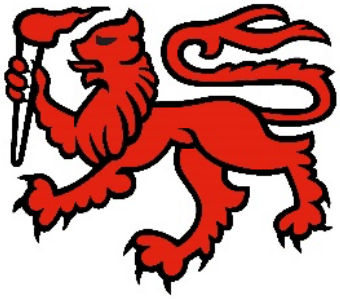

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Understanding and minimising microbiological spoilage of chilled vacuum-packed lamb

Laura Ellen Rood

Bachelor of Science with Honours

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

University of Tasmania, Australia

April, 2022

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Statement of Co-Authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Candidate: Laura Rood, Tasmanian Institute of Agriculture

Author 1: Chawalit Kocharunchitt, Tasmanian Institute of Agriculture

Author 2: John Bowman, Tasmanian Institute of Agriculture

Author 3: Tom Ross, Tasmanian Institute of Agriculture

Author 4: Joanne Pagnon, Tasmanian Institute of Agriculture

Author 5: Ross Corkrey, Tasmanian Institute of Agriculture

Author 6: David Nichols, Central Science Laboratory

Author 7: Erin D' Agnese, Tasmanian Institute of Agriculture

Author 8: Shareen Wen Ting Yang, Tasmanian Institute of Agriculture

Author 9: Mandeep Kaur, Tasmanian Institute of Agriculture

Paper 1: located in Chapter 2

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Author contributions:

Conceived and designed experiments: Candidate, Author 1, Author 9

Performed experiments/lab work: Candidate, Author 1, Author 4

Data analysis/results: Candidate, Author 1, Author 3, Author 5,

Wrote manuscript: Candidate

Input in manuscript: Author 1, Author 2, Author 3

Paper 2: located in Chapter 3

Rood, L., Bowman, J.P., Ross, T., Corkrey, R. Pagnon, Wen Ting Yang, S., and Kocharunchitt, C. Surface glucose treatment extends the shelf life of vacuum-packed lamb, *Meat Science. In review.*

Author contributions:

Conceived and designed experiments: Candidate, Author 1

Performed experiments/lab work: Candidate, Author 4, Author 8,

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Wrote manuscript: Candidate

Input in manuscript: Author 1, Author 2, Author 3

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Rood, L., Bowman, J.P., Ross, T., Nichols D., D' Agnese, E., Corkrey, R. and Kocharunchitt, C. Effects of additional glucose as a surface treatment on the microbial community and associated volatilome of vacuum-packed lamb. *Manuscript in preparation*.

Author contributions:

Conceived and designed experiments: Candidate, Author 1

Performed experiments/lab work: Candidate, Author 6

Data analysis/results: Candidate, Author 5, Author 7

Wrote manuscript: Candidate

Input in manuscript: Author 1, Author 2, Author 3

We, the undersigned, endorse the above stated contribution of work undertaken for each of the published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed:

Laura Rood
Candidate
Tasmanian Institute of Agriculture,
University of Tasmania

Date: 30/8/21

Professor Tom Ross
Primary Supervisor
Tasmanian Institute of Agriculture,
University of Tasmania

Date: 30/8/21

Professor Michael Rose
Interim Director
Tasmanian Institute of Agriculture,
University of Tasmania

Date: 30/8/21

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. Ethics reference number: H0017213.

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20 August, 2021

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Table of Contents

Chapter 1

Literature Review

Chapter 2

Spoilage potential of bacterial species isolated from chilled vacuum-packed lamb

Chapter 3

Surface glucose treatment extends the shelf-life of vacuum-packed lamb

Chapter 4

Effects of additional glucose as a surface treatment to influence the microbial community, shelf-life and associated volatilome of vacuum-packed lamb

Chapter 5

List of Figures

List of Tables

Abstract

Beef and lamb produced in Australia typically have shelf-lives of 26 and 12 weeks respectively when vacuum-packed (VP) and stored at temperatures of -1.5 to 0°C. The faster rate of quality loss of VP lamb is thought to be mainly due to its intrinsically higher pH (~ 0.4 pH units *cf.* beef) from large amounts of adipose tissue compared to beef. Meat with a higher pH increases bacterial growth rate, including the growth of spoilage bacteria. The inherently shorter shelf-life of lamb compared to beef meat can present a challenge in long distance export supply chains in which value loss may occur due to quality being compromised from unforeseen delays and temperature fluctuations. Therefore, the primary objectives of the research reported in this thesis are to provide insights into microbial spoilage mechanisms of chilled VP lamb, and to investigate potential interventions to extend the shelf-life. This was initially approached by investigating whether representative bacteria of meat spoilage communities independently play important roles in spoilage of chilled VP lamb. Subsequent studies evaluated the potential for glucose surface treatments to extend the shelf-life of VP lamb products. The latter experiments were expanded upon by investigating the effects of glucose treatments on the meat microbial community and the associated meat volatilome.

There have only been a limited number of studies that have investigated the spoilage mechanisms of chilled VP lamb, as the focus has been on beef due to the larger size of that export market. However, the biochemistry of beef and lamb is different and by extension so are the spoilage processes. Therefore, an initial study was undertaken to establish whether particular organisms independently play important roles in the rate of spoilage of chilled VP lamb. The spoilage potential of 13 representative bacterial strains derived from the spoilage community of VP lamb was investigated through a series of shelf-life trials. Each isolate was

individually inoculated onto sterile and non-sterile (i.e., containing natural microbiota) VP lamb meat. Meat quality was assessed over time by measuring sensorial qualities, bacterial growth and pH. Among all test organisms, *Clostridium* spp. had the highest spoilage potential and independently played a major role in spoilage. Specifically, *Clostridium estertheticum* caused premature 'blown pack' spoilage; however, the onset of this spoilage was delayed in the presence of the meat microbial community. *Clostridium putrefaciens* and *Clostridium algidicarnis* also caused premature spoilage of VP lamb both independently and in the presence of the meat microbial community. Of the facultative anaerobes tested *Carnobacterium divergens* and *Serratia* spp. were capable of spoiling meat at a faster rate when present in a community. These results suggest that these species have high potential to form metabolic by-products that lead to off-odours indicative of spoilage. The community-related responses observed also suggests that spoilage is implicitly affected by community member interactions including competition and exchange of metabolites. Overall, these studies highlighted that *Clostridium* spp. are important for causing quality loss of chilled VP lamb. Such knowledge helps to inform approaches that can enable shelf-life extension.

Due to the relatively short shelf-life of lamb and the associated challenges it presents to exporters, there is an impetus to develop cost-effective interventions to extend the shelf-life of Australian lamb products. Therefore, a component of this research was to evaluate the efficacy of glucose (0.5%, 1%, 2.5%, 5% and 10% w/v) as a surface treatment for shelf-life extension of VP bone-in and boneless lamb. This was investigated through a series of trials, in which the dynamics of the sensorial qualities, bacterial growth, pH, residual glucose and lactic acid were measured. Added glucose extended the shelf-life of bone-in and boneless lamb, by 8% to 76% (2 to 9 days) relative to the control, with the 5% treatment being the

most effective in both cases. Glucose treatments reduced meat pH, potentially affecting the microbial community composition as well as the rate and type of accumulation of spoilage metabolic by-products. The results indicate that the application of glucose has the potential to be developed for shelf-life extension of VP lamb.

Subsequent studies were undertaken to investigate the effects of glucose surface treatment to influence the microbial community and associated volatilome of VP lamb. To achieve this, changes in the microbial community composition (16S rRNA gene amplicon sequencing) and volatilome (solid phase micro-extraction method coupled with gas chromatography-mass spectrometry) of bone-in and boneless lamb shoulder were assessed as dictated by storage time, glucose treatments (0.5%, 1%, 2.5%, 5% and 10% w/v), and odour scores. Glucose treatments had a significant effect on the microbial community composition on both meat cuts. The most effective glucose treatments for bone-in (1% and 5%) and boneless (5%) lamb led to significantly lower numbers of various *Vagococcus* amplicon sequence variants, and all tested glucose concentrations on bone-in lamb had significantly lower numbers of *S. proteamaculans*. Additional glucose is likely to alter the microbial community composition by shifting substrate utilisation and the accumulation of acidic end-products, having an overall impact on the rate and type of spoilage metabolic by-products. However, no difference was detected in the composition of organic volatile compounds between glucose treatments for either bone-in or boneless lamb. There was a significant increase in a range of sulphur, ester and carboxylic acid compounds later in storage, indicative of microbial and possibly *post-mortem* metabolism that contributes to a decline in the odour quality of the meat. These findings provide an insight into the microbiological mechanisms underpinning shelf-life

extension achieved by glucose addition; however, further investigation is required to determine how it influences the manifestation of spoilage metabolites.

Overall, the findings of this PhD thesis provide fundamental knowledge of the microbiological spoilage mechanisms of chilled VP lamb produced in Australia and highlights the importance of *Clostridium* spp. in spoilage rate. These findings can help to inform opportunities for shelf-life interventions targeting organisms with 'high' spoilage potential. The results also indicate that the application of glucose has the potential to be developed for shelf-life extension of VP lamb.

Chapter 1

Literature Review

1.1 Introduction

All meat products possess finite storage shelf-lives. Shelf-life represents the duration, under specified storage conditions, where a food product such as meat is expected to be microbiologically safe, and of acceptable quality for human consumption before spoilage occurs (Nychas et al., 2008). The spoilage process comprises the development of off-odours, off-flavours, discolouration and physical defects (Gram et al., 2002). Spoilage of fresh meat is most commonly caused by microbial growth and their metabolism of meat tissues, resulting in the accumulation of spoilage metabolites (Ercolini et al., 2006; Gram et al., 2002). The rate at which spoilage manifests is dependent on the microbial community growth rate and metabolic activity under the storage conditions applied (Ercolini et al., 2011; Lambert et al., 1991). A combination of factors influence the growth and development of the microbial community, foremostly, the pre- and post- slaughter contamination, the intrinsic properties of meat (especially pH), packaging used, and storage conditions (Bacon et al., 2000; Mossel, 1971).

Vacuum-packaging combined with chilled storage temperatures (-1.5 to 0°C) are used to maximise the shelf-life of fresh red meat products, owing to the economic, logistical, and quality preservation benefits (Huynh et al., 2016). These conditions select for distinctive microbial communities of slower growing psychrotrophic facultative anaerobes, dominated by lactic acid bacteria. These communities can reach maximum population densities weeks before the onset of spoilage owing to the relatively innocuous metabolic end-products lactic

acid bacteria typically form, combined with their competitive and antagonistic behaviours against higher potential spoilers.

The expected shelf-life of 'larger' primal cuts produced in Australia is 26 weeks for beef and 12 weeks for lamb when vacuum-packed (VP) and stored at chilled temperatures (-1.5 – 0°C) (Kaur et al., 2017b; Kaur et al., 2021; Kiermeier et al., 2013; Small et al., 2012). These long shelf lives have allowed Australia to consolidate a large international export market. Consequently, the red meat export trade makes a significant contribution to the Australian economy, valued at $10.8 billion for beef and $4.1 billion for lamb in 2019 (MLA, 2020a, b). However, the nominal 12-week shelf-life of VP lamb can present a challenge to exporters. This is because the total travel time required for transportation and distribution can be lengthy (> 90 days) for distant markets (Huynh et al., 2016). The predilection to more rapid spoilage compared to VP beef meat thus also makes VP lamb exports more vulnerable to quality loss during distribution from unplanned shipping delays and temperature fluctuations (Sumner, 2016). These potential issues in supply chain logistics can lead to undesired product downgrades and rejection as well as contributing to food waste.

The rate of quality loss of lamb is due to bacterial growth rates and community composition, which directly reflects the inherent biochemistry of lamb meat, of which pH is of particular importance (Kaur et al., 2021; Kiermeier et al., 2013). However, the study of microbial spoilage mechanisms of VP red meat has primarily focused on beef and consequently there have been assumptions that these mechanisms also apply to lamb meat (Gribble et al., 2014). This review focuses on understanding the main factors that influence the microbial spoilage of chilled VP red meat produced in Australia. It proceeds to discuss factors that determine the nature of spoilage of lamb, and the challenges this presents to Australian lamb exporters.

1.2 Spoilage mechanisms of red meat

Meat spoilage is a complex process that can be caused by oxidative, *post-mortem* enzymatic, and microbiological processes, resulting in off-odours, off-flavours, discolouration and physical defects (Dave and Ghaly, 2011; Gram et al., 2002). Enzymatic spoilage of meat is a natural process in which meat components (carbohydrates, fats and proteins) are broken down into simpler units by natural meat enzymes (Dave and Ghaly, 2011). These enzymatic processes can result in softening and discolouration of the meat, and the degradation products can enable growth of microorganisms (Dave and Ghaly, 2011; Toldrá, 2006). However, degradation of protein by meat enzymes, such as cathepsins, calpains, peptidases and aminopeptidases, is an important process that contributes to eating quality, such as tenderisation and flavours (Kemp et al., 2010). This is known as *post-mortem* ageing. Meat spoilage can also be caused by oxidation of lipids, which can be either enzymatic or non-enzymatic. It is caused by oxygen reacting with unsaturated fatty acids via a free radical mechanism forming peroxides as primary products (Dominguez et al., 2019). These processes can cause further deterioration of meat quality typically by the development of rancid odours and flavours (Dominguez et al., 2019).

Microbial spoilage of meat is the most common form of spoilage and dictates the shelf-life of meat products. It is caused by microbial growth and metabolism, owing to the high abundance of readily available nutrients and the high water-activity of meat (Ercolini et al., 2006; Gram et al., 2002). The rate and type of meat spoilage is dependent on the growth of the microbial community and their metabolic activity that can lead to the accumulation of compounds associated with spoilage (Doulgeraki et al., 2012; Ercolini et al., 2011; Lambert et al., 1991). Specifically, to build biomass microbes metabolise available nutrients in various

orders depending on their affinity for substrates within the meat ecosystem (Casaburi et al., 2015). This creates a complicated array of volatile and non-volatile metabolites that accumulate within the meat matrix. The volatile component of microbial metabolites can impact the odour quality of meat. Volatile compounds can include a range of sulphur, ketone, alcohol, aldehyde, ester, and carboxylic acid compounds (Casaburi et al., 2015; Ercolini et al., 2009; Ercolini et al., 2006; Iulietto et al., 2015; Nychas et al., 2008). Depending on their odour thresholds and interactions, they can manifest as off-odours, the main criterion for lamb meat spoilage sensorial assessment (Casaburi et al., 2015; Kaur et al., 2017b).

1.3 Red meat microbiology during processing

Despite rigorous hygiene standards throughout slaughter and processing, the development of a microbial community on fresh meat is inevitable due to aerial and surface contamination within the processing facility (Kaur et al., 2017a; Stellato et al., 2016). There are many pre-slaughter and processing factors that can influence the initial microbial community composition on meat and the subsequent safety and shelf-life of the product. As a result, good hygiene practices are strictly adhered to, and decontamination processes can be implemented.

1.3.1 Pre-slaughter factors

Abattoir processing regimes have a major impact on the microbiology of meat by influencing the initial microbial loading and affecting meat pH. A major focus of these processing regimes is to control pathogen occurrence on meat by minisming contamination on the livestock that can transfer to the carcase. The main factors contributing to meat contamination from farm production to carcase processing are outlined in Figure 1.1.

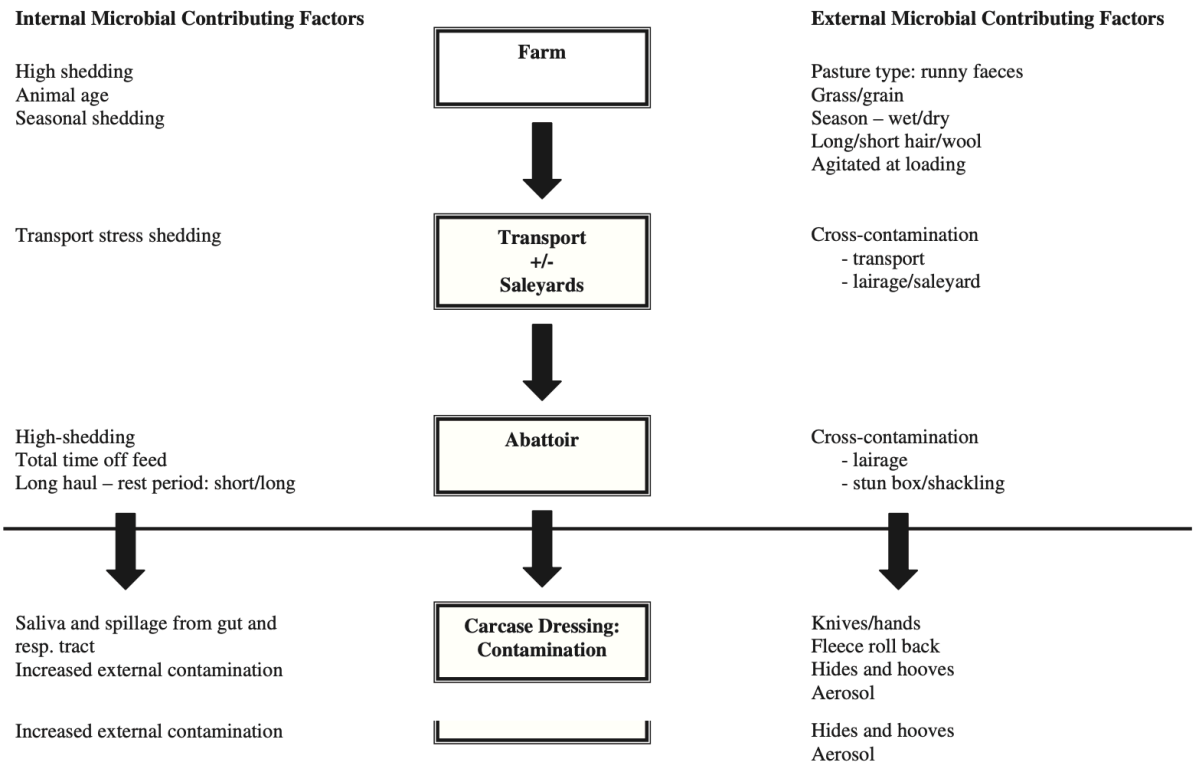


Figure 1.1 Factors contributing to animal and carcase contamination (Pointon et al., 2012).

Hide contamination of livestock is a major source of most contamination on carcases (Tucker and Klepper, 2005). Highlighting the importance of stock cleanliness, the Australian Standard for the Hygienic Production and Transportation of Meat and Meat Products for Human Consumption section AS 4696:2007 states that "*animals that are not clean are not passed for slaughter or slaughtered subject to conditions that ensure they do not contaminate animals, carcases*". Bacteria on hides, in faeces and on lairage material have been found to survive for more than one week, indicating the risks of potential cross-contamination of 'dirty' animals from different herds over time (Small et al., 2002). Washing of live animals prior to slaughter can be employed to reduce fleece and hide contamination. However, this is not commonly practised or advised due to concerns about welfare and animal stress (Tucker and Klepper, 2005). Heightened animal stress levels can impact the eating quality and pH of red meat and

by extension bacterial growth rates (Gill and Newton, 1979). Increasing animal stress levels reduces the glycogen reserves that are required for *post-mortem* acidification (Gardner et al., 2014; Scheffler and Gerrard, 2007). The biochemistry of this process and the impact it can have on bacteria is explained in more detail in Section 1.4.1.1.

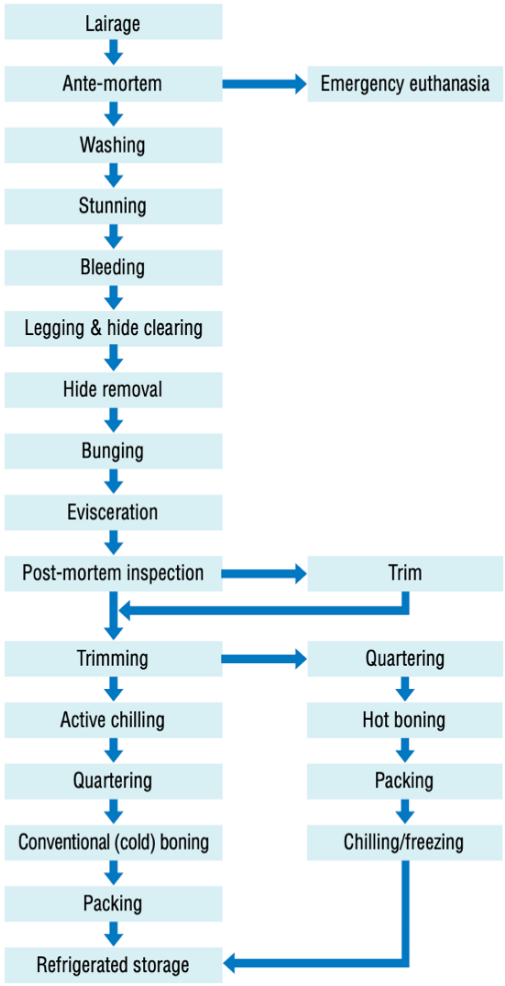
The degree of hide contamination and animal stress has been shown to increase with the time it takes to travel to the abattoir, duration of withholding feed and water prior to slaughter, and lairage holding times (Pointon et al., 2012). Efforts are therefore made to minimise travel distances to abattoir. Withholding feed before slaughter is practiced in Australia to reduce soiling during transportation, thereby aiding hygienic dressing (Pointon et al., 2012). However, withholding feed increases rumen pH by reducing the production of volatile fatty acids, thereby facilitating the growth of enteric pathogens such as pathogenic *Escherichia coli* and *Salmonella* (Brownlie and Grau, 1967). As a result, increasing the duration of time off feed potentially increases pathogen populations in the gastrointestinal tract; therefore, great emphasis is placed on avoidance of ingesta spillage during evisceration (Pointon et al., 2012).

1.3.2 Processing factors

There are many potential sources of microbial contamination during slaughter and processing of meat. Figure 1.2 provides an overview of beef and lamb processing in Australia in which the main sources of contamination are from hide removal, evisceration and boning (Bacon et al., 2000; Bell and Hathaway, 1996; Small et al., 2002). Cross contamination between livestock/carcases can also occur by the process workers and from the processing environment itself. During hide removal, large numbers of bacteria may be deposited on the carcase, allowing contamination with a mixture of bacteria derived from pre-slaughter

environments, such as faecal, soil, water and feed origin (Bell and Hathaway, 1996). Contamination during hide removal occurs with each incision through the hide, transferring microbes to underlying tissue (Koutsoumanis and Sofos, 2004). Further contamination of the carcase can occur by contact with fleece or hide, in addition to dust and aerosols generated from the hide removal process and cross-contamination of process workers and the environment (Koutsoumanis and Sofos, 2004; Schmidt et al., 2012).

a)



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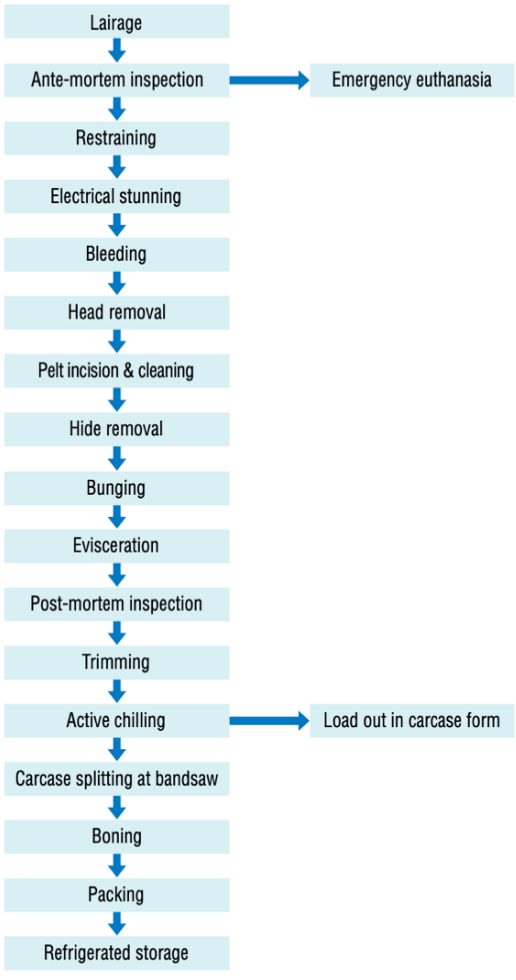

Figure 1.2 Flow chart of a) beef and b) sheep processing for lairages, slaughter floors and boning rooms in Australian abattoirs (Huynh et al., 2016).

Evisceration may contaminate the carcase with microbes from the gastrointestinal and respiratory tracts; however, it can be carried out with minimal contamination provided organs are not punctured or ruptured (Bell and Hathaway, 1996). 'Closing off' the oesophagus and rectum are operations used to minimise contamination when removing viscera (Huynh et al., 2016; Nesbakken et al., 1994). The carcase is also inspected after evisceration in which visible contamination and excess fat are removed. Contamination can also occur during boning, when carcases are processed into primals, specific cuts and trims, and packaged. The main sources of contamination during boning are via aerosols, cross-contamination of other carcases, hands of workers, knives and equipment, and surfaces over which the product passes (Bakhtiary et al., 2016; Huynh et al., 2016; Jericho et al., 2000).

1.3.3 Initial microbial community composition

The Australian red meat industry has conducted four national baseline surveys to determine the initial microbiological quality of lamb and beef to help monitor and inform hygiene aspects of the industry (Phillips et al., 2012; Phillips et al., 2013). These studies assessed 1,144 beef primals from 29 establishments, and 1, 226 lamb primals from 12 establishments (Phillips et al., 2012; Phillips et al., 2013). The results showed the total viable count at the time of packaging is typically 1.3 – 1.5 $\log_{10}$ CFU/cm^2 for beef and lamb 2.02 – 2.29 $\log_{10}$ CFU/cm^2 when strict hygienic practises are implemented (Phillips et al., 2012; Phillips et al., 2013). Table 1.1 identifies the main bacterial genera cultured and identified within the initial communities of VP red meat (Dainty and Mackey, 1992; Doulgeraki et al., 2012; Kaur et al., 2021), and their potential origins (Chaillou et al., 2015; De Filippis et al., 2013; Stellato et al., 2016).

Table 1.1 Major genera that contaminate red meat during processing and their potential origin (Chaillou et al., 2015; De Filippis et al., 2013; Stellato et al., 2016).

	Genera	Potential contamination sources
Gram-positives	*Brochothrix*	Water and soil and plant reservoirs
	Carnobacterium	Water and soil
	Clostridium	Environment, animal skin, nostrils and gastrointestinal tracts
	Enterococcus	Animal skin, nostrils and gastrointestinal tracts
	Lactobacillus	Water, feeds, animal skin, nostrils and gastrointestinal tracts
	Lactococcus	Animal skin, nostrils and gastrointestinal tracts
	Leuconostoc	Water and plant derived feeds
	Vagococcus	Water and plant derived feeds
Gram-negatives	*Acinetobacter*	Water and soil
	Aeromonas	Water
	Enterobacter	Water, soil, and intestinal tracts
	Hafnia	Water and soil
	Janthinobacterium	Water and soil
	Morganella	Environment and animal intestinal tracts
	Providencia	Environmental and animal intestinal tracts
	Pseudomonas	Water and soil
	Rahnella	Environment
	Serratia	Water and soil
	Yersinia	Environmental
	Shewanella	Water and soil

The initial microbial community composition on red meat can vary significantly within and between abattoirs depending on the pre-slaughter and processing factors. For instance, Kaur et al. (2017a) and Kaur et al. (2021) observed differences in the initial community structure on meat between geographically distinct Australian abattoirs. These dissimilarities could be due to differences in geographical origin of the animal processed, processing protocols and

abattoir specific resident microbial communities (De Filippis et al., 2013; Hultman et al., 2015; Kaur et al., 2017a). Furthermore, the initial microbial community composition can also vary depending on the cut type (De Filippis et al., 2013). Cuts from the forequarters of the carcass are recognised as the most contaminated part of the carcase. This difference is due to carcases typically being hung up by the hind legs; therefore, contamination in meat exudates and wash water move from the posterior to the anterior sections (Bell, 1997; Eisel et al., 1997; Yalcin et al., 2001).

Pathogenic bacteria such as *Campylobacter* spp., *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus* and pathogenic *Escherichia coli,* typically have low prevalence on red meat produced in Australia. The presence of pathogens on carcasses during processing are tested for regularly (Phillips et al., 2012; Phillips et al., 2013). For instance, the national baseline survey did not detect *E. coli* O157:H7, *Campylobacter* or *Salmonella* on beef primals, and only 0.1% were positive for *Listeria* spp. at a concentration of ≥ 1 CFU/cm^2 (Phillips et al., 2012). Similarly, sheep primals had a low prevalence of *E. coli* O157:H7, which was detected on 0.3% leg and 0.2% of shoulder samples. *Campylobacter* was only detected on shoulders at a prevalence of 0.2%. *Salmonella* was detected on 2.8% of leg and 0.8% of shoulder samples, while 0.2% of leg samples were positive for *Listeria* spp. (Phillips et al., 2013).

1.3.4 Minimising microbial contamination during processing

Australian abattoirs are required to document all dressing procedures, which the Controlling Authority in Australia needs to approve and regularly audits compliance (Huynh et al., 2016). This has helped to ensure the strict application of correct hygienic practises during processing (Koutsoumanis and Sofos, 2004). Carcase chilling is a procedure that is strictly

implemented to critical limits that are set by Australian Standard AS4696:2007 and in the Export Control (Meat and Meat Products) Order (Huynh et al., 2016). This is to prevent potential growth of enteric pathogens during processing, while improving eating quality and production yield. Primary chilling, also known as active chilling (Fig. 1.2), is the process of cooling carcasses immediately after slaughter from body temperatures to refrigeration temperatures (Koutsoumanis and Taoukis, 2005). This process is required to be relatively rapid to prevent growth of pathogenic and spoilage bacteria. However, if the carcasses temperature reduces to less than 15 – 16°C before the completion of rigor, it can induce the muscles to contract (cold shortening), resulting in tough meat (Savell et al., 2005). Handling and processing of meat during boning and packaging can facilitate growth of bacteria if temperatures increase; therefore, secondary chilling is implemented where temperatures are typically reduced to below 10°C (Huynh et al., 2016; Koutsoumanis and Taoukis, 2005).

Australian processors can also implement decontamination processes to reduce carcase contamination throughout processing. These include physical interventions (e.g., knife trimming, washing), thermal interventions (hot water, steam pasteurisation) and chemical interventions (e.g., organic acid washes) (MLA, 2015). The implementation of such interventions is dependent on a range of factors specific to the plant, such as logistical constraints of the premises, the processes used, and environmental management of waste disposal (Gill and Baker, 1998; 2015). It is also dependent on the constraints of the market and the regulatory status of the intervention. For instance, Europe will not accept products with non-approved interventions, whereas the US encourages the use of interventions to reduce and eliminate *E. coli* O157:H7 (Huynh et al., 2016).

1.4 Red meat microbiology during storage

Subsequent growth of the initial community on the meat matrix is dependent on many intrinsic (meat biochemistry and physicochemical properties) and extrinsic (packaging and storage conditions) factors (Mossel, 1971; Nychas et al., 2008). These factors in combination enforce a selection pressure on the microbial community, allowing those bacteria that are best to adapt to the environment to outcompete others and reach high numbers, which ultimately dictates the shelf-life of meat (Doulgeraki et al., 2012).

1.4.1 Intrinsic factors

1.4.1.1 Meat pH

Meat pH is one of the most important intrinsic factors as it can have profound impacts on the microbial community and subsequent shelf life. Beef typically has a pH range of 5.5 – 5.8, and lamb has a pH range of 5.6 – 6.8 (Kaur et al., 2021). *Post-mortem* acidification of muscle tissue is dependent on the concentration of residual glycogen-glucose at the time of slaughter; therefore, adequate muscle reserves (45 – 55 mmol/kg) are necessary (Gardner et al., 2014; Immonen and Puolanne, 2000). This process is known as *post-mortem* glycolysis, in which stored glycogen-glucose is catabolized to provide ATP and subsequently generates lactate and H^+, thereby reducing meat pH (Scheffler and Gerrard, 2007). Glycolysis ceases when ultimate pH (pH 5.5) is reached, therefore post-rigor muscle usually contains residual glycogen-glucose (Gill, 1986). Reaching ultimate pH ensures that meat is of acceptable sensory qualities including redness, flavour, and tenderness (Gardner et al., 2014; Jacob and Pethick, 2014). Furthermore, the pH reduction has a large effect on the microbial community composition by slowing bacterial growth rates, and inhibition of some spoilage bacteria (EFSA, Panel on Biological Hazards (BIOHAZ), 2016; Gill and Penney, 1985; Yang et al., 2014).

Previous studies have reported meat of higher pH can facilitate the growth of *B. thermosphacta* (> 5.8 pH units), *Clostridia* (> 6), members of the *Enterobacteriaceae* family, such as *Serratia*, *Hafnia* and *Rahnella* (> 5.8), and *Shewanella* (> 6) (Borch et al., 1996; Brightwell and Clemens, 2012; Ercolini et al., 2011; Gribble and Brightwell, 2013; Kaur et al., 2021; Nowak et al., 2012; Pin et al., 2002; Ross et al., 2016; Yang et al., 2009; Yang et al., 2014; Youssef et al., 2014a; Youssef et al., 2014b). In contrast, stressed animals have muscle with low residual glycogen-glucose concentration. This produces meat with a higher pH (≥ 6) as the premature depletion of glycogen-glucose during glycolysis results in insufficient lactic acid to reduce the pH of the meat (Gill, 1986). In addition to allowing increased bacterial growth rates, this condition is also associated with meat with dark, firm, dry sensory qualities (DFD meat) (Newton and Gill, 1978).

1.4.1.2 Nutrient composition

The main constituents of meat are fat, protein, minerals, and a small portion of carbohydrates (Iulietto et al., 2015). Meat nutrients enable the growth of many microorganisms assuming they can grow under the storage conditions applied (Doulgeraki et al., 2012; Iulietto et al., 2015). Most bacteria likely build biomass by rapidly growing on efficiently metabolizable low-molecular weight compounds (Perrin et al., 2020), such as glucose, (Gill, 1986; Nychas et al., 1988). As storage time progresses, the catabolism or conversion of other substrates increases, such as glucose-6-phosphate, glycogen, lactate, gluconate, pyruvate, propionate, formate, ethanol, acetate, amino acids, nucleotides, urea and lipid compounds (Gill, 1986). There is evidence to suggest that when multiple nutrients are present, such as in the meat matrix, bacteria can use both diauxic and co-utilisation strategies simultaneously (Perrin et al., 2020). As a result, the order of nutrient uptake is

partially driven by the biomass yield possible from the substrate (Perrin et al., 2020). This creates a complicated array of metabolic activities that ultimately leads to spoilage (Casaburi et al., 2015; Ercolini et al., 2011; Ercolini et al., 2009; Ercolini et al., 2006; Nychas et al., 2008).

1.4.1.3 Lactic acid

Lactic acid is a natural constituent of red meat, as *post-mortem* acidification of muscle tissue occurs by converting glycogen/glucose to lactic acid via glycolysis. If glycogen reserves are adequate in meat, lactic acid is attained at a concentration of 90 to 100 mM (Gill and Newton, 1982). Lactic acid has a significant effect on bacterial growth due to the undissociated acid and the low pH (Gill and Newton, 1977, 1979; Grau, 1981; Signorini et al., 2006b). The bactericidal and bacteriostatic properties of lactic acid are pH dependent. Lower pH results in a higher concentration of undissociated lactic acid (Gill and Newton, 1982). It is the solubility of the undissociated lactic acid within the cytoplasm and the insolubility of dissociated acid, leading to acidification of the cytoplasm (Reis et al., 2012). This creates a trans-membrane pH gradient, ultimately decreasing the amount of available energy for cell growth (Oda et al., 2002; Wee et al., 2006). Lactic acid bacteria (LAB), which are an unavoidable contaminant of fresh meat during processing, also produce lactic acid as a by-product of their metabolism of carbohydrates (Reis et al., 2012). Growth of LAB can therefore impact lactic acid content, and subsequent pH of meat.

1.4.1.4 Water activity

Water activity (a_w) is the measure of the available water in food for the growth of microbes. Adequate water activity is crucial for bacteria to undertake enzymatic reactions, synthesising cellular materials and other biochemical reactions (Iulietto et al., 2015). The required a_w for

bacteria is dependent on the organism. For instance, *Enterobacteriaceae* are inhibited below 0.94 a_w, while *L. monocytogenes* is inhibited below 0.92, and *S. aureus* below 0.87 (ICMSF, 1996). Raw meat has high a_w of 0.98 – 0.99, which supports the growth of most bacteria (Aymerich et al., 2002). The surface a_w of a carcase during primary chilling has shown to be equivalent to the relative humidity of the surrounding environment; therefore, can be lowered to reduce surface a_w (Mills et al., 2014). However, a_w is also influenced by the packaging conditions during storage, for example, vacuum-packaging meat (see section 1.4.2.1) prevents further drying at the surface, allowing moisture within the meat to equilibrate surface a_w to above 0.98 (Bell, 2001).

1.4.2 Extrinsic factors

1.4.2.1 Packaging gaseous atmosphere

The growth of bacteria is greatly affected by the gaseous composition of the atmosphere surrounding meat during storage. The three most common types of packaging for red meat are aerobic, modified atmosphere and vacuum, all of which enforce different selection pressures (Table 1.2). The high O_2 availability under aerobic conditions can allow growth of a broad range of bacteria (Casaburi et al., 2015). More importantly, it increases the rate of spoilage by selecting for fast growing spoilage bacteria, such as *Pseudomonas* and *Shewanella* (Enfors et al., 1979 ; Lambropoulou et al., 1996; Newton and Gill, 1978). Vacuum, and modified atmosphere packaging with low O_2 and higher CO_2 concentrations select for slower growing facultative anaerobic populations, which delays the onset of spoilage (Borch et al., 1996; Pennacchia et al., 2011; Yost and Nattress, 2002). Packaging with restricted O_2 content also prevents other forms of spoilage, such as browning from metmyoglobin formation and lipid oxidation spoilage (Dave and Ghaly, 2011).

Table 1.2 Genera of bacteria commonly found on raw meat under different packaging types (Reproduced from Casaburi et al., 2015).

Gram-positive	Storage conditions			Gram-negative	Storage conditions		
	Air	MAP	VP		Air	MAP	VP
Bacillus	+		+	*Achromobacter*	+		
Brochothrix	+	+	+	*Acinetobacter*	+	+	+
Carnobacterium	+	+	+	*Aeromonas*	+		+
Corynebactenum	+			*Alcaligenes*	+	+	+
Clostridium			+	*Alteromonas*	+	+	+
Enterococcus	+	+		*Campylobacter*	+		
Kocuria	+			*Chromobacterium*	+		
Kurthia	+			*Citrobacter*	+	+	
Lactobacillus	+	+	+	*Enterobacter*	+	+	
Lactococcus	+			*Escherichia*	+		
Leuconostoc	+	+	+	*Flavobacterium*	+		
Listeria	+	+		*Hafnia*	+	+	+
Microbacterium	+	+	+	*Klebsiella*	+		
Micrococcus	+	+		*Kluyvera*	+		
Paenibacillus	+			*Moraxella*	+		
Staphylococcus	+	+	+	*Pantoea*	+		+
Streptococcus	+	+		*Proteus*	+	+	
Weissella	+	+	+	*Providencia*	+	+	+
				Pseudomonas	+	+	+
				Serratia	+	+	+
				Shewanella	+		
				Vibrio	+		
				Yersinia	+		+
				Moraxella	+		

Modified atmosphere packaging involves replacing the air in the headspace of the pack with modified gaseous composition using different ratios of O_2, N_2 and CO_2 (Narasimha and Sachindra, 2002). Combinations of gas mixtures with reduced O_2 and increased CO_2 have been shown to significantly extend the shelf-life of meat products, and facilitate colour stability (Huynh et al., 2016; Narasimha and Sachindra, 2002). However, there are food safety concerns about the potential growth of psychrotrophic pathogens capable of growing at high CO_2 at chilled temperatures, such as *Clostridium botulinum*, *Listeria monocytogenes* and *Yersinia enterocolitica* (Huynh et al., 2016). In comparison, vacuum packaging involves

removing the air from the packaging prior to heat sealing, resulting in an atmosphere that typically contains < 0.5% O_2, approximately 20 – 40% CO_2, with the remainder being N_2 (Huynh et al., 2016). This atmosphere is maintained by using packaging with low O_2 permeability (~18.6 cm^3/m^2/24 h at 23 °C) (Kiermeier et al., 2013). The small amount of oxygen that enters the pack is consumed by the muscle tissue and contaminating microbes (Gill and Penney, 1985; Kropf, 2004). It is common industry practise to 'heat-shrink' vacuum-packed (VP) meat, which involves dipping the packs in water at 85 – 90°C, for 2 – 3 s immediately after sealing (Moschonas et al., 2011). This process enhances vacuum seal, improves appearance, and further reduces O_2 permeability as well as drip loss (Bell, 2001).

Vacuum-packaging is commonly used in Australia for primal cuts due to its ability to effectively extend the shelf-life, in addition to its logistical and economic advantages (Small et al., 2011). For instance, vacuum packages are more compact and durable than modified atmosphere packaging, making them easier to handle during the distribution process (Hur et al., 2013; Jayasingh et al., 2001; Kiermeier et al., 2013). Modified atmosphere is also typically more expensive than vacuum packaging due to the cost of gases, and gas packaging machinery that ensures correct gas mixtures (Hur et al., 2013).

1.4.2.2 Temperature

Storage temperature is one of the most important factors dictating the shelf-life of meat and the risk of meat-borne pathogens (Bell, 2001; James, 2002). Temperatures below an organisms' optimum for growth increases the lag phase and generation time, and can reduce the final population (Labuza and Fu, 1993; Mataragas et al., 2006). Lower refrigeration temperatures (< 4 °C) therefore reduce bacterial growth rates and alters community

structure, and by extension increases the shelf-life of meat products (Kaur et al., 2017b; Nychas et al., 2008).

The majority of meat-borne pathogens are mesophilic and are able to grow at temperatures above 7°C (Bell, 2001). Therefore, storage temperatures below 5°C significantly reduce the food safety risks (James, 2002). As a result, stringent temperature control is implemented throughout all stages of the supply chain (Nychas et al., 2008). Low temperatures can also enhance the effect of packaging; for instance, the O_2 transmission rate of vacuum and modified atmosphere packaging decreases as temperature is reduced, especially at temperatures below 0°C (Lambden et al., 1985; Newton and Rigg, 1979).

The most common temperature control methods implemented during the supply chain are chilling and freezing. Australian regulations do not specify a temperature for frozen products; however, many international customers require frozen meat to be delivered at a temperature below -18°C (Huynh et al., 2016). Despite inhibition of bacterial growth, the quality of frozen meat products, such as palatability, odour and colour, can still deteriorate due to lipid oxidation and autolytic enzymatic degradation (Aidani et al., 2014). Meat quality can also be substantially impacted by the methods used to freeze and thaw products (Aidani et al., 2014; Dave and Ghaly, 2011; Kaale et al., 2011). Furthermore, the cost and logistical requirements to freeze meat products throughout the export supply chain is extensive, especially during transportation (Kaale et al., 2011).

Chilling red meat products between temperatures of -1.5 – 0°C is commercially feasible and can effectively extend the shelf-life of meat products, while preserving premium quality (Gill et al., 1988). At chilled temperatures a range of psychrophilic and psychrotrophic bacteria, such as LAB, *Pseudomonas, Brochothrix* and *Enterobacteriaceae,* are able to grow on meat,

which can still lead to spoilage (Ercolini et al., 2007; Gill and Newton, 1977). However, chilled storage temperatures combined with further selection pressures introduced by the type of packaging can reduce microbial diversity and greatly extend the shelf-life of meat products (Nychas et al., 2008).

1.5 Spoilage communities of chilled vacuum-packed red meat

Vacuum-packaging combined with chilled storage temperatures are the most common methods to extend the shelf-life of VP red meat, providing up to 26 weeks of shelf-life for some types of red meat (Small et al., 2012). Over time these conditions select for distinctive microbial communities of psychrophilic and psychrotrophic facultative/obligate anaerobes with relatively low spoilage potential (Doulgeraki et al., 2012). As a result, the onset of spoilage occurs weeks after these communities have reached maximum population (Fig. 1.3) (Gill, 2004; Kaur et al., 2017b). The main members of these spoilage communities are LAB, members of the family *Enterobacteriaceae*, *Clostridium*, *Pseudomonas*, *Brochothrix* and *Shewanella* (Brightwell et al., 2007; Ercolini et al., 2006; Fontana et al., 2006; Jones, 2004; Jones et al., 2008; Kaur et al., 2017a; Kaur et al., 2017b; Kaur et al., 2021; Pennacchia et al., 2011).

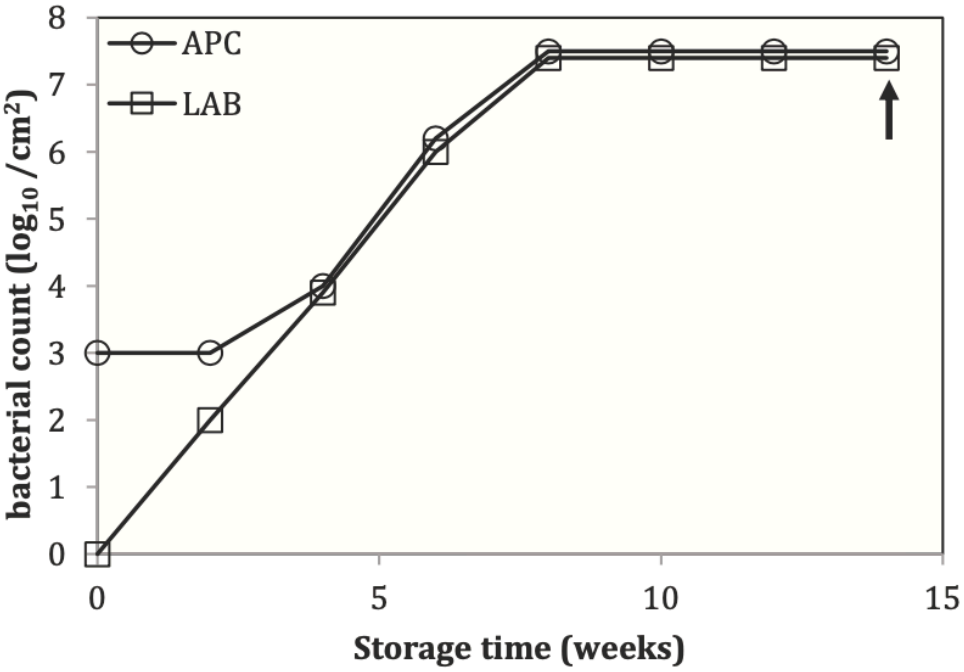

Figure 1.3 Increase in aerobic plate count (APC) and LAB counts on VP beef primals stored at 0 °C, arrow shows time at which product was considered spoiled (Sumner et al., 2021).

1.5.1 Lactic acid bacteria (LAB)

LAB constitutes a group of Gram-positive bacteria (order *Lactobacillales*) that are rod- and coccus-shaped, usually non-motile and non-spore forming (Reis et al., 2012). They grow mainly by carbohydrate fermentation and form organic acids as end-products (Reis et al., 2012). Their ability to grow well under chilled anaerobic conditions allows them to dominate the microbial community of chilled VP red meat (Borch et al., 1996). At the time of packaging LAB constitutes only a small proportion of the microbial community (1 – 2 $\log_{10}$ CFU/cm^2), but dominates within the first few weeks of storage at 0°C, and after approximately 10 weeks reaches a maximum population of 7 – 8 $\log_{10}$ CFU/cm^2 (Figure 1.3) (Egan, 1983; Jones, 2004; Kaur et al., 2017b; Kaur et al., 2021). The main LAB genera found in VP meat communities are *Carnobacterium*, *Enterococcus, Lactococcus, Leuconostoc,* and *Vagococcus* (Brightwell et al.,

2009; Castellano et al., 2004; Kaur et al., 2017a; Kaur et al., 2017b; Kaur et al., 2021; Pennacchia et al., 2011; Yost and Nattress, 2002; Youssef et al., 2014b). Among these taxa, some of the most common species are *Carnobacterium divergens, Carnobacterium maltaromaticum, Lactobacillus curvatus*, *Lactobacillus sakei,* and *Lactococcus piscium* (Casaburi et al., 2011; Ercolini et al., 2010a; Ercolini et al., 2011; Laursen et al., 2005; Sakala et al., 2002b).

Different LAB strains produce mostly innocuous fermentation products. This depends on their metabolism, substrates fermented and storage conditions (Leisner et al., 1996; Pothakos et al., 2015). A majority of LAB found on VP red meat are heterofermentative, meaning that they degrade available substrates into a number of metabolites, such as different organic acids, acetate, acetoin, ethanol, CO_2 and various aromatic compounds (Fig. 1.4a) (John et al., 2007). This can increase the array of metabolites that potentially contribute to meat spoilage (Kröckel, 2013). A number of studies have reported different strains of LAB to be implicated with the production of a range of malodourous metabolites associated with meat spoilage, such as butyric acid, butanol, and dimethyl sulphide, and methyl thioacetate (Casaburi et al., 2011; Ercolini et al., 2011; Ercolini et al., 2009; Ferrocino et al., 2013; Hernandez-Macedo et al., 2012; Huis in't Veld, 1996; Jääskeläinen et al., 2013; Jones, 2004; Labadie, 1999). However, it has been reported that LAB do not cause spoilage of VP meat until after the attainment of maximum population, which is due to the accumulation of metabolites over time (Bailey et al., 1992)

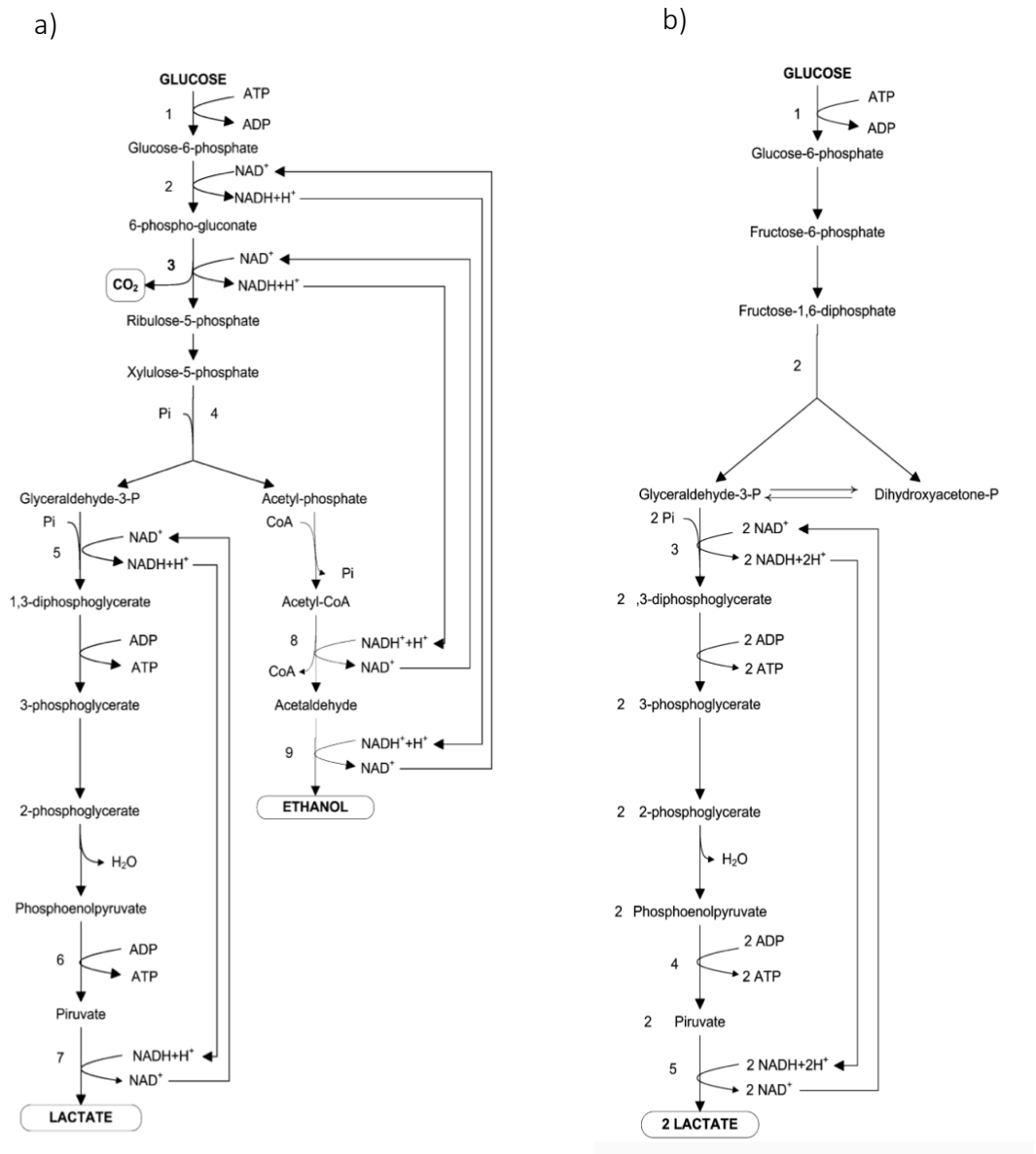

Figure 1.4 LAB fermentation pathways a) homolactic fermentation (glycolysis, Embden-Meyerhof-Parnas pathway); b) heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway) (Reis et al., 2012).

In comparison, LAB can be homofermentative, where they convert available substrates almost completely into lactic acid by pyruvate (Fig. 1.4b). This pathway generates less metabolites as it is equilibrated by the redox reaction (John et al., 2007; Reis et al., 2012). Over time this can result in a slightly acidic confinement odour, which usually disappears after opening the pack (Kröckel, 2013). It is difficult to establish the spoilage potential of LAB as fermentation types are not exclusive. For instance, *Lactobacillus* spp. under glucose

limitation can switch from being homofermentative to being heterofermentative (Borch et al., 1991).

1.5.2 Enterobacteriaceae

The *Enterobacteriaceae* are a large family, generally consisting of Gram-negative facultatively anaerobic rods that are non-spore forming, and are usually motile by flagella (Farmer et al., 2010). The most common *Enterobacteriaceae* found on chilled VP red meat are of the genera *Serratia*, *Hafnia*, *Rahnella*, and *Yersinia* (Kaur et al., 2017b; Kaur et al., 2021; Sakala et al., 2002a; Youssef et al., 2014a; Youssef et al., 2014b). *Hafnia alvei*, *Rahnella aquatilis*, *Serratia liquefaciens*, and *Serratia proteamaculans* are among the most frequently reported species occurring at the end of storage (Sade et al., 2013). Under CO_2 enriched packaging, such as VP, *Enterobacteriaceae* numbers usually remain relatively low in comparison to LAB, especially under optimum chilled storage temperatures (-1.5 – 0°C) (Kaur et al., 2017b; Sade et al., 2013; Sakala et al., 2002a). Under these conditions, it is likely that LAB inhibit the growth of *Enterobacteriaceae* due to competition for substrates required for growth (Matamoros et al., 2009; Youssef et al., 2014b). However, the prevalence of *Enterobacteriaceae* increases on VP meat with a higher pH (> 5.8) and at temperatures above those optimal for storage (Ross et al., 2016; Youssef et al., 2014a; Youssef et al., 2014b).

Enterobacteriaceae may play a key role in spoilage of chilled VP meat despite the relatively low initial abundance under optimum storage conditions. This is because under glucose limitations they can begin to utilise amino acids, giving rise to a range of malodourous metabolites, such as amines, dimethyl sulphide, mercaptans, and ammonia, and also contributing to meat pH changes (Bell, 2001; Casaburi et al., 2015; Gill, 1986; Mills et al.,

2014). *Enterobacteriaceae* have also been implicated with gaseous distention (Brightwell et al., 2007; Chaves et al., 2012) and green discoloration (Dainty et al., 1989b). Greening by *Enterobacteriaceae* is caused by the production of H_2S, which subsequently forms green sulphmyoglobin under anaerobic conditions (Fox, 1966).

1.5.3 Clostridium

Members of the genus *Clostridium* are Gram-positive, spore forming, obligate anaerobes belonging to the phylum *Firmicutes* (Broda et al., 2000). A range of psychrophilic and psychrotrophic *Clostridium* species have commonly been implicated in spoilage of chilled VP red meat, such as *C. algidicarnis*, *C. putrefaciens*, *C. estertheticum*, *C. frigidicarnis*, and *C. gasigenes* (Adam et al., 2010; Broda et al., 2003; Broda et al., 2009; Cavill et al., 2011). Their ability to produce environmentally resilient endospores that germinate under growth permissive conditions allow to them to easily contaminate meat during processing (Broda et al., 2000; Clemens et al., 2010). Spores on VP meat must germinate to grow and cause spoilage (Fig. 1.5). Activation of spores can occur by exposure to high temperatures during heat-shrinking applied during vacuum packaging (Bell, 2001; Moschonas et al., 2011). Heat shrinking has been shown to decrease the time taken for gas-distention ('blown pack') in packs inoculated with spores of *C. estertheticum* (Bell, 2001). Following activation, the spores will germinate, and the resulting vegetative cells will start to grow (Adam et al., 2010).

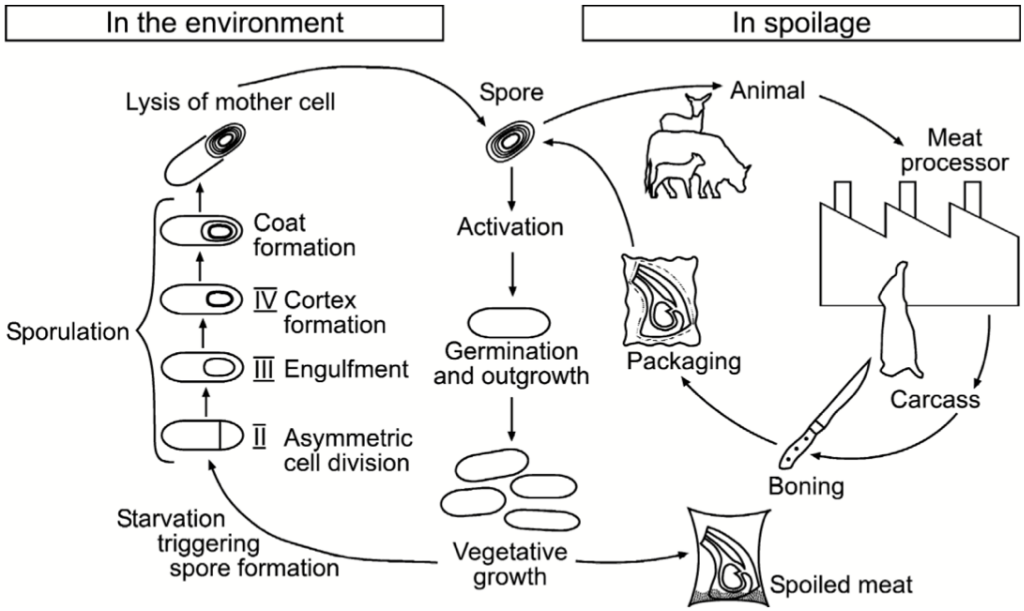

Figure. 1.5 The life cycle of spore forming bacteria in the environment and as spoilers of vacuum-packaged chilled meats. Under appropriate conditions transfer of spores of spoilage causing organisms results in spoiled meat. Stages of spore formation based on Paredes et al. (2005), diagram from Adam et al. (2010).

Chilled VP meat provides optimal growth conditions for vegetative cells of *Clostridia*, especially on meat with higher pH (> 6) (Brightwell and Clemens, 2012; Kaur et al., 2021; Yang et al., 2009; Yang et al., 2014). *Clostridia* are also well equipped to utilise a variety of intramuscular carbohydrates found in meat, as evidenced by the main spoilage species possessing an extensive range of degradative carbohydrate-active enzymes (Palevich et al., 2021; Yu et al., 2016). Their metabolic by-products include butyric acid, butanol, carbon dioxide and hydrogen, which are associated with malodours, discoloration and gas-production (Adam et al., 2010; Broda et al., 1996b; Casaburi et al., 2015; Dainty et al., 1989a; Spring et al., 2003; Yang et al., 2011).

1.5.4 Brochothrix thermosphacta

B. thermosphacta is a Gram-positive, rod-shaped facultative anaerobe of the family *Listeriaceae* commonly found on red meat under a range of meat packaging types and temperatures (Pin et al., 2002). Under anaerobic conditions *B. thermosphacta* is typically homofermentative, thereby preferentially converts sugars to lactic acid, in addition to producing short chain fatty acids and ethanol (Ercolini et al., 2010b; Ercolini et al., 2011; Pennacchia et al., 2011; Pin et al., 2002). These volatiles can contribute cheesy or dairy odours to the spoilage volatilome (Casaburi et al., 2015). Under substrate limitations, it can shift to heterofermentation, increasing the array of spoilage metabolites (Pin et al., 2002). *B. thermosphacta* has also been shown to metabolise proteins and lipids, although this is dependent on the strain and typically occurs at higher storage temperatures (Casaburi et al., 2014; Labadie, 1999).

B. thermosphacta usually does not grow to high populations on VP red meat when stored at optimum chilled conditions (Kaur et al., 2017b). This is because *B. thermosphacta* requires higher storage temperatures, the presence of residual O_2, and relatively high meat pH (> 5.8) (Ercolini et al., 2011; Gribble and Brightwell, 2013; Nowak et al., 2012; Pin et al., 2002). At lower temperatures the prevalence of LAB may inhibit *B. thermosphacta* due to the accumulation of undissociated organic acids (Mohsina et al., 2020; Russo et al., 2006). Therefore, *B. thermosphacta* typically contributes to quality loss of VP meat under sub-optimal conditions that can arise throughout the supply chain.

1.5.5 Pseudomonas

Pseudomonads are mostly aerobic Gram-negative, rod-shaped, non-spore forming, motile bacterium of family *Pseudomonadaceae* and are commonly associated with relatively rapid aerobic spoilage of meat even at low temperatures (Dainty and Mackey, 1992; Ercolini et al., 2007; Gill, 2004; Labadie, 1999). *Pseudomonas* are known to be highly competitive under aerobic conditions as they are able to rapidly build biomass by catabolism of glucose due to their high affinity to O_2 (Gill and Newton, 1977). When glucose is exhausted *Pseudomonas* spp. can begin to metabolise amino acids with consequent production of sulphides, esters and amines, resulting in offensive off-odours and formation of slime (Nychas et al., 1988; Nychas et al., 2008). *Pseudomonas* spp. are still detected on chilled VP meat; however, at a relatively low population due to restricted oxygen levels (Kaur et al., 2017a; Kaur et al., 2017b; Kaur et al., 2021; Kiermeier et al., 2013; Mills et al., 2014). *Pseudomonas* spp. is problematic if vacuum seals are compromised or punctured during distribution (Kaur et al., 2017b).

1.5.6 Shewanella

Shewanella (family *Shewanellaceae*), a genus related to *Pseudomonas* within class Gammaproteobacteria, constitutes a range of facultatively anaerobic Gram-negative, motile, rod-shaped bacterial species (Doulgeraki et al., 2012). *Shewanella* spp., such as *S. putrefaciens* and *S. baltica* are recognised as major spoilers of meat due to the ability to form H_2S at refrigeration temperatures under anaerobic conditions (Doulgeraki et al., 2012; Kamenik, 2013; McMeekin, 1982; Molin and Ternstrom, 1982). In addition to the offensive odour, H_2S can react with myoglobin forming sulphmyoglobin resulting in meat developing a green discolouration (Gill, 1986; Molin and Ternstrom, 1982; Nychas et al., 2008). However,

to grow to sufficient levels to cause spoilage, *Shewanella* requires some access to O_2 (or alternative electron acceptors) and meat of high pH (> 6) (Borch et al., 1996). Modern industry standards, temperature control and low O_2 permeability packaging has reduced the occurrence of sulphmyoglobin spoilage of VP meats caused by *Shewanella* spp. (Huynh et al., 2016). Recent studies on VP red meat have detected low populations of *Shewanella* species under a range of temperatures (Kaur et al. 2017,2021). To further minimise the risk of sulphmyoglobin spoilage, it is industry practise to not vacuum pack DFD primals due to their high pH, which may allow growth of *Shewanella* (Huynh et al., 2016).

1.5.7 Community interactions

The rate and type of meat spoilage is dependent on the order that meat substrates are metabolised by the microbial community to produce different catabolic by-products associated with spoilage. This is dictated by the composition of the microbial community and their complex interactions (Casaburi et al., 2015; Gram et al., 2002; Jørgensen et al., 2000). A range of antagonistic and synergistic interactions can occur between bacteria within the meat ecosystem (Gram et al., 2002; Zhang et al., 2015). These interactions can impede or facilitate the growth of an organism within the meat ecosystem. Interactive mechanisms can involve quorum sensing, exchange of metabolites, contact-dependent inhibition, substrate competition and production of inhibitors such as bacteriocins, antibiotics, and organic acids (Aoki et al., 2005; Blana and Nychas, 2014; Cotter et al., 2013; Deriu et al., 2013; Dubey and Ben-Yehuda, 2011; Nychas et al., 2009).

Antagonistic and competitive interactions are typically the most common, where the growth of one organism is impeded by another (Zhang et al., 2015). Within the microbial communities of chilled VP red meat, LAB exhibit a range of antagonistic and competitive

behaviours, allowing them to dominate the meat matrix relatively quickly. Combined with their rather innocuous fermentation products, the inhibitory activity of LAB can aid shelf-life extension by suppressing growth of higher potential spoilers. A number of studies have reported different strains of LAB have inhibited growth of spoilers, such as *Pseudomonas* spp. and *B. thermosphacta*, as well as potential meat pathogens, *L. monocytogenes*, *Salmonella* and *E. coli* (Chaillou et al., 2014; Fall et al., 2012; Hilgarth et al., 2018; Jones et al., 2008; Jones et al., 2009; Leisner et al., 1996; Saraoui et al., 2016; Signorini et al., 2006a; Vasilopoulos et al., 2010; Yang et al., 2011). Studies have also shown that various LAB were able to inhibit growth of *C. estertheticum* and consequently prevent 'blown pack' spoilage of VP meat (Jones et al., 2008; Jones et al., 2009; Yang et al., 2011). Inhibitory activity of LAB can likely be attributed to their ability to reduce meat pH caused by the accumulation of their acidic metabolites (Fall et al., 2010; Hilgarth et al., 2018; Saraoui et al., 2016). Other inhibitory mechanisms include competition for simple, preferred substrates, and/or production of antagonistic molecules such as bacteriocins (Jones et al., 2008; Jones et al., 2009; Vermeiren et al., 2006; Yang et al., 2011). The inhibition mechanisms of LAB within the meat ecosystem are not yet fully understood and may be multifactorial.

Synergistic interactions, where an organism facilitates the growth of another, have been less frequently reported. However, Zhang et al. (2015) observed cell free supernatant from *Pseudomonas* spp. cultures promoted the growth of *C. divergens* and *C. maltaromaticum in vitro*. Therefore, in the early stages of storage, when residual O_2 is still present, the growth-promoting effects of *Pseudomonas* spp. may influence the composition of the subsequent microbial community that will develop over time (Zhang et al., 2015). Further, Nychas et al. (2009) found quorum-sensing compounds from meat increased the growth rate of *Serratia*

sp. and *Pseudomonas* sp.. Quorum-sensing is advantageous as it allows bacteria to communicate information about cell density within their environment, enabling expression of genes that are beneficial to that environment (Keller and Surette, 2006). Exchange of metabolites between bacteria can also occur within microbial communities. For example, *E. coli* was found to physically connect to other bacteria cells via nanotubes derived from the membrane, which allowed reciprocal exchange of amino acids (Pande et al., 2015). These intercellular connections were not evident when amino acids were abundant (Pande et al., 2015).

The interactive mechanisms of bacteria within the meat ecosystem are complex and currently not well understood. More research is required to gain a better understanding and how this can influence the manifestation of spoilage.

1.6 Shelf-life of Australian vacuum-packed red meat

Vacuum-packaging combined with chilled storage temperatures are the most common methods used to extend the shelf-life of premium Australian red meat products, owing to the economic, logistical, and meat quality advantages. These methods, combined with advances in hygiene and the cold chain, have resulted in significant increases in the shelf lives of beef and lamb (Egan et al., 1988). To this end, beef and lamb primal cuts produced in Australia have shelf-lives of 26 and 12 weeks respectively when VP and stored at chilled temperatures of -1.5 to 0°C (Kiermeier et al., 2013; Small et al., 2012). These relatively longer shelf lives of chilled VP meat have allowed Australia to consolidate a large international export market (Small et al., 2012). The value of this export industry makes a significant contribution to the Australian economy; in 2019 beef exports were valued at $10.8 billion (1.23 million tonnes) and $4.1 billion for lamb (281,518 tonnes) and mutton (183,833 tonnes) (MLA, 2020a, b).

The main export markets of Australian beef and lamb are China, USA and Korea (MLA, 2020a, b). Other major export markets include Japan and Indonesia for beef, United Arab Emirates (UAE) and Qatar for lamb (MLA, 2020a, b). Figure 1.6 illustrates the percentage exported to the major international markets in 2019 relative to the total exported, which made Australia the largest exporter of sheep meat (lamb and mutton) and the second largest for beef (MLA, 2020a, b).

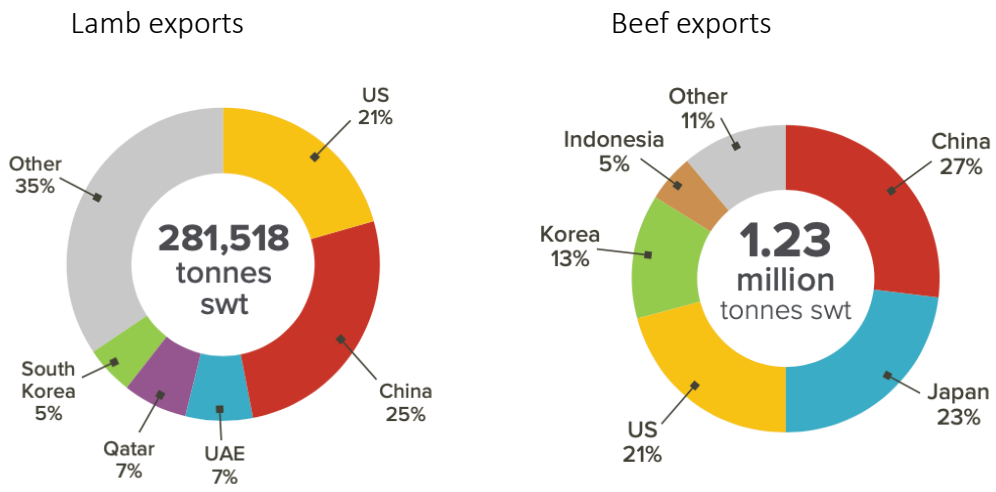

Figure 1.6 The percentage exported to the main international markets in 2019 relative to total exported (MLA, 2020a, b).

1.6.1 Industry challenges associated the shorter shelf-life of lamb

A majority of red meat exports are by sea-freight due to the economic and environmental costs associated with faster transportation such as air-freight (Mercier et al., 2017; MLA, 2017). However, the shorter shelf-life of lamb can present a challenge to lamb exporters. This is because shipping voyages to more distant markets can be lengthy, exacerbated by the additional time required by the importing country for further processing, distribution, and retailing. Consequently, it can significantly reduce the available shelf-life for the purchaser,

and in some instances the shelf-life can already be exhausted (Huynh et al., 2016; Mills et al., 2014).

The shorter shelf-life also makes lamb more vulnerable to potential issues that may arise during transportation and distribution, such as delays in total travel time and temperature fluctuations (Sumner, 2016). These problems in the supply chain logistics can result in product downgrades and rejection. It can also lead to failure to meet technical trade requirements that are set by a number of international markets (Huynh et al., 2016). For instance, Middle Eastern countries require chilled VP lamb to have a shelf-life ranging between 70 – 90 days after slaughter (Huynh et al., 2016). Extended trans-shipment times can substantially increase the total travel time. For instance, tracking information of an Australian shipment of meat destined for Belgium showed that trans-shipment in Tanjung Pelepas took five days and a further five days was required for clearance at the destination port, thereby reducing the shelf-life available to the purchaser by 10 days (Sumner, 2016). Other factors that may contribute to longer travel times include industrial disputes and slow steaming (Sumner, 2016). Pressure on shipping companies to reduce energy cost and environmental impact has resulted in slow steaming whereby vessel engines are slowed by up to 20% (Kloch, 2013; Psaraftis and Kontovas, 2013). Mills et al. (2014) highlighted the impact of slow steaming on exports of VP lamb primals from New Zealand to Europe, which extended the voyage by a week and substantially decreased the shelf-life available to the purchaser. In addition, small temperature increases above optimal (-1.5°C) during distribution can have a significant impact on the rate of quality loss of VP lamb (Gill et al., 1995). For instance, a shelf-life reduction of approximately 30, 50 and 70% can occur when temperatures increase to 0°C, 2°C and 5°C (Figure 1.7) (Sumner and Jenson, 2011). Products

can increase in temperature as a result of inadequate temperature control aboard the vessel, and when loaded and unloaded at dockside (Sumner, 2016). The temperature at which products are loaded is also important because if they are loaded at higher temperatures of 2 – 3°C it can take some time to reduce to optimum chilled temperatures below 0°C (James and James, 2004; Sumner, 2016).

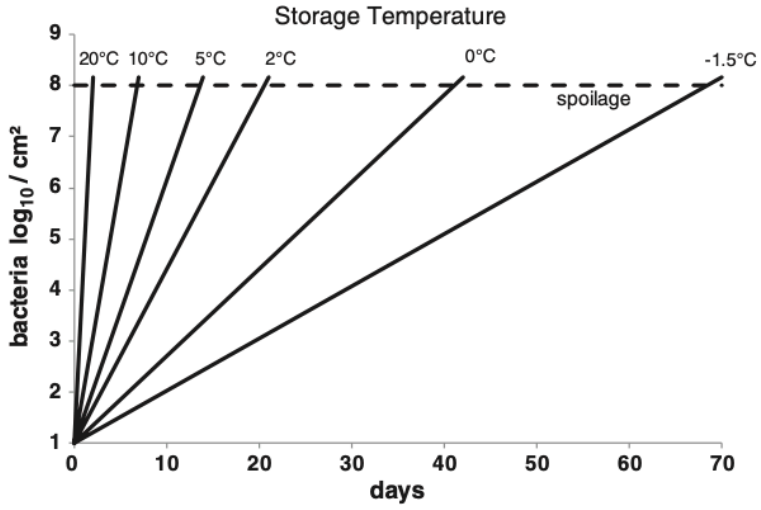

Figure 1.7 Effect of storage temperature on the time to spoilage as a consequence of the anaerobic growth on high pH lamb strain psychrotrophic *Serratia liquifaciencs* (Mills et al., 2014).

1.6.2 Factors impacting the shelf-life of lamb

1.6.2.1 Meat biochemistry

The substantial shelf-life difference between beef and lamb is likely a consequence of the biochemistry of the meat type, and the subsequent impacts on bacterial selection and growth capabilities (Kaur et al., 2021). Beef has a pH range of 5.5 – 5.8, whereas lamb typically is 0.4 pH units higher, ranging between 5.6 – 6.8 (Kaur et al., 2021; Kiermeier et al., 2013). This difference is due to the large amount of adipose tissue distributed through lamb

muscle, as well as the biochemistry of the meat types, in which glycogen and lactic acid are of particular importance (Carse and Locker, 1974; Gill, 2004; Gill and Penney, 1985; Kaur et al., 2021).

The higher pH of lamb may alter bacterial growth capabilities, which is likely a consequence of lower concentrations of undissociated lactic acid (Gill and Newton, 1978). Accordingly, bacterial growth rates on lamb have been reported to be approximately twice that of beef (Kaur et al., 2021; Tamplin, 2009). However, there are very few studies that have compared the effect of pH on the growth and spoilage capabilities of bacteria on VP lamb and beef, and the current information is variable (Gribble et al., 2014). For instance, inoculation shelf-life trials showed that *Brochothrix thermosphacta* and *Serratia proteamaculans* had the ability to grow and spoil VP lamb cuts of high (5.9 to 6.4) and low (5.4 to 5.8) pH at chilled temperatures of -1.5°C, whereas previous inoculation trials using VP beef detected no growth of these organisms below pH 5.8 (Grau, 1980; Grau, 1981; Gribble and Brightwell, 2013; Gribble et al., 2014). It was therefore suggested that the levels of substrates present at low and high pH may also affect microbial growth (Bell, 2001; Gill and Newton, 1978; Gribble et al., 2014). Substrate composition could vary between beef and lamb but the overall effect on bacterial growth rates, rates of quality loss (indicated by odour change) and community structure does not support this conclusion (Gribble et al., 2014; Kaur et al., 2021). Growth rate is linearly correlated to hydrogen ion concentration and maximum population densities achieved on VP beef and lamb are observed to be similar suggesting chemical composition are also relatively similar with pH proposed as the leading intrinsic factor (Kaur et al. 2021). At this stage, there is a paucity of data reported on the substrate composition of lamb and

the rate they are or become available to bacteria at chilled temperatures, therefore further investigation is required.

Beef has been the primary model for spoilage mechanisms of VP red meat and most information has consequently been extrapolated to lamb (Gribble et al., 2014; Mills et al., 2014). However, it is clear that the biochemistry of beef and lamb is different and by extension so is the bacterial selection, growth and metabolic capabilities. Kaur et al. (2021) recently highlighted that the spoilage community composition of chilled VP lamb was significantly different and more diverse than that of beef. Lamb had significantly higher proportions of *Yersinia*, *Carnobacterium*, *Clostridium*, *Pseudomonas*, *Rahnella*, *Morganella*, *Janthinobacterium*, *Acinetobacter, Providencia*, and *Aeromonas*. In comparison, beef had significantly higher abundance of *Serratia, Lactococcus, Leuconostoc, Enterobacter, Brochothrix, Vagococcus* and *Lactobacillus.* Another shelf-life study by Kaur et al. (2017b) also found *Carnobacterium*, *Yersinia* and *Clostridium* spp. dominated VP lamb at -1.2°C, and the main spoilage community expanded to *Hafnia*, *Lactococcus* and *Providencia* spp. at 8°C. However, the metabolic capabilities of these taxa on chilled VP lamb and their potential contribution to the faster rate of quality loss is yet to be reported.

1.6.2.2 Initial microbial diversity and counts

Another factor contributing to the shorter shelf-life of lamb than beef may be the differences in slaughter and dressing processes, resulting in lamb typically having a higher initial bacterial count (Bell and Hathaway, 1996; Huynh et al., 2016; Whyte et al., 2002). In 2012/2013, the Australian national baseline surveys indicated that beef had an initial total viable count of 1.3 – 1.5 $\log_{10}$ CFU/cm^2 while lamb was 2.02 – 2.29 $\log_{10}$ CFU/cm^2 (Phillips et al., 2012; Phillips et al., 2013). Initial microbial counts can be influenced by cleanliness of lamb fleece and

whether it is unshorn versus shorn (Hauge et al., 2011; Whyte et al., 2002). The initial counts on primals are unavoidably variable due to many pre- and post-slaughter contamination factors. However, higher initial bacterial counts on lamb can increase the rate of quality loss, especially combined with potential fluctuations in temperature and increased travel time, as it presents the greatest opportunity for bacterial growth (Mills et al., 2014; Sumner, 2016).

In addition to having higher initial counts, lamb has been found to have a significantly more diverse starting community composition compared to beef (Kaur et al., 2021). Kaur et al. (2021) found this to be true for abattoirs that produced both beef and lamb, as well as in those abattoirs that produce beef and lamb separately. Similarly, Bakhtiary et al. (2016) found bacterial diversity of environmental samples to be significantly higher in sheep slaughter lines compared to those of cattle. This was attributed to the more hygienic conditions evident in the cattle slaughter lines, such as, performing slaughter on a production line and automatic hide pullers. However, overtime the microbial communities of VP red meat become less diverse and restricted due to the storage conditions (i.e., VP atmosphere and chilled temperatures) that constrain the growth of many bacteria (Kaur et al., 2021). Further research is required to assess the differences in initial microbial community composition of VP lamb and if it has an influence on the rate of spoilage.

1.6.2.3 Packaging

Increased difficulty packing bone-in lamb primals has also been reported due to the smaller size and bone shapes (Sumner et al., 2021). This has the potential to compromise anaerobic conditions as a result of higher levels of residual O_2 in the pack, thereby reducing inhibition of aerobic spoilers such as *Pseudomonas* (Ščetar et al., 2010). Residual O_2 in packs can also be

caused by the larger surface of adipose tissue of lamb combined with only limited oxygen-scavenging potential (Gill, 2004; Huynh et al., 2016).

1.7 Thesis objectives

The primary objectives of the research reported in this thesis is to provide insights into microbial spoilage mechanisms of chilled VP lamb, and to investigate potential interventions to aid shelf-life extension of VP lamb products. This research will build upon the research literature on the microbiology of VP lamb which is currently limited. It will also assist the development of potential cost-effective interventions to increase the shelf-life of VP lamb. The following research aims are presented to achieve the primary objectives:

1. *to investigate the spoilage potential of bacterial species isolated from chilled VP lamb*

2. *to evaluate the potential for glucose surface treatments to extend the shelf-life of VP lamb*

3. *to investigate the effects of additional glucose as a surface treatment on the microbial community, shelf-life and associated volatilome of VP lamb*

1.8 Thesis structure

The structure of this thesis reflects the logical order of the research aims. Chapter 1 provides a background of the current research literature regarding the microbiology of red meat during processing, and the microbial spoilage of VP lamb and the challenges it presents to the industry. This identifies the knowledge gaps that this research intents to build upon in Chapters 2 – 4. These chapters are presented in sequence to allow them to build upon the findings of the previous chapter. All experimental chapters have been prepared with the aim for submission to the international refereed published literature. As a result of this formatting, and in some instances the use of similar methodology, there is some repetition within and between the experimental chapters. However, each chapter seeks to address a unique aim relating to the primary objectives of this thesis. Specifically, Chapter 2 provides insights into the microbial spoilage mechanisms of VP lamb at low temperatures. Chapter 3 establishes the potential of glucose surface treatments to successfully extend the shelf-life of VP lamb. Chapter 4 builds upon this by investigating the effects of glucose surface treatment on the microbial community and associated volatilome of VP lamb.

Chapter 2

Spoilage potential of bacterial species isolated from chilled vacuum-packed lamb

2.1 Introduction

The main process driving meat spoilage is microbial growth and metabolism. This is due to meat's high water-activity and the abundance of readily available nutrients (Ercolini et al., 2006; Gram et al., 2002). Despite rigorous hygiene standards within processing facilities, the surface of meat is inevitably contaminated with a diverse range of bacteria derived from slaughter, processing, and livestock (Mills et al., 2014; Nychas et al., 1988; Nychas et al., 2008; Stellato et al., 2016). The 'spoilage potential' of a given bacterial species on meat is determined by its ability to grow sufficiently on the meat matrix, and to produce metabolites that contribute to spoilage under the storage conditions (Dainty and Mackey, 1992; Ercolini et al., 2010a; Koutsoumanis et al., 2006; Lambert et al., 1991). Storage environments with minimal oxygen, such as vacuum packaging, in combination with low storage temperatures can effectively extend the shelf-life of meat products by selecting for facultative anaerobes with relatively low spoilage potential (Borch et al., 1996; Doulgeraki et al., 2012; Mills et al., 2014; Pennacchia et al., 2011). Specifically, these communities are typically dominated by lactic acid bacteria (notably *Carnobacterium* spp., *Lactococcus* spp. and *Leuconostoc* spp.), which outcompete the main organisms that dominate aerobic spoilage, such as *Pseudomonas spp*., *Enterobacteriaceae* and *Brochothrix thermosphacta* (Doulgeraki et al., 2012; Nissen et al., 1996).

Vacuum-packed (VP) lamb produced in Australia has a shelf-life of 12 weeks when stored at - 1.5 to 0°C, whereas beef has a shelf-life of 26 weeks under the same conditions (Kiermeier

et al., 2013; Small et al., 2012). The difference in their shelf-life is mainly attributed to the intrinsically higher pH of lamb as a higher pH can enable faster growth rates of bacteria including high potential spoilers (EFSA, Panel on Biological Hazards (BIOHAZ), 2016; Gill and Penney, 1985; Kaur et al., 2021). For instance previous studies have reported meat of higher pH can facilitate the growth of *B. thermosphacta* (> 5.8 pH units), *Clostridia* (> 6), members of the *Enterobacteriaceae* family, such as *Serratia*, *Hafnia* and *Rahnella* (> 5.8), and *Shewanella* (> 6) (Borch et al., 1996; Brightwell and Clemens, 2012; Ercolini et al., 2011; Gribble and Brightwell, 2013; Kaur et al., 2021; Nowak et al., 2012; Pin et al., 2002; Ross et al., 2016; Yang et al., 2009; Yang et al., 2014; Youssef et al., 2014a; Youssef et al., 2014b).

Beef has been the primary model for spoilage mechanisms of VP red meat and most information has consequently been extrapolated to lamb (Gribble et al., 2014; Kaur et al., 2017b; Mills et al., 2014). However, the biochemistry of beef and lamb is different and by extension so is the bacterial selection, growth and spoilage potential. For instance, Kaur et al. (2021) recently found the abundance of some of the main taxa on chilled VP red meat significantly differed between beef and lamb. Lamb had significantly higher abundance of *Yersinia*, *Carnobacterium*, *Clostridium*, *Pseudomonas*, and *Rahnella* compared to beef. However, the metabolic capabilities of taxa on VP lamb and their potential contribution to the faster rate of quality loss is yet to be reported (Kaur et al., 2017b). Therefore, the primary objective of this study was to establish whether specific organisms play important roles in the rate of spoilage of VP lamb at chilled temperatures. This was done by assessing the spoilage potential of each representative organism from the spoilage community of VP lamb through a series of shelf-life trials, in which they were individually inoculated onto sterile lamb meat, as well as fresh lamb meat containing its natural microbial community. Meat quality was then

assessed over time by measuring sensorial attributes, bacterial numbers and pH. This is the first published study that has assessed the spoilage potential of each representative 'spoilage' organism through inoculation challenge studies on sterile and non-sterile chilled VP lamb. Such knowledge will help to provide insights into the spoilage mechanisms of VP lamb and may assist with the development of shelf-life extension methods.

2.2 Materials and methods

2.2.1 Isolate selection

Representative bacterial colonies were selected from tryptone soya agar (TSA) (CM0131, Oxoid, Australia) stored at 20°C for 5 days from lamb samples near the end of the predicted shelf life that showed sensory decline. These colonies were streaked on brain heart infusion (BHI) (CM1135, Oxoid, Australia) agar and incubated at 25°C for 5 days to obtain pure cultures. The whole biomass from the pure culture plate was transferred to BHI glycerol broth and stored at -80°C. A total of 133 pure isolates were obtained. Pure cultures were grown in BHI broth (25°C for 48 h), followed by genomic DNA extraction using UltraClean Microbial DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer's instructions. Extracted DNA was PCR amplified using 16S rRNA universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG – 3') and 519R (5' - GWA TTA CCG CGG CKG CT – 3'). Each PCR reaction mixture was 25 µL containing 0.20 µM of each primer, 10 µL of ImmoMix Red ™ (Bioline, Australia), 12 µl of ultra-pure 18.2 MΩ DNAase/RNAase free water (Bioline, NSW, Australia) and 2 µL of DNA. Amplifications were performed in a C1000 Thermal Cycler (Bio-Rad, USA) under the following conditions: initial denaturation for 1 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. The final extension was for 10 min at 72°C. To check the size and

purity of PCR amplicons, 5 µl of each reaction was run in a 1.5% agarose gel (w/v). Amplicons were purified and sequenced by Big Dye Sanger sequencing on an Applied Biosystem 3730xl System (Macrogen Inc., Seoul, Korea). The sequences were edited using BioEdit (Hall, 1999), which were compared to the GenBank data using the BLASTn algorithm (Altschul et al., 1990).

Random amplified polymorphic DNA (RAPD) was conducted to biotype different strains using M13 (5'-GAGGGTGGCGGTTCT-3'), XD5 (5'-CTGGCGGCTG-3'), 239 (5'-CTGAAGCGGA-3'), 795 (5'-GAGACGCACA-3'), HLWL85 (5'-ACAACTGCTC-3') and P16 (5'-TCGCCAGCCA-3') primers.

Ten representative bacterial isolates were identified (Table 2.1). Due to partial sequence analysis, full identification could not be made for *Enterococcus* sp., *Pseudomonas* sp. and *Serratia* spp. However, these isolates were related to organisms that are commonly found on chilled VP meat such as *Enterococcus malodoratus*, *Pseudomonas fragi* and *Serratia proteamaculans*. Three *Clostridium* species commonly associated with lamb spoilage were also used in this study (Broda et al., 2009; Cavill et al., 2011), which were purchased as freeze dried cultures from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. This included *C. algidicarnis* (DSM 15099), *C. estertheticum* subsp. *estertheticum* (DSM 8809) and *C. putrefaciens* (DSM 1291). *Clostridium* isolates were revived from freeze dried cultures as per culture collection guidelines and stored at -80°C on storage beads (TS/80-MX, Technical Service Consultants Ltd, UK).

Table 2.1 Representative isolates of the microbial community of VP lamb organised into Gram stain and respiration mode. Bacterial combinations for the three cocktail inocula are highlighted.

Gram stain	Isolate	Respiration	
Gram-positive	*Clostridium algidicarnis*	Obligate anaerobe	
	Clostridium estertheticum		
	Clostridium putrefaciens		
Cocktail II	*Brochothrix thermosphacta*	Facultative anaerobe	Cocktail I
	Carnobacterium divergens		
	Carnobacterium maltaromaticum		
	Enterococcus sp.		
	Lactococcus lactis		
	Lactococcus piscium		
Gram-negative Cocktail III	*Hafnia paralvei*		
	Serratia spp.		
	Yersinia intermedia		
	Pseudomonas sp.	Aerobic	

2.2.2 Sample collection and storage

Two sets of trials were conducted in which all named bacterial isolates were individually inoculated onto sterile meat (irradiated, *see* below for more details), and non-sterile meat (i.e., containing natural microbiota). For both trials, lamb eye of loin (n = 192 in total) was used due to its consistent shape and size. Meat was freshly sourced from a local abattoir (Tasmania, Australia) from a single herd of animals slaughtered three days prior to commencement of each trial. The abattoir split each eye of loin into two pieces across the cut and vacuum packed (VP) each half in a barrier bag TBG4620 (O_2 transmission rate of 20 cc/m^2/24 h at 23 °C, 0% relative humidity). This made a total of 216 samples for the sterile meat trial and 168 samples for the non-sterile meat trial (~ 8 x 7 x 2 cm). All shelf-life trials were conducted at 2°C. This temperature was chosen because it would shorten the expected

time to spoilage, but without significantly altering the microbial community composition compared to storage at 0°C (Kaur et al. 2021).

2.2.3 Inoculum treatments

As described above, all the named bacterial isolates were individually inoculated onto sterile meat (n = 216), and non-sterile meat (n = 168). Both sterile and non-sterile meat trials had an untreated control (i.e., not irradiated or inoculated) which provided a reference point for spoilage. The sterile trial also included inocula of bacterial cocktails. Cocktail I included all facultative anaerobes and *Pseudomonas* sp., and Cocktail II consisted of Gram-positives (excluding *Clostridium* species), whereas Cocktail III included all Gram-negatives (Table 2.1). The sterile meat trial also had a positive control, which involved inoculating sterile meat with the natural microbial community of untreated meat. This was to test whether irradiation of meat influenced other (endogenous) processes of spoilage.

Meat samples used in the sterile trial were gamma irradiated (Steritech Pty Ltd, Victoria, Australia) at a dosage of 10 kGy to inactivate the meats' natural microbial community. These samples were airfreighted (~12 days) to Victoria, Australia, at refrigeration temperatures, during which the untreated controls were stored at -1°C to minimise further growth of the microbial community but without freezing the samples. Preliminary trials were conducted to validate the irradiation process, which showed no bacterial growth on irradiated meat (see microbial enumeration section 2.6) (data not shown).

2.2.4 Isolate revival and culture preparation

To prepare bacterial inocula, representative facultative anaerobes and *Pseudomonas* sp. were revived from stock cultures by streaking onto tryptone soya agar (TSA) (CM0131, Oxoid,

Australia) followed by incubation at 25°C for 5 days. Pure colonies were grown in brain heart infusion (BHI) broth (CM1135 Oxoid, Australia) at 25°C for 48 h. For *C. putrefaciens* and *C. algidicarnis*, these isolates were revived from stock cultures by adding a bead to 1 ml of BHI in a filter cap tissue culture flask (Greiner Bio-One, Austria) followed by incubation at 25°C for 48 h in sealed containers under an anaerobic atmosphere, generated by an Anaerogen compact pouch (AN0020D, Oxoid, Australia). Stationary phase cultures were streaked onto Columbia sheep blood agar (PB0123, Oxoid, Australia) and incubated under anaerobic conditions at 25°C for 5 days. Pure colonies were transferred to 1 ml of BHI in a filter cap tissue culture flask and incubated for a further 48 h under anaerobic conditions at 25°C. *C. estertheticum* was revived from a stock culture under anaerobic conditions, where a bead was added to Cooked Meat Medium (CMM) (CM0081, Oxoid, Australia) followed by incubation at 10°C for 4 weeks. The broth culture was anaerobically streaked onto Columbia sheep blood agar and incubated for a further 4 weeks at 10°C, after which pure colonies were transferred to anaerobic Clostridium Reinforcement Medium (CRM) (CM0149, Oxoid, Australia) and incubated for 3-4 weeks at 10°C. To determine stationary phase of *Clostridium* spp., cultures were measured by optical density at 600 nm with a Spectrostar Nano microplate reader (BMG Labtech, Germany) until cultures had a reading of 1.0 (± 0.05).

To ensure growth of correct isolates, pure colonies from all isolates were identified by colony morphology, Gram-staining, as well as being periodically confirmed by DNA sequencing (see section 2.2.1 for methods).

2.2.5 Inoculum preparation and inoculation protocol

To prepare inocula of individual bacterial strains, 1 ml of stationary phase working culture (approximately 10^9 CFU/ml, as previously described above) was centrifuged for 5 mins at

5000 *x g*, the supernatant was removed, and cells were re-suspended in 1 ml of 0.1% peptone water (LP0037, Oxoid, Australia). This was repeated twice. All individual cell suspensions were diluted with 0.1% peptone water to achieve an inoculum concentration of approximately 10^6 CFU/ml.

To prepare the bacterial cocktails, 1 ml of all required cell suspensions were combined. From this, the expected concentration was calculated, followed by appropriate dilution to achieve a concentration of approximately 10^6 CFU/ml. To obtain the natural microbial community for the positive control, meat rinsates were collected from fresh meat samples (n = 3) by massaging with 50 ml of sterile 0.1% peptone water for 1 min. The meat rinsates were stored anaerobically at 2°C while meat samples were in transit for irradiation (~ 12 days), and by the commencement of the trials concentration was 10^6 CFU/ml, which was determined by spread plating onto TSA (see section 2.2.6).

Both sides of the meat surfaces were inoculated through the vacuum-pack with 0.5 ml of the appropriate inoculum via 1cc/ml syringes with a 25g x 5/8" needle (Terumo, Japan) to achieve a final concentration of approximately 10^4 CFU/cm^2. To maintain anaerobic conditions, syringe needles were inserted through a septum sealing strip (10 x 20 x 20 mm) (Novatech, Australia), which were evenly placed over the vacuum-pack to maximize inoculum spread over the meat surface.

2.2.6 Sensory analyses, surface pH and microbial enumeration

Storage samples were periodically tested for odour, colour, pH and microbial counts after 0, 21, 48 and 68 days. For all treatments, three replicates were analysed at each timepoint. The sensory assessment was a blind evaluation, conducted by a panel of 4 – 6 volunteers (Gill and

Badoni, 2002; Small et al., 2012; Yang et al., 2021). Panelists were selected based on their expertise in food microbiology and included a range of different cultural backgrounds. Prior to the trials, all panelists were required to attend a training session, in which samples at different stages of shelf-life were used for practice assessment. Persistent odour was assessed using an 9-point categorical hedonic scale (0 = extreme off-odour, spoiled to 8 = no odour, normal meaty odour), and colour was determined using a visually anchored scale (0 = other colour e.g. green colouration; 2 = very poor bloom, grey colouration; 4 = poor bloom, some greyness; 6 = bloom, light red colouration; 8 = bloom, red colouration) (Small et al., 2012). Any score of $\leq$ 4 was considered as commercially unacceptable. Sensory assessment was conducted approximately 10 minutes after packs were opened to ensure accurate assessment of colour and persistent odour.

Meat surface pH was measured using a handheld pH meter (Model 206-pH2, Testo, Australia) at four random locations on the meat. The pH meter was calibrated prior to sampling according to manufacturer's instructions. To enumerate bacteria, each meat sample was transferred to sterile bags and massaged by hand in 50 ml of sterile 0.1% peptone water for 1 min to obtain meat rinsate. Serial dilutions were prepared from the meat rinsate in sterile 0.1% peptone water and spread plated on TSA followed by incubation at 25°C for 5 days.

2.2.7 Statistical analysis

The datasets were analysed to determine differences in bacterial counts ($\log_{10}$ CFU/cm^2), pH and sensory scores between the inoculum treatments and the untreated control. For all datasets, quadratic regressions were fitted to each inoculum treatment and the control. The model was in the form of: $outcome = A_j + B_j \, x \, time + C_j \, x \, time^2$, where A_j is a constant, B_j is a

linear effect for time, C_j is the effect for the quadratic term in time, and *j* refers to the *j*th treatment. The levels of the factor treatment are the microbe species and untreated control. An analysis of variance approach was used to test whether the coefficients A_j, B_j and C_j differed by treatment. Where significant effects were found, differences between the fitted quadratic curve for the control and the curves for treatments were then calculated at each timepoint (0, 21, 47 and 68 days). Sterile and non-sterile trials were analysed separately, using the untreated control respective of the trial for each analysis. The *p*-values were adjusted for multiplicity using simulation (Westfall et al., 2011). Statistical analyses were done using Proc Mixed in SAS 9.4. Initial and maximum bacterial populations (log_{10} CFU/cm^2) were determined for each treatment group using the D Model (Baranyi and Roberts, 1994).

Persistent odour and colour scores were used to determine the quality shelf-life duration of meat samples. The end of shelf-life was estimated based on the time taken for samples to reach a sensory score of ≤ 4 at a given timepoint and subsequent timepoints. This was calculated from the fitted curve of the quadratic regression. To account for the inherent variability of meat spoilage, treatment groups were not considered different to the untreated control if they spoiled between 90 – 110% of the observed shelf-life of the untreated controls.

2.3 Results

2.3.1 Sensory assessment and shelf-life duration

Initially the irradiated meat samples (Fig. 2.1, 2.2, 2.4 and 2.5) had lower odour and colour scores compared to the untreated control. Sensory scores of irradiated meat samples were also initially more variable between treatments compared to that of fresh meat samples in

the non-sterile meat trial (Fig. 2.3, 2.6). These results are due to the irradiation process causing a slight 'metallic' odour and dull colour. However, the slight loss of sensory quality of meat caused by irradiation was rated as acceptable by the sensory panel and therefore not problematic for the purpose of this study.

2.3.1.1 Odour

In the sterile meat trial, the untreated control (containing natural microbiota and not inocualted) reached the end of shelf-life (odour score ≤ 4) by 60 days (Fig. 2.1). This was similar to the positive control (sterile lamb inoculated with natural meat rinsate) that reached an odour score of ≤ 4 by 52 days, indicating that inoculated irradiated meat samples spoil similarly to the non-irradiated lamb with an analogous natural microbial community (Fig. 2.2). Sterile lamb samples that were inoculated with individual isolates of *C. putrefaciens* and *C. algidicarnis* also reached the end of shelf-life at similar times to the untreated control, at 58 and 65 days, respectively (Fig. 2.1). However, meat inoculated with *C. estertheticum* only maintained quality shelf-life duration for up to 30 days, approximately half that of the control in the sterile meat trial. *C. estertheticum* had significantly lower odour scores than the untreated control (t_{126}= 4.71, p = .0004) as early as within 21 days of the trial. In contrast, sterile meat inoculated with individual facultative anaerobes and *Pseudomonas* sp. were still rated as acceptable (odour score > 4) beyond the end of the trial (i.e., more than 68 days), and had significantly higher odour scores compared to the untreated control (p < .0001 to .0245). Similarly, sterile meat inoculated with the bacterial cocktail I (t_{45} = 3.29, p = .0256), cocktail II (t_{45} = 5.37, p = < .0001) and cocktail III (t_{45} = 6.22, p = < .0001) were rated as acceptable beyond the end of the trials, all of which had significantly higher odour score than the untreated control (Fig. 2.2).

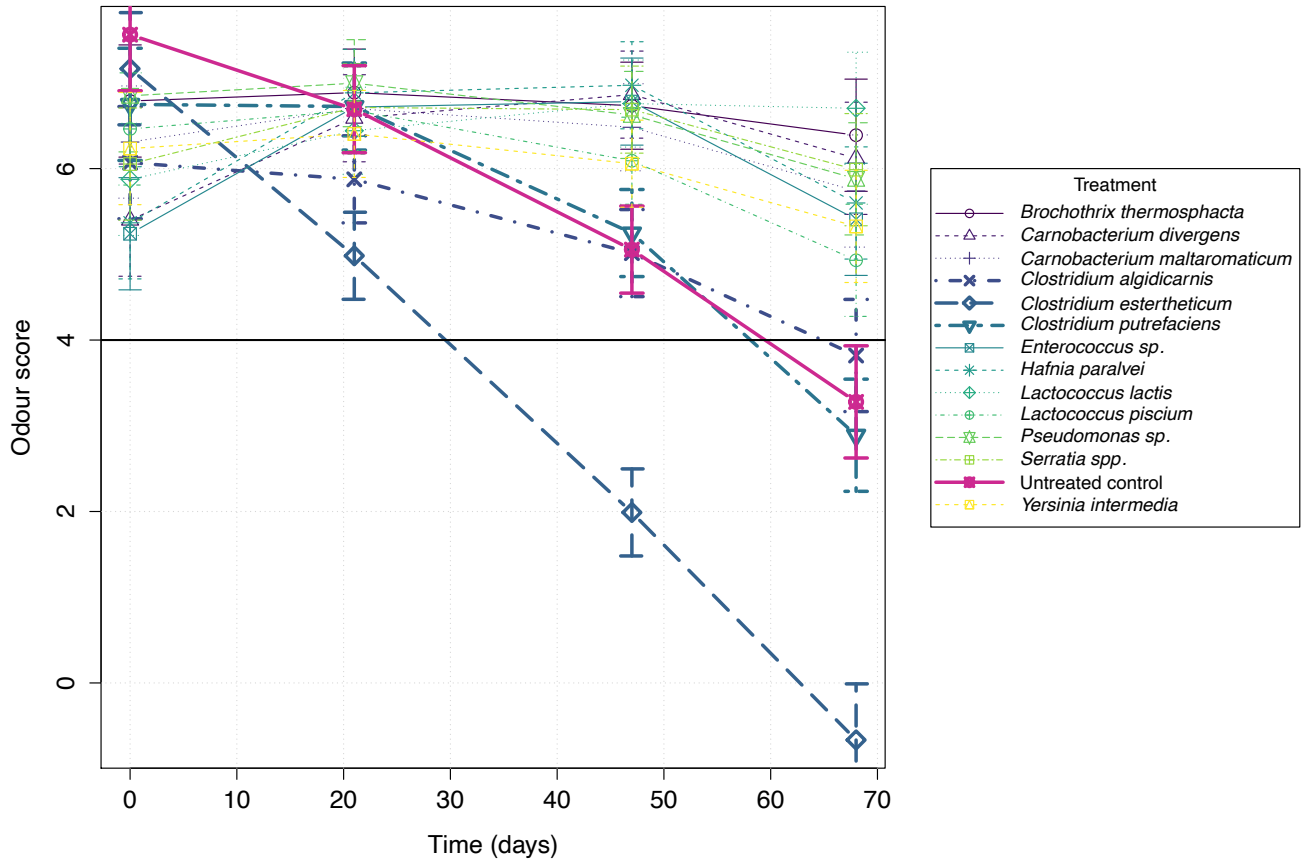

Figure 2.1 Odour scores and 95% CI of sterile vacuum-packed lamb meat stored at 2°C after inoculated with individual bacterial isolates. End of shelf-life is demarcated by an odour score of ≤4, highlighted by the horizontal line. Treatments with bold lines reached end of shelf-life.

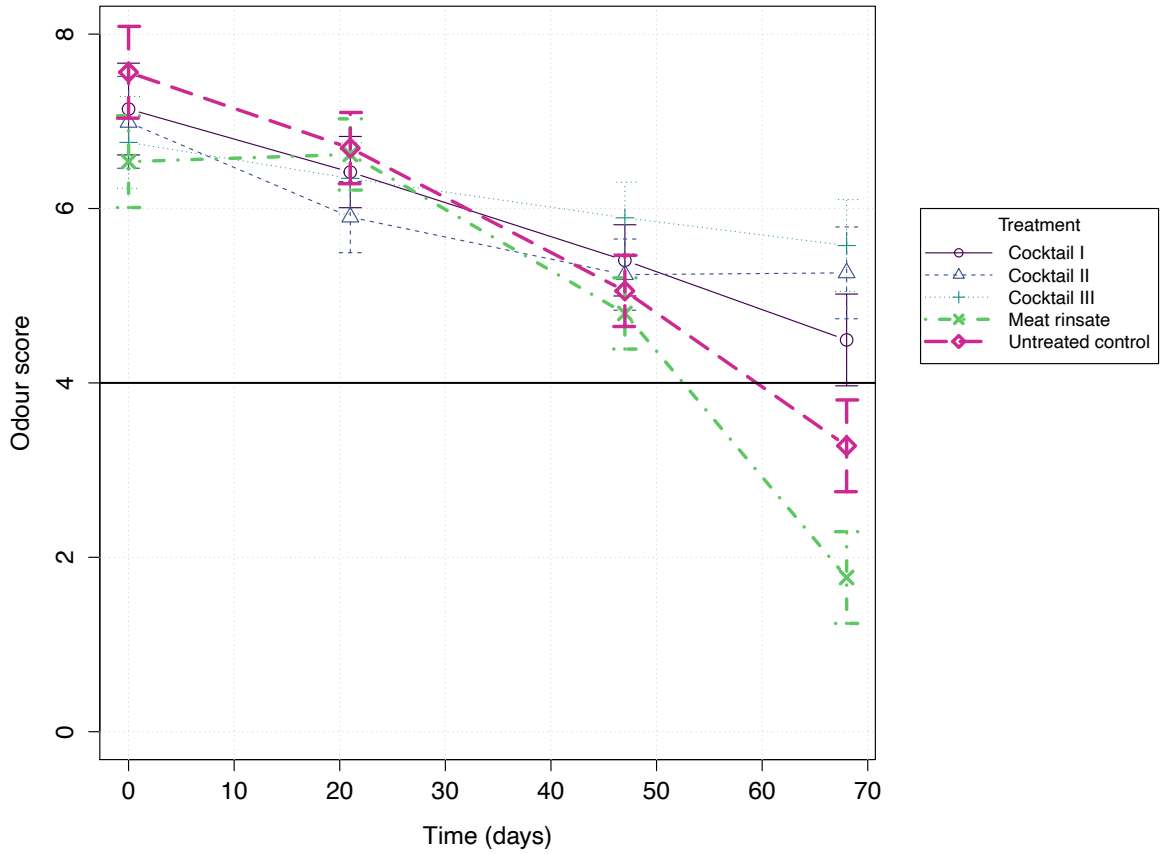

Figure 2.2 Odour scores and 95% CI of sterile vacuum-packed lamb meat stored at 2°C after inoculated with bacterial cocktails. Cocktail I included all facultative anaerobes and *Pseudomonas* sp., cocktail II included Gram-positives (excluding *Clostridium*), and Cocktail III included all Gram-negatives. End of shelf-life is demarcated by an odour score of ≤4, highlighted by the horizontal line. Treatments with bold lines reached end of shelf-life.

In the non-sterile meat trial, the shelf-life of the untreated control was up to 65 days (Fig. 2.3). All treatment groups reached an odour score of ≤ 4. However, the time taken to spoil varied between inoculum treatments. Similar to the sterile meat trial, *C. putrefaciens* and *C. estertheticum* were the first treatment groups to reach an unacceptable odour score, which occurred by 49 and 50 days, respectively. This was followed by *C. divergens* at 52 days, and *Serratia* spp. at 54 days. All of these treatment groups had significantly lower odour scores compared to the untreated control at the end of the trial (*C. estertheticum:* $t_{126} = 4.81$,

p = .0002; *Serratia spp*: $t_{126} = 4.00$, *p* = .0060; *C. putrefaciens*: $t_{126} = 3.93$, *p* = .0073; and *C. divergens:* $t_{126} = 3.30$, *p* = .047). *C. algidicarnis* reached the end of shelf-life by 55 days, however, the odour score was not significantly different from the control by the end of the trial. Meat inoculated with all other organisms spoiled between 58 – 68 days. This fell within 90% - 110% of the observed shelf-life of the untreated control; therefore, not considered to be significantly different. The odour scores for these treatments were also not significantly different compared to the control.

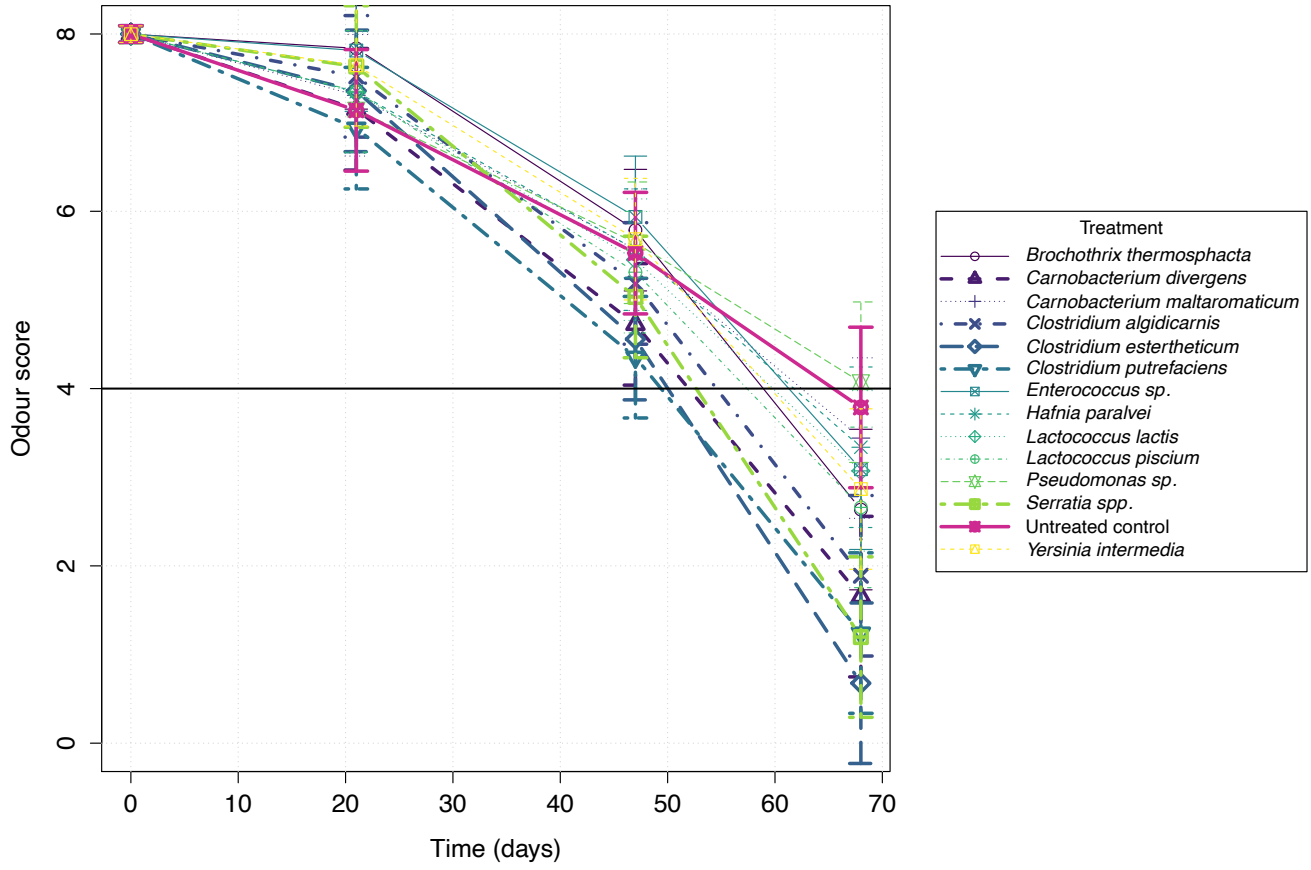

Figure 2.3 Odour scores and 95% CI of non-sterile vacuum-packed lamb meat stored at 2°C after inoculated with individual bacterial isolates. End of shelf-life is demarcated by an odour score of ≤4, highlighted by the horizontal line. Treatments with bold lines had a significantly lower odour score and a shorter shelf-life compared to the control.

Both sterile and non-sterile lamb meat inoculated with *C. estertheticum* showed signs of pack distension associated with 'blown pack' spoilage. Pack distension was not evident for *C. algidicarnis* or *C. putrefaciens*. This study did not measure pack distension. However, odour and colour assessment were considered to be adequate indicators of meat sensory qualities in this study, as pack distension was observed past shelf-life termination, as dictated by odour.

2.3.1.2 Colour scores

The colour of the untreated control in the sterile meat trial had an initial score of 8, which was still ≥ 6 by the end of the trial (Fig. 2.4). At day 68 of the trial, all individual bacterial treatments, except for the *Clostridium* spp., had colour scores ranging between 7 – 5.3, none of which were significantly different from the untreated control or below the acceptable limit (≤ 4) (Fig. 2.4). However, significantly lower colour scores were evident in the *Clostridium* treatment groups as early as day 21 of the trial (*C. algidicarnis:* $t_{126} = 4.74$, $p = .0002$; *C. estertheticum*: $t_{126} = 5.16$, $p = < .0001$; C*. putrefaciens:* $t_{126} = 5.46$, $p = < .0001$). This trend continued for the remainder of the trial and colour scores were below the acceptable threshold between 47 – 68 days. The odour score for all *Clostridium* treatments had already reached an unacceptable odour score of ≤ 4 at these times, therefore were already considered spoiled.

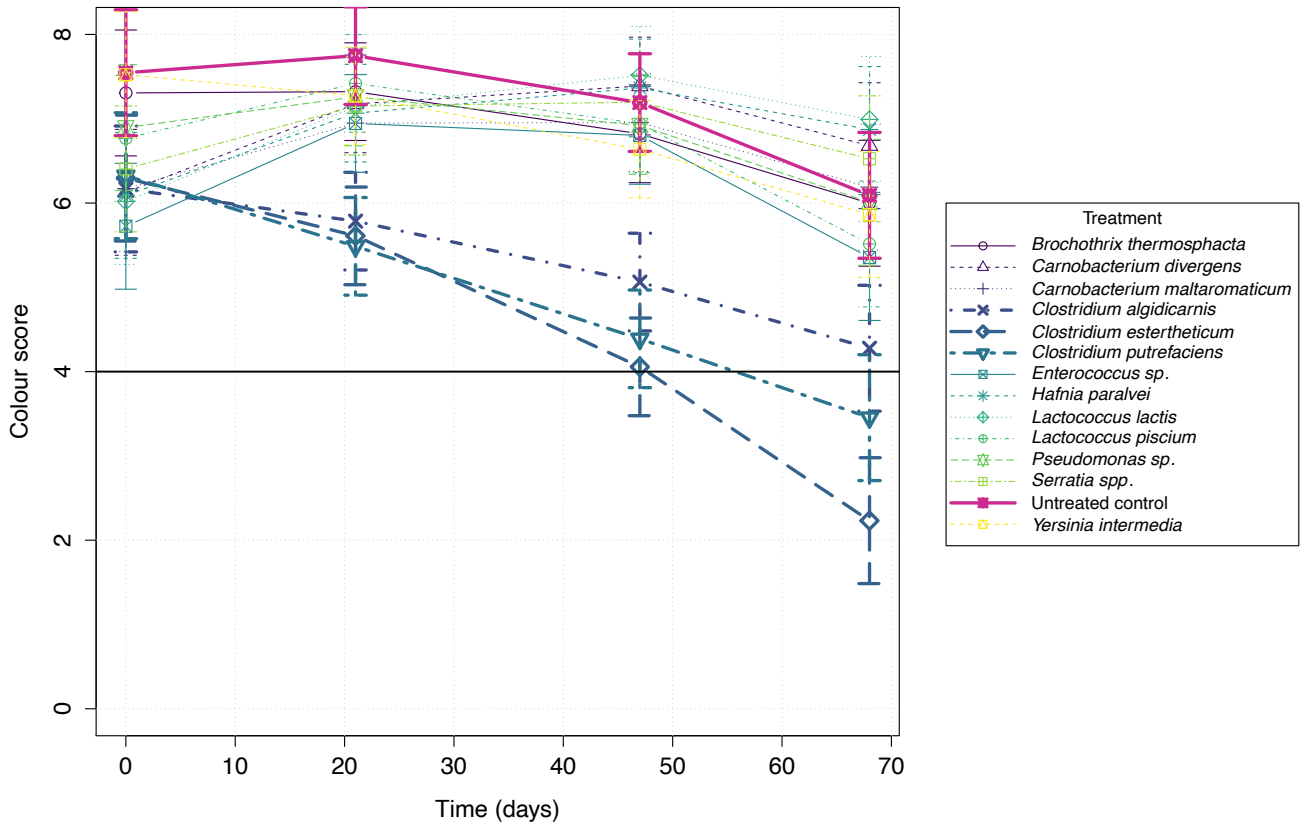

Figure 2.4 Colour scores and 95% CI of sterile vacuum-packed lamb meat stored at 2°C after inoculated with individual bacterial isolates. End of shelf-life is demarcated by a colour score of ≤4, highlighted by the horizontal line. The control is bolded as well as treatments that reached end of shelf-life.

Meat inoculated with bacterial Cocktail III had similar colour scores to the untreated control, with an initial score of 6.5, which decreased to a score of 5.9 by the end of the trial (Fig. 2.5). Cocktails I and II were similar to the untreated control until day 68, after which the colour scores decreased to 4 ($t_{126} = 6.46$, $p = < .0001$) and 4.8 ($t_{126} = 4.20$, $p = .0029$), respectively (Fig. 2.5). Cocktail I had not reached an odour score of ≤4 by this time; therefore, the colour score was indicative of spoilage at 68 days (Fig. 2.5). The positive control also reached a colour score of 4 by 68 days; however, the odour score had already reached an unacceptable score (Fig 2.5).

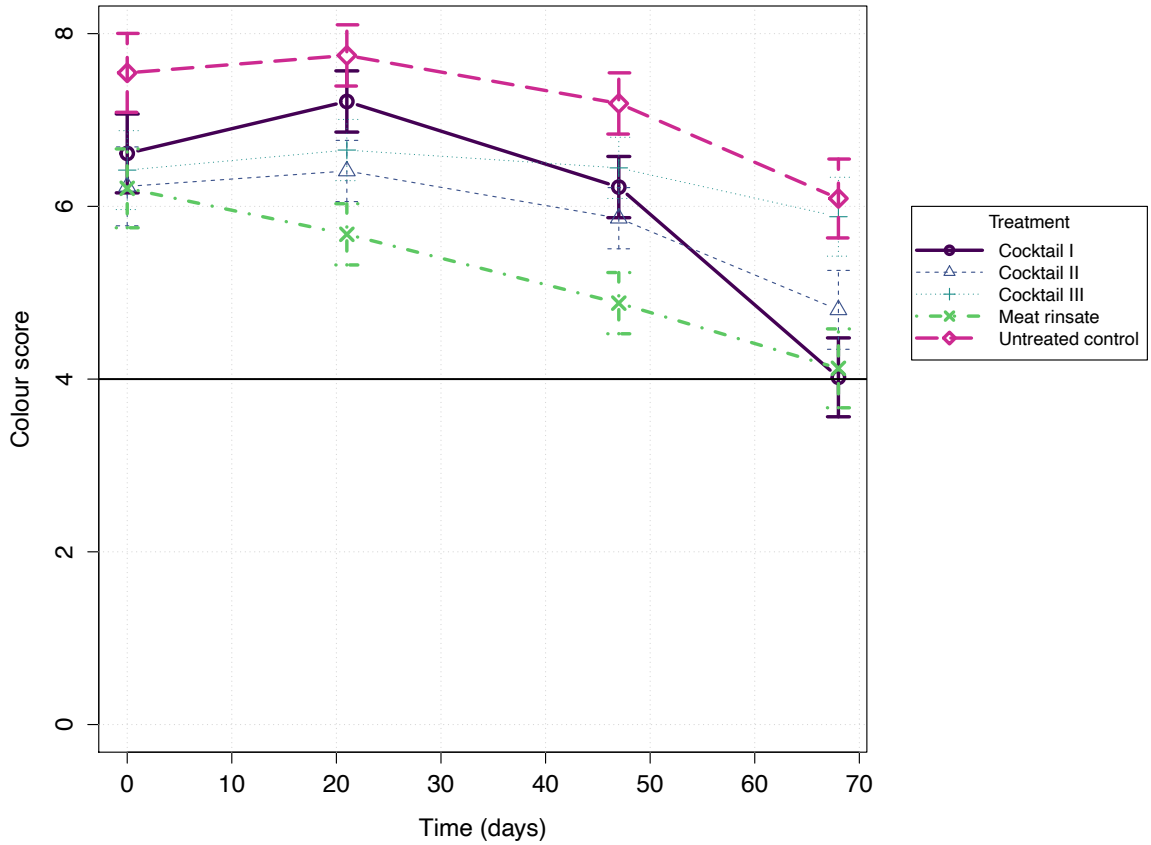

Figure 2.5 Colour scores and 95% CI of sterile vacuum-packed lamb meat stored at 2°C after inoculated with bacterial cocktails. Cocktail I included all facultative anaerobes and *Pseudomonas* sp., Cocktail II included Gram-positives (excluding *Clostridium* species), and Cocktail III included all Gram-negatives. End of shelf-life is demarcated by a colour score of ≤4, highlighted by the horizontal line. The control is bolded as well as treatments that reached end of shelf-life.

In the non-sterile trial, the initial colour score for all samples was 8 and most treatment groups decreased to a colour score of 6.5 – 5.5 by the end of the trial, which was similar to the untreated control (Fig 2.6). The colour score reached ≤ 4 by 53 days for *C. estertheticum*, 66 days for *Y. intermedia*, 67 days for *L. piscium* and 68 days for *C. putrefaciens*, all of which were significantly lower than colour scores of the untreated control (*C. estertheticum*: $t_{123} = 11.30$, $p < .0001$; *L. piscium*: $t_{123} = 8.12$, $p < .0001$; *C. putrefaciens*: $t_{123} = 7.02$, $p < .0001$) (Fig 2.6). However, the odour score had already reached ≤4 for each of these treatment groups.

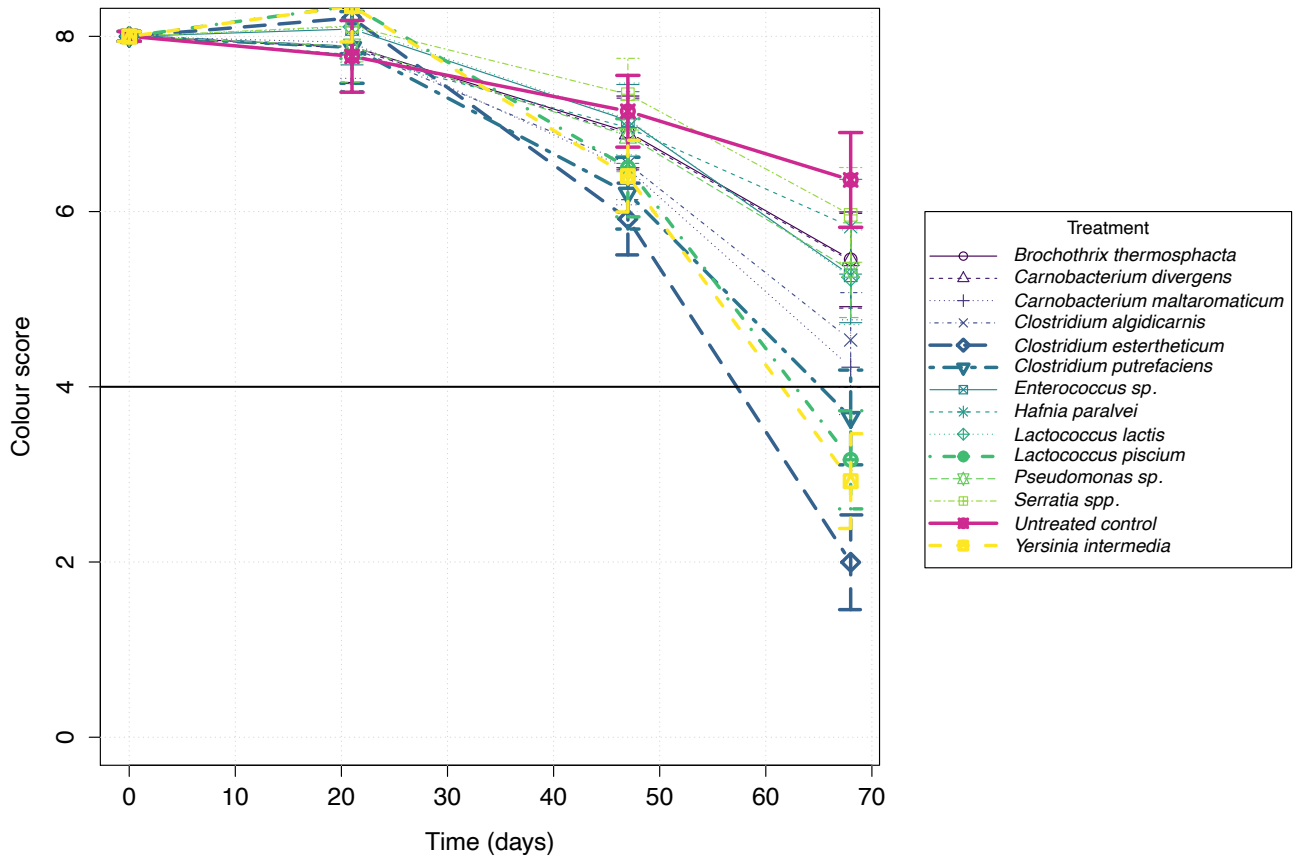

Figure 2.6 Colour scores and 95% CI of non-sterile vacuum-packed lamb meat stored at 2°C after inoculated with individual bacterial isolates. End of shelf-life is demarcated by a colour score of ≤4, highlighted by the horizontal line. The control is bolded as well as treatments that reached end of shelf-life.

2.3.2 Viable growth dynamics of the isolates

In the sterile meat trial, the initial total viable count (TVC) for the untreated control was 2.23 $\log_{10}$ CFU/cm^2 and reached a maximum population of 7.59 $\log_{10}$ CFU/cm^2 (Fig 2.7). Sterile meat samples that were inoculated with 4 log CFU/cm^2 of specific bacterial inocula had initial counts ranging between 2.80 – 4.54 $\log_{10}$ CFU/cm^2. This indicates that there was some variation with the inoculation procedure (Fig. 2.7). Despite this variation, initial counts for all individual strain treatment groups were not significantly different to the initial TVC of the untreated control. *C. divergens, C. maltaromaticum, Hafnia* spp.*, L. piscium, Serratia* spp., and

Y. intermedia had similar TVCs to untreated controls throughout the whole trial (Fig. 2.7). These isolates reached maximum populations between 6.44 – 7.21 $\log_{10}$ CFU/cm^2.

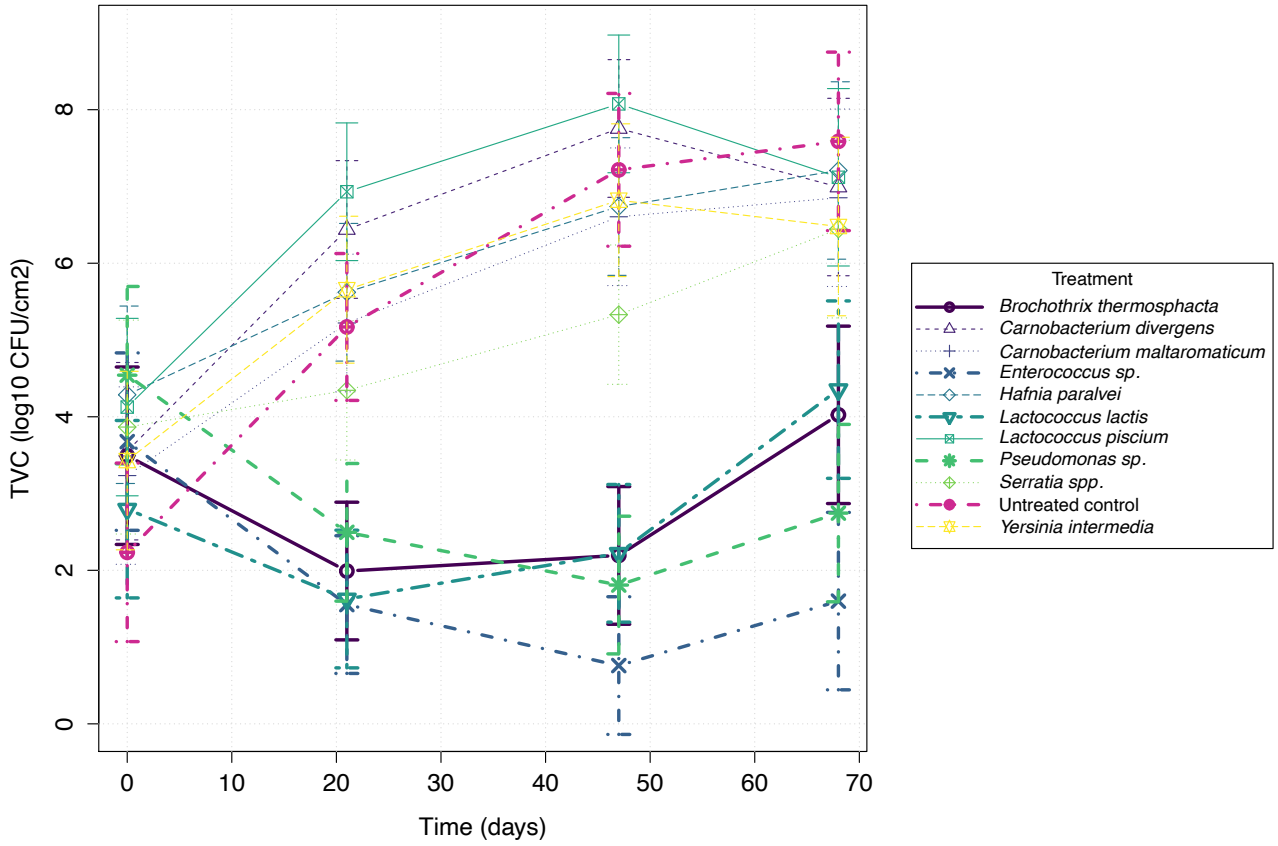

Figure 2.7 Bacterial growth ($\log_{10}$ CFU/cm^2) and 95% CI of sterile vacuum-packed lamb meat stored at 2°C after inoculated with individual bacterial isolates. The control is bolded as well as isolates that did not grow on meat.

Among all the test isolates, *B. thermosphacta*, *Enterococcus* sp., *L. lactis,* and *Pseudomonas* sp. did not grow when individually inoculated onto sterile meat. There was no net increase in their numbers throughout the shelf-life (Fig. 2.7). *Clostridium* isolates were not successfully enumerated using culture-dependent methods. However, spread plates from sterile meat inoculated with *Clostridium* showed no growth of any bacteria, confirming meat samples were not contaminated with other bacteria. Sterile samples inoculated with natural meat rinsate (positive control) and Cocktail I, had similar TVC to the untreated control at all

timepoints; with an initial count of 2.76 and 3.07 $\log_{10}$ CFU/cm^2, respectively (Fig. 2.8). Both treatments reached a maximum population of 6.77 $\log_{10}$ CFU/cm^2. The initial TVC for the Cocktails II and III were 4.17 and 4.01 $\log_{10}$ CFU/cm^2, which was significantly higher than the untreated control by 1.94 (t_{44} = 5.89, p = < .0001) and 1.77 $\log_{10}$ CFU/cm^2 (t_{44} = 5.39, p = < .0001), respectively (Fig. 2.8). The difference was less pronounced by day 21, and both treatments had a similar TVC to the control by day 47 and for the remainder of the trial.

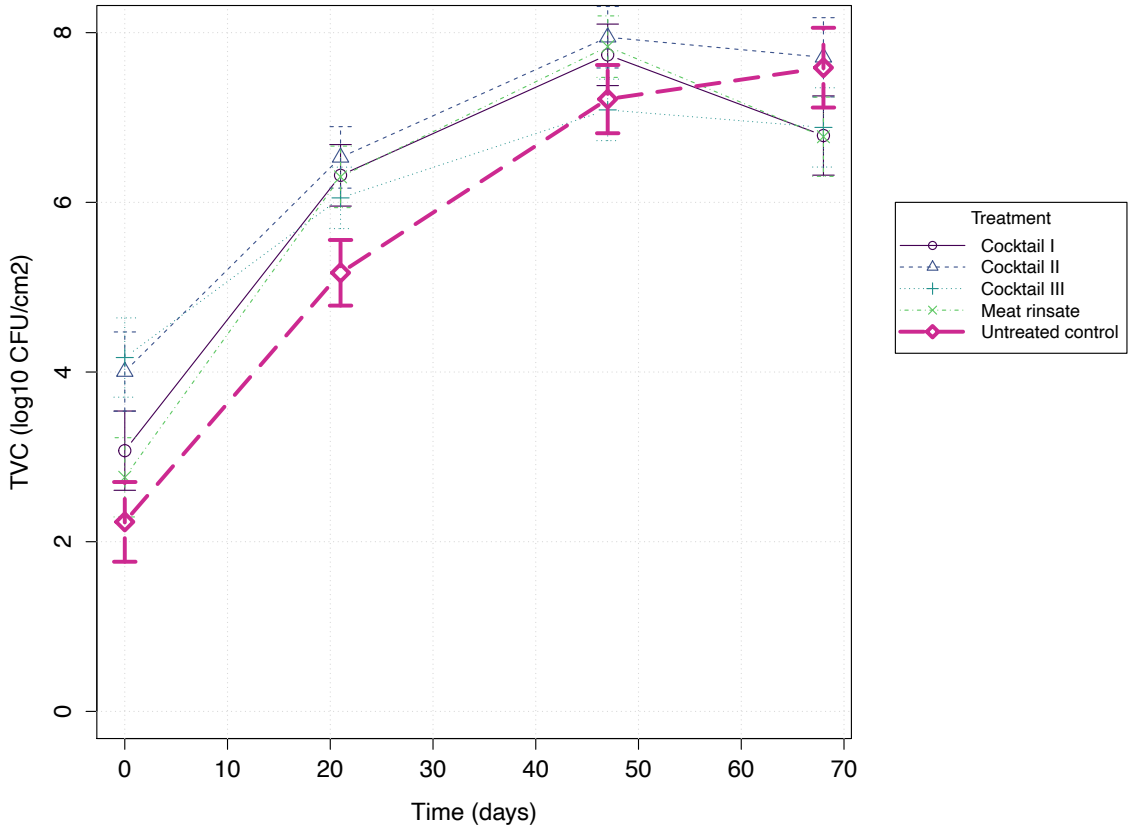

Figure 2.8 Bacterial growth ($\log_{10}$ CFU/cm^2) and 95% CI of sterile vacuum-packed lamb meat stored at 2°C after inoculated with bacterial cocktails. Cocktail I included all facultative anaerobes and *Pseudomonas* sp., Cocktail II includes Gram-positives (excluding *Clostridium*), and Cocktail III includes all Gram-negatives.

In the non-sterile trial, TVC for the untreated control was 2.83 $\log_{10}$ CFU/cm^2 and reached a maximum population of 7.43 $\log_{10}$ CFU/cm^2 (Fig. 2.9). After the meat samples had been inoculated with 10^4 CFU/cm^2 of the target isolates, the initial counts ranged from 2.72 – 3.51 $\log_{10}$ CFU/cm^2, none of which were significantly different from the initial TVC of the untreated control (Fig. 2.9). Due to the indigenous microbial community, counts of the specific inoculated isolates could not be measured; however, TVC was measured. To that end, the maximum population of the treated meat ranged from 7.14 – 7.63 $\log_{10}$ CFU/cm^2 and was not significantly different from the untreated control (Fig. 2.9).

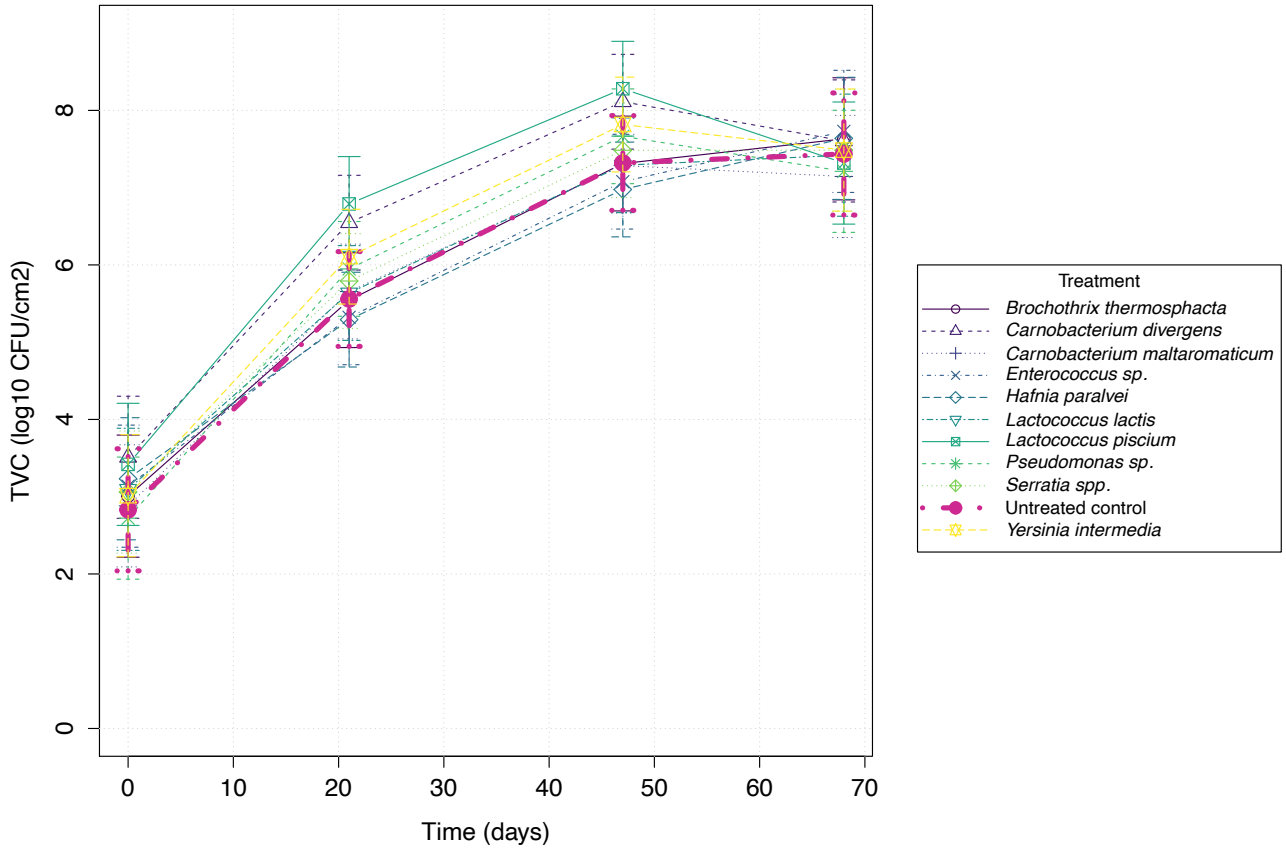

Figure 2.9 Bacterial growth ($\log_{10}$ CFU/cm^2) and 95% CI of sterile vacuum-packed lamb meat stored at 2°C after inoculated with individual bacterial isolates.

2.3.3 pH dynamics

The initial pH of fresh eye of loin for the sterile and non-sterile trials was similar, ranging from 5.6 – 5.9 and 5.7 – 6, respectively (Fig. 2.10 and 2.11). Throughout the sterile meat trial, the pH of the control and most treatment groups remained relatively similar to the initial pH in the meat, and by day 68 ranged from 5.4 – 5.8 (Fig. 2.10). There was no significant difference in the pH of meat samples between the control and most single inoculum treatment groups at any timepoint, except for the *L. piscium* treatment, which was 0.4 pH units less than the control at day 47 ($t_{126} = 5.18$, $p < .0001$), and had a marginally significant difference of 0.3 pH units less at day 68 ($t_{126} = 3.21$, $p = 0.058$) (Fig. 2.10).

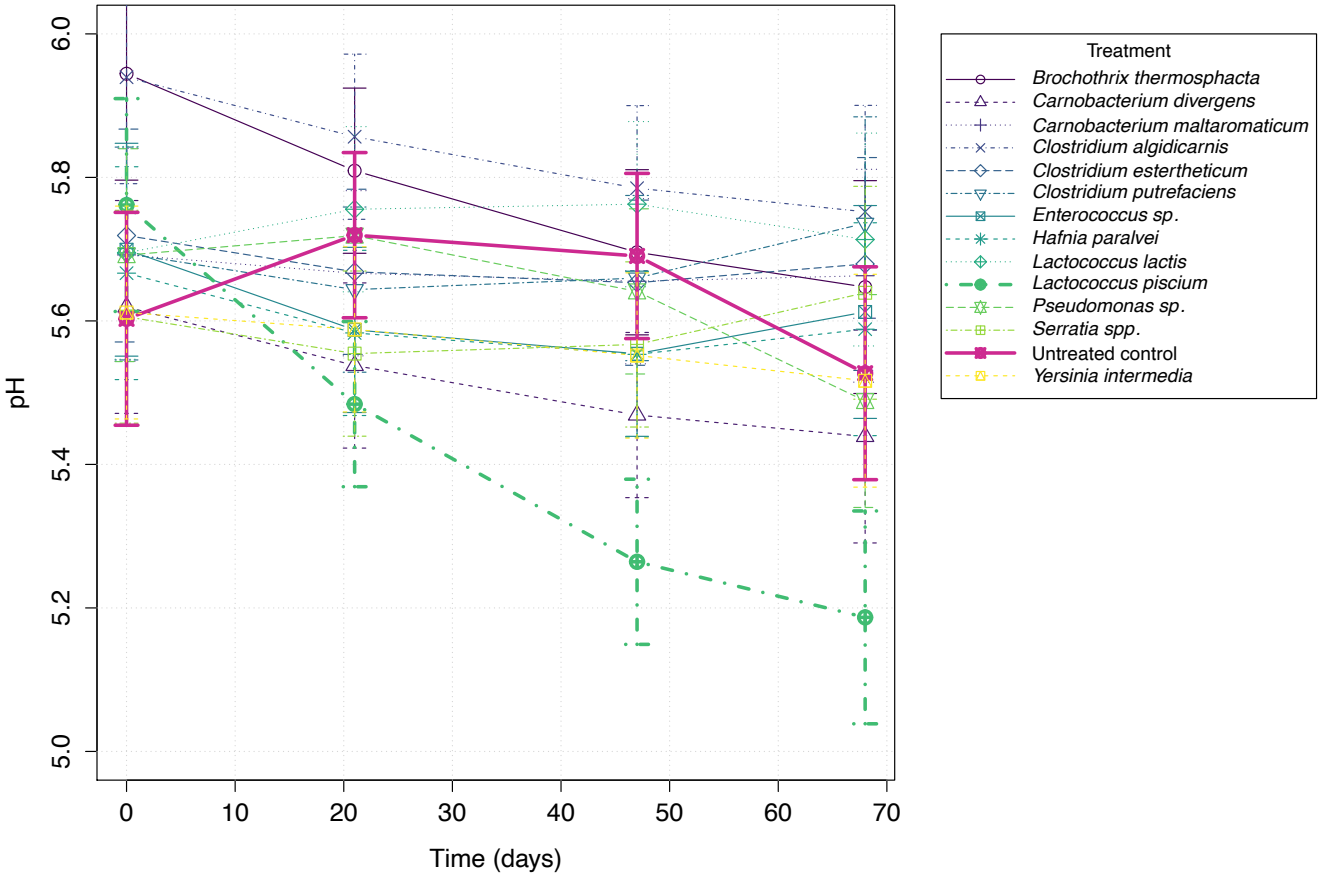

Figure 2.10 pH and 95% CI of sterile vacuum-packed lamb meat stored at 2°C after inoculated with individual bacterial isolates. The control is bolded as well as treatments that significantly reduced meat pH.

By day 47, Cocktail treatments I and II also produced a significantly lower pH by 0.24 units (t_{45} = 3.29, p = .026) and 0.38 units (t_{45} = 5.26, p = < 0.0001) respectively compared to the untreated control (Fig. 2.11). These differences were more pronounced by day 68, when the Cocktail II treatment was 0.41 units less than the untreated control (t_{45} = 4.48, p = .0004) while Cocktail I was 0.31 pH units less (t_{45} = 3.39, p = .0187) (Fig. 2.11). In the non-sterile trial, all treatment groups and the control showed a slight decrease in pH by day 21 and 47, which typically increased subsequently to a range between 5.4 – 5.9 (Fig. 2.12). There was no significant difference between the treatment groups and the control at any time points in the non-sterile trial.

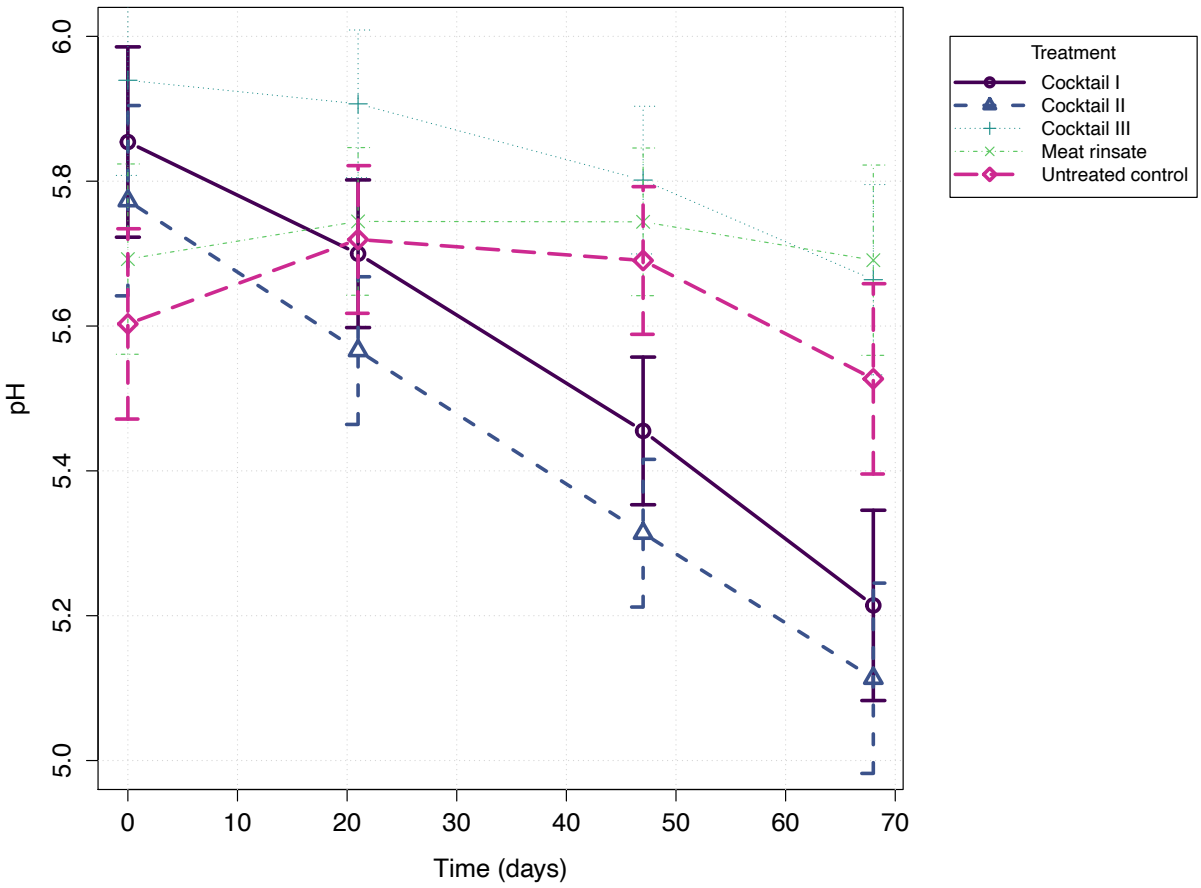

Figure 2.11 pH and 95% CI of sterile vacuum-packed lamb meat stored at 2°C after inoculated with bacterial cocktails. Cocktail I included all facultative anaerobes and *Pseudomonas* sp., Cocktail II included Gram-positives (excluding *Clostridium*), and Cocktail III includes all Gram-negatives.

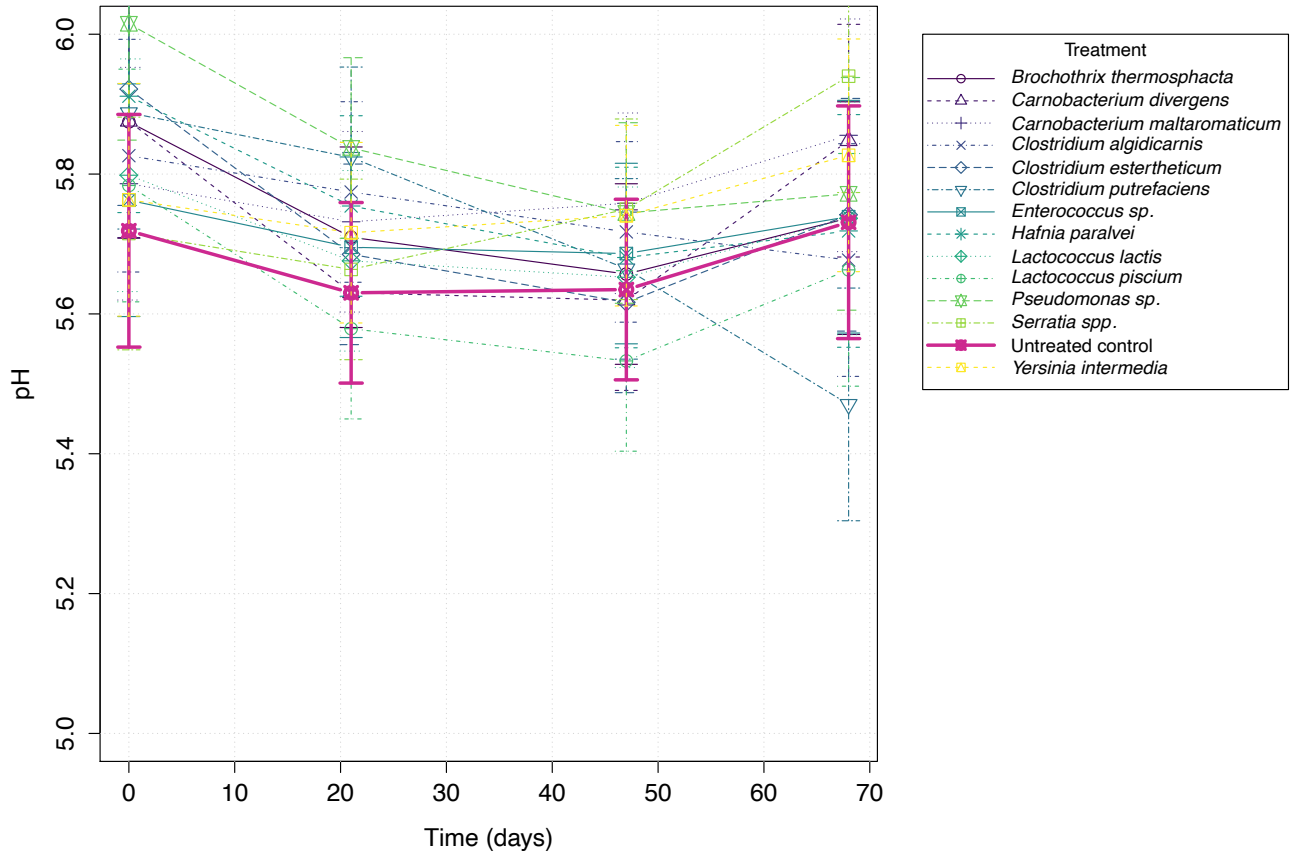

Figure 2.12 pH and 95% CI of non-sterile vacuum-packed lamb meat stored at 2°C after inoculated with individual bacterial isolates

2.4 Discussion

This study showed that among all the test isolates, *Clostridium* spp. had the highest spoilage potential, and independently played an important role in the spoilage rate of chilled VP lamb. *Clostridium* were the only bacterial group capable of spoilage when inoculated individually onto sterile lamb meat. It also caused a faster rate of quality loss when inoculated onto VP lamb containing its natural microbiota (non-sterile). These findings suggest that *Clostridium* might be the main taxa driving the faster rate of quality loss of chilled VP lamb compared to beef. Psychrophilic and psychrotrophic *Clostridium*, such as those used in this study, have been implicated in various types of red meat spoilage, such as 'blown pack', 'surface spoilage', and 'bone taint' (Adam et al., 2010). This can be attributed to their ability to grow

well at the optimal storage conditions for VP red meat, such as anaerobic conditions at temperatures between -1.5 and 2°C (Brightwell and Clemens, 2012). Genomic studies have also shown that *Clostridium* species associated with meat spoilage possess an extensive range of carbohydrate catabolising enzymes (Palevich et al., 2021; Yu et al., 2016). This includes a range of glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, glycosyl transferases, and carbohydrate-binding protein modules (Palevich et al., 2021). *Clostridium* species have different profiles of these carbohydrate-active enzymes allowing them to employ different carbohydrate metabolism strategies for growth on meat (Palevich et al., 2021; Yu et al., 2016). This would allow *Clostridium* to maintain their growth within the community and become a dominant species without requiring exchange of metabolites from other microbes (Palevich et al., 2021). Furthermore, despite odour scores demarcating shelf-life termination, all *Clostridium* isolates showed a significant decline in colour scores compared to the untreated control and all facultative anaerobes in both trials. The ability of *Clostridium* to discolour meat is most likely due to the production of butyric acid, butanol, carbon dioxide and hydrogen (Broda et al., 1996b; Casaburi et al., 2015; Dainty et al., 1989a).

Most published studies have focused on psychrophilic *Clostridium* as causative agents of 'blown pack' spoilage due to their sporadic occurrence and the subsequent economic losses for the industry (Adam et al., 2010; Moschonas et al., 2010). The present study found that *C. estertheticum* was the only *Clostridium* isolate that showed signs of pack distention associated with 'blown pack' spoilage, and it resulted in half the shelf-life of the control and other *Clostridium* species when inoculated individually onto sterile meat. This is consistent with the published literature, as *C. estertheticum* is the most commonly detected *Clostridium* species in 'blown pack' spoilage of chilled VP red meat and often results in premature

spoilage (Adam et al., 2010; Cavill et al., 2011; Silva et al., 2016; Yang et al., 2014). The production of hydrogen, carbon dioxide, butyrate, and 1-butanol contribute to the distinctive odour and gases associated with 'blown pack' spoilage caused by *C. estertheticum* (Adam et al., 2010; Dainty et al., 1989a; Spring et al., 2003; Yang et al., 2011).

The present study found that the spoilage potential of *C. estertheticum* was hindered when it was inoculated onto meat containing natural microbiota (non-sterile meat trails). This suggests that the natural microbial community on meat may reduce the spoilage potential of *C. estertheticum* through antagonistic interactions. The number and types of competitive members of the bacterial community on meat has previously been reported as an important factor affecting the onset of blown pack spoilage (Húngaro et al., 2016). For instance, *C. estertheticum* growth is limited by glucose availability; therefore, among other microbiota its spoilage potential is dependent on its ability to compete for glucose, as most bacteria initially prefer to utilise glucose as an energy source (Nychas et al., 1988; Yang et al., 2009, 2011). Other studies have also shown that various lactic acid bacteria, such as *Lactobacillus sakei*, *Leuconostoc carnosum, Leuconostoc mesenteroides,* and *Lactococcus lactis,* inhibited *C. estertheticum* by the production of bacteriocin-like inhibitory molecules, as well as reducing meat pH to levels that inhibit *C. estertheticum* growth through lactic acid production (Jones et al., 2008; Jones et al., 2009; Yang et al., 2011). The results of this study highlight the need for future research to investigate the commercial practicality of the use of bioprotective cultures to delay and/or inhibit spoilage blown pack spoilage caused by *C. estertheticum*.

Less is known about *C. putrefaciens* and *C. algidicarnis* and their role in spoilage of chilled VP red meat. However, they have been implicated as a cause of stifle joint odour, also known as 'bone taint', and surface spoilage of chilled VP lamb meat (Adam et al., 2010; Broda et al.,

2009; Broda et al., 1996a; Broda et al., 1996b; Cavill et al., 2011). The data revealed that *C. putrefaciens* and *C. algidicarnis* can independently have an important effect on the rate of surface spoilage of chilled VP lamb. Both isolates spoiled sterile meat at a similar rate to the untreated control, whereas most other test isolates did not accelerate spoilage under these conditions. Furthermore, meat containing natural microbiota typically spoiled faster when *C. putrefaciens* or *C. algdicarnis* had been added compared to the untreated control or addition of other test isolates. The potential importance of *Clostridium* in surface spoilage of chilled VP lamb was also highlighted by Kaur et al. (2017b), who found *Clostridium* reached maximum population at the onset of surface spoilage. These results were generated using 16S rRNA amplicon sequence data to estimate absolute bacterial numerical data, allowing assessment of *Clostridium* across a logarithmic scale of abundance. Other studies using 16S rRNA amplicon sequence data have revealed that *Clostridium* was consistently the main taxa that dominated lamb meat with low sensory scores, and was more abundant on VP lamb than VP beef (Kaur et al., 2021). This was attributed to the growth capability of *Clostridium* under the higher pH conditions of lamb meat (Yang et al., 2009; Yang et al., 2014). The findings of this study suggest that *Clostridium* might be the main taxa driving the faster rate of quality loss of chilled VP lamb compared to beef. Such knowledge is of fundamental importance to the meat industry because it can inform strategies for shelf-life extension that target *Clostridium*. Assessing the growth of *Clostridium* on VP lamb using molecular techniques, such as qPCR, may also provide an alternative approach to evaluate the efficacy of potential shelf-life interventions as opposed to using total viable count reductions.

This study did not successfully enumerate *Clostridium* due to its fastidious nature using traditional culture-dependent techniques (Brightwell and Clemens, 2012; Broda et al.,

1996a). However, it is unlikely that the apparent spoilage of meat previously inoculated with *Clostridium* were not a result of the growth of *Clostridium*. This is because microbial sensory degradation was evident for sterile meat samples that were inoculated with individual *Clostridium* isolates, and it was confirmed that there was no other bacterial contamination that could account for this. However, an appropriate molecular technique, such as qPCR, are required to confirm this (Brightwell and Clemens, 2012). Furthermore, future studies could use 16S rRNA amplicon data to estimate absolute bacterial numbers or assess differential abundances of tested isolates within the microbial community (Kaur et al., 2017b). This would also allow a rigorous assessment of how each bacterial inoculum influenced the microbial community, which was not assessed in this study.

This study found that *C. divergens* grew to maximum population density on sterile meat but did not have the metabolic capability to spoil meat independently. However, this isolate spoiled meat containing natural microflora at a faster rate compared to the control and most other facultative anaerobes. These results may suggest that the metabolic by-products of *C. divergens* are different in a community setting, having more organoleptic impact. This could be due to antagonistic and synergistic interactions with other members of the microbial community, such as competitive exclusion and/or exchange of metabolites (Zhang et al., 2015) or interactions among resulting metabolites. Similar to this study, Ercolini et al. (2011) found that the growth of *C. divergens* within the microbial community of VP beef was implicated in the formation of butyrate when stored at 4 °C. Butyrate can be derived from the metabolism of free amino acids and carbohydrates and is commonly associated with meat spoilage (Jones, 2004). However, the spoilage potential of *Carnobacterium* spp. on meat remains controversial as they have been reported as successful protective cultures to

enhance quality, as well as implicated in the spoilage of chilled meat products (Ercolini et al., 2009; Laursen et al., 2005; Leisner et al., 2007). The consensus is that spoilage caused by *Carnobacterium* is dependent on intraspecies and interspecies variation (Yang et al., 2021). This is supported by this study as *C. maltaromaticum* had low or no spoilage potential on sterile and non-sterile chilled VP lamb despite the observed increase in their numbers. Casaburi et al. (2011) also found that different strains of *C. maltaromaticum* had low spoilage potential when grown on beef at low temperatures in both aerobic and anaerobic conditions.

Similar to *C. divergens*, *Serratia* spp. caused premature spoilage when inoculated on meat containing natural microflora but not onto sterile meat, despite reaching maximum population. *Serratia* spp. are commonly reported to have high spoilage potential due to their ability to metabolise protein and amino acids for growth, which typically results in metabolic by-products with offensive odours (Fougy et al., 2017; Nychas et al., 1988). Gribble et al. (2014) also reported that *Serratia* spp. have the ability to spoil VP lamb meat prematurely when inoculated onto meat containing its natural microbial community. *Serratia* spp. have been found to be significantly more abundant on lamb samples that had low sensory scores compared to those samples with acceptable odour scores (Kaur et al., 2021). In mixed communities, glucose/saccharides depletion is faster due to competition. As a result *Serratia* spp. is likely to begin proteolysis and contribute to spoilage (Nychas et al., 2008).

The results showed that *H. paralvei, L. piscium*, and *Y. intermedia* had low or no spoilage potential on chilled VP lamb despite the observed increase in their numbers. Sterile samples inoculated with these isolates showed no sensory degradation despite growing to maximum population densities during the trials. Furthermore, when these isolates were inoculated onto meat containing its natural microbial community, they did not spoil meat at a faster rate

than the untreated control. The reported spoilage potential of *L. piscium* is variable within the research literature and has been reported to be dependent on the strain and food matrix (Saraoui et al., 2016). However, the observed inability of *L. piscium* to cause spoilage agrees well with the study of Hilgarth et al. (2018). They found that meat inoculated with *L. piscium* enhanced meat quality by suppressing the growth of high potential spoilers without undesirable sensory alterations to the meat. The basis of the antimicrobial mechanisms of *L. piscium* are unknown and could potentially be multifactorial (Hilgarth et al., 2018). However, one mechanism could be the acidification of meat by lactic acid production via glucose fermentation (Fall et al., 2010; Hilgarth et al., 2018; Saraoui et al., 2016). This is in keeping with the present observation that *L. piscium* presence significantly reduced sterile meat pH by up to 0.4 units. However, this was not evident when inoculated onto meat containing its natural microbial community. This could be because lactic acid production by *L. piscium* was limited by available glucose within a community setting, as it is preferentially utilized by most bacteria present (Ellis and Goodacre, 2001).

Few studies have investigated the role of *H. paralvei* and *Y. intermedia* in meat spoilage, despite being abundant taxa within the spoilage community of different meat types (Holl et al., 2016; Kaur et al., 2017b). This study found no evidence to suggest that *H. paralvei* and *Y. intermedia* have high spoilage potential on chilled VP lamb. It has also been previously reported that *Hafnia* and *Yersinia* may not play a major role in spoilage of VP lamb. For instance, *Yersinia* was significantly less abundant on VP lamb with low sensory scores compared to VP beef (Kaur et al., 2021). Further, the spoilage capability of *Hafnia* may be strain dependent as its association with lamb of low sensory scores was dependent on where the meat was sourced (Kaur et al., 2021).

B. thermosphacta, Enterococcus sp., *Pseudomonas* sp. and *L. lactis* were considered to have low spoilage potential as they did not have the ability to grow well on sterile meat. Non-sterile meat inoculated with each of these isolates also did not spoil faster than the untreated control. However, it is unknown whether these isolates were able to grow well in a community setting. Alternatively, these isolates might enter a long lag phase due to the differences in pre-inoculation conditions before commencing growth. Low spoilage potential due to the inability to grow in vacuum pack was expected for *Pseudomonas* as its growth is limited by low oxygen environments (Doulgeraki et al., 2012). *B. thermosphacta* also does not typically grow to high populations on VP red meat when stored at optimum chilled conditions either due to low O_2 levels, substrate limitation and other forms of implicit competition (Ercolini et al., 2011; Gribble and Brightwell, 2013; Kaur et al., 2017b; Mohsina et al., 2020; Nowak et al., 2012; Pin et al., 2002).

Enterococcus has previously been reported as a causal bacterium for meat souring, bone-taint, slime production and blown pack spoilage in a range of different meat products under various storage conditions (Hernandez-Macedo et al., 2012; Nychas et al., 2008). Few studies have investigated its role in the spoilage of chilled VP red meat, because it has predominately been detected in aerobic and some modified atmosphere packaging (Casaburi et al., 2015; Jay et al., 2003). However, it was found that *Enterococcus* had little impact on spoilage of VP lamb and did not grow on sterile meat. These results may suggest that *Enterococcus* spp. in general are not well equipped to grow in anaerobic conditions at chilled temperatures. Similarly, Bjorkroth et al. (2005) found that *Enterococcus* had low growth potential and limited impact on meat spoilage when stored in low oxygen environments at 6°C. It was also evident that under these conditions *Carnobacterium* and *Lactococcus* outcompeted

Enterococcus. Furthermore, *Carnobacterium* is known to produce bacteriocins that can effectively inhibit other lactic acid bacteria, such as *Enterococcus* (Stoffels et al., 1992).

L. lactis is considered to be innocuous in food spoilage as it is widely applied as protective cultures for a variety of foods, including meat products, to inhibit the growth of spoilage bacteria and food pathogens (Comi et al., 2016; Jones et al., 2008; Sarika et al., 2012). However, the present study found *L. lactis* did not grow in axenic culture on sterile meat which may indicate that it requires synergistic interactions with other members of the VP lamb microbiota, such as exchange of metabolites, in order to grow on the meat matrix (Zhang et al., 2015). Such interactions are evident for *L. lactis* when grown in milk. Specifically, proteinase-negative variants of *L. lactis* are limited by the concentration of free amino acids in milk, yielding a low final population. However, when proteinase-negative strains are cocultured with proteinase-positive strains, growth is stimulated as non-proteolytic strains can utilise the proteolysis products released by other strains (Juillard et al., 1996).

This study also assessed the spoilage potential of assemblages of different bacterial groups (excluding *Clostridium*) on sterile meat, in the form of cocktails, as detailed in Table 2.1. Meat inoculated with these bacterial cocktails were still considered as commercially acceptable by the end of the trial, despite all reaching a maximum population. Cocktails I and II significantly reduced meat pH by up to 0.31 and 0.41 units. This is expected as both cocktails contained mainly lactic acid bacteria, which primarily produce acidic end-products such as lactic acid, acetic acid and formic acid via glucose metabolism (Borch et al., 1996). These results highlight the potential use of lactic acid bacterial cocktails as bioprotective cultures to reduce

meat pH to slow/inhibit growth of higher potential spoilers that prefer higher pH environments, such as *Clostridium*.

2.5 Conclusions

This study established that *Clostridium* spp. had the highest spoilage potential and had a major effect on the spoilage rate of chilled VP lamb. These results highlight that *Clostridium* could be one of the main taxa driving the faster rate of quality loss of chilled VP lamb as compared to beef. Such knowledge is of fundamental importance to the meat industry because it can inform strategies for shelf-life extension that target *Clostridium*. Assessing the growth of *Clostridium* on VP lamb using molecular techniques may also provide an alternative approach to evaluate the efficacy of potential shelf-life interventions as opposed to using total viable count reductions. In contrast, all tested facultative anaerobes and aerobes had no impact on the quality of VP lamb, except for *C. divergens* and *Serratia* spp. which spoiled meat containing natural microbiota at a faster rate compared to the control. This suggests that the latter isolates produced different metabolic by-products in a community setting due to antagonistic and synergistic interactions with other members of the microbial community. Future studies require molecular techniques, such as qPCR, to estimate absolute isolate numbers in a community setting. This will provide a more sensitive approach to assess *Clostridium* growth dynamics. Other molecular techniques, such as 16S rRNA amplicon sequencing, may provide in-depth assessment of bacterial inocula within the microbial community.

Chapter 3

Surface glucose treatment extends the shelf-life of vacuum-packed lamb

3.1 Introduction

All meat products are given a specific storage shelf-life, which is the duration, under specified storage conditions, that it is expected to be microbiologically safe, and of acceptable quality for human consumption (Nychas et al., 2008). The loss of organoleptic quality of meat at the end of shelf-life, also known as spoilage, is a complex process that is caused by a variety of chemical, enzymatic and microbiological processes (Dave and Ghaly, 2011). The main process that drives meat spoilage is the implicit effects arising from the meat's microbial community, which is dependent on the community composition and their ability to produce compounds associated with spoilage (Doulgeraki et al., 2012; Ercolini et al., 2011; Lambert et al., 1991). Despite rigorous hygiene standards throughout slaughter and processing of meat, the development of a microbial community is inevitable due to aerial and surface contamination within the processing facility (Kaur et al., 2017b; Stellato et al., 2016). However, the time taken for spoilage to occur is dependent on many extrinsic (packaging and storage conditions), implicit (i.e., the interactions between organisms), and intrinsic factors (meat biochemistry and physicochemical properties), in addition to animal husbandry and processing (Mossel, 1971; Nychas et al., 2008).

As discussed in chapter 1, the most common extrinsic applications to improve the shelf-life of fresh meat is vacuum packaging in combination with rigorous temperature control (Mills et al., 2014). Vacuum packaging removes air prior to sealing the meat in the pack. The small amount of air that remains or can enter the pack is respired by the muscle tissue and

contaminating microbes, which creates an environment with low oxygen and elevated carbon dioxide levels, thereby suppressing the growth of microorganisms with high spoilage potential such as *Pseudomonas* spp. (Gill and Penney, 1985; Kropf, 2004). Furthermore, lower storage temperatures reduce bacterial growth rates, which reduces microbial diversity since only a restricted range of bacterial taxa can grow under vacuum-packed (VP) conditions (Dave and Ghaly, 2011; Kaur et al., 2017b).

Beef and lamb produced in Australia typically have shelf-lives of 26 and 12 weeks respectively when VP and stored at temperatures of -1.5 to 0°C (Kiermeier et al., 2013; Small et al., 2012). The substantial shelf-life difference between beef and lamb is thought to be due mainly to differences in intrinsic pH (Kaur et al., 2021). Beef has a pH range of 5.5 – 5.8, whereas lamb typically is 0.4 pH units higher, ranging between 5.6 – 6.8 (Kaur et al., 2021; Kiermeier et al., 2013). This difference is due to the large amount of adipose tissue distributed through lamb muscle, as well as the biochemistry of the meat types, in which glycogen and lactic acid are of particular importance (Carse and Locker, 1974; Gill and Penney, 1985; Kaur et al., 2021). Meat with a higher pH increases bacterial growth rates and can facilitate the growth of spoilage bacteria (EFSA, Panel on Biological Hazards (BIOHAZ), 2016; Kaur et al., 2021; Yang et al., 2014).

The relatively long shelf-lives of fresh beef and lamb have allowed Australia to consolidate a large international export market (Small et al., 2012). However, the shorter shelf-life of lamb can be problematic when exporting to international markets as shipping voyages can be >90 days transit time (Huynh et al., 2016; Mills et al., 2014). Therefore, to avoid economic losses and unnecessary wastage through downgrades or rejection, the shelf-life of vacuum-packed lamb must be sufficient to allow for shipping transportation and distribution within

international markets, while still meeting quality standards that can successfully compete with local produce and provide logistical chain agility (Huynh et al., 2016; Mills et al., 2014). There is also a need for exporters to meet technical trade requirements that are set by a number of countries (Huynh et al., 2016). For instance, Middle Eastern countries have an expiry date for VP meats ranging between 70 – 90 days after slaughter (Huynh et al., 2016). As a result, research is needed to assist the development of safe and cost-effective interventions to extend the shelf-life of lamb products (Mills et al., 2014).

Previous studies have found that the application of additional glucose to the surface of meat helped to extend the shelf-life of different meat products (ranging between 3 – 10 days extension) under aerobic conditions at a range of different temperatures (from 5 to 25°C) (Kumudavally et al., 2010; Lambropoulou et al., 1996; Nychas et al., 1988; Shelef, 1977). Glucose is utilized by many bacterial species able to grow on VP meat; however, many other substrates, due to the nutrient complexity of meat, are likely utilized as well, such as amino acids and lipids. It has been found that bacteria will consume many substrates with preferences and the relative order of uptake driven by the biomass yield possible from the substrate (Perrin et al., 2020). This creates a complicated array of metabolic activities that ultimately leads to spoilage, although metabolism of some compounds is more likely to lead to organoleptically detectable spoilage (Casaburi et al., 2015; Ercolini et al., 2011; Ercolini et al., 2009). Applying glucose to meat is thought to affect the access to favoured catabolic substrates, thereby impacting the community composition as well as the rate and type of accumulation of spoilage metabolic by-products.

Despite the reports successfully demonstrating that glucose surface treatments can help extend the shelf-life of meat products in aerobic conditions (Kumudavally et al., 2010;

Lambropoulou et al., 1996; Newton and Gill, 1978; Shelef, 1977), the effects of this treatment are yet to be reported under anaerobic/VP conditions in combination with low temperatures used in commercial storage i.e., 0 to 4°C. Therefore, the primary objective of this study was to determine whether additional glucose as a surface treatment has the potential for extending the shelf-life of vacuum-packed lamb at 4°C. This was investigated through a series of shelf-life trials, in which the dynamics of the sensorial qualities, bacterial growth, pH, and residual glucose and lactic acid were measured over time.

3.2 Materials and Methods

3.2.1 Sample collection

Two sets of trials were conducted in May and August 2019, using vacuum-packed (VP) bone-in (n = 36) and boneless (n = 39) lamb shoulders, respectively. The shoulders were freshly sourced from a local abattoir (Tasmania, Australia) from a single herd of animals. All shoulders were cut into 6 pieces and VP using appropriate commercial packaging films by the abattoir making a total of 216 bone-in and 234 boneless samples (~13 x 7 x 4 cm). The abattoir cut the bone-in lamb shoulders transverse to the shoulder blade. Both trials commenced three days after slaughter due to processing and transportation.

3.2.2 Glucose treatment and storage

Various D-glucose (LP0071 Oxoid, Australia) solutions (0.5%, 1%, 2.5%, 5% and 10% w/v) were prepared and added to the surface of the meat samples on trial commencement. Specifically, each pack was opened, and the meat sample was placed into a fresh pack (Therma-sorb bone-in barrier bag E86: O_2 transmission rate of <10 cc/m^2/ 24 h at 23°C, 85% relative humidity; barrier bag TBG4620: O_2 transmission rate of 20 cc/m^2/24 h at 23°C, 0%

relative humidity). Either sterile distilled water (control) or glucose solutions were evenly aliquoted onto both sides of the meat in the fresh pack to achieve a level of 0.01 ml/cm^2. The concentrations of the glucose that were added to the meat were 0.23 mmol/kg for 0.5%, 0.46 mmol/kg for 1%, 1. 16 mmol/kg for 2.5%, 2.32 mmol/kg for 5%, and 4.64 mmol/kg for 10%. After the addition of glucose, the packs were vacuum sealed using a table-top food vacuum sealer (T60 - T100 Technovac, Italy) under the following conditions: 0% gas, 99.9% vacuum, and 2 s sealing time. Samples were stored at 4°C and temperature was monitored using data loggers.

3.2.3 Meat slurry preparation

To account for the inherent pH variability of meat, a third trial using meat slurry was conducted in parallel to the bone-in trial. Meat samples were weighed and measured for its surface area to calculate the surface area to weight ratio. Meat slurries were prepared aseptically by blending (Model 7010S, Waring Commercial, America) a meat sample with sterile distilled water (1:2). A mass of 10 g was treated with either distilled water (control) or glucose at 0.01 ml/cm^2 (concentrations as above). All treated samples were vacuum packed and stored at 4°C. There were three independently prepared samples per treatment.

3.2.4 Microbial and sensory analyses

Samples for all treatments were periodically tested for total viable counts (TVC), lactic acid bacteria (LAB) numbers, sensory characteristics (excluding meat slurry samples), glucose and lactic acid concentrations, and pH over the course of the trials. The shelf-life of VP lamb stored at 4°C was assumed to 20 – 22 days based on prior data (Huynh et al., 2016; Kaur et al., 2017b). Accordingly, for the initial shelf-life trial using bone-in lamb shoulder, samples were tested after 0, 3, 7, 10, 13, 17, 20, 22, 25, 28, and 30 days. The bone-in trial was

conducted with at least two replicates for each treatment at all timepoints. The experiment was repeated in a second trial using boneless lamb shoulder. Based on the results of the bone-in trial the sampling regime for the boneless trial were modified to 0, 7, 12, 20, 22, 27, 29, 32, and 35 days. Further, it was conducted with five biological replicates for each treatment at all timepoints.

The sensory assessment was a blind evaluation, conducted by a panel of between 4 and 6 volunteers (Gill and Badoni, 2002; Small et al., 2012; Yang et al., 2021). Panelists were selected based on their expertise in food microbiology and included a range of different cultural backgrounds. Prior to the trials, all panelists were required to attend a training session, in which samples at different stages of shelf-life were used for practice assessment. Odour was assessed using an 9-point categorical hedonic scale (0 = extreme off-odour, spoiled; 8 = no odour, normal meaty odour), and colour was determined using a visually anchored scale (0 = other colour e.g. green colouration; 2 = very poor bloom, grey colouration; 4 = poor bloom, some greyness; 6 = bloom, light red colouration; 8 = bloom, red colouration)(Small et al., 2012). Any score $\leq$ 4 was considered as commercially unacceptable.

Meat surface pH was measured using a handheld pH meter (Model 206-pH2, Testo, Australia) at four random locations on the meat sample. The pH meter was initially calibrated according to manufacturer's instructions. To enumerate bacteria, each meat sample was transferred to a sterile bag and massaged by hand in 100 ml of sterile 0.1% peptone water (LP0037, Oxoid, Australia) for 1 min. Serial dilutions were prepared in sterile 0.1% peptone water and spread plated. Total viable counts were enumerated on tryptone soya agar (TSA) (CM0131, Oxoid, Australia), and lactic acid bacteria on de Man-Rogosa-Sharpe agar (MRS) (CM0361, Oxoid, Australia) under anaerobic conditions using Anaerogen Compact pouches

(Anaerogen Compact pouch, Oxoid, Australia). Both TSA and MRS were incubated at 25°C for 5 days.

3.2.5 Glucose and lactic acid assays

At each time point, 10 g of meat was aseptically sliced from the surface of each sample and stored at -20°C. These samples were used for glucose and lactic acid assays upon completion of the trial. To prepare meat samples for both assays, the meat was homogenized in phosphate buffered saline (1:2) with a homogeniser (T 25 Ultra-Turrax, IKA, China). For lactic acid assays, 0.1 g of meat homogenate was hydrolyzed in 1.88 ml of 0.1 M HCl at 100°C for 2 h, after which the pH was adjusted to approximately 10 (± 0.2) using 1 M NaOH (Immonen and Puolanne, 2000). For glucose assays, 0.7 g of homogenate was hydrolyzed in 1 ml of 0.1 M HCl at 100°C for 2 h, the pH was adjusted to approximately 5.0 (± 0.2) using 1 M acetate buffer. The content of L- and D-lactic acid was determined spectrophotometrically by measuring the increase of NADH at 340 nm using the Megazyme D-/L- Lactic acid Kit (Megazyme International Ireland Ltd, Ireland), which has a lower detection limit of 1.44 mmol/kg. Glucose content was determined spectrophotometrically (540 nm) using the Glucose Oxidase (GO) Assay Kit (Sigma-Aldrich, USA), having a lower detection limit of 0.44 mmol/kg.

3.2.6 Statistical analysis

The datasets were analysed to determine differences in trends of the response variables over time between the treatments and the control. The response variables were total viable bacterial counts and lactic acid bacterial counts (log_{10} CFU/cm^2); odour and colour scores; residual glucose and lactic acid concentrations (mmol/kg); meat pH; and ratio of LAB to TVC. Odour and colour scores were used to determine the quality of the meat throughout the

shelf-life. The end of shelf-life for each treatment group was the time taken for the average odour score of the independent replicates to reach a score of ≤ 4 at a given timepoint and subsequent timepoints. The rate of exponential growth for TVC and LAB was calculated using the D-model (Baranyi and Roberts, 1994).

For all datasets, spline curves were fitted to the trend of the response variables to smooth the data for all treatments within the datasets. The fitted spline had a cubic B-spline basis with three equally spaced knots positioned between the minimum and maximum values of the time variable. The interaction of treatment with the spline effect indicated which trends differed per treatment. The significance of the interaction was determined using type III F tests. A random effect was included for the sensory panelists for odour and colour. For residual lactic acid concentrations, there were too few timepoints for the spline approach; therefore, no spline was fitted, and simple linear trends were applied to the data. Differences between the control and treatments were tested at each timepoint for all datasets. As there were five pairs of comparisons, the *p*-values were adjusted using Monte Carlo simulation (Westfall et al., 2011). The ratio of LAB over TVC was calculated by LAB - TVC as the data were on the log scale. Bone-in and boneless trials were analysed separately as they were conducted independently. All analyses were done using PROC GLIMMIX and PROC PLM in SAS/STAT version 9.4 (SAS Institute Inc., Cary, NC, USA).

3.3 Results

3.3.1. Sensory assessment and shelf-life duration

The odour trends differed significantly between treatments in the bone-in trial ($F_{35,753}$ = 33.62, $p < .0001$) (Fig. 3.1). The control group reached the end of shelf-life at 17 days, which was approximately 25% less than assumed shelf-life (22 days) based on previous studies

(Huynh et al., 2016; Kaur et al., 2017b). In all cases, bone-in samples treated with glucose had a longer shelf-life compared to the control, as measured by odour (Table 3.1). However, different levels of glucose appeared to have different effects on shelf-life extension. The 2.5% and 10% glucose treatments both reached end of shelf-life by 22 days, which was an increase of 29% compared to the observed shelf-life of the control. The 0.5% glucose treatment reached end of shelf-life by 28 days, which was a 65% shelf-life extension in comparison to the control. However, 1% and 5% treatment groups still had acceptable odour scores of 6.0 by the end of the bone-in trial (30 days). This was equivalent to a >76% shelf-life increase relative to the control.

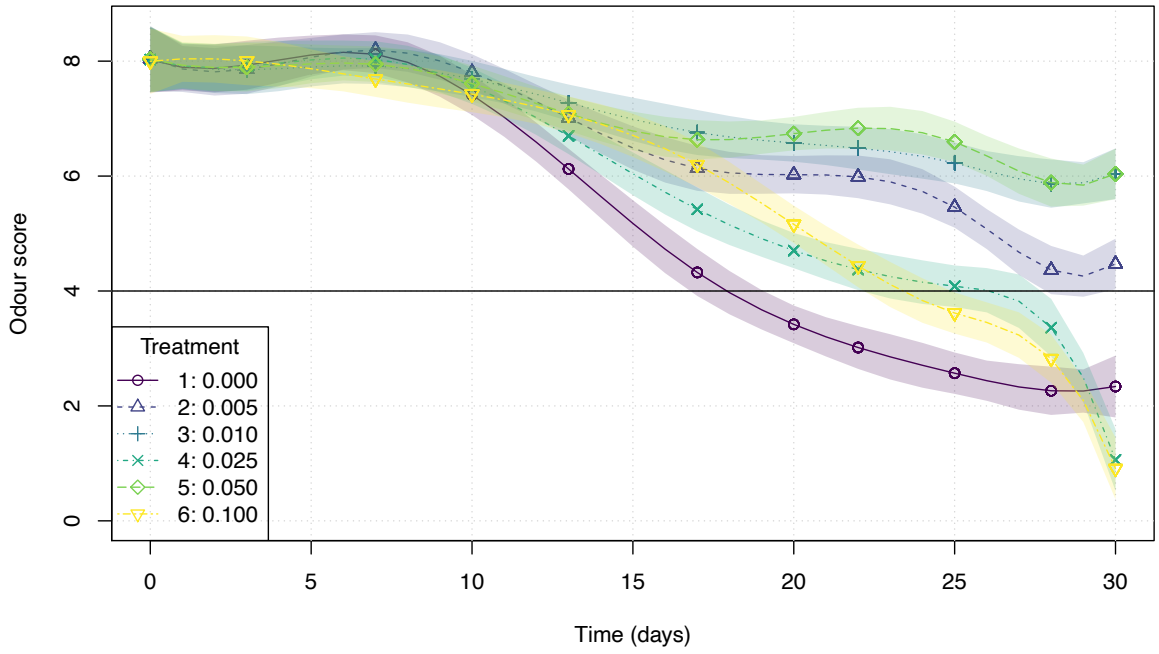

Figure 3.1 Fitted spline curves and 95% CI for odour scores of VP bone-in lamb shoulders stored at 4°C after surface treated with different glucose concentrations. End of shelf-life is demarcated by an odour score of ≤4 highlighted by the horizontal line.

Table 3.1 Observed shelf-life of VP lamb shoulders stored at 4°C after surface treated with glucose concentrations.

	Bone-in lamb		Boneless lamb	
Glucose concentration	Observed shelf-life (days)	Shelf-life extension (%)[a]	Observed shelf-life (days)	Shelf-life extension (%)
0%	17	-	26	-
0.5%	28	65%	31	19%
1%	>30[b]	>76%	31	19%
2.5%	22	29%	31	19%
5%	>30[b]	>76%	35	35%
10%	22	29%	28	8%

a. Shelf-life extension (%) was determined in relative to the observed shelf-life of the control samples.
b. The samples were still rated as acceptable to consumers even after the end of the trials (i.e., more than 30 days)

Odour trends also differed significantly between treatments in the boneless trial ($F_{35,1212}$ = 16.61, $p < .0001$) trial (Fig. 3.2). All samples treated with glucose had a longer shelf-life compared to the control. The control group in the boneless trial reached the end of its shelf-life by 26 days (Fig. 3.2). This was approximately 15% longer than the expected shelf-life based on previous data (Huynh et al., 2016; Kaur et al., 2017b). The 10% glucose treatment group was the first to reach the end of shelf-life, which was at 28 days, increasing the shelf-life by 8% relative to the control (Table 3.1). This was followed by glucose treatments 0.5%, 1% and 2.5%, which reached the end of shelf-life by 31 days, with a shelf-life extension of 19% compared to the control. The 5% glucose was also the most effective treatment in the boneless trial, reaching the end of shelf-life after 35 days. This was equivalent to a 35% shelf-life extension compared to the control. In all cases, the colour sensory score of samples did not decrease below the acceptable limit before the end of shelf-life based on the odour score (Figs 3.3 and 3.4).

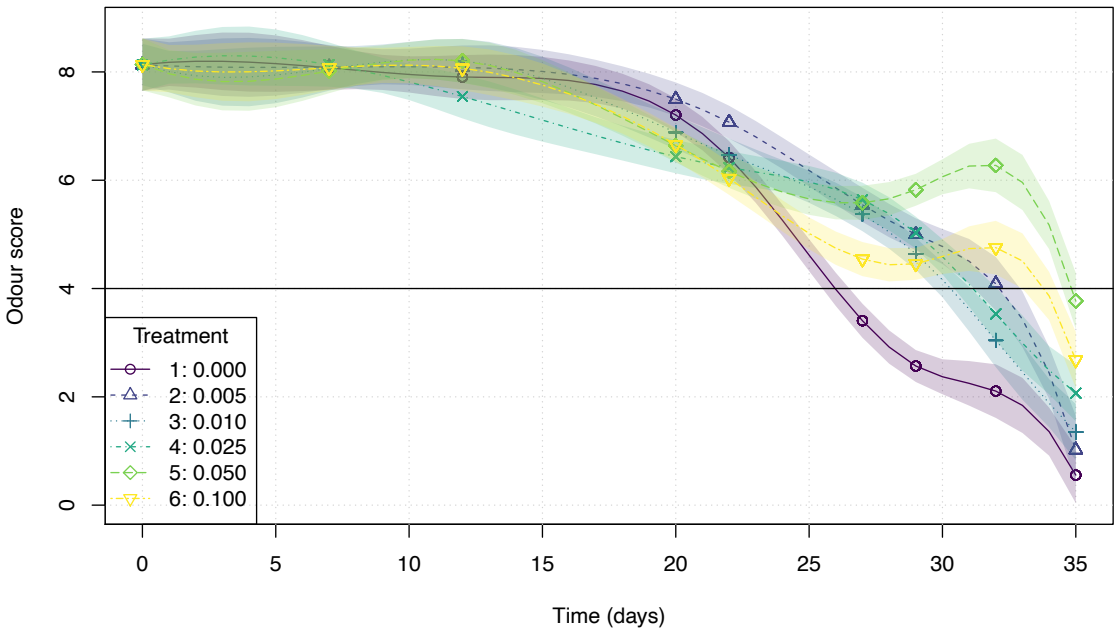

Figure 3.2 Fitted spline curves and 95% CI for odour scores of VP boneless lamb shoulders stored at 4°C after surface treated with different glucose concentrations. End of shelf-life is demarcated by an odour score of ≤4, highlighted by the horizontal line.

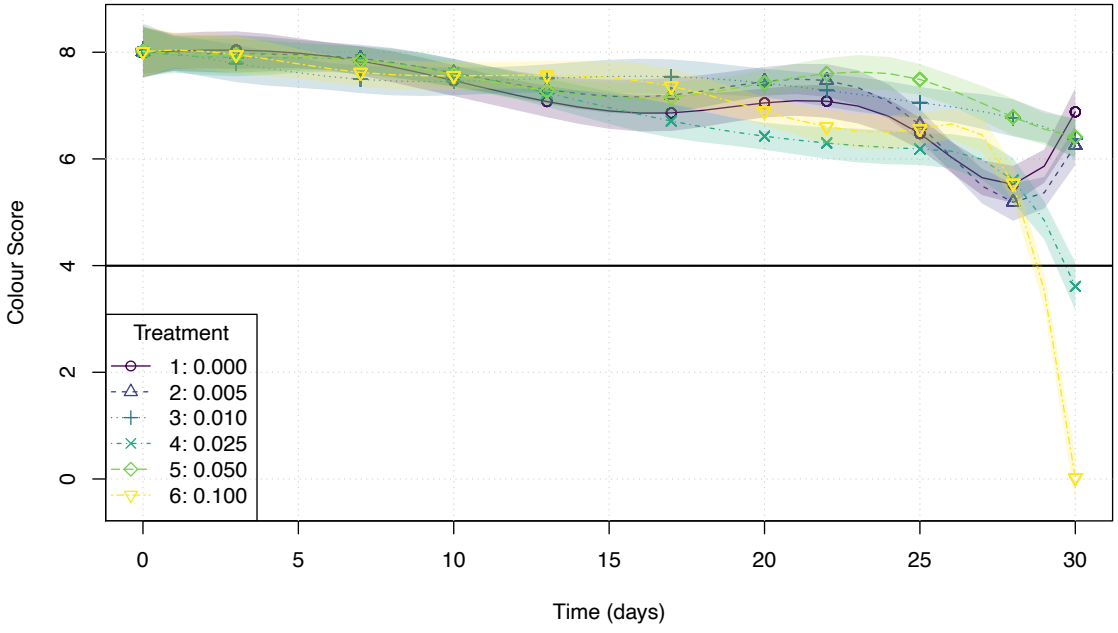

Figure 3.3 Fitted spline curves and 95% CI for colour scores of VP bone-in lamb shoulders stored at 4°C after surface treated with different glucose concentrations. End of shelf-life is demarcated by an odour score of ≤4, highlighted by the horizontal line.

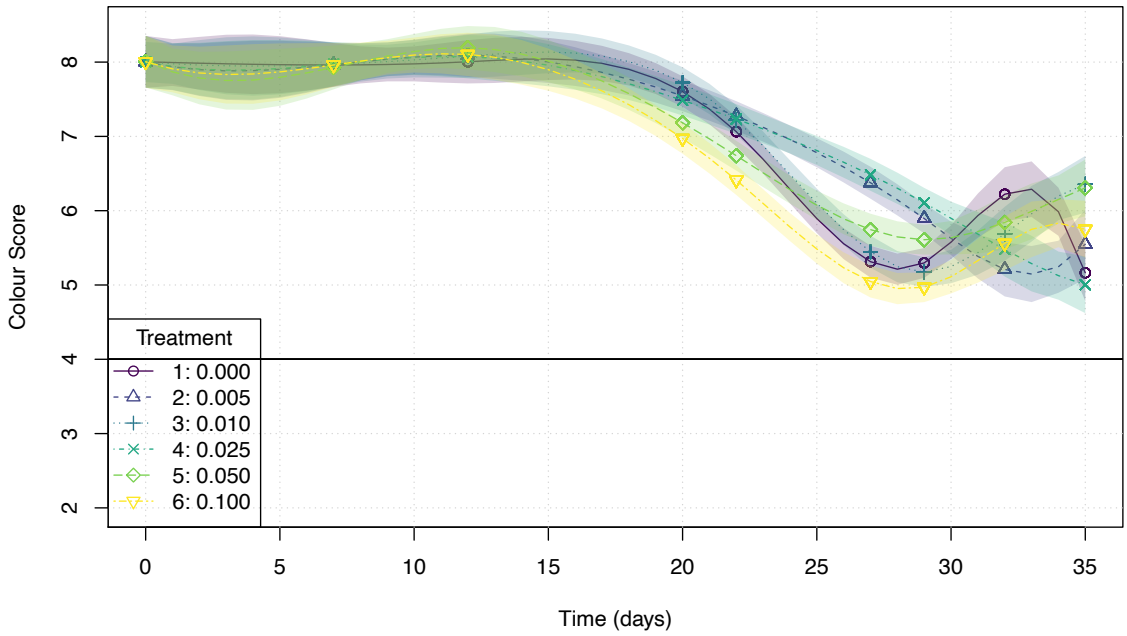

Figure 3.4 Fitted spline curves and 95% CI for colour scores of VP boneless lamb shoulders stored at 4°C after surface treated with different glucose concentrations. End of shelf-life is demarcated by an odour score of ≤4, highlighted by the horizontal line.

3.3.2. Viable bacterial growth dynamics on lamb samples

Initial total viable counts (TVC) of fresh bone-in lamb shoulder ranged from 2.64 – 2.96 $\log_{10}$ CFU/cm^2 and reached a maximum population of 7.65 – 7.86 $\log_{10}$ CFU/cm^2 (Fig. 3.5). The fresh boneless lamb initial TVC was 3.64 – 3.98 $\log_{10}$ CFU/cm^2. Boneless meat reached a maximum population of 7.66 – 7.81 $\log_{10}$ CFU/cm^2 similar of that observed in bone-in samples (Fig. 3.6). The rate of exponential TVC growth in the bone-in and boneless trials ranged from 0.97 – 1.23, and 0.76 – 0.97 $\log_{10}$ CFU/cm^2 per day, respectively. Growth rate and final cell density did not differ significantly between control and treatments in either trial.

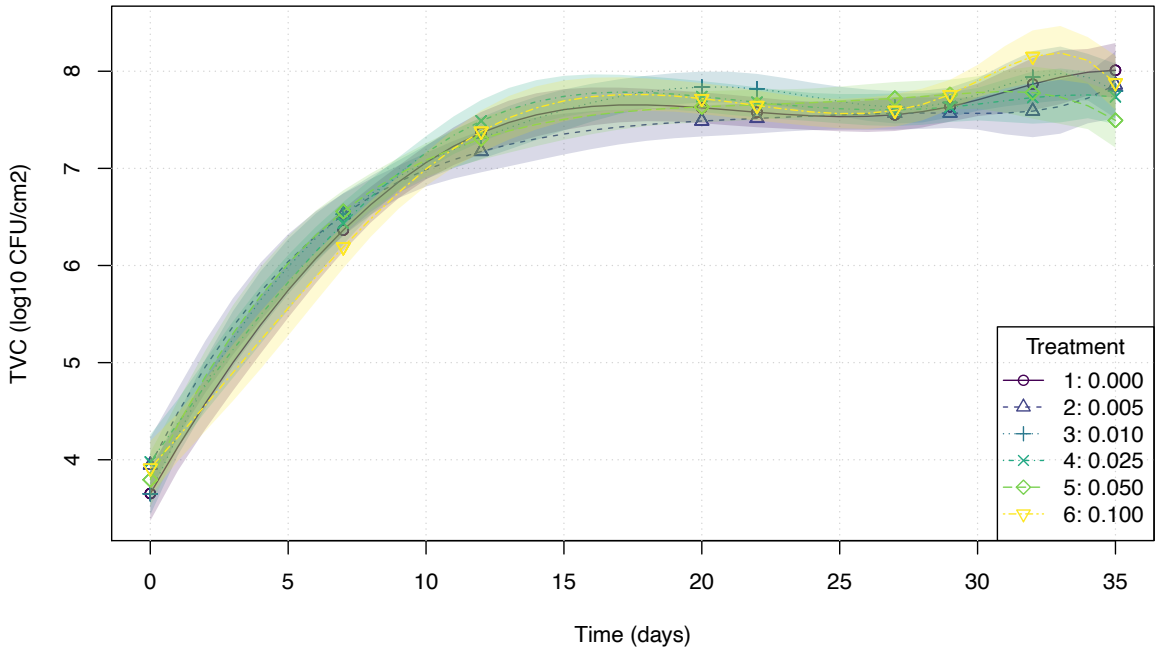

Figure 3.5 Fitted spline curves and 95% CI for bacterial growth (log_{10} CFU/cm^2) as TVC on VP bone-in lamb shoulder stored at 4°C after surface treated with different glucose concentrations.

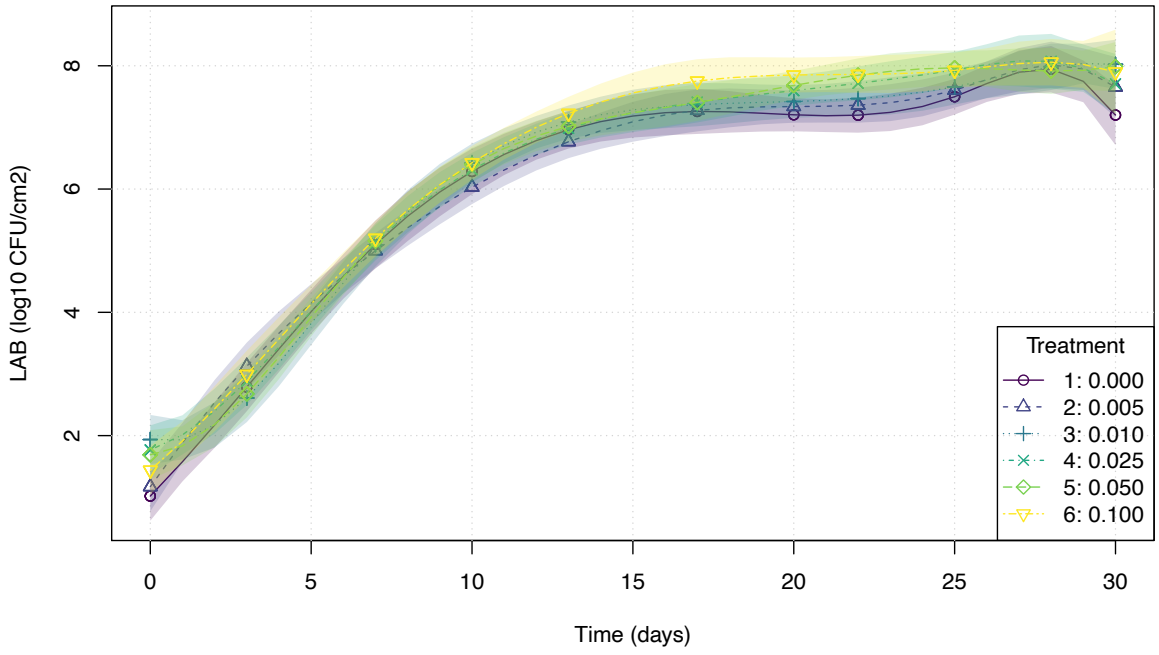

Figure 3.6 Fitted spline curves and 95% CI for bacterial growth (log_{10} CFU/ cm^2) as TVC on VP boneless lamb shoulder stored at 4°C after surface treated with different glucose concentrations.

Initial lactic acid bacteria (LAB) count on fresh bone-in lamb shoulder was 0.62 – 2.15 $\log_{10}$ CFU/cm^2 (Fig. 3.7). Boneless samples had a higher initial count of 2.53 – 4.1 $\log_{10}$ CFU/cm^2 (Fig. 3.8). Both bone-in and boneless trials reached similar maximum populations of 7.22 – 8.26 $\log_{10}$ CFU/cm^2 and 7.07 – 8.37 $\log_{10}$ CFU/cm^2, respectively. The rate of exponential LAB growth ranged from 1.29 – 1.58 $\log_{10}$ CFU/cm^2 per day in the bone-in trial and 0.99 – 1.08 $\log_{10}$ CFU/cm^2 per day in the boneless trial. The growth rate and final cell density of LAB did not differ significantly between control and treatments in either trial.

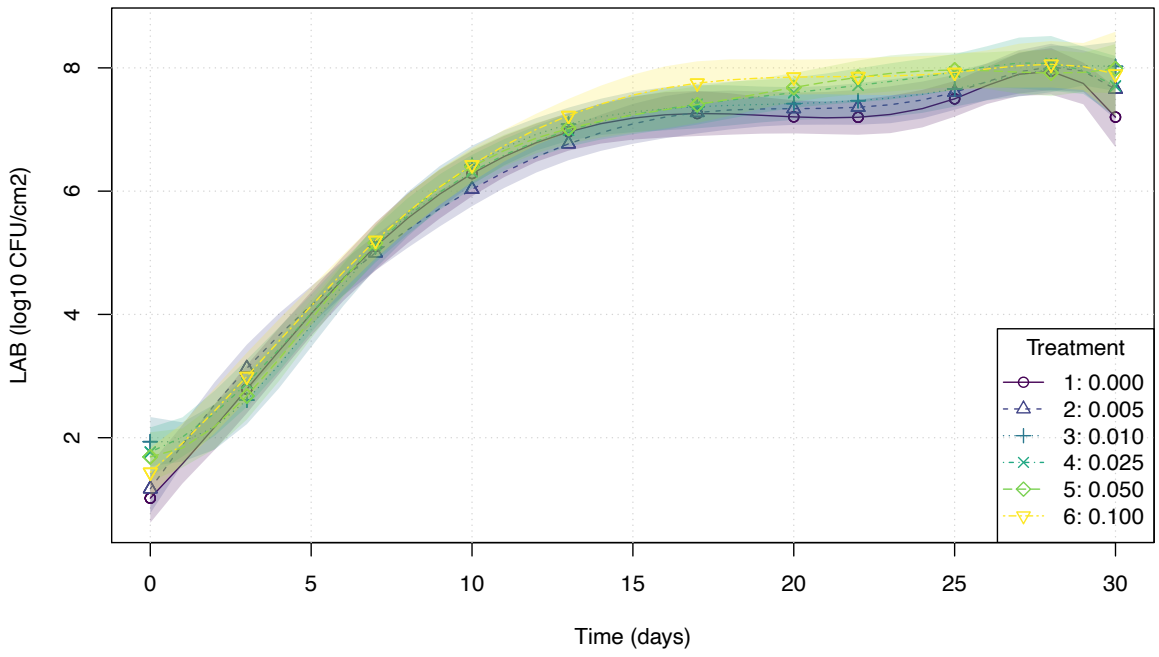

Figure 3.7 Fitted spline curves and 95% CI for lactic acid bacterial growth ($\log_{10}$ CFU/ cm^2) on VP bone-in lamb shoulder stored at 4°C after surface treated with glucose concentrations.

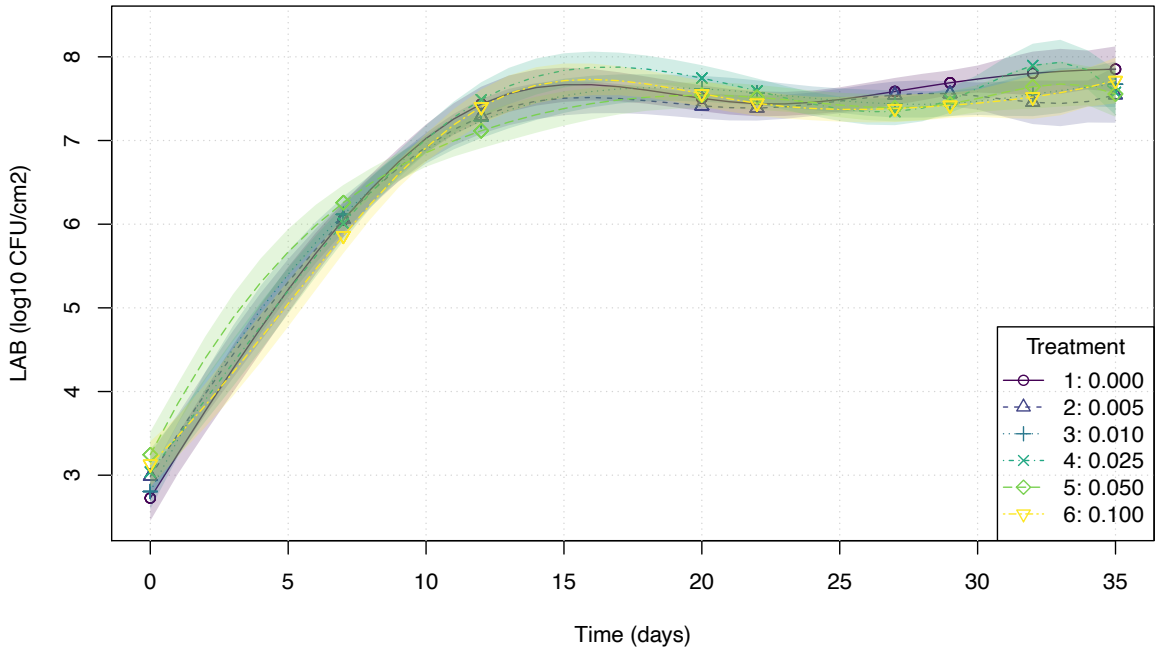

Figure 3.8 Fitted spline curves and 95% CI for lactic acid bacterial growth (log_{10} CFU/cm^2) on VP boneless lamb shoulder stored at 4°C after surface treated with different glucose concentrations.

3.3.3. pH dynamics of VP lamb shoulders

The initial pH of fresh bone-in and boneless lamb shoulder were similar, ranging from 5.82 – 6.12 (Fig. 3.9) and 5.95 – 6.1 (Fig. 3.10), respectively. By the end of the bone-in trial (30 days) meat pH ranged from 5.8 – 6.1, while meat pH at the end of the boneless trial (35 days) was more variable, ranging from 5.9 – 6.4.

In the boneless trial, the pH trends differed significantly between treatments ($F_{35,193}$ = 1.78, p = 0.008). The 10% glucose treatment showed a significant reduction in meat pH compared to the control, which was evident by 22 days (t_{193} = 2.75, p = 0.03) (Fig. 3.10). This trend continued for the remainder of the trial, and by 35 days it showed the largest pH difference of 0.43 units less than the control (t_{193} = 2.76, p = 0.03). There was no significant difference in meat pH between all other tested glucose treatments and the control.

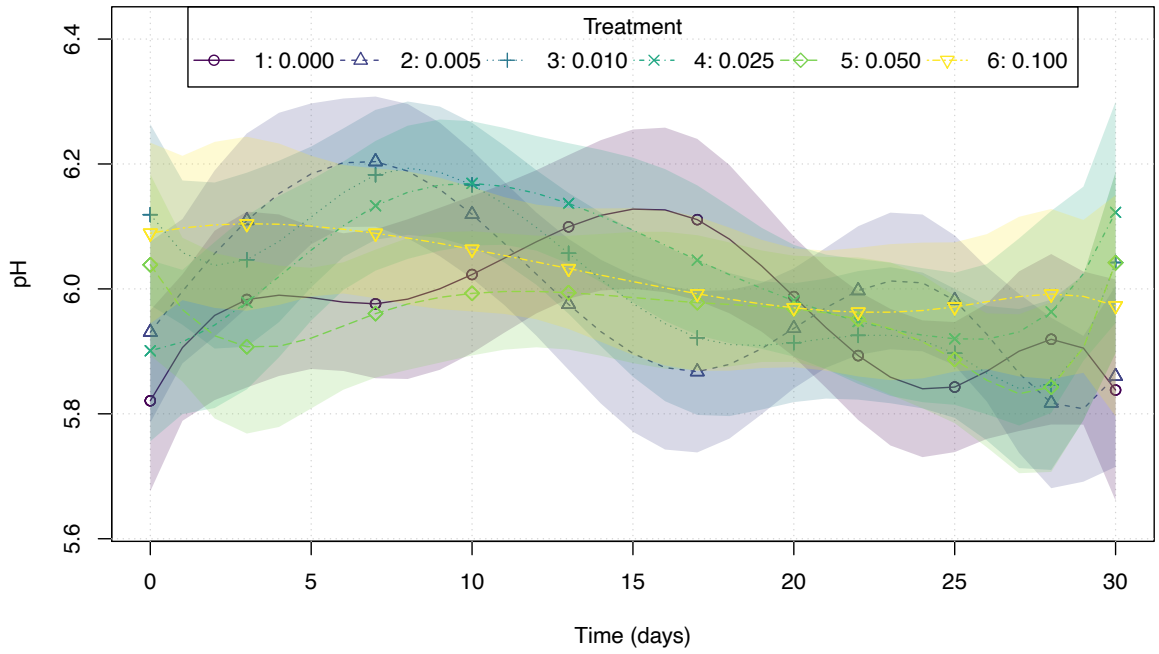

Figure 3.9 Fitted spline curves and 95% CI for pH of bone-in lamb shoulder stored at 4°C after surface treated with different glucose concentrations.

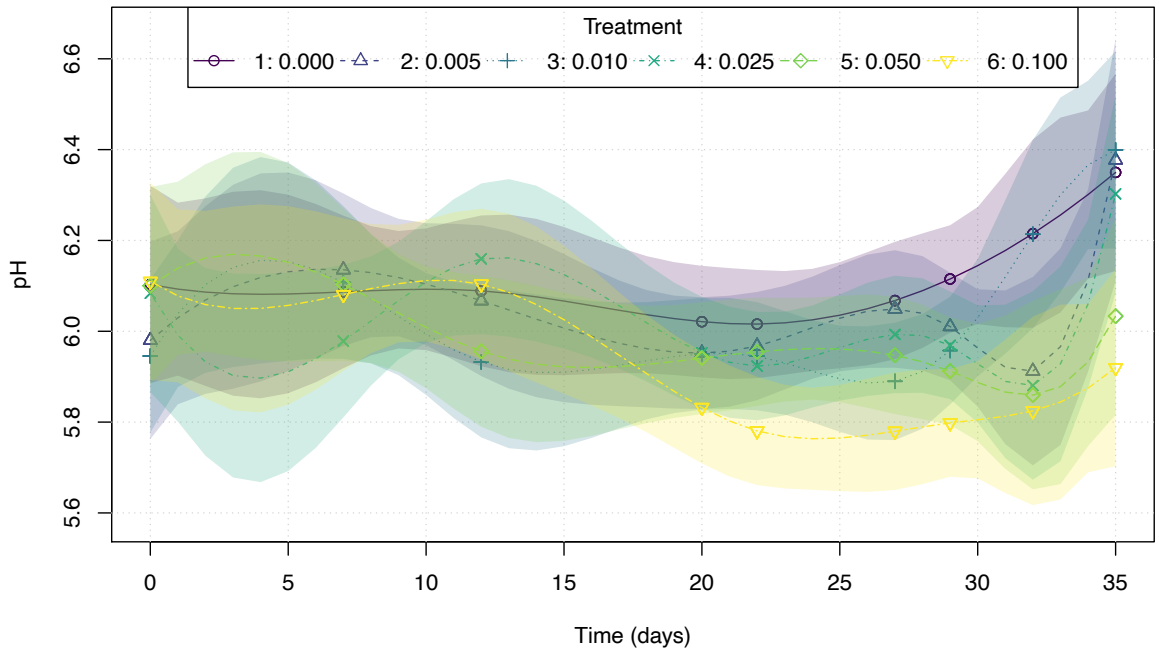

Figure 3.10 Fitted spline curves and 95% CI for pH of boneless lamb shoulder stored at 4°C after surface treated with different glucose concentrations.

The pH of the bone-in samples was highly variable and not consistent with the boneless data (Figs. 3.9 and 3.10). However, the meat slurry trial, which was run in parallel to the bone-in trial showed a similar significant trend between treatments to the boneless trial ($F_{35,141}$ = 5.31, $p < .0001$) (Fig. 3.11). The 10% glucose treatment showed the biggest reduction in meat slurry pH compared to the control, which was evident by 13 days (10%: $t_{141} = 5.23$, $p < .0001$). This trend continued for all subsequent timepoints, ranging between 0.24 and 0.36 pH units less than the control. The 5% glucose also had a significant impact on meat slurry pH, which was significantly less than the control by 13 days ($t_{141} = 3.45$, $p < .0039$). The pH of the 5% treatment group remained significantly lower than the control by 0.16 to 0.20 units until 28 days, when it had a similar pH to the control. The 2.5% treatment had less of an effect on meat slurry pH, having significantly lower pH than the control by day 13 ($t_{141} = 2.94$, $p = .01$); however, by day 22 it was similar to the control. No significant pH reduction was evident for samples treated with the lower glucose concentrations (0.5% and 1%).

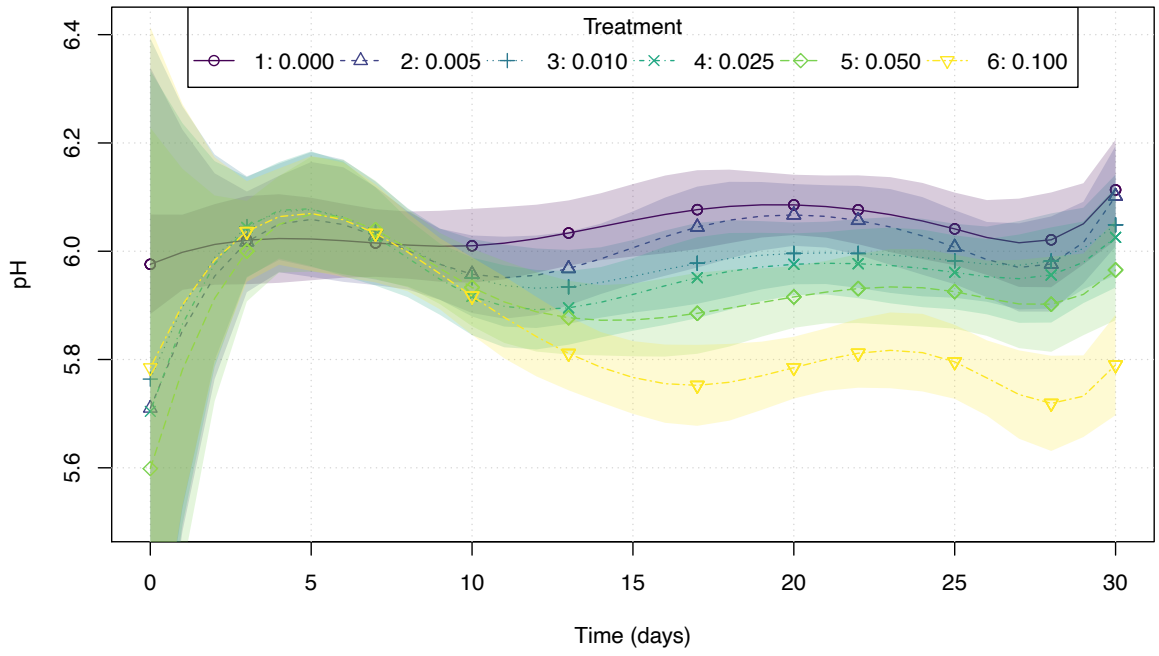

Figure 3.11 Fitted spline curves and 95% CI for pH of lamb meat slurry stored at 4°C after surface treatment with different glucose concentrations.

3.3.4 Residual glucose and lactic acid of lamb samples

The residual glucose concentration of fresh bone-in and boneless lamb shoulder ranged from 0.65 – 1.68 mmol/kg and < 0.44 – 1.89 mmol/kg, respectively (Figs. 3.12 and 3.13). After glucose treatments were applied, residual glucose was highest for the 10% treatment (boneless 5.63; bone-in 6.90 mmol/kg), followed by 5% (boneless 2.40; bone-in 2.82 mmol/kg). Glucose treatments 0.5%, 1% and 2.5% had similar starting glucose concentrations to the control in both trials.

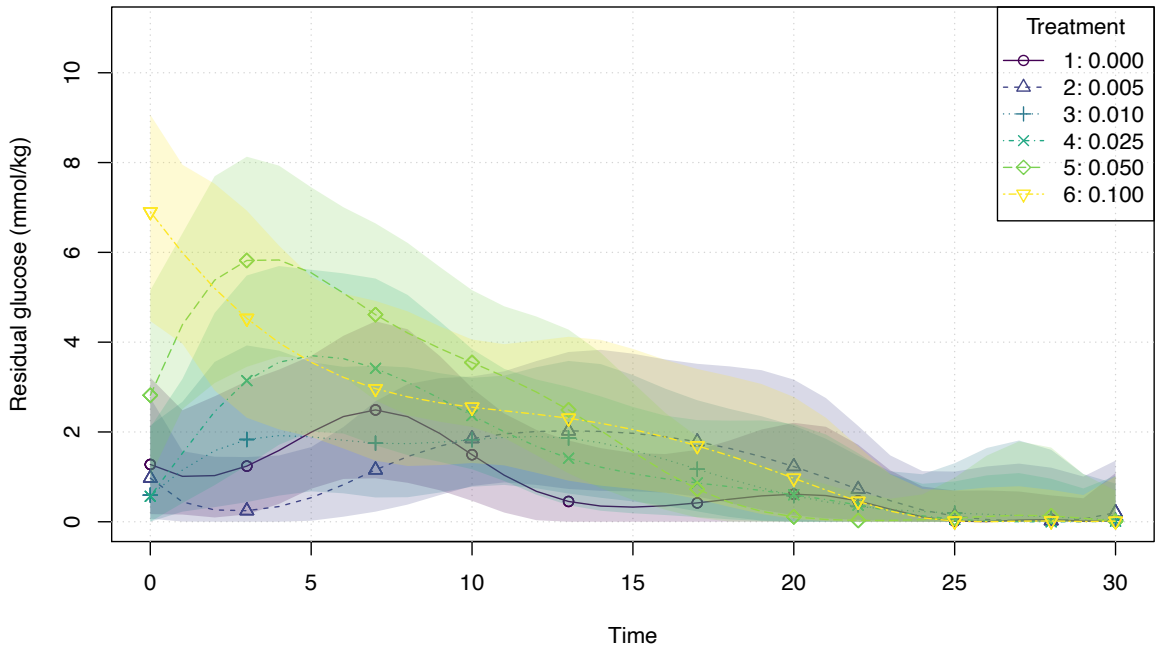

Figure 3.12 Fitted spline curves and 95% CI for residual glucose concentration (mmol/kg) of VP bone-in lamb shoulder stored at 4°C after surface treated with different glucose concentrations. Lower detection limit was 0.44 mmol/kg.

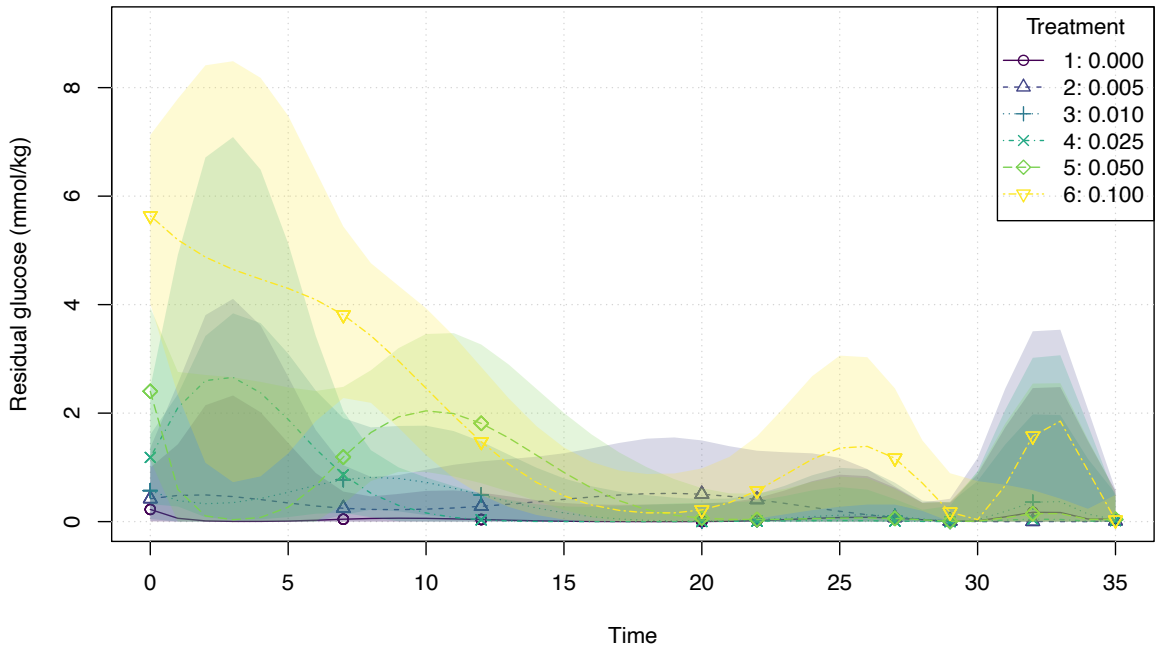

Figure 3.13 Fitted spline curves and 95% CI for residual glucose concentration (mmol/kg) of VP boneless lamb shoulder stored at 4°C after surface treated with different glucose concentrations. Lower detection limit was 0.44 mmol/kg.

Residual glucose trends differed significantly between treatments in the bone-in ($F_{35,296}$ = 1.43, p = 0.005) trial (Fig. 3.12). The 5% and 10% glucose treated samples were significantly higher ($p < 0.05$) compared to the control until 7 days, at which there were no significant differences detected between any glucose treatment and the control. This trend continued for the remainder of the trial; however, there was a large amount of variation between treatments replicates, as indicated by the confidence intervals in Fig. 3.12. The control was below the detection limit (0.44 mmol/kg) from day 13. The 5% glucose treatment was below detection limit by 20 days, while all other glucose treatments reached below the detection limit by 22 – 25 days.

Residual glucose trends also differed significantly between treatments for the boneless trial ($F_{35,83}$ = 3.14, $p < .0001$) (Fig. 3.11). Similar to the bone-in trial, there was large amount of

variation between treatment replicates, as indicated by the confidence intervals in Fig. 3.13. The 5% and 10% glucose treatments had significantly higher residual glucose ranging between 2.35 – 5.85 mmol/kg higher ($p < 0.05$) compared to the control until 12 days. However, by 20 days both the 5% and 10% glucose treatments were below the limit of detection (0.44 mmol/kg). The control and 0.5% glucose treatment were below the detection limit (0.44 mmol/kg) throughout all of the trial (Fig. 3.13). By day 12 the residual glucose concentration for the 1% and 2.5% glucose treatments were also below the detection limit.

The concentration of lactic acid in the bone-in meat ranged from 28.00 – 97.02 mmol/kg and 26.26 – 70.8 mmol/kg (Fig. 3.14 and 3.15) for the boneless sample. There was no trend over time, and there was no detectable difference in concentration between the control and treatment groups for either trial.

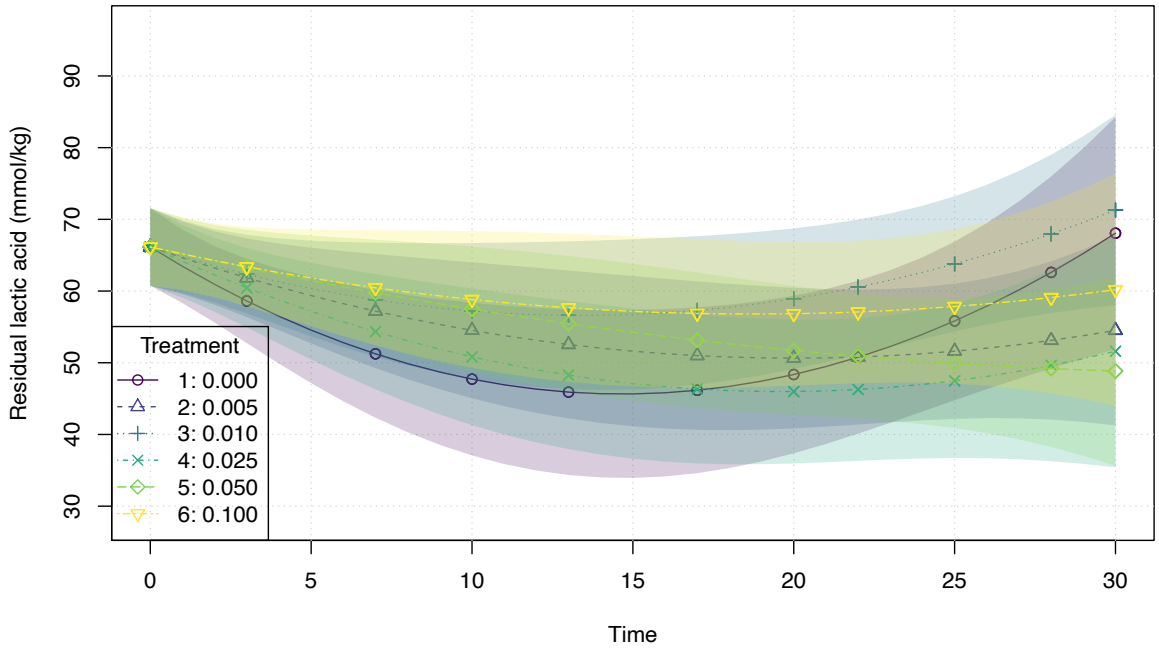

Figure 3.14 Fitted spline curves and 95% CI for residual lactic acid (mmol/kg) of VP bone-in lamb shoulder stored at 4°C after surface treated with different glucose concentrations. Lower detection limit was 1.44 mmol/kg.

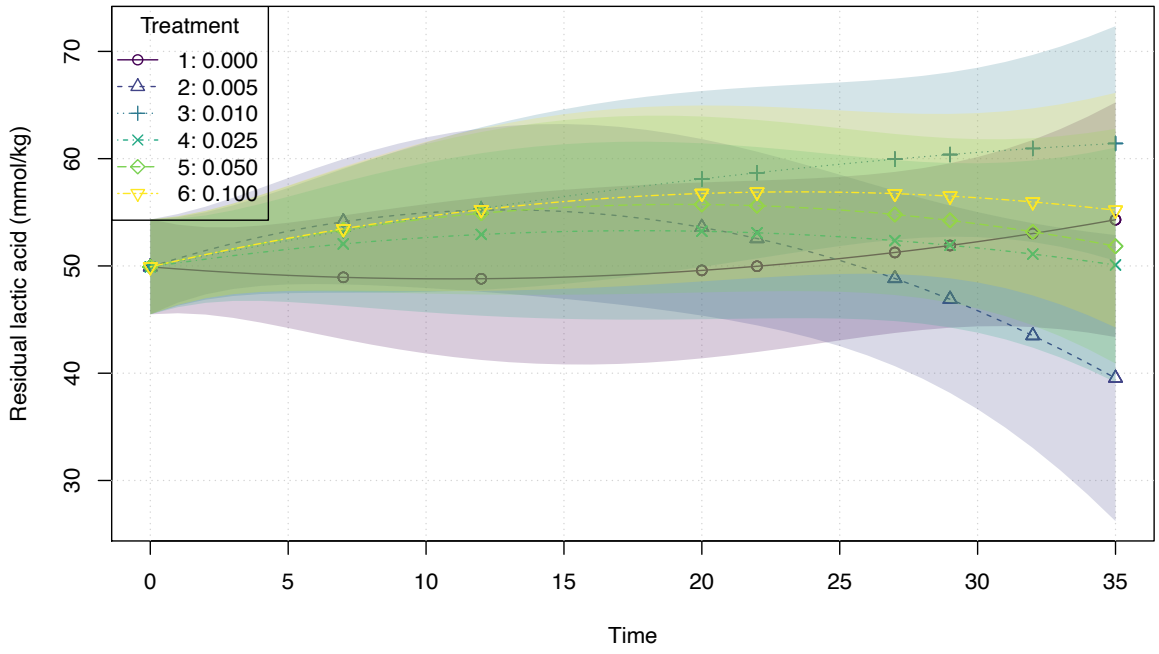

Figure 3.15 Fitted spline curves and 95% CI for residual lactic acid (mmol/kg) of VP bone-in lamb shoulder stored at 4°C after surface treated with different glucose concentrations. Lower detection limit was 1.44 mmol/kg.

3.4 Discussion

This study found that the surface application of all test concentrations of glucose (up to 10%) extended the shelf-life of vacuum-packed (VP) lamb shoulder stored at 4°C. The length of the shelf-life extension ranged from 8% to 76% (2 – 13 days) relative to the observed shelf-life of the control. These results were dependent on the glucose concentration and varied between the bone-in and boneless products, however the shelf-life extension was not directly proportional to the glucose concentration. The success of glucose for shelf-life extension of meat products has previously been reported in the research literature for red meat stored aerobically. For instance, Kumudavally et al. (2010) found that when mutton was surface treated with 5% (w/v) glucose solution, containing 1,000 ppm potassium sorbate to inhibit moulds and yeasts, it extended the shelf-life of the product from 1 day to 3 days when stored

aerobically at 25 °C. Shelef (1977) found that ground beef samples containing 2 – 10% (w/v) glucose prolonged the shelf-life by 5 – 10 days when stored aerobically at 5 °C. Furthermore, Newton and Gill (1978) found that the application of 100 µg/g (0.56 mmol/kg) glucose to beef striploins that were completely devoid of glucose extended the shelf-life from 2 days to 4 days under aerobic conditions. It has, however, been suggested by Gill (1986) that adding glucose to VP meat with a higher pH (≥ 6), like lamb, can potentially cause premature spoilage by stimulating the growth of *Shewanella putrefaciens*. This and related species, such as *S. baltica*, can produce H_2S at refrigeration temperatures, causing premature malodours and colour defects, such as greening (Doulgeraki et al., 2012; Kamenik, 2013). This study found no evidence of greening colour defects or premature malodours, suggesting that under the conditions of this study the organism was not able to reach the population density required to cause spoilage (Kaur et al., 2017b). Furthermore, molecular studies on VP beef and lamb detected little or no of growth of *Shewanella* species under a range of temperatures (from -0.5˚C to 8˚C) (Kaur et al. 2017; 2021).

Under aerobic conditions, shelf-life extension by additional glucose is thought to be due to the delay of meat proteolysis and the subsequent production of off-odours attributable to *Pseudomonas* spp. (Kumudavally et al., 2010; Lambropoulou et al., 1996; Newton and Gill, 1978; Shelef, 1977). Specifically, meat bacteria likely build biomass by rapidly growing on efficiently metabolizable low-molecular weight compounds (Perrin et al., 2020), such as glucose (Ellis and Goodacre, 2001; Ercolini et al., 2011). As time progresses, the catabolism of nitrogenous and lipid compounds increases the concentration of an array of metabolites that can include those with volatile and malodourous properties (Casaburi et al., 2015; Ercolini et al., 2011; Ercolini et al., 2009; Ercolini et al., 2006; Nychas et al., 2008).Thus, the first signs of

meat spoilage typically correspond with the depletion of glucose (Boers and Dijkmann, 1994; Byun et al., 2003; Gill, 1986; Kumudavally et al., 2010; Nychas et al., 1988; Shelef, 1977). This agrees well with the observations of this study that both control and treatment groups had glucose levels below detection limits before the onset of spoilage. It was also evident in both trials that the control was the first treatment group to spoil, as well as the first to become depleted of glucose. In the bone-in trial, the control was below the glucose detection limit after 13 days, whereas glucose treated samples were below the detection limit after 20 – 25 days, all of which had significantly longer shelf-lives (by at least 29%) than the untreated controls. Similar results were observed in the boneless trial. Meat samples treated with glucose reached a glucose concentration below the detection limit 12 – 20 days after the control, in addition to having significantly longer shelf-lives (by at least 8%) relative to the control. These results suggest that the observed increase in shelf-life of meat by additional glucose might be due to the delayed usage of other substrates by spoilage organisms.

The results of this study showed variability, and the relationship between timing of glucose depletion and the onset of spoilage was not clearly definable. For example, the control and 0.5% treatment had a glucose concentration below the detection limit from the start of the boneless trial, but spoilage was not evident until day 25 and 30, respectively. Furthermore, the 0.5% glucose treatment had a significantly longer shelf-life (by 20%) than the control, despite having residual glucose concentration similar to that of the control throughout the trial. The results were also variable in the bone-in trial, i.e., the control spoiled ~4 days after glucose concentration was below the detection limit, whereas the 5% glucose treated samples had no detectable glucose from 20 days but were still rated as acceptable past 30 days. These inconsistent results may be due to the limitations and sensitivity associated with

the enzymatic assay used to detect glucose. The lower detection limit of the assay was 0.44 mmol/kg, which was relatively high as the initial concentration of residual glucose in fresh lamb shoulder ranged from 0.44 mmol/kg – 1.89 mmol/kg. As a result, in this work, the reported glucose depletion in meat was only an approximation.

It is important to note that the effects of glucose on meat under aerobic conditions were expected to be different to those reported under anaerobic conditions. VP meat-associated microbial communities are different to that found under aerobic conditions thus affecting the rate and type of the spoilage process (Doulgeraki et al., 2012). For example, the microbial community of chilled VP lamb is dominated by a number of different anaerobic heterofermentative lactic acid bacteria (LAB) with low spoilage potential, notably *Carnobacterium* spp., *Lactococcus* spp. and *Leuconostoc* spp. (Kaur et al., 2017b). Some meat associated species are proteolytic; however, their amino acid catabolic pathway can be vastly different from other bacteria, producing less offensive metabolites (Fernandez and Zuniga, 2006). Therefore, proteolysis might not be as clearly related to the onset of spoilage under anaerobic conditions especially given the nutrient complexities of meat. Further investigation using more sensitive methods, such as metabolomics or volatilomics, are required to determine the effect of additional glucose on glucose depletion, substrate utilisation and the onset of spoilage under anaerobic conditions.

Additional glucose may extend the shelf-life of meat by reducing meat pH. It was found that as glucose concentration increased, the larger the pH decline was over time. This effect was relatively clear in the boneless trial; however, the results from the bone-in trial did not show a pH-related trend. The pH variability of meat samples may be attributed to the inherent variability of meat pH (Bendall, 1978). Such variability was controlled for by conducting the

meat slurry trial, the results clearly illustrate that as glucose concentrations increase, the larger the pH reduction is over time. Reducing meat pH may contribute to longer shelf-lives of VP lamb by inhibiting and slowing the growth rate of bacteria with higher spoilage potential (EFSA, Panel on Biological Hazards (BIOHAZ), 2016). For instance, *Clostridium*, *Yersina* and *Rahnella* species are of particular interest for VP lamb spoilage, as they are typically more abundant on lamb meat compared to beef being constrained by pH < 6.0 (Kaur et al., 2021). The additional glucose most likely reduces meat pH due to the accumulation of organic acid end-products as a result of glucose fermentation (Barua and Shelef, 1980; Leisner et al., 2007; Shelef, 1977). Lactic acid bacteria, which make up a significant proportion of the microbial community on VP red meat (Kaur et al., 2021), are most logically the main drivers of the pH decline, due to formation of lactic acid and other acids. However, this study found no detectable change in lactic acid/lactate over time between the control and glucose treatment groups. This could be because the enzymatic method used was not sensitive enough to detect lactic acid changes that were a result of microbial fermentation, or the population of lactic acid bacteria was not high enough to have intensive lactic acid production. Alternatively, lactic acid might not have been the only organic acid produced by lactic acid bacteria that could contribute to a pH reduction. For instance, lactic acid bacteria can be obligatory heterofermentative, meaning glucose metabolism can produce other acidic compounds, such as acetic acid and formic acid (Borch et al., 1996; Casaburi et al., 2015). For instance, *C. divergens* and *C. maltaromaticum,* which are dominant lactic acid bacteria species on meat, can in some instances produce substantially more acetic acid relative to lactic acid (Laursen et al., 2006; Leisner et al., 2007).

This study found no correlation between glucose concentration and shelf-life extension in either trial. The highest glucose concentration (10%) was one of the least effective treatments in both boneless and bone-in trials, extending the shelf-life by 8% (2 days) and 29% (5 days) relative to the control, respectively. However, the 10% glucose treatment also had the largest pH reduction (up to 0.36 units in meat slurry trial) for the longest duration (day 13 onwards). Similarly, Shelef (1977) claimed glucose concentrations of 10% had the largest pH reduction but developed a sour odour after prolonged storage, which was not present in glucose treatments of lower concentration. This suggests that at a concentration of ≥10% the amount of acidic metabolic by-products of glucose metabolism results in noticeable sensory degradation. In addition, relatively high concentrations of glucose (55 mM) have also been reported to facilitate the growth of some spoilage bacteria associated with VP meat, such as *Brochothrix thermosphacta* (Mohsina et al., 2020). Therefore, the addition of large amounts of glucose seems inappropriate for commercial application for shelf-life extension of VP meat. The 5% glucose treatment, however, had less influence on meat pH compared to the 10% treatment, reducing meat pH by 0.20 units (meat slurry trial) from day 13 to day 28. Furthermore, it was the most effective treatment, resulting in the longest shelf-life extension of 35% and >76% (9 and >13 days) in both boneless and bone-in trials, respectively.

All of the lower glucose concentrations (0.5%, 1% and 2.5%) increased the shelf-life of meat in both trials, ranging from 20% to > 76% increase relative to the control. Although, Shelef (1977) found glucose concentrations below 2% had no effect on meat sensory qualities and also did not reduce meat pH, this study found that the lower glucose concentrations also had less influence on meat pH, while increasing meat shelf-life. The pH of 2.5% glucose

treatments was significantly lower than the control for ~ 9 days in the meat slurry trial. However, 0.5% and 1% glucose treatments had no effect on meat pH in any trial. The results suggests that pH reduction is not the only mechanism driving shelf-life extension when meat is treated with glucose but could also be the accumulation of organic acids and an increase in concentration of undissociated organic acids.

We found the application of glucose did not have an effect on the population density and growth rate of lactic acid bacteria and total viable bacteria. Furthermore, there was no difference in the ratio of lactic acid bacteria and total viable bacteria between the controls (i.e., no added glucose) and glucose-treated meat, indicating that additional glucose did not cause a detectable shift in the microbial community using culture-dependent methods. This agrees well with the results of Kumudavally et al. (2010) who also found no difference in total viable bacterial counts. In contrast, Shelef (1977) found glucose treatments had an effect on microbial composition, as lactic acid bacteria constituted less than 0.2% of the total bacterial population in the control samples, but nearly 8% in the glucose treated meat. However, all previous work has relied on culture-dependent enumeration methods, which has inherent drawbacks, such as selective recovery (Brightwell et al., 2009; Giorgio and Neviani, 2001). Culture-dependent techniques will not detect subtle differences in the microbial community composition, as the effects of glucose might be at a species level. Therefore culture-independent techniques, such as 16S rRNA amplicon sequencing, should be utilised in future studies to better understand the effects of glucose on meat microbiology.

Bone-in samples were consistently found to have a greater shelf-life extension compared to the analogous boneless samples for all tested glucose concentrations. However, direct comparisons cannot be made between the bone-in versus boneless products as the trials

were independent experiments. They were conducted on two separate occasions, and the meat came from different herds of animals. Rather, the purpose of this study was to determine if the effects of glucose were consistent under two different experimental trials. It is still noteworthy that the bone-in samples consistently had the longest shelf-life extension relative to the boneless samples to see if future experiments show the same trend. The differences in reproducibility could be due to bone-in control spoiling at fast rate, since in this study it spoiled by 75% of the expected shelf-life (Huynh et al., 2016; Kaur et al., 2017b). The basis of this variance remains to be elucidated. It is also worth noting that the initial total bacterial count was lower in the bone-in trial. This potentially suggests that glucose treatments may be more effective if the microbial community is less established. Further investigations involving a more detailed analysis of meat from a range of sources is required to confirm this. Despite the variability between the bone-in and boneless trials, the results highlight the success of glucose for shelf-life extension of VP lamb shoulder at 4°C. Further research is therefore required to investigate the efficacy of this treatment using other types of meat cuts and at lower commercial storage temperatures.

3.5 Conclusion

Glucose surface treatments, ranging from concentrations of 0.5% - 10% (w/v) extended the shelf-life of both bone-in and boneless vacuum-packed lamb shoulder stored at 4°C. The 5% treatment was the most effective in all cases, increasing the shelf-life of bone-in lamb by > 76% and boneless lamb by 35% relative to the control. Glucose treatments had no effect on meat colour, lactic acid concentration, bacterial population density or growth rates. There was no clear relationship between the onset of meat spoilage and glucose depletion.

However, glucose treatments reduced meat pH, potentially affecting the microbial community composition as well as the rate at which malodorous compounds accumulate.

Chapter Four

Effects of additional glucose as a surface treatment on the microbial community, shelf-life and associated volatilome of vacuum-packed lamb

4.1 Introduction

Spoilage of fresh meat is most commonly caused by growth of microbes and their metabolism of meat tissues, resulting in the accumulation of spoilage metabolites (Ercolini et al., 2006; Gram et al., 2002). The rate at which spoilage manifests is dependent on the microbial community growth rate, composition, and metabolic activity (Ercolini et al., 2011; Lambert et al., 1991). A combination of factors influence the growth and development of the microbial community, such as the pre- and post- slaughter contamination, the intrinsic properties of meat (especially pH), packaging used and storage conditions (Bacon et al., 2000; Mossel, 1971).

As discussed in chapter 1, beef and lamb produced in Australia typically have shelf-lives of 26 and 12 weeks respectively when vacuum-packed (VP) and stored at temperatures of -1.5 to 0°C (Kiermeier et al., 2013; Small et al., 2012). The comparatively shorter shelf-life of lamb is thought to be mainly due to its intrinsically higher pH (~ 0.4 pH units) from large amounts of adipose tissue compared to beef (Gill and Penney, 1985; Kaur et al., 2021). Meat with a higher pH increases bacterial growth rates, including the growth of a more diverse array of spoilage bacteria (EFSA, Panel on Biological Hazards (BIOHAZ), 2016; Kaur et al., 2021; Yang et al., 2014). As highlighted in earlier chapters, the faster rate of quality loss of lamb can present a challenge to lamb exporters due to lengthy shipping times and potential issues that may arise during distribution, such as travel delays and temperature fluctuations (Huynh et al., 2016; Sumner, 2016). Therefore, research is needed to assist the development of safe

and cost-effective interventions to extend the shelf-life of lamb products (Huynh et al., 2016; Mills et al., 2014).

The potential application of additional glucose for shelf-life extension of VP lamb shoulder was previously established in chapter 3. It was found that glucose surface treatments ranging from 0.5% - 10% (w/v) significantly extended the shelf-life of both bone-in and boneless lamb shoulder. This ranged from 8% to 76% (2 to 9 days) increase relative to the control, with the 5% treatment being the most effective in all cases. It was evident that the glucose treatments reduced meat pH by up to 0.43 units less than the control. This was most likely due to the accumulation of organic acid end-products from glucose fermentation.

Glucose is primarily utilized by most bacterial species on VP meat (see chapter 3); however, as glucose depletes many other substrates are likely utilized as well, such as amino acids and lipids. It is evident that bacteria will preferentially consume substrates in a relative order of uptake, which is largely dependent on the biomass yield possible from the given substrate (Perrin et al., 2020). The presumed meat community's multi-auxic behaviour manifests as a complicated array of metabolic activities that ultimately leads to spoilage. Although metabolism of some compounds is more likely to lead to organoleptically detectable spoilage (Casaburi et al., 2015; Ercolini et al., 2011; Ercolini et al., 2009). This study hypothesised that additional glucose alters the microbial community composition and the rate and type of accumulation of spoilage metabolic by-products. This hypothesis was investigated by assessing the changes in the microbial community and volatilome due to glucose treatment. To achieve this, 16S rRNA amplicon sequencing was used to characterise the microbial community composition, and headspace solid phase micro-extraction method coupled with

gas chromatography/mass spectrometry (HS-SPME GC/MS) was used to investigate the volatilome from meat samples.

4.2 Materials and Methods

4.2.1 Sample collection

All samples used in this study were described in chapter 3, which evaluated the potential for glucose surface treatments to extend the shelf-life of VP lamb. Specifically, throughout the shelf-life trials, meat rinsates (50 ml) and subsamples (10 g) were collected from meat samples following microbiological and organoleptic assessment. Meat rinsates (n = 183 bone-in; n = 161 boneless) were used for microbial community sequence analysis in this study. Rinsates were obtained by massaging each meat piece by hand with 100 ml of sterile 0.1% peptone water for 1 min, of which 50 ml was collected and stored at -20°C. Meat subsamples were aseptically sliced from each sample (n = 183 bone-in; n = 161 boneless) and stored at -20°C and were used for volatile compound analysis.

4.2.2 Microbial community structure analysis

4.2.2.1 DNA extraction from meat rinsate

Frozen meat rinsates were thawed for 30 min at 37°C, mixed and aliquoted into 5 × 2 ml subsamples in microcentrifuge tubes. Samples were centrifuged at 5000 *x g* for 10 min at 4°C, the supernatant was removed, the cell pellet was resuspended in the remaining supernatant, and the five subsamples were combined. The combined samples were centrifuged at 5000 *x g* for 10 min, and the supernatant removed. Extraction of DNA from cell pellets was performed using High Pure PCR Template Preparation Kit (Roche Diagnostics NZ Ltd.) as per the manufacturer's protocol. DNA concentration was measured using a

NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) (all DNA samples were then kept at -20°C).

4.2.2.2 16S rRNA gene amplicon sequencing and sequence identification

Samples were sequenced using 16S rRNA amplicon MiSeq Illumina analysis at the Ramaciotti Centre for Genomics (Sydney, NSW, Australia). The sequencing was carried out on the amplified product from implementation of the 27F/519R universal primers for the V1-V3 region of the 16S rRNA gene using standard protocols to generate 300 bp pair-ended reads. Primers 27F/519R were selected to minimize amplification of mammalian host DNA (Kaur et al., 2021). Quality control, library preparation, and normalization was carried out before high-throughput sequencing using the MiSeq platform. Subsequent preparation of the sequence data was performed in the SEED2 pipeline (Větrovský and Baldrian, 2013). For this process FASTQ files were joined, trimmed and quality filtered using routines within the FASTX toolkit (http://hannonlab.cshl.edu/fastx_ toolkit/index.html). Sequences with a PHRED scores with a mean PHRED score of < 30 were discarded. Alignment, clustering and chimera removal was then performed using USEARCH (Edgar, 2013). Amplicon sequence variant (ASV) clusters were defined with a 98% sequence similarity radius. Singletons were excluded to reduce risk of spurious reads resulting from errors in PCR/sequencing. ASV clusters were taxonomically classified using megaBLAST against the SILVA 138 SSU reference database using SEED2.

4.2.3 Volatile compound analysis

4.2.3.1 Sample preparation and headspace SPME

The volatile compounds of meat were isolated by the headspace solid phase micro-extraction method (HS–SPME). The fibre selected for absorption of volatiles was 50/30 µm DVB –

Divinylbenzene/CAR – Carboxen / PDMS – Polydimethylsiloxane Stable Flex 23ga (Supelco, Bellefonte, USA) based on its capability to absorb a wide range of volatile compounds.

Meat samples were prepared by thawing at room temperature for 15 minutes followed by homogenising (T 25 Ultra-Turrax, IKA, China) 5 g of diced lamb shoulder with 8 ml of 30% NaCl solution in 20 ml Headspace vials (23 x 75 mm) (Microanalytix, Sydney, Australia) (Argyri et al., 2015). Samples were spiked through a vial septum with 20 µl of internal standard (IS) solution using a gas-tight syringe (25 µl SGE, Melbourne, Australia) and vortexed. The IS solution contained a mixture of compounds from different representative classes of volatile organic compounds. This was to account for inherent differences in fibre adsorption abilities between the classes. Representative compounds were 6-undecanone (ketone) (68738, Sigma-Aldrich, Australia), cyclohexanol (alcohol) (44113, Sigma-Aldrich, Australia), undecanoic acid methyl ester (ester) (U0250, Sigma-Aldrich, Australia), pentadecanal (aldehyde) (P1869, ChemSupply Australia), and pentadecanoic acid (carboxylic acid) (P6125, Sigma-Aldrich, Australia). These compounds were selected based on their absence in meat, as well as their Kovats Retention Index to ensure elution did not interfere with meat analytes. The linearity between concentration and peak area was assessed for each representative compound to select appropriate concentrations to spike onto samples. Based on these results the IS contained a final concentration of 125 µg/g for cyclohexanol and pentadecanoic acid, and 1.2 µg/g for 6-undecanone, undecanoic acid methyl ester, and pentadecanal.

HS-SPME sampling was performed using an automated Gerstel Multi-Purpose Autosampler (Gerstel Gmbh & Co, Mullheim an der Ruhr, Germany) equipped with SPME and heated agitator modules. Samples were heated at 80°C for 30 min with an agitator program of 5 sec on, 1 sec off at a speed of 400 rpm. Thereafter, the pre-conditioned fibre (prepared as

described above) was inserted into the sample vial and exposed for 20 min to capture headspace volatiles. The fibre was then inserted into the GC injector for thermal desorption of volatiles for 4 min. A blank vial was included in all runs to assess impurities and carry over from samples.

4.2.3.2 Gas chromatography/mass spectrometry

Analysis was conducted using a Varian CP3800 GC (Bruker Corporation, USA) coupled to a Bruker 300MS triple quadrupole MS with a split/splitless injector in splitless mode. Compound separation was carried out using Agilent DB-5MS capillary column 30 m x 0.25 mm x 0.25 mm film thickness (Agilent Scientific, USA) with the following conditions: carrier gas (helium) with a flow rate of 1 mL/min, injector temperature 270°C. Oven initial temperature 40°C, held for 4 min, to 80°C at 6°C/min, to 250°C at 8°C/min and to 290°C at 25°C/min. The MS was operated in full scan analysis (m/z) 35 - 350. Source temperature was 220°C and electron impact ionisation (EI) at 70 eV was used.

4.2.3.3 Library building and identification of compounds

MS Workstation version 7 (Bruker Corporation, USA) was used to plot and process chromatogram and spectra obtained. Compounds were identified by comparing: (i) Kovats Retention Index (KI) based on a homologous series of n-alkanes (C6–C40, Supelco, Bellefonte, USA) with those of analyte compounds and by comparison with literature data; and (ii) mass spectrometry data with reference spectra of compounds obtained from the National Institute of Standards and Technology (NIST, USA) library (NIST/EPA/NIH Mass Spectral Library with Search Program, data version NIST 2017). All identified compounds were added to a library which was used to identify compounds in other samples. For this process the quantifying ion was set at Reconstructed Ion Chromatogram, while each chromatogram was integrated by

filtering the peak at 5 smooth points with an integration window set at 0.150 min, peak width (sec ½HZ) and peak size rejection (counts) set at 5000. This was manually screened to ensure correct integration. The volatile compounds were semi-quantified by dividing the peak areas of the compounds of interest by the peak area of the relevant IS compound class. This ratio was multiplied by the concentration of the relevant IS and divided by the mass of the sample (expressed as µg/g). The peak area of IS undecanoic acid methyl ester was used for those identified compounds that were not represented by the compounds classes that were included as IS.

4.2.4 Statistical analysis

Prior to analysis the sequence abundance data was transformed with a square root function (Jeffries et al., 2015; Osborne, 2002) and the semi-quantitative volatilome data was standardised by total concentrations following a fourth root transformation. The boneless and bone-in trials were analysed separately for both the sequence and volatilome datasets using PRIMER v7 with PERMANOVA + software (Primer-E Ltd., Massey University, Auckland, New Zealand). A distance matrix using Bray Curtis coefficient was calculated for each dataset (Bray and Curtis, 1957). PERMANOVA (permutational multivariate ANOVA) was used to test for differences in bacterial communities and volatile components under fixed (time, treatment) and random (odour score) factors (Anderson and Gribble, 1998). Monte Carlo simulations were used to test significance and interactions with analysis designs assumed type III partial sums of squares. Differences were considered significant at $p < 0.01$ (using 9999 unrestricted permutations). Canonical analysis of principal coordinates (CAP) was used to visualize and confirm community differences in relation to measurable factors (time,

treatment and odour score category) with data plotted along simple axes expressing similarity and dissimilarity among samples (Anderson and Willis, 2003).

Diversity indices analysis was conducted on sequence data by calculating Shannon Weiner index (H', log base e), Pielou's evenness (J'), and Fisher's α-diversity in PRIMER-7 according to time and treatment. Sequence data was also visualised according to time by relative abundance plots using the top 100 most abundant sequences, representing 99.8 – 99.4% of total sequences for bone-in and boneless samples, respectively. Sequences were summed by genus, and replicates within each timepoint were averaged. Each timepoint was standardised by total sequences. The relative abundance plots only include the control sequence data. This is because the results (see section 4.3.1) indicate that glucose treatments only had a subtle effect on community composition therefore no differences were evident at a genus level.

4.2.4.1 Analysis of composition of microbiomes

Analysis of composition of microbiomes (ANCOM) was used to find taxonomic differences associated with spoilage of VP lamb, and in particular the effects of glucose treatments. Taxonomic differences were determined between groups of the variables tested, which included storage time, glucose treatment and odour score category. Meat odour scores were categorised into the following groups for analysis: none/slight (odour score $6 \geq x \leq 8$), slight/moderate (odour score $4 > x < 6$), moderate/strong (odour score $2 > x \leq 4$), strong/extreme (≤ 2). Significant differences in community composition was defined as W-statistic > 0.9. Storage time was included as a covariate for all ANCOMs to account for the interaction between time and tested factors. All analyses were performed using ANCOM v2.1 (https://github.com/FrederickHuangLin/ANCOM) in RStudio (v. 4.0.3) and the following packages: 'nlme', 'tidyverse', 'compositions', and 'ggplot2'.

4.2.4.2 Estimating growth kinetics from sequence analysis

The growth kinetics of selected ASVs were estimated from sequence data to determine the effects of glucose treatments over storage duration. The ASVs that were considered for this analysis were those that were significantly different between glucose treatments according to the ANCOM analysis. The most abundant ASVs for *Clostridium algidicarnis*, *Serratia proteamaculans*, and *Carnobacterium divergens* were also included due to their significant contribution to the spoilage rate of VP lamb established in chapter 2 of this study. *Clostridium esthertheticum* and *Clostridium putrefaciens* were not included in this analysis, despite their significant role in spoilage (see chapter 2). This is because neither were major components of the microbial community on either bone-in or boneless VP lamb in this experiment. Furthermore, *Clostridium putrefaciens* was not detected in any of the samples.

Estimating absolute numbers from the sequence data was calculated in accordance with Kaur et al. (2017b). Briefly, the number of reads classified for a given ASV was first normalised by the total number of reads per respective meat sample. The TVC for each sample was then used to convert the normalised fraction into predicted $\log_{10}$ estimates of abundance. The growth rates, lag phases and maximum populations of ASVs of interest were calculated using the D-model (Baranyi and Roberts, 1994).

4.2.4.3 Volatile analysis

Linear Discriminant Analysis (LDA) was used to identify which volatiles were the best predictors for storage times. This was achieved by using the greedy Wilks function calculated using R library klaR (Weihs et al., 2005) to select predictive volatiles followed by LDA using the selected predictors. The LDA was calculated using the LDA function in the R MASS library (Venables and Ripley, 2002). Analysis of Variance (ANOVA) was used to determine how the

concentration of the time predictor volatiles changed over time using Proc Mixed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

4.3 Results

4.3.1 Microbial community structure of bone-in and boneless VP lamb

There was a total of 5,945,295 sequences that were assigned to 10,434 ASVs representing 10 phyla, of which 98% belonged to phyla *Firmicutes* and *Proteobacteria*. Most sequences (~ 95%) belonged to 17 ASVs representing 12 genera (*Aeromonas, Buttiauxella, Brochothrix, Carnobacterium, Clostridium, Ewingella, Lactobacillus, Pseudomonas, Rahnella, Serratia, Vagococcus* and *Yersinia*). Bone-in meat samples (n = 183) had a total of 2,358,920 sequences and a mean number of 12,890 (min: 34 – max: 34,306) sequences per sample. Boneless meat samples (n = 161) had a total of 3,586,375 sequences with an average of 22,276 (min: 1,720 – max: 563,448) sequences per sample.

PERMANOVA showed that storage time ($p = 0.0001$) had a significant effect on bacterial community structure of bone-in and boneless lamb. Pair-wise analysis for time showed significant differences ($p < 0.01$) in community structure between most timepoints for both bone-in and boneless lamb. CAP analysis further confirmed this observation with overall 60.1% and 60.2% exact classifications of bone-in and boneless lamb samples, respectively, into the different timepoints (Figs. 4.1 and 4.2).

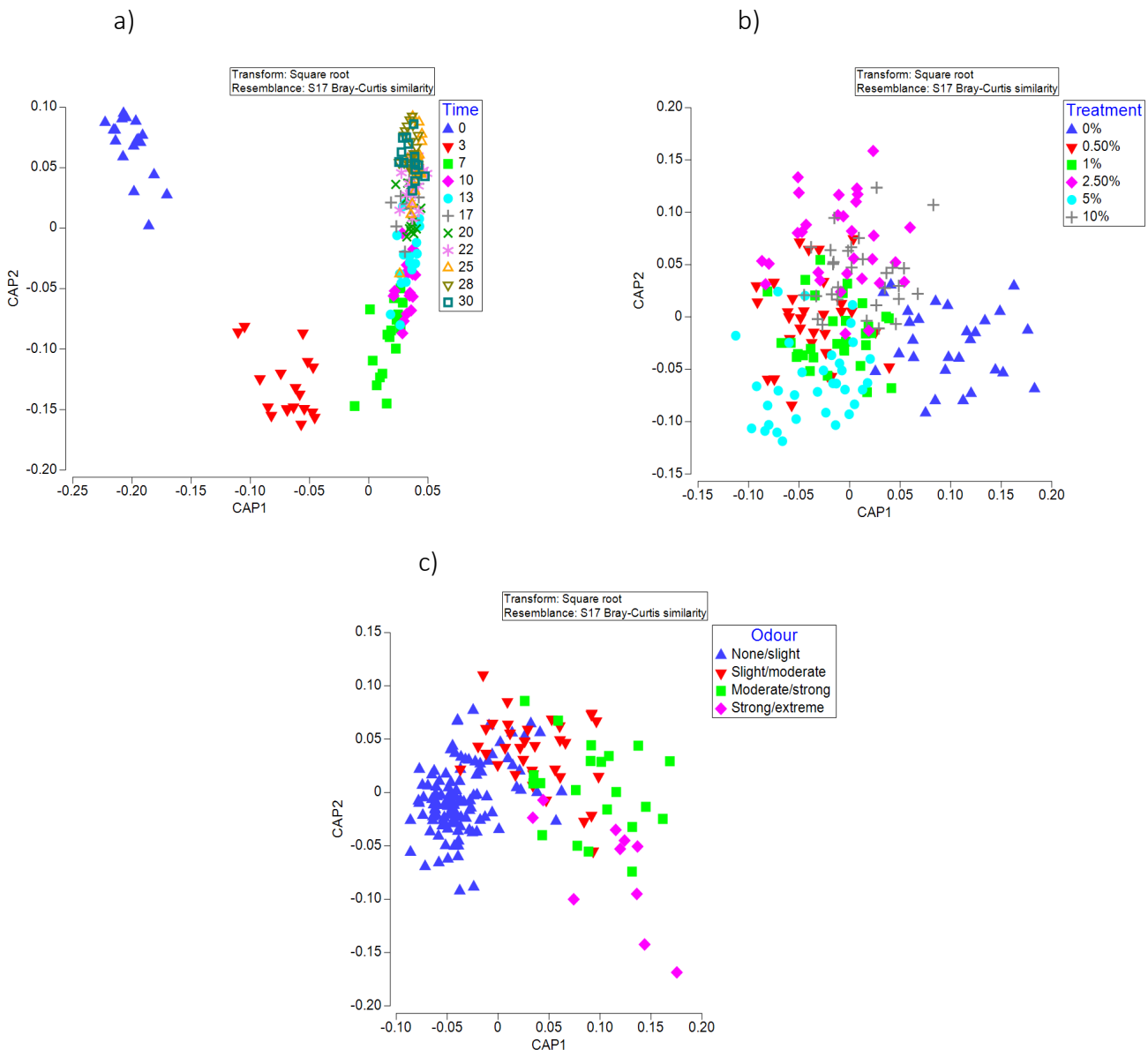

Figure. 4.1 Canonical analysis of principal coordinates (CAP) plots of bacterial composition data from vacuum packaged bone-in lamb shoulder samples stored at 4°C. The CAP plots are based on (a) storage time, (b) treatment, (c) off-odour. Colours denote the sample storage time, glucose treatment or odour. PERMANOVA significance tests are reported in the text.

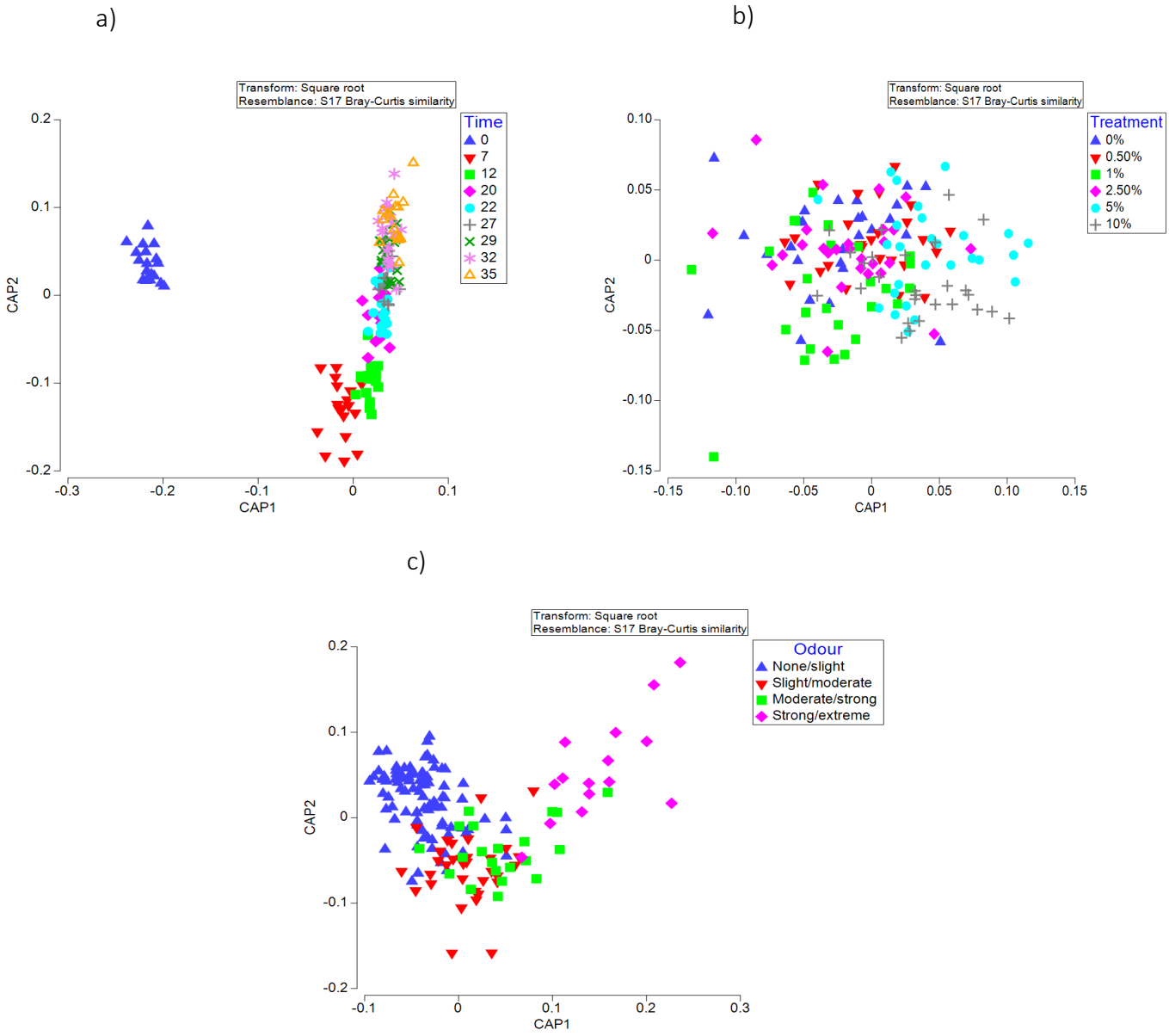

Figure 4.2 Canonical analysis of principal coordinates (CAP) plots of bacterial composition data from vacuum packaged boneless lamb shoulder samples stored at 4°C. The CAP plots are based on (a) storage time, (b) treatment, (c) off-odour. Colours denote the sample storage time, glucose treatment or odour. PERMANOVA significance tests are reported in the text.

Diversity indices and relative abundance plots were used to visualise the sequence data according to time for bone-in and boneless lamb. It was found that the diversity indices for the microbial communities on bone-in and boneless lamb converged as storage time progressed. Diversity indices analysis illustrated how much more diverse, rich and even the initial community was compared to the community that developed over the storage period under VP conditions for both bone-in and boneless samples (Figs 4.3 and 4.4). Species diversity, evenness and richness declined during the first 7 days of storage and plateaued for the remainder of the trial for both bone-in and boneless samples, indicating the development of a stable community.

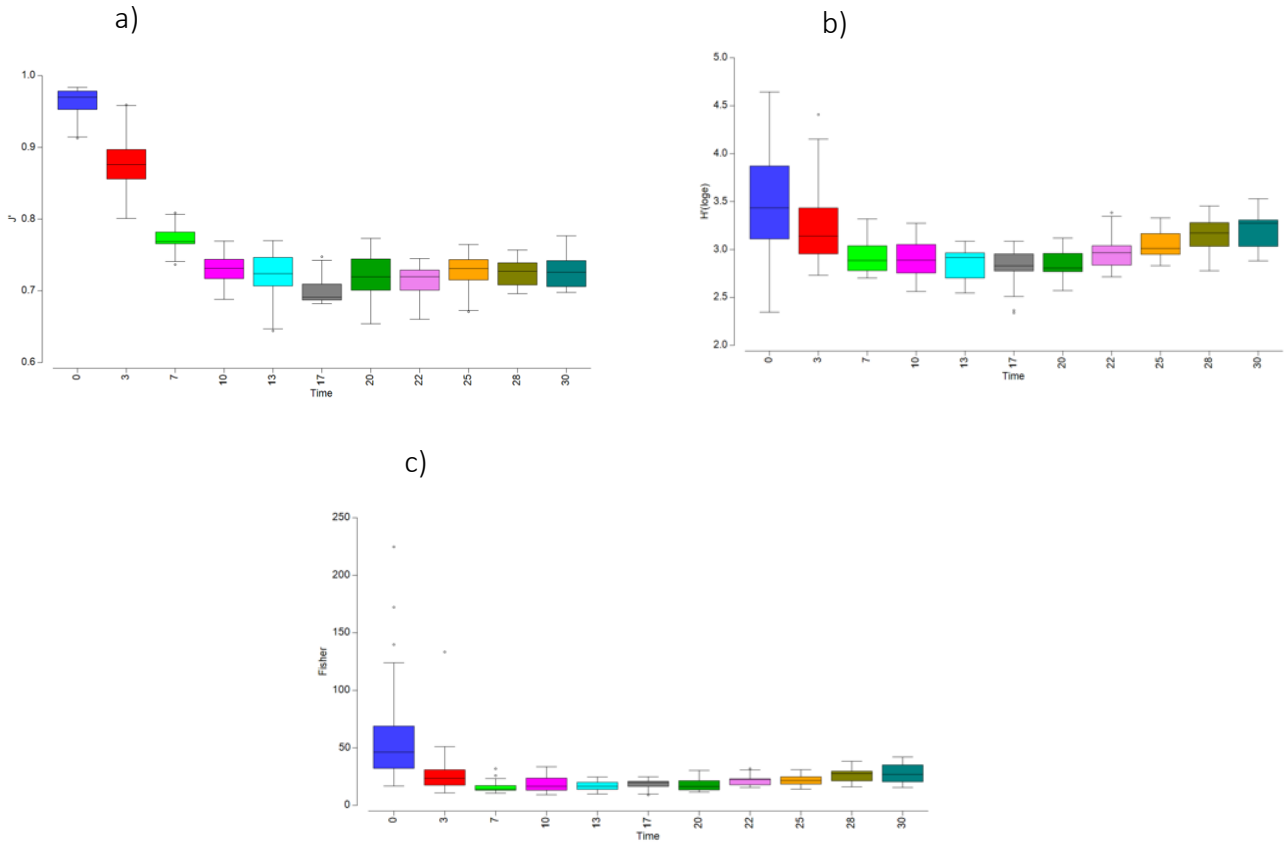

Figure 4.3 Diversity indices for the microbial community on VP bone-in lamb samples plotted according to storage time at 4°C: a) Pielou's evenness (J'); b) Shannon-Weiner index (H', log base e) and c) Fisher's alpha diversity.

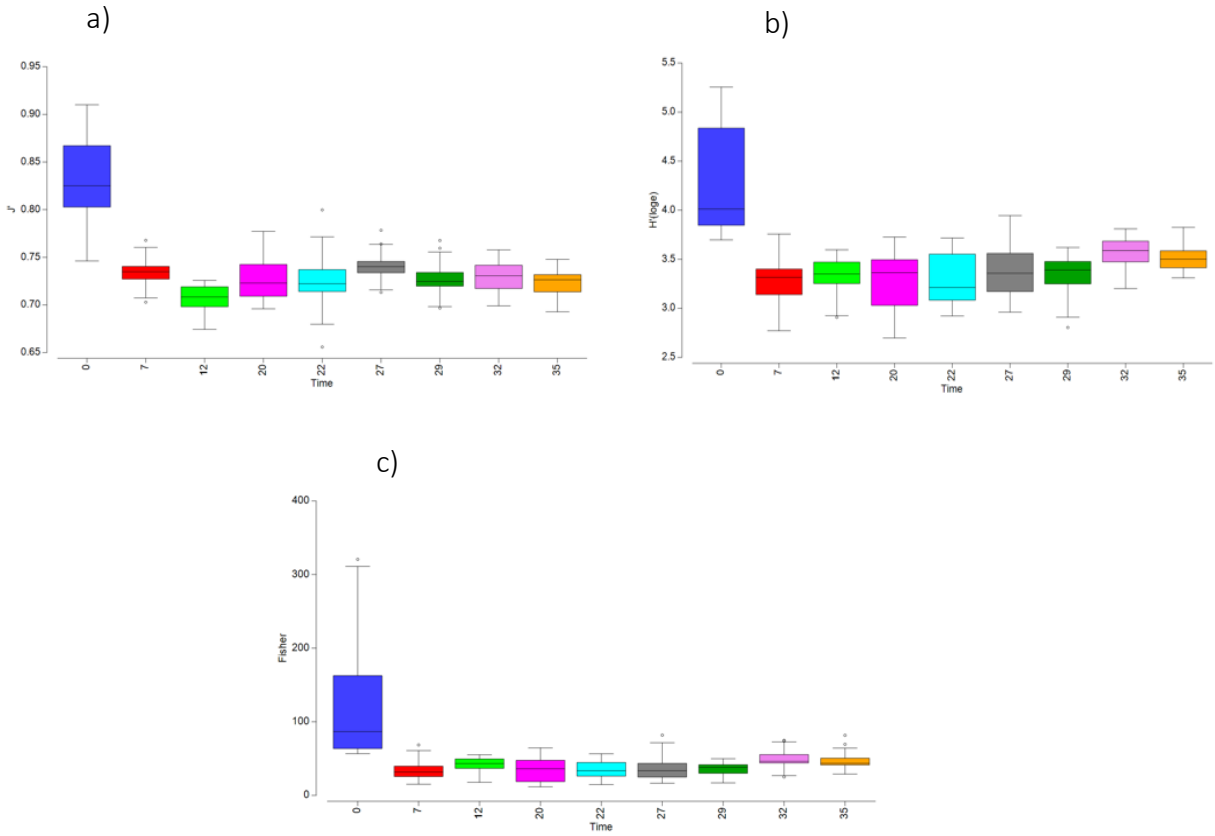

Figure 4.4 Diversity indices for the microbial community on VP boneless lamb plotted samples according to storage time at 4°C: a) Pielou's evenness (J'); b) Shannon-Weiner index (H', log base e) and c) Fisher's alpha diversity.

The major community components of the bone-in samples that had an increasing relative abundance from day 0 to the end of the trial (day 30) were *Carnobacterium* (38.2 –55.9%), *Ewingella* (3.9 – 15.9%), *Serratia* (4.2 – 11.5%), *Clostridium* (0.1 – 4.0%), *Rahnella* (0.2 – 2.8%) and *Yersinia* (0.3 – 2.1 %) (Fig. 4.5). In contrast, the major genera of the initial community that declined in relative abundance over the storage period on the bone-in control samples were *Aeromonas* (9.5 – 2.1%), *Buttiauxella* (2.3 – 1.9%), *Vagococcus* (7.8 – 1.8%), *Brochothrix* (11.7 – 0.7%), *Shewanella* (4.4 – 0.38%), *Pseudomonas* (4.5 – <0.1%) and *Lactococcus* (2.4 – 0.1%).

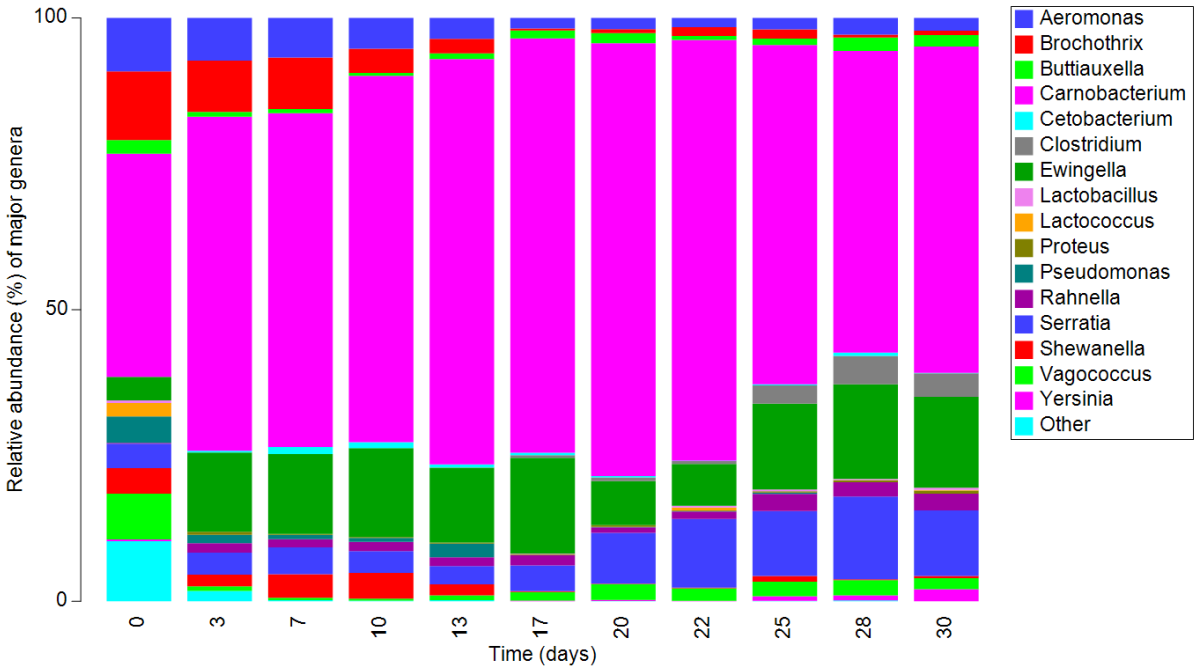

Figure 4.5 The relative abundance (%) of the major genera on VP bone-in lamb shoulder samples represented over storage time at 4°C.

The major genera of the boneless samples which increased in relative abundance from 0 to 35 days were *Clostridium* (< 0.1 – 21.8%), *Buttiauxella* (0.3 – 16.2%), *Vagococcus* (0.1 – 14.9%), *Serratia* (0.2 – 3%), *Ewingella* (< 0.1 – 3.3%), *Lactococcus* (0.1 – 1.6%), *Yersinia* (< 0.1 – 1.4%), *Rahnella* (0.1 – 1.3%) and *Moellerella* (< 0.1 – 1.1%) (Fig. 5). *Carnobacterium* was the most relatively abundant genus on boneless lamb at all timepoints, although it reduced from an initial relative abundance of 52.8% to 24.4% by the end of the trial. The genera that initially had a relatively high abundance but declined overtime were *Brochothrix* (26.6 – 7.9%), *Pseudomonas* (11.9 – < 0.1%) and *Shewanella* (1.5 – 0.2%).

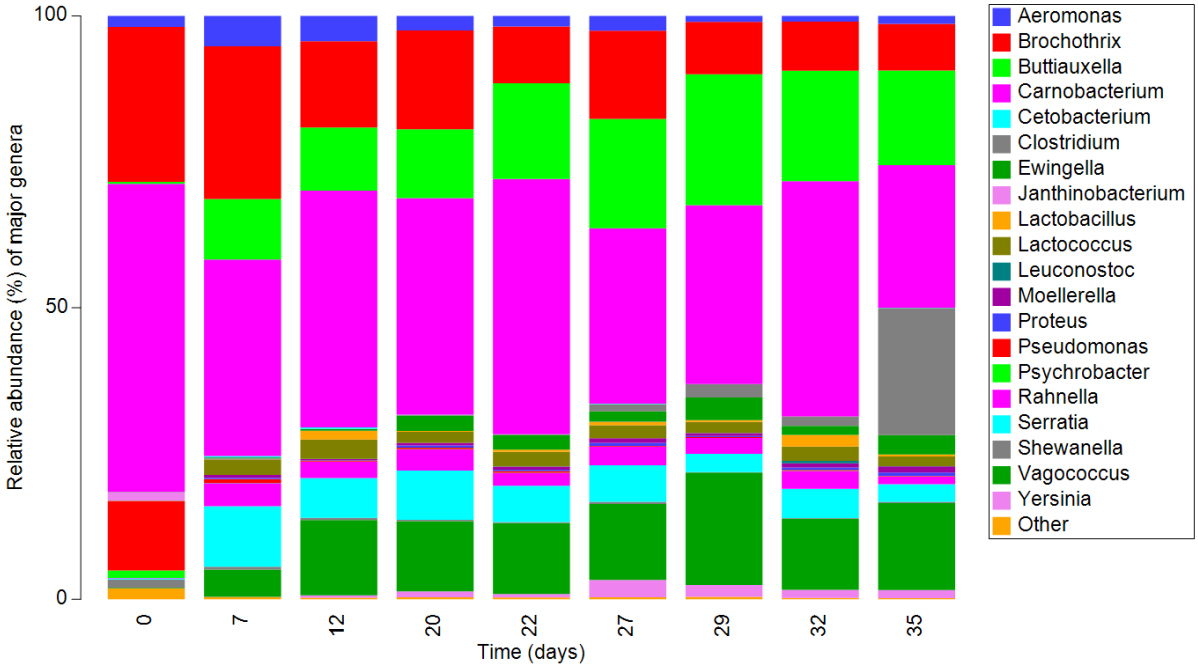

Figure 4.6 The relative abundance (%) of the major genera on VP boneless lamb shoulder samples represented storage time at 4°C.

PERMANOVA showed that glucose treatments also had a significant effect on community structure for the bone-in samples (p = 0.0012). Pair-wise analysis showed that the greatest difference was between 5% and the control (p = 0.0053), while differences between the other glucose treatments and the control were more marginally significant (0.5% p = 0.0148; 1% p = 0.0128; 2.5% p = 0.0297; 10% p = 0.0481). All glucose treatments appeared to have less of an effect on microbial community structure for the boneless samples, showing an overall marginal significant difference (p = 0.0149). Pair-wise analysis for the boneless samples also showed marginal differences between the control and glucose treatments 1% (p = 0.031), 5% (p = 0.0421) and 10% (p = 0.0322). No difference was evident between the control compared to 0.5% and 2.5% glucose treatments. The subtle effects that the glucose treatments had on the bacterial community structure was further highlighted by CAP analysis, which showed only 33.9% and 36.1% exact classifications into treatment groups for

bone-in and boneless samples, respectively (Figs. 4.1 and 4.2). In accordance, diversity indices did not differ between treatments. PERMANOVA pairwise analysis revealed that for the bone-in meat samples, the slight/moderate odour category was marginally significantly different between moderate/strong odour (p = 0.011) and strong/extreme odour (p = 0.047). Slight-moderate odour was also marginally significantly different from strong-extreme odour (p = 0.012) for the boneless meat samples. In accordance, CAP analysis showed clustering between odour categories, and had an overall 71% and 68% exact classification of bone-in and boneless lamb samples, respectively, into the different categories (Figs 4.1 and 4.2).

4.3.2 Analysis of composition of microbiomes

ANCOM was used to find compositional taxonomic differences between timepoints, glucose treatments, and odour scores. The results presented in the heatmaps show the taxonomic assignments of ASVs represented in the meat microbial community that significantly differed in sequence number between groups (Figs. 4.7 to 4.9). Treatment replicates were pooled together in the ANCOM for time. This was because treatments showed no differences in diversity indices, relative abundance, and only a marginal significant difference in community composition. The analysis revealed 36 and 65 ASVs that were significantly different over the storage period for the bone-in and boneless lamb samples, respectively (Figs. 4.7a and 4.7b). The ASVs that changed in sequence number most significantly on bone-in and boneless meat over storage time were *Clostridium algidicarnis, Carnobacterium divergens, Ewingella americana*, *Buttiauxella* sp. (ASV09208), *Aeromonas salmonicida*, *Lactobacillus sakei*, *Vagococcus* sp. (ASV06133), *Yersinia* sp. (ASV10657), *Serratia proteamaculans*, *Carnobacterium* sp. (ASV01923), *Vagococcus fessus, Proteus vulgaris*, *Brochothrix* sp. (ASV04681), and *Rahnella aquatilis*. A major difference between the bone-in and boneless

meat was that *Pseudomonas* sp. (ASV08426) read number significantly increased on the bone-in samples over the storage period (Fig. 4.7a), whereas on boneless samples it significantly decreased (Fig. 4.7b). The rest of the significant ASVs are presented in Figs. 4.7a and 4.7b, a majority of which were initially present but declined over the storage period.

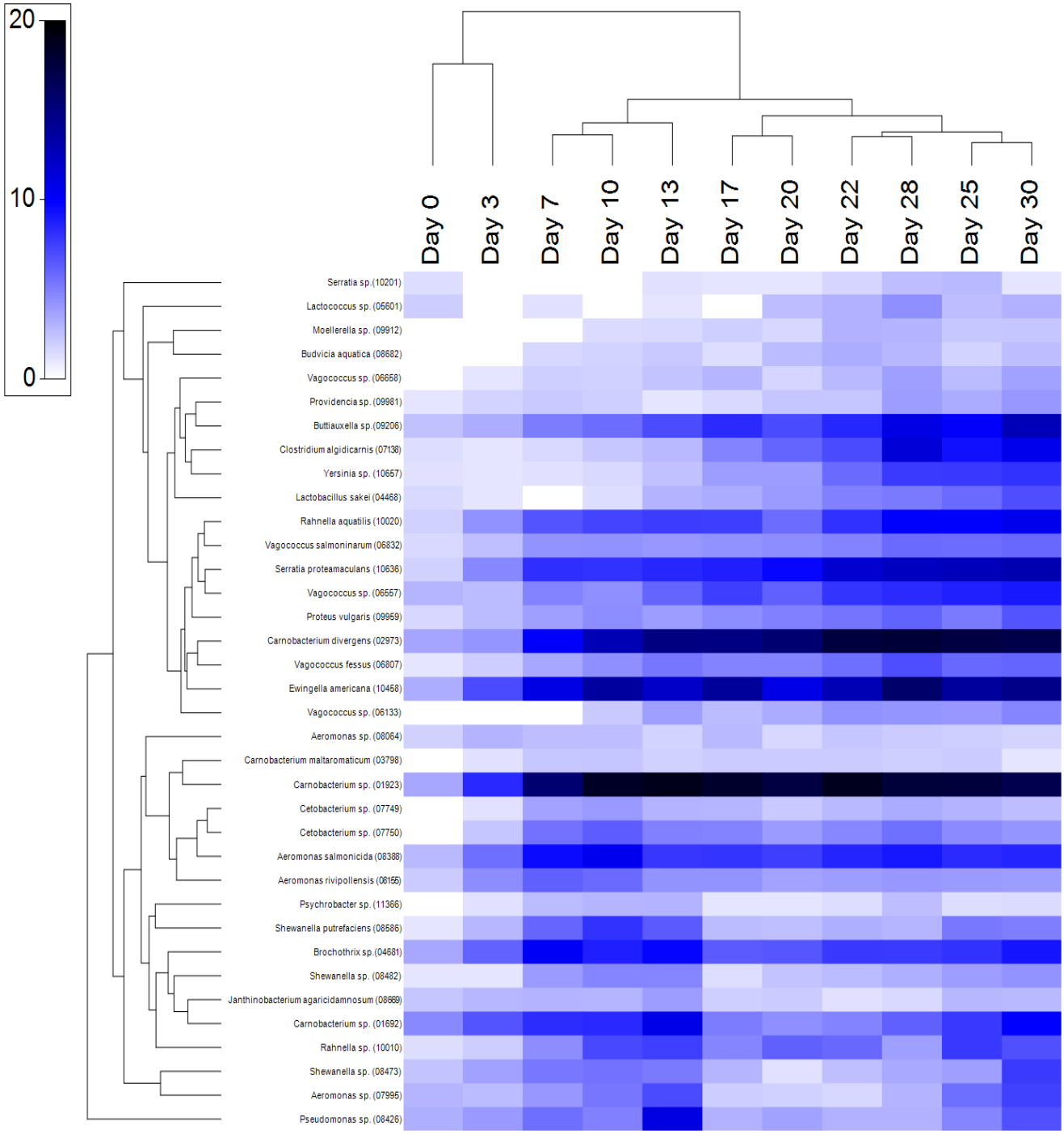

Figure. 4.7a Heatmap showing relative sequence abundance of ASVs that were significantly different between storage time (days) on bone-in VP lamb shoulder samples stored at 4°C. ASV identification number are in brackets. Raw sequence data was normalised by applying square root transformation.

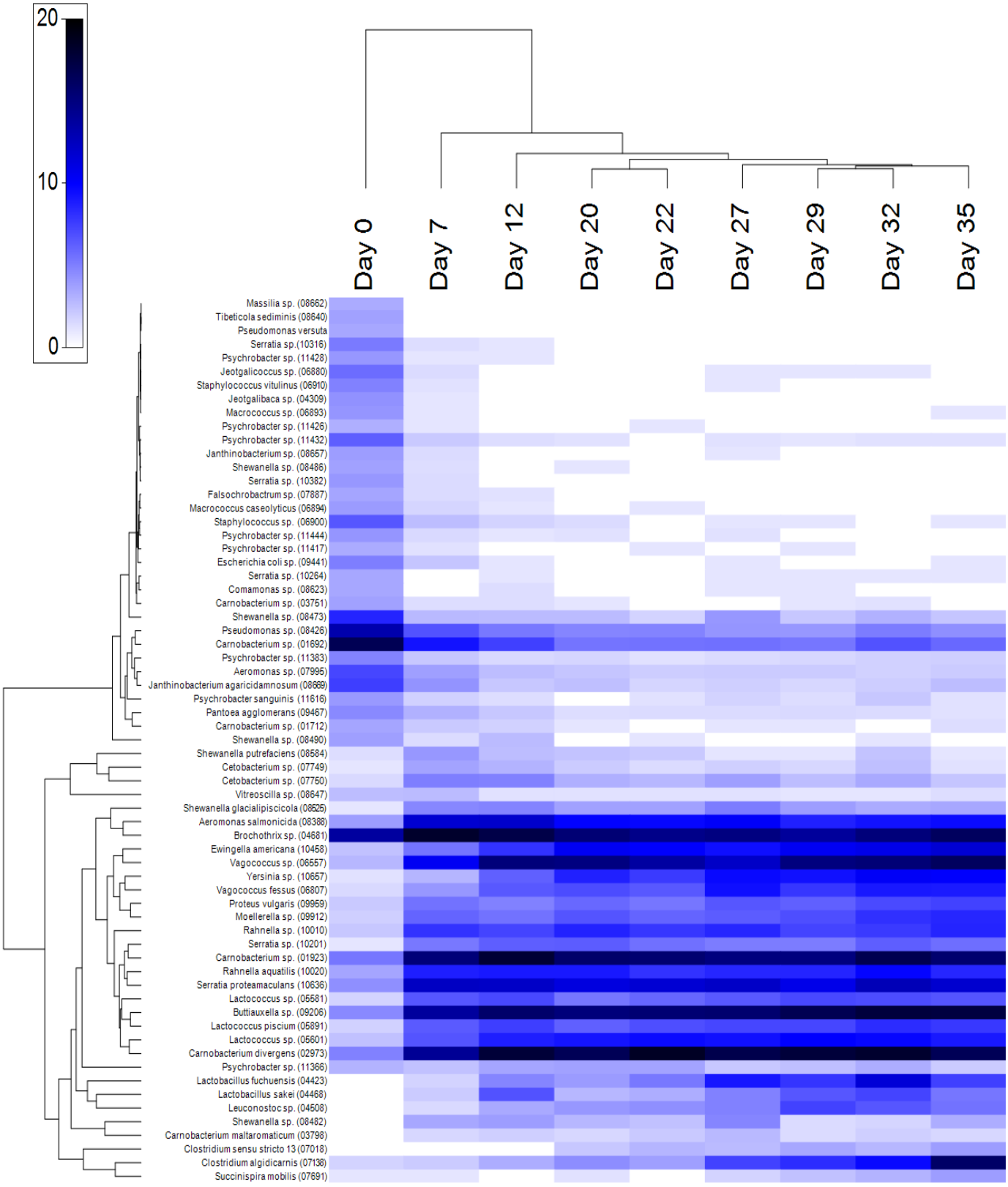

Figure 4.7b Heatmap showing relative sequence abundance of ASVs that were significantly different between storage time (days) on boneless VP lamb shoulder samples stored at 4°C. ASV identification number are in brackets. Raw sequence data was normalised by applying square root transformation.

All ANCOMs that used treatment or odour as the main variable included storage time as a covariate to account for the variation that can be ascribed to storage. The analysis revealed 6 and 3 ASVs that were significantly different between glucose treatment groups for the bone-in and boneless lamb samples, respectively (Figs. 4.8a and 4. 8b.). For the bone-in meat samples, the control had a significantly higher read number of *Serratia proteamaculans* compared to all glucose treatment groups (Fig 4.8a). The 0.5%, 1% and 5% glucose treatments formed a cluster, all of which had significantly lower read numbers of *Vagococcus* sp. (ASV06133) and *V. fessus* compared to the control, 2.5% and 10% glucose treatments. Similarly, *Pseudomonas* sp. (ASV08426) had significantly lower read numbers in the 0.5%, 1% and 5% glucose treatments, of which the 1% and 5% had the lowest read number. The 5% glucose treatment had the highest read number of *Shewanella* sp. (ASV08469) compared to all other treatments and the control. The 2.5% and 10% glucose treatments were clustered together, both having significantly higher read numbers of *Pseudomonas* sp. and *Buttiauxella* sp. (ASV09206) compared to the control and other glucose treatments. *Pseudomonas* sp. read number was especially high in the 2.5% glucose treatment.

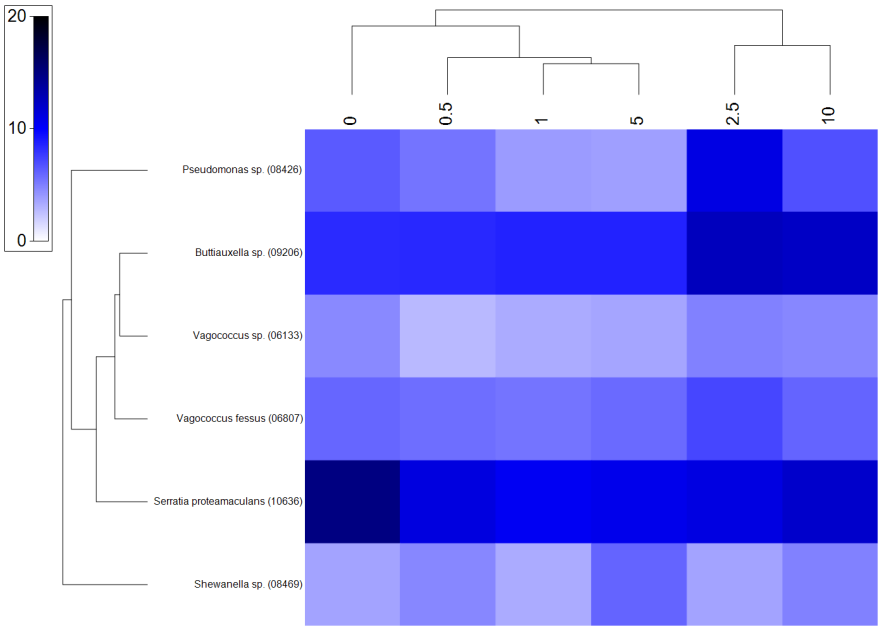

Figure 4.8a Heatmap showing relative sequence abundance of ASVs that were significantly different between glucose treatments (%) on bone-in VP lamb shoulder samples stored at 4°C. ASV identification number are in brackets. Raw sequence data was normalised by applying square root transformation.

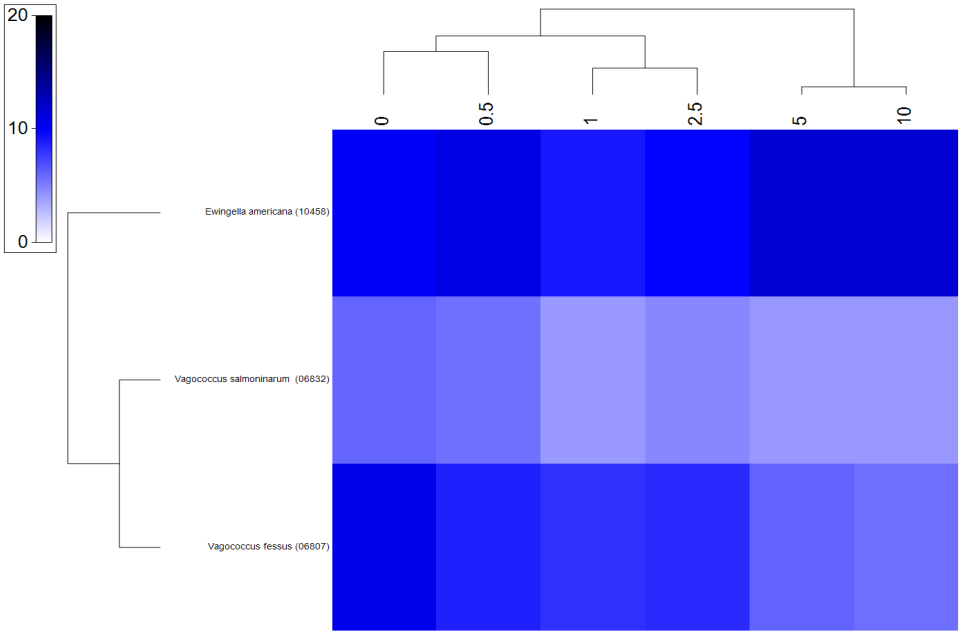

Figure 4.8b Heatmap showing relative sequence abundance of ASVs that were significantly different between glucose treatments (%) on boneless VP lamb shoulder samples stored at 4°C. ASV identification number are in brackets. Raw sequence data was normalised by applying square root transformation.

For the boneless meat samples, the control and 0.5% glucose treatments clustered together, both having significantly higher read numbers of *Vagococcus salmoninarum* and *V. fessus* compared to all other glucose treatments (Fig 4.8b). The 5 and 10% glucose treatments clustered together and had the lowest read number of *V. salmoninarum* and *V. fessus* compared to all other treatment groups and the control. The 5 and 10% glucose treatments also had significantly higher read numbers of *E. americana*.

ANCOM revealed 25 and 20 ASVs that were significantly different between odour categories for the bone-in and boneless lamb samples, respectively (Figs. 4.9a and 4.9b). The hierarchical clustering shows that read numbers of significant ASVs are most different between 'none to slight' and 'strong to extreme' odours, while 'slight to moderate' and 'moderate to strong' off odours were more similar, which agrees with the CAP analysis for both bone-in and boneless meat (section 4.3.1).

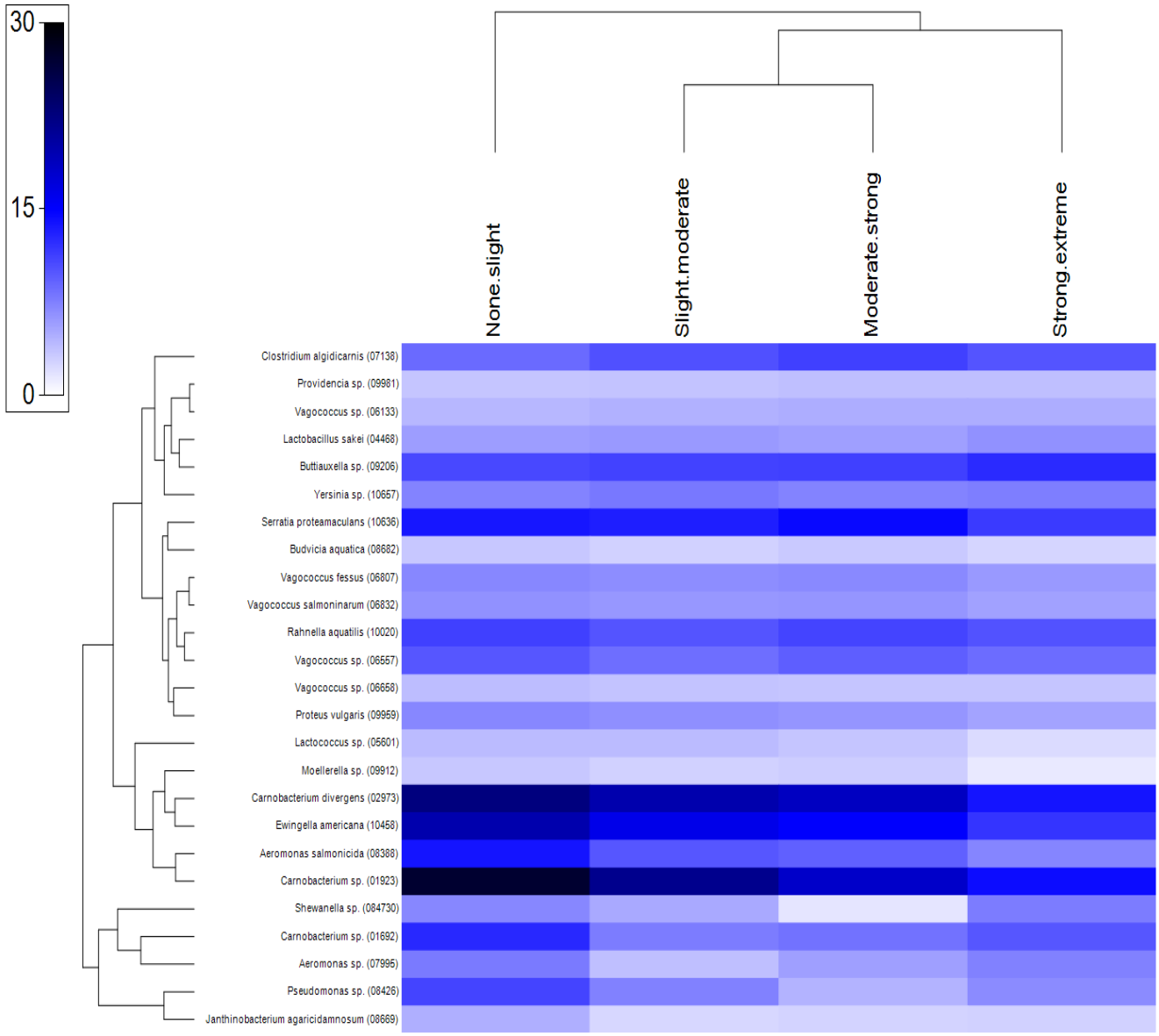

Figure 4.9a Heatmap showing relative sequence abundance of ASVs that were significantly different between off-odour categories of bone-in VP lamb shoulder samples stored at 4°C. ASV identification number are in brackets. Raw sequence data was normalised by applying square root transformation.

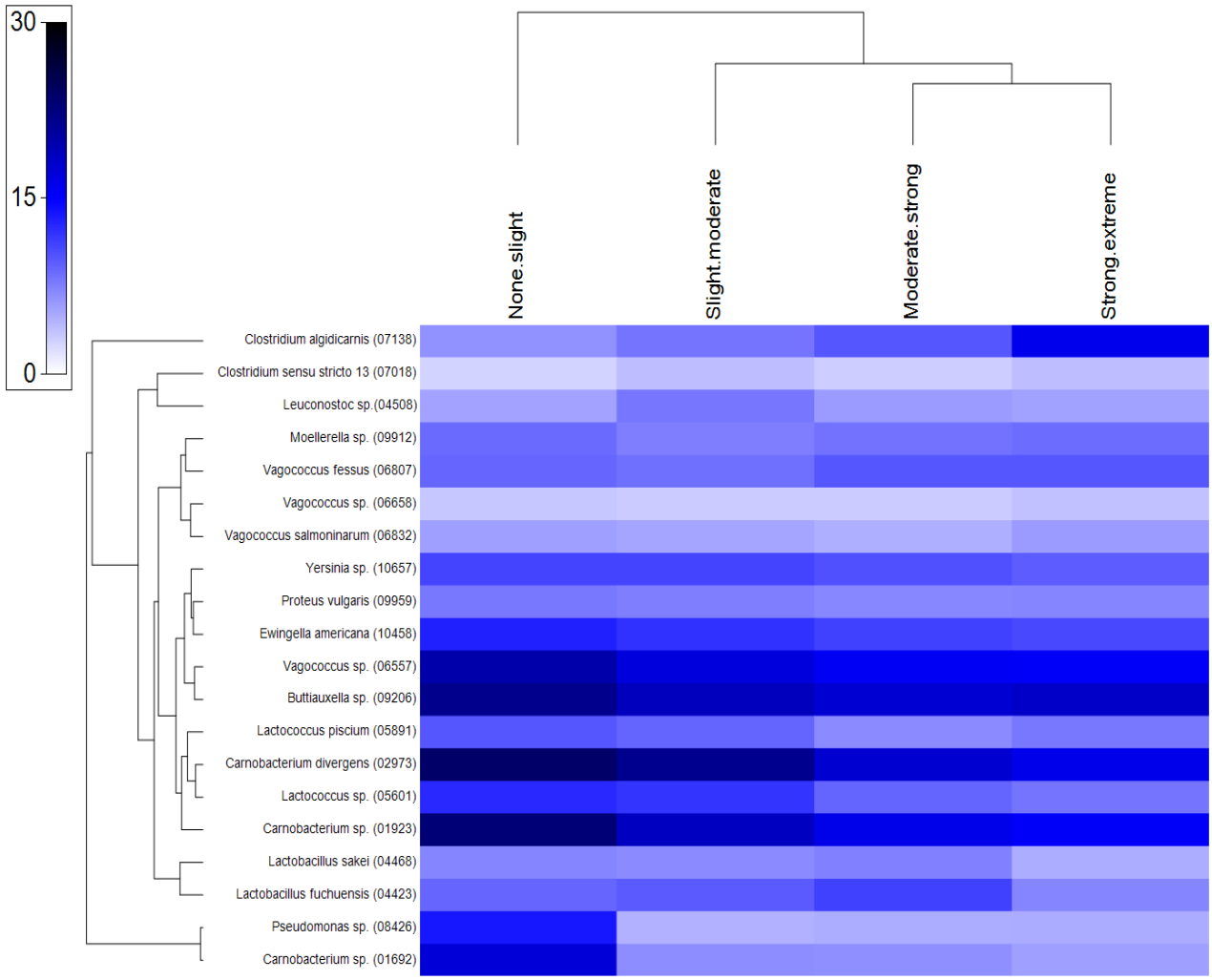

Figure 4.9b Heatmap showing relative sequence abundance of ASVs that were significantly different between off-odour categories of boneless VP lamb shoulder samples stored at 4°C. ASV identification number are in brackets. Raw sequence data was normalised by applying square root transformation.

For the bone-in samples, *C. algidicarnis* and *Buttiauxella* sp. (09206) were the most significant ASVs associated with moderate to strong (odour score $2 > x \leq 4$), and strong to extreme off odour (≤ 2) (Fig. 4.9a). *S. proteamaculans* also had a significantly higher read number in the moderate to strong off odour category compared to all other odour categories. In contrast, *C. divergens*, *Carnobacterium* sp. (ASV01692), *E. americana*, *Proteus vulgaris*, and *Vagococcus* sp. (ASV06557) were significantly more associated with meat of

high sensory quality (odour score 6 ≥ x ≤ 8). The rest of the significant ASVs are presented in Figure 4.9a.

For the boneless lamb samples, the most significant ASVs associated with moderate to strong (2 > x ≤ 4) and strong to extreme off (≤ 2) odours were *C. algidicarnis* and *V. fessus* (Fig. 4.9b). *Lactobacillus fuchuensis* also had a significantly higher read number in the moderate to strong off odour category compared to all other odour categories. The ASVs that were significantly more associated with meat of high sensory quality were *Yersinia* sp. (ASV10657), *E. americana*, *C. divergens*, *Buttiauxella* sp. (09206), *Vagococcus* sp. (ASV06557), *Proteus vulgaris*, and *Lactococcus* sp. (ASV01692).

4.3.3 Growth kinetics of representative bacteria

The growth kinetics of selected ASVs were estimated from sequence data to determine the effects of glucose treatments on growth of potential spoilage organisms over the storage period. Growth rates, lag phases and maximum populations for ASVs of interest from the control and the 5% glucose treatment are shown in Table 4.1 and 4.2. The growth data for representative ASVs for all glucose treatments were also plotted according to time and are included in Appendix A.

For the bone-in lamb samples, growth kinetics could not be accurately calculated for *Shewanella* sp. (ASV08469), *Vagococcus* sp. (ASV06133), and *Pseudomonas* sp. (ASV08426) due to poor fit to the model (r^2 = < 0.5) (Table 4.1). *Buttiauxella* sp. also had similar growth kinetics for the control and the 5% glucose treated samples, both having a growth rate of 0.4 $\log_{10}$ CFU/cm^2/day, no lag phase, and reached maximum populations of 5.6 – 5.8 $\log_{10}$ CFU/cm^2. In contrast, the growth kinetics of *V. fessus* were notably different between the control and the 5% glucose treatment group. Specifically, *V. fessus* appeared to have a faster

growth rate of 0.9 ± 0.2 $\log_{10}$ CFU/cm^2/day and smaller maximum population density of 4.6 ± 0.2 $\log_{10}$ CFU/cm^2 for the 5% glucose treatment group compared to the control which had a growth rate of 0.3 ± 0.1 $\log_{10}$ CFU/cm^2/day and maximum population of 5.2 ± 0.2 $\log_{10}$ CFU/cm^2. Further, *V. fessus* had a lag phase of 5.4 ± 1.0 days on meat treated with 5% glucose whereas no lag was evident for the control. *S. proteamaculans* had a growth rate of 0.5 $\log_{10}$ CFU/cm^2/day for both the 5% glucose treatment and the control. However, a lag phase of 7.7 ± 3.0 days was evident for the 5% glucose treated samples while no lag was evident for the control. Further, *S. proteamaculans* had a smaller maximum population density of 6.3 ± 0.1 $\log_{10}$ CFU/cm^2 on the 5% glucose treated meat compared to the control (6.8 ± 0.1 $\log_{10}$ CFU/cm^2).

The ASVs that were included to represent those species that had a significant effect on spoilage rate in chapter 2 showed no difference in growth kinetics between the control and glucose treatments. Specifically, *C. divergens* had a growth rate of 0.6 - 0.7 ± 0.1 $\log_{10}$ CFU/cm^2/day, a lag phase of 2.2 – 3.2 ± 1.0 days and reached a maximum population of 7 ± 0.1 $\log_{10}$ CFU/cm^2. *C. algidicarnis* had a growth rate of 0.4 ± 0.1 $\log_{10}$ CFU/cm^2/day, a lag phase of 5.4 – 5.7 ± 2.1 days and reached a maximum population of 6.2 – 6.7 $\log_{10}$ CFU/cm^2.

Table 4.1 Growth kinetics (± 95% CI) of representative ASVs relative to the TVC on VP bone-in lamb meat stored at 4°C estimated directly from 16S rRNA gene sequence data. Growth kinetics were calculated for the control and the most effective glucose treatment (5%) for shelf-life extension.

	Growth rate (log_{10} CFU/cm^2/day)		Lag (days)		Maximum population (log_{10} CFU/cm^2)	
	0%	5%	0%	5%	0%	5%
Vagococcus fessus (ASV06807) [a]	0.3 ± 0.1	0.9 ± 0.2	No lag	5.4 ± 1.0	5.2 ± 0.2	4.6 ± 0.2
Buttiauxella (ASV09206) [a]	0.4 ± 0.0	0.4 ± 0.0	No lag	No lag	5.8 ± 0.1	5.6 ± 0.2
Serratia proteamaculans [a,b] (ASV10636)	0.5 ± 0.1	0.5 ± 0.2	No lag	7.7 ± 3.0	6.8 ± 0.1	6.3 ± 0.1
Carnobacterium divergens [b] (ASV02973)	0.6 ± 0.1	0.7 ± 0.1	2.2 ± 1.4	3.2 ± 1.0	7.1 ± 0.1	7.0 ± 0.1
Clostridium algidicarnis [b] (ASV07138)	0.4 ± 0.0	0.4 ± 0.1	5.4 ± 2.1	5.7 ± 2.0	6.7 ± 0.4	6.2 ± 0.3

a. ASVs that were significantly different between treatment groups according to ANCOM results.
b. Most abundant ASVs for species that significantly contributed to the spoilage rate of VP lamb in chapter 2 of this study.

For the boneless lamb samples, *V. fessus* and *V. salmoninarum* had faster growth rates of 0.4 ± 0.1 and 0.5 ± 0.1 $\log_{10}$ CFU/cm^2/day, respectively, on the 5% glucose treated lamb compared to that of the control (0.2 ± 0.1 and 0.4 ± 0.1 $\log_{10}$ CFU/cm^2/day) (Table 4.2). *V. fessus* and *V. salmoninarum* also had a lower maximum population density of 4.3 ± 0.3 and 3.8 ± 0.2 $\log_{10}$ CFU/cm^2, respectively, compared to the control which had a maximum population of 6.1 ± 0.4 $\log_{10}$ CFU/cm^2 for *V. fessus* and 4.9 ± 0.4 $\log_{10}$ CFU/cm^2 for *V. salmoninarum. E. americana* had a similar growth rate of 0.4 ± 0.1 – 0.5 ± 0.1 $\log_{10}$ CFU/cm^2/day for both the control and 5% glucose treated lamb. However, *E. americana* had a higher maximum population density of 6.2 ± 0.2 $\log_{10}$ CFU/cm^2 in the 5% glucose treated samples compared to the control where it had a maximum population of 5.6 ± 0.2 $\log_{10}$ CFU/cm^2.

S. proteamaculans, *C. divergens*, and *C. algidicarnis*, which were included to represent those species that had a significant effect on spoilage (as described in chapter 2), also showed similar growth kinetics between the control and the 5% glucose treatment. However, there was more variability within these sequence datasets, as evidenced by the relatively large confidence intervals. *S. proteamaculans* and *C. divergens* both had a growth rate of 0.6 ± 0.1 $\log_{10}$ CFU/cm^2/day and reached a maximum population between 5.3 ± 0.5 – 6.9 ± 0.1 $\log_{10}$ CFU/cm^2. In comparison, *C. algidicarnis* had a slower growth rate of 0.2 ± 0.0 $\log_{10}$ CFU/cm^2/day and reached a maximum population ranging between 5.6 ± 0.3 – 8.5 ± 6.5 $\log_{10}$ CFU/cm^2 for the 5% glucose treatment and the control, respectively.

Table 4.2 Growth kinetics (± 95% CI) of representative ASVs relative to TVC on VP boneless lamb meat stored at 4°C estimated directly from 16S rRNA gene sequence data. Growth kinetics were calculated for the control and the most effective glucose treatment (5%) for shelf-life extension.

	Growth rate (log_{10} CFU/cm^2)		Lag (days)		Maximum population (log_{10} CFU/cm^2)	
	0%	5%	0%	5%	0%	5%
Vagococcus fessus (ASV06807) [a]	0.2 ± 0.1	0.4 ± 0.1	No lag	No lag	6.1 ± 0.4	4.3 ± 0.3
Vagococcus salmoninarum (ASV06832) [a]	0.3 ± 0.1	0.5 ± 0.1	No lag	No lag	4.9 ± 0.4	3.8 ± 0.2
Ewingella american (ASV10458) [a]	0.5 ± 0.1	0.4 ± 0.1	No lag	No lag	5.6 ± 0.2	6.2 ± 0.2
Serratia proteamaculans (ASV10636) [b]	0.6 ± 0.1	0.6 ± 0.1	No lag	No lag	6.9 ± 0.1	5.3 ± 0.5
Carnobacterium divergens (ASV02973) [b]	0.6 ± 0.1	0.7 ± 0.1	No lag	No lag	6.9 ± 0.1	6.9 ± 0.1
Clostridium algidicarnis (ASV07138) [b]	0.2 ± 0.0	0.2 ± 0.0	No lag	No lag	8.5 ± 6.5	5.6 ± 0.3

a. ASVs that were significantly different between treatment groups according to ANCOM results.
b. Most abundant ASVs for species that significantly contributed to the spoilage rate of VP lamb in chapter 2 of this study

4.3.4 Volatile compound composition of bone-in and boneless VP lamb

A total of 150 volatiles were identified between both trials, all of which were identified in the boneless trial, while 145 of those were identified in the bone-in trial. PERMANOVA showed that storage time (p = 0.0001) had a significant effect on the volatile composition of bone-in and boneless lamb. Pair-wise analysis for time showed significant differences (p < 0.0001) in volatile composition between all timepoints for both meat cuts. CAP analysis further confirmed this observation with overall 90.4% and 90.2% exact classifications of bone-in and boneless lamb samples, respectively, into the different timepoints (Figs. 4.10 and 4.11). Like the sequence data, PERMANOVA pairwise analysis showed a marginal significant difference between slight/moderate and strong/extreme odour categories for both bone-in (p = 0.099) and boneless meat samples (p = 0.041). Further, the CAP analysis for odour categories showed clustering, and had an overall 66.1% and 75.8% exact classification of bone-in and boneless lamb samples, respectively, into the different odour categories (Figs 4.10 and 4.11).

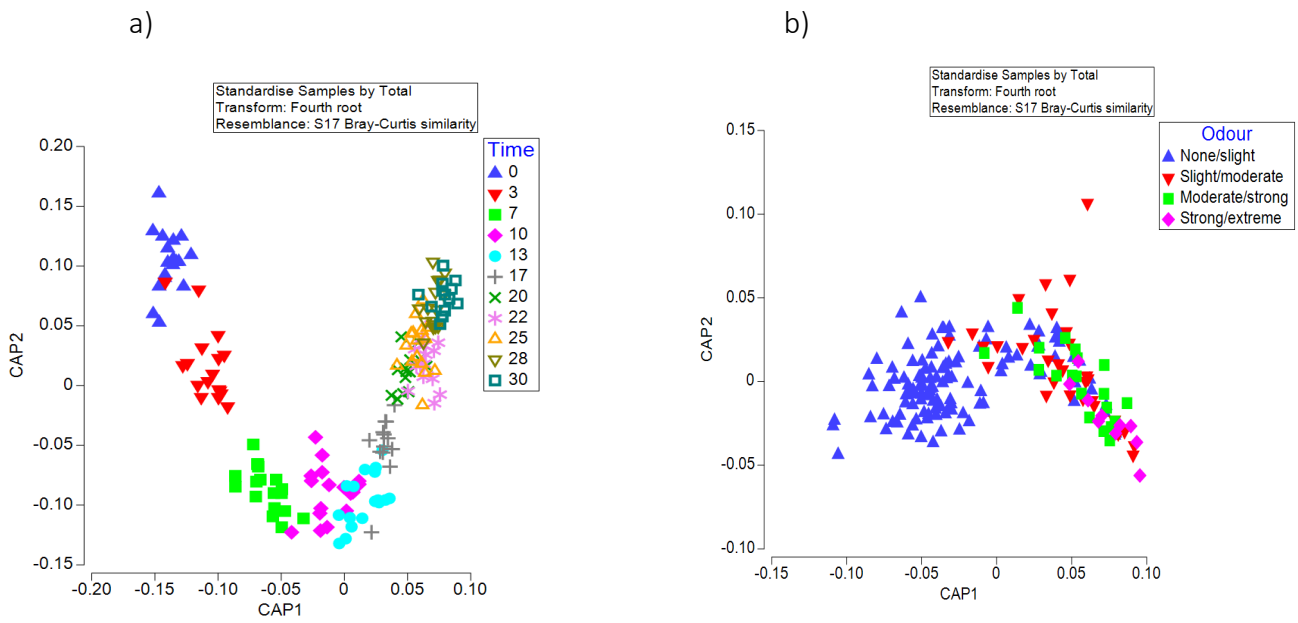

Figure 4.10. Canonical analysis of principal coordinates (CAP) plots of volatile composition data from vacuum packaged bone-in lamb shoulder stored at 4°C. The CAP plots are based on (a) time and (b) off-odour. Colours denote the sample storage time or odour. PERMANOVA significance tests are reported in the text.

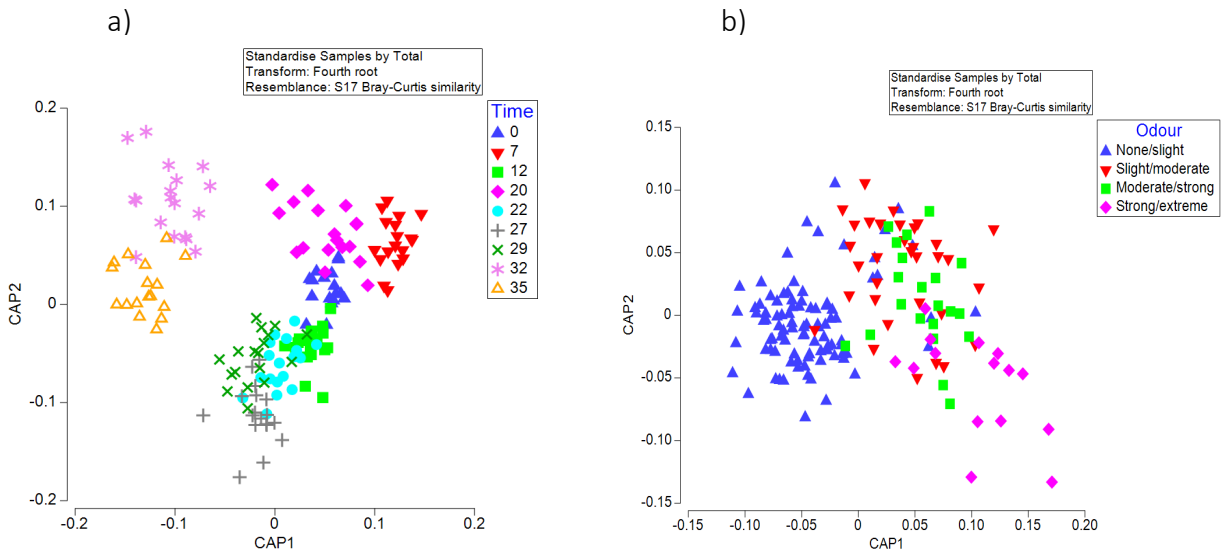

Figure 4.11 Canonical analysis of principal coordinates (CAP) plots of volatile compound composition data from vacuum packaged boneless lamb shoulder samples stored at 4°C. The CAP plots are based on (a) storage time and (b) off-odour. Colours denote the sample storage time or odour. PERMANOVA significance tests are reported in the text.

4.3.5 Volatile compound predictors for time

Linear Discriminant Analysis (LDA) was used to determine the volatiles that showed significant trends over storage time. As PERMANOVA results did not detect a significant difference in volatile composition between treatments, samples for each treatment were pooled together at each timepoint (n = 14 to 18 per timepoint) and the average concentration and standard deviation were calculated and are presented in the text. The significant volatile compounds for time are shown in heatmaps for bone-in and boneless meat, which hierarchically clusters the compounds according to trends over time.

4.3.5.1 Bone-in lamb volatile predictors for time

Linear Discriminant Analysis (LDA) found 37 volatile compounds that showed significant trends throughout the shelf-life of bone-in lamb, with a prediction accuracy of 99.4% (Fig 4.12a). These significant volatiles included 10 esters, 10 aldehydes, 7 alcohols, 6 hydrocarbons, 3 carboxylic acids, and a sulphur compound. The most significant trends are described below.

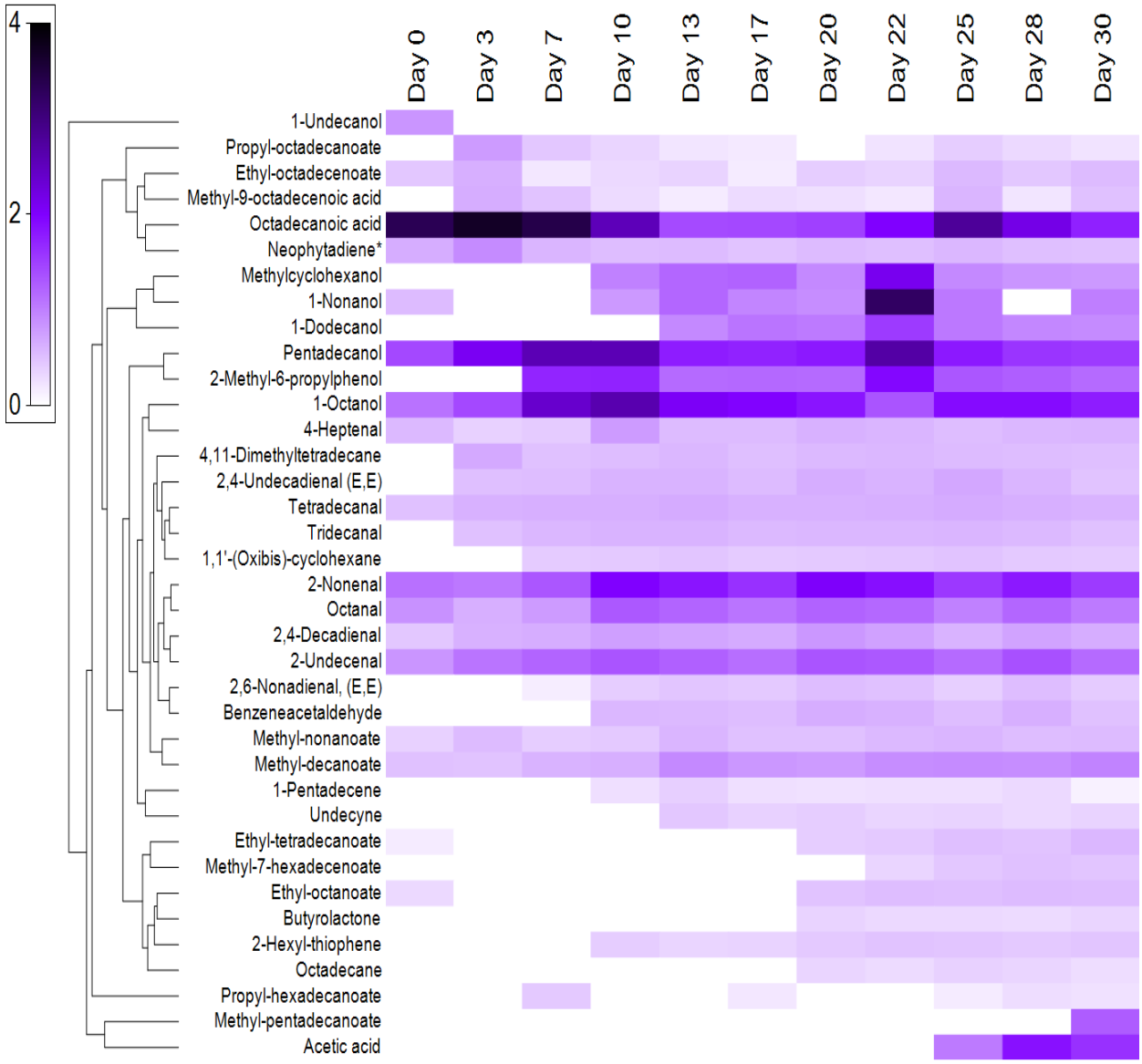

Figure 4.12a Heatmap showing relative concentrations (µg/g) for volatile compounds that showed significant trends over the storage time for bone-in VP lamb shoulder samples stored at 4°C. Data was normalised by applying forth root transformation * Tentative identification.

A range of aldehydes were initially detected in bone-in samples such as 2-nonenal (1.54 ± 2.15 µg/g), octanal (0.53 ± 0.80 µg/g), 2-undecenal (0.46 ± 0.46 µg/g), tetradecanal (0.06 ± 0.07 µg/g), and 2, 4 decadienal (0.04 ± 0.03 µg/g). All the aforementioned compounds significantly increased ($p < 0.0001$) to respective concentrations of 16.36 ± 14.01 µg/g, 2.89 ±

2.99 μg/g, 3.43 ± 2.28 μg/g, 0.19 ± 0.11 μg/g and 0.44 ± 0.34 μg/g between 3 – 10 days of storage and remained stable for the rest of the trial. Other aldehyde compounds, such as tridecanal and 2,4-undecadienal (E, E), were detected after 3 days of storage at concentrations of 0.20 ± 0.13 μg/g, and 0.06 ± 0.04 μg/g respectively, and remained similar for the rest of storage. Similarly, benzeneacetaldehyde (0.12 ± 0.12 μg/g), and 2,6-nonadienal (E, E) (0.04 ± 0.07 μg/g) were not detected until after 10 – 13 days of storage, after which they did not significantly differ for the remainder of the trial.

There were a range of ester compounds that were detected in bone-in meat and typically increased over storage period. Methyl-decanoate and methyl-nonanoate were detected at the beginning of the trial at respective concentrations of 0.05 ± 0.03 μg/g and 0.02 ± 0.01 μg/g. After 3 -10 days storage, methyl-decanoate significantly increased to a concentration of 0.86 ± 0.75 μg/g and methyl-nonanoate significantly increased to 0.11 ± 0.08 μg/g ($p <$ 0.0001) and thereafter both remained similar. A number of esters were detected after 20 to 22 storage days, including ethyl-octanoate (0.05 ± 0.09 μg/g), ethyl-tetradecanoate (0.02 ± 0.03 μg/g), methyl-7-hexadecenoate (0.15 ± 0.15 μg/g) and butyrolactone (0.01 ± 0.02 μg/g). The detection and concentration of some esters varied during storage, as a result there was no clear trend over time. These esters included ethyl-octadecenoate, which ranged between 0.01 ± 0.5 to 0.09 ± 0.13 μg/g between all timepoints. Propyl-octadecanoate was not detected at 0 or 20 days of storage but ranged between 0.01 ± 0.01, to 0.38 ± 0.14 μg/g at all other timepoints. Similarly, methyl-octadecenoate was not detected at the initial timepoint but ranged between 0.17 ± 0.21 to 0.01 ± 0.01 μg/g at all subsequent timepoints. Lastly, propyl-hexadecanoate was detected at 0, 17 25, 28 and 30 days of storage at a concentration ranging between 0.01 ± 0.01 to 0.03 ± 0.03 μg/g.

Alcohols that were detected at the beginning of the trial included 1- octanol at a concentration of 1.40 ± 1.06 µg/g, pentadecanol at 4.10 ± 1.78 µg/g, and 1-undecanol at 0.49 ± 1.31 µg/g. After three days of storage 1- octanol increased to a concentration of 4.12 ± 1.8 µg/g but did not significantly change for the rest of the trial, expect for day 22, when it was similar to the initial concentration. Pentadecanol significantly ($p < 0.0001$) increased to a concentration of 43.11 ± 22.36 µg/g by day 7, however declined after 10 days to range between 5.55 ± 1.70 and 10.58 ± 12.98 µg/g for the remainder of the trial. In contrast, 1-undecanol was only detected in meat samples at the start of the trial. Other alcohol compounds increased over the storage period, for instance 2-methyl-6-propylphenol, was detected at a concentration of 8.39 ± 8.71 µg/g after 7 days of storage and remained at a similar concentration for the rest of storage. Further, methylcyclohexanol, 1- dodecanol and 1- nonanol were detected at concentrations of 0.94 ± 2.69 µg/g, 0.73 ± 0.83 µg/g, and 0.40 ± 0.75 µg/g, respectively after 10 to 13 days of storage. These compounds did not significantly differ for the remainder of the trial, with the exception of 1-nonanol which decreased to below detectable limits from 25 – 28 days of storage.

There were a number of carboxylic acids that showed significant trends throughout the trial. Octadecanoic acid had the highest concentration compared to all compounds. For the first 7 days of the trial, it was detected at a concentration ranging between 123.30 ± 104.20 and 184.82 ± 74.13 µg/g. After 10 days of storage octadecanoic acid significantly decreased ($p < 0.01$) to 40.59 ± 64.13 µg/g and was not significantly different for the remainder of the trial. Several carboxylic acids were detected at the end of storage. For instance, acetic acid was detected after 25 days at a concentration of 1.12 ± 4.48 µg/g, and the concentration significantly ($p < 0.0001$) increased to 11.83 ± 12.22 µg/g by day 28. Methyl-pentadecanoate

was detected at the last timepoint of the trial (30 days) at a concentration of 2.54 ± 2.3 µg/g. A similar trend was observed for sulphur compound, 2-hexyl-thiophene. It was detected after 10 days of storage at a concentration of 0.02 ± 0.03 µg/g, and significantly increased (p < 0.0001) to a concentration of 0.05 ± 0.02 µg/g by 17 storage days and remained similar for the rest of the trial.

The tentatively identified hydrocarbon neophytadiene was initially detected in bone-in meat at a concentration ranging between 0.11 ± 0.05 to 0.68 ± 0.41 µg/g for the first 7 days of storage. After 10 days it significantly decreased (p < 0.0001) to a concentration of 0.07 ± 0.05 µg/g and did not significantly differ for all further timepoints. Hydrocarbon, 4,11-dimethyltetradecane, was detected after 3 storage days at concentrations of 0.06 ± 0.04 µg/g respectively, and it did not significantly change for the remainder of the trial. Similarly, 1,1'-(oxibis)-cyclohexane, was detected after 7 days of storage at a concentration of 0.03 ± 0.03 µg/g and remained similar for the rest of the storage. All other hydrocarbons were not detected until later in storage. For instance, undecyne was detected after 10 – 13 days at a concentration of 0.04 ± 0.05 µg/g and did not significantly differ for the remainder of the trial. Further, octadecane was detected by 20 to 22 days of storage and ranged between a concentration of 0.01 ± 0.01 to 0.02 ± 0.0 µg/g for the remainder of storage.

4.3.4.2 Boneless lamb volatile predictors for time

Linear Discriminant Analysis (LDA) showed 37 volatile compounds in boneless lamb that showed significant trends over storage time, with a 99.4% prediction accuracy (Fig. 4.12b). This included nine esters and carboxylic acids, eight hydrocarbons, four aldehydes, three alcohols, and two sulphurs and aromatics. The most significant trends are described below.

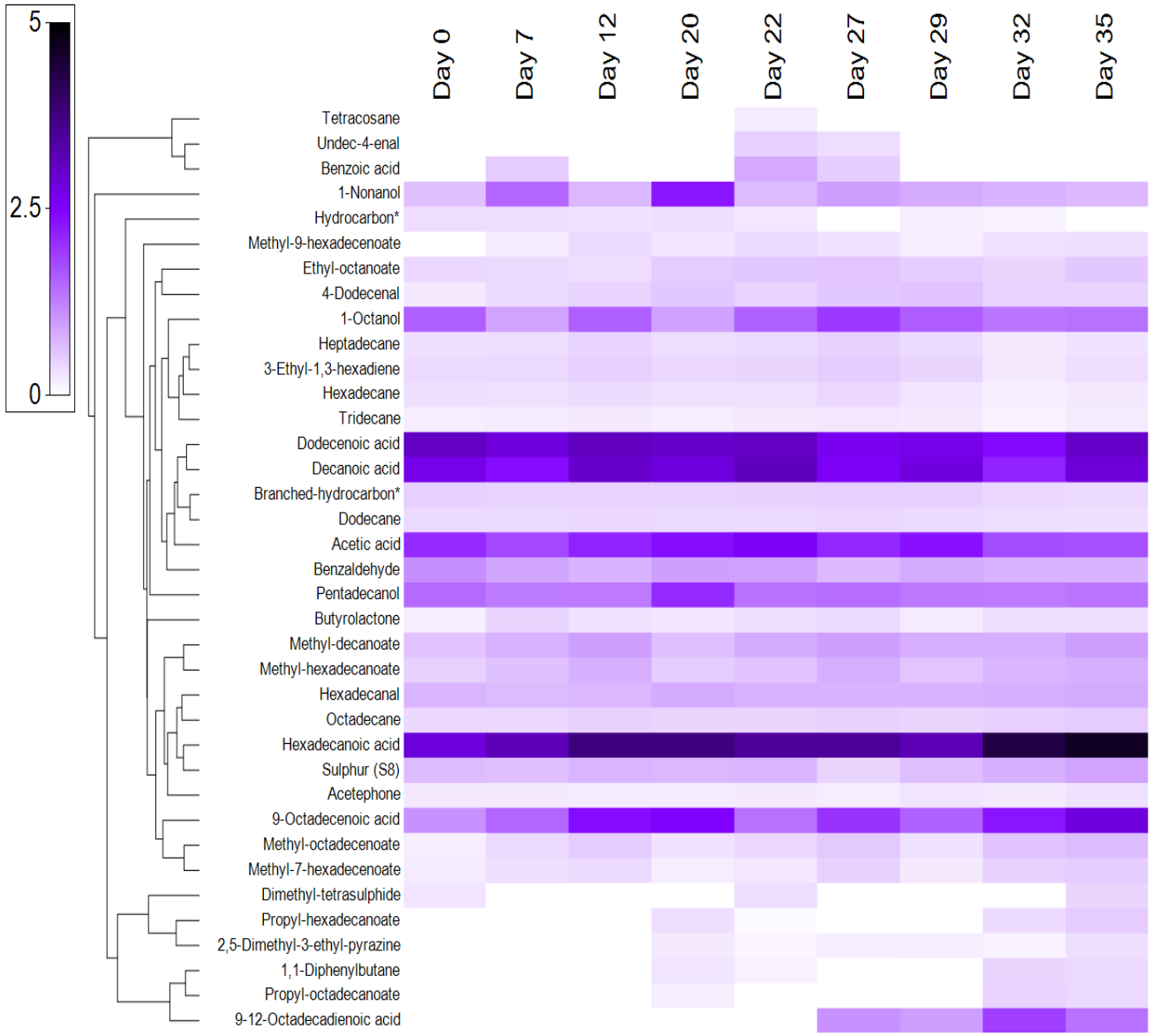

Figure 4.12b Heatmap showing relative concentrations (µg/g) for volatile compounds that showed significant trends over the storage time for boneless VP lamb shoulder samples stored at 4°C. Data was normalised by applying forth root transformation * Tentative identification.

A range of esters were detected in boneless meat at the beginning of the trial, and typically increased in concentration throughout storage period. These included methyl-decanoate, methyl-hexadecanoate, methyl-octadecenoate, and methyl-7-hexadecenoate which were initially detected at respective concentrations of 0.12 ± 0. 10 µg/g, 0.04 ± 0. 02 µg/g, 0.0005 ±

0.0001 µg/g and 0.001 ± 0.001 µg/g. After 12 days of storage, all of the aforementioned compounds significantly increased ($p < 0.0001$) to concentrations of 0.78 ± 0. 48 µg/g, 0.36 ± 0.22 µg/g, 0.02 ± 0.03 µg/g, and 0.03 ± 0.01 µg/g, respectively. Most of these compounds did not significantly differ after 12 days, except for methyl-octadecenoate, and methyl-7-hexadecenoate, which significantly reduced ($p < 0.0001$) at 22 and 29 days of storage to a concentration similar to the initial timepoint. Butyrolactone was also initially detected at the start of the trial at a concentration of 0.001 ± 0.001 µg/g and significantly increased to 0.03 ± 0.01 µg/g after 12 days and remained similar for the rest of the trial. In contrast, 3-ethyl-1,3-hexadiene decreased from an initial concentration of 0.01 ± 0.001 µg/g at the start of the trial to 0.0024 ± 0.006 µg/g after 32 days of storage ($p < 0.0001$).

There were several esters that were not detected at the beginning of the trial but were detected as storage time increased. This included methyl-9-hexadecenoate, which was detected after 7 days of storage at a concentration of 0.002 ± 0.004 µg/g. It significantly increased ($p < 0.0001$) to a concentration of 0.02 ± 0.02 µg/g after 12 days of storage and remained similar for rest of storage. Other ester compounds, such as propyl-octadecanoate, propyl-hexadecanoate, and 2,5-dimethyl-3-ethyl-pyrazine were also detected at 20 days of storage at low concentrations of 0.001 ± 0.002 µg/g, 0.01 ± 0.02 µg/g and 0.002 ± 0.004 µg/g, respectively. 2,5-Dimethyl-3-ethyl-pyrazine significantly increased ($p < 0.0001$) to a concentration of 0.009 ± 0.01 µg/g by 35 days of storage. In contrast, both propyl-octadecanoate and propyl-hexadecanoate were not detected again until 32 days of storage at significantly higher concentrations of 0.03 ± 0.03 µg/g and 0.07 ± 0.05 µg/g, respectively. A similar trend was observed for aromatic compound 1,1-diphenylbutane. It was detected at

low concentrations 0.004 ± 0.005 µg/g after 20 days of storage, and significantly increased ($p < 0.0001$) to a concentration of 0.03 ± 0.02 µg/g after 32 days.

A number of carboxylic acids were detected in boneless meat and typically increased during storage. Hexadecanoic acid was the most abundant compound, initially detected at a concentration of 63.60 ± 47. 85 µg/g at the start of the trial. It significantly increased ($p < 0.0001$) to 99.82 ± 93. 06 µg/g by day 7, and then to concentrations of 344.56 ± 205.13 µg/g at 32 days and 455.89 ± 143.46 µg/g at 35 days. 9-octadecenoic acid was detected in meat at the start of the trial at a concentration of 1.35 ± 5.72 µg/g and significantly increased ($p < 0.0001$) to 31.95 ± 28.39 µg/g after 12 days of storage, and then remained similar for the rest of the storage period. Decanoic acid and dodecenoic acid were two of the most abundant compounds detected in boneless meat throughout the whole trial. However, the concentration varied over time; decanoic acid ranged between 21.31 ± 18.30 to 97.89 ± 89.86 µg/g and dodecenoic acid ranged between 33.0 ± 20.21 to 91.40 ± 73.40 µg/g. As a result, there was no clear trend overtime. In contrast, acetic acid was detected at a concentration of 18.30 ± 21.47 µg/g at the beginning of the trial but after 32 days of storage it significantly ($p < 0.0001$) decreased in concentration to 9.03 ± 6.25 µg/g. Carboxylic acid, 9-12- octadecadienoic acid, was not detected until 27 days of storage at a concentration of 1.36 ± 5.76 µg/g but remained similar until the end of storage period.

There were a variety of hydrocarbons that were detected at the beginning of the trial and decreased throughout storage. This included heptadecane (0.01 ± 0.01 µg/g), dodecane (0.02 ± 0.01 µg/g), hexadecane (0.01 ± 0.01 µg/g), and a branched hydrocarbon (0.05 ± 0.02 µg/g). All of the aforementioned hydrocarbons significantly ($p < 0.0001$) decreased to respective

concentrations of 0.004 ± 0.02 μg/g, 0.009 ± 0.021 μg/g, 0.002 ± 0.008 μg/g and 0.02 ± 0.02 μg/g between 29 to 35 days of storage.

Aldehyde compounds, 4-dodecenal and benzaldehyde, were present at the beginning of the trial at respective concentrations of 0.002 ± 0.008 μg/g and 1.51 ± 0.98 μg/g. After 7 days of storage, 4-dodecenal significantly increased (p = 0.002) to a concentration of 0.02 ± 0.02μg/g and remained similar for the rest of storage. In contrast, benzaldehyde significantly decreased (p < 0.0001) to a concentration of 0.31 ± 0.18 μg/g after 12 days and it did not significantly differ for the remainder of the trial.

Two sulphur compounds were detected in boneless meat that showed significant trends over time. Octasulphur compound was detected at the beginning of the trial at a concentration of 0.20 ± 0.18 μg/g. It was detected at similar concentrations at most timepoints, except for day 27 when it had a significantly lower concentration of 0.03 ± 0.09 μg/g (p < 0.0001). Dimethyl-tetrasulphide was only detected at 0, 22 and 35 days of storage at concentrations of 0.004 ± 0.008 μg/g, 0.013 ± 0.016 μg/g, and 0.028 ± 0.05 μg/g, respectively.

Alcohol compounds, 1-nonanol and 1-octanol were detected at the start of the trial at concentrations of 0.14 ± 0.37 μg/g and 6.03 ± 2.47 μg/g. 1-nonanol had significantly higher (p < 0.0001) concentrations at 7 (4.90 ± 2.59 μg/g) and 20 (26.50 ± 76.04 μg/g) days of storage. However, after 22 days the concentration of 1-nonanol decreased to similar concentrations as the initial timepoint. In contrast, 1-octanol was significantly lower in concentration (p < 0.0001) at days 7 (0.63 ± 0.25 μg/g) and 20 (0.734 ± 1.614 μg/g) compared to all other timepoints.

4.4 Discussion

The overall objective of this study was to investigate the potential mechanisms underpinning the shelf-extension of meat samples after surface treatment with glucose. To achieve this, changes in the microbial community composition and volatilome of VP bone-in and boneless lamb shoulder influenced by glucose treatments, storage time and odour scores were assessed.

4.4.1 Microbial community composition of VP lamb

The results clearly indicate that storage time had the most significant influence on the microbial community of bone-in and boneless samples. The members of the lamb meat microbial community that are observed in this study agree well with what has previously been reported (Casaburi et al., 2015; Doulgeraki et al., 2012; Kaur et al., 2017b; Kaur et al., 2021). Similar to Kaur et al. (2017b) and Kaur et al. (2021), it was observed that the differences in the microbial community were more pronounced at the beginning of the trial followed by a less diverse restricted community presumably driven by intrinsic (meat pH and available nutrients), implicit (*i.e.,* the characteristics of the organisms) (Zhang et al., 2015) and extrinsic factors (VP atmosphere and temperature) that constrain the growth of many bacteria. Both composition and modelled growth rates both indicated the results are generally consistent between the bone-in and boneless samples.

One of the major differences between meat cuts was that *Pseudomonas* sp. grew on the bone-in samples, whereas it did not on the boneless samples. The observed growth of *Pseudomonas* sp. on bone-in samples may have been due to higher levels of residual oxygen in the pack. Vacuum-packaging can sometimes be problematic for bone-in and irregular shaped cuts due to poor sealing, potentially compromising the anaerobic conditions (Ščetar

et al., 2010). It was also observed that boneless samples had substantially more ASVs that were initially present but did not grow over time compared to bone-in samples. Despite controlling the conditions and time between slaughter and trial commencement similarly in the bone-in and boneless trials, variation in the initial microbial community composition is expected as these trials were conducted on two separate occasions and the meat came from different herds of animals (Kaur et al., 2021). For instance, the shelf-life data detailed in chapter 3 of this research showed that the boneless samples had a higher initial bacterial count, ranging between 3.64 – 3.98 $\log_{10}$ CFU/cm^2, compared to the bone-in samples, ranging between 2.64 – 2.96 $\log_{10}$ CFU/cm^2. Seasonal influence is likely to a be driving some of the variation in the initial microbial communities, as the boneless trial was conducted in winter (August) while the bone-in trial was conducted in autumn (May). Winter is generally associated with higher microbial contamination on livestock due to greater moisture content in manure than the drier seasons (Moriarty et al., 2011; Tucker and Klepper, 2005).

Glucose treatments had a statistically significant influence on the microbial community composition; although, as expected it was a more subtle effect compared to storage time. These results support the hypothesis that applying glucose to meat (manipulating access to favoured catabolic substrates) alters the microbial community composition, and thereby the subsequent rate and type of spoilage metabolic by-products.

The bone-in controls had significantly higher read numbers of *S. proteamaculans* compared to all test glucose concentrations. Accordingly, the growth kinetic data revealed that *S. proteamaculans* had a lag of 7.7 days in the most effective glucose treatment (5%) for shelf-life extension, whereas no lag was evident for the control. *S. proteamaculans* also had a smaller maximum population density in the 5% glucose treatment compared to the control.

An extended lag and smaller maximum population densities of *S. proteamaculans* are likely to contribute to the shelf-life extension evident in glucose treated meat, as this organism is commonly implicated in the spoilage of VP red meat due to its high proteolytic capability and subsequent production of malodourous compounds (Fougy et al., 2017; Nychas et al., 1988). This study also found that *S. proteamaculans* was significantly associated with bone-in samples of low odour quality (odour score ≤ 4). These results agree with Gribble et al. (2014) and those reported in chapter 2, which both reported that *Serratia* spp. spoiled VP lamb meat prematurely in inoculation experiments. Further, Kaur et al. (2021) found *Serratia* to be significantly more abundant on lamb samples that had low sensory scores.

The application of glucose on meat may have slowed/inhibited the growth of *S. proteamaculans* and, by extension, the formation of its spoilage metabolites, due to the accumulation of organic acid end-products resulting from glucose metabolism. Mohsina et al. (2020) reported growth inhibition of *Serratia* when exposed to undissociated lactic acid (1.12 mM) in simulated meat medium under VP conditions. Indeed, chapter 3 of this research found that meat treated with glucose concentrations (2.5%, 5% and 10%) showed significant reductions in meat pH (0.16 – 0.36 pH units less than untreated control), which could be attributed to the higher levels of undissociated organic acid and/or organic acid metabolites that resulted from glucose metabolism. The lower glucose concentration treatments (0.5% and 1%) did not show a significant reduction in meat pH, despite extending the shelf-life and having lower numbers of *S. proteamaculans*. However, it was expected that the concentration of organic acids was higher in these treatment groups compared to the untreated control, but not at a concentration that was sufficient to alter the meat pH due to the buffering capacity of meat (Puolanne and Kivikari, 2000). As evident in chapter 3, there

was no detectable difference in lactic acid between glucose treatments, suggesting the production of other organic acids such as acetic or formic.

The bone-in samples had significantly higher numbers of unclassified *Pseudomonas* sp. and *Buttiauxella* sp. associated with 2.5% and 10% glucose treatments. Both glucose treatment groups were the least effective for shelf-life extension of bone-in samples; although, still resulted in a shelf-life extension compared to the untreated control. Growth kinetics could not be determined for *Pseudomonas* sp. due to poor fit to the model ($r^2 < 0.5$) suggesting inconsistent/poor growth throughout storage. *Pseudomonas* are known to be highly competitive due to their ability to catabolise a range of compounds; however this is attributed to their higher affinity for oxygen, which is a limiting factor in VP conditions (Gill and Newton, 1977). This study found that *Pseudomonas* sp. was not associated with low sensory quality, which also suggests their population density was limited to a level that was not sufficient to cause spoilage. It was evident that the growth kinetics of *Buttiauxella* spp. were similar between the control and the 5% glucose treatment, despite having significantly higher read numbers in the 2.5% and 10% glucose treatments. These results suggest that this organism did not contribute significantly to shelf-life extension. *Buttiauxella* spp. have been associated with the spoilage community of various meats at refrigeration temperatures; although less is known about its role in spoilage of VP red meat (Ercolini et al., 2009; Mace et al., 2012; Sade et al., 2013). However, this study also found that *Buttiauxella* sp. was significantly more associated with strong to extreme off odour scores (≤ 2). This could be the reason why those glucose treatments which had a higher abundance of *Buttiauxella* sp. were the least effective for shelf-life extension. However, further research is required to determine the spoilage potential of *Buttiauxella* sp. on VP lamb, and the effects of glucose.

The most effective glucose treatments for shelf-life extension for both bone-in and boneless lamb had significantly fewer read numbers of *Vagococcus* spp. Specifically, the bone-in samples had significantly lower read numbers of an unidentified *Vagococcus* sp. and *V. fessus* in the 0.5%, 1% and 5% treatments. Further, the boneless trial had significantly lower read numbers of *V. fessus* and *V. salmoninarum* in the 5% treatment. Growth kinetics could not be obtained for *Vagococcus* sp. from the bone-in trial due to poor fit to the model ($r^2 < 0.5$) suggesting inconsistent/poor growth throughout storage. However, in the bone-in trial *V. fessus* had a longer lag phase, a faster growth rate and a smaller maximum population density in the 5% glucose treatment group compared to the control. Interestingly, *V. fessus* and *V. salmoninarum* also had faster growth rates and smaller maximum population densities in the 5% glucose treatment compared to the control in the boneless trial.

Culture-independent techniques have recently identified the genus *Vagococcus*, a group of lactic acid bacteria, to be present in the spoilage community on a variety of meat types (Alfaro and Hernandez, 2013; Calliauw et al., 2016; Hernandez-Macedo et al., 2012; Kaur et al., 2021; Liu et al., 2019; Powell and Tamplin, 2012; Svanevik and Lunestad, 2011). There are however limited studies that have investigated the spoilage potential of *Vagococcus* on VP red meat (Jääskeläinen et al., 2020). This study found that *Vagococcus* spp. had no clear association with low odour quality of VP lamb, despite having significantly lower numbers in the glucose treatments that were the most successful for shelf-life extension. These results suggest that *Vagococcus* spp. could be contributing to spoilage implicitly through interactions with other bacteria within the community, such as the exchange of metabolites and nutrients (Gram et al., 2002; Jørgensen et al., 2000; Mayo et al., 2021; Morin et al., 2018), and potentially metabolite to metabolite interactions with those formed by other bacteria

(Françoise and Jean-Jacques, 2011). It is unlikely that additional glucose inhibited *Vagococcus* spp. by acidic metabolic by-products, as lactic acid bacteria are generally more tolerant to acidic conditions since they can maintain a lower intracellular pH (Adams and Nicolaides, 1997). This is supported by the results of this study, as *Vagococcus* spp. had significantly higher numbers on the 10% glucose treated bone-in meat, despite the 10% treatment having the largest reduction in pH.

A possible explanation for the significantly lower numbers of *Vagococcus* spp. on the most successful glucose treatments could be due to the resulting shift in substrate utilisation by the microbial community at these concentrations. Specifically, by manipulating the access to favoured catabolic substrates it likely provides a competitive advantage for those bacteria with a higher affinity for such substrates. This is likely to affect the complex antagonistic and synergistic interactions between members of the microbial community, thereby altering metabolism and potential success of some bacteria (Françoise and Jean-Jacques, 2011; Gram et al., 2002; Mayo et al., 2021; Morin et al., 2018; Zhang et al., 2015). The success of *Vagococcus* sp. through interactions within a microbial community has previously been reported in sour milk fènè (Wullschleger et al., 2018). Specifically, the growth of different *Vagococcus* strains was dependent on the synergistic interactions within the complex fènè microbial community (Wullschleger et al., 2018).

It was observed that *E. americana* was significantly associated with the boneless samples that were treated with higher glucose concentrations (5% and 10%). In accordance, the growth kinetic data revealed it had a higher maximum population in the 5% glucose treatment compared to the control. The presence of this *Enterobacteriaceae* species is not as common on VP lamb as most of the other genera that were identified (Helps et al., 1999; Kaur et al.,

2017b; Kaur et al., 2021). Therefore, limited information is available for *E. americana* spoilage capabilities in VP meat. However, our results showed that *E. americana* was significantly more associated with samples of high sensory quality for both bone-in and boneless samples. Further, *E. americana* has been found to produce organic acids from glucose in other food matrices and cultures (Grimont et al., 1983; Reyes et al., 2004). This may indicate that higher concentrations of additional glucose created favourable conditions for *E. americana*, and likely contributed to the accumulation of organic acids and pH reduction.

Gill (1986) suggested that adding glucose to VP meat with a higher pH (≥ 6), like lamb, can potentially cause premature spoilage by stimulating the growth of *Shewanella putrefaciens*. This and its related species, such as *S. baltica*, can produce H_2S at refrigeration temperatures, causing premature malodours and colour defects, such as greening (Doulgeraki et al., 2012; Kamenik, 2013). Earlier results (see chapter 3) found no evidence of greening colour defects or premature malodours for any glucose-treated meat samples with the observed pH of ≥ 6, suggesting that under the VP shelf-life conditions these microorganisms were unable to reach the population densities required to cause spoilage, as also reported by Kaur et al. (2017b). These results were confirmed, and it was found that *S. putrefaciens* was not among the identified ASVs that was significantly associated with any glucose treatment. Although, this study found an unclassified *Shewanella* ASV that was significantly associated with the 5% and 10% glucose treatments in the bone-in trial; however, growth kinetics could not be established due to poor fit of model ($r^2 < 0.5$) suggesting relatively poor growth. Further, other molecular studies on VP beef and lamb barely detect evidence of growth of any *Shewanella* species under a range of temperatures (Kaur et al., 2017b; Kaur et al., 2021).

This study also found that none of the tested glucose concentrations stimulated the growth of *Brochothrix thermosphacta.* This differed from Mohsina et al. (2020), who found a glucose concentration of 5.55 mM increased maximum population density of *B. thermosphacta* in monocultures in anaerobic simulated beef medium, and at higher concentrations (55 mM) increased the growth rate. The rate of glucose consumption by *B. thermosphacta* is affected by O_2 and CO_2; which is likely the limiting factor evident in this study (Iulietto et al., 2015).

The effects of glucose treatments on the microbial community were different between bone-in and boneless samples. The ASVs that significantly differed between treatments were different between the meat cuts, although *Vagococcus* spp. were evident in both and similar differences in growth kinetics were observed. Interestingly, as reported in chapter 3, bone-in samples resulted in a longer shelf-life extension compared to the boneless samples. This variation is likely to be a result of the differences in the initial community composition that are notable in the relative abundance plots and diversity indices in section 4.3.1, and the heatmaps showing sequence abundance over storage time in section 4.3.2. This reaffirms the conclusion that glucose application is potentially more successful when applied to meat that has lower initial counts as the community is less established. Further investigations involving a more detailed analysis of meat from a range of sources is required to confirm this.

4.4.2 Organic volatile compound composition of VP lamb

There was no detectable significant difference in volatile composition between glucose treatments for either bone-in or boneless meat. There were only marginal significant differences detected in the volatile composition between odour categories, despite both bone-in and boneless samples clustering into clear groups in the CAP analysis like the microbial community. These results might be due to lack of resolution resulting from the

large amount of variation in volatile profiles at each timepoint. The variation within and between the bone-in and boneless samples was expected as meat is a highly heterogeneous product, the samples came from multiple animals (Frank et al., 2020), and the bone-in and boneless cuts were conducted as two independent experimental trials. Further, the sensory impact of volatile compounds is not easily defined as interactions between molecules can create unpredictable off-odours, in addition to the interactions with the microbial community (Casaburi et al., 2015). However, storage time had a significant effect on volatile compound composition for both bone-in and boneless lamb. The volatile compounds that were identified agree well with what has previously been reported for red meat, comprising a range of alcohols, aldehydes, ketones, esters, carboxylic acids, sulphur and aromatic hydrocarbons compounds (Argyri et al., 2015; Casaburi et al., 2015; Corral and Flores, 2017; Ercolini et al., 2011; Ercolini et al., 2009; Frank et al., 2020; Jääskeläinen et al., 2016; Kosowska et al., 2017; Reis et al., 2016). Of the 150 volatile compounds that were identified, there were 37 that showed significant trends over the storage period for both the bone-in and boneless cuts. This provides general insights into the metabolic activities, both microbial and endogenous, occurring at the various timepoints (as discussed further below).

4.4.3 Bone-in lamb volatile predictors for time

Many of the volatile compounds that were identified as predictors for time in the bone-in samples significantly increased in concentration within the first 13 days of the trial and did not change with further storage. This included several aldehydes, alcohols, as well as some esters and hydrocarbons. Aldehydes and alcohols can form through hydrolysis of triglycerides, and metabolism of glucose, fatty acids and amino acids (Casaburi et al., 2015; Insausti et al., 2002; Montel et al., 1998; Moretti et al., 2004). Both classes of compounds are

key intermediates in branched-chain amino acid catabolism, and can have various functions, such as the formation of branched-chain fatty acids for cell membrane synthesis in different microbial species (Beck et al., 2004; Montel et al., 1998). The most common aldehydes and alcohols that have been identified in red meat under VP storage conditions are known to be produced by a range of Gram-positive and Gram-negative bacteria, such as *Carnobacterium, Clostridium, Hafnia, Lactobacillus, Leuconostoc, Pseudomonas* and *Serratia* (Broda et al., 2000; Casaburi et al., 2011; Ercolini et al., 2009; Hernandez-Macedo et al., 2012; Jääskeläinen et al., 2016; Jääskeläinen et al., 2013).

All other volatiles that showed significant trends over time increased later during the storage period of bone-in samples. Interestingly, day 17 corresponded to the increase in concentration of sulphur compounds as well as the onset of spoilage of the untreated controls. Sulphur volatiles have previously been linked to low odour quality and the onset of spoilage due to their putrid odour and low perception thresholds (Casaburi et al., 2015; Curioni and Bosset, 2002; Mayr et al., 2003). The formation of sulphur compounds typically originate from the degradation of sulphur containing amino acids, specifically through cleavage of a bond between carbon and sulphur by amino acid demethiolase (Yvon and Rijnen, 2001). This potentially indicates an increase in microbial proteolytic activity around day 17 of storage. Several *C. maltaromaticum* strains have been associated with the production of thiophene under VP storage conditions (Casaburi et al., 2011).

The concentration of ethyl-octanoate, ethyl-tetradecanoate, and methyl-7-hexadecenoate significantly increased between 20 to 22 days of storage. Further, methyl pentadecanoic acid significantly increased by 30 days. These ester compounds likely contribute to the spoilage volatilome of bone-in samples with their characteristic fruity off-odours (Marco et al., 2007;

Takeoka et al., 1996). The occurrence of esters later in storage is expected as they are formed from microbial esterification activity of other metabolites, such as carboxylic acids and alcohols (Talon et al., 1998; Toldra, 1998). Production of esters in VP meat has previously been linked with *Carnobacterium* spp., *C. maltaromaticum*, *B. thermosphacta* and *S. proteamaculans* (Casaburi et al., 2015; Ercolini et al., 2009). Significant increases in the concentration of esters may likely be a result of increasing interactions between members of the microbial community, such as exchange of carboxylic acids, due to limitations of preferred substrates (Gram et al., 2002; Jørgensen et al., 2000; Mayo et al., 2021; Morin et al., 2018). To further support this, the increase in esters corresponds to the time (> 20 days) at which all bone-in samples had glucose levels below the detection limit (0.44 mmol/kg), as evident in chapter 3.

Other compounds such as an unidentifiable hydrocarbon, octadecane and butyrolactone also significantly increased between 20 to 22 days. Hydrocarbons can originate from degradation of fatty acids, methyl ketones and secondary alcohols (Mottram, 1998). Although, some hydrocarbons found in raw meat might not be a result of microbial metabolism, but potentially from the chemical changes in the meat itself during storage (Casaburi et al., 2015). Butyrolactone has previously been associated with red meat aroma, characterised as having a 'sulphur' odour (Frank et al., 2020; Resconi et al., 2013; Shahidi et al., 1986). However, many lactones are known to produce desirable flavours and aromas of meat, which are mainly formed by meat oxidation during storage (Matsuishi et al., 2004; Watanabe et al., 2012). Acetic acid was also detected by day 25 and significantly increased in concentration by 28 days where it remained stable for further storage. High concentrations of acetic acid can result in sharp acidic odours, altering the sensory quality of meat (Casaburi et al., 2015;

Curioni and Bosset, 2002; Pothakos et al., 2015). The production of acetic acid and other carboxylic acids are common under VP storage conditions. This can be attributed to a microbial community dominated by lactic acid bacteria, a majority of which are heterofermentative and produce lactic, acetic and formic acid through glucose metabolism (Borch et al., 1996; Laursen et al., 2006; Leisner et al., 2007; Pothakos et al., 2015). Acetic acid has also previously been associated with hydrolysis of triglycerides and amino acid degradation, which may explain the significant increase that was found later in storage (Montel et al., 1998). Further, acetic acid has been identified as one of the main volatile end-products associated with *Clostridium* spp. fermentation and has been suggested as potential spoilage marker for this genus (Broda et al., 2000; Hernandez-Macedo et al., 2012). This agrees well with this study, as *C. algidicarnis* significantly increased (by an order of magnitude) between 20 to 28 days. Further, *C. algidicarnis* was the most significant ASV that was associated with low sensory quality for both the bone-in and boneless, which agrees well with chapter 2 of this research, Kaur et al. (2017b) and Kaur et al. (2021).

4.4.4 Boneless lamb volatile predictors for time

The volatile compounds that showed significant trends over time in the boneless samples were quite different from those for bone-in samples. However, as mentioned above, this is probably expected due to the heterogeneity of meat and because the bone-in and boneless trials were conducted as independent trials and meat was sourced from different herds of animals. Many of these volatiles significantly increased in concentration within the first 12 days of storage and did not change with further storage. This included an aldehyde (4-dodecenal), several esters (see results), a fatty acid (9-octadecenoic acid), and a lactone (butyrolactone). The significant increase in the concentration of esters in boneless samples

earlier in storage compared to the bone-in samples could potentially be a result of the higher microbial loading found in boneless samples, and the subsequent increasing microbial interactions due to competition for preferred substrates. Similar to the bone-in samples, this is supported by the significant increase in esters corresponding to the time in which all boneless samples reached residual glucose levels below the detection limit (between 12 – 20 days) (see chapter 3). A number of volatile compounds were initially present and decreased at the end of the storage, indicating a shift in substrate utilisation by some bacteria. This included octasulphur, an ester (3-ethyl-1,3-hexadiene), alcohols (1-nonanol and 1-octanol), hydrocarbons (benzaldehyde, heptadecane, dodecane, and hexadecane) and carboxylic acids (decanoic acid, dodecenoic acid and acetic acid). In contrast, aromatic hydrocarbons 2,5-dimethyl-3-ethyl-pyrazine and 1,1-diphenylbutane were both detected from 20 days, and significantly increased in concentration by 32 days. As mentioned above, aromatic hydrocarbons in raw meat may be a result of changes taking place in the meat itself during storage, rather than microbial metabolism (Casaburi et al., 2015). The compound 2,5-dimethyl-3-ethyl-pyrazine contributes to the desired aroma of grilled red meat and is usually formed during heating (Frank et al., 2016; Frank et al., 2020; Shahidi et al., 1986). The detection of 2,5-dimethyl-3-ethyl-pyrazine compounds in boneless samples here could potentially be a by-product of the SPME methods as it requires heating of samples before the fibre is exposed to headspace. However, this does not explain the significant increase found in samples during storage. The metabolic contribution of this volatile remains to be further elucidated.

Volatile fatty acids, including 9-12-octadecadienoic acid, propyl-octadecanoate, and propyl-hexadecanoate were initially detected at low concentrations at 20 days, and the latter two

significantly increased in concentration by >27 days. Further, 7-hexadecanoic acid increased by an order of magnitude by 32 days of storage. The production of volatile fatty acids in meat can be derived from hydrolysis of triglycerides and phospholipids, as well as amino acid degradation (Toldra, 1998), therefore, suggesting increased metabolism of fats and proteins by day 27 of our study. Volatile fatty acids typically have dairy/cheese and acidic odours; however, their contribution to spoilage is dependent on concentration as well as olfactory thresholds (Casaburi et al., 2015). Butanoic acid is one the main carboxylic acids associated with meat spoilage (Jones, 2004) and has been implicated with many of the major taxa we found in our study, such as *Carnobacterium*, *Clostridium* and *Leuconostoc* (Broda et al., 2000; Casaburi et al., 2011; Ercolini et al., 2011; Ercolini et al., 2006; Jääskeläinen et al., 2013). However, we did not detect butanoic acid or any of its derivatives, which may be due to SPME GC/MS conditions used in this study. For instance, detection of short-chain fatty acids (from 2 – 6 carbons) may require further optimisation, such as selection of a fibre more suitable for low molecular weight compounds, as well as optimisation of extraction temperatures (Douny et al., 2019).

4.5 Conclusion

Glucose treatments (0.5% - 10% w/v) had a significant impact on the microbial community composition of VP bone-in and boneless lamb. The most effective treatments for bone-in (1% and 5%) and boneless (5%) lamb had significantly lower numbers of various *Vagococcus* ASVs, and all tested glucose concentrations had significantly lower numbers of *S. proteamaculans* on bone-in lamb. Accordingly, growth kinetics over storage revealed that the various *Vagococcus* ASVs had a faster growth rate, smaller maximum population densities and in some cases a longer lag in the 5% glucose treatment compared to the control in both

bone-in and boneless shelf-life trials. Similarly, *S. proteamaculans* had an extended lag and a smaller maximum population density in the 5% glucose treatment compared to the control. Additional glucose likely alters the microbial community composition by shifting substrate utilisation and the accumulation of acidic end-products, having an overall impact on the formation rate of various spoilage metabolic by-products. However, there was no detectable difference in the composition of organic volatile compounds between glucose treatments for either bone-in or boneless lamb, which may be due to the variability between samples. There was a significant increase in a range of sulphur compounds, esters and carboxylic acids later in storage, indicating an increase in microbial degradation of amino acids, lipids and other catabolic by-products, reducing sensory quality of meat. To better understand the mechanisms behind shelf-life extension of VP meat by glucose further studies are required to investigate its effects on microbial substrate utilisation and manifestation of spoilage metabolites, such as measurement of non-volatile metabolites. Chapter 5 will provide a synthesis of the findings from experimental chapters 2, 3 and 4.

Chapter 5

Overall Synthesis

The faster rate of quality loss of chilled VP lamb can present a challenge to Australian lamb exporters. This is because the total travel time required for transportation and distribution can be lengthy (> 90 days) when exporting to more distant markets (Huynh et al., 2016). It also makes lamb more vulnerable to quality loss during distribution arising from potential delays that can increase travel time and possible temperature fluctuations (Sumner, 2016). Despite having a shorter shelf-life, there are few studies that have investigated the spoilage mechanisms of chilled VP lamb, as beef has been the primary model with most information consequently extrapolated to lamb (Gribble et al., 2014; Kaur et al., 2017b; Mills et al., 2014). However, the biochemistry of beef and lamb is to an extent different and by extension so is the bacterial selection, growth and metabolic capability. Therefore, the primary objectives of this thesis were to provide insights into microbial spoilage mechanisms of chilled VP lamb, and to investigate potential interventions to aid shelf-life extension. This was initially approached by investigating whether representative bacteria (Table 5.1) of meat spoilage communities independently play important roles in the rate of spoilage of chilled VP lamb (chapter 2). Subsequent studies evaluated the potential for glucose surface treatments to extend the shelf-life of VP lamb products (chapter 3). This was expanded upon by investigating the effects of glucose treatments on the microbial community and associated volatilome (chapter 4).

Table 5.1 Representative isolates of the microbial community of VP lamb organised into Gram stain and respiration mode.

Gram stain	Isolate	Respiration
Gram-positive	*Clostridium algidicarnis*	Obligate anaerobe
	Clostridium estertheticum	
	Clostridium putrefaciens	
	Brochothrix thermosphacta	Facultative anaerobe
	Carnobacterium divergens	
	Carnobacterium maltaromaticum	
	Enterococcus sp.	
	Lactococcus lactis	
	Lactococcus piscium	
Gram-negative	*Hafnia paralvei*	
	Serratia spp.	
	Yersinia intermedia	
	Pseudomonas sp.	Aerobic

The work described in chapter 2 showed that *Carnobacterium maltaromaticum*, *Hafnia paralvei, Lactococcus piscium*, and *Yersinia intermedia* had low or no spoilage potential on chilled VP lamb despite observing an increase in their numbers throughout storage. This was observed when inoculated onto VP lamb samples that were sterile, and for those that contained its natural microbiota during storage at 2°C. The 16S rRNA amplicon sequence results reported in chapter 4 of this study showed similar results at a higher storage temperature (4°C), as ASVs within each of these genera were typically associated with better odour scores of VP lamb. Previous studies have also shown that *H. paralvei* and *Y. intermedia* were typically less abundant on chilled VP lamb samples that had low sensory scores (Kaur et al., 2021). Further, *C. maltaromaticum* and *L. piscium* have been reported to have the ability to enhance the shelf-life of VP red meat by inhibition of higher potential spoilers and also due

to their relatively innocuous fermentation products (Hilgarth et al., 2018; Laursen et al., 2005; Youssef et al., 2014a; Zhang et al., 2015; Zhang et al., 2017).

This study found *Brochothrix thermosphacta, Enterococcus* sp., *Pseudomonas* sp. and *Lactococcus lactis* to have low spoilage potential, as they did not have the ability to successfully grow on chilled VP lamb that was sterile. Additionally, they did not spoil lamb faster when inoculated onto meat containing natural microbiota. It was not established whether these isolates were able to grow well in a community setting in these initial experiments. However, the 16S rRNA amplicon sequence data in chapter 4 illustrated that *Pseudomonas* sp. had limited growth capabilities within the natural ecosystem of VP lamb, most likely due to O_2 limitations. The growth capabilities of *Pseudomonas* sp. on bone-in lamb cuts were, however, higher than in boneless samples. This may have been a result of higher levels of residual oxygen in the pack, as vacuum-packaging can sometimes be problematic for bone-in and irregular shaped cuts due to poor sealing (Ščetar et al., 2010; Sumner et al., 2021).

The 16S rRNA amplicon sequence data in chapter 4 established that *Brochothrix* sp. had the ability to grow on VP lamb, although, this might be due to the higher storage temperature used in these experiments (4°C). *Brochothrix* spp. has been reported to not usually grow to high populations on VP red meat when stored at optimum chilled conditions (~0.5°C), as it requires higher temperatures, presence of residual O_2, and relatively high meat pH (> 5.8) (Ercolini et al., 2011; Kaur et al., 2017b; Nowak et al., 2012; Pin et al., 2002). However, Gribble and Brightwell (2013) and Gribble et al. (2014) found that *B. thermosphacta* was able to grow on, and spoil, VP lamb cuts at both high (5.9 to 6.4) and low (5.4 to 5.8) pH at chilled temperatures of -1.5°C (Gribble and Brightwell, 2013; Gribble et al., 2014). Consistently,

previous trials using beef indicate that the growth of this organism is not possible below pH 5.8 under chilled anaerobic conditions (Grau, 1980; Grau, 1981). It was therefore suggested that the levels of substrates present at low and high pH, such as lactic acid etc., affect the microbial growth rather than the pH itself, which is likely to differ between lamb and beef (Bell, 2001; Gill and Newton, 1978; Gribble et al., 2014). However, there is little information reported on the substrate composition of lamb and the rate they become available to bacteria at chill temperatures. Therefore, this requires further investigation (Gribble et al., 2014).

Similar to the findings of chapter 2, it has previously been reported that *Enterococcus* are not well equipped to grow under anaerobic conditions at low temperatures; therefore, they have limited impact on meat spoilage (Bjorkroth et al., 2005; Kaur et al., 2021). The results presented in chapter 4 of this study also supports these earlier findings, as *Enterococcus* 16S rRNA amplicon sequences were not detected on VP lamb samples stored at 4°C. It has been suggested that growth of *Enterococcus* under these conditions may be impeded by *Carnobacterium* due to the production of bacteriocin compounds (Stoffels et al., 1992).

The lack of growth evident by *L. lactis* when inoculated onto sterile meat was unexpected due to their ability to grow in anaerobic conditions at low temperatures. These results suggest that *L. lactis* requires synergistic interactions with other members of the microbial community, such as exchange of metabolites or growth promotors, in order to grow on the meat matrix (Zhang et al., 2015). Such interactions have dictated the growth of *L. lactis* in other food matrices, such as milk (Juillard et al., 1996).

In chapter 2 it was shown that *C. divergens* and *Serratia* spp. did not have the metabolic capability to spoil lamb independently despite growing to maximum population on sterile

meat. However, these isolates spoiled lamb containing its natural microbiota at a faster rate compared to the control and all other facultative anaerobes and aerobes. These results may suggest that the metabolic by-products of *C. divergens* and *Serratia* spp. are different in a community setting, having more organoleptic impact. For instance, *C. divergens* is heterofermentative, and therefore can ferment available substrates into a number of metabolites that may contribute to spoilage, such as different organic acids, acetate, acetoin, ethanol, and CO_2 (John et al., 2007). However, the spoilage potential of *Carnobacterium* spp. is variable and is dependent on intraspecies and interspecies interactions (Yang et al., 2021). For instance, in chapter 4 it was shown that *Carnobacterium* ASVs were significantly associated with VP lamb samples of high sensory quality, despite having the largest relative abundance throughout the whole storage period.

Similar to the findings in chapter 2, *Serratia* spp. have been reported to spoil VP lamb prematurely when inoculated onto meat containing its natural microbial community at storage temperatures as low as -1.5°C (Gribble et al., 2014). In mixed communities, glucose/saccharides depletion is faster due to competition, and as a result *Serratia* spp. are likely to begin proteolysis earlier and be a contributor to spoilage (Nychas et al., 2008). *Serratia* spp. have also been shown to be significantly more abundant on lamb samples that had low sensory scores compared to those samples with acceptable odour scores (Kaur et al., 2021). In accordance, experiments described in chapter 4 also found *S. proteamaculans* was significantly associated with VP bone-in samples of poor odour quality.

These studies also established that *Clostridium* had the highest spoilage potential among all the test isolates, and in pure culture on sterile VP lamb samples caused spoilage. *Clostridium* was found to be the only bacterial group capable of spoilage when inoculated onto sterile

lamb meat. It also caused lamb to spoil at a faster rate when inoculated onto meat containing its natural microbiota. *Clostridia* species, such as those used in chapter 2 (Table 5.1), have commonly been implicated in various types of red meat spoilage, especially meat with a higher pH (> 6) (Adam et al., 2010). This is due to their ability to grow well under conditions that are optimal for storage/distribution of VP red meat and to utilise a variety of intramuscular carbohydrate stores (Brightwell and Clemens, 2012; Palevich et al., 2021; Yu et al., 2016).

Most studies that have focused on *Clostridium* spp. as meat spoilage organisms have been concerned with *Clostridium* spp. as causative agents of 'blown pack' spoilage due to its sporadic occurrence resulting in economic losses (Adam et al., 2010; Moschonas et al., 2010). The studies described in chapter 2 found that *C. estertheticum* caused 'blown pack', resulting in half the expected shelf-life when inoculated onto sterile lamb. The aggressive spoilage potential of *C. estertheticum* is likely due to their unique enzyme profiles that enable a degree of specialisation in their ability to metabolise a variety of simple to complex saccharides (Palevich et al., 2021). However, the results of that study also showed that when *C. estertheticum* was inoculated onto lamb with natural microflora the rate of quality loss was reduced. This finding is likely a result of antagonistic interactions within the microbial community, including competition for available carbohydrates, and inhibition by LAB that would reduce pH and could produce inhibitory molecules (Jones et al., 2008; Jones et al., 2009; Yang et al., 2011). For example, this study also found that sterile VP lamb inoculated with the lactic acid bacterial species *Lactococcus piscium*, reduced the pH of meat by 0.4 units compared to the untreated control after 47 days of storage.

Less is known about *C. putrefaciens* and *C. algidicarnis* and their role in spoilage of chilled VP lamb. Chapter 2 found that when these species were inoculated onto sterile VP lamb the rate of quality loss was similar to that of the untreated control with natural microflora. It was also evident, however, that when these species were inoculated onto VP lamb with natural microbiota spoilage occurred more quickly. The potential importance of *Clostridium* in driving the rate of quality loss of chilled VP lamb was also highlighted by Kaur et al. (2017b). Using 16S rRNA amplicon sequence data to estimate absolute bacterial numbers, they found *Clostridium* reached maximum population at the time of onset of spoilage. Further, using 16S rRNA amplicon sequence data, Kaur et al. (2021) revealed that *Clostridium* was consistently the main taxa that dominated lamb meat with low sensory scores, and was more abundant on lamb compared to beef. Similarly, the 16S rRNA amplicon sequence data in chapter 4 found that *C. algidicarnis* was the most significant ASV that was associated with VP lamb of low sensory quality. The growth capability of *Clostridium* on VP lamb is most likely attributable to lamb's intrinsically higher pH (~0.4 units higher than beef) (Kaur et al., 2021; Yang et al., 2009; Yang et al., 2014). However, further studies are required to specifically determine the effect of meat pH on ecology of *Clostridium* spp. associated with spoilage of VP lamb.

Chapters 2 and 4 establish the significance of *Clostridium* spp. in the spoilage rate of VP lamb. These results highlight the importance of abattoir hygiene, and the potential benefit of implementing interventions against *Clostridium* within these processing facilities, such as potential use of sporicidal chemical agents on equipment, and hot/cold water carcase washing to delay spoilage by *Clostridium* (Adam et al., 2013). Further, these results also

emphasise the need to minimise faecal contamination of carcases as high numbers of *Clostridium* spp. have been detected in faeces (Moschonas et al., 2009).

In chapter 3 of this research, the potential of glucose as a surface treatment for extending the shelf-life of VP lamb was investigated. Despite published reports demonstrating that glucose surface treatments can help extend the shelf-life of meat products in aerobic conditions (Kumudavally et al., 2010; Lambropoulou et al., 1996; Newton and Gill, 1978; Shelef, 1977), the effects of this treatment were yet to be reported under anaerobic/VP conditions in combination with low temperatures used in commercial storage and distribution i.e., 0 to 4°C. This study found that glucose surface treatments, ranging from concentrations of 0.5% - 10% (w/v) extended the shelf-life of both bone-in and boneless vacuum-packed lamb shoulder stored at 4°C. The 5% treatment was the most effective in all cases, increasing the shelf-life of bone-in lamb by > 76% and boneless lamb by 35% relative to the control.

The glucose treatments had no effect on meat colour, measured lactic acid concentration, total viable bacterial population density or growth rates. There was also no clear relationship between the onset of meat spoilage and glucose depletion. However, glucose treatments reduced meat pH by up to 0.43 units compared to the control; as glucose concentration increased, the larger the pH decline that occurred over time. The observed reduction in meat pH by additional glucose most likely reduces meat pH due to the accumulation of organic acid end-products via glucose fermentation (Barua and Shelef, 1980; Leisner et al., 2007; Shelef, 1977). This may contribute to longer shelf-lives of VP lamb by affecting the microbial community composition and the rate at which malodorous metabolites accumulate. Studies described in chapter 4 confirmed that glucose treatments had a significant effect on the

microbial community composition of VP bone-in and boneless lamb. The most effective treatments for bone-in (1% and 5%) and boneless (5%) lamb had significantly lower numbers of various *Vagococcus* amplicon sequence variants, and all glucose concentrations resulted in significantly lower numbers of *S. proteamaculans* on bone-in lamb. In accordance, growth kinetics over storage revealed that the various *Vagococcus* ASVs had a faster growth rate, smaller maximum population densities, and in some cases, a longer lag in the 5% glucose treatment compared to the control in both bone-in and boneless shelf-life trials. Similarly, *S. proteamaculans* had an extended lag and a smaller maximum population density in the 5% glucose treatment compared to the control. There are few studies that have investigated the spoilage potential of *Vagococcus* on VP red meat (Jääskeläinen et al., 2020). This research found that *Vagococcus* spp. had no clear association with low odour quality of VP lamb. This suggests that *Vagococcus* spp. could be contributing to spoilage implicitly through interactions with other bacteria within the community, such as exchange of metabolites and nutrients (Gram et al., 2002; Jørgensen et al., 2000; Mayo et al., 2021; Morin et al., 2018), and potentially metabolite to metabolite interactions with those catabolites formed by other bacteria (Françoise and Jean-Jacques, 2011). It is unlikely that additional glucose inhibited *Vagococcus* spp. by acidic metabolic by-products, as lactic acid bacteria are generally acidic tolerant (Adams and Nicolaides, 1997). This is supported by the observation that *Vagococcus* spp. grew to significantly higher numbers on the 10% glucose treated bone-in meat compared to other glucose treatments, despite the 10% treatment having the largest reduction in pH.

A possible explanation for the significantly lower numbers of *Vagococcus* spp. on the most successful glucose treatments could be due to the resulting shift in substrate utilisation by

the microbial community at these concentrations. Specifically, by manipulating microbial access to favoured catabolic substrates it is possible to provide a competitive advantage for those bacteria with a higher affinity for such substrates, therefore altering the order of utilisation and subsequent catabolic by-products within the meat ecosystem (Casaburi et al., 2015; Doulgeraki et al., 2012; Gram et al., 2002). This is also likely to affect the complex antagonistic and synergistic interactions within the microbial community, therefore altering the metabolism and potential success of some bacteria (Françoise and Jean-Jacques, 2011; Gram et al., 2002; Mayo et al., 2021; Morin et al., 2018; Zhang et al., 2015). The success of *Vagococcus* sp. through interactions within a microbial community has also previously been reported in sour milk fènè (Wullschleger et al., 2018).

This study detected no significant differences in the composition of organic volatile compounds between glucose treatments for either bone-in or boneless lamb, which may be due to the variability between samples. Further, the sensory impact of volatile compounds is not easily defined as interactions between molecules can create unpredictable off-odours, in addition to the interactions with the microbial community (Casaburi et al., 2015). However, there was a significant increase in the range of sulphur compounds, esters and carboxylic acids later in storage, indicating an increase in microbial degradation of amino acids, lipids and other catabolic by-products, reducing sensory quality of meat (Argyri et al., 2015; Casaburi et al., 2015; Corral and Flores, 2017; Ercolini et al., 2011; Ercolini et al., 2009; Frank et al., 2020; Jääskeläinen et al., 2016; Kosowska et al., 2017; Reis et al., 2016).

This research has demonstrated that the application of glucose has the potential to be developed for shelf-life extension of VP lamb. Further research is required to investigate the efficacy of this treatment using other types of meat cuts and at lower commercial storage

temperatures. To better understand the mechanisms behind glucose shelf-life extension of VP meat further studies are required to investigate its effects on microbial substrate utilisation and manifestation of spoilage metabolites, such as measurement of non-volatile metabolites.

In conclusion, the research and results described in this thesis has extended the research literature on the microbiology of VP lamb. It reinforced that *Clostridium* plays a significant role in the faster rate of quality loss of VP lamb. It also highlighted the complexity of microbial spoilage mechanisms and the need to gain a better understanding of the interactive mechanisms of bacteria within the meat ecosystem. This research has also assisted with the development of a safe and cost-effective intervention, which has the potential to minimise the challenges lamb exporters face due to lamb's faster rate of quality loss.

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Appendix A

Supplementary figures of Chapter 4

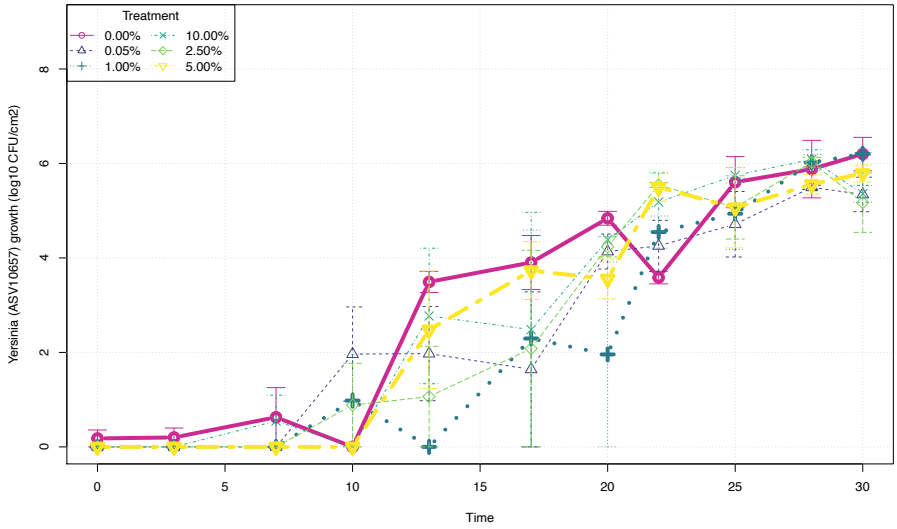

Figure A.1 Growth (± SD) of *Yersinia* sp. (ASV10657) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

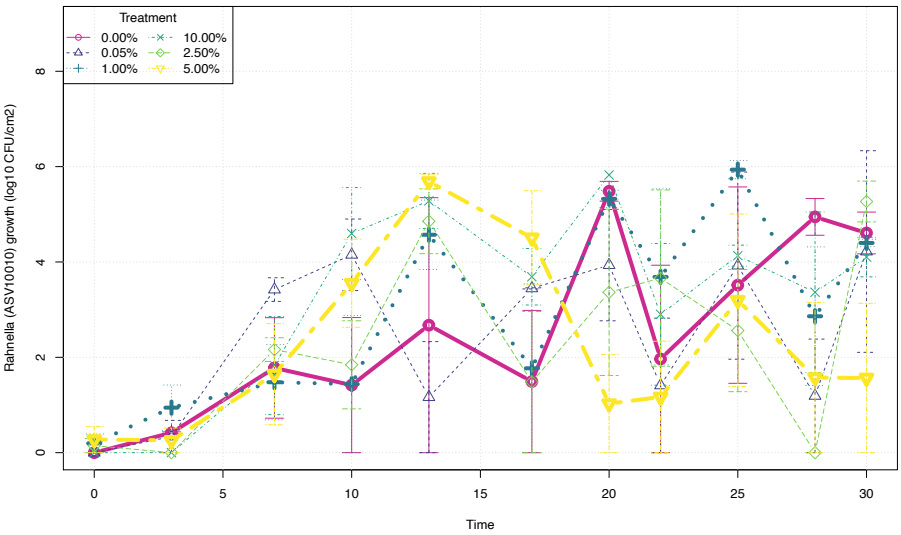

Figure A.2 Growth (± SD) of *Rahnella* sp. (ASV10010) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

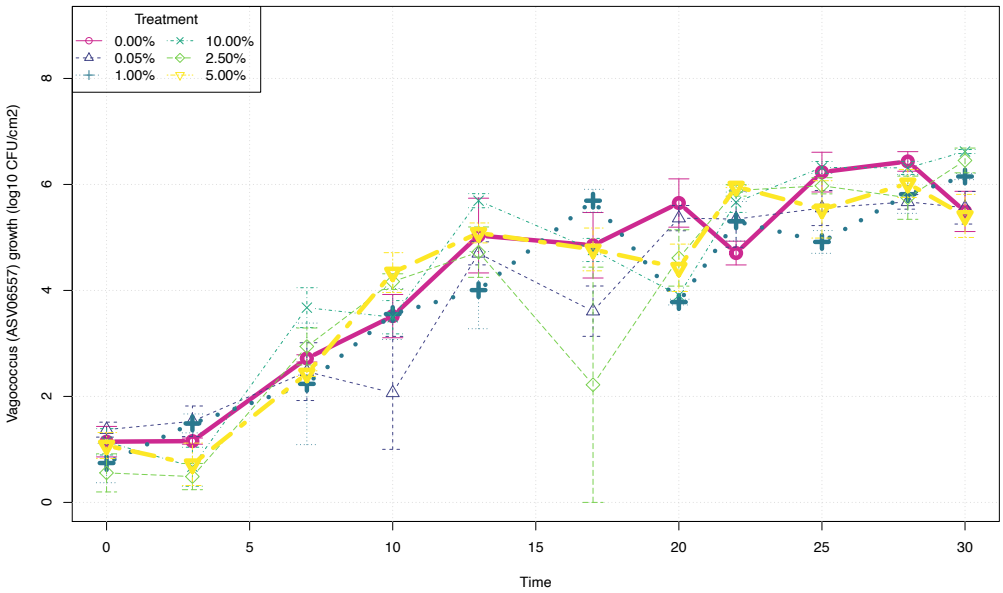

Figure A.3 Growth (± SD) of *Vagococcus* sp. (ASV06557) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

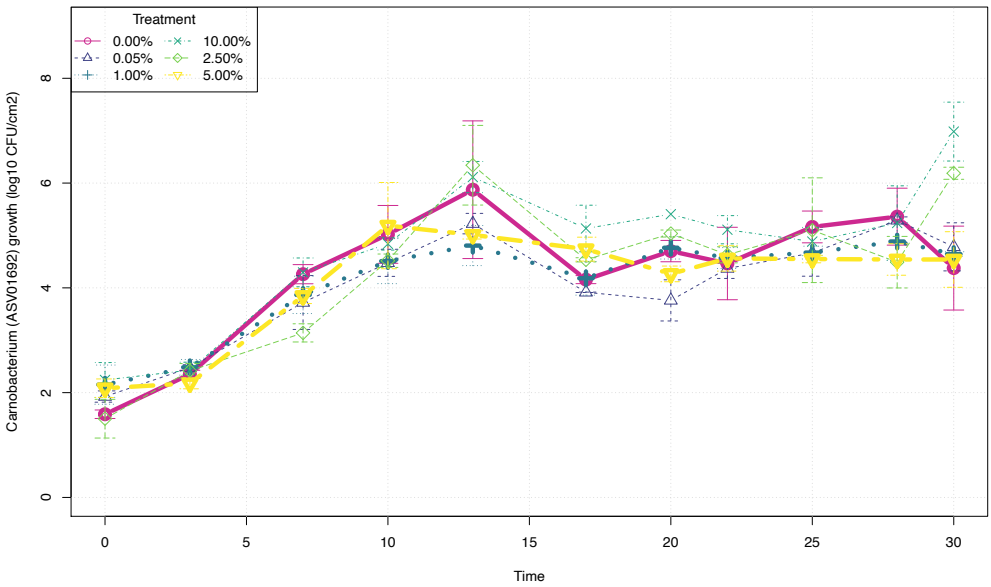

Figure A.4 Growth (± SD) of *Carnobacterium* sp. (ASV01692) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

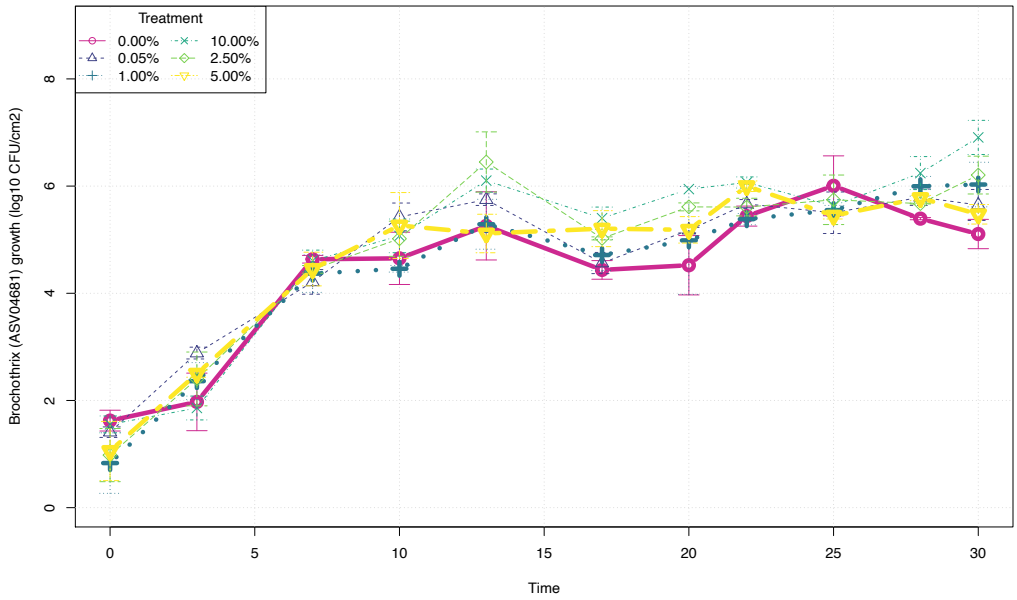

Figure A.5 Growth (± SD) of *Brochothrix* sp. (ASV04681) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

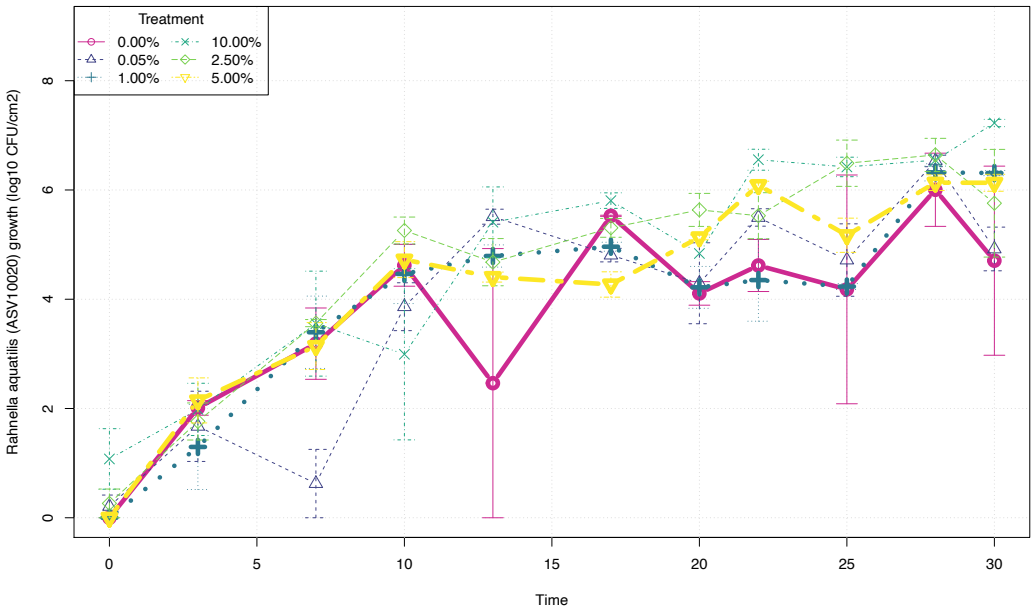

Figure A.6 Growth (± SD) of *Rahnella aquatilis* (ASV10020) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

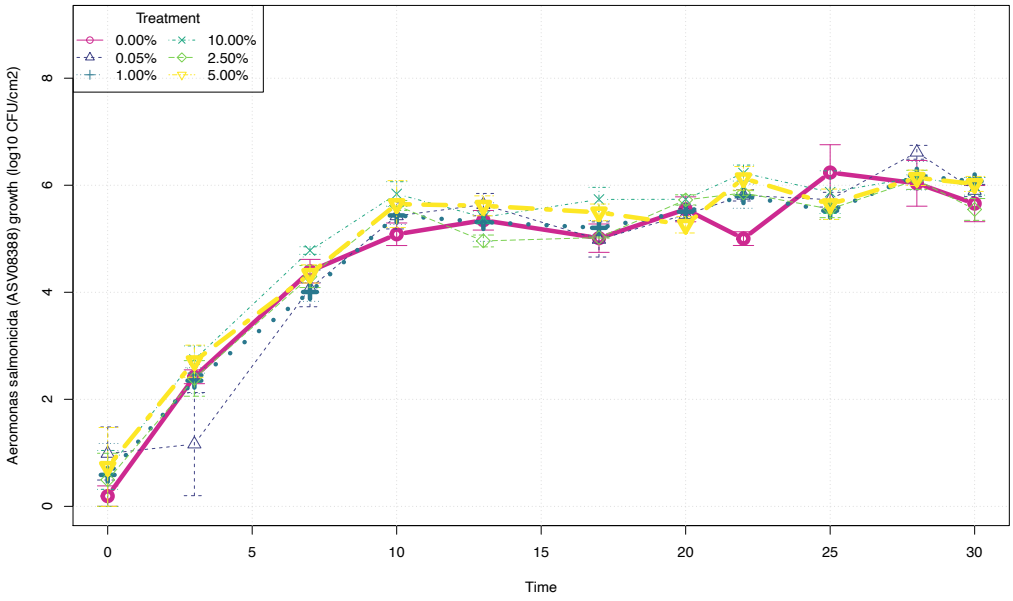

Figure A.7 Growth (± SD) of *Aeromonas salmonicida* (ASV08388) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

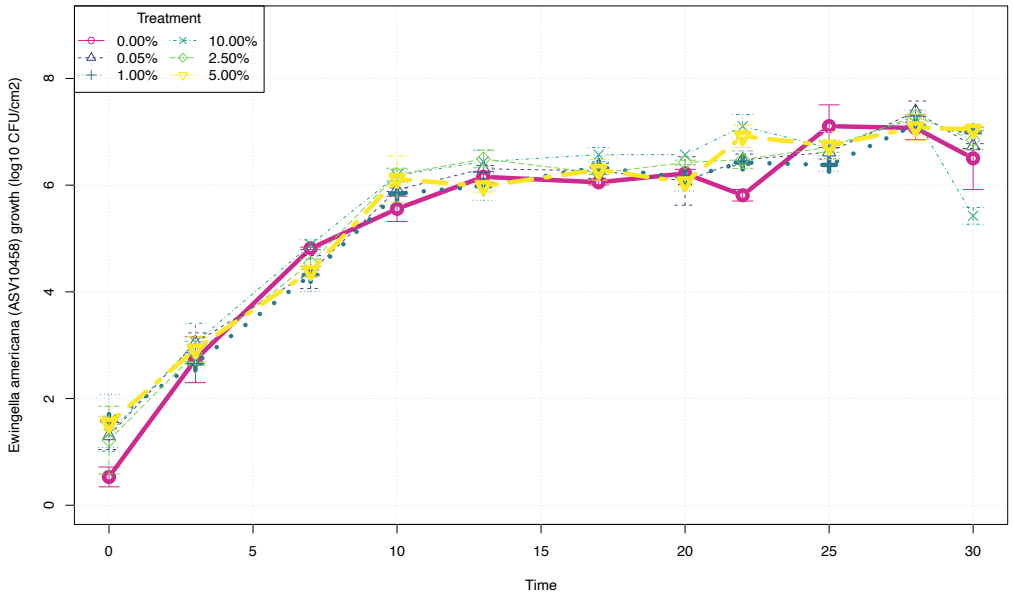

Figure A.8 Growth (± SD) of *Ewingella americana* (ASV10458) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

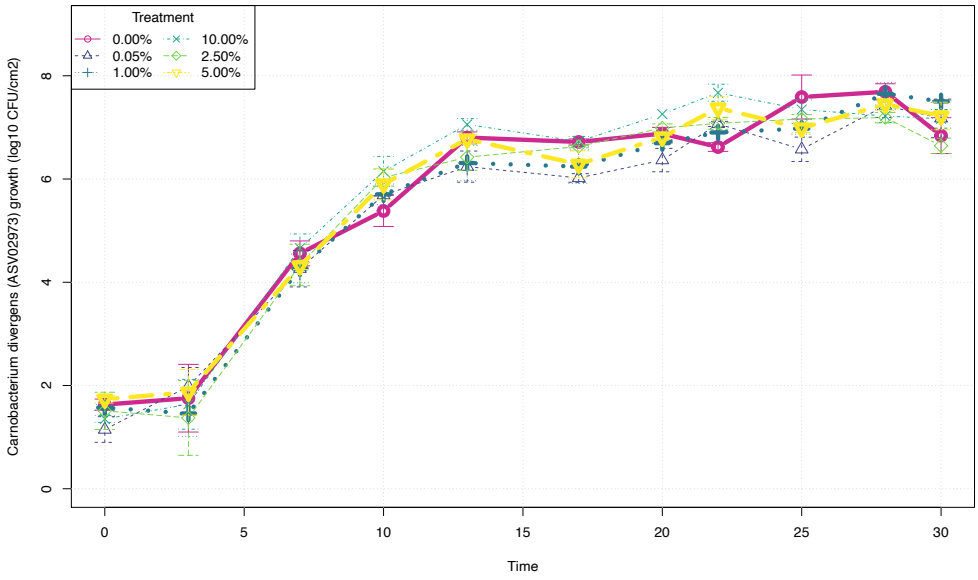

Figure A.9 Growth (± SD) of *Carnobacterium divergens* (ASV02973) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

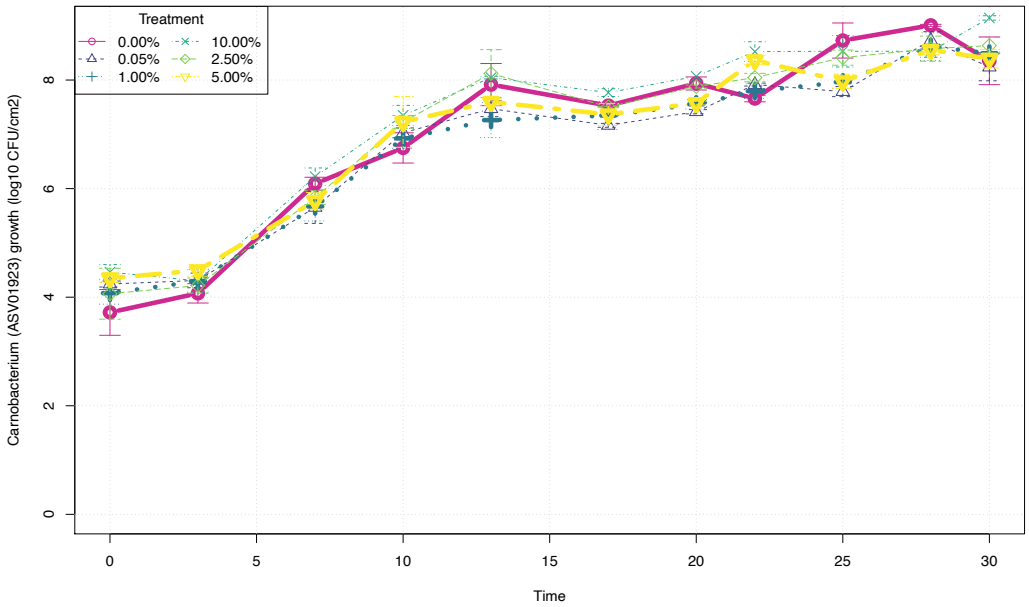

Figure A.10 Growth (± SD) of *Carnobacterium* sp. (ASV01923) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

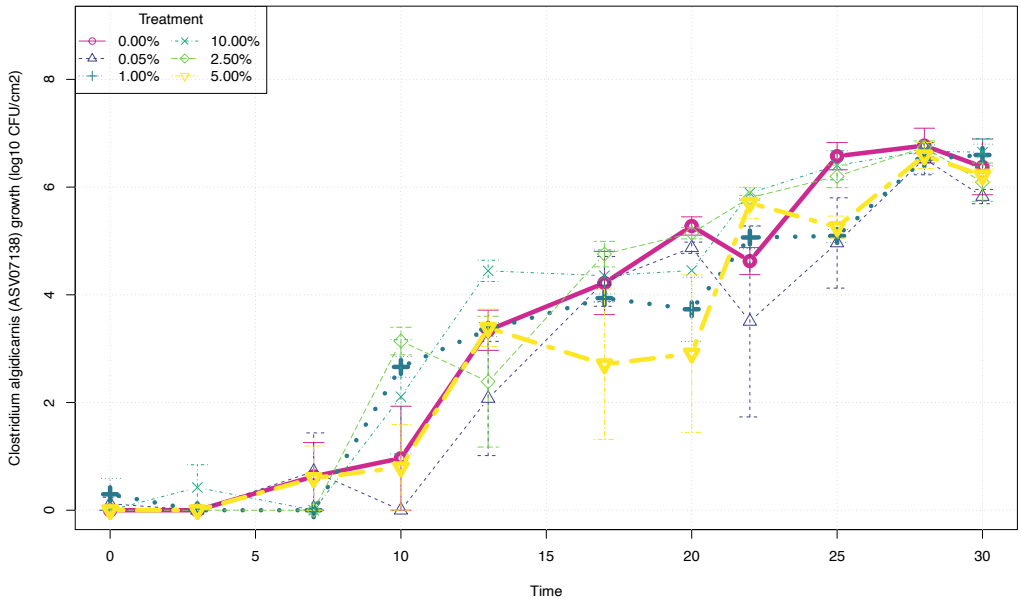

Figure A.11 Growth (± SD) of *Clostridium algidicarnis* (ASV07138) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

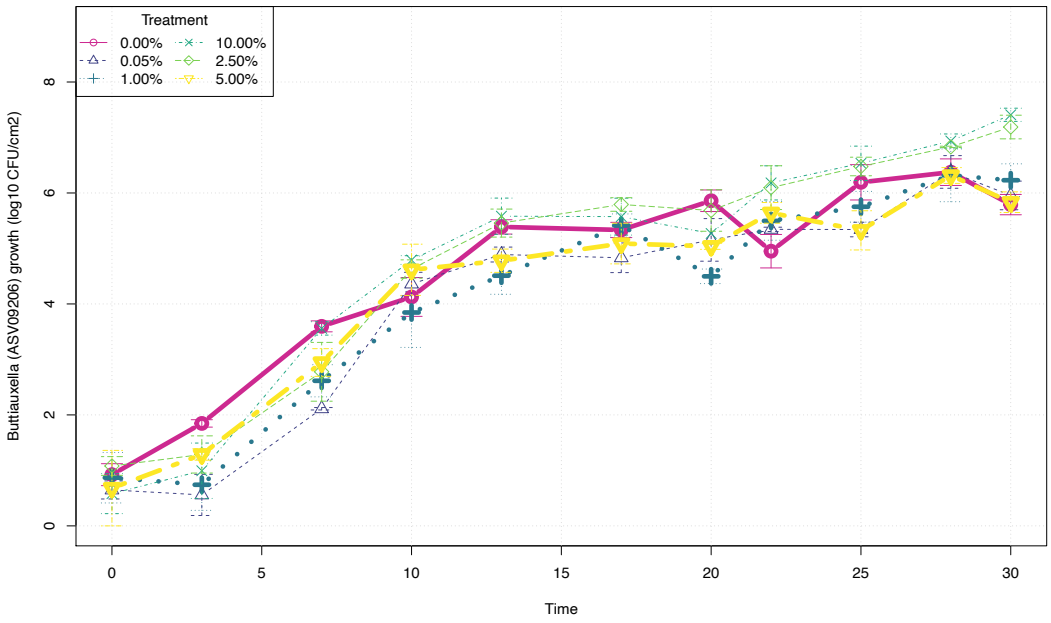

Figure A.12 Growth (± SD) of *Buttiauxella* sp. (ASV09206) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

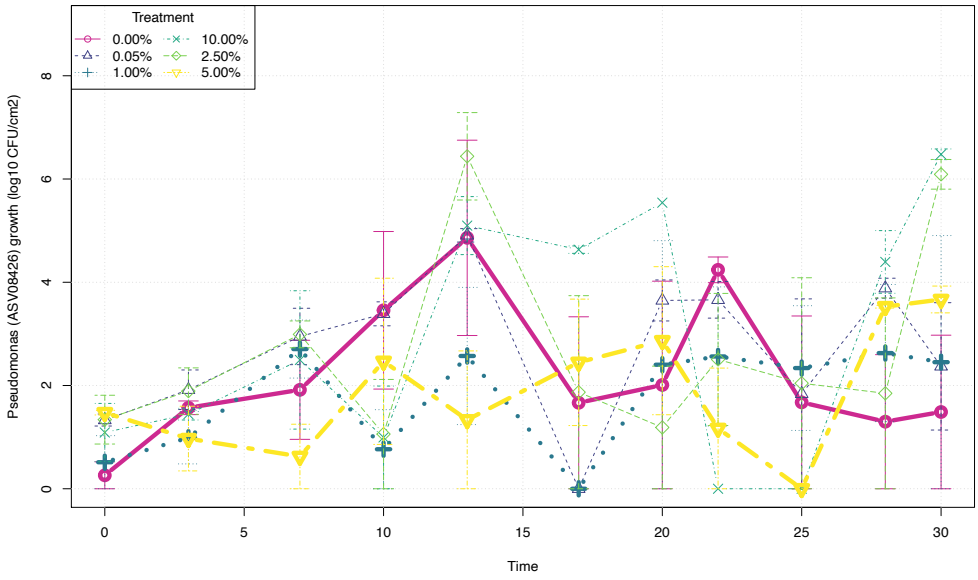

Figure A.13 Growth (± SD) of *Pseudomonas* sp. (ASV08426) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

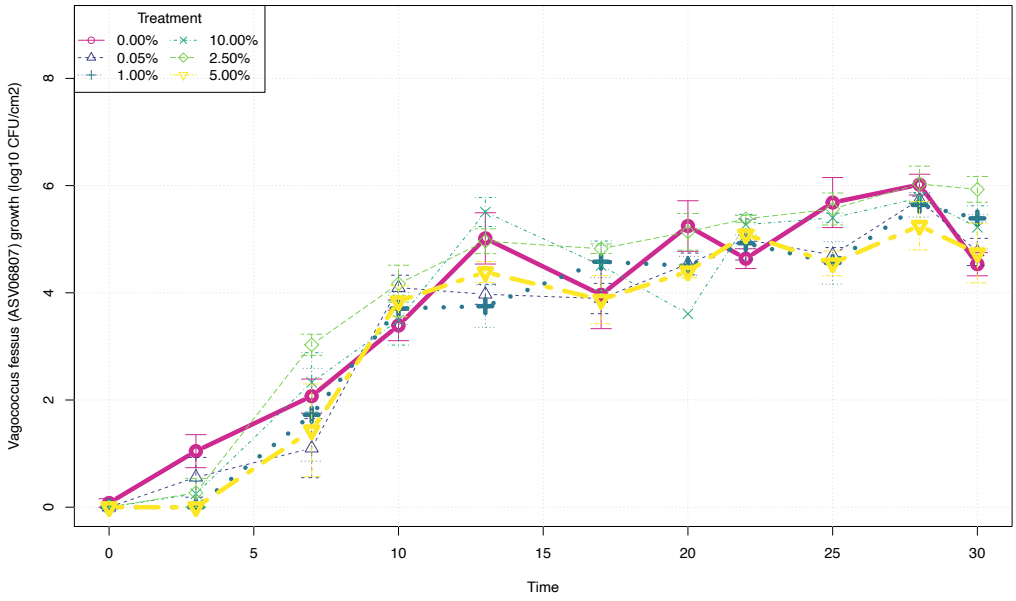

Figure A.14 Growth (± SD) of *Vagococcus fessus* (ASV06807) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

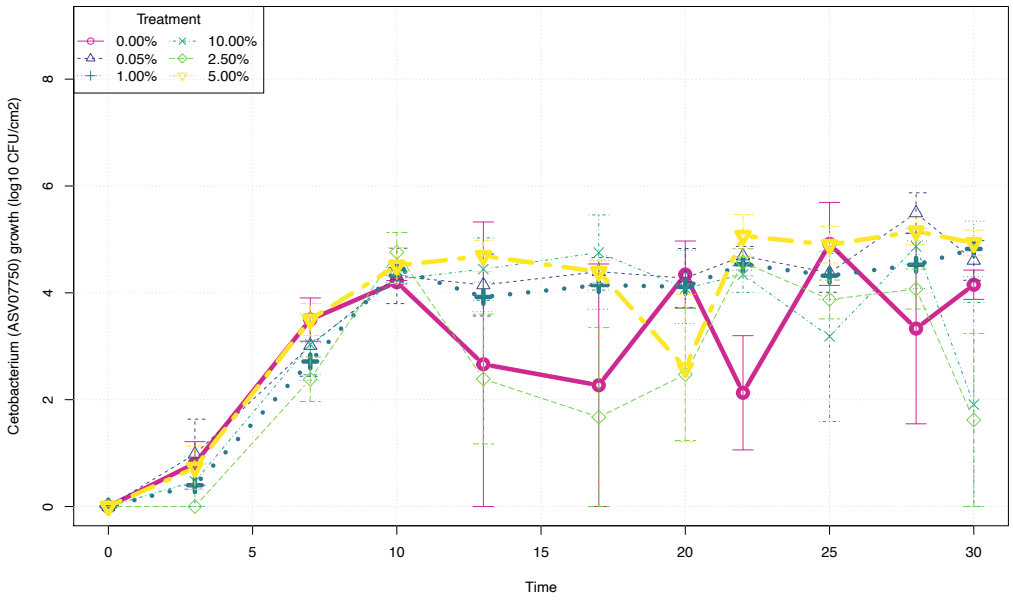

Figure A.15 Growth (± SD) of *Cetobacterium* sp. (ASV07750) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

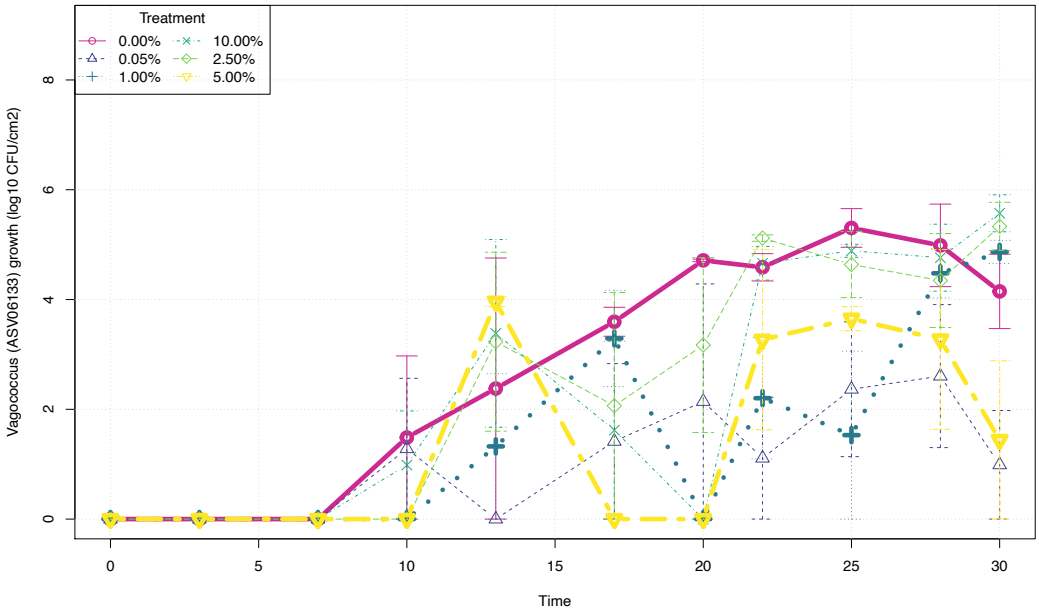

Figure A.16 Growth (± SD) of *Vagoccocus* sp. (ASV06133) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

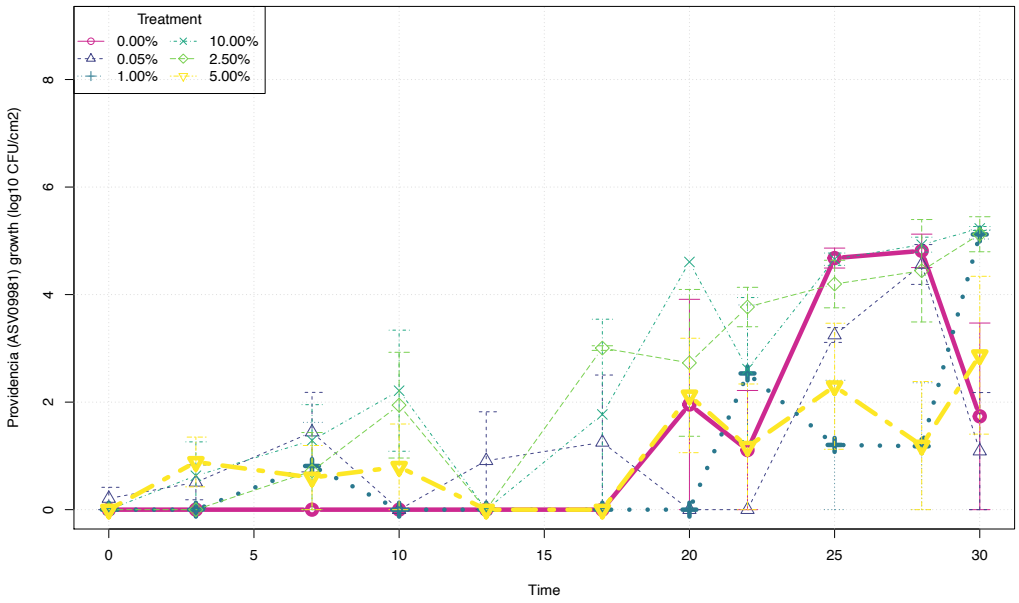

Figure A.17 Growth (± SD) of *Providencia* sp. (ASV09981) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

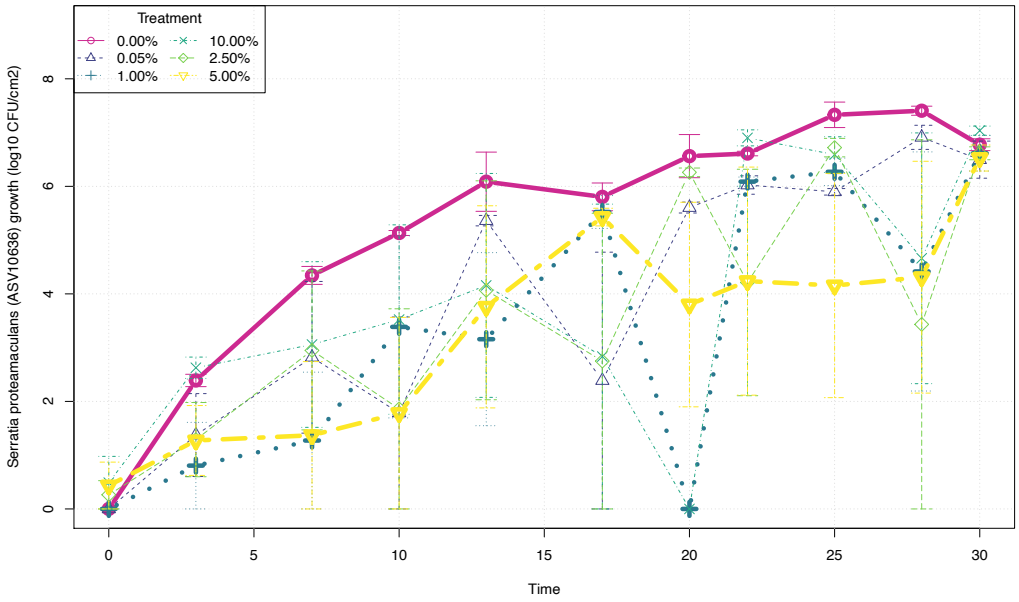

Figure A.18 Growth (± SD) of *Serratia proteamaculans* (ASV10636) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

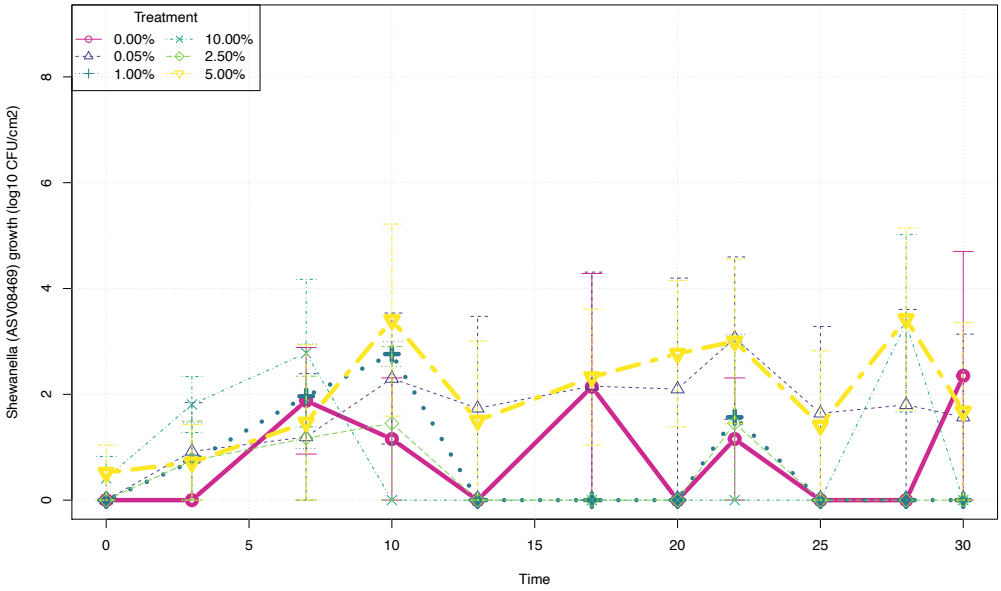

Figure A.19 Growth (± SD) of *Shewnella* (ASV08469) on VP bone-in lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (1% and 5%) for shelf-life extension are bolded.

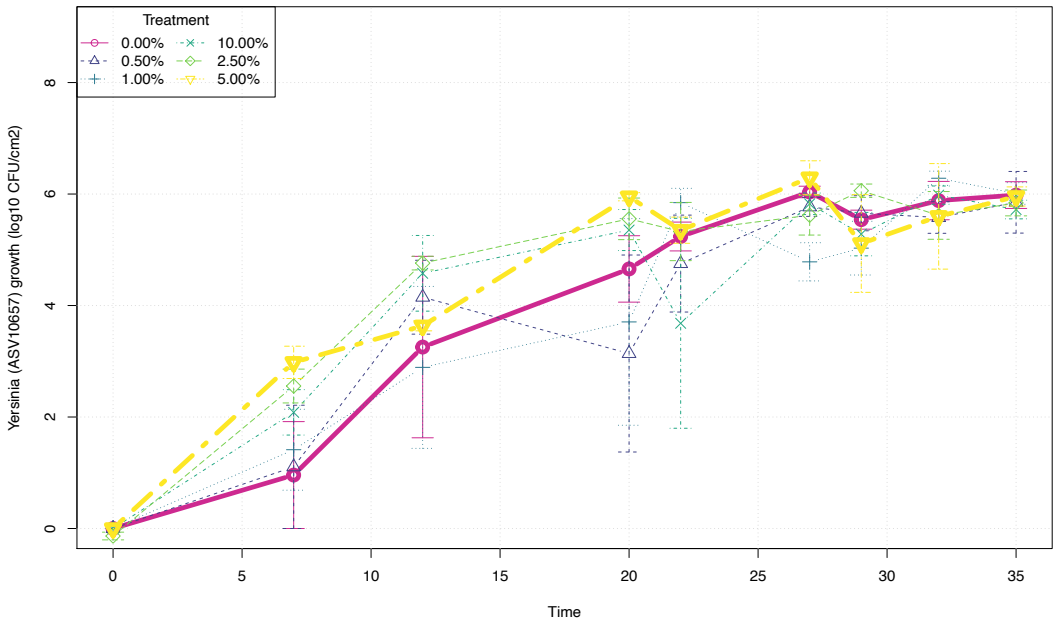

Figure A.20 Growth (± SD) of *Yersinia* sp. (ASV10657) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

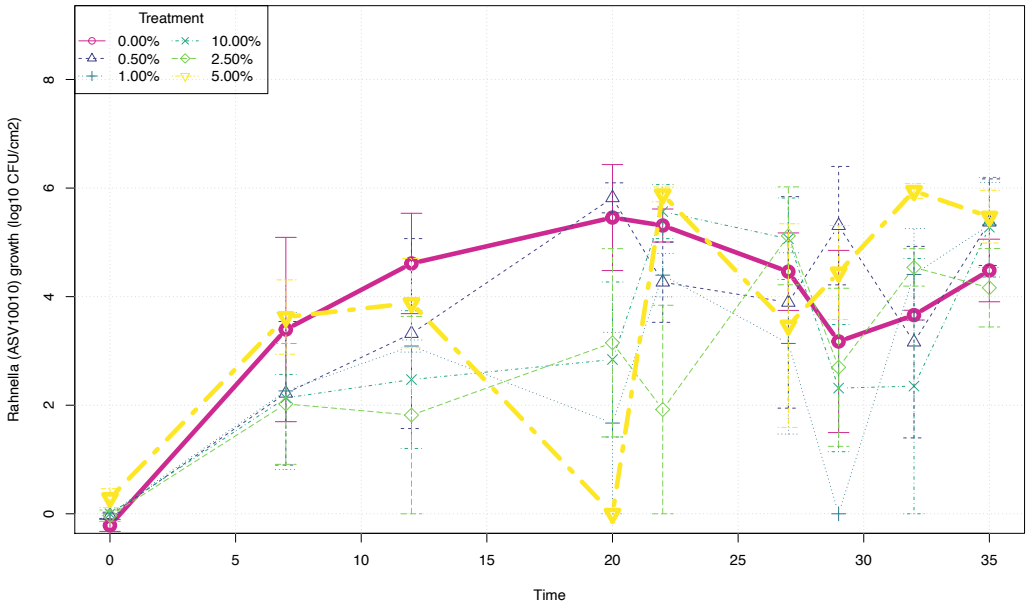

Figure A.21 Growth (± SD) of *Rahnella* sp. (ASV10010) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

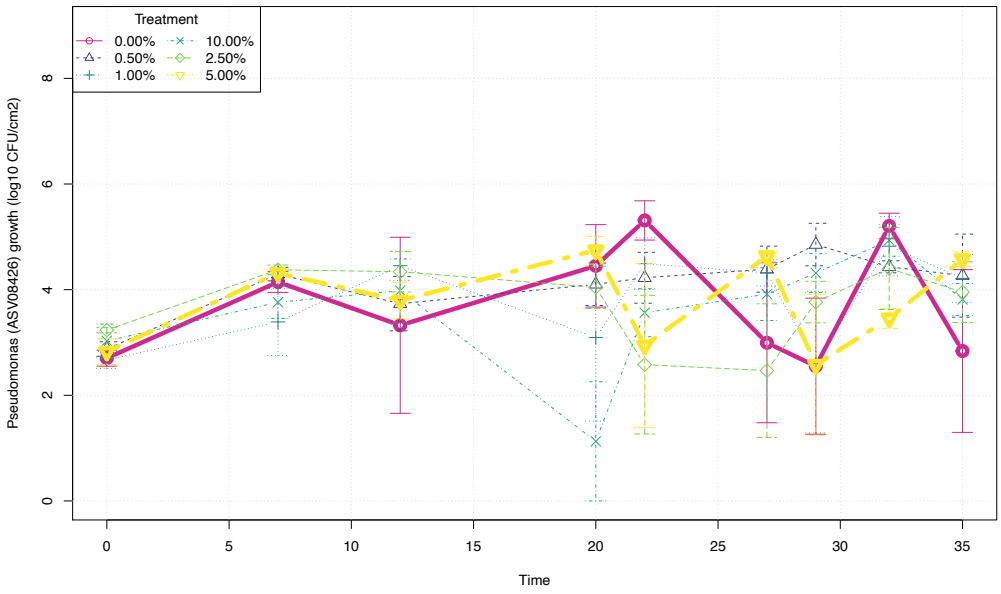

Figure A.22 Growth (± SD) of *Pseudomonas* sp. (ASV08426) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

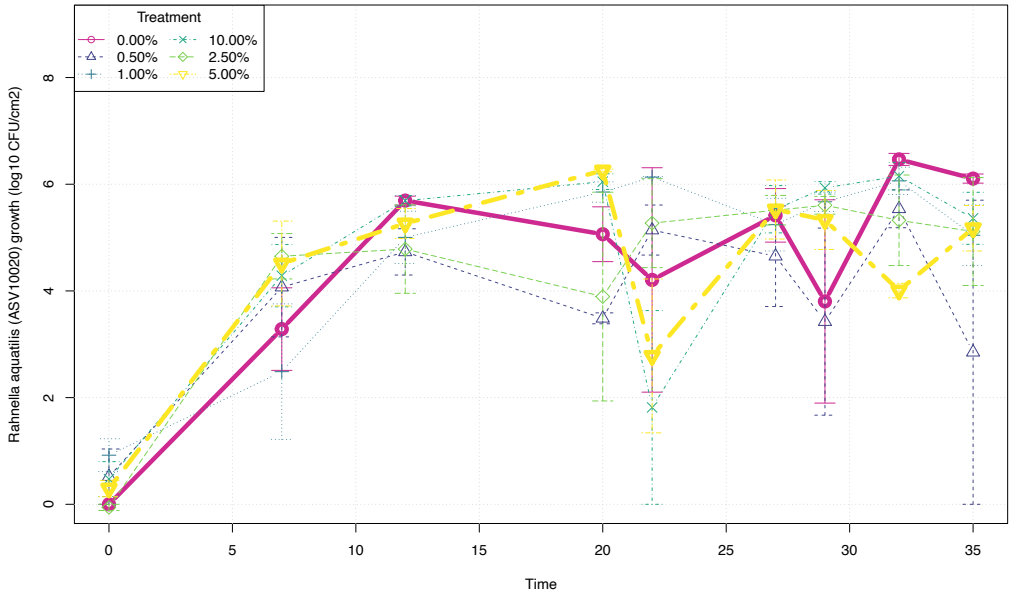

Figure A.23 Growth (± SD) of *Rahnella aquatilis* (ASV10020) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

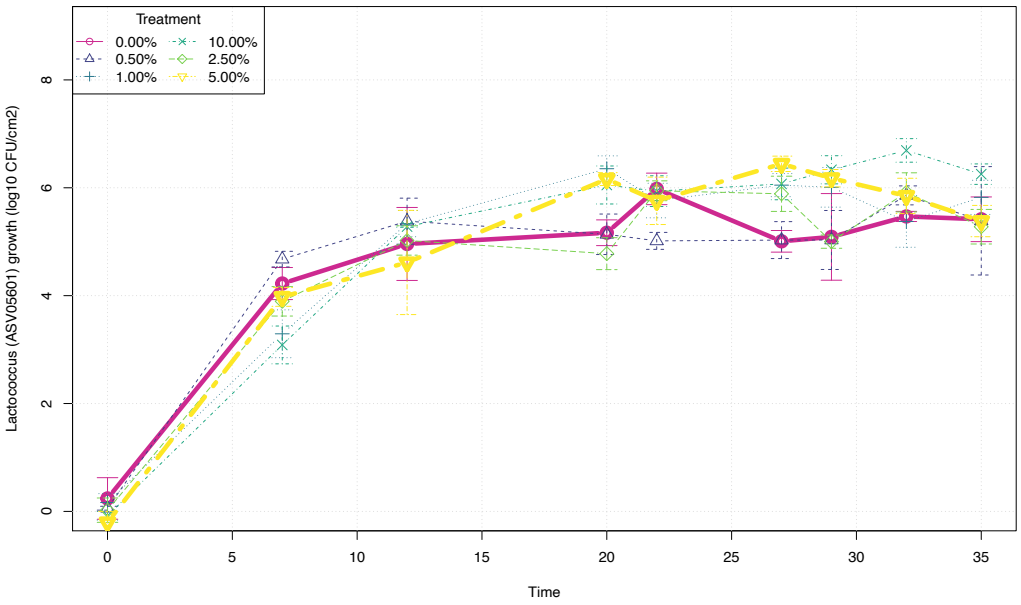

Figure A.24 Growth (± SD) of *Lactcoccus* sp. (ASV05601) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

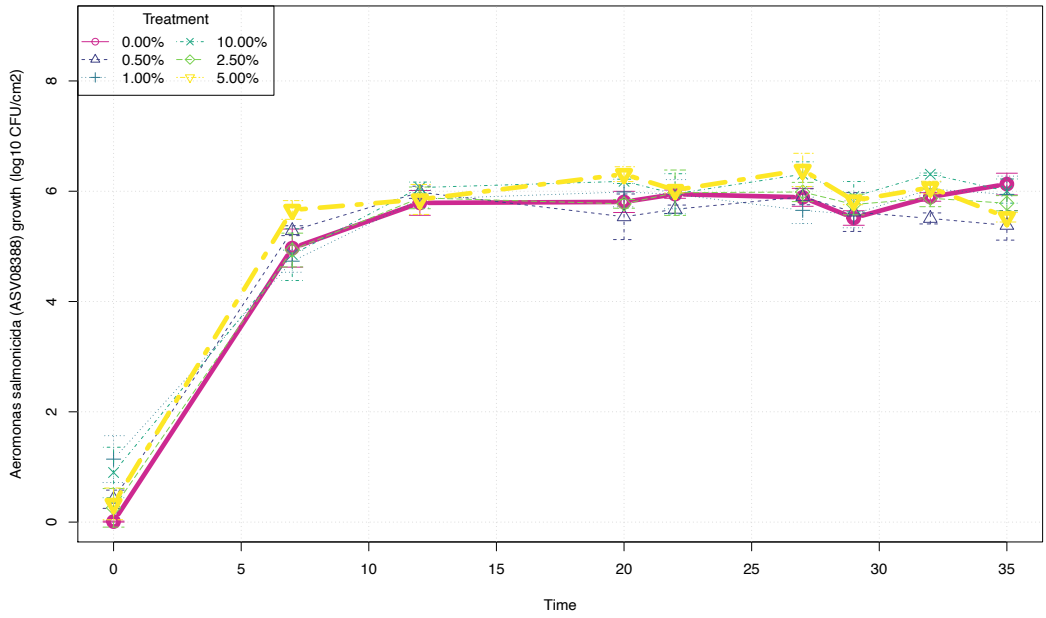

Figure A.25 Growth (± SD) of *Aeromonas salmonicida* (ASV08388) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

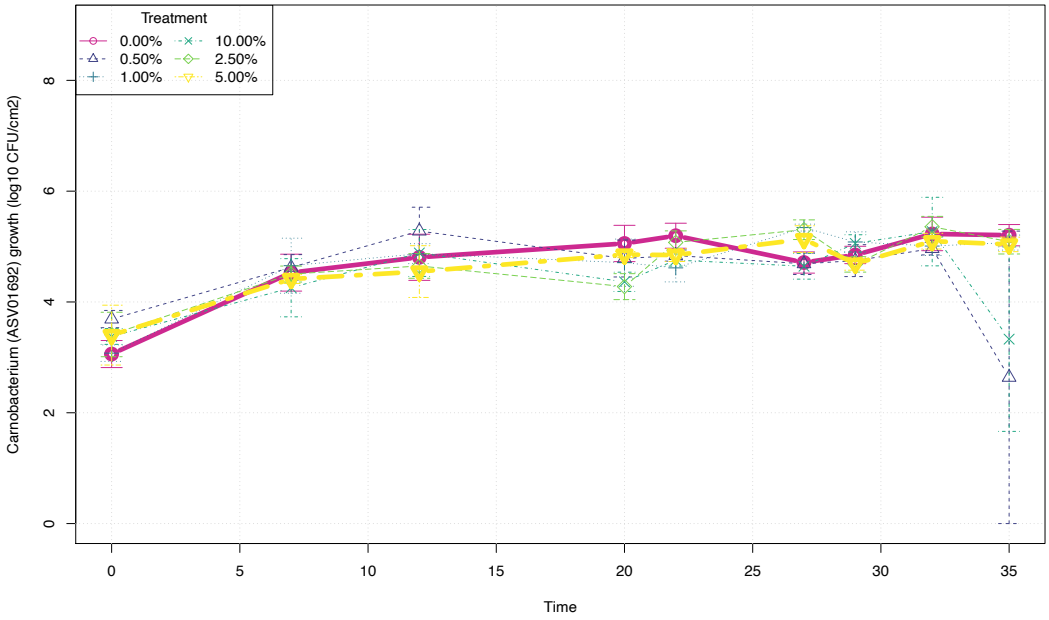

Figure A.26 Growth (± SD) of *Carnobacterium* sp. (ASV01692) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

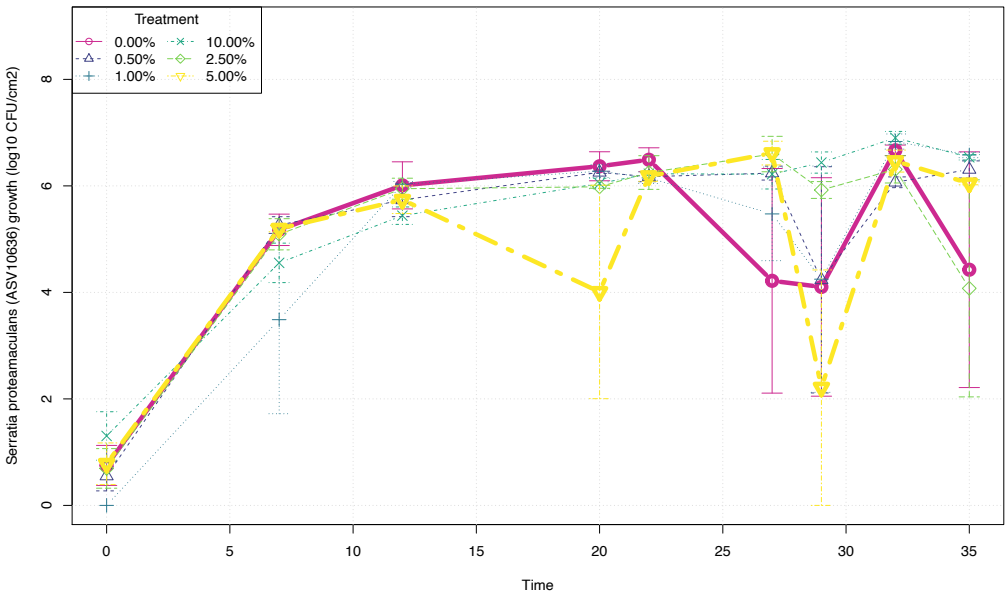

Figure A.27 Growth (± SD) of *Serratia proteamaculans* (ASV10636) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

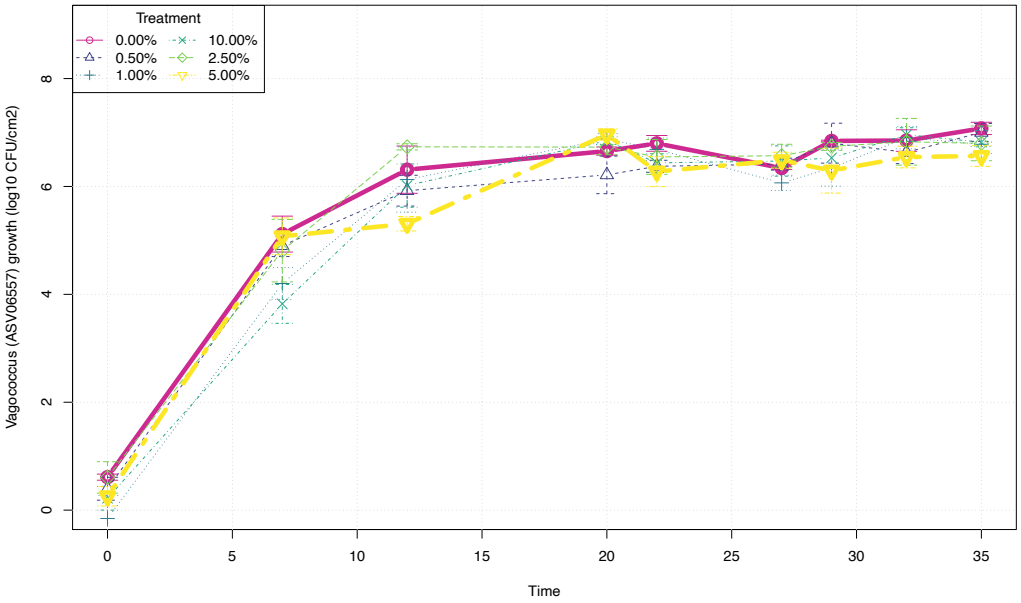

Figure A.28 Growth (± SD) of *Vagococcus* sp. (ASV06557) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

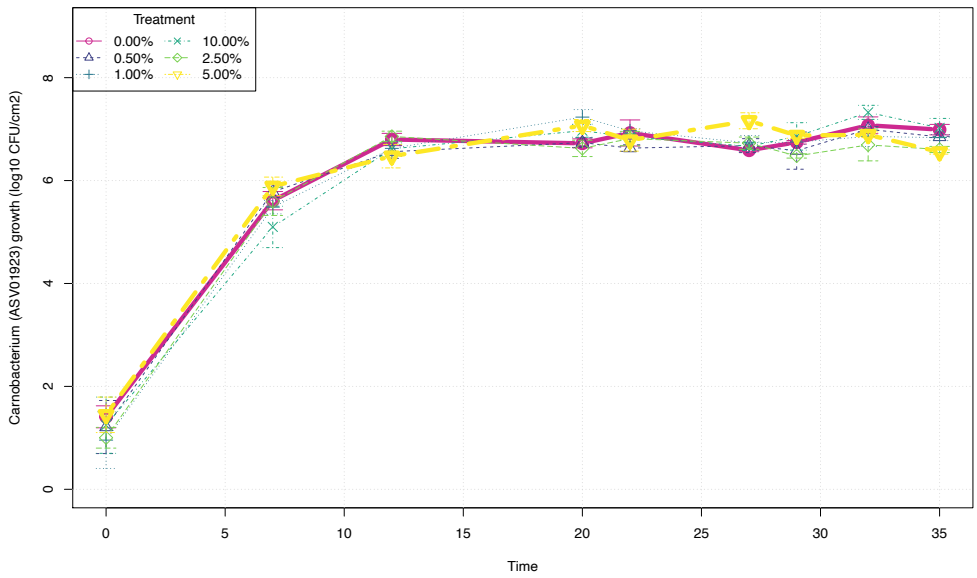

Figure A.29 Growth (± SD) of *Carnobacterium* sp. (ASV01923) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

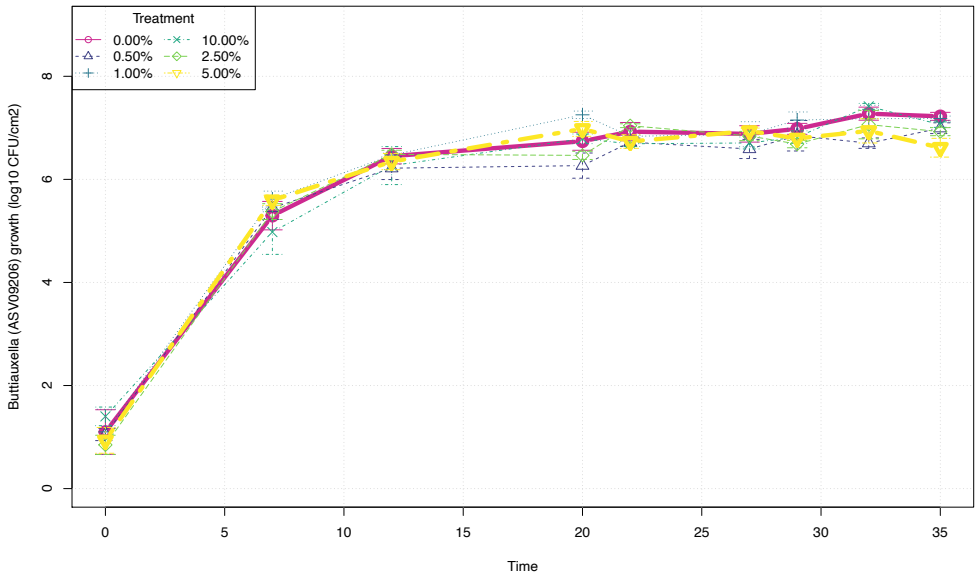

Figure A.30 Growth (± SD) of *Buttiauxella* sp. (ASV09206) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

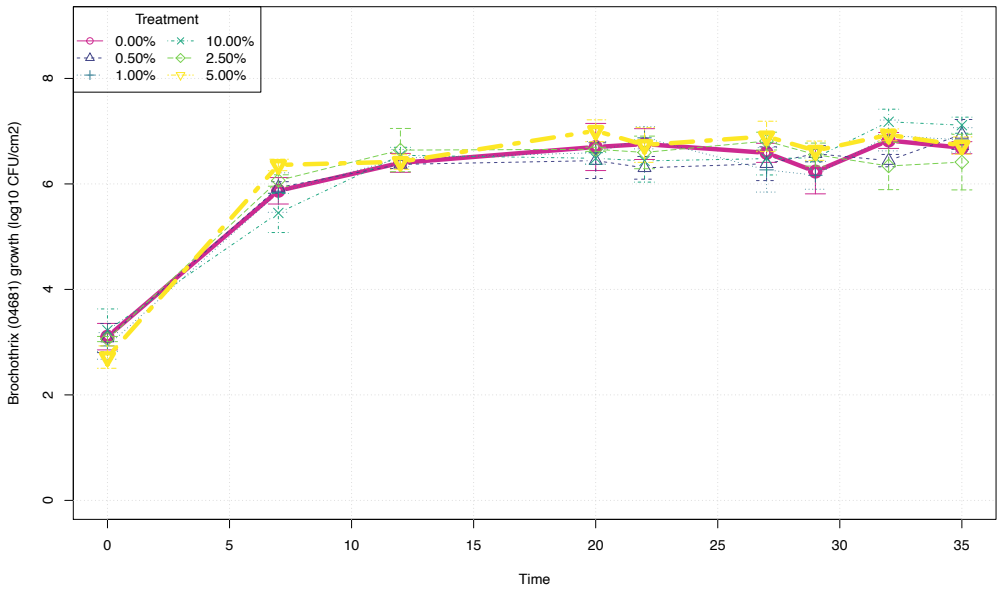

Figure A.31 Growth (± SD) of *Brochothrix* sp. (ASV04681) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

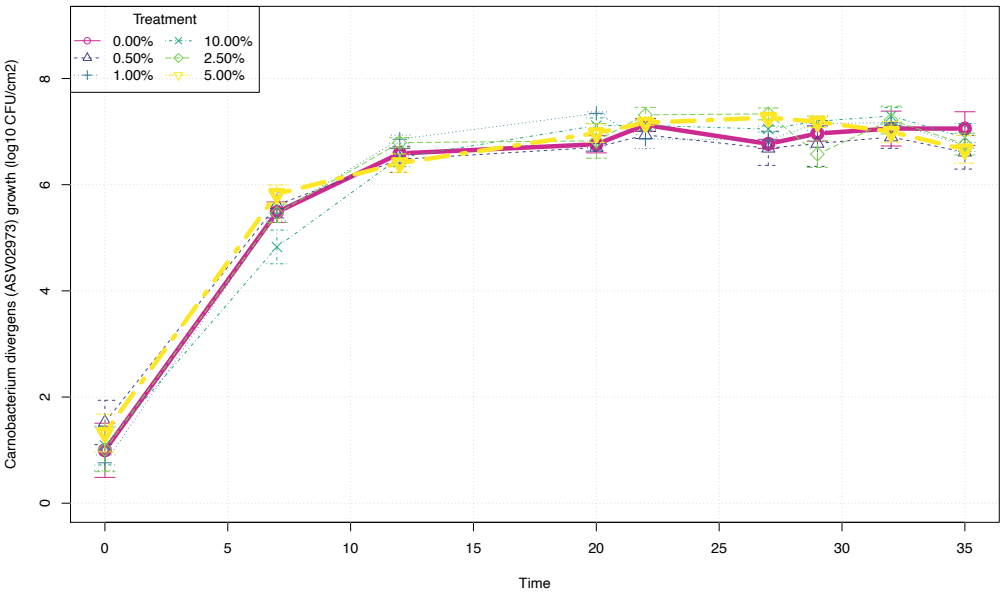

Figure A.32 Growth (± SD) of *Carnobacterium divergens* (ASV02973) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

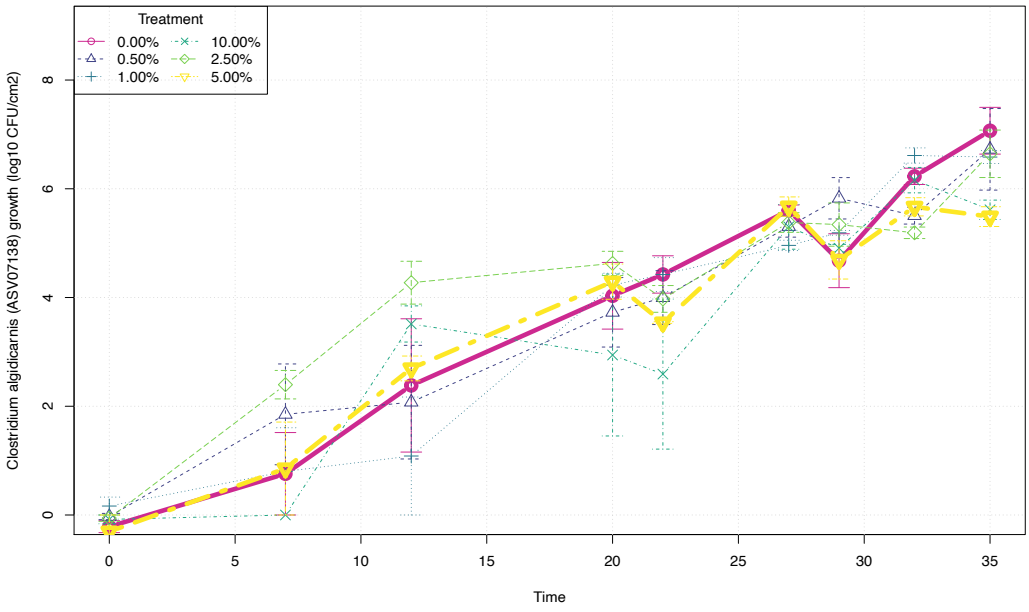

Figure A.33 Growth (± SD) of *Clostridium algidicarnis* (ASV07138) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

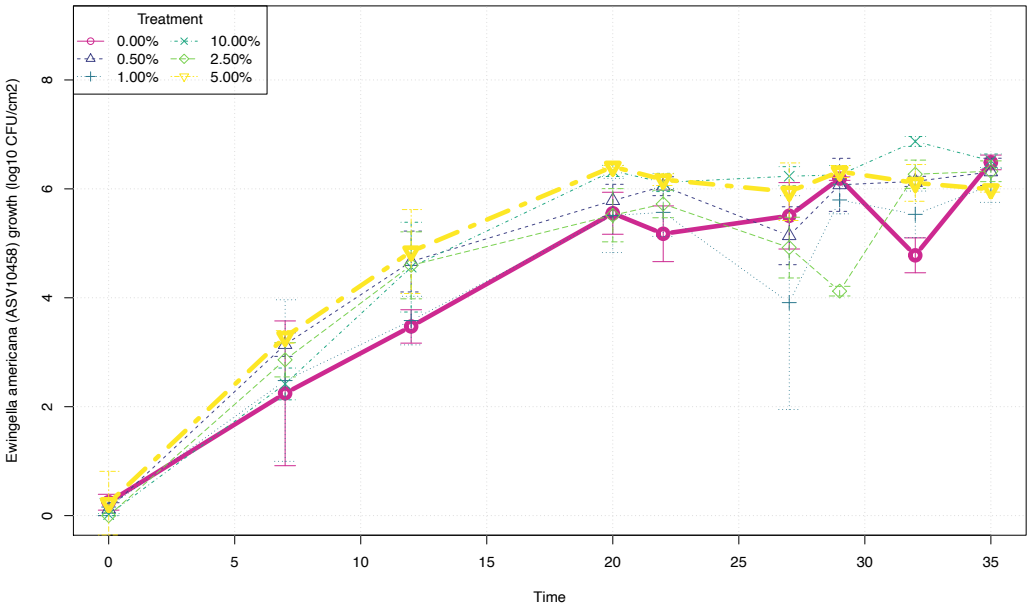

Figure A.34 Growth (± SD) of *Ewingella americana* (ASV10458) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

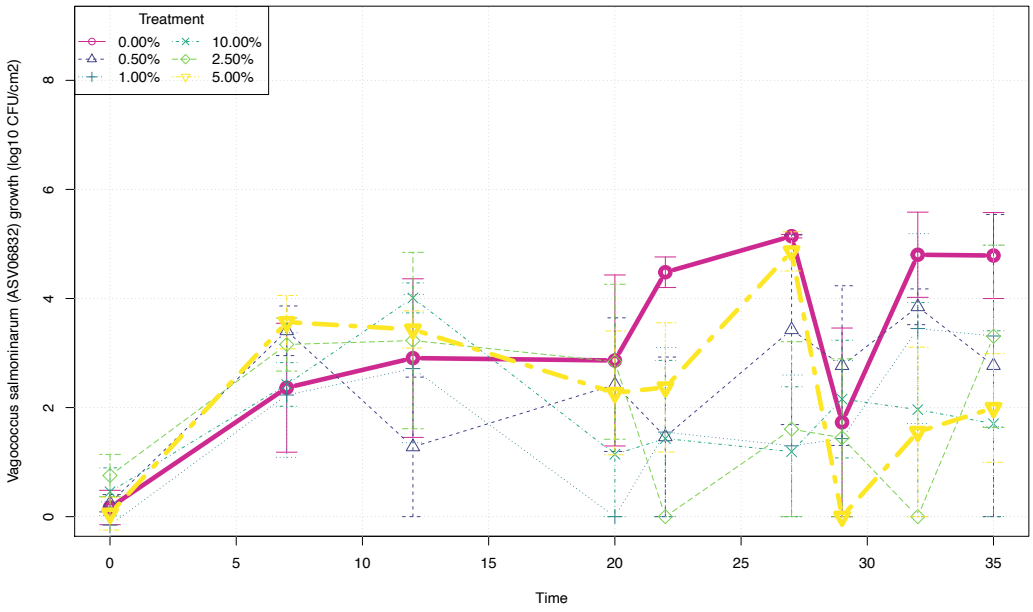

Figure A.35 Growth (± SD) of *Vagocuccus salmoninarum* (ASV06832) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.

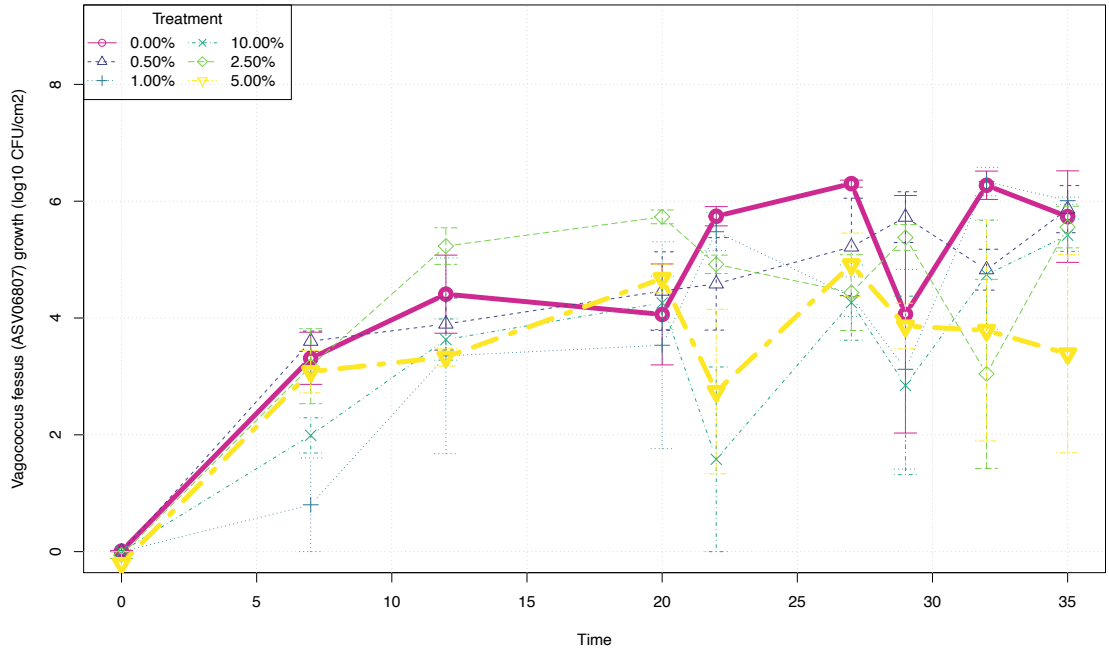

Figure A.36 Growth (± SD) of *Vagococcus fessus* (ASV06807) on VP boneless lamb shoulder over storage time (days) at 4°C after surface treated with different glucose concentrations. Growth curves are relative to TVC estimated directly from 16S rRNA gene sequence data. The control and the most effective glucose treatments (5%) for shelf-life extension are bolded.