Dry-ageing of lean beef and lamb: Effect of processing and storage factors on product quality and acceptability

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgments), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Signature of candidate

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Abstract

Dry-ageing is a traditional post-mortem processing technique for the improvement of tenderness and flavour of meat.

Most dry-aged meat is produced from well-marbled prime beef cuts rather than lean beef. The use of lean beef may offer advantages for storage stability and reduced off-flavours arising from oxidative and microbial spoilage. Lamb is another important red meat with characteristic flavour profile favoured by consumers around the world. The quality of wet-aged lamb has been studied with limited knowledge of its dry-aged equivalents.

The current study aimed to produce dry-aged lean beef and lamb using in-bag dry-ageing (BD) technique. The study consisted of two stages: (1) Determine the impact of processing factors, including ageing air velocities, stepwise ageing and ageing time, and frozen storage on the quality and acceptability of dry-aged lean beef; and (2) Investigate the impact of BD on the quality, acceptability and biochemical changes of lamb compared to its widely available wet-aged equivalents.

Lean bull beef *longissimus lumborum* (n = 30) were stepwise in-bag dry-aged in water-permeable ageing bags at 2 °C, RH 75% under three different air velocities: 0.5, 1.5 and 2.5 $m.s^{-1}$ for 7 days followed by wet-ageing (W) for 14 days. The impact of processing factors and frozen storage on the quality and acceptability of the in-bag dry-aged lean beef was compared to the straight-dry-aged equivalents at 0.5 $m.s^{-1}$ as a control. Increased air velocities accelerated dehydration process with no negative impact on all the quality traits determined in this study, including physicochemical quality, sensory acceptability, *in vitro* digestibility and metabolite profile. A total of 1705 metabolites were identified in dry-aged beef using Rapid Evaporative Ionisation Mass spectrometry (REIMS) fingerprinting. Stepwise ageing resulted in 692 metabolites which differed ($P < 0.05$) from the control. Stepwise processed dry-aged beef had equivalent quality, palatability, lipids and proteins oxidative stabilities, and lower level of surface microorganisms and higher yield compared to the control ($P < 0.05$). Free amino acids, small peptides and metabolites arising from lipid and protein oxidation increased significantly with ageing time in fresh beef, regardless of ageing regimes (straight BD/stepwise ageing). Frozen storage for 12 months had little or no effect on the quality and acceptability of the dry-aged lean beef. Oxidative stabilities and protein digestibility

of in-bag dry-aged beef were improved during the frozen storage (-18 °C) compared to the unaged.

Lamb hindlegs (Ram, n = 60) were used to produce the in-bag dry-aged lamb products in this study. Dry-aged lamb legs were produced using water-permeable ageing bags at 2 °C, 0.5 $m.s^{-1}$ air velocity and RH 75%, for 21 days. The quality, acceptability and biochemical changes of in-bag dry-aged lamb were compared to the widely available wet-aged equivalents at -1.5 °C for 21 days. The dry- and wet-aged lamb were equally preferred by the consumer panellists suggesting the niche nature of dry-ageing products. Dry-aged lamb had harder and chewier texture profiles and lower colour attributes (L*, a* and b*) than the wet-aged ($P < 0.05$). BD resulted in higher yeast and lipid oxidation (TBARS) levels compared to the wet-aged ($P < 0.05$). There was no difference in the SDS-PAGE protein profile, fatty acids and protein carbonyl content between two ageing treatments. The gastric digestibility of dry-aged lamb was greater than wet-aged. A total of 1704 metabolites was identified using REIMS fingerprinting. There were 663 metabolites that differed ($P < 0.05$) between the ageing methods mainly arise from proteolysis and lipid metabolism. Different peptide profiles (< 10 kDa) were observed using peptidomic profiling between the two ageing techniques. Small peptides released from creatine kinase, LIM domain binding 3, nebulin, sHSP and myozenin were significantly higher for dry-aged, suggesting the potential as signature biomolecules to distinguish in-bag dry-aged from wet-aged.

Outcomes of this study demonstrate the feasibility of producing dry-aged lean beef and lamb using BD technique. Ageing time was the main factor affecting the quality of in-bag dry-aged products rather than ageing air velocities which had no impact. Stepwise ageing regime could produce dry-aged lean beef products with acceptable sensory quality, improved oxidative stability and digestibility during long-term frozen storage up to 12 months. The use of REIMS to monitor processing in meat science is a novel application suggesting the potential as a rapid food authentication screening and quality control tool in the food industry. Outcomes in the present study could also have implications for industry to produce value-added dry-aged beef and lamb which are safe microbiologically, easy to handle and free of trimming for local and export markets.

Co-authored publications

The manuscripts and conference proceedings from my PhD work are listed below. All the submissions were to international peer-reviewed journals and conferences.

Peer-reviewed journal papers:

Published papers

- **Paper I: Zhang, R.**, Yoo, M. J. Y., & Farouk, M. M. (2019). Quality and acceptability of fresh and long-term frozen in-bag dry-aged lean bull beef. *Journal of Food Quality, 2019*, 1975264. (Impact factor 1.763) – **Chapter 3**
- **Paper II: Zhang, R.**, Yoo, M. J. Y., & Farouk, M. M. (2020). Oxidative stability, proteolysis, and *in vitro* digestibility of fresh and long-term frozen stored in-bag dry-aged lean beef. *Food Chemistry, 344*, 128601. (Impact factor 6.306) **– Chapter 4**
- **Paper III: Zhang, R.**, Ross A.B., Yoo, M. J. Y., & Farouk, M. M. (2021). Use of Rapid Evaporative Ionisation Mass Spectrometry fingerprinting to determine the metabolic changes to dry-aged lean beef due to different ageing regimes. *Meat Science* special issue: Objective Measure, article number 108438. (Impact factor 3.644) – **Chapter 5**
- **Paper IV: Zhang, R.**, Yoo, M. J. Y., Realini, C.E., Staincliffe, M., & Farouk, M. M. (2020). In-bag dry *vs.* wet aged lamb: Quality, consumer acceptability, oxidative changes, and *in vitro* digestibility. *Foods, 10*(1), 41. (Impact factor 4.092) – **Chapter 6**
- **Paper V: Zhang, R.**, Ross A.B., Yoo, M. J. Y., & Farouk, M. M. (2021). Metabolic fingerprinting of in-bag dry- and wet-aged lamb with Rapid Evaporative Ionisation Mass Spectroscopy. *Food Chemistry, 347*, 128999. (Impact factor 6.306) – **Chapter 7**

Submitted manuscripts

- **Paper VI: Zhang, R.**, Yoo, M. J. Y., Ross A.B., & Farouk, M. M. (2020). Mechanisms and strategies to tailor dry-aged meat flavour. *Trends in Food Science & Technology* (Impact factor 11.077) – **Chapter 2**
- **Paper VII: Zhang, R.**, Maes, E., Noble, A., Yoo, M. J. Y., Farouk, M. M. & Deb-Choudhury S. (2020). A peptidomic approach to understand potential biomolecular signatures in dry *vs.* wet aged lamb. *Foods* (Impact factor 4.092) – **Chapter 8**

Other publication based on PhD work

- **Paper VIII:** Ross, A.B., Brunius, C., Chevallier, O., Dervilly, G., Elliott, C., Guitton, Y., Prenni, J., Savolainen, O., Hemeryck, L., Vidkjaer, N.H., Scollan, N., Stead., **Zhang, R.**, Vanhaecke, Lynn. (2020, in press). Making complex measurements of meat composition fast: Application of Rapid Evaporative Ionisation Mass Spectrometry to measuring meat quality and fraud. *Meat Science* special issue: Objective Measure, article number 108333. (Impact factor 3.644, my contribution to this review is based on my PhD work)

Peer-reviewed conference proceedings and presentations:

- **Zhang, R.**, Yoo, M.J., Craigie, C.R., Staincliffe, M., Realini, C.E., McEwan, J.C., Farouk, M.M. (2018). Session 7: Next generation technologies to assess carcase and meat quality. *Oral presentation in the 64th International Congress of Meat Science and Technology**, 12-17 Aug 2018, Melbourne, Australia.
- **Zhang, R.**, Yoo, M.J., Mungure, T.E., Bekhit, A.E.D., Farouk, M.M. (2018). Stepwise in-bag dry-ageing of lean beef. Session 7: Next generation technologies to assess carcase and meat quality. *Poster presentation in the 64th International Congress of Meat Science and Technology*, 12-17 Aug 2018, Melbourne, Australia.
- **Zhang, R.**, Ross A.B., Yoo, M. J. Y., & Farouk, M. M. (2019). Effect of step-wise dry-ageing and trimming on the metabolite profiles of dry-aged bull beef. P-11-04. *Poster presentation in the 65th International Congress of Meat Science and Technology*, 4-9 Aug 2019, Potsdam, Germany.
- **Zhang, R.**, Yoo, M. J. Y., Frost, D.A., & Farouk, M. M. (2019). Aroma profile of dry-aged lamb and bull beef using Real-Time Selected Ion Flow Tube Mass Spectrometry (SIFT-MS). P-07-14. *Poster presentation in the 65th International Congress of Meat Science and Technology*, 4-9 Aug 2019, Potsdam, Germany.
- Deb-Choudhury, S., **Zhang, R.**, Maes, E., Haines, S., Thomas, A., Yoo, M.J., Farouk, M.M. (2019). Peptidomic comparison of dry aged *vs* a novel stepwise aged lean bull beef. P-11-02. *Poster presentation in the 65th International Congress of Meat Science and Technology*, 4-9 Aug 2019, Potsdam, Germany.

*The International Congress of Meat Science and Technology (ICoMST) is a high impact, annual global scientific conference for meat scientists and technologist to present the latest research addressing in all aspects of the production and distribution of animal protein food products.

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Appendix

List of Abbreviations

AA	amino acids
ANOVA	analysis of variance
APC	aerobic bacteria plate count
AT	ageing time
ATP	adenosine triphosphate
AV	air velocity
A_w	water activity
BD	in-bag dry-ageing
BF	*m. biceps femoris*
Cer	ceramides
D	traditional dry-ageing
DG	diacylglycerols
EAA	essential amino acids
E coli	*Escherichia coli*
FAAs	free amino acids
FAs	fatty acids
IMF	intramuscular fat
IMP	inosine monophosphate
LAB	lactic acid bacteria
LDB3	LIM domain binding 3 protein
LSD	least significant differences
MDA	malondialdehyde
MUFA	mono-unsaturated fatty acids
MW	molecular weight
NR	not reported
OPLS-DA	orthogonal projection to latent structures-discriminant analysis
PA	phosphatidic acids
PC	phosphatidylcholines
PCA	principal component analysis
PE	phosphatidylethanolamines
PG	phosphatidylglycerols
PI	glycerophosphoinositols
PS	phosphatidylserines
PUFAs	poly-unsaturated fatty acids
REIMS	rapid evaporation ionisation mass spectrometry
RF	*m. rectus femoris*
RH	relative humidity
SA	stepwise ageing
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SED	standard error of a difference between means
SFAs	saturated fatty acids
sHSP	small heat shock proteins
SM	*m. semimembranosus*
TG	triacylglycerols
TPA	texture profile analysis
TBARS	thiobarbituric acid reactive substances
VL	*m. vastus lateralis*
W	wet-ageing

Chapter 1: Introduction

1.1 Background

Red meat (beef and lamb) sector is the largest manufacturing industry in New Zealand with about 88% of beef and 94 % of sheep meat production are exported to over 120 countries. The export of New Zealand red meat is dominated by beef (48%), followed by lamb (44%) and other meat products (8%). In the year ending September 2019 alone, the total value of beef and sheep exports was worth $9.2 billion NZD (Beef + Lamb New Zealand, 2020). Value-adding and innovative production to provide consistently high-quality meat products to consumers is the long-term strategy for meat sector to meet growing demand of exceptional eating experience (Coriolis, 2017).

Ageing is a post-harvest processing technique widely used in the meat industry for the improvement of tenderness and flavour of meat. Post-mortem ageing involves a series of biochemical and physicochemical processes, including proteolysis, lipolysis, oxidation and changes in water permeability of membranes. Ageing process is generally accomplished by subjecting carcasses, primal or sub-primal cuts to controlled storage conditions of air circulation, relative humidity (RH) and refrigeration (-1.5 - 5 °C) in the commercial practice (Devine, 2014). Various forms of ageing have been practised over the last decades, such as carcass hanging, ageing of primal/sub-primal cuts in vacuum barrier packages – also called wet-ageing (W), and out-of-bag ageing which is known as traditional dry-ageing (D).

W technique was developed in the 1960s, and over 90% of the beef marketed was wet-aged by the 1980s (Savell, 2008). W is generally carried out using oxygen- and water-impermeable vacuum bags for maturation of meat under anaerobic conditions. This technique is widely adopted in the meat industry owing to the ease and flexibility for storage and transport, and its ability to extend shelf life and substantially improve the palatability of meat (Kim et al., 2018). Most primal cuts are wet-aged in commercial practice, especially during shipping and storage, before they are fabricated into steaks or roasts (Sitz, Calkins, Feuz, Umberger, & Eskridge, 2006).

Dry-ageing is another post-mortem ageing technique which has been used by butchers for preservation and tenderisation of beef for centuries before the adoption of W technique in meat industry. Since then, such traditional post-mortem handling technique has mainly been used by butchers and upscale restaurants for the gourmet markets. Dry-ageing is an aerobic maturation of meat with moisture evaporation which has been

considered superior to those of W by meat purveyors owing to the characteristic flavours from the process (Campbell, Hunt, Levis, & Chambers, 2001; Kim, Kemp, & Samuelsson, 2016; Li et al., 2014). Dry-ageing of beef generally requires 3 to 4 weeks of ageing time to generate unique eating quality and therefore are commonly marketed as artisan products with a higher price tag compared to the wet-aged equivalents (Kim et al., 2018; Savell, 2008). Interests by meat industry have risen to explore the potential of dry-ageing to provide alternative merchandising opportunities to satisfy the expectation of discerning consumers for premium meat products. However, D is a costly process due to the excessive weight and trim losses, proliferation of microorganisms and requirements for ageing facilities to have a critical control of temperature, RH and air velocity, which limits the feasibility to produce dry-aged meat for export (Parrish, Boles, Rust, & Olson, 1991; Smith et al., 2008). Inconsistent quality of dry-aged meat produced by D process have been reported and the key factors which have profound impacts on the quality of dry-aged products have not been well understood.

1.2 Motivations

Dry-aged beef is mostly produced from well-marbled premium beef cuts, such as loins and ribs, from prime steers or heifers with high intramuscular fat (IMF). The aerobic maturation process of dry-ageing promotes the oxidation of lipid which is more evident in marbled beef compared to lean (Domínguez et al., 2019). Severe oxidative damage of lipid may lead to detrimental effects on the sensorial and nutritional quality of beef (Domínguez et al., 2019; Gray, Gomaa, & Buckley, 1996). Lean bull beef, on the contrary, may demonstrate a low oxidative potential during post-mortem processing and storage due to the low IMF content. However, this type of beef cuts is generally rated as low-value cuts and processed into sausages, patties and other processed meat rather than dry-aged due to the reduced juiciness and tough texture compared to well-marbled equivalents. Previous study by Stenström, Li, Hunt, and Lundström (2014) produced dry-aged beef from young bull with approx. 2% IMF which was preferred by the consumers over the wet-aged equivalents, suggesting the opportunity to produce dry-aged lean beef with acceptable eating quality.

On the other hand, most dry-aged beef is consumed locally rather than exported and fresh rather than frozen-thawed. Therefore, questions regarding whether dry-aged beef can be frozen or not, how the quality will change over the frozen storage and how long the frozen storage can be continued without deterioration in quality remain unknown. Answers to these questions are required if the commercial export of dry-aged meat is the

target for meat industry where freezing of meat during storage and distribution is commonly practised for export market. Further, the impacts of dry-ageing on meat quality, microbial safety and sensory characteristics have been studied and compared to the wet-aged equivalents (Kim et al., 2018). However, there remains a need to determine the underlying biochemical changes, such as oxidative stability, proteolysis, and protein functionality, and to understand their roles in the quality changes during dry-ageing and storage.

Given that the D is a costly process, a regime called "stepwise ageing" was proposed by combining two ageing techniques as a stepwise dry-/wet-ageing process to mitigate the excessive costs from the operation of ageing facilities and the low product yield from long dry-ageing process. The eating qualities of the dry-aged beef after 21 days of ageing have been reported to remain stable during the extended W storage up to 16 days (Campbell et al., 2001). The combination of dry-ageing for 10 days followed by W for additional 7 days resulted in similar sensory quality whereas improved water-holding capacity and shear force tenderness compared to the straight-dry-ageing for 17 days (Kim, Meyers, Kim, Liceaga, & Lemenager, 2017). Thus, substitution of extended dry-ageing period with W as a stepwise ageing regime may provide advantages over the straight-dry-ageing to reduce the need for dry-ageing facilities and to increase product yield without compromising the eating quality.

A novel dry-ageing technique called "in-bag dry-ageing" (BD) has been developed over the last decade to address the concerns over the economic and microbial impacts of D. BD is the integration of D with W to dry age inside a water-permeable ageing bag which allow moisture evaporation from the meat while regulating the access of oxygen. The TUBLIN® plastic bag is a commercially available ageing bag which has been reported to result in the increase of product yield and reduce lipid oxidation, cook loss and microbial contamination without compromising eating quality compared to traditional out-of-bag dry-ageing regime (Ahnström, Seyfert, Hunt, & Johnson, 2006; DeGeer et al., 2009; Li et al., 2014; Li, Babol, Wallby, & Lundström, 2013; Stenström et al., 2014). The barrier function of the ageing bags can protect the meat from contamination by the surroundings and reduce oxidation and proliferation of microorganisms. Therefore, the use of ageing bags demonstrates the potential to produce dry-aged meat with consistent premium quality at a lower cost. In light of previous studies, a novel ageing regime is proposed in this study by combining BD with W as a "stepwise in-bag ageing" regime which may have the potential for industrial application to produce

microbiologically safe dry-aged meat with more consistent premium quality and a higher product yield.

As described above, various forms of dry-ageing have been carried out concentrating on beef muscles with limited information about the other types of muscles. New Zealand is the dominant producer and exporter of lamb meat and represents more than one third of global trade (Coriolis, 2017). Lamb meat is well known for its nutritional values and characteristic sensory properties favoured by the consumers across the world. Lamb is mainly wet-aged in the commercial practice to produce premium quality thus there remains an opportunity to add values to lamb meat by converting it into dry-aged products. Sheep/lamb meat consumers are familiar with the flavour produced from W technique, however, their acceptability towards the equivalent dry-aged lamb remains unknown.

1.3 Objectives

The main objective of this research is to produce quality acceptable dry-aged lean beef and lamb using BD technique. The effects of processing conditions and storage factors on the quality, acceptability and biochemical changes including oxidative stability, proteolysis, digestibility and metabolite profile of in-bag dry-aged lean beef and lamb were investigated. An overview of this research including research questions, primary aim and objectives is shown in Figure 1.1.

The specific objectives of this research are as follows:

- Objective 1: To produce in-bag dry-aged lean beef using “stepwise in-bag ageing” regime and compared with straight-dry-ageing;
- Objective 2: To determine the effect of processing conditions including ageing air velocities and ageing times on the in-bag dry-aged lean beef;
- Objective 3: To investigate the effect of frozen storage and its interactions with the different processing regimes on the in-bag dry-aged lean beef;
- Objective 4: To determine the quality and biochemical changes of in-bag dry-aged lean beef following one-year frozen storage;
- Objective 5: To determine the effect of BD on the quality and acceptability of lamb compared to the wet-aged equivalents;
- Objective 6: To investigate the underlying biochemical changes between BD and W;

- Objective 7: To investigate the potential of Rapid Evaporative Ionisation Mass Spectrometry (REIMS) for identification and discrimination of dry-aged lean beef and lamb due to different process regimes;
- Objective 8: To explore the potential biomolecular signatures using peptidomic profiling of small peptides to discriminate the in-bag dry-aged lamb from the wet-aged equivalents.

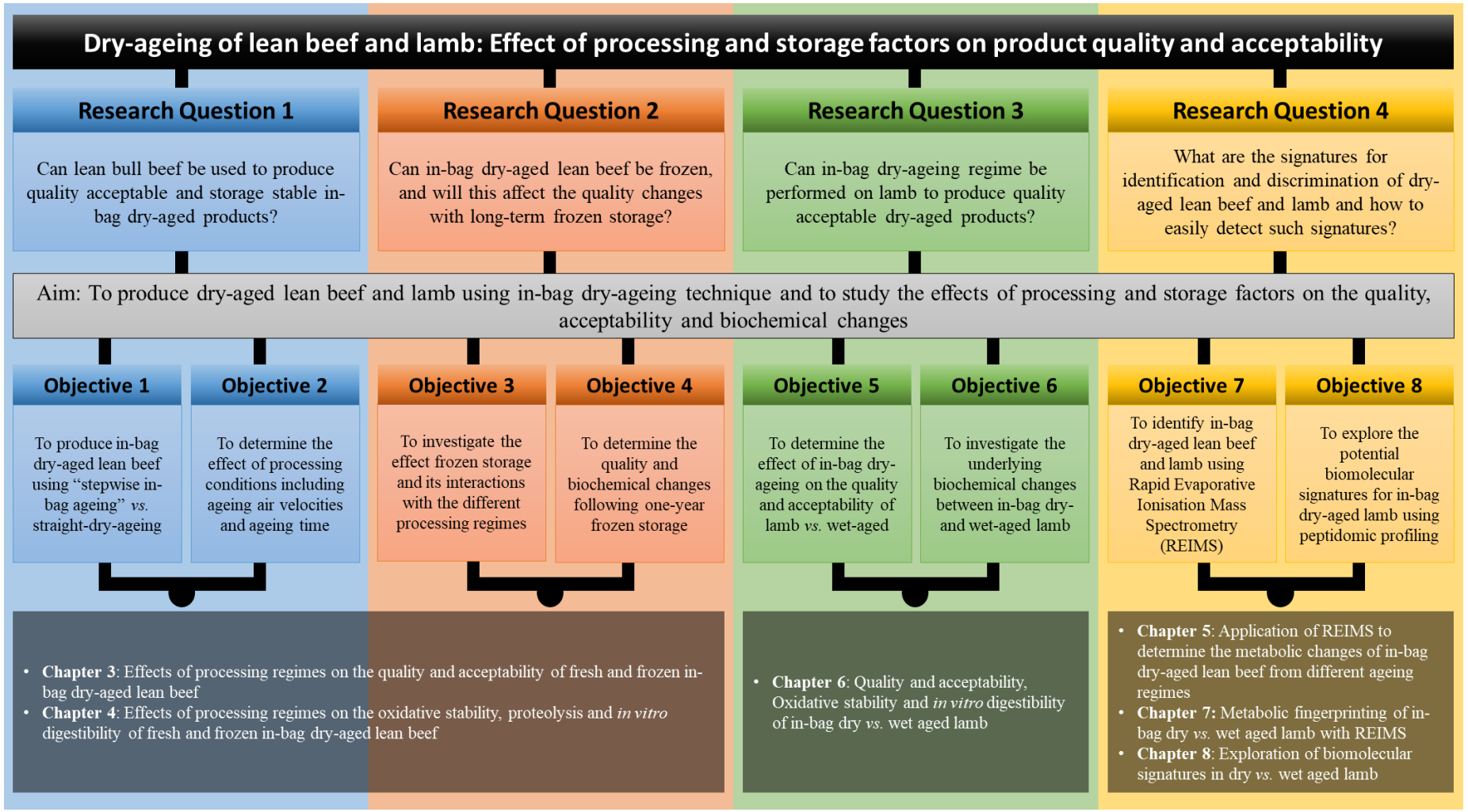


Figure 1.1 Overview of thesis with summary of the research questions, primary aim and objectives, and the associated chapters where they are addressed.

1.4 Contributions

In this research, I have made some original contributions to the research area by addressing the objectives and therefore laying the groundwork for further improvements. The primary contributions of this research are summarised as follows:

1) I developed the "stepwise in-bag ageing" regime which demonstrates the potential for industrial application to improve the microbial safety, storage stability and product yield. Such regime can also shorten the turnover time needed for dry-ageing facilities because the W process can be accomplished during the distribution with minimum loss of quality. Consequently, in-bag dry-aged lean beef produced using stepwise regime has similar quality compared to the straight-dry-ageing. Further, trimming of dehydrated surface following ageing is not necessary for stepwise in-bag ageing regime due to the moisture difference between the surface and centre resulting from dry-ageing can be equilibrated during W process and thus improve the product yield.
2) To my best knowledge, this is the first research that investigated the effect of long-term frozen storage on quality, acceptability and storage stability of in-bag dry-aged lean beef. Outcomes of this research demonstrated the possibility to frozen store in-bag dry-aged lean beef for a year without significant impact on the quality. The in-bag dry-aged lean beef was considered acceptable by consumer panel with improved oxidative stability and digestibility compared to the unaged counterparts.
3) To date, there was only two reports published on dry-ageing of lamb (Burvill, 2016a, 2016b) with no peer-reviewed publication available. In this study, I applied in-bag dry-ageing technique on lamb muscles to produce quality acceptable dry-aged lamb products which were highly preferred by consumers. Such outcomes demonstrated the feasibility to add values to lamb meat through dry-ageing technique.
4) I determined the biochemical changes of in-bag dry-aged lean beef and lamb, including the oxidative stability of lipid and protein, proteolytic pattern, peptidomic profiling of small peptides and digestibility which impart deeper insights to understand the underlying biochemical mechanisms responsible for the quality changes and flavour development of dry-aged meat products.
5) I used BD of lean beef and lamb as a platform to establish the relationships between (a) the percentage of ageing weight loss and ageing time; (b) the initial

weight and the final product weight; and (c) the initial weight and percentage of ageing weight loss. Such relationships can be further applied to the meat industry in designing the ageing strategies and estimating product yields and leading to the consistent premium quality.

6) I evaluated the relative digestibility of in-bag dry-aged lean beef and lamb using three methods (a) free amino acids profiling across the simulated digestion process; (b) 1D SDS-PAGE gel electrophoresis of protein profile; and (c) available protein content in the final hydrolysates. Combining three methods could impart a more comprehensive insights into the determination of protein digestibility compared to a single method which may overlook some significant findings due to the limitation of the assay. Additionally, multiple determinations of relative digestibility could also facilitate the understanding of biochemical processes during the digestion of meat products especially in the upper gastrointestinal tract, which is important when the bioaccessibility of the products is considered.
7) I have successfully employed REIMS as a real time metabolic fingerprinting tool to identify and discriminate in-bag dry-aged lean beef and lamb due to different processing regimes. This is a novel application of REIMS in the area of food science to discriminate the metabolic changes due to the processing factors. Such application has great significances for the purpose of quality control and preventing fraud where dry-aged meat is concerned due to different ageing regimes and various lengths of ageing time. I have also contributed knowledge towards the understanding the underlying biochemical mechanisms responsible for the discrimination between different processing regimes and discover the potential biomolecular signatures for in-bag dry-aged meat products.

1.5 Thesis outline

This thesis involves the BD of lean beef and lamb to determine the effects of processing regimes and storage factors on the quality and acceptability of dry-aged products and to understand the underlying biochemical mechanisms contributing to the quality changes. The thesis consists of nine chapters briefly summarised as follows:

- **Chapter 2 (Paper VI)**: Comprehensively reviews the published literatures on dry-aged beef and explores the mechanisms responsible for the development of

characteristic dry-aged flavour. An integrated mechanism is proposed for tailoring the dry-aged flavour.

- **Chapter 3 (Paper I)**: Presents the effect of processing regimes including stepwise ageing, air velocities and ageing time on the meat quality and acceptability of fresh and long-term frozen in-bag dry-aged lean beef.
- **Chapter 4 (Paper II)**: Further investigates the effects of processing regimes on the biochemical changes of fresh and long-term in-bag dry-aged lean beef.
- **Chapter 5 (Paper III)**: Shows the application of REIMS as a rapid metabolic fingerprinting tool to discriminate in-bag dry-aged lean beef due to different processing regimes.
- **Chapter 6 (Paper IV)**: Investigates the meat quality and consumer acceptability, oxidative stability, and *in vitro* digestibility of in-bag dry-aged lamb compared to the wet-aged equivalents.
- **Chapter 7 (Paper V)**: Compares the metabolic profiles of in-bag dry- and wet-aged lamb using REIMS and the underlying biochemical mechanisms responsible for the differences are reported.
- **Chapter 8 (Paper VII)**: Explores the biomolecular signatures of in-bag dry-aged lamb through the peptidomic profiling of small peptides (< 10 kDa).
- **Chapter 9**: Concludes this thesis by providing a summary of the outcomes and contributions of this research. Suggestions and directions for the future research in the field are also included.

Chapter 2: Mechanisms and strategies to tailor dry-aged meat flavour (Literature review)

2.1 Introduction

Post-mortem ageing of fresh meat improves the eating quality of beef, especially tenderness (Nishimura, 1998; Watanabe et al., 2015), which is one of the most important sensory qualities, along with flavour (Feuz, Umberger, Calkins, & Sitz, 2004; Shackelford et al., 2001). Post-mortem ageing is accomplished by subjecting carcasses, primal or sub-primal cuts, particularly those from ribs and loins, to controlled storage conditions of air circulation, relative humidity (RH) and refrigeration. Typically 10 to 20 days are required to reach desirable degree of tenderness through proteolysis (Smulders, Toldrá, Flores, & Prieto, 1992). During post-mortem ageing, proteolysis and lipolysis breakdown large, flavour-absent molecules into smaller flavoursome fragments, inducing changes in sugars, lipids, organic acids, vitamins, sugar phosphates and nucleotide-bound sugars in meat. These degraded compounds either provide specific aroma and flavour or become important flavour precursors to drive complex biochemical reactions during cooking, such as Maillard reaction, oxidation and their interactions (Zamora & Hidalgo, 2011; Zamora, Navarro, Aguilar, & Hidalgo, 2015). The flavour of cooked meat is largely dependent on these flavour compounds generated from cooking (Campo, Sañudo, Panea, Alberti, & Santolaria, 1999; Van Boekel, 2006).

The term, ‘post-mortem ageing’, commonly refers to wet-ageing (W) in the literature and in the meat industry. W process can be defined as anaerobic maturation in vacuum barrier packages under refrigerated storage condition. It is considered as the most practical method of ageing by the meat industry owing to it being a minimal processing technique and negligible yield loss. Dry-ageing is another post-mortem ageing technique which was traditionally used by butchers prior to the mass adoption of vacuum packaging by the meat industry. Dry-ageing remains in use by a small number of meat purveyors for upscale restaurants and gourmet markets (Savell, 2008). In the past decade, re-emergence of traditional dry-ageing (D) technique was observed as an alternative merchandising opportunity in the global meat market (Laster et al., 2008). D process is described as ageing an entire carcass, sub-primal or wholesale cuts without covering or packaging for a period of time under critically controlled conditions of temperature, RH and air velocity. The exposure of meat surface to the ageing environment allows the aerobic maturation to create the unique flavour profile of dry-aged meat. Dry-aged beef products are considered premium foods with intensified buttery, nutty, beefy, roasted and earthy flavour profile,

resulting in a higher sale price compared to the wet-aged equivalents (DeGeer et al., 2009; Savell, 2008; Warren & Kastner, 1992). D is a costly process compared to W due to the loss in weight from the dehydration, unavoidable trimming, reduced saleable yield, increased risk of contamination from microbial spoilage and extra requirements for ageing facilities (Parrish et al., 1991; Smith et al., 2008).

To overcome the issue of low product yield and microbial contamination, an innovative dry-ageing method, called in-bag dry-ageing (BD), has been developed (Ahnström et al., 2006; Berger et al., 2018; DeGeer et al., 2009; Dikeman, Obuz, Gök, Akkaya, & Stroda, 2013; Lee et al., 2017; Shi, Zhang, & Zhou, 2020; Stenström et al., 2014; Zhang, Yoo, & Farouk, 2019). TUBLIN® plastic film is a relatively new type of bag with high permeability to water vapour, which allows moisture to evaporate during D, while restricting the access of oxygen and microorganisms from the environment as a protective barrier (Ahnström et al., 2006; Li et al., 2013). BD results in a higher product yield over the D by reducing losses from excessive trimming and microbial contamination without compromising tenderness and eating quality (Ahnström et al., 2006; DeGeer et al., 2009; Li et al., 2014; Li et al., 2013; Stenström et al., 2014; Zhang et al., 2019). With a small additional cost of the bags, improvement of the eating qualities of meat through BD has a great potential to bring better economic returns to the meat industry through new premium products for high-value markets.

The impacts of dry-ageing (D/BD) and ageing time on the meat quality and sensorial characteristics of beef have been extensively reported, but with inconsistent results (Table 2.1). A small number of studies have reported a positive impact of dry-ageing on flavour development. To date, signatures to define the dry-aged products remain unknown and the mechanisms responsible for the development of signature dry-aged flavour are not thoroughly understood. Since the majority of dry-ageing studies have been carried out on beef muscles, the current review will use mainly beef as the exemplar to critically assess the published literature on the impacts of dry-ageing and ageing time on the eating qualities and flavour development. Then, we will elaborate on the mechanisms and associated factors responsible for the development of signature dry-aged flavour based on experimental evidence. Finally, we will propose an integrated mechanism and illustrate strategies to produce dry-aged products with consistent quality and tailorable flavour profile.

2.2 Impact of dry-ageing on eating qualities: current findings

2.2.1 Texture quality – tenderness

2.2.1.1 Ageing techniques

Of the sensory qualities of meat, tenderness plays the most important role in determining the liking and acceptability of meat by the affluent western consumers (Platter et al., 2003). Positive impact of dry-ageing on tenderness has been determined by instrumental measures, mainly by Warner-Bratzler shear force (WBSF) (Jose, Jacob, & Gardner, 2020; Shi et al., 2020), and by sensory trials (Berger et al., 2018; Campbell et al., 2001; Li et al., 2014; Li et al., 2013; Stenström et al., 2014), though only in a few studies (Table 2.2). Most of the studies have reported that dry-ageing (D/BD) resulted in a similar degree of tenderness on beef compared to the wet-aged equivalent, when determined by instrumental and sensory trials (Table 2.2). This may be explained by there being a similar primary tenderisation pathway across all the ageing techniques (D/BD/W) where enzymatic proteolysis plays a central role. Post-mortem proteolysis of myofibrillar and associated proteins by endogenous proteases, especially the calpains (mainly μ-calpain and m-calpain), is responsible for tenderisation of beef during ageing (Ouali, 1990; Ouali et al., 2013). Calpains trigger degradation of structural and cytoskeletal proteins, such as troponins-T and I, and C-protein, actin and myosin filaments (Dransfield, 1994; Herrera-Mendez, Becila, Boudjellal, & Ouali, 2006; Hwang, Park, Kim, Cho, & Lee, 2005), leading to the improvement of tenderness. Another group of enzymes named cathepsins (mainly B, D and L) are also associated with proteolysis during the extended post-mortem ageing (Ouali et al., 2006). Similar protein degradation profile has been reported across different ageing techniques (D/W) using myofibril fragmentation index and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Choe, Park, Lee, & Jo, 2020; Parrish et al., 1991). Thus, D may not impose advantages in tenderisation of meat over the widely employed W technique. On the other hand, W has been suggested to present commercial advantages to suppliers in terms of ease in handling and storage, product quality, yield and inventory management, if the improvement of tenderness was the only goal (Lepper-Blilie, Berg, Buchanan, & Berg, 2016).

Table 2.1 Summary of dry-ageing parameters and suggested mechanisms for the development of dry-aged flavour reported in the literature. D: Traditional dry-ageing, W: Wet-ageing, BD: In-bag dry-ageing, NR = Not reported.

Ageing treatments compared in the study	Meat source	Marbling/fat content (%)	Temperature (°C)	RH (%)	Air velocity ($m.s^{-1}$)	Ageing time (d)	Moisture loss (%)	Trimming	Dry-aged flavour development	Mechanisms of ageing			Sources
										Dehydration	Oxidation	Microorganism	
D *vs.* W	Short loins & ribs	USDA Choice	0/4.4	81/84	NR	7/15	7d: ~3 15d: ~6	NR	No	-	-	-	(Minks & Stringer, 1972)
D *vs.* W	Heifer striploins	USDA Standard to Good	1-3	NR	NR	14	NR	Yes	No	-	-	-	(Miller, Davis, & Ramsey, 1985)
D *vs.* W	Striploins	Slight	2		NR	7	4.62	Yes	No	-	-	-	(Oreskovich et al., 1988)
D *vs.* W	Short cut striploins & ribs	Prime vs. Choice vs. Select	0-1	80-85	0.5-2.5	W: 21 D: 14/21	7d: ~3-5 21d: ~4-6	~5-6	No	-	-	-	(Parrish et al., 1991)
D *vs.* W	Striploins	USDA Choice or better	3.1-3.6	78	NR	0/11	~14	NR	Yes	-	-	-	(Warren & Kastner, 1992)
D *vs.* W	Steers ribs	USDA Choice	4		NR	W: 28 D: 14	NR	NR	Yes	-	√	-	(King, Matthews, Rule, & Field, 1995)
W-D-W	Angus short/striploins	NR	2	75	NR	W:7/14+D: 7/14/21+W: 2/9/16	NR	Yes	Yes	√	√	-	(Campbell et al., 2001)
D *vs.* BD	Angus striploins	~7	2.5-2.6	87	NR	14/21	21d: ~9-10	21d: ~15-18	No	-	-	-	(Ahnström et al., 2006)
D-W *vs.* W	Angus striploins	USDA Prime vs. Choice	1	NR	NR	D: 30+W: 7 W:37	NR	NR	No	-	-	-	(Sitz et al., 2006)
D *vs.* W	Ribeye roll, striploin & top sirloin	USDA Top Choice vs. Select	0.6	78	NR	14/21/28/35	6-11	~17-24	No	-	-	-	(Laster et al., 2008)
D *vs.* W	Steers fore-loins & fore-ribs	~1.5-3	1	NR	NR	19	NR	NR	No	-	-	-	(Richardson, Nute, & Wood, 2008)
D *vs.* W	Short loins	USDA Select vs. Choice	1	83	NR	14/21/28/35	~5-8	Yes	No	-	-	-	(Smith et al., 2008)
D *vs.* BD-W	Striploins & strip shell loins	USDA Modest-high marbling	2.2	50	NR	21/28+W: 7	~11-19	~22-34	No	-	-	-	(DeGeer et al., 2009)
D *vs.* W	Steers (*longissimus* & *triceps*)	Choice	2	NR	NR	14	NR	NR	No	-	-	-	(Jiang et al., 2010)
D	Flank	NR	1-3.5	70-100	NR	14	NR	NR	No	-	-	-	(Knudsen, Sommer, Sørensen, Olsen, & Aabo, 2011)
D *vs.* W	Heifer (*longissimus*)	Distinctive marbling	1	90	Intensive	1/14/28/42	NR	NR	No	-	-	-	(Lautenschlaeger, 2012)
D *vs.* BD *vs.* W	Beef (*longissimus lumborum*)	USDA Choice (~4) & Select (~3)	2.2		Minimal	21	~13-16	~24-26	No	-	-	-	(Dikeman et al., 2013)
BD *vs.* W	Heifer (*gluteus medius*)	High fat	2.9	91	NR	14	15.2	~7	Yes	-	-	-	(Li, Babol, Wallby, & Lundström, 2013)

Table 2.1 (continued)

Ageing treatments compared in the study	Meat source	Marbling/fat content (%)	Temperature (°C)	RH (%)	Air velocity ($m.s^{-1}$)	Ageing time (d)	Moisture loss (%)	Trimming	Dry-aged flavour development	Mechanisms of ageing			Sources
										Dehydration	Oxidation	Microorganism	
D *vs.* BD *vs.* W	Steers& heifer (*longissimus thoracis et lumborum*)	~3	2.9	NR	NR	8/19	8d: ~6-7 19d: ~14-15	8d: ~22 19d: ~27-30	Yes		√		(Li et al., 2014)
D *vs.* W	Cow striploin	Slight^{+} to modest^{+}	2.2	NR	NR	2/23	~6-8	~24	No	-	-	-	(Obuz et al., 2014)
D *vs.* W	Ribeye & top sirloin butts	USDA Choice	4	98	NR	35	~15	~21	No	-	-	-	(Smith et al., 2014)
D *vs.* BD *vs.* W	Bull (*longissimus thoracis et lumborum*)	~2	1.6	NR	NR	13	~4-5	Yes	Yes	-	-	-	(Stenström et al., 2014)
D *vs.* W	Cow (*longissimus dorsi*)	EUROP 3	4	NR	NR	0/7/14/21	NR	21d: ~38	No	-	-	-	(Gudjónsdóttir et al., 2015)
D *vs.* W	Beef loin (*longissimus dorsi*)	~3-5	2.2	NR	Minimal	15/25	~14	~23	No	-	-	-	(Velotto et al., 2015)
D	Ribeye (*longissimus thoracis*)	High marbling (~7)	1-4	80-90	NR	1/8/17/27/37/47/57	NR	NR	No	-	-	-	(Iida et al., 2016)
D *vs.* W	Steers short loins (*longissimus lumborum*)	NR	1/3	49/55/73/76	0.2/0.5	21	~9-14	Yes	Yes	-	-	-	(Kim et al., 2016)
D *vs.* W	Short/striploin	USDA Slight to Small (~3.5-5)	1	70	0.2	11/18/25/32/39/46	~24-38	Yes	Yes	-	-	-	(Lepper-Blilie et al., 2016)
W-D *vs.* W	Striploins	USDA Prime, Premium Choice, Low Choice & Select	1-2	77	NR	W: 14/46 W: 16/17+D: 30	NR	Yes	Yes	-	-	-	(O'Quinn et al., 2016)
D *vs.* D-W	Steers short loin (*longissimus lumborum*)	USDA Low Choice	1	78	1.5	D: 10 d+W: 7 d D: 17 d	NR	NR	No	-	-	-	(Kim, Meyers, Kim, Liceaga, & Lemenager, 2017)
D *vs.* BD *vs.* W	Grass-fed heifer (*longissimus lumborum*)	USDA Select	2	78	<2	28	~9-14	~7-9	Yes	-	-	-	(Berger et al., 2018)
D (mould inoculation)	Cow beef lumps	NR	2	80	NR	0/7/14/21/28	~16-18	~12-14	Yes	-	√	-	(Hanagasaki & Asato, 2018a)
D *vs.* W	Cow beef round	NR	2	80	No air flow	0/7/14/21/28	~18	~15-18	Yes	√	-	-	(Hanagasaki & Asato, 2018b)

Table 2.1 (continued)

Ageing treatments compared in the study	Meat source	Marbling/fat content (%)	Temperature (°C)	RH (%)	Air velocity ($m.s^{-1}$)	Ageing time (d)	Moisture loss (%)	Trimming	Dry-aged flavour development	Mechanisms of ageing			Sources
										Dehydration	Oxidation	Microorganism	
D	Angus (bull and heifer) loin	2-3	1	85	0.5	12-36	21d: ~3	NR	No	-	-	-	(Hulánková, Kameník, Saláková, Závodský, & Borilova, 2018)
D *vs.* W *vs.* W-D	Heifer & steers (*longissimus thoracis et lumborum*)	NR	<0.5	<80	0.5-2.0	7/21/35/56	56d: ~16-18	Yes	Yes	√	-	-	(Ha et al., 2019)
D *vs.* W	Holstein steer striploin (*longissimus lumborum*)	Low grade	4	75	2.5	0/7/14/21/28	NR	Yes	Yes	√	-	√	(Lee et al., 2019a)
D	Holstein steer rump (*middle gluteal*)	Low grade	4	75	0/2.5/5	0/14/21/28	NR	Yes	Yes	√	-	√	(Lee et al., 2019b)
D	Holstein steer (*longissimus lumborum*)	Low grade	0-4	75	0/2.5/5	0/14/21/28	NR	NR	Yes	-	-	√	(Oh, Lee, Lee, Jo, & Yoon, 2019)
D	Nellore steer (*longissimus thoracis*)	~	2	75	NA	0/14/28	14d: ~10 28d: ~20	Yes	No	-	-	-	(Passetti et al., 2019)
BD *vs.* BD-W	Holstein-Friesian bull (*longissimus lumborum*)	~1	2	75	0.5/1.5/2.5	BD: 21 BD: 7+W: 14	7d: ~10 21d: ~20	Yes	No	-	-	-	(Zhang, Yoo, & Farouk, 2019)
D *vs.* W	Droughtmaster (Bos indicus) steer short loin	NR	1.8	87	0.3	28	~18-20	NR	No	-	-	-	(Jose, Jacob, & Gardner, 2020)
D *vs.* BD *vs.* W	Luxi bull (*longissimus thoracis*)	NR	2	85	1.5	0/7/14	14d: ~2-8	Yes	No	-	-	-	(Shi, Zhang, & Zhou, 2020)
D *vs.* W	Holstein bull (*longissimus*)	Low grade	4	75	2.5	0/28	NR	Yes	Yes	√	-	√	(Choe, Park, Lee, & Jo, 2020)

Table 2.2 Summary of the impact of dry-ageing and increasing the dry-ageing time on instrumental tenderness and eating qualities of beef muscles. D: Traditional dry-ageing, W: Wet-ageing, BD: In-bag dry-ageing, NR: Not reported.

Qualtiy attributes	Impact of dry-ageing	Impact of dry-ageing time
Instrumental tenderness (shear force)	*Unchanged* D=BD=W (Ahnström et al., 2006; DeGeer et al., 2009; Dikeman et al., 2013; Gudjónsdóttir et al., 2015; Lautenschlaeger, 2012; Lee et al., 2017; Lepper-Blilie et al., 2016; Li et al., 2013; Miller et al., 1985; Minks & Stringer, 1972; Oreskovich, et al., 1988; Parrish et al., 1991; Sitz, et al., 2006; Smith et al., 2008; Velotto et al., 2015); BD=BD-W (Zhang et al., 2019) *Decreased* D<D-W (Kim et al., 2017); D<W (Jose et al., 2020; Shi et al., 2020)	*Decreased* For 7-14 d, then decreased slightly (Ahnström et al., 2006; Gudjónsdóttir et al., 2015; Laster et al., 2008; Lautenschlaeger, 2012; Lepper-Blilie et al., 2016; Minks & Stringer, 1972; Obuz et al., 2014; Shi et al., 2020); For 28 d (Passetti et al., 2019) *Unchanged* (Campbell et al., 2001; DeGeer et al., 2009; Hulánková et al., 2018)
Sensory quality		
Tenderness	*Improved* D/BD>W (Berger et al., 2018; Campbell et al., 2001; Li et al., 2014; Li et al., 2013; Richardson, Nute, & Wood, 2008; Stenström et al., 2014); D>D-W>W (Ha et al., 2019) *Unchanged* D=BD=W (Ahnström et al., 2006; DeGeer et al., 2009; Dikeman et al., 2013; George, 2011; Kim et al., 2016; Laster et al., 2008; Lepper-Blilie et al., 2016; Miller et al., 1985; Minks & Stringer, 1972; Oreskovich et al., 1988; Smith et al., 2014; Smith et al., 2008; Stenström et al., 2014; Velotto et al., 2015; Warren & Kastner, 1992); D=D-W (Kim et al., 2017); BD=BD-W (Zhang et al., 2019) *Decreased* W>D (Obuz et al., 2014; Parrish et al., 1991; Sitz et al., 2006)	*Improved* For 2-3 weeks (Li et al., 2014; Minks & Stringer, 1972; Obuz et al., 2014; Warren & Kastner, 1992), then unchanged (Campbell et al., 2001; Lepper-Blilie et al., 2016) *Unchanged* (DeGeer et al., 2009; Iida et al., 2016; Laster et al., 2008; Smith et al., 2008)

Table 2.2 (continued)

Qualtiy attributes	Impact of dry-ageing	Impact of dry-ageing time
Juiciness	*Improved* D/BD>W (Berger et al., 2018; Campbell et al., 2001; Li et al., 2013; Richardson et al., 2008; Stenström et al., 2014); D>D-W>W (Ha et al., 2019) *Unchanged* D=BD=W (Ahnström et al., 2006; DeGeer et al., 2009; Dikeman et al., 2013; Kim et al., 2016; Laster et al., 2008; Lepper-Blilie et al., 2016; Li et al., 2014; Miller et al., 1985; Minks & Stringer, 1972; Obuz et al., 2014; Oreskovich et al., 1988; Parrish et al., 1991; Sitz et al., 2006; Smith et al., 2008; Velotto et al., 2015); D=D-W (Kim et al., 2017); BD=BD-W (Zhang et al., 2019)	*Improved* (Campbell et al., 2001; Li et al., 2014; Smith et al., 2008) *Unchanged* (DeGeer et al., 2009; Iida et al., 2016; Laster et al., 2008; Lepper-Blilie et al., 2016; Minks & Stringer, 1972; Obuz et al., 2014) *Decreased* (Obuz et al., 2014)
Overall flavour	*Improved* D>W (Kim et al., 2016); D>D-W>W (Ha et al., 2019) *Unchanged* D=BD=W (Berger et al., 2018, Dikeman et al., 2013; Laster et al., 2008; Lee et al., 2017; Miller et al., 1985; Minks & Stringer, 1972; Parrish et al., 1991; Smith et al., 2008); D=D-W (Kim et al., 2017); BD=BD-W (Zhang et al., 2019) *Decreased* D<W (Sitz et al., 2006; Smith et al., 2014)	*Unchanged* (Iida et al., 2016; Laster et al., 2008; Minks & Stringer, 1972; Smith et al., 2008)
Overall liking	*Improved* D=BD>W (Kim et al., 2016; Lee et al., 2017; Li et al., 2013; Smith et al., 2014; Stenström et al., 2014; W-D>W (O'Quinn et al., 2016); D>D-W>W (Ha et al., 2019) *Unchanged* D=BD=W (Berger et al., 2018; Parrish et al., 1991; Smith et al., 2008); D=D-W (Kim et al., 2017); BD=BD-W (Zhang et al., 2019) *Decreased* D<W (Miller et al., 1985; Parrish et al., 1991; Sitz et al., 2006)	*NR*
Off-flavour	*Unchanged* D=BD=W (Dikeman et al., 2013; Oreskovich et al., 1988; Velotto et al., 2015); BD=BD-W (Zhang et al., 2019) *Decreased* D<W (Obuz et al., 2014)	*Unchanged* (Obuz et al., 2014)

2.2.1.2 Ageing time

Ageing of beef sub-primal for 7 to 14 days in general favours the improvement of (instrumental) tenderness regardless of ageing treatments (D/BD/W) compared to the unaged meat (Table 2.2). Extended ageing time may continue to improve the tenderness, but not significantly (Ahnström et al., 2006; Campbell et al., 2001; Gudjónsdóttir et al., 2015; Laster et al., 2008; Lepper-Blilie et al., 2016; Obuz, Akkaya, Gök, & Dikeman, 2014; Shi et al., 2020). Gudjónsdóttir et al. (2015) reported a significant decrease of WBSF in loin steaks through ageing time of 7 to 14 days, compared to those unaged steaks. Similar results were also reported for loins and ribs (Minks & Stringer, 1972) and striploins (Obuz et al., 2014) using D/W. Prolonged ageing periods of 14 to 35 days had slight but no significant effect on WBSF of beef loins irrespective of ageing techniques (D/BD/W) (Ahnström et al., 2006; Gudjónsdóttir et al., 2015; Laster et al., 2008; Smith et al., 2008). Similar WBSF values were also reported for beef loins dry-aged for 21 and 28 days (DeGeer et al., 2009).

Consumer preference of tenderness for dry-aged beef increased with ageing time for 14 to 21 days, then the improvement in tenderness slowed down or stopped with longer time-periods (Campbell et al., 2001; Lepper-Blilie et al., 2016). A higher rating on tenderness was detected from beef aged for 7 and 14 days (Minks & Stringer, 1972), 11 days (Warren & Kastner, 1992) and 23 days (Obuz et al., 2014) compared to unaged controls and those aged for 1-2 days only. Interestingly, Iida et al. (2016) has reported that sensory tenderness (trained panels) of highly marbled beef remained unchanged throughout the D for a period of 57 days. They concluded that uniformly distributed fat through marbling made the beef significantly tender (Ueda et al., 2007) that the effect of ageing time seemed negligible.

2.2.2 Flavour and other eating qualities

2.2.2.1 Ageing techniques

Flavour is another important sensory attribute which determines the eating quality and preference of meat. Often consumers relate meat flavour to the flavour of wet-aged meat, as it is the most commonly available choice in the market. As summarised in Tables 2.2 and 2.3, sensory assessments of eating qualities of dry-aged beef remain inconsistent in the literature, even when the same ageing technique (D/BD) was used. Due to the evaporation occurring during dry-ageing (D and BD), flavour becomes more intense compared to that of W. According to DeGeer et al. (2009), the intense and distinct flavour

of dry-aged beef becomes a preferred option over the wet-aged once consumers are accustomed to it. Juiciness is another highly contributing factor for driving preference for dry-aged beef (D/BD) compared to the wet-aged (Berger et al., 2018; Campbell et al., 2001; Li et al., 2014; Li et al., 2013; Richardson, Nute, & Wood, 2008; Stenström et al., 2014). Despite the moisture loss occurring during dry-ageing process (D/BD), some studies have shown no variation in juiciness of beef, between D and W (Kim et al., 2016; Laster et al., 2008; Lepper-Blilie et al., 2016; Miller, Davis, & Ramsey, 1985; Minks & Stringer, 1972; Obuz et al., 2014; Oreskovich, Mckeith, Novakofski, & Bechtel, 1988; Parrish et al., 1991; Sitz et al., 2006; Smith et al., 2008; Stenström et al., 2014; Velotto et al., 2015).

From the literature, it is premature to conclude the impact of dry-ageing on the overall desirability of beef, as some have reported negative (Miller et al., 1985; Parrish et al., 1991; Sitz et al., 2006) or no impacts compared to W beef (Berger et al., 2018; Parrish et al., 1991; Smith et al., 2008). Though improvement of palatability has been reported with the use of dry-ageing (D/BD) (Ha et al., 2019; Kim et al., 2016; Li et al., 2013; O'Quinn et al., 2016; Smith et al., 2014; Stenström et al., 2014), its effect on overall desirability and flavour intensity was also dependent on quality grade of beef (e.g. USDA quality grade). Consumers could not differentiate between D and W for USDA Choice beef while for USDA Prime beef, W was preferred over D for the overall desirability and flavour intensity (Sitz et al., 2006).

A summary of flavour attributes reported in the literature is shown in Table 2.3. Warren and Kastner (1992) observed positive impact of dry-ageing on the improvement of flavour attributes such as beefy and brown/roasted, while its equivalent W had higher intensities of undesirable flavour notes such as sour, bloody, and metallic flavour. A higher overall flavour and/or intensity of aged-flavour was also detected in dry-aged beef over the wet-aged counterparts (Ha et al., 2019; Kim et al., 2016; Lepper-Blilie et al., 2016). However in some studies, either small (Minks & Stringer, 1972) or no impact of D was observed for the intensity of aged-flavour when compared to BD (Ahnström et al., 2006; DeGeer et al., 2009) and W techniques (Jiang et al., 2010; Laster et al., 2008; Richardson et al., 2008; Smith et al., 2014; Smith et al., 2008; Velotto et al., 2015). According to King, Matthews, Rule, and Field (1995), a greater proportion of esters and heptane in the volatile compounds from D than W beef may contribute towards the development of dry-aged flavour. Improvements of butter-fried odour, umami taste and nutty/roasted nut taste have also been reported in dry-aged beef with decreased level of

negative flavour notes such as metallic and liver odour, grassy, hay-like, gamey, livery and fishy flavour (Li et al., 2014; O'Quinn et al., 2016). According to Lepper-Blilie et al. (2016), flavour attributes, such as beefy, bloody/serumy, brown/roasted and sour flavour, were similar between D and W, and they speculated that the higher fat content in dry-aged beef due to reduced moisture content could be accountable for the enhanced beefy flavour.

2.2.2.2 Ageing time

With prolonged ageing time of greater than 3 to 4 weeks, aged-flavour becomes more intense for dry-aged beef. The intensified flavour is favoured by some restaurants or butchery stores and such products are sold at premium price. Effect of ageing time on the sensory qualities of dry-aged beef has been investigated while results remain inconsistent (Table 2.2). Improvement of juiciness with an increase in dry-ageing time has been reported (Campbell et al., 2001; Li et al., 2014; Smith et al., 2008). This is explained by the concentration effect arising from moisture evaporation where the subsequent increase of fat content in the dry-aged final product is associated with the level of juiciness (Jeremiah, Dugan, Aalhus, & Gibson, 2003). Again, inconsistent findings related to this matter are evident in the literature where juiciness of dry-aged beef (D/BD) remain unchanged over the ageing time of 15 days (Minks & Stringer, 1972), 46 days (Lepper-Blilie et al., 2016) and 57 days (Iida et al., 2016).

No significant improvement of overall flavour liking with extended ageing period (beyond 21 days in general) has been reported (Iida et al., 2016; Laster et al., 2008; Minks & Stringer, 1972; Smith et al., 2008). A few studies have demonstrated an improvement of flavour attributes such as sweet, bitter, umami and bouillon taste, butter-fried meat and aged flavour (Campbell et al., 2001; Lepper-Blilie et al., 2016; Li et al., 2014) with dry-ageing time. Most of the other reported flavour attributes of dry-aged beef did not change, irrespective of ageing time (Table 2.3). Similar results were also reported by DeGeer et al. (2009) that there was no significant difference in flavour attributes for beef loins aged by dry-ageing techniques (D/BD) for 21/28 days. The extended ageing beyond 21 days seemed not to improve unique dry-aged flavour. From the study of free amino acids, significant increase within 7 days of ageing was observed, regardless of the ageing methods, followed by accumulation at a lower rate over the next 21 days of ageing (Lee, Yoon, Kim, Oh, Yoon, & Jo, 2019). While the degradation of inosine monophosphate (IMP) and increase of reducing sugars with ageing time were observed for both D and W,

of up to 21 days of ageing, extending the ageing time to 28 days (Lee et al., 2019b) and 50 days (Iida et al., 2016) only had an impact on further degradation of IMP, for D.

Characteristic flavour of dry-aged beef is likely to have resulted from an increase in proteolytic activity by the microorganisms (mould and yeast) growing on the crust (Lee, et al., 2019a). This is further supported by the presence of higher trimethylamine content in dry-aged beef compared to the wet-aged, where its production is known to be proportional to the microbial activity. Another study by Li et al. (2014) has reported positive impact of dry-ageing on the flavour development with ageing time. Improved sweet, bitter, umami and bouillon taste and butter fried meat flavour was observed in dry-aged beef aged for 19 days compared to 8 days of ageing. A longer ageing time could result in a higher degree of proteolysis and thus increase the concentration of free amino acids and other flavour precursors such as glutamic acid, which is responsible for umami taste (Iida et al., 2016; Koutsidis et al., 2008). Li et al. (2014) has also suggested that in order to obtain significant interactions between ageing treatments and eating qualities of aged beef, sufficient ageing time is required. However, extra costs will be incurred from the operation cost of ageing facilities with extended dry-ageing time, on top of further weight (moisture) loss and trim loss leading to lower saleable yield. Thus, understanding the mechanisms responsible for the development of dry-aged flavour is necessary for the optimisation of ageing process in order to produce consistent dry-aged products with guaranteed premium flavour at a lower cost.

2.3 Mechanisms for development of dry-aged flavour

This section assesses three main factors, including, microbial activity, oxidation and dehydration, that drive the mechanisms for development of dry-aged flavour during the dry-ageing process.

2.3.1 Microbial activity

Microorganisms have been proposed to contribute to the unique dry-aged flavour, especially the proliferation of moulds and yeasts on the surface of meat during dry-ageing. Dry-ageing process is generally associated with the proliferation of spoilage microorganisms such as aerobic bacteria, lactic acid bacteria (LAB), moulds and yeasts (Table 2.4) (Berger et al., 2018; DeGeer et al., 2009; Gudjónsdóttir et al., 2015; Li et al., 2014; Li et al., 2013). However, Knudsen, Sommer, Sørensen, Olsen, and Aabo (2011) suggested D could provide advantage over the W from its capability to reduce the growth of pathogens, such as *Salmonella*. This could be attributed to the decrease in water activity

arising from dehydration during dry-ageing, which creates an adverse environment for the microbial growth (Szczesniak, 1997). A negative correlation between moisture level and the growth of microorganisms (total aerobic count, yeasts and moulds) was also reported (Gudjónsdóttir et al., 2015). Proliferation of LAB may cause sour taste due to the generation of lactic acid, slime and CO_2 during W process which may have detrimental effects on meat quality (Gram et al., 2002; in't Veld, 1996; Mills, Donnison, & Brightwell, 2014). A lower LAB count was observed in dry-aged beef (D/BD) compared to the W technique (Gudjónsdóttir et al., 2015; Li et al., 2013) as the anaerobic environment for W favours the growth of LAB (Gram et al., 2002; in't Veld, 1996).

Moulds (*Pilaira anomala*) and yeasts (*Debaryomyces hansenii*) were the primary microorganisms detected on the surface of dry-aged beef (Lee et al., 2019b; Oh, Lee, Lee, Jo, & Yoon, 2019). It is well-known that moulds and yeasts could be applied as a starter culture to contribute to flavour development during curing and fermentation process (Flores & Toldrá, 2011; Toldrá, 2008). *D. hansenii* has been extensively reported to play a key role in creating the distinctive flavour profile of dry-cured/fermented meat products (Flores, Durá, Marco, & Toldrá, 2004; Flores & Toldrá, 2011). Yeast species have the ability to grow at low-water activity environment in general which could account for higher yeast counts in the meat aged by dry-ageing techniques (D/BD) compared to W (Ahnström et al., 2006; Gudjónsdóttir et al., 2015; Li et al., 2014).

A more recent study by Oh et al. (2019) used inoculation to investigate the impact of yeasts and moulds on the flavour development of beef. Results demonstrated the potential to inoculate yeast and/or mould to accelerate dry-ageing process from the proteolytic and lipolytic capability of both microbes to tenderise beef as well as to enhance flavour (Flores & Toldrá, 2011). However, inconsistent findings were reported in their study on the changes of shear force tenderness, free amino acids and free fatty acids, throughout the ageing compared to the non-inoculated control. This may have been caused by the enzymes produced from yeasts and moulds, affecting development of distinct flavour. The enzyme activities in *P. anomala* have been suggested to be more effective over *D. hansenii* for lipolysis and degradation of complex protein structures into primary polypeptides and protein fragments (Lee et al., 2019b). Exogenous enzymes originated from *D. hansenii* may have more profound effects on the further cleavage of large polypeptides into smaller compounds such as small peptides and free amino acids.

Table 2.3 Summary of the impact of dry-ageing and increasing the dry-ageing time on flavour attributes of beef muscles. D: Traditional dry-ageing, W: Wet-ageing, BD: In-bag dry-ageing, NR: Not reported.

Flavour attributes	Impact of dry-ageing	Impact of dry-ageing time
Aged flavour	*Improved* D>W (Lepper-Blilie et al., 2016) *Unchanged* D=BD=W (Ahnström et al., 2006; DeGeer et al., 2009; Jiang et al., 2010; Laster et al., 2008; Minks & Stringer, 1972; Richardson et al., 2008; Smith et al., 2014; Smith et al., 2008; Velotto et al., 2015)	*Improved* (Campbell et al., 2001; Lepper-Blilie et al., 2016) *Unchanged* (DeGeer et al., 2009)
Beefy/brothy flavour	*Improved* D>W (Warren & Kastner, 1992); W-D>W (O'Quinn et al., 2016) *Unchanged* D=BD=W (Ahnström et al., 2006; Dikeman et al., 2013; Laster et al., 2008; Lepper-Blilie et al., 2016; Obuz et al., 2014; Oreskovich et al., 1988; Smith et al., 2008; Velotto et al., 2015) *Decreased* D<W (Smith et al., 2014)	*Unchanged* (Campbell et al., 2001; DeGeer et al., 2009; Laster et al., 2008; Lepper-Blilie et al., 2016; Obuz et al., 2014; Smith et al., 2008)
Browned/roasted flavour	*Improved* D>W (Warren & Kastner, 1992); W-D>W (O'Quinn et al., 2016) *Unchanged* D=BD=W (Ahnström et al., 2006; Lepper-Blilie et al., 2016)	*Unchanged* (Campbell et al., 2001; DeGeer et al., 2009; Lepper-Blilie et al., 2016)
Buttery/beef fat flavour	*Improved* D=BD>W (Li et al., 2014); W-D>W (O'Quinn et al., 2016) *Unchanged* D=W (Warren & Kastner, 1992)	*Improved* (Li et al., 2014)

Table 2.3 (continued)

Flavour attributes	Impact of dry-ageing	Impact of dry-ageing time
Astringent	*Unchanged* D=BD (Ahnström et al., 2006)	*Unchanged* (Campbell et al., 2001; DeGeer et al., 2009) *Decreased* (Ahnström et al., 2006)
Bloody/metallic flavour	*Improved* D>W (Smith et al., 2014) *Unchanged* D=BD=W (Ahnström et al., 2006; Lepper-Blilie et al., 2016) *Decreased* D<W (Warren & Kastner, 1992); W-D<W (O'Quinn et al., 2016)	*Unchanged* (Campbell et al., 2001; DeGeer et al., 2009; Lepper-Blilie et al., 2016; Li et al., 2014)
Off-odour (musty, sour, yeasty, putrid)	*Improved* BD/W<D (Lepper-Blilie et al., 2016; Li et al., 2014) *Unchanged* D=W (Dikeman et al., 2013) *Decreased* D<W (Obuz et al., 2014)	*NR*
Other flavour attributes	*Improved* Butter-fried odour, umami taste (Li et al., 2014), nutty/roasted nut taste (O'Quinn et al., 2016) *Unchanged* Animal and boiled meat odour, bitter, sweet, liver, boiled meat and bouillon taste, crumbly and fibrous texture (Li et al., 2014), earthy and mushroom flavour (O'Quinn et al., 2016) *Decreased* Metallic and liver odour (Li et al., 2014), grassy, haylike, gamey, livery and fishy flavour (O'Quinn et al., 2016)	*Improved* Animal and butter fried meat odour, sweet, bitter, umami and bouillon taste, crumbly texture (Li et al., 2014) *Unchanged* Metallic/liver odour, fatty and livery taste (Li et al., 2014) *Decreased* Boiled meat odour, fibrous texture (Li et al., 2014)

Table 2.4 Summary of the impact of dry-ageing and increasing the dry-ageing time on physicochemcial and microbial properties of dry-aged beef muscles. D: Traditional dry-ageing, W: Wet-ageing, BD: In-bag dry-ageing, NR: Not reported.

Attributes	Impact of dry-ageing	Impact of dry-ageing time
% Moisture (raw)	*Unchanged* D=BD=W (Ahnström et al., 2006; DeGeer et al., 2009; Ha et al., 2019; Oreskovich, et al., 1988; Parrish et al., 1991); BD=BD-W (Zhang et al., 2019) *Decreased* D<BD<W (Berger et al., 2018; DeGeer et al., 2009; Gudjónsdóttir et al., 2015); D=BD<W (Lee et al., 2019a; Li et al., 2014; Li et al., 2013; O'Quinn et al., 2016; Obuz et al., 2014; Sitz et al., 2006; Velotto et al., 2015)	*Increased* (DeGeer et al., 2009; Gudjónsdóttir et al., 2015) *Unchanged* (Ahnström et al., 2006; Hulánková et al., 2018; Iida et al., 2016); to 21 d, then decreased at 28 d (Lee et al., 2019a) *Decreased* (Ha et al., 2019; Lee et al., 2019a; Zhang et al., 2019)
% Moisture (cooked)	*Unchanged* D=BD=W (Dikeman et al., 2013; Velotto et al., 2015)	*NR*
% Cook loss	*Unchanged* W=BD=D (Ahnström et al., 2006; Berger et al., 2018; Dikeman et al., 2013; Gudjónsdóttir et al., 2015; Kim et al., 2016; Lautenschlaeger, 2012; Oreskovich et al., 1988; Stenström et al., 2014; Velotto et al., 2015; Warren & Kastner, 1992); BD=BD-W (Zhang et al., 2019) *Decreased* D<BD=W (DeGeer et al., 2009; Dikeman et al., 2013), D<W (Laster et al., 2008; Li et al., 2013)	*Unchanged* (Ahnström et al., 2006; DeGeer et al., 2009; Laster et al., 2008) *Decreased* (Zhang et al., 2019)
Lipid oxidation	*Increased* BD<D (DeGeer et al., 2009); D>W (Ha et al., 2019) *Unchanged* D=W (Jiang et al., 2010; Oreskovich et al., 1988; Parrish et al., 1991)	*Increased* (Ha et al., 2019; Oreskovich et al., 1988) *Unchanged* (DeGeer et al., 2009)
Microbial analysis		
Total bacteria	*Increased* D>BD>W (Li et al., 2014; Li et al., 2013; Minks & Stringer, 1972)	*Increased* (Li et al., 2014)
Lactic acid bacteria	*Unchanged* D=BD=W (Ahnström et al., 2006; DeGeer et al., 2009; Li et al., 2014; Shi et al., 2020) *Decreased* BD<W (Li et al., 2013); BD=W>D (Berger et al., 2018; Gudjónsdóttir et al., 2015)	*Increased* (Shi et al., 2020); in BD, unchanged in D (Gudjónsdóttir et al., 2015; Li et al., 2014) *Unchanged* For 14 d, then increased (Campbell et al., 2001)

Table 2.4 (continued)

Attributes	Impact of dry-ageing	Impact of dry-ageing time
Lactic acid bacteria	*Not detected* BD=BD-W (Zhang et al., 2019)	*Decreased* (Ahnström et al., 2006; DeGeer et al., 2009)
Yeast	*Increased* D>BD>W (Ahnström et al., 2006; Li et al., 2014; Li et al., 2013; Shi et al., 2020); BD=D>W (Ahnström et al., 2006; Berger et al., 2018; Gudjónsdóttir et al., 2015; Li et al., 2014) *Unchanged* BD=BD-W (Zhang et al., 2019)	*Increased* (Ahnström et al., 2006; DeGeer et al., 2009; Gudjónsdóttir et al., 2015; Li et al., 2014; Shi et al., 2020; Zhang et al., 2019)
Moulds	*Increased* D>W (Gudjónsdóttir et al., 2015; Shi et al., 2020) *Unchanged* BD=D=W (Berger et al., 2018; DeGeer et al., 2009; Li et al., 2014; Li et al., 2013); *not detected* (Lee et al., 2017; Zhang et al., 2019)	*Increased* (DeGeer et al., 2009; Gudjónsdóttir et al., 2015; Shi et al., 2020) *Unchanged* (Li et al., 2014; Li et al., 2013)
Aerobic count	*Increased* BD>D>W (Berger et al., 2018; Gudjónsdóttir et al., 2015); D>BD>W (Shi et al., 2020) *Unchanged* D=BD (Ahnström et al., 2006; DeGeer et al., 2009; Lee et al., 2017); BD=B-W (Zhang et al., 2019)	*Increased* (DeGeer et al., 2009; Gudjónsdóttir et al., 2015; Shi et al., 2020); for 7 d, then unchanged (Campbell et al., 2001) *Unchanged* (Ahnström et al., 2006) *Decreased* BD (Zhang et al., 2019)
Enterobacteriaceae	*Increased* D>BD>W (Li et al., 2014) *Unchanged* BD=W (Li et al., 2013) *Not detected* BD=BD-W (Zhang et al., 2019)	*Increased* (Li et al., 2014)
Escherichia coli/coliforms	*Unchanged* BD=D (DeGeer et al., 2009) *Not detected* BD=BD-W (Zhang et al., 2019)	

The proliferation of moulds and yeasts could be interconnected with several other factors, such as dry-ageing techniques (D/BD), ageing conditions (temperature, air velocities, RH) and ageing time. D has a higher chance of proliferation of microorganisms compared to the BD technique, due to the direct contact with the surroundings during ageing. Similar level of yeast and mould may be achieved for the two dry-ageing techniques (D and BD) due to a good hygiene practice from slaughter to product processing (Berger et al., 2018; DeGeer et al., 2009; Li et al., 2014; Li et al., 2013).

Different air velocities could also change the composition of microorganisms during ageing. Moulds (mainly *P. anomala*) were the predominant microorganism detected on the crust of beef dry-aged for 28 days with no air flow (Lee, et al., 2019). Increasing air velocities to 2.5 and 5 $m.s^{-1}$ favoured the proliferation of yeast (*D. hansenii*) probably due to reduced water activity on the surface (Dave & Ghaly, 2011). There was no or a low level of yeasts and moulds detected after 2 to 3 weeks of dry-ageing regardless of air velocities and ageing techniques (Lee et al., 2019b; Li et al., 2014; Li et al., 2013). Extending dry-ageing to 3 to 4 weeks could enhance the proliferation of yeasts and moulds, enabling the microorganisms to contribute more meaningfully to flavour development of dry-aged meat. A recent study by Choe et al. (2020) suggests that post-mortem ageing of 28 days generally resulted in an increase of low molecular weight protein fragments (< 15 kDa) and small peptides (< 3 kDa) compared to the unaged equivalents, regardless of ageing techniques. No difference of SDS-PAGE protein profile in trimmed meat between ageing techniques was observed in their study suggesting that the proteolytic pattern did not differ. However, the crust formed on the surface of dry-aged meat had different protein and small peptide profiles compared to the edible trimmed part and unaged equivalents (Choe et al., 2020). More small protein fragments (< 15 kDa) were detected from the crust with a longer period of dry-ageing and this could be associated with the proteolytic activities arising from microbial enzymes. Therefore, we hypothesise that the primary proteolytic and lipolytic processes at early stage (1 to 2 weeks) of dry-ageing are dominated by endogenous enzymes (mainly calpains and lipases). Growth of yeasts and moulds produces exogenous enzymes and trigger the secondary degradation of lipids and proteins during extended ageing time. Such enzymatic reactions may result in distinct metabolite profiles (e.g. peptides and amino acids) as flavour precursors, contributing to the evolution of characteristic dry-aged flavour. Decrease of IMP and increase of reducing sugar, free amino acids and free fatty acids with dry-ageing up to 28 days have also been reported (Lee et al., 2019a, 2019b).

The role of moulds and yeasts in the improvement of dry-aged flavour, and interaction with air velocity warrants further investigation.

2.3.2 Oxidation

Post-mortem ageing has been reported to negatively affect the colour and flavour of beef products owing to the oxidative damage to the colour pigments (myoglobin), lipid and protein (Ha et al., 2019; Lund, Hviid, & Skibsted, 2007; Martinaud et al., 1997; Parrish et al., 1991; Sante-Lhoutellier, Engel, Aubry, & Gatellier, 2008). Dry-ageing of meat with its exposure of meat to an oxygen-rich environment has a higher risk of oxidative damage, and deterioration of meat quality (Rysman, Van Hecke, Van Poucke, De Smet, & Van Royen, 2016; Xiong, 2000).

The impact of dry-ageing on the oxidative changes to lipids and proteins and its role in the development of dry-aged flavour is not well understood. Limited studies (Table 2.4) have reported that dry-ageing process enhances lipid oxidation (thiobarbituric acid reactive substances, TBARS) of beef (Ha et al., 2019; Parrish et al., 1991; Passetti et al., 2019). However, the measurement of lipid oxidation reported in their studies was to evaluate its role in quality deterioration instead of flavour development. Dry-ageing is an aerobic maturation process which involves a mild extent of lipid oxidation. Such oxidative reactions of lipids produce oxidative intermedia which may react with other flavour precursors (e.g. sugars, peptides and amino acids) during cooking to develop signature dry-aged flavour.

Lipids are an important constituent of meat and play an important role in the quality and flavour development of meat. Degradation of lipids (triglycerides and phospholipids) into free fatty acids during dry-ageing is mainly associated with the activities of lipolytic enzymes from both endogenous and exogenous sources (Hierro, de la Hoz, & Ordóñez, 1997; Toldra, 1998). As described in the previous section, proliferation of the microorganisms (mainly yeasts and moulds) during dry-ageing process produces lipolytic enzymes and enhance the degradation of lipids (Lee et al., 2019b; Oh et al., 2019). A series of lipid-derived volatile compounds is responsible for development of desired meat flavour, such as alcohols, aldehydes, aliphatic hydrocarbons, carboxylic acids, esters and ketones (Mottram, 1998). Both intact and free forms of lipids are susceptible to oxidation and generate a series of oxidative by-products. These oxidative products are involved during cooking to produce a range of volatiles such as aldehydes, hydrocarbons, ketones and lactones, that contribute to the flavour intensity of meat (Khan, Jo, & Tariq, 2015;

Mottram, 1998; Watanabe et al., 2015). The lipid-derived free radicals and reactive carbonyls produced from the oxidation of unsaturated fatty acids could also react with free amino acids and peptides from proteolysis, to produce Strecker aldehydes, α-keto acids, amines, pyridines and pyrrole derivatives and give rise to new flavours (Zamora & Hidalgo, 2011; Zamora et al., 2015). The final volatile profile depends on the initial oxidised lipid, which depends on the source of meat (fat level and type) and dry-ageing process (aerobic conditions and microbe profile). These are the factors that could be tailored for the development of unique dry-aged flavour.

2.3.3 Dehydration

Higher loss in weight and trimming cannot be avoided when using dry-ageing technique (Ahnström et al., 2006; Berger et al., 2018; DeGeer et al., 2009; Dikeman et al., 2013; Gudjónsdóttir et al., 2015; Kim et al., 2016; Laster et al., 2008; Lepper-Blilie et al., 2016; Li et al., 2014; Li et al., 2013; Obuz et al., 2014; Smith et al., 2014; Smith et al., 2008; Stenström et al., 2014; Velotto et al., 2015). A significant weight loss occur mainly at the early stage of dry-ageing (within 3 weeks), however as the time lapse, the rate of weight loss decreases significantly (Ahnström et al., 2006; DeGeer et al., 2009; Gudjónsdóttir et al., 2015; Lepper-Blilie et al., 2016; Li et al., 2014; Minks & Stringer, 1972; Parrish et al., 1991). During dry-ageing, the primary moisture evaporation occurs on the outer layer of meat. It is followed by the formation of a crust with extended ageing time at the outer layer of the meat which is the dehydrated and discoloured surface where microorganisms grow (Lee et al., 2019b; Smith et al., 2014). The crust acts as a barrier for moisture retention inside the meat and improve the juiciness (Berger et al., 2018; Campbell et al., 2001; Li et al., 2013; Richardson et al., 2008; Stenström et al., 2014). The moisture content of dry-aged meat may therefore be significantly reduced on the outer layer of the meat with only minor changes occurring inside (Li et al., 2014; Li et al., 2013). Trimming of the crust after ageing is a common practice reported in the literature (Table 2.1). When trimming was applied, moisture content of the aged meat showed no difference across the different ageing techniques (D/BD/W) (Table 2.4) (Ahnström et al., 2006; DeGeer et al., 2009; Lee et al., 2017; Oreskovich et al., 1988), in other words, similar eating quality can be maintained (Table 2.2).

Moisture evaporation during the dry-ageing process results in concentration of flavour compounds, which has been associated with the development of distinctive dry-aged flavour (Kim et al., 2016; Kim et al., 2018; Lee et al., 2019a). A positive impact of dehydration on dry-aged flavour development has only been observed when the moisture

content of trimmed dry-aged meat was considerably reduced. Some studies have attempted to associate the level of dehydration with the resultant changes in eating qualities. A more recent study by Lee et al. (2019a) suggested that the moisture evaporation could partially explain the significant increase of taste-active compounds after dry-ageing. A significant decrease in the moisture content was only observed after 21 days of dry-ageing in their study which coincided with the significant increase of free amino acids and reducing sugars. Thus, the development of dry-aged flavour with intensified flavour notes could be simply achieved by manipulating the levels of moisture evaporation.

The role of dehydration in the improvement of dry-aged flavour is not well elucidated in the literature. This is due to the moisture content in fresh meat being similar prior to the ageing treatments (D/BD/W). The higher moisture loss from dry-ageing results in lower cook loss compared to the W (DeGeer et al., 2009; Dikeman et al., 2013; Laster et al., 2008; Li et al., 2013), thereby producing cooked meat with a similar water content and eating quality irrespective of the ageing techniques (D/BD/W) (Dikeman et al., 2013; Velotto et al., 2015). By correlating the improvement in dry-aged flavour with the changes in moisture content of cooked meat due to different ageing regimes, the impact of dehydration on flavour development may be better elucidated.

2.4 Other factors affecting dry-aged flavour

The final flavour could be influenced by several other factors based on the mechanisms, including meat sources (e.g. compositions, cuts and species), control of microorganism growth, ageing parameters (temperature, air velocity and RH) and ageing time, as shown in Figure 2.1a. These factors are further elaborated in the following sections.

2.4.1 Meat sources

2.4.1.1 Animal factors

Animal factors, such as species, breeds (Campo et al., 1999), age and sex (Lucero-Borja et al., 2014), diet of animals (Jiang et al., 2010), extent of exercise (related to cuts) and pre-slaughter stress (Gruber et al., 2010; Helser, Nelson, & Lowe, 2017; Warner et al., 2005) have been suggested to affect the sensory quality of meat. These factors are also interconnected with dry-ageing process and contribute to development of distinct flavour profiles. For example, meat from different breeds vary in the amount of fat, degree of unsaturation in fatty acid profile, quantity and solubility of collagen and activities of

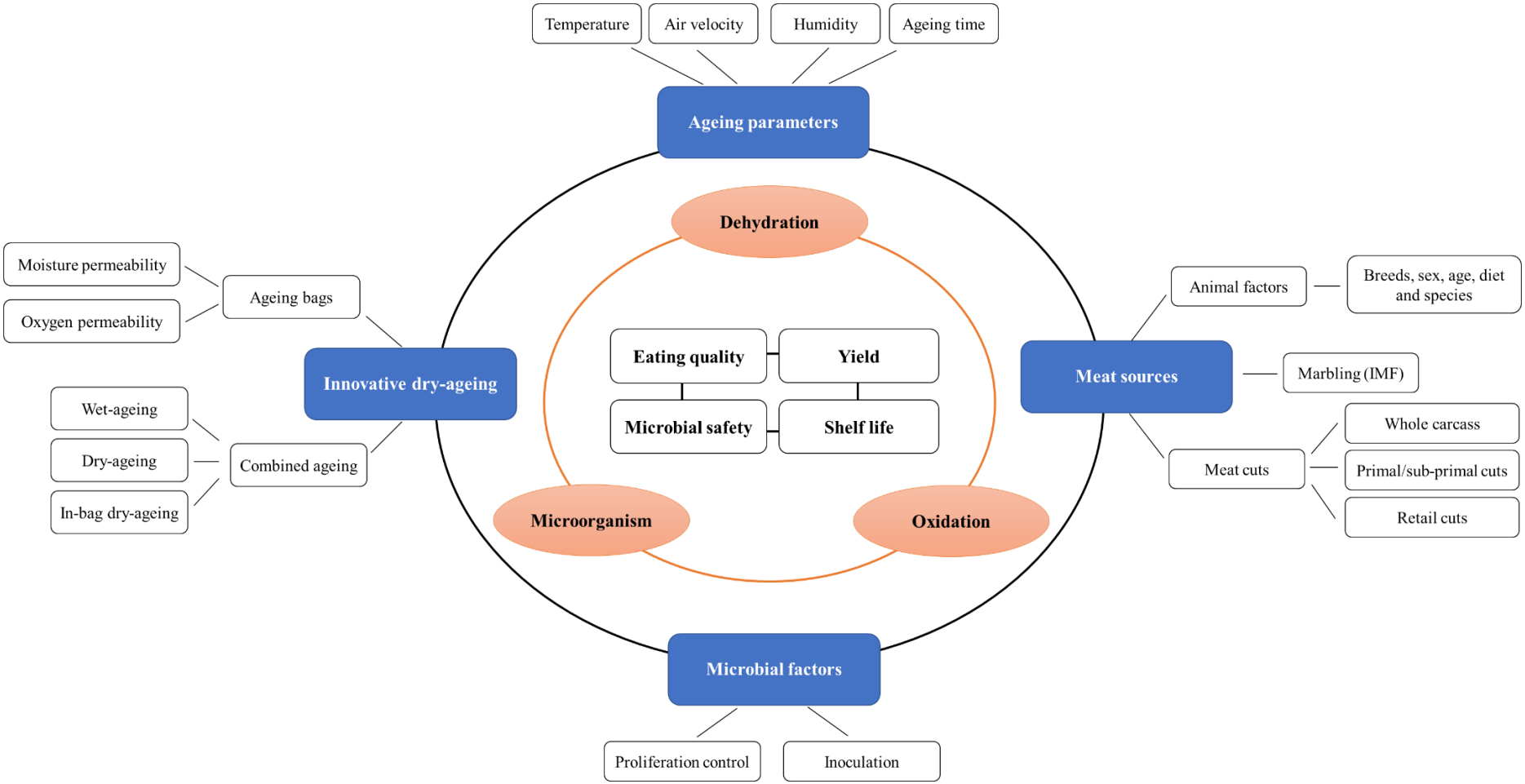
endogenous proteases, which may interact differently to the biochemical changes occurring during dry-ageing and resulting in distinct flavour (Khan et al., 2015).

By utilising meat from different animal species, such as sheep (lamb and mutton), goat, pork and game meat, the range of dry-aged products can be further expanded. We speculate that the unique animal-specific flavours that are naturally present, particularly those of lipid-derived flavours (Mottram, 1998), could be enhanced or as precursors to produce new flavour profile through the dry-ageing process. However, availability of diverse animal sources, development of animal-specific ageing regimes, consumer acceptability and purchase willingness in the local and international markets need to be understood to determine the market opportunity for a diverse range of dry-aged meats.

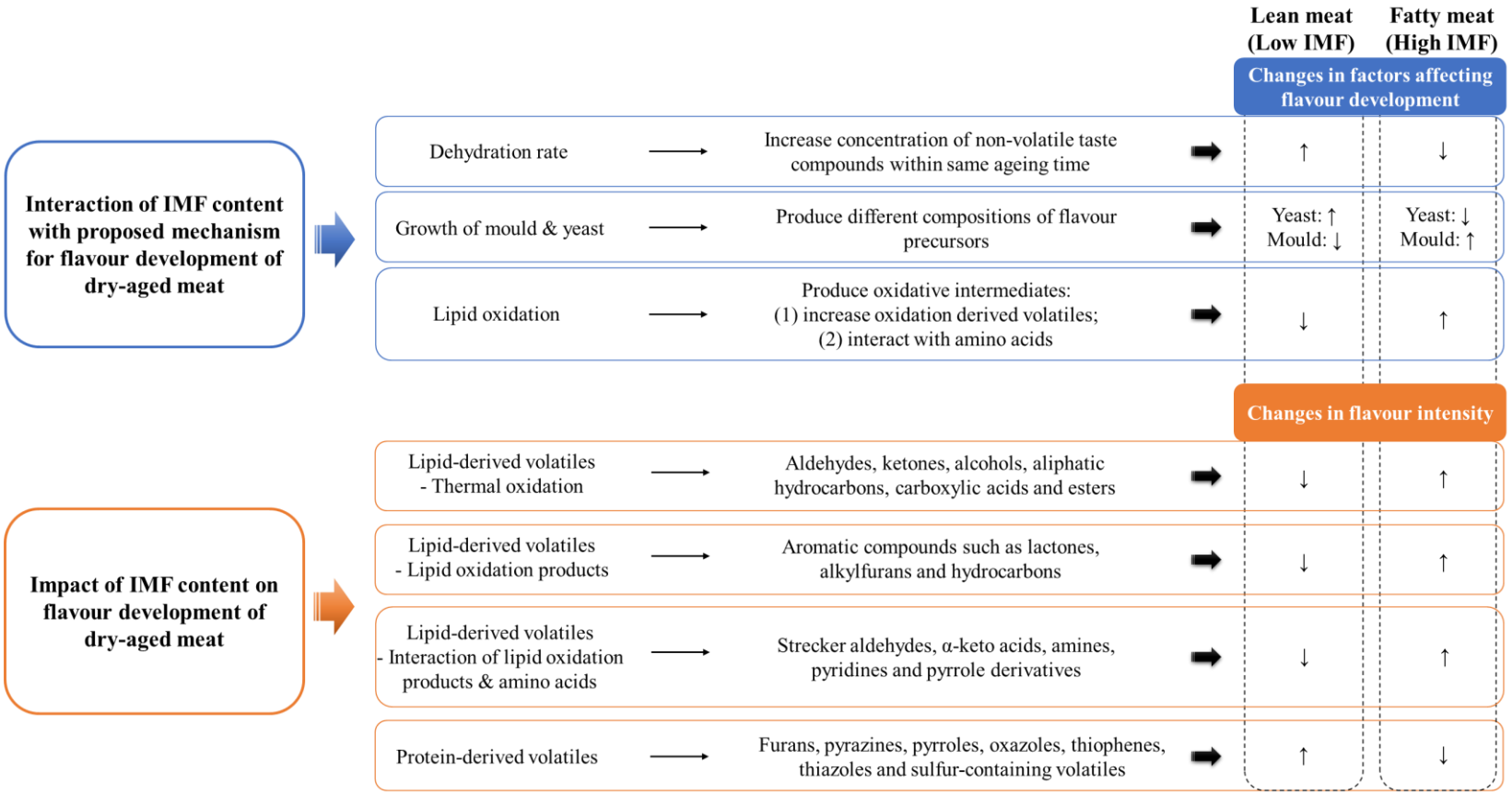
2.4.1.2 Marbling grade (IMF content)

The animal factors could eventually affect the quantity and quality of fat in the carcass. Marbling or IMF content is one of the important characteristics which affect consumers' purchase willingness and satisfaction of the meat due to its positive effect on juiciness, tenderness and flavour (Nishimura, Hattori, & Takahashi, 1999; Platter et al., 2003). Some countries use quality grading system (e.g. USDA quality grade) to classify meat with different marbling/IMF levels.

The IMF content and the degree of fatty acid unsaturation could possibly become key attributes associated with the development of characteristic dry-aged flavour. A wide range of quality grades/IMF contents from lean bull beef to highly marbled Prime cuts have been used for dry-ageing of beef (Table 2.1), while the role of IMF in the development of dry-aged flavour has not been well-elucidated. Aged beef with a higher quality grade generally had greater intensity of beefy/brothy, brown/grilled flavour and buttery/beef fat flavours, and reduced negative flavour attributes such as bloody/serumy, livery/organy and grassy, regardless of ageing methods (D/BD/W) (Emerson, Woerner, Belk, & Tatum, 2013; O'Quinn et al., 2016). Previous studies have failed to observe positive impact of dry-ageing on the eating quality of beef which had different USDA quality grades, when compared to wet-aged equivalent (Dikeman et al., 2013; Laster et al., 2008; O'Quinn et al., 2016; Parrish et al., 1991; Sitz et al., 2006; Smith et al., 2008). This could be explained by the positive impact of IMF content on the generation of distinctive dry-aged flavour, which is mainly based on the oxidation mechanism as described in section 2.3.2.


Temperature
Air velocity
Humidity
Ageing time
Ageing parameters
Dehydration
Moisture permeability
Oxygen permeability
Ageing bags
Innovative dry-ageing
Wet-ageing
Dry-ageing
In-bag dry-ageing
Combined ageing
Eating quality
Yield
Microbial safety
Shelf life
Microorganism
Oxidation
Animal factors
Breeds, sex, age, diet and species
Meat sources
Marbling (IMF)
Meat cuts
Whole carcass
Primal/sub-primal cuts
Retail cuts
Microbial factors
Proliferation control
Inoculation

(a)



(b)

Figure 2.1 Diagram summarising (a) strategies to tailor signature dry-aged flavour and (b) the interaction of intramuscular fat (IMF) with three key factors (dehydration, growth of mould and yeast and lipid oxidation), and its impact on flavour development of dry-aged meat.

The possible impact of IMF content on the development of dry-aged flavour is summarised in Figure 2.1b. Meat cuts with a higher IMF content may be more susceptible to lipid oxidation during dry-ageing and produce higher amount of peroxides which could interact with amino acids and peptides and give rise to higher level of oxidised-lipid derived volatiles (section 2.3.2). Volatile compounds produced from lipid and/or lipid oxidation could further interact with water-soluble precursors (e.g. protein, sugars and vitamins) which are dominated in lean tissues and result in the unique flavour profile. However, such unique volatile profile may not be easily detected by consumers because the odour threshold values for lipid-derived volatile compounds are generally higher than those generated from water-soluble precursors (Mottram, 1998). Further, the increase of IMF content reduces the volatility of most volatile aroma compounds due to their lipophilic nature, which impedes the release of these compounds during consumption of meat (Frank, Kaczmarska, Paterson, Piyasiri, & Warner, 2017). For example, the significant increase of volatile content was only observed for beef samples with IMF content of greater than 10%, whereas no difference was detected in a lower IMF content of 5 to 10% (Frank et al., 2017). Thus, the positive impact of IMF on the improvement of dry-aged flavour may require the significant increase of IMF level in meat.

The flavour profiles of lean meat, on the other hand, are dominated by protein-derived flavours through Maillard reaction and Strecker degradation routes and generate volatile compounds such as furans, pyrazines, pyrroles, oxazoles, thiophenes, thiazoles and sulphur-containing volatiles (Mottram, 1998; Zamora & Hidalgo, 2011). The production of these volatile compounds depends on the profile of flavour precursors including peptides and amino acids which are interconnected with dehydration and microorganism mechanisms during dry-ageing. A lower impact of lipid on the volatility of flavour compounds released from lean meat tissues could also contribute to a distinctive flavour profile perceived by consumers compared to fatty meat. Therefore, there could be opportunities to produce new dry-aged products from lean meat. For example, meat cuts from bull generally contain a low IMF (less marbled) which are usually processed into sausages, patties or other low-valued products instead of premium products such as dry-aged meat. However, the low IMF could account for greater weight loss during ageing (Dikeman et al., 2013), which could be suitable for dry-ageing process when rapid dehydration is required. The use of lean portion of meat cuts may also offer advantages over fatty cuts for storage stability and reduced off-flavours due to its low oxidation potential (Wood et al., 2008). A recent study by Zhang et al. (2019) suggested the use of

lean bull beef (1-2% IMF) could produce dry-aged beef with acceptable quality and stability during frozen storage for 12 months.

2.4.2 Ageing parameters

Extensive studies have reported on the effect of ageing techniques on the quality of dry-aged meat with limited information provided on the influence of ageing parameters, such as temperature, RH and air velocity used for the ageing chamber (Table 2.1). These parameters need to be carefully considered when designing the dry-ageing process to produce desired eating quality and characteristic dry-aged flavour. As shown in Table 2.1, a wide range of settings for dry-ageing have been used across the studies, the common practice is to dry-age in a refrigerated room for 21 to 28 days at 0 - 4 ℃ and 60 - 85% RH, with an air velocity of 0.5 to 2.5 $m.s^{-1}$. Manipulation of these parameters may change the beneficial impact of dry-ageing on the meat quality.

Some studies have reported that elevating the temperature of dry-ageing could accelerate the ageing process by reducing the turnover time and associated costs (Kim et al., 2016). Higher ageing temperature favours the primary protein degradation by endogenous enzymes and accelerates the tenderisation process and flavour development (Huff-Lonergan, Zhang, & Lonergan, 2010). However, the increase of temperature also enhances the proliferation of spoilage microorganisms. A low ageing temperature (below freezing temperature, e.g. -2 to -3 °C) on the other hand could reduce the proliferation of microorganisms and retard enzymatic ageing processes.

The role of RH and air velocity in the ageing chamber for flavour development has not been well defined. The basic mechanism is the dehydration process, which could be accelerated through enhancing air circulation and/or reducing the RH level in the ageing chamber. A RH of 70% to 85% is generally used to prevent excessive moisture loss and slow down the proliferation of microorganisms. A high RH level will favour the microbial growth and a lower RH will cause excessive dehydration of dry-aged products. D is performed in direct contact with the ambient environment, where there is a high risk of microbial contamination. Air circulation acts as a medium for moisture removal from the meat surface during ageing. Insufficient air velocity will allow excessive moisture to condense on the product, to result in spoilage and generation of off-flavours. A high air velocity, on the other hand, could cause excessive surface drying and greater weight and trim losses. Increasing air velocity from 0 and 2.5 to 5 $m.s^{-1}$ significantly reduced the moisture level of dry-aged beef rump after trimming, but no change was observed with

air velocity between 0 and 2.5 $m.s^{-1}$ (Lee et al., 2019b). Use of different air velocities could also result in different microorganism profiles, which in turn will affect the secondary enzymatic reactions and consequently the final flavour. According to Oh et al. (2019), the use of a moderate air velocity of 2.5 $m.s^{-1}$ resulted in a more diverse microbial profile compared to lower (no air flow) or higher air velocities (5 $m.s^{-1}$). As described in section 2.3.1, mould (*P. anomala*) is the dominant microbe detected on dry-aged beef with the absence of air circulation, though proliferation of both mould and yeast (*D. hansenii*) on the meat surface was observed with the use of higher air velocities (2.5 and 5 $m.s^{-1}$) (Lee et al., 2019b).

Another study by Zhang et al. (2019) suggested that the increase of air velocity from 0.5 to 2.5 $m.s^{-1}$ accelerated the moisture loss but with no detrimental impact on the quality of dry-aged lean beef if BD technique was applied. The water and oxygen permeability of dry-ageing bags are the main factors interconnected with dry-ageing parameters to regulate the rates of moisture evaporation and oxidation and consequently affect the quality of in-bag dry-aged meat. However, the role of these two factors in development of dry-aged flavour has not been well understood. A recent study by Shi et al. (2020) suggested that BD using a higher moisture permeability ageing bag could accelerate the dehydration and also subsequently enhance the proliferation of yeast after 14 days of ageing. BD technique has also been suggested to selectively favour the growth of yeast rather than moulds (Li et al., 2013; Shi et al., 2020; Zhang et al., 2019) which could also modify the final flavour profile of dry-aged products.

2.4.3 Ageing time

The length of ageing time is one of the primary factors in the dry-aged flavour development due to its close interconnection with enzymatic reactions during dry-ageing (Velotto et al., 2015; Zhang et al., 2019). Determining the length of ageing time is of great significance from the economic perspectives due to the considerable cost from operating ageing chamber, dehydration, trimming and spoilage. Sufficient ageing time should be allowed for the enzymatic reactions to positively convert the meat into a “well-aged” product with distinctive dry-aged flavour (Li et al., 2014). A short period of dry-ageing for 1 to 2 weeks could favour tenderisation of meat and produce the dry-aged product with tenderness level similar to its wet-aged equivalent (Campbell et al., 2001; Lepper-Blilie et al., 2016; Warren & Kastner, 1992). However, it is the unique dry-aged flavour rather than tenderness that defines dry-aged products. Therefore, to design a product which can be labelled as “dry-aged”, enough ageing time should be allowed for the

development of unique dry-aged flavour. On the other hand, it is also important to prevent the meat from being over-aged, to avoid mushy texture and off-flavour.

The relationship between dry-aged flavour development and length of ageing time is influenced by processing parameters (as discussed in section 2.4.2), animal species and types of meat cut (e.g. various IMF fat content and fibre types), because they provide different metabolic responses to the ageing treatments (Ithurralde et al., 2018; Ouali & Talmant, 1990). For instance, meat samples obtained from different carcasses generally have variable size and weight prior to ageing which could affect the rate of weight loss during the dry-ageing process. A sample with higher initial weight and larger size could require a longer time to reach the same level of weight loss compared to those smaller sized cuts as shown in the weight loss of lamb samples (hindlegs) with variable initial weights from 15% to 30% processed under the same ageing conditions for 21 days (Figure 2.2, unpublished observations). Sample sizes (whole leg with shank-on *vs.* leg section without shank) will also have an impact on the rate of weight loss after the same period of dry-ageing. Therefore, dry-ageing of meat with various sizes/weights under the same conditions could give rise to significantly different moisture levels and result in distinct quality profiles based on the dehydration mechanism. There remains a need to investigate the interactions of these factors with different dry-ageing techniques (D/BD) and ageing time to fully understand their impact on the eating quality.

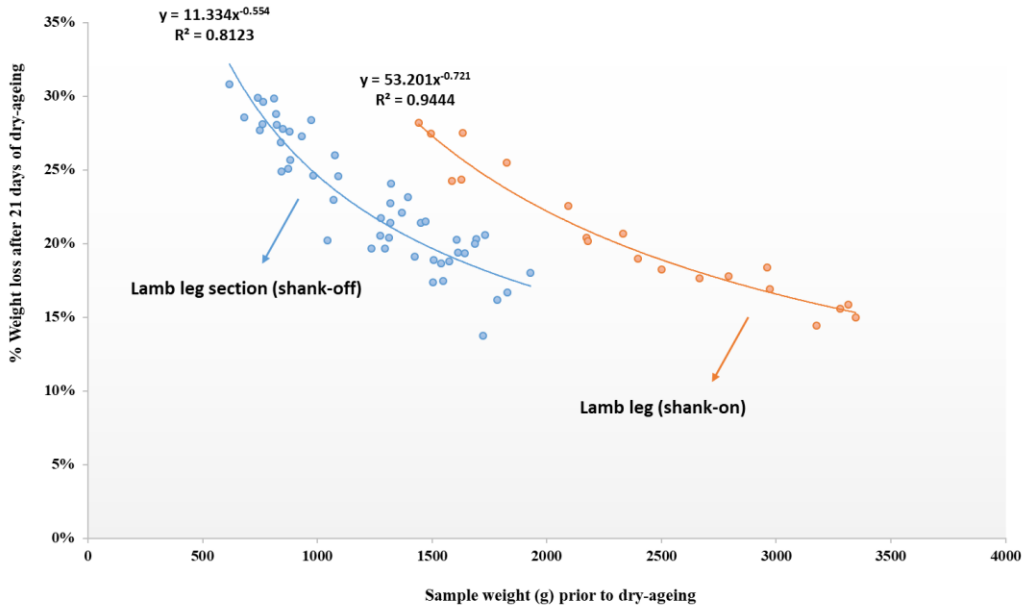


Figure 2.2 Relationship between % weight loss after 21 days of dry-ageing and initial weight of lamb legs.

2.4.4 Combined ageing regime

To overcome the high costs and to improve the efficiency of dry-ageing process, scientists have attempted to combine dry-ageing techniques (D/BD) with W as a stepwise ageing regime. The stepwise process produced aged beef products with equivalent or improved qualities compared to straight aging process as shown in Table 2.2 and 2.3 (Campbell et al., 2001; DeGeer et al., 2009; Kim et al., 2017; O'Quinn et al., 2016; Sitz et al., 2006; Zhang et al., 2019). The flavours, such as aged/beefy, brown/roasted, bloody/serumy, metallic, and astringent flavour, were stable during 16 days of W following D for 21 days (D-W) (Campbell et al., 2001). Similar sensory quality has been observed between stepwise regime (10 days D + 7 days W) and straight D only for 17 days (Kim et al., 2017). Thus, the substitution of extended D with W could be applied to improve the efficacy of dry-ageing with no detrimental impact on the flavour quality. Another approach by O'Quinn et al. (2016) using W-D regime suggested that the application of D technique after the W could improve the intensity of the browned/grilled flavour. The stepwise regime (W-D) gave rise to higher ratings of beefy/brothy, browned/grilled, and buttery/beef fat flavours and overall flavour desirability, and lower ratings of bloody/metallic, gamey and fishy flavours and sour and bitter taste, when compared to the straight W only. Dry-aged beef using stepwise regime (21 days W + 35 days D) was preferred for flavour with higher overall liking by consumers with no difference in the liking of tenderness and juiciness compared to W only for 56 days (Ha et al., 2019). The eating quality of dry-aged beef for 56 days was rated slightly higher by consumers in their study over the stepwise regime and W only, while the retail yield of D (66.06%) reduced significantly compared to the stepwise regime (38.36%). Based on the mechanisms discussed in this review (section 2.3), the biochemical reactions by endogenous enzymes are similar between the two ageing methods (D and W). Generation of dry-aged flavour through oxidation and microorganism proliferation occurs mainly during the extended period of dry-ageing (> 2 to 3 weeks) due to the lipid oxidation requiring a longer period of ageing time to develop (Watanabe et al., 2015). Thus, substitution of D with W at the early stage of ageing (i.e. W-D) could produce similar precursors which could produce equivalent dry-aged products compared to straight-D. A higher retail yield then can be achieved using W-D regime due to the reduced weight loss by dehydration. The resultant flavour profile from W-D regime may differ from the D-W regime as the dehydration is the primary contributor to the dry-aged flavour in later part of the regime instead of oxidation and microbial mechanisms.

A new approach called ‘stepwise in-bag ageing’, which combines BD with W as the stepwise regime (7 days BD + 14 days W), has been reported to produce similar eating quality with lower weight loss compared to straight BD (Zhang et al., 2019). The in-bag ageing system used incorporated water-permeable ageing bags to accelerate the dehydration process, while reducing oxidation and microbial growth. The potential advantage for this ageing regime is an increase in salable yield of dry-aged products by eliminating post-ageing trimming process. The crust formed from dry-ageing disappear due to the equilibration of moisture between the inner and outer layers of meat during the subsequent W process. Thus, no trimming would be necessary after the ageing process. This could considerably increase the product yield. Furthur, crust has been suggested to possess functional properties, such as flavour enhancement (Park, Yong, Choe, & Jo, 2018) and antioxidants and possess Angiotensin I-Converting Enzyme (ACE) inhibitory activity (Choe et al., 2020). However, the crust is normally trimmed off owing to the proliferation of microorganisms and discolouration during D process (Table 2.1).

2.5 Proposed mechanism and strategies to tailor dry-aged flavour

Proliferation of microorganisms, oxidation and dehydration are three key interconnected elements to the development of unique features of dry-aged products. These three mechanisms are used (as shown in Figure 2.3) to suggest strategies to tailor the flavours of dry-aged meat. The strategies are predicated on the understanding that during the dry-ageing of meat, proteins and lipids undergo primary degradation into polypeptides and small amount of free fatty acids by endogenous enzymes within 1 to 2 weeks of dry-ageing to achieve acceptable tenderness; the enzymatic reactions then slow down due to the autolysis of endogenous enzymes which reduces the enzyme activities with extended dry-ageing (> 2 - 3 weeks); rapid dehydration at the early stage of dry-ageing reduces the water activity on the outer layer of meat which favours the proliferation of certain microorganisms, with yeasts and moulds being potentially the most influential on flavour; the exogenous enzymes produced from yeasts and moulds trigger the secondary enzymatic proteolysis and lipolysis giving rise to unique profiles of smaller metabolites, such as small peptides, free amino acids and free fatty acids; these metabolites serve as important precursors for the development of dry-aged flavour. Dehydration and lipid oxidation are involved throughout the entire dry-ageing process, contributing to the development of intense signature taste and aroma of dry-aged meat.

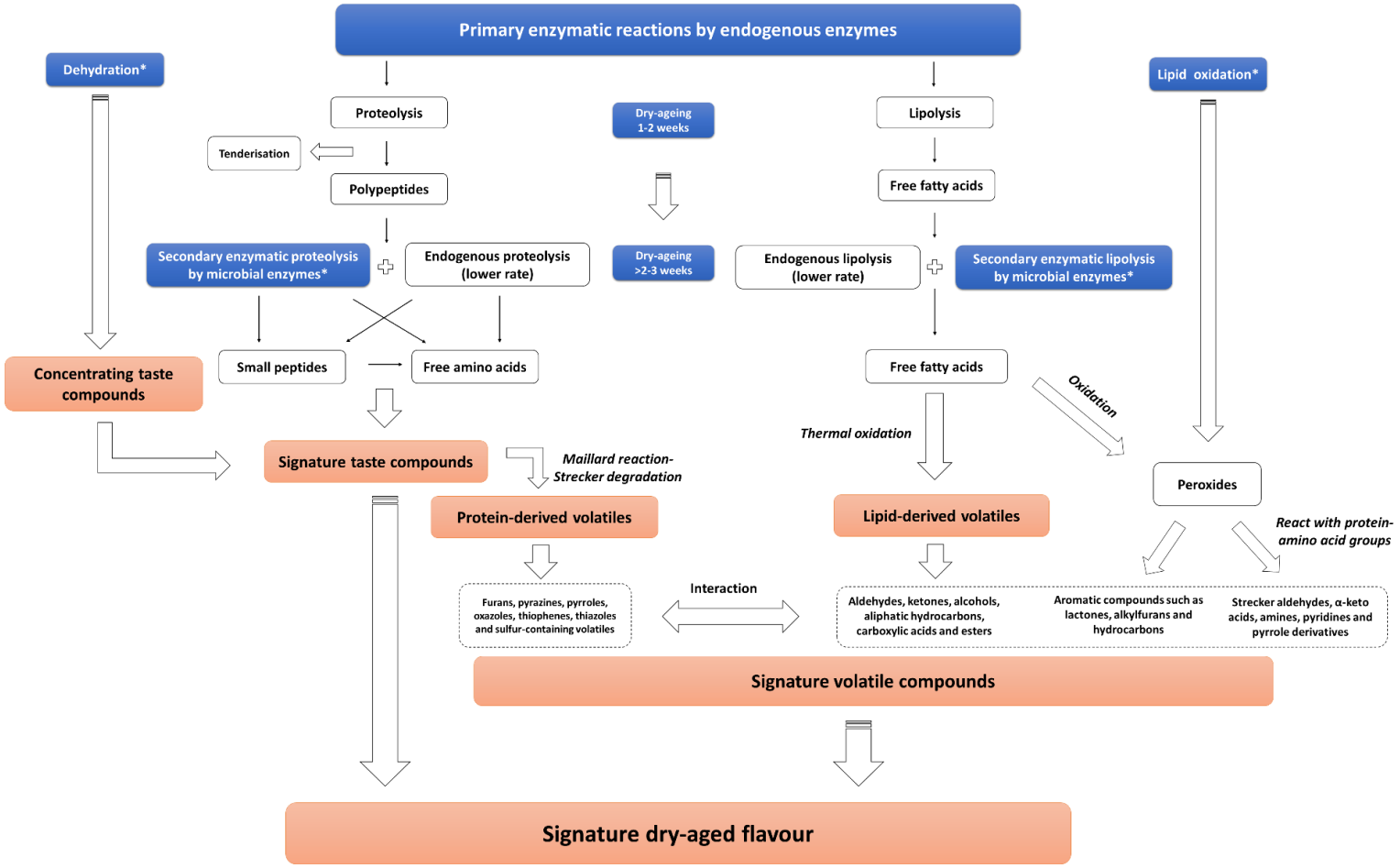


Figure 2.3 Proposed mechanism for the development of signature dry-aged flavour. * denotes factors contributing to the development of dry-aged flavour.

A distinctive dry-aged flavour can be tailored through manipulating the associated factors as discussed in section 4 (Figure 2.1), which would modify the profile of flavour precursors and therefore modify the final flavour. The success of processing design for dry-aged flavour needs to consider other attributes including overall eating quality, product yield, microbial safety and shelf life. For example, extensive dehydration may produce concentrated flavour compounds while resulting in a low product yield. The following are examples of how the three mechanisms can be practically used to tailor the flavours of dry-aged meat:

(1) Strategy for creating flavours through microbial activity (section 2.3.1)

- Select meat with intermediate to low level of marbling to allow rapid dehydration (section 2.4.1.2);
- Meat surface is inoculated with yeast strain (*D. hansenii*) and packaged in a water-permeable bag with higher water and oxygen permeability to dehydrate at intermediate to low air velocity (e.g. 1 to 2 $m.s^{-1}$) and low RH (e.g. 65% to 75%) for 2 to 3 weeks (section 2.3.1, 2.4.2 and 2.4.3);
- Repackage in vacuum bags without trimming and hold at low temperature (e.g. -1.5 °C) for 1 to 2 weeks to allow moisture to equilibrate, thereby the dehydrated surface from dry-ageing would disappear (section 2.4.4);
- Portion into retail-cuts.

This ageing strategy induces proteolytic activity by yeast which provide unique profile of flavour compounds and precursors consisting of small metabolites such as short-chain peptides, free fatty acid and free amino acids, etc. The use of inoculation of yeast accelerates the flavour development through microbial activity which normally requires a longer dry-ageing time (> 3 weeks) to effectively change the flavour profile of dry-aged products.

(2) Strategy for manipulating flavour profile mainly through oxidation mechanism (section 2.3.2):

- Select high marbled meat (high IMF with fat cap off) to generate the production of lipid-derived flavour (section 2.4.1.2);
- Wet-age (e.g. 2 °C) for 2 weeks to achieve maximum proteolytic changes by endogenous enzymes (section 2.4.3);
- Remove from vacuum bag and package in water-permeable bag with low water permeability and high oxygen permeability to commence dry-ageing

at low air speed (e.g. 0.1 $m.s^{-1}$) and intermediate humidity (e.g. 85%) for 2 weeks to encourage lipid oxidation (section 2.3.2 and 2.4.4);

- Repackage in vacuum bags without trimming and hold at low temperature for 1 to 2 weeks to allow moisture to equilibrate (section 2.4.4);
- Portion into retail-cuts.

Lipid oxidation and dehydration are two key mechanisms involved in the dry-ageing processing described above. We hypothesise the resultant flavour profile produced from this strategy is dominated by lipid and lipid oxidation derived volatiles (Figure 2.1b) and enriched with concentrated taste active compounds (e.g. peptides and free amino acids).

(3) Strategy for acceleration of dry-ageing process to produce shelf stable dry-aged products primarily through dehydration mechanism (section 2.3.3):

- Select lean meat (low IMF without subcutaneous fat) to reduce the oxidative potential (section 2.3.2 and 2.4.1.2);
- Vacuum package in water impermeable bag and wet age for 4 to 6 weeks (e.g. -1.5 °C) to allow proteolysis and some lipolysis to occur under controlled environment;
- Remove from impermeable bag and repackage in a water-permeable bag with high moisture permeability and low oxygen permeability to allow rapid moisture evaporation and reduced lipid oxidation (section 2.4.4);
- Dry-age (2 °C) at high air velocity (e.g. 5 $m.s^{-1}$) and lower RH (e.g. 55% - 60%) to accelerate moisture evaporation and concentrate desired flavour compounds (section 2.4.2);
- Dry-age for up to 7 days until the desired level of moisture loss is achieved (10% - 15%);
- Remove from water permeable bag without trimming and package in vacuum barrier bag to commence W (-1.5 °C) for 2 to 3 weeks to allow moisture to equilibrate (section 2.4.4);
- Portion into retail-cuts or frozen for export.

The dry-ageing process described above favours the moisture evaporation while improving oxidative stability and microbial safety. Such strategy will reduce the cost for operating dry-ageing facility, minimise product handling, and increase product yield. Due

to the use of lean meat, the flavour profile produced from this strategy will be dominated by protein-derived volatiles (Figure 2.1b)

2.6 Emerging areas and conclusions

Biochemical reactions arising from endogenous and exogenous pathways contribute to the flavour profile of dry-aged products, though no specific signatures for what is desirable has been defined. Developing a 'premium dry-aged' signature or signatures will allow the meat industry to design ageing process for producing dry-aged products with consistent quality and highest possible yield. The signature could be defined by some features, such as the level of dehydration (Lee et al., 2019a), and/or potential biomarkers such as formation of unique volatile compounds (King et al., 1995; O'Quinn et al., 2016) and metabolites such as peptides and free amino acids (Kim et al., 2016). Dry-ageing of beef has been suggested to produce more abundant free amino acids including tryptophan, phenylalanine, valine, tyrosine, glutamic acid, isoleucine, leucine and histidine, compared to those wet-aged for 3-4 weeks (Kim et al., 2016; Lee et al., 2019a). Based on the proposed mechanism, the distinct metabolites derived from oxidation and microorganism activity may be the suitable biomarkers, such as some unique peptides, amino acids, aldehydes and other fermentation-related compounds. On the other hand, incorporation of omics technologies, such as metabolomics, peptidomics, and lipidomics, could help to identify the biomarkers of dry-aged flavour and also detect non dry-aged meat sold as premium dry-aged meat and related fraud (Bendixen, 2005; D'Alessandro & Zolla, 2013; Ellis, Muhamadali, Allen, Elliott, & Goodacre, 2016; Picard et al., 2015; Ross et al., 2020).

Emerging non- or minimally-invasive technologies such as hyperspectral imaging system (Elmasry, Barbin, Sun, & Allen, 2012; Reis et al., 2018), near-infrared spectroscopy (NIR) (Prieto, Roehe, Lavín, Batten, & Andrés, 2009), Raman spectroscopy (Herrero, 2008; Yang & Ying, 2011) or rapid evaporative ionisation mass spectrometry (Ross et al., 2020) should be utilised when developing new regimes for dry-ageing, as they enable monitoring of the ageing process easily (e.g. extent of dehydration). Use of these advanced technologies will allow rapid minimally-invasive measurement of individual samples to prevent over-ageing or under-ageing from occurring.

The shelf life of dry-aged products under chilled or frozen storage is another area which will become relevant particularly when dry-aged meats are to be exported. For example, how long a dry-aged product can be stored fresh and frozen? What factors could be associated with limiting the storage, such as ageing time, storage conditions, packaging

formats and freezing methods? Some studies have investigated the storage stability of dry-aged beef in both fresh and frozen forms and suggested the possibility of frozen storage after dry-ageing process (Kim et al., 2017; Zhang et al., 2019). A strategy called, ‘aged-then-frozen’ has been developed to provide solution over the storage for premium aged-meat products, where improved stability during frozen storage has been observed. This strategy will facilitate the export of frozen premium meat to maintain comparable eating quality to the fresh-never-frozen meat (Farouk, Wiklund, Stuart, & Dobbie, 2009a, 2009b; Kim et al., 2018; Zhang et al., 2019). Yet, its application on the dry-aged products has not been explored. More research is warranted to determine the efficacy of these strategies and approaches in improving the development of dry-aged flavour.

Chapter 3: Quality and acceptability of fresh and long-term frozen in-bag dry-aged lean bull beef

This chapter determines the effects of processing regimes including air velocities of the ageing chamber, stepwise in-bag ageing, and ageing time on the quality and acceptability of fresh and frozen in-bag dry-aged lean beef. In-bag dry-aged lean beef was produced using a stepwise in-bag ageing process. Lean bull beef striploins (m. longissimus lumborum) were dry-aged at 2 °C, RH 75% under three different air velocities: 0.5, 1.5 and 2.5 m.s^{-1} for 7 days followed by wet-ageing for 14 days. The quality and acceptability of the dry-aged beef was compared with equivalent beef dry-aged for 21 days at 0.5 m.s^{-1} which served as a control. Two portions of the dry-aged beef (7/21 days) were randomly selected and held frozen at -18 °C for 12 months to study the quality changes following a long-term frozen storage.

Results showed that the shear force, drip and cook loss decreased significantly with dry-ageing time. Increased air velocities accelerated dehydration process with no negative impact on the meat quality, microbiological safety and consumer acceptability compared to the control. Frozen storage for 12 months had little or no effect on the quality and acceptability of the dry-aged lean beef. Thus, dry-aged lean beef of equivalent quality and palatability, with lower level of surface microorganisms and higher yield compared to the control, could be produced using stepwise ageing process.

This chapter is organised as follows: Section 3.1 provides an introduction which briefly overviews the background, motivations and objectives of the study presented in this chapter. Section 3.2 describes the meat samples, ageing process, and the experimental work performed in this study. Results and discussion for addressing the objectives are given in Section 3.3. Section 3.4 provides brief summary of this chapter.

3.1 Introduction

Post-mortem ageing of fresh beef for retail and foodservice is essential in meeting the high demands and expectations of discerning consumers seeking exceptional eating experience. Ageing improves tenderness (Campbell et al., 2001; Smith et al., 2008) and flavour (Feuz et al., 2004). Ageing of beef up to 14 days has been reported to increase the fatty and distinct aged-flavours (beefy, brothy, sweet and browned caramel) and these contribute positively towards the consumers' liking of premium beef cuts from the loin and rib (Sitz et al., 2006). The most widely used ageing practice is wet-ageing (W), which refers to ageing of a piece of meat in a moisture and air impermeable vacuum package bag under chilled storage conditions. Traditional dry-ageing (D) involves aging of the primal and sub-primal cuts without the use of any packaging or ageing bags to produce meat considered superior to wet-aged meat by purveyors due to the intense beefy and

roasted flavour from the process (Warren & Kastner, 1992). Dry-aged beef is typically described as having a buttery, rich, nutty, and/or earthy flavour profile (Savell, 2008).

Dry-ageing requires critical control of processing parameters including temperature, air velocity and relative humidity, to prevent excessive weight loss and growth of microorganism. A new method called “in-bag dry-ageing” (BD) has emerged over the last decade to address the concerns associated with D process (Lee et al., 2017; Li et al., 2014). The dry-ageing bags allow loss of moisture from the meat in a similar way to that of the D method. The dry-ageing bags act as an oxygen barrier to reduce oxygen exchange with the surrounding air, thereby limiting oxidation and its associated consequences, which include oxidative deterioration which produces rancid or off-flavour (DeGeer et al., 2009). The bags also act as protective barriers to prevent contamination from the surroundings and reduce the proliferation of spoilage microorganisms during the ageing process (Li et al., 2013), which in turn, reduces the need for excessive trimming. Excessive trimming cannot be avoided with the D method and often causes a loss to the meat purveyors. A novel dry-ageing strategy called “smart-ageing” proposed by Kim et al. (2018) has shown improvement in meat quality and value through modification of specific post-mortem ageing conditions. Stepwise ageing is one of the smart-ageing strategies which combines different ageing methods to produce dry-aged beef of equivalent quality compared with those using dry-ageing only (Kim et al., 2017).

Most dry-aged beef is produced from well-marbled premium beef cuts from prime steers or heifers with high intramuscular fat (IMF) and consumed locally rather than exported and fresh rather than thawed. Lean bull beef on the other hand is characterised as low value beef with fat content of 1-2%, reduced juiciness and tough texture. As a result, it is usually processed to sausages, patties and other further processed meat products and hardly used for premium products such as the dry-aged products. Recently, dry-aged *Longissimus thoracis et lumborum* from young bull (IMF around 2%) was produced and rated to be preferred over the wet-aged counterparts by the consumers (Stenström et al., 2014), suggesting the potential to produce dry-aged beef products from lean cuts. The use of lean beef may offer further advantages over the prime fatty beef for storage stability and reduced off-flavours associated with high fat content and the interaction of the latter with storage time and temperature.

Long-term storage of dry-aged beef may need to be considered if the meat industry was to produce dry-aged meat for commercial export as it currently does for chilled and frozen wet-aged meat. Freezing of meat at -18 °C during storage is a practice in the meat

industry, particularly for the export market. A processing strategy called “aged and then frozen” was of great interest over the last decade. This strategy refers to applying a certain period of W (2 to 4 weeks) prior to the frozen storage. It has been proven to improve the colour stablility, tenderness and water-holding capacity without negative impact on the meat quality (Coombs, Holman, Collins, Friend, & Hopkins, 2017a; Coombs et al., 2018b; Farouk et al., 2009a, 2009b; Kim, Liesse, Kemp, & Balan, 2015). Kim et al. (2017) reported on the improvements of water-holding capacity and shear force tenderness with no impact on colour and sensory quality of stepwise aged beef (D 10 days + W 7 days) followed by frozen storage for 6 months. However, whether dry-aged lean beef can be frozen without deterioration in quality or not and how long the frozen storage can be continued without deterioration in quality remain unknown. Answers to these questions are required if dry-aged meat was to be commercially traded globally and particularly to growing markets in countries like China where frozen rather than chilled meat imports is the norm.

This study aimed to investigate the effects and interactions of air velocities, ageing time and long-term frozen storage on the meat quality and acceptability of in-bag dry-aged striploins from lean bull beef using stepwise ageing regime. Current study was carried out to test the hypothesis that the combination of BD at higher air velocities for shorter ageing time followed by W in vacuum barrier bags would produce dry-aged meat of equivalent quality, long-term frozen stability and acceptability to BD meat produced using longer dry-ageing time and with no W involved.

3.2 Materials and Methods

3.2.1 Sample collection and dry-ageing procedure

A total of 15 pairs (n = 30) of beef striploin (*m. longissimus lumborum*) from bull (≈ 2 years old, boneless) beef carcasses were obtained on the slaughter day from a local meat processing plant. All the loins were held at 12 °C for 12 hrs until they entered rigor and then randomly assigned to four treatments (Figure 3.1): (T1) straight-dry-ageing: BD for 21 days (control, n = 6) at air velocity of 0.5 $m.s^{-1}$; (T2) - (T4) stepwise in-bag ageing: BD for 7 days + wet-aged for 14 days at air velocity of 0.5 $m.s^{-1}$ (T2); 1.5 $m.s^{-1}$ (T3) and 2.5 $m.s^{-1}$ (T4) (n = 8 for each stepwise ageing treatment). The paired loins were assigned to different treatments with 6 single loins of different carcasses for T1; the remaining 24 loins of 15 carcasses (6 single loins + 18 paired loins) were then randomly allocated to treatments T2-T4 with each treatment allocated 8 loins from different carcasses. All the loins were vacuum packaged in dry-ageing bags (TUBLIN® 10, 50 μm thick, polyamide

mix with water vapour transmission rate 2500 g/50 µ/m^2/24 h at 38 °C, 50% RH, TUB-EX ApS, Denmark) and laid out on wire racks inside a dry-ageing chamber set at 2 ± 0.5 °C and relative humidity of 75 ± 5%. Samples were weighed during the ageing process (0, 3, 5, 7, 13, 16, 18 and 21 days) and the weights were used to calculate the % weight loss: % Weight loss = [(Initial weight of sample before ageing - Weight at a given time point)/Initial weight before ageing] × 100.

A thin layer of dried and discoloured surface (including subcutaneous fat) was trimmed off from the striploins aged for 7 days and 21 days and then fabricated into 2 cm thick steaks. Minimum three steaks were taken from each loin of each treatment (T1 - T4) and at ageing time point (0, 7 and 21 days) for further analysis of fresh beef. No subsample was taken at 7 days of ageing time for the control (T1). Another three fresh steaks (minimum) were obtained from each loin of each treatment and ageing time point, as described above, vacuum packed immediately after ageing, and stored frozen at -18 °C for 12 months to determine the effect of long-term frozen storage on the quality of dry-aged lean beef.

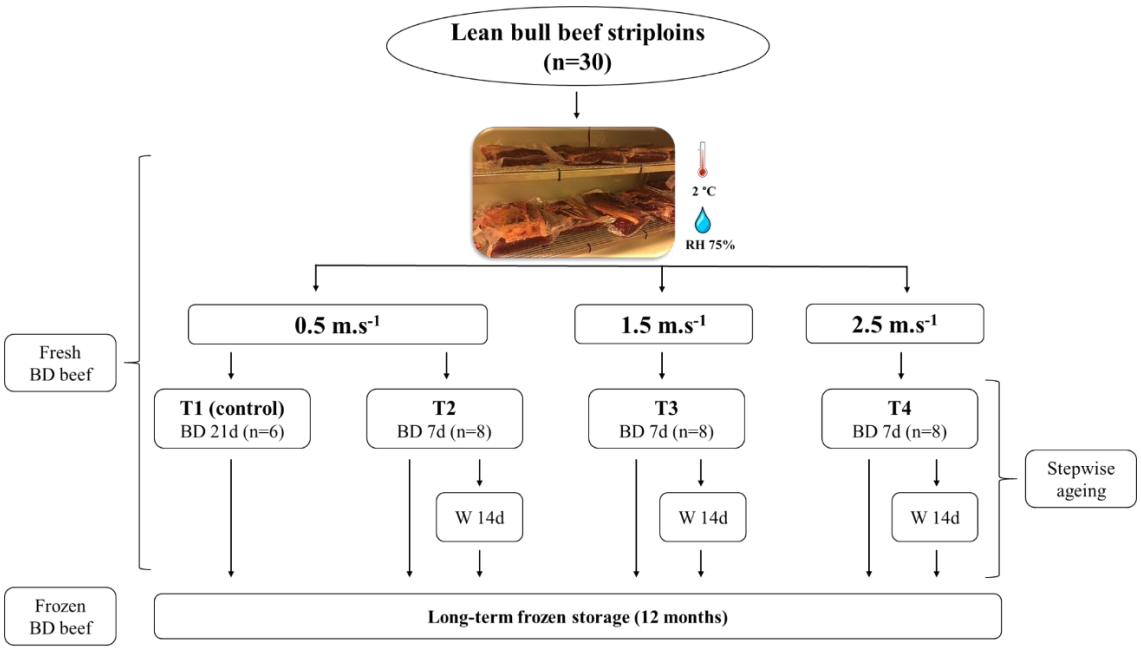


Figure 3.1 Schematic illustration of the ageing process and treatment combinations in the current study. BD: In-bag dry-ageing; W: Wet-ageing; T1: BD at 0.5 m.s^{-1} for 21 days; T2: BD at 0.5 m.s^{-1} 7 days + W for 14 days; T3: BD at 1.5 m.s^{-1} for 7 days + W for 14 days; T4: BD at 2.5 m.s^{-1} for 7 days + W for 14 days.

3.2.2 Surface microbial growth and water activity (A_w)

Microorganisms from the untrimmed surface of fresh (unfrozen) beef samples were enumerated before (0 day) and after the ageing process (21 days) for all four treatments

using standard methods in the Compendium of Methods of Microbiological Examination of Foods (Downes & Ito, 2001) for *Escherichia coli* (*E coli*, Chapter 8.91), aerobic bacteria (Chapter 7.62), lactic acid bacteria (Chapter 19.522), *Enterobacteriaceae* (Chapter 8.63), moulds and yeast (Chapter 20.51).

A_w of the fresh beef samples (one steak per loin) collected on 0 and 21 days of ageing from all four treatments was measured in duplicate at 20 °C using a water activity meter (Aqua lab CX-2, Decagon Devices, Inc., Washington, US). The water activity meter was calibrated using a saturated potassium sulphate solution and water at 20 °C.

3.2.3 pH and proximate content

The pH of fresh and frozen (thawed at 4 °C overnight) in-bag dry-aged beef samples (T1 - T4 at all ageing time points, one steak per loin) obtained from *Longissimus lumborum* was measured by inserting a calibrated pH probe (Hanna 99,163 pH meter with a FC232D combined temperature and pH insertion probe, Rhode Island, USA) directly into the beef samples. Triplicate measurements were taken and averaged.

Beef steaks from pH measurement were minced individually after trimmed off intramuscular fat and subsamples were collected for proximate analysis. Moisture content was measured using the oven drying method described in AOAC 950.46 (AOAC, 2010). Crude fat content was measured using Soxhlet extraction method of AOAC 960.39 (AOAC, 2010). The extraction of total muscle protein was as described by Lomiwes, Farouk, Wu, and Young (2014) using an extraction buffer consisting of 50 mM Tris-HCl (pH=5.8), 10% glycerol, 2% SDS and 2% β-mercaptoethanol. Protein content of total muscle extracts was determined using a RC-DC protein assay kit (Bio-Rad® Laboratories, Hercules, CA, USA) based on Lowry assay (Lowry, Rosebrough, Farr, & Randall, 1951). Muscle protein concentrations were determined from the standard curve prepared with bovine serum albumin of concentrations from 0 to 2.0 $mg.mL^{-1}$.

3.2.4 Instrumental colour

The steaks (one steak per loin) from fresh (0, 7 and 21 days) and frozen (7 and 21 days, thawed at 4 °C overnight) in-bag dry-aged beef loins (T1 - T4) were placed on a polypropylene foam tray lined with moisture absorbent pads, then overwrapped with polyvinyl chloride (PVC) film and allowed to bloom for 30~60 min under simulated retail display light at 4 °C. Surface colour was measured using a Minolta Chroma Meter (CR-400; Konica Minolta Photo Imaging Inc., Mahwah, NJ, USA) that had been calibrated using a standard white tile. CIE L* (lightness), a* (redness) and b* (yellowness) values

were measured (Illuminant D65, 8 mm diameter aperture, 10° standard observer) through the PVC film at three random locations on each steak.

3.2.5 Water-holding capacity

The water-holding capacity was evaluated in the form of % drip loss for the fresh beef (7 and 21 days of in-bag dry-aged), % thaw + drip loss for the frozen beef (7 and 21 days of in-bag dry-aged) and % cook loss for both the fresh and frozen samples (one steak per loin).

3.2.5.1 Drip loss

The bag drip method by Honikel (1987) with some minor changes proposed by Kim et al. (2016) was used on the collected samples from 7 days and 21 days of ageing time for both the fresh and frozen in-bag dry-aged beef samples. The % drip loss was calculated as: % Drip loss = [(Initial weight – weight after hanging for 48 hrs)/Initial weight] × 100. The drip loss of frozen storage samples was measured from the frozen state. Therefore, the drip loss was expressed as the total loss from thawing and suspension in drip bags which was calculated as % thaw + drip loss = [(Initial weight at frozen – weight after suspension for 48 hrs)/Initial weight at frozen] × 100.

3.2.5.2 Cook loss

Fresh beef portions (one portion per loin, 6 cm thickness) with weight of approximately 400 g from 7 days and 21 days of ageing were cooked in a boiling water bath (99 °C) to the internal temperature of 70 °C. Frozen steaks (one steak per loin, thaw at 4 °C overnight) of 2 cm thickness from 7 days and 21 days of ageing time were cooked *sous vide* at 70 °C for 1 hr. Immediately after cooking, the cooked samples were transferred into an ice bath for 30 min to prevent further cooking, blotted dry and weighed. The % cook loss was calculated as: % Cook loss = [(Initial weight – cooked weight)/initial weight] × 100.

3.2.6 Instrumental texture

Cooked steaks from the above (section 3.2.5.2) were further analysed for instrumental texture. Tenderness of cooked fresh beef were measured with a MIRINZ tenderometer (Chrystall & Devine, 1991). A 10 mm × 10 mm cross section was cut from each cooked steak to measure the force required to shear through the sample at a right angle to the fibre axis. The results were expressed as shear force (N) from the average shear force values of ten replicates for each of the cooked sample. A tender meat is

defined as the meat of shear force value ≤ 88 N (9 kgF) measured by MIRINZ tenderometer (Chrystall & Devine, 1991).

The texture profile of long-term frozen in-bag dry-aged lean beef was analysed using Texture Profile Analysis (TPA) according the procedure described by Zhang, Yoo, Gathercole, Reis, and Farouk (2018). Briefly, a cube (1 cm^3) was measured using Stable Micro System TA.HD Plus texture analyser (Surry, UK) with a 50 mm cylinder probe at 50% strain setting using test speed of 5.0 $mm.s^{-1}$. Maximum load force of 50 kg was used with trigger force of 5 g at auto mode. A minimum of ten replicates from each steak were measured and averaged.

3.2.7 Consumer sensory testing

3.2.7.1 Cooking and sample preparation

Fresh in-bag dry-aged steaks (21 days, minimum three steaks per loin) were cooked in a conventional oven at 170 °C until the core temperature reached 70 °C. Frozen in-bag dry-aged steaks (21 days, minimum three steaks per loin) were thawed at 4 °C chiller overnight and pre-cooked *sous vide* at 70 °C for 1 hr and reheated on a grill set at 230 °C for 90 s on each side. Once cooked, each steak was cut across the grain into a 1.3 × 1.3 × 2.0 cm piece and randomly assigned to a plastic cup. All the cups were pre-labelled with unique codes made of panellist number (1, 2, 3, etc.) × sample ID (A, B, C and D). Each panellist was asked to taste one sample each time in the order from A to D. Each sample ID (A - D) of the same panellist corresponded to one of the ageing treatments (T1 - T4). The panellists may have tasted the same sample from the same steak more than once due to the randomised design model. Water and water crackers were provided as palate cleansers. Consumers were asked to take a bite of cracker, rinsed their mouths and rest for 30 s between the samples. Consumers were informed that swallowing was allowed but not compulsory.

3.2.7.2 Sensory evaluation of fresh and long-term frozen beef

(1) Fresh in-bag dry-aged beef

The aim of the first sensory session was to determine the effect of stepwise ageing and ageing chamber air velocity on the palatability of fresh in-bag dry-aged lean bull beef. A total of 44 untrained panellists (20 to 65 years old) who are frequent meat consumers and familiar with sensory evaluation of various meat products participated in the study. Each consumer was provided with a computer to accomplish the webpage-based questionnaire. Consumer panels were asked to evaluate the acceptance and liking of the

steak samples in terms of aroma, tenderness, juiciness, flavour and overall liking on a scale of 1 - 100, where 1 represented ‘extremely dislike’, and 100 represented ‘extremely like’. Consumers were also asked to rate the degree of off-flavours where 1 represented ‘not detected’ and 100 represented ‘detected very strongly’. After tasting every two samples (A and B, or C and D), panellists were also asked to rank for a preferred sample.

(2) Frozen in-bag dry-aged beef

The aim of the second sensory session was to determine the acceptability of long-term frozen stored (for 12 months) of in-bag dry-aged lean bull beef. A total of 72 panellists (20 to 65 years old), consisting of 40% female and 60% male, participated in this study. Each panellist evaluated four samples from four different ageing treatments in a random order. Panellists were asked to rate their acceptance on a 9-point hedonic scale using a printed questionnaire where 1 represented ‘extremely dislike’ and 9 represented ‘extremely like’. Detection of off-flavour was evaluated on a 5-point hedonic scale where 1 represented ‘not detected’ and 5 represented ‘detected very strongly’.

3.2.8 Statistical analysis

An incomplete randomised block design was used in this study with 30 striploins from 15 beef carcasses (n = 30) which were unevenly assigned to four different treatment combinations (1 control dry-ageing; T1; n = 6 and 3 stepwise ageing regimes; T2 - T4; n = 8 for each). Linear mixed effect regression analyses were performed on the data using R (version 3.4.1), with “lme4” package to determine the difference between four treatment combinations across the ageing time. The ageing treatments (T1 - T4) and ageing time (0, 7 and 21 days) were included as fixed effects, where the sample ID (loin number) was included as a random effect to account for the uneven number of samples between the control and other treatments. One-way analysis of variance (ANOVA) was performed to determine the effect of air velocity, stepwise ageing and ageing time on the fresh and frozen in-bag dry-aged beef. The effect of air velocity was determined by the comparison between T2, T3 and T4 at 7 days of ageing time. The effect of stepwise ageing was determined by the comparison between T1 and T2 at 21 days of ageing time. The interaction of stepwise ageing and air velocity was determined by the comparison between all four treatments at the 21 days of ageing time. The effect of ageing time on each treatment was analysed separately by the comparing across the ageing time from 0 to 21 days. The effect of frozen storage on the proximate content (0, 7 and 21 days), pH (7 and 21 days) and instrumental colour (7 and 21 days) of in-bag dry-aged beef was analysed by comparing fresh and frozen beef samples aged for the same ageing time

individually. Post-hoc comparison of means was performed using Fisher's least significant differences (LSD) and Tukey's (HSD) test at 5% significance level.

3.3 Results and Discussions

3.3.1 Effect of dry-ageing chamber air velocity on the physicochemical properties of fresh and long-term frozen in-bag dry-aged beef

3.3.1.1 Fresh in-bag dry-aged beef

Weight loss of lean beef during BD increased ($P < 0.05$) with the increase in air velocity over the first 7 days of ageing time as shown in Figure 3.2. The highest weight loss was associated with the highest air velocity of 2.5 m.s^{-1} (T4, 11.19%) compared with those of medium (T3, 10.57%) and low velocity (T2, 9.37%). Significantly higher weight loss with increased velocity from 0.2 to 0.5 m.s^{-1} after 21 days of ageing time has been reported for dry-aged prime steer beef loins (Kim et al., 2016).

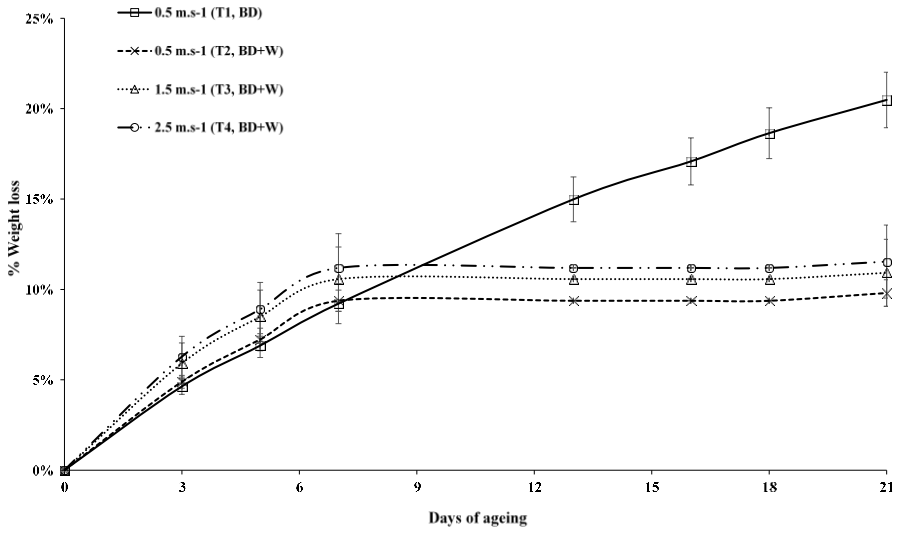


Figure 3.2 Average % weight loss of lean beef striploins of four different ageing treatments across different ageing time (days). BD: In-bag dry-ageing; W: Wet ageing. T1: BD at 0.5 m.s^{-1} for 21 days; T2: BD at 0.5 m.s^{-1} 7 days + W for 14 days; T3: BD at 1.5 m.s^{-1} for 7 days + W for 14 days; T4: BD at 2.5 m.s^{-1} for 7 days + W for 14 days.

A lower ($P < 0.05$) moisture content was found in beef that was in-bag dry-aged at medium velocity of 1.5 m.s^{-1} (T3, Table 3.1). However, the % moisture differences were less than 1% between different air velocities. Therefore, the effect of ageing chamber air velocity on the moisture content of in-bag dry-aged beef was minimum. This could be explained by the moisture loss during dry-ageing being mainly on the surface and the outer section of the beef at higher velocities forming "crust-like" dry skin faster compared to lower velocities, which might have reduced moisture loss. Hence, the higher lost at

medium velocity compared to lower or higher velocities. The difference ($P < 0.05$) detected in crude fat content was likely to be caused by the variations of moisture content.

Meat colour is widely used by consumers to determine the freshness of the meat products (Miller, Kerry, & Ledward, 2002). Colour is closely associated with pH values of the meat. As shown in Table 3.2 and Table 3.3, increase of air velocity had no impact on the pH and instrumental colour of in-bag dry-aged beef except for the lightness (L*). L* decreased ($P < 0.05$) with the increase of velocity to 2.5 $m.s^{-1}$. Similar findings have been reported with the increase in air velocity from 0.2 to 0.5 $m.s^{-1}$ in dry-aged beef (Kim et al., 2016). The reduced lightness could be attributed to the moisture loss on the surface of the meat, resulting in more light absorption and darker colour (Kim & Hunt, 2011).

Water-holding capacity (Table 3.4) and shear force tenderness (Table 3.5) of fresh in-bag dry-aged samples were not influenced by the increase of air velocity ($P > 0.05$) which agreed with the findings reported by Kim et al. (2016) on dry-aged beef.

3.3.1.2 Frozen in-bag dry-aged beef

There was no interaction ($P > 0.05$) between air velocity and frozen storage on the quality and physicochemical properties of in-bag dry-aged lean bull beef. Air velocity had no effect on the proximate content, pH, water-holding capacity, instrumental colour and instrumental texture of in-bag dry-aged beef (7 days of ageing, T2 - T4) stored frozen at -18 °C for 12 months. This suggests that the use of higher air velocity in the dry-ageing of lean bull beef would not negatively influence the meat quality of in-bag dry-aged lean beef frozen for 12 months.

Table 3.1 Effect of ageing treatments, ageing time and frozen storage on proximate content of in-bag dry-aged lean bull beef.

Attributes/storage type	Ageing time	Treatments				SED	*P*-AV	*P*-SA	*P*-SA × AV	*P*-storage (unaged)	*P*-storage (7 d ageing)	*P*-storage (21 d ageing)
		T1	T2	T3	T4							
% Moisture												
Fresh	0d	76.22x	76.29x	75.84x	75.81x	0.43	0.029	0.532	0.450	0.561	0.727	0.810
	7d		75.83ay	74.77by	75.84ay							
	21d	74.93y	75.19z	74.67z	74.33z							
	***P*-ageing time**	0.001	***	***	***							
Frozen	0d	76.27	76.37	76.55	75.69	0.82	0.002	0.824	0.494			
	7d		76.22a	74.83b	75.69a							
	21d	74.93	75.09	74.57	74.08							
	***P*-ageing time**	0.241	0.145	0.068	0.075							
% Crude fat												
Fresh	0d	0.69	0.64	1.10	1.17	0.26	0.018	0.796	0.163	0.201	0.370	0.832
	7d		0.63a	1.21b	0.62a							
	21d	0.66	0.69	1.05	1.25							
	***P*-ageing time**	0.799	0.654	0.298	0.146							
Frozen	0d	1.09	0.99	0.88	2.00	0.33	0.056	0.394	0.222			
	7d		0.73a	1.28b	0.85a							
	21d	0.68	0.78	1.13	1.08							
	***P*-ageing time**	0.232	0.541	0.600	0.116							
% Muscle protein												
Fresh	0d	22.31x	22.17x	22.26x	22.26x	0.29	0.948	0.709	0.236	***	***	***
	7d		22.65y	23.21y	22.77y							
	21d	23.48y	23.33z	23.70z	23.77z							
	***P*-ageing time**	0.003	<0.001	***	***							
Frozen	0d	19.01x	18.25x	18.65	20.34	1.04	0.198	0.874	0.432			
	7d		19.78xy	21.37	20.12							
	21d	21.35y	21.23y	21.11	22.11							
	***P*-ageing time**	0.043	0.044	0.125	0.058							

BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 $m.s^{-1}$ for 21 days; T2: BD at 0.5 $m.s^{-1}$ 7 days + W for 14 days; T3: BD at 1.5 $m.s^{-1}$ for 7 days + W for 14 days; T4: BD at 2.5 $m.s^{-1}$ for 7 days + W for 14 days. $P < 0.0001$ presented as ∗∗∗ for level of significance. Different letters of "x, y or z" within the same column mean results are significantly different from each other ($P < 0.05$). Different letters of "a, b or c" within the same row mean results are significantly different from each other ($P < 0.05$).

Table 3.2 Effect of ageing treatments, ageing time and frozen storage on pH of in-bag dry-aged lean bull beef.

Attributes/storage type	Ageing time	Treatments				SED	*P*-AV	*P*-SA	*P*-SA × AV	*P*-storage (7 d ageing)	*P*-storage (21 d ageing)
		T1	T2	T3	T4						
pH											
Fresh	0d	5.34x	5.34x	5.36x	5.32x	0.04	0.082	0.232	0.035	0.226	0.654
	7d		5.74y	5.60y	5.69y						
	21d	5.66ay	5.62abz	5.64ay	5.58bz						
	***P*-ageing time**	***	***	***	***						
Frozen	7d		5.66	5.65	5.62	0.03	0.420	0.644	0.525		
	21d	5.63	5.62	5.61	5.6						
	***P*-ageing time**		0.068	0.299	0.588						

BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 $m.s^{-1}$ for 21 days; T2: BD at 0.5 $m.s^{-1}$ 7 days + W for 14 days; T3: BD at 1.5 $m.s^{-1}$ for 7 days + W for 14 days; T4: BD at 2.5 $m.s^{-1}$ for 7 days + W for 14 days. P <0.0001 presented as ∗∗∗ for level of significance. Different letters of "x, y or z" within the same column mean results are significantly different from each other ($P < 0.05$). Different letters of "a, b or c" within the same row mean results are significantly different from each other ($P < 0.05$).

Table 3.3 Effect of ageing treatments, ageing time and frozen storage on instrumental colour of in-bag dry-aged lean bull beef.

Attributes/storage type	Ageing time	Treatments				SED	*P*-AV	*P*-SA	*P*-SA × AV	*P*-storage (7 d ageing)	*P*-storage (21 d ageing)
		T1	T2	T3	T4						
*L**											
Fresh	0d	33.51x	33.34x	33.05x	32.65x	0.62	0.022	0.588	0.411	***	***
	7d		40.78ay	41.28ay	38.54by						
	21d	39.80y	39.46z	39.46z	38.85y						
	***P*-ageing time**	<0.001	***	***	***						
Frozen	7d		35.99	36.52	35.44	0.97	0.492	0.712	0.926		
	21d	36.22	35.86	36.08	36.48						
	***P*-ageing time**		0.897	0.678	0.232						
*a**											
Fresh	0d	18.05	18.21x	17.44x	17.47x	0.90	0.797	0.927	0.084	0.004	***
	7d		15.25y	15.29y	14.62y						
	21d	18.45a	18.55ax	17.06abx	16.38bz						
	***P*-ageing time**	0.232	<0.001	0.004	0.001						
Frozen	7d		13.37	11.79	13.82	0.81	0.075	0.312	0.742		
	21d	13.77	13.02	13.34	13.04						
	***P*-ageing time**		0.686	0.303	0.157						
*b**											
Fresh	0d	11.84x	11.74x	11.44	11.31x	0.49	0.059	0.53	0.018	***	***
	7d		10.81y	11.39	10.23y						
	21d	12.93ay	12.69az	11.98ab	11.44bx						
	***P*-ageing time**	0.005	0.001	0.298	0.058						
Frozen	7d		6.56	5.53	6.87	0.58	0.116	0.53	0.471		
	21d	7.43	6.93	7.17	6.55						
	***P*-ageing time**		0.547	0.154	0.355						

Table 3.3 (continued)

Attributes/storage type	Ageing time	Treatments				SED	*P*-AV	*P*-SA	*P*-SA × AV	*P*-storage (7 d ageing)	*P*-storage (21 d ageing)
		T1	T2	T3	T4						
Chroma											
Fresh	0d	21.59x	21.68x	20.87x	20.82x	0.99	0.585	0.347	0.047	***	***
	7d		18.70y	19.08y	17.84y						
	21d	22.54ay	22.48ax	20.86abx	19.98bx						
	***P*-ageing time**	0.034	<0.001	0.048	0.005						
Frozen	7d		14.9	13.03	15.44	0.94	0.078	0.994	0.653		
	21d	15.66	14.00	15.15	14.6						
	***P*-ageing time**		0.888	0.236	0.169						
Hue											
Fresh	0d	33.36x	32.85x	33.34x	32.93x	0.75	0.267	0.885	0.775	***	***
	7d		35.42y	36.79y	35.01y						
	21d	35.25y	34.42z	35.14y	34.95y						
	***P*-ageing time**	0.01	<0.001	0.001	***						
Frozen	7d		26.04	24.89	26.38	1.22	0.401	0.442	0.544		
	21d	28.34	27.92	28.2	26.67						
	***P*-ageing time**		0.061	0.158	0.755						

BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 $m.s^{-1}$ for 21 days; T2: BD at 0.5 $m.s^{-1}$ 7 days + W for 14 days; T3: BD at 1.5 $m.s^{-1}$ for 7 days + W for 14 days; T4: BD at 2.5 $m.s^{-1}$ for 7 days + W for 14 days. $P < 0.0001$ presented as ∗∗∗ for level of significance. Different letters of "x, y or z" within the same column mean results are significantly different from each other ($P < 0.05$). Different letters of "a, b or c" within the same row mean results are significantly different from each other ($P < 0.05$).

Table 3.4 Effect of ageing treatments, ageing time and frozen storage on water holding capacity of in-bag dry-aged lean bull beef.

Attributes/storage type	Ageing time	Treatments				SED	*P*-AV	*P*-SA	*P*-SA × AV
		T1	T2	T3	T4				
% Drip loss									
Fresh	7d		3.86x	3.35x	3.26x	0.29	0.226	0.152	0.257
	21d	1.35	1.56y	1.58y	1.92y				
	***P*-ageing time**		<0.001	0.001	0.001				
% Thaw + drip loss									
Frozen	7d		12.38x	10.36x	11.75x	1.41	0.335	0.322	0.443
	21d	5.79	7.49y	5.86y	7.33y				
	***P*-ageing time**		0.009	0.013	0.003				
% Cook loss									
Fresh	7d		30.22	29.46	32.18x	1.69	0.519	0.979	0.204
	21d	27.77	27.74	25.98	28.69y				
	***P*-ageing time**		0.240	0.120	0.020				
Frozen	7d		31.81	31.81	33.04x	1.04	0.174	0.498	0.644
	21d	29.60	30.29	30.59	29.40y				
	***P*-ageing time**		0.103	0.371	0.003				

BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 $m.s^{-1}$ for 21 days; T2: BD at 0.5 $m.s^{-1}$ 7 days + W for 14 days; T3: BD at 1.5 $m.s^{-1}$ for 7 days + W for 14 days; T4: BD at 2.5 $m.s^{-1}$ for 7 days + W for 14 days. Different letters of "x, y or z" within the same column mean results are significantly different from each other ($P < 0.05$).

3.3.2 Effect of stepwise ageing on the physicochemical properties and acceptability of fresh and long-term frozen in-bag dry-aged beef

3.3.2.1 Effect of stepwise ageing on the physicochemical properties

Stepwise ageing involving the in-bag dry-aging of lean bull beef for 7 days followed by 14 days of W (T2) reduced ($P < 0.0001$) % weight loss of the dry-aged meat compared to the control (T1, straight-dry-ageing for 21 days). In this study, stepwise ageing had no effect on the proximate content (Table 3.1), pH (Table 3.2), and instrumental colour (Table 3.3) of fresh in-bag dry-aged beef ($P > 0.05$) as was previously reported (Ahnström et al., 2006; O'Quinn et al., 2016; Oreskovich et al., 1988; Parrish et al., 1991). The water-holding capacity (measured as drip and cook losses) of fresh in-bag dry-aged lean beef (21 days, T1 and T2) were not affected by the stepwise ageing regimes as shown in Table 3.4. Conflicting findings were reported by Kim et al. (2017) that stepwise ageing (D 10 days + W 7 days) significantly decreased the drip loss but did not differ in cook loss as compared to dry-aged only. They attributed the higher drip loss from the dry-aged only beef loins to the possible higher protein oxidation in the samples which resulted in the decrease of water-holding capacity. The lack of difference in cook loss between ageing regimes observed in the current study was in line with the outcomes of most of the previous studies on dry-aged beef (Dikeman et al., 2013; Gudjónsdóttir et al., 2015; Kim et al., 2016; Stenström et al., 2014).

The instrumental tenderness (shear force) of dry-aged lean beef produced using stepwise ageing did not differ with its control counterpart produced by straight-dry-ageing of lean beef for 21 days (Table 3.5). This result contradicts the findings reported by Kim et al. (2017) that stepwise ageing produced lower ($P < 0.05$) shear force compared with D. The difference between our finding and that of Kim et al. (2017) could be the type of beef used in the studies particularly the difference in the fat content of our lean bull beef and the beef (USDA low choice) used in Kim et al. (2017).

There was no effect of stepwise ageing on the measured quality parameters of frozen in-bag dry-aged lean beef (21 days, T1 and T2). Therefore, the use of stepwise ageing produced in-bag dry-aged lean beef of equivalent quality to those of straight-dry-ageing without adverse effect, even after a long-term frozen storage.

3.3.2.2 Interaction between air velocity and stepwise ageing

A higher ($P < 0.05$) average % weight loss (20.48%) was found in the control (straight-dry-ageing, T1) at low velocity of 0.5 $m.s^{-1}$ compared to the other treatments

(T2 = 9.80%, T3 = 10.92% and T4 = 11.54%) as shown in Figure 3.2. There was no weight loss ($P > 0.05$) arising from the extended W process for T2, T3 and T4 regardless of the velocity as expected. The pH, b* and Chroma of stepwise ageing were similar to those of dry-ageing only beef samples (Table 3.2 and 3.3). Although the values slightly ($P < 0.05$) decreased at the high velocity (T4), the difference was negligible and should not impact meat quality. The other colour attributes, proximate content and water-holding capacity of fresh in-bag dry-aged beef (21 days, T1 - T4) did not differ between the treatment combinations of air velocity and stepwise ageing ($P > 0.05$). As discussed above, air velocity and stepwise ageing process mainly influenced the water fraction on the surface of the samples.

A higher ($P < 0.05$) amount of yeast was detected in the control (straight-dry-ageing) compared to those from stepwise ageing, although the growth of surface microorganisms in all samples was low (Table 3.6). The results are supported by the outcomes of other studies that reported higher yeast counts in the beef samples of BD as compared to the wet-aged counterparts (Gudjónsdóttir et al., 2015; Li et al., 2014; Li et al., 2013). The lactic acid bacteria, *Enterobacteriaceae, E. coli* and moulds did not differ between the treatment combinations of air velocity and stepwise ageing because the proliferation of these microorganisms was low and below the detection limit (Table 3.6). The use of different ageing regimes (D/W) had no impact on growth of *Enterobacteriaceae* and moulds (Li et al., 2014; Li et al., 2013) and *E. coli* (DeGeer et al., 2009). Some studies have suggested that W may contribute to a higher amount of lactic acid bacteria and lower aerobic bacteria count (Berger et al., 2018; DeGeer et al., 2009; Gudjónsdóttir et al., 2015; Li et al., 2014; Li et al., 2013) post ageing compared with (in-bag) dry-ageing due to the anaerobic ageing condition favouring the proliferation of lactic acid bacteria and supressing that of aerobic bacteria. The aerobic bacteria count tended to decrease with the air velocity in stepwise aged beef samples (T2 - T4, Table 3.6). Current findings of lactic acid bacteria and aerobic bacteria could be explained by the faster dehydration on meat surface at higher air velocities playing a major role in creating an adverse environment for the growth of both bacteria.

The quality parameters of a long-term frozen stored in-bag dry-aged lean beef (21 days, T1 - T4) were not affected by the treatment combinations of air velocity and stepwise ageing. The differences in colour (a*, b* and Chroma) observed in the fresh in-bag dry-aged beef due to ageing treatments disappeared after long-term frozen storage

(Table 3.3). This could be due to the biochemical changes of muscle cells and myoglobin during the frozen storage.

3.3.2.3 Sensory quality of fresh and long-term frozen in-bag dry-aged beef

(1) Acceptability of fresh in-bag dry-aged beef

The consumer panel could not detect any difference ($P > 0.05$) between the samples produced using the four ageing treatment combinations in terms of aroma, texture, tenderness, juiciness, flavour and overall liking (Table 3.7). The lack of differences in the consumer acceptability of the tenderness and texture of the in-bag dry-aged samples (21 days) from the four ageing treatments agreed with the instrumental shear force measurement of tenderness (Table 3.5). Kim et al. (2017) also found no difference in the sensory acceptability of dry-aged beef loins when comparing the stepwise ageing regimes with those traditional dry-aged only.

Debate on the consumer preference and acceptability of dry-aged beef over the equivalent wet-aged beef is ongoing. Some of the studies found no significant difference in the consumer acceptability of the tenderness and juiciness of dry-aged as compared to the wet-aged beef (Dikeman et al., 2013; Kim et al., 2016; Lepper-Blilie et al., 2016; Stenström et al., 2014; Velotto et al., 2015); others reported higher preference of (in-bag) dry-aged beef compared to the wet-aged in terms of tenderness and juiciness (Campbell et al., 2001; Li et al., 2014; Li et al., 2013; Richardson et al., 2008; Stenström et al., 2014); while others suggested the tenderness of wet-aged beef was more acceptable/preferred than the dry-aged counterparts (Obuz et al., 2014; Parrish et al., 1991; Sitz et al., 2006). The differences arising from ageing time, ageing conditions and muscle types across the studies of dry-ageing may contribute to the inconsistent findings in consumer acceptability.

For the overall liking and the flavour liking, findings from previous studies were also controversial. Thus, the conclusion over the most effective ageing method to maximise palatability cannot be easily drawn. In current study, consumers gave similar ratings of overall liking to all four samples, however, different findings were observed when they were asked to express their preference between samples. As shown in Table 3.7, about 94% of the panellists were able to discriminate the difference between four treatment combinations. The control (straight-dry-ageing for 21 days) was the most preferred by the consumers (28.41%) in the current study compared to other ageing treatments which were equally preferred (20-22%).

(2) Acceptability of frozen dry-aged beef

For the first time, we report the sensory quality of dry-aged beef that were frozen for 12 months. Despite the negative impression over the frozen storage of beef, the sensory quality of the in-bag dry-aged beef (21 days) of all the ageing treatments, including aroma, tenderness, flavour and overall liking, was rated to be higher than that of the fresh (never frozen) in-bag dry-aged counterparts (Table 3.7). No difference ($P > 0.05$) was found in tenderness, between the four ageing treatments, which is in agreement with the instrumental texture profile analysis of the corresponding frozen in-bag dry-aged samples. Panellists failed to differentiate the control straight dry-aged beef (T1) and stepwise aged samples except for those aged at low ageing chamber air velocity (T2), and this was the least preferred by the consumers at a significant level ($P = 0.006$). This may have been caused by lower rating of juiciness ($P < 0.05$), which is further supported by higher % thaw + drip loss and % cook loss compared to the other ageing treatments (Table 3.4).

Rancidity, noted as off-flavour, is another important indicator for the consumers to determine the freshness of cooked meat. Low mean values of off-flavour were found in the cooked steaks of all the treatments suggesting the difficulty in recognising the rancid flavour from the frozen in-bag dry-aged (21 days) lean beef samples. The rancidity note generated from the meat is mainly caused by the oxidation and hydrolysis of the fat in meat. The meat samples containing higher fat (such as prime cuts and wagyu meat) were more susceptible to oxidation and consequently give off rancid flavour. It is worth noting that the beef samples used in this study were lean bull beef, which only contained approximately 1% of IMF (Table 3.1). Therefore, the low level of rancidity in the beef samples post long-term frozen storage was expected. Kim et al. (2017) recently reported no effect of short-term (1 month) freezing on the sensory quality of beef loins (USDA low choice) dry-aged by stepwise ageing process. However, how the sensory quality would change if the storage time was extended (e.g. ≥ 6 - 12 months) and how the long-term frozen storage would affect the sensory quality of dry-aged beef with different IMF contents have not been explored. Answers to these questions would be of great significance for the export of frozen dry-aged beef.

3.3.3 Effect of dry-ageing time on the physicochemical properties of fresh and long-term frozen in-bag dry-aged beef

3.3.3.1 Fresh in-bag dry-aged beef

The length of dry-ageing time affects the % weight loss ($P < 0.05$) observed in the present study as expected (Figure 3.2). There was a significant decrease in moisture content with the increased ageing time (Table 3.1). This finding is consistent with the outcomes of some previous studies (Ahnström et al., 2006; Iida et al., 2016), and contradicted others (DeGeer et al., 2009). The variation in the outcomes reported in the literature may have arisen from the different sampling methods used in the studies, though often these were not clearly stated in the studies. The slight increase in the protein content ($P < 0.05$) with ageing time observed in the current study (Table 3.1) was more likely to be due to the decrease in moisture content ($P < 0.05$) in the in-bag dry-aged samples (T1 - T4) over the ageing time.

Overall, the pH of in-bag dry-aged striploins increased ($P < 0.05$) after 21 days of ageing (Table 3.2) as compared to unaged counterparts which was in agreement with other studies on dry-aged beef (Ahnström et al., 2006; Kim et al., 2016; Obuz et al., 2014). Within the first 7 days of BD, the pH increased from an average of 5.34 to 5.74 (T2), 5.60 (T3) and 5.69 (T4). Although a slight decrease ($P < 0.05$) of pH values during the extended 14 days of W was detected in T2 and T4 samples, the actual difference was minimal (0.1 units). The increase of pH values post dry-ageing could be associated with the generation of nitrogenous compounds caused by proteolysis as suggested by Aksu, Kaya, and Ockerman (2005).

After BD for 21 days (T1, Table 3.3), all the colour parameters (L*, b*, Chroma and hue) measured in this study increased ($P < 0.05$) except for the a* which did not change. However, a* has been reported in other studies to increase with ageing time on dry-aged beef using ageing bags (Li et al., 2014) and traditional (no-bag) dry-ageing (Gudjónsdóttir et al., 2015). The stepwise ageing process affected the colour properties differently from the control across the ageing time. The first 7 days of BD process increased ($P < 0.05$) the L* and hue angle, decreased the a* and Chroma compared with unaged (0 day) counterparts (T2 - T4, Table 3.3). The extended W from 7 days to 21 days of ageing slightly decreased the L* (T2 and T3) and hue (T2) but increased ($P < 0.05$) the a* and Chroma (T2 - T4) to similar levels as unaged counterparts. The inconsistent changes of a* and Chroma over the stepwise ageing process is unclear. It could be associated with the difference of metmyoglobin reducing ability and stability of myoglobin at different

ageing time. The increase of L* has been linked with the myofibrillar protein denaturation which consequently gave rise to a tighter and more opaque structure (Hector, Brew-Graves, Hassen, & Ledward, 1992). The lack of change in L*, a* and b* values over the dry-ageing process was also reported in previous studies (Li et al., 2014). The stepwise ageing has been reported to have no negative effect on the instrumental colour quality (Kim et al., 2017). However, to the best of our knowledge, the effect of stepwise ageing time on the instrumental colour of in-bag dry-aged beef has not been explored in previous studies.

Drip loss decreased ($P < 0.05$) in in-bag dry-aged beef at 21 days compared to 7 days regardless of the ageing treatment combinations (Table 3.4). This could be due to the significant amount of moisture lost by evaporation after 21 days of ageing thereby reducing the amount of moisture that could be lost as drip compared to 7 days, as discussed above. The reduced drip loss at 21 days could also be attributed to the higher muscle protein breakdown with 21 days of ageing compared to 7 days resulting in the "sponge effect" proposed by Farouk *et al.* (2012), which physically entrap the water and improve the water-holding capacity by lowering the water loss by gravity. The water-holding capacity of lean beef increased with ageing time, particularly in terms of the decrease in drip loss. The cook loss, on the other hand, could be another indicator of the water-holding capacity under extreme conditions, i.e. heating. The cook loss of in-bag dry-aged lean beef decreased with the ageing time from 7 days to 21 days in general, but the significant decrease was only seen in the beef aged at highest air velocity (T4). Other studies also did not find any difference in cook loss of dry-aged beef from 14 to 35 d (Ahnström et al., 2006; DeGeer et al., 2009; Laster et al., 2008).

Shear force values decreased ($P < 0.05$) with dry-ageing time at the first 7 days from an average of approximately 120 N (untender) to 80 N (tender), and then further decreased slightly ($P > 0.05$) during the extended 14 days of W period to a similar level (approx. 70 N), regardless of the ageing treatments (Table 3.5). The majority of studies on dry-aged beef reported no difference of shear force between W, D and BD of beef (DeGeer et al., 2009; Lepper-Blilie et al., 2016; Li et al., 2013; Sitz et al., 2006; Smith et al., 2008), which further support the findings of the current study. Therefore, it was the ageing time rather than the ageing methods that played the key role in tenderisation of beef. A significant decrease of shear force occurring within the first 14 days of ageing time has been observed by Gudjónsdóttir et al. (2015). Extension of ageing time beyond 4 weeks showed a minor effect on the improvement of tenderness (Ahnström et al., 2006; Laster

et al., 2008; Lepper-Blilie et al., 2016; Obuz et al., 2014). Therefore, the most rapid improvement of in-bag dry-aged beef tenderness occurs within the first 7 to 14 days of ageing time. This could be explained by the activity of endogenous enzymes (mainly μ-calpain) which plays a significant role in the tenderisation through proteolysis (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010). The activity is decreased significantly after 7 days of ageing time (Gil, Hortós, & Sárraga, 1998). A more recent study by Velotto et al. (2015) observed slightly faster decline of μ-calpain activity in dry-aged beef compared to the wet-aged over 15 days of ageing time. Further tenderisation during extended ageing time was mainly affected by more stable lysosomal proteases (mainly cathepsin B and B + L), though the rate of tenderisation decreased. Measurement of shear force at 0 and/or 7 days of ageing time is absent in many of the studies to date, which may have contributed to overlooking of the fact that the longer ageing time is not necessary for the improvement of tenderness. Though tenderisation is well known to improve the eating quality of meat, it should be critically controlled to avoid over-tenderisation and loss of texture and mouth appeal.

A_w is one of the most important parameters to indicate the shelf life of a food product. A_w of beef samples from all four treatments decreased ($P < 0.05$) from an average value of 0.993 to a similar level of 0.987 after 21 days of ageing time as shown in Table 3.6. In general, a low level of growth of surface microorganism was found prior and post ageing. The decrease ($P < 0.05$) of the aerobic bacteria counts in the in-bag dry-aged beef of T1 and T4 may be associated with the decline of A_w due to the surface being dried. The proliferation of aerobic bacteria with the increase of dry-ageing time has been reported (Campbell et al., 2001; DeGeer et al., 2009; Lee et al., 2017). However, contradictory findings were reported by Ahnström et al. (2006) that no difference of aerobic bacteria counts on dry-aged beef regardless of ageing methods and ageing time. Yeast in the present study increased with ageing time ($P < 0.05$) in the control in-bag dry-aged beef (T1) which is in line with other studies on (in-bag) dry-aged beef reported by DeGeer et al. (2009). The increase of yeast could be attributed to yeast species being able to grow on the dry meat surface with low moisture content compared to other microorganisms.

Table 3.5 Effect of ageing treatments, ageing time and frozen storage on instrumental texture of in-bag dry-aged lean bull beef.

Attributes/storage type	Ageing time	Treatments				SED	*P*-AV	*P*-SA	*P*-SA × AV
		T1	T2	T3	T4				
Shear force (N)									
Fresh	0d	132.00x	116.70x	131.81x	114.25x	12.45	0.071	0.932	0.919
	7d		89.34xy	69.24y	83.95y				
	21d	72.67y	71.98y	68.26y	70.61y				
	***P*-ageing time**	0.001	0.011	***	0.007				
Texture profile analysis									
Frozen									
Hardness (kg)	7d		2.49	2.81	2.72	0.37	0.348	0.601	0.920
	21d	3.02	2.88	3.11	3.10				
	***P*-ageing time**		0.404	0.362	0.193				
Springiness	7d		0.53	0.51	0.54	0.02	0.678	0.634	0.969
	21d	0.53	0.53	0.53	0.52				
	***P*-ageing time**		0.778	0.051	0.582				
Cohesiveness	7d		0.55	0.53	0.55	0.41	0.313	0.609	0.410
	21d	0.55	0.54	0.54	0.54				
	***P*-ageing time**		0.380	0.388	0.537				
Chewiness (kg)	7d		0.72	0.75	0.81	0.12	0.394	0.547	0.944
	21d	0.89	0.83	0.89	0.89				
	***P*-ageing time**		0.440	0.159	0.509				
Resilience	7d		0.23	0.22	0.22	0.01	0.522	0.909	0.276
	21d	0.23	0.22	0.22	0.22				
	***P*-ageing time**		0.181	0.473	0.773				

BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 $m.s^{-1}$ for 21 days; T2: BD at 0.5 $m.s^{-1}$ 7 days + W for 14 days; T3: BD at 1.5 $m.s^{-1}$ for 7 days + W for 14 days; T4: BD at 2.5 $m.s^{-1}$ for 7 days + W for 14 days. $P < 0.0001$ presented as *** for level of significance. Different letters of "x, y or z" within the same column mean results are significantly different from each other ($P < 0.05$).

Table 3.6 Effect of ageing treatments and ageing time on the A_w and surface microbial growth of fresh-n-never frozen in-bag dry-aged lean bull beef.

Attributes	Ageing time	Treatments				SED	*P*-SA × AV
		T1	T2	T3	T4		
A_w	0d	0.992x	0.993x	0.994x	0.992x	0.001	0.325
	21d	0.987y	0.987y	0.988y	0.986y		
	***P*-ageing time**	0.005	***	0.003	***		
Microbial load (log cfu/g)							
APC	0d	4.21x	2.62	3.09	2.74x	0.61	0.281
	21d	2.50y	3.29	2.56	2.00y		
	***P*-ageing time**	0.038	0.459	0.494	***		
LAB	0d	1.15	ND	1.50	ND	0.07	-
	21d	ND	ND	ND	ND		
	***P*-ageing time**	-	-	-	-		
Mould	0d	1.39	ND	ND	ND	0.05	-
	21d	ND	1.00	ND	ND		
	***P*-ageing time**	-	-	-	-		
Yeast	0d	2.21x	ND	1.48	1.39	0.40	0.003
	21d	4.06ay	1.57b	2.12b	2.41b		
	***P*-ageing time**	0.007	-	0.237	0.076		
Enterobacteriaceae	0d	3.16	1.84	1.38	1.71	0.71	-
	21d	ND	2.69	ND	1.35		
	***P*-ageing time**	-	-	-	-		
E. coli (MPN/g)	0d/21d	ND	ND	ND	ND	-	-

ND: Not detected; BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 $m.s^{-1}$ for 21 days; T2: BD at 0.5 $m.s^{-1}$ 7 days + W for 14 days; T3: BD at 1.5 $m.s^{-1}$ for 7 days + W for 14 days; T4: BD at 2.5 $m.s^{-1}$ for 7 days + W for 14 days. APC, LAB, *E. coli* refer to aerobic bacteria plate counts, lactic acid bacteria counts and *Escherichia coli*, respectively. $P < 0.0001$ presented as *** for level of significance. Different letters of "x, y or z" within the same column mean results are significantly different from each other ($P < 0.05$). Different letters of "a, b or c" within the same row mean results are significantly different from each other ($P < 0.05$).

Table 3.7 Effect of ageing treatment combinations and frozen storage on sensory acceptability of in-bag dry-aged lean beef for 21 days.

Attributes	Storage type	Treatments				SED	*P*-SA × AV
		T1	T2	T3	T4		
	Fresh						
Aroma		56.53	55.58	55.78	56.60	2.81	0.977
Tenderness		53.62	48.73	45.84	55.01	4.88	0.224
Juiciness		54.39	53.64	48.35	47.80	4.12	0.244
Flavour		44.85	44.76	41.87	42.95	4.35	0.881
Off flavour		20.68	16.73	19.16	17.79	3.66	0.727
Overall liking		59.77	58.20	52.88	57.17	3.33	0.218
Preference ranking (%)		28.41	22.73	22.73	20.45		
	Frozen						
Aroma		5.75	5.81	5.81	6.00	0.19	0.548
Tenderness		5.74	5.46	5.75	6.02	0.30	0.397
Juiciness		5.86a	4.55b	5.24c	5.91a	0.26	***
Flavour		6.12	5.76	5.96	6.33	0.23	0.126
Off flavour		1.71	1.89	1.85	1.78	0.12	0.401
Overall liking		6.03a	5.37b	5.89a	6.25a	0.24	0.006

BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 $m.s^{-1}$ for 21 days; T2: BD at 0.5 $m.s^{-1}$ 7 days + W for 14 days; T3: BD at 1.5 $m.s^{-1}$ for 7 days + W for 14 days; T4: BD at 2.5 $m.s^{-1}$ for 7 days + W for 14 days. Fresh samples were evaluated on a scale of 1-100, frozen samples were evaluated on a hedonic scale of 1-9. $P < 0.0001$ presented as ∗∗∗ for level of significance. Different letters of "a, b or c" within the same row mean results are significantly different from each other ($P < 0.05$).

There was no difference in lactic acid bacteria, *Enterobacteriaceae* and moulds across ageing time in the current study. *E. coli* was not detected in all the samples. Inconsistent results have been reported that *Enterobacteriaceae* (Li et al., 2014), *E. coli* (DeGeer et al., 2009) and moulds (Gudjónsdóttir et al., 2015) increased with ageing time. The decreased (Ahnström et al., 2006; DeGeer et al., 2009) or unchanged (Campbell et al., 2001) counts of lactic acid bacteria were also observed on dry-aged beef across the ageing. The proliferation of microorganisms has been observed in D process which could cause the spoilage of meat and some toxin-producing pathogens may lead to serious food poisoning and even death (Mills et al., 2014). On the other hand, microorganisms could facilitate the deterioration of meat quality and generation of off-flavours such as cheesy and dairy (Egan, Eustace, & Shay, 1988) and discolouration (Li et al., 2015). Therefore, the strict control of the processing hygiene and monitoring of the level of microorganism contamination are extremely important in terms of meat quality and food safety assurance. Current dry-ageing treatment combinations were able to produce microbiologically safe dry-aged lean beef products after 21 days of ageing time. This enables the meat industry to produce dry-aged products that satisfies the food safety standard for both local and export markets.

3.3.3.2 Frozen in-bag dry-aged beef

After long-term frozen storage, the proximate content of in-bag dry-aged beef from all four ageing treatments did not differ across the ageing time except for the muscle protein content which increased with ageing (Table 3.1). Increase of muscle protein content was seen in T1 and T2 ($P < 0.05$) which was likely to be attributed to the decrease of moisture content with ageing time. The water-holding capacity of frozen in-bag dry-aged samples increased ($P < 0.05$) with ageing time in terms of % thaw + drip loss which was also evident in the fresh counterparts (Table 3.4). In general, the cook loss of frozen in-bag dry-aged samples decreased with ageing, though the decrease ($P < 0.05$) was only found in the samples aged at highest air velocity (T4). Therefore, the increased water-holding capacity with ageing time was stable over the long-term frozen storage, which was in agreement with Farouk, Wieliczko, and Merts (2004).

The pH, colour, texture profile of in-bag dry-aged lean beef was not affected by ageing time ($P > 0.05$) after long-term frozen storage. The differences of pH, instrumental colour and texture detected in fresh in-bag dry-aged samples between 7 days and 21 days of ageing were not observed after long-term storage. The changes in texture could be explained by two theories suggested by Dransfield (1994): (1) proteolysis during long-

term frozen storage although the rate was slow because of calpain activity being suppressed; (2) a more rapid proteolysis occurred when thawing than prior to freezing due to the re-activation of calcium-depend proteases. Another possible explanation could be the interaction between ageing time and frozen storage as suggested by Vieira, Diaz, Martínez, and García-Cachán (2009) that the beef aged for 3 days showed significant decrease on shear force during longer frozen storage time as compared to those aged for 7 days which did not change. Therefore, understanding the texture profile of in-bag dry-aged lean beef which has been in long-term frozen storage is more important than the shear force tenderness due to the frozen storage could further tenderise the meat as discussed above. The changes of other texture properties are not able to be determined by single shear force measurement.

3.3.4 Effect of frozen storage on lean beef dry-aged for different ageing times

The pH of in-bag dry-aged beef was not affected by long-term frozen storage ($P > 0.05$). This supports the outcomes of the 6 months frozen storage of stepwise dry-aged beef reported by Kim et al. (2017). Proximate content of in-bag dry-aged beef at all three time points (0, 7 and 21 days) was not affected by the frozen storage except for the muscle protein content which decreased ($P < 0.0001$) after frozen storage. A consistent moisture and fat content over the frozen storage of up to 52 weeks was also reported by Holman, Coombs, Morris, Kerr, and Hopkins (2017) on beef, with W for up to 5 weeks prior to freezing. The muscle protein content in this study was measured from the extracted muscle protein solution. The decrease of muscle protein content was more likely to be attributed to the decrease of protein solubility in the extraction buffer due to protein denaturation after long-term frozen storage which has also been reported by Farouk et al. (2004).

Frozen storage had the major effect on the instrumental colour of in-bag dry-aged beef (Table 3.3). All colour attributes have decreased ($P < 0.05$) for in-bag dry-aged samples (7 and 21 days) after the frozen storage. Thawed in-bag dry-aged beef in current study became darker but was still within the consumer acceptable range (approx. 35 - 40 of L*) (Ponnampalam et al., 2017). The a* declined to around 13.0 which was slightly below the threshold of 14.5, according to their study. However, a lesser brown colour (lower hue) as compared to the fresh BD beef was detected. Decrease of hue in the current study was attributed to the significant decrease in b*. The change in colour observed in this study due to long-term frozen storage could be partially explained by the damage of muscle cells which altered the optical properties of the meat (Perez-Chabela & Mateo-

Oyague, 2004). This may support the loss of lightness and redness of meat colour. The decreases in b* (Vieira et al., 2009) and hue (Farouk et al., 2004) of beef over the frozen storage have also been reported previously. The decrease in b* may have been caused by the migration of oxygenated layer to a deeper position due to the reduced oxygen consumption rate over long-term frozen storage, and this may have resulted in a delay of the oxidation of myoglobin to metmyoglobin. Another possible reason may be that due to the low fat content of the lean bull beef used in this study, a lower level of lipid oxidation could contribute less to the generation of metmyoglobin and yellowness of beef (Faustman, Sun, Mancini, & Suman, 2010). A significant decrease of L* and a* and increase of b* and hue have also been reported by Kim et al. (2017) on dry-ageing/stepwise aged-then-frozen beef loins.

The water-holding capacity of frozen in-bag dry-aged beef decreased due to the extra fluid loss upon thawing (Table 3.4). Cook loss also tended to increase after frozen storage which may be associated with the decrease of juiciness (Lagerstedt, Enfält, Johansson, & Lundström, 2008). However, there was no clear decline in the juiciness rating of frozen BD samples in this study (Table 3.7), which was also observed on beef (Vieira et al., 2009) and lamb of other studies (Muela, Sañudo, Campo, Medel, & Beltrán, 2012).

3.4 Conclusion

The increase of dry-ageing chamber air velocity accelerated the weight loss of in-bag dry-aged lean bull beef but had no other negative effects on meat quality, microbiological safety and consumer palatability. Ageing time on the other hand, played a more important role in improving the quality of the dry-aged products. Combining BD with traditional W as a stepwise in-bag ageing strategy was able to produce dry-aged lean beef of equivalent quality compared to those of straight-dry-ageing for the same period of ageing time, but with lower weight loss/higher yield. In-bag dry-aged lean bull beef products could be long-term frozen stored for up to 12 months and still be acceptable to consumers.

3.5 Epilogue

This chapter assessed the impact of processing regimes and storage factors on the quality and acceptability of in-bag dry-aged lean beef. Outcomes of this study demonstrated the potential to produce shelf stable dry-aged beef which is acceptable to consumers when fresh or following long-term frozen storage for a year. Stepwise in-bag

ageing regime could be implemented by the meat industry to produce dry-aged products with more consistent quality and high product yield for the local and export markets.

In the next chapter, the effect of processing and storage factors on biochemical changes, including oxidative stability of lipid and protein, proteolysis and digestibility, were further studied to understand the mechanisms responsible for the quality changes observed in this chapter.

Chapter 4: Oxidative stability, proteolysis, and *in vitro* digestibility of fresh and long-term frozen stored in-bag dry-aged lean beef

This chapter performs a deeper investigation to determine the effect of air velocity, stepwise in-bag ageing and ageing time on the oxidative stability, proteolysis and digestibility of fresh and long-term frozen-stored dry-aged lean beef. Oxidative changes of lipid and protein due to different processing regimes and storage factors were studied. Proteolytic pattern of in-bag dry-aged lean beef was determined using gel electrophoresis (SDS-PAGE) and free amino acids (FAAs) profiling. The changes in digestibility due to ageing and frozen storage were evaluated through the simulated gastrointestinal digestion (2 hours pepsin digestion + 2 hours pancreatin digestion).

Outcomes of this research showed that the increased air velocities and stepwise ageing regime had no effect on dry-aged beef lipid and protein oxidative stabilities and proteolysis pattern compared to control straight-dry-ageing. TBARS, protein carbonyl and FAAs increased with ageing time. In-bag dry-ageing of beef improved its lipid and protein oxidative stability during long-term frozen storage compared to unaged beef. Improvement in beef protein digestibility was observed through increased release of FAAs and appearance of small protein fragments from SDS-PAGE in dry-aged samples compared to the unaged. The high lipid and protein oxidative stability of long-term frozen lean beef produced using stepwise in-bag ageing process suggested potential for the process to be used for producing dry-aged meat for export.

This chapter starts with the introduction to state the motivations and objectives of this research. The processing regimes and the biochemical analyses included in this study are presented in Section 4.2. Section 4.3 describes the findings of this research and Section 4.4 discusses current findings in terms of oxidative stability, proteolysis, frozen storage and digestibility. The summary of this chapter is presented in Section 4.5.

4.1 Introduction

Dry-ageing is a post-mortem practice for tenderisation and flavour improvement of beef. Strategies to produce quality stable dry-aged products should be considered especially if the products were to be traded globally where a long period of chilled/frozen storage is usually required. Success of dry-aged products requires not only a critically controlled ageing chamber, but also a well-designed ageing regime (Kim et al., 2016). This is the reason effort is being put in developing new techniques to improve the quality, yield and stability of dry-aged meats. For instance, the adoption of water permeable ageing bags to produce dry-aged products is mainly to reduce microbial contamination, lipid oxidation and trim loss when compared to the traditional out-of-bag dry-ageing technique (DeGeer et al., 2009). Another technique that combines in-bag dry-ageing (BD) with wet-ageing (W) as a stepwise process, known as “stepwise in-bag ageing”, is

recently proposed to enable the production of microbiologically safe dry-aged beef with maximised saleable meat yield and with minimum or no post-production trimming required (Zhang et al., 2019).

Traditional dry-ageing (D) regime requires ageing of well-marbled beef cuts with subcutaneous fat covering the surface, which acts as a barrier to protect the beef from excessive moisture (weight) loss. However, lipid is susceptible to oxidation and generating a series of oxidative by-products, which may also trigger oxidative damage to myoglobin and other proteins (Park, Xiong, Alderton, & Ooizumi, 2006). Protein oxidation during ageing and long-term frozen storage results in the formation of oxidative by-products including protein carbonyls (aldehydes and ketones) from the side chains of amino acids (Martinaud et al., 1997; Sante-Lhoutellier et al., 2008). These have been used to predict the oxidative damage of proteins (Estévez, 2011). Severe oxidative damage of lipids and proteins may cause adverse effects on sensorial and nutritional quality of meat products, including the generation of off-flavour, decrease in tenderness and digestibility, loss of unsaturated fatty acids (UFAs), amino acids and other functionalities of proteins (Xiong, 2000).

We recently produced dry-aged beef from very lean meat and stored the product for 12 months at -18 ºC (Zhang et al., 2019). The dry-aged lean beef product was found to be acceptable in meat quality and sensory palatability in terms of low off-flavours following the 12 months frozen storage. The frozen stability of the dry-aged product following long-term storage necessitates further investigation to understand the mechanisms for the stability and the other biochemical changes observed.

Therefore, the aim of this study was to determine the effects and interactions of air velocities, ageing time and long-term frozen storage on oxidative stability, proteolysis and *in vitro* digestibility of in-bag dry-aged lean bull beef. We hypothesise that "stepwise in-bag ageing" regime accompanied by increased air velocities for dry-ageing could produce dry-aged product of equivalent quality and long-term storage stability to the straight-dry-aged meat with no W involved.

4.2 Materials and methods

4.2.1 Sample collection and study design

Bull beef striploins (Holstein-Friesian, *m. longissimus lumborum*, paired loins, n = 30) were collected from an abattoir on the day of slaughter and held at 12 °C for approx. 12 hours until they entered rigor. A randomised trial was designed to determine the impact

of air velocity, stepwise ageing regime and ageing time on dry-aged lean bull beef. Four ageing treatment combinations consist of one straight-dry-ageing as control (T1, n = 6) and three stepwise in-bag ageing regimes (T2 - T4, n = 8 for each treatment): (T1) dry-ageing in water permeable ageing bags (TUBLIN® 10, 50 μm thick, polyamide mix with water vapor transmission rate 920 g/50 μ/m^2/24 h at 7 °C, 50% RH, and oxygen transmission rate 660 g/m^2/24 h at 7 °C, 50% RH, TUB-EX ApS, Denmark.) for 21 days (control, n = 6) at air velocity of 0.5 $m.s^{-1}$; (T2) - (T4) BD for 7 days followed by W in water-impermeable vacuum barrier bags for 14 days at air velocity of 0.5 $m.s^{-1}$ (T2); 1.5 $m.s^{-1}$ (T3) and 2.5 $m.s^{-1}$ (T4), respectively. All the samples were evenly distributed in a dry-ageing chamber set at 2 ± 0.5 °C and 75 ± 5% RH, to allow sufficient air circulation around each sample. The paired loins were assigned to different treatments with 6 single loins of different carcasses for T1; the remaining 24 loins of 15 carcasses (6 single loins + 18 paired loins) were then randomly allocated to treatments T2-T4 with each treatment allocated 8 loins from different carcasses.

Fresh beef steaks taken from assigned ageing treatments (T1-T4) before ageing were analysed for the baseline determination as compared to aged samples. Following 7 days of ageing time, sub-samples (2 cm steaks) were taken from ageing treatments T2 - T4 further analyses to determine the effect of air velocity. Remaining striploins (T2 - T4) were then vacuum packed in water impermeable barrier bags for another 14 days of W under the same conditions. On 21 days of ageing time, steaks from the four treatments (T1 - T4) were obtained and three effects were determined include: (i) the impact of stepwise ageing regime (T1 *vs.* T2); (ii) the interaction of air velocity and stepwise ageing (T1 - T4) ; and (iii) the effect of ageing time (0, 7 and 21 days).

A thin layer (approx. 5 mm) of the dry surface from steak sample was trimmed on 7 and 21 days. Trimmed steak samples from 0 day (unaged, T1 - T4), 7 days (T2 - T4) and 21 days (T1 - T4) of ageing times were then vacuum packed in water-impermeable barrier bags and immediately stored in a freezer at -18 °C for 12 months to determine the effect of frozen storage on in-bag dry-aged lean beef. All steak samples (fresh and frozen) were minced individually for further analyses. 4.2.2 Oxidative stability of lipids

Oxidative stability of lipids was evaluated by determining the generation of thiobarbituric acid reactive substances (TBARS) and changes in fatty acid (FA) profile during ageing (0, 7 and 21 days of T1 - T4) and following the frozen storage. Concentrations of TBARS were measured according to Buege and Aust (1978). The amount of TBARS was expressed as mg of malondialdehyde (MDA)/kg meat. FA profile

was determined from lyophilised and ground samples following the procedures described by Zhang et al. (2018). The FA profile was expressed as % FAs in total FAs. Both measurements were performed in duplicate.

4.2.3 Oxidative stability of proteins

Whole muscle proteins were extracted using an extraction buffer which consisted of 50 mM Tris-HCl (pH = 5.8), 10% glycerol, 2% SDS and 2% β-mercaptoethanol, as described by Zhang et al. (2019). The amount of protein carbonyl was measured using 2,4-dinitrophenylhydrazine (DNPH) assay proposed by Levine et al. (1990). Absorbance of carbonyl solution was read at 370 nm using a UV-spectrophotometer after suspending in 6 M guanidine HCl (in 20 mM sodium phosphate buffer, pH = 2.3) against a blank, which followed the same preparation steps but with the protein extraction buffer. The measurement was performed in duplicate.

4.2.4 SDS-PAGE gel electrophoresis

Extracted whole muscle proteins (section 4.2.3) from fresh and frozen in-bag dry-aged beef samples of four ageing treatment combinations at three ageing times (0, 7 and 21 days) were diluted with SDS-PAGE sample loading buffer which consisted of 50 mM Tris-HCl (pH = 5.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.002% bromophenol blue. The diluted samples were heated for 5 min at 95 °C and centrifuged at 10,000 g for 5 min at 4 °C. Supernatant (equivalent to 40 µg protein) was loaded onto each well of a Novex™ NuPAGE™ 10% Bis-Tris Midi Protein Gels (Invitrogen™, UK). An 8 µl of Novex™ Sharp Pre-stained protein standard (Invitrogen™, UK) was loaded to determine the size of proteins and peptides from 260 kDa to 3.5 kDa. Protein fragments were separated at room temperature (22 ºC) in a Bio-Rad® Criterion cell system equipped with PowerPac™ HC High-Current Power Supply (Bio-Rad® Laboratories, Hercules, CA, USA) using 150 V constant voltage. SimplyBlue SafeStain (Invitrogen™) was used for gel staining at room temperature for 4 hours followed by washing in distilled water for 3 hours. Images of stained gels were captured using a GS900 densitometer scanner (Bio-Rad® Laboratories, Inc.). Identification and quantification of protein bands were performed using Image Lab™ software (Version 6.0, Bio-Rad® Laboratories, Inc.). Identified protein bands were grouped into six molecular weight (MW) groups; G1: 110 - 260 kDa, G2: 60 - 110 kDa, G3: 50 - 60 kDa, G4: 30 - 50 kDa, G5: 20 - 30 kDa and G6: 3.5 - 20 kDa. The % relative optical quantity of six protein band groups to the total optical quantity of all bands was calculated. Protocol for the identification of protein bands

followed Farouk, Wu, Frost, Clerens, and Knowles (2014) and Kaur, Maudens, Haisman, Boland, and Singh (2014).

4.2.5 Free amino acids (FAAs) analysis

FAAs were extracted from the lyophilised (freeze-dried) fresh and frozen in-bag dry-aged beef samples to eliminate the influence of moisture. Approximately 0.5 g of lyophilised and ground sample was homogenised with 5 mL of 80 % methanol at 14,000 rpm (IKA Labortechnik, Germany) for 20 s in ice bath. Supernatant containing extracted FAAs was obtained after centrifugation at 15,000 g for 10 min at 4 °C. The AAs extract was spiked with 10 $mg.L^{-1}$ d_4-alanine as an internal standard (1 : 1, v/v). Pre-column derivatisation of FAAs was performed using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (2.8 mg/mL in dry acetonitrile, Apollo Scientific, UK) according to Salazar, Armenta, and Shulaev (2012). The FAA derivatives were neutralised with 10% formic acid (1 : 3, v/v) and analysed by Agilent 1260 Infinity HPLC system equipped with Agilent 6420 Triple Quadrupole LC/MS system (Agilent Technologies, New Zealand). Derivatised AAs were separated on a C18 core shell Kinetex LC column (100 × 2.1 mm with a 1.7 µm particle size, 00D-4726-AN, Kinetex®) with flow rate of 0.2 $mL.min^{-1}$ and temperature at 22.6 °C. The separation gradient used was: 0 to 8.0 min (13.0% A and 87.0% B), 8.0 to 16.0 min (17.0% A and 83.0% B), 16.0 to 17.0 min (80.0% A and 20.0% B), and 18.0 to 18.5 min (1.5% A and 98.5% B). Buffers A and B were prepared with 0.1% formic acid in acetonitrile and 0.6% formic acid in MilliQ water, respectively. Quantitation of AAs was carried out using positive electrospray ionisation (ESI+) mode with MRM-MS method by selecting the most sensitive parent-daughter ion transition of m/z $[M-H]^+ > 171$. The ionisation source temperature was set at 325 °C with gas flow rate at 6.0 $L.min^{-1}$ and capillary voltage of 2.0 kV. Standard mix of AAs (10 µM) was prepared with 37 AA standard mixture (A9906, Sigma), asparagine (A0884, Sigma) and glutamine (G3126, Sigma), which was serially diluted to 0.078 µM to obtain an eight-point standard curve for identification and quantification of FAAs using MassHunter software (Agilent Technologies). The FAAs content of in-bag dry-aged beef sample was expressed as mg FAAs/g dry matter.

4.2.6 *In vitro* digestion of frozen stored in-bag dry-aged beef

In vitro digestibility of frozen stored in-bag dry-aged beef samples was evaluated using a two-stage static *in vitro* digestion model which consisted of simulated gastric (120 min) and intestinal/pancreatic (120 min) digestion, modified from Zhang et al. (2018). Enzyme solutions of porcine pepsin (P6887, Sigma) and pancreatin (ACROS Organics™,

Thermo Fisher Scientific) were prepared to the final enzyme : substrate ratio of 1 : 278 and 1 : 100, respectively (Farouk et al., 2014). Minced beef samples (approx. 4 g, exact weight was recorded). At 0, 30 and 120 min of gastric digestion and 120 min of pancreatic digestion, the protein hydrolysates were collected for further analyses which included (1) protein and peptide profile using SDS-PAGE electrophoresis (section 4.2.4); (2) release of FAAs which was extracted from the hydrolysates using methanol (1 : 2, $v_{hydrolysate}/v_{methanol}$) and determined following the procedure described in section 2.5; and (3) protein content in the hydrolysates using RC-DC protein assay kit (Bio-Rad® Laboratories, Hercules, CA, USA), to determine relative digestibility of frozen in-bag dry-aged beef. Relative digestibility (%) was calculated based on three analyses:

(1) Relative digestibility $_{SDS\text{-}PAGE} = \left(1 - \frac{\text{Optical intensity of protein bands} < 10\ kDa}{\text{Optical intensity of all protein bands}}\right) \times 100\%$

(2) Relative digestibility $_{FAAs} = \left(\frac{\text{Total FAAs (g) at 240 min} - \text{total FAAs (g) at 0 min}}{\text{Protein content of the sample}}\right) \times 100\%$

(3) Relative digestibility $_{Protein\ content} = \left(1 - \frac{\text{Protein content (g) in hydrolysate at 240 min}}{\text{Protein content of the sample}}\right) \times 100\%$

4.2.7 Statistical analysis

An incomplete randomised block design was used in this study, as described in chapter 3. Linear mixed effect regression analyses were performed on the current data matrix using R (version 3.4.1) with the "lme4" and "predictmeans" packages to determine the effect of air velocities, stepwise ageing strategy and ageing time on the fresh in-bag dry-aged lean beef. The same analysis was also carried out following the long-term frozen storage for 12 months to determine the interaction of frozen storage with ageing treatments. The impact of frozen storage on dry-aged beef was further determined by comparing fresh and frozen stored samples which have been aged for the same period of ageing time: 0, 7 and 21 days, respectively. Ageing treatments and ageing time were included as the fixed effects, carcass ID and sides were included as the random effects. One-way analysis of variance (ANOVA) was performed with post-hoc comparison of predicted means using Fisher's least significant differences (LSD) and Tukey's (HSD) test at 5% significance level. Pearson's correlation coefficient comparison (r value) was performed to investigate the relationship between lipid (TBARS) and protein (carbonyl) oxidation, and the relationship between SDS-PAGE, FAAs and available protein content on relative digestibility.

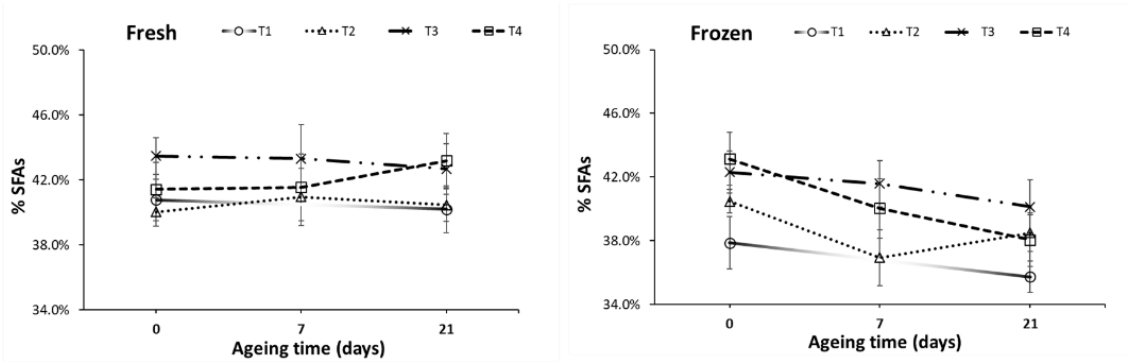
4.3 Results

4.3.1 Effect of ageing treatments on the oxidative stability and proteolysis of fresh and frozen in-bag dry-aged lean beef

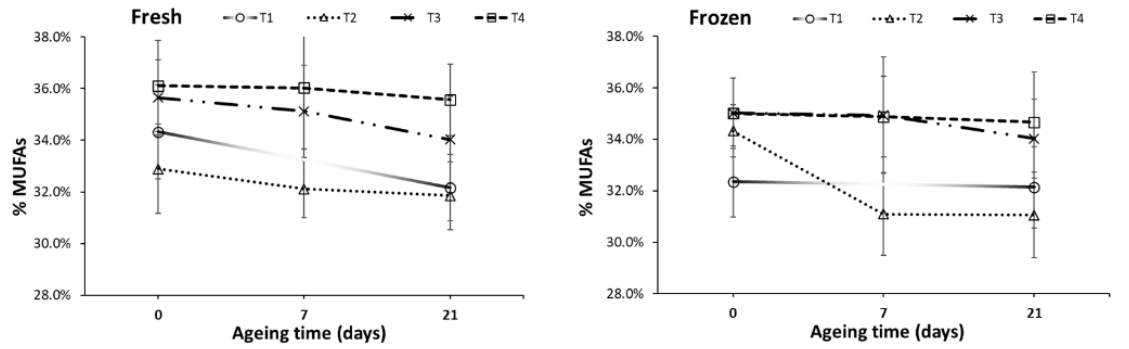
4.3.1.1 Dry-ageing air velocities (0.5 m.s^{-1}; 1.5 m.s^{-1} and 2.5 m.s^{-1})

A total of 14 fatty acids (FAs) were detected in lean beef striploins prior to ageing (0 day, Table 4.1). The FAs were comprised of 41.42% of saturated fatty acids (SFAs), 34.75% of monounsaturated FAs (MUFAs) and 23.83% of polyunsaturated FAs (PUFAs). Palmitic acid (21.18%) was the predominant SFA followed by stearic acid (16.60%). Unsaturated FAs (UFAs) were dominated by MUFAs (mainly oleic and elaidic acids, 31.67% combined) and Omega 6 PUFAs (linoleic acid, 13.00%). Composition of UFAs did not differ ($P > 0.05$) between T2, T3 and T4 at different air velocities following 7 days of BD (Table 4.2 and Figure 4.1) suggesting that the oxidative stability of FAs was not affected. This result was further supported by the lipid oxidation products (TBARS) measurements, which did not differ ($P > 0.05$) between T2, T3 and T4 at 7 days ageing time (Table 4.2 and Figure 4.2). A similar level ($P > 0.05$) of protein carbonyl was detected in T2, T3 and T4 at 7 days of ageing, suggesting that the oxidative stability of proteins was also not affected by the increase of air velocity for dry-ageing.

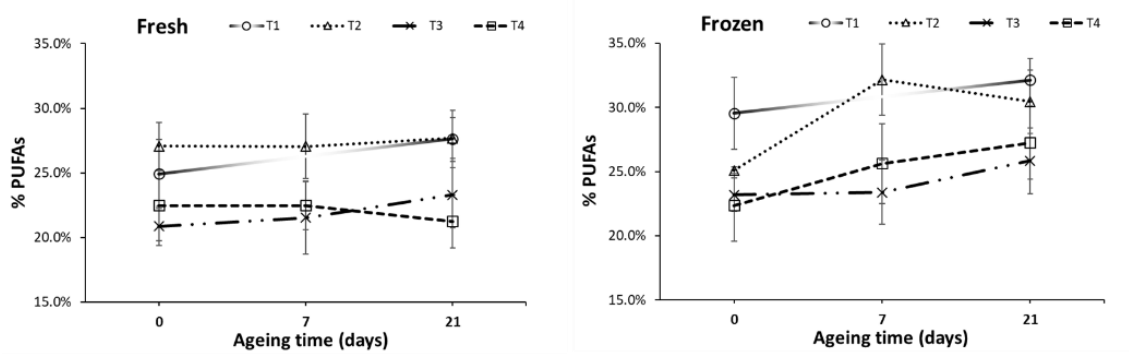
From the separation of whole muscle proteins of lean beef samples by SDS-PAGE gel electrophoresis (Figure 4.3a), no qualitative change of protein bands was observed between T2, T3 and T4 after 7 days of BD under different air velocities. The relative quantity of six summative protein groups (G1 - G6) did not change with the increase of air velocity ($P > 0.05$). A total of 20 FAAs was detected from dry-aged lean beef (Table 4.3), which consisted of 9 essential and 11 non-essential AAs. There was no difference in FAAs profile ($P > 0.05$) of the fresh dry-aged samples after 7 days of ageing time (T2 - T4), regardless of the air velocity used.



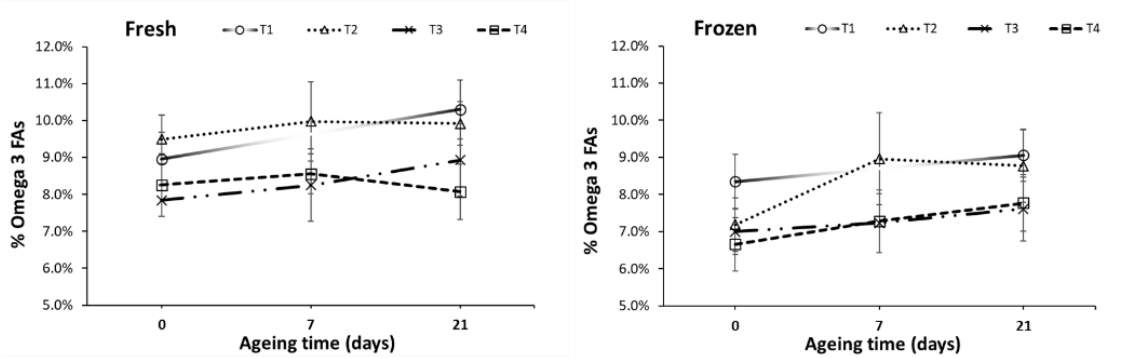
% SFAs



% MUFAs



% PUFAs



Omega 3 FAs

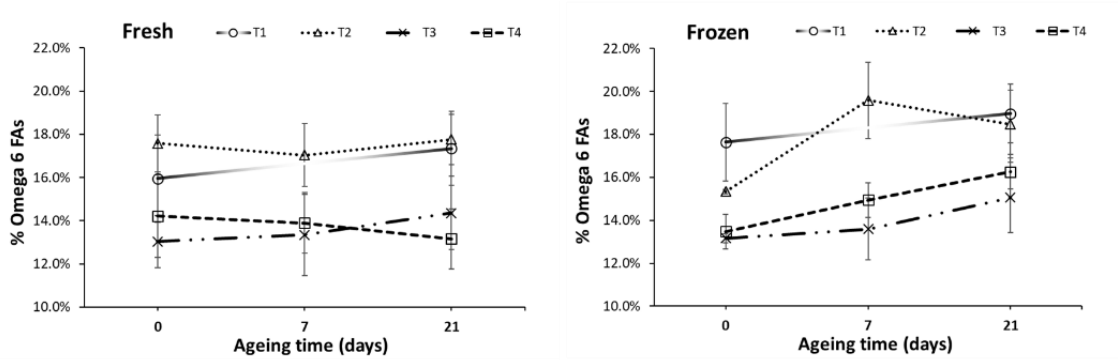


Figure 4.1 Fatty acids, (Means ± SEM) of in-bag dry-aged bull beef by four ageing treatments (T1 - T4) for three ageing time (0, 7 and 21 days) followed by two storage types: fresh (never frozen, left) and frozen for 12 months (right). BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 m.s^{-1} for 21 days; T2: BD at 0.5 m.s^{-1} for 7 days + W for 14 days; T3: BD at 1.5 m.s^{-1} for 7 days + W for 14 days; T4: BD at 2.5 m.s^{-1} for 7 days + W for 14 days.

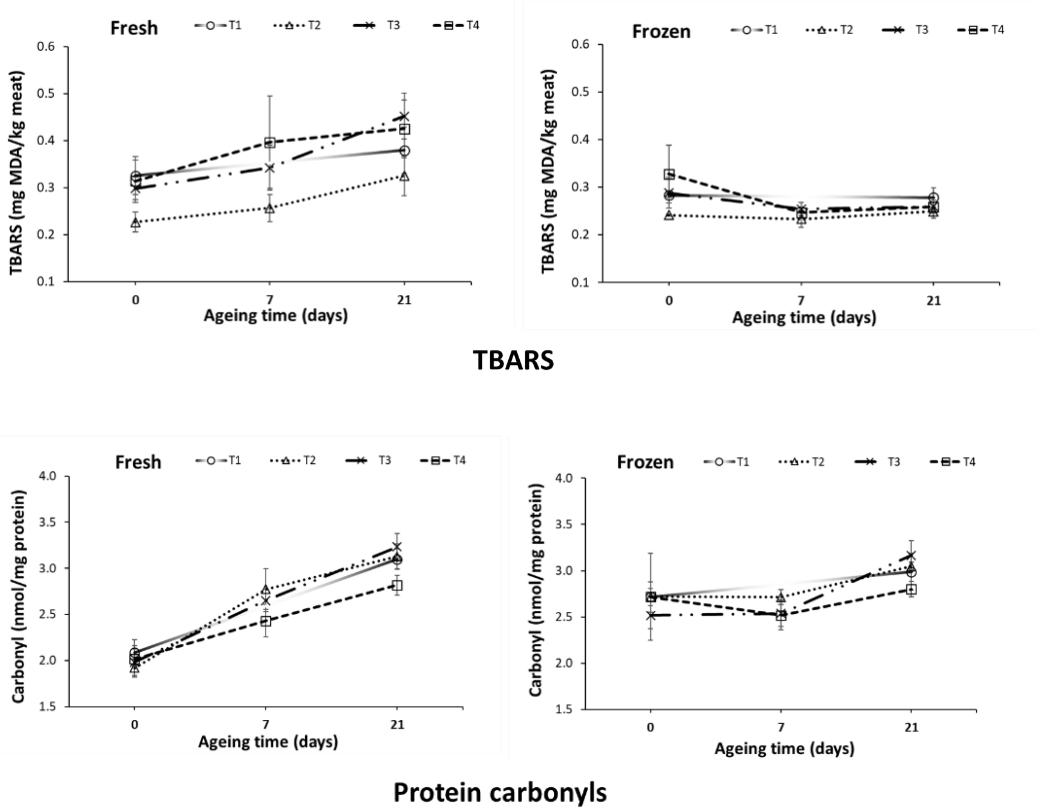


Figure 4.2 TBARS, and protein carbonyls content (Means ± SEM) of in-bag dry-aged bull beef by four ageing treatments (T1 - T4) for three ageing time (0, 7 and 21 days) followed by two storage types: fresh (never frozen, left) and frozen for 12 months (right). BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 m.s^{-1} for 21 days; T2: BD at 0.5 m.s^{-1} 7 days + W for 14 days; T3: BD at 1.5 m.s^{-1} for 7 days + W for 14 days; T4: BD at 2.5 m.s^{-1} for 7 days + W for 14 days.

The air velocity used for dry-ageing had no impact ($P > 0.05$, Table 4.2) on the oxidative stability of lipids (FAs and TBARS) and proteins except for the higher level ($P = 0.033$) of Omega 6 FAs detected in T2 following 12 months of frozen storage (Figure 4.1). There was no change ($P > 0.05$, Table 4.3) in FAAs profile and protein profiles (Figure 4.3b) observed between T2, T3 and T4 after 7 days of BD. There was an increase in small sized proteins (< 20 kDa) with increase in the air velocities used in dry-aging.

Table 4.1 Fatty acids profile of fresh lean beef in four assigned treatments before ageing (0 day).

Abbreviation	Common name	Concentration (% total fatty acids)
SFAs		41.42
14:0	Myristic acid	1.14
16:0	Palmitic acid	21.18
17:0	Margaric acid	0.63
18:0	Stearic acid	16.60
24:0	Lignoceric acid	1.87
MUFAs		34.75
16:1 (n-7)	Palmitoleic acid	2.56
17:1	Heptadecenoic acid	0.52
18:1 (n-9)	Oleic (*cis*) - Elaidic (*trans*) acid	31.67
PUFAs		23.83
18:2 (n-6)	Linoleic acid	13.00
18:3 (n-3)	α-Linolenic acid	5.40
20:3 (n-3)	Eicosatrienoic acid	0.46
20:5 (n-3)	Eicosapentaenoic acid (EPA)	2.53
20:3 (n-6)	Dihomo-*γ*-linolenic acid (DGLA)	2.19
22:6 (n-3)	Docosahexaenoic acid (DHA)	0.25

SFAs, MUFAs, PUFAs and n denote to Saturated Fatty Acids, Mono-Unsaturated Fatty Acids, Poly-Unsaturated Fatty Acids and Omega, respectively.

4.3.1.2 Stepwise ageing regime

Levels of UFAs, TBARS, protein carbonyl (Table 4.2), SDS-PAGE protein profile (Figure 4.3) and FAAs profile (Table 4.3) did not change between T1 (control) and T2 after 21 days of ageing time, suggesting that the oxidative stability and proteolysis pattern of in-bag dry-aged bull beef were not affected by the current stepwise ageing regime.

Also, there was no impact ($P > 0.05$, Table 4.2) of stepwise ageing regime on the FAs composition, TBARS and protein carbonyl content following the frozen storage for 12 months.

4.3.1.3 Interaction between air velocity and stepwise ageing regime

Combination of air velocity and stepwise ageing regime (T1 - T4, 21 days) had no impact on the oxidative stability of lipids and proteins (Figure 4.2 and Table 4.2), and the proteolysis pattern of dry-aged lean beef as seen in SDS-PAGE protein (Figure 4.3b) and FAAs profiles (Table 4.3). The oxidative stability of lipids and proteins, and the FAAs profile of the long-term frozen stored dry-aged lean beef was also not affected ($P > 0.05$) by the treatment combinations of air velocity and stepwise ageing strategy. Increased air velocity combined with stepwise ageing process produced more low MW proteins (< 20 kDa) following long-term frozen storage (Figure 4.3b-3).

4.3.2 Effect of ageing time on the oxidative stability and proteolysis of fresh and frozen in-bag dry-aged lean beef

The length of ageing time of up to 21 days had no impact on FAs composition except for the decrease in MUFAs content ($P = 0.043$) found in T3 (Figure 4.1). A significant increase in TBARS level with ageing time was observed in T2 - T4, suggesting the presence of lipid oxidation (Table 4.2 and Figure 4.2). Similar trend was also found in production of protein oxidation products – the carbonyl level increased ($P < 0.05$) over 21 days of ageing time.

As shown in Figure 4.3a-1, no clear change of protein profile was observed with the increase of ageing time in large sized proteins (G1 - G2), with changes in medium to small sized proteins (G3 - G6). Total FAAs and essential AAs content in the dry-aged beef samples increased ($P < 0.01$) following 21 days of ageing compared to 7 days and unaged counterparts (Table 4.3). It is worth noting that the significant increase of FAAs content was driven by essential AAs. The % essential AAs content (in total FAAs) increased ($P < 0.0001$) to about three times by 21 days of ageing compared to the unaged. AAs carrying sweet tastes, for example, alanine, glycine, proline, serine and threonine, increased about 1.5 times after 21 days of ageing compared to the unaged. AAs which contribute to umami and meaty taste, such as aspartic acid, asparagine, glutamic acid, methionine and lysine, increased at least 3 times after 21 days of ageing than the unaged counterparts. Another group of taste active AAs associated with bitter and astringent tastes, including isoleucine, leucine, methionine, phenylalanine, tryptophan and tyrosine, showed large increase of greater than 3 folds compared to the unaged. The largest increase was found in methionine (greater than 20 folds increase compared to the unaged).

Ageing time did not affect the FAs profile and TBARs of stepwise dry-aged beef following the long-term frozen storage ($P > 0.05$, Table 4.2), while the protein carbonyl concentration showed an increasing trend with ageing time (Figure 4.2). Following frozen storage, no clear change of protein profile with the increase of ageing time was seen in medium to large sized proteins (MW 50 - 260 kDa, G1 - G3). While the further cleavage of medium to small sized proteins (G5 - G6) into finer protein hydrolysates (MW < 10 kDa) was observed with ageing time (Figure 4.3b-3).

4.3.3 Effect of frozen storage on oxidative stability and proteolysis of in-bag dry-aged lean beef for different ageing times

Long-term frozen storage had no negative impact on the FAs composition of dry-aged lean beef that were aged for 0, 7 and 21 days, as shown in Table 4.2. There was a decrease ($P = 0.001$) of SFAs with an increase ($P = 0.022$) of PUFAs in the frozen dry-aged samples (21 days ageing) compared to the fresh unfrozen counterparts regardless of the ageing treatments. However, the decrease in SFAs were only detected in T1 and T4 ($P < 0.05$). Frozen storage resulted in a reduced level ($P < 0.05$) of TBARS in beef samples dry-aged for 7 and 21 days compared to the fresh counterparts (Table 4.2). Protein carbonyl content of the unaged samples had increased ($P < 0.0001$) whereas that of samples dry-aged for 7 or 21 did not change after the long-term frozen storage.

As shown in Figure 4.3b, the optical density of medium to small sized proteins (G4 - G6) from in-bag dry-aged beef samples increased following the long-term frozen storage. A significantly increased level of total FAAs was observed in frozen dry-aged beef samples after 7 and 21 days of ageing with no difference ($P = 0.056$) detected in unaged samples (Table 4.3). Overall, the long-term frozen storage reduced ($P < 0.05$) the level of essential AAs in beef samples of unaged and dry-aged for 7 days (data not shown). The FAAs composition of dry-aged beef samples for 21 days increased ($P < 0.05$) after frozen stored for 12 months except for some marginal changes that were found in phenylalanine, alanine and glutamine. Lysine and proline decreased ($P < 0.05$) following the frozen storage regardless of ageing treatments and time.

4.3.4 *In vitro* digestibility of long-term frozen in-bag dry-aged lean beef

The air velocity employed in dry-ageing and the stepwise ageing regime had no effect on the digestibility of the dry-aged beef held frozen for one year at -18 °C ($P > 0.05$). Thus, further analysis focused on the effect of ageing time on the digestibility of the frozen in-bag dry-aged lean beef.

Table 4.2 Statistical significance (*P*-values) of the effect of ageing time and frozen storage on the oxidative stability of in-bag dry-aged lean beef.

Attributes	Storage type	*P*-ageing time				*P*-AV	*P*-SA	*P*-SA × AV	*P*-storage (0 day)	*P*-storage (7 days)	*P*-storage (21 days)	SED
		T1	T2	T3	T4							
% SFAs	*Fresh*	0.773	0.585	0.656	0.412	0.458	0.879	0.369	0.757	0.117	**0.001**	1.93
	Frozen	0.294	0.468	0.553	0.293	0.146	0.107	0.266				2.70
% MUFAs	*Fresh*	0.356	0.317	**0.043**	0.856	0.306	0.874	0.251	0.589	0.451	0.688	2.01
	Frozen	0.730	0.167	0.747	0.991	0.195	0.610	0.415				2.64
% PUFAs	*Fresh*	0.448	0.819	0.206	0.726	0.208	0.988	0.103	0.832	0.106	**0.022**	2.89
	Frozen	0.441	0.287	0.623	0.638	0.073	0.658	0.320				4.58
% Omega 3 FAs	*Fresh*	0.245	0.514	0.171	0.854	0.528	0.705	0.226	0.127	0.221	0.096	1.02
	Frozen	0.485	0.351	0.903	0.686	0.595	0.825	0.576				1.45
% Omega 6 FAs	*Fresh*	0.612	0.649	0.262	0.623	0.124	0.838	0.099	0.585	0.310	0.175	2.02
	Frozen	0.500	0.310	0.558	0.602	**0.033**	0.826	0.318				2.86
TBARS	*Fresh*	0.248	**0.012**	**0.002**	**0.049**	0.543	0.422	0.320	0.892	**0.032**	***	0.06
	Frozen	0.867	0.531	0.784	0.086	0.733	0.238	0.566				0.03
Protein carbonyl	*Fresh*	**0.002**	***	***	***	0.340	0.879	0.100	***	0.806	0.464	0.18
	Frozen	0.986	0.165	**0.045**	0.275	0.520	0.783	0.275				0.26

BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity. T1: BD at 0.5 $m.s^{-1}$ for 21 days; T2: BD at 0.5 $m.s^{-1}$ 7 days + W for 14 days; T3: BD at 1.5 $m.s^{-1}$ for 7 days + W for 14 days; T4: BD at 2.5 $m.s^{-1}$ for 7 days + W for 14 days. SED is the standard error of a difference between means. SFAs, MUFAs and PUFAs refer to Saturated Fatty Acids, Monounsaturated Fatty Acids and Polyunsaturated Fatty Acids, respectively. $P < 0.0001$ presented as *** for level of significance.

Table 4.3 Free amino acids content (mg/g dry matter) of fresh and frozen in-bag dry-aged lean beef after frozen storage for 12 months.

	Fresh in-bag dry-aged lean beef																		Frozen in-bag dry-aged lean beef			*P*-storage		
	T1		*P*-AT	T2			*P*-AT	T3			*P*-AT	T4			*P*-AT	*P*-AV	*P*-SA	*P*-AV × SA	SED	*P*-AV	*P*-SA	*P*-AV × SA	0d	7d	21d
	0d	21d		0d	7d	21d		0d	7d	21d		0d	7d	21d											
Essential amino acids																									
Histidine	0.05a	0.09b	**0.019**	0.05a	0.05a	0.11b	***	0.05a	0.05a	0.13b	**	0.04a	0.05a	0.11b	**0.001**	0.904	0.368	0.456	0.02	0.904	0.456	0.368	0.584	**0.002**	***
Isoleucine	0.08a	0.34b	**0.001**	0.08a	0.12a	0.37b	**	0.06a	0.13b	0.43c	***	0.07a	0.12a	0.35b	***	0.385	0.481	0.333	0.03	0.385	0.333	0.481	**0.007**	**0.030**	***
Leucine	0.12a	0.64b	**0.001**	0.12a	0.23b	0.71c	***	0.11a	0.26b	0.82c	***	0.11a	0.22a	0.69b	***	0.356	0.438	0.286	0.06	0.356	0.286	0.438	**0.004**	**0.019**	***
Lysine	0.17a	0.48b	**0.001**	0.15a	0.17a	0.48b	***	0.16a	0.20a	0.60b	***	0.16a	0.20a	0.47b	**	0.504	0.978	0.443	0.07	0.504	0.443	0.978	**0.010**	***	**0.011**
Methionine	0.02a	0.24b	**	0.01a	0.07b	0.27c	***	0.01a	0.08a	0.31b	***	0.02a	0.07a	0.29b	***	0.581	0.254	0.374	0.03	0.581	0.374	0.254	0.866	0.385	***
Phenylalanine	0.05a	0.30b	**	0.06a	0.11b	0.35c	***	0.05a	0.11a	0.41b	***	0.05a	0.11a	0.38b	***	0.487	0.330	0.332	0.04	0.487	0.332	0.330	**0.003**	**0.002**	0.494
Threonine	0.11a	0.25b	**0.001**	0.11a	0.13a	0.26b	***	0.10a	0.14a	0.33b	***	0.10a	0.13a	0.28b	***	0.213	0.781	0.320	0.03	0.213	0.320	0.781	**0.062**	**0.001**	***
Tryptophan	0.02a	0.07b	**0.001**	0.02a	0.03a	0.08b	***	0.02a	0.03a	0.09b	***	0.02a	0.03a	0.09b	***	0.152	0.232	0.370	0.04	0.152	0.370	0.232	0.912	0.256	***
Valine	0.17a	0.45b	**0.001**	0.17a	0.20a	0.47b	***	0.15a	0.22a	0.55b	***	0.15a	0.21a	0.48b	***	0.259	0.712	0.327	0.04	0.259	0.327	0.712	**0.002**	**0.007**	***
Non-essential amino acids																									
Alaine	0.89a	1.09b	**0.028**	0.85a	0.86a	1.07b	***	0.81a	0.90a	1.17b	***	0.75a	0.81a	1.08b	***	0.307	0.926	0.876	0.10	0.307	0.876	0.926	**0.007**	0.800	**0.028**
Arginine	0.19	0.36	0.059	0.18a	0.22a	0.40b	***	0.15a	0.22a	0.46b	***	0.15a	0.20a	0.40b	***	0.788	0.533	0.669	0.06	0.788	0.669	0.533	0.121	**0.011**	***
Asparagine	0.03a	0.08b	***	0.03a	0.04b	0.09c	***	0.03a	0.04a	0.11b	***	0.02a	0.04a	0.09b	***	0.168	0.615	0.395	0.01	0.168	0.395	0.615	**0.026**	***	***
Aspartic acid	0.01a	0.07b	**0.008**	0.01a	0.03a	0.07b	**	0.01a	0.02a	0.08b	**	0.01a	0.01a	0.05b	**	0.441	0.926	0.388	0.02	0.441	0.388	0.926	**0.009**	***	***
Glutamine	1.94	2.11	0.141	1.95a	1.99ab	2.17b	**0.030**	1.87a	1.83a	2.29b	**0.040**	1.67a	1.73ab	2.13b	**0.030**	0.721	0.854	0.965	0.28	0.721	0.965	0.854	**0.034**	***	0.458
Glutamic acid	0.04a	0.15b	**0.005**	0.04a	0.07b	0.17c	***	0.03a	0.07a	0.21b	***	0.03a	0.06a	0.16b	***	0.709	0.461	0.216	0.02	0.709	0.216	0.461	***	***	***
Glycine	0.28a	0.39b	**	0.29a	0.30a	0.39b	***	0.29a	0.30a	0.43b	***	0.27a	0.28a	0.40b	**	0.464	0.923	0.637	0.03	0.464	0.637	0.923	0.439	***	***
Hydroxyproline	0.01	0.02	0.585	0.01	0.01	0.01	0.623	0.01	0.01	0.02	0.684	0.01	0.01	0.01	0.247	0.316	0.858	0.960	0.00	0.316	0.960	0.858	***	***	***
Proline	0.11a	0.18b	**0.004**	0.11a	0.10a	0.16b	**	0.10a	0.11a	0.21b	***	0.09a	0.11a	0.16b	***	0.373	0.667	0.332	0.02	0.373	0.332	0.667	**0.004**	***	**0.002**
Serine	0.16a	0.41b	**0.001**	0.22a	0.23a	0.44b	**0.001**	0.14a	0.21a	0.50b	***	0.13a	0.19a	0.44b	***	0.633	0.609	0.474	0.05	0.633	0.474	0.609	0.145	***	***
Tyrosine	0.07a	0.31b	**0.001**	0.09a	0.11a	0.35b	**0.011**	0.06a	0.12a	0.42b	***	0.06a	0.11a	0.37b	***	0.233	0.426	0.942	0.02	0.233	0.942	0.426	0.126	0.073	***
EAAs	0.78a	2.85b	**0.001**	0.78a	1.11a	3.11b	***	0.73a	1.21a	3.67b	***	0.72a	1.14a	3.15b	***	0.356	0.536	0.301	0.31	0.578	0.536	0.820	**0.014**	**0.019**	***
% EAAs	17.29a	35.62b	***	17.10a	21.76b	36.89c	***	17.30a	23.96b	38.43c	***	18.37a	24.10b	37.32c	***	0.552	0.607	0.488	1.75	0.870	0.723	0.670	**	***	**0.012**
Total FAAs	4.51a	8.00b	**0.001**	4.56a	5.10a	8.43b	***	4.22a	5.05a	9.55b	***	3.92a	4.73a	8.44b	***	0.485	0.671	0.558	0.79	0.530	0.503	0.963	0.056	**0.003**	***

BD: In-bag dry-ageing; W: Wet ageing; SA: Stepwise Ageing; AV: Air Velocity; AT: Ageing Time. SED is the standard error of a difference between means. T1: BD at 0.5 $m.s^{-1}$ for 21 days; T2: BD at 0.5 $m.s^{-1}$ 7 days + W for 14 days; T3: BD at 1.5 $m.s^{-1}$ for 7 days + W for 14 days; T4: BD at 2.5 $m.s^{-1}$ for 7 days + W for 14 days. $P < 0.0001$ presented as *** for level of significance. $P < 0.001$ presented as ** for level of significance. Different letters of "a, b or c" within the same row mean results are significantly different from each other ($P < 0.05$).

Table 4.4 Effect of ageing time on the release of FAAs (mg/g protein) of frozen dry-aged lean beef at different digestion stages: undigested phase (0 min), 30 min, 120 min and the gastric phase of the digestion simulation (240 min).

	0 min			SED	*P*-AT	30 min			SED	*P*-AT	120 min			SED	*P*-AT	240 min			SED	*P*-AT
	0d	7d	21d			0d	7d	21d			0d	7d	21d			0d	7d	21d		
Essential amino acids																				
Histidine	0.07a	0.11a	0.20b	0.02	***	0.01	0.01	0.02	0.00	0.402	0.01a	0.02ab	0.02b	0.00	**0.043**	0.02	0.02	0.02	0.00	0.214
Isoleucine	0.09a	0.16a	0.58b	0.04	***	0.09a	0.15a	0.63b	0.04	***	0.11a	0.19a	0.59b	0.04	***	0.88a	0.92a	1.48b	0.07	***
Leucine	0.15a	0.30b	1.04c	0.06	***	0.18a	0.32a	1.15b	0.07	***	0.26a	0.43a	1.14b	0.07	***	8.41a	8.11a	9.28b	0.19	**
Lysine	0.07a	0.09a	0.57b	0.05	***	0.15a	0.19a	0.74b	0.06	***	0.22a	0.27a	0.72b	0.07	***	3.06	3.22	3.46	0.18	0.094
Methionine	0.04a	0.12b	0.48c	0.02	***	0.05a	0.15b	0.62c	0.04	***	0.10a	0.24b	0.66c	0.04	***	1.33a	1.40a	1.87b	0.06	***
Phenylalanine	0.07a	0.15a	0.48b	0.03	***	0.13a	0.21a	0.67b	0.04	***	0.55a	0.45b	0.88c	0.04	***	10.20a	10.22a	11.22b	0.22	***
Threonine	0.10a	0.21a	0.63b	0.06	***	0.15a	0.19a	0.63b	0.05	***	0.23a	0.23a	0.59b	0.04	***	0.54a	0.62a	1.30b	0.09	***
Tryptophan	0.03a	0.05a	0.14b	0.01	***	0.05a	0.06a	0.21b	0.01	***	0.08a	0.09a	0.21b	0.01	***	2.27a	2.28a	2.61b	0.08	***
Valine	0.16a	0.25a	0.79b	0.05	***	0.17a	0.25a	0.85b	0.05	***	0.18a	0.32b	0.83c	0.06	***	0.65a	0.72a	1.29b	0.06	***
Non-essential amino acids																				
Alaine	0.98a	1.21ab	1.27b	0.12	**0.033**	1.26a	1.37a	2.00b	0.11	***	1.17a	1.71b	2.18c	0.13	***	1.53a	1.62a	2.24b	0.08	***
Arginine	0.25a	0.35a	0.66b	0.04	***	0.19a	0.28a	0.66b	0.06	***	0.18a	0.26a	0.54b	0.04	***	9.77	10.33	10.83	0.73	0.247
Asparagine	0.05a	0.09b	0.24c	0.01	***	0.07a	0.10a	0.35b	0.03	***	0.08a	0.14b	0.37c	0.03	***	0.18a	0.21a	0.44b	0.03	***
Aspartic acid	0.03a	0.04a	0.15b	0.02	***	0.03a	0.03a	0.17b	0.02	***	0.05a	0.08a	0.18b	0.03	**	0.08a	0.09a	0.23b	0.02	***
Glutamine	2.74	2.93	2.84	0.17	0.494	3.25	3.24	3.83	0.35	**0.068**	3.70	3.33	3.71	0.35	0.747	5.29	5.80	5.84	0.82	0.730
Glutamic acid	0.14a	0.22a	0.44b	0.07	***	0.15a	0.21a	0.47b	0.03	***	0.35a	0.22b	0.50c	0.03	***	0.56a	0.70a	1.49b	0.11	***
Glycine	0.40a	0.44a	0.60b	0.03	***	0.46a	0.44a	0.84b	0.05	***	0.45a	0.54a	0.82b	0.06	***	0.64a	0.64a	0.98b	0.06	***
Hydroxyproline	0.04ab	0.05a	0.04b	0.00	**0.016**	0.10a	0.12a	0.29b	0.02	***	0.10a	0.14a	0.28b	0.02	***	0.35a	0.37a	0.57b	0.03	***
Proline	0.09a	0.10a	0.20b	0.02	***	0.09a	0.10a	0.20b	0.02	***	0.12a	0.12a	0.18b	0.02	**	0.16a	0.17a	0.29b	0.03	***
Serine	0.19a	0.34b	0.77c	0.04	***	0.23a	0.32a	1.00b	0.07	***	0.26a	0.40a	0.96b	0.07	***	0.35a	0.48a	1.11b	0.09	***
Tyrosine	0.11a	0.19a	0.59b	0.04	***	0.13a	0.19a	0.69b	0.05	***	0.19a	0.27a	0.73b	0.04	***	8.13a	7.87a	8.84b	0.19	**0.001**
EAAs	0.63a	1.18a	4.12b	0.33	***	0.81a	1.29a	4.66b	0.44	***	1.56a	1.92a	4.81b	0.39	***	26.71a	26.78a	31.24b	0.91	***
% EAAs	10.88a	15.79b	32.38c	1.11	***	11.75a	16.05b	29.10c	1.70	***	18.62a	20.14a	29.88b	1.81	***	48.98	48.00	47.75	1.04	0.199
Total AAs	5.79a	7.48b	12.72c	0.65	***	6.93a	8.04a	16.02b	1.03	***	8.38a	9.51a	16.11b	0.98	***	54.53a	55.79a	65.43b	1.75	***

AAs, EAAs and AT refer to Amino Acids, Essential Amino Acids and Ageing Time, respectively. SED is the standard error of a difference between means. Different superscript letters "a, b or c" within the same row at the same digestion time mean results significantly differed from each other ($P < 0.05$); $P < 0.0001$ presented as *** for level of significance. $P < 0.001$ presented as ** for level of significance.

4.3.4.1 SDS-PAGE gel electrophoresis

The representative SDS-PAGE protein profile of frozen dry-aged beef samples following 4 hours (0, 30, 120 and 240 min) of simulated gastrointestinal digestion is shown in Figure 4.4a. For quantitative analysis, the protein bands were further grouped into three lots: 30 - 260 (high), 10 - 30 (intermediate) and 3.5 - 10 kDa (low). Fold changes relative to 0 min of the optical density of the three protein groups during the simulated digestion are shown in Figure 4.4b. Overall, the high MW (30 - 260 kDa) protein fragments including filamin, titin and nebulin (> 250 kDa) and myosin heavy chain (MHC, > 220 kDa), were digested rapidly by pepsin within the first 30 min of gastric digestion into smaller proteins including myomesin family (156 kDa) and other proteins < 110 kDa. Most of the intermediate protein fragments (10 - 30 kDa) were cleaved into low MW peptides in the first 30 min of digestion. Also, the optical density of intermediate and high MW protein fragments from 30 min of gastric digestion was reduced ($P < 0.05$) to less than a half (fold change < 0.5) of the undigested counterparts along with an increase (fold change > 40) in low MW peptides (Figure 4.4a). After 120 min of gastric digestion, there were only five major protein bands observed in the high MW protein group, which may include α-actinin (91 kDa), myosin family (71 kDa), tropomyosin-β-chain (38 kDa), troponin T (36 kDa) and tropomyosin-α-chain (34 kDa). The intensity of intermediate proteins significantly decreased while that of smaller peptides increased by 120 min of gastric digestion (Figure 4.4b).

Following 120 min of pancreatic digestion, large protein fragments were further cleaved by pancreatic enzymes into four main protein bands (91, 73, 68 and 38 kDa) and several bands with low densities which were detected between 10 and 60 kDa (Figure 4.4a). The optical density of low MW peptides decreased significantly at the end of the simulated digestion compared to those at 30 and 120 min. There was no difference of protein profile observed between different ageing time except for the progressive cleavage of protein fragments at 91 kDa (α-actinin) and accumulation of smaller proteins from myosin family at 72 and 68 kDa with increase of the ageing time (Figure 4.4a).

4.3.4.2 Release of FAAs

FAAs were progressively released over the gastric digestion and increased ($P < 0.05$) by the end of pancreatic digestion (Table 4.4). The hydrolysates collected at the end of pancreatic digestion were dominated by essential AAs, including leucine, lysine, methionine, phenylalanine and tryptophan, and non-essential AAs such as alanine, arginine glutamine and tyrosine. FAAs content from 21 days of ageing was higher ($P <$

0.05) than that of the 7 days and unaged counterparts across the digestion simulation except for glutamine and histidine which did not differ between the ageing time. The % essential AAs increased significantly with the ageing time during the gastric digestion, but not at the end of pancreatic digestion.

4.3.4.3 Protein content in the digesta

The protein content (protein fragments and peptides) in the final digesta following 240 min simulated digestion was determined. About 60% of the proteins were hydrolysed with about 40% of the hydrolysates remaining in the digests (Figure 4.3c). Ageing for 7 and 21 days reduced ($P < 0.05$) the protein content in the digesta compared to the unaged equivalents.

4.3.4.4 Relative protein digestibility (%)

An increase of relative protein digestibility ($P < 0.05$) with ageing time was observed when calculated in three different ways, as shown in Figure 4.4c. The use of SDS-PAGE quantitation resulted in higher relative digestibility results (~70%) compared to the protein content (~60%) with the lowest values shown in FAAs (~5%). However, similar findings were observed from all three methods that longer dry-ageing time (21 days) improved ($P < 0.05$) protein digestibility. A moderate but significant correlation was detected between the relative digestibility determined from SDS-PAGE and final protein content ($r = 0.42$, $P = 0.005$) and release of FAAs ($r = 0.43$, $P = 0.004$). However, there was no correlation found between the release of FAAs and final protein content ($P > 0.05$).

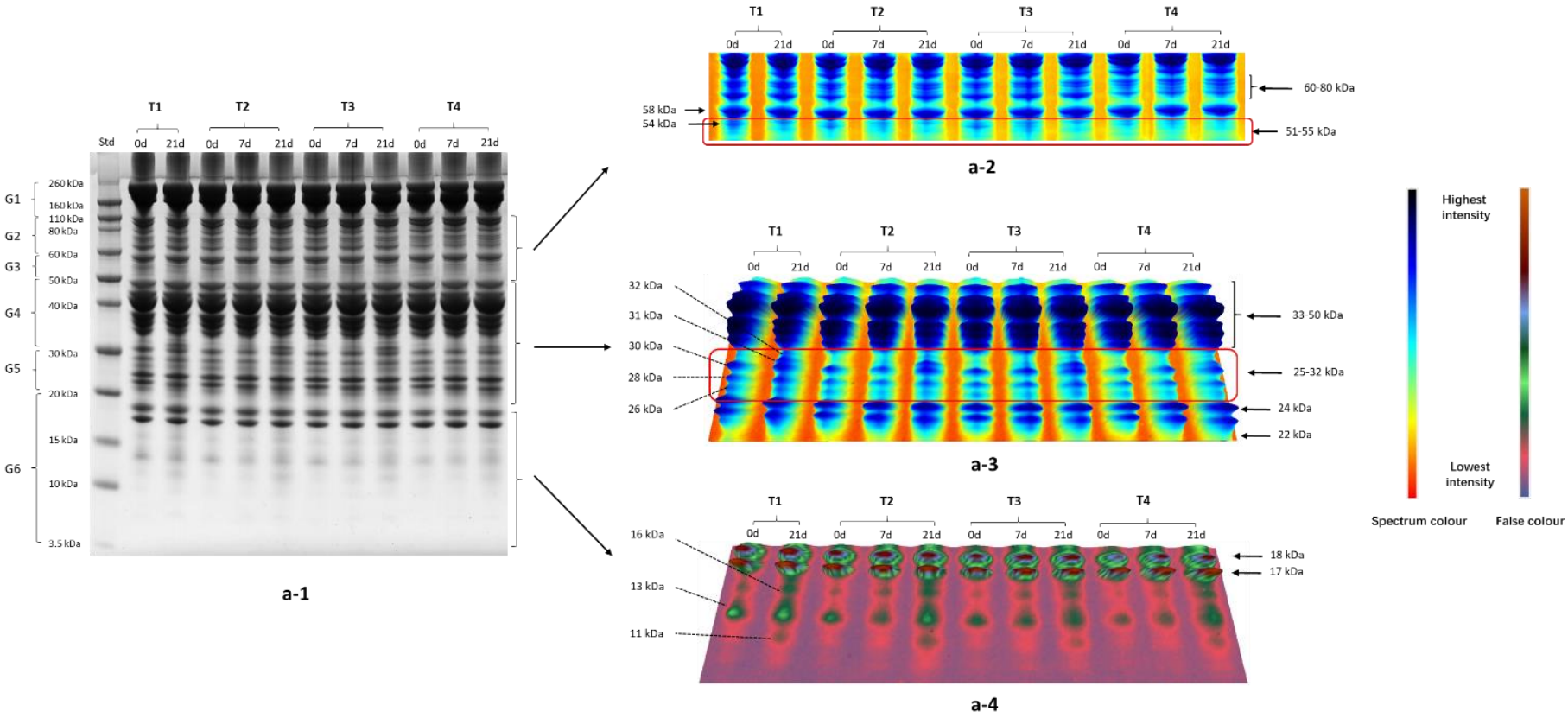
4.4 Discussion

4.4.1 Oxidative stability

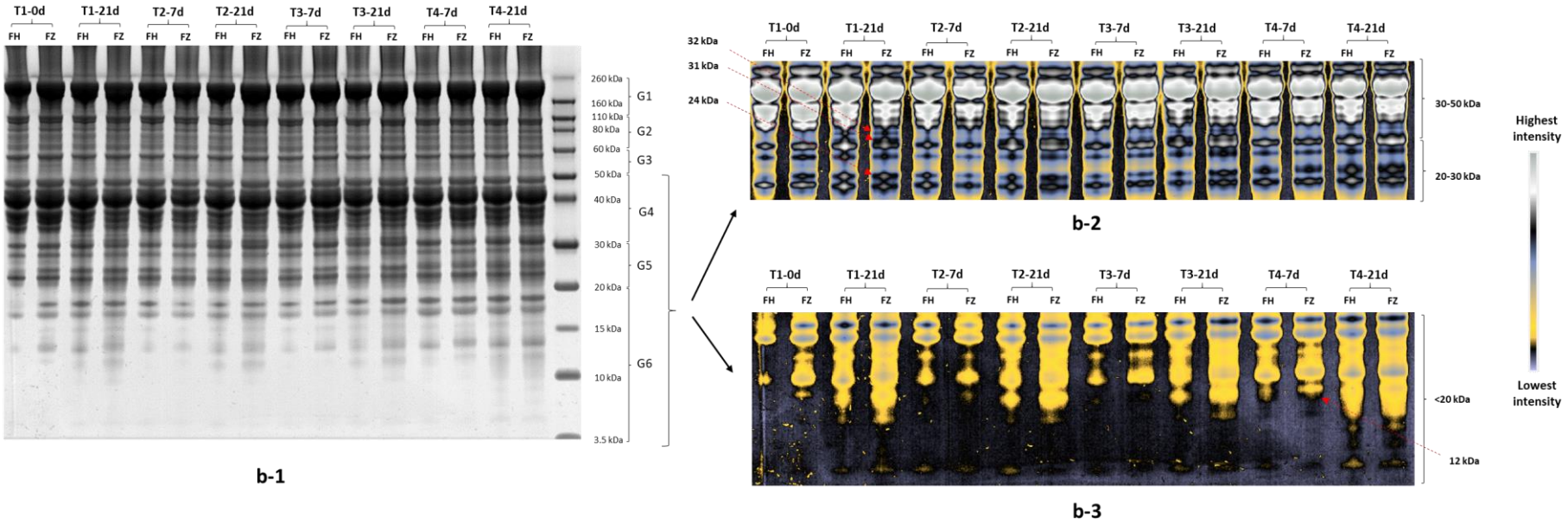
Limited and inconsistent findings on the effect of dry-ageing on oxidative stability of beef products have been reported (DeGeer et al., 2009; Ha et al., 2019; Jiang et al., 2010). In the present study, increasing the air velocity for dry-ageing and the use of stepwise ageing treatment had no significant impact on the oxidative stability of lipids and proteins. This may be due to the barrier function of the dry-ageing bag, as oxygen availability becomes lower, thereby reducing the potential of oxidation. Lower TBARS levels have been reported for in-bag dry-aged beef loins compared to the traditional dry-aged counterparts (DeGeer et al., 2009), suggesting the prevention of oxidative deterioration with the use of ageing bags. Additionally, lean beef (approx. 1% fat) was used in this study, which reduced the availability of reactive substrates (UFAs) and the

risk of oxidation. This could consequently improve the oxidative stability of the dry-aged products during storage. An increase of oxidative potential with ageing time through the increase of TBARS content was also observed, though the overall lipid oxidation level remained low in this study. Ageing has been reported to contribute towards lipid oxidation (TBARS) of dry-aged beef (Ha et al., 2019; Parrish et al., 1991). The increase in the level of MDA may be due to the oxidation of MUFAs, which had reduced with ageing time in this study (Figure 4.1). Decrease of MUFAs could be associated with lipolysis during ageing which releases free MUFAs and being easier to be oxidised compared to the bounded PUFAs.

Protein carbonyl is a natural constituent of live animal tissues usually at a level of 1-2 nmol/mg. Protein carbonylation during rigor mortis and post-mortem (wet) ageing of beef has been reported (Martinaud et al., 1997; Sante-Lhoutellier et al., 2008). In this study, a significant increase of total carbonyl content of in-bag dry-aged lean beef with ageing time was observed. This could be attributed to the release of catalytic iron and oxidizing enzymes, modification of cellular compartmentalisation and propagation of lipid oxidation (Estévez, 2011). Beef is known to be more susceptible to protein carbonylation than pork and chicken as the higher levels of iron and myoglobin in beef promote protein carbonylation (Lund et al., 2007). The protein carbonyl content ranged between 2 and 3 nmol/mg in this study, which was still at a low level compared to the other studies on beef *longissimus lumborum* (3.1~5.1 nmol/mg) and *diaphragma pedialis* (4.8~6.9 nmol/mg) from 10 days of ageing (Martinaud et al., 1997). On the other hand, the concomitant occurrence of lipid and protein oxidation suggests the potential interaction between the two processes as seen in the current study – with a significant correlation ($r = 0.45$, $P < 0.0001$) between TBARS and carbonyl content (Mercier, Gatellier, & Renerre, 1995; Park et al., 2006). The relationship between lipid and protein oxidation remains unclear; however, it can be speculated that the reactive and non-reactive species between lipids and proteins derived from oxidative damage under free-radical chain reaction mechanism may be involved (Estévez, 2011; Gardner, 1979). Once the autoxidation of lipids and proteins commences, lipids would undergo faster oxidative degradation and produce reactive oxygen species, such as peroxyl radicals ($ROO^{\cdot}$) and hydroperoxides, which would contribute towards the protein carbonylation by attacking susceptible amino acid side chains (Park et al., 2006).

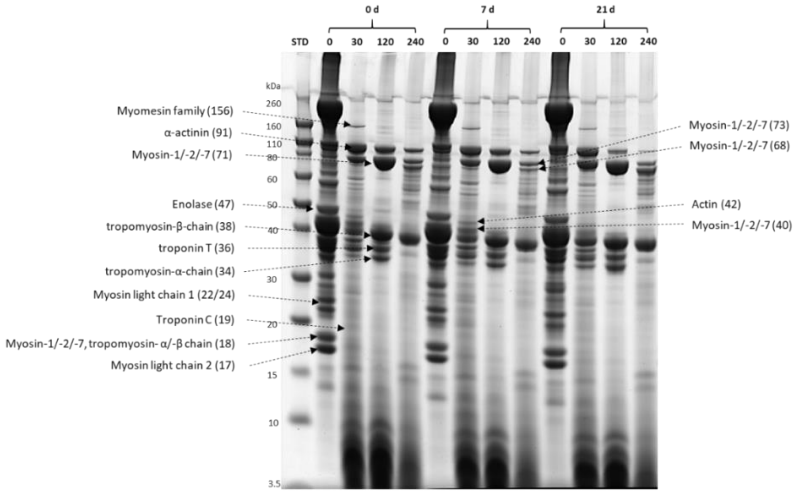


a. Fresh in-bag dry-aged lean beef

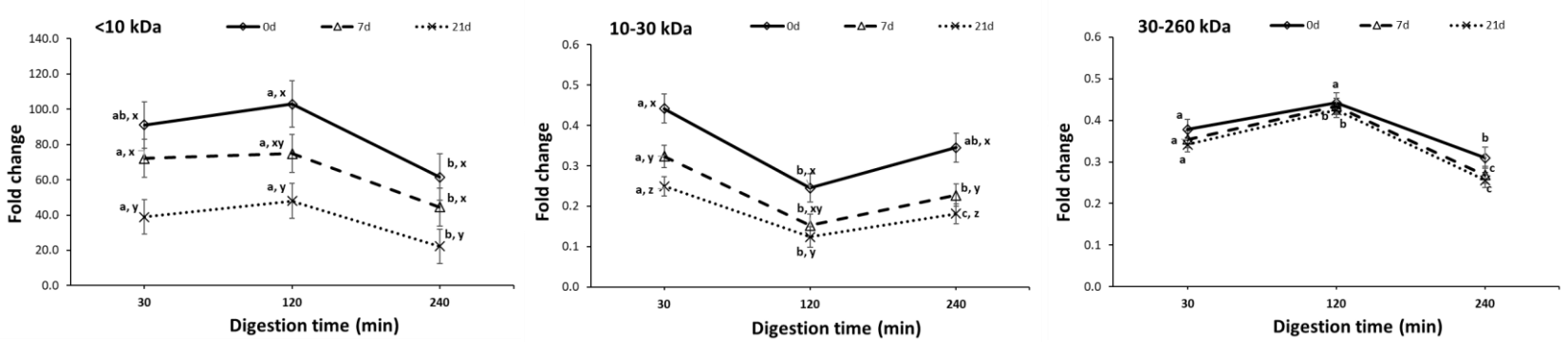


b. Fresh *vs.* frozen in-bag dry-aged lean beef

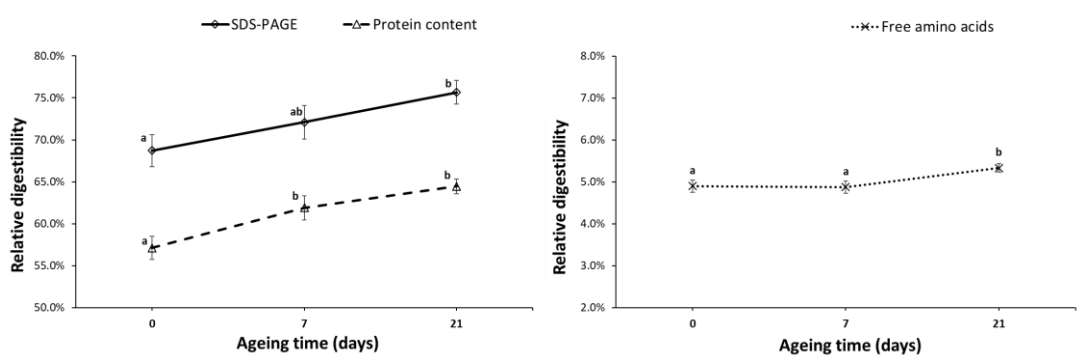
Figure 4.3 (a) Representative SDS-PAGE images of whole muscle proteins of fresh in-bag dry-aged lean beef by four ageing treatments (T1 - T4) across three ageing time (0, 7 and 21 days) and BD: in-bag dry ageing, W: wet-ageing. T1 = BD at 0.5 m.s^{-1} for 21 days; T2 = BD at 0.5 m.s^{-1} for 7 days + W for 14 days; T3 = BD at 1.5 m.s^{-1} for 7 days + W for 14 days; T4 = BD at 2.5 m.s^{-1} for 7 days + W for 14 days. Six summative protein regions were used for analysis (G1 - G6). Protein region G2-G5 in a-2 and a-3 were presented in spectrum colour, and G6 (a-4) was presented in false colour. A 3-D imaging model was applied in a-3 and a-4. (b) Whole muscle proteins of frozen (FZ) in-bag dry-aged lean beef as compared to the fresh (FH) counterparts. Protein region G4-G6 in b-2 and b-3 were presented in gold silver colour.



a. *In vitro* digestion of frozen in-bag dry-aged lean beef



b. Fold change of protein band optical intensity



c. Relative digestibility

Figure 4.4 (a) Representative SDS-PAGE images of proteins profile of in-bag dry-aged lean beef by three ageing time (0, 7 and 21 day) at four sampling time points (0, 30, 120 and 240 min) across the gastrointestinal digestion simulation. (b) Fold change of predicted means (± SEM) of protein band optical intensity in three molecular weight groups (<10 kDa, 10-30 kDa and 30-260 kDa) between three ageing time (0, 7 and 21 days) over the digestion period (30, 120 and 240 min). Different letters of "a, b or c" within the same line (between digestion time) mean results are significantly different from each other ($P < 0.05$). Different letters of "x, y or z" within the same digestion time (between ageing time) mean results are significantly different from each other ($P < 0.05$). (c) Relative digestibility (% predict means ± SEM) of in-bag dry-aged bull beef by three ageing time (0, 7 and 21 days) followed by frozen storage for 12 months. Different letters of "a, b or c" within the same line (between ageing time) mean results are significantly different from each other ($P < 0.05$).

4.4.2 Proteolysis

Meat undergoes proteolysis during post-mortem ageing resulting in improved tenderness and flavour. In this study, air velocities and stepwise ageing had no impact on the post-mortem protein degradation (SDS-PAGE). Ageing time was the critical factor in degradation of structural and cytoskeletal proteins. Appearance of protein fragments of approximately 32 kDa and 31 kDa after ageing are associated with enzymatic degradation of troponin T (Negishi, Yamamoto, & Kuwata, 1996) and actin (Longo, Lana, Bottero, & Zolla, 2015). These two protein fragments may be used as markers to indicate the degree of tenderness achieved. Degradation of desmin (54 kDa) during 21 days of ageing was observed in this study, which is known to be concomitant with meat tenderisation (Lomiwes et al., 2014).

Post-mortem ageing results in a significant increase in the level of FAAs (Feidt, Petit, Bruas-Reignier, & Brun-Bellut, 1996) which was also observed in the current study. Dry-ageing has been reported to produce significantly higher level of FAAs compared to the wet-aged (Kim et al., 2016; Lee et al., 2019a). However, it is more likely to be due to dehydration during the dry-ageing process, as it concentrates the FAAs content. The stepwise ageing process showed no significant difference on the protein degradation compared to straight-dry-ageing regime, as this was measured on dry basis. Both dry- and wet-ageing have therefore undergone similar enzymatic protein degradation process and generated equivalent amounts of degradation products within the same period of ageing time. Ageing time, on the other hand, played the major role in generation of FAAs during post-mortem storage. Increased ($P < 0.05$) level of FAAs was only found at 21 days of ageing compared to 7 days and unaged samples. Similar finding was also reported by Koutsidis et al. (2008) that the FAAs content did not change within the first 7 days of W. Our previous study suggested that 7 days of ageing could be sufficient to improve the tenderness (Zhang et al., 2019); however, extended ageing time is required to improve the flavour intensity of dry-aged products. FAAs are important flavour precursors which contribute towards the improvement of taste and flavour intensity of meat products (Koutsidis et al., 2008). Increase of umami and buttery taste in beef positively correlate with increased FAAs released from longer (21 days) ageing time (Koutsidis et al., 2008). Significant increases of sweet (alanine, glycine, proline, serine and threonine) and umami taste FAAs (aspartic acid, asparagine, glutamic acid, methionine and lysine) observed in the current study would have contributed towards enhancing the beef flavour (Pereira-Lima, Ordoñez, de Fernando, & Cambero, 2000). The largest increases in AAs were

associated with the bitter taste AAs after ageing for 7 and 21 days. These AAs mainly consisted of essential AAs (isoleucine, leucine, methionine, phenylalanine, tryptophan) and tyrosine. Bitterness is also an important constituent to balance the desirable taste and flavour of meat (Khan et al., 2015).

Another important role of FAAs in the desirable meat flavour formation is being the crucial precursors of volatile flavour compounds during cooking, where the degradation of FAAs, or interaction with other flavour precursors such as reducing sugars, peptides and organic acids are involved (Spanier, Flores, McMillin, & Bidner, 1997). A sulfur-containing AA – methionine – showed the largest increment (fold change > 20) during ageing. Methionine breaks down during cooking and generates sulfur-containing volatiles, such as pyrazines, oxazoles, thiophenes, thiazoles and other heterocyclic sulfur containing compounds, which contributes to the cooked beef flavour. Other aroma compounds, such as Strecker aldehydes (2- and 3-methylbutanals) and pyrazines, are formed from the flavour precursors consisting of isoleucine, leucine, serine, threonine, valine and phenylalanine. These amino acids increased significantly following ageing.

4.4.3 Frozen storage

"Aged then frozen" strategy has been reported to provide beef products with acceptable meat and sensorial quality (Coombs, Holman, Friend, & Hopkins, 2017b). However, the combination of ageing and freezing should be considered with caution to obtain optimum quality of frozen products, as the length of ageing time could affect the meat quality and oxidative stability during long-term frozen storage (Holman, Coombs, Morris, Bailes, & Hopkins, 2018). In this study, improved oxidative stability of lipids and proteins was observed in frozen stored beef samples that have been aged for 7 and 21 days compared to the unaged counterparts. The current findings agreed with our previous study that long-term frozen storage had no negative impact on the quality and sensory acceptability of in-bag dry-aged lean beef (Zhang et al., 2019).

Frozen storage may result in a slower but ongoing lipid oxidation compared to the ageing process. Lipids (UFAs) undergo initiation and early propagation stages of auto-oxidation and produce peroxides which can subsequently break down by the action of peroxidase enzymes to form lower MW secondary products, such as ketones, epoxides and aldehydes including MDA, which is measured as TBARS. The overall TBARS level (0.23 - 0.33) found in both unaged and aged lean beef products following the long-term frozen storage was lower than the threshold for consumer perception of rancidity (above 1 - 2.6) and results reported in other studies on beef (approx. 0.5 - 1.7) (Holman et al.,

2018). This could be explained by the low-fat content (approx. 1%) of lean bull beef used in the present study which reduced the level of oxidizable substrates (UFAs) during ageing and frozen storage. Another possible reason could be the use of oxygen-limiting packaging during ageing (ageing bag) and frozen storage (vacuum barrier bag) which reduced the rate of oxidation by reducing the oxygen concentration in the meat environment (Rogers, 2007).

TBARS have been reported to peak following long chilled storage (3~4 weeks) (Coombs et al., 2018b; Farouk & Wieliczko, 2003) or frozen period up to 9 months (Muela, Monge, Sañudo, Campo, & Beltrán, 2015), then decrease or stabilise after a longer period of ageing or frozen storage. The decrease of TBARS could be explained by the reduced level of lipid peroxidation product – MDA in the sample matrix. During storage, MDA may further degrade into organic alcohols and acids, or attach to FAAs and proteins as MDA-AAs complex (Farouk & Wieliczko, 2003), or as a precursor to protein oxidation (Xiong, 2000). These changes cannot be detected using the TBARS assay. Therefore, the variation of TBARS during ageing and frozen storage observed in the current and other studies (Coombs et al., 2018b; Holman et al., 2018) could have resulted from different reaction rates between generation and degradation of MDA during the storage, which may be affected by the availability of oxidising substrates (fat, oxygen and iron), muscle types and storage conditions (temperature and time).

Protein carbonylation of dry-aged products following the long-term frozen storage has not been studied. Meat proteins would undergo carbonylation during frozen storage and this may be due to the freezing practice which causes irreversible damage to the protein ultrastructure, resulting in release of mitochondrial and lysosomal enzymes and iron which triggers protein carbonylation (Xiong, 2000). Generation of oxidising lipids could also contribute to the increased level of protein carbonyl during frozen storage. No significant changes of protein carbonyl content in aged then frozen lamb samples have been reported throughout different combinations of ageing (up to 8 weeks) and frozen storage (up to 52 weeks) times (Coombs et al., 2018a). Similar findings were also observed in the current study that there were only marginal changes in the dry-aged lean beef samples (7 and 21 days) with increment of protein carbonyl content detected in the unaged counterparts. This may be explained by delayed oxidative reaction in unaged samples compared to the aged counterparts, caused by the frozen storage resulting in slower oxidation process than the chilled. The overall protein carbonyl contents of frozen stored lean beef samples in this study remained at low level (< 3 nmol.mg^{-1} protein). Thus,

the effect of protein oxidation on the meat quality is negligible. It could be attributed to the low-fat content of lean beef samples used in this study. As suggested by Utrera, Morcuende, and Estévez (2014), the increase of fat content could significantly promote the oxidative damage of proteins during frozen storage.

Proteolysis pattern of dry-aged then frozen meat products has not been reported in the literature. Frozen storage has been suggested to cause degradation of myofibrillar proteins by proteolysis and result in an increase in instrumental tenderness (reduced shear force) and variations of protein profile (SDS-PAGE) (Ali et al., 2015). However, the increase of proteolysis rate (interpreted by the improvement of tenderness) seems to be associated with the tenderisation by proteases during thawing process (Crouse & Koohmaraie, 1990). Large MW proteins (> 110 kDa) significantly decreased after long-term frozen storage with accumulation of lower MW protein fragments (< 50 kDa), especially the potential proteolysis biomarkers degraded from troponin T at 32 kDa and actin at 31 kDa, suggesting the occurrence of proteolysis post frozen storage. This is likely to be due to the formation of intracellular ice crystals between degraded myofibrillar proteins, weakening the protein structure and making it to be more susceptible to proteolysis during storage (Coombs et al., 2017b). Therefore, protein fragmentation would be more evident in meat proteins that have been aged prior to frozen storage. There was significant increase of FAAs following long-term frozen storage in dry-aged compared to non-aged samples in the present study as evidenced in the aged beef samples (7 and 21 days) compared to the unaged. The loss of some FAAs (mainly EAAs) in unaged and in short-term aged samples (7 days) could be attributed to the oxidative damage that took place especially in unaged samples.

4.4.4 Digestibility of frozen stored dry-aged beef

Protein oxidation has been suggested to affect meat digestibility in different ways, mainly depending on the extent of the oxidative damages. The decrease of protein digestibility due to oxidative damage could result from protein polymerisation that reduced the vulnerability of proteins to the digestive enzymes (Rysman et al., 2016). However, a mild protein oxidation is generally believed to improve the digestibility by minor modifications and partial unfolding of protein structures, which increase enzyme accessibility to protein cleavage sites. This basis may partially explain the similar digestibility observed between unaged and dry-aged beef samples for 7 days.

Ageing, on the other hand, can improve the digestibility of meat protein (Farouk et al., 2014). The fold change of protein bands intensity decreased with the ageing time for

all three protein groups in our study. The significant increments observed at intermediate (10 - 30 kDa) and low (< 10 kDa) MW protein groups can be explained by the extent of proteolysis with ageing time prior to digestion. BD for longer ageing time (21 days) resulted in significantly increased level of low MW peptides (< 10 kDa) before digestion as compared to the unaged counterparts. However, the intensity of intermediate and large protein groups (30 - 260 kDa) remained unchanged across the ageing time. Following digestion, dry-aged beef proteins were more susceptible to digestive enzymes and resulted in earlier cleavage of degraded protein by pepsin into lower MW peptides and FAAs. This is well supported by significantly reduced levels of intermediate and small sized proteins with the increment of FAAs found in beef samples dry-aged for 21 days. Lack of significant change in digestibility with ageing time has been reported on beef aged for a short period of time (< 7 days) (Rysman et al., 2016). Sante-Lhoutellier et al. (2008) also reported that the digestibility of lamb loins (chill stored at 4 ºC for 7 days using air-permeable wrapping) remained unchanged during gastric digestion, but significantly improved by the digestion of pancreatic proteases (trypsin and α-chymotrypsin). They attributed the improvement of digestibility with the oxidative state of proteins after storage, where more recognition sites favourable for the cleavage by pancreatic proteases were provided. Extended ageing time to 21 days is known to improve the digestibility of beef (Farouk et al., 2014), which was also evident in our study. A significant increase of relative protein digestibility of dry-aged lean beef for 21 days was detected using three different measurements in this study as compared to unaged counterparts. Therefore, it can be concluded that improvement of digestibility by ageing requires sufficient period of ageing time.

Inconsistent findings on protein digestibility by other studies could have been associated with variations arising from different protocols of digestion simulation (e.g. enzyme concentration) and methods of identifying and quantifying proteins, peptides and FAAs. Three measurements were carried out to verify the digestibility findings in this study. The relative digestibility evaluated by SDS-PAGE were positively correlated with protein and FAAs content suggesting the application of these measurements to estimate the relative protein digestibility is reasonable and valid. However, three methods evaluated different aspects of digestion process. Thus, it would be beneficial to include at least two measurements instead of using only one to determine the digestibility when complex multivariate factors are involved.

4.5 Conclusion

Current study supported our hypothesis that the air velocities used in producing dry-aged lean beef had no effect on the oxidative stability of the finished dry-aged product. “Stepwise in-bag ageing” regime can be used to produce dry-aged lean beef products with equivalent oxidative stability and proteolysis patterns to the straight in-bag dry-aged equivalent. The “in-bag ageing” strategy used in this study improved the oxidative stability of lipids and proteins and consequently improved their digestibility following long-term storage at -18 ºC for 12 months.

4.6 Epilogue

This chapter provided deeper insights into the effects of processing regimes and storage conditions on the biochemical changes of in-bag dry-aged lean beef. Outcomes of this chapter supported the previous findings in Chapter 3 that the BD regime can result in a shelf stable dry-aged lean beef product. Air velocities, stepwise ageing and long-term frozen storage had no detrimental impact on the quality and acceptability of in-bag dry-aged lean beef due to the low level of lipid and protein oxidation occurring during the ageing. Ageing time is the primary factor for the quality improvement of in-bag dry-aged lean beef. The proteolytic degradation with ageing time tenderises the meat and releases FAAs which contributes towards the development of unique eating quality.

In the next chapter, the impact of processing factors on the metabolic changes was evaluated by REIMS fingerprinting, as an alternative to the conventional measurements used in Chapters 3 and 4, to explore the potential for real time identification and discrimination of in-bag dry-aged lean beef produced from different processing parameters.

Chapter 5: Use of Rapid Evaporative Ionisation Mass Spectrometry fingerprinting to determine the metabolic changes to dry-aged lean beef due to different ageing regimes

This chapter explores the application of REIMS fingerprinting to perform rapid identification and discrimination of in-bag dry-aged lean beef arising from different processing factors, including air velocity, stepwise in-bag ageing, ageing time and trimming. Different metabolic profiles were found for ageing regimes (stepwise in-bag ageing vs. straight-dry-ageing), ageing time (0 vs. 7 vs. 21 days) and sampling locations (surface meat vs. trimmings vs. trimmed meat), with no difference for air velocities. The underlying biochemical mechanisms responsible for the metabolic changes were also investigated. Small metabolites such as dipeptides and amino acids resulted from proteolysis were more abundant, especially on the surface of untrimmed lean beef, following 21 days of straight-dry-ageing. These small metabolites may be the candidate signatures for the unique quality of dry-aged lean beef.

This chapter is organised as follows. Motivations and aims of this research are described in section 5.1. The ageing regimes, REIMS sampling and data analysis are presented in Section 5.2. Section 5.3 compares the metabolite profile of in-bag dry-aged lean beef arise from different processing regimes using multivariate analysis (PCA and OPLS-DA) of total 1705 metabolites. Metabolites of interests are identified, and the underlying biochemical mechanisms are discussed in Section 5.4. Section 5.5 summarises this chapter.

5.1 Introduction

Dry-ageing is a traditional post-mortem processing technique for the preservation, tenderisation and flavour development in meat. Dry-ageing of beef, which would normally require an ageing period of 3 to 4 weeks, are commonly marketed as artisan meat with a higher price tag and intense flavour profile compared to the wet-ageing (W) equivalents (Iida et al., 2016; Kim et al., 2018). Strong interest in dry-aged meat from meat industries and consumers has led to a need to better understand what occurs during dry-ageing and how this contributes to a value-premium meat product (Laster et al., 2008). Previous studies have demonstrated the improvement of sensory quality using dry-ageing technique, including the generation of distinct and intense buttery, nutty and meaty flavours (Li et al., 2014; Savell, 2008). However, the impact of different ageing conditions (air velocity, temperature and relative humidity) on the quality of dry-aged products is not well understood. Limited studies have reported variations in the quality and microbial compositions of dry-aged beef owing to air velocity (Lee et al., 2019b), temperature and relatively humidity (Kim et al., 2016). It is important to be able to easily

determine these variations for the purpose of quality control and preventing fraud where dry-aged meat is concerned compared to the wet-aged.

The biochemical and biophysical signatures which define and discriminate dry-ageing process from W are yet to be fully determined. Through metabolic profiling, it could be possible to identify compounds that are formed solely from dry-ageing processes, with specifications related to when these compounds appear in relation to the ageing period. Such information would guide both consumers and manufacturers over what to expect for dry-aged beef and provide a reference point for regulation, process improvement and authenticity. The potential signature could include the level of weight loss, certain volatile compounds (O'Quinn et al., 2016) and/or certain groups of metabolites such as unique peptides and amino acids (Kim et al., 2016). Several amino acids, such as tryptophan, phenylalanine, valine, tyrosine, glutamic acid, isoleucine and leucine have been reported to be more abundant in dry-aged beef than the wet-aged presumably due to a greater extent of proteolysis during dry-aging (Kim et al., 2016).

Recently, the use of biomarker/signature-driven food testing to verify food authenticity and to assure quality, has gained traction. Several analytical platforms have been used for metabolic characterisation of meat products, mainly LC-MS and GC-MS mass spectrometric technologies (Trivedi et al., 2016) and isotope ratio mass spectrometry (IRMS) (Piasentier, Valusso, Camin, & Versini, 2003), Fourier transform infrared (FT-IR) spectroscopy (Ellis, Broadhurst, Kell, Rowland, & Goodacre, 2002), and nuclear magnetic resonance (NMR) (Kim et al., 2016). However, the major disadvantages of these techniques are that they often require extensive sample preparation time and labour and require >10 minutes for sufficient data to be acquired, limiting the applicability of the methods outside of the laboratory environment. A high per-sample cost also inhibits the use of these methods for product development and quality control. Further, the measurements undertaken must be able to account for the complexity of meat composition due to the factors including species, breed, age, sex and type of muscle, which will require a panel of biomarker compounds rather than a single marker to adequately characterise the meat products.

One potential tool for performing rapid meat composition measurements is the Rapid Evaporative Ionization Mass Spectrometry (REIMS). REIMS has been used earlier in meat research for proof of principle work including identifying meat from different species and breeds (Balog et al., 2016), authentication of minced beef (Black et al., 2019) and the detection of boar taint in an abattoir setting (Verplanken et al., 2017). The

underlying principle of REIMS is the thermal ablation of tissue samples by electric currents using a surgical knife (also called iKnife) to generate an aerosol, which is ionised allowing the ablated compounds to be detected by mass spectrometry. A real-time (2 - 3 s) determination is possible with no sample preparation needed and results in a reproducible metabolic fingerprint consisting of > 1000 detected features.

Stepwise ageing is a processing regime proposed for industrial application in order to reduce the costs associated with the traditional dry-ageing (D) process without compromising the quality of the dry-aged products (Campbell et al., 2001; Kim et al., 2018). Our previous study assessed the quality and sensory acceptability of lean beef produced using stepwise ageing through combining in-bag dry-ageing (BD) with W (BD 7 days + W 14 days), and compared it to straight-dry-ageing for 21 days and found that the quality attributes measured were similar (Zhang et al., 2019). Thus, the aim of this study was to explore the potential of REIMS to determine the biomolecular difference between in-bag dry-aged lean beef produced using a stepwise-ageing process with different ageing chamber air velocities, compared with equivalent straight-dry-aged lean beef.

5.2 Materials and methods

5.2.1 Ageing regimes

This study was carried out complementary to Zhang et al. (2019) and the duplicities of the experimental design and sample set are acknowledged. Thirty striploins (*Longissimus lumborum*, 1 - 2% crude intramuscular fat, Holstein-Friesian, ~2 years old) from fifteen bull beef carcasses were collected on the day of slaughter from an abattoir and held at 12 °C until rigor. The striploins all reached normal ultimate pH range (5.32-5.36) as previously reported by Zhang et al. (2019). The 30 striploins were assigned to four ageing regimes with a control dry-ageing (T1, n = 6) and three stepwise ageing regimes (T2 - T4, n = 8 for each treatment). The paired loins were assigned to different treatments with 6 single loins of different carcasses for T1 (n = 6); the remaining 24 loins of 15 carcasses (6 single loins + 18 paired loins) were then randomly allocated to treatments T2-T4 with each treatment allocated 8 loins from different carcasses (n = 8). The Control (T1) was a straight-dry-ageing of beef using water-permeable ageing bag (TUBLIN® 10, 50 μm thick, polyamide mix with water vapor transmission rate 920 g/50 μ/m^2/24 h at 7 °C, 50% RH, and oxygen transmission rate 660 g/m^2/24 h at 7 °C, 50% RH, TUB-EX ApS, Denmark) at 2 ± 0.5 °C, 0.5 $m.s^{-1}$ air velocity and relative humidity of 75 ± 5%, for 21 days; The Stepwise-ageing at three air velocities (T2) - (T4) involved:

in bag dry-ageing for 7 days using the same conditions as T1, followed by W in vacuum barrier bags (Cryovac® A600 barrier bag, Sealed Air®, New Zealand) for 14 days using air velocity of 0.5 $m.s^{-1}$ (T2); 1.5 $m.s^{-1}$ (T3) and 2.5 $m.s^{-1}$ (T4). Steak samples (2 cm thickness) were taken from unaged (day 0, T1 - T4), day 7 (T2 - T4) and day 21 (T1 - T4) of ageing. Trimming of the dry surface (approximately 5 mm) was only performed on the steak samples on day 7 or 21 of ageing. To determine the effect of sampling locations on the metabolic profile of lean beef, another five striploins (n = 5) were assigned to T1 ageing treatment but without trimming of dehydrated surface following 21 days of straight-dry-ageing. All samples were stored at -80 °C until analysis.

5.2.2 REIMS

REIMS measurements were carried out using an electronic monopolar surgical knife coupled to a Waters Xevo® G2 qToF mass spectrometer with REIMS interface (Waters Corp. Wilmslow, UK). The surgical knife was set to 'cut' mode and 15 W power (Electrosurgical pencil and Erbe VIO 50C generator, Erbe Medical UK Ltd, UK). Cuts, approximately 2 mm deep and 15 mm long, and 3 seconds per cut, were made directly in thawed meat samples. Five technical replicates for each meat sample were acquired, with a 10 second delay between cut to allow signal to return to baseline and for the electronic knife to be cleaned by wiping with a clean tissue wetted with deionised water and scraping to avoid loss of conductivity. To determine the effect of trimming on metabolic profile, three different sampling locations were used from untrimmed steaks (n = 5): (1) surface meat/crust, but not on the silver skin and fat, (2) trimmings: the area where post-ageing trimming is usually carried out (within 1 cm away from the surface) and (3) the centre of the steak. Samplings for the other treatments (T1 - T4) were carried out at the centre of the steak. The use of five technical replicates provides both better reproducibility and spatial coverage of the meat sample. The resulting aerosol/smoke was evacuated from the sample through a transfer line into the mass spectrometer for ionisation and subsequent analysis. The mass spectral data were acquired in untargeted mode with negative ionisation between mass range of m/z 50-1500 with a scan rate of 0.5 seconds per scan. The instrument was calibrated prior to the sampling using sodium formate and run in sensitivity mode with a mass resolution of 40,000. LC-MS grade isopropanol was infused into the REIMS interface at 100 $\mu L.min^{-1}$ during the measurements.

REIMS data was processed using ProGenesis Bridge (Waters) which splits sample files into new files with each individual replicate and subtracts the baseline of ambient air. Masses were aligned to the m/z for fatty acid C18:1 $[M-H]^-$ ion (281.2481) which was

one of the major ions present in all samples. Data were then processed using ProGenesis QI (Waters) to correct for noise and match the masses against the Human Metabolome Database (http://www.hmdb.ca) and Lipid Maps database (www.lipidmaps.org) with 5 ppm mass error. These database-based identifications can only be tentative as compound identification based on high resolution mass alone and only meets level 2 criteria for identification in metabolomics (Sumner et al., 2007). The identification of the compounds cannot be considered to be strong, yet are useful for understanding what compound classes are impacted by different meat aging methods and time. Here we have reported the m/z values for the significant compounds and discussed those compounds which have been identified and are known to be related to mammalian metabolism. In several cases there are many potential identifications, especially in the case of lipids where there are many isomers. In such cases we have focused on the lipid class rather than identification of individual lipids.

5.2.3 Statistical analysis

An incomplete randomised block design was used in this study with 30 striploins from 15 beef carcasses (n = 30) which were unevenly assigned to four different treatment combinations (1 control dry-ageing; T1; n = 6 and 3 stepwise ageing regimes; T2 - T4; n = 8 for each). Additional 5 loins were collected to determine the effect of sampling locations on REIMS fingerprints. REIMS data analysis was carried out to determine (1) the effect of ageing time (0, 7 and 21 days); (2) the effect of stepwise-ageing (T1 *vs.* T2, 21 days); (3) the effect of air velocities (T2 - T4, 7 days); (4) the interactions of air velocities with stepwise-ageing (T1 - T4, 21 days); and (5) the effect of trimming from three sampling locations.

The normalised abundance of the identified ions (1705 ions) produced from the technical replicates were combined into an average abundance for each sample. Differences between ageing treatments and sampling locations were determined using a combination of multivariate and univariate statistics on the identified ions (total 1705 ions). Principal Component Analysis (PCA), an unsupervised multivariate analysis tool, was used to determine if there was any underlying structure in the data that was not related to the research question (e.g. due to the analysis). Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA), a supervised multivariate analysis tool was used to determine the differences between different ageing treatments and times as this allows the determination of those molecular features which differ the most between treatments. Both PCA and OPLS-DA were done using SIMCA 16 (Umetrics, Sweden).

For OPLS-DA, R^2 (cumulative) and Q^2 (cumulative) scores were used to describe the robustness and accuracy of the present statistical model. R^2 describes the overall variation explained by all components in the model and Q^2 is the measure of robustness of the prediction. OPLS-DA models were of interest if the Q^2 score for predictability was > 0.2. A model with Q^2 score > 0.5 was considered as a good prediction of clusters (Broadhurst & Kell, 2006). Data were scaled to unit variance before OPLS-DA modelling to give all features equal weight in the model irrespective of their abundance.

The normalised mass intensities between treatments were compared using one-way ANOVA with t-test to separate the means at $P < 0.05$. To account for false positive results due to the high number of variables detected, it was assumed that of the results 5 % would have $P < 0.05$ by chance. Of the m/z values that were $P < 0.05$, 5 % of the total number of ions detected were removed (for example, if 1000 ions were detected, and 100 had $P < 0.05$, only the 50 with the lowest P values were considered to be different). Due to the high number of variables that differed between groups, we focused on two groups of ions: (1) dominant metabolites with highest average abundance (minimum threshold = 100); (2) high fold change metabolites with highest (> 1.1) and lowest (< 0.9) fold changes compared to corresponding treatment.

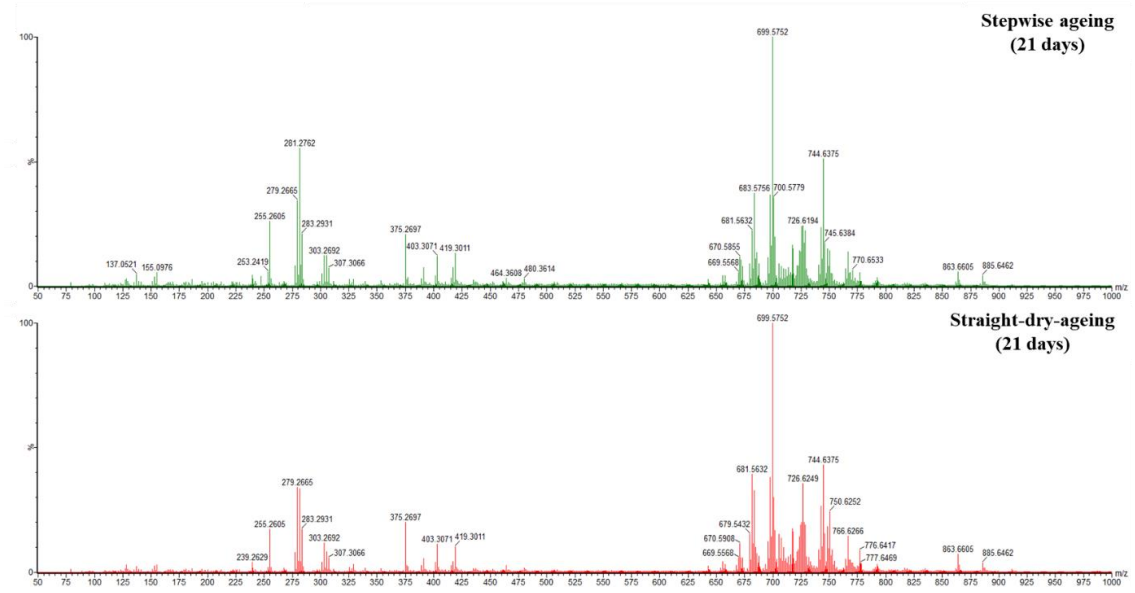
5.3 Results

A total of 1705 ions were detected and tentatively identified from dry-aged beef samples using REIMS. Thus, the current study focused on the ions which have variance ($P < 0.05$) between treatments with greatest fold change and abundance in the sample matrix. Some of the high molecular weight ions (> m/z 600), mainly lipids (Figure 5.1), have only been assigned to one or more lipid classes due to the large number of potential isobaric and isomeric lipids associated with a single high-resolution m/z value. As REIMS does not include any chromatographic separation and nor is there the possibility to do MS/MS fragmentation during standard REIMS operation, it was not possible to further identify these ions. The lipids detected were putatively assigned to a wide range of lipid classes including ceramides (Cer), diacylglycerols (DG), phosphatidic acids (PA), phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG), glycerophosphoinositols (PI) phosphatidylserines (PS) and triacylglycerols (TG). It should be noted that for the purposes of identifying markers related to meat processing, compound identification is not necessary. However, this is an important step for understanding the underlying biological reasons for the changes.

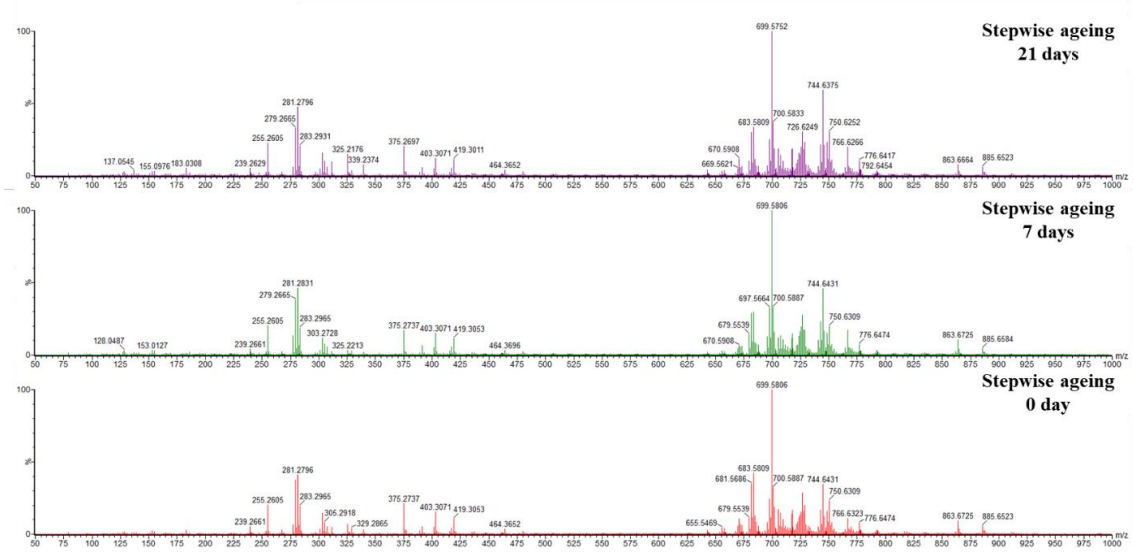
5.3.1 Effect of ageing methods (straight-dry-ageing *vs*. stepwise-ageing)

There was no clear clustering observed between ageing treatments using PCA modelling, and no apparent systematic trend due to run order or batch (data not shown). In-bag stepwise-ageing and straight-dry-ageing had clearly different metabolite profiles based on OPLS-DA modelling (Figure 5.2), with a Q^2 value of 0.85 suggesting that the naive model was robust with good predictability. Around 41% of metabolites differed significantly between the two ageing treatments. Out of those identified metabolites, 194 metabolites (approx. 11.38%) were significantly more abundant in stepwise-aged beef. In the straight-dry-aged beef, 498 metabolites (approx. 29.21%) were significantly more abundant, with the dominant metabolite ions tentatively identified as amino acids, dipeptides, amines and metabolites produced from glycerophospholipid metabolism (Table 5.1). The fold changes of these metabolites over stepwise-ageing were around 1.2 - 1.3 except for glutamic acid (1.80, m/z 128.0, $[M-H_2O-H]^-$) and tyramine (1.41, m/z 410.3, $[3M-H]^-$) in straight-dry-aged beef. Amines and glycerophospholipids were the predominant metabolites in straight-dry-aged beef with significant changes (fold change 1.4 - 1.8, $P < 0.05$) compared to those stepwise-aged equivalents. On the other hand, the dominant metabolites with significant changes (fold change > 2.0) in stepwise-aged beef were low molecular weight ions (< m/z 400, Table 5.1).

There was no difference ($P > 0.05$) between the three air velocities in the stepwise treatment after 7 days of dry-ageing (T2 - T4). Further comparison between the two ageing methods was carried out by identifying the recurrent metabolites detected across the three stepwise-ageing treatments (T2 - T4), which significantly differed from straight-dry-ageing method (T1). There were 6 metabolites consistently more abundant ($P < 0.05$) in stepwise-aged beef regardless of air velocities compared to the straight-dry-aged equivalents. All 6 metabolites were low molecular weight (m/z 100 - 350) and 5 of them were recurrent ions also observed when the comparison was performed between the T1 and T2 at the same air velocity of 0.5 $m.s^{-1}$ (Table 5.1). Higher level of asparagine-lysine dipeptide (m/z 281.1, $[M+Na-2H]^-$) was also detected in stepwise-aged beef compared to straight-dry-aged counterpart. On the other hand, straight-dry-ageing resulted in consistently increased content of larger ions (> m/z 500) than stepwise-ageing (T2 - T4), including m/z 369.2 ($[M+H_2O-H]^-$), 869.6 ($[M+K-2H]^-$), 893.6 ($[M+H_2O-H]^-$), 1201.8 ($[M+Na-2H]^-$) and 1290.9 ($[M+H]^-$). These ions were putatively identified as metabolites produced from lipid metabolism including glycerophospholipids and sphingolipids.



(a)



(b)

Figure 5.1 Representative mass spectra (m/z 50 - 1000 Da) acquired from REIMS analysis of in-bag dry-aged lean beef by different ageing regimes. (a) Straight-dry-ageing *vs.* stepwise ageing following 21 days of ageing period; (b) Stepwise ageing for different ageing time: 0, 7 and 21 days

5.3.2 Effect of ageing time (0, 7 & 21 days)

A good predictive model with Q^2 value of 0.53 was obtained for differentiating between three ageing time points regardless of the ageing methods (Figure 5.3). Lean beef samples aged for 21 days were clearly separated from the unaged (0 day) and dry-aged for 7 days.

5.3.2.1 0 vs. 7 days dry-ageing

There were 9 recurrent metabolites detected in beef samples following 7 days of BD and these differed (P < 0.05) from the unaged beef equivalents, regardless of air velocities. Dry ageing for 7 days resulted in significantly reduced level of some small ions (6 ions, < m/z 500) than unaged beef; these include fatty acid esters, sugar alcohols and long chain polyunsaturated fatty acids. Another three ions matching oleic acid (m/z 324.2, $[M+K-2H]^-$), lucidenic acid J (m/z 527.2, $[M+K-2H]^-$) and glycerophosphoinositol (m/z 895.6, $[M+Na-H]^-$) were also consistently more abundant in dry-aged beef (7 days, T1-T4) compared to its unaged equivalent.

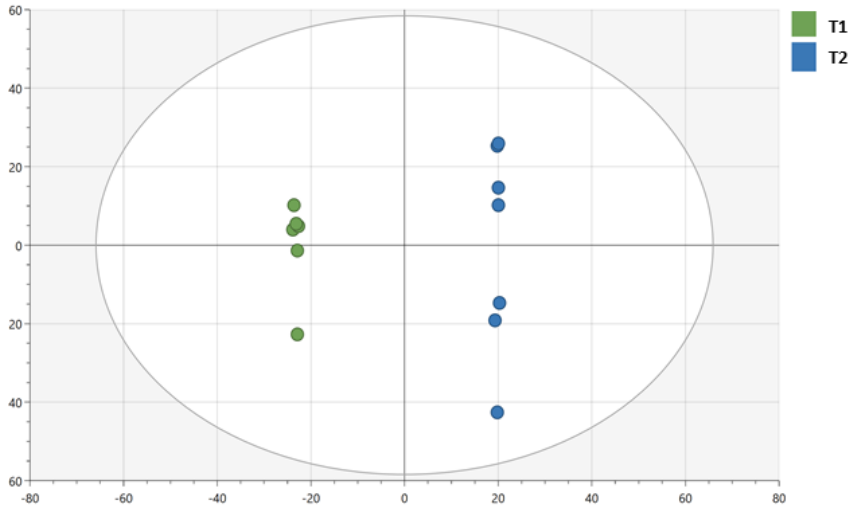


Figure 5.2 OPLS-DA score plot of the comparison between in-bag straight-dry-ageing (T1) and stepwise ageing (T2) (R^2X = 0.79, Q^2 = 0.85) of lean beef.

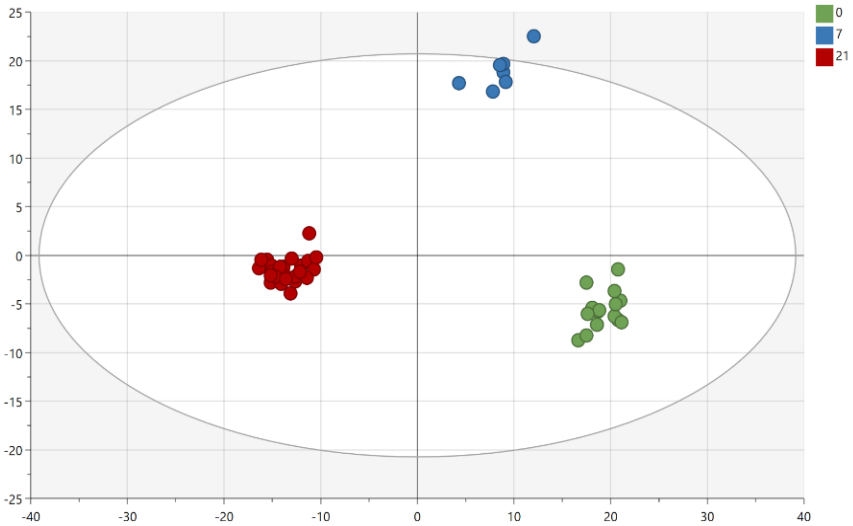


Figure 5.3 OPLS-DA score plot of the effect of ageing time (0, 7 and 21 days) on lean beef regardless of ageing methods (R^2X: 0.73, Q^2 = 0.53).

5.3.2.2 0 vs. 21 days straight-dry-ageing

Meat composition was clearly different following 21 days of straight-dry-ageing in comparison to the equivalent unaged beef (Q^2 = 0.95), with 411 metabolites (24.11%) differing between the dry-aged and unaged meat. Out of these 411 metabolites, 164 compounds were more abundant at 21 days of straight-dry-ageing. As shown in Table 5.2, all the dominant metabolites decreased after 21 days of straight-dry-ageing period (fold change < 1.0). Straight-dry-ageing of lean beef for 21 days also produced significantly increased levels of low-medium molecular weight ions (< m/z 500), which are speculated to be dipeptides, free amino acids and fatty acids, and their derivatives. Another group of metabolites tentatively identified as amino acid derivatives, amines, heterocyclic fatty acids, and glycerophospholipids were found to be more abundant in unaged beef (0 day) with high fold changes compared to the dry-aged (Table 5.2).

5.3.2.3 0 vs. 21 days stepwise-ageing

The metabolic profile of stepwise-aged beef for 21 days differed significantly from the unaged equivalents with an OPLS-DA Q^2 value of 0.77, 0.71 and 0.71 for T2, T3 and T4, respectively. More than 40% of metabolites detected changed significantly after 21 days of stepwise-ageing process (690, 796 and 706 metabolites for T2 - T4, respectively).

The dominant metabolites which changed significantly across the ageing time regardless of air velocities and which were found to be more abundant in unaged beef compared to those stepwise-aged for 21 days are shown in Table 5.3. There were 10 metabolites made of mainly medium-high molecular weight (> m/z 400 - 900) diglycerols and glycerophospholipids observed in unaged beef. Stepwise-ageing, on the other hand, produced significantly higher level of low-medium molecular weight metabolites compared to unaged equivalents.

5.3.3 Sampling locations

As shown in the PCA score plot (Figure 5.4a), the metabolic profile of intact dry-aged beef with untrimmed surface separated from the trimmed equivalent and from the trimmings (Q^2 = 0.40). This was further validated by an OPLS-DA model with three sampling locations (Figure 5.4b) and acceptable prediction (Q^2 = 0.40). Stronger predictive models were generated when untrimmed meat surface was compared to the trimmings (Q^2 = 0.94) and the trimmed meat equivalent (Q^2 = 0.73). The modelling between trimmed meat and the trimmings resulted in a negative Q^2 value (Q^2 = -0.12) suggesting no significant difference existed between these two sampling locations.

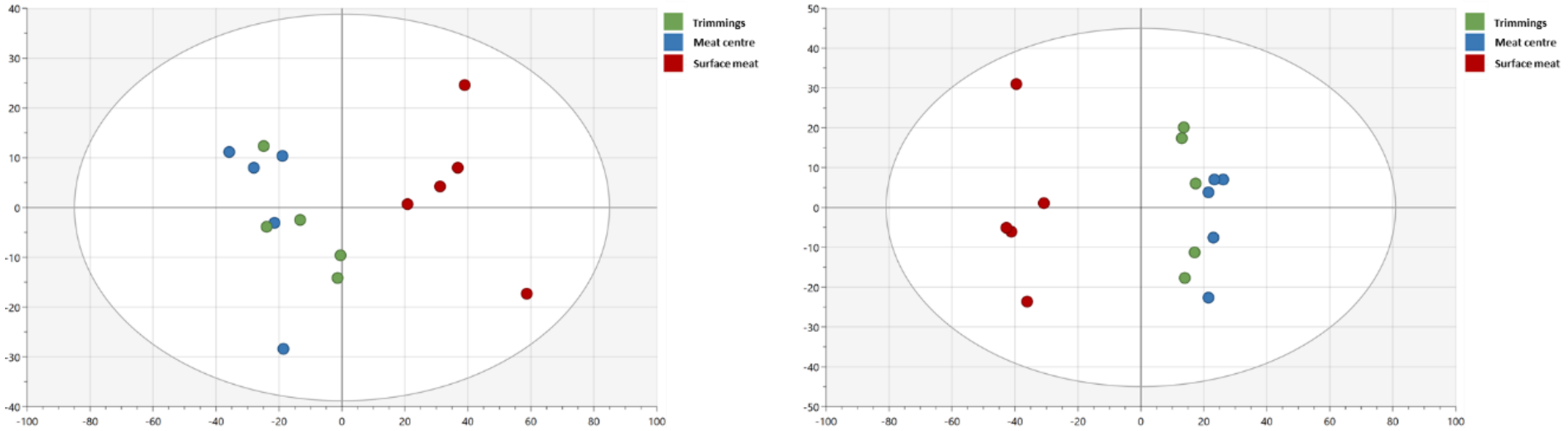


Figure 5.4 Effect of sampling locations (surface of untrimmed dry-aged lean beef, trimmings from dry-aged beef equivalent, and centre of a trimmed equivalent: a. PCA score plot of three sampling locations ($R^2X = 0.62$, $Q^2 = 0.40$); b. OPLS-DA score plot of three sampling locations ($R^2X = 0.61$, $Q^2 = 0.40$) on straight-dry-aged lean beef.

More than 50% of metabolites differed ($P < 0.05$) between untrimmed surface, trimmings (963 metabolites, 56.48%) and trimmed centre of the meat (1074 metabolites, 62.99%). The dominant metabolites which were also more abundant ($P < 0.05$) in untrimmed surface meat mainly consisted of low-medium molecular weight ion, such as amino acids, dipeptides, amines, fatty alcohols, and aldehydes (Table 5.4). The medium-high molecular weight metabolites (mainly glycerophospholipids) were present at higher levels in trimmings and trimmed meat centre compared to untrimmed surface meat.

5.4 Discussion

In this study, stepwise-ageing was carried out by combining BD for 7 days followed by W for 14 days. This method of drying may differ in proteolytic pathway with its straight-dry-ageing equivalent. Three recurrent ions of m/z 245.0 ($[M+Na-2H]^-$), 283.1 ($[M+K-2H]^-$) and 299.0 ($[M+K-2H]^-$ or $[M+Cl]^-$), suspected to be dipeptides, were significantly more abundant in stepwise-aged beef for 21 days compared to straight-dry-aged equivalents (Table 5.1). This group of ions was also observed to increase after 21 days of straight-dry-aging (Table 5.2), suggesting that some of the primary proteolytic pathways by endogenous proteases (mainly calpain and calpastatin) is likely to be similar between the two ageing methods (Kim et al., 2018).

The ion m/z 127.1 ($[M-H_2O-H]^-$) which is tentatively identified as an alanine-glycine dipeptide, decreased significantly after 21 days of straight-dry-ageing (Table 5.2), and was further reduced for the equivalent stepwise-aged meat as shown in Table 5.1. We hypothesise that this variation in dipeptide profiles could be due to one of two factors. Firstly, a more complete proteolysis took place in stepwise-ageing to cleave alanine-glycine into free amino acids due to the endogenous enzyme activities being higher than in straight-dry-ageing. A faster decline of μ-calpain activity has been observed in dry-aged beef compared to the wet-aged over 15 days of ageing time (Velotto et al., 2015). This could be associated with the dehydration during dry-ageing process affecting the enzyme activity (Toldrá, 2006b) and resulting in slower enzymatic proteolysis. Secondly, a different proteolytic pathway (e.g. microbial proteolysis) may have occurred during the stepwise-ageing process, particularly during the W step, consequently resulting in different protein metabolism products. W of beef in vacuum packaging has been suggested to produce significantly higher lactic acid bacteria counts than those (in-bag) dry-aged (Li et al., 2014). Lactic acid was putatively identified (m/z 89.0, Table 5.3) following 21 days of stepwise-ageing and this could be an indicator for the microbial proteolysis by lactic acid bacteria under anaerobic conditions during 14 days of W period.

However, the intensity and fold change (< 1.3) of this ion compared to unaged equivalent was low. This could be due to the low level of lactic acid bacteria on the stepwise-aged lean beef (Zhang et al., 2019).

Different amines have been detected in the dry-aged lean beef regardless of ageing methods (straight-dry-ageing or stepwise-ageing). These include biogenic amines, N-acyl amines, amino acid derived amines and glycerophosphoethanolamines. Tyramine is a common biogenic amine found in animal biological systems, and it has been reported to increase with ageing time for wet-aged beef (Smith, Kenney, Kastner, & Moore, 1993). In the current study, a higher level of m/z 410.3 ($[3M-H]^-$), which is assumed to be tyramine, has been observed in lean beef samples that were straight-dry-aged compared to the stepwise-aged equivalents (Table 5.1). This could be associated with proteolysis and decarboxylation of tyrosine by yeast which resulted in the elevated levels of tyramine (Gardini, Özogul, Suzzi, Tabanelli, & Özogul, 2016). An increase in tyramine was also detected following 21 days of stepwise-ageing (*vs.* unaged) (Table 5.3), although the concentration was significantly lower than in the straight-dry-aged equivalents. As discussed above, the proliferation of lactic acid bacteria under anaerobic environment of W process could have contributed to the tyrosine metabolism process and produced significant amount of tyramine with ageing time. A low level of lactic acid bacteria was previously observed by Zhang et al. (2019), which could have accounted for the reduced level of tyramine detected in stepwise-aged beef compared to the straight-dry-aged equivalents.

Another ion of interest is m/z 201.0 ($[M-Na-2H]^-$) which was tentatively identified as a hexose. Many monosaccharides such as glucose, mannose, fructose or galactose have the same molecular weight and cannot be conclusively identified using REIMS (Table 5.1). Post-mortem glycolysis within the first 24 to 48 hrs of slaughter is the primary biochemical pathway to produce sugar-related metabolites such as hexoses and trioses (Matarneh, England, Scheffler, & Gerrard, 2017). Hexoses are important water-soluble precursors contributing directly to sweet taste and also subsequently participating in Maillard reaction to produce desirable beef flavour (Koutsidis et al., 2008; Nishimura, Rhue, Okitani, & Kato, 1988). Hexoses (glucose and mannose) have been reported previously by Kim et al. (2016) in aged beef regardless of ageing methods (wet-/dry-ageing).

Table 5.1 Effect of dry-ageing methods on the metabolic profile of dry-aged lean bull beef striploins.

Dominant metabolites					High fold change metabolites				
m/z	**Ion/Adduct**	**Putative compounds/Lipid class**	***Fold change**	***P*-values**	**m/z**	**Ion/Adduct**	**Putative compounds/Lipid class**	***Fold change**	***P*-values**
Straight-dry-ageing (T1) for 21 days									
127.1	$[M-H_2O-H]^-$	Alanine-glycine dipeptide	0.87	0.031	211.1	$[M-H]^-$	2-Amino-3,4-dimethylimidazo[4,5-f] quinoline	0.54	<0.001
128.0	$[M-H_2O-H]^-$	Glutamic acid	0.55	0.001	241.1	$[M-H_2O-H]^-$	Cyclo (Leucyl- Phenylalanine)	0.69	<0.001
410.3	$[3M-H]^-$	Tyramine	0.71	0.004	319.2	$[M+Cl]^-$	Retinoid	0.65	0.003
436.3	$[M-H_2O-H]^-$	Phosphatidylethanolamine	0.83	0.029	320.2	$[M-H]^-$	Arginine phenylalanine dipeptide	0.57	<0.001
453.3	$[M+K-2H]^-$ $[M+Cl]^-$	Sterols Secosteroid	0.82	0.008	389.2	$[M-H]^-$	Pre-putrebactin	0.62	0.003
467.3	$[M-H_2O-H]^-$	N-(2'-(4-benzenesulfonamide)-ethyl) arachidonoyl amine	0.80	0.028	408.2	$[M+K-2H]^-$	Anandamide (22:6, n-3)	0.70	0.001
479.3	$[M+Cl]^-$	Vitamin D3 and derivative	0.83	0.030	440.3	$[M-H]^-$	N-arachidonoyl histidine	0.63	0.016
481.3	$[M+Cl]^-$	Sterol	0.81	0.010	448.2	$[M+Cl]^-$	Sterol lipid	0.67	0.004
493.3	$[M+K-2H]^-$	Secosteroid	0.83	0.036	717.6	$[M+K-2H]^-$	Sterol ester	0.70	0.009
701.5	$[M-H]^-$	Ceramide phosphoethanolamine	0.81	0.045	719.4	$[M+K-2H]^-$	Phosphatidic acid	0.61	0.015
749.5	$[M-H]^-$ $[M+Cl]^-$	Phosphatidylglycerol Ceramide phosphoethanolamine	0.77	0.043	828.5	$[M-H]^-$	Oxidized glycerophosphocholine	0.69	0.004
764.5	$[M+K-2H]^-$	Glucosylceramide (d18:1/18:0)	0.75	0.028	829.6	$[M-H_2O-H]^-$	Glycerophosphoinositol	0.68	0.003
765.5	$[M+Na-2H]^-$	Phosphatidylcholine Phosphatidylethanolamine	0.74	0.018	872.6	$[M+K-2H]^-$	LacCer(d14:0/18:0)	0.65	0.010
Stepwise-ageing (T2, dry-aging for 7 days then wet ageing for 14 days)									
137.0	$[M+Na-2H]^-$	2-Methyl-1-methylthio-2-butene	3.08	<0.001	139.0	$[M+Na-2H]^-$	Hydroxy fatty acids	9.14	<0.001
153.0	$[M-H_2O-H]^-$	Glycerol 3-phosphate	1.17	0.004	141.0	$[M-H_2O-H]^-$	Thymine glycol	19.61	0.001
161.0#	$[M+Na-2H]^-$	3,4-Diethylthiophene	3.64	<0.001	191.0	$[M+Na-2H]^-$	S-(2-Furanylmethyl) propanethioate	14.48	<0.001
163.1	$[M-H_2O-H]^-$	1,2-dihydrostilbene	2.57	<0.001	200.0	$[M+Na-2H]^-$	2-Amino-3,4-dihydroxypentanedioic acid	8.66	<0.001
175.1	$[M-H_2O-H]^-$	1-Hydroxyphenanthrene	2.76	<0.001	223.1	$[M+Cl]^-$	Branched Fatty Acid	16.03	<0.001
177.0	$[M+Na-2H]^-$	2-[(Isopropylthio)methyl]furan	5.62	<0.001	227.0	$[M+Na-2H]^-$	Lipoic acid	16.40	<0.001
189.1#	$[M+Na-2H]^-$	2-Hexylthiophene	2.35	<0.001	233.0	$[M+K-2H]^-$	5-Hydroxyconiferyl alcohol	11.98	<0.001
201.0	$[M+Na-2H]^-$	Hexose	5.38	<0.001	235.0	$[M-H_2O-H]^-$ $[M-H]^-$	5-L-Glutamyl-taurine Aspartate-cysteine dipeptide	9.62	<0.001
203.1	$[M-H_2O-H]^-$	Heptadec-1,7,9-trien-11,13,15-triyne	8.52	<0.001	245.0	$[M+Na-2H]^-$	Cysteinyl-cysteine	5.66	<0.001
217.1	$[M+Na-2H]^-$	1-(2-Thienyl)-1-heptanone	3.06	<0.001	245.1	$[M+Cl]^-$	2-Ethyl-1-hexanol sulfate	9.83	<0.001
221.1#	$[3M-H]^-$	Glycinamide	3.97	<0.001	281.0	$[M+K-2H]^-$	Fucose 1-phosphate	8.57	<0.001
283.1	$[M+K-2H]^-$	Methionine-proline dipeptide	4.75	<0.001	299.0	$[M+K-2H]^-$ $[M+Cl]^-$	Hydroxypropyl-methionine/Methionyl-Hydroxyproline Aspartate-Methionine/Methionyl-Aspartate	41.16	<0.001
340.2#	$[M+K-2H]^-$	Arachidonoyl amine	1.46	0.008	303.0	$[2M-H]^-$	1,4-Dithiane-2,5-diol	8.22	<0.001
365.2#	$[M+Na-2H]^-$	Estrane steroid	1.29	0.001	319.0	$[2M-H]^-$	4-Fluoromuconolactone	10.65	<0.001
					358.9	$[M+Cl]^-$	Trichloroethanol glucuronide	6.71	<0.001

Table 5.2 Effect of ageing time (0 *vs.* 21 days) on the metabolic profile of straight-dry-aged (T1) lean bull beef striploins.

m/z	Ion/Adduct	Putative compounds/Lipid class	*Fold change	*P*-values
Dominant metabolites				
138.1	$[M-H_2O-H]^-$	3-Methylcrotonylglycine	0.70	0.018
250.1	$[M+Cl]^-$	N-Nonanoylglycine	0.46	0.010
344.1	$[M+Cl]^-$	Lysine-tyrosine dipeptide	0.69	<0.001
455.2	$[M-H]^-$	Phytyl diphosphate	0.62	<0.001
518.4	$[M-H_2O-H]^-$	Phosphatidylcholine Phosphatidylethanolamine	0.84	0.022
527.3	$[M+K-2H]^-$	N-oleyl-alanyl-histidine	0.43	0.001
598.3	$[M+Cl]^-$	1-(9Z-octadecenoyl)-2-(4-oxobutryl)-sn-glycero-3-phosphatidylethanolamines	0.72	0.001
708.4	$[M+Cl]^-$	1-(9Z-octadecenoyl)-2-(5,8-dioxo-6E-octenoyl)-sn-glycero-3- phosphatidylcholines	0.70	0.006
738.5	$[M-H]^-$ $[M-H_2O-H]^-$	Phosphatidylcholine Phosphatidylethanolamine	0.69	0.018
763.5	$[M+Cl]^-$	PA(P-20:0/19:1(9Z))	0.65	<0.001
873.4	$[M+K-2H]^-$	Hebevinoside VII Hebevinoside II	0.68	0.019
1066.6	$[M+Cl]^-$	MIPC(t18:0/20:0(2OH)) MIPC(t20:0/18:0(2OH))	0.74	0.010
1165.8	$[2M+Hac-H]^-$	1-(2-methoxy-docosanyl)-sn-glycero-3-phosphatidylethanolamine	0.66	<0.001
Abundant at 21 days				
98.1	$[M-H_2O-H]^-$	Valine	1.70	0.005
124.1	$[M-H_2O-H]^-$	Proline betaine	1.50	0.011
135.1	$[M+Cl]^-$	Fatty alcohol	1.73	0.005
245.0	$[M+Na-2H]^-$	Cysteinyl-cysteine	1.52	0.008
281.1	$[M+Na-2H]^-$	Asparagine-lysine dipeptide	2.07	0.012
283.1	$[M+K-2H]^-$	Methionine-proline	1.90	0.006
299.0	$[M+K-2H]^-$ $[M+Cl]^-$	Hydroxyproline-methionine dipeptide Aspartate-methionine dipeptide	2.73	0.003
353.2	$[2M-H]^-$	Proline- or lysine-derived Maillard product	1.31	0.036
489.4	$[M-H_2O-H]^-$	2'-Apo-beta-carotenal	2.26	0.011
545.3	$[2M+Hac-H]^-$	Lysine-proline dipeptide	1.65	0.009
551.4	$[M-H]^-$ $[M-H_2O-H]^-$ $[M-H_2O-H]^-$	Monoterpenoid Diterpenoid Phosphatidylethanolamine	1.49	0.018
768.5	$[M+Na-2H]^-$ $[M+K-2H]^-$	Phosphatidylcholine Phosphatidylserine	2.14	0.003
843.6	$[M+K-2H]^-$	PG(O-18:0/21:0) PG(O-20:0/19:0)	1.55	0.011
957.7	$[M+Cl]^-$	PI(O-20:0/21:0)	1.33	0.007
1029.8	$[M+Cl]^-$	Triacylglycerol	1.70	0.013
Abundant at 0 day				
127.1	$[M-H_2O-H]^-$	Alanine-glycine dipeptide	0.37	0.005
176.1	$[M+Cl]^-$	1-(1-Pyrrolidinyl)-2-butanone/3-(1-Pyrrolidinyl)-2-butanone	0.03	<0.001
300.1	$[M+K-2H]^-$	Hydroxy-alpha-sanshool	0.45	0.010
326.2	$[M-H_2O-H]^-$	3-hydroxyundecanoyl carnitine	0.21	0.001
504.3	$[M+K-2H]^-$	N-Arachidonoyl tyrosine	0.40	<0.001
517.3	$[M+Na-2H]^-$	PG(17:1(9Z)/0:0)	0.41	0.002
531.4	$[2M-H]^-$	Heterocyclic fatty acid	0.19	0.001
739.4	$[2M-H]^-$	5-Megastigmen-7-yne-3,9-diol 9-glucoside	0.45	0.001
751.5	$[M+Cl]^-$	Phosphatidic acid	0.45	0.011
761.5	$[2M-H]^-$	Sphinganine 1-phosphate	0.35	0.002
771.7	$[M+Na-2H]^-$	Triacylglycerol	0.25	<0.001
804.6	$[M+Na-2H]^-$	Phosphatidylethanolamine	0.39	0.002
839.6	$[3M-H]^-$	Heterocyclic fatty acid	0.18	0.002
1363.9	$[2M+Hac-H]^-$	PG(O-16:0/12:0)	0.39	0.003
1377.9	$[M-H_2O-H]^-$	Cardiolipin	0.37	0.003
1389.9	$[M+K-2H]^-$	Cardiolipin	0.27	0.004

Post-mortem ageing (W) has also been reported to significantly increase the hexose (glucose, fructose and mannose) level in beef, of up to 21 days of ageing time (Koutsidis et al., 2008). The significant increase in hexoses after 7 days of ageing could be associated with the enzymatic hydrolysis of glycoproteins (Koutsidis et al., 2008). In this study, slightly different biochemical reactions may have occurred during the stepwise-ageing compared to the straight-dry-ageing as discussed above, consequently resulting in the variations in the metabolic profiles between the two ageing methods. Further analysis using chromatography is needed to validate this finding.

Table 5.3 Effect of ageing time (0 *vs.* 21 days) on the metabolic profile of stepwise-aged lean bull beef striploins.

m/z	Ion/Adduct	Putative compounds/Lipid class
Abundant at 21 days, P < 0.05		
89.0	$[M-H]^-$	Lactic acid
293.2	$[M+Cl]^-$	2-Acetyl-3,5,5,6,8,8-hexamethyl-5,6,7,8- tetrahydronaphthalene
363.3	$[M-H_2O-H]^-$ $[M+Cl]^-$	Cholesterol and derivative Fatty alcohol
365.2	M+Na-2H	Estrane steroid
388.3	$[M+K-2H]^-$	Anandamide (20:4, n-6)
393.3	$[M-H_2O-H]^-$	Cholesterol and derivative Vitamin D and derivative
410.3	$[3M-H]^-$	Tyramine
Abundant at 0 day, P < 0.05		
404.3	$[M+Cl]^-$	Macamide
715.5	$[M+Cl]^-$ $[M-H_2O-H]^-$	Diacylglycerol Phosphatidic acid
717.5	$[M+Cl]^-$ $[M-H_2O-H]^-$	Diacylglycerol Phosphatidic acid
767.5	$[M+K-2H]^-$ $[M+Cl]^-$	Phosphatidic acid Phosphatidylglycerol
769.5	$[M+K-2H]^-$	PE-Cer(d14:1(4E)/24:0(2OH)) PE-Cer(d16:1(4E)/22:0(2OH))
771.6	$[2M+Hac-H]^-$ $[M-H_2O-H]^-$	Tetracosahexaenoic acid Phosphatidylglycerol
845.6	$[3M-H]^-$ $[M-H_2O-H]^-$	Retinoid Glycerophosphoinositol
863.6	$[M-H]^-$	PG(21:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) PG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/21:0)
864.6	$[M-H_2O-H]^-$ $[M+K-2H]^-$ $[M+Cl]^-$	Phosphatidylcholine Phosphatidylethanolamine Phosphatidylserine
865.6	$[M+Na-2H]^-$	Phosphatidylglycerol

Ageing time has been suggested to be one of the essential factors in the tenderisation and flavour development of meat (Kim et al., 2018). Enough ageing time should be allowed for biochemical reactions to occur to convert meat to a "well-aged" product. In the current study, more putative ions identified as free amino acid valine (m/z 98.1, $[M-H_2O-H]^-$) and six dipeptides (ion mass of m/z 245.0, 281.1, 283.1, 299.0, 352.2 and 545.3, Table 5.2) increased in lean beef after 21 days of straight-dry-ageing. The significant increase of free amino acids (including valine) with ageing time up to 28 days has also been reported in other studies (Kim et al., 2016; Lee et al., 2019a).

It is well recognised that free amino acids and peptides are produced from enzymatic proteolysis during post-mortem ageing (Fu, Young, & Therkildsen, 2017; Nishimura, 1998). A post-mortem ageing period of around 3 to 4 weeks has been suggested to be necessary to significantly improve meat flavour (Campbell et al., 2001; Kim et al., 2018; Watanabe et al., 2015). This could be explained by the enzymatic proteolysis at refrigerated temperature (0 - 4 °C) being slow (Bechtel & Parrish, 1983), and consequently requiring a longer time to produce significant amount of taste active metabolites and flavour precursors that contribute towards unique aged flavour (Kim et al., 2018). Koutsidis et al. (2008) suggested ageing time of over 7 days to produce significantly higher level of water-soluble flavour precursors including free amino acids and reducing sugars. In this study, no significant change of these flavour precursors following 7 days of BD has also been observed. Therefore, a short period of ageing time for 7 to 10 days could have produced quality acceptable dry-aged products, but mainly due to the improvement of tenderness as suggested in our previous study (Zhang et al., 2019). A longer period of ageing time of up to 3 to 4 weeks will produce premium niche products with the 'well-aged' signature including significantly more abundant free amino acids and small peptides being produced, as detected by REIMS in current study, and contributing to the flavour.

Increase of air velocities during traditional out-of-bag dry-ageing process has been suggested to result in different microbial compositions (mainly mould and yeast) and consequently modify flavour (Lee et al., 2019a). However, our previous work has shown that increase of air velocity had not affected the quality and sensory acceptability of in-bag dry-aged lean beef (Zhang et al., 2019). The REIMS metabolic fingerprints where no impact of air velocity has been observed, support our previous findings. The use of water-permeable ageing bags for dry-ageing process protects the meat from microbial contamination by the surroundings therefore limits the growth of microorganisms. BD of beef has been reported to enhance the proliferation of yeast while having no impact on moulds, regardless of air velocities. Thus, the role of air velocity in the biomolecular changes of in-bag dry-aged beef is different from those out-of-bag regime.

In this study, three different sampling locations were examined on intact straight dry-aged lean beef after 21 days of ageing, to determine the impact of trimming practice on the metabolic profile of dry-aged beef. Straight-dry-ageing for 21 days resulted in significantly higher levels of small ions, such as amino acids (glutamic acid, m/z 128.0, $[M\text{-}H_2O\text{-}H]^-$) and dipeptides including alanine-glycine (m/z 127.1, $[M\text{-}H_2O\text{-}H]^-$), glycine-

methionine (m/z 205.1, $[M-H]^-$) and lysine-methionine (m/z 830.4, $[3M-H]^-$) on the untrimmed meat surface compared to the sampling locations from trimmings and trimmed meat centre (Table 5.4). Glutamic acid was one of the predominant metabolites detected on the untrimmed meat surface with about 4-fold higher than the trimmings and meat centre (Table 5.4). Glutamic acid is an important taste active amino acid contributed towards improving saltness and umami taste, and these are believed to be the signature of dry-aged flavour (Campbell et al., 2001; Kim et al., 2018; Savell, 2008). A significantly higher level of glutamic acid was also observed in straight-dry-aged beef compared to the stepwise-aged equivalents as shown in Table 5.1, suggesting that straight-dry ageing for 21 days is able to produce more intense dry-aged flavour via the production of higher amounts of free glutamic acid. Similar finding was reported by Kim et al. (2016) that significantly higher (foldchange = 1.51) amount of glutamic acid was observed in dry-aged beef compared to the wet-aged equivalents. The increase of glutamic acid on meat surface could be due to be the proteolytic reaction resulting from yeast on the untrimmed meat surface. The yeast level has been reported to increase significantly on the untrimmed surface of in-bag dry-aged lean beef (Zhang et al., 2019). The proliferation of yeast on the surface during dry-ageing has also been reported to be significantly higher than in the wet-aged (Li et al., 2014; Li et al., 2013). This hypothesis is further supported by the more abundant (foldchange > 1.10) metabolite ions observed on the trimmings over the meat centre, including putative dipeptides such as alanine-glycine (m/z 127.1, $[M-H_2O-H]^-$), and hydroxyproline-proline (m/z 209.1, $[M-H_2O-H]^-$). This latter dipeptide may be specifically be coming from the breakdown of collagen during the dry-ageing process. Therefore, post dry-ageing trimming practice could result in loss of some biomolecular signatures for dry-ageing process such as small peptides and free amino acids which could be contributing to the distinct flavour of dry-aged meat.

Table 5.4 Effect of sampling locations (surface meat, trimmings and meat centre) on the metabolic profile of in-bag dry-aged untrimmed lean bull beef.

Dominant metabolites			High fold change metabolites		
m/z	**Ion/Adduct**	**Putative compounds/Lipid class**	**m/z**	**Ion/Adduct**	**Putative compounds/Lipid class**
Abundant at surface meat, P < 0.05					
109.0	$[M-H_2O-H]^-$	4-amino-4-cyano-butanoic acid 2-amino-4-cyano-butanoic acid	81.0	$[M-H]^-$	Sulfite
127.1	$[M-H_2O-H]^-$	Alanine-glycine dipeptide	105.0	$[M-H]^-$	Glyceric acid
128.0	$[M-H_2O-H]^-$	Glutamic acid	105.0	$[M+Na-2H]^-$	3-Methyl-2-butenal
135.1	$[M+Cl]^-$	Fatty alcohol	179.0	$[M-H]^-$	Nicotinuric acid
154.1	$[M-H]^-$	Histidine	205.1	$[M-H]^-$	Glycine-methionine dipeptide
181.1	$[M+Cl]^-$ $[M+K-2H]^-$	Fatty alcohol	221.1	$[M+Cl]^-$	3-Oxodecanoic acid
241.1	$[M-H_2O-H]^-$	Cyclo(Leu-Phe)	222.1	$[M+Cl]^-$	7-oxo-8-amino-nonanoic acid
354.2	$[M+K-2H]^-$	N-methyl arachidonoyl amine	226.1	$[M+Na-2H]^-$	1-(2,3-Dihydro-1H-pyrrolizin-5-yl)-1,4-pentanedione
369.2	$[M+Cl]^-$	Anandamide (18:4, n-3)	271.2	$[M-H]^-$	16-Hydroxy hexadecanoic acid
615.2	$[M-H_2O-H]^-$	Hydroperoxy fatty acid	363.2	$[M+Na-2H]^-$	11-Hydroxy-9,15,16-trioxooctadecanoate
649.4	$[M+Na-2H]^-$	Phosphatidic acid	413.3	$[M-H_2O-H]^-$	Vitamin D3 and derivative
703.5	$[M-H]^-$	Ceramide phosphoethanolamine	587.2	$[2M-H]^-$	Fatty acyl glycosides of mono- and disaccharide
799.7	$[M-H]^-$	Triacylglycerol	830.4	$[3M-H]^-$	Lysine-methionine dipeptide
Abundant in trimmings and meat centre, P < 0.05					
305.3	$[M+Cl]^-$	Octadecanol	488.3	$[3M-H]^-$	Erythro-5-hydroxy-L-lysinium(1+)
669.5	$[M+Na-2H]^-$	Sphingomyelins(d18:0/12:0)	667.5	$[M+Na-2H]^-$	Sphingomyelins(d18:1/12:0)
671.5	$[M-H_2O-H]^-$	Diacylglycerol	668.5	$[M-H_2O-H]^-$	Phosphatidylcholine Phosphatidylethanolamine
679.5	$[M+Na-2H]^-$	Ceramide phosphoethanolamine	705.5	$[M+Na-2H]^-$	PE-Cer(d14:2(4E,6E)/22:1(13Z))
681.5	$[M-H_2O-H]^-$	PE-Cer(d16:2(4E,6E)/20:1(11Z)(2OH))	707.5	$[M+Na-2H]^-$	Ceramide phosphoethanolamine
683.5	$[3M-H]^-$	11-Hydroxy-9-tridecenoic acid	711.5	$[M+K-2H]^-$ $[M-H_2O-H]^-$	Diacylglycerol Phosphatidic acid
685.5	$[M-H_2O-H]^-$	Phosphatidic acid	752.5	$[M+K-2H]^-$ $[M+Cl]^-$	Phosphatidylcholine Phosphatidylethanolamine
695.5	$[2M+Hac-H]^-$	Xylene	761.5	$[2M-H]^-$	Sphinganine 1-phosphate
699.5	$[M+K-2H]^-$	Cholesterol fatty acid ester	815.6	$[M+K-2H]^-$ $[M+Na-2H]^-$	Phosphatidic acid Phosphatidylglycerol
709.5	$[M+Na-2H]^-$	Sphingomyelins(d16:1/17:0)	816.6	$[M-H_2O-H]^-$	Phosphatidylcholine Phosphatidylethanolamine
713.5	$[M+K-2H]^-$	PE-Cer(d14:1(4E)/22:0)	818.6	$[M+Na-2H]^-$	Phosphatidylcholine
721.5	$[M-H]^-$ $[M+Na-2H]^-$	Phosphatidylglycerol Diacylglycerol	898.6	$[M-H]^-$ $[M+Cl]^-$	Phosphatidylserine Phosphatidylcholine Phosphatidylethanolamine
766.6	$[M+K-2H]^-$ $[M+Cl]^-$	Phosphatidylcholine Phosphatidylethanolamine	1483.9	$[M+Cl]^-$	Cardiolipin

Post-ageing trimming is a common practice in manufacturing dry-aged products using traditional out-of-bag process to remove discolouration, dehydration and microbial contamination, which mainly take place on the surface (Kim et al., 2018). These are not visually appealing to consumers and can also cause food safety issues. In this study, in-bag ageing process (i.e. stepwise ageing) was developed using water-permeable ageing bags for dry-ageing and vacuum barrier bags for the W. The use of ageing bag accelerates moisture evaporation and prevents microbial contamination (Ahnström et al., 2006; Li et al., 2013; Zhang et al., 2019). The moisture difference between the surface and the centre of dry-aged meat is equilibrated during the extended W process thereby minimising the detrimental effect of surface dehydration associated with traditional out-of-bag dry-ageing process, thus, removing the need for trimming the surface of the dry aged meat.

The findings of the current study have demonstrated that REIMS can successfully differentiate the impact of different ageing treatments, ageing times and sampling locations on the metabolic profile of lean bull beef. As an exploratory approach, our results found that different dry-ageing regimes led to very different molecular fingerprints. To summarise, increase of dry-ageing time produced more small sized ions (< m/z 500) which consists of dipeptides, amino acids, fatty alcohol and organic acids. Production of small sized ions were also more evident on the surface/crust of dry-aged beef compared to the meat centre. Biomolecular changes by straight-dry-ageing resulted in more large sized ions, which were produced from phospholipid metabolism (e.g. oxidation) whereas for the stepwise ageing, 99% of the significant increase was driven by small sized ions.

To date, most assessments of dry-ageing methods and processes have used standard meat quality measures, including sensory testing, measurements of pH, proximate content, instrumental colour and texture analysis. We have found previously that these were insufficient to differentiate between ageing methods (Zhang et al., 2019). In this study, REIMS results differed markedly between the different dry-ageing methods and has given some insights into some of the biological differences that may influence final dry-aged meat quality. As a relatively new metabolic fingerprinting technique in the area of food science, REIMS can perform close to real time profiling of thousands of metabolite ions to distinguish the samples based on their inherent properties and external processing treatments (Balog et al., 2016; Black et al., 2019). As no sample preparation is necessary there is scope for REIMS and similar technologies to be applied within the meat industry and meat research for daily routine sampling per carcass/cut as a quality control measure. For example, pilot studies on the use of REIMS to detect boar-taint in an abattoir have

been successfully carried out (Verplanken et al., 2017). Compared to chromatography-mass spectrometry methods (GC-MS and LC-MS) which are commonly applied for metabolic study of meat, REIMS has limitations of not being suitable for quantitative detection and real time qualification of identified molecules due to the difficulty in consistently introducing standard or reference molecules to meat samples. Further studies are required to establish the relationship between REIMS fingerprinting and conventional omics profiling (e.g. lipidomics and metabolomics) to validate current identified compounds.

5.5 Conclusion

REIMS was successfully applied in this study as a real time metabolic fingerprinting technique to detect the impact of different ageing treatments applied on lean beef samples. Ageing methods, ageing time and sampling locations have been clearly differentiated based on the 1705 tentatively identified metabolite ions. More abundant low molecular weight metabolites (dipeptides and amines) were associated with stepwise-ageing of lean beef compared to straight-dry-ageing for 21 days. Glutamic acid and several dipeptides could become candidate biomarkers for in-bag dry-aged lean beef. REIMS could also potentially be a suitable tool for identifying food fraud issues associated with dry-aged meat products when a definition for dry-aged meat is developed and widely accepted.

5.6 Epilogue

This chapter demonstrated the novel application of REIMS for discrimination of in-bag dry-aged lean beef by different processing parameters. REIMS is a sensitive tool which is able to discriminate the metabolic changes between stepwise in-bag ageing and straight-dry-ageing, ageing time, and different sampling locations based on 1705 identified metabolite ions, which would be impossible to detect using conventional analyses as described in Chapters 3 and 4. It also provides deeper insights into the biochemical mechanisms beyond the common meat quality measurements, which could be used to quickly identify the metabolic signatures responsible for specific processing regimes.

A comprehensive study to determine the effect of processing regimes and frozen storage on the quality, acceptability and biochemical changes of in-bag dry-age lean beef using conventional analyses and emerging technology is presented in Chapters 3, 4 and 5. Some key findings are summarised as follows:

- Increase in air velocities of the ageing chamber only accelerated dehydration rate with no impact on other quality attributes of the final product;
- Stepwise in-bag ageing resulted in different metabolic profiles and a higher yield with comparable quality and stability compared to straight-dry-ageing;
- Quality, stability and digestibility of in-bag dry-aged lean beef was improved with ageing time, along with an increase in proteolysis and oxidation of lipid and protein;
- In-bag dry-aged lean beef can be frozen stored for up to a year at -18 °C with little or no detrimental impacts on quality and acceptability;
- REIMS can be used as an emerging tool for rapid metabolic fingerprinting of meat arising from different processing regimes.

As summarised in Table 2.1, dry-ageing was predominantly performed on beef muscles with limited knowledge of other meat sources, like lamb/sheep meat. Additionally, growing interests from the meat industry to explore the value-adding strategies for lamb products motivated the next stage of this research to explore the feasibility to produce dry-aged lamb. Therefore, for the first time, this line of work was continued to investigate the effect of BD on the quality and consumer acceptability of lamb legs to compare with the widely available wet-aged equivalents.

Chapter 6: In-bag dry- *vs.* wet-aged lamb: Quality and consumer acceptability, oxidative changes, and *in vitro* digestibility

This chapter aims to apply the in-bag dry-ageing technique to hindlegs of lamb to create a new dry-aged product. A comprehensive study was performed to compare in-bag dry-aged lamb with its wet-aged equivalents in terms of meat quality, microbiological safety, consumer acceptability, oxidative stability and in vitro digestibility. Significantly higher pH, weight loss and reduced cook loss were observed in dry-aged lamb compared to the wet-aged. Dry-aged lamb had harder and chewier texture profiles and lower colour attributes (L, a* and b*) than the wet-aged. The dry-aged and wet-aged lamb were equally preferred (about 40% each) by the consumer panel demonstrating the merchandising potentials of dry-aged meat. Both ageing regimes resulted in low microbial counts. Significantly higher yeast and TABRS levels were observed in dry-aged lamb compared to wet-aged. There was no difference in fatty acid profile, protein carbonyl content and pattern of proteolysis between ageing regimes. Ageing regimes had no impact on the overall digestibility, however, a greater gastric digestibility was observed in the dry-aged lamb, which was observed through the increased release of FAAs compared to the wet-aged. Outcomes of this study demonstrated, for the first time, the possibility of producing dry-aged lamb legs of acceptable quality, oxidative stability and superior digestibility compared to the equivalent wet-aged lamb.*

This chapter starts with the motivations and aims in Section 6.1 followed by Section 6.2 describing the lamb samples, ageing process and analyses performed in this chapter. Results and discussion are given in Section 6.3. Section 6.4 provides a brief summary of this chapter.

6.1 Introduction

Dry-ageing is a processing technique for adding value to meat products. Dehydration, proteolysis, lipolysis and oxidation take place during dry-ageing to produce unique and intensified aged, sweet, brothy, nutty, buttery and roasted flavours compared to the widely practiced wet-aged equivalents (Li et al., 2014; Savell, 2008). Extensive studies have been carried out on various forms of dry-ageing of beef (Ahnström et al., 2006; Kim et al., 2018; Zhang et al., 2019) with comparatively limited research carried out on lamb, although lamb is consumed widely around the world for its nutritional and sensorial qualities. Lamb is a good dietary source of nutrients, including iron, vitamins, high quality protein and polyunsaturated fatty acids (PUFAs), especially the Eicosapentaenoic acid (20:5, EPA) and Docosahexaenoic acid (22:6, DHA) which are the essential omega-3 PUFAs linked to health-promoting functions (Benatti, Peluso, Nicolai, & Calvani, 2004). Dry-aged beef is mostly produced from the middle cuts, particularly the loin, cube roll and tenderloins (Kim et al., 2018). The equivalents of these cuts in lamb

are too small to be profitably dry-aged for commercial purposes. The part of a lamb carcass that could be viably used for dry-ageing is the hindleg, known to consumers as lamb/leg chops at retail and when cooked and served in restaurants. Sheep/lamb meat consumers are familiar with wet-aged lamb chops, however, their acceptability towards the dry-aged equivalent remains unknown.

Many factors contribute to variations in the eating quality of dry-aged meat; some of these include ageing conditions (Kim et al., 2016), level of moisture evaporation (Lee et al., 2019a) and proliferation of microorganisms (Lee et al., 2019b; Oh et al., 2019). Pathways responsible for the development of characteristic dry-aged quality remain to be fully explored. One of the features with dry-ageing is the direct exposure of meat to oxygen in the atmosphere, which could trigger oxidative damage to lipids and proteins, producing oxidative by-products, such as lipid/protein derived carbonyls (Martinaud et al., 1997; Park et al., 2006; Sante-Lhoutellier et al., 2008). Oxidation has been suggested to modify the protein ultrastructure and result in protein carbonylation and aggregation (Lund, Heinonen, Baron, & Estevez, 2011). Such modifications of proteins may eventually affect the solubility and functionality, leading to the decrease of proteolytic degradation and aggregation during ageing (Berardo, Claeys, Vossen, Leroy, & De Smet, 2015) and gastrointestinal digestion (Sante-Lhoutellier, Aubry, & Gatellier, 2007). Interactions between oxidative changes arising from the use of different ageing regimes, proteolytic pattern and protein digestibility have not been explored.

Severe oxidative damage to lipids and proteins results in detrimental impacts on sensorial, nutritional and functional qualities of meat (Domínguez et al., 2019; Resconi et al., 2018). To overcome these issues, dry-ageing in a moisture-permeable ageing bag, so called “in-bag dry-ageing” (BD), has been recently developed. With the use of an ageing bag as a barrier to the atmosphere, improvements in microbiological safety and product yield were also achieved when compared to the traditional out-of-bag dry-ageing (Ahnström et al., 2006; Li et al., 2013).

The aim of the present study was to produce dry-aged lamb legs using water permeable ageing bags and to compare the dry-aged chops to their wet-aged equivalents in terms of quality, consumer acceptability, oxidative stability and *in vitro* digestibility.

6.2 Materials and methods

6.2.1 Sample collection and ageing regimes

Sixty lamb legs were collected from thirty carcasses (Ram, approx. 46 weeks and 26 kg carcass weight, n = 60) at a local abattoir on the day of slaughter. Each leg (bone-in, rump muscles on, shank-off) was further portioned into three parts as shown in Figure 6.1. The main section, after cutting off the rump muscles and 4 cm from the shank side, was used for ageing. The left or right side of lamb leg (from the same animal) was randomly assigned to two ageing regimes: (1) BD using water-permeable ageing bag (TUBLIN® 10, 50 µm thick, polyamide mix with water vapor transmission rate 920 g/50 µ/m^2/24 h at 7 °C, 50% RH, and oxygen transmission rate 660 g/m^2/24 h at 7 °C, 50% RH, TUB-EX ApS, Denmark) at 2 ± 0.5 °C, 0.5 $m.s^{-1}$ air velocity and relative humidity of 75 ± 5%; (2) wet-ageing (W) in water-impermeable barrier bags (Cryovac® A600 barrier bag, oxygen transmission rate 20-50 g/m^2/24 h at 23 °C, Sealed Air®, New Zealand) at -1.5 ± 0.5 °C as the control. The ageing chamber comprised of two compartments, as illustrated by Kim et al. (2016): (1) an environmental test chamber (walk-in) as the main chilling chamber with control of temperature, humidity and air velocity; (2) four tunnel chambers located inside the chilling chamber with heating elements at one end and exhaust fan at the other end of the chamber to render more precise control of temperature, humidity and air velocity. Samples of both treatments were aged for 21 days. The sample weight before and after ageing was recorded to calculate the % weight loss from 21 days of ageing. Aged samples from both treatments were fabricated into chops (1.5 cm thickness) with no trimming of dry surface for further quality and sensory analyses. After removing the bone, whole lamb chop which contains multiple muscles was minced and subsamples were taken from the same chop for chemical analysis.

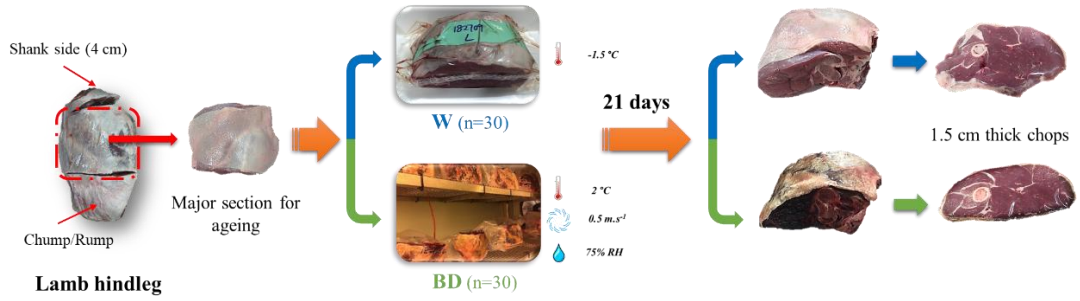


Figure 6.1 A schematic diagram outlining the ageing process and chops to produce in-bag dry-aged (BD) and wet-aged (W) lamb chops.

6.2.2 Weight loss, pH and proximate content

6.2.2.1 Ageing weight loss (%)

Sample weight was recorded at an interval of three days to calculate % weight loss from ageing: % Ageing weight loss = [(Initial weight of sample before ageing - Weight at a given time point)/Initial weight before ageing] × 100. A correlation study between the initial sample weight and the weight after 21 days of ageing was also carried out to estimate the yield of dry-aged lamb under current processing conditions based on the initial weight.

6.2.2.2 Cook loss and percentage total loss (%)

Dry- and wet-aged lamb chops were vacuum packaged and cooked *sous vide* in vacuum barrier bags at 72 °C for 1 hour, then cooled in an ice bath for 1 hour before they were transferred to 0 °C chiller overnight. The sample weight before and after cooking were recorded to calculate the percentage of cook loss. The percentage of total loss was calculated by combining the percentage of ageing loss and the percentage of cook loss.

6.2.2.3 pH

pH of lamb samples before and after the ageing treatments were measured by inserting a calibrated pH probe (Hanna 99,163 pH meter with a FC232D combined temperature and pH insertion probe, Rhode Island, USA) directly into the meat. The calibration of pH meter was carried out with 2-point standard buffer solution of 7.01 and 4.01 at ambient temperature. Measurement was carried out in duplicate for each sample.

6.2.2.4 Proximate content

The lamb chop for proximate analysis was minced and subsamples were taken from the same chop (n = 60). Moisture and crude fat content were determined using standard methods of AOAC 950.46 and AOAC 960.39, respectively (AOAC, 2010). The total muscle proteins were extracted following the method described by Zhang et al. (2019) using total muscle extraction buffer (50 mM Tris-HCl, pH 5.8; 10% glycerol; 2% SDS and 2% 2-mercaptoethanol). The concentration of protein solution was determined using RC-DC protein assay kit (Bio-Rad® Laboratories, Hercules, CA, USA).

6.2.3 Surface microorganism enumeration

Enumeration of microorganisms growing on the surface of the untrimmed meat surface post ageing was determined following standard methods in Compendium of Methods of Microbiological Examination of Foods (Downes & Ito, 2001) for *Escherichia*

coli (*E. coli*, Chapter 8.91), aerobic plate count (Chapter 7.62), lactic acid bacteria (Chapter 19.522), *Enterobacteriaceae* (Chapter 8.63), mould and yeast (Chapter 20.51).

6.2.4 Instrumental colour

Freshly cut lamb chops from BD or W were placed in a polypropylene foam tray with absorbent meat pad and overwrapped with polyvinyl chloride film and allowed to bloom at 4 °C for 1 hour. Instrumental colour was measured on the surface of four major muscles: *m. semimembranosus* (SM), *m. biceps femoris* (BF), *m. vastus lateralis* (VL), and *m. rectus femoris* (RF) using a calibrated Minolta Chroma Meter (CR-400; Konica Minolta Photo Imaging Inc., Mahwah, NJ, USA). Three random positions were measured on each muscle. The colour coordinates of CIE L* (lightness), a* (redness) and b* (yellowness) were measured using Illuminant D65 with 8 mm diameter aperture. Chroma and hue angle were further calculated to describe the colour properties of the samples according to Zhang et al. (2018).

6.2.5 Instrumental texture profile analysis

Texture profile analysis was performed on four major muscles (SM, BF, VL and RF) of the cooked lamb chops from section 6.2.2.2. Compression test was carried out using Stable Micro System TA.HD Plus texture analyser (Surry, UK) with a maximum loading force of 50 kg. Each muscle was cut into 1 cm^3 cubes (minimum 6 cubes per muscle) and measured against the grain using a 50 mm cylinder probe. The compression test was performed at 50% strain with the test speed of 5.0 $mm.s^{-1}$ and trigger force of 5 g. At least 6 measurements were taken for each muscle.

6.2.6 Consumer sensory evaluation

Lamb chops (1.5 cm) were *sous vide* cooked in vacuum barrier bags at 65 °C for 75 minutes and then grilled for 60 seconds each side at approx. 180 °C to obtained core temperature of 72 °C. The samples were then cut across the muscles to obtain two slices per chop. Each consumer was served with two slices of lamb, both slices were from the same carcass but different ageing regimes. The lamb slices were placed on a plate which was coded with two randomly selected IDs for dry- or wet-aged lamb. Consumers were informed that the two samples were from different ageing regimes and served in a random order. A group of 114 high income (≥ $70,000/year NZD) consumer panel who could afford dry-aged products, participated in the consumer sensory evaluation. Panellists were asked to rate the degree of liking using a 9-point hedonic scale (1 = Dislike extremely to 9 = Like extremely), and rate the eating quality using a 5-point hedonic scale (1 =

Unsatisfactory as an everyday product, 2 = Good everyday product, 3 = Slightly better than an everyday product, 4 = Almost a premium product and 5 = A premium product). The procedures used for consumer sensory evaluation in this study has been approved by Auckland University of Technology Ethics Committee.

Table 6.1 Interview guide for the focus group of in-bag dry-aged lamb chops.

1)	Introduction Welcome and introduce participants.
	Explain research objectives and samples information.
	Explain the focus group procedure.
2)	Consent of voluntary participation Inform about recording equipment and how confidentiality will be protected.
	Obtain verbal consent from each of the participants.
3)	Cooking and eating All the participants will be encouraged to observe the meat being cooked and note the aroma and general appearance of lamb chops.
	Lamb presented on a table with other food accompaniments.
	Participants invited to serve themselves one or more lamb chops.
4)	Discussion on why like or dislike dry-aged lamb Do you like or dislike the lamb chops?
	Why do you like or dislike the lamb chops?
	What do you like/dislike about the cooking process and aroma?
	What do you like/dislike about the aroma?
	What do you like/dislike about the appearance of cooked lamb chops?
	What do you like/dislike about the texture while eating?
	What do you like/dislike about taste?
	Do you like or dislike the flavour?
	Do you think this product has a strong sheep meat (mutton) flavour?
	What else do you like/dislike about the flavour?

A focus group is recommended to generate perceptions and/or hypotheses for an area when previous knowledge about the area is limited (McLafferty, 2004; Powell & Single, 1996). A six-member focus group was organised as a complementary study to explore the sensory descriptors of in-bag dry-aged lamb. A group of 12 persons were selected from the consumer sensory panel who have participated the acceptability evaluation of dry-aged lamb, and then were subjected to further screening. They were screened according to the following criterion: 1) High income; 2) Age range of 25 to 70 years; 3) High level of education (Degree or higher), 4) confident to verbally express their opinions (from the 12 we selected 6 that voiced a strong positive or negative opinion on the product). The focus group discussion followed a semi-structured interview protocol according to Rabiee (2004) which is summarised in Table 6.1.

The focus group was directed by three people, which comprised of one moderator and two assistants. The moderator facilitated the discussion and the assistants took notes. The session lasted approximately 60 minutes, and the discussion was recorded on an audio recorder. All the participants were seated together in a relaxed environment at home to mimic the type of environment where the product is likely to be consumed. The lamb was cooked (the same cooking method as the consumer study) in the kitchen, and then presented on a table with other food accompaniments (e.g. sauces, breads and vegetables). The participants were invited to serve themselves as much lamb and other items that they wanted. They were able to serve themselves more lamb at any time during the session. Participants were informed that there were no right or wrong answers to the questions and that we were interested in gaining a better understanding of their perceptions of the product. They were encouraged to freely express their attitudes regarding the product.

6.2.7 Protein carbonyl content

Whole muscle protein of lamb samples was extracted using method as described in section 6.2.2.4. Concentrations of protein carbonyl groups generated from protein oxidation were measured using 2,4-dinitrophenylhydrazine (DNPH) method proposed by Levine et al. (1990). The extracted total muscle proteins were used, and the protein extraction buffer served as a blank to react with DNPH. Absorbance of carbonyl solution was read at 370 nm using a UV-spectrophotometer after suspending in 6 M guanidine HCl (in 20 mM sodium phosphate buffer, pH = 2.3) against the blank. The measurement was performed in duplicate.

6.2.8 Lipid oxidation

6.2.8.1 Lipid oxidation

Extent of lipid oxidation was determined based on the content of thiobarbituric acid reactive substances (TBARS) according to Buege and Aust (1978).

6.2.8.2 Fatty acids (FAs) profile

FAs profile of lamb samples was determined by the procedure described by Zhang et al. (2018).

6.2.9 *In vitro* digestion of lamb chops

In vitro digestion of dry- and wet-aged lamb chops was performed using a static enzymatic digestion method modified from Zhang et al. (2018). A two-stage digestion of 240 minutes (120 minutes of gastric digestion and 120 minutes of intestinal/pancreatic

digestion) was simulated in a bioreactor in a shaking water bath (Thermo Haake DC 10, Karlsruhe, Germany) set at 37 ± 0.2 °C and 80 rpm. Simulated solutions of Gastric Fluid (SGF, pH = 3) and Intestinal Fluid (SIF, pH = 7) were prepared with pepsin (P6887, Sigma) and pancreatin (ACROS Organics™, Thermo Fisher Scientific), respectively. Lamb chops were minced and approximately 4 g (exact weight was recorded) of sub-sample was homogenised with 5 mL of simulated salivary fluid (pH = 7) at 22,000 rpm (IKA Labortechnik, Germany) for 20 seconds, twice to simulate mastication. Pepsin solution was added to the bioreactor to initiate gastric proteolysis (enzyme to substrate ratio of 1 : 278, pH = 2) (Farouk et al., 2014). At 0, 2, 10, 60, and 120 minutes of gastric digestion, two aliquots of 250 µL hydrolysates were removed and immediately mixed with either methanol (1 : 2, v/v) for free amino acids (FAAs) analysis, or SDS-PAGE sample loading buffer (1 : 1, v/v, 50 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% 2-mercaptoethanol and 0.02% bromophenol blue) for SDS-PAGE gel electrophoresis. Aliquots for gel electrophoresis were heated at 95 °C for 5 minutes and then stored at -80 °C until further analysis. Pancreatin solution of enzyme to substrate ratio of 1 : 100 was added to initiate the intestinal digestion at pH 7 for 120 minutes. Aliquots of hydrolysates were removed from the reactor and treated the same as those collected in the gastric phase.

6.2.9.1 SDS-PAGE gel electrophoresis

Hydrolysates collected from the digestion simulation were loaded on to a Novex™ NuPAGE™ 10% Bis-Tris Midi Protein Gels (Invitrogen, UK), by 40 µg proteins per well and separated at ambient temperature in a Bio-Rad Criterion cell system at 150 V using PowerPac™ HC High-Current Power Supply (Bio-Rad® Laboratories, Hercules, CA, USA). An 8 µl aliquot of Novex™ Sharp Pre-stained protein standard (Invitrogen, UK) was used to determine molecular weight (MW) of different protein sizes from 3.5 kDa to 260 kDa. Following electrophoresis, gels were stained in a SimplyBlue SafeStain (Invitrogen™) for 4 hours. Stained gels were then washed with distilled water and images were captured with a GS900 calibrated densitometer scanner (Bio-Rad® Laboratories). The gels were loaded following the order which enabled the time-course of digestion (0, 2, 10, 60, 120, and 240 minutes) to be visualised and for BD and W treatments to be compared.

6.2.9.2 Analysis of free amino acids (FAAs)

Aliquots for FAAs profile were centrifuged at 10,000 g for 10 minutes. The supernatant containing FAAs extract was quantified by LC-MS/MS according to Salazar

et al. (2012) with some modifications. The FAAs extract was diluted and spiked (1 : 1, v/v) with d_4-alanine (10 $mg.L^{-1}$) as an internal standard. The spiked FAAs sample of 10 µL was derivatised with 10 µL of 2.8 $mg.mL^{-1}$ 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (in dry acetonitrile, Apollo Scientific, UK) in 70 µL of 200 mM sodium tetraborate decahydrate buffer (pH of 8.8, Pure Science Ltd, New Zealand) at 55 °C for 15 minutes. The amino acid (AA) derivatives of 50 µL were neutralised with 450 µL of formic acid (10%) before the analysis by the Agilent 1260 Infinity HPLC system equipped with Agilent 6420 Triple Quadrupole LC/MS system (Agilent Technologies New Zealand Limited, New Zealand). The AA standard solution (100 µM) of 40 amines (included internal standard) prepared with the 37 AA standard mixture (A9906, Sigma), asparagine (A0884, Sigma) and glutamine (G3126, Sigma) was serially diluted to 0.78 µM to generate a standard curve for the identification and quantification of FAAs using MassHunter software (Agilent Technologies). The final concentration of FAAs was expressed as mg/g protein.

Derivatised AAs were separated on a C18 core shell Kinetex LC column (100 × 2.1 mm with a 1.7 µm particle size, 00D-4726-AN, Kinetex®) using a gradient of 0 - 8.0 minutes (13.0% A and 87.0% B), 8.0 - 16.0 minutes (17.0% A and 83.0% B), 16.0 - 17.0 minutes (80.0% A and 20.0% B), and 18.0 - 18.5 minutes (1.5% A and 98.5% B). The working eluent A was 0.1% formic acid in acetonitrile, and B was 0.6% formic acid in MilliQ water. The column flow rate was 0.2 $mL.min^{-1}$ and column temperature was 22.6 °C. A positive electrospray ionisation (ESI+) mode with MRM-MS method was employed for the quantitation of AAs by selecting the most sensitive parent-daughter ion transition of m/z $[M-H]^+ > 171$. Ionisation source temperature was 325 °C, gas flow rate at 6.0 $L.min^{-1}$ and capillary voltage of 2.0 kV.

6.2.9.3 Relative protein digestibility (%)

The relative protein digestibility following 240 minutes of simulated gastrointestinal digestion was determined by three methods; (1) the protein profile using SDS-PAGE electrophoresis (section 6.2.9.1), (2) the release of FAAs (section 6.2.9.2), and (3) the protein content (protein fragments and peptides) in the hydrolysates after pancreatic digestion using RC-DC protein assay kit (Bio-Rad® Laboratories, Hercules, CA, USA). Relative digestibility (%) was calculated as follows:

(1) $\text{Relative digestibility}_{\text{SDS-PAGE}} = \left(1 - \frac{\text{Optical intensity of protein bands} < 10\ \text{kDa}}{\text{Optical intensity of all protein bands}}\right) \times 100\%$

(2) $\text{Relative digestibility}_{\text{FAAs}} = \left(\frac{\text{Total FAAs (g) at 240 min} - \text{total FAAs (g) at 0 min}}{\text{Protein content of the sample}}\right) \times 100\%$

(3) Relative digestibility $_{\text{Protein content}} = (1 - \frac{\text{Protein content (g) in hydrolysate at 240 min}}{\text{Protein content of the sample}}) \times 100\%$

6.2.10 Statistical analysis

A randomised control trial was designed with 30 pairs of lamb legs (n = 60) which were evenly assigned to two different treatments: BD and W. A model including the fixed effect of ageing treatments and the random effect of carcass ID and carcass sides was used for chemical analysis of minced chops. For instrumental colour and texture profile analysis, the ageing treatments and muscles were considered as fixed effects, and carcass ID and carcass sides were considered as random effects fitted in the model. For the sensory evaluation analysis, the ageing treatments were included as the fixed effects, panellists, carcass ID and muscles of lamb chops were included as the random effect in this model. Linear mixed effect regression analyses were performed on the data using R (version 3.4.1), with "lme4" and "predictmeans" packages to determine the difference between ageing treatments. Analysis of variance (ANOVA, one-way) was used to investigate the effect of different ageing treatments with a post-hoc comparison of means performed using Fisher's least significant differences (LSD) and Tukey's (HSD) test at 5% significance level. A power curve was generated to describe the relationship between % ageing weight loss and ageing time under the current dry-ageing process. The Chi-squared test was performed on the eating quality rating of two ageing methods at 5% significance level.

6.3 Results and discussions

6.3.1 Meat quality

6.3.1.1 Weight losses

The weight losses during ageing and cooking were measured to estimate the yield of dry-aged lamb chops from processing to the point of consumption. The losses from both treatments consisted of weight loss from ageing and cook loss only, as no trimming of the dry-aged lamb was necessary. BD resulted in a significantly ($P < 0.0001$) higher weight loss than W, which was expected in dry-aged meat products. The change in weight loss from ageing over the ageing time is shown in Figure 6.2a. In general, about 20% of moisture was lost from dry-aged lamb after 21 days of ageing time as compared to the control with an average of 0.71% purge loss. Various levels of ageing loss (approx. 10 - 40%) have also been reported on dry-aged beef products in other studies which were mainly attributed to the various ageing conditions and length of ageing time used. The

relationship (R^2 = 0.9983) between % weight loss from ageing and ageing time under the current dry-ageing process is shown as follows:

$$Y = 0.0552X^{0.6783} \times 100$$

Y = % weight loss from ageing;

X = Days of ageing

The correlation between the initial weight (applicable range: 0.5 - 2 kg) and dry-aged weight (21 days) was calculated to predict the yield of dry-aged lamb when the initial weight was known (Figure 6.2b). A close to linear relationship (R^2 = 0.9844) was found as follows:

$$Y = 0.9044X - 151$$

Y = Dry-aged weight (g) at 21 days of ageing;

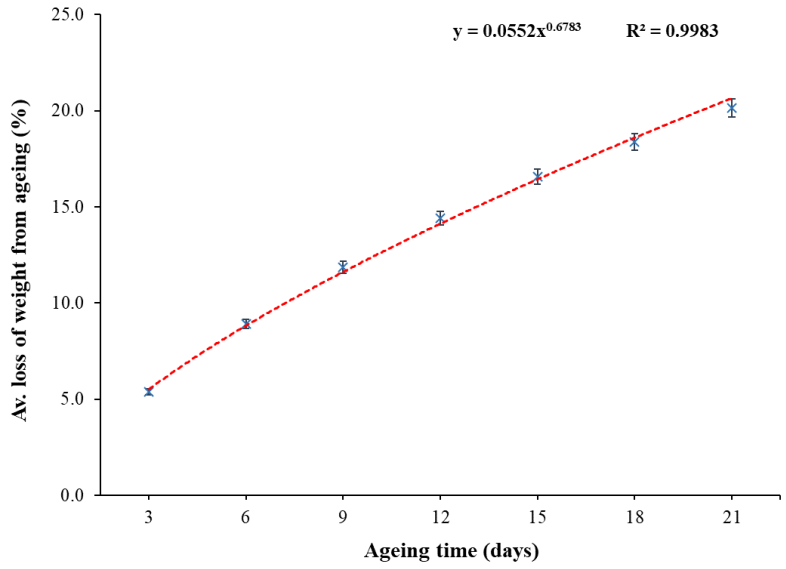
X = Initial weight (g) before ageing

Cook loss of dry-aged lamb was significantly reduced to an average value of 16.94% compared to the wet-aged control (27.79%, Table 6.2). Similar findings were reported on beef products where cook loss was lower in the dry-aged beef than wet-aged counterparts (DeGeer et al., 2009; Li et al., 2013). Although the total loss (ageing weight loss + cook loss) remained significantly higher in dry-aged samples (36.52%) than the control (28.48%), the difference between the two ageing treatments was reduced to less than 10%. Dikeman et al. (2013) also observed that similar moisture levels were detected in cooked beef regardless of ageing method, although a significantly higher moisture content was found in the wet-aged samples prior to cooking compared to the dry-aged, as observed in this study.

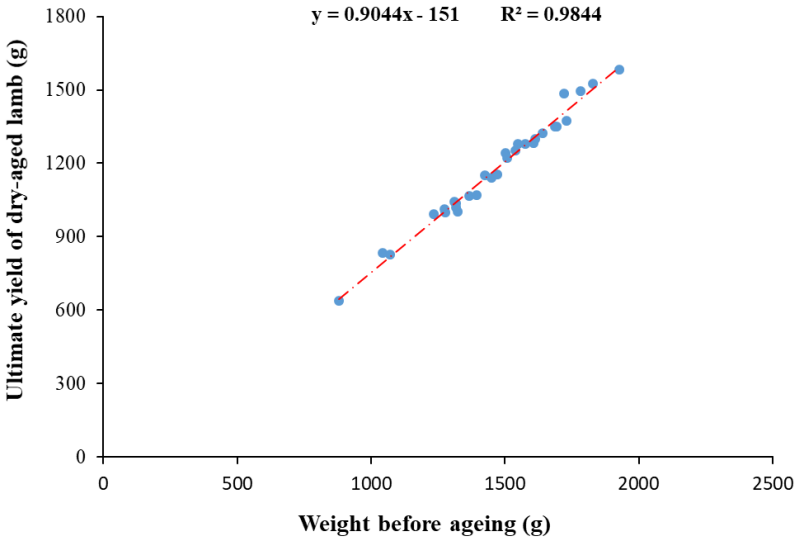
6.3.1.2 Proximate content and pH

The moisture content of dry-aged lamb (67.30%) was lower ($P < 0.05$) than the wet-aged counterparts (73.25%) which contradicts the outcomes of other studies that found no difference in moisture content between wet and dry-ageing treatments (Ahnström et al., 2006; DeGeer et al., 2009). This could be attributed to dehydration as part of dry-ageing process mainly occurring on the outer surface of the meat. Most studies have trimmed the surface crust of dry-aged meat prior to sampling for moisture content analysis thus eliminating the difference that would have been observed (Zhang et al., 2019). With the use of dry-ageing bags, no trimming was necessary in this study. Lower

moisture levels in dry-aged lamb were expected owing to the significantly higher weight loss in the in-bag dry-aged sample compared to wet-aged after 21 days of ageing.



(a)



(b)

Figure 6.2 (a) Average loss of weight from ageing (%) observed in in-bag dry-aged lamb across 21 days of ageing time. A trendline was added to establish the power relationship between the weight loss from ageing with ageing time. Error bars represent standard errors (n = 30). (b) Initial weight (g) of lamb legs before ageing and the ultimate yield (g) of in-bag dry-aged lamb (n = 30). A trendline was added to predict the linear relationship between the weight of lamb samples before and post in-bag dry-ageing.

Dry-aged lamb had higher (P = 0.058) crude fat (6.82%) and protein (20.22%) contents compared to the wet-aged control (5.93% fat and 16.54% protein), which agreed

with the outcomes of other studies on beef (DeGeer et al., 2009; Dikeman et al., 2013). The increase of fat and protein content in the current study may be attributed to the significant decrease in moisture content, which concentrated the other components of lamb.

Both ageing methods increased ($P < 0.05$) the pH values of lamb from the average value of 5.83 (before ageing) to 5.92 (wet-aged) and 6.04 (dry-aged), respectively (Table 6.2). The increase of pH after ageing has been well reported on beef (Kim et al., 2017; Obuz et al., 2014; Zhang et al., 2019), which could be associated with the production of nitrogenous compounds by proteolysis. Dry-ageing has been reported to increase the pH of beef loins (Li et al., 2013) with ageing time and for the dry-aged samples to have a higher pH value than the wet-aged counterparts (Li et al., 2014).

6.3.1.3 Surface microorganism growth

Overall, the surface microorganism counts were low in both dry- and wet-aged lamb (Table 6.2). BD increased ($P < 0.05$) the level of yeast and lowered aerobic bacterial counts compared to W. This could be associated with the low moisture environment on the surface of dry-aged meat favouring the proliferation of yeast which consumed the available oxygen on meat surface, and consequently outcompeted the aerobic bacteria. A higher yeast level was also observed in dry-aged beef than the wet-aged counterparts (Li et al., 2014; Li et al., 2013; Zhang et al., 2019). There were no *E. coli* and moulds detected in either treatment which was also observed in a previous study on beef (Hulánková, Kameník, Saláková, Závodský, & Borilova, 2018). Lactic acid bacteria and *Enterobacteriaceae* were found on the surface of wet-aged lamb samples but negligible on the dry-aged. Similar findings of lactic acid bacteria were also reported by Li et al. (2013) between wet- and dry-aged beef which could be attributed to the anaerobic environment of W favouring the proliferation of lactic acid bacteria. Contradictory results have been reported on the *Enterobacteriaceae* counts of dry-aged beef being higher (Li et al., 2014) or similar (Li et al., 2013) compared to the wet-aged control.

6.3.1.4 Instrumental colour

The ageing process affected the colour properties of lamb. All the colour attributes were lower ($P < 0.05$) in dry-aged lamb than the wet-aged except for hue angle and VL a* ($P > 0.05$) (Table 6.3). Overall, BD generated a slightly darker, less red and less yellow colour which could be associated with the moisture loss during the ageing process. The dehydration of dry-aged lamb reduced the light reflection and concentrated colour

components including myoglobin and iron that result in changes in the meat colour. Similar effects of dry-ageing on beef colour was reported by Kim et al. (2016). Colour plays an essential role in consumer acceptability of lamb. The minimum thresholds for L* and a* for consumer colour acceptability has been reported as 34 to 35 and 9.5, respectively (Ponnampalam et al., 2017). The colours of the lamb samples from both ageing treatments were within the acceptable colour range, thus, BD had no negative effect on the colour quality of lamb after 21 days of ageing period.

There was no difference in the way the ageing treatments affected the colour of the four muscles of lamb chops, though an exception was seen for the VL muscle of dry-aged lamb, which was less brown (lower hue) than the wet-aged control ($P < 0.05$, Table 6.3). The impact of different muscles on the colour properties in the current study may be associated with the inherent variations across muscles including pH, the content of fat, iron and myoglobin (Seideman, Cross, Smith, & Durland, 1984) and also the oxidative stability of myoglobin (Renerre & Labas, 1987).

Table 6.2 Effect of ageing treatments on the physico-chemical and microbial properties of lamb chops.

	W	**BD**	**SED**	***P*-ageing**
pH	5.92[a]	6.04[b]	0.02	< 0.0001
% Moisture	73.25[a]	67.30[b]	0.37	< 0.0001
% Crude fat	5.93	6.82	0.46	0.058
% Muscle protein	16.54[a]	20.22[b]	0.24	< 0.0001
% Cook loss	27.79[a]	16.94[b]	0.67	< 0.0001
% Total loss	28.48[a]	36.52[b]	0.56	< 0.0001
Meat surface microbial count (log cfu/g)				
APC	5.16[a]	2.68[b]	0.75	0.030
LAB	2.64	< 1.00	-	-
Moulds	< 1.00	< 1.00	-	-
Yeast	2.38[a]	3.75[b]	0.47	0.043
Enterobacteriaceae	2.36	< 1.00	-	-
E. coli (log MPN/g)	< 0.48	< 0.48	-	-

W and BD refer to wet-ageing and in-bag dry-ageing, respectively. SED is the standard error of a difference between means. APC, LAB, *E. coli* refer to aerobic bacteria plate counts, lactic acid bacteria counts and *Escherichia coli*, respectively. Different superscript letters "a, b" within the same row indicates results significantly differed from each other ($P < 0.05$).

Table 6.3 Effect of ageing treatments and muscle types on instrumental colour of lamb chops.

	W	BD	SED	*P*-ageing	*P*-muscle (Across treatments)	*P*-ageing (Across muscles)
L*						
SM	44.01^{ax}	42.57^{b}	0.56	0.017	< 0.0001	< 0.0001
BF	44.52^{ax}	42.66^{b}		< 0.0001		
VL	45.78^{ay}	43.40^{b}		< 0.0001		
RF	45.70^{ay}	43.63^{b}		0.002		
P-muscle	0.002	0.202				
a*						
SM	14.92^{axy}	13.84^{bx}	0.52	0.041	< 0.0001	< 0.0001
BF	15.95^{az}	14.40^{bxy}		0.007		
VL	15.78^{xz}	14.97^{y}		0.092		
RF	14.39^{ay}	12.43^{bz}		0.001		
P-muscle	0.004	< 0.001				
b*						
SM	12.62^{ax}	11.66^{bx}	0.39	0.011	< 0.0001	< 0.0001
BF	13.59^{ay}	12.32^{bxy}		0.003		
VL	13.75^{ay}	12.64^{by}		0.003		
RF	13.13^{ax}	11.17^{bz}		< 0.0001		
P-muscle	0.009	0.003				
Chroma						
SM	19.56^{ax}	18.11^{bx}	0.63	0.020	< 0.0001	< 0.0001
BF	20.96^{ay}	18.96^{bxy}		0.004		
VL	20.93^{ay}	19.59^{by}		0.024		
RF	19.49^{ax}	16.73^{bz}		< 0.001		
P-muscle	0.009	< 0.001				
Hue						
SM	40.34^{x}	40.20^{x}	0.47	0.803	< 0.0001	0.216
BF	40.47^{x}	40.67^{x}		0.611		
VL	41.11^{ax}	40.25^{bx}		0.017		
RF	42.49^{y}	42.12^{y}		0.501		
P-muscle	< 0.0001	< 0.001				

W and BD refer to wet-ageing and in-bag dry-ageing, respectively. SM, BF, VL and RF refer to *m. semimembranosus*, *m. biceps* femoris, *m. vastus lateralis* and *m. rectus femoris*, respectively. SED is the standard error of a difference between means. Different superscript letters "a, b" within the same row indicates results significantly differed from each other ($P < 0.05$). Different superscript letters "x, y, *z*" within the same column indicates results significantly differed from each other ($P < 0.05$).

Table 6.4 Effect of ageing treatments and muscle types on the instrumental texture profile analysis of the lamb chops.

	W	BD	SED	*P*-ageing	*P*-muscle (Across treatments)	*P*-ageing (Across muscles)
Hardness (N)						
SM	21.14^{a}	25.27^{b}	1.66	0.015	0.366	< 0.001
BF	22.53	26.50		0.067		
VL	22.48^{a}	27.19^{b}		0.004		
RF	22.17	24.45		0.120		
P-muscle	0.680	0.465				
Chewiness (N)						
SM	7.47	8.46	0.69	0.134	0.017	< 0.001
BF	8.22^{a}	9.89^{b}		0.048		
VL	7.67^{a}	9.46^{b}		0.014		
RF	7.27	7.87		0.309		
P-muscle	0.414	0.051				
Springiness						
SM	0.62^{xy}	0.63^{x}	0.02	0.514	< 0.0001	0.427
BF	0.65^{x}	0.65^{x}		0.979		
VL	0.61^{y}	0.62^{xy}		0.463		
RF	0.59^{y}	0.60^{y}		0.657		
P-muscle	0.012	0.010				
Cohesiveness						
SM	0.56^{a}	0.53^{b}	0.01	0.002	0.597	0.001
BF	0.55	0.54		0.360		
VL	0.55	0.54		0.497		
RF	0.55	0.53		0.057		
P-muscle	0.373	0.354				
Adhesiveness						
SM	-11.56	-18.13	4.10	0.229	0.054	0.335
BF	-9.18	-8.35		0.229		
VL	-10.93	-12.31		0.736		
RF	-7.08	-8.44		0.593		
P-muscle	0.439	0.127				
Resilience						
SM	0.23a	0.21b	0.01	0.017	0.284	0.048
BF	0.22	0.22		0.845		
VL	0.22	0.22		0.465		
RF	0.22	0.21		0.356		
P-muscle	0.123	0.443				

W and BD refer to wet-ageing and in-bag dry-ageing, respectively. SM, BF, VL and RF refer to *m. semimembranosus, m. biceps femoris, m. vastus lateralis* and *m. rectus femoris*, respectively. SED is the standard error of a difference between means. Different superscript letters "a, b" within the same row indicates results significantly differed from each other ($P < 0.05$). Different superscript letters "x, y, z" within the same column indicates results significantly differed from each other ($P < 0.05$).

6.3.1.5 Instrumental texture profile analysis

Most of the study on dry-aged beef products focused on the tenderness of the meat as compared to the wet-aged. However, the texture profile of dry-aged products has not been well explored, particularly for dry-aged lamb. The majority of studies on beef failed to detect any significant difference in shear force between dry- and wet-aged beef (Dikeman et al., 2013; Kim et al., 2016; Li et al., 2013; Sitz et al., 2006), suggesting that improvement in tenderness was not an advantage of dry-aged products over wet-aged, considering both ageing methods can effectively tenderise the meat to similar levels.

As shown in Table 6.4, both ageing treatments produced lamb samples which were tender and easy to chew (low force values for hardness and chewiness). A higher ($P < 0.05$) hardness and chewiness was detected in dry-aged lamb compared to wet-aged likely as a result of dehydration of the samples which caused shrinkage and resulted in a firm texture. Cohesiveness and resilience of dry-aged lamb were slightly ($P < 0.05$) lower as compared to the wet-aged control. However, the difference between the two treatments was numerically too small to have a significant impact on the textural properties of the meat.

Lamb legs consist of multiple muscles. Different muscles were affected differently by the ageing treatments. As shown in Table 6.4, the type of muscle only influenced the hardness, chewiness and springiness of lamb samples. The difference ($P < 0.05$) of hardness between ageing treatments was seen in SM and VL muscles. Dry-aged lamb was slightly chewier than the control, however, the increase ($P < 0.05$) was only observed in BF and VL muscles.

6.3.2 Sensory quality

6.3.2.1 Consumer acceptability

The palatability of in-bag dry-aged lamb has not been previously evaluated. As shown in Table 6.5, both lamb samples were equally preferred ($P > 0.05$) by the consumer panellists as a 'better than everyday product'. The average degree of preference for both samples (in-bag dry-aged = 6.68, wet-aged = 6.75) was close to "like moderately" which was score 7.

The sensory quality of in-bag dry-aged beef compared to wet-aged has been extensively studied but the results were inconsistent. For instance, in-bag dry-aged beef samples have been reported to be preferred by consumers to the wet-aged counterparts (Kim et al., 2016; Li et al., 2013; Stenström et al., 2014); no difference (Berger et al., 2018; Smith et al., 2008); or wet-aged products preferred (Sitz et al., 2006). The conflicting findings may have risen from the use of different ageing process, types of muscles and breeds.

Dry-aged meat is a niche product which is expected to be only preferred by a certain group of consumers. Therefore, the debate on different ageing methods should be focused on how many (more) consumers would prefer dry-/wet-aged products instead of which products were more palatable than the other because they are all palatable. In the current study, 44.74% of consumers rated wet-aged lamb higher in terms of overall preference.

This was more likely owing to their familiarity with the wet-aged lamb products. It was promising to observe a similar number (40.35%) of consumers that preferred in-bag dry-aged lamb to the wet-aged counterparts (44.74%). There was only a small group (14.95% on overall preference) of consumers who could not distinguish between in-bag dry-aged and wet-aged lamb. In eating quality rating, there was no significant difference between the average score of in-bag dry-aged and wet-aged lamb. Both in-bag dry- and wet-aged samples were considered by most of the consumers (71.68% and 68.14%, respectively) as slightly higher than an everyday product (score 3). Considering these were lamb chops and not lamb loins that were assessed, this overall level of acceptability is highly promising for this lamb cut. No difference (chi-square; P = 0.670) was found in the distribution of response along the 5-point scale consumer rating of eating quality between two ageing methods (Table 6.5). Therefore, the current findings suggest a niche market for dry-aged products and a great market potential for in-bag dry-aged lamb.

Table 6.5 Effect of ageing treatments on the sensory quality of lamb chops.

	W	**BD**	**SED**	***P*-ageing**
Degree of liking	6.75	6.68	0.19	0.682
% preferred[1]	44.74	40.35		
Eating quality rating	3.14	3.10	0.13	0.750
% rating[2]				0.670
Unsatisfactory as an everyday product	2.65	4.42		
Good everyday product	29.20	23.89		
Slightly better than an everyday product	31.86	37.17		
Almost a premium product	24.78	26.55		
A premium product	11.50	7.96		

W and BD refer to wet-ageing and in-bag dry-ageing, respectively. SED is the standard error of a difference between means. % preferred[1] was calculated as % consumers rated W or BD sample higher than its equivalent. % rating[2] was calculated as % consumers rated on the five-point scale eating quality groups of W and BD samples.

6.3.2.2 Focus group perceptions of in-bag dry-aged lamb chops

In the present study, a focus group was used phenomenologically to strengthen the outcomes of the quantitative survey with the targeted consumers due to the paucity of information on dry-aged lamb. Members of the focus group in the present study discussed and described the characteristics of in-bag dry-aged lamb chops based on their eating experience of lamb products. Out of the six members, only one member did not like the

in-bag dry-aged lamb chop because of the drier texture, though the same person perceived the flavour of the meat as pleasant.

Appearance and aroma: The "lean (not fatty)" appearance of in-bag dry-aged lamb chops was considered as an advantage. "Dry (not juicy)" appearance was another attribute of in-bag dry-aged lamb which may be one of the key features of dry-aged products. The dehydration process of dry-ageing caused a certain level of moisture loss and resulted in a "dry look". All the focus group panellists strongly liked the (cooked meat) aroma of the in-bag dry-aged lamb and described it as "fine/pleasant aroma" and "less mutton smell (unpleasant) than the normal lamb".

Texture: All of the group members agreed that the texture of in-bag dry-aged lamb was "tender", "spongy" and "less fibrous". These attributes were considered as positive descriptors for the texture characteristics of in-bag dry-aged lamb chops. These attributes were also observed from consumer sensory session that the consumers who preferred in-bag dry-aged lamb liked the "chewier" texture rather than too soft/tender texture of the wet-aged lamb.

Taste/flavour: The taste/flavour of in-bag dry-aged lamb was considered as nutty, sweet with aftertaste, venison-like (gamey), stronger lamb flavour (pleasant) but no mutton flavour (unpleasant), no fatty taste or greasy aftertaste (even when cold). A stronger umami and sweet taste, buttery, nutty, brothy and roasted flavours were also detected previously in (in-bag) dry-aged beef samples as compared to the wet-aged counterparts (Li et al., 2014; O'Quinn et al., 2016). The suggestion by the focus group that dry-aged lamb left no greasy coating is highly significant as one of the issues consumers have with lamb meat is the coating of the mouth and throat often experienced on eating lamb due to the high melting temperatures of lamb fat (Wood, 1984; Young, Reid, Smith, & Braggins, 1994). This quality change as a result of dry-ageing need to be further ascertained and if confirmed should be used to differentiate dry-aged lamb from its wet-aged equivalent.

Table 6.6 Effect of ageing treatments on the oxidative changes of lipid and protein of lamb chops.

	W	BD	SED	*P*-ageing
Protein carbonyl (nmol/mg protein)	2.20	2.31	0.08	0.151
TBARS (mg MDA/kg meat)	0.38[a]	1.30[b]	0.13	< 0.0001
Fatty acids profile (mg/g dry matter)				
10:0	0.18	0.17	0.02	0.631
12:0	0.35	0.33	0.03	0.539
14:0	2.56	2.44	0.22	0.580
14:1	0.09	0.09	0.01	0.490
15:0	0.27	0.25	0.02	0.390
16:0	12.71	12.44	0.72	0.706
16:1	1.00	1.01	0.07	0.879
17:0	1.10	1.07	0.05	0.542
17:1	0.38	0.37	0.02	0.490
18:0	10.89	10.60	0.57	0.606
18:1 (n-9, *cis* & *trans*)	18.37	18.22	1.06	0.887
18:2 (n-6, *cis* & *trans*)	2.00	2.02	0.07	0.715
18:3 (n−6)	0.21	0.21	0.02	0.848
18:3 (n-3)	1.31	1.29	0.05	0.713
20:0	0.19	0.20	0.01	0.272
20:2 (n-6)	0.15	0.16	0.01	0.429
20:6 (n-6)	0.23	0.23	0.01	0.873
20:5 (n-3)	0.69	0.70	0.02	0.801
22:0	0.27	0.27	0.01	0.900
24:0	0.20	0.20	0.01	0.946
22:6 (n-3)	0.36	0.35	0.01	0.725
UFAs	24.80	24.65	1.24	0.905
SFAs	28.72	27.96	1.47	0.609
MUFAs	19.84	19.68	1.14	0.890
n-3	2.36	2.34	0.05	0.756
n-6	2.59	2.62	0.08	0.734
PUFAs	4.95	4.96	0.12	0.928
% UFAs	46.40	46.92	0.47	0.266
% SFAs	53.60	53.08	0.47	0.266
% MUFAs	36.87	37.15	0.54	0.609
% n-3	4.56	4.64	0.22	0.721
% n-6	4.96	5.14	0.20	0.390
% PUFAs	9.52	9.77	0.40	0.534

W and BD refer to wet-ageing and in-bag dry-ageing, respectively. SED is the standard error of a difference between means. UFAs, SFAs, MUFAs, PUFAs and n refer to Unsaturated Fatty Acids, Saturated Fatty Acids, Mono-Unsaturated Fatty Acids, Poly-Unsaturated Fatty Acids and Omega, respectively. Different superscript letters "a, b" within the same row indicate results significantly differed from each other ($P < 0.05$).

6.3.3 Protein and lipid oxidation

6.3.3.1 Protein carbonyl content

Assessment of protein carbonyl content has been widely used to estimate the extent of protein oxidation. As shown in Table 6.6, overall, the protein carbonyl content was low in both lamb samples ($P > 0.05$). The protein carbonyl naturally exists in the animal tissues at a level of 1 - 2 nmol.mg^{-1} and increases during rigor mortis (Martinaud et al., 1997; Sante-Lhoutellier et al., 2008). Oxidative damage (e.g. oxidation, cross-link formation and aggregation) of protein has been linked to the loss of protein functionality (for example, solubility, gelation, emulsifying and water-holding capacity), nutritional values (including the loss of essential AAs and protein digestibility) (Sante-Lhoutellier et al., 2008) and sensory qualities (mainly texture/tenderness and juiciness). It has been reported that the post-mortem ageing may promote the carbonylation of meat proteins (Sante-Lhoutellier et al., 2008). However, the influence was highly dependent on the source of meat, type of muscle and, particularly the storage conditions (Estévez, 2011). Storage under high-oxygen atmosphere at higher temperature or exposure to reactive agents such as light, transition metals (Fe^{3+}/Cu^{2+}) and oxidising lipids could also trigger the protein oxidation. In the current study, the barrier function of the ageing bag limited oxygen availability in the meat (Li et al., 2013), and reduced the potential for the carbonylation of protein and resulted in a similar carbonyl level as the control.

6.3.3.2 TBARS and FAs profile

Lipid is more susceptible to oxidative damage than protein during post-mortem storage (Park et al., 2006). TBARS has been widely used to evaluate the extent of lipid oxidation by measuring the content of lipid peroxidation products, malondialdehyde (MDA), present in the samples (Min & Ahn, 2005). The oxidative damage of lipids is linked to a deteriorative impact on the meat quality in the form of 1) nutritional values including the loss of polyunsaturated fatty acids (PUFAs) and production of pro-oxidants that initiate protein oxidation (Estévez, 2011), and 2) sensory qualities, such as discoloration and generation of rancid and oxidised flavours. As shown in Table 6.6, a significantly higher TBARS value was detected in dry-aged lamb (1.30 mg MDA/kg meat) compared to the wet-aged control (0.38 mg MDA/kg meat). However, the TBARS level in the current study was below the rancidity threshold of 2.0 mg MDA (Campo et al., 2006). Jiang et al. (2010) found no significant difference in TBARS between dry- and wet-aged beef samples. BD was reported to lower lipid oxidation (lower TBARS) in beef products compared to the D (DeGeer et al., 2009). As described earlier, dry-ageing is an

aerobic maturation process involving a certain degree of oxidation and resulting in oxidative by-products. These oxidative products participate later in cooking and produce a range of volatiles such as aldehydes, hydrocarbons, ketones and lactones, which may contribute towards the flavour intensity of meat (Khan et al., 2015; Mottram, 1998; Watanabe et al., 2015).

The FAs content were not affected ($P > 0.05$, Table 6.6) by the ageing treatments suggesting that the BD did not negatively affect the nutritional value of FAs in lamb. It is worth noting that BD did not adversely affect PUFAs, including EPA and DHA. Therefore, the nutritional value of lamb was not affected by the current BD process in terms of FAs profile, although an increase of TBARS level in the in-bag dry-aged lamb was observed. The increase in oxidative potential (TBARS), on the other hand, may be associated with the flavour development of dry-aged products as discussed above.

6.3.4 Proteolysis

Protein profiles (SDS-PAGE) of in-bag dry- and wet-aged lamb are shown in Figure 6.3 (0 min). No difference of protein profiles between the ageing regimes was observed, suggesting a similar proteolytic pattern occurred due to the activities of endogenous proteases regardless of the ageing regimes. Similar findings were also reported by Choe et al. (2020) that similar protein profile (SDS-PAGE) was detected in beef muscles aged by traditional out-of-bag dry-ageing and W regimes. Peptides of smaller than < 3 kDa were present in higher amounts in dry-aged beef compared to the wet-aged in their study. Their observation could be due to the proliferation of microorganisms (mould and yeast) during dry-ageing, which contributed towards the proteolytic activity of peptidases and resulted in different compositions of FAAs compared to the wet-aged (Lee et al., 2019a, 2019b). In this study, the release of FAAs following BD was more evident than that of W where the increases ($P < 0.05$) were only detected in isoleucine, lysine, aspartic acid and proline (Table 6.7, 0 min). Such changes in FAAs compositions could be explained by two mechanisms: the activity of aminopeptidases due to the growth of yeast (Table 6.2) and a higher ageing temperature used for the BD process. The use of ageing bags has been suggested to mainly result in a higher yeast level because the moisture evaporation during dry-ageing lowers the water activity in meat and favours the proliferation of yeast (Li et al., 2014; Zhang et al., 2019). The activity of aminopeptidases by yeast is known to contribute towards the release of FAAs in dry cured/fermented meat products as well (Flores & Toldrá, 2011). On the other hand, different ageing temperatures (2 *vs.* -1.5 °C) used in the current study may also have contributed to the changes in the FAAs profile

between two ageing regimes. A significantly higher level of tryptophan, phenylalanine, valine, tyrosine, glutamate, isoleucine and leucine was detected in dry-aged beef at 3 °C compared to the wet-aged equivalents at 1 °C by Kim et al. (2016). However, such contributory effect of ageing temperature on the activity of proteases was not supported by the findings of protein profile between two ageing regimes in this study. Thus, there remains a need for future study to assess the actual impact of the slight increase in ageing temperature on the proteolytic pattens of in-bag dry-aged lamb.

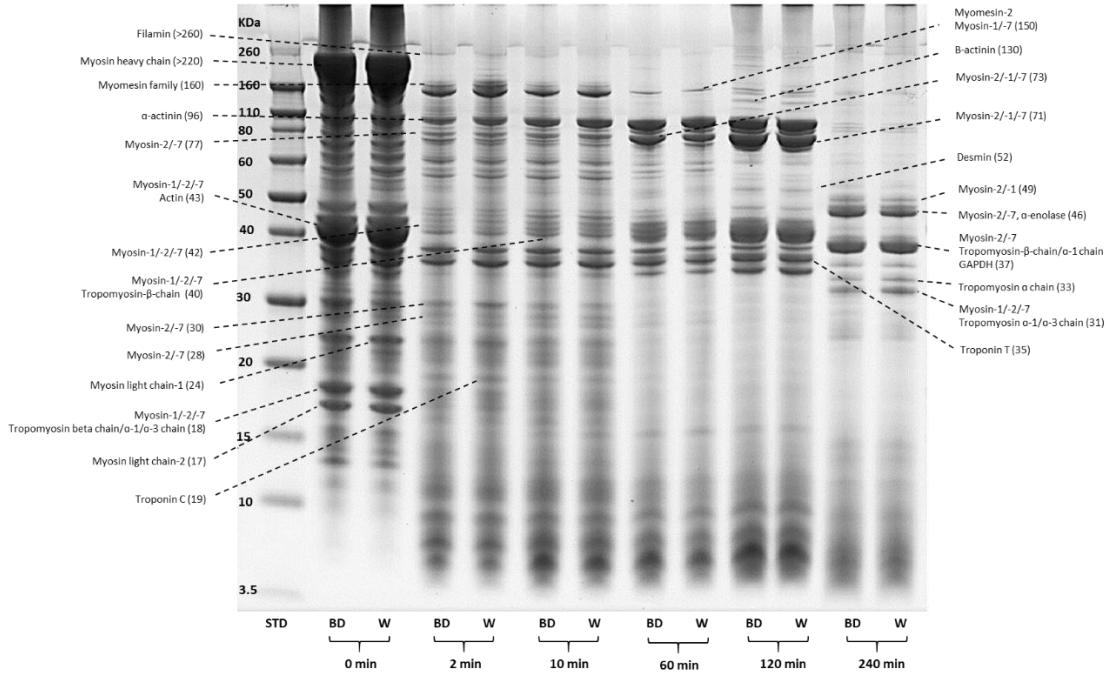


Figure 6.3 SDS-PAGE protein profile of *in vitro* gastrointestinal digestion of in-bag dry-aged (BD) and wet-aged (W) lamb chops; protein identification was according to Farouk et al. (2014); Kaur et al (2014), and Wu et al. (2014).

6.3.5 *In vitro* digestibility

In vitro protein digestibility was measured in three ways: (1) by using SDS-PAGE to determine the changes in protein profiles with four hours of digestion time simulating peptic and pancreatic digestions in the upper and lower gastrointestinal tract; (2) by measuring the changes in FAAs with the digestion simulation; and (3) by measuring the protein content in the final hydrolysate.

6.3.5.1 Changes in protein profiles (SDS-PAGE)

The representative SDS-PAGE protein profiles of in-bag dry- and wet-aged lamb at five sampling time points (0, 2, 10, 60, 120, and 240 minutes) during four hours of simulated digestion process are shown in Figure 6.3. The % relative quantity [(% RQ = optical density of the protein fragments/optical density of total proteins) × 100%] was

used to compare the quantitative differences of protein fragments between the ageing treatments. Protein fragments of different MW were grouped into seven MW groups (> 110, 60 - 110, 50 - 60, 40 - 50, 30 - 40, 20 - 30 and < 20 kDa) for statistical analysis as shown in Figure 6.4. There was no difference ($P > 0.05$) in the % RQ of the seven MW groups between in-bag dry- and wet-aged lamb over the digestion period. Therefore, statistical analysis of these protein groups was performed on the average % RQ of the two ageing treatments.

Most of the large size proteins including filamin, titin and nebulin (>250 kDa) and myosin heavy chain (MHC, > 220 kDa) were digested rapidly by pepsin and the protein bands disappeared within 10 minutes of pepsin digestion (Figure 6.3) (Farouk et al., 2014; Wu, Clerens, & Farouk, 2014). Myomesin and myosin family proteins of MW of 150 - 160 kDa were progressively cleaved through the gastric digestion with accumulation of lower MW (60 - 110 kDa) protein fragments. This was also seen with the % RQ results where large MW proteins (>110 KDa) were reduced ($P < 0.05$) over the gastric digestion process with increased ($P < 0.05$) protein fragments of MW between 60 and 110 kDa (Figure 6.4).

Protein fragments of MW 50-60 kDa were quickly digested within the first 2 minutes of gastric digestion with continuous cleavage throughout the digestion period (Figure 6.3). Another group of protein fragments with significant changes in protein profiles ranged from 30 to 50 kDa, which mainly consisted of myosin family, actin, and tropomyosin family (Farouk et al., 2014; Wu et al., 2014). These protein fragments underwent rapid partial digestion by pepsin at the initial stage (2 min). The broken fragments became resistant to pepsin and accumulated throughout the gastric digestion, as evidenced by the significant decrease in the % RQ of proteins (40 - 50 kDa) within the first 10 minutes with no further changes afterward (Figure 6.4). The low MW protein fragments (10 - 30 kDa) included myosin-1, -2 and -7, myosin light chain-1 and -2, Troponin C, which were readily digested within 60 minutes of gastric digestion. The % RQ of low MW proteins (20 - 30 kDa) significantly reduced over the gastric digestion while the smaller protein fragments (< 20 kDa) increased ($P < 0.05$) within 2 minutes then varied slightly throughout the pepsin digestion time of 120 minutes.

The protein fragments that resisted gastric digestions (30 - 50 kDa) were further hydrolysed to smaller fragments during subsequent 120 minutes of pancreatic digestion (Figure 6.3). These fragments may include proline-containing peptides that are reported to resist digestion by pancreatic enzymes (Sitrin, 2014). This is further supported by the

relatively unchanged amount of proline in the hydrolysates over the digestion simulation period (Table 6.7). Farouk et al. (2014) also observed the resistance of these protein fragments to pancreatic digestion in beef samples. The % RQ of proteins above 60 kDa decreased ($P < 0.05$) at the end of pancreatic digestion (240 min) with increase ($P < 0.05$) in the % RQ of 30 - 40 kDa proteins.

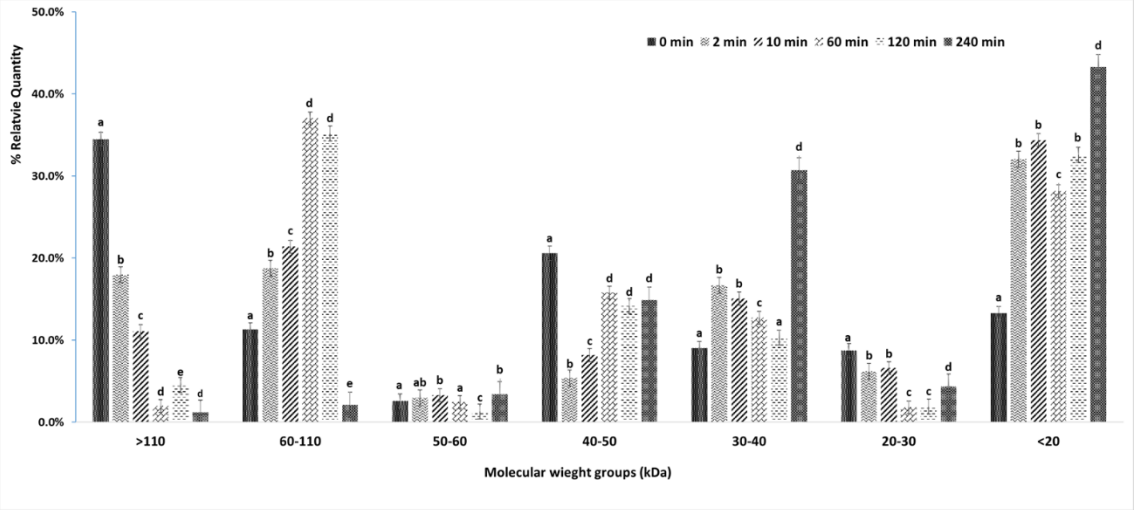


Figure 6.4 Relative quantity changes (mean ± standard error) of seven major molecular weight protein groups across the gastrointestinal digestion process (0, 2, 10, 60, 120 and 240 minutes.). The relative quantity of protein fragments significantly differed across the digestion process when means showed different letters "a, b, c, d, e" within the same molecular weight group ($P < 0.05$).

6.3.5.2 Changes in FAAs

The release of FAAs over the four hours of gastrointestinal digestion is shown in Table 6.7. The FAAs level increased to about three times by the end of pancreatic digestion (240 minutes) compared to the gastric phase levels (120 minutes). The percentage of EAAs in the total AAs (% EAAs) decreased in the first 2 minutes of digestion, then remained unchanged throughout the gastric digestion process. However, the increase of % EAA was observed at the end of the gastrointestinal digestion simulation. Current findings indicated that the main role of gastric enzymes (pepsin) was to break down large proteins into smaller fragments ready for the more complete digestion by pancreatic enzymes and the concomitant release of FAAs. Pepsin is an endopeptidase which plays a role in the cleavage of peptide bonds within molecules in P1 and P1' positions and breakdown large protein molecules into smaller peptides. The pancreatic enzymes, including endopeptidases (trypsin, chymotrypsin and elastase), exopeptidases (carboxypeptidase A and B), and other proteases are the major sources of proteases in the human digestive system. It is well known that pancreatic enzymes contribute more (about

40%) than pepsin (10 - 20%) for the proteolysis during human gastrointestinal digestion (Krehbiel & Matthews, 2003).

A significant increase in EAAs at the end of digestion was observed. These consisted of leucine, lysine, phenylalanine, tyrosine and arginine, as previously observed in dry-cured ovine meat (Zhang et al., 2018). This can be explained by the selective cleavage of peptide bonds which link carboxyl side of basic AAs (e.g. lysine and arginine) by trypsin (Krehbiel & Matthews, 2003), and the preferential cleavage of the linkage between aromatic AAs (e.g. phenylalanine and tyrosine) by chymotrypsin (Sitrin, 2014). Elastase is another pancreatic endopeptidase responsible for the cleavage of carboxyl side of aliphatic AAs, which included alanine, glycine, isoleucine, leucine, and valine (Sitrin, 2014). The exposure of these AAs can act as substrates to be further digested by exopeptidases (Carboxypeptidase A and B), which may explain the significant increase of these AAs after 120 minutes of pancreatic digestion observed in the current study (Table 6.7).

There was no significant difference between the ageing treatments within the first 10 minutes of gastric digestion except for a higher ($P < 0.05$) content of cysteine in dry-aged lamb. With time, EAAs including isoleucine, lysine, threonine, and valine, and non-essential AAs (non-EAAs) comprising of arginine, aspartic acid, cysteine, histidine, and proline, increased in dry-aged lamb more than the wet-aged equivalents (Table 6.7, 60 minutes, $P < 0.05$). The significant difference in FAAs observed between ageing treatments at 120 minutes of gastric digestion was not observed following the 120 minutes of pancreatic digestion ($P > 0.05$), probably due to the greater cleavage of protein fragments by pancreatic enzymes that resulted in similar levels of FAAs being released in both ageing treatments.

When considering the FAAs level between ageing treatments across the digestion process, three EAAs (isoleucine, threonine and valine) and most of the non-EAAs (except for hydroxyproline and arginine) were higher ($P < 0.05$) in dry-aged lamb as compared to the wet-aged control. Therefore, based on the release of FAAs during gastric digestion, dry-aged lamb was more digestible than the wet-aged equivalent. The higher digestibility of dry-aged lamb products could also be attributed to the higher ($P < 0.05$) yeast counts in the dry-aged sample compared to the control (3.75 *vs.* 2.38 log cfu/g). The presence of yeast in meat products has been associated with the increase of pH, production of peptides, free fatty acids, FAAs, and flavour compounds (Choe et al., 2020; Flores, Corral, Cano-García, Salvador, & Belloch, 2015; Flores et al., 2004). Thus, it is our assumption that a

mild fermentation induced by yeast may have occurred during dry-ageing process that could have contributed to the higher FAAs and smaller peptides in dry-aged lamb. Further studies are required to isolate and identify the yeast strain, and to determine its role in the improvement of proteolysis and digestibility.

6.3.5.3 Protein content in hydrolysate

The protein content (protein fragments and peptides) in the final hydrolysates following 240 minutes simulated digestion was determined and no difference ($P > 0.05$) between the two ageing treatments was found (Table 6.8). Only 70% of the proteins were hydrolysed with about 30% of protein hydrolysates remaining in the digests. This could be explained by the limitation of pancreatic proteases which accounts for only 30 to 40% of protein hydrolysis occurring in the human gastrointestinal digestion (Fricker & Drewe, 1996; Sitrin, 2014).

6.3.5.4 Overall relative protein digestibility (%)

The relative protein digestibility of dry-aged lamb products compared to the wet-aged control was determined by three methods. As shown in Table 6.8, both ageing regimes had similar digestibility regardless of the method used. Although the overall relative protein digestibility did not differ when the full 240 minutes of gastric and pancreatic digestions were considered together, it is important to note that from the analysis of the FAAs, we were able to determine that dry-aged meat had a higher gastric digestibility compared to wet-aged. This information is particularly relevant because the increased digestibility of meat at the upper gastrointestinal tract will minimise the chances of larger fragments of proteins reaching the lower gastrointestinal tract where they may be fermented to produce metabolites that could contribute to bowel ailments in the elderly and those with compromised guts (Corpet et al., 1995; Silvester & Cummings, 1995). Thus, we recommend that a combination of assessment methods – such as the three used in the current study – be employed in order to fully determine the extent of digestibility of meat.

Table 6.7 Effect of ageing treatments on the release of free amino acids (mg/g protein) of lamb chops at different digestion stages: undigested phase (0 minute), 2, 10, 60, and 120 minutes of the gastric phase, and the end of small intestinal phase (240 minutes) of the *in vitro* digestion process.

	0 min			2 min			10 min			60 min			120 min			240 min			SED	*P*-ageing (across digestion time)
	W	BD	*P*-ageing	W	BD	*P*-ageing	W	BD	*P*-ageing	W	BD	*P*-ageing	W	BD	*P*-ageing	W	BD	*P*-ageing		
Essential amino acids																				
Histidine	0.36	0.46	0.252	0.35	0.41	0.202	0.37	0.40	0.481	0.42^{a}	0.60^{b}	0.021	0.38^{a}	0.57^{b}	0.001	0.73^{a}	0.87^{b}	0.034	0.06	<0.0001
Isoleucine	1.24^{a}	1.57^{b}	0.018	0.87	0.94	0.570	0.87	0.92	0.458	0.97^{a}	1.21^{b}	0.049	1.00^{a}	1.31^{b}	0.005	1.80	2.01	0.153	0.10	<0.0001
Leucine	1.01	0.98	0.907	1.49	1.60	0.574	1.51	1.59	0.507	1.77	2.18	0.075	1.89^{a}	2.49^{b}	0.005	12.13	12.75	0.591	0.50	0.150
Lysine	1.01^{a}	1.19^{b}	0.001	1.27	1.39	0.528	1.46	1.59	0.463	1.42^{a}	2.01^{b}	0.021	1.37^{a}	2.10^{b}	0.002	13.56	13.36	0.922	0.88	0.505
Methionine	0.66	0.72	0.491	0.66	0.71	0.571	0.59	0.59	0.996	0.73	0.82	0.410	0.79^{a}	0.97^{b}	0.030	1.89	2.05	0.438	0.11	0.063
Phenylalanine	1.60	2.00	0.144	1.00	1.03	0.669	0.99	1.01	0.632	1.25	1.38	0.180	1.46^{a}	1.70^{b}	0.025	10.41	10.91	0.632	0.44	0.218
Threonine	1.34	1.73	0.153	0.56	0.63	0.285	0.63	0.77	0.305	0.67^{a}	0.98^{b}	0.045	0.65^{a}	1.01^{b}	0.007	0.82^{a}	1.05^{b}	0.004	0.14	<0.0001
Tryptophan	0.24	0.30	0.238	0.53	0.55	0.424	0.55	0.56	0.704	0.63	0.68	0.125	0.67^{a}	0.75^{b}	0.011	2.55	2.58	0.898	0.11	0.372
Valine	1.01	1.27	0.087	1.16	1.26	0.518	1.23	1.29	0.532	1.32^{a}	1.70^{b}	0.038	1.36^{a}	1.85^{b}	0.005	1.92^{a}	2.34^{b}	0.028	0.14	<0.0001
Non-essential amino acids																				
Alanine	1.97	2.21	0.547	3.25	3.57	0.405	3.38	3.51	0.771	3.78	4.36	0.242	3.92^{a}	4.93^{b}	0.034	3.91	4.60	0.101	0.39	0.004
Arginine	1.35	1.63	0.152	1.04	1.16	0.380	1.02	1.08	0.599	1.25^{a}	1.49^{b}	0.034	1.40	1.64	0.155	20.02	20.12	0.963	0.91	0.643
Asparagine	0.45	0.59	0.124	0.46	0.53	0.227	0.52	0.55	0.585	0.82	0.82	0.768	1.24^{a}	1.73^{a}	0.004	1.46^{a}	1.84^{b}	0.008	0.11	<0.0001
Aspartic acid	0.35^{a}	0.64^{b}	0.043	0.49	0.52	0.688	0.70	0.76	0.446	0.60^{a}	1.00^{b}	0.004	0.63^{a}	1.15^{b}	0.000	0.67^{a}	1.11^{b}	0.000	0.09	<0.0001
Cysteine	0.04	0.04	0.236	0.69^{a}	0.83^{b}	0.014	0.85	0.98	0.412	0.91^{a}	1.19^{b}	0.046	0.91^{a}	1.30^{b}	0.002	1.18	1.38	0.233	0.11	<0.0001
Glutamic acid	0.93	1.16	0.354	1.13	1.24	0.534	1.25	1.17	0.630	1.36	1.59	0.139	1.47^{a}	1.81^{b}	0.007	1.36^{a}	1.69^{b}	0.044	0.16	0.005
Glutamine	3.67	4.18	0.447	2.97	3.26	0.567	2.94	3.22	0.672	3.68	4.14	0.367	3.43	4.58	0.056	3.68	4.50	0.258	0.59	0.017
Glycine	1.19	1.28	0.603	2.80	2.92	0.659	2.71	2.91	0.254	2.94	3.19	0.232	2.92^{a}	3.50^{b}	0.008	3.29	3.78	0.054	0.20	0.001
Hydroxyproline	0.11	0.11	0.847	0.04	0.05	0.302	0.04	0.04	0.580	0.06	0.05	0.218	0.05	0.06	0.094	0.05	0.06	0.067	0.01	0.051
Proline	0.50^{a}	0.71^{b}	0.045	0.42	0.45	0.534	0.47	0.51	0.468	0.50^{a}	0.65^{b}	0.031	0.60^{a}	0.85^{b}	0.007	0.50^{a}	0.67^{b}	0.014	0.06	<0.0001
Serine	1.20	1.46	0.259	1.38	1.50	0.530	1.50	1.56	0.668	1.62	2.04	0.055	1.70^{a}	2.24^{b}	0.003	1.69^{a}	2.17^{b}	0.008	0.17	<0.0001
Tyrosine	0.93	1.13	0.361	0.93	0.99	0.603	0.93	0.98	0.384	1.11	1.29	0.129	1.15^{a}	1.43^{b}	0.008	8.63	9.01	0.657	0.37	0.203
EAAs	8.48	10.23	0.092	7.88	8.52	0.498	8.20	8.72	0.460	9.23	11.56	0.044	9.56	12.75	0.005	45.81	47.92	0.682	2.24	0.063
% EAAs	40.43	40.65	0.895	33.53	33.39	0.874	33.56	33.73	0.877	33.46	34.55	0.484	32.98	33.57	0.569	49.51	48.53	0.258	1.15	0.790
Total AAs	21.17	25.36	0.211	23.48	25.54	0.458	24.51	25.99	0.547	27.82	33.37	0.052	28.99^{a}	37.96^{b}	0.003	92.25	98.84	0.499	4.59	0.014

W and BD refer to wet-ageing and in-bag dry-ageing, respectively. SED is the standard error of a difference between means. AAs and EAAs refer to Amino acids and Essential amino acids. Different superscript letters "a, b" within the same row indicates results significantly differed from each other ($P < 0.05$).

Table 6.8 Overall relative digestibility (%) of lamb chops after 240 minutes *in vitro* digestion.

	W	BD	*P*-ageing	SED
Relative digestibility $_{\text{SDS-PAGE}}$	63.96	65.24	0.776	4.36
Relative digestibility $_{\text{FAAs}}$	7.11	7.35	0.802	0.92
Relative digestibility $_{\text{protein content}}$	70.18	70.75	0.570	0.95

W and BD refer to wet-ageing and in-bag dry-ageing, respectively. SED is the standard error of a difference between means.

6.4 Conclusion

This is the first time in-bag dry-aged lamb was produced, and a systematic study was performed to compare the product with its wet-aged equivalents in terms of meat quality, consumer acceptability, oxidative stability and digestibility considerations. The current findings demonstrated that BD process can be used to produce highly acceptable and microbiologically safe lamb products with comparable meat quality and oxidative stability, and with improved gastric digestibility compared to the wet-aged equivalents. The outcomes also confirmed the niche nature of dry-aged meat compared to the equivalent wet-aged lamb and provided a formula for determining the yield of dry-aged lamb legs from its initial wet weight.

A combination of SDS-PAGE, FAAs and total protein measurement of digests enabled better assessment of meat digestibility than the use of each method alone. The use of FAAs profiling in conjunction with SDS-PAGE will help to better understand the biochemical processes during the digestion of meat products particularly in the upper gastrointestinal tract, which is important when the bioaccessibility of the products is of interest.

6.5 Epilogue

In this chapter, in-bag dry-aged lamb were produced using standard dry-ageing conditions of 2 °C, 0.5 $m.s^{-1}$ and RH 75% as described in Chapter 3 for straight-dry-ageing of lean beef. Similar level of weight loss (20% for 21 days) and low microbial counts were observed for both lean beef and lamb using the same ageing parameters suggesting that adoption of BD technique is able to produce consistent quality dry-aged products. In-bag dry- and wet-aged lamb were equally preferred by consumers which further demonstrated the potential for dry-aged lamb to be produced as an alternative to the widely available wet-aged equivalents to satisfy the growing demands of consumers for premium meat products with exceptional eating quality.

A higher TBARS level was detected in-bag dry-aged lamb, indicating the presence of aerobic maturation using BD technique. This finding was in line with that of lean beef as described in Chapter 4. Lamb contains more IMF (6 *vs*. 1%) than lean beef, and therefore may have produced more oxidative by-products during dry-ageing, resulting in a higher TBARS (1.3 *vs.* 0.4) compared to that of the lean beef. However, the protective function of ageing bags limits the access of oxygen, thereby preventing the meat from severe oxidative damage during ageing. Thus, no significant changes were observed in FA profile and protein carbonyl content of in-bag dry-aged lean beef and lamb, regardless of the processing regimes.

Similar protein patterns were observed previously (chapter 4) between stepwise in-bag ageing and straight-dry-ageing of lean beef, and these were once again found in the current chapter. These findings suggest that the primary proteolysis by endogenous enzymes was similar between BD and W regimes. While in-bag dry-aged lamb produced more FAAs than the wet-aged in the current research, this may be due to the proliferation of yeast during BD leading to the secondary proteolysis by peptidase activity of yeast. Such activity may have also improved the gastric digestibility of in-bag dry-aged lamb.

In the next chapter, metabolic fingerprinting of in-bag dry- and wet-aged lamb was performed using REIMS to determine the metabolic changes between two ageing regimes to further identify different ageing regimes based their biomolecular features.

Chapter 7: Metabolic fingerprinting of in-bag dry- and wet-aged lamb with Rapid Evaporative Ionisation Mass Spectroscopy (REIMS)

This chapter demonstrates the use of REIMS fingerprinting to determine the effect of in-bag dry- and wet-ageing on metabolite profiles of lamb legs. Four main muscles were used for the REIMS analysis which were m. semimembranosus, m. biceps femoris, m. vastus lateralis, and *m. rectus femoris*. *Total of 1705 metabolite ions were identified with 663 metabolites which significantly differed between ageing regimes, mainly resulting from proteolysis and lipid metabolism. Dry-ageing produced more of smaller-sized metabolites including dipeptides and FAAs and lipid oxidation metabolites compared to the wet-aged equivalents. Different muscles had distinct REIMS metabolic profiles. Proteolysis patterns, FAAs profile and lipid oxidation (TBARS) were also determined to support the findings from REIMS.*

This chapter firstly introduces the motivations and objectives of this study followed by the experimental work, which is described in Section 7.2. Section 7.3 presents the findings for discrimination of in-bag dry- and wet-aged lamb based on metabolic profile of 1705 metabolites, and other determinations including proteolysis and lipid oxidation. The underlying biochemical mechanisms responsible for the metabolic changes between the two ageing regimes are discussed in Section 8.4 followed by conclusion of this chapter in Section 7.5.

7.1 Introduction

Demand for meat products with premium quality is increasing to meet the expectations of an exceptional eating experience. Post-mortem ageing is a widely used processing technique in the meat industry to improve meat quality, in terms of tenderness and characteristic aged flavour (Koutsidis et al., 2008; Nishimura, 1998). Post-mortem ageing commonly refers to wet-ageing (W), an anaerobic maturation process accomplished in the water-impermeable vacuum packaging. Dry-ageing is another form of post-mortem ageing which allows for moisture evaporation during ageing process under aerobic conditions for developing distinctive dry-aged flavour, such as intense nutty, buttery, and meaty flavour notes with umami taste (Li et al., 2014; O'Quinn et al., 2016). Extensive studies have been carried out using beef samples to investigate the differences in meat quality, microbial safety, sensory and flavour profiles arising from the use of different ageing treatments, but rarely on lamb (Kim et al., 2018; Li et al., 2014; O'Quinn et al., 2016; Sitz et al., 2006; Zhang et al., 2019). Lamb is favoured by the consumers across the world due to its characteristic flavour profile. However, the impact of different ageing regimes on the biochemical and metabolic changes of lamb and the underlying mechanisms for such changes have not been explored.

Metabolic fingerprinting is an emerging approach for understanding of the changes in biological samples arising from inherent properties and/or external factors (Kosmides, Kamisoglu, Calvano, Corbett, & Androulakis, 2013). For example, the dehydration process during dry-ageing could be associated with metabolic changes to the meat that contributed towards the development of distinct flavour profile. Determining such changes could be useful for understanding the dry-ageing process, and identifying biomarkers or fingerprints as the signature for a distinct product or associated with a unique response to a processing treatment. In the case of dry-ageing, the potential signature could be related to the level of dehydration (Lee et al., 2019a), formation of unique volatile compounds (O'Quinn et al., 2016) and/or metabolites such as peptides and free amino acids (Kim et al., 2016). Dry-ageing of beef has been suggested to produce more abundant free amino acids including tryptophan, phenylalanine, valine, tyrosine, glutamic acid, isoleucine, leucine and histidine, compared to those wet-aged for 3 to 4 weeks (Kim et al., 2016; Lee et al., 2019a).

Rapid evaporative ionisation mass spectrometry (REIMS) is a relatively novel mass spectrometry-based metabolic fingerprinting technique which has demonstrated accurate and reliable differentiation of meat according to their species and breeds (Balog et al., 2016). Through the use of REIMS, rapid determination of whole tissue samples is possible to enable detection of adulteration in minced beef (Black et al., 2019), which would impose a significant merit in addressing food safety issues. Compared to other metabolomics techniques, such as liquid or gas chromatography – mass spectrometry and nuclear magnetic resonance (Kosmides et al., 2013), no sample preparation is required, which removes this as a source of error, and allows a much higher throughput of samples. This reduces cost per sample and can allow many more samples to be analysed in the same timeframe as conventional metabolomics methods, potentially improving statistical power for detecting small changes. The trade-off is less certainty around compound identification and ion suppression, as well as the production of chemical artefacts due to the thermal process required for volatilising samples for REIMS (Ross et al., 2020).

The main purpose of this study was to use REIMS fingerprinting (1) to test the hypothesis that in-bag dry-ageing (BD) of lamb would produce different metabolic profiles compared to W; (2) to understand the underlying biochemical mechanisms which differed between BD and W of lamb, as observed by lipid oxidation, protein and free amino acids profiles.

7.2 Materials and methods

7.2.1 Ageing treatments

Lamb legs for ageing were collected from a local abattoir (bone-in, shank and chump off, three pairs, n = 6). Paired legs from the same animal were randomly assigned to two ageing methods: BD and W. BD was carried out in a water permeable ageing bag (TUBLIN® 10, 50 μm thick, polyamide mix with water vapor transmission rate 920 g/50 μ/m^2/24 h at 7 °C, 50% RH, and oxygen transmission rate 660 g/m^2/24 h at 7 °C, 50% RH, TUB-EX ApS, Denmark) at 2 ± 0.5 °C, 0.5 $m.s^{-1}$ air velocity and relative humidity of 75 ± 5%. Wet ageing of lamb legs using water impermeable barrier bags (Cryovac® A600 barrier bag, oxygen transmission rate 20-50 g/m^2/24 h at 23 °C, Sealed Air®, New Zealand) was performed at -1.5 ± 0.5 °C. After 21 days of ageing, the lamb samples from both ageing methods were fabricated into chops (1.5 cm thickness) for further analyses. Lamb chops used for metabolic profiling by REIMS were frozen at -80 °C immediately after ageing for further analysis.

7.2.2 pH and proximate analysis, and lipid oxidation

The pH of fresh lamb chops after the ageing treatments was measured by inserting a calibrated pH probe (Hanna 99,163 pH meter with a FC232D combined temperature and pH insertion probe, Rhode Island, USA) directly into the meat. A 2-point standard buffer solution of 7.01 and 4.01 was used for calibration of the pH meter at ambient temperature. Four measurements were taken from four random positions of each sample.

Following ageing experiment, lamb chops consisted of multiple muscles were deboned and finely minced in order to obtain a representative sample for the whole chop. Subsamples (approx. 5 g) were taken for measurement of moisture content using oven drying (AOAC 950.46) and crude fat content by Soxhlet extraction (AOAC 960.39) according to the AOAC standard analytical methods (AOAC, 2010). The extent of lipid oxidation arising from different ageing methods was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) in the minced lamb samples following the method described by Buege and Aust (1978). The TBARS results were expressed as μg of malondialdehyde (MDA)/g fat. All measurements were carried out in triplicate.

7.2.3 REIMS

REIMS analysis was carried out on four major muscles of lamb chops: *m. semimembranosus* (SM), *m. biceps femoris* (BF), *m. vastus lateralis* (VL), and *m. rectus*

femoris (RF). Metabolic profiling of lamb samples was performed using an electronic monopolar surgical knife (Electrosurgical pencil, Erbe Medical UK Ltd, UK) coupled to a Waters Xevo® G2 qToF mass spectrometer (Waters Corp., UK). The surgical knife was used to directly cut the thawed lamb chops (each cut or 'burn' was approx. 2 mm deep and 15 mm long) with 3 seconds per cut at 15 W power in cutting mode (Erbe VIO 50C generator, Erbe Medical UK Ltd, UK). Five technical replicates were carried out per muscle with 10 seconds delay between each cut. To avoid loss of conductivity and carry-over effects, the knife was cleaned by scraping off the residues and wiping with a clean tissue (Kimwipes, Kimtech Science™) which had been dampened with distilled water. Aerosol produced from the samples was infused with isopropanol (LC-MS grade, Fisher scientific) at a constant flow rate of 100 $\mu L.min^{-1}$ into the spectrometer. The mass spectra were acquired between the mass range of m/z 50 - 1500 using a negative ionisation mode with a scan rate of 0.5 seconds per scan. In-house prepared minced lamb sample was analysed for every 10-cuts cycle for quality control and accuracy of the collected data.

REIMS data was processed first using ProGenesis Bridge (Waters UK) which converts each individual REIMS measurement ('burn') into a separate file and at the same time subtracts the background signal from ambient air and performs mass alignment, in this case to m/z 281.25 for fatty acid C18:1 $[M-H]^-$ which was one of the main ions observed in all samples. Data were then further processed using ProGenesis QI (Waters, UK) to group adducts belonging to the same compound and perform tentative identification based on matching the measured masses with the Human Metabolome Database (http://www.hmdb.ca) and Lipid Maps database (www.lipidmaps.org), with a mass error of 5 ppm. Here, we have focused on the m/z values with significant variations between treatments and identified those masses which were associated with mammalian metabolism. There were several potential identifications assigned to one high-resolution m/z value, especially for the lipid compounds which have large number of potential isobaric and isomeric forms. For this reason, we have reported the lipid class rather than individual lipids.

The identification of metabolites based on database search met level 2 criteria for identification in metabolomics (Sumner et al., 2007). While it is useful for understanding the underlying biochemical mechanisms arising from different ageing treatments, and/or other potential factors involved. Measurements of lipid oxidation (TBARS), protein and free amino acids profile were also performed to support the present findings from REIMS.

7.2.4 SDS-PAGE gel electrophoresis

Whole muscle protein of minced lamb chops (as described in 7.2.1) was extracted with a buffer made of 50mM Tris-HCl (pH = 5.8, Tris base, ACS reagent grade, Millipore®), 10% glycerol (AR grade, Fisher Scientific), 2% Sodium dodecyl sulfate (ACS grade, Sigma-Aldrich®), and 2% β-mercaptoethanol (AR grade, 99.0% purity, Sigma Aldrich®) according to the procedures described by Zhang et al. (2019). The protein content in the extract was determined by a commercial protein assay kit (RC-DC™ protein assay, Bio-Rad® Laboratries, Hercules, USA). The protein extract was diluted to a constant concentration of 5 $\mu g.\mu L^{-1}$ with the extraction buffer then loaded to a Novex™ NuPAGE™ 10% Bis-Tris Midi Protein Gels (Invitrogen, UK), by 8 µL aliquot protein extract (equivalent to 40 µg protein) per well. Electrophoresis separation was carried out at ambient temperature in a Bio-Rad Criterion cell system at 150 V equipped with a PowerPac™ HC High-Current Power Supply (Bio-Rad® Laboratories, Hercules, CA, USA). An 8 µl aliquot of Novex™ Sharp Pre-stained protein standard (Invitrogen, UK) was used to assist with identification of the protein molecular weight ranging from 3.5 kDa to 260 kDa. Staining was carried out in a SimplyBlue SafeStain (Invitrogen™) for 4 hours. The stained gels were washed twice with distilled water for 2 hours before the images were captured with a GS900 calibrated densitometer scanner (Bio-Rad® Laboratories). Identification and semi-quantitative analysis of protein bands were carried out in Image Lab™ software (Version 6.0.1, Bio-Rad® Laboratories, Inc.). Seven molecular weight regions were grouped according to the protein standards, including < 20, 20 - 30, 30 - 40, 40-50, 50-60, 60-110 and > 110 kDa. Relative optical density of protein groups was calculated by % relative optical density = (optical density of the protein bands in the group/total density of all the bands) × 100%. The analysis was carried out in duplicate.

7.2.5 Free amino acids analysis

Free amino acids were extracted from minced chops with 80% methanol (HPLC grade, Sigma-Aldrich®), as described in 7.2.1. Sub-samples (5 g) were taken from the minced chop as described in section 2.2 and homogenised with 80% methanol (15 mL) using an ultra-turrax (Ultra-turrax, IKA) at 14,000 rpm for 20 s in an ice bath. The clear supernatant containing free amino acids was obtained after centrifuging at 15,000 g for 10 min at 4 °C. Chromatographic separation and quantification of free amino acids were performed by LC-MS/MS according to Salazar et al. (2012) with some adaptions. In brief, aliquots of free amino acids extract were spiked with 10 $mg.L^{-1}$ d_4-alanine (DL-Alanine-

2,3,3,3-d4, Sigma-Aldrich®) as an internal standard (1 : 1, v/v), and derivatised (1 : 1, v/v) with 2.8 mg.mL^{-1} 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (in dry acetonitrile, Apollo Scientific, UK) in a 70 µL of 200 mM sodium tetraborate decahydrate buffer (pH = 8.8, Pure Science Ltd, New Zealand) at 55 °C for 15 min. The amino acid derivatives were then neutralised with of 10% formic acid (1 : 9, v/v) prior to the chromatographic analysis by the Agilent 1260 Infinity HPLC system integrated with Agilent 6420 Triple Quadrupole LC/MS system (Agilent Technologies New Zealand Limited, New Zealand). A C18 core shell Kinetex LC column (100 × 2.1 mm, 1.7 µm particle size, 00D-4726-AN, Kinetex®) was used for the separation of amino acid derivatives using separation gradient as following: 0 - 8.0 minutes (13.0% A and 87.0% B), 8.0 - 16.0 minutes (17.0% A and 83.0% B), 16.0 - 17.0 minutes (80.0% A and 20.0% B), and 18.0 - 18.5 minutes (1.5% A and 98.5% B). The working buffer A was prepared with 0.1% formic acid in acetonitrile, and B with 0.6% formic acid in MilliQ water. Constant column flow rate (0.2 mL.min^{-1}) and set temperature (22.6 °C) were used. Identification of free amino acids was carried out using MRM-MS with positive electrospray ionization (ESI+) mode to select the most sensitive parent-daughter ion transition of m/z $[M-H]^+ > 171$. Ionisation source settings in mass spectrometry included the temperature at 325 °C, capillary voltage of 2.0 kV and gas flow rate of 6.0 L.min^{-1}.

Quantitative analysis of free amino acids was performed using external standard calibration. The standard solution was prepared with 40 amines including 37 amines mix, asparagine, glutamine (A9906, A0884 and G3126, Sigma-Aldrich®) and d_4-alanine as an internal standard. The amino acid standard (100 µM) was then serially diluted to 0.78 µM to generate a standard curve for the identification and quantitation of free amino acids using MassHunter software (Agilent Technologies). The final concentration of free amino acids was expressed as two forms on wet basis and dry basis. Measurements of each lamb samples were performed in duplicate.

7.2.6 Statistical analysis

This study was carried out complementary to Chapter 6, and the duplicities of the experimental design and sample set are acknowledged. The statistical analyses of pH, proximate content, TBARS, free amino acids and SDS-PAGE were carried out using linear mixed effect regression analyses in R software (version 3.4.1) using “lme4” and “predictmeans” packages. The ageing treatments were considered as a fixed effect, and carcass IDs and sides were random effects fitted into the model. Analysis of variance (ANOVA, one-way) was used to investigate the changes in lamb chops due to different

ageing treatments with a post-hoc comparison of means performed using Fisher's least significant differences (LSD) and Tukey's (HSD) test at 5% significance level.

The normalised abundance of the identified ions generated from REIMS were collected for determining the effect of ageing treatments, carcass sides and types of muscle on the metabolic profiles of lamb chops. Five technical replicates were combined into an average abundance for each sample/muscle. Differences on metabolic profiles were determined using Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) (SIMCA, Umetrics, Sweden) on the abundance of identified ions in each sample/muscle. R^2 (cumulative) and Q^2 (cumulative) scores were used to describe the robustness and accuracy of the statistical model used in this study. An OPLS-DA model with the Q^2 score > 0.2 is suggested to be of interest due to a predictability of separation. The normalised abundance between treatments were compared using one-way ANOVA with t-test to separate the means at $P < 0.05$. As a high number of identifications with significant changes in abundance were observed in the present study, we have focused on two groups of ions: (1) dominant metabolites with the highest average abundance (minimum threshold = 100); (2) high fold change metabolites with the largest fold changes between two treatments (fold change > 1.1, or < 0.9).

7.3 Results

7.3.1 Proximate analysis, pH and lipid oxidation (TBARS)

As shown in Table 7.1, BD of lamb resulted in a lower ($P = 0.034$) moisture content than the wet-aged equivalents. No difference ($P = 0.856$) in fat content was found between the ageing methods. Higher pH ($P = 0.016$) and TBARS ($P = 0.007$) levels were observed in in-bag dry-aged lamb compared to the wet-aged equivalents.

Table 7.1 Effect of in-bag dry- and wet-ageing on pH, moisture, crude fat and lipid oxidation (TBARS) of lamb legs.

	In-bag dry-ageing	Wet-ageing	SED	*P*-values
$pH_{\text{ageing 0d}}$	5.84	5.80	0.05	0.510
$pH_{\text{ageing 21d}}$	5.98	5.87	0.03	0.016
Moisture (%)	68.46	72.52	1.28	0.034
Crude fat (%)	6.49	6.04	2.34	0.856
TBARS (μg MDA/g fat)	14.51	5.81	1.68	0.007

SED is the standard error of a difference between means.

7.3.2 Metabolic profile

In this study, the REIMS metabolic fingerprinting of in-bag dry- and wet-aged lamb resulted in the detection of approximately 4000 features and the identification of 1705 ions. Due to the large number of identified compounds, the current study focused on the ions which showed the greatest ($P < 0.05$) fold change and abundance in the sample matrix between the two ageing treatments (Table 7.2 and Table 7.3). The standard operation of REIMS does not include chromatographic separation and MS/MS fragmentation. Thus, several lipid molecules with high ion mass (> m/z 600) have been assigned to one or more lipid classes instead of a specific lipid, owing to a large number of isobaric and isomeric forms which cannot be distinguished by REIMS. The lipid classes putatively identified in this study include ceramides (Cer), diacylglycerols (DG), phosphatidic acids (PA), phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG), glycerophosphoinositols (PI), phosphatidylserines (PS) and triacylglycerols (TG).

7.3.2.1 BD vs. W

A clear separation was observed between the metabolite profile of in-bag dry- and wet-aged lamb using OPLS-DA model, with the Q^2 value of 0.86 (Figure 7.1a). Out of 1705 identified metabolites, 663 metabolites (38.89%) differed significantly between the two ageing treatments. There were 318 metabolites (18.65%), which were significantly more abundant in dry-aged lamb over the wet-aged equivalents. These mainly consisted of low ion mass metabolites (178 ions, < m/z 500), including amino acids, dipeptides and amines (Table 7.2). Wet ageing, on the other hand, produced significantly more abundant high molecular weight metabolites (284 of total 345 ions, > m/z 500), mainly associated with phospholipids metabolism.

7.3.2.2 Carcass sides and different muscles

A negative Q^2 value ($Q^2 = -0.06$) was observed using OPLS-DA modelling between the carcass sides suggesting there was no significant variation in metabolic profiles arising from using the different sides of carcasses, regardless of the ageing methods (data not shown). Only 1.94% of the detected metabolites differed ($P < 0.05$) between the carcass sides, well within what would be expected by random chance.

A weak OPLS-DA model was seen between the four major muscles (SM, BF, VL and RF) of the lamb chops with Q^2 value of 0.12. As shown in Figure 7.1b, muscle SM and BF and muscles RF and VL were separated into two groups in the OPLS-DA model

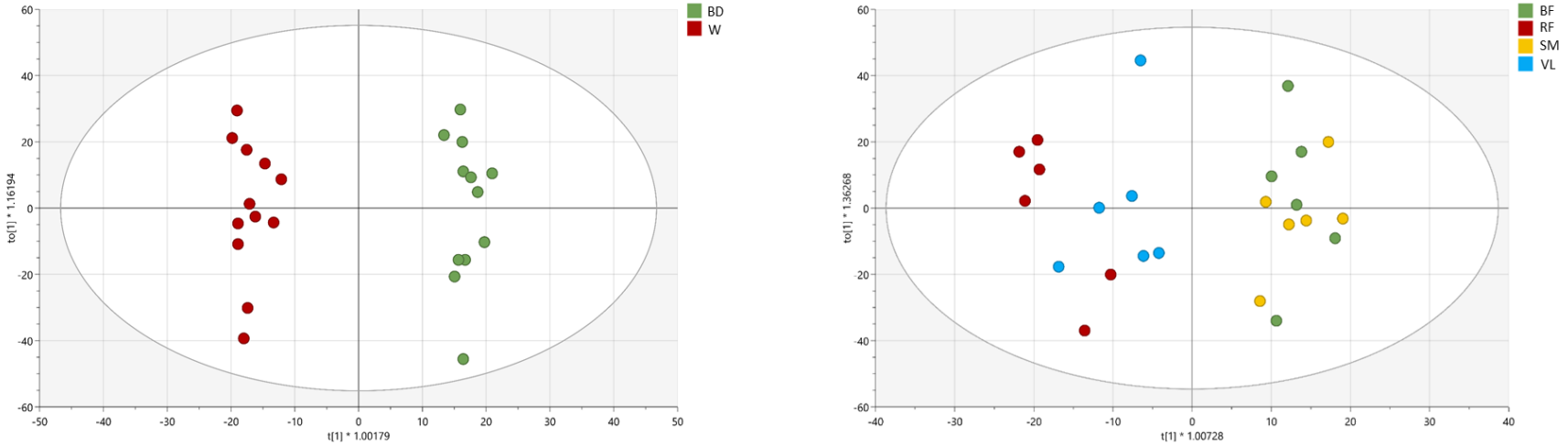
with a Q^2 value of 0.62, suggesting that a good prediction of different metabolic profiles between the two muscle groups was observed. Through further quantitative analysis, difference ($P < 0.05$) in 418 metabolites (24.52%) was observed. There were 313 metabolites (18.36%) being significantly more abundant in SM + BF muscles, where around 44.41% (139 ions) of the metabolites had low-medium ion mass (< m/z 500). Another 105 metabolites (6.16%) were significantly more abundant in RF + VL muscles and most of them (88.57%) had high ion masses (> m/z 500).

7.3.3 SDS-PAGE gel electrophoresis

As shown in Figure 7.2a, there was no clear difference observed in SDS-PAGE protein profile between the two ageing methods. This finding was further confirmed by semi-quantitative analysis on the relative optical quantity of seven molecular weight groups (< 20, 20 - 30, 30 - 40, 40 - 50, 50 - 60, 60 - 110 and > 110 kDa) to determine the changes in protein degradation arising from different ageing methods. As shown in Figure 7.2b, no significant difference ($P > 0.05$) was observed for all seven groups. The large size (> 110 kDa) protein fragments were the most predominant group in both ageing methods, followed by the intermediate size (30 - 50 kDa), together accounting for about 70% of the total proteins. Around 10% of the total proteins were small sized protein fragments, which may be associated with protein degradation, and these were also observed in both aged lamb samples.

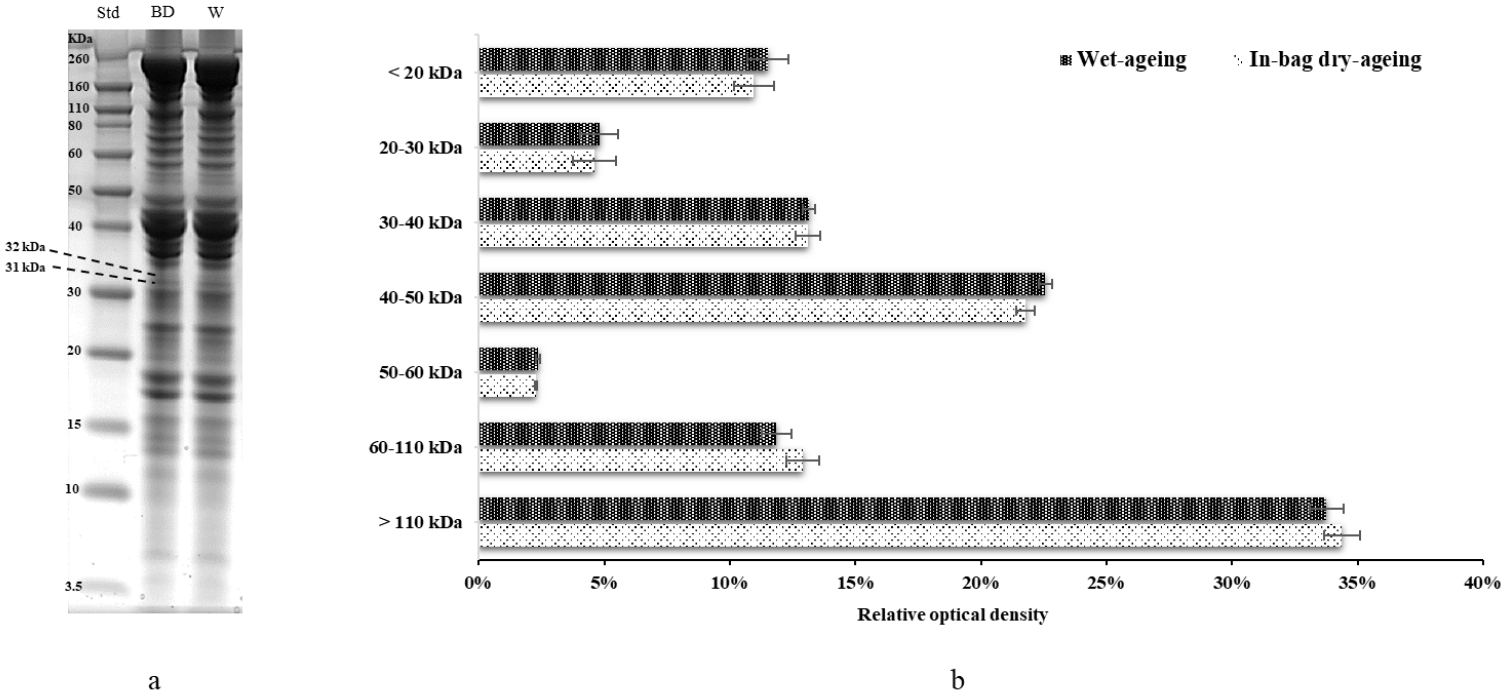
7.3.4 Free amino acids analysis

Total of 21 free amino acids have been detected in both ageing methods including 9 essential amino acids, as shown in Table 7.4. BD resulted in a higher ($P = 0.049$) level of total free amino acids compared to the wet-aged. The most predominant amino acid released from the aged lamb samples was glutamine, followed by alanine, leucine, threonine, arginine and serine, accounting for over 50% of total free amino acids. There were no differences ($P > 0.05$) observed in most of the dominant free amino acids (> 5% of total free amino acids) except for arginine ($P = 0.001$, fold change = 1.43), leucine ($P = 0.005$, fold change = 1.43) and valine ($P = 0.017$, fold change = 1.41), which were more abundant in dry-aged lamb. However, there was no significant difference in all the amino acids when the concentrations were determined based on dry weight (Table 7.4). Dry-ageing process tended to result in higher levels (fold change > 1.0) of free amino acids than the wet-aged even after accounting for the differences in moisture content of the samples from two methods.



a b

Figure 7.1 OPLS-DA score plot: (a) in-bag dry-ageing (BD) *vs.* wet-ageing (W) ($R^2X = 0.64$, $R^2Y = 0.98$, $Q^2 = 0.86$); (b) comparison between four muscles: *m. semimembranosus* (SM), *m. biceps femoris* (BF), *m. vastus lateralis* (VL), and *m. rectus femoris* (RF): $R^2X = 0.52$, $R^2Y = 0.33$, $Q^2 = 0.12$, and for muscle groupings SM + BF muscles and VL + RF muscles: $R^2X = 0.52$, $R^2Y = 0.92$, $Q^2 = 0.62$.



a b

Figure 7.2 (a) Representative SDS-PAGE protein profile of in-bag dry-ageing (BD) and wet-ageing (W) of lamb chops; (b) Relative optical density (mean ± standard error) of seven major molecular weight protein groups from SDS-PAGE gel electrophoresis of in-bag dry- and wet-aged lamb samples.

Table 7.2 Effect of in-bag dry-ageing and wet-ageing on metabolic profile of lamb legs.

Dominant metabolites					High fold change metabolites				
m/z	**Ion/Adduct**	**Putative compounds/Lipid class**	***P*-values**	**[#]Fold change**	**m/z**	**Ion/Adduct**	**Putative compounds/Lipid class**	***P*-values**	**[#]Fold change**
More abundant in in-bag dry-aged lamb									
98.0	$[M-H_2O-H]^-$	L-2-amino-3-oxobutanoic acid	**	1.22	110.1	$[M+Na-2H]^-$	Histamine	*	1.42
127.1	$[M-H_2O-H]^-$	Alanine-glycine dipeptide	0.008	1.14	116.1	$[M-H]^-$	Valine	**	1.55
128.0	$[M-H_2O-H]^-$	Glutamic acid	*	1.55	130.1	$[M-H]^-$	Leucine/isoleucine	**	1.32
135.0	$[M-H]^-$	Hypoxanthine	0.002	1.18	148.0	$[M+Cl]^-$	Creatinine	0.008	2.28
135.1	$[M+Cl]^-$	Hexanal	0.011	1.20	175.0	$[M+Na-2H]^-$	1-(2-Thienyl)-1-butanone	*	1.69
141.1	$[M-H_2O-H]^-$	Alanyl-alanine	0.024	1.12	201.0	$[M+Na-2H]^-$	Hexoses (glucose/fructose/mannose/galactose)	0.021	1.40
154.1	$[M-H]^-$	Histidine	0.015	1.17	205.1	$[M-H]^-$	Glycine-methionine dipeptide	**	2.73
179.1	$[M+Cl]^-$	1-Nonanol	0.005	1.17	217.0	$[M-H_2O-H]^-$	Aspartate-cysteine dipeptide	*	1.49
194.1	$[M+Cl]^-$	2-Aminooctanoic acid	0.010	1.19	217.1	$[M+Na-2H]^-$	1-(2-Thienyl)-1-heptanone	**	1.59
369.2	$[M-H_2O-H]^-$	Hydroperoxy fatty acid	0.006	1.21	219.1	$[M-H]^-$	Alanine-methionine dipeptide Cysteine-valine dipeptide	0.005	1.33
452.3	$[M+Cl]^-$	N-acyl amine	0.012	1.17	222.1	$[M+Cl]^-$	7-oxo-8-amino-nonanoic acid	*	1.81
482.3	$[M-H]^-$	PS(O-16:0/0:0)	0.005	1.21	231.0	$[M-H_2O-H]^-$	Cysteine-glutamate dipeptide	*	1.78
524.4	$[M+Na-2H]^-$	Tetracosatetraenoyl carnitine	**	1.99	231.1	$[M-H_2O-H]^-$	Methionine-threonine dipeptide	0.020	1.35
552.3	$[M-H]^-$	PS(20:0/0:0)	0.012	1.23	237.1	$[M-H_2O-H]^-$	Histidine-threonine dipeptide	*	1.34
614.3	$[M+Na-2H]^-$	PC(16:0/5:0(CHO))	0.019	1.22	245.1	$[M-H]^-$	Methionine-proline dipeptide	0.027	1.13
757.6	$[M-H]^-$	Cholesteryl-6-O-myristoyl-alpha-D-glucoside	0.003	1.27	260.1	$[M-H_2O-H]^-$ $[M-H]^-$	Asparagine-phenylalanine dipeptide Glycine-tryptophan dipeptide	0.005	1.25
796.5	$[M+K-2H]^-$ $[M+Na-2H]^-$	Phosphatidylcholine Phosphatidylethanolamine	0.003	1.36	275.1	$[M+Na-2H]^-$	Histidine-valine dipeptide	0.007	1.26
798.6	$[M-H_2O-H]^-$	Phosphatidylcholine	0.008	1.27	332.1	$[M-H_2O-H]^-$	Phenylalanine-tryptophan dipeptide	0.021	1.30
828.5	$[M-H]^-$	Oxidized glycerophosphocholine	*	1.31	570.3	$[M-H]^-$	Arginyl-histidyl-prolyl-tyrosine	*	1.29
854.6	$[M+Cl]^-$	Phosphatidylcholine	0.002	1.16	609.3	$[2M-H]^-$	Arginine-methionine dipeptide	0.003	1.43

Table 7.2 (continued)

Dominant metabolites					High fold change metabolites				
m/z	**Ion/Adduct**	**Putative compounds/Lipid class**	***P*-values**	**[#]Fold change**	**m/z**	**Ion/Adduct**	**Putative compounds/Lipid class**	***P*-values**	**[#]Fold change**
More abundant in wet-aged lamb									
655.5	$[M-H]^-$	Ceramide phosphoethanolamine	*	1.34	123.0	$[M+K-2H]^-$	But-2-enoic acid	**	1.34
657.5	$[M-H]^-$	Ceramide phosphoethanolamine	**	1.31	160.0	$[M+Na-2H]^-$	Histidinal	0.004	1.44
671.5	$[M-H_2O-H]^-$	DG(20:3(5Z,8Z,11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	**	1.42	196.1	$[M-H]^-$	N-Acetylhistidine	0.008	1.52
673.5	$[M+K-2H]^-$	1-(8-[3]-ladderane-octanyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerol	**	1.30	270.1	$[M-H_2O-H]^-$	Arginine-aspartic acid dipeptide	0.015	1.53
679.5	$[M+Na-2H]^-$	Ceramide phosphoethanolamine	**	1.58	433.3	$[2M-H]^-$	Alanine-lysine dipeptide	**	1.31
680.5	$[M+Cl]^-$	CerP(d18:1/18:0)	**	1.57	445.3	$[M-H_2O-H]^-$	7-Dehydrocholesterol-3-sulfate ester	**	1.71
681.5	$[M-H_2O-H]^-$	PE-Cer(d14:2(4E,6E)/22:1(13Z)(2OH)) PE-Cer(d16:2(4E,6E)/20:1(11Z)(2OH))	**	1.54	463.3	$[2M-H]^-$	3-Mercaptohexyl hexanoate	0.006	1.20
683.5	$[3M-H]^-$	11-Hydroxy-9-tridecenoic acid	**	1.47	489.3	$[M-H]^-$	Saccharolipid	**	1.35
685.5	$[M-H_2O-H]^-$	Phosphatidic acid	*	1.42	643.5	$[2M-H]^-$	15(S)-Hydroxyeicosatrienoic acid	**	1.49
695.5	$[2M+Hac-H]^-$	Xylene	**	1.49	677.5	$[M+Na-2H]^-$	PE-Cer(d14:2(4E,6E)/20:1(11Z))	**	1.45
699.5	$[M+K-2H]^-$	Ceramide	**	1.41	773.5	$[3M-H]^-$	Tetradecanedioic acid	**	1.62
701.5	$[M-H]^-$	Ceramide phosphoethanolamines	0.007	1.16	816.6	$[M-H]^-$	Phosphatidylcholine	**	1.45
705.5	$[M+Na-2H]^-$	PE-Cer(d14:2(4E,6E)/22:1(13Z)) PE-Cer(d16:2(4E,6E)/20:1(11Z))	**	1.75	830.6	$[M-H]^-$	Phosphatidylserine	**	1.64
711.5	$[2M-H]^-$	Tetracosahexaenoic acid	*	1.58	842.6	$[M-H_2O-H]^-$	Glycosphingolipid	**	1.45
713.5	$[M+K-2H]^-$	Ceramide	0.001	1.35	844.6	$[M-H]^-$	Phosphatidylserine	**	1.64
721.5	$[M+Na-2H]^-$	Phosphatidylglycerol Diacylglycerol	0.004	1.28	845.6	$[M-H_2O-H]^-$	Glycerophosphoinositol	**	1.46
733.5	$[M+Na-2H]^-$	PE-Cer(d14:2(4E,6E)/24:1(15Z)) PE-Cer(d16:2(4E,6E)/22:1(13Z))	**	1.73	847.6	$[M+Cl]^-$	Phosphatidic acid	**	1.51
818.6	$[M+Na-2H]^-$	Phosphatidylcholine	*	1.34	850.6	$[M-H_2O-H]^-$	Phosphatidylserine	**	1.43
863.6	$[M-H]^-$	PG(21:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	**	1.40	874.6	$[M+Cl]^-$	Phosphatidylcholine Phosphatidylethanolamine	**	1.47
886.6	$[M+K-2H]^-$	Phosphatidylcholine Phosphatidylethanolamine	**	1.26	876.6	$[M-H_2O-H]^-$	Phosphatidylserine	**	1.48

"*" denotes $P < 0.001$, "**" denotes $P < 0.0001$. [#]Fold change is calculated as the ratio of metabolite intensity between in-bag dry- and wet-aged lamb legs.

Table 7.3 Comparison of the metabolite profiles of the two muscle groupings *m. semimembranosus* + *m. biceps femoris* and *m. vastus lateralis* + *m. rectus femoris*.

m/z	Ion/Adduct	Putative compounds/Lipid class	*P*-values	#Fold change
More abundant in muscle group SM + BF				
124.0	$[M\text{-}H_2O\text{-}H]^-$	Vinylacetylglycine	0.004	1.20
144.1	$[M\text{-}H]^-$	4-Guanidinobutanoic acid	0.009	1.14
169.1	$[M+Na\text{-}2H]^-$	1-Methyl-4-(1-methylpropyl)-benzene	0.005	1.18
210.1	$[M+K\text{-}2H]^-$	9-amino-nonanoic acid	0.006	1.22
250.2	$[M+Cl]^-$	12-amino-dodecanoic acid	0.006	1.12
338.2	$[M+Na\text{-}2H]^-$	3-hydroxynonanoyl carnitine	0.002	1.31
354.2	$[M+K\text{-}2H]^-$	N-methyl arachidonoyl amine	0.001	1.22
370.3	$[M+K\text{-}2H]^-$	2,4,12-Octadecatrienoic acid isobutylamide	*	1.28
396.2	$[M+Cl]^-$	N-Arachidonoyl glycine	*	1.27
410.2	$[3M\text{-}H]^-$	Tyramine	0.009	1.20
411.2	$[M+Cl]^-$	10'-Apo-beta-carotenal	0.005	1.20
467.3	$[M\text{-}H_2O\text{-}H]^-$	N-(2'-(4-benzenesulfonamide)-ethyl) arachidonoyl amine	*	1.25
614.3	$[M+Na\text{-}2H]^-$	PC(16:0/5:0(CHO))	0.002	1.28
620.4	$[3M\text{-}H]^-$	Phenyl-leucine dipeptide	0.003	1.33
651.4	$[M+H]^-$	Phosphatidylglycerol	0.001	1.33
665.4	$[M+K\text{-}2H]^-$	PE-Cer(d14:2(4E,6E)/18:1(9Z))	*	1.32
768.5	$[M\text{-}H_2O\text{-}H]^-$ $[M+Na\text{-}2H]^-$	Glycerophosphoethanolamine Diacylglycerophosphocholine	0.010	3.39
More abundant in muscle group VL + RF				
332.3	$[M\text{-}H]^-$	Docosahexaenoic acid	*	0.65
388.2	$[M+Cl]^-$	Hexadecaphinganine-1-phosphate	0.010	0.76
388.3	$[M+K\text{-}2H]^-$	Anandamide	0.005	0.66
657.5	$[M\text{-}H]^-$	Ceramide phosphoethanolamine	0.017	0.83
698.5	$[M\text{-}H_2O\text{-}H]^-$ $[M\text{-}H]^-$	Phosphatidylcholine Phosphatidylethanolamine	*	0.78
701.5	$[M\text{-}H]^-$	Ceramide phosphoethanolamine	0.008	0.86
703.5	$[M\text{-}H]^-$	Ceramide phosphoethanolamine	0.006	0.83
712.5	$[M\text{-}H_2O\text{-}H]^-$	N-tryptophanyl-35-aminobacteriohopane-32,33,34-triol	0.006	0.78
714.5	$[M\text{-}H_2O\text{-}H]^-$ $[M\text{-}H]^-$	Phosphatidylcholines Phosphatidylethanolamine	0.004	0.78
724.5	$[M\text{-}H]^-$	Phalluside-1	0.003	0.79
726.6	$[M\text{-}H]^-$	Cerebroside B	0.002	0.79
727.6	$[M\text{-}H]^-$	Diacylglycerol	*	0.76
808.6	$[M+Na\text{-}2H]^-$	Phosphatidylcholine Phosphatidylethanolamine	0.007	0.84
979.8	$[M+Cl]^-$ $[M+K\text{-}2H]^-$	Triacylglycerol	0.001	0.72
981.8	$[M+Cl]^-$ $[M+K\text{-}2H]^-$	Triacylglycerol	0.010	0.72
989.8	$[M+K\text{-}2H]^-$	Triacylglycerol	0.011	0.76
991.8	$[M+K\text{-}2H]^-$ $[M+Na\text{-}2H]^-$	Triacylglycerol	*	0.71

"*" denotes $P < 0.001$, "**" denotes $P < 0.0001$. #Fold change is calculated as the ratio of metabolite abundance between *m. semimembranosus* (SM) + *m. biceps femoris* (BF) and *m. vastus lateralis* (VL) + *m. rectus femoris* (RF).

7.4 Discussion

A series of biochemical changes occur post-mortem. These mainly include energy depletion, degradation and oxidation of lipids and proteins, which play a key role in determining the meat quality (Matarneh et al., 2017). It is vital to understand how these biochemical changes interact with the different processing techniques in order to assure the

meat quality. In this study, our hypothesis that in-bag dry-aged lamb would produce different metabolic profiles compared to wet-aged lamb was confirmed by REIMS fingerprinting. The following discussion was divided into four sections focusing on the biochemical (proteolysis, energy metabolism and oxidation) and physiological (type of muscles) mechanisms which resulted in the observed changes of metabolites in this study. Results of lipid oxidation and protein and free amino acid profiles were used to aid the understanding of the underlying biochemical changes behind the different ageing methods.

7.4.1 Proteolysis

Enzymatic proteolysis by the calpain system during post-mortem ageing has been widely accepted as the primary pathway to produce free amino acids and peptides (Nishimura, 1998). BD resulted in significantly increased levels of metabolites contributed to developing the flavour profile of dry-aged products, including peptides, amino acids and amines. As shown in Table 7.2, there were 20 metabolites produced from proteolytic degradation and 18 of them were found significantly more abundant in dry-aged lamb over the wet-aged equivalents. These suggest that different proteolytic processes may have occurred in those two ageing methods. These metabolites were identified as a tetrapeptide, 15 dipeptides and 4 free amino acids. W of lamb resulted in significantly higher levels of 2 dipeptides – arginine-aspartic acid and alanine-lysine – compared to dry-aged equivalents.

Small peptides and free amino acids are the primary taste-active compounds and/or flavour precursors which considerably contribute to the flavour and other sensory properties of meat (Mottram, 1998). BD of lamb produced significantly increased level of free amino acids with ion masses of m/z 116.1 ($[M-H]^-$), 128.0 ($[M-H_2O-H]^-$), 130.1 ($[M-H]^-$) and 154.1 ($[M-H]^-$). These were identified as valine, glutamic acid, leucine/isoleucine and histidine, respectively (Table 7.2). The current findings have been further supported by chromatographic profiling of free amino acids, as shown in Table 7.4 (wet basis). Although there was no statistically significant difference observed when determined on dry basis, numerically dry-ageing tended to produce higher levels of free amino acids compared to the wet-aged. The increase of small peptides and free amino acids in dry-aged samples could be partially associated with dehydration during dry-ageing process, where the components were concentrated due to the significantly reduced moisture level in dry-aged lamb. The impact of moisture evaporation may account for about 4% of the differences observed in REIMS metabolites. As shown in Table 7.2 and Table 7.3, over 10% of variations in metabolites were

observed suggesting other factors may contribute towards the changes between two ageing treatments. Previous studies on beef have suggested that dry-ageing may have a higher rate of protein hydrolysis than W (Kim et al., 2016), that could also support the present findings. Significantly increased level of the FAAs glutamic acid, valine, leucine, isoleucine and histidine in dry-aged beef has been reported in comparison to wet-aged equivalent (Kim et al., 2016; Lee et al., 2019a). These amino acids contribute directly towards the meat flavour or participate later in the Maillard reactions or via the Strecker degradation to produce flavour compounds (Koutsidis et al., 2008).

Glutamic acid is one of the most important taste active compounds produced from ageing process and contributed towards the improvement of umami taste, which are associated with distinctive flavour of dry-aged products (Kim et al., 2018; Li et al., 2014). Post-mortem ageing has been reported to release a significant amount of bitter taste amino acids, including valine, leucine and isoleucine. These amino acids can also act as important flavour precursors participating in the production of meat aroma compounds, including 2-methylbutanal, 3-methylbutanal and pyrazines, through the Strecker degradation with dicarbonyl compounds generated in the Maillard reaction (Koutsidis et al., 2008). The increased level of 2- and 3-methylbutanal have been associated with pronounced browned/grilled, buttery and nutty flavour of dry-aged beef (O'Quinn et al., 2016). Histidine is another amino acid with bitter taste found in more abundance in dry-aged lamb. Free histidine in meat products may come from the enzymatic proteolysis during ageing process as described above. Another pathway could be associated with the histidine biosynthesis through enzymatic oxidation of histidinol by histidinol dehydrogenase from yeast to produce histidine (Kulis-Horn, Persicke, & Kalinowski, 2014). This hypothesis could be supported by the significantly reduced level of histidinal (m/z 160.0, $[M+Na-2H]^-$), which was observed in dry-aged lamb (P = 0.004, Table 7.2). Histidinal is an important intermediate product acting as a substrate for the biosynthesis of histidine (Kulis-Horn et al., 2014).

Table 7.4 Effect of ageing methods on the release of free amino acids in lamb legs.

	Wet basis (mg/100g meat)					Dry basis	
	In-bag dry-ageing	**Wet-ageing**	**SED**	***P*-values**	**[#]Fold change**	***P*-values**	**[#]Fold change**
Essential amino acids							
Histidine	13.58	8.19	0.97	0.005	1.66	0.206	1.23
Isoleucine	34.02	23.57	5.29	0.120	1.44	0.451	1.24
Leucine	47.21	33.03	2.52	0.005	1.43	0.085	1.20
Lysine	24.71	19.64	1.18	0.013	1.26	0.363	1.11
Methionine	17.70	13.93	1.17	0.032	1.27	0.252	1.12
Phenylalanine	26.91	19.46	3.39	0.093	1.38	0.188	1.21
Threonine	42.13	29.00	5.24	0.066	1.45	0.131	1.27
Tryptophan	7.26	5.08	0.77	0.046	1.43	0.128	1.26
Valine	30.36	21.56	2.24	0.017	1.41	0.073	1.24
Non-essential amino acids							
Alanine	52.06	41.49	10.66	0.378	1.25	0.744	1.08
Arginine	39.62	27.80	1.45	0.001	1.43	0.138	1.18
Asparagine	14.14	9.26	1.41	0.026	1.53	0.058	1.30
Aspartic acid	13.93	6.93	0.82	0.001	2.01	0.170	1.37
Cysteine	0.84	0.85	0.09	0.948	0.99	0.137	0.87
Glutamine	84.43	69.62	17.19	0.438	1.21	0.831	1.05
Glutamic acid	27.80	16.84	3.74	0.043	1.65	0.542	1.18
Glycine	29.68	24.94	3.91	0.292	1.19	0.746	1.04
Hydroxyproline	2.39	2.13	0.16	0.180	1.12	0.843	0.98
Proline	16.53	11.00	1.84	0.039	1.50	0.281	1.24
Serine	35.04	24.96	4.35	0.081	1.40	0.284	1.18
Tyrosine	29.10	20.22	3.61	0.070	1.44	0.265	1.20
% Essential amino acids	41.65	40.71	2.77	0.752	1.02	0.698	1.03
Total free amino acids	589.45	429.50	57.24	0.049	1.37	0.282	1.16

SED is the standard error of a difference between means. [#]Fold change is calculated as the ratio of amino acid levels between in-bag dry- and wet-aged lamb legs.

The changes in protein degradation due to ageing methods are shown in Figure 7.2. Similar protein profile has been observed in dry- and wet-aged lamb. Appearance of fragments with molecular weight of 32 and 31 kDa following post-mortem ageing has been associated with enzymatic degradation of troponin T (Negishi et al., 1996) and actin (Longo et al., 2015). No significant difference in these two protein fragments has been detected between the two ageing methods. Therefore, the primary protein degradation due to endogenous enzymes (mainly calpains and cathepsins) are very likely to be similar between the two ageing methods throughout 21 days of ageing time. However, the secondary

proteolysis pathway due to the proliferation of microorganisms may differ between ageing methods and this may have consequently led to the variations in the metabolites observed in REIMS. As shown in Table 7.1, significantly reduced moisture content was found in lamb samples following 21 days of BD. The decrease in water activity due to the dehydration along with the exposure to oxygen during dry-ageing process could enhance the proliferation of microorganism, such as yeast and moulds. The growth of yeast has been reported in dry-aged beef using both traditional out-of-bag dry-ageing and BD regimes (Li et al., 2014; Zhang et al., 2019). The proteolytic activities of yeast on meat during dry-ageing process could account for the increased levels of small peptides and free amino acids in dry-aged lamb observed in this study.

Several amines have also been detected in the dry- and wet-aged lamb, including biogenic amine, N-acyl amines and glycerophosphoethanolamines, as shown in Table 7.2. Histamine (110.1, $[M+Na-2H]^-$) is a biogenic amine found in meat and meat products (Ruiz-Capillas & Jimenez-Colmenero, 2005). A significantly higher level ($P < 0.001$, fold change = 1.42) of histamine was detected in dry-aged lamb over the wet-aged equivalents. The production of histamine during post-mortem processing of meat has been associated with the decarboxylation of histidine by microorganisms including yeast (Gardini et al., 2016). As described above, the significantly more abundant histidine observed in dry-aged lamb could also act as substrate to impart the formation of histamine.

Another ion of interest was m/z 241.1 ($[M-H_2O-H]^-$) which was identified as cyclo(Leu-Phe). This is a cyclodipeptide commonly found in biological tissues and it is mainly produced from protein metabolism by microorganisms, including bacteria and fungi (yeast) (Prasad, 1995). Several cyclodipeptides have been reported previously in cooked dry-aged beef (Chen et al., 2009). As shown in Table 7.2, significantly more abundant cyclo(Leu-Phe) found in dry-aged lamb could be attributed to the action of yeast during the ageing process, as described above.

7.4.2 Energy metabolism

Some metabolites associated with energy metabolism have also been identified; these include the metabolism of glycogen, adenosine triphosphate (ATP) and creatine (Table 7.2). Sugar-related metabolites in meat, including hexoses and trioses, are mainly produced from the post-mortem glycolysis within the first 24 to 48 hours of slaughter (Matarneh et al., 2017).

Hexose has been identified with ion mass of 201.0 ($[M+Na-2H]^-$) as shown in Table 7.2. Many monosaccharides (e.g. glucose, mannose, fructose and galactose) have the same molecular weight which cannot be differentiated based on high resolution mass alone. Glucose and mannose have been reported in aged beef while no significant difference was found between D and W (Kim et al., 2016). In this study, an increased level (P = 0.021, fold change = 1.40) of hexose has been observed in dry-aged lamb compared to the wet-aged equivalents. Hexose (glucose, fructose and mannose) content in beef has also been reported to increase significantly during the extended post-mortem ageing up to 21 days (Koutsidis et al., 2008). Hexose are the taste-active compounds contributing towards the sweet taste of meat and are also important water-soluble precursors in the Maillard reaction formation of flavour volatiles (Koutsidis et al., 2008). Hypoxanthine is another water-soluble flavour precursor in meat produced from the metabolism of ATP within 24 h post-mortem. Upon the exhaustion of ATP, hypoxanthine has been suggested to accumulate during the extended ageing period over 3-4 weeks, due to the degradation of inosine monophosphate (IMP) (Koutsidis et al., 2008). Hypoxanthine was identified with ion mass of m/z 135.0 ($[M-H]^-$) as shown in Table 7.2. An increased level of (P = 0.002, fold change = 1.18) hypoxanthine was observed in the dry-aged lamb. The enhanced levels of hexose and hypoxanthine observed in dry-aged lamb could have resulted from the slightly different biochemical reactions caused by the activities of microorganisms during dry-ageing compared to the W, as described above.

Another metabolite associated with energy metabolism was identified to be creatinine with ion mass of m/z 148.0 ($[M+Cl]^-$). More abundant (P = 0.008, fold change = 2.28) creatinine was observed in dry-aged lamb compared to the wet-aged equivalents (Table 7.2). The formation of creatinine in meat could be due to the non-enzymatic cyclisation of creatine (Dvořák, 1981). Creatine is the key component participating in the post-mortem energy metabolism of skeletal muscles upon slaughter (Toldrá, 2006a). Post-mortem ageing has been suggested to significantly increase the level of creatinine in meat products (Koutsidis et al., 2008). The increased levels of creatinine in dry-aged lamb in this study could be explained by the significant increase of pH value following BD (5.98 *vs.* 5.87), which may have played a role in the conversion of creatine to creatinine during post-mortem processing (Mora, Hernández-Cázares, Sentandreu, & Toldrá, 2010). Another possible biosynthesis

pathway of creatinine from creatine could be associated with the activity of yeast under limited oxygen level inside ageing bags (Soda, Yoshida, & Oikawa, 1953).

7.4.3 Oxidation

As shown in Table 7.2, BD of lamb resulted in significantly more abundant metabolites arising from lipid oxidation than the wet-aged equivalents. These metabolites included aldehydes, acylcarnitine, aryl alkyl ketones, fatty alcohols and oxidised glycerophosphocholine. Hexanal (m/z 135.1, $[M+Cl]^-$) is one of the main aldehydes formed from the oxidation of linoleic and arachidonic acids (Tamura, Kitta, & Shibamoto, 1991). Hexanal has a low odour threshold which could be suggested as an indicator for oxidative stability and changes in meat flavour during the post-mortem handling (Shahidi & Pegg, 1994). Tetracosatetraenoyl carnitine (m/z 524.4, $[M+Na-2H]^-$) is an acylcarnitine which could result from the β-oxidation of fatty acid followed by esterification with carnitine in the mitochondria (Reuter & Evans, 2012). The production of ketones (m/z 175.0 and 217.1, $[M+Na-2H]^-$) and fatty alcohol (1-Nonanol, m/z 179.1, $[M+Cl]^-$) observed in this study could have been associated with β-oxidation of fatty acid (Toldra, 1998).

Another oxidation-derived metabolite has been observed with higher level in wet-aged lamb over the dry-aged equivalents ($P < 0.0001$, fold change = 1.62), which is tetradecanedioic acid (m/z 773.5, $[3M-H]^-$). Tetradecanedioic acid is a saturated dicarboxylic fatty acid, which has been associated with the oxidation of long chain polyunsaturated fatty acids (Passi et al., 1993). However, increased levels ($P < 0.05$) of unsaturated fatty acids, including but-2-enoic acid (m/z 123.0, $[M+K-2H]^-$) and tetracosahexaenoic acid (m/z 711.5, $[2M-H]^-$), have also been observed using wet-aging regime due to the lipolysis.

Therefore, lipid oxidation occurred in both ageing methods, with higher oxidative changes using BD regime. This was in line with the significantly higher level of TBARS observed in dry-aged lamb over the wet-aged equivalents, as shown in Table 7.1. Lipid is susceptible to oxidative reactions during post-mortem storage, especially under aerobic conditions (Ladikos & Lougovois, 1990). In this study, dry-ageing of lamb was carried out in a water permeable bag with limited level of oxygen present to simulate the aerobic maturation of traditional out-of-bag dry-ageing regime. Therefore, a moderate oxidation level is expected in the dry-aged products which may be one of the key contributors to the signature dry-aged flavour. The severe oxidative damage of lipid has been reported to result in

deterioration in sensory quality and generation of toxic products (Min & Ahn, 2005). However, a moderate level of lipid oxidation has also been suggested to produce flavour precursors that may contribute to the unique aroma during ripening stage of dry-cured meat production (Domínguez et al., 2019). Similar process could be speculated to occur during dry-ageing of meat which may account for the distinctive flavour of dry-aged meat.

7.4.4 Type of muscles

The hindleg of lamb consist of multiple muscles. The compositions and properties of different muscles may differ due to their unique roles in supporting the physiological activities of the animals. In this study, the formation of two muscle groups (SM + BF and VL + RF, Figure 7.1b) suggested a certain level of similarity of metabolic profile within the group. As shown in Table 7.3, SM + BF muscles had significantly increased level of metabolites associated with metabolic reactions of proteins and lipids, mainly including proteolysis (m/z 620.4), decarboxylation (m/z 410.2) and lipid degradation and oxidation (m/z 124.0, 338.2, 354.2, 396.2 and 467.3). Different muscles have variable metabolic responses to the post-mortem ageing treatments owing to muscle fibre types (Ithurralde et al., 2018; Ouali & Talmant, 1990). All four muscles analysed in this study consisted of multiple fibre types, mainly including slow oxidative fibres (type I) and fast glycolytic fibres (type IIA and IIB). SM and BF in lamb generally contain higher levels of slow oxidative fibres than VL and RF which predominantly consist of fast glycolytic fibres (Hou et al., 2013; Ithurralde et al., 2018). In general, muscles made of predominantly fast glycolytic fibres are more susceptible to post-mortem biochemical changes than those dominated by slow oxidative fibres. This could be attributed to the increase in activity of calpastatin in slow oxidative fibres which has inhibitory effects on calpains, resulting in a slower response to the ageing treatments (Ouali & Talmant, 1990). Therefore, we hypothesise the primary enzymatic protein metabolism due to the endogenous enzymes (mainly calpains) may be slow in SM and BF, while these muscles could be susceptible to oxidation and secondary biochemical reactions by microorganisms.

7.5 Conclusion

The effect of in-bag ageing methods on the metabolic profiles of lamb legs was successfully determined using REIMS based on 1705 identified metabolite ions. These metabolites did not differ between carcass sides as expected but did differ between muscles.

The primary enzymatic degradation of proteins in the current lamb samples was similar between the two ageing methods as observed using gel electrophoresis. BD resulted in significantly more abundant dipeptides and free amino acids compared to the wet-aged equivalents. The peptides and amino acids which differed between the ageing methods could potentially be used as the biomarkers for in-bag dry-aged lamb. A moderate level of lipid oxidation caused by the BD process might contribute to the distinctive flavour of in-bag dry-aged lamb.

7.6 Epilogue

This chapter successfully employed REIMS as a rapid metabolic fingerprinting to discriminate the aged lamb products, produced from two different ageing regimes. Such technique provided an in-depth determination of the metabolic changes arising from the ageing regimes and these had not been detected before using traditional meat quality measurements. Thus, REIMS demonstrated the potential to be utilised by the meat industry for quality control purpose and to detect fraud arising from the dry-ageing process. Further, REIMS can also help to identify the metabolites responsible for the metabolic changes, which in turn, will help to understand the underlying biochemical mechanisms. The findings reported in this chapter provide the groundwork for applying REIMS for meat research, however, further validation may be required.

Significant differences in small peptides observed from the in-bag dry-aged lamb suggest that the biomolecular signatures differ between the two different ageing regimes. Therefore, in the next chapter, a deeper exploration of small peptides produced from two ageing regimes was performed using a peptidomic approach to discover the biomolecular signatures for in-bag dry- and wet-aged lamb.

Chapter 8: A peptidomic approach to understand potential biomolecular signatures in dry *vs.* wet aged lamb

This chapter shows peptidomic profiling of small peptides to explore the biomolecular signatures for in-bag dry- and wet-aged lamb. Low molecular weight peptides (< 10 kDa) were extracted from lamb produced by the two ageing regimes. The peptide profile was identified using LC-MS/MS and the quantification of peptides was performed using a label-free peptidomic approach. Results showed that two ageing regimes had significantly different peptide profiles. Out of 395 peptides identified in both treatments, 59% of variation was explained by the ageing treatment (PC1), and a further 23% by the extent of lipid oxidation. Peptides released from creatine kinase, LIM domain binding 3, nebulin, sHSP and myozenin were significantly higher for dry-aged, suggesting the potential as signature biomolecules to distinguish the dry-aged from wet-aged. Dry-ageing of lamb promoted the growth of yeast and lipid oxidation which may affect proteolysis and resulted in distinct peptide profiles compared to the wet-aged equivalents.

This chapter is organised as follows: Section 8.1 briefly states the motivations and objectives of this study. Lamb samples, ageing process, extraction and peptidomic profiling of peptides are described in Section 8.2. The comparison of peptides profiles between two ageing regimes are shown in Section 8.3. Section 8.4 presents the peptides with extreme changes between two regimes followed by the discussion on their role as potential signature biomolecules for in-bag dry- and wet-aged lamb. A brief summary is included in Section 8.5.

8.1 Introduction

Dry-ageing of fresh meat has become a value-adding strategy for the meat industry to explore alternative merchandising opportunities (Laster et al., 2008; Zhang et al., 2019). Dry-aged meat is a niche product favoured by meat purveyors for its enhanced buttery, nutty and meaty flavour (Li et al., 2014; Savell, 2008) compared with the widely available wet-aged equivalents. As a common post-mortem tenderisation practice, wet-ageing (W) is accomplished in moisture-impermeable vacuum bags under refrigerated conditions. The typical processing for dry-ageing is carried out in a chamber/chiller with well-controlled conditions of temperature, humidity and air velocity for 3 to 4 weeks.

A series of physical and biochemical reactions occur during dry-ageing, which includes dehydration, proteolysis, lipolysis and oxidation. However, the biomolecular signatures responsible for the distinct dry-aged quality have not been well explored. The extent of dehydration (Lee et al., 2019a) and the generation of volatile compounds (King et al., 1995;

O'Quinn et al., 2016) and metabolites such as free amino acids and peptides from the dry-ageing process (Kim et al., 2016; Lee et al., 2019a) are the main drivers in the formation of the potential biomolecular signatures. As meat ages, significant changes in the composition of flavour precursors is observed (Koutsidis et al., 2008). Dry-aged flavour results from the release of free amino acids, peptides and the breakdown of ribonucleotides, to yield inosine monophosphate (IMP), guanosine monophosphate, inosine, and hypoxanthine (Khan et al., 2015; Koutsidis et al., 2008; Lee et al., 2019a). Many of these changes are due to the activity of various hydrolases, such as the calcium-dependent calpain proteases, cathepsins and peptidases implicated in the production of flavour peptides (Nishimura, 1998; Spanier, Flores, McMillin, & Bidner, 1997). Several amino acids, including tryptophan, phenylalanine, valine, tyrosine, glutamate, isoleucine and leucine, have been reported to be more abundant in the dry-aged than the wet-aged beef (Kim et al., 2016). These breakdown products further contribute to the intense meaty, nutty and roasted flavour of dry-aged beef when cooked (Kim et al., 2016; Li et al., 2014).

Previous studies have reported the use of peptides as markers for monitoring ageing (Fu et al., 2017) and curing process of meat products (Mora, Gallego, Aristoy, Fraser, & Toldrá, 2015). Peptides have better stability to thermal treatments than intact proteins. Hence they allow the identification of ageing-specific markers which helps to understand biochemical changes (Choe et al., 2020). Distinct peptide profiles (< 3 kDa) between dry- and wet-aged beef have been observed suggesting the potential of using peptides as biomolecular signatures for dry-ageing (Choe et al., 2020). Detection of marker peptides produced from enzymatic hydrolysis using label free relative quantification through liquid chromatography coupled to mass spectrometry (LC-MS) has been successfully applied for the determination of species-specific peptides from meat, with high sensitivity and efficiency (Montowska, 2017; Montowska & Fornal, 2017; Sentandreu, Fraser, Halket, Patel, & Bramley, 2010). Thus, it is feasible to employ such technique for peptidomic profiling of the biomolecular signatures of dry-aged meat.

The aim of the present preliminary study is to determine whether peptidomic profiling using label free quantification of the low molecular weight peptides(< 10 kDa), obtained from in-bag dry-aged lamb and compared with wet-aged equivalents could potentially be used for developing a biomolecular signature profile of dry-aged lamb.

8.2 Materials and methods

8.2.1 Ageing process

Four lamb hindlegs were collected from two lamb carcasses (Ram, shank and chump off) on the day of slaughter. Paired legs (left and right) from the same animal were randomly assigned to two ageing treatments: in-bag dry-ageing (BD) and W. BD was carried out in water permeable ageing bags (TUBLIN® 10, 50 μm thick, polyamide mix with water vapor transmission rate 920 g/50 μ/m^2/24 h at 7 °C, 50% RH, and oxygen transmission rate 660 g/m^2/24 h at 7 °C, 50% RH, TUB-EX ApS, Denmark) at 2 ± 0.5 °C, 0.5 $m.s^{-1}$ air velocity and relative humidity of 75 ± 5%. W of lamb legs using water impermeable barrier bags (Cryovac® A600 barrier bag, oxygen transmission rate 20 - 50 g/m^2/24 h at 23 °C, Sealed Air®, New Zealand) was performed at -1.5 ± 0.5 °C. After 21 days of ageing time, lamb chops (1.5 cm thickness) were taken from the same anatomical position of the lamb legs (both wet- or dry-aged), with no further trimming of surface. The lamb legs were fabricated into chops then freeze-dried (deboned, ~3 days) then ground into fine powder for the peptidomic analysis.

8.2.2 Extraction and quantification of peptides

Peptide extracts were obtained from lyophilised and pulverised meat powder from both ageing treatments. Approx. 20 mg of meat powder was mixed with appropriate volume of MilliQ water on a vortex mixer for 60 s then homogenised in a sonicator for 5 min to obtain a sample mixture with the final concentration of 20 $mg.mL^{-1}$. The homogenate was then spun at 1,000 rpm for 10 min. The liquid fraction (top layer) was obtained and mixed with 5% acetonitrile (1 : 3, v/v) before centrifuged at 15,000 rpm for 10 min. An aliquot of clear supernatant containing water soluble fraction of lamb samples was transferred in to a Nanosep® 10K Omega™ tube (Pall Corporation, USA) and spun at 5,000 g for 90 min at 4 ºC. The peptide concentration of the ultrafiltrate was determined using a peptide assay kit (Pierce™ quantitative colorimetric peptide assay, Thermo Fisher Scientific, San Jose, CA, USA). The peptide concentration was calculated from an average of triplicate measurements. An aliquot of the peptide solution containing 38 μg peptides was taken and spun in a vacuum evaporator at 40 ºC to remove liquid fraction. The peptide sample was re-suspended in 50 μL formic acid (0.1%) for label free quantification.

8.2.3 Peptide profiling

8.2.3.1 Mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) was performed on a nanoflow Ultimate 3000 UPLC (Dionex) coupled to an Impact HD mass spectrometer equipped with a CaptiveSpray source (Bruker Daltonik, Bremen, Germany). For each sample, 1 µL of the sample was loaded on a C18 PepMap100 nano-Trap column (300 µm ID x 5 mm, 5 micron 100Å) at a flow rate of 3000 $Nl.min^{-1}$. The trap column was then switched in line with the analytical column ProntoSIL C18AQ (100 µm ID x 150 mm 3 micron 200Å). The reverse phase elution gradient was from 2% to 20% to 45% B over 60 min, total 84 min at a flow rate of 1000 $nL.min^{-1}$. Solvent A was LCMS-grade water with 0.1% Formic acid; solvent B was LCMS-grade Acetonitrile with 0.1% Formic acid.

The LC was directly interfaced with a captive spray ion source (3.0 $L.min^{-1}$ dry gas, operated at 1500 V) to a high-resolution Impact HD quadrupole-time-of-flight (Q-TOF) (Bruker Daltonics) mass spectrometer. To profile protein expression patterns, the analytes were detected via MS-only mode in positive ion mode, with a mass range between 130 – 2200 m/z and a sampling rate of 2 Hz. To link the expression levels with identifications, a pool of per treatment was created, and these pooled samples were run via LC-MS/MS with data-dependent auto-MS/MS mode with the following settings: the same LC parameters as described before, a full scan spectrum, with a mass range of 150 - 2200 m/z, was followed by a maximum of ten collision-induced dissociation (CID) tandem mass spectra at a sampling rate of 2 Hz for MS scans and 1 to 20 Hz for MS/MS. Precursors with charges 1+ to 5+ were preferred for further fragmentation and a dynamic exclusion of 60 sec was set.

Following the LC-MS run, the Q-TOF data were further analysed with Compass DataAnalysis 4.4 software (Bruker Daltonics) to evaluate the LC chromatogram and the overall quality of both MS1 and MS2 spectra.

8.2.3.2 Peptide identification

The PEAKS X+ Studio data analysis software package (Bio informatic Solutions Inc) was used to analyse the LC-MS/MS data. The raw data were refined by a built-in algorithm which allows association of chimeric spectra. The peptides were identified with the following parameters: a precursor mass error tolerance of 10 ppm and fragment mass error tolerance of 0.05 Da were allowed, the Uniprot_Ovis aries database (v2018.07, 27885 sequences) was

used, the minimum peptide length was set to 3 amino acids long and an unspecific digestion was specified, as no enzyme was added to generate the peptides. Oxidation (M), phosphorylation (STY) and deamidation (NQ) were chosen as variable modifications. A maximum of 3 post-translational modifications (PTMs) per peptide was permitted. False discovery rate (FDR) estimation was made based on decoy-fusion. An FDR of < 5% on peptide spectrum match level and a PTM A-score of 100 was considered adequate for confident peptide identification.

8.2.3.3 Label free quantification

To quantify the peptide expression levels, label-free quantification (LFQ) was performed using the quantitation node of Peaks Studio X+ software. Here, peptide expression levels between all samples were compared. The following parameters were included: a mass tolerance error of 15 ppm and a retention time shift tolerance of 2 min was allowed. To determine the relative peptide abundance in the RT aligned samples, peptide feature based quantification was performed. Relative comparison between samples was based on the area under the curve at the MS1 level after TIC normalisation.

8.2.4 Statistical analysis

This preliminary study was carried out complementary to Chapter 6 and the duplicities of the experimental design are acknowledged. Two pairs of lamb legs determined in this study were selected from the sample set of 60 lamb legs as described in Chapter 6. Peptide information from lamb chops (consisting of four main muscles: *m. semimembranosus, m. biceps femoris, m. vastus lateralis*, and *m. rectus femoris*) obtained from two different animals with varying degrees of lipid oxidation as determined by TBARS assay (Buege & Aust, 1978) and two ageing techniques, either wet or dry, were used as variables for PCA analysis. A multivariate statistical method, principal component analysis (PCA), was used to identify variability in these samples in a multivariate data matrix.

A principal component analysis (PCA) was carried out using standardised, centred and scaled, variables. On inspection of the first and second principal components the four groups separated on both of the axes in Figure 8.1. An analysis of variance was carried out on the first principal component and there were differences between the groups (P = 0.006). A cluster analysis was also carried out using Euclidean distances and Ward linkage to identify peptides that were in different groups. The two most extreme groups were inspected and the

peptides in them were further investigated. All analysis was carried out in R software (version 3.6.0).

8.3 Results

In order to elucidate differences at the molecular level between dry- and wet-aged lamb, we performed a preliminary study on a limited number of samples to discover whether the analysis of endogenous peptide profiles in these meat samples have the potential in discriminating between two ageing techniques and whether predisposition of the meat to higher lipid oxidation could also have an influence.

When looking at the overall peptide profiles (i.e. the peptide abundance levels of all 395 peptides in each sample) via multivariate analysis, different clusters of samples could be discriminated. Here, PC1 comprised of the type of ageing treatments used, whereas PC2 was characterised by the predisposition of these animals to a higher level of lipid oxidation. The loading plot revealed coefficients of variation with a high correlation between two ageing treatments. The first principal component explains 59.4% of the variability in the data and the second (results from animals with or without the predisposition to a higher level of lipid oxidation) explained a further 22.6% (Figure 8.1).

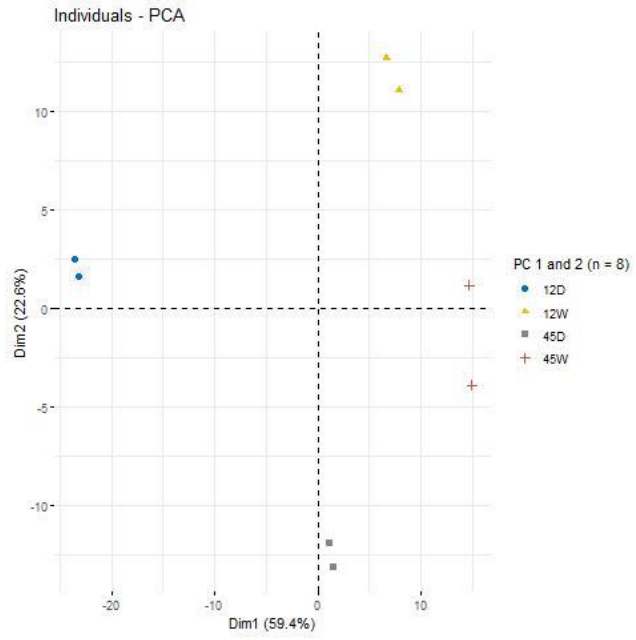


Figure 8.1 A multivariate analysis using principal component analysis based on the peptide profiles of the samples indicates that a clear discrimination between the different experimental groups is found based on the abundance differences of the 395 peptides detected in this experiment.

PCA transformed the variables into 6 principal components (PCs). The analysis revealed that about 59% of the total variation is explained by the first component and 82% by the first two components (Table 8.1). In other words, a high percentage of the total variance in the four considered variables can be condensed into two new variables (PCs) with a major contribution obtained from just the type of ageing used (PC1). Additionally, this also means that knowledge on the predisposition of meat to lipid oxidation after ageing process is crucial, as it can cause variation within a dry-aged or wet-aged experimental group.

Table 8.1 Summary of principal components identified by principal component analysis of total peptide profile.

Component	Eigenvalues	% of variance	Cumulative variance %
1	2.348255e+02	59.45	59.45
2	8.912755e+01	22.56	82.01
3	3.855814e+01	9.76	91.77
4	1.280490e+01	3.24	95.01
5	8.454533e+00	2.14	97.16
6	5.942573e+00	1.50	98.66

Next, an analysis of variance was carried out on the first principal component and showed that, based on endogenous peptide profiles, a significant difference between two ageing treatments can be found (P = 0.00636). Although a limited number of samples were used in this study, this test gave a good indication that peptide profiling potentially could elucidate the differences between dry- and wet-aged meat. These peptides that varied significantly were identified using a cluster analysis using Euclidean distances and Ward linkage (Figure 8.2). A further investigation on the parent proteins responsible for the generation of these endogenous peptides was performed. A list of these peptides, along with their proteins of origin are summarised in Table 8.2.

8.4 Discussion

The ultimate tenderness and improvement of flavour of aged meat is closely related to the activity of endogenous proteolytic enzymes (Hopkins & Geesink, 2009). The greatest activity of proteolytic enzymes, especially Ca^{2+} dependent proteases (calpain system), has been shown in the first seven days of ageing (Velotto et al., 2015) and by the fourteenth day, the greatest gains in tenderness have usually been achieved (Gudjónsdóttir et al., 2015; Zhang et al., 2019). However, proteolytic enzymes continue acting on muscle proteins and their effect on quality traits such as flavour and juiciness is not well understood (Lian, Wang, &

Liu, 2013). In this preliminary study, dry-aged *vs* wet-aged lamb carcasses were compared to determine whether these two treatments of the lamb meat resulted in differences in their respective peptide profiles. Although the replicate numbers were low, a number of peptides were found to be at the extremes of an Euclidian distance tree, indicating a potential difference in the two treatment groups. An overview of these peptides found in these extreme clusters, and which could be linked back to their parent protein, are shown in Table 8.2. A possible linkage between these peptides and the ageing process is described below.

Several peptides were found to be higher in the dry-aged sample compared to its wet-aged equivalent, for example, one peptide from LIM domain binding 3 protein (LDB3) was present in higher quantities which indicate that this protein may be subjected to a different enzymatic proteolysis pathway occurring from the dry-ageing process. LDB3 is known to stabilise the sarcomere through interactions with actin and is predominantly expressed in striated muscles as it forms multiprotein complexes that play an important role in the structural integrity of sarcomeres (Zhou et al., 2001). LDB3 protein is essential for stabilising Z line structure by binding to its major component alpha-actinin thus helping to maintain muscle integrity (Seto et al., 2011). LDB3 proteins, also known as Z band alternatively spliced PDZ-motif protein (ZASP), are found to be localised at integrin adhesion sites and therefore changes to this protein results in disruption of integrin interactions (Jani & Schöck, 2007). Although as a minor component of the Z line, ZASP plays an important role in the development of myofibrils and the conversion of mechanical stimulus to chemical signals during muscle contraction (Leung, Hitchen, Ward, Messer, & Marston, 2013). LDB3 interacts with protein kinase C via its three C-terminal LIM domains and via its N-terminal PDZ domain with α-actinin-2, thus anchoring the myofibrillar actin filaments, providing mechanical strength to the Z lines which acts as an interface between the contractile apparatus and the cytoskeleton connecting myofibrils to the sarcolemma membrane (Klaavuniemi, Kelloniemi, & Ylänne, 2004). It has been reported earlier that post-mortem ageing over 72 h caused proteolytic cleavage of constitutive proteins of the Z line, resulting in the weakening of the Z line (Morzel et al., 2004). It has been established that LDB3 protein sequences are mostly acted upon by aminopeptidases, carboxypeptidases and cathepsins (Gallego, Mora, Fraser, Aristoy, & Toldrá, 2014). Extensive proteolysis has been reported during processing of dry-cured ham due to the action of endo- and exo- peptidases (Gallego et al., 2014; Toldrá, Aristoy, & Flores, 2000; Toldrá & Flores, 1998). Activity of exopeptidases, such as

aminopeptidases and dipeptidyl peptidases exhibited a good stability along the curing process (Toldrá et al., 2000).

Proteolysis of myozenin 1 produced peptide PVDY which was more abundant in dry-aged lamb compared to the wet-aged. Myozenin binds to gamma-filamin or the alpha-actinin in the Z line which is a major protein responsible for cross-linking antiparallel actin filaments from opposite sarcomeres (Takada et al., 2001). Therefore, proteolysis of myozenin can also contribute to the weakening of the Z line during ageing process (Morzel et al., 2004). Several studies have provided convincing evidence that most of the tenderness development during ageing is the result of post-mortem proteolysis of key structural proteins (Lametsch et al., 2003) and the disruption of sarcomere structure due to Z-disks degradation within a few days of ageing (Morzel et al., 2004).

Peptide DPDVPQ from nebulin was also determined to be higher in quantity in the dry-aged lamb samples. Previous studies have shown a high rate of nebulin degradation by calpains which are Ca^{2+}-dependent proteases endogenous to skeletal muscle cells (Bhat, Morton, Mason, & Bekhit, 2018). The degradation of nebulin leads to the disruption of the myofibrillar structure resulting in meat tenderisation (Huff-Lonergan et al., 2010).

LPPGVDP is a peptide derived from small heat shock protein (sHSP) family B which has been observed significantly more abundant in dry-aged lamb. Biochemical changes to muscle tissue post-mortem, driven by the termination of nutrient and oxygen supply results in muscles becoming increasingly ischaemic and acidic, triggering apoptosis of muscle cells (Honikel, 2014). In response to impending cell death, sHSPs are synthesised to combat apoptosis (Beere, 2005) and maintain muscle cell homeostasis. sHSPs are chaperone proteins that are abundantly expressed in muscles after slaughter (Jia, Hollung, Therkildsen, Hildrum, & Bendixen, 2006; Pulford et al., 2008). sHSPs are known to regulate actin polymerisation and actin–myosin interactions (Mounier & Arrigo, 2002). Thus, the expression of sHSPs prevent the aggregation of myofibrillar proteins and contribute towards the proteolytic degradation and tenderisation during ageing. A higher abundance of the peptide from sHSP in the dry-aged sample probably suggests a higher rate of degradation of the sHSPs thus reducing its chaperon effect. Such changes in turn favour the proteolytic degradation of actin and myosin leading to more tender meat during the dry-ageing process (Berger et al., 2018; Li et al., 2014).

Table 8.2 List of peptides detected by Euclidean distances with the most extreme difference between dry- and wet-ageing of lamb.

Peptide sequence	Protein Name	Carcass 1 peptide intensity (dry-aged)		Carcass 1 peptide intensity (wet-aged)		Carcass 2 peptide intensity (dry-aged)		Carcass 2 peptide intensity (wet-aged)	
		Repeat 1	Repeat 2	Repeat 1	Repeat 2	Repeat 1	Repeat 2	Repeat 1	Repeat 2
L.DDVIQTG.V	Creatine kinase, M-type	1.14E+06	1.06E+06	9.04E+05	7.77E+05	7.88E+05	7.95E+05	7.57E+05	7.39E+05
V.DNPGHPF.I	Creatine kinase, M-type	1.24E+05	1.16E+05	1.25E+05	1.13E+05	8.83E+04	8.81E+04	9.59E+04	9.62E+04
Q.SPLPVIP.H	LIM domain binding 3	8.47E+05	8.21E+05	5.09E+05	5.07E+05	4.94E+05	4.82E+05	4.28E+05	3.91E+05
Q.SPLPVIPHQK.D	LIM domain binding 3	6.25E+05	6.29E+05	1.58E+06	1.49E+06	1.26E+06	1.34E+06	1.58E+06	1.46E+06
L.DPDVPQ.F	Nebulin	1.55E+05	1.53E+05	7.45E+04	6.47E+04	8.66E+04	8.56E+04	6.50E+04	5.83E+04
R.LPPGVDP.P	Heat shock protein family B (small) member 1	8.79E+04	9.00E+04	6.13E+04	5.81E+04	3.01E+04	4.88E+04	4.80E+04	4.60E+04
E.PVDY.N	Myozenin 1	1.56E+05	1.83E+05	1.03E+05	9.07E+04	1.76E+05	1.72E+05	1.05E+05	1.15E+05

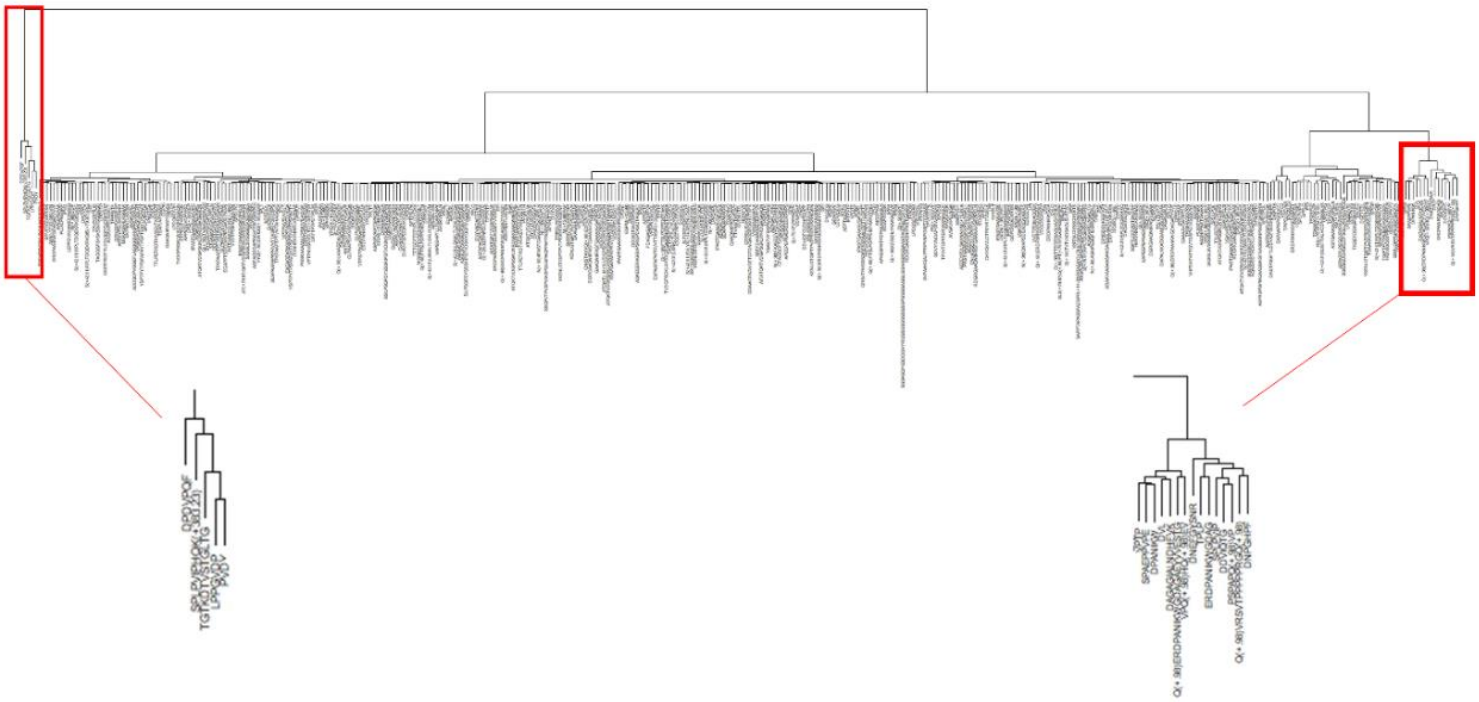


Figure 8.2 Cluster analysis using Euclidean distances of all 395 peptides detected in the samples. Peptides of interest clustered as outgroups to the main cluster of peptides. The outgroup clade with 5 peptides contained the peptides DPDVPQF (Nebulin), SPLPVIPHQK (LIM domain binding 3), PVDY (Myozenin 1), and LPPGVPD (Heat Shock Protein family B (small) member 1). DDVIQTG (Creatinase kinase, M-Type), DNPGHPF (Creatinase kinase, M-Type) and SPLPVIP (LIM domain binding 3) clustered at the extreme end of the main cluster of proteins.

Two peptides DDVIQTG and DNPGHPF from creatine kinase were also more abundant in the dry-aged samples. Creatine kinase is an important enzyme for the *in vivo* energy metabolism of skeletal muscles. Creatine kinase is one of the most abundant sarcoplasmic proteins in skeletal muscles but is also a substrate for proteases involved in the enzymatic proteolysis during ageing (Lametsch, Roepstorff, & Bendixen, 2002) and curing/fermentation (López, Bru, Vignolo, & Fadda, 2015; Zhang et al., 2018). A total of 16 creatine kinase peptides were observed previously in dry-cured lamb and mutton (Zhang et al., 2018). Further, sarcoplasmic proteins have been suggested to be more susceptible to proteolysis compared to myofibrillar fractions (López et al., 2015). Creatine kinase is mainly degraded by endogenous muscle proteases (Martín, Córdoba, Rodríguez, Núñez, & Asensio, 2001), including μ-calpain during the early post-mortem (1 - 2 weeks) (Purintrapiban, Wang, & Forsberg, 2001) and cathepsins B and L during the extended storage and processing period (Mora, Sentandreu, Fraser, Toldra, & Bramley, 2009). During the post-mortem storage of porcine muscle, it has been observed that both structural as well as metabolic proteins are degraded (Lametsch et al., 2002). The accumulation of proteolytic fragments from metabolic proteins, including creatine kinase were the most abundant. Therefore, creatine kinase peptides resulting from its proteolysis may be employed as useful markers of meat quality (Daroit & Brandelli, 2008).

The lack of difference in the eating quality (Kim et al., 2018; Laster et al., 2008; Smith et al., 2008) and protein profile (SDS-PAGE) (Choe et al., 2020) between dry- and wet-aged beef suggest the role of endogenous proteases in proteolysis is similar between the two ageing techniques. Different peptide levels resulting from the proteolysis of LDB3, myozenin-1, nebulin and sHSP between two ageing techniques could be associated with two factors: (1) higher ageing temperature for dry-ageing process compared to the wet-aged; and (2) activity by microbial enzymes. The slightly higher ageing temperature (2 *vs* -1.5 °C) used for dry-ageing compared to the W in this study could have contributed towards an improved proteolytic activity of muscle proteases, resulting in an increased level of small peptides. A higher rate of proteolysis was suggested by Kim et al. (2016) for dry-aged beef at 3 °C compared to equivalent wet-aged at 1 °C due to the increased level of free amino acids in the dry-aged beef. However, no significant difference in the shear force and sensory tenderness between the two ageing temperatures was observed in their study. Therefore, the actual impact of the slight increase in ageing temperature on the proteolytic activity was

inconclusive. Such an increase of amino acid concentration could be associated with moisture evaporation instead of the ageing temperature during dry-ageing (Lee et al., 2019a). Further, a study by Velotto et al. (2015) reported a faster autolysis of μ-calpain in beef during 14 days of dry-ageing compared to W, suggesting a lower rate of proteolysis during dry-ageing. Thus, the slight increase of ageing temperature in dry-ageing process may only have a minor contribution towards the increased level of some peptides observed in this study. Another factor could be the action of endopeptidases and exopeptidases by microorganisms (Mora, Sentandreu, & Toldrá, 2010), especially those originating from mould and yeast (Choe et al., 2020). Proliferation of mould and yeast has been observed on the crust of dry-aged beef using traditional no-bag regime with extended ageing period for over 3 to 4 weeks (Lee et al., 2019b; Oh et al., 2019). The BD regime applied in this study has been shown to result in a significant increase of yeast with no impact on mould (Li et al., 2014; Zhang et al., 2019). Activities of peptidases from yeast and mould have been reported to produce a unique profile of small peptide on the crust of dry-aged beef compared to the unaged and wet-aged equivalents (Choe et al., 2020). Proliferation of yeast on the surface of current dry-aged lamb samples was also observed (Table 6.2). Absence of post-ageing trimming in this study allowed peptide profiling to be performed on the intact lamb chops, including peptides that may have been produced by peptidases from the moulds and yeast. Exogenous peptidases from yeast (*Debaryomyces hansenii*) have been previously shown to generate a range of small peptides and free amino acids during the dry-curing process (Lee et al., 2019b; Mora et al., 2015).

8.5 Conclusion

The peptidomic profiling of small peptides was performed and a total of 395 peptides (< 10 kDa) were identified in both in-bag dry- and wet-aged lamb. Peptides released from creatine kinase, LDB3, nebulin, sHSP and myozenin were observed in the extremes of an Euclidian distance tree, indicating differences in peptide profiles and thus suggesting the potential of these peptides as signature molecules for discriminating between two ageing techniques. Further studies are however required to validate current observations of the potential of using these peptides as biomolecular signatures for in-bag dry-aged lamb.

Chapter 9: Conclusions and future work

Previous chapters have demonstrated a comprehensive research towards the main goal of this study which was to produce quality acceptable dry-aged lean beef and lamb using in-bag dry-ageing technique. The effects of processing regimes (stepwise ageing, air velocity and ageing time) and storage factors (fresh and frozen) on their quality, acceptability and biochemical changes were systematically investigated. To achieve this goal, eight specific objectives (Figure 1.1) were established to address four research questions which are listed as follows:

1) *Can lean bull beef be used to produce quality acceptable and storage stable in-bag dry-aged products? (Chapter 3 & 4)*
    - *Objective 1: To produce in-bag dry-aged lean beef using "stepwise in-bag ageing" regime and compared with straight-dry-ageing;*
    - *Objective 2: To determine the effect of processing conditions including ageing air velocities and ageing times on the in-bag dry-ageing lean beef;*
2) *Can in-bag dry-aged lean beef be frozen, and will this affect the quality changes with long-term frozen storage? (Chapter 3 & 4)*
    - *Objective 3: To investigate the effect frozen storage and its interactions with the different processing regimes on the in-bag dry-aged lean beef;*
    - *Objective 4: To determine the quality and biochemical changes of in-bag dry-aged lean beef following one-year frozen storage;*
3) *Can in-bag dry-ageing regime be performed on lamb to produce quality acceptable dry-aged products? (Chapter 6)*
    - *Objective 5: To determine the effect of in-bag dry-ageing on the quality and acceptability of lamb compared to the wet-aged equivalents;*
    - *Objective 6: To investigate the underlying biochemical changes between in-bag dry-ageing and wet-ageing;*
4) *What are the signatures for identification and discrimination of dry-aged lean beef and lamb and how to easily detect such signatures? (Chapter 5, 7 & 8)*
    - *Objective 7: To investigate the potential of Rapid Evaporative Ionisation Mass Spectrometry (REIMS) for deification and discrimination of dry-aged lean beef and lamb due to different process regimes;*
    - *Objective 8: To explore the potential biomolecular signatures using peptidomics profiling of small peptides to discriminate the in-bag dry-aged lamb from the wet-aged equivalents.*

This chapter is organised as follows: Section 9.1 presents the research summary with an overall discussion of current outcomes. Section 9.2 is the conclusion of this thesis. Suggestion for the future improvement is included in 9.3.

9.1 Research summary and discussion

9.1.1 Effect of stepwise in-bag ageing regime (Objective 1)

This research proposed a novel ageing regime, called "stepwise in-bag ageing", which was performed with BD for 7 days followed by W for 14 days. The impacts of stepwise in-bag ageing on quality, acceptability and biochemical changes of lean bull beef striploin (*m. longissimus lumborum*) were investigated to compare with straight-dry-ageing using BD for 21 days without W component. Outcomes of this research showed that using stepwise in-bag ageing regime had no detrimental effects on the physicochemical properties, microbial safety and eating quality of in-bag dry-aged lean beef as compared to the equivalent straight-dry-aged beef (Chapter 3). Higher amounts of yeast were found on the surface following 21 days of straight-dry-ageing. This is the unique feature of BD regime, which has also been observed for in-bag dry-aged lamb (Chapter 6). The proliferation of yeast could be attributed to yeast species being able to grow on the dry meat surface with low moisture level compared to other microorganisms.

Low levels of TBARS and protein carbonyl were detected, and no significant difference was found between two ageing regimes suggesting that both ageing regimes demonstrated similar oxidative stability during ageing. Stepwise in-bag ageing resulted in a similar proteolytic pattern compared to the straight-dry-ageing when evaluated by SDS-PAGE protein profile and FAAs level (Chapter 4). This finding is further supported by the similar profile of small peptides (< 10 kDa) observed between two ageing regimes (Appendix A5). Thus, both ageing regimes followed a similar proteolytic pathway and produced in-bag dry-aged lean beef with similar eating quality, microbial safety and oxidative stability. However, stepwise in-bag ageing provided advantages over the straight-dry-ageing through reducing the weight loss from ageing (10% *vs.* 20%) and costs of operating dry-ageing facilities (7 days *vs.* 21 days). Therefore, stepwise in-bag ageing regimes have demonstrated the potential to be used for industrial application to produce quality acceptable dry-aged beef with improved product yield and reduce the product cost overall.

9.1.2 Effect of air velocities (Objective 2)

Three ageing chamber air velocities of 0.5, 1.5 and 2.5 $m.s^{-1}$ were used for stepwise in-bag ageing of lean beef. The effects of air velocities on the quality, acceptability and biochemical changes were determined following 7 days of BD. Increase of air velocities from

0.5 to 2.5 $m.s^{-1}$ increased the dehydration rate and resulted in the significantly increased weight loss after 7 days of BD. There was no significant change between air velocities observed for physicochemical properties, oxidative stability and proteolytic pattern, suggesting that the increase of air velocities mainly improved the dehydration rate with no detrimental effect on the quality and stability of lean beef following 7 days of BD (Chapter 3 and 4).

The interactions of air velocities with ageing regimes (straight-dry-ageing and stepwise in-bag ageing) were also investigated following 21 days of ageing by comparing straight-dry-ageing at 0.5 $m.s^{-1}$ with stepwise in-bag ageing using different air velocities. Combination of air velocity and stepwise ageing regime had no impact on the quality, acceptability, oxidative stability of lipids and proteins and proteolysis pattern (SDS-PAGE protein and FAAs profiles) of dry-aged lean beef. Therefore, stepwise in-bag ageing with an increased air velocity could accelerate the dry-ageing process to obtain dry-aged meat with desired level of dehydration, with an increase in the product yield.

9.1.3 Effect of ageing time (Objective 2)

In-bag dry-aged lean beef following 7 and 21 days of ageing were compared with the un-aged (0 day) counterparts to study the effect of ageing time on the quality, acceptability and biochemical changes (Chapters 3 and 4). In-bag dry-ageing of lean beef for 21 days resulted in significant increase of pH, water-holding capacity (i.e. decrease in drip and cook loss) with decrease in A_w regardless of the treatment combinations (stepwise ageing and air velocity).

Interactions of ageing regimes with ageing time were observed for instrumental colour and microbial properties. All the colour parameters (L*, b*, Chroma and hue) increased ($P < 0.05$) with straight-dry-ageing for 21 days except for the a* which did not change. The colour properties in beef changed differently across the stepwise in-bag ageing process, suggesting that the BD process could result in different colour features compared to the W. Current findings can be further supported by the different colour properties observed between BD and W of lamb (Chapter 6). There was no significant change in microbial counts observed following 21 days of stepwise in-bag ageing compared to the unaged counterparts. While straight-dry-ageing for 21 days improved the proliferation of yeast on the meat surface and this could be attributed to the surface drying during BD as described above (section 9.1.1).

An increase of oxidative potential with ageing time through the increase of TBARS and protein carbonyl content were also observed, though the overall oxidation level remained low. This was further supported by observing no significant change of FAs compositions following 21 days of BD regardless of processing treatments (air velocities and ageing regimes). Low levels of oxidative damage of lipid and protein may be due to the barrier function of the dry-ageing bag, as oxygen availability becomes lower, thereby reducing the potential of oxidation. Additionally, lean beef (approx. 1% fat) was used in this study, which has reduced availability of reactive substrates (UFAs) and therefore the reduced risk of oxidation. This could consequently improve the oxidative stability of the dry-aged products during storage.

Further, degradation of protein with ageing time improved tenderness and flavour regardless of ageing treatments. Shear force values decreased significantly following 7 days of BD, then further decreased slightly during the extended 14 days of W period to a similar level compared to straight-dry-ageing for 21 days. Therefore, it was the ageing time rather than the ageing methods that played the key role in tenderisation of beef. Further, the most significant improvement of in-bag dry-aged beef tenderness occurs within the first 7 to 14 days of ageing time. Extended ageing time may continue improving the tenderness but at a lower rate. On the other hand, proteolytic degradation resulted in higher levels of low MW protein fragments (SDS-PAGE protein profile) and FAAs. These may contribute to the flavour development of in-bag dry-aged lean beef. Total FAAs and essential AAs content in the dry-aged beef samples increased significantly following 21 days of ageing compared to 7 days and unaged counterparts. It is worth noting that the significant increase of FAAs content was driven by essential AAs. The % essential AAs content (in total FAAs) significantly increased to about three times by 21 days of ageing compared to the unaged. Therefore, BD of lean beef for 1 to 2 weeks is sufficient to improve the tenderness; however, extended ageing time is required to improve the flavour intensity of dry-aged products.

9.1.4 Effect of frozen storage (Objectives 3 & 4)

(1) Interaction of frozen storage with processing regimes (Objective 3)

In-bag dry-aged lean beef following 7 and 21 days of ageing was frozen stored at -18 °C for 12 months. The impact of frozen storage and its interaction with processing regimes (stepwise ageing, air velocity and ageing time) were investigated (Chapter 3 and 4). There

was no interaction between frozen storage with stepwise in-bag ageing and air velocity on the physicochemical properties, acceptability, oxidative stability of in-bag dry-aged lean bull beef. This suggests that the use of higher air velocity and stepwise in-bag ageing for dry-ageing of lean beef would not negatively influence the meat quality of in-bag dry-aged lean beef frozen for 12 months.

After long-term frozen storage, the proximate content, pH, instrumental colour, texture profile of in-bag dry-aged beef did not differ across the ageing time regardless of ageing treatments. The differences of pH, instrumental colour and texture detected in fresh in-bag dry-aged samples between 7 days and 21 days of ageing were not observed after the long-term storage. The water-holding capacity improved with ageing time, but it became stable over the long-term frozen storage. Ageing time did not affect the FAs profile and TBARs of stepwise dry-aged beef following the long-term frozen storage, while the protein carbonyl concentration showed an increasing trend with ageing time. BD of beef improved its lipids and protein oxidative stability during long-term frozen storage compared to unaged beef. Improvement in beef protein digestibility was observed through increased release of FAAs and appearance of small protein fragments from SDS-PAGE in dry-aged samples compared to the unaged.

(2) Frozen vs. fresh in-bag dry-aged lean beef for different ageing times (Objective 4)

The impact of frozen storage on lean beef dry-aged for different ageing times was also determined (Chapters 3 and 4). Proximate content, pH of in-bag dry-aged beef at all three time points (0, 7 and 21 days) were not affected by the frozen storage. Frozen storage had the major effect on the instrumental colour and resulted in discoloration of in-bag dry-aged beef. All colour attributes have significantly decreased for in-bag dry-aged samples (7 and 21 days) after the frozen storage. Thawed in-bag dry-aged beef became darker and less red. The water-holding capacity of frozen in-bag dry-aged beef also decreased due to the extra fluid loss upon thawing.

Long-term frozen storage had no negative impact on the FAs composition of dry-aged lean beef that were aged for 0, 7 and 21 days. Frozen storage resulted in a significantly reduced level of TBARS in beef samples dry-aged for 7 and 21 days compared to the fresh counterparts. Protein carbonyl content of the unaged samples had significantly increased

whereas that of samples dry-aged for 7 or 21 days did not change after the long-term frozen storage.

Therefore, in-bag dry-aged lean bull beef products could be stored frozen for long-term, up to 12 months, with acceptable quality and oxidative stability. This demonstrates the potential to provide dry-aged beef products for export markets.

9.1.5 In-bag dry- *vs.* wet-aged lamb (Objectives 5 & 6)

In-bag dry-aged lamb was produced and the meat quality, consumer acceptability and *in vitro* digestibility of the meat compared to the wet-aged equivalent (control) were determined (Chapter 6). Both ageing regimes increased ultimate pH following 21 days of ageing which was also observed in BD of lean beef (section 9.1.3). Significantly higher pH and weight loss from ageing were observed in dry-aged lamb with a reduced cook loss compared to the wet-aged. The in-bag dry- and wet-aged lamb were equally preferred (about 40% each) by the consumer panel which underpins the niche nature of dry-aged meat. The harder and chewier texture profiles and lower instrumental colour attributes were observed for in-bag dry-aged lamb compared to the wet-aged which may have resulted from the moisture evaporation during dry-ageing. BD of lamb resulted in a significant increase of TBARS level compared to the wet-aged whereas with no impact on FAs profile and protein carbonyl content was observed. The same observations were made in in-bag dry-aged lean beef (section 9.1.1) as well. No significant difference in SDS-PAGE protein profile was observed and most of the FAAs resulted from proteolysis suggesting both ageing regimes follow the similar proteolytic pathway, which are in agreement with the outcomes from lean beef (section 9.1.1).

Further, low microbial counts were detected for both ageing regimes with significantly higher amount of yeast observed in dry-aged lamb. As described in previous sections (section 9.1.1 & 9.1.3), proliferation of yeast during BD process could be the unique feature which may contribute towards the biochemical changes of in-bag dry-aged lean beef and lamb. Ageing regimes had no impact on overall digestibility, however, a greater gastric digestibility was observed in dry-aged lamb through the increased release of FAAs compared to the wet-aged, which could be due to the proteolytic activity by yeast.

Therefore, outcomes of this study have demonstrated the potential of consistently producing premium quality dry-aged lamb using ageing bags. BD process is able to produce

microbiologically safe lamb products with comparable meat quality and oxidative stability, and with improved gastric digestibility compared to the wet-aged equivalents.

9.1.6 Exploration of biomolecular signatures for different processing regimes (Objective 7 & 8)

(1) Application of REIMS for metabolic fingerprinting of in-bag dry-aged lean beef and lamb

The metabolic changes of in-bag dry-aged lean beef and lamb arising from different processing parameters were determined using REIMS (Chapter 5 and 7). The comparison of processing factors included: (a). stepwise in-bag ageing *vs.* straight-dry-ageing; (b). air velocities (0.5, 1.5 and 2.5 $m.s^{-1}$); (c). ageing times (0, 7 and 21 days); (d). post-ageing trimming (three sampling locations); (e). BD *vs.* W; (f). four muscles: SM, BF, VL and RF and (g). carcass sides (left *vs.* right side). About 4000 metabolic features were detected and 1705 metabolite ions were identified and used for further multivariate analysis to compare different processing factors. OPLS-DA models based on 1705 identified m/z features were found for (a). stepwise in-bag ageing *vs.* straight dry-ageing ($Q^2 = 0.85$), (c). ageing time (0 *vs.* 21 days, $Q^2 = 0.95$) and (d). sampling locations (surface meat *vs.* trimmings, $Q^2 = 0.94$; surface meat *vs.* trimmed meat, $Q^2 = 0.73$), (e). BD *vs.* W ($Q^2 = 0.86$) and (f). muscles groups SM + BF *vs.* VL + RF ($Q^2 = 0.62$). No significant difference in metabolites were found due to (b). air velocities and (g). carcass sides.

BD of lean beef resulted in more of small metabolites (< m/z 500) such as dipeptides, amino acids, fatty alcohol and organic acids, compared to the un-aged counterparts. Significantly more abundant small metabolites were found on the surface/crust of untrimmed lean beef compared to the trimmed meat following 21 days of straight-dry-ageing due to the dehydration and proliferation of yeast which mainly occurred on the surface of meat. Biomolecular changes arising from straight-dry-ageing resulted in more of large sized ions, which were produced from phospholipid metabolism (e.g. oxidation) whereas for the stepwise ageing, 99% of the significant increase was driven by small sized ions.

Out of 1705 metabolites identified, 663 metabolites (38.89%) differed significantly between in-bag dry-and wet-aged lamb with 318 metabolites (18.65%) being more abundant in dry-aged lamb over the wet-aged. These mainly consisted of small sized metabolites (178 ions, < m/z 500), including amino acids, dipeptides, amines and lipid oxidation derived products, such as aldehydes and ketones. W, on the other hand, produced significantly more

abundant large sized metabolites (284 of total 345 ions, > m/z 500), mainly associated with phospholipids metabolism. Further, the metabolic profile of in-bag dry-aged lamb was affected by dehydration and proliferation of yeast, leading to concentration of meat constitutes and secondary proteolysis by microorganisms resulting in more abundant metabolites from proteolysis.

Four main muscles of lamb chops were determined by REIMS and the metabolite profiles of these muscles were separated into two groups (SM + BF and VL + RF) in the OPLS-DA model. SM + BF muscles had significantly increased level of metabolites associated with metabolic reactions of proteins and lipids, mainly including proteolysis, decarboxylation and lipid degradation and oxidation. The differences between the two muscle groups may have resulted from different fibre types, which had different biochemical responses to the ageing process. SM and BF in lamb generally contain higher levels of slow oxidative fibres than VL and RF which predominantly consist of fast glycolytic fibres. In general, muscles predominantly made of fast glycolytic fibres are more susceptible to post-mortem biochemical changes than those dominated by slow oxidative fibres. Therefore, it was hypothesised that the primary enzymatic protein metabolism due to the endogenous enzymes (mainly calpains) may be slow in SM and BF, while these muscles could be susceptible to oxidation and secondary biochemical reactions by microorganisms.

This research demonstrated the novel application of REIMS as a rapid metabolic fingerprinting tool for identification and discrimination of in-bag dry-aged lean beef and lamb based on different processing regimes. The peptides and amino acids which differed between the ageing regimes could potentially be used as the biomolecular signatures. Further investigation on profiling of small peptide from BD and W of lamb is described in the next section.

(2) Peptidomic profiling of small peptides to unlock the biomolecular signatures

Peptidomic profiling of low molecular weight peptides (< 10 kDa) was performed to explore the biomolecular signatures responsible for BD and W of lamb (Chapter 8). Lamb samples (two pairs) used for peptidomics analysis were selected from the dry-ageing trial with 30 pairs of lamb legs (Chapter 6) which had extreme differences of lipid oxidation (TBARS) between carcasses and ageing regimes. Different clusters of samples were discriminated by PCA and overall peptide profiles significantly differed between two ageing

regimes. Out of 395 peptides identified in both treatments, 59% of variation was explained by ageing treatment (PC1), and a further 23% by extent of lipid oxidation (PC2). Peptides released from creatine kinase, LIM domain binding 3, nebulin, sHSP and myozenin were significantly higher for dry-aged, suggesting the potential as signature biomolecules to distinguish in-bag dry-aged from wet-aged. BD of lamb promoted the growth of yeast and lipid oxidation (section 9.1.5) which may have affected proteolysis and resulted in distinct peptide profiles compared to the wet-aged equivalents.

9.2 Conclusions

Dry-aged lean beef and lamb were produced using BD technique. A comprehensive study has been performed to determine the effects of processing regimes (stepwise in-bag ageing, air velocity and ageing time) and storage factors (fresh *vs.* frozen) on lean beef and lamb in terms of meat quality, microbial safety, consumer acceptability, oxidative stability, proteolytic pattern, digestibility and metabolite profile. This research answers all the research questions and addresses all eight objectives. This thesis significantly contributes to the current knowledge of dry-ageing process which impacts not only the qualitative changes but also the in-dept understanding of the biochemical and metabolic changes of in-bag dry-aged lean beef and lamb due to different processing and storage factors. The highlights of this research are listed as follows:

- In-bag dry-aging of lean beef and lamb can provide quality consistent and storage stable dry-aged meat with improved eating quality and microbiological safety.
- A novel ageing regime called “stepwise in-bag ageing” used in this study demonstrates potential for industrial application to improve product yield while shortening the time needed for dry-ageing facilities to produce premium quality, cheaper.
- Increase of air velocities in the ageing chamber for BD only accelerates the dehydration rate with no impact on other quality attributes. This finding is important when dehydration is the main pathway for developing signature dry-aged flavour.
- In-bag dry-aged lean beef can be frozen up to 12 months with no major impact on the quality. This would mean that the ageing regime can be applied in meat industry to provide dry-aged beef products for export markets.

- Ageing time plays the key role in dry-ageing process and contributes towards improvement of eating quality, oxidative stability and digestibility.
- REIMS as a real-time metabolic fingerprinting technique, can be used to detect the metabolic changes of lean beef and lamb arising from different processing parameters, including ageing regimes, ageing time, post-ageing trimming and different muscles.
- Microbial activity, lipid oxidation and dehydration are the three main features for dry-ageing process responsible for the development of characteristic quality of dry-aged meat. A novel pathway which integrates three elements is proposed for future study to develop and tailor signature dry-aged flavour.

9.3 Future work

This work advances the understanding of dry-ageing process. Processing factors have been studied and their effects on dry-ageing process have been better understood. There remains a need for continued study to implement the current outcomes and to generate new knowledge in this research area. Some suggested directions are as follows:

- This study has proposed an integrated pathway involving the growth of microorganisms, lipid oxidation and dehydration collectively contributing to the development of unique features during dry-ageing process. Future work is required to assess and validate the roles of the three factors in the development of unique qualities for dry-ageing. To achieve such goal, identification of signatures for the characteristic features of dry-ageing is necessary. This will allow meat industry to produce dry-aged meat with guaranteed premium quality and tailorable flavour profile. Some candidate signatures have been proposed previously, including the degree of moisture evaporation, generation of unique volatile compounds, and release of FAAs.

  In this study, REIMS was used for metabolic fingerprinting to explore the potential biomolecules to discriminate dry-ageing from the other ageing regimes and associated processing parameters. Metabolic changes following BD were driven by small sized metabolites, such as small peptides, FAAs and lipid oxidation derived products which may be candidate biomolecular signatures for in-bag dry-aged meat.

Further validation using omics techniques (e.g. lipidomics, peptidmoics and metabolomics) will be necessary to strengthen and validate the current findings.

This study has also performed an exploratory research using peptidomic profiling (Chapter 8) on in-bag dry- and wet-aged lamb to test the outcomes from REIMS. Small peptides produced from creatine kinase, LIM domain binding 3, nebulin, sHSP and myozenin demonstrated great potential as biomolecules for discrimination between ageing regimes, which require future work to validate their roles as signatures for dry-ageing process.

- Further, minimally-invasive technologies, such as REIMS, direct analysis in real time mass spectrometry (DART-MS), hyperspectral imaging system, near-infrared spectroscopy and Raman spectroscopy can be utilised when developing new regimes for dry-ageing, as they enable monitoring of the ageing process at ease. Use of these advanced technologies will allow rapid minimally-invasive measurement of individual samples to ensure an optimal product is produced avoiding over- or under-ageing during the process.
- The shelf life of dry-aged products under chilled or frozen storage is another emerging area which will become relevant when the efficiency of dry-ageing has been improved and the production for local and export markets becomes possible. This study has demonstrated the possibility to freeze and store in-bag dry-aged lean beef for one year and still be acceptable to consumers. Further clarifications may be required for this concept. For example, how long a dry-aged product can be stored fresh and frozen and how the quality changes over the storage period? What factors could be associated with limiting the storage factors, such as ageing time, storage conditions, packaging formats and freezing methods? Another approach called ‘aged-then-frozen’ strategy (Farouk, et al., 2009a, 2009b; Kim et al., 2018) has shown promising results to produce premium meat with comparable quality to the fresh-never-frozen meat. More research is warranted to determine the efficacy of these strategies and approaches in the improvement of dry-aged flavour.

Supplementary materials

Appendix A: Conference papers

Appendix A1: Oral presentation in the 64th International Congress of Meat Science and Technology, session 7: next generation technologies to assess carcase and meat quality. 12-17 Aug 2018, Melbourne, Australia.

Quality and consumer acceptability of in-bag dry- and wet-aged lamb

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Introduction

Dry-aged meat is a niche product favoured by meat purveyors for mostly local upscale restaurants and gourmet markets. The distinct flavour of dry-aged meat commands a higher price in the marketplace, yet very little dry-aged meat is exported from its country of origin. Lamb is consumed widely around the world, and New Zealand is the major producer and exporter of wet-aged lamb globally. Dry-ageing of beef has been well studied over the last decade (Li et al., 2013; Kim et al., 2016) with comparatively limited research carried out on lamb. There is an opportunity to develop commercial dry-aged lamb for high value export markets. The aim of the present study was to compare the quality and consumer acceptability of in bag, dry- and wet-aged lamb.

Materials and methods

A total of 30 pairs (n=60, left and right) of lamb legs (bone-in, chump on, shank-off) were collected from a local abattoir. Each leg was further portioned into 3 parts as shown in Figure A.1. The middle part, after cutting off the chump and about 4 cm from the shank side, was used for ageing. The left or right side of lamb was randomly assigned to two ageing treatments: (1) in-bag dry ageing (BD) using water-permeable ageing bag at 2 °C, 0.5 m/s air velocity and 75% humidity; (2) wet-ageing (W) at -1.5 °C for 21 d. Surfaces of BD and W aged samples were swabbed for microbial enumeration using a commercial analytical laboratory. Following ageing, samples were cut into 1.5 cm thick chops, vacuum packaged and stored at -1.5 °C for further analyses. pH values and sample weights pre- and post-ageing were recorded, and instrumental colour was measured using Minolta Colour Meter as described by Kim et al. (2016). Wet- and dry-aged samples were *sous vide* cooked at 72 °C for 1 h and their texture measured using Texture Profile Analysis (TPA) according to (Farouk et al., 2018). Cook loss was calculated as percent weight loss before and after cooking and combined with ageing weight loss to obtain total loss. Samples of chops for sensory analysis were *sous vide* cooked at 65°C for 75 min, then grilled for 60 s each side. Two slices per chop for wet and dry-aged samples were served on coded plates to a high income (≥ $70K) group of 114 consumer panellists, who were asked to express overall preference on a 9-point hedonic scale (1 = Dislike extremely to 9 = Like extremely), and eating quality rating on a 5-point hedonic scale (1 = Unsatisfactory as an everyday product to 5 = A premium product).

Data were analysed using one-way ANOVA and Tukey's honest significant difference was used to separate the means at $P < 0.05$.

Results and Discussion

In-bag dry-aged lamb had less total aerobic microbial count compared to the wet-aged equivalent ($P < 0.05$, Table A.1). The pH and % total loss of BD lamb was significantly higher than its W counterparts (Table A.1). W lamb had higher instrumental colour values compared to BD, except for hue angle (Table A.1). BD had harder and chewier texture than their W equivalents. There was no significant difference in the number of consumers who preferred BD or W, and dry- and wet-aged products were rated equally in their overall eating qualities. However, about 40% of the consumers preferred BD lamb over W, most likely due to its unique taste/flavour (average score difference = 1.91), while 45% of consumers liked W over BD, most likely due to its tenderness and more familiar flavour (average score difference = 1.90). Consumers scored the eating quality of their preferred aged lamb as slightly better than an everyday product up to a premium product, and rated their less preferred lambs 1.4 and 1.5 points lower than for those who preferred BD and W, respectively. Results suggest that in-bag dry-aged lamb could be produced as a value-added product targeted to a significant percentage of high-income earning consumers (around 40% in this study), encouraging commercial development for high value lamb export markets.

Table A.1. Quality attributes and consumer acceptability of in-bag dry- and wet-aged lamb.

Quality Parameter		W	BD	SED	P-values
pH		5.92	6.04	0.02	<0.001
Colour	L*	45.00	43.07	0.39	<0.001
	a*	15.26[a]	13.91[b]	0.32	<0.001
	b*	13.27[a]	11.95[b]	0.25	<0.001
	Chroma	20.23[a]	18.35[b]	0.39	<0.001
	Hue angle°	41.10	40.81	0.32	0.370
Total moisture loss (%)		27.5[a]	36.5[b]	1.03	<0.001
APC (log cfu/g)		5.16	2.68	0.75	<0.03
Texture profile analysis	Hardness (kg)	2.25[a]	2.64[b]	0.09	<0.001
	Chewiness (kg)	0.78[a]	0.91[b]	0.03	<0.001
	Springiness	0.62	0.62	0.01	0.451
	Adhesiveness (g/sec)	-9.72	-11.55	2.18	0.402
	Cohesiveness	0.55[a]	0.54[b]	0.01	0.002
	Resilience	0.22	0.21	0.01	0.071
Sensory analysis	Overall preference	6.75	6.68	0.19	0.681
	Eating quality rating	3.14	3.10	0.13	0.750

BD = in-bag dry-aged lamb, W = wet-aged lamb, SED = standard error of difference of means. APC = total aerobic microbial count

Figure A.1 Dry-ageing process: (a) Portion of the lamb leg dry or wet aged; (b) BD in dry-ageing chamber; (c) BD (left) and W (right) paired lamb legs after 21 d of ageing; (d) BD (left) and W (right) chops from a pair of lamb legs.

Conclusion

In-bag dry-aged lamb was strongly preferred by 40% of consumers over its wet-aged equivalent. Results confirm the niche nature of dry-aged meat and suggest that in bag dry-aged lamb can be targeted to high-income consumers and has potential as a value-added product for export.

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Appendix A2: Poster presentation in the 64th International Congress of Meat Science and Technology, session 7: next generation technologies to assess carcase and meat quality. 12-17 Aug 2018, Melbourne, Australia.

Stepwise in-bag dry-ageing of lean beef

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Introduction

Beef is commonly dry-aged out-of-bag in a refrigerated room for 21 to 28 days at 0-2°C and 60-85% relative humidity, with an air flow of 0.5-2.5 $m.s^{-1}$. Dry-ageing (D) enhances tenderization and flavour of beef (Warren et al., 1992) though a close monitoring and adjustment of processing parameters are required to minimize microbial growth and weight loss. Increasing the air velocity may accelerate the moisture loss from beef to speed up the dry-ageing process. However, the resultant quality of the dry-aged beef has not been explored fully. Recently, Li et al. (2013) attempted to use water-permeable bags for dry-ageing and has reported lower thawing and cooking losses than D with no negative effects on sensory or other quality attributes. Kim et al. (2017) combined dry-ageing with wet-ageing as a stepwise ageing process to produce equivalent and/or improved qualities in beef compared to using a single ageing method. The aim of this study was to determine the effect of stepwise in-bag dry-ageing (BD) on the quality and acceptability of lean beef for BD for one week at higher air velocities, followed by additional two weeks of wet ageing time compared to BD only for three weeks as a control.

Materials and Methods

A total of 30 striploins from 15 beef carcasses (bull; ≈ 2 yr olds) were randomly assigned to 4 different ageing regimes after 24hr post-mortem: (1). BD for 21 days at 0.5 m/s air velocity (BD21, Control, n = 6); (2) - (4). BD for 7 days at 0.5/1.5/2.5 m/s air velocity (n=8), respectively, followed by 14 days of wet ageing. All ageing was performed in an ageing chamber at 2 °C and relative humidity of 75% (Figure A.2). Weight loss, pH and lipid oxidation (Thiobarbituric Acid Reactive Substances; TBARS) (Jo et al., 1998) were measured. Cook loss was measured using 6 cm thick steaks cooked individually in a boiling water bath to an internal temperature of 70°C. Cooked meat samples with 10mm x 10mm cross section were analysed for shear force using MIRINZ tenderometer.

All measurements were taken in triplicate. An in-house consumer panel (n=45) determined the acceptability of the steaks. Steaks from the 4 treatments were cooked in an oven to internal temperature of 70°C and cut into 1.27 × 1.27 × 2.54cm pieces and placed in a closed plastic container labelled with a random 3-digit code. The samples were held at 50°C (less than 10min) before serving to the panellists. Panellists evaluated the aroma, texture, tenderness, juiciness, flavour, and overall liking of the cooked steaks on a scale of 0 to 100,

where 0 = dislike extremely, and 100 = like extremely. Data was analyzed using one-way ANOVA and Tukey's honest significant difference was used to separate the means at $P < 0.05$.

Results and Discussion

As shown in Table A.2, approximately 20% of moisture was lost from control BD bull beef striploin. Increase of air velocity increased the weight loss from samples that were BD for 7 days. Dry-ageing process significantly ($P < 0.05$) increased pH values and cook loss of samples from all treatment combinations. pH significantly ($P < 0.05$) decreased with the increase of air velocity of the ageing process. Total microbial counts (data not shown), cook loss, shear force and TBARS were not affected by air velocity and treatment combinations ($P > 0.05$), but they were affected by ageing time. With the increase of ageing time, shear force significantly decreased ($P < 0.05$) and cook loss significantly increased ($P < 0.05$) regardless of the ageing treatment.

Lipid oxidation (TBARS) increased with BD ageing time for samples dry-aged at higher air velocities. Due to the low lipid content of the sample (<1%), the level of lipid oxidation was overall low. For sensory, there was no difference ($P > 0.05$) found in all attributes between the four treatment combinations. Therefore, BD for 7 days followed by 14 days of wet-ageing could produce beef of similar sensorial quality to those BD for 21 days, regardless of air velocity.

Figure A.2 Photos taken from BD process: (a) striploins laid out in the controlled chamber; (b) Striploin BD for 7d; (c) Cut surface of the BD striploin (21d); (d) BD loin steak (21d); (e) Cooked BD steak (21d)

Conclusion

Increased air velocity during BD of bull beef striploin increased moisture loss, but had no effect on pH, cook loss, shear force, lipid oxidation (TBARs) and consumer acceptance of the beef. Current ageing strategies using stepwise ageing process could produce quality equivalent dry-aged lean beef to those BD only for same period of time, but with lower weight loss.

Table A.2 Effects of treatment combinations on the quality and consumer acceptance of in-bag dry-aged lean beef

	0.5 m/s (21d BD)	0.5 m/s (7d BD+14d W)	1.5 m/s (7d BD+14d W)	2.5 m/s (7d BD+14d W)	SED	*P*-treatment
Weight loss%						
21d	20.48ay	9.80b	10.92bc	11.54c	0.0071	<0.0001
P-ageing	<0.0001	0.2210	0.7064	0.7243		
pH						
0d	5.34x	5.34x	5.36x	5.32x	0.032	0.7949
21d	5.66ay	5.62aby	5.64ay	5.58by		0.0349
P-ageing	<0.0001	<0.0001	<0.0001	<0.0001		
Cook loss%						
0d	18.92%x	17.30%x	19.43%x	17.14%x	0.018	0.6615
21d	27.77%y	27.74%y	25.98%y	28.69%y		0.2035
P-ageing	0.0005	0.0001	0.0018	<0.0001		
Shear force (kgF)						
0d	13.46x	11.90x	13.44x	11.65x	1.27	0.5745
21d	7.41y	7.34y	6.96y	7.20y		0.9193
P-ageing	<0.0001	0.0069	<0.0001	0.0076		
TBARS						
0d	0.32	0.23	0.30x	0.31x	0.062	0.1887
21d	0.38	0.33	0.45y	0.42y		0.4750
P-ageing	0.4146	0.05579	0.0138	0.1658		
Consumer sensory 21d						
Aroma	56.53	55.58	55.78	56.6	2.81	0.9769
Texture	58.07	52.92	49.14	58.1	4.24	0.1131
Tenderness	53.62	48.73	45.84	55.01	4.88	0.2236
Juiciness	54.39	53.64	48.35	47.8	4.12	0.2439
Flavor	44.85	44.76	41.87	42.95	4.35	0.8813
Overall liking	59.77	58.2	52.88	57.17	3.33	0.2175

Different letter “a, b, c” within same row means results are significantly different from each other. Different letter “x, y, z” within same column means results are significantly different from each other.

Acknowledgements

This work was supported by Ministry of Science and Innovation, New Zealand, and from the internal Strategic Science Investment Fund of AgResearch Limited (contract A19113). The first author would like to acknowledge AUT University for his PhD scholarship.

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Appendix A3: Poster presentation in the 65th International Congress of Meat Science and Technology, P-11-04. 4-9 Aug 2019, Potsdam, Germany. In Proceeding of 65th International Congress of Meat Science and Technology, Potsdam, Germany.

Effect of step-wise dry-ageing and trimming on the metabolite profiles of dry-aged bull beef

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Introduction

Dry-ageing of beef has been extensively studied in the last decade, but the metabolite profile remains relatively unknown. Several amino acids (tryptophan, phenylalanine, valine, tyrosine, glutamate, isoleucine and leucine) have been reported to be more abundant in the dry-aged beef than the wet-aged (Kim, et al., 2016). Post-ageing trimming is a common practice to remove the dry and discoloured surface which results in a great loss. The combination of dry-ageing with wet-ageing as a step-wise dry-ageing process has been reported to produce equivalent and/or improved qualities in beef compared to dry-ageing alone (Kim, et al., 2017). Mass spectrometry-based metabolic fingerprinting using Rapid Evaporative Ionization Mass Spectrometry (REIMS) (Balog, et al., 2016) was carried out to investigate the difference in the metabolite profiles arising from the different ageing methods and trimming practices.

Materials and Methods

Bull beef striploins were dry-aged using water-permeable ageing bags (TUBLIN® 10, Denmark) at a chamber set at 2 ˚C, 0.5 $m.s^{-1}$ air velocity and 75% humidity. Ageing treatment 1 (T1, n=6) used a step-wise dry-ageing method with 7 d of dry-ageing, followed by 14 d of wet-ageing, and ageing treatment 2 (T2, n=11) used straight dry-ageing for 21 d. Approximately 5 mm of the dry surface was trimmed from both T1 and T2. Untrimmed samples from T2 (n=5) was used to determine the impact of trimming. Samples were taken from three different positions; silver skin, trim (1 cm of the surface) and the centre of the meat sample for metabolomic profiling by REIMS on Waters Xevo® G2 QToF (Waters Corp., UK). The meat surface was electrosurgically cut with an electronic surgical knife (depth 5 mm × length 3 cm, five replicates per sample and sampling position) with 15 W power setting. The aerosol produced from the samples was analysed in negative ion mode with the mass range of 50−1500 m/z. The accurate mass of detected ions was measured and used to tentatively identify the chemical identity of the ions. Identification, mass peak integration, and normalisation against total abundance were performed using Progenesis QI (Waters, UK) and differences between methods and sampling sites determined using Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) (SIMCA, Umetrics, Sweden). OPLS-DA models were considered to be of interest if the Q^2 score for

predictability was >0.2. The mass peak areas between treatments were compared using one-way ANOVA with t-test to separate the means at $P < 0.05$.

Results and Discussion

A clear separation was observed between T1 and T2 with Q*2* value of 0.852 suggesting that the current model was robust with good predictability towards data matrix (Fig A.3a). Out of 1704 metabolites that were detected and tentatively identified (mainly amide derivatives of amino acids and lipids, sulphur-containing compounds and monosaccharide/sugar alcohol), approximately 11%, were significantly ($P < 0.05$) more abundant in T1 than T2 (Table A.3). This may have resulted from the metabolism of lipids and proteins by bacteria or fungus (yeast) in T1 during wet-ageing process. About 29% of metabolites from glycerophospholipid metabolism and/or lipid peroxidation, proteolysis (glutamate) and tyrosine metabolism (tyramine), were significantly ($P < 0.05$) more abundant in T2 than T1.

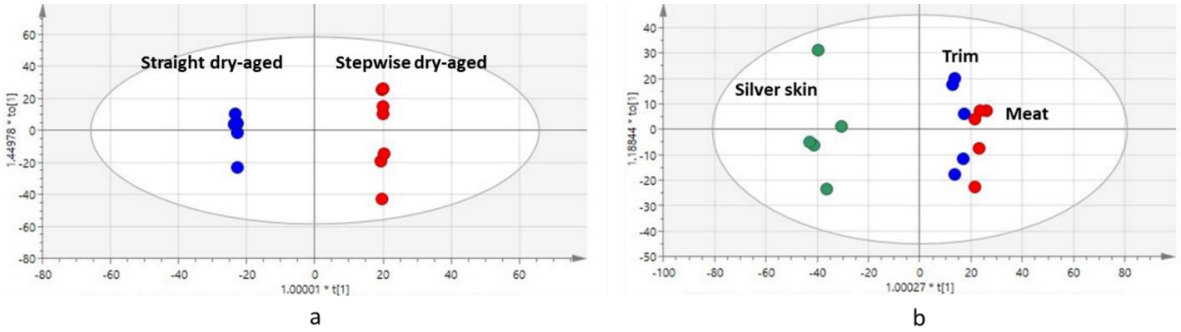

Figure A.3 OPLS-DA score plots: a. Straight dry-aged bull beef as compared to stepwise dry-aged counterparts; b. Three sampling positions on the centre of meat, trim and silver skin of straight dry-aged bull beef

Sampling position affected the metabolite profiles of dry-aged beef. Metabolites found from silver skin differed from that of the meat centre and the trim (Figure A.3b) with a Q2 value of 0.401 suggesting an acceptable prediction was made in current data matrix. Metabolites from proteolysis and amino acids metabolism differentiated the metabolite profiles of silver skin from the meat centre. Only 6% of metabolites were significantly different ($P < 0.05$) between the trim and the centre of meat. Meat centre was dominated by lipid metabolites. A higher level of beef flavour enhancing compounds (glutamate, glutamine and histidine) was found on the trim compared to the meat.

Table A.3 Putative metabolites abundant in dry-aged sample with significant changes between ageing treatments and sampling positions.

	Putative compounds	Fragments (m/z)	Adducts	Formula	Fold change
Stepwise dry-ageing *vs.* Straight dry-ageing					
Higher in stepwise dry-ageing (T1, p<0.01)	2-Methyl-1-methylthio-2-butene	137.0	M+Na-2H	$C_6H_{12}S$	3.1
	Glycinamide	221.1	3M-H	$C_2H_6N_2O$	4.0
	Arachidonoyl amine	340.2	M+K-2H	$C_{20}H_{33}NO$	1.5
	Hexoses/Inositol	201.0	M+Na-2H	$C_6H_{12}O_6$	5.4
Higher in straight dry-ageing (T2, control, p<0.05)	Phosphatidylcholine/ Phosphatidylethanolamine	764.5	M+FA-H	$C_{39}H_{78}NO_8P$	1.3
	Glycerophosphates	765.5	M+Na-2H	$C_{42}H_{81}O_8P$	1.4
	Glutamate	128.0	M-H_2O-H	$C_5H_9NO_4$	1.8
	Tyramine/m-tyramine	410.2	3M-H	$C_8H_{11}NO$	1.4
Silver skin *vs.* Meat					
Higher in silver skin (p<0.01)	Glutamate	128.0	M-H_2O-H	$C_5H_9NO_4$	3.8
	Hexanoylglycine	208.1	M+Cl	$C_8H_{15}NO_3$	5.2
	L-Glutamine	127.1	M-H_2O-H	$C_5H_{10}N_2O_3$	1.8
	L-Histidine/2-Oxoarginine	154.1	M-H/M-H_2O-H	$C_6H_9N_3O_2$/$C_6H_{11}N_3O_3$	2.0
Higher in meat (p<0.0001)	Cholesteryl ester/cholesterol ester	699.5	M+K-2H	$C_{45}H_{74}O_3$	3.6
	Ceramide phosphoethanolamines	683.5	M-H/M-H_2O-H	$C_{38}H_{73}N_2O_6P$/$C_{38}H_{75}N_2O_7P$	3.9
	Ceramide phosphoethanolamines	681.5	M-H_2O-H	$C_{38}H_{73}N_2O_7P$	5.5
	Glycerophosphates	685.5	M-H_2O-H/M-H	$C_{39}H_{77}O_8P$/$C_{39}H_{75}O_7P$	3.1

Metabolomic profiling in this study indicated that the step-wise dry-ageing may result in different metabolite profiles compared to dry-aging alone. Current metabolite profile of straight dry-aged beef may have resulted from metabolism of microorganisms and lipid oxidation. Further, trimming after dry-ageing may result in loss of several compounds that contribute to the flavour of dry-aged beef.

Acknowledgements

This work was supported by Ministry of Science and Innovation, New Zealand, and from the internal Strategic Science Investment Fund of AgResearch Limited (contract A19113). The first author would like to acknowledge AUT University for his PhD scholarship.

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Appendix A4: Poster presentation in the 65th International Congress of Meat Science and Technology, P-07-14. 4-9 Aug 2019, Potsdam, Germany.

Aroma profile of dry-aged lamb and bull beef using Real-Time Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)

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Introduction

Dry-ageing produces meat of superior eating quality and unique flavour (Warren, et al., 1992) compared to that produced from wet-ageing. The distinct flavour and aroma compounds generated from dry-ageing as a result of proteolysis, lipolysis, and oxidation remain relatively unexplored (O'Quinn, et al., 2016). Some ketones (Diacetyl and acetoin) and aldehydes (2-methylbutanal and 3-methylbutanal) have been reported to be positively associated with the intense dry-aged flavour, for example, buttery, nutty, browned/grilled and sweet (Calkins, et al., 2007). The aim of this study was to investigate the effect of ageing methods and animal species on the aroma profile of lamb and bull beef using real time Selected Ion Flow Tube Mass Spectrometry (SIFT-MS).

Materials and Methods

Three biological replicates of lamb (paired legs, bone-in, shank-off) and bull beef striploins were dry-aged in water-permeable ageing bags (TUBLIN® 10, Denmark) at 2 ˚C, 0.5 m/s air velocity and 75% humidity as compared to wet-aged lamb in vacuum barrier bags, for 21 d. The aged lamb (bone-out, untrimmed) and beef (trimmed) samples were minced and analysed for moisture and fat contents and lipid oxidation (Thiobarbituric Acid Reactive Substances; TBARS) using standard methods. The aged sample (5 g) was placed in a sealed reagent bottle and cooked at 75 °C for 30 min. Immediately post-cooking, the sample headspace was extracted into SIFT-MS Voice 200 Ultra (Syft Technologies, Christchurch, NZ) in a positive full scan mode with direct sample inlet. The sample flow rate was 0.3 tls with tube pressure of 0.66 Torr and temperature of 119 °C. The adducts with H_3O^+, NO^+, O_2^+ in the mass range of 15-400 m/z were measured. Identification and quantitation of odorants were performed by Labsyft software (Syft Technologies). Statistical analysis was performed using R (version 3.2.2). Identified odorants were analysed by Principal Component Analysis (PCA) with moisture, fat and TBARS to investigate the differences between ageing methods and animal species and the factors that contributed to the differences. Results of moisture, fat and TBARS were analysed using one-way ANOVA and Tukey's honest significant difference to separate the means at $P < 0.05$.

Results and Discussion

A significantly lower ($P < 0.05$) amount of moisture was detected in dry-aged lamb compared to the wet-aged (Table A.4). Both dry- and wet-aged lamb had significantly higher ($P < 0.05$) fat content compared to the dry-aged beef, with the highest TBARS (*P*

= 0.10) detected in the dry-aged lamb. A total of 38 odorants were identified, with some unique odorants presented in each sample (Table A.4). A clear separation of the three samples was observed by the score plot of PCA (Figure A.4a), suggesting that the difference in aroma profiles resulted from both the ageing methods and the animal species. PC1 explained 66.49% of the variation and clearly differentiated the beef samples from the lamb regardless of the ageing methods. PC2 explained 33.51% of the variation which separated the dry-aged lamb from the wet-aged lamb. In the loading plot (Figure A.4b), four odorants (2-methyl-2-butanol, 3-methyl pentane, methyl glyoxal, and pentanal) and higher fat content differentiated the lamb aroma from the beef which was dominated by hexanal, propanal and unique odorants in PC1. Increased TBARS level and the unique odorants (Table 1) may have contributed to the distinct aroma profile (e.g. nutty, sweet and buttery) of the dry-aged lamb in PC2. The aroma profile of wet-aged lamb may be associated with the higher moisture content, ethyl mercaptan, dimethyl sulfide and dimethyl amine. Dimethyl sulfide, reported to have bloody/metallic flavour of wet-aged meat (Calkins & Hodgen, 2007), was also detected.

Table A.4 Moisture, fat and TBARS and the unique odorants of dry- and wet-aged bull beef and lamb samples.

	Wet-aged lamb	Dry-aged lamb	Dry-aged beef	SED	P-values
% Moisture	73.15	67.98	74.68	0.99	0.001
% Fat	5.64	6.20	0.71	0.70	<0.001
TBARS (mg MDA/kg meat)	0.30	1.44	0.38	0.48	0.102
Unique odorants	1-methylpyrrolidine allyl methyl sulfide carbon disulfide formic acid ammonia trimethylamine	2-methyl-2-propanol 3-methylbutanal	acrolein carbonyl sulphide ethanol octanoic acid methyl mercaptan diethyl amine ethanolamine 2-methyl-1-butanamine 2-methyl naphthalene		

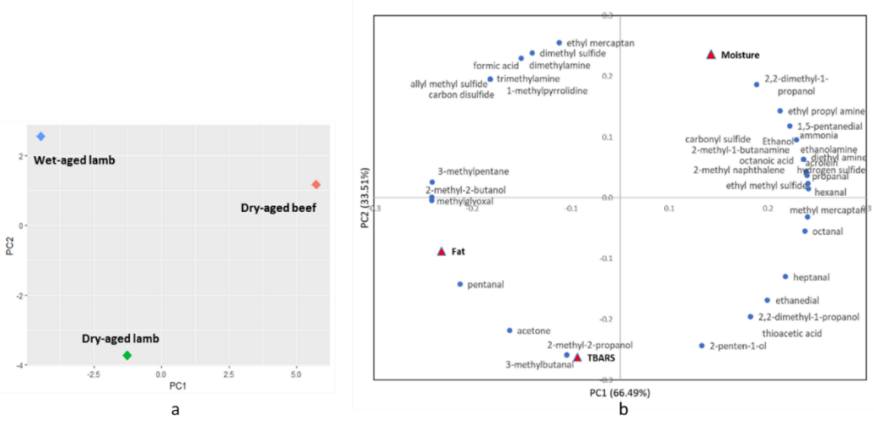

Figure A.4 Score plot (a) and loading plot (b) of Principle Component Analysis of odorants, moisture, fat and TBARS of dry- and wet aged lamb and bull beef.

A clear differentiation between the aroma profiles of dry- and wet-aged lamb and bull beef was detected by real-time SIFT-MS. The amount of fat and lipid oxidation were the major contributors to the distinct aroma profile of lamb and dry-aged lamb, respectively.

Acknowledgements

This work was supported by Ministry of Science and Innovation, New Zealand, and from the internal Strategic Science Investment Fund of AgResearch Limited (contract A19113). The first author would like to acknowledge AUT University for his PhD scholarship.

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Appendix A5: Poster presentation in the 65th International Congress of Meat Science and Technology, P-11-02. 4-9 Aug 2019, Potsdam, Germany.

Peptidomic comparison of dry aged vs a novel stepwise aged lean bull beef

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Introduction

Dry-ageing (D) is a traditional ageing process for the tenderisation and preservation of meat. The advent of vacuum packaging and efficiency of transportation has resulted in a decline in the use of D. However, recently there has been a resurgence of this technique due to the unique and concentrated flavours generated from the D process which are favoured by discerning consumers. In general, both wet-ageing (W) and DA can produce intense aged flavours and tenderise the meat (Warren et al., 1992). During WA, meat is vacuum sealed in packages and stored in a refrigerated environment whereas during DA, the meat is aged using controlled temperature, relative humidity and air flow in a refrigerated environment but without the use of packaging. Although D has been extensively studied over the last few decades, the unique flavours of dry-aged meat and the precursors contributing to the "dry-aged flavours" have not been well explored. The aim of this study was to compare the peptidomic profiles of meat that had undergone a novel D (D for 7 days followed by WA for 14 days) vs traditional D (D for 21 days) process to determine any impact on product quality.

Methods

Sample preparation: Lean bull beef striploins (pH = 5.3) were D in water permeable ageing bags (TUBLIN® 10, Denmark) for 21 d at 2 ˚C, 0.5 $m.s^{-1}$ air speed and 75% humidity and compared with stepwise aged samples (D 7 d + W 14 d). The unaged samples served as controls.

LC-MS/MS: Peptides were extracted from beef samples using 5% acetonitrile. The extracts were ultrafiltered using a 10 kDa cut-off filter and the peptides analysed using a nanoflow LC-MS directly interfaced to a maXis impact HD Q-TOF mass spectrometer (Bruker). Runs with MS/MS data acquired using automated data-dependent acquisition in CID mode were included for peptide identification.

Identification: Fragmented compounds data were imported into PEAKS Studio 8.5 (Ma et al., 2003) and interrogated without any enzyme specificity against the *Bos taurus* taxonomy of the UniProt database. A semi-quantitative approach using spectral counting of peptides was used to determine the differences between the two ageing treatments and compared against an unaged control sample. Two biological replicates were used per sample and a one-sample t-test was performed to establish statistical significance using R (version 3.2.2).

Results

Peptide analysis: Spectral counting for semi-quantitation of proteins was used to evaluate differences in peptide numbers derived from each protein from the aged beef samples in comparison to unaged controls. The rationale was firstly to identify differences in the peptide profiles that may have arisen due to the different conditions used for ageing the samples and to determine if the stepwise ageing procedure was comparable to the D only. The higher spectral counts of the aged samples compared to the unaged control (Figure A.5) reflects enhanced proteolytic cleavage of meat proteins during the ageing processes. Both ageing procedures displayed very similar degrees of hydrolysis of a range of proteins, indicating comparable proteolytic activity (Figure A.5).

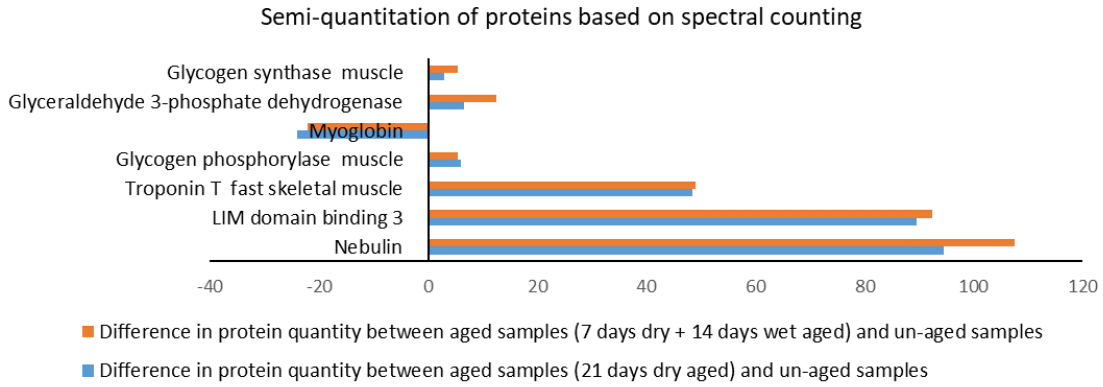

Figure A.5 Semi-quantitation of proteins, based on spectral counting, between two different aged beef samples and un-aged control samples

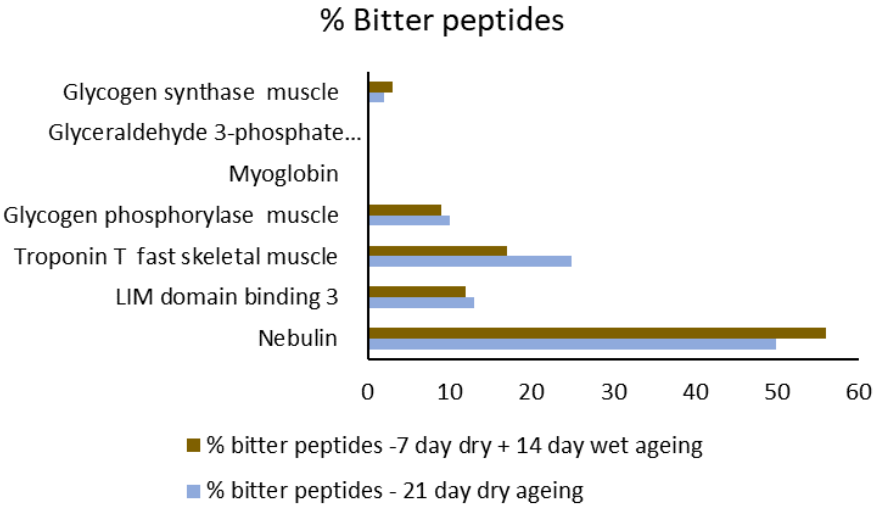

Figure A.6 Percent bitter peptides released from the proteins that differ significantly between the aged and the unaged samples

Taste analysis: As a proxy for sensory testing of taste properties, the bitterness index based on average hydrophobicity or Q value (Murray et al., 2013) of peptides from proteins with significant differences between the aged and unaged samples, was calculated. Overall only 5% of peptides from the D only and 6.5% from the stepwise aged samples had Q values > 1400 cal/residue, indicating bitterness. Peptides from nebulin contributed most towards the bitterness index, followed by troponin T (Figure A.6).

Conclusion

The peptidomic results strongly indicate that a reduction in the D time from 21 d to 7 d followed by a W period for 14 d can produce comparable peptide profiles, possibly indicating similar product qualities and greater economic viability for the stepwise ageing technique.

Acknowledgement

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Appendix B: AUTEC approvals for sensory studies

Appendix B1: AUTEC approval for dry-aged beef loins

AUTEC Secretariat

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17 July 2017

Michelle Yoo
Faculty of Health and Environmental Sciences

Dear Michelle

Re Ethics Application: **17/78 Consumer sensory evaluation of dry-aged beef loins**

Thank you for providing evidence as requested, which satisfies the points raised by a subcommittee of the Auckland University of Technology Ethics Committee (AUTEC).

Your ethics application has been approved for three years until 17 July 2020.

Standard Conditions of Approval

1. A progress report is due annually on the anniversary of the approval date, using form EA2, which is available online through http://www.aut.ac.nz/researchethics.
2. A final report is due at the expiration of the approval period, or, upon completion of project, using form EA3, which is available online through http://www.aut.ac.nz/researchethics.
3. Any amendments to the project must be approved by AUTEC prior to being implemented. Amendments can be requested using the EA2 form: http://www.aut.ac.nz/researchethics.
4. Any serious or unexpected adverse events must be reported to AUTEC Secretariat as a matter of priority.
5. Any unforeseen events that might affect continued ethical acceptability of the project should also be reported to the AUTEC Secretariat as a matter of priority.

Non-Standard Conditions of Approval

Please quote the application number and title on all future correspondence related to this project.

AUTEC grants ethical approval only. If you require management approval for access for your research from another institution or organisation then you are responsible for obtaining it. You are reminded that it is your responsibility to ensure that the spelling and grammar of documents being provided to participants or external organisations is of a high standard.

For any enquiries, please contact ethics@aut.ac.nz

Yours sincerely,

Kate O'Connor
Executive Manager
Auckland University of Technology Ethics Committee

Cc: zhangrenyu01@126.com

Appendix B2: AUTEC approval of amendments to include lamb samples

Auckland University of Technology Ethics Committee (AUTEC)

Auckland University of Technology
D-88, Private Bag 92006, Auckland 1142, NZ
T: +64 9 921 9999 ext. 8316
E: ethics@aut.ac.nz
www.aut.ac.nz/researchethics

14 December 2018

Michelle Yoo
Faculty of Health and Environmental Sciences

Dear Michelle

Re: Ethics Application: **17/78 Consumer sensory evaluation of dry-aged beef loins**

Thank you for your request for approval of amendments to your ethics application.

The amendment to the data collection protocols (lamb testing) is approved.

I remind you of the **Standard Conditions of Approval**.

1. A progress report is due annually on the anniversary of the approval date, using form EA2, which is available online through http://www.aut.ac.nz/research/researchethics.
2. A final report is due at the expiration of the approval period, or, upon completion of project, using form EA3, which is available online through http://www.aut.ac.nz/research/researchethics.
3. Any amendments to the project must be approved by AUTEC prior to being implemented. Amendments can be requested using the EA2 form: http://www.aut.ac.nz/research/researchethics.
4. Any serious or unexpected adverse events must be reported to AUTEC Secretariat as a matter of priority.
5. Any unforeseen events that might affect continued ethical acceptability of the project should also be reported to the AUTEC Secretariat as a matter of priority.

Please quote the application number and title on all future correspondence related to this project.

AUTEC grants ethical approval only. If you require management approval for access for your research from another institution or organisation then you are responsible for obtaining it. If the research is undertaken outside New Zealand, you need to meet all locality legal and ethical obligations and requirements.

For any enquiries please contact ethics@aut.ac.nz

Yours sincerely,

Kate O'Connor
Executive Manager
Auckland University of Technology Ethics Committee

Cc: zhangrenyu01@126.com

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