to be a much cheaper option, up to 7.5 times cheaper than chlorine dioxide. Similar studies have also shown promising results with using PAA. These studies have reported 2-3.5 log reductions of *E. coli* O157:H7 on beef samples (Davenport 2016; Penney et al. 2007). They used higher concentrations of PAA (400 ppm), than the current study of 200 ppm. The difference possibly highlights the importance of treatment timing, during initial chilling, to increasing efficacy.

Chlorine dioxide is a strong oxidant, highly soluble in water at cold temperatures and is known to be highly effective over a wide range of pH (5-10) and thus appropriate for carcase chilling conditions (LeChevallier et al. 2004). It has been used to control iron, manganese, taste and odour causing compounds in many European countries as a disinfectant of water (LeChevallier et al. 2004). A major disadvantage of ClO_2 is that it is highly reactive with blood, potentially affecting the quality of meat and meat products. In the current study some lightening of the fat was observed and a darkening of blood vessels. However, these affects did not seem to be a widespread problem, and the whitening may enhance appearance during storage. Other issues of concern are health and safety risks associated with ClO_2, its de-gassing and strong oxidising properties.

4.5. Conclusion and future work

Both oxidants reduce *E. coli* significantly, though PAA is repeatedly seen to be more effective. PAA comes with less safety issues, restrictions on use and is easier to apply without requiring an activation step. Therefore, PAA is an attractive potential for industry with little to no structural changes in the plant. With further optimising of the application process around 3 log

reductions seem a feasible outcome within industrial applications. In the current study we allowed for differing factors on the behaviour of the artificial inoculated cultures compared to naturally found contamination. Allowances were made for growth conditions, strain variation and attachment time. The results highlight the potential for the oxidants to be an intervention on natural *E. coli* populations, although broth-based cultures cannot replicate the complex physiological state of *E. coli,* growing anaerobically in the presence of organic acids, of the gastrointestinal tract of cows. However, the current experiment being limited by the artificial application of *E. coli* still warrants further investigation on natural populations. The previous study (Chapter 3) observed the potential for >4 log reduction of *E. coli* compared to the potential >2 log cfu/cm^2 reduction, in this current study. However, laboratory-based trials are small scale and easily modified for efficiency. A carcase is a more complex structure, with a large surface area compared to a striploin with a flat lean and the fat side, site variation is to be expected. We suggest further investigations into larger scale experiments, other locations (possible with less highly technical infrastructure) and different strains.

Although no adverse effects to the quality of the meat was seen, further studies will be required to examine any negative effects on quality and shelf life in more detail over long storage times. With shelf-life up to 6 months for VP beef meat potential loss of profit/value may occur if shelf-life is reduced or if any detrimental quality effects occur.

Chapter 5

Shelf-life of vacuum packed beef after oxidant spray chilling.

Abstract

The international export of red meat and other perishable food products relies on the ability to maintain long shelf-lives. Beef cuts have extended shelf-lives of over 140 days when VP and stored at low temperatures. The anaerobic condition of VP inhibits aerobic bacteria with the meat microbial community then dominated by lactic acid bacteria, in particular the genus *Carnobacterium*. The meat population structure potentially slows spoilage microorganism growth and therefore allows for a longer shelf-life. Consumer safety and economic implications are an important consideration when any new antimicrobial intervention is introduced in the processing chain. Therefore, the current study investigated any negative effects on VP striploins and its shelf-life after trials of oxidant spray chilling in an export abattoir. The oxidants used were chlorine dioxide (35 ppm) and peroxyacetic acid (200 ppm). Sensory and microbial data were collected and compared to non-treated samples to determine if the oxidants influenced shelf-life. Any negative effects could lower the quality and value of the meat product and shorten the shelf-life, which would result in a decrease in industry productivity. In this investigation no detrimental effects were detected for either sensory or microbial data. However, samples treated with ClO_2 differed from the control after extended storage, from 180 days, for pH and for consumer preference. There were no conclusive results to suggest that either oxidant causes a loss in quality or shelf-life, though more extensive studies should be undertaken to assess consumer responses.

5.1. Introduction

Australia's export of red meat is an important economic business for the country. Ensuring the export of high quality and long-lasting meat products is essential for this industry to remain one of Australia biggest export income earners. Shelf-life of meat has been extensively studied, beef is famed for having excellent stability with the use of VP and non-freezing storage temperatures. The use of VP allows overseas shipment which requires at least 90 days storage and where meat is kept chilled at approximately -0.5°C (Bell 1994, Yousef 2014).

Long shelf-lives of over 140 days have been reported for beef (Youssef et al. 2014), and Australian beef can be stored over 180 days in some cases (Small et al. 2012). VP extends shelf-life by modifying the atmosphere and hence changes the growth environment of bacteria, leading to population changes. The storage of meat under these conditions results in the bacterial population being less diverse over time and maintains a high proportion of lactic acid bacteria (LAB) (Kaur et al. 2017a; Sade et al. 2017).

Pseudomonas sp., *Brochothrix* sp., *Psychrobacter* sp., *Lactobacillus* sp., and *Acinetobacter* sp. have been identified as major species contaminating raw meat before processing treatments. They are commonly found in processing environments (De Filippis et al. 2018; Stellato et al. 2017). Once meat becomes VP the microbiota changes with the changing environmental conditions. Oxygen levels are low and restrict the growth of *Pseudomonas* sp. and other aerobic spoilage bacteria (Mansur et al. 2019). The carbon dioxide level also becomes high (up to 40%) enough to prevent growth of gram negative bacteria (Narasimha and Sachindra 2002). This change allows Gram-positive bacteria such as LAB to become the dominant microbiota. In VP meats, LAB grows slowly and uses glucose or glycogen which gets converted to lactic acid (Casaburi et al. 2011). The production of lactic acid can cause souring, though this causes less obvious spoilage (Casaburi et al. 2011). Therefore, the total count of bacteria on VP meat can reach 8 log CFU/cm^2 without the meat being obviously spoiled.

Carnobacterium maltaromaticum and *Carnobacterium divergens* are the main species associated with VP beef (Casaburi et al. 2011). Various cold-tolerant Lactobacillus, *Lactococcus* and *Leuconostoc* species may also be present (Liang et al. 2016; Zhang et al. 2018). Members of the family *Enterobacteriaceae* species are also present, in particular *Serratia* and *Hafnia* spp., and some species are linked to early spoilage (Youssef et al. 2014). It is thought some bacteria on meat may contribute to implicit effects on meat spoilage. For example, some strains of *C. maltaromaticum* grow efficiently on VP meat stored at low temperatures with low sensory impacts and spoilage overall (Casaburi et al. 2011; Kaur et al. 2017a). These strains have also been linked to inhibiting the growth of other species, potentially assisting in controlling spoilage (Zhang et al. 2015).

Decontamination treatments during carcase processing are thought to have a major effect on the microbiome, in both numbers and diversity (Youssef et al. 2014). The improvement of decontamination treatments over time has led to not only safer products but potentially also longer shelf-lives (Yang et al. 2012). Therefore, any change in the processing environment has the potential to substantially affect the microbial population and hence affect shelf-life (Gill 1996). This variation in populations under VP conditions has been seen for different cuts, treatments, and processing environments. Therefore, logically the application of antimicrobial treatments, as conducted previously (Chapter 4) requires sensory studies to ensure product quality and stability is maintained or indeed enhanced. Thus, in the current study, the shelf-life and sensory effects of VP beef was assessed after antimicrobial spray chilling of an oxidant, using either chlorine dioxide or PAA. The goal was to determine whether these antimicrobial treatments, aimed at increased pathogen elimination, detrimentally impact sensory and/or shelf-life or instead have no sensorial impact and also achieve an improved shelf-life.

5.2. Material and Methods

5.2.1 Test site

The carcase treatment trials were undertaken at an export processing plant in Tasmania, Australia. These trials involved the use of beef carcase sides in a chiller receiving an industrial spray chilling application over a weekend period (as detailed in Chapter 4).

5.2.2 Preparation and treatment of test carcases

Carcases were either subjected to the normal process of spray chilling (potable water), or to the application of an oxidising agent (ClO_2 at 35 ppm or PAA at 200 ppm) during spray chilling. The chillers were programmed to spray in cycles as for a regular weekend chilling regime (62 h). This regime consists of 45 s sprays at 10 min intervals for the first 13 sprays, followed by 30 s sprays every 15 min for the remaining 52 sprays. Further details of the antimicrobial interventions and outcomes as described in Chapter 4.

5.2.3 Shelf-life sample collection and storage

The shelf-life trial was conducted on striploin pieces cut in half and VP on the processing site, from treated carcases (ClO_2 or PAA) and the control group (potable water). The striploins were cut and VP within a local export abattoir, after 62 h of chilling. Half striploins were individually VP in Thermasorb bag E86 (O_2 transmission rate of <10 cc/m^2/24 h at 23 °C, 85% relative humidity). Fifty-six meat samples were prepared and stored at -1°C for up to 194 days. Storage temperature was monitored using DS1921G Thermochron i-button temperature logger (iButtonLink, WI, USA).

5.2.4 Vacuum packed beef sensory analyses

Sensory tests were performed to determine meat quality including whether testing panellists were able to differentiate PAA or ClO_2 treated samples from controls. For each sensory analysis time point 2 replicates from the control and 2 replicated from the treatment group (either PAA or ClO_2) were analysed. Samples were subsequently tested periodically up to 194 days of storage. For the sensory assessment, an untrained panel of six was used to assess meat quality based on personal preference, visual appeal prior to opening, followed by an assessment of odour and colour 5 min after opening. Personal preference and buying preference were a yes or no score to each individual meat pieces. Odour after 5 min was scored as follows: 3 (no odour), 2.5 (mild odour), 2 (sour odour), 1.5 (strong sour odour), 1 (off odour) or 0.5 (strong off odour). Colour after 5 min was scored as follows: 3 (red), 2 (greyness) or 1 (other). The appearance, tightness of vacuum and seal quality was also noted. In addition, the surface pH was measured using a digital surface pH meter (ORION model 250A) for both fat and lean sides.

5.2.5 Vacuum packed beef microbiological analyses

The surface area of the meat including both lean and fat surfaces was calculated to allow for determination of colony forming unit per unit area. After sensory analysis, the individual pieces of meat were transferred to sterile bags and massaged in 100 mL of sterile 0.1% peptone water for 2 min. Serial dilutions were prepared in sterile 0.1% peptone water and spread plated. Total viable counts were enumerated using tryptone soya agar (TSA) (CM0131 Oxoid, Australia). Lactic acid bacteria (LAB) counts were performed on de Man-Rogosa-Sharpe (MRS) agar (CM0361 Oxoid, Australia). All agar plates were incubated at 20°C for 5 days in sealed jars, under an anaerobic atmosphere generated by an AnaeroGen sachet (AN0035 Oxoid, Australia).

5.2.6 Statistical analysis

Sensory data was analysed by categorical data analysis, converting to percentages and chi-square tests done. When the analysis of treatment effects indicated a significant difference ($P < 0.05$), mean separation was conducted using the Cochran-Mantel-Haenzel test using SAS statistical package (version 9.4; SAS Institute, Cary, NC, USA). Microbiological counts were transformed into log data before statistical analysis with analysis of variance and Proc Mixed in SAS v 9.4.

5.3. Results

5.3.1 Sensory analysis over shelf-life

5.3.1.1 Visual and consumer preference of VP striploins

The visual and buying preferences for the ClO_2 trials are detailed in Figure 5.1, where preference scores are shown as percentage of total scores. Overall, both preferences, visual and buying, for control and ClO_2 treated striploins were found to be significantly different ($P < 0.05$). Analysing each time point found only the later points (180 days and later) to be significantly different for both visual and buying preferences.

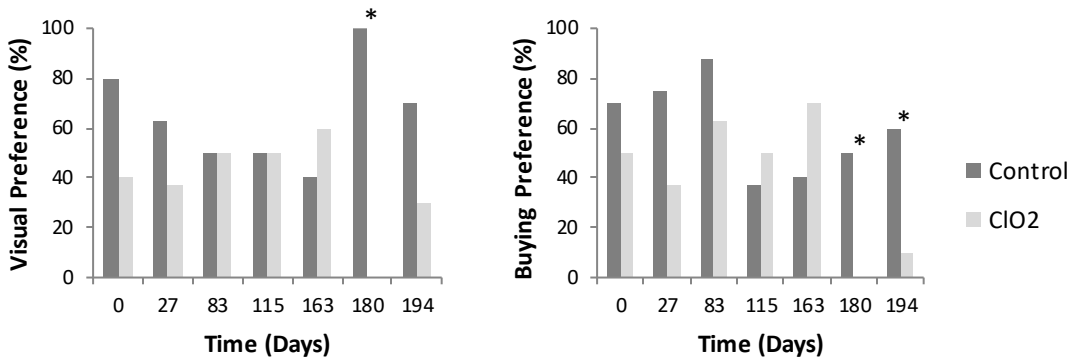


Figure 5.1. Visual and buying preference of VP striploins after treatment with 35 ppm ClO_2, over storage at -1°C. * Denotes control and ClO_2 treated samples are significantly different at the corresponding time point ($P < 0.05$).

The visual and buying preferences for the PAA trials are detailed in Figure 5.2, where preference scores are shown as percentage of total scores. Overall visual preferences were not significantly different between control and PAA treated striploins ($P = 0.2515$), though buying preference difference was marginally significant ($P = 0.0495$). Therefore, visual preference was not analysed further. Analysing each time for buying preference found only 90 days to be significantly different. Therefore, PAA was not considered to have significant detrimental effect on shelf-life related visual appearance or for consumer preference.

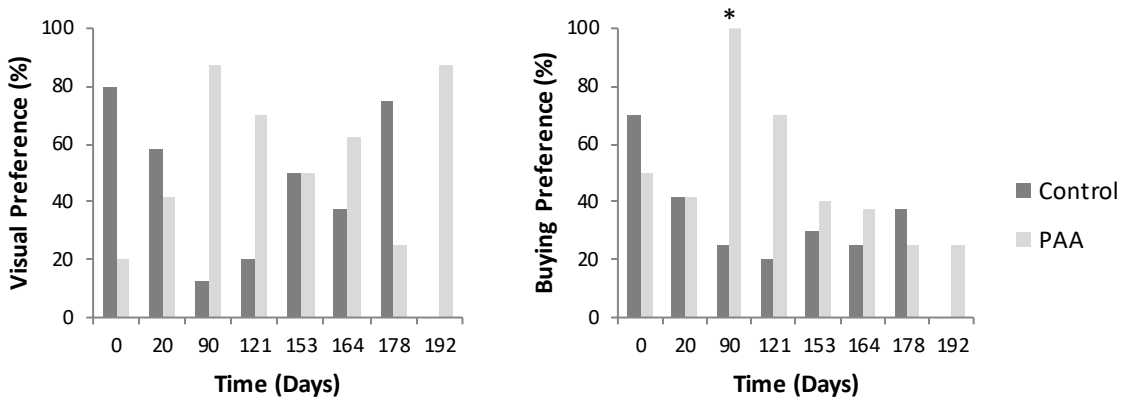


Figure 5.2. Visual and consumer preference of VP striploins after treatment with 200 ppm PAA, over storage at -1°C. * Denotes control and PAA treated samples are significantly different at the corresponding time point ($P < 0.05$).

5.3.1.2 Odour sensory data

There was no apparent detrimental effect of the ClO_2 treatment on odour (Figure 5.3). ClO_2 may have received a stronger odour score initially with 20% of panellists scoring 'Strong Off Odour' though 20% of the panellists also scored the control as having an 'Off Odour'. This shows the objective nature of this sensory test, as the meat was considered fresh by most other panellists. Adjusting for the outlier sensory panellists, the Cochran-Mantel-Haenszel statistics showed there is no overall significant difference of odour score proportions between control and treatment samples over time ($P = 0.1$). Therefore, individual time points were not assessed. However, trends of increasing odour over time, as expected towards the end of shelf-life for both control and treated samples, were seen after 163 days.

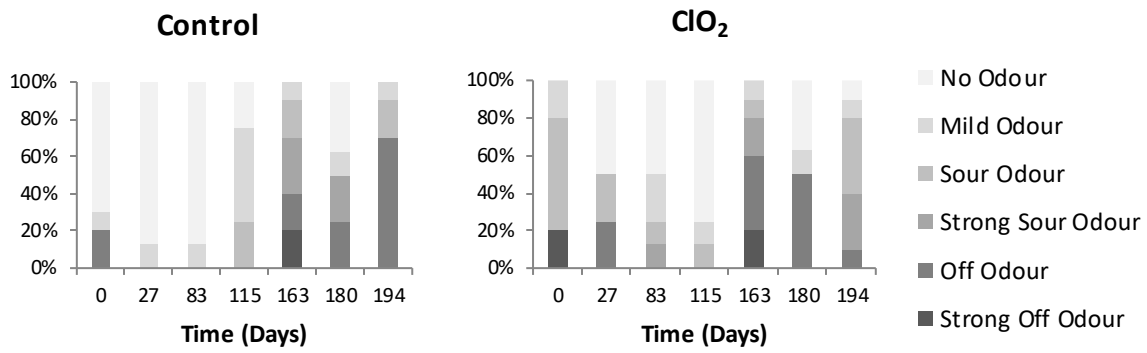


Figure 5.3. Sensory panel proportion scores (as percentage) on odour, control samples vs 35 ppm ClO_2 treated samples of VP striploins, over storage at -1°C.

There was also no apparent detrimental effect of the PAA treatment on odour (Figure 5.4). Control samples received a high percentage of 'Off Odour' scores from 121 days which may affect the comparisons. Adjusting for panellist variations the Cochran-Mantel-Haenszel statistics showed there was an overall significant difference of odour score proportions between control and treatment samples over time ($P < 0.0001$). Therefore, individual time points were assessed. Time points of Day 0 ($P = 0.0016$), 121 ($P = 0.0004$), 153 ($P = 0.0009$) and 178 ($P = 0.0124$) were significantly different between control and PAA treatment proportions. However, the trend of increasing odour over time, as expected towards the end of shelf-life is significantly more dramatic for control samples rather than treated samples. This result infers no detrimental effect from PAA treatment, though panellist preference for odour notably varied.

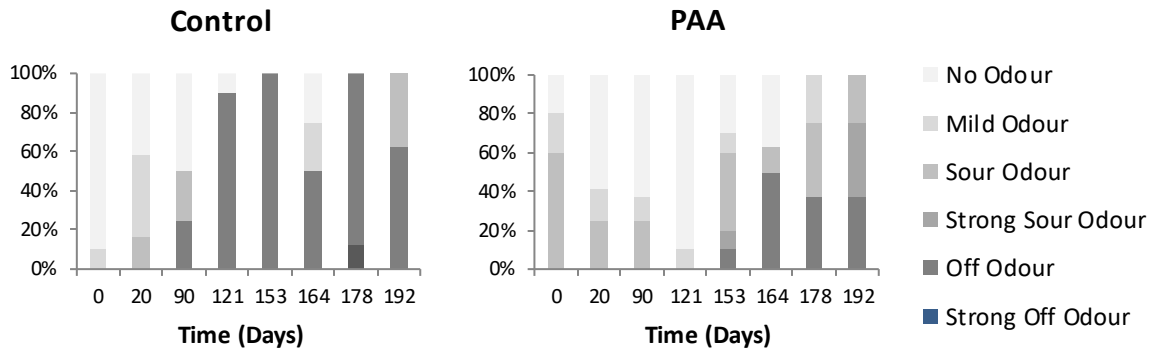


Figure 5.4. Sensory panel proportion scores (as percentage) on odour, control samples vs 200 ppm PAA treated samples of VP Striploin, over storage at -1°C.

5.3.1.3 Colour

The colour of VP striploins was scored on redness, greyness or other. There was no significant difference between control and ClO_2 treated meat pieces for colour ($P = 0.077$). All samples were scored red, apart from one time point (Day 27), where both control and treatment samples scored only 87.5% and 62.5% respectively. There was also no clear detrimental effect of the PAA treatment on colour. The only colour scored for 'Greyness' was the control samples at 178 days with 25% proportion of the score. Adjusting for panellist variations, the Cochran-Mantel-Haenszel statistics showed there was no overall significant difference of colour score proportions between control and PAA treatment samples over time ($P > 0.1904$).

5.3.2 pH dynamics on VP packed striploins

There was no significant difference ($P > 0.05$) between control and ClO_2 treated samples initially in terms of pH (Figure 5.5). As expected, the fat and lean sides had a different pH at the beginning of storage. Fat sides had a pH 6.12 ± 0.03 and lean sides pH 5.73 ± 0.05, for both the control and ClO_2 treated samples. However, ClO_2 treated samples, both fat and lean sides, had a noticeable increase in pH close to the end of shelf-life. Although there was higher pH in the treated samples there was also an increase in variation between samples and replicates, with significantly different results to the controls.

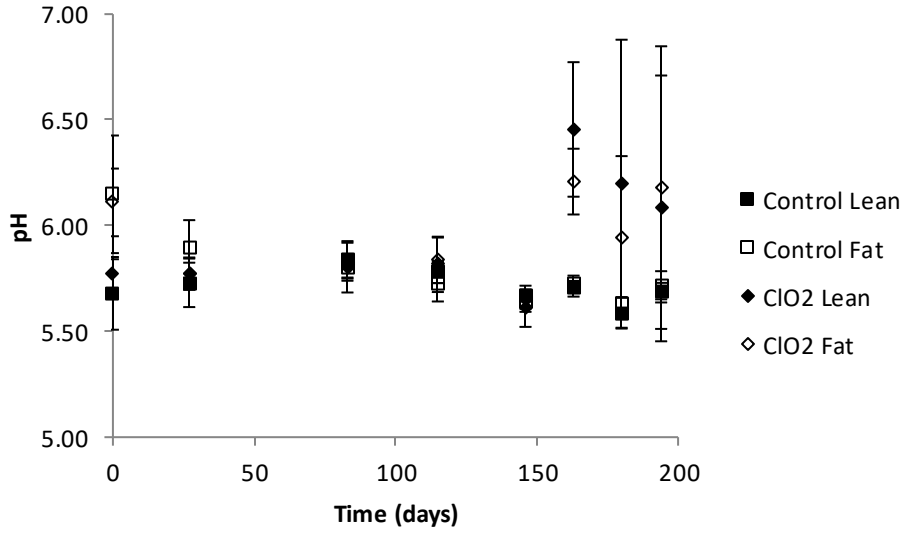


Figure 5.5. The pH of VP striploin halves stored at -1°C, treated with potable water or ClO_2 at 35 ppm.

PAA treatments produced the same trend as ClO_2 for initial pH (Figure 5.6). At day 0 the fat and lean sides were different, pH 5.95 - 6.17 compared to 5.6 respectively, for both control and PAA treated samples. However, over storage at -1°C there was no significant difference between control and treated sample ($P > 0.5$), for either lean or fat sides, remaining at pH 5.65

± 0.05 throughout shelf-life. The only effect on pH was incubation time (P values: fat < 0.0002, lean < 0.0001) overall.

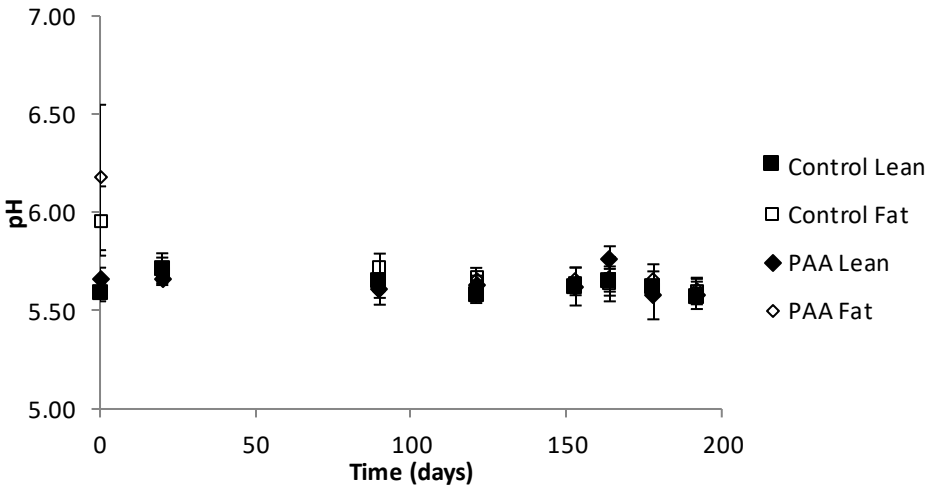


Figure 5.6. The pH of VP striploin halves stored at -1°C, after treatment with potable water or PAA at 200 ppm.

5.3.3 Bacterial viable population on VP striploin samples

5.3.3.1 ClO_2 treated bacterial results

The growth curves of TVC and LAB from treated and non-treated ClO_2 VP striploins are plotted in Figure 5.7, over their shelf-life at -1°C. Initial TVC counts are 3.9 ± 0.19 log CFU/cm^2 for the control samples and significantly lower ($P < 0.05$) for ClO_2 treated sample at 2.6 ± 0.11 log CFU/cm^2. The LAB counts had the same pattern with 1.9 ± 0.68 log CFU/cm^2 for control samples and 0.7 ± 0.30 log CFU/cm^2 for ClO_2 treated samples. LAB counts, as expected are a lower percentage of the initial TVC counts with 50% of the control counts on day 0 ($P < 0.05$), and 26.9% of the treated samples ($P < 0.05$). Over storage the TVC and LAB

counts for both control and treated samples become similar, with no significant differences ($P > 0.05$). LAB dominates the TVC until it becomes the major population at 83 days after the maximum population. The maximum population after 83 days reaches on average 6.7 ± 0.4 log CFU/cm^2 and remains around this level for the 194 days storage period. Despite the initial time point tested there was no significant difference between the control and treated samples after the maximum population is reach ($P > 0.05$).

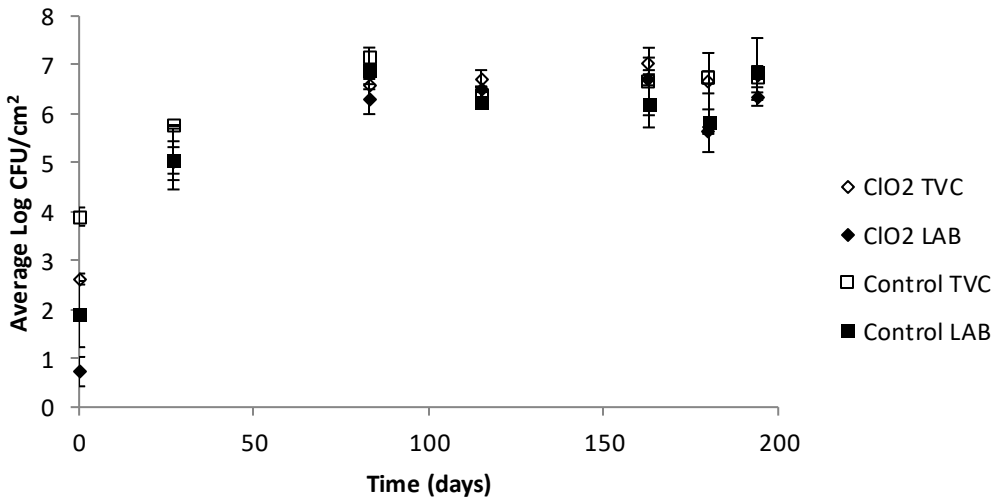


Figure 5.7. Total viable count (TVC) and lactic acid bacteria (LAB) of VP striploin halves stored at -1°C, over their expected shelf-life, after spray chilling with water or ClO_2 at 35 ppm. Samples treated under normal spray conditions (water) are displayed as TVC control (□) or LAB control (■), and samples treated with ClO_2 35 ppm are TVC ClO_2 (◇) or LAB ClO_2 (◆).

5.3.3.2 PAA treated bacterial results

The growth curves of TVC and LAB from treated and non-treated PAA VP striploins show a similar trend to ClO_2 (Figure 5.8). Initial TVC counts are 3.9 ± 0.19 log CFU/cm^2 for the control samples and significantly lower ($P < 0.05$) for PAA treated sample with 2.6 ± 0.11 log CFU/cm^2. The LAB counts reflect the same pattern with 2.7 ± 0.43 log CFU/cm^2 for control samples and 1.6 ± 0.05 log CFU/cm^2 for PAA treated samples. The initial TVC counts for PAA were the same as ClO_2 for both control and treated samples. However, LAB counts were 0.9 log CFU/cm^2 higher than the ClO_2 treatment LAB counts for both controls and treated samples. LAB counts, as expected, also represent a lower percentage of the TVC counts initially with > 60% of the control and treatment population ($P < 0.05$). Over the storage period the TVC and LAB counts for both control and treated samples also become similar, with no significant differences ($P > 0.05$). LAB dominates the TVC and it becomes the dominant population at 90 days. The maximum population after 90 days reaches on average 7.19 ± 0.57 log CFU/cm^2 and remains below this average count for the 194 days storage period. The maximum population numbers are on average 0.5 log lower than the previous trial (ClO_2 experiments) and resulted in a lot more variability in the LAB counts for both control and treated samples. Despite the initial time point tested and the LAB variability towards the end of storage, there was no significant difference ($P > 0.05$) between the control and treated samples after the maximum population is reached.

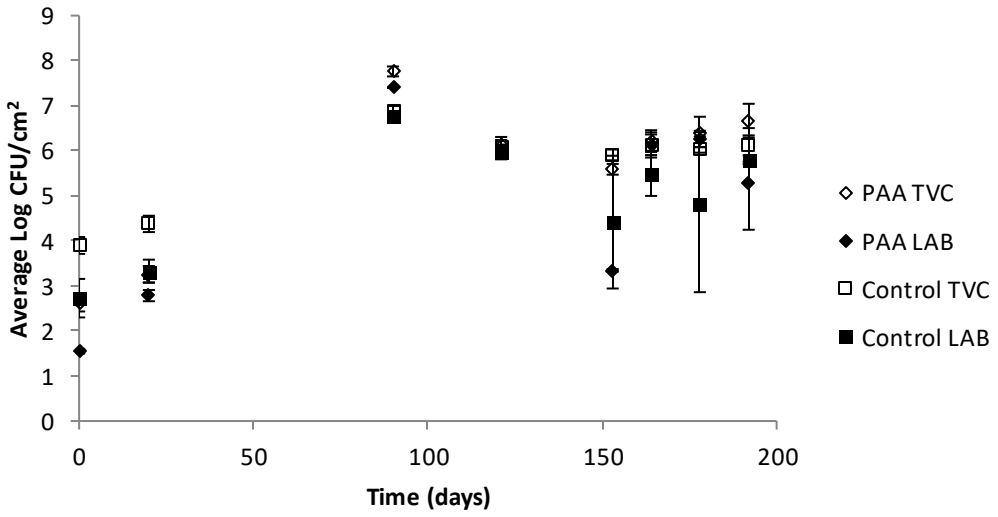


Figure 5.8. Total viable count (TVC) and lactic acid bacteria (LAB) of VP striploin halves stored at -1°C, over their expected shelf-life time, after spray chilling with potable water or PAA at 200 ppm. Samples treated under normal spray conditions (water) are displayed as TVC control (□) or LAB control (■), and samples treated with PAA 200 ppm are TVC ClO_2 (◇) or LAB ClO_2 (◆).

5.4. Discussion

5.4.1 Sensory analysis over the duration of shelf-life

The impact of sanitisers applied during spray-chilling on consumer perceptions of VP striploin quality were assessed based on meat appearance, particularly colour, and package integrity followed by odour on opening. Therefore, the sensory characteristics assessed in this study were directed at assessing preferences on appearance which would affect buying preference. This was followed by assessment of odour and colour after opening, assessments more useful for ascertaining the occurrence of spoilage and more overt loss of consumer acceptability.

The type of packaging influences consumer preferences and subsequently purchasing decisions. Reduced acceptance can be associated with darker meat colouration that can be enhanced in VP meat. In addition, the presence of excess purge liquid in the package can be discouraging to some customers. This is linked to package sealing issues causing muscle fibre compaction and liquid expulsion (McMillin 2017). The packaging used in this study did have some limitations due to the small-sized lesser quality cuts of half striploins that would not normally be acceptable for VP for export. Therefore, some poor packaging may have influenced the results. Also smaller cut sizes can affect bag vacuum integrity and thus may cause shorter shelf-life than what is expected for typical export grade striploins (Lagerstedt et al. 2011). Although, some individual pieces may have been affected the comparisons with control samples should account for this issue and did not appear to influence the overall results. In general, the treatments did not have substantial detrimental effects on visual and buying preference. However, there was a perceived trend of quality loss towards the end of storage for ClO_2 treated samples, possibly due to a darkening of the meat prior to opening along with occurrence of increased purge. The significantly low scoring preferences occurred only after 160 days storage, and although some studies report extended shelf-lives of over 190 days (Small et al. 2012), for the lesser quality meat used in this trial this difference does not imply or suggest detrimental impacts of ClO_2, even if the product might not be considered ideal for an especially discerning consumer market.

Over the storage of the VP meat (typically 190 days at -1°C) no poor odour was scored for either treatment, ClO_2 or PAA, in comparison to the control samples. However, there is some evidence that PAA treated samples had better odour scores in comparison to the control samples. Further studies would have to be instigated to see if this effect is reliable, with in depth detailed analysis of the bacterial community differences. It should also be mentioned that odour was the most subjective sensory characteristic in this study. The scoring panel was

untrained and initially gave negative scores for both control and treated samples even though the meat was fresh (Day 0). Over time with regular comparisons the scoring became more realistic to the meat odour characteristics, with an increase in negative scoring in tandem with increasing storage duration. A persistent off-odour score was given from 160 days in contrast to other studies where persistent off odours were detected after 80-110 days (Bell et al. 1994) and after 190 days of storage (Small et al. 2012). The meat used was not of export quality and this may have influenced the shelf-life due to packing being possibly not optimal.

5.4.2 Physicochemical characteristics

Anaerobic environments, such as VP can greatly increase shelf-lives, though have been linked to some sensory effects, *e.g.*, due to the lack of oxygen the meat can appear darker (Polkinghorne et al. 2018). However, colour in the end was not an issue for sensory results in the current study overall. Colour rating was consistently red except for the occasional samples with no overall trend present. Over time there was no greyness or browning scored. Poor packaging can lead to increased browning colour (Polkinghorne et al. 2018), though that was not seen in this study even though some packaging issues were seen, such as loss of vacuum and increased purge in some samples. On opening VP meat samples routinely bloomed bright red with the interaction of oxygen binding to the myoglobin (Cruz-Romero et al. 2017). Although this study shows no major adverse effect of the treatments, it is a preliminary trial requiring a more rigorous study with a trained panel or larger untrained panel. It would also be recommended to use a more detailed colour scale scoring system such as the AUS-MEAT colour references or colorimeter (AUS-MEAT 2019; Trinderup et al. 2013).

The fat and lean sides of meat have very different compositions and are known to differ in pH. The adipose tissue surface is generally reported as having a higher pH (pH 6 ± 0.7) than lean surfaces *i.e.*, 5.6 ± 0.2 (McGilchrist et al. 2019; Reid et al. 2017). Over storage under VP conditions the environment equalises and the pH becomes less variable due to liquid mixing over the whole surface (Reid et al. 2017). This was observed in the current trials where after only 20 days storage the pH was not significantly different for control and treatment samples. However, for the ClO_2 treated samples the pH differed after 160 days storage, this timing also matched the buying preference of panellists and therefore may have led to a visual indication in loss of shelf-life. High correlations have been reported between the initial values of pH, fat and the rate of spoilage (Blixt et al. 2002). Increased meat pH has been associated with more rapid spoilage, and linked to the growth of specific spoilage organisms such as *Serratia liquefaciens* and *Shewanella putrefaciens* (Vanderzant et al. 1983).

5.4.3 Bacterial population on VP striploin samples

The spray chilling treatments, using ClO_2 and PAA, had no significant differences compared to the control over the entire shelf-life of VP striploins for cultivable bacterial populations. Bacterial counts reached 6-7 log CFU/cm^2 maximum population after 80 days of storage, as reported by other studies (Frank et al. 2019). The combined effects of meat pH, temperature, and CO_2 concentration are believed to create conditions where microbial growth is limited and is unable to reach counts above 7 log CFU/cm^2 (Small et al. 2012). Over storage LAB becomes the dominant population of bacteria and helps preserve spoilage populations and extend shelf-life (Signorini et al. 2006). Many studies show this change in population with species diversity decreasing to a stable community well before the end of shelf-life (Kaur et al. 2017b; Sade et al. 2017).

Meat microbial communities are widely reported as being dynamic and depending on the hygiene of the processing environment, can considerably vary including the numbers of spoilage species present (Sade et al. 2017). Antimicrobial treatments have the potential to alter the microbiome composition in either a beneficial or negative way (Ferrocino et al. 2016). This outcome is determined by the susceptibility of the bacteria to the particular treatment and the composition of the initial bacterial population (Botta et al. 2018; Ferrocino et al. 2016). Studies with PAA have shown positive results with significantly less *Enterobacteriaceae* present compared to untreated meat and instead dominated by *Carnobacterium* spp. (Brightwell et al. 2009).

Decontamination of carcases may result in meat carrying different species both in terms of numbers and community compositions (Youssef et al. 2014). Therefore, identifying species would help understand the effect of the new treatments. Initial spoilage bacterial load can explain the subsequent increase in microbial counts and its relationship with the deterioration of shelf-life of the products observed. Additionally, it suggests that differences in the processes, storage and transportation conditions can affect shelf-life.

5.5. Conclusion and future work

Personal preference can be very subjective in sensory analysis with large variation between panellists. Large scale trials with expert panels to eliminate some of the subjective sensory preferences and characteristics would help clarify some variable results between panellists. However, further studies into the change in microbial communities during shelf-life of treated and non-treated samples would also give a better understanding of the effect on the natural microbiota. Comparing the microbial community with treated samples would identify changes in the population of potentially beneficial bacteria or conversely the increase in spoilage-related species. The preliminary trials in this study show promise with oxidant treatments and allows for further studies to extend spray treatment during chilling, with preference towards PAA treatments.

Chapter 6

Thesis discussion and summary

6.1. Overview

The Australian red meat industry is worth $18 billion in gross domestic product (RMAC 2017). It employs thousands of people and affects nearly everybody in Australia. Australians eat, on average, 26 kg red meat per person per year. The consumption is even higher in the United States (Greenwood et al. 2018). The USA receives approximately 225,000 tonnes of red meat every year from Australia (Whitnall 2019). Australia is one of the largest export countries of beef with 69% of production for the export market (MLA 2018). Even though Australia only produces 7% of global production of beef it is one of the top three largest exporters and regarded as producing the highest quality products (MLA 2018). The Australian meat industry has built a long-standing export commitment by meeting all relevant global importation requirements. To achieve this economically, Australia's products must be of high quality and meet their regulations, including those intended for the United States which implements a "zero tolerance" policy for seven strains of potentially pathogenic *E. coli*.

To maintain Australia's export industry, research is constantly funded to improve and prevent any loss to this trade. Industry-funded research has been extensive over recent decades in many countries to deliver the meat quality and safety and reputation the Australian meat industry has today. Hence this thesis had, as its overall purpose, to help the Australian meat industry resolve export problems. In doing so, this contributes to a greater knowledge about industry applications, and innovations, toward minimising pathogen risk for public health globally.

6.2. What it means for current research and research in the future

Research into meat processing has been intensive over the years. Studies range from on farm practices to packing technology, as reviewed in Chapter 1. The decontamination practices include physical, chemical and sanitation processes. Many studies have led to in-house practices and are responsible for the high-quality products produced today, and ultimately the reputation the multi-billion-dollar industry is built on. However, most interventions or control measures are broad decontamination techniques, either minimising visual contamination or with the general aim to decrease microbial populations, and hence pathogens, in the process. This series of studies is novel and differs from past research, done in various countries, *e.g.*, South and North Americas and European countries, in that it targets specific enteric pathogens, including *E. coli* O157:H7, responsible for many large outbreaks and public health concerns around the world. The current research draws on physiological understanding of the pathogens stress response under forced air abattoir chilling conditions, and therefore is a targeted antimicrobial approach. Future research should take advantage of modern technologies to reveal a deeper understanding of pathogen physiology. Pathogenic bacteria have important health implications when ingested with food and investigations into their physiological sensitivities would be a beneficial way to exploit their weaknesses, to produce new tailored antimicrobial interventions.

6.3. Thesis purpose

The primary objective of this research was to enhance the microbiological safety of red meat by improved technologies and innovations in red meat processing, and in an "industry acceptable" practice. Drawing on previous knowledge, in which the physiological responses of *E. coli* to cold, reduced water activity, and combinations of these stresses led to the hypothesis tested in this thesis.

In particular, this research follows on from Kocharunchitt (2012), where the physiological response of *E. coli* O157:H7 Sakai, under conditions relevant to carcase chilling in cold air, was extensively studied. The effect of cold stress, osmotic stress, and their combination on *E. coli* O157:H7 Sakai cells metabolic pathways, in particular gene expression and protein expression, was detailed. Of particular interest to this set of work was the downshift in temperatures from 37°C (i.e., mammalian body temperature), to chilling and the corresponding water activity change (14°C, a_w 0.967). Kocharunchitt (2012) found that cold temperature was responsible for the oxidative stress shown by *E. coli* and that it was a consequence of the cold adaptation response. As the oxidative sensitivity was induced by the cold adaption alone the use of an oxidant during either conventional or spray chilling was hypothesised to be potentially useful as an antimicrobial intervention. However, conventional chilling would require modification to abattoir processes and/or infrastructure. Ultimately this research shows that existing chilling conditions could potentially be adapted to use an oxidant treatment as an enhanced antimicrobial intervention.

6.4. Testing the hypothesis

The initial part of the research was to determine if *E. coli* cells were in fact more sensitive to an oxidant at earlier stages during carcases chilling conditions (Chapter 2). Previous studies had detailed a window of susceptibility to oxidative stress through gene and protein expression (Kocharunchitt 2012). *E. coli* O157:H7 Sakai cultures decreased expression of stress response proteins responsible for oxidative stress only earlier in the recovery stages after the shock of chilling conditions. These in-vitro studies allowed the effects on *E. coli* O157:H7 Sakai to be observed under controlled conditions without other interfering factors, such as a complex matrix or additional organic compounds. The experiments, detailed in Chapter 2, allowed description of the detailed response to an oxidant being added to *E. coli* at different times after chilling. The results showed *E. coli* was, in fact, more sensitive to the oxidant at earlier stages during chilling. Timing was found to be critical for maximum efficiency of inactivation and this works on a variety of species, strains, and serotypes, in particular targeting *E. coli* O157:H7 Sakai.

Different oxidants also caused different growth responses of the *E. coli* cultures. Hydrogen peroxide caused a milder response only affecting growth with the earliest addition, giving evidence of critical timing for maximum efficacy. Chlorine dioxide, in contrast, was more effective at inactivating *E. coli* at a wider range of addition times. It did, however, confirm the earlier addition is most effective, as shown by the rate at which inactivation occurred. The earlier additions caused considerably faster rates with the rates slowing over time at each further addition. These results gave promise to further the work in which the efficacy of an oxidant was tested on the complex meat environment/matrix.

6.5. Confirmation of efficacy

In-vitro studies are a cost-effective and a relatively easy first step in testing of the hypothesis of *E. coli* having an oxidant susceptibility "window": conditions can be controlled and allow for the basic concept to be resolved. However, there are many factors that affect the outcomes of a potential antimicrobial process. Hence, in Chapter 3 meat studies were undertaken to test the feasibility of an oxidant on the complex meat surface, where structure of the meat matrix, blood and other factors could interfere with the efficacy of the oxidant. Meat is also complex in structural composition, its tissues varies with fat, sinew, and lean muscle. The current studies highlight this difference with efficacy being less on the lean meat surfaces than adipose surfaces. Fortunately, from the perspective of microbial food safety most of a carcase constitutes adipose and connective tissues, which were more effectively sanitised in the laboratory-based trials. The meat studies demonstrated that the application of an oxidant, ClO_2 or PAA, was very effective in eliminating *E. coli* and *Salmonella* on beef meat. Such effects were more pronounced when applied during spray chilling than when applied before chilling. Therefore, even in the complex matrix of meat, timing was confirmed to have a critical role for maximum efficiency of disinfections. Also, these trials showed no adverse effects to the meat tissue, which would render the oxidant not useful, as sensory attributes are vital in the marketing of beef products. This highlights the potential of an oxidant-based application during spray chilling to be developed and evaluated further as an antimicrobial intervention for beef carcases in commercial settings, to minimise the risks of enteric pathogens associated with beef meat.

6.6. Industry compatibility

The application of an oxidant to an already existing spray chilling system was an easy and effective way to add an antimicrobial intervention in an existing industry setting (Chapter 4). More considerations would be required to introduce the step into conventional (forced air) chilling, such as hand spraying or implementing a spraying system. Both oxidants, ClO_2 and PAA, reduced pathogenic *E. coli* significantly, though PAA was repeatedly seen to be more effective. Some optimising of the spray system would improve the results as the oxidant had less effect on the lower regions of the carcase. This was most likely due to the lack of contact with the oxidant in those areas, such as seen in the neck and foreleg region.

Overall, PAA introduces less safety issues and is easier to apply. PAA was also found to be more economical and easier to use, without requiring an activation step. Therefore, PAA is a more attractive option for industry with little to no structural changes required in the plant. With further optimising of the application process around 3 log reductions seem a feasible outcome for industry and warrants further large-scale investigation.

Although no adverse effects on the quality of the meat were observed, further studies were undertaken to assess whether any negative effects on quality and shelf-life were observed over storage. It would be detrimental to profit/value if the existing long shelf-life was reduced or if the packaged product had less quality as a result of the oxidant treatment.

6.7. Quality and shelf-life

Finally, the effect of adding an oxidant during carcase spray chilling on shelf-life and quality was assessed in Chapter 5. The research was to ensure no adverse effects on the final product would occur, by either shortening the storage time or by visual and odour evaluation. The shelf-life of the products was not found to be affected, though some minor discolouration was noted with the use of chlorine dioxide, but it did not affect the sensory scoring. Ultimately there was no change seen in this series of studies on the quality of the meat after oxidant treatments were applied. PAA had no significant difference from the control over the shelf-life. However, there were individual differences between pieces of meat from both control and treated groups. The difference is attributed to the integrity of the packaging and the tightness of the VP seals, and most likely is responsible for the varied result in LAB counts. Although ClO_2 did seem to affect pH and consumer preference, possibly due to a change in population dynamics, because samples treated with ClO_2 differed from the control after extended storage (180 d). There were no conclusive results to suggest that either oxidant causes a loss in quality or shelf-life. Further studies into the change in microbiological populations, in particular between the treatments over time, would help us to better understand if any detrimental changes are occurring which may affect the stability of the product towards the end of shelf-life. Along with investigations into microbial community changes further quality analyses should be undertaken. Although the current study assessed visual and odour qualities, other quality parameters should be assessed. The "eating qualities" such as taste, juiciness and tenderness were not investigated in this thesis. The current sensory testing was small and employed an untrained panel. Therefore, a more extensive sensory experiment should be conducted as sensory assessment is highly subjective and influenced by consumer perceptions.

6.8. Concluding remarks

In conclusion, this research has contributed to a greater knowledge of *E. coli* physiological weakness during carcase chilling and how this sensitivity to oxidants can be practically exploited. Such knowledge can now be taken advantage of commercially with minimal change to the current processes of Australian meat export plants. The implementation of the practices in this current research will lead to safer and more economical meat products. From this research, PAA appears to be the "best fit" as an oxidant for the Australian meat processing industry, though other oxidants such as ClO_2 should also be effective. Direct outcome for the industry is a cheap, effective, and safe additive as an antimicrobial intervention and without the need for regulatory approval in many countries as it is an existing food grade wash (*e.g.*, it is approved in the poultry industry). These new applications can be used in conjunction with other antimicrobial interventions along the processing chain to greatly enhance the overall safety of the end products. If PAA, or its equivalent, is added to the usual series of interventions normally used in abattoirs, the likelihood of pathogenic *E. coli* still contaminating meat for consumption would be considerably lower.

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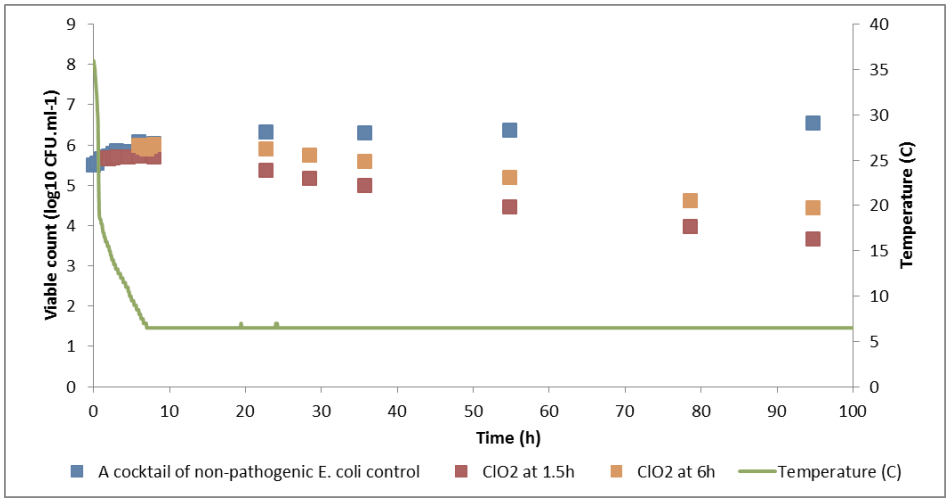
Appendix

A1. Referenced section 2.2.6

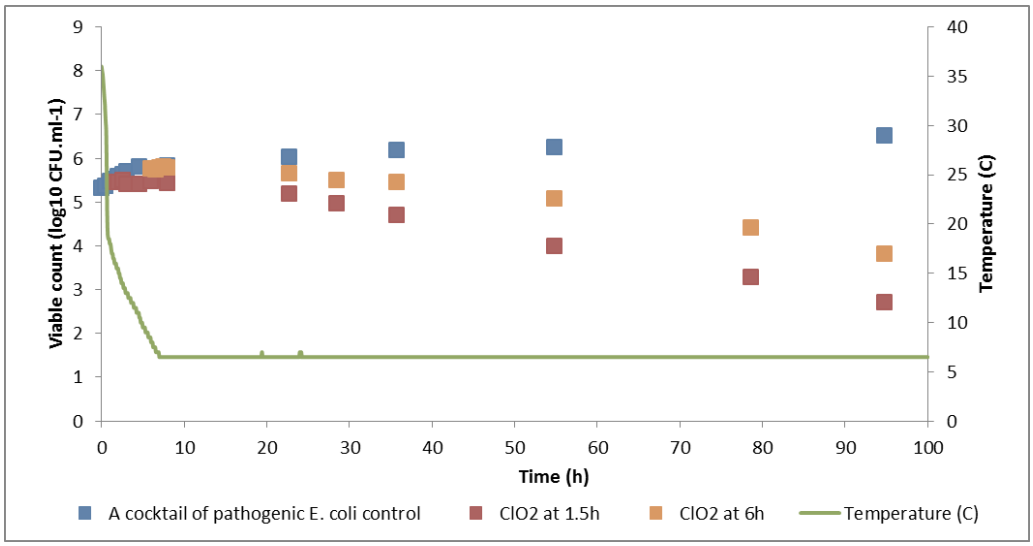
Preliminary studies to determine levels of ClO_2 which had no detrimental effects on *E. coli* O157:H7 Sakai under optimal growth conditions, at relevant physiological states (A = exponential growth phase, B = stationary phase cells prior to lawn spreads on BHI agar) incubated at 37°C

ClO_2	Inhibition zones			
PPM	A1	A2	B1	B2
1000	++	++	++	++
500	++	++	++	++
250	+-	+-	+-	+-
200	+-	+-	+-	+-
150	+-	+-	+-	+-
100	+-	+-	--	--
75	--	--	--	--
50	--	--	--	--
25	--	--	--	--
10	--	--	--	--
1	--	--	--	--

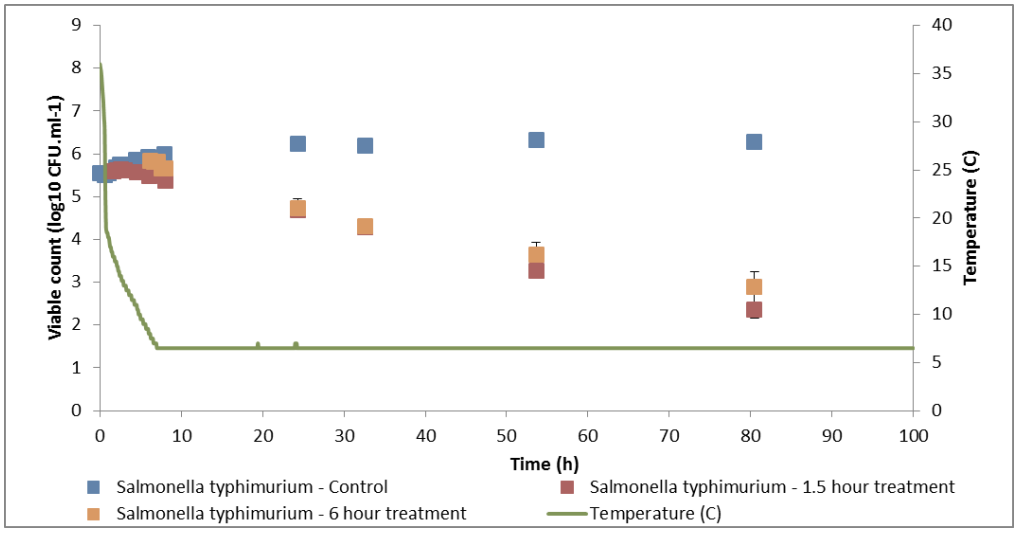
A2. Reference section 2.3.3



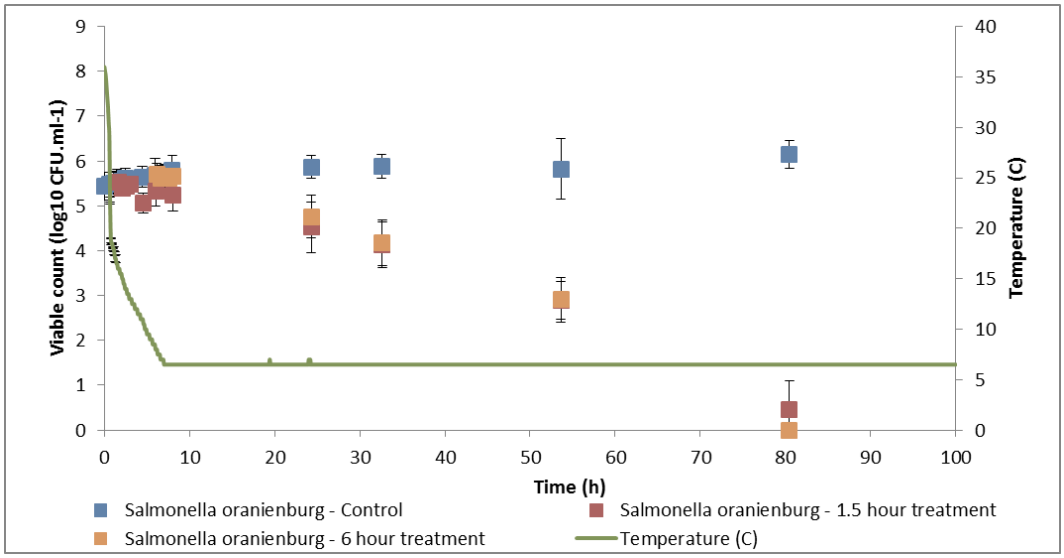
Population changes of stationary-phase cultures of a five-strain cocktail of non-pathogenic *E. coli* during exposure to dynamic changes in temperature as occur during spray chilling and when 150 ppm ClO_2 was applied at different time points.



Population changes of stationary-phase cultures of a five-strain cocktail of pathogenic *E. coli* during exposure to dynamic changes in temperature as occur during spray chilling and when subjected to 150 ppm ClO_2 was applied at different time points.



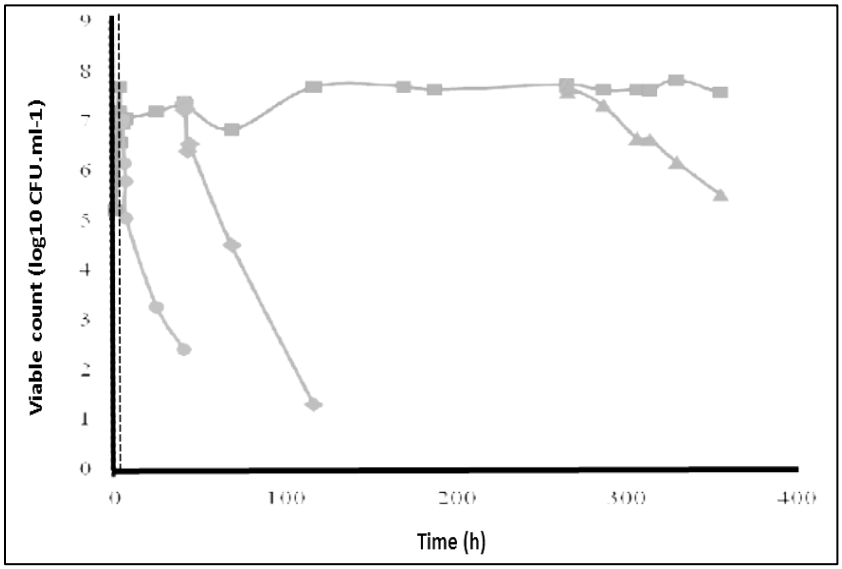
Population changes of stationary-phase cultures of *S.* Typhimurium during exposure to dynamic changes in temperature as occur during spray chilling of carcases and when 150 ppm ClO_2 was applied at different time points.



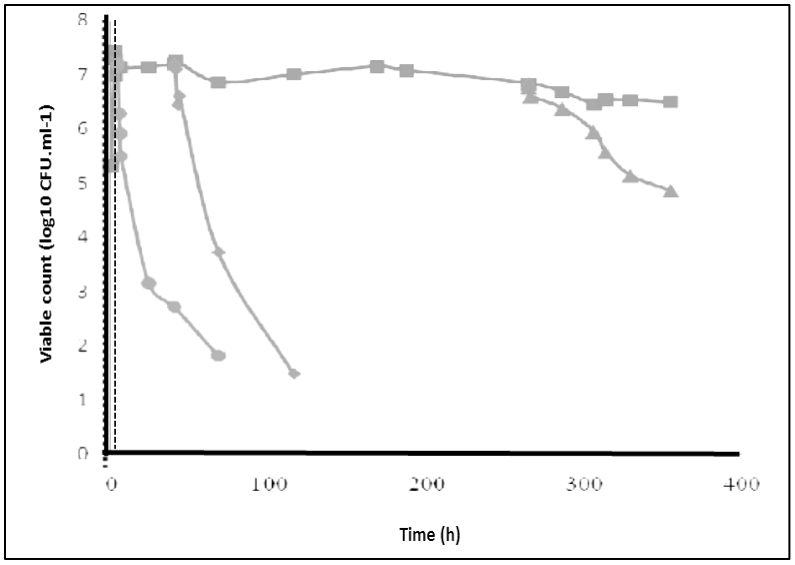
Population changes of stationary-phase cultures of *S.* Oranienburg during exposure to dynamic changes in temperature as occur during spray chilling of carcases and when 150 ppm ClO_2 was applied at different time points.

A3. Reference section 2.4.2

Salmonella strains showed a similar complex pattern of population kinetics when subjected to abrupt downshifts in temperature and water activity.



Population changes of S. Typhimurium after simultaneous downshifts in temperature and water activity from 35°C a_w 0.993 to 10°C a_w 0.967 and when subjected to ClO_2 treatment. The time at which simultaneous downshifts in temperature and a_w were applied is indicated by a dotted line. 200 ppm ClO_2 was added at 1.5 h (●), 37.5 h (◆), and 262 h (▲) after imposition of the shifts.



Population changes of *S.* Oranienburg after simultaneous downshifts in temperature and water activity from 35°C a_w 0.993 to 10°C a_w 0.967 and when subjected to ClO_2 treatment. The time at which simultaneous downshifts in temperature and a_w were applied is indicated by a dotted line. 200 ppm ClO_2 was added at 1.5 h (●), 37.5 h (◆), and 262 h (▲) after the shifts.