School of Public Health

Bacterial Community Diversity as a Predictor of Beef Safety

Seong-San Kang

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Seong-san Kang

ABSTRACT

Beef is an important agricultural product for Australia with exporting of product playing an important role for the industry. The industry must produce beef products that are safe from pathogenic bacteria such as Shiga toxin-producing *Escherichia coli* (STEC) while preserving quality in order to maintain their trades in overseas markets. Understanding microbial contamination through production of beef in previous studies have provided a fundamental concept of contamination, however this has been limited to a basic level of counting cells by using conventional cultivation methods. There is currently a lack of in-depth analysis of microbial contamination through processing phases of beef (slaughter and boning). The main objective of this study was to use a genetic-based technique, 16S rRNA amplicon sequencing, to expand the understanding of microbial contamination and search for ways to use the genetic information to predict the presence of STEC through Australian beef processing.

Profiles of bacterial 16S rRNA gene were analyzed at an abattoir that received cattle from sources using similar production system (A: integrated) and at an abattoir that processed cattle from multiple different sources (B: fragmented). Despite the differences in the supply chain, both abattoirs shared similar bacterial profiles. Analysis of 16S rRNA sequences demonstrated that different practices can have varying effect on contamination of carcases during slaughter. For example, transmission of contaminants from hide to carcase was more prominent in abattoir B which used an upward hide pulling system. As a result, microflora on carcase after slaughter was heavily influenced by the hide bacterial communities in abattoir B. On the other hand, abattoir A used a downward hide pulling system and hide to carcase contamination was minimized. Sequencing analysis showed that cross-contamination between environmental surfaces and carcases contributed more to the final microflora on carcases in abattoir A.

Next stage in the beef processing is the boning phase where the slaughtered carcases are manufactured into smaller beef trim and products. Interestingly, the 16S rRNA analysis showed that chilling of post-slaughter carcases overnight prior to boning did not alter the bacterial communities on the carcases. There were substantial differences in compositional changes within the beef microflora between the abattoirs throughout the boning room. Enterobacteriaceae dominated the community in beef trim from abattoir B whereas Clostridiales, Lactobacillales and Pseudomonadales were predominant in abattoir A. More

importantly, the results demonstrated that contamination of beef trim was a result of accumulation of contaminants from the environment (e.g. conveyor belts) and that the boning phase largely transferred the bacterial populations from post-slaughter onto the final products. This indicated that processors at slaughter phase should focus to reduce and control carcase contamination and the boning phase should emphasize maintaining good hygiene levels throughout the process.

Sequencing has enabled more descriptive analysis of contamination through slaughter and boning of beef. The next step was to evaluate if these ecological changes in bacterial community structure were associated with presence of Top 7 STEC (O26, O45, O103, O111, O121, O145 and O157). This study showed that the changes in bacterial diversity was not correlated with STEC regardless of sample types (e.g. carcases or beef trim) and could not be used as a bioindicator to predict the presence of STEC through beef processing. In addition, there were no operational taxonomic units (OTUs) that differentiated the bacterial community composition when the samples were clustered by STEC status and appeared to be randomly assorted. The processing phase may not be the best stage in the supply chain to investigate STEC and bacterial communities, especially in Australia, due to lower prevalence of STEC in comparison to other countries and the fact that the processing environment is under constant exposure to change which can be a hostile condition for stable communities to develop.

Analysis of changes in 16S rRNA during processing was not helpful to anticipate STEC prevalence. However, using this tool to study the development of bacterial communities through enrichment (as part of the current STEC detection system) provided useful insights to optimize the detection process. The results showed that enrichment had varying effects on the bacterial populations in four different enrichment media used in this study (buffered peptone water, MP, mEHEC and PDX-STEC) and that MP may be less effective in comparison to the other media. In general, *Clostridium* and *Escherichia* dominated the community after enrichment by taking up between 59 – 99% of the composition combined. The study demonstrated that it is important to maximise *Escherichia* populations (including STEC) by inhibiting competitive background bacteria such as *Clostridium* to increase the chance of detecting STEC in enrichment broths.

The thesis demonstrated that sequencing technology provided a new set of tools to understand microbiology and contamination in the Australian beef industry. It enabled to describe how the bacterial communities move and changes, allowing much deeper

understanding of contamination. The findings in this project is important for the beef industry to enhance microbiological quality and safety by providing microbiological changes that are specific to the environment within individual abattoirs. Meaning that the processors can now control microbial contamination using strategies that are most efficient for their abattoirs. The 16S rRNA analysis of enrichment proved useful in identifying possible approaches to optimize detection of STEC by understanding the changes in bacterial communities through the enrichment process. Increasing efficiency in the detection system can potentially save the processors substantial time and cost invested during confirmation of Top 7 STEC in their products.

Recommendations for future studies include investigating more abattoirs in different geographical locations of Australia and explore a diverse a range of contamination sources. It would be interesting to test if some of the species from the beef microflora in a community structure has an influence on STEC and can potentially be used to control the presence. Future enrichment studies should aim to inhibit competitive background bacterial groups (e.g. *Clostridium*) and evaluate the enrichment process.

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STATEMENT OF ATTRIBUTION

The following tables describe the contribution made by each author towards the research outputs from this thesis and the co-authors were listed in no particular order.

Paper 1: located in Chapter 2

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	Conception and Design	Acquisition of Data and Method	Data Conditioning and Manipulation	Analysis and Statistical Method	Interpretation and Discussion	Reviews and Feedback	Final Approval
Seong-san Kang	70%	50%	100%	90%	75%	0%	60%
Co-Author 1 Acknowledgement: I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate Signed: September 2020							
Gary Dykes	0%	0%	0%	0%	5%	30%	10%
Co-Author 2 Acknowledgement: I acknowledge that this index represents my contribution to the above research output Signed: September 2020							
Ranil Coorey	0%	0%	0%	0%	5%	10%	10%
Co-Author 3 Acknowledgement: I acknowledge that this index represents my contribution to the above research output Signed: September 2020							
Joshua Ravensdale	0%	0%	0%	0%	5%	10%	10%
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Robert Barlow (CSIRO)	30%	50%	0%	10%	10%	50%	10%
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Seong-san Kang	60%	80%	100%	90%	75%	0%	60%
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Gary Dykes	5%	0%	0%	0%	5%	30%	10%
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Ranil Coorey	5%	0%	0%	0%	5%	10%	10%
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PUBLICATIONS

Kang, S., Ravensdale, J., Coorey, R., Dykes, G.A and Barlow, R. 2019. A Comparison of 16S rRNA Profiles Through Slaughter in Australian Export Beef Abattoirs. *Frontiers in Microbiology* 10: 2747. https://doi.org/10.3389/fmicb.2019.02747.

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CONFERENCE PRESENTATIONS

Poster presentations

Kang, S., Ravensdale, J., Coorey, R., Dykes, G.A and Barlow, R. 2018. Variability of top 7 Shiga toxin-producing *Escherichia coli* (STEC) and microbial populations through slaughter in Australian beef export abattoirs. Australian Society of Microbiology 2018 Annual Scientific Meeting, Brisbane, Australia.

Kang, S., Ravensdale, J., Coorey, R., Dykes, G.A and Barlow, R. 2019. Prevalence of top 7 Shiga toxin-producing *Escherichia coli* and microbial populations in Australian beef export abattoirs. Australian Institute of Food Science & Technology Convention 2019, Sydney, Australia.

Oral presentations

Kang, S., Ravensdale, J., Coorey, R., Dykes, G.A and Barlow, R. 2017. Bacterial community diversity and composition as a predictor of beef safety and quality. Australian Meat Processor Corporation 2017 Annual Forum, Perth, Australia.

Kang, S., Ravensdale, J., Coorey, R., Dykes, G.A and Barlow, R. 2018. Prevalence of Top 7 Shiga toxin-producing *Escherichia coli* through slaughter in Australian beef abattoirs. Australian Meat Processor Corporation 2018 Annual Forum, Melbourne, Australia.

Kang, S., Ravensdale, J., Coorey, R., Dykes, G.A and Barlow, R. 2019. Metagenomics profile and presence of STEC throughout beef processing in Australia. Australian Meat Processor Corporation 2019 Annual Forum, Brisbane, Australia.

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ABBREVIATIONS

AHL	N-acylhomoserine lactone
ANOSIM	Analysis of similarity
AUD	Australian dollars
bp	Base pair
BPW	Buffered peptone water
C. perfringens	*Clostridium perfringens*
°C	Celsius degrees
CFU	Colonies forming unit
cm	Centimetres
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DHP	Downward hide pulling
DNA	Deoxyribonucleic acid
eae	Intimin gene
E. coli	*Escherichia coli*
e.g.	Example
ehxA	Enterohaemolysin gene
Fig.	Figure
FSIS	Food Safety Inspection Services
g	Grams
GIT	Gastrointestinal tract
GMPs	Good manufacturing practices
h	Hour
H7	H7 flagella antigen
HC	Haemorrhagic colitis
HUS	Haemolytic uremic syndrome
i.e.	id est
IMS	Immunomagnetic separation
kg	Kilograms
L	Litres

log_{10}	Logarithm, to the base of 10
MAP	Modified atmosphere packaging
mg	Milligrams
mL	Millilitres
MLG	Microbiology Laboratory Guidebook
mRBA	Modified rainbow agar
N	Normality
n	Sample size
NaOH	Sodium hydroxide
NGS	Next generation sequencing
NNDSS	National Notifiable Diseases Surveillance System
nM	Nanomolar
nMDS	Non-metric multidimensional scaling
O26	O-antigen belonging to O26
O45	O-antigen belonging to O45
O103	O-antigen belonging to O103
O111	O-antigen belonging to O111
O121	O-antigen belonging to O121
O145	O-antigen belonging to O145
O157	O-antigen belonging to O157
OTU	Operational taxonomic units
PCR	Polymerase chain reaction
pM	Picomolar
PP	Potential positive
R-value	Correlation coefficient value
ROPES	Manilla rope pen testing device
rcf	Relative centrifugal force
rRNA	Ribosomal ribonucleic acid
SI	Simpson's index
spp.	Species
STEC	Shiga toxin-producing *Escherichia coli*
stx	Shiga toxin gene

TSA	Tryptic soy agar
TSC	Typtose Sulphite Cycloserine agar
TVC	Total viable count
UHP	Upward hide pulling
UK	United Kingdom
µL	Microlitres
US	United States
USD	United States dollars
USDA	United States Department of Agriculture
v/v	Volume over volume
w/v	Weight over volume

Chapter 1. Literature Review

1.1 Introduction

The Australian beef industry is one of the world's largest beef exporters and relies heavily on export as approximately 72% of beef and veal production is exported to more than 70 countries (Meat & Livestock Australia., 2019). The industry must comply with food safety regulations that are in place by the importing countries in order to gain and maintain market access. For instance, the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) in 1994 officially declared O157:H7 Shiga toxin-producing *Escherichia coli* (STEC) as an adulterant in raw non-intact beef following a major foodborne outbreak in multiple states of the US associated with undercooked ground beef containing O157 in 1993 (Taylor, 1994). An adulterant can compromise the safety of consumers leading to serious life-threatening infection and the need for rapid sensitive detection methods became apparent (Baker et al., 2016). More outbreaks caused by several non-O157 pathogenic *E. coli* were reported as diagnostic methods and awareness for other serovars improved (Brooks et al., 2005). Subsequently, six non-O157 STEC serogroups (O26, O45, O103, O111, O121 and O145) were added to the list of adulterants in 2012 (Dewsbury et al., 2015, Magwedere et al., 2013, Mellor et al., 2016). O157 and six non-O157 serogroups are commonly referred to as the 'Top 7' STEC in food safety (Browne et al., 2018, Fratamico et al., 2017).

Microbiological profiles at different stages of the supply chain of beef cattle have been investigated over the last few decades to understand the transmission of STEC through processing of beef. Previous studies examined the presence of STEC in faeces, hides, carcases and manufacturing beef using conventional cultivation techniques (Barlow et al., 2006, Bosilevac et al., 2005, Fegan et al., 2009, Nou et al., 2003, Pointon et al., 2012, Stromberg et al., 2015a, Sumner et al., 2003). From these studies, it was hypothesized that the microbial contamination of beef carcases primarily occurs by transmission of contaminants from hides with faecal contamination to the carcases during removal of the hides (Elder et al., 2000, Kennedy et al., 2014, McEvoy et al., 2000, Svoboda et al., 2013). Subsequently, the carcases become a vehicle carrying contaminants and the quality of the final products decreases (Arthur et al., 2010, Fegan et al., 2005, Koohmaraie et al., 2005). The findings from the previous studies have assisted in the implementation of regulatory

practices and anti-microbial interventions employed throughout beef processing to minimise STEC contamination of carcases (Vanderlinde et al., 2005).

Knowledge of microbial contamination during processing of beef cattle has been restricted to a basic level of understanding by inherent limitations of conventional cultivation methodology. These methods are limited to the detection of microbes that can be grown under laboratory conditions. Culturable bacteria only represents a small proportion of the diverse consortium of microbes found on beef and in the environment (Cao et al., 2017, De Filippis et al., 2013). In recent years, advancement of high throughput sequencing techniques has enabled greater depth and resolution for characterising microbial communities without the biased results of cultivation-based methods (Singer et al., 2016, Yang et al., 2016). There has been a number of studies that analysed the changes in bacterial 16S ribosomal ribonucleic acid (rRNA) profiles in relation to the presence of STEC and suggested that there is an association between higher bacterial diversity and lower prevalence of STEC in pre-harvest hides and faeces (Chopyk et al., 2016, Zhao et al., 2013). The previous studies investigated changes in microbial ecology in the presence of STEC prior to slaughter and there is currently a lack of sequencing data analysis of contamination in relation to STEC presence during slaughter and boning of beef cattle.

This review covers the importance of Top 7 STEC in the Australian beef industry and the current understanding of contamination. This chapter will also discuss the application of sequencing technology to facilitate more in-depth investigation of how ecological changes can influence STEC and other contaminants through slaughter and boning of beef. While there are multiple sequencing platforms and protocols for microbiome analysis, this review focused on studies that have implemented 16S rRNA amplicon sequencing. The use of bacterial 16S rRNA sequencing technology as a tool to examine beef safety by analysing changes in bacterial communities in relation to the presence of Top 7 STEC is also discussed.

1.2 Emergence of STEC

First described in 1885 by Theodor Escherich, *E. coli* is a Gram-negative, rod-shaped facultative anaerobic bacterium that is a part of the normal microbiota in the gastrointestinal tract of healthy humans and animals (Kaper et al., 2004, Tenaillon et al., 2010). While some strains are harmless, others have evolved pathogenic mechanisms through random genetic drift and phage-mediated events to cause disease in humans and animals (Clements et al.,

2012, Hussein, 2007, Lim et al., 2010). Infections by pathogenic *E. coli* may be restricted to intestinal mucosal linings and cause diarrheal disease or can propagate throughout the body and result in sepsis/meningitis, respiratory illnesses or urinary tract infection (Nataro & Kaper, 1998). STEC is one of many pathotypes that belong to a diverse group of *E. coli* and is the one that causes the most concern in the red meat industry.

Over 380 serotypes of STEC have been isolated from the gastrointestinal tract of diseased humans and animals. However, only a small number of serotypes can cause diarrheal illnesses such as haemorrhagic colitis (HC) and life-threatening haemolytic uremic syndrome (HUS) (Karmali et al., 2010, Nguyen and Sperandio, 2012). Konowalchuk et al. (1977) first identified STEC and cytopathic toxins from culture filtrates obtained from infants with diarrhoea, and extrapolated that the toxins were responsible for diarrheal manifestations. After a few years, O'Brien and LaVeck (1983) characterised a lethal polypeptide subunit toxin that was produced by a strain of STEC and this toxin had similar structure and biological activity to Shiga toxins produced by *Shigella dystenteriae* type 1. The group subsequently described it as Shiga-like toxins. The cytotoxicity of Shiga toxin makes STEC a lethal pathogen, especially in younger and older age groups, immuno-compromised individuals or pregnant women (Castro et al., 2017). Shiga toxin is the main virulence factor of STEC and the toxin gene (*stx*) is generally carried by prophages (Kruger and Lucchesi, 2015, Mellor et al., 2012). This means that another pathotype like enteropathogenic *E. coli* (EPEC) that contains the gene encoding the surface attachment protein, intimin (*eae*) can possibly gain a *stx* gene and enhance their pathological ability (Afset et al., 2003, Chen and Frankel, 2005).

STEC has emerged as a foodborne pathogen of significant public health concern across the globe since the first recognized outbreaks in the states of Michigan and Oregon in 1982 (Hiramatsu et al., 2002). Unusual gastrointestinal illness was characterised by initially watery diarrhoea followed by bloody diarrhoea with little or no fever and intense abdominal cramps which resulted in approximately a week of hospitalization (Riley et al., 1983). The diarrheal illness was indicated as HC, a newly recognized syndrome at the time (Nataro & Kaper, 1998). The aetiologic agent was traced backed to undercooked contaminated hamburger patties from a single fast-food chain when two groups of scientists isolated a 'rare' *E. coli* serotype with O-antigen 157 and H-antigen 7 from stool of affected or recovered individuals, and burger patties sold at the suspected fast-food establishment (Riley et al. et al. 1983, Wells

et al. et al. 1983). *E. coli* O157:H7 was deemed as the causative agent in both studies. The mechanism of pathogenesis remained unclear at the time, but the strain was later found to produce Vero toxins, also known as Shiga toxins.

In the same year, Karmali et al. (1983) investigated the aetiology of HUS and discovered an association between several serotypes of STEC, including O157, and the conditions of the little known disease. Karmali and colleagues (1983) found Shiga toxin in faecal filtrates of sporadic HUS patients and speculated that the Shiga toxins had a significant role in the genesis of HUS. The exact pathogenic mechanism of STEC at this time was unknown, but the hypothesis was that Shiga toxin was responsible for the cause of a triad of clinical symptoms of HUS (acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia) due to its cytotoxic activity on endothelial cells. Thus, the two key epidemiological studies led to recognition of previously unrecognized pathogenic *E. coli*, especially the O157 STEC (Karmali et al., 1983, Riley et al., 1983). Since its discovery, STEC became increasingly more recognized as a foodborne pathogen and public interest in O157 STEC and other serotypes grew exponentially with more outbreaks occurring (Brooks et al., 2005, Centres for Disease Control and Prevention., 1994, Rangel et al., 2005, Vally et al., 2012).

Other food types such as leafy vegetables, dairy products, fruits and other meat products (pork) have also been associated with Top 7 STEC infections and outbreaks (Luna-Gierke et al., 2014). Ruminants (e.g. cattle, sheep, goats) are asymptomatic carriers as they lack the receptors for Shiga toxins. This group of animals is the main reservoir of STEC meaning that pathogenic strains of STEC can be found in the gastrointestinal tract of dairy and beef cattle. However, beef cattle is considered the most common zoonotic vehicle for foodborne transmission of STEC (Fegan et al., 2005).1.3 STEC in the beef industry

STEC has caused numerous outbreaks and sporadic cases of foodborne illnesses over the past 20 years with clinical manifestations ranging from watery or bloody diarrhoea to life threatening HC and HUS (Barlow et al., 2006, Delannoy et al., 2012, Karmali et al., 1983). Infections caused by STEC have been associated with beef products as the gastrointestinal tract (GIT) of beef cattle are major reservoirs of STEC (Lim et al., 2007). In 1993, *E. coli* O157:H7 became broadly recognised as a significant pathogen when a large multistate foodborne outbreak in the US was caused by undercooked ground beef patties containing a strain of *E. coli* O157:H7 sold from a fast-food chain restaurant (Rangel et al., 2005). More

than 700 infections were confirmed with *E. coli* O157:H7, 178 victims had permanent kidney or irreversible neurological damage and four associated deaths occurred during this outbreak (Bell et al., 1994, Centres for Disease Control and Prevention., 1994, Tuttle et al., 1999).

The United States Department of Agriculture (USDA) Food Safety and Inspection Services (FSIS) officially declared O157 STEC as an adulterant in raw non-intact beef in 1994 and asserted that adulterated beef products will not be accepted for commerce in the US market (Barkocy-Gallagher et al., 2003). More foodborne-related cases of non-O157 STEC infections has resulted in expansion of the official list of adulterants by the FSIS to regulate additional STEC serotypes such as O26, O45, O103, O111, O121 and O145 in 2012 (Food Safety Inspection Safety, 2011). These 7 serotypes (O157 plus non-O157 serogroups) are collectively referred to as the 'Top 7' STEC hereafter. US receives approximately 21.9% of Australian beef and veal exports and is one of the top three customers of the industry (Meat & Livestock Australia., 2019). This means that to gain and maintain access to the US market, the processors must ensure that raw non-intact beef (referred to as manufacturing beef) is deemed free of Top 7 STEC.

Keeping in mind that there may be differences in techniques and surveillance protocols for STEC infections, the estimated incident rates in Australia are comparatively lower than the US and other countries (Vally et al., 2012). STEC outbreaks are far less common in Australia and were found to be less associated with foodborne transmission. Between 2000 and 2014, the annual notified rate of STEC illness was 0.4 cases per 100,000 and confirmed O157 STEC incidence rate was 0.12 cases per 100,000 (Meat & Livestock Australia, 2015). Detection of STEC is heavily influenced by practices by jurisdictions regarding screening of stool specimens, and South Australia has the most comprehensive screening practices across all jurisdictions in Australia. As a result, South Australia typically has the highest notification rate of STEC infections (OzFoodNet Working Group, 2007). There were 0.7 cases per 100,000 STEC infections in 1997 – 2009 in South Australia (Vally et al., 2012). In 2015, the incidence rate increased to 2.6 cases per 100,000 infections while the other states and territories reported between 0.4 and 0.7 cases per 100,000 (NNDSS Annual Report Working Group, 2019). NNDSS Annual Report Working Group (2019) reported the serotypes that were identified from the notified STEC infections in 2015 and the most common serotypes in the order of prevalence were O157, O26 and O111. The annual cost of STEC infections in

Australia is estimated to be approximately AUD$2.6 million in a year (McPherson et al., 2011, NNDSS Annual Report Working Group, 2019).

In comparison to Australia, the US has relatively higher incidence rate at 1.69 cases per 100,000 in 2016 with 0.72 cases per 100,000 confirmed as O157 serotype and it was reported that similar serotypes were found to be responsible for STEC infections; O157, O26, O103, O111 and O121, in the order of frequency (Centres for Disease Control and Prevention., 2018). The total economic burden of STEC in the US is estimated to be more than USD$300 million per year (Meat & Livestock Australia, 2015). Hoffmann et al. (2015) from USDA reported that health cost of STEC O157 can range from USD$25 million to $1.2 billion per year, and from $1.6 million to $88 million dollars per year for non-STEC infections. Other countries such as New Zealand had a relatively higher incidence of human STEC cases with 8.9 cases per 100,000 reported in 2016 (Browne et al., 2018). STEC incidence rates varied across the European regions with Austria at 2.0 cases per 100,000, Denmark at 3.7 cases per 100,000 and Ireland with the highest at 15.6 cases per 100,000 in 2016 (European Centre for Disease Prevention and Control, 2018).

1.4 STEC contamination of beef

STEC can be present in high numbers in faeces of beef cattle and can transfer to hides of the animals during transport or in the holding pens at the abattoir (Dewsbury et al., 2015, Fegan et al., 2009). Beef cattle can carry STEC in the distal portion of the terminal rectoanal junction without showing any clinical symptoms that would normally manifest during infections in humans. Chase-Topping et al. (2008) suggested that colonisation in the epithelium of the terminal rectum are associated with higher levels and longer duration of STEC excretion in the faeces. Concentration of STEC in the faeces can differ between animal to animal and can vary between 10^1 and 10^5 CFU per gram of faeces (Bonardi et al., 2001, Hussein, 2007). Individual animals that shed STEC at higher than 10^4 CFU/g are deemed as ‘super shedders’ (Matthews et al., 2006, Xu et al., 2014). Studies have shown that the super shedders can constitute a small proportion of the herd (8 – 9%) but are responsible for a substantial amount of O157 STEC transmission in the abattoirs (Castro et al., 2017, Chase-Topping et al., 2008). It has been reported that reducing stress levels in animals during handling and transport can reduce super shedding and assist in minimising the contamination of hides (Arthur et al., 2010, Fegan et al., 2009).

Cattle hides with faecal contamination are considered a major source of STEC contamination of beef carcases during beef slaughter (Arthur et al., 2004, Barkocy-Gallagher et al., 2003, Elder et al., 2000). Transfer of STEC from hide to carcase is highly likely to occur during the vigorous process of removing the hide (Bosilevac et al., 2005, Collis et al., 2004). STEC contamination of the carcases can also occur during evisceration of the carcases where visceral content may leak. In general, the processors can deal with lower numbers of STEC entering the abattoir using rigorous sanitisation protocols and Good Manufacturing Practices (GMPs) coupled with intervention strategies such as hot water decontamination or chemical treatment as necessary (Desmarchelier et al., 2007, Meat & Livestock Australia., 2020). However, the presence of super shedders can overwhelm contamination control strategies and may lead to increased concentrations of STEC on the carcases and throughout the abattoir. As the super shedders are asymptomatic carriers and there are no visual cues upon inspection in the holding pens, it is difficult to detect the oncoming STEC threat earlier in the slaughtering process (Baker et al., 2016).

Using manilla ropes, or ROPES, has been suggested as a risk management strategy for detection of STEC at feedlot pens (Renter et al., 2008, Smith et al., 2004). These ropes are secured and hung on the inside of pen rails for the animals to lick, chew or rub on the devices. Confirmation of STEC presence is performed using these ropes and the pens with at least one rope that STEC is recovered from are confirmed as ROPES-positive. Smith et al. (2004) found that this pen test device was a useful tool to monitor presence of STEC (82% sensitivity, 92% specificity on average) and ROPES-positive pens were more likely to contribute to environmental transmission of STEC (e.g. faeces, water tanks) within commercial cattle feeding systems (Smith et al., 2005). ROPES were tested for detection of STEC in the Australian feedlot system. Meat & Livestock Australia (2007) also found that the ropes were an effective indicator of STEC shedding level of cattle herds and suggested that using the pen test devices a week before slaughter will provide enough to determine STEC shedding status prior to transport and processing. These studies suggested that STEC testing at the pen-level is useful to identify feedlot production practices that are associated with greater risk for contributing STEC into the food supply (Smith et al., 2008). It also showed that there may be reliable methods to detect super shedding of STEC before the animals enter the slaughter phase. Early detection of super shedders could provide processors

with time to strategize against higher risk of increased STEC contamination through processing.

Investigation of microbial contaminants using conventional cultivation methods within the boning room suggested that the bacterial populations from slaughter can remain on the carcases post-slaughter and after sanitation (Bell, 1997, Stellato et al., 2016). These bacteria can be transferred onto personnel and processing equipment that ultimately contribute to contamination of beef products (Brightwell et al., 2006, Yang et al., 2017). However, transmission of contaminants including STEC through the boning room is not well documented.

1.5 Detection of Top 7 STEC

1.5.1 FSIS guideline

The FSIS has established guidelines for testing manufacturing beef to regulate the presence of Top 7 STEC for exporters to meet market requirements in the US. The guideline is comprised of a series of molecular techniques that target genetic markers associated with virulence of STEC (*stx*, *eae* and gene fragments specific for individual serogroups of Top 7 STEC) by polymerase chain reaction (PCR) followed by selective cultivation (Food Safety Inspection Services, 2019a). Typically, STEC constitutes a small proportion of the bacterial community composition and the enrichment step is an essential process to support the growth of STEC to reach higher numbers in the bacterial community to exceed the limit of PCR detection (Stromberg et al., 2015b, Vimont et al., 2006b). The enriched samples that test positive for *stx*, *eae* and one or more of the Top 7 serotypes indicate that STEC may be present and are treated as potential positives (PPs).

STEC isolation from PPs is performed using immunomagnetic separation (IMS; paramagnetic beads targeting Top 7 O-antigens). The bead-bacteria complexes are selectively cultivated to collect single colonies for additional characterisation (Drysdale et al., 2004, Wasilenko et al., 2014). The bead-bacteria complexes at the completion of IMS are plated onto modified Rainbow Agar (mRBA; supplemented with cefixime trihydrate, potassium tellurite and sodium novobiocin) (Food Safety Inspection Services, 2019a). Morphologically distinct colonies on mRBA are picked and tested the presence of O-antigens using an agglutination test. The O-antigen positive colonies are streaked onto tryptic soy agar with 5% sheep blood for confirmation by genetic assays (for *stx* and *eae*) and biochemical

identifications. The sample is confirmed positive when pure single colonies are positive for O serotypes that belongs to the Top 7 serogroups with *stx* and *eae*, and a biochemical profile of *E. coli* (Food Safety Inspection Services, 2019a).

1.5.2 Detection of STEC in Australia

In Australia, the Department of Agriculture, Water and Environment has approved a list of microbiological methods that can be used for detection and isolation of STEC in beef products relating to export (Department of Agriculture Water and Environment., 2019). These techniques are deemed equivalent with the FSIS methods and can be used interchangeably. From this list, DuPoint Qualicon BAX® System Real-Time PCR and Assurance GDS® MPX Top 7 STEC are the two most commonly used methods for rapid screening of Top 7 STEC in the Australian beef industry.

Australia uses N60 sampling methods to detect STEC in manufacturing beef (Kiermeier et al., 2011). This means that 12 cartons of beef are randomly selected from a single production lot. Five samples are taken from each carton to produce a composite of 375 g. The composite sample is then tested for presence of Top 7 STEC using BAX or GDS system. Both techniques have similar enrichment condition prior to screening with dilution of the sample in 1.5L of pre-warmed medium and enrichment at 42 ±1°C for a minimum of 10 hours. Nevertheless, the enrichment broth used in each system is different. For instance, BAX uses MP enrichment medium whereas GDS uses mEHEC. Isolation of STEC using BAX and GDS systems have been investigated or compared to other common enrichment media in previous studies (Feldsine et al., 2016, Fratamico et al., 2017, Guerini et al., 2006, Wasilenko et al., 2014), however, these two media have not been directly compared in parallel.

1.5.3 Limitations of conventional methods

The current FSIS protocols for detection of STEC uses a combination of technologies (e.g. real-time PCR, IMS and differential media), there are opportunities for improvement. The process from enrichment to characterisation is often time-consuming, laborious and the conversion rate of PPs to confirmed positives is often low meaning that it may not be cost-effective (Brusa et al., 2016, Delannoy et al., 2016). In addition, the system relies on conventional microbiological methods that requires cultivation of bacterial populations in laboratory conditions. The methods that were used in the past to study prevalence of STEC in the beef supply chain are similar to the regulatory methods that are used to detect the

presence of STEC in manufacturing beef. Cultivation of microorganisms can produce biased results caused by selective pressure during *in vitro* incubation (Bakhtiary et al., 2016). This type of technique can be useful for STEC prevalence surveys but can be limited in terms of understanding complex shifts and changes in microbial communities that may be linked to the presence of STEC during contamination of beef throughout processing or during STEC enrichment. The enrichment process changes the composition of the community to encourage a higher population density of STEC, meaning that the structure of the bacterial populations from the original niche is altered (Ge and Meng, 2009, Hussein et al., 2008). This means that the ecological changes that may contribute to lower or higher presence of STEC can be overlooked. On top of that, selectively culturing all viable microorganisms to understand the overall ecological shifts in response to STEC abundance pre- and post-enrichment is not possible as some of the bacterial groups are not culturable in a laboratory (Wang and Salazar, 2016).

1.6 Bacterial population analysis using 16S rRNA gene

Sequencing of 16S rRNA gene has been commonly used to investigate compositional changes within bacterial communities in microbiology. The hypervariable regions of the 16s rRNA gene in bacterial DNA extracted from samples is replicated using PCR. Each sample may contain varying concentrations of the 16s rRNA amplicons from different bacterial species (i.e. concentrations of bacterial populations). Hence, it is important to distinguish such differences in for bacterial population analysis. The amplicons from each sample are tagged with primers that contain a unique barcode. PCR products are then normalised and combined to create a pooled library of the amplicons. This library is sequenced using high throughput sequencing tools where the unique barcodes allow de-multiplexing of the amplicons, meaning that the origin of raw 16S rRNA sequences can be identified.

The raw sequences of 16S rRNA amplicons are analysed using bioinformatic software where sequencing and PCR errors (e.g. repetitive sequences, chimeras) can be removed. Improved sequences are classified into Operational Taxonomic Units (OTUs) by comparing to a large database of bacterial 16S rRNA gene. It is critical to utilise frequently updated 16S rRNA database to ensure accurate classification of bacteria. The OTUs can be used to measure diversity of bacterial communities within each sample (alpha diversity) or between different samples (beta diversity).

1.7 Analysis of 16S rRNA bacterial profiles in the beef industry

The use of bacterial 16S rRNA gene sequences is becoming a common technique in meat industries to study bacterial phylogeny and taxonomy (Chen et al., 2020, Handley et al., 2018, Hultman et al., 2015, Rothrock et al., 2016, Zwirzitz et al., 2020). Unlike culture-dependent techniques, sequencing technologies can be used to provide more in-depth understanding of the beef microflora by describing the changes in structure and composition of bacterial communities. In addition, these methods are believed to overcome inherent weaknesses associated with cultivation techniques and avoid interpretation of results that are produced from a small subset of microorganisms (Armougom and Raoult, 2009, Janda and Abbott, 2007). There is a limited number of studies with sequencing information of beef microbiomes in the literature, and even fewer attempted to link ecological population shifts to the presence of STEC. The studies that analysed beef microbiomes have demonstrated how 16S rRNA results can be used to understand movement of contaminants in ways that were not possible with conventional microbiological methods.

1.7.1 Bacterial communities through beef processing

After an initial study that evaluated the applicability of 16S rRNA sequencing for contamination analysis (Chandry, 2013), an industry report for Meat & Livestock Australia was produced which monitored 16S rRNA profiles through Australian beef processing in an attempt to track sources of contamination (Chandry, 2016). Chandry (2016) demonstrated that bacterial communities on hides correlated with the bacterial profiles on matching carcases throughout the slaughter phase. This study also investigated air contamination at three different locations (near the hide puller, in the chiller and outside the slaughter floor) and demonstrated that more than 80% of bacteria in the air samples were derived from the hides. A small proportion of carcases had an almost identical 16S rRNA profile with air while some carcases had low or minimal contribution of contamination suggesting that air was not always the main route of transferring hide contaminants to the carcases.

It was interesting that the hide contaminants in the air was found in areas further away from hide removing station (in the chiller and outside the slaughter floor) and contributed to the microflora on the carcase during slaughter. The study also reported that the carcase microflora consisted of commensal bacteria from the rumen and oral cavity of cattle such as *Fusobacterium* (Chandry, 2016). Droplets from the tongue or the mouth may have reached

the hides during the removal of the head and passed onto the carcases during the removal of hides. The findings from this study suggested that control of air movement and bacteria derived from the mouth may be helpful to minimise microbial contamination of carcases during processing.

1.7.2 Bacterial communities in beef products

Stellato et al. (2016) investigated the influence of environment on initial fresh meat contamination by comparing 16S rRNA profiles of beef cuts and environmental swabs (knife, chopping boards, meat handler's hands) at multiple butcheries in small-scale and large-scale retail distribution facilities. The 16S rRNA analysis showed that approximately 80% of the samples shared core microflora consisting of common beef-associated bacterial groups such as *Acinetobacter* spp., *Brochothrix* spp., *Pseudomonas* spp., *Psychrobacter* spp., and *Streptococcus* spp (Ercolini et al., 2006, Nychas et al., 2008, Reid et al., 2017). Clustering of OTUs separated the microflora into meat and environmental communities, with higher abundance of *Proteobacteria* in meat. This group of bacteria includes zoonotic pathogens such as *E. coli*, *Salmonella* and *Campylobacter* and high abundance of *Proteobacteria* have been associated with dysbiosis in the gut (Auffret et al., 2017, Gallardo et al., 2017). Findings from the study highlighted the importance of microbial levels in the initial environment that the fresh beef cuts are exposed to and that the different type of retail facility did not have a significant impact on meat contamination.

A number of studies examined changes in microbiomes of different types of beef products under various conditions (De Filippis et al., 2013, Ercolini et al., 2011, Weinroth et al., 2019). One study used 16S rRNA amplicon sequencing to test the effect of antimicrobial agents (peroxyacetic acid, sulfuric acid and sodium sulfate blend) and storage duration in a retail display case (4°C) on the microbiome of ground beef (Weinroth et al., 2019). The results suggested that antimicrobial treatment had a substantial impact on bacterial community diversity within (alpha) and between (beta) samples, whereas storage duration only altered alpha diversity. It appeared that implementation of antimicrobial agents significantly changed ($P<0.05$) the relative abundance of *Enterobacteriaceae* which was the second most abundant group after lactic acid bacteria in all samples. Weinroth et al. (2019) also identified rare bacterial populations that are not typically associated with ground beef (*Geobacillus, Thermus* and *Sporosarcina*) and added that sequencing analysis allowed investigation of the entire microflora, not just a subset of indicator microorganisms in meat.

Another study explored the microbiome of beef steaks (from three beef cuts) before and after storage at 4°C in aerobic conditions as well as the composition of bacterial communities on the parts of carcases where the steaks were acquired from, and the environment where the beef was handled (De Filippis et al., 2013). The study found a significant correlation ($P<0.05$) between beef microflora and specific beef cuts, suggesting that different cuts of the same carcase can have a substantial effect on microbial contamination of beef steaks. Microbial complexity of beef steaks decreased through storage as *Pseudomonas* sp. and *Brochothrix thermosphacta* became the main contaminants. De Filippis et al. (2013) detected *Enterobacteriaceae* and *Psychrobacter* which were also found in a previous study by Weinroth et al. (2019) during 25-day aerobic storage of ground beef at 4°C. Also, abundance of the same OTUs that were found in beef steaks increased in the environment (equipment and hands of operator, chopping board), suggesting that the microorganisms originated from carcases and became resident microflora near the meat-handling area. It appeared that these bacteria linger in the environment and subsequently contribute to spoilage via contamination of beef during storage (De Filippis et al., 2013, Doulgeraki et al., 2012, Zwirzitz et al., 2020).

Ercolini et al. (2011) analysed 16S rRNA amplicon sequences from beef chops stored at 4°C under different conditions for 45 days; air, modified-atmosphere packaging (MAP), vacuum packaging, and bacteriocin-activated antimicrobial packaging. The analysis demonstrated that *B. thermosphacta* was the most dominant bacteria during the early stages of storage in aerobic and MAP beef samples, while *Pseudomonas* spp. dominated the bacterial communities during further storage in air-packaged samples. However, changing the packaging condition (active antimicrobial substances and vacuum packaging) inhibited the growth of *B. thermosphacta*, *Pseudomonas* spp., and *Enterobacteriaceae* (Ercolini et al., 2006, Mohebi and Marquez, 2015). Interestingly, the study found some bacterial groups that are commonly associated with soil in aerobic and antimicrobial packaging (Ercolini et al., 2011). Lactic acid bacteria, especially *Carnobacterium divergens,* eventually became the most dominant group of bacteria during the late stages of storage in antimicrobial packaging. Through this study, Ercolini et al. (2011) demonstrated that the meat microbiota can be substantially influenced by storage conditions by showing changes in 16S rRNA profiles in a shelf-life study of beef chops.

1.8 Bacterial diversity analysis in correlation to the presence of STEC

There has been increased research into the structural changes in bacterial communities present at various stage in the beef chain and how this may relate to the prevalence of Top 7 STEC in recent years. Some studies suggested that higher bacterial diversity correlates with lower prevalence of STEC while others suggest that the shifts in bacterial composition are not directly related to the presence of STEC. These studies are reviewed below to compare the findings on the relationship between STEC and bacterial diversity.

1.8.1 STEC and bacterial diversity on pre-harvest cattle hides

Understanding how the bacterial populations change within the beef microflora could provide strategies to control STEC in processing of beef cattle and can potentially be used to identify biomarkers for risk assessment of STEC. One study collected samples from cattle faeces and hides over a 3-month period from a large commercial feedlot and established a cohort of bacterial populations that were specific for pre-harvest hides by eliminating faecal 16S rRNA gene OTUs from the OTUs found within each hide 16S rRNA library (Chopyk et al., 2016). Then, the differences in bacterial community composition were compared between communities with and without the presence of STEC. Their analysis showed that alpha and beta bacterial diversity in STEC negative groups deviated from groups that contained O157 alone or both O157 and non-O157s. Despite STEC being a small proportion of the community on the hide and in faeces, the study suggested that presence of STEC populations may respond to different community compositions. The final conclusion was that cumulative prevalence of Top 7 STEC was associated with lower bacterial community diversity on pre-harvest cattle hides. Chopyk et al. (2016) added that this correlation was also found in collective assembly of total 16S rRNA OTUs (faeces and hides).

Although the previous study was an observational study of the 16S rRNA amplicons and would require further investigation, Chopyk et al. (2016) hypothesized that a diverse bacterial community may prevent colonisation of Top 7 STEC by producing antimicrobial substances such as organic acids and bacteriocin or by occupying ecological niches on the carcases that would otherwise be taken up by STEC in communities with reduced diversity (De Filippis et al., 2013, Wei et al., 2016, Yang et al., 2016). *Solibacillus* and *Streptococcus* were commonly found at higher relative abundance in the absence or lower prevalence of STEC in samples with reduced bacterial diversity. On the other hand, these bacteria were found at lower

relative abundance in STEC positive samples. The study suggested that *Solibacillus* may be inhibiting STEC colonisation of hides. It has been shown that some *Solibacillus* species are able to degrade quorum sensing molecules (N-acylhomoserine lactones; AHLs) that *E. coli* O157 uses to assess the environment and modulate survival gene expression (Sperandio, 2010, Van Houdt et al., 2006). *Streptococcus* species are part of a normal skin microflora that are known to produce lactic acid which can lower the pH and permeabilize the outer membrane of *E. coli* O157 (Alakomi et al., 2000, Brashears et al., 2003, Chopyk et al., 2016). This may be linked to lower prevalence of STEC in samples with higher abundance of *Solibacillus* and *Streptococcus*, however this would require further investigation to confirm. Other OTUs were compared between the groups containing different Top 7 STEC serogroups but it appeared to be difficult to identify a list of OTUs that were commonly predominant in the STEC positive hide-specific dataset with higher bacterial diversity (i.e. large list of unique OTUs with each having low relative abundance) (Chopyk et al., 2016).

1.8.2 Faecal bacterial communities and O157 STEC shedding

In a study by Xu et al. (2014), the structure of faecal bacterial communities in O157 super shedders was compared with that of non-STEC shedders to identify OTUs that characterised each group via 454 pyrosequencing of bacterial 16S rRNA genes (V1 and V3 regions). Faeces were collected via rectal palpation from confirmed super shedding steers and non-shedding pen mates at a commercial feedlot in Canada. Findings from the study suggested that super shedders and non-shedders harboured faecal bacterial communities that were distinctly different. Five different methods were individually used for calculation of OTU clustering and diversity (Mothur, Espirit-tree, CROP, UPARSE and Otupipe) to reduce bias analysis caused by varying clustering algorithms used for binning raw sequences into OTUs (Schloss and Westcott, 2011). Xu et al. (2014) reported that bacterial diversity in faecal microflora of super shedders was higher than the faecal communities of non-shedders regardless of the clustering methods used to process the sequence data.

Super shedders had 72 OTUs with differing relative abundance in comparison to non-shedders and 55 of these OTUs were more abundant in the super shedding communities. These OTUs mostly belonged to *Bacteroidales* and *Clostridiales*. Some members of *Bacteroidales* have been reported to produce AHL and Xu et al. (2014) suggested that it would be interesting to see if these OTUs are associated with AHL production (Romero et al., 2010). Production of AHL can contribute to survival of *E. coli* O157:H7 within the acidic

rumen and promote colonisation in the GIT through modulation of gene expression (Hughes et al., 2010, Sperandio, 2010). The study found that the bacterial communities of the super shedders shared high similarity and were generally clustered together on non-metric multidimensional scaling plots, suggesting that specific microbial community structures may allow the presence of O157 STEC (Xu et al., 2014). It appeared there were no shared predominant taxa between individual super shedders and less abundant bacterial groups may have played a role in the development of super shedding state.

1.8.3 Composition changes in the intestine of O157:H7 super shedders

A study by Zaheer et al. (2017a) used 16S rRNA gene pyrosequencing to investigate the structure of faecal bacterial communities at several locations from the duodenum to the rectal-anal junction in super shedding and non-shedding steers. In this study, faecal samples were removed through excision of the gastrointestinal tract (GIT) at 8 different locations in anatomical order (duodenum, proximal jejunum, mid jejunum, distal jejunum, cecum, spiral colon, descending colon and rectum at the rectoanal junction) within 10 minutes of slaughter. Zaheer et al. (2017a) found that the *E. coli* O157:H7 shedding status did not have an effect on the overall structure and diversity of the bacterial community along the GIT. Diverse commensal microflora in the gut has been implicated in prevention of colonisation from invading pathogens and may be mediated by numerous mechanisms including direct killing, competition for limited nutrients and enhanced immune response (Baumler and Sperandio, 2016, Pickard et al., 2017). Disruption of the endogenous microflora in the GIT can potentially promote infection by pathogenic bacteria of epithelial cells in the intestinal lining of the host (Gallardo et al., 2017).

STEC can typically be found in the lower regions of GIT within the mucosa of the rectoanal junction of super shedders which is their preferred site of colonisation in beef cattle (Cobbold et al., 2007, Lim et al., 2007). The previous study showed that the analysis of 16S rRNA pyrotags in lower GIT had greater bacterial diversity and richness in comparison to the upper GIT (Zaheer et al., 2017a). However, Zaheer et al. (2017a) did not find significant differences in the bacterial community diversity between the super shedders and non-shedders at this location of the GIT. In general, several bacterial groups including *Prevotella*, *Treponema, Ruminococcocus, Selenomonas, Campylobacter* and *Streptococcus* were abundant in different proportions between the two cattle groups. Some of these bacteria are commonly found in faecal communities and are relatively abundant within the rumen and lower GIT of

cattle (Callaway et al., 2010, Dowd et al., 2008, Xu et al., 2014). It appears that the complex ecological interactions throughout the GIT resulted in varying bacterial community composition along the tract and more accurate sequencing technologies are required to describe the structure of STEC positive communities at the lowest taxonomic classes. Interaction between bacterial species and strains may provide important correlation between commensal bacterial populations and pathogens which could be overlooked at higher taxa.

1.9 Knowledge gap in the literature

Currently, there is a lack of understanding how the composition of bacterial communities affect the presence of top 7 STEC throughout processing of Australian beef cattle (slaughter and boning). The previous studies that have implemented next generation sequencing (NGS) in understanding the effect of surrounding microbial communities on the presence of STEC has focused on analysis in the phases before processing within the beef supply chain (Chopyk et al., 2016, Xu et al., 2014, Zaheer et al., 2017a). In addition, observations from these studies have provided mixed observations on the status of bacterial diversity in which some studies suggested a correlation between higher bacterial diversity and lower STEC prevalence (Chopyk et al., 2016) while some found the structure of bacterial communities in STEC positive groups was more diverse (Xu et al., 2014), or did not find significant ecological differences between the STEC positive and negative groups (Zaheer et al., 2017a). In general, there is currently a limited amount of studies to compare and contrast the microflora in response to the presence of STEC. However, the previous studies have demonstrated the capability of 16S rRNA sequencing approach to evolve the understanding of contamination from enumeration of viable and culturable cells to being able to describe the changes in bacterial populations. Combining cultivation and sequencing techniques provide more descriptive analysis of transmission of microbial contaminants that could not be achieved with cultivation methods alone.

It is important to note that there were some variations in experimental designs and analytical tools between the previous studies that applied 16S rRNA amplicon sequencing (Chopyk et al., 2016, Xu et al., 2014, Zaheer et al., 2017a). Bacterial 16S rRNA gene profiling was executed using different sequencing platforms, bioinformatic tools and algorithms, and the samples were collected from different locations. Measurement of bacterial diversity is dependent on the sensitivity and accuracy of computational algorithms used during processing of 16S rRNA sequences and subsequent analysis of bacterial community structure

may give varying results (Schloss and Westcott, 2011). The quality of 16S rRNA gene amplicons, regions of 16S rRNA used and preparation of pooled 16S rRNA libraries can also have an impact on the analysis downstream (Lagkouvardos et al., 2016, Wei et al., 2016). This suggests that it may be difficult to directly compare results of the previous studies despite using samples with a similar microbiological profile such as cattle faeces. Nonetheless, the difference in sample type should not produce contrasting results if the correlation between higher bacterial diversity and lower prevalence of Top 7 STEC truly exists.

The meat processing environment of beef cattle is dynamic and the microorganisms in the abattoirs are more prone to ecological shifts and physical stress (e.g. temperature changes, physical alteration of carcases) throughout the course of transforming animals into food products (Stellato et al., 2016, Yang et al., 2017). It would be no surprise that the bacterial communities on carcases through processing are continuously changing through contact with the residential bacteria in the environment or between carcases and boned products that are processed on the same day. Contamination of carcases through aerosols can also play a role in contributing to the final microflora on the carcases and subsequently in the products (Chandry, 2016, Sheridan et al., 1992). Counts of microorganisms throughout processing (e.g. carcases, personnel equipment, environmental surfaces) can fluctuate depending on level of sanitation and cross-contamination in the abattoir (Arthur et al., 2004, Kim and Yim, 2017, Madden et al., 2004). Previous studies that examined the presence of pathogens and other indicator organisms typically utilised cultivation methodology meaning that the groups of bacteria were often individually analysed and reported. This indicates that the dynamic flow of beef contamination including STEC during processing has been limited to a basic understanding. Sequencing-based techniques could be a useful tool to comprehend the diverse ecological shifts and the potential influence the changes may have on the prevalence of Top 7 STEC.

1.10 Conclusion and the thesis objectives

Seven serogroups belonging to STEC that are commonly referred to as the Top 7 STEC have been an important group of pathogens for public health and the global beef trade since the first outbreak in 1982. It became a regulatory requirement to test non-intact, raw beef products (e.g. manufacturing beef) that are exported to the US market for the presence of all serogroups of Top 7 STEC in 2012 (Food Safety Inspection Safety, 2011). The current

system for detection and confirmation of STEC in manufacturing beef relies on conventional microbiological methods that are the best and most efficient methods, but with limitations in their sensitivity and specificity. Similar methods have been used to investigate STEC contamination of beef in the past which has inherently restricted the understanding of contamination to a basic level. With the advent of NGS and sequencing of specific target genes such as bacterial 16S rRNA, researchers are able to expand the knowledge of microbial contamination through the supply chain of beef cattle from enumeration of viable counts to more in-depth analysis of changes in composition of bacterial communities.

There is increasing evidence that bacterial community diversity may be associated with the prevalence of Top 7 STEC in cattle faeces and hides (Chopyk et al., 2016, Xu et al., 2014). In saying that, sequencing analysis of bacterial populations in the beef supply chain is generally lacking. There are currently no studies that closely examine the potential relationship between bacterial diversity and STEC within the processing stages of beef cattle. Previous studies that utilised 16S rRNA profiles in pre-processing environments to investigate the presence of STEC demonstrated that sequencing hypervariable regions of the 16S rRNA gene can be useful in analysing changes in bacterial community structure and composition (Zaheer et al., 2017a). Profiling of 16S rRNA allowed the researchers to describe ecological shifts in bacterial communities and link these diverse changes to the presence of Top 7 STEC serogroups which was not achievable with conventional cultivation methods. Understanding how bacterial populations circulate in abattoirs using sequencing analysis may expand our understanding of microbial contamination of beef products and provide new strategies to effectively control the presence of important foodborne pathogens such as STEC.

In this project, sequencing technology were used to for in-depth analysis of bacterial communities through the beef processing chain. The project utilised 16S rRNA amplicon sequencing in combination with conventional microbiology methods to achieve the following objectives:

- To understand transmission of microbial contaminants by monitoring changes in composition of bacterial communities through slaughter at two abattoirs with an integrated (abattoir A) and a fragmented (abattoir B) supply chain
- To understand the flow of contaminants through observations of ecological shifts within bacterial communities on beef trim and environmental surfaces in the boning room at abattoir A and B

- To investigate correlation between bacterial community diversity and presence of Top 7 STEC (O26, O45, O103, O111, O121, O145 and O157) through processing of beef cattle and evaluate the potential application of 16S rRNA sequencing to predict the presence of Top 7 STEC
- To compare and contrast how the presence of STEC may affect the structure of bacterial communities throughout enrichment of manufacturing beef.

Chapter 2: A comparison of 16S rRNA profiles through slaughter in Australian export beef abattoirs

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2.1 Introduction

The general route of contamination involves faecally contaminated hides transferring faecal matter and contaminants onto carcases during slaughter and processing of the animals (Arthur et al., 2004, Barkocy-Gallagher et al., 2003, Bosilevac et al., 2005, McEvoy et al., 2000). Transfer of microbial contaminants from the hide to carcase is likely to occur during hide removal resulting in the contaminated carcases becoming a vector for transmission of microorganisms including pathogens to different cuts of beef throughout the supply chain (Arthur et al., 2010, Chopyk et al., 2016, Fegan et al., 2009). Such movement and prevalence of microbial contaminants including the regulatory important pathogens in the beef supply chain is well documented in the literature (Antic et al., 2010, Barlow and Mellor, 2010, Collis et al., 2004, Elder et al., 2000, Fegan et al., 2005, Fegan et al., 2009, Stromberg et al., 2015a, Svoboda et al., 2013).

Australian beef processing abattoirs may operate as an integrated or a fragmented supply chain. Currently, fragmented supply chains appear to be the dominant type of supply chain in the Australian beef industry (Australian Meat Processor Corporation, 2017). In an integrated supply chain, beef cattle are received by processors from the same suppliers using consistent production strategies resulting in herds of animals arriving with minor variation in traits. In a fragmented supply chain, the processors receive animals from multiple different producers which may result in herds of cattle with variations in physical traits such as weight, feed type and breed as well as physiological and stress levels (Australian Competition and Consumer Commission, 2016). Therefore, one could argue that higher fragmentation in the supply chain may contribute to increased variability in the microbial composition due to beef cattle

arriving from multiple different sources potentially carrying diverse endogenous microbial populations.

The use of traditional microbiological techniques to analyse contamination of beef carcases in previous studies focused the understanding of contamination on easily culturable microorganisms. These techniques have assisted in understanding the general movement of microbial contaminants from faeces to hides and ultimately to the carcases during beef slaughter (Arthur et al., 2010, Bell, 1997, Fegan et al., 2005, Pointon et al., 2012, Stromberg et al., 2015a). The advent of high throughput, next-generation sequencing (NGS) has enabled the understanding of the biodiversity in a given environment to be explored in much finer detail and complexity (Cao et al., 2017, Vollmers et al., 2017, Zaheer et al., 2018). It has been utilised to explore the microbiome in beef abattoirs in recent years and demonstrated that 16S rRNA sequencing acts as a viable tool to monitor sources of microbial contamination (Bakhtiary et al., 2016, Chopyk et al., 2016, De Filippis et al., 2013, Yang et al., 2017). Research in the Australian beef industry has typically focused on the general microbial contamination levels of chilled beef carcases and beef products, pathogen surveillance and antimicrobial resistance (Barlow and Mellor, 2010, Barlow et al., 2015, Fegan et al., 2009, Jordan et al., 2007, Mellor et al., 2016, Phillips et al., 2012, Sumner et al., 2003). In the current literature, there are two industry project reports that describes the bacterial profiles in different sample types collected from an Australian export beef abattoir using 16S rRNA amplicon-based approach (Chandry, 2013, Chandry, 2016). There are however no published articles on the use of amplicon-based studies in Australian red meat production systems.

This study was designed to map and compare the flow of bacterial profiles during slaughter in two Australian beef export abattoirs with a varying level of supply chain integration using 16S rRNA amplicon sequencing. Fragmentation in the supply chain is hypothesized to have an effect on microbial ecology present during slaughter by introducing variation in the microbial load entering the abattoir. This hypothesis is tested by comparison analysis of 16S rRNA profiles between two Australian beef export abattoirs with varying integration in the supply chain. Additionally, the abattoirs are examined to provide an insight to recognising parts of the slaughtering process that contribute the most to carcase contamination and understand the movement of contaminants with natural variations in the process.

2.2 Materials and methods

2.2.1 Sample collection and experimental design

Samples were collected from two abattoirs with an integrated and a fragmented supply chain in Australia. The integrated abattoir (A) employed downward hide pull (DHP) system where the fragmented abattoir (B) used upward hide pull (UHP) system. Neither abattoir employed specific antimicrobial interventions and the locations and frequency of trimming was comparable between the two abattoirs. Each abattoir was each visited twice with a period of three months between the visits (between January – July 2018) and 90 samples were collected per visit. Abattoir A and B had similar processing line speed at 80 – 100 carcases per hour. The samples collected consisted of 10 faecal samples, 15 hide samples prior to hide-pull, 15 carcase samples immediately after hide-pull, 15 carcase samples post-evisceration, 15 carcase samples immediately before chilling (pre-chill) and 20 environmental samples throughout the slaughter floor. For the environmental samples, 10 sites were chosen, and each site was periodically sampled twice. The samples were collected after sanitation and within an hour of slaughter commencement in the morning of the day. Each and every sampling visit was ensured to collect samples at this time of the slaughter day. The sampling sites were sequentially matched in a spatial order of slaughter process but were not identical between the abattoirs.

Large area sampling was used as previously described (Chandry, 2013). In brief, hide and carcase samples were collected aseptically by swabbing forequarter of the animals using large area (3000 cm^2) sampling technique. Environmental samples were obtained from swabbing an area approximately equivalent to 900 cm^2. Faecal samples were collected from the internal content of freshly deposited faecal pats in holding pens before slaughter. Swabbing of carcase and environmental samples were performed using Whirl-Pak® Speci-Sponge® (Nasco, Wisconsin, US) pre-moistened in 25 mL of sterile buffered peptone water (BPW; Oxoid, Hampshire, UK). Faecal samples were collected in individual yellow capped plastic jars (Sarstedt, Numbrecht, Germany) using a sterile spoon. All samples were immediately placed on ice and transferred to the laboratory within four hours of collection. At the laboratory, all samples collected with a sponge had an additional 75 mL of BPW added prior to being stomached for 30 seconds at four strokes per second (Interscience, Saint Nom, France). Faecal samples (approx. 10 g) were diluted 1 in 10 with BPW and stomached for a minute. Following stomaching, aliquots from the samples were kept for 16S rRNA

sequencing analysis at -80°C and the remaining portion of the bacterial-BPW suspension was used for microbiological analysis.

2.2.2 Microbiological examination of samples

Bacterial-BPW suspensions were used for total viable count (TVC). TVC were obtained by plating 100 µL aliquots of serial 10-fold dilutions prepared in saline (Oxoid) onto tryptic soy agar (TSA; Oxoid) and incubated at 25°C for 72 – 96 hours. Statistical analysis by *t* test comparing two means for continuous data was performed using GraphPad Prism version 8.0 for Windows, https://www.graphpad.com/.

2.2.3 Analysis of 16S rRNA amplicon sequencing

2.2.3.1 DNA extraction

A 1 mL aliquot from hide and faecal samples were centrifuged to pellet the cells. For carcase and environmental samples, a 40 mL aliquot was used to pellet the cells. The pellets were washed two times using ultrapure H_2O prior to DNA extraction using QIAamp PowerFecal DNA kit (Qiagen, California, US) following the manufacturer's instructions with a modified bead beating step. The bead beating procedure involved a total of three minutes of beating with a minute rest after the first and second minute of beating. The bead tubes were then placed in heating blocks at 65°C for 10 minutes and the bead beating was repeated.

2.2.3.2 Preparation of 16S rRNA library

Extracted DNA was utilised to construct a sequencing library targeting the V4 region of 16S rRNA gene using a previously published protocol (Kozich et al., 2013). Briefly, an aliquot of 5 µL from each DNA template was amplified with dual-index primers via PCR and the amplicons were manually normalised. Each template contained specific barcode sequences at the 5' and 3' of the PCR amplicon to allow stratification among each other in the pooled library on the Illumina sequencing platform. The concentration of the amplicons was estimated by visually comparing the intensity of the DNA band against the GeneRuler 100 bp Plus DNA Ladder (ThermoFisher, Victoria, Australia) stained with ethidium bromide in 2% agarose gel under ultraviolet light. A final 16S amplicon concentration of 50 ng from each sample was combined to generate a pooled library. Additionally, ZymoBIOMICS™ microbial community DNA standard (Zymo Research, California, US) was normalised and added to the library. After normalisation, an aliquot of approximately 200 µL was used for

purification via Agencourt AMPure XP magnetic beads (Beckman Coulter, California, US) using the manufacturer's instruction. The purity and concentration of DNA were estimated pre- and post-purification using a NanoPhotometer® (Implen, California, US) and Qubit® 2.0 Fluorometer (Life technologies, California, US), respectively.

2.2.3.3 Sequencing using Illumina MiSeq

Sequencing of the amplicons was conducted at University of New South Wales (UNSW) using the Illumina MiSeq platform (Illumina, California, US) with a paired-end 300 base pair sequencing protocol. A pooled library (20 nM) and a PhiX control v3 (20 nM; Illumina) were mixed with 0.2 N fresh NaOH and HT1 buffer (Illumina) to produce the final concentration at 12 pM each. The resulting library was mixed with the PhiX control v3 (5%, v/v; Illumina) and 600 µL loaded on a MiSeq1 v2 (500 cycles) Reagent cartridge for sequencing. All sequencing procedures were monitored through the Illumina BaseSpace® website.

2.2.3.4 Bioinformatic analysis

Both de-multiplexed R1 and R2 sequencing read (approximately 250 bp in length) files were acquired from the Illumina BaseSpace® website and data processing was performed using the v1.40.5 MOTHUR pipeline (Schloss et al., 2009) following the MiSeq standard operating procedures (Kozich et al., 2013). Paired end reads were generated and clustered prior to assembly into Operational Taxonomic Units (OTUs) tables with 97% identity. Representative sequences from the SILVA 16S rRNA gene database (v132) were used to classify reads into the respective taxonomical level from domain to genus. Subsequent bacterial community structure and similarity were measured using the PRIMER-7 (version 7.0.13, Primer-E, Ivybridge, UK) software package. For analysis of the community data, Bray-Curtis similarity matrix and non-metric multidimensional scaling (nMDS) was used within PRIMER-7. For analysis of transition of microbial communities, average of relative abundance for OTUs for each sample type was calculated in Excel. OTUs that were less than 0.5% of the total 16S rRNA sequence reads were excluded from the list. The OTUs that were above 0.5% approximately represented 98% of the total within each sample group.

2.3 Results

2.3.1 Presence of microorganisms throughout the slaughter floor and abattoir environment

Total viable counts (TVC) of faeces, hides and carcases at three different stages (post-hide pull, post-evisceration and pre-chill) were determined and are shown in Figure 2.1. The mean TVC was the highest in faecal samples for both abattoirs with a mean count of 7.3 $\log_{10}$CFU/g for the integrated (A) and fragmented (B) abattoir. Mean TVC in hide samples was 4.9 and 4.3 $\log_{10}$CFU/cm^2 in abattoir A and B, respectively. Abattoir A removed the hides using a downward hide pull (DHP) system resulting in mean TVC in post-hide pull carcases of 0.4 $\log_{10}$CFU/cm^2. On the other hand, abattoir B removed the hides utilising an upward hide pull (UHP) system and the mean TVC in hides samples was significantly higher ($P < 0.05$) than post-hide pull carcases samples from abattoir A at 1.2 $\log_{10}$CFU/cm^2.

The counts of post-evisceration carcases from abattoir B were also significantly higher ($P < 0.05$) than abattoir A while there was no statistical difference ($P < 0.05$) in mean TVC of pre-chill carcase samples between the abattoirs (Fig. 2.1). Abattoir A and B showed opposing trends of mean TVC in carcases as they progressed through slaughter after the hide was removed. In abattoir A, counts of the carcases significantly increased ($P < 0.05$) moving through slaughter shown by the mean TVC of 0.4 $\log_{10}$CFU/cm^2 in post-hide pull carcases increasing to 1$\log_{10}$CFU/cm^2 in pre-chill carcases. In contrast, the counts of carcases significantly decreased ($P < 0.05$) through the slaughtering process in abattoir B from the mean TVC of 1.2 $\log_{10}$CFU/cm^2 in post-hide pull carcases to 0.70 $\log_{10}$CFU/cm^2 in pre-chill carcases.

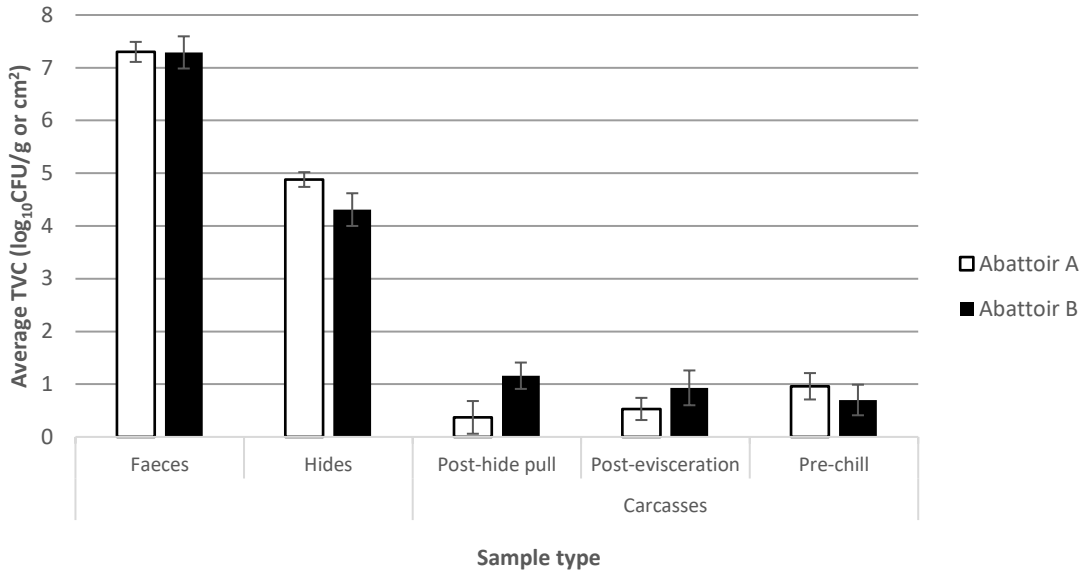

Figure 2.1 Mean total viable count (TVC) of faecal, hide, and post-hide pull, post-evisceration and pre-chill carcase samples collected from the slaughter floor of an integrated (abattoir A) and a fragmented (abattoir B) Australian beef supply chain. Mean TVC of faecal samples are reported in log_{10}CFU/g with the hide and carcase samples reported in log_{10}CFU/cm^2. Each column represents the mean of X samples with error bars representing the standard deviation from the mean.

Direct numerical comparison of TVC in environmental samples between the abattoirs cannot be drawn due to the fact that the sampling sites were not identical. There was a noticeable trend of higher counts in abattoir A from areas surrounding the hide removal station in comparison to the corresponding areas in abattoir B (1 – 3, Fig. 2.2). The mean TVC in the environment between pre- to post-hide pull area was 3.8 and 2.5 log_{10}CFU/cm^2 in abattoir A and B, respectively. Higher mean TVC were observed towards the end of slaughter in abattoir A compared to abattoir B. Indeed, the overall trend for TVC in environmental samples from abattoir A was to increase with proximity to the chillers whereas abattoir B demonstrated reductions in TVC through the processing line. There may be a number of factors for this disparity in counts (e.g. processing line design, speed of the rail, trimming technique, aerosol control management) and the trend cannot accurately compare the realistic differences in counts at each stage. Nonetheless, the highlighting point is that the changes in TVC in the environment similarly reflected the trend of TVC observed in carcases throughout slaughter within each abattoir and that the trends in changes of TVC in abattoir A did not share common patterns with abattoir B.

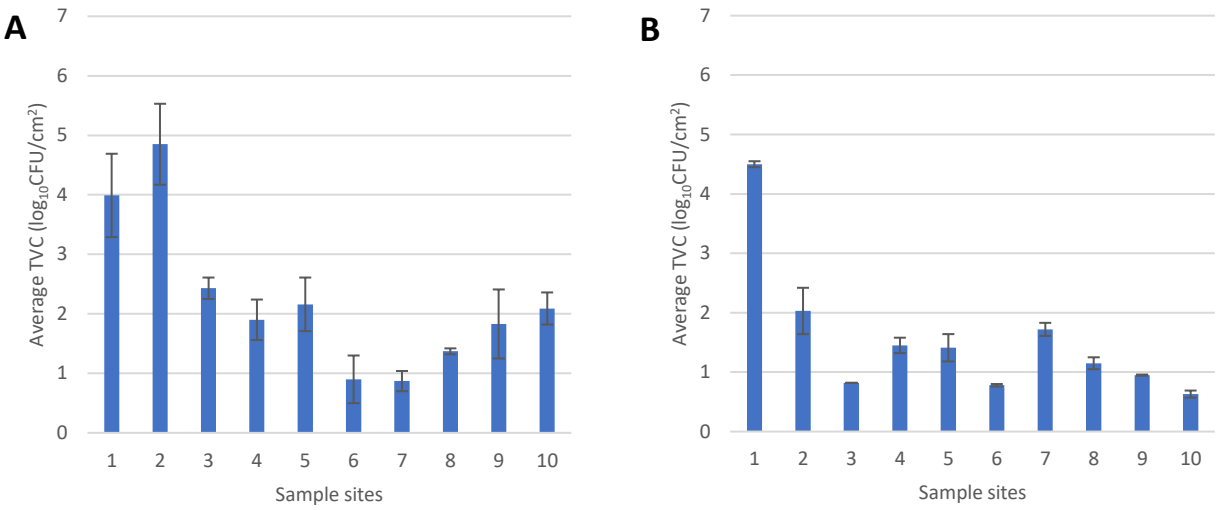

Figure 2.2 Mean total viable count of environmental samples sequentially arranged throughout the slaughter floor in abattoir A (A) and abattoir B (B). In A); 1 & 2 = pre-hide pull, 3 = post-hide pull, 4 = post-evisceration, 5 = pre-scales, 6 & 7 = post-scales, 8 = pre-trim, 9 = trimming, 10 = chiller. In B); 1 = pre-hide, 2 & 3 = post-hide, 4 = pre-evisceration, 5 = post-evisceration, 6 = pre-scales, 7 = post-scales, 8 & 9 = post-trim, 10 = chiller entry.

2.3.2 Changes in composition of the microbiota in faecal, hide and carcase samples

A total of 12 million 16S rRNA gene sequence reads were obtained and were clustered into 122 operational taxonomic units (OTUs) at the Order level. OTUs that were present at less than 0.5% of the total sequence reads within each group were excluded from the list for the following analysis. The 18 most abundant OTUs across all sample types represented at least 98% of the total sequence reads and were plotted to assess changes and transition in the diversity of the microbial community from faeces in the holding pen to the carcases in the chiller. Relative abundance of OTUs in each sample was calculated and the average value of relative abundance for each sample type is shown in Figure 2.3. It is important to highlight that relative abundance of OTUs hereafter is used or referred to as a measurement to describe the change in sequence reads within or between groups.

Similarities in relative abundance of 18 OTUs and changes in community composition in hide and carcase samples between abattoir A and B are shown in Figure 2.3. The hide sample groups in abattoir A were predominantly comprised of Clostridiales (A: 45.6% and B: 41.4%) and Bacteroidales (A: 32.1% and B: 31.6%) while the other OTUs remained below 10% (Fig. 2.3A and 2.3B). In abattoir B, Clostridiales (C: 29.9% and D: 27.0%) and Bacteroidales (C: 19.9% and D: 15.5%) were also predominant but an increase in the presence of Pseudomonadales (C: 25.0% and D: 34.3%) was a notable feature in the hide microbial community (Fig. 2.3C and 2.3D). Additionally, a contrasting trend of Pseudomonadales

between the abattoirs was observed with Pseudomonadales in carcase microflora from abattoir A generally increasing as slaughter progressed (A: 16.1% to 26.4% and B: 22.0% to 30.4%) but was either maintained or decreased in abattoir B as slaughter progressed (C: 24.3% to 24.5% and D: 59.4% to 41.5%). The opposite pattern occurred in the trend of changes in carcase Lactobacillales levels where it decreased in abattoir A (A: 32.6% to 26.7% and B: 16.3% to 9.5%) but increased in abattoir B (C: 17.0% to 27.9% and D: 18.5% to 33.4%).

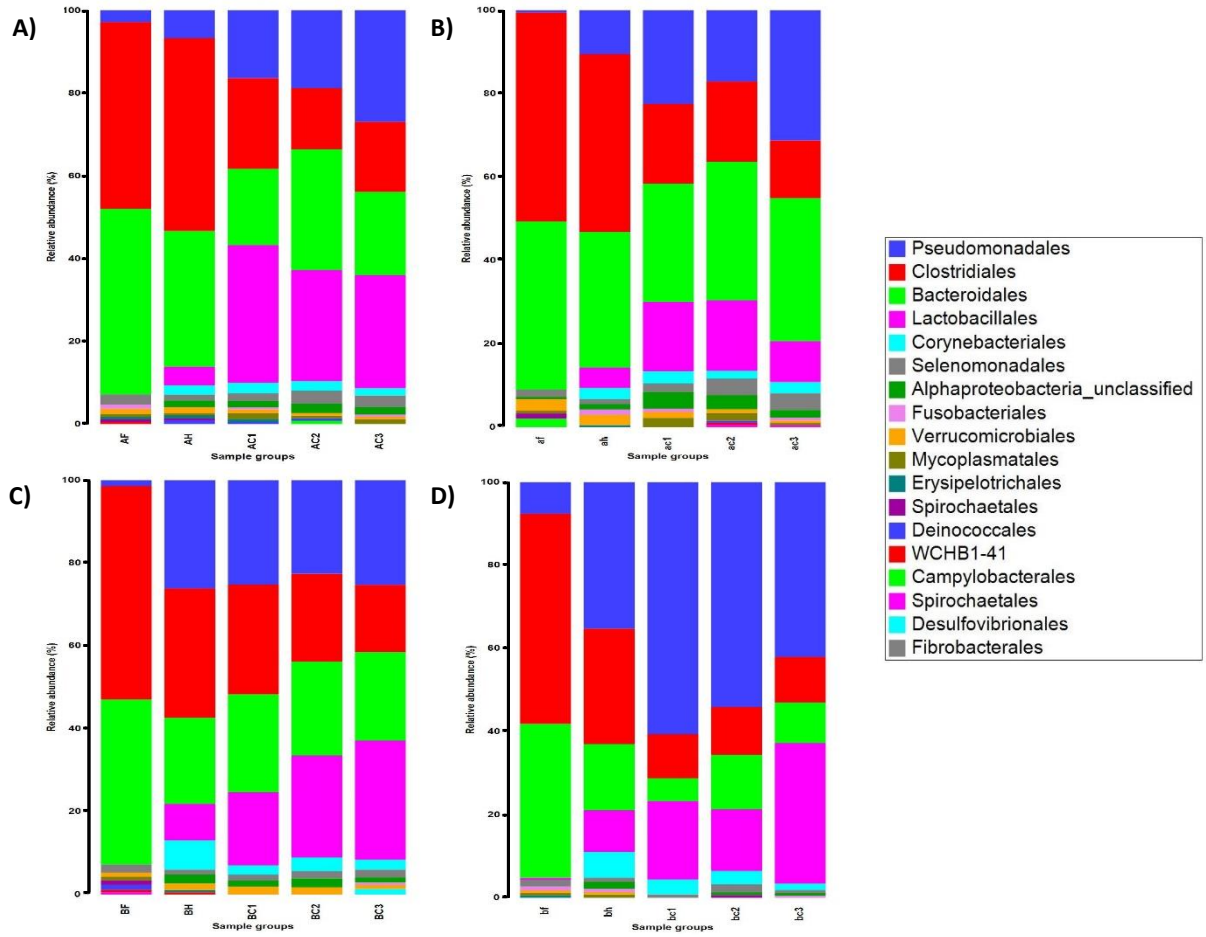

Figure 2.3 Relative abundance (%) of 18 OTUs at the Order taxonomy in faecal, hide and carcase groups. A) first visit to abattoir A; B) second visit to abattoir A; C) first visit to abattoir B and; D) second visit to abattoir B. The groups are labelled in upper and lower case for the first and second visit to the abattoir, respectively. There are three sample groups; faecal (F or f), hide (H or h) and carcase (C or c) groups. C1, C2 and C3 represents post-hide pull, post-evisceration and pre-chill carcases, respectively.

An interesting trend was observed in the transition of microbial contaminants from the hide (BH) to the carcase groups (BC1, BC2 and BC3) in abattoir B which was not observed in abattoir A. On the first visit, BH and post-hide pull carcase group (BC1) shared almost identical community structures. Lactobacillales was the only OTU that had more than 5%

change in the relative abundance; 8.4% to 17.0% (Fig. 2.3C). Relative abundance of Clostridiales and Corynebacteriales decreased by 4.3% and 4.5% from hides to post-hide pull carcases (29.9% to 25.6% and 6.7% to 2.2%), respectively, while the remaining OTUs altered between 0.3% to 2.9%. The changes in bacterial community composition from the hide to post-hide pull carcase in the second visit was more apparent in comparison. Pseudomonadales increased from 34.3% in hide to 52.3% in carcase while Lactobacillales increased from 9.7% to 18.5% (Fig. 2.3D). Bacteroidales and Clostridiales decreased 10.1% and 16.6%, respectively and the relative abundance of remaining OTUs was altered by less than 5%.

2.3.3 Differences in bacterial composition and diversity between all sample types

A curated list of all OTUs at the order level was utilised to generate non-metric multidimensional scaling (nMDS) plots. This ordination physically represents the similarity in composition of the bacterial community between all sample groups (faeces, hides, carcases and environmental) from different sites using the Bray-Curtis distance matrix. For this analysis, OTUs that were present at more than 0.5% of the total sequences in each sample group were selected and added to the list of OTUs. A final list of 27 OTUs that covered 97% of total sequence reads in each sample group was generated. Environmental samples provided a wider range of OTUs in comparison to the three groups above (faeces, hides and carcases). Here, the effect of fragmentation in the supply chain on the indigenous microflora between and within the abattoirs is shown by direct comparison of beta diversity (visualised by nMDS) and bacterial community structure. This subsequently allowed investigation of the relationship between the environment and the carcases during slaughter.

Lactobacillales in both visits to abattoir A and B was predominantly present in the carcase groups and a number of common environmental sites as shown in Figure 2.4. Pseudomonadales was predominantly distributed in the environment in the first visit to abattoir B and in the carcases as well as in the environment in the second visit (Fig. 2.4E and 2.4G). In contrast to abattoir A, Corynebacteriales and Alphaproteobacteria_unclassified were predominantly present in hides, carcases and environmental sites. Intriguingly, three environmental sites (BE4, BE9 and BE10) from the first abattoir B visit were the only sample groups to have Fusobacteriales contributing a high proportion within the communities at 36.5%, 14.5% and 57.2%, respectively (Fig. 2.4C).

In abattoir A, the faecal groups shared 66% (A) and 59% (B) similarity with the composition of bacterial communities in the other sample groups (Fig. 2.4A and 2.4B). The hide group was placed in the closest proximity to the faecal group on nMDS in both visits indicating that the hide group shared the most similar microbiological profiles with the faecal group. In abattoir B, the faecal group shared less similar community composition with the rest of the samples in comparison to abattoir A (C: 54% and D: 56%, Fig. 2.4). Some consistency between the sample groups was observed across abattoir A visits where the environmental sites 1 and 2 were more than 80% similar to the hide groups (Fig. 2.4A and 2.4B). In the first abattoir A visit, all three carcase groups were approximately 70% similar to the composition of the hide group and were more than 80% similar to environmental sites 5, 7 and 8 (Fig. 2.4A). On the second visit, the hide group shared at least 80% similarity in the bacterial community composition with the carcase groups in addition to environmental sites 5 and 7 (Fig. 2.4B). In abattoir B, the hide groups were found in the closest proximity to the carcase groups in nMDS during both visits sharing more than 80% similarity in community composition (Fig. 2.4C and 2.4D). Interestingly, all sample groups excluding the faecal group were at least 70% similar on the second visit to abattoir B meaning that three environmental sites (1, 3 and 5) were consistently found to be more than 70% similar (75 – 76 %) to the carcase groups in abattoir B (Fig. 2.4D).

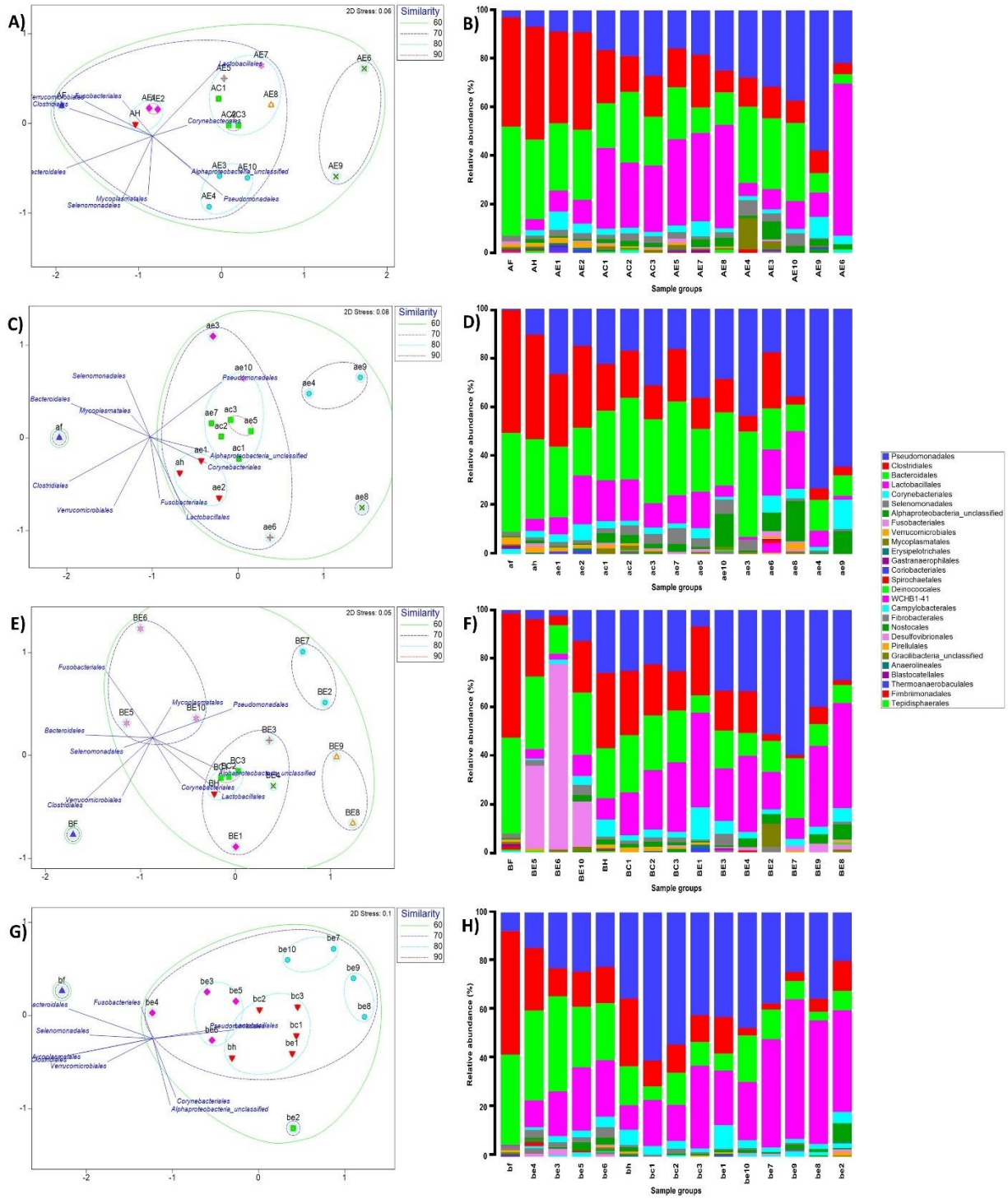

Figure 2.4 Non-metric multidimensional scaling (nMDS) ordination showing the similarity in bacterial community composition using Bray-Curtis and overlay vectors of the top 10 OTUs (left) and relative abundance of 27 Order OTUs in all sample groups (right). First abattoir A visit is shown in A) and B); and second visits in C) and D). First visit to abattoir B in C) and D); and second visit in E) and F). Physical distance between each group in the ordination indicates the similarity of the two or multiple groups. Displacement of the OTUs in the same ordination represents the distribution of each OTU. Faecal (F or f), hide (H or h), carcase (C or c) and environmental (E or e) groups are labelled in upper and lower case for the first and the second visit for each abattoir, respectively.

2.4 Discussion

This was the first study to utilise 16S rRNA sequence-based in a combined effort with traditional microbiology to monitor the flow of bacterial communities and compare the microflora between two Australian beef abattoirs with a different supply chain. Chandry et al. (Chandry, 2016) carried out studies in Australia using 16S rRNA amplicon sequencing and traditional methods where the group tracked and collected samples from matching hides and carcases in addition to collection of airborne bacteria at three different sites near the sampling stations. The study found that a substantial amount of airborne microflora derived from the hide and the aerosols played a role in contamination of the carcases (Burfoot et al., 2006, Cenci-Goga et al., 2007, Madden et al., 2004). There are other studies that have used NGS techniques to investigate sources of contamination in beef processing abattoirs (De Filippis et al., 2013, Hultman et al., 2015, Chandry, 2013, Chandry, 2016). However, none of these studies have monitored flow and transition of microbial populations between the animals the abattoir environment throughout beef processing. Therefore, this study is the first of its kind and is aimed at understanding the flow of bacterial communities through the first stage of beef processing with an emphasis on the level of supply chain integration.

The combined results of bacterial community transition and composition similarities demonstrated that the movement of contaminants in abattoir B conformed to the traditional pathway of contamination more than abattoir A, i.e. faeces to hides to carcases (Arthur et al., 2010, Collis et al., 2004, Fegan et al., 2005, Barkocy-Gallagher et al., 2003). These abattoirs harboured similar groups of beef-associated microbes such as Pseudomonadales, Lactobacillales, Corynebacteriales, Clostridiales and Bacteroidales despite abattoir B receiving herds of beef cattle from multiple producers with varying traits as opposed to continuous supply of cattle with comparably consistent production traits (De Filippis et al., 2013, Habimana et al., 2010, Zaheer et al., 2017b). However, the microbiological differences between the abattoirs were evident in the prevalence of these OTUs within the bacterial community. For instance, the hide samples from abattoir B had higher level of Pseudomonadales than abattoir A. This may have contributed to higher dominance of Pseudomonadales in carcase samples from abattoir B due to the close relationship that the hide group had with the carcase groups. Pseudomonadales and Lactobacillales were consistently found to be one of the predominant OTUs in carcase, hide and environmental samples in abattoir B compared to abattoir A. It wasn’t surprising to find these OTUs at such

high proportion because of multiple beef spoilage bacteria such as *Pseudomonas* spp., *Lactobacillus* spp. and *Leuconostoc* spp. belong to the OTUs and that Pseudomonadales are ubiquitous soil bacteria that can spread in the processing environment (Ercolini et al., 2006, Koutsoumanis et al., 2006, Nychas et al., 2008).

The two abattoirs utilised different hide pulling systems with abattoir A employing a DHP system in their chain whereas abattoir B has an UHP system. Carcases at both abattoirs were suspended on a single processing line by the hind limbs. DHP pulls the hide down from the hind leg to the neck after initial opening near the rump region and UHP removes the hide up from the shoulder to the hind leg. The general consensus within the meat industry is that UHP leads to increased microbial load on the carcasses in comparison to DHP. UHP provides an opportunity for microorganisms to attach to otherwise sterile muscle tissue as the hide is vigorously plucked over the carcase. There is a lack of published data regarding this aspect, but different studies have shown that hide contamination generally is transferred to the carcases during processing (Bell, 1997, Chopyk et al., 2016, Svoboda et al., 2013). A study by Kennedy et al. (2014) investigated the microbial effect of the two hide pulling systems using TVC and found that the overall contamination of the carcase forequarter was not substantially affected by changing the direction of hide pull. Contamination increased in the flank and chuck after UHP and the shin and brisket after DHP leading to different products being contaminated by the change in the direction of hide pull. Implementation of hygienic hide removal practices (e.g. washing hands and knives between hide/carcase contact) were concluded as a more important factor for preventing overall forequarter contamination than the hide pulling method (Kennedy et al., 2014).

This study produced an opposite outcome to the previous study and suggested that UHP system may induce increased microbial contamination in the forequarter of de-hided carcases in comparison to DHP system at least by half a log per cm^2 on average. Here, the analysis of 16S rRNA profiles revealed that there is a profound and stronger microbial community similarity between the carcase and the hide groups in abattoir B (with UHP) than A (with DHP). Some studies have found that hindquarter of beef carcases harboured higher concentration of microbes (Charlebois et al., 1991, Phillips et al., 2012, Gill et al., 1998), meaning that greater differences may have been detected if sampling of hindquarters was conducted as part of this study. It is possible that airborne contamination in such a dynamic working vicinity could be playing a part and it would be beneficial to consolidate the findings

from this study (Burfoot et al., 2006, Cenci-Goga et al., 2007, Madden et al., 2004, Chandry, 2016).

It is clear that carcases in abattoir A accumulated more bacteria on the forequarter of the carcases as they moved through slaughter than abattoir B. Such a trend of TVC on carcases throughout slaughter in abattoir B suggests that the abattoir is less efficient at minimising the transfer of microbial contaminants from hide to carcase but may have counteractive management practices downstream to reduce the carcase bacterial load before chilling. Abattoir B was able to simultaneously reduce and maintain the level of microorganisms in the environment throughout slaughter after the hide pulling station. Analysis of 16S gene amplicons indicated that the microbes in the slaughter environment are likely to contribute to the carcase microflora. There is an ecological interaction between the bacterial communities in the carcase and the environment, but it is implausible to conclude that the microbes from the sampled environmental sites were the only contributor to the change of the carcase microflora in abattoir A or B (Kim and Yim, 2017, Yang et al., 2017). With that in mind, comparably lower levels of TVC in the environment in abattoir B may have contributed to progressive lowering of the carcase count in abattoir B. In contrast, removal of the hide in abattoir A leads to less contamination of the carcase than in abattoir B. However, the abattoir appeared to be less efficient at maintaining lower TVC in carcases and the environment throughout slaughter.

2.5 Conclusion

The results from this study demonstrated that common meat-associated microorganisms are found throughout slaughter regardless of the level of integration in the supply chain of beef cattle. Microbiological differences were observed in the composition of the bacterial communities, especially in the environment, and the relationships between the different sample types were characteristic to individual abattoirs. The integrated abattoir showed efficient control of microbial contamination at the hide puller but was less efficient at controlling contamination as the carcases moved through the remainder of the slaughter process. In contrast, contamination of carcases in the fragmented abattoir conformed more to the traditional route of contamination (from faeces to hides then to carcases) and it is likely that the direction of hide pull in abattoir B enhances the transfer of bacteria from hides to carcases. This study demonstrated that 16S rRNA amplicon-based analysis can be a powerful tool for understanding microbial ecology and specific interactions of microbial contaminants

in commercial beef processing settings that may ultimately assist in controlling contamination events during slaughter. In a broad sense, 16S rRNA gene sequencing can potentially be used to manipulate or increase the presence of favoured non-harmful cohort of bacteria or identify bioindicator/s for rapidly determining the microbiological quality of carcases. It is important to highlight that the microbiome at the end of slaughter is likely to be additionally affected as the carcases are processed further within a boning room. Further studies investigating the complete slaughter process including chilling and boning would facilitate greater understanding of the role of microbial ecology in the beef industry.

Chapter 3. Bacterial community analysis using 16S rRNA amplicon sequencing in the boning room of Australian beef export abattoirs

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3.1 Introduction

A better understanding of bacterial contamination through beef cattle processing can greatly enhance the quality and safety of the beef products. Processing of beef cattle can be divided into two phases; slaughter and boning, with chilling of the carcases providing the link between the two. During slaughter, live animals are processed into carcases then stored in the chiller overnight for temperature no more than 7°C on all carcase surfaces as regulated (Australian and New Zealand Food Regulation Ministerial Council, 2007). The chilled carcases are then further processed into beef products during the boning phase by removing or cutting portions off of the carcase into smaller parts. The processing of cattle employs good hygienic practices in an attempt to reduce carcase and product contamination as the product moves from slaughter through to final product. There are events throughout the process (e.g. hide pulling) where cross-contamination may occur. Transmission of microbial contaminants through slaughter has been extensively investigated by enumeration of microbes and/or selected groups of indicator organisms using traditional microbiology techniques (Elder et al., 2000, Fegan et al., 2005, Koohmaraie et al., 2005, Nou et al., 2003, Stromberg et al., 2015a). Changes in the bacterial numbers throughout slaughter suggested that faecal microbial contaminants on hides transferred onto carcases and led to cross-contamination between the carcases during slaughter. Microbial contamination through boning has also been examined by observing the presence of microbes on meat, environmental surfaces and personnel equipment (Charlebois et al., 1991, Sheridan et al., 1992). The findings in the boning room indicated that contact with environmental surfaces generally associated with higher bacterial counts in the beef trim. Hygienic practices and

thorough sanitation of the equipment were concluded as an important factor to controlling microbial contamination in the boning room. It is inevitable that slaughtered carcases and beef products are continuously exposed to microbial contamination until the meat products are packaged for distribution (Bell, 1997, Eisel et al., 1997).

Microbial contamination of carcases and boned products throughout beef processing has been reported using total viable counts (TVCs) and counts of selected groups of bacteria such as coliform count, *Campylobacter*, *Escherichia coli*, *Pseudomonas*, *Salmonella* and *Listeria* spp. (Phillips et al., 2006, Pointon et al., 2012, Sheridan et al., 1992, Svoboda et al., 2013). Culture dependent techniques can inadequately define complex ecological and structural functions or symbiotic relationships of beef associated microbiota (Kergourlay et al., 2015, Lagkouvardos et al., 2016). These conventional microbiological methods could allow some bacterial species to become dominant and suppress growth of other species. In contrast, culture independent techniques that utilise high throughput sequencing technologies can efficiently detect the presence of microbes in a given environment with a more accurate representation of the true proportions of bacterial populations (Bakhtiary et al., 2016). Sequencing of a target sequence (e.g. hypervariable regions of 16S rRNA) from DNA extracted from raw samples avoids biases of selective growth of the cells (Brightwell et al., 2006, Kang et al., 2019). Additionally, transmission of bacteria at the population level and changes in community composition can be described in greater detail by identifying 16S rRNA profiles and monitoring the changes in relative abundance of operational taxonomic units (OTUs) (Cao et al., 2017, Schloss et al., 2009).

In a previous study, the movement of bacterial populations through the slaughter phase in an integrated and a fragmented Australian beef export abattoir was assessed by detecting the changes in frequency of 16S rRNA sequences (Kang et al., 2019). Full integration in the beef supply chain ensures that the abattoir has consistency in the animals being processed by receiving cattle that were produced under the same production system. Fragmented abattoirs operate by processing of cattle from multiple different producers. The previous study found that upward hide pulling systems contributed to the bacterial communities on carcases at the end of slaughter by transferring hide contaminants onto the carcases. Bacterial communities on environmental surfaces had a major contribution to final microbiota on carcases, whereas hide to carcase contamination was minimised by downward hide pull. These findings indicated that processing practices can have a substantial influence on both the concentration

and composition of bacterial population on pre-chill carcases at the end of slaughter. The bacterial communities on pre-chill carcases may or may not contribute to the microbiological ecology in the final beef trim, but the relationship is yet unknown.

The study by Kang et al. (2019) investigated microbial community structure on carcases through slaughter that represented the initial phase of beef processing. The previous study demonstrated that fragmentation in the chain did not lead to the presence of different types of bacterial populations throughout slaughter phase. However, changes in composition of the communities varied between the fragmented and integrated abattoirs (Kang et al., 2019). The effect of fragmentation in the supply chain on the microbial ecology in the boning room is yet unknown. This study was aimed to analyse changes in bacterial communities and describe the transmission of contamination through the boning room in an integrated and a fragmented Australian beef abattoir. Results from this current study expand on the previous study by providing a greater insight into how the bacterial communities change during boning and what may contribute to these compositional changes by monitoring 16S rRNA sequences.

3.2 Materials and Methods

3.2.1 Sample collection and experimental design

Samples were collected from the boning room at two abattoirs with different levels of integration in the supply chain. An integrated abattoir (abattoir A) receives the animals that were produced using the same production regime and from a small number of sources and a fragmented abattoir (abattoir B) processes cattle that derived from multiple producers. Abattoir A and B were each visited twice over a period of five months (November 2018 – March 2019) and each visit comprised of two sampling days collecting 90 samples in total. The first sampling day was spent collecting 15 pre-chill carcase samples. Samples collected on the second day consisted of 15 chilled carcases, five forequarters, five hindquarters, 30 beef trim (manufacturing beef) and 20 environmental samples in the boning room. In abattoir A, trim samples from forequarters (n=12) and hindquarters (n=12) and boxes of manufacturing beef (n=6) were collected. In abattoir B, trim samples were collected from forequarters (n=16) and hindquarters (n=14). Trim samples with larger than 100 cm^2 surface area was obtained. Carcase and beef trim samples were not matched but the samples were collected from the same lot of animals. The structure of the processing line was comparable between the two abattoirs with similar reduction of carcases throughout boning.

Environmental sampling surfaces were in sequential order through the boning process but were not identical between the abattoirs. Ten environmental surfaces were sampled twice in abattoir A but only five surfaces (four samples per surface, n=20) were selected in abattoir B due to availability of space. The samples in the boning room were collected after sanitation at the beginning of a typical boning shift in the morning. Sanitation procedures were not recorded and directly compared between the abattoirs but were similarly carried out between the abattoirs using sprays and surface disinfectant.

Carcase samples were collected aseptically using large area (3000 cm^2) sampling technique as previously described (Chandry, 2016). Environmental samples were obtained by swabbing an area covering approximately 900 cm^2. For beef trim, approximately 100 cm^2 of the most outer fat surface was collected in a large stomacher bag. Swabbing of the surface samples was carried out using Whirl-Pak® Speci-Sponge® (Nasco, Wisconsin, US) pre-moistened in 25 mL of sterile buffered peptone water (BPW; Oxoid, Hampshire, UK). All samples were immediately placed on ice and stored at 4°C until arrival at the laboratory. Samples collected with a sponge had an additional 75 mL of BPW added prior to being stomached for 30 seconds at eight strokes per second (Interscience, Saint Nom, France). Approximately 100 cm^2 of the most outer layer of trim samples was excised and added to 100 mL of BPW. The samples were then stomached for a minute. Following stomaching, aliquots from the samples were kept for 16S rRNA sequencing analysis at -80°C and the remaining portion of the bacterial-BPW suspension was used for microbiological analysis.

3.2.2 Enumeration of microbial cells

Bacterial-BPW suspensions were used for TVC by plating 100 µL of serial 10-fold dilutions prepared in 0.85% saline (Oxoid) onto tryptic soy agar (TSA; Oxoid) and incubated at 25°C for 72 – 96 hours. Statistical significance between two means for continuous data was calculated by using *t* test via GraphPad Prism version 8.0 for Windows, https://www.graphpad.com/.

3.2.3 Preparation and analysis of 16S rRNA amplicons

3.2.3.1 DNA extraction

A 40 mL aliquot from the bacterial-BPW suspension of each sample was centrifuged to pellet the cells. The pellets were washed twice with ultrapure H_2O prior to DNA extraction using the QIAamp PowerFecal DNA kit (Qiagen, California, US) following the manufacturer's

protocol with modifications in bead beating step. The bead beating step was adopted from Kang et al. (2019). Briefly, the bead beating cycle involved a total of three minutes of beating with a minute rest after the first and second beating. During that minute of rest, the bead tubes were placed in a heat block at 65°C. After the three minutes of beating, the bead tubes were incubated for 10 mins at 65°C in a heat block and the bead beating cycle was repeated.

3.2.3.2 Preparation of 16S rRNA library

Extracted DNA was used to construct a library of 16S rRNA amplicons targeting the V4 region using the protocol from a previous study (Kozich et al., 2013). Briefly, 5 µL from each DNA template was amplified with dual-index primers via polymerase chain reaction (PCR) and the amplicons were manually normalised by comparing the intensity of the DNA band against the GeneRuler 100 bp Plus DNA Ladder (ThermoFisher, Victoria, Australia). The DNA materials were stained with ethidium bromide and visualised under ultraviolet light in 2% agarose gel under ultraviolet light. Each template contained barcode sequences at the 5' and 3' of the PCR amplicon to enable demultiplexing of sequence reads. Approximately 50 ng of rRNA DNA amplicon from each sample was combined to create a pooled library for sequencing. A microbial community DNA standard, ZymoBIOMICS™ (Zymo Research, California, US), was normalised using the same approach and added to the library. The library was purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, California, US) following the manufacturer's instruction. The concentration of amplicons was measured before and after purification using the Qubit® 2.0 Fluorometer (Life technologies, California, US). A NanoPhotometer® (Implen, California, US) was used to measure the purity and the final purified pooled library was sent for sequencing.

3.2.3.3 Sequencing using Illumina MiSeq

Sequencing of the pooled library of amplicons was performed at the Ramaciotti Centre for Genomics, University of New South Wales using the Illumina MiSeq platform (Illumina, California, US) with a paired-end 300 base pair sequencing protocol. A pooled library (20 nM) and a PhiX control v3 (20 nM; Illumina) were mixed with 0.2 N fresh NaOH and HT1 buffer (Illumina) to produce the final concentration of 12 pM each. The resulting library was mixed with the PhiX control v3 (5% v/v; Illumina) and 600 µL loaded on a MiSeq1 v2 (500 cycle) reagent cartridge for sequencing.

3.2.3.4 Bioinformatic analysis

Demultiplexed sequencing read (approximately 250 bp in length) files were retrieved from the Illumina BaseSpace® website. Bioinformatic processing of raw sequence data was performed using the v1.40.5 MOTHUR pipeline (Schloss et al., 2009) following the MiSeq standard operating procedures (Kozich et al., 2013). The paired end reads were clustered and assembled into Operational Taxonomic Unit (OTU) tables with 97% as the cut-off for identification. The taxonomic classification of OTUs was performed using SILVA 16S rRNA database (v132). For analysis of relationships between bacterial structures, multivariate function and non-metric multidimensional scaling (Bray-Curtis similarity matrix) were executed using PRIMER-7 (version 7.0.13, Primer-E, Ivybridge, UK) software package. For analysis of transition in bacterial community composition, average relative abundance for OTUs was calculated in Microsoft Excel (v16.0) and used to create bar plots within PRIMER-7.

3.3 Results and discussion

3.3.1 Level of contamination on beef carcases, trim and environmental surfaces

Total viable count on carcases and beef trim are displayed in Figure 3.1 and changes in TVC on the environmental surfaces through boning are shown in Figure 3.2 (abattoir A) and 3.3 (abattoir B). Abattoir A utilises spray chilling and abattoir B uses air chilling. Both abattoirs produced pre-chill carcases with similar mean TVC of 1.2 and 0.9 $\log_{10}$ CFU/cm^2 in abattoir A and B respectively. Also, post-chill carcases had a similar mean TVC of 0.8 and 0.7 $\log_{10}$ CFU/cm^2 in abattoir A and B, respectively. This indicated that chilling had no significant reduction on the count regardless of the different chilling methods. Kinsella et al. (2006) reported similar findings by testing a commercial beef carcase chill unit fitted with a water spraying cycle that was comparable to the spray chilling method of abattoir A. The group showed that there was 0.2 $\log_{10}$ CFU/cm^2 reduction in TVC on post-chill carcase and that no significant differences was found between the control and spray chilled carcases. A study by

Reid et al. (2017) reported that chilling of carcases had reduced the TVC by an average of 0.4 $\log_{10}$ CFU/cm^2 over 24 hours.

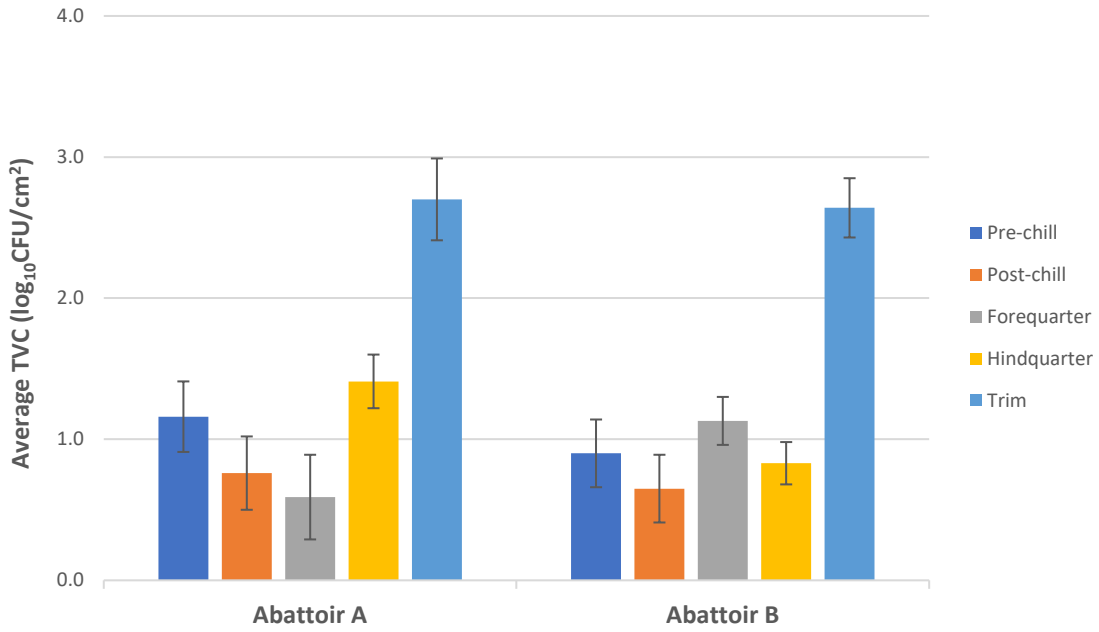
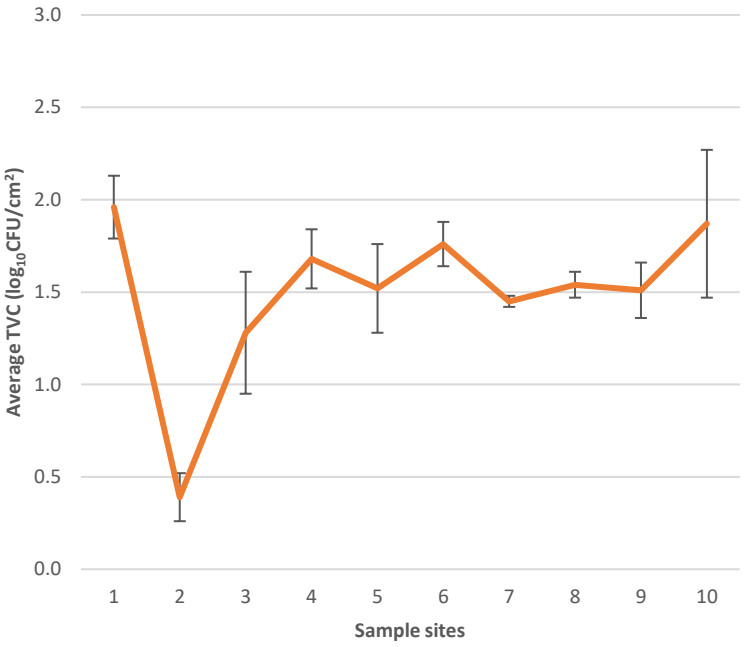


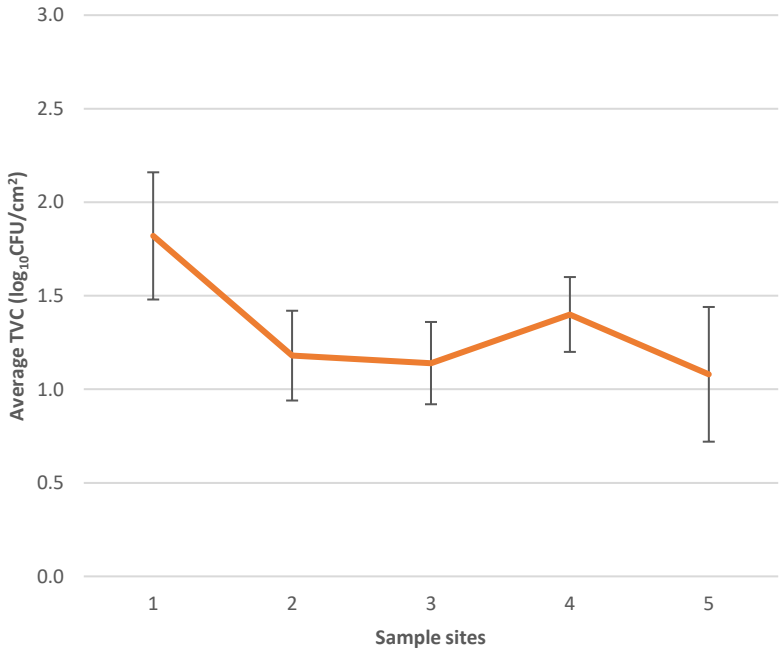
Figure 3.1 Total viable count of pre-chill, post-chill, fore-quarter, hind-quarter carcases and beef trim in abattoir A (integrated supply chain) and abattoir B (fragmented supply chain). Average count in each sample group is presented in $\log_{10}$CFU/cm^2.

Mean TVC on environmental surfaces ranged from 0.4 – 2.0 $\log_{10}$ CFU /cm^2 in abattoir A and 1.1 – 1.8 $\log_{10}$ CFU /cm^2 in abattoir B (Fig. 3.2 and 3.3). The level of microbial contamination on the environmental surfaces was considerably lower than the other studies in which TVC ranged from 1 – 6 $\log_{10}$ CFU/cm^2 (Kim and Yim, 2017, Sheridan et al., 1992). Observations from the previous studies generally indicated that surfaces that regularly came in contact with the animals (e.g. stainless steel saws, trimming boards, personal equipment) were likely to have higher TVC's than surfaces with minimal contact (e.g. walls) (Cetin et al., 2006, Eisel et al., 1997, Widders et al., 1995). Widders et al. (1995) found that relative contribution of the environmental surfaces to beef contamination increased as the level of contaminants on carcases decreased. More recent studies demonstrated that increasing numbers of bacteria on environmental surfaces were reflected by the increasing trend of TVC on the carcases through processing in Australian beef export abattoirs with varying integration level in the supply chain (Kang et al., 2019).



Sequential order	Sample site
1	Grading stand
2	Pre-quartering wall
3	Conveyor belt - South
4	Conveyor belt – North
5	Primal belt – South
6	Primal belt – North
7	Ribs conveyor belt
8	Manufacturing beef conveyor belt
9	Manufacturing beef packaging bench
10	Primal pre-packaging belt

Figure 3.2 Total viable count on environmental surfaces in the boning room of integrated abattoir (A) and the list of environmental sites in sequential order with standard deviation. The conveyor belt carrying the carcases into the boning room split into two separate lanes; South and North. The lanes emerged back into a single lane before pre-packaging section (10).



Sequential order	Sample site
1	Quartering station tiles
2	Boning table 1
3	Table between boning table 2 and 3
4	Boning table 6
5	End of line conveyor

Figure 3.3 Total viable count on environmental surfaces in the boning room of fragmented abattoir (B) with standard deviation and the list of environmental sample sites in sequential order.

In this study, manufacturing beef samples contained the highest TVC in both abattoirs. Abattoir A and B produced beef trim with similar mean TVC at 2.7 and 2.6 $\log_{10}$ CFU/cm^2, respectively (Fig. 3.1). These levels of contamination were in a similar range with counts in boneless beef from previous Australian national surveys and imported beef from other countries such as New Zealand and Uruguay indicating that the counts were typical for Australia (Bosilevac et al., 2007, Phillips et al., 2012). Abattoir A and B were able to minimise contamination of post-chill carcases coming into the boning room to below 1.0 $\log_{10}$ CFU/cm^2 and managed to maintain the level of microbial contaminants at below 2.0 $\log_{10}$ CFU/cm^2 during the first few hours of the boning process. This suggests that the increased count in beef trim samples could be due to accumulative contamination from surfaces that are consistently in contact with the meat products during boning (e.g. boning tables, conveyor belts, packing benches). Frequent sanitation of the surfaces may contribute to reduction in count on finished products by minimising build-up of microbial concentration.

3.3.2 Changes in the composition of bacterial communities on beef carcases, trim and environmental surfaces through boning

Raw 16S rRNA sequence data resulted in approximately 11 million reads across all abattoir visits. The sequence reads at the Order taxonomy level gave a total of 242 operational taxonomic units (OTUs). OTUs that contributed more than 0.5% of the total sequences in each sample group were selected to generate a list of the most abundant OTUs. The list contained 26 OTUs for abattoir A and 21 OTUs for abattoir B. These OTUs represented approximately 97% of total sequence reads in each sample group. The relative abundance of OTUs in each sample was calculated and average value of relative abundance for each sample type (carcase and trim) was used to generate bar plots (Fig. 3.4). It is important to note that the OTUs may be generated from both living and dead cells, and that the 16S amplicon data is not an absolute representation. The focus of this study was placed on application of using 16S rRNA sequencing to investigate beef contamination by observing general changes in bacterial populations through the boning room. Classification at the Order level for the following analysis was elected to provide continuity with a previous study that examined the slaughter phase (Kang et al., 2019). The approach taken in this study does not permit the identification of specific bacteria of food safety interest, however it is expected that the results may yield insights into how pathogens may move through cattle processing and therefore how best to avoid this occurring.

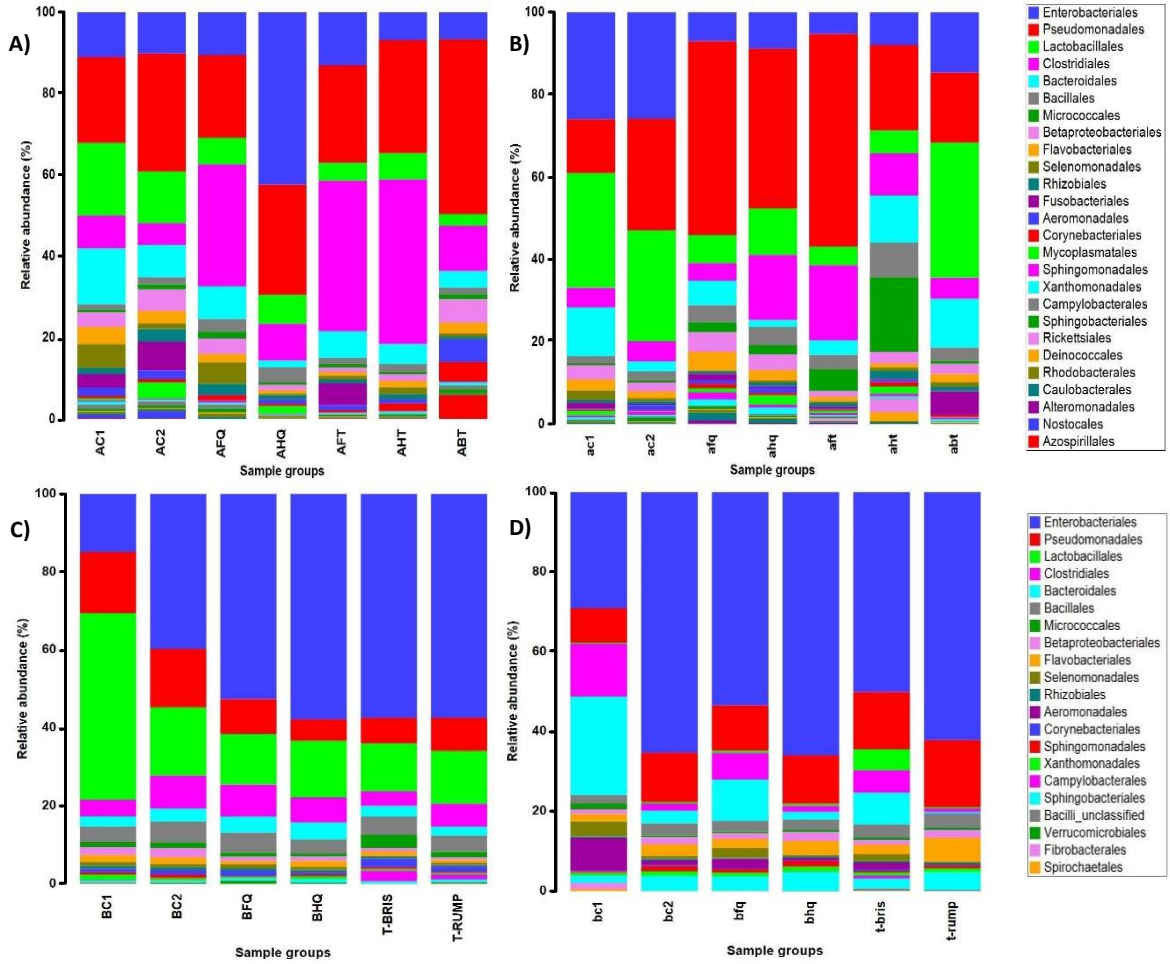


Figure 3.4 Relative abundance of OTUs that presented at a prevalence greater than 0.5% in each sample group in the boning room from the; A) 1st abattoir A visit, B) 2nd abattoir A visit, C) 1st abattoir B visit, and D) 2nd abattoir B visit. Abattoir A visits shared 26 OTUs (top right) and abattoir B visits had 21 OTUs (bottom right). The groups were labelled in capital and lower case to distinguish the first and second visit to the abattoirs, respectively, and A (integrated) or B (fragmented) was used as a prefix for classification of abattoirs. The sample groups are; pre- (C1) and post-chill (C2) carcases, fore- (FQ) and hind-quarter (HQ) carcases, and different beef trim groups. Meat from forequarter trim (FT), hindquarter trim (HT) and boxed trim (BT) were collected in abattoir A. Trim from brisket (T-BRIS) and rump (T-RUMP) were collected in abattoir B.

Kang et al. (2019) investigated the movement of 16S rRNA profiles and microbial populations throughout slaughter at the same abattoirs. Pre-chill carcases at the end of slaughter were also collected in this study from the same abattoirs to provide continuity and comparative analysis of bacterial contamination between the slaughter floor and the boning room. Subsequently, a combined set of population data throughout slaughter and boning would provide a comprehensive map of bacterial flow in the Australian beef processing industry. Enterobacteriales may have been less prominent on the carcases during the slaughter visits in the previous study by Kang et al. (2019) but the top 4 most dominant OTUs

on pre-chill carcases in the previous study were also frequently present on carcases and manufacturing beef in high proportions in this study. Analysis of 16S sequencing data indicated that bacterial populations from slaughtering process can survive through chilling on the carcases and contribute to microbiological make-up in beef trim. It also demonstrated that there are microbiological variations between the abattoirs and that these compositional variations early in the boning room play an important role in determining the final microbiota in the trim (Fig. 3.4).

An explanation for the dominance of Enterobacteriales in abattoir B remains uncertain. As previously mentioned, this study did not include sampling of personnel equipment and aerosols or other potential factors through chilling and boning that could be contributing to these contamination trends. Other vectors of contamination such as cutting knives, processing floor and contaminated water sources have been suggested as factors to microbial contamination of beef carcases and trim (Burfoot et al., 2006, Chandry, 2016, Eisel et al., 1997, Madden et al., 2004). Inclusion of a larger pool of factors coupled with 16s rRNA amplicon sequencing in future investigations of microbiological communities in beef processing may assist in understanding these trends in greater depth.

In abattoir A, relative abundance of Bacteroidales, Clostridiales, Enterobacteriales, Lactobacillales and Pseudomonadales represented 70% and 64% of the pre-chill carcases' community in the first and second visit respectively (Fig. 3.4A and 3.4B). In the first visit to abattoir B, Enterobacteriales, Pseudomonadales and Lactobacillales made up 75% of the community on pre-chill carcases (Fig. 3.4C). In the second visit, Aeromonadales, Bacteroidales, Clostridiales, Enterobacteriales and Pseudomonadales contributed 80% of the community (Fig. 3.4D). Spray chilling did not cause a substantial variation in the transition of bacterial populations from pre-chill to post-chill carcases (>80% similarity) in both visits to abattoir A (Fig. 3.5C and 3.5D). The dominant OTUs (Bacteroidales, Clostridiales, Enterobacteriales, Lactobacillales and Pseudomonadales) continued to contribute high proportions in the communities on post-chill carcases (Fig. 3.4A and 3.4B). Air chilling in abattoir B had no effect on the overall structure of communities on post-chill carcases on the first visit (Fig. 3.5C). However, pre- and post-chill carcases shared less similarity in comparison on the second visit which may be due to a substantial increase in relative abundance of Enterobacteriales from 28% to 62% (Fig. 3.4D and 3.5D).

Enterobacteriales was the most predominant group on post chill-carcases from abattoir B with a mean relative abundance of 38% and 62% in the first and second visit, respectively (Fig. 3.4C and 3.4D). Carcases and beef trim shared greater than 80% similarity in the bacterial community composition in both abattoir B visits, with Enterobacteriales as the most dominant OTU (Fig. 3.4 and 3.5). The changes in composition of communities through boning in abattoir A fluctuated in comparison. For instance, Clostridiales, Lactobacillales and Pseudomonadales continued to be predominant in carcases and trim samples but changes in relative abundance differed between the visits (Fig. 3.4A and 3.4B). Differences in day-to-day sanitation condition could result in variations in the residential bacteria on a daily basis throughout the boning room as these bacterial groups possess varying resistance to different methods of sanitation (Habimana et al., 2010, Moretro and Langsrud, 2017). This may explain why variations in bacterial load were observed on the same surfaces on different sampling days after sanitation (Stellato et al., 2016). For instance, Pseudomonadales were present at higher levels near trimming on the first visit (Fig. 3.4A) but higher near quartering stations on the second visit (Fig. 3.4B). The OTU plot in abattoir B suggests that the carcases carried in Enterobacteriales as the bacteria dominated the carcase microflora. It is likely that Enterobacteriales became also dominant residential bacteria in the environment and contributed in accumulation of contamination in the beef products throughout boning in both visits.

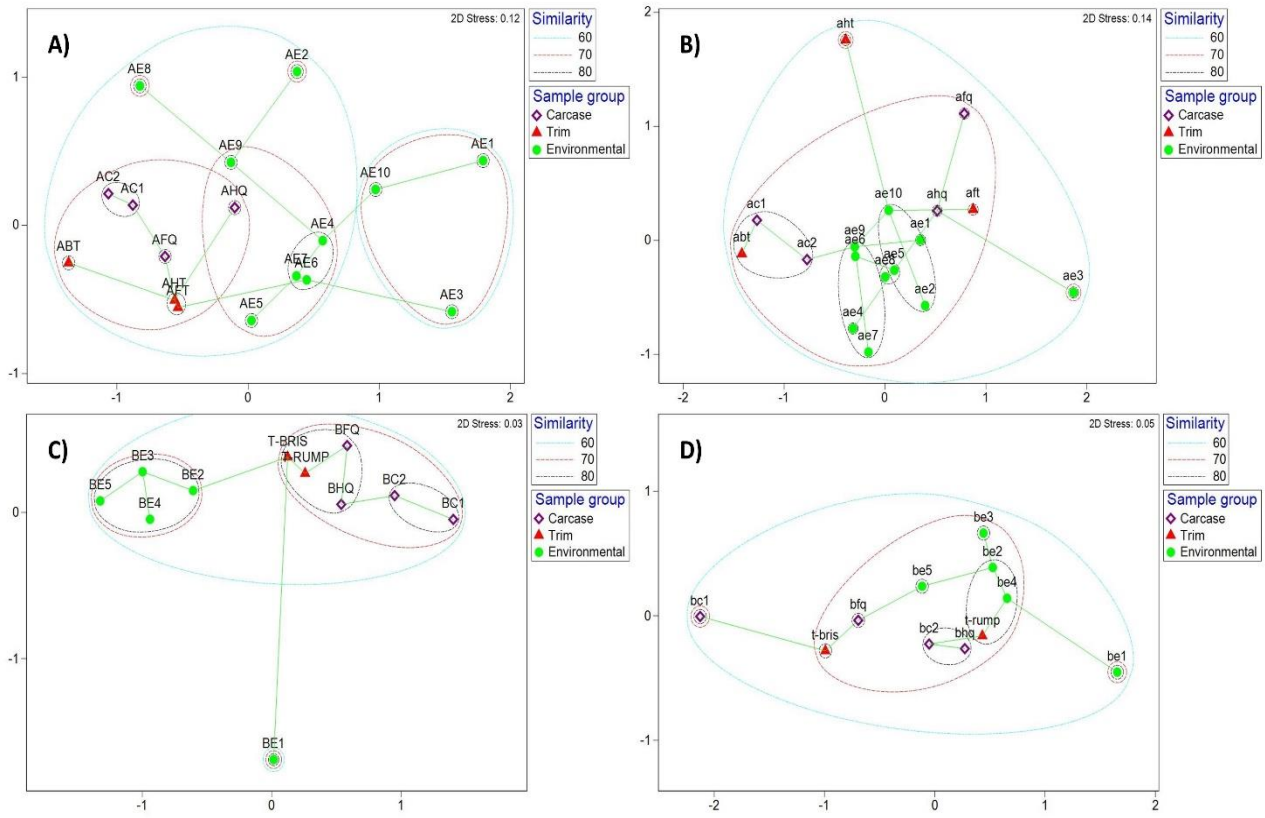


Figure 3.5 Non-metric multidimensional scaling (nMDS) plot of beta diversity (Bray-Curtis similarity matrix) in carcase, trim and environmental sample groups from boning room visits to abattoir A (A and B) and B (C and D). Samples are positioned based on the percentage of similarity in their community composition using light blue (≥60%), red (≥70%) and black (≥80%). Abattoir A samples are labelled; AC1: pre-chill carcases, AC2: post-chill carcases, AFQ: forequarter carcases, AHQ: hindquarter carcases, AFT: forequarter trim, AHT: hindquarter trim, ABT: boxed trim, and AE1 – 10: environmental sites. Abattoir B samples are labelled; BC1: pre-chill carcases, BC2: post-chill carcases, BFQ: forequarter carcases, BHQ: hindquarter carcases, T-BRIS: brisket trim, T-RUMP: rump trim and BE1 – 5: environmental sites. All samples are labelled in capital (first visit) and lower case (second visit).

3.3.3 Contamination of beef trim by environmental surfaces through the boning process

A list of all OTUs at the Order level was used to generate non-metric multidimensional scaling (nMDS) ordinations (Fig. 3.5). nMDS ordinations are used to visualise the similarity in composition and structure of the bacterial community between the sample groups (carcases, trim and environmental surfaces) using the Bray-Curtis similarity matrix. For this analysis, nMDS with two-dimensions and stress level below 0.15 is used to accurately describe the differences in community composition (beta diversity) between the samples (Schloss et al., 2009).

Analysis of 16S rRNA profiles using nMDS suggested that the contamination of beef trim may be a result of accumulated contamination from the environmental surfaces. However, the consistency of environmental contamination of beef trim varied depending on the day of boning. In the first visit to abattoir A, the carcase (AC1, AC2, AFQ and AHQ) and beef trim (AFT and AHT) samples shared approximately 65% similarity in community composition with seven of the environmental samples (AE2, AE4, AE5, AE6, AE7, AE8 and AE9) while the remaining sites (AE1, AE3 and AE10) shared less than 60% to the rest (Fig. 3.5A). In the second visit, there was moderately higher percentage of similarity (>70%) between the carcase, trim and all of the environmental samples but one (ae3, Fig. 3.5B). Some environmental sites (2, 4, 5, 6, 7, 8, 9, Fig. 3.2) appeared to consistently contribute to beef trim community across the two visits. Sampling after sanitation increased the chances of capturing residential bacteria in the environment (Stellato et al., 2016). Unlike abattoir B, there was not a single group of bacteria that dominated the microflora throughout the abattoir. Additionally, the level of microbial contaminants in the environment was similar. Therefore, it may be that the residential bacterial populations in abattoir A are comparably transient and determined by the exchange between microbial load on the carcases on the day of boning. This observation would benefit from complementary data that captures the changes in 16S profile on individual carcases and in diverse contamination sources in the boning room under time-controlled sampling.

In abattoir B, there was a substantial difference in community composition between the first and the second visit. Most noticeably, the degree of beef trim contamination from the environmental surfaces in the first visit showed a minor variation in comparison to the second visit. In the first visit, the environmental samples (BE2, BE3, BE4 and BE5) shared approximately 66% similarity with the meat samples (Fig. 3.5C). On the second visit, the environmental samples from the same locations (be2, be3, be4 and be5) shared 78% similarity with the meat samples excluding pre-chill carcases (Fig. 3.5D). In addition, bacterial communities on boning table 1 and 6 (be2 and be4) were more than 80% comparable to the communities in the beef trim from rump (t-rump). Quartering station tile (BE1 and be1) was consistently found to share 66 – 69% similarity in composition with the carcase and trim samples in both visits (Fig. 3.5C and 3.5D). Overall, the relationship between the bacterial community structure on the carcases, beef and environmental surfaces supported the previous idea that Enterobacteriales were generally dominant throughout

boning in abattoir B. The 16S rRNA data suggests that these environmental sites could potentially be targeted to reduce the concentration of predominant contaminants that are present in the trim communities (Fig. 3.4 and 3.5).

Use of genetic sequencing techniques for characterisation of microbial communities has gained popularity in meat microbiology but no studies have investigated contamination of beef throughout the boning process using this approach. Microbial prevalence in the environment of boning rooms has been linked with the level of hygiene and microbiological load of beef in the past and the association was generally accepted by comparing microbial count between the surfaces and beef trim throughout the boning process (Cetin et al., 2006, Charlebois et al., 1991). Analysis of 16S rRNA gene profiles have added in-depth understanding of environmental contamination of beef trim by monitoring the shifts in bacterial communities between the environment and beef. Each abattoir had characteristic trends within the boning room. Similarities in bacterial composition visualised through nMDS ordination suggested that some environmental surfaces were more consistent factors of accumulative contamination in beef trim (Fig. 3.5). Future investigations with a bigger pool of environmental contamination sources could enable specific areas to be targeted for strategic control of contamination and enhance microbiological quality and safety in beef products. Targeting multiple regions of 16S rRNA would be useful to describe beef contamination at the species level that can assist in controlling specific spoilage and pathogenic bacteria in beef processing. Shotgun metagenomics permits analysis at even greater resolution than 16S and would potentially allow for pathogens to be tracked through beef processing. The abattoirs have better opportunity to control contamination by understanding cross-contamination points in greater detail to offset these events. The outcome of doing so would likely result in reduced opportunities for pathogens of food safety concern contaminating final product.

3.4 Conclusion

Australian beef export-certified abattoirs with varying levels of integration in the supply chain produced beef trim that are in similar range of total viable count (TVC) but vary in ecological make-up. Analysis of 16S rRNA sequencing showed that the dominant bacterial populations throughout boning in the abattoirs are similar. However, the changes in community composition during the boning process were different between the abattoirs. Conventional counting of colonies demonstrated reduced microbial concentrations on

carcases, manufacturing beef and environmental surfaces that were regularly in contact with beef products when compared with previously published studies. Monitoring 16S rRNA profiles enabled deeper understanding of beef contamination by analysing the interactions between bacterial communities in different samples throughout the boning process. The results from this study complements the analysis of 16S bacterial profiles in a previous study that investigated the slaughter phase in the same abattoirs. Results from both studies indicated that there are specific variations within individual abattoirs that drive the transmission of microbial communities. Profiles of 16S rRNA in the boning room revealed that the microflora on the carcases at the end of slaughter had similar profiles to carcases entering the boning room. However, the community composition in manufacturing beef differed from chilled carcases in every visit. Common beef associated populations were found but Enterobacteriales dominated abattoir B whereas other groups such as Bacteroidales, Clostridiales, Lactobacillales and Pseudomonadales contributed more to community populations in abattoir A in general. Although the sequencing method in this study could not definitively determine all contributions to 16S profiles at varying points of the boning process, it has proved to be a useful tool for analysing the importance of ecological changes that occur during the entirety of beef processing.

Chapter 4. Analysis of bacterial diversity in relation to presence of Top 7 Shiga toxin-producing *Escherichia coli* (STEC) throughout Australian beef abattoirs

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4.1 Introduction

Beef cattle are identified as a natural reservoir of Shiga toxin-producing *Escherichia coli* (STEC) and beef products have been implicated in STEC infections and multiple foodborne outbreaks across the globe over the last few decades (Barlow et al., 2006, Browne et al., 2018, Fegan et al., 2004). Since then, stringent STEC screening procedures have been implemented in the Australian red meat industry to meet regulatory standards for beef export. The United States Department of Agriculture, Food Safety Inspection Services (FSIS) currently list seven serotypes (O26, O45, O103, O111, O121, O145 and O157 known as the Top 7 STEC) as adulterants in ground beef (Food Safety Inspection Safety, 2011). The regulatory procedure demands that raw non-intact beef products for the United States are deemed free of the Top 7 STEC in order to gain and maintain access to the market.

Pathogenesis of STEC relies on attachment to host epithelial cells in the intestinal lining (*eae* genes) and release of the cytotoxic Shiga toxins (*stx* genes) (Castro et al., 2017, Ferdous et al., 2015, Nguyen and Sperandio, 2012, Paton and Paton, 1998b). Researchers have used these virulence genes to understand the STEC prevalence and subsequently the contamination of beef in the supply chain (Barlow and Mellor, 2010, Bosilevac et al., 2007, Elder et al., 2000, Fegan et al., 2005, Fegan et al., 2009, Mellor et al., 2016, Stromberg et al., 2015a, Svoboda et al., 2013, Vanderlinde et al., 1998). These studies typically relied on molecular analysis and cultivation of STEC using selective media. The findings contributed to the hypothesis that microbial contaminants transfer from faecally contaminated hides onto the

carcases and ultimately onto beef product (Arthur et al., 2010, Barlow and Mellor, 2010, Elder et al., 2000, Fegan et al., 2005, Pointon et al., 2012). More recently, high throughput sequencing technologies and bioinformatic software enabled researchers to understand STEC contamination of beef at resolutions that were not achievable with conventional microbiological methods (Brightwell et al., 2006, Kang et al., 2019, Kergourlay et al., 2015, Lagkouvardos et al., 2016). Advances in high throughput sequencing and population analysis techniques has given researchers the capability to investigate the ecological patterns in beef microflora that may indicate the presence of STEC within the community.

There are a number of studies exploring bacterial diversity and prevalence of STEC in the beef supply chain between production and slaughter of the animals. Chopyk et al. (2016) examined the association between STEC and corresponding differences in bacterial diversity on pre-harvest hides. The study reported that a correlation exists between prevalence of STEC serotypes and low bacterial community diversity in hide-specific cohorts of bacterial populations. It also showed that there were variations in bacterial community composition depending on combinations of top seven serotypes. Zhao et al. (2013) found that high levels of STEC shedding was associated with lower faecal bacterial diversity, especially in younger calves with lower butyrate-producing bacteria in the lower gastrointestinal tract. In another study, local bacterial diversity differences were present along the digestive tract in super shedders and non-super shedders but no correlation was found between faecal bacterial diversity and O157 shedding status (Zaheer et al., 2017a). A study by Xu et al. (2014) demonstrated that faecal bacterial richness and diversity was higher in super shedders compared to non-super shedders. These studies were able to describe the diversity differences observed in response to the presence of STEC by analysing 16S rRNA amplicon sequences in combination with enumeration of STEC.

We recently reported bacterial community profiles from two Australian abattoirs in relation to indictor bacterial counts (Kang et al., 2019, Kang et al., 2020). In the present study, we examine the metagenomic data from the two abattoirs in light of STEC and the influence of diversity on Top 7 STEC. Knowledge of the relationship between STEC and the structure of beef microflora during slaughter and boning of the animals is currently lacking. The present study utilised 16S rRNA amplicon sequencing in combination with cultivation methods and PCR to examine the fundamental changes in bacterial diversity through processing of beef cattle. The hypothesis is that there is no correlation between bacterial diversity and presence

of the Top 7 STEC, therefore the emphasis of the analysis was placed on diversity differences between samples that tested positive and negative for STEC virulence markers. The findings from this study could contribute to the understanding of the ecological changes that surrounds the Top 7 STEC in the beef supply chain.

4.2 Materials and methods

4.2.1 Sample collection

Samples were collected from two abattoirs with differing levels of integration in the supply chain. In brief, an integrated abattoir (abattoir A) receives beef cattle that have been through the same production system and typically from a small number of sources. A fragmented abattoir (abattoir B) processes cattle that are provided from multiple producers. Abattoir A and B were each visited four times over a period of 14 months. Each abattoir was visited twice for collection of slaughter samples between January – July 2018 and twice again for boning room samples between November 2018 – March 2019. A total of 90 samples were collected from the slaughter floor per visit. The samples collected consisted of 10 faecal samples, 15 hide samples prior to hide-pull, 15 carcase samples immediately after hide-pull, 15 carcase samples post-evisceration, 15 carcase samples immediately before chilling (pre-chill) and 20 environmental samples throughout the slaughter floor. For the boning room, 90 samples were collected over two sampling days. The first sampling day was spent collecting 15 pre-chill carcase samples. Samples collected on the second day consisted of 15 chilled carcases, 5 forequarters, 5 hindquarters, 30 beef trim that were boned from the carcases (manufacturing beef) and 20 environmental surfaces. In abattoir A, trim samples from forequarters (n=12) and hindquarters (n=12) and boxes of manufacturing beef (n=6) were collected. In abattoir B, trim samples were collected from forequarters (n=16) and hindquarters (n=14). Carcase and beef trim samples were not matched but the samples were collected from the same lot of animals.

4.2.2 Sampling method and preparation

Faecal samples were collected from internal content of freshly excreted faecal pats in holding pens prior to slaughter using sterile yellow capped plastic jars (Sarstedt, Numbrecht, Germany) and a sterile spoon. Large area sampling technique was used for sampling of the carcases as previously described (Chandry, 2016). Briefly, the carcase samples were collected aseptically using large area sampling technique (3000 cm^2). Environmental samples

were obtained from swabbing an area covering approximately 900 cm^2. For beef trim, approximately 100 cm^2 of the most outer fat surface was collected in a large stomacher bag. Swabbing of the surface samples was performed using Whirl-Pak® Speci-Sponge® (Nasco, Wisconsin, US) pre-moistened in 25 mL of sterile buffered peptone water (BPW; Oxoid, Hampshire, UK). All samples were immediately placed on ice for transport to the laboratory. At the laboratory, samples collected with a sponge had an additional 75 mL of BPW added prior to being stomached for 30 seconds at eight strokes per second (Interscience, Saint Nom, France). Faecal samples were diluted 10-fold by adding 90 mL of BPW to 10 g of faeces. Approximately 100 cm^2 of the most outer layer of trim samples was excised and added to 100 mL of BPW. The samples were then stomached for a minute. Following stomaching, aliquots from the samples were kept for 16S rRNA sequencing analysis at -80°C and the remaining portion of the bacterial-BPW suspension was used for detection of STEC.

4.2.3 Confirmation of Top 7 STEC

All samples were processed for the presence of Top 7 STEC following the export regulatory guideline for meat products by the FSIS (Food Safety Inspection Services, 2019a). The guideline evolved around detection of *stx, eae* and gene fragments that were specific for the Top 7 serotypes throughout the methods. In brief, enriched bacterial-BPW suspensions were tested for detection of Top 7 STEC virulence markers. The samples that contained the markers were indicative of STEC presence and these samples were processed for isolation of colonies. The samples were confirmed as positives if the isolated colonies were pure and tested positive for all STEC virulence markers.

4.2.3.1 Detection of STEC serotypes

Bacterial-BPW suspensions were enriched overnight at 37°C using BPW as the enrichment media. Stepwise PCR based approach was used to screen the enriched samples using ViiA™ 7 Real-Time PCR System (Applied Biosystems, Victoria, Australia) for all PCR tests. The initial phase of PCR detection of STEC involved screening for *stx* and *eae* markers. The samples that were *stx* and *eae* positive were subsequently tested for the presence of genetic loci specific for individual O-serotypes of Top 7 STEC (O26, O45, O103, O111, O121, O145 and O157). DNA was extracted from enriched sampled using the Wizard® Genomic DNA Purification Kit (Promega, New South Wales, Australia). Extracted DNA was used as a template using the PCR procedures and sequences of primers and probes from previously

published methods (Food Safety Inspection Services, 2019b, Perelle et al., 2004). Protocols for O157 and non-0157 STEC were adopted from Perelle et al. (2004) and FSIS Microbiology Laboratory Guidebook (MLG) 5C Appendix 4.00 (Food Safety Inspection Services, 2019a). Samples that were positive for *stx, eae*, and one or more STEC serotypes were regarded as potential positive (PPs) and continued to the isolation and characterisation phase.

4.2.3.2 Isolation and characterisation of STEC from slaughter

Isolation of Top 7 STEC was carried out for PPs from the slaughter room visits using immunomagnetic separation (IMS) with commercially available Assurance GDS® MPX Top 7 STEC (AMSL, New South Wales, Australia) beads following the manufacturer's instructions. The bead-bacteria complexes at the completion of IMS for O157 were plated onto three different media; sorbitol MacConkey with cefixime-tellurite supplement (0.05 mg/l cefixime and 2.5 mg/l potassium tellurite; Oxoid), Rainbow Agar (Cell Biosciences, Victoria, Australia) supplemented with 0.05 mg/l cefixime trihydrate, 0.15 mg/l potassium tellurite and 5.0 mg/l sodium novobiocin; and CHROMAgar™ STEC (Dutec Diagnostics, New South Wales, Australia). Non-O157 bead-bacteria complexes were plated on modified Rainbow Agar (as above) and CHROMAgar™ STEC (Dutec Diagnostics). All media were incubated for 18 – 24 hours at 37°C.

Following IMS, up to 5 phenotypically distinct colonies from each plate were streaked onto TSA (Oxoid) and incubated for 18 – 24 hours at 37°C. All of the isolates were initially tested for *stx*$_1$, *stx*$_2$, *eae* and *ehxA* using conventional multiplex PCR as described by Paton and Paton (Paton and Paton, 1998a). The isolates with *stx* and *eae* were then characterised targeting the serotypes that were captured during detection using real-time PCR. PCR master mix without DNA material was used as negative control and known pure Top 7 STEC isolates were used as positive control for all PCR tests.

4.2.4 Sequencing analysis of bacterial 16S rRNA genes

4.2.4.1 DNA extraction for 16S rRNA amplicon sequencing

A 40 mL aliquot of pre-enriched bacterial-BPW suspension from each sample was centrifuged at 10,000 rcf for 5 minutes to pellet the cells. The pellets were washed twice with BPW prior to DNA extraction using QIAamp PowerFecal DNA kit (Qiagen, California, US)

following the manufacturer's protocol with modifications in the bead beating step as previously described by Kang et al. (2019).

4.2.4.2 Preparation of 16S rRNA library

Extracted DNA was used to construct a library of 16S rRNA amplicons targeting the V4 region using the protocol from a previous study by Kozich et al. (2013). Briefly, 5 µL from each DNA template was amplified with dual-index primers by PCR and the amplicons were manually normalised by comparing the intensity of the DNA band against the GeneRuler 100 bp Plus DNA Ladder (ThermoFisher, Victoria, US). The DNA materials were stained with ethidium bromide and visualised under ultraviolet light in 2% agarose gel under ultraviolet light. Each template contained barcode sequences at the 5' and 3' of the PCR amplicon to enable demultiplexing of sequence reads. Each template acted as a positive and negative control for each PCR reaction. Approximately 50 ng of rRNA DNA amplicon from each sample was combined to create a pooled library for sequencing. A microbial community DNA standard, ZymoBIOMICS™ (Zymo Research, California, US) was normalised using the same approach and added to the library. The library was purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, California, US) following the manufacturer's instruction. The concentration of amplicons was measured before and after purification using the Qubit® 2.0 Fluorometer (Life technologies, California, US). A NanoPhotometer® (Implen, California, US) was used to measure the purity of the library and the final purified library was sent for sequencing.

4.2.4.3 Sequencing using Illumina MiSeq

Sequencing of the pooled library of amplicons was performed at the Ramaciotti Centre for Genomics (University of New South Wales) using the Illumina MiSeq platform (Illumina, California, US) with a paired-end 300 base pair sequencing protocol as previously described (Kang et al., 2019). All sequencing procedures were monitored through the Illumina BaseSpace® website.

4.2.4.4 Bioinformatic analysis of 16S rRNA profile

Both de-multiplexed R1 and R2 sequencing read (approximately 250 bp in length) files were acquired from the Illumina BaseSpace® website. Bioinformatic processing of raw sequence data was performed using the v1.40.5 MOTHUR pipeline (Schloss et al., 2009) following the MiSeq standard operating procedures (Kozich et al., 2013). The paired end reads were

generated and clustered prior to assembly into Operational Taxonomic Unit (OTU) tables with 97% as the cut-off for identification. The taxonomic classification of OTUs was performed using SILVA 16S rRNA database (v132). The 16S rRNA sequences were converted into relative abundance for each sample in Microsoft Excel (v16.0) and used for the subsequent analysis. For investigation of bacterial diversity within groups (alpha diversity), Simpson's index and number of OTUs at Genus level were calculated using ANALYSE→DIVERSE tool on PRIMER-7 version 7.0.13 (Primer-E, Ivybridge, UK) and used to create bar plots in Microsoft Excel. Simpson's index is scaled from 0 to 1 where 1 indicates high diversity within the community. For analysis of bacterial diversity between sample groups (beta diversity), non-metric multidimensional scaling (nMDS) and analysis of similarity (ANOSIM) were used. Relative abundance of Genus OTUs were used to create similarity ranks (resemblance matrix) using Bray-Curtis similarity. The matrix was then used for nMDS ordination and ANOSIM (with >999 permutations) on ANALYSE tools within PRIMER-7. The samples were assigned with sample type and STEC testing status as a function of diversity during estimation of bacterial population composition.

4.3 Results and discussion

4.3.1 STEC potential positives and confirmed isolates

The presence of PPs during slaughter at the abattoirs were similar and ranged between 64 – 69% (58 – 62/90) with the exception of the second visit to abattoir B that had 81% (73/90) of the samples being PPs (Table 4.1). The number of PPs generally decreased in the boning room and abattoir B had a substantially lower percentage of PPs at 7% (6/90) and 2% (2/90) compared to 23% (21/90) and 48% (43/90) in abattoir A samples despite having equivalent or higher levels of STEC in the slaughter phase. From a total of 360 samples from slaughter, ten STEC isolates belonging to O157 and O111 serotypes were obtained (Table 4.2). Two O157 isolates were retrieved from the first abattoir A visit in a hide and a faecal sample with the same profile of virulence markers (*stx*$_2$, *eae* and *ehxA*). The remaining eight isolates were from the visits to abattoir B. O157 and O111 STEC were isolated from faecal and hide samples from the first visit but only the hide samples from the second visit had O157 and O111. Three O157s were also isolated from carcase and environmental samples from the second visit. Prevalence of the Top 7 STEC in the Australian beef industry is low and O157 is the most common STEC while non-O157 STECs such as O26 and O111 have also been identified but are less common (Barlow and Mellor, 2010, Mellor et al., 2016, Vally et al.,

2012). Thus, it was not surprising to find as few as ten isolates that were O157 and O111 STEC from the slaughter samples.

Table 4.1 Sources and virulence markers (*stx*, *eae* and *ehxA*) of Top 7 STEC (O26, O45, O103, O111, O121, O145 and O157) isolated from samples collected on the slaughter floor at an integrated (A) and a fragmented abattoir (B).

Distribution of PPs (PPs/no. of samples)					
	Slaughter				
Abattoir	**Faeces**	**Hides**	**Carcases**	**Environment**	**Total**
A - visit 1	10/10	15/15	28/45	10/20	62/90
- visit 2	10/10	15/15	22/45	11/20	58/90
B - visit 1	10/10	15/15	22/45	12/20	58/90
- visit 2	10/10	15/15	37/45	14/20	73/90
	Boning				
	Carcases	**Beef trim**	**Environment**		**Total**
A - visit 1	10/40	6/30	5/20		21/90
- visit 2	17/40	15/30	11/20		43/90
B - visit 1	5/40	0/30	1/20		6/90
- visit 2	1/40	1/30	0/20		2/90

Table 4.2 suggests that there were possibly higher proportions of STEC contamination within abattoir B on the second visit and the contamination control strategies were not able to manage the subsequent increase in STEC concentration through slaughter. It also suggests that the transfer of STEC from faeces to hides was minimised during the other three visits. From hereafter, PPs and non-PPs are used to compare and analyse the changes in bacterial community diversity. The hypothesis is that lower bacterial diversity should be observed in samples that are indicative of STEC presence if higher bacterial diversity is truly correlated with lower prevalence of STEC.

Table 4.2 Sources and virulence markers (*stx*, *eae* and *ehxA*) of Top 7 STEC (O26, O45, O103, O111, O121, O145 and O157) isolated from samples collected on the slaughter floor at an integrated (A) and a fragmented abattoir (B).

Abattoir	Supply chain	Visit no.	Source	Serotype	STEC virulence markers
A	**Integrated**	1	Hide	O157	*ehxA, eae, stx*$_2$
A	**Integrated**	1	Faeces	O157	*ehxA, eae, stx*$_2$
B	**Fragmented**	1	Hide	O157	*ehxA, eae, stx*$_{1\ \&\ 2}$
B	**Fragmented**	1	Hide	O111	*ehxA, eae, stx*$_{1\ \&\ 2}$
B	**Fragmented**	1	Faeces	O111	*ehxA, eae, stx*$_1$
B	**Fragmented**	2	Hide	O111	*ehxA, eae, stx*$_1$
B	**Fragmented**	2	Hide	O157	*ehxA, eae, stx*$_2$
B	**Fragmented**	2	Carcase	O157	*ehxA, eae, stx*$_{1\ \&\ 2}$
B	**Fragmented**	2	Environment	O157	*ehxA, eae, stx*$_2$
B	**Fragmented**	2	Carcase	O157	*ehxA, eae, stx*$_{1\ \&\ 2}$

4.3.2 Alpha diversity by STEC PCR in slaughter samples

The alpha diversity (Simpson's index; SI) was not significantly different (P>0.05) between STEC positive and negative samples regardless of the processing stage or sample type (Fig. 4.1). All the faecal samples from abattoir A were PPs as opposed to the faecal samples from abattoir B which had PPs and non-PPs (Fig. 4.1A and 4.1B). Regardless of STEC status, the faecal group in both abattoirs had similar OTUs and SI. Faecal PPs in abattoir A had average OTUs of 148 and SI of 0.95. In abattoir B, faecal PPs had an average of 152 OTUs and SI of 0.93 while the negative group had an average of 140 OTUs and SI of 0.92, showing no significant differences (P>0.05) in alpha diversity (Fig. 4.1B). Previous studies have analysed bacterial diversity in faecal samples by comparing community structure in faeces from animals with different levels of STEC shedding (Xu et al., 2014, Zaheer et al., 2017a, Zhao et al., 2013). Conclusions on the correlation of diversity and STEC prevalence varied between these studies. In this study, the comparison of diversity in faecal samples would have benefited from having a larger pool of both STEC positive and negative samples to consolidate and compare the findings to the previous studies.

Hide samples from the slaughter floor had the highest average number of OTUs and SI values in comparison to the other samples in both abattoirs (Fig. 4.1A and 4.1B). Hide samples from abattoir A and B had an average of 257 and 312 OTUs with an average SI of 0.96 and 0.94 respectively. Chopyk et al. (2016) investigated bacterial diversity in pre-harvest hide samples and found that STEC presence correlated with lower bacterial diversity. The study eliminated

faecal OTUs from hides and created a hide-specific 16S profile. The authors then used the hide-specific communities to measure diversity within and between sample groups. Chopyk et al. (2016) added that the bacterial composition in hides without STEC varied from the hide communities with the presence of one, two, three or more STEC serotypes. Comparison of diversity in hide microflora between the previous study and this study may provide an insight into the differences in ecological niches that ultimately can have an impact on diversity difference with STEC as a function of diversity. However, there were insufficient amount of positive and negative hide samples in this study to draw statistically significant conclusions or comparisons with the previous study (Chopyk et al., 2016). It is important to emphasise that the aim of this study was not to compare changes in bacterial diversity at specific regions of processing (faeces or hides) to the previous findings but to observe bacterial diversity in light of the Top 7 STEC through the processing of beef cattle.

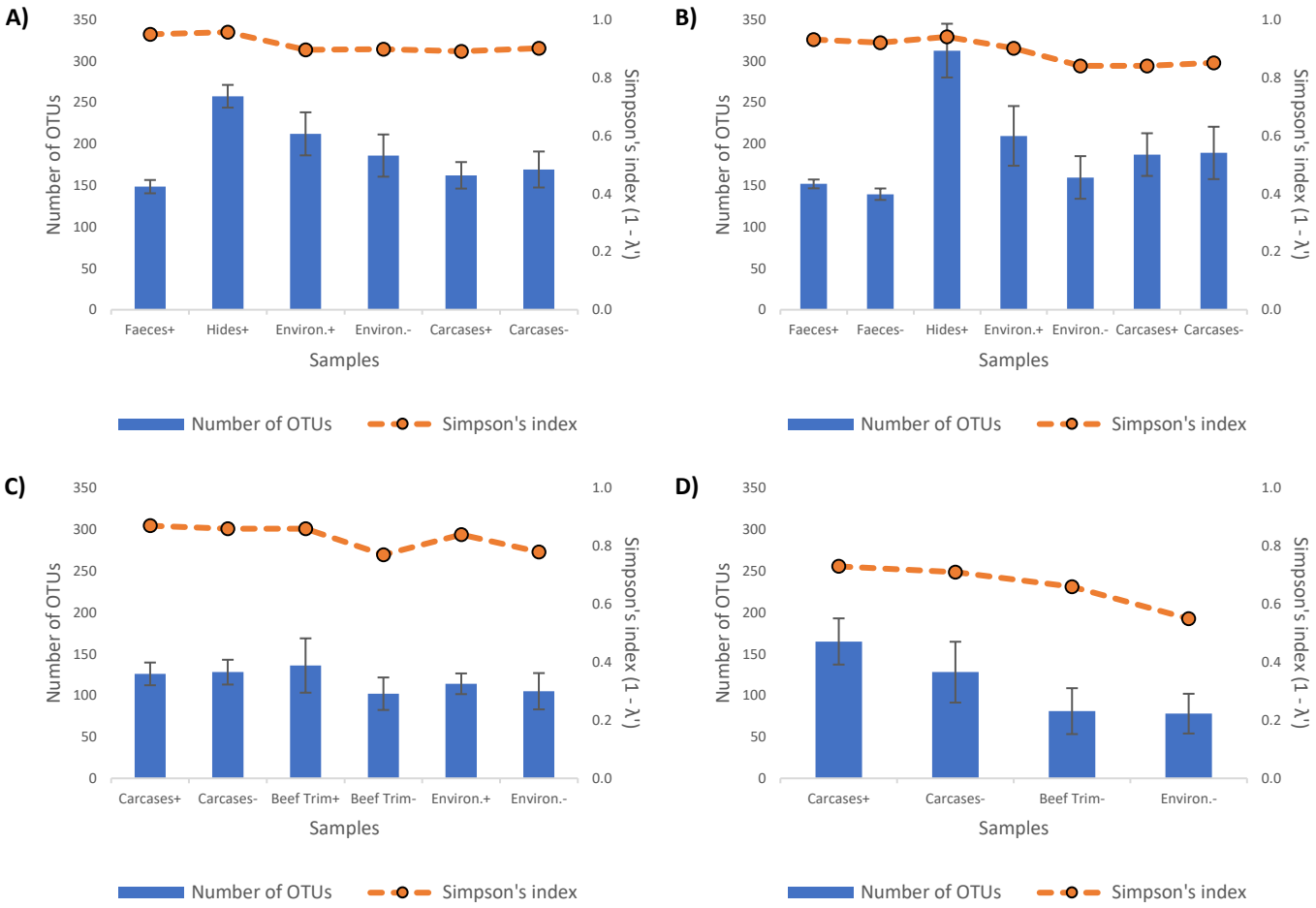

Figure 4.1 Number of OTUs at Genus level and Simpson's index (alpha diversity) in different sample groups in slaughter and boning of an integrated (A) and a fragmented (B) Australian beef abattoir. Abattoir A visits to slaughter and boning are shown in A) and C), respectively. Abattoir B visits to slaughter and boning are shown in B) and D), respectively. Sample groups are categorised by STEC qPCR signals. STEC status of the groups are labelled '+' and '–'; some groups do not show both due to absence of the signal in the samples. Alpha diversity within each group is represented in Simpson's index.

In abattoir A, the average number of OTUs in PP environmental samples (212) was marginally higher than the non-PP samples (186) but both groups had an average SI of 0.90 (Fig. 4.1A). Environmental PPs in abattoir B had a higher number of OTUs by more than 50 and a higher SI value by 0.06 than the negative group, but the difference in alpha diversity was not statistically significant ($P>0.05$). In abattoir A, the average number of OTUs and SI were slightly higher in the non-PP group. However, the alpha diversity between the two groups was not significantly different ($P>0.05$).

Presence of PPs in carcase samples from the boning phase did not have any significant effect ($P>0.05$) on the alpha diversity in both abattoirs. In abattoir A, carcase PPs had an average SI

of 0.87 whereas the non-PPs had an average of 0.86 (Fig. 4.1C). In abattoir B, the SI was noticeably lower in comparison to abattoir A at 0.73 in the positive group and 0.71 in the negative group (Fig. 4.1D). PPs from beef trim samples had an average SI value of 0.86 while the STEC negative group had an average of 0.77 (Fig. 4.1C). However, the difference in alpha diversity between the two groups was not statistically significant ($P>0.05$). The apparent numerical difference in alpha diversity of beef trim PPs and non-PPs from abattoir B may have been due to a wide range of SIs that were observed across the beef trim samples. Only one sample from beef trim (3%) and environmental surfaces (5%) tested positive for STEC and therefore comparison of alpha diversity in different STEC testing groups of beef trim B was not valid.

This study showed that alpha diversity in the boning room was generally lower in comparison to slaughter in both abattoirs (Fig. 4.1). In abattoir A, the average SI decreased from 0.91 on the slaughter floor to 0.83 in the boning room. In abattoir B, the reduction in alpha diversity across the samples was more substantial; from 0.89 in slaughter to 0.66 in boning. This correlated with a substantial decrease in the number of OTUs from slaughter to boning rooms in both abattoirs; 189 to 118 in abattoir A and 193 to 113 in abattoir B. Changes in bacterial community composition in the boning room was investigated in a different study (data not shown). In that study, the community structure throughout the boning room in abattoir A had a number of predominant bacterial populations (Clostridiales, Enterobacteriales, Lactobacillales and Pseudomonadales) in contrast to the ecological make-up in abattoir B where Enterobacteriales was the most dominant (Kang et al., 2020). Relative abundance of Enterobacteriales averaged at 39.5% and 51.2% in carcases, and 56.1% and 53.9% in beef trim from two visits to the boning room in abattoir B. In abattoir A, the four groups (Clostridiales, Enterobacteriales, Lactobacillales and Pseudomonadales) dominated 65.5 – 72.8% of carcase composition and 63.6 – 73.3% in beef trim. Yang et al. (2016) demonstrated that the proportion of *E. coli* amongst other bacterial groups increased in the beef trim community as diversity decreased. These findings shed light on the observation of alpha diversity in trim samples from abattoir B in this study and indicate that communities dominated by a small number of species can limit the richness of bacterial populations in beef microflora. Despite a large proportion of the community being Enterobacteriales in the boning room of abattoir B, the number of STEC PPs was comparably lower than abattoir A which suggests that the relative abundance of STEC in that population was minimal.

Previous studies have hypothesized that the degree of competitive exclusion increased as the microflora became more diverse and reduced the chance of STEC becoming prevalent within the community (Chopyk et al., 2016, Fujikawa and Sakha, 2014). Another hypothesis was there may be a specific composition of bacterial populations that either outcompete or produce inhibitory substances that prevent STEC from becoming prevalent in beef microflora (Arthur et al., 2010, Chopyk et al., 2016, Matthews et al., 2006, Xu et al., 2014, Zaheer et al., 2017a). Observations from this study do not provide evidence to validate these hypotheses. On the contrary, results from this study and previous studies appear to show that higher alpha diversity does not affect the STEC prevalence throughout beef processing (Kang et al., 2019, Kang et al., 2020).

4.3.3 Relationship between STEC and beta diversity

Figure 4.2 shows the differences in bacterial diversity between the sample groups (beta diversity) in relation to the presence of STEC. The statistical significance of STEC markers as a function of diversity in bacterial communities is shown in Figure 4.3. Prior to the analysis of diversity in PPs and non-PPs, the samples with confirmed O157 and O111 STEC were compared with non-STEC samples and no differences were observed. The confirmed positives were included as PPs during comparison of bacterial diversity in PP and non-PP groups and the two groups did not show significant differences in bacterial diversity through slaughter ($P>0.05$, Fig. 4.3). In abattoir A, all faecal and hide samples were PPs and most of these samples overlapped on nMDS plots indicating that the bacterial diversity in the two sample groups were similar (Fig. 4.2A). In abattoir B, the faecal and hide samples were separated from the rest on the nMDS plot with hide samples being closer to the carcase samples (Fig. 4.2B). The faecal group from both abattoirs shared commonality, in that they were ranked away from the other sample types on nMDS plots. The carcase and environmental samples from slaughter rooms in both abattoirs were predominantly grouped

without any distinguishable trends between the groups regardless of the STEC status (Fig. 4.2A and 4.2B).

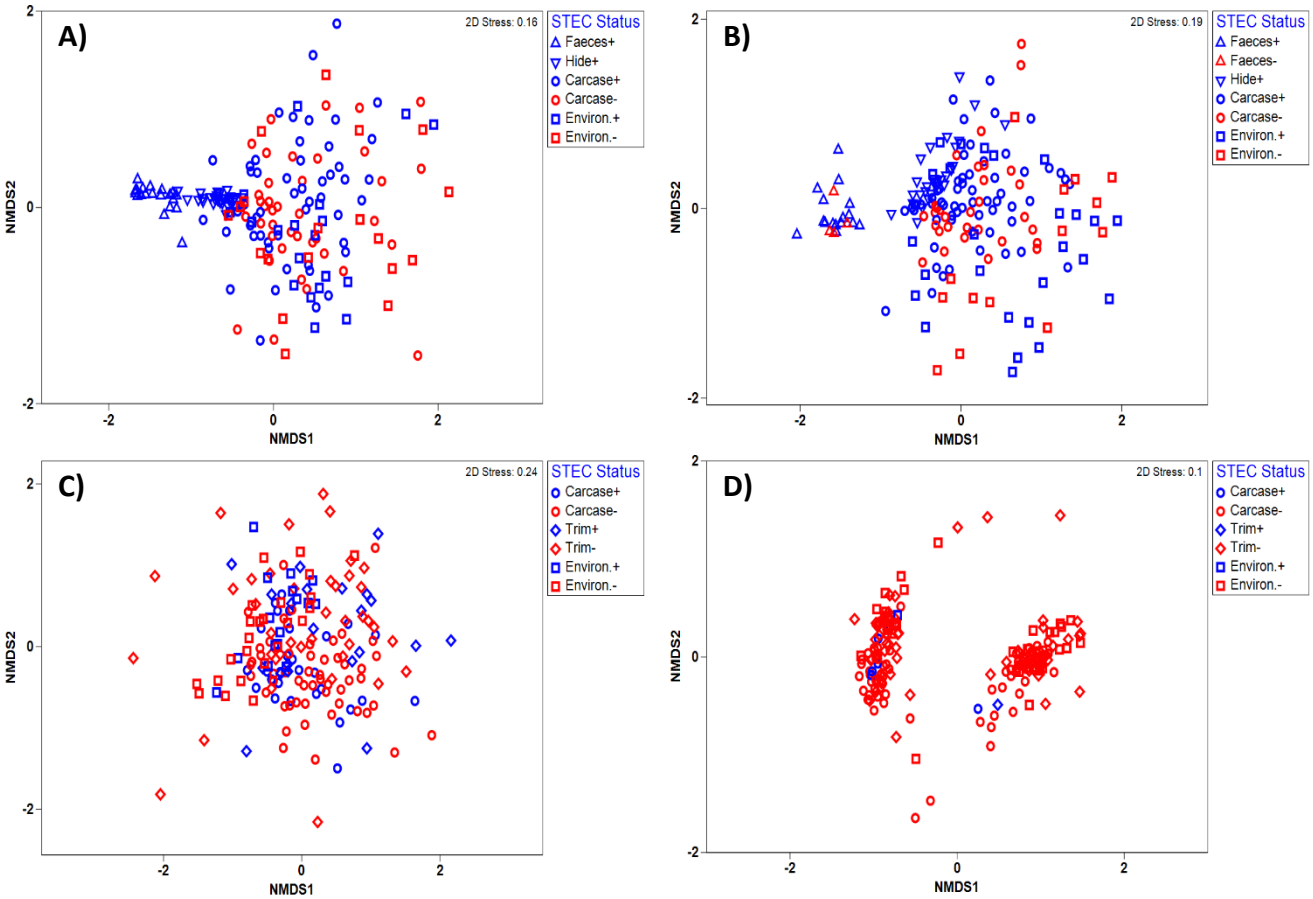

Figure 4.2 Comparison of beta diversity of bacterial communities in all samples by STEC status using non-metric multidimensional scaling (nMDS). Slaughter samples (faecal, hide, carcase and environmental) from abattoir A and B are shown in A) and B), respectively. C) and D) represent the samples from the boning room (carcase, trim and environmental) in abattoir A and B, respectively. Blue labels indicate samples that tested positive for STEC and red for negative STEC samples. Two-dimensional stress of nMDS ranged from 0.10 – 0.24.

The boning room samples provided a similar outcome with no ecological differences observed between STEC PP and non-PP groups. In abattoir A, the carcase and environmental samples clustered relatively close to each other and the distribution of PPs and non-PPs on nMDS appeared to be randomly placed (Fig. 4.2C). Some non-PPs of beef trim were separated from the rest of the trim samples suggesting that the bacterial structure was slightly different in comparison to the other samples but was not statistically significant (P>0.05, Fig. 4.3C). Carcase, trim and environmental PPs from the boning room of abattoir B were grouped into one tight cluster with non-PPs separating into two defined areas with some outliers (Fig. 4.2D). The distribution of the samples in two areas may be due to differences

observed in bacterial community composition between the boning room samples from visit one and two (data not shown). The samples that were grouped in the left population on nMDS were from visit one and the other population represented samples from the second visit (Fig. 4.2D). Therefore, the clustering of the samples on the nMDS plot may more be a function of variability between sampling days than of differences in beta diversity.

Analysis of similarity (ANOSIM) demonstrated that differences in beta diversity between sample groups, regardless of STEC testing status, processing phase or supply chain was not significant ($P>0.05$, Fig. 4.3). R-values in the slaughter phase from both abattoirs (A: 0.314, B: 0.395) showed greater differences in beta diversity (higher R-value) in comparison to the boning phase (A: 0.134, B: 0.064). As discussed above, the faecal group in both abattoirs shared the least similarity in bacterial composition to the other groups (Fig. 4.2A and 4.2B). The higher R-values indicate that the bacterial composition of faeces is substantially different to the other sample groups (Fig. 4.2A and 4.2B), which increased the overall diversity differences during ANOSIM calculation. Therefore, this result is indicative of the ecological influence of the faecal groups being responsible for the stretch of the R-value (Fig. 4.3A and 4.3B), but not necessarily the hypothesized impact of STEC presence on bacterial diversity (Chopyk et al., 2016, Zhao et al., 2013). Previous STEC and diversity correlation studies have explored the diversity in a single sample type (i.e. faeces or hide) and this correlation does not necessarily apply across different sample types (Chopyk et al., 2016, Xu et al., 2014, Zaheer et al., 2017a, Zhao et al., 2013). Kang et al. (2019) demonstrated that the faecal bacterial populations shared the least similarity in bacterial composition (<60%) with carcase

and environmental samples from slaughter rooms. This supports the impact of faecal bacteria on the analysis of overall diversity differences between slaughter groups in this study.

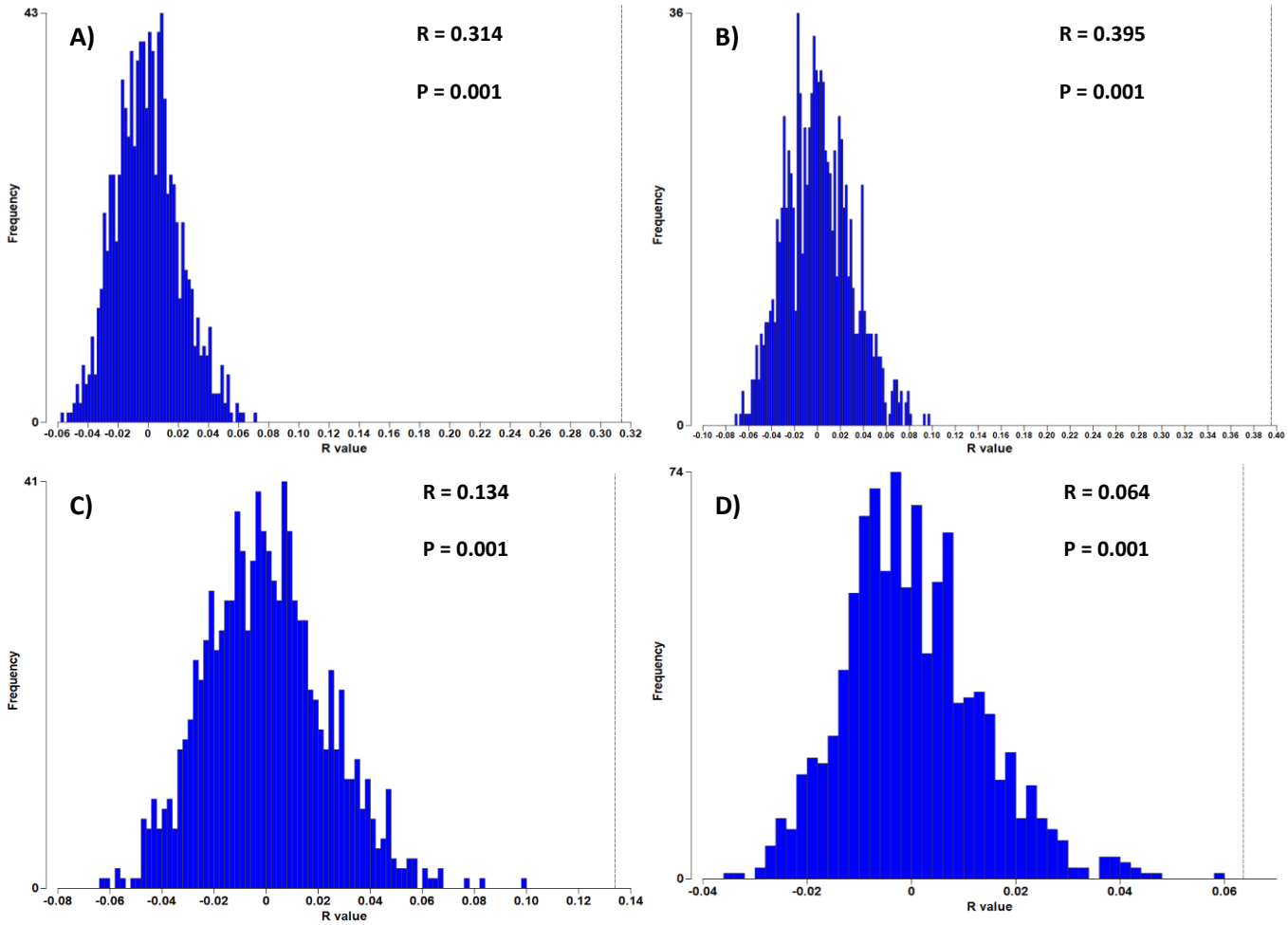

Figure 4.3 One-way analysis of similarity (ANOSIM) using a combined factor (STEC markers and sample type) for comparing bacterial diversity within and between sample groups. Slaughter visits for abattoir A and B are shown in A) and B); boning room visits for abattoir A and B are shown in C) and D). R-value for each ordination is indicated by the dotted vertical line. All the plots had a P-value of 0.001 showing significance for the difference between frequency of permutated samples and R-value from actual samples.

ANOSIM of bacterial diversity in the boning room added statistical confidence to the hypothesis of this study; that there is no correlation between STEC prevalence on bacterial diversity throughout beef processing. The histogram showing the frequency of permutations were similar between the abattoirs regardless of the processing phase but the R-values from the actual samples in the boning room reflected the higher level of similarity in diversity in comparison to slaughter rooms (Fig. 4.2 and 4.3). The R-values for abattoir A and B were 0.134 and 0.064, respectively, and both analyses had a P-value less than 0.05 (P=0.001, Fig. 4.3C and 4.3D). A previous study by Kang et al. (2020) demonstrated that carcase and trim

communities in the boning room consistently shared more than 70% similarity in composition and more than 50 – 70% with environmental microflora. This study took STEC testing status into consideration and produced an outcome where the distribution of high and low ranks of dissimilarity within and between carcase, trim and environmental groups was closer to being even. This indicated that the bacterial diversity of samples collected throughout the slaughter and boning process could not be used to predict the presence or absence of STEC.

4.4 Conclusion

In conclusion, correlation between bacterial diversity and the presence of the Top 7 STEC did not appear to exist through slaughter and boning phases in two Australian beef abattoirs with a different level of fragmentation in the supply chain. The major difference of this study to the previous studies is that this study investigated the processing stages in the beef supply chain. It is possible that the previous studies may have observed correlations of diversity or dominant bacterial species in response to the top seven STEC presence due to the fact that there were opportunities for bacterial populations to establish a community in the samples that were collected (faeces and hides in feedlots). A stable community is less likely to grow and become fully established on the meat in the processing environment as microbial groups within the microflora are consistently changing and exposed to intervention strategies employed in the processing facilities (Arthur et al., 2004, Brashears and Chaves, 2017, Kang et al., 2019, Kennedy et al., 2014). This study used 16S rRNA amplicon sequencing to show that the processing environment may not be the optimal part of the beef chain to better understand the possible relationship between STEC and diversity. Future studies using techniques with increased resolution such as shotgun metagenomics would be useful to provide a deeper understanding of the relationship between STEC presence and microbial diversity.

Chapter 5. Changes in bacterial communities during enrichment of manufacturing beef in selective and non-selective media

5.1 Introduction

Seven serogroups of Shiga toxin-producing *Escherichia coli* (STEC) commonly referred as the 'Top 7' STEC (O26, O45, O103, O111, O121, O145 and O157) have been declared as adulterants of raw non-intact beef in the United States. Manufacturing beef exported to the US and other North American markets must be deemed free of the Top 7 STEC to be accepted (Fegan et al., 2009, Fratamico et al., 2017, Kiermeier et al., 2011). Screening for STEC involves a stepwise PCR test of manufacturing beef for Shiga toxin (*stx*) and attaching and effacing (*eae*) gene, followed by testing for O-serogroup specific gene fragments (Food Safety Inspection Services, 2019a). The enrichment step prior to PCR screening is an important process to resuscitate injured and stressed cells in order to increase the concentration of STEC within the beef microflora that has a high level of interfering background flora (Ge and Meng, 2009, Guerini et al., 2006). An enriched sample that tests positive for *stx*, *eae* and one or more O-serotypes is deemed as a potential positive (PP). The PPs are processed to confirm the presence of STEC by isolating pure single colonies that are *stx*, *eae* and Top 7 O-serotype positive using immunomagnetic separation and PCR (Hussein et al., 2008).

Challenges remain as the conversion rate of PPs to confirmed positives is relatively low and the isolation step is not always successful (Bosilevac et al., 2007, Delannoy et al., 2016, Wang et al., 2013). This process is also time-consuming and laborious, identifying a strong need for optimisation in STEC detection protocols (Baker et al., 2016, Brusa et al., 2016, Vimont et al., 2006b). In recent years, sequencing-based technologies have been utilised to investigate the changes in bacterial diversity of microflora in relation to the presence of STEC prior to slaughter in the beef supply chain (Xu et al., 2014, Zaheer et al., 2017a). A correlation between lower prevalence of STEC and higher bacterial community diversity was reported in cattle hide (Chopyk et al., 2016) and faecal samples (Zhao et al., 2013). These studies demonstrated that high throughput sequencing techniques can be useful to understand the ecological dynamics occurring within the beef microflora in greater depth. Currently, the

changes in bacterial communities during the enrichment of manufacturing beef and the impact of the development of enrichment communities on the growth of STEC is not well understood.

The aim of this study was to use 16S rRNA amplicon sequencing to gain a better understanding of how bacterial community composition change during the enrichment of manufacturing beef by monitoring 16S rRNA profiles throughout the enrichment process. The underlying concept behind this study is to optimise the process of STEC detection and subsequently enhance the efficacy of STEC confirmation. Understanding the background bacterial communities that may affect enrichment of STEC populations will guide optimisation of selective enrichment strategies for detection of STEC.

5.2 Materials and methods

5.2.1 Experimental design and sample preparation

Boxes of frozen beef trim were obtained from an integrated and a fragmented Australian beef abattoir. The integrated abattoir sent two boxes of trim from manufacturing beef lots with unknown STEC status and the fragmented abattoir sent four boxes from lots that were confirmed positive for STEC (Table 5.1). All the boxes originated from independent processing days. Surface trim slices were removed to obtain 2 kg from each box from the integrated abattoir. For the fragmented abattoir, approximately 1 kg from each of two boxes was extracted and combined to make a bag containing 2 kg of trim. The same method was carried out for the other two boxes. Samples from the integrated abattoir were labelled Sample A and B. Samples from the fragmented abattoir were labelled Sample C and D. From each sample, 375 g was extracted using sterile technique and enriched in 1.5 L of four different enrichment media; buffered peptone water (BPW; Oxoid, Hampshire, UK), Assurance GDS MPX top 7 STEC mEHEC® medium (AMSL, New South Wales, Australia), BAX® *E. coli* O157:H7 MP medium (Oxoid) and PDX-STEC (Paradigm Diagnostics Inc., Minnesota, US). The enrichment media were pre-warmed at 42°C before the enrichment. After the addition of beef trim into the media, the enrichment broths (1:5 w/v ratio) were massaged gently for a minute prior to incubation at 42°C for a total of 23 hours without shaking, comprising 5 hours of pre-incubation and 18 hours of incubation. The 5 hours of pre-incubation allows for the temperature of the enrichment to return to 42°C following the addition of the beef trim sample. Samples are then incubated for 18 hours of continuous

enrichment at 42°C. A 30 mL aliquot was extracted at the commencement and completion of pre-enrichment and at 6-, 12- and 18-hour intervals of enrichment. The resulting samples were labelled -5, 0, 6, 12 and 18 hours. Approximately 5 mL was used for microbiological analysis and quantitative PCR, and the remaining volume was kept at -80°C for 16S rRNA sequencing analysis.

Table 5.1 Production and Shiga toxin-producing *Escherichia coli* (STEC) testing information for boxes of manufacturing beef from an integrated and a fragmented Australian beef abattoir.

	Abattoir	**Production Date**	**# of cartons received from lot**	**Lot status (if known)**
A	Integrated	13/11/2019	1	Unknown
B	Integrated	14/11/2019	1	Unknown
C	Fragmented	04/11/2019	2	STEC positive (O157)
D	Fragmented	05/11/2019	2	STEC positive (O157 & O26)

5.2.2 Analysis of 16S rRNA amplicon sequencing

5.2.2.1 DNA extraction for 16S rRNA amplicon sequencing

A 20 mL aliquot of each enrichment broth was centrifuged at 10,000 rcf for 5 minutes to pellet the cells. The pellets were washed twice with BPW (Oxoid) prior to DNA extraction using QIAamp PowerFecal DNA kit (Qiagen, California, USA) using the manufacturer's protocol with modifications in the bead beating step (Kang et al., 2019). Briefly, the bead beating cycle involved a total of three minutes of beating with a minute rest after the first and second beating. After three minutes of beating, the bead tubes were placed in a heat block for 10 mins at 65°C and the bead beating cycle was repeated.

5.2.2.2 Preparation for 16S rRNA sequencing

A 16S rRNA amplicon library was constructed from the extracted DNA targeting the V4 region using the protocol from a previous study Kozich et al. (2013). In brief, 5 µL from each DNA template was amplified with dual-index primers by PCR and the amplicons were normalised by comparing the intensity of the DNA band against the GeneRuler 100 bp Plus DNA Ladder (ThermoFisher, Victoria, Australia). The DNA materials were stained with ethidium bromide and visualised under ultraviolet light in 2% agarose gel under ultraviolet

light. Each template contained barcode sequences at the 5' and 3' end of the amplicon to enable demultiplexing of sequence reads. Approximately 50 ng of rRNA amplicon from each sample was combined to create a pooled library for sequencing. A microbial community DNA standard, ZymoBIOMICS™ (Zymo Research, California, US), was normalised using the same approach and added to the library. The library was purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, California, US) following the manufacturer's instruction. The concentration of amplicons was measured before and after purification using the Qubit® 2.0 Fluorometer (Life technologies, California, US). A NanoPhotometer® (Implen, California, US) was used to measure the purity and the final library was sent for sequencing.

5.2.2.3 Sequencing using Illumina MiSeq

The 16S rRNA pooled amplicon library was sequenced using the Illumina MiSeq platform (Illumina, California, US) with a paired-end 300 base pair sequencing protocol by Ramaciotti Centre for Genomics, University of New South Wales, Australia. A pooled library (20 nM) and a PhiX control v3 (20 nM; Illumina) were mixed with 0.2 N fresh NaOH and HT1 buffer (Illumina) to produce the final concentration of 12 pM each. The resulting library was mixed with the PhiX control v3 (5% v/v; Illumina) and 600 µL loaded on a MiSeq1 v2 (500 cycle) reagent cartridge for sequencing. All sequencing procedures were monitored through the Illumina BaseSpace® website, https://basespace.illumina.com.

5.2.2.4 Bioinformatic analysis of 16S rRNA profile

Both de-multiplexed R1 and R2 sequencing read (approximately 250 bp in length) files were acquired from the Illumina BaseSpace® website. Bioinformatic processing of raw sequence data was performed using the v1.40.5 MOTHUR pipeline (Schloss et al., 2009) following the MiSeq standard operating procedures (Kozich et al., 2013). The paired end reads were generated and clustered prior to assembly into operational taxonomic unit (OTU) tables with 97% as the cut-off for identification. The taxonomic classification of OTUs was performed using the SILVA 16S rRNA database (v132). For investigation of bacterial diversity between samples (beta diversity), relative abundance of Genus OTUs were used to create similarity ranks (resemblance matrix) using Bray-Curtis similarity. The matrix was used to create non-metric multidimensional scaling (nMDS) and analysis of similarity (ANOSIM). nMDS ordination and ANOSIM (with >999 permutations) were produced using ANALYSE tools

within PRIMER-7. For analysis of community composition, relative abundance at the Order level was used to create the heat map. Six OTUs represented 98% of the total sequences and the OTUs on average covered 97% of the sequences in each sample.

5.2.3 Microbiological analysis of total viable count, *Escherichia coli* and *Clostridium perfringens*

Total viable counts (TVCs) were obtained by plating 100 µL of enrichment broth samples in serial 10-fold dilutions prepared in saline (Oxoid) onto tryptic soy agar (TSA; Oxoid). The plates were incubated at 25°C for 72 – 96 hours. Enumeration of *Clostridium perfringens* was obtained by plating 100 µL of enrichment broth samples in serial 10-fold dilutions onto Tryptose Sulphite Cycloserine (TSC) agar and overlaid with Perfringens agar base (TSC without egg yolk) when dried. The agar plates were incubated at 35°C for 18 – 24 hours in an anerobic chamber containing Anaerogen sachets (ThermoFisher). Black colonies with an opaque white halo were enumerated and recorded as presumptive *C. perfringens*. The *E. coli* count was determined by plating 1 mL of enrichment broth samples in serial 10-fold dilutions onto Petrifilm™ *E. coli*/Coliform count plate (3M™, New South Wales, Australia). Colonies were counted as per the manufacturer's instructions.

5.2.4 STEC isolation and characterisation

Isolation of Top 7 STEC was performed with commercially available Assurance GDS® MPX Top 7 STEC beads (AMSL) following the manufacturer's instructions. The bead-bacteria complexes at the completion of IMS were plated onto two different media; sorbitol MacConkey with cefixime-tellurite supplement (0.05 mg/L cefixime and 2.5 mg/L potassium tellurite; Oxoid) and CHROMAgar™ STEC (Dutec Diagnostics, New South Wales, Australia). All media were incubated for 18 – 24 hours at 37°C.

Following IMS, phenotypically distinct colonies from each plate were streaked onto TSA (Oxoid) and incubated for 18 – 24 hours at 37°C. All of the isolates were initially tested for *stx*$_1$, *stx*$_2$, *eae* and *ehxA* using conventional multiplex PCR as described by Paton and Paton (Paton and Paton, 1998a). The isolates with *stx* and *eae* were then characterised for Top 7 O-serotypes using conventional PCR according to Microbiology Laboratory Guidebook (MLG) 5C (Food Safety Inspection Services, 2019a).

5.2.5 Quantitative PCR analysis

A 1 mL aliquot of enrichment broth was centrifuged in an Eppendorf tube at 13,000 rcf for three minutes. The cell pellet was resuspended in 500 mL of ultrapure water after discarding the supernatant. The resuspension was placed in a heating block set at 100°C for ten minutes. After cooling to room temperature, the tubes were centrifuged at 13,000 rcf for three minutes. The boiled cell lysates were used to quantify O157 and *eae* markers alongside known concentrations of standard curves. PCR data were converted into cell concentration (log_{10}CFU/mL) in Microsoft Excel (v16). ViiA™ 7 Real-Time PCR System (Applied Biosystems, Victoria, Australia) was used for all PCR tests. Sequences of primers and probes used for the PCR tests were adopted from Perelle et al. (2004) and FSIS MLG 5C Appendix 4.00 (Food Safety Inspection Services, 2019b).

5.3 Results and discussion

5.3.1 Changes in bacterial diversity during enrichment of manufacturing beef

Enrichment samples were plotted on non-metric multidimensional scaling (nMDS) to compare the changes in bacterial community diversity and composition within each sample through the enrichment process of beef trim in four different media (Figure 5.1). Analysis of similarity (ANOSIM) indicated that there were no significant differences (P=0.001) in bacterial community diversity between samples (A, B, C and D) that were enriched in different enrichment media (BPW, mEHEC, MP and PDX-STEC; Figure 1B). It appeared that the samples at the beginning of enrichment (in the first 6 hours) were generally grouped in the left region on the ordination and shifted away into the right side after enrichment, regardless of the enrichment broth used. The results demonstrated that the samples were randomly spread after 6 hours of enrichment with no obvious differences separating the dispersed samples (Figure 5.1A).

The nMDS demonstrated that diversity in bacterial community did not substantially change after 6 hours of enrichment and that the structure within the bacterial community after this point generally remained similar throughout the remaining period of enrichment (Figure 5.1). The recommended duration of enrichment for the enrichment media used in this study does vary, however, all require a minimum of 10 hours enrichment at 42°C (Feldsine et al., 2016, Guerini et al., 2006, Wasilenko et al., 2014). The similarities of bacterial communities across

the enrichment media after the first 6 hours of enrichment would indicate that the suggested incubation timeframes are likely to provide similar community outcomes provided the pre-enrichment time is taken into consideration. Similar diversity profiles between the samples throughout enrichment may be due to particular bacterial members becoming dominant in the enrichment community after 6 hours into enrichment and limiting diversification within the community (Brusa et al., 2016, Vimont et al., 2006b).

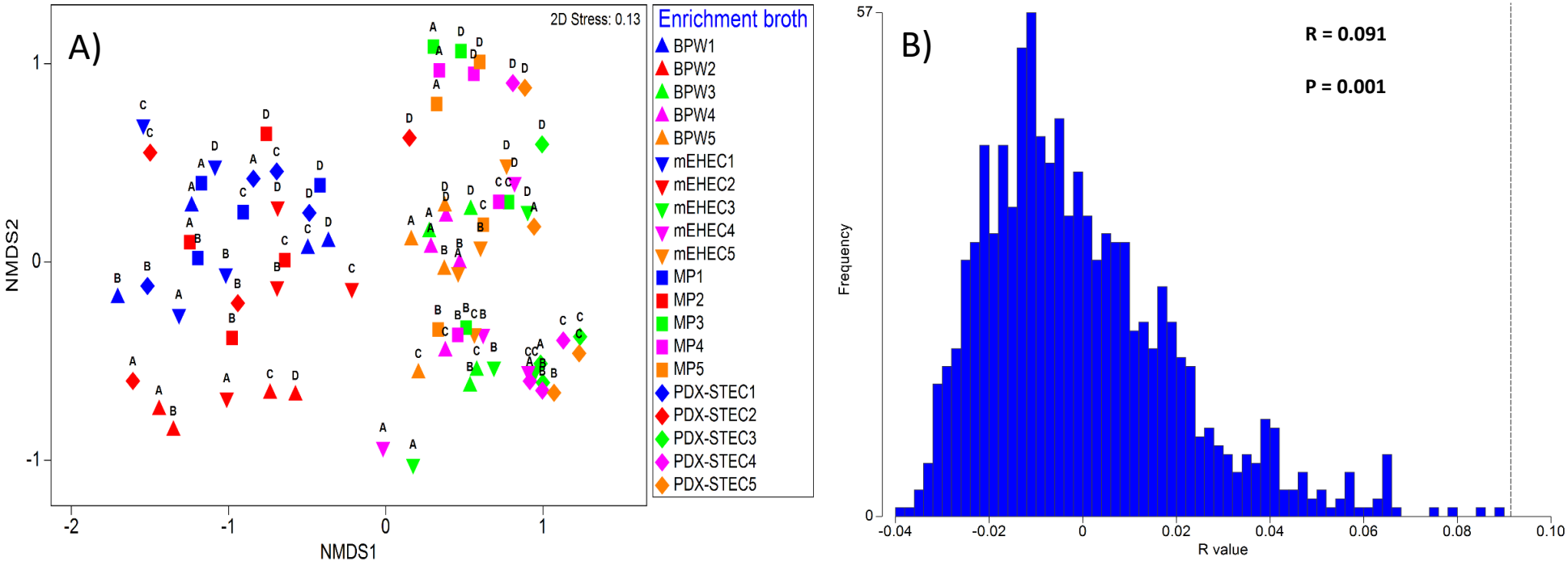

Figure 5.1 Differences in beta community diversity of samples from an integrated (A and B) and a fragmented (C and D) abattoir through enrichment in four different enrichment media are shown in A) non-metric multidimensional scaling (nMDS). Each enrichment broth (BPW, mEHEC, MP and PDX-STEC) is assigned with numbers from 1 to 5; 1 = -5 hours, 2 = 0 hours, 3 = 6 hours, 4 = 12 hours and 5 = 18 hours. In B), analysis of similarity (ANOSIM) with 999 permutations using enrichment media as a function of diversity produced an R-value of 0.091 and a P-value of 0.001.

5.3.2 Composition of the bacterial communities during enrichment

Previous studies investigated the effect of enrichment by comparing counts of a few bacterial groups after enrichment, limiting the understanding of the enrichment process to a basic level (Hussein et al., 2008, Stromberg et al., 2015b, Vimont et al., 2007). For this analysis, the 16S rRNA amplicon sequences were analysed at the Order level in order to understand the effect of enrichment on the relative abundance of Enterobacteriales as a collective population, including STEC, and general ecological shifts within the community throughout enrichment. Figure 5.2 indicates that the composition of the communities in the enrichment broths before the enrichment was similar, and that Enterobacteriales or Clostridiales generally dominated the bacterial community in the latter stages of enrichment. An interesting pattern was observed in the changes of bacterial populations in samples from both abattoirs. In general, the relative abundance of Enterobacteriales was inversely associated with the relative abundance of Clostridiales. After 6 hours of enrichment in mEHEC, two samples from the integrated abattoir (Sample A and B) and one sample from the fragmented abattoir (Sample C) had a high level of Enterobacteriales with a low level of Clostridiales while Sample D had slightly higher level of Clostridiales (Figure 5.2). PDX-STEC broths showed similar results where Enterobacteriales dominated the population with the exception of Sample D in which Clostridiales abundance was substantially higher than Enterobacteriales after 5 hours of pre-enrichment.

Bacterial communities in MP broths had variations between the samples and these discrepancies varied more in comparison to the other selective enrichment media. In general, Enterobacteriales was dominant in the enrichment broths of samples A, B and C (Figure 5.2). However in MP enrichment broths, Clostridiales was the most predominant in Sample A and the relative abundance of Enterobacteriales and Clostridiales were similar during enrichment of Sample C. MP enrichment medium is frequently used during enrichment for detection of STEC using the BAX® PCR system in Australia which is a method approved for rapid screening of STEC by the Department of Agriculture Water and Environment. (2019). The observations from this study suggest that MP may facilitate the growth of Clostridiales better than the other enrichment media and that enrichment with MP medium may not provide the best solution for the purpose of optimising STEC enrichment and detection.

The 16S rRNA profile throughout the slaughter phase in the same abattoirs as this study indicated that residential microflora on environmental surfaces had an impact on the carcase microflora in the integrated abattoir (Kang et al., 2019). Observations from the previous study suggested that control of Clostridiales in the environment of the slaughter floors in the integrated abattoir could contribute to reducing the level of Clostridiales throughout processing and ultimately in the manufacturing beef. Abattoir A used a downward hide puller whereas abattoir B used an upward hide puller. Kang et al. (2019) suggested that the upward hide pulling at abattoir B may have contributed more to transfer of hide contaminants onto the carcases. The samples from abattoir B (Samples C and D) in this study had higher relative abundances of Clostridiales before enrichment in comparison to the integrated abattoir (Samples A and B) suggesting that robust hygienic practices during hide removal may be a good strategy to control the level of Clostridiales in the beef microflora in the fragmented abattoir.

BPW is a generic nutrient media that provides a supportive nutritional environment for the growth of microorganisms while mEHEC, MP and PDX-STEC are enrichment media that selectively promote the growth of STEC (Eggers et al., 2018, Feldsine et al., 2016, Guerini et al., 2006, Wasilenko et al., 2014). However, observations from this study suggested that the presence of Enterobacteriales (and potentially the STEC populations) could be masked by Clostridiales through enrichment despite using selective enrichment media. Enterobacteriales represent a large number of enteric bacterial groups and the changes in abundance of Enterobacteriales does not necessarily mean that the populations of Top 7 STEC change in the same proportion within the bacterial community (Nguyen and Sperandio, 2012). However, this study indicated that further investigation of the two groups during enrichment is necessary to expand the understanding of changes in the level of STEC and the background microflora during enrichment.

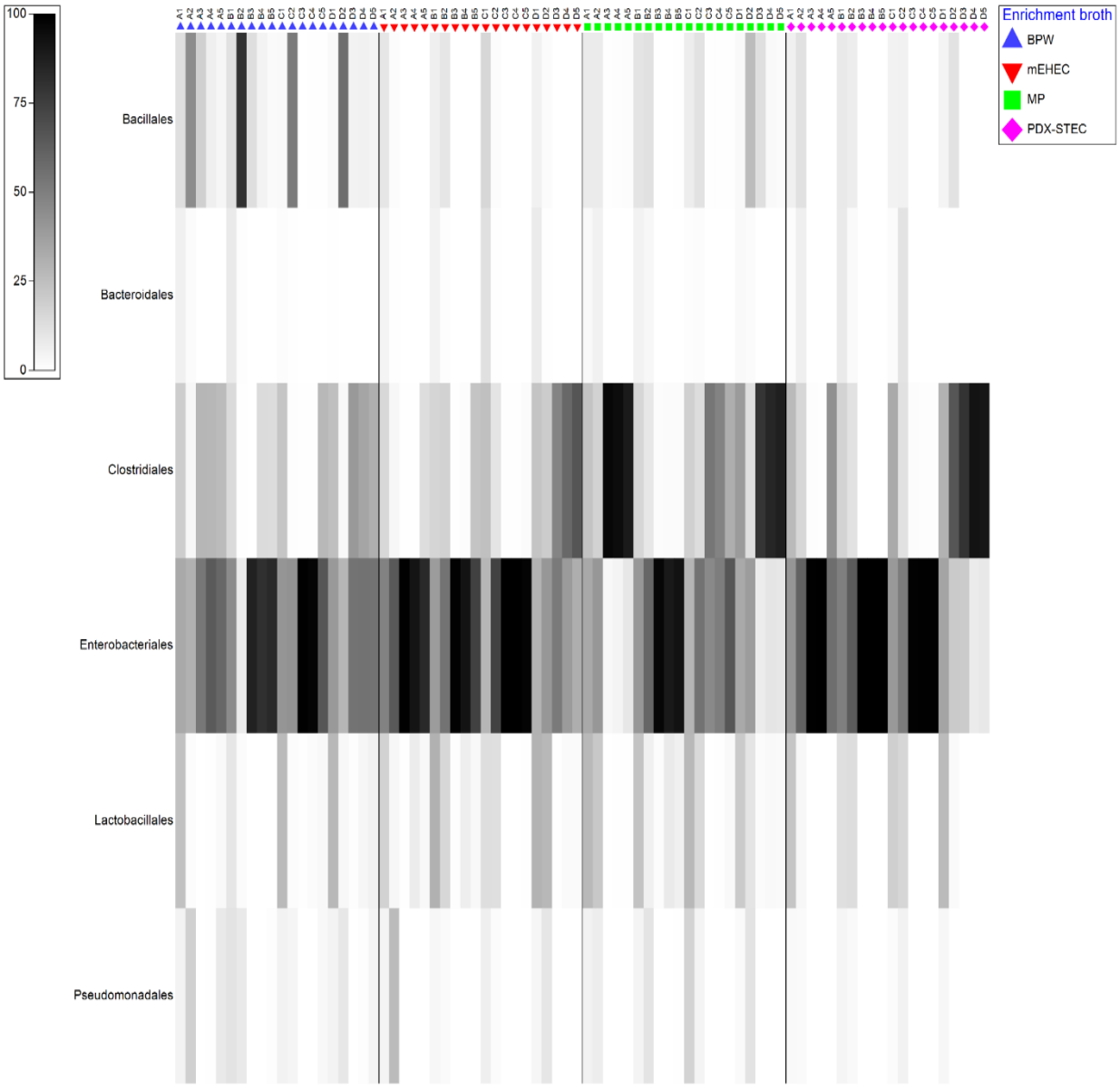

Figure 5.2 Heat map showing the dominance of Clostridiales and Enterobacteriales in the bacterial communities of four samples through enrichment in different media (BPW, mEHEC, MP and PDX-STEC). The relative abundance of each OUT is indicated by the scale (on the left) with the highest ratio at 100. Taxonomic affiliations at the Order level are shown on the left of the heat map. On average, two OTUs covered 97% of total sequence reads in each sample and represented 98% of the total reads. Samples from an integrated (A and B) and a fragmented (C and D) abattoir are numbered from 1 to 5 (1 = -5 hours, 2 = 0 hours, 3 = 6 hours, 4 = 12 hours and 5 = 18 hours) to indicate the enrichment period.

5.3.3 Changes in relative abundance of *Clostridium* and *Escherichia* in different enrichment media

Analysis of 16S rRNA sequences at the Genus level demonstrated that *Clostridium* and *Escherichia* had a similar relative abundance at the beginning of enrichment within each

sample but varied by the end of the enrichment process (Table 5.2). *Clostridium* represented 95% of 16S rRNA sequences belonging to Clostridiales populations while 84% of Enterobacteriales was *Escherichia* (data not shown). A substantial proportion of the community in BPW-enriched Sample B was dominated by *Escherichia* (80.6%) but this group was less prominent in Sample A (48.2%), C (58.5%) and D (53.3%, Table 5.2). *Clostridium* made up 25.1%, 13.1% 0.3% and 30.7% of the post-enrichment community in Samples A, B, C and D, respectively. In Sample A and D, the remaining proportion (26.7% and 16.0%) of the community was occupied by *Acinetobacter, Kurthia* and unclassified *Bacillaceae* and *Enterobacteriaceae* (data not shown). Sample C was the only sample to contain *Veillonella* at 27.1% after enrichment. These groups of bacteria have been reported in previous studies during analysis of microorganisms in beef products (Corry, 2006, Foster et al., 1977) and in the rumen of beef cattle (Titgemeyer and Nagaraja, 2006). It was not surprising to find these OTUs in the post-enrichment communities in BPW considering that BPW is a non-selective enrichment media (Baker et al., 2016, Drysdale et al., 2004). The BPW-enriched samples had higher relative abundance of 16S rRNA sequences of bacterial groups that were not observed in mEHEC, MP and PDX-STEC at the same level. BPW is preferred in some studies and it is commonly used as an enrichment broth (Baker et al., 2016). Isolation of STEC can become more challenging and time-consuming in the presence of high numbers of interfering background microflora (Brusa et al., 2016, Foster et al., 2003, Vimont et al., 2006a). The analysis of 16S rRNA profile in BPW broths suggested that enrichment in BPW can result in higher levels of background microflora in comparison to the selective enrichment media used in this study. This can potentially have a negative effect on the isolation procedures downstream.

In general, more than 90% of the bacterial community in the enrichment broths was dominated by *Escherichia* and *Clostridium* at the end of enrichment (Table 5.2). *Escherichia* was consistently found as the dominant group with the highest relative abundance in the post-enrichment community of Samples B and C regardless of the enrichment media. Enrichment of Samples B and C in PDX-STEC gave the highest relative abundance of *Escherichia* at 97.7% and 99.2%, respectively while the proportion of *Clostridium* was minimal at 0.1% and 0.7%, respectively. Sample C had *Escherichia* as the most dominant in the community after enrichment in MP at 64.9% but was substantially less in mEHEC (95.2%) and PDX-STEC (99.2%). The relative abundance of *Clostridium* and *Escherichia* in the bacterial communities

of Sample A and D enriched in mEHEC, MP and PDX-STEC varied. In these samples, relative abundance of *Clostridium* and *Escherichia* at the beginning of pre-enrichment varied in different enrichment media but was relatively similar within each medium (Table 5.2). *Clostridium* before pre-enrichment in MP broth was lower than *Escherichia* by 4.5% but reached 87.2% after enrichment becoming the most dominant in Sample C while *Escherichia* was at 8.1%. *Escherichia* was the most dominant group after enrichment in mEHEC and PDX-STEC with a relative abundance of 79.6% and 53.9%, respectively. *Clostridium* was the most dominant group in Sample D in the selective enrichment broths at varying proportion between the broths, with the highest abundance in PDX-STEC at 90.6% and the least in mEHEC broth at 65.5%.

Table 5.2 Changes in relative abundance (%) of *Clostridium* and *Escherichia* in four samples (A, B, C and D) during enrichment in different media (BPW, mEHEC, MP and PDX-STEC). Relative abundance of *Clostridium* and *Escherichia* in enrichment samples from an integrated (A and B) and a fragmented (C and D) abattoir is reported at -5 hours, 0 hours, 6 hours, 12 hours and 18 hours of enrichment.

	Sample A					**Sample B**					**Sample C**					**Sample D**				
	-5h	**0h**	**6h**	**12h**	**18h**	**-5h**	**0h**	**6h**	**12h**	**18h**	**-5h**	**0h**	**6h**	**12h**	**18h**	**-5h**	**0h**	**6h**	**12h**	**18h**
Genus	**BPW**																			
Clostridium	12.8	0.9	26.9	27.0	25.1	3.6	0.2	0.0	13.7	13.1	19.6	0.3	0.2	0.3	0.3	21.3	1.2	39.0	34.4	30.7
Escherichia	14.7	1.0	42.7	50.5	48.2	3.7	3.7	85.2	79.1	80.6	21.2	19.9	88.4	88.2	58.5	23.8	27.5	53.8	53.2	53.3
	mEHEC																			
Clostridium	7.8	2.7	0.4	0.1	13.3	9.3	11.8	0.2	1.3	22.9	10.9	9.5	0.4	0.4	0.6	13.9	11.8	47.5	58.2	65.5
Escherichia	10.4	2.7	10.6	12.0	79.6	14.0	21.5	95.6	89.6	71.6	15.9	51.3	97.7	97.3	95.2	14.5	29.2	51.5	38.7	31.4
	MP																			
Clostridium	9.3	9.3	95.7	94.1	87.2	10.5	4.6	0.7	0.3	0.3	12.7	5.7	52.8	47.1	30.2	32.7	7.4	77.3	81.5	80.2
Escherichia	13.8	14.2	1.6	3.6	8.1	16.0	13.1	95.3	87.6	89.7	20.5	45.9	45.8	50.2	64.9	21.7	34.9	7.2	10.1	9.1
	PDX-STEC																			
Clostridium	22.1	7.3	0.7	0.4	43.0	6.2	7.0	0.1	0.1	0.1	27.3	3.8	1.2	0.9	0.7	24.9	64.4	79.4	91.9	90.6
Escherichia	23.9	3.7	96.7	94.9	53.9	10.2	15.8	98.0	97.2	97.7	28.4	31.9	98.7	98.9	99.2	23.3	18.9	19.8	7.5	8.8

The analysis of 16S rRNA sequences demonstrated that the population of *Escherichia* can vary in the bacterial community after enrichment using different enrichment media. Initial relative abundances of *Clostridium* and *Escherichia* were similar within the samples but the group with marginally higher proportion typically became the most dominant after the enrichment in the broths. *Clostridium* is an obligate anaerobe but some *Clostridium* species are known to tolerate low concentrations of oxygen in the environment with a longer lag phase and generation time (Juneja et al., 1994). It is possible that the conditions in some enrichment broths became more favourable for *Clostridium* as oxygen concentration in the broth decreased by cellular respiration and suffocation of head space in the enrichment bag from accumulation of beef trim fat debris on the surface (Zhou et al., 2011). *Clostridium* is known to survive and grow in beef during storage at higher temperatures under microoxic conditions (Al-Qadiri et al., 2015). Introducing shaking during incubation may be worth investigating as it could prevent the potential effect of suffocation and any chance of biofilm formation on the meat. Nevertheless, this study suggests that controlling the level of *Clostridium* during beef enrichment could potentially increase the chance of detecting STEC by enabling *Escherichia* to reach greater relative abundance in the post-enrichment community.

5.3.4 Bacterial counts during enrichment in BPW, mEHEC, MP and PDX-STEC

The enrichment samples were measured for total viable count (TVC), *E. coli* and *Clostridium perfringens* (Figure 5.3). TVC exponentially increased and reached greater density than 8 $\log_{10}$CFU/mL after the enrichment process (Figure 5.3). *E. coli* counts showed similar results where the bacteria were undetected at -5h and reached an average count of 8.2 $\log_{10}$CFU/mL in BPW, 7.6 $\log_{10}$CFU/mL in mEHEC, 7.8 $\log_{10}$CFU/mL in MP and 8.0 $\log_{10}$CFU/mL in PDX-STEC at 6h (Figure 5.3). The count of *E. coli* continued to remain high at 10^{8-9} $\log_{10}$CFU/mL at 12h and 18h. It was not surprising for *E. coli* to reach high numbers during enrichment as *E. coli* is frequently found in beef products and is capable of rapid proliferation in nutrition rich environments (Barlow and Mellor, 2010, McEvoy et al., 2004, Kang et al., 2019, Vimont et al., 2006b). Unlike TVC and *E. coli*, counts of *C. perfringens* varied throughout enrichment. In BPW, mEHEC and PDX-STEC, the average count of *C. perfringens* ranged between 3.0 – 4.4 $\log_{10}$CFU/mL after 6h (Figure 5.3A, 5.3B and 5.3D). In

MP, *C. perfringens* increased to an average of 8.1 log_{10}CFU/mL at 6h and decreased to 6.2 log_{10}CFU/mL at 12h (Figure 5.3C).

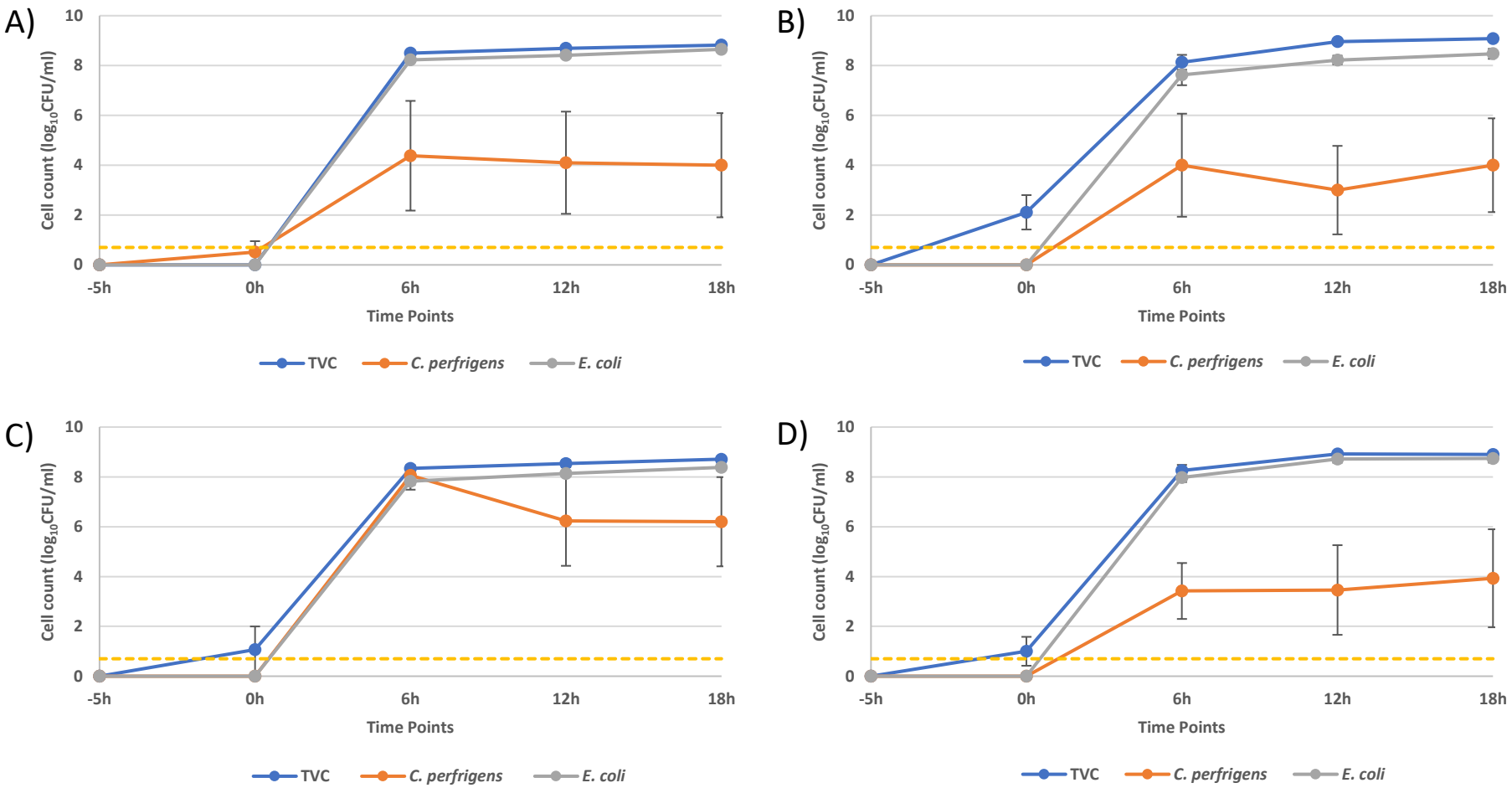

Figure 5.3 Analysis of total viable count (TVC), *Clostridium perfringens* and *Escherichia coli* in four different enrichment broths through enrichment; A) BPW, B) mEHEC, C) MP and D) PDX-STEC. Microbiological count was carried out at -5 hours, 0 hours, 6 hours, 12 hours and 18 hours through enrichment. The yellow dotted line represents the limit of detection at <0.7 log_{10}CFU/mL for count.

The numbers of *E. coli* did not show any differences in enrichment media with high or low levels of *C. perfringens*. This suggested that the count of *E. coli* was unlikely to be affected by the presence of *C. perfringens*. *C. perfringens* is sensitive to free oxygen radicals that may have been produced from aerobic respiration by *E. coli* and other species which most likely had an impact on counts of *C. perfringens* in BPW, mEHEC and PDX-STEC enrichment broths (Akhtar et al., 2009, Juneja et al., 2006, Kamber et al., 2007). The counts showed different trends in results to the sequence analysis and it is possible that different species of *Escherichia* and *Clostridium* were accountable for the trends observed in the 16S rRNA analysis. Another aspect to consider is that the counts of *C. perfringens* and *E. coli* was obtained using different cultivation methods meaning that competition for limited nutrients and space between the two groups within a single ecosystem was eliminated.

5.3.5 Analysis of STEC markers and confirmed isolates

The enrichment samples were tested for O157 and *eae* markers using quantitative PCR and the results are shown in Figure 5.4. No O157 and *eae* markers were detected in the enrichment broths of Sample A (below the limit of detection at 2.5 $\log_{10}$CFU/mL, Fig. 5.4A). Figure 5.4B indicates that there may be a population of *eae*-negative O157 *E. coli* in Sample B enriched in PDX-STEC (3.5 $\log_{10}$CFU/mL). Sample C was the only sample to contain both O157 and *eae* markers after enrichment in all media. The detection of the O157 marker ranged from 4.8 – 5.8 $\log_{10}$CFU/mL and detection of the *eae* marker varied between 5.8 – 6.5 $\log_{10}$CFU/mL (Figure 4C). In Sample D, the O157 marker was not detected in any of the enrichment broths but the *eae* marker was detected in BPW (6.5 $\log_{10}$CFU/mL), mEHEC (5.5 $\log_{10}$CFU/mL) and MP (4.8 $\log_{10}$CFU/mL) broths after enrichment.

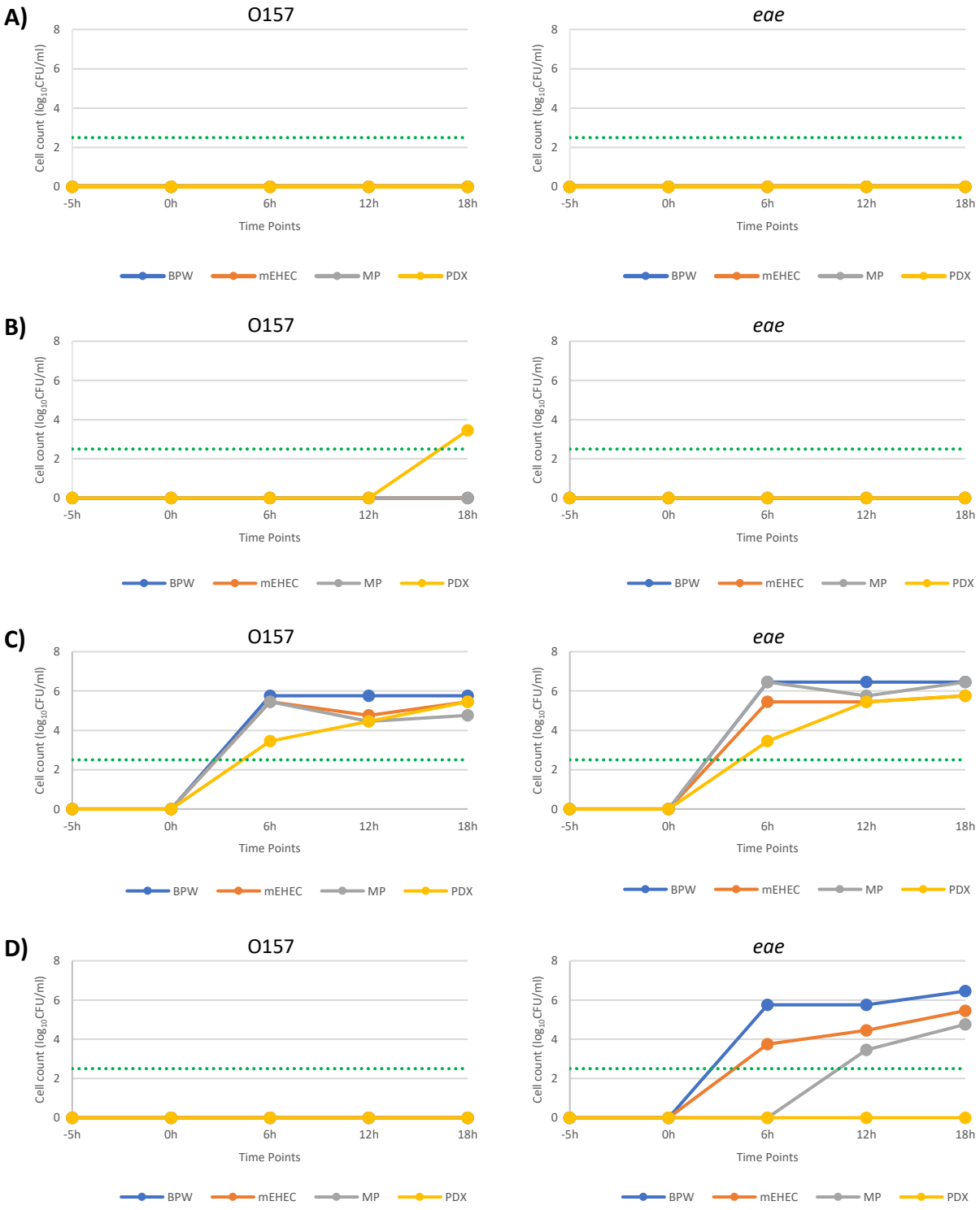

Figure 5.4 Quantitative PCR analysis of O157 and *eae* STEC markers in samples from an integrated (A and B) and a fragmented (C and D) abattoir during enrichment in four different media; BPW, mEHEC, MP and PDX-STEC. The markers were quantified at -5 hours, 0 hours, 6 hours, 12 hours and 18 hours of enrichment. Known concentrations of standard curves for each marker was used to convert PCR results into cell count reported in log_{10}CFU/mL. The green dotted lines in the graphs represent the limit of detection at 2.5 log_{10}CFU/mL.

Sample C came from O157 STEC positive lot while Sample D derived from O26 and O157 STEC positive lot (Table 5.1). Sample C tested positive for *eae* and O157 markers, and O157 STEC isolates were recovered (Fig. 5.4C). In contrary, STEC could not be isolated from Sample D despite PCR evidence for the presence of *eae* (Fig. 5.4D). The challenges in isolation of STEC from positive lots of manufacturing beef was demonstrated in a study by Kiermeier et al. (2011) which highlighted the difficulty associated with recovering O157 STEC from manufacturing beef cartons that had previously generated a confirmed positive result. In Australia, N60 sampling is used to test for the presence of STEC in manufacturing beef. In this process, five samples are collected from each of 12 cartons to form a composite 375 g samples which is subsequently tested for STEC. This means that although the cartons used in this study came from a STEC positive lot, it is possible that they did not contribute to the original positive lot result (Kiermeier et al., 2011). It is highly likely that the two boxes that Sample D represented in this study had no or low concentrations of O157 and O26 STEC after enrichment due to the nature of sampling and testing system of STEC. The 16S rRNA and PCR analysis of Sample C demonstrated that detection of STEC could be enhanced by maximising the growth of *Escherichia* by using appropriate selective enrichment media during the enrichment step.

5.4 Conclusion

Currently, there is a need for improvement in the enrichment and subsequent isolation of STEC in the beef industry. Enrichment increases the chance of detecting the presence of Top 7 STEC by selectively favouring STEC populations. However, growth of different bacterial populations through enrichment is poorly understood. This study demonstrated that there are variations in the growth of *Escherichia* in different selective media during enrichment. Enrichment conditions can create competition between *Escherichia* and other groups of bacteria in the background, such as *Clostridium.* Comparison of 16S rRNA profiles in beef trim samples revealed that the profiles of bacterial communities were similar regardless of the source through pre-enrichment. It appeared that stable populations developed through enrichment that were typically dominated by *E. coli* and *Clostridium*. Evidence of O157 and *eae* markers were detected in Sample C and O157 STEC isolates were only obtained from Sample C. Sample D also originated from a STEC positive lot but no isolates were obtained. The analysis suggested that Sample D most likely did not contain Top 7 STEC and the

selective enrichment media were not responsible for the failure of STEC detection. Currently, no single method has been identified as the best system for detection of Top 7 STEC. Sequencing analysis of the ecological dynamics during enrichment can provide an insight into the effect of enrichment protocols on the microflora during enrichment of manufacturing beef, which was not possible with conventional microbiology methods. This study evaluated sequencing technology as a useful tool to provide strategies to increase efficiency in detection of STEC by identifying potential background populations that may interfere with proliferation of the target group. This could subsequently optimise the process of STEC confirmation and contribute to controlling the presence of STEC in the beef industry.

Chapter 6. General Discussion

6.1 General conclusions

The Australian beef industry is globally recognized for producing premium, high quality products that are safe with long shelf-life (Phillips et al., 2012). The industry is one of the world's largest exporters of beef and veal, exporting approximately 72% of its total production to over 70 countries in 2018-19 (Meat & Livestock Australia., 2019). Ensuring the safety of beef is a high priority for the industry in order to protect public health and meet international requirements for export (Fegan et al., 2009, Pointon et al., 2012). Beef cattle is a major reservoir of a foodborne pathogen called Shiga toxin-producing *Escherichia coli* (STEC) which has occasionally contaminated beef products, causing outbreaks around the world. Previously, microbial contamination of beef products has been studied using conventional methods by cultivating a selected group of bacteria including pathogens and indicator microorganisms (Barlow et al., 2015, Bosilevac et al., 2005, Dewsbury et al., 2015, Koohmaraie et al., 2005). The advent of next-generation sequencing technologies has enabled a new set of tools that provide deeper analysis of changes in bacterial community composition and contamination pathways for the industry to gain greater understanding and opportunities to control transmission of contaminants.

Sequencing techniques, such as 16S rRNA amplicon sequencing, can be used to describe the flow of bacterial populations through processing phases of beef cattle (slaughter and boning). This provides an opportunity to investigate relationships between bacterial community structure and the potential for STEC to be present with greater resolution than was previously available. Additionally, understanding how the bacterial communities on beef products affect the ability to detect and isolate STEC may provide additional insights that enable optimisation of methods for STEC detection and isolation. Ultimately, the goal is to prevent STEC from getting onto the carcases and subsequently ending up in beef products. The objectives of this project focused on the application of 16S rRNA analysis to enable greater understanding of the microbial communities that prevail and persist within beef processing environments, and to permit the development of strategies that may assist in controlling contamination events during beef processing.

6.2 Chapter 2: Slaughter phase

The first objective was to investigate the changes in the bacterial communities and determine if the ecological shifts could provide deeper understanding of microbial contamination through the slaughter phase of beef processing. The slaughter phase is essentially the first stage of processing where the processors have an opportunity to limit contamination of carcases from faeces and hides as live animals are transformed into carcases (Arthur et al., 2017, Stromberg et al., 2015a). Better understanding of contamination at this stage can be important to the industry by providing greater insights of carcase contamination, giving them opportunities to preserve microbiological quality. An integrated (A) and a fragmented (B) abattoir had similar bacterial 16S rRNA profiles that were predominant through slaughter (e.g. Bacteroidales, Clostridiales, Lactobacillales and Pseudomonadales) despite the differences in the level of integration within their supply chains; abattoir A received cattle produced under a consistent and defined production system whereas abattoir B received cattle from many different sources and production pathways. Both abattoirs significantly reduced total viable counts (TVC) on carcases after hide removal ($P<0.05$) but showed different trends in transmission of contaminants during the process.

Previous studies using only conventional cultivation methods hypothesized that microbial contaminants are transferred from hides containing faecal contaminants onto carcases and the carcases become a vector of cross-contamination that ultimately affects the quality and safety of beef products (Antic et al., 2010, Kennedy et al., 2014, Sumner et al., 2003). Chapter 2 suggested that hide to carcase contamination was more prominent in abattoir B and that the carcase microflora in abattoir A was more influenced by the residential bacterial populations on the environmental surfaces. Abattoir B was using an upward hide pull system and the general consensus is that transmission of contaminants from hide to carcases is increased with this mechanism in comparison to downward pulling which was the preferred system in abattoir A (Kennedy et al., 2014). The analysis of 16S rRNA profiles suggested that the upward hide pulling was likely to have transferred hide bacterial communities onto the carcases, however further evaluation would be required to confirm that the direction of the hide pull system was the main driving factor by specifically targeting this procedure.

It was assumed that the processing environment can have an impact on the overall contamination of a carcase, but it was difficult to define this connection using conventional cultivation methods (Galland, 1997, Yang et al., 2017). However, the 16S rRNA analysis in

Chapter 2 (and 3) highlighted the importance of environmental contamination of beef through the processing phases. Environmental contaminants had more influence on the carcases in abattoir A where hide to carcase contamination was minimised. It was interesting to find that the 16S rRNA profiles on carcases and environmental surfaces shared high similarity while TVC on both carcases and surfaces increased throughout slaughter. This suggested that controlling contamination pathway from the environment to the carcases during slaughter in abattoir A can potentially help to produce carcases with lower microbial loads. The main outcomes of this Chapter highlighted the importance of maintaining good hygiene via regular sanitation and how certain practices (e.g. hide pulling) can have more impact on the microbiological status of carcase during slaughter.

6.3 Chapter 3: boning phase

The second objective was to examine the bacterial communities through the second phase of beef processing; boning phase. In addition, this study was also designed to observe if post-slaughter microflora continued through to the boning phase by collecting samples from slaughtered carcases that were subsequently boned the next day. This phase of beef processing is where carcases are refined into primal cuts and manufacturing beef are produced. Knowledge of changes in microbial communities during this process is generally lacking in the literature. Profiling of 16S rRNA amplicons showed, although compositional changes varied between the abattoirs, the core members of the microflora initially present on post-slaughter carcases persisted through to the beef products at the end of boning.

It is inevitable that the meat will be in contact with surfaces during the boning process as the trimmed pieces from the carcases move through on conveyor belts and frequently contact other surfaces due to the oscillation of the belts. Sequencing analysis demonstrated that residential microflora on contact surfaces throughout the boning room interacted with the beef microflora in varying consistency depending on the day of boning. The results also suggested that microbial contamination of manufacturing beef (average TVC of 2.6 – 2.7 $\log_{10}$CFU/cm^2) appeared to be a result of accumulative contamination from the environment through the boning process. The findings from this chapter indicated that the boning phase is essentially transferring the incoming contamination from slaughter onto the beef products. This means that maintenance of a good hygiene level and control of contaminants from the beginning of the entire beef processing chain is important to improve microbiological quality of beef products.

6.4 Chapter 4: STEC and bacterial diversity

The analysis of 16S rRNA sequences permitted the ecological changes that occurred during processing of Australian beef cattle to be described in detail (Chapter 2 and 3). The third objective of this study was to use this information to search for bacterial groups within the beef microflora that correlate with the presence of STEC. Previous studies have explored this relationship using sequencing techniques, however, the conclusions varied and the 16S rRNA profiles were obtained from pre-processing environments and not during slaughter or boning (Chopyk et al., 2016, Xu et al., 2014, Zaheer et al., 2017a). This study sought to determine if there is a correlation between changes in bacterial diversity and presence of STEC through the beef processing phases in an attempt to define bacterial composition or groups that can predict the presence of STEC.

The findings presented in Chapter 4 suggested that bacterial diversity may not be a valid option as a bioindicator of STEC presence during the processing of Australian beef cattle. Regardless of the sample type, bacterial diversity had no significant effect on the presence of Top 7 STEC (O26, O45, O103, O111, O121, O145 and O157) through an integrated and a fragmented Australian beef abattoir. Simpson's index was measured for each sample group to compare alpha diversity (species diversity within a sample group) and no significant differences were identified when using STEC testing status as a function of diversity. The samples were plotted on non-metric multidimensional scaling (nMDS) and the groups did not cluster by STEC testing status. Analysis of similarity (ANOSIM) indicated that the differences in bacterial diversity between the sample groups (beta diversity) were not significantly affected by the presence of STEC. Bacterial populations in STEC positive and negative samples were compared but there were no bacteria that were differentially abundant between the two groups in this study. The samples generally shared similar bacterial community structures (e.g. types and relative abundance of bacterial groups) when the samples were divided by STEC testing status.

Previous studies that investigated the correlation between bacterial diversity and STEC examined hide and faecal samples prior to processing (e.g. commercial feedlot and holding pens) and reported varying conclusions on the relationship. Xu et al. (2014) showed that higher bacterial diversity was found in the faeces of *E. coli* O157 super shedders (animals that shed $>10^4$ CFU/g of *E. coli* O157 in faeces) than non-shedders while Chopyk et al. (2016) found higher bacterial diversity in pre-harvest hides with lower prevalence of Top 7

STEC. Samples collected in the previous studies may not have been exposed to constant physical and chemical stress that is present in the processing environment. This may have provided more opportunities for bacterial communities to develop and establish a consortium of bacteria that may have contributed to or prevented the presence of STEC. This study suggested that the processing phase may not be the optimal part of the beef chain to investigate correlations between STEC and the beef microflora due to the variable nature of this environment.

The limitation of the findings in Chapter 4 is that the analysis of bacterial diversity between STEC positive and negative groups was executed based on the results of STEC potential positives (samples with *stx, eae* and one or more Top 7 serotypes after enrichment) due to there being a low number of samples from which STEC was isolated. Additionally, the findings are limited to abattoirs in two geographical regions. It may be difficult and impractical to obtain a large sample pool of confirmed positive samples through Australian beef processing environment as the prevalence of STEC in Australia is very low (Barlow and Mellor, 2010, Fegan et al., 2009).

6.5 Chapter 5: bacterial communities during enrichment

The previous chapter could not identify specific organisms or detect changes at the community level that could predict the presence of STEC during processing phases of beef. Therefore, the next step was to investigate if the current STEC detection system provides the best chance at detecting and isolating STEC from beef products. The fourth objective was to use sequencing methods to understand the development of bacterial communities through STEC enrichment of manufacturing beef. Better understanding of the enrichment process may provide new strategies to maximise the growth of STEC during enrichment. This can provide greater insights to optimise the detection process and subsequently increase the efficiency of isolating STEC.

The findings in this chapter showed that enrichment in different media had resulted in bacterial communities with varying composition at the end of the enrichment process. The 16S rRNA analysis indicated that high background microflora can potentially reduce the population density of *Escherichia* within the enrichment community. STEC is a small population of the genus *Escherichia*, thereby it's important to maximise the growth of *Escherichia* to optimise the process of STEC detection downstream (Kaper et al., 2004).

Clostridium was particularly more abundant in MP broths indicating that this medium may not be as effective as other selective media (mEHEC and PDX-STEC) and *Clostridium* could interfere with enrichment of *Escherichia*. This study suggests that further evaluation of this media is required to determine if the effect observed in this study is common during the enrichment process.

It's worth mentioning that STEC isolates were only obtained from one (Sample C) of two samples that originated from STEC positive lots at abattoir B. Results from Chapter 5 demonstrated that this does not necessarily mean that the enrichment process for the other positive sample (Sample D) was unsuccessful. In Australia, N60 sampling is used to test manufacturing beef for the presence of STEC (Kiermeier et al., 2011). During the sampling process, five samples are collected from each of 12 cartons to form a 375 g sample that represents a single lot and is subsequently tested for STEC. Meaning that it is likely that the boxes of manufacturing beef used to make Sample D did not contribute to the positive results from the test of the original lot at the abattoir.

A limitation of findings in Chapter 5 is that bacterial populations within the enrichment communities were identified at genera-level. *Escherichia* and *Clostridium* were the two predominant groups ranging between 59 – 99% in post-enrichment communities. Future studies are recommended to increase the sequencing power (i.e. shotgun metagenomics or full sequence of 16S rRNA gene by third generation sequencing) to investigate deeper into these groups and analyse development of enrichment communities at lower taxa. Effect of enrichment at species-level can provide greater insights to improving selective growth of STEC populations thereby increasing efficiency of STEC detection and isolation protocols. This can be useful to the industry in a way that it can effectively save the industry processing time and cost.

In conclusion, this project showed that genetic-based approaches such as 16S rRNA amplicon sequencing with appropriate bioinformatic analysis is a useful tool to understand microbial contamination events occurring during beef processing by allowing in-depth observation of the changes in bacterial communities. Findings in this study suggested that certain practices (e.g. hide pulling system) and residential bacteria on environmental surfaces can have an important role in carcase contamination, which subsequently affected the microbiology of the resultant beef products. One of the objectives of this study was to evaluate if 16S rRNA profiling could be used to predict the presence of Top7 STEC throughout processing of

Australian beef cattle. This study was not able to identify any bacterial diversity changes or organisms that could potentially be used as a bioindicator of Top 7 STEC. However, sequencing analysis enabled detailed analysis of the development of bacterial communities during enrichment of manufacturing beef. The changes in 16S rRNA profiles demonstrated that minimising competing background bacteria such as *Clostridium* may assist in increasing the chances of detecting and isolating STEC.

This study highlighted the revolution of science in meat microbiology; from counting indicator microorganisms to being able to describe changes in bacterial populations and community structure. Sequencing of 16S rRNA amplicons is not suitable for identification and characterisation of pathogens (Brightwell et al., 2006, Cao et al., 2017). Meaning that it should not replace but be used in combination with conventional cultivation methods to obtain greater understanding of microbiological quality of beef and movement of Top 7 STEC. The findings presented in this thesis are novel and show strong relevance to the beef industry. The ultimate goal would be to utilise 16S rRNA signals to manipulate the bacterial community composition to favour an ecological niche or diversity and control the presence of Top 7 STEC. However, this technique can also be applied to ensure safe and hygienic environment in a range of different food industries which use processing chains susceptible to contamination in order to enhance microbiological quality of their products.

6.6 Future recommendations

Recommendations for future studies include creating a bacterial community that can be applied to inhibit growth of STEC. It would be a worthy investigation to monitor the changes in STEC levels throughout the incubation period after spiking a cohort of bacterial populations that were predominant in beef microflora with known strains of STEC. Different combinations could be tested and there may be a specific combination of bacterial species that could assist or prevent colonization of Top 7 STEC through direct or indirect biological mechanisms. This would initially be *in vitro* but could be investigated further for application in beef production and could be applied to control rapid increase in STEC concentrations (e.g. appearance of super shedders leading to heavy STEC contamination) prior to beef processing.

It would be beneficial to explore more abattoirs in different regions of Australia. This study has demonstrated that particular practices within the processing environment (e.g. hide

pulling system) can have an impact on the microflora of carcases and ultimately the beef products. Although there are commonalities across the beef production system in Australia, animals arrive at the abattoir with a range of different production attributes (e.g. geography, production pathways, breed) and 16S rRNA profiling will allow to evaluate outcomes of contamination during processing procedures that are specific to the environment in individual abattoirs.

Previous studies suggested that aerosols can play a role in contamination of beef (Chandry, 2016, Sheridan et al., 1992). Therefore, understanding how movement of animals and personnel, and impact of abattoir design (e.g. heavy machineries generating wind flow, movement of carcases in unilateral direction) on contamination of carcases would be beneficial in future contamination studies.

Future STEC enrichment studies should consider inhibition of competitive background microflora such as *Clostridium* by addition of antimicrobial agents or minimisation of oxygen depletion during enrichment. The use of a static enrichment procedure results in build-up of beef fat at the top of the enrichment broths and potentially reducing the flow of oxygen into the enrichment media which may have aided the growth of anaerobes such as *Clostridium*. The effect of constant and gentle shaking of the enrichment broths during incubation on the level of bacterial populations would be worth investigating.

In addition, it would be useful to review the enrichment media tested in this study and evaluate the efficiency of the media for optimal enrichment of STEC. MP enrichment medium is commonly used for detection of STEC in the Australian beef industry using the DuPoint Qualicon BAX® System. It would be important for the industry to fully understand the effect it has on bacterial communities during enrichment and subsequently, on the performance of STEC testing.

Chapter 7. References

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