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Ollscoil na hÉireann, Corcaigh
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**Investigation of the effects of emerging technologies
on the kinetics of glycolytic and proteolytic enzymes
relevant for meat quality**

Thesis presented by

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For the degree of

Doctor of Philosophy – Food Science and Technology

Under the supervision of

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Table of Contents	i
Acknowledgments.....	v
Declaration	vii
Abstract	viii
Abbreviations	xii
List of Figures	xv
List of Tables.....	xix
List of Publications emerging from this thesis	xx
List of Presentations in International Conferences.....	xxi
Schematic representation of thesis structure	1
Chapter 1. Factors affecting the endogenous enzymatic systems in early post-mortem muscle relevant for final meat tenderness.....	2
1.1 Introduction.....	3
1.2 Glycolysis	4
1.2.1 Glycolytic potential and glycolytic capacity	5
1.2.2 Glycolytic enzymes: regulation and pH effect	7
1.2.3 Other factors affecting post-mortem glycolytic kinetics	15
1.3 Proteolysis	16
1.3.1 Main enzymes involved in proteolysis	17
1.4 Protein post-translational modifications of endogenous enzymes in post-mortem muscle	36
1.4.1 Phosphorylation	36
1.4.2 S – Nitrosylation	37
1.5 Future work and conclusions	40
Chapter 2. Carcass interventions and their ability to impact endogenous enzymatic systems and quality of meat.....	41
2.1 Introduction.....	42
2.2 Traditional Carcass Interventions/Handling Procedures	45
2.2.1 Hanging, hot boning and aging method	45
2.2.2 Electrical Stimulation (ES)	48
2.3 Innovative Technologies.....	51

2.3.1	Pulse electric fields (PEF)	52
2.3.2	Ultrasound (US)	60
2.3.3	High Pressure Processing (HPP)	69
2.4	Conclusion	75
Chapter 3. Assessing the impact of ultrasound on the rate and extent of early post-mortem glycolysis in bovine <i>Longissimus thoracis et lumborum</i>		
3.1	Introduction.....	78
3.2	Materials and methods	79
3.2.1	Chemicals and reagents	79
3.2.2	Muscle preparation and treatment	79
3.2.3	Ultrasound treatment	80
3.2.4	<i>In vitro</i> buffer system	81
3.2.5	Analysis of metabolites in muscle prior to ultrasound treatment and the <i>in vitro</i> glycolytic buffer/muscle homogenate model system during incubation at 25 °C for 1440 min	82
3.2.6	Statistical analysis	83
3.3	Results and discussion.....	83
3.3.1	Biochemical status of muscle pre-ultrasound treatment.....	83
3.3.2	pH decline in muscle and <i>in vitro</i> buffer system model systems.....	84
3.3.3	Metabolites in the <i>in vitro</i> system	87
3.4	Conclusion	92
Chapter 4. The impact of ultrasound treatment on glycolytic enzymes when applied to crude extracts from early post-mortem bovine muscle.....		
4.1	Introduction.....	94
4.2	Materials and methods	95
4.2.1	Chemicals and reagents	95
4.2.2	Animal and sample preparation.....	96
4.2.3	Preparation of supernatants for determination of initial metabolite concentrations in muscles	96
4.2.4	Ultrasound treatment	97
4.2.5	<i>In vitro</i> glycolytic buffer model system.....	98
4.2.6	Preparation of supernatants for metabolite analysis of the <i>in vitro</i> glycolytic buffer model system.....	99

4.2.7	Determination of concentration of metabolites in supernatants	99
4.2.8	Statistical analysis	99
4.3	Results	100
4.3.1	Biochemical status of muscle prior to ultrasound treatment.....	100
4.3.2	pH decline.....	100
4.3.3	Concentration of metabolites in the <i>in vitro</i> glycolytic buffer model system	102
4.4	Discussion	105
4.5	Conclusion	108
Chapter 5. Evaluating high pressure processing as a strategy to accelerate water loss and its effect on eating quality in dry aged beef: A preliminary study.		110
5.1	Introduction.....	111
5.2	Materials and Methods	112
5.2.1	Chemicals and reagents	112
5.2.2	Animal and sample preparation.....	112
5.2.3	High pressure processing and dry aging	113
5.2.4	pH and proximate analysis.....	114
5.2.5	Aging loss.....	115
5.2.6	Drip loss.....	115
5.2.7	Cook loss and Warner-Bratzler shear force	115
5.2.8	Colour measurements.....	116
5.2.9	Lipid oxidation	117
5.2.10	Myofibrillar fragmentation index.....	117
5.2.11	Calpain activity	118
5.2.12	Statistical analysis	119
5.3	Results and Discussion	119
5.3.1	pH	119
5.3.2	Weight loss and proximate composition	120
5.3.3	Drip loss and cook loss	123
5.3.4	Warner-Bratzler Shear Force (WBSF) values	124
5.3.5	Bloomed colour	126
5.3.6	Lipid oxidation	130

5.3.7	Myofibrillar fragmentation index (MFI)	131
5.3.8	Calpain activity	133
5.4	Conclusion	134
Chapter 6. Evaluating the impact of a high pressure pre-treatment on dry aged bovine <i>Longissimus thoracis et lumborum</i> using SDS-PAGE and untargeted LC-MS metabolomics.....		135
6.1	Introduction.....	136
6.2	Materials and Methods	138
6.2.1	Chemicals and reagents	138
6.2.2	Animal and sample preparation.....	138
6.2.3	High pressure processing and dry aging	138
6.2.4	One dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).....	139
6.2.5	Untargeted metabolomics	140
6.2.6	Data analysis	140
6.3	Results and discussion	142
6.3.1	SDS-PAGE profile	142
6.3.2	Untargeted metabolomics	143
6.4	Conclusion	157
Chapter 7. General Discussion and Conclusions.....		158
7.1	General Discussion	158
7.2	Conclusion and future investigations.....	169
References.....		172
Appendix 1 – RStudio Code for Section 6.2.6		203

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Declaration

I, Mary Ann Kent, hereby declare that this submitted thesis is my work and effort and has not been submitted for another degree at either the National University Ireland, Cork, or elsewhere. All the external references and other sources of information have been used, and they have been acknowledged. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

A handwritten signature in black ink that reads "Mary Ann Kent". The script is cursive and fluid, with the first letters of each name being capitalized and prominent.

Mary Ann Kent

Abstract

Tenderness, along with flavour and juiciness, are important factors that influence the overall consumer satisfaction of meat. These quality parameters are influenced by two key biochemical processes related to the conversion of muscle to meat: anaerobic glycolysis and proteolysis. Glycolysis involves a series of metabolic reactions, where glycogen is broken down to glucose and subsequently converted to lactic acid, which is concomitant with a decline in pH post-mortem. Glycolysis is regulated by three key rate limiting enzymes: glycogen phosphorylase, phosphofructokinase and pyruvate kinase. Proteolysis involves the degradation of key structural muscle proteins such as desmin and titin, which contributes to the tenderisation of muscle. Enzymes involved in proteolysis include calpains and cathepsins. Carcass interventions offer the potential to modulate these biochemical processes that are key to determining meat quality. Several interventions are currently used by the meat industry, including aging (wet and dry), hanging and hot boning. Electrical stimulation is another well established carcass intervention that has been applied to improve meat quality through its ability to accelerate the rate of glycolysis and modify proteolysis, post-mortem. Innovative technologies such as ultrasound and high pressure processing have also been investigated to determine their effects on meat quality, with varying results and further studies in this area are warranted. Therefore, the objectives of the research were to investigate the impact of innovative technologies on post-mortem biochemical processes in bovine *Longissimus thoracis et lumborum*. It examined the impact of ultrasound on glycolytic enzyme activity in pre-rigor muscle, *in vivo* and *in vitro*, as well as in muscle homogenates (prepared from pre-rigor muscle). Additionally, it evaluated the effects of a high pressure pre-treatment on weight loss, eating quality (Warner-Bratzler shear force values, colour, lipid oxidation), proteolysis (MFI, SDS-PAGE, calpain activity), and metabolite profiles during dry aging.

Ultrasound was applied to pre-rigor (2 to 4 h post-mortem) bovine *Longissimus thoracis et lumborum*, using varying treatment parameters such as treatment duration and frequency (intact steaks in Chapter 3: 25/45 kHz, 15/30/45 min; and homogenates in Chapter 4: 60/100% amp, 15/30 min). The impact of ultrasound on

glycolytic enzyme activity was determined within the muscle and *in vitro* using a glycolytic buffer system, which allows determination of the inherent enzyme activity by monitoring changes in pH and the concentration of key metabolites, namely, glycogen, reducing sugars and lactic acid. When the effect of ultrasound (25/45 kHz, 15/30/45 min) was investigated on the rate of pH decline in pre-rigor bovine *Longissimus thoracis et lumborum*, it was found that ultrasound frequency (25/45 kHz) did not have a significant ($P > 0.05$) effect on the rate of pH. However, treatment duration (15/30/45 min) had a significant ($P < 0.05$) effect on the rate of pH decline, in particular the 30 min treatment where the pH decline in muscle was accelerated. However, no significant ($P > 0.05$) effect on the ultimate pH was observed between the treatments at 24 h post-treatment. Using the *in vitro* glycolytic buffer system, it was found that ultrasound frequency and treatment duration did not have a significant effect ($P > 0.05$) on the inherent glycolytic enzyme activity, measured via changes in pH and content of glycogen, reducing sugars and lactic acid over a 24 h incubation period. It was postulated that the change in pH decline observed in muscle due to the 30 min treatment was potentially due to environmental factors within the muscle. Overall, it was hypothesised that the lack of effect on glycolysis was due to the ultrasound treatments applied not being strong enough to affect enzymatic activity, or that the matrix of the muscle provided protection to the glycolytic enzymes. To examine this further in Chapter 4, the matrix of pre-rigor bovine *Longissimus thoracis et lumborum* was disrupted by powdering the muscle using liquid nitrogen, from which crude extracts were prepared for ultrasound treatment (60/100% amp (11/19 W/cm²), for 15/30 min). When analysed using the same *in vitro* glycolytic buffer system, it was found that the degradation of glycogen was not significantly ($P > 0.05$) affected by ultrasound treatment. However, the rate of pH decline and changes in the content of reducing sugars and lactic acid over time were significantly ($P < 0.05$) affected by the 100% amp, 30 min treatment. Following this treatment, there was an accumulation of reducing sugars and delayed production of lactic acid and decline in pH within the first 4 h of incubation compared to the other treatments. However, following 24 h of incubation, no significant ($P > 0.05$) differences in pH and the content of reducing sugars and lactic acid were observed between treatments or between treatments and controls. From the results of

Chapter 3 and 4, it was concluded that ultrasound had limited potential to modify enzymatic activity within pre-rigor bovine muscle.

High pressure processing (200 MPa, 20 min, 8 °C) was explored as a pre-treatment to dry aging bovine *Longissimus thoracis et lumborum* (2 °C, 80% relative humidity, air flow 0.5-2.0 m/s) for up to 28 days. In Chapter 5, the effects of high pressure pre-treatment on weight loss, quality parameters (shear force values, colour, lipid oxidation) and aspects of proteolysis (calpain activity, myofibrillar fragmentation index (MFI)) were investigated, while Chapter 6 explored the impact of the same treatment on the protein (SDS-PAGE) and metabolomic profile (UPLC-Q-TOF/MS). High pressure pre-treatment significantly ($P < 0.05$) increased weight loss over the 28 day dry aging period, allowing for the potential concentration of flavour related compounds. When compared to the control, it was found that high pressure significantly ($P < 0.05$) affected quality parameters such as Warner-Bratzler shear force values, lipid oxidation and colour, in particular lightness (L^*). Lightness (L^*) was significantly ($P < 0.05$) higher at all time points examined, when compared to the control. However, differences in colour between control and high pressure pre-treatment could be minimised following cooking. Warner-Bratzler shear force values of the HPP pre-treated samples were significantly ($P < 0.05$) higher than the control for the first 21 days of dry aging, however, following 28 days of dry aging, no significant ($P > 0.05$) differences were present between control and treated samples. High pressure significantly ($P < 0.05$) increased lipid oxidation, although the TBARS values were within the consumer's limit of acceptance. HPP pre-treatment did not significantly ($P > 0.05$) affect drip and cook loss. When its impact on aspects of proteolysis was investigated, high pressure pre-treatment was found to significantly ($P < 0.05$) decrease calpain activity, however, myofibrillar fragmentation index significantly ($P < 0.05$) increased with high pressure pre-treatment and over dry aging time. When examined further using SDS-PAGE, high pressure pre-treatment led to a significant ($P < 0.05$) reduction in the relative density (%) of myosin heavy chain (MHC) over time, with a corresponding increase ($P < 0.05$) in the 160 kDa band, identified in the literature as a degradation product of MHC; indicating that the high pressure pre-treatment altered the degradation profile of muscle over time.

Metabolomics was used as a tool to examine the effects that the high pressure pre-treatment and dry aging over time have on the metabolomic profile of bovine muscle. When metabolic profiles were examined using PCA and PLS-DA analysis, no differences between control and HPP pre-treated muscle were observed immediately post-treatment; however differences were evident following 14, 21 and 28 days post-treatment. On the other hand, OPLS-DA was able to distinguish between all age time points within and between treatments. High pressure pre-treatment altered the metabolomic profile of bovine muscle at all aging time points (0, 14, 21 and 28 days post-treatment), resulting in an increase in the abundance of peptides and phospholipids, while dry aging time resulted in the increase in peptide content and altered nucleotide concentrations. Metabolites such as peptides, phospholipids and nucleotides may act as precursors to flavour compounds. Hence, alterations to the weight loss, lipid oxidation and metabolomic profile following high pressure pre-treatment have the potential to alter the flavour of dry aged beef.

In conclusion, ultrasound, under the experimental conditions used in this research, appears to have limited potential for altering the rate of glycolysis of pre-rigor bovine *Longissimus thoracis et lumborum*. However, the results indicate that high pressure processing pre-treatment can accelerate moisture loss of dry aged muscle, increase lipid oxidation and alter the metabolomic profile of bovine muscle, without having a detrimental effect on the shear force values following 28 days of aging, potentially enhancing the flavour of dry aged beef. Further detailed investigations are warranted to determine the impact of HPP pre-treatment on the flavour of dry aged bovine meat and potential consumer preferences.

Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C	Control
DA	Dry aging
DAHPP	Dry aging with high pressure processing
DFD	Dark, firm, dry
DNS	Dinitrosalicylic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ES	Electrical stimulation
FC	Fold change
GC	Gas chromatography
GP	Glycogen phosphorylase
GSNO	S – nitroglutathione
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPP	High pressure processing
HSP	Heat shock proteins
HVES	High voltage electrical stimulation
ICoMST	International Congress of Meat Science and Technology
IFSTI	Institute of Food Science and Technology of Ireland
IMP	Inosine monophosphate
INIA	National Agricultural Research Institute of Uruguay
KOH	Potassium hydroxide
L – NAME	N ω – nitro – L – arginine methyl ester hydrochloride
LC	Liquid chromatography

LTL	Longissimus thoracis et lumborum
LVES	Low voltage electrical stimulation
MDA	Malondialdehyde
MFI	Myofibrillar fragmentation index
MHC	Myosin heavy chain
MLC	Myosin light chain
MS	Mass spectrometry
N	Nitrogen
NAD ⁺	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principal component analysis
PCA	Perchloric acid
PEF	Pulsed electric fields
PFK	Phosphofructokinase (-1)
PFK - 2	Phosphofructokinase – 2
pHu	Ultimate pH
PK	Pyruvate kinase
PLS-DA	Partial least squares discriminant analysis
PSE	Pale, soft, exudative
PTFE	Polytetrafluoroethylene
Q-TOF	Quadrupole time - of - flight
QC	Quality control
RFN	Red, firm and non - exudative
RFU	Relative fluorescence units
rpm	Rotations per min
SAS	Statistical analysis system
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error mean
sHSP	Small heat shock proteins

TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
UPLC	Ultra - high performance liquid chromatography
US	Ultrasound
UV	Ultraviolet
VIP	Variable importance in projection
WBSF	Warner-Bratzler Shear Force

List of Figures

Figure 1-1. A schematic of the anaerobic glycolytic pathway, where glucose, from the breakdown of glycogen, is converted into lactic acid. Key rate limiting enzymes are highlighted.	5
Figure 1-2. pH – temperature relationship illustrating the windows for hot and cold shortening, adapted from Gutzke et al. (2014).	16
Figure 1-3. The myofibrillar structure of muscle with the main contractile and cytoskeletal fibres labelled, adapted from Kemp et al. (2010).	17
Figure 2-1. Different methods of hanging carcasses post-slaughter via A) the Achilles tendon and B) pelvic suspension, adapted from Ahnström (2008).	45
Figure 2-2. Schematic of the application of electrical stimulation to bovine carcasses, (A) via low voltage electrical stimulation (LVES), and (B) high voltage electrical stimulation (HVES), adapted from Polidori and Vincenzetti (2017) and AMPC (2015).	49
Figure 2-3. Effect of PEF on the structure of cell membranes, showing the A) intact cell membrane, B) compression during PEF, C) reversible and D) irreversible breakdown of the cell structure following treatment, adapted from Gómez et al. (2019).	53
Figure 2-4. The compression and rarefaction of microbubbles, with eventual implosion as a result of US treatment, adapted from Astráin-Redín et al. (2021). ...	61
Figure 2-5. A schematic of applying HPP in a pressurised vessel with water as the medium for transmitting pressure to the product, adapted from Chacha et al. (2021).	70
Figure 3-1. The setup for ultrasound treatment using the water bath system.	81
Figure 3-2. The average decline in pH over the course of 1440 min in the <i>in vitro</i> glycolytic buffer. N = 4. Data is mean $\pm$ SEM.	87
Figure 3-3. The average degradation of glycogen (mM) over the course of 1440 min in the <i>in vitro</i> glycolytic buffer. N = 4. Data is mean $\pm$ SEM.	88
Figure 3-4. The average content of reducing sugars over the course of 1440 min in the <i>in vitro</i> glycolytic buffer. N = 4. Data is mean $\pm$ SEM.	89

Figure 3-5. Reducing sugar content (mM) plotted against pH of the buffer, which appear to be exponentially related to each other. All samples points plotted. N = 4.	90
Figure 3-6. The average production of lactic acid (mM) over the course of 1440 min in the <i>in vitro</i> glycolytic buffer. N = 4. Data is mean $\pm$ SEM.	91
Figure 4-1. The setup for ultrasound treatment using the probe system.	98
Figure 4-2. pH decline of the <i>in vitro</i> glycolytic reaction buffer over 1440 min (24 h) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60% amp for 15 min (US60 15), 60% amp for 30 min (US60 30), 100% amp for 15 min (US100 15), 100% amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SEM.	101
Figure 4-3. Glycogen concentration (mM) of the <i>in vitro</i> glycolytic reaction buffer over 1440 min (24 h) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60% amp for 15 min (US60 15), 60% amp for 30 min (US60 30), 100% amp for 15 min (US100 15), 100% amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SEM.	103
Figure 4-4. Glucose (and other reducing sugars) concentration (mM) of the <i>in vitro</i> glycolytic reaction buffer over 1440 min (24 h) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60% amp for 15 min (US60 15), 60% amp for 30 min (US60 30), 100% amp for 15 min (US100 15), 100% amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SEM.	104
Figure 4-5. Lactic acid concentration (mM) of the <i>in vitro</i> glycolytic reaction buffer over 1440 min (24 h) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60% amp for 15 min (US60 15), 60% amp for 30 min (US60 30), 100% amp for 15 min (US100 15), 100% amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SEM.	105
Figure 4-6. Glucose (and other reducing sugars) content (mM) plotted against pH of the buffer. All samples points plotted. N = 4. The grey • correspond to the points from buffer systems containing muscle treated with 100% amp US for 30 min for the first 240 min (where abnormal pH decline took place), while the black •	

corresponds to the buffers following a normal pH decline. The blue and green dotted lines are the respective lines of best fit.	107
Figure 5-1. The sampling plan for sections following dry aging for 0, 14, 21 and 28 days.	114
Figure 5-2. . Effect of aging time and HPP on the weight loss (%) of dry aged bovine LTL over a 28 day dry aging period. Results are mean values of four biological replicates.	121
Figure 5-3. The effect of aging time and HPP on the A) drip loss (%) and B) cook loss (%) of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.	124
Figure 5-4. The effect of aging time and HPP on the WBSF values of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.	125
Figure 5-5. The effect of aging time and HPP on the TBARS values (mg MDA/kg) of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.	131
Figure 5-6. The effect of aging time and HPP on the MFI values of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.	132
Figure 5-7. The effect of aging time and HPP on calpain activity of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.	133
Figure 6-1. A representative 1D SDS-PAGE (4-20% gel) profile of bovine <i>Longissimus thoracis et lumborum</i> following dry aging (DA) and dry aging with high pressure processing (DAHPP) at four different aging times (D0, D14, D21, D28).	142
Figure 6-2. PCA score plot of treatment/time combinations for A) positive and B) negative ionisation mode.	143
Figure 6-3. PLS-DA graph of A) all treatment/ time combinations and B) treatment groups of positive ionisation mode data.	145
Figure 6-4. PLS-DA graph of A) all treatment/ time combinations and B) treatment groups of negative ionisation mode data	145
Figure 6-5. OPLS-DA model distinguishing D0 and D28 for A) DA and B) DAHPP treatments and C) overall effect of DA and DAHPP from the positive ionisation mode	147

Figure 6-6. OPLS-DA model distinguishing D0 and D28 for A) DA and B) DAHPP treatments and C) overall effect of DA and DAHPP from the negative ionisation mode	148
Figure 6-7. Volcano plots illustrating the effect of aging and DAHPP on metabolites. A) aging with DA, B) aging with DAHPP, C) overall effect of DAHPP compared to DA, and D) DAHPP v DA at individual times points. Data from the positive ionisation mode.	150
Figure 6-8. Volcano plots illustrating the effect of aging and DAHPP on metabolites A) aging with DA, B) aging with DAHPP samples, C) overall effect of DAHPP compared to DA, D) DAHPP v DA at individual time points. Data from the negative ionisation mode.	151
Figure 6-9. Top 15 identifiable significant differential metabolites ($VIP > 1$, $P < 0.05$) between DA – D0 and DA – D28, A) positive and B) negative ionisation modes. ...	152
Figure 6-10. Top 15 identifiable significant differential metabolites ($VIP > 1$, $P < 0.05$) between HPP – D0 and HPP – D28, A) positive and B) negative ionisation modes.....	153
Figure 6-11. Top 15 identifiable significant differential metabolites ($VIP > 1$, $P < 0.05$) between DA and DAHPP treatments, A) positive and B) negative ionisation modes.....	154

List of Tables

Table 1-1. The effect of pH and temperature on the activity of key glycolytic enzymes in muscle	13
Table 1-2. The effect of pH and temperature on the activity of key proteolytic enzymes in muscle	32
Table 2-1. Traditional and innovative post-mortem interventions applied on muscle, categorised based on their mechanism of action.....	44
Table 3-1. Average pH values over the course of 1440 min post-ultrasound treatment when measured in the muscle. N = 4. Data is mean $\pm$ SEM. The <i>P</i> – values from the F – Test, following a three way analysis of variance (ANOVA) are included.	85
Table 5-1. The effect of aging time and HPP on the pH of dry aged bovine LTL. Results are the mean values of four biological replicates $\pm$ SEM.....	120
Table 5-2. The effect of aging time and HPP on the proximate composition of dry aged bovine LTL.	122
Table 5-3. Effect of aging time and high pressure processing on <i>L*</i> , <i>a*</i> , <i>b*</i> values of dry aged bovine LTL. Results are the mean of four biological replicates $\pm$ SEM.	128
Table 5-4. Effect of aging time and high pressure processing on <i>L*</i> , <i>a*</i> , <i>b*</i> values of cooked dry aged bovine LTL. Results are the mean of four biological replicates $\pm$ SEM.	129

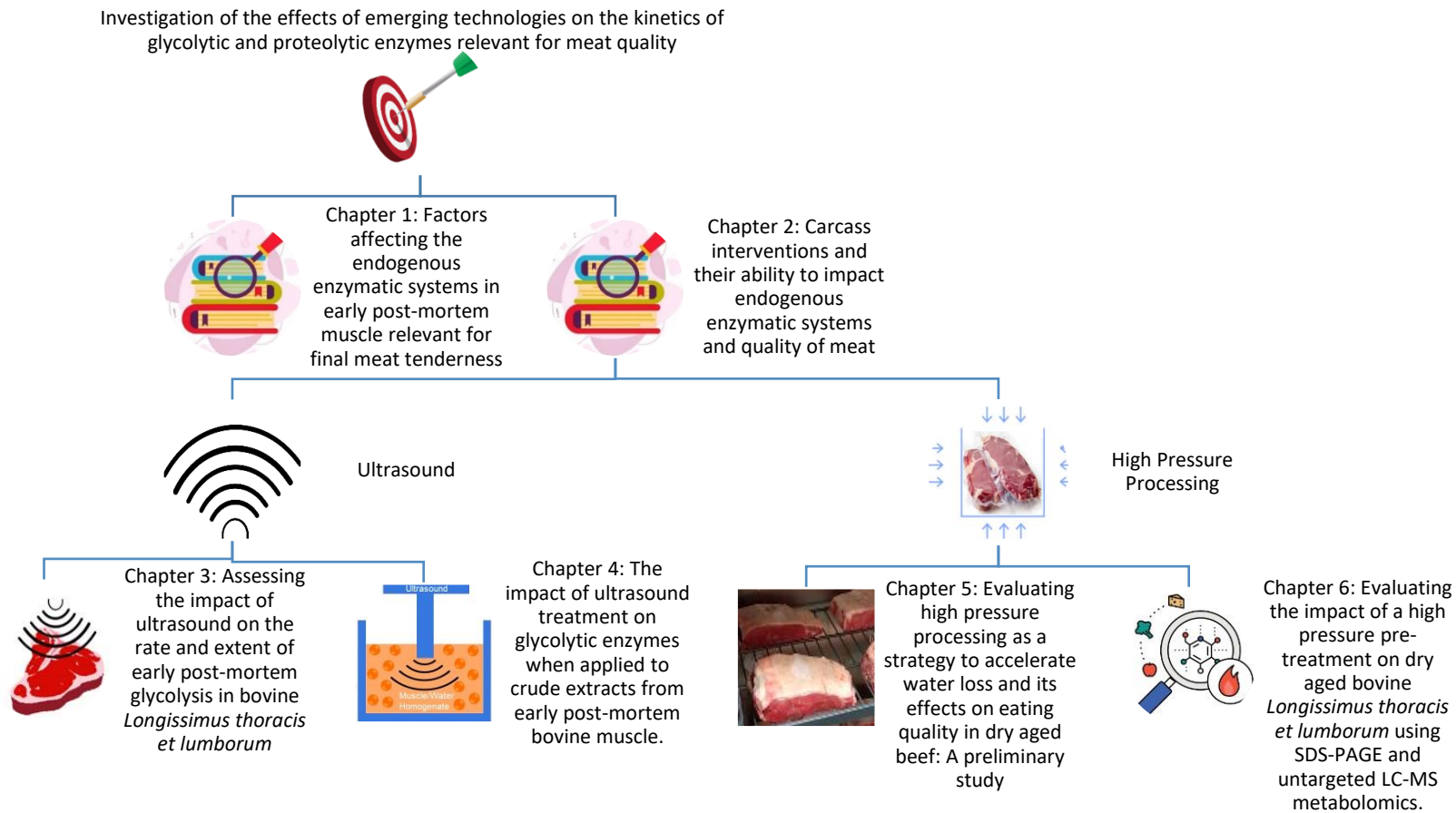
List of Publications emerging from this thesis

- **Kent, M. A.,** Mullen, A. M., O'Neill, E. & Álvarez, C. (2024). Assessing the impact of ultrasound on the rate and extent of early post-mortem glycolysis in bovine *Longissimus thoracis et lumborum*. *Meat Science*, 214, 109531, <https://doi.org/10.1016/j.meatsci.2024.109531>
- **Kent, M. A.,** Mullen, A. M., O'Neill, E. & Álvarez, C. (2024). The impact of ultrasound treatment on glycolytic enzymes when applied to crude extracts from early post-mortem bovine muscle. *Ultrasonics Sonochemistry*, 104, 106842. <https://doi.org/10.1016/j.ultsonch.2024.106842>
- **Kent, M. A.,** Cabral, E. M., Mullen, A. M., O'Neill, E. & Álvarez, C. (2025). Evaluating high pressure processing as a strategy to accelerate water loss and its effect on eating quality in dry aged beef: A preliminary study. *Applied Food Research*, 5(1), 100786. <https://doi.org/10.1016/j.afres.2025.100786>
- **Kent, M. A.,** Cabral, E. M., Brahmachari, R., Frías-Celayeta, J. M., O'Neill, E. & Álvarez, C. Evaluating the impact of a high pressure pre-treatment on dry aged bovine *Longissimus thoracis et lumborum* using SDS-PAGE and untargeted LC-MS metabolomics. Manuscript submitted.

List of Presentations in International Conferences

1. **Kent, M. A.**, Mullen, A. M., O' Neill, E., & Álvarez, C. (2022). Investigating the effect of ultrasound on glycolysis in early postmortem bovine muscle. ICoMST, 68th International Congress of Meat Science and Technology, Kobe, Japan (22-25th August 2022).
2. **Kent, M. A.**, Mullen, A. M., O' Neill, E., & Álvarez, C. (2023). Does ultrasound have the capacity to modulate the glycolytic metabolism kinetics? An *in vitro* study. ICoMST, 69th International Congress of Meat Science and Technology, Padua, Italy (20-25th August 2022).
3. **Kent, M. A.**, Mullen, A. M., O' Neill, E. & Álvarez, C. (2023). An investigation on the effect on high pressure processing on the water holding capacity of dry aged beef. Institute of Food Science and Technology of Ireland (IFSTI), 51st Annual Food Science and Technology Conference, Dublin, Ireland (6th and 7th December 2023).
4. **Kent, M. A.**, Mullen, A. M., O' Neill, E., & Álvarez, C. (2023). Under pressure: High pressure processing and quality attributes of dry aged beef. Institute of Food Science and Technology of Ireland (IFSTI), 51st Annual Food Science and Technology Conference, Dublin, Ireland (6th and 7th December 2023).
5. **Kent, M. A.**, Cabral, E. M., Brahmachari, R., Frías-Celayeta, J. M., O' Neill, E., & Álvarez, C. (2024). The effect of a high pressure pre-treatment on the metabolomics profile of bovine muscle following dry aging. Institute of Food Science and Technology of Ireland (IFSTI), 52nd Annual Food Science and Technology Conference, Dublin, Ireland (11th and 12th December 2024).
6. **Kent, M. A.**, **Cabral, E. M.**, Brahmachari, R., Frías-Celayeta, J. M., O' Neill, E., & Álvarez, C. (2025). The effect of high-pressure pre-treatment and dry aging on the metabolomic profile of bovine muscle. ICoMST, 71st International Congress of Meat Science and Technology, Girona, Spain (3rd – 8th August 2025).

Schematic representation of thesis structure



Chapter 1. Factors affecting the endogenous enzymatic systems in early post-mortem muscle relevant for final meat tenderness

Overview

This literature review explores the biochemical factors that influence the endogenous enzymatic systems responsible for the tenderisation of early post-mortem muscle, with a focus on the glycolytic and proteolytic processes and their interaction with environmental factors. Glycolysis results in the decline in pH, early post-mortem, while proteolysis leads to the degradation of proteins and tenderisation. These processes are impacted by different factors such as pH, temperature and protein post-translational modifications. This chapter will present an in-depth discussion regarding the key enzymes responsible for glycolysis and proteolysis and the factors that influence their activity.

1.1 Introduction

Tenderness is one of the principal quality parameters that determine consumers' overall enjoyment of meat. However, ensuring that meat is consistently tender is challenging due to the large number of pre- and post-slaughter factors that influence this quality attribute. Very briefly, pre-slaughter factors include the breed of the animal, genetic factors, age at slaughter, gender and diet of the animal, all of which are determined at farm level, as well as the muscle location and type of fibre predominant in the muscle (Hwang et al., 2010, Chriki et al., 2013, Gagaoua et al., 2018a, Gagaoua et al., 2018b, Soulat et al., 2018, Cafferky et al., 2019, Lebedová et al., 2021). Other pre-slaughter factors such as transport and lairage conditions also impact the final eating quality of meat (Reiche et al., 2019, Steel et al., 2021, Hultgren et al., 2022, Sullivan et al., 2024). Post-slaughter variables include how animals are slaughtered and the use of carcass interventions, such as hot boning and electrical stimulation, the method of hanging and aging conditions (White et al., 2006a, Pinto Neto et al., 2013, Lang et al., 2016, Crownover et al., 2017, Pogorzelski et al., 2023). The above mentioned factors may also have an impact on the biochemical processes that take place in the conversion of muscle to meat.

Among the different biochemical processes taking place after slaughter, two deserve special attention: anaerobic glycolysis (Frylinck et al., 2013) and proteolysis during aging or maturation (Marino et al., 2013). Anaerobic glycolysis involves complex metabolic pathways, where glycogen is mobilised to be transformed to glucose via glycogenolysis. This glucose is metabolised to lactic acid which accumulates in the muscle with a concomitant drop in pH. These events are triggered when the oxygen supply to the muscle is interrupted after exsanguination and energy metabolism shifts from aerobic to anaerobic pathways (Álvarez et al., 2019). As will be discussed later, the rate of lactic acid production and accumulation, in combination with muscle temperature, is critical in determining final meat tenderness (O'Halloran et al., 1997a, Hannula and Puolanne, 2004). The degradation of proteins, such as troponin-T, nebulin and titin, by endogenous proteolytic enzymes also has a major impact on the final tenderness of muscle. The activity of proteolytic enzymes is influenced by

the pH and temperature conditions observed throughout aging (Huff-Lonergan et al., 1996, Hwang et al., 2004).

Due to the importance of the events happening in the early post-mortem period (glycolysis, *rigor* onset or activation of proteolytic enzymes, among others), there is significant interest from the meat industry in understanding the biochemical mechanisms involved, and in developing strategies to manipulate these events with the aim of reducing variability and to consistently improve the overall eating quality of meat. Proposed strategies may be focused on mechanical interventions affecting the structure of the muscle and/or avoiding muscle shortening (hanging methods (Sørheim et al., 2001, Lundesjö Ahnström et al., 2012, Moran et al., 2021) or hot boning followed by Pi-Vac wrapping (O'Sullivan et al., 2003, Taylor et al., 2012, Taylor et al., 2013), for example); or on manipulating the activity of the enzymes involved in the glycolytic and proteolytic processes (such as electrical stimulation (Hwang et al., 2003, Kim et al., 2013, Jaspal et al., 2021), chilling regimes (Hwang and Thompson, 2001b, Hannula and Puolanne, 2004, White et al., 2006a) or pulsed electric fields (Suwandy et al., 2015b, Suwandy et al., 2015a, Bhat et al., 2019b).

This review will comprehensively discuss the metabolic process of glycolysis including the impact of external and internal factors on the activity of key enzymes involved and the importance of glycolysis in relation to the eating quality of meat. The key proteolytic enzymes along with the main factors that affect their activity will also be discussed and their importance in relation to meat quality, in particular, tenderness.

1.2 Glycolysis

Energy is stored in muscle as glycogen (glucose monomers linked linearly by α -1, 4 bond, and branching by α -1, 6 glycosidic bonds) which is depolymerised to glucose when muscle requires energy. Glycolysis, a metabolic pathway consisting of a ten step series of biochemical events, involves the conversion of glucose to pyruvate (Figure 1-1). Under aerobic conditions, pyruvate is transported to the mitochondria, oxidised to acetyl CoA and utilised in Krebs cycle to generate ATP (Ferguson and Gerrard, 2014, Tondo et al., 2015). However, when oxygen availability is low, pyruvate generated from glycolysis cannot enter the Krebs cycle and is converted to lactic acid following an alternative and less efficient pathway to generate ATP.

Anaerobic glycolysis is carried out in an effort to maintain homeostasis in the muscle, and maintain the levels of ATP in the absence of oxygen. As a consequence of glycogen degradation, the concentration of lactate and H^+ increases over time with a resultant decline in pH. Under normal conditions, anaerobic glycolysis stops when glycogen (Ponnampalam et al., 2017) or ATP (England et al., 2016) is no longer available, or following the pH inactivation of enzymes (England et al., 2014).

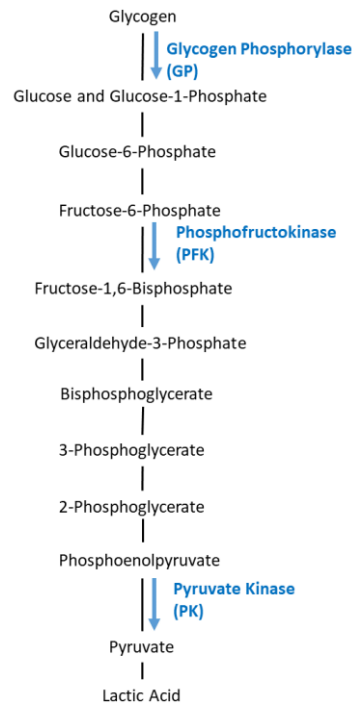


Figure 1-1. A schematic of the anaerobic glycolytic pathway, where glucose, from the breakdown of glycogen, is converted into lactic acid. Key rate limiting enzymes are highlighted.

1.2.1 Glycolytic potential and glycolytic capacity

The glycolytic potential of muscle refers to the amount of glycogen available in muscle prior to slaughter, which can be converted to lactate during glycolysis. This influences the ultimate pH of muscle and can be calculated according to Equation 1-1 (Monin and Sellier, 1985, Ferguson and Gerrard, 2014, McKeith et al., 2016, Kirkpatrick et al., 2023):

$$\text{Glycolytic potential (mmol lactate/g)} = [\text{lactate}] + 2([\text{glycogen}] + [\text{glucose}] + [\text{glucose} - 6 - \text{phosphate}]) \quad \text{Eq. 1-1}$$

For normal pHu (pH at 24 h post-mortem) ($\text{pH} < 5.8$) to be reached in bovine muscle, 45-57 mM glycogen/kg muscle is the critical concentration of glycogen required (Tarrant, 1989), although this can vary depending on other factors such as the buffering capacity and glycolytic capacity of the muscle (Chauhan et al., 2019). The ultimate pH (pHu) influences the characteristics of meat, including tenderness, colour and water holding capacity (Holdstock et al., 2014, Barón et al., 2021, Khatri and Huff-Lonergan, 2023).

When pre-slaughter glycogen concentrations are low, muscle has a lower glycolytic potential and lactic acid production is reduced post-mortem, leading to a higher pHu, resulting in dark, firm, dry (DFD) muscle (Wulf et al., 2002). Immonen and Puolanne (2000) reported that there is a curvilinear relationship between residual carbohydrate (glucose and glycogen) content at 48 h post-mortem and ultimate pH in bovine *Longissimus dorsi*, *Gluteus medius* and *Semimembranosus*. In muscle where the ultimate pH was normal ($\text{pH} \leq 5.75$), it was found that in general, there was a high residual carbohydrate content, with the range being quite broad. The residual carbohydrate content in muscle with an ultimate pH of 5.4 to 5.75 ranged from 10 to 83 mM/kg with corresponding glycogen concentrations at slaughter of 55 mM/kg to 125 mM/kg. There was a lower residual carbohydrate concentration in muscle with a higher ultimate pH ($\text{pH} > 5.75$) than in muscle with a normal pH range ($\text{pH} \leq 5.75$), indicating that the glycogen content of the muscle was diminished at the time of slaughter (Immonen and Puolanne, 2000). When investigating the effects of pre-slaughter conditions on the glycogen content of bovine *Longissimus lumborum*, Apaoblaza et al. (2017) observed that fasting prior to slaughter caused a decrease in glycogen reserves pre-slaughter, increasing the risk of producing muscle with a high ultimate pH. However, it was found that having high glycogen content did not guarantee an ultimate pH below 5.8. Furthermore, studies have shown that the concentration of glycogen in muscle at the time of death is not always the limiting factor in determining the extent of glycolysis, particularly in muscles with a high ultimate pH. In oxidative muscles, the glycolytic capacity, defined as the muscle's ability to convert glucose to lactate or pyruvate (Mookerjee et al., 2016, Chauhan et al., 2019), appears to have a major influence on the ultimate pH (England et al.,

2016). Results of *in vivo* and *in vitro* studies indicate that glycolysis in oxidative muscles of porcine, bovine, ovine and poultry origin stops sooner and at a higher ultimate pH than glycolytic muscles, despite there being an adequate concentration of glycogen present (England et al., 2016, Chauhan et al., 2019). In the case of oxidative muscles, such as porcine *Masseter*, it has been suggested that ATP is the limiting factor responsible for the termination of post-mortem glycolysis (England et al., 2016). This comes about as the rate of ATP hydrolysis in the oxidative muscle exceeds the re-phosphorylation of ADP, causing glycolysis to prematurely stop (Chauhan et al., 2019). In glycolytic muscles, such as porcine *Longissimus thoracis et lumborum*, glycolysis appears to be limited by the activity of phosphofructokinase-1, whose activity declines as the pH decreases below pH 5.9 (England et al., 2014).

Deviations from the rate and extent of normal glycolysis and pH decline can have a negative impact on meat quality. DFD, which occurs predominantly in bovine muscle, results in muscle that has a dark colour, firm texture and dry surface, inferior flavour and decreased shelf life (Mahmood et al., 2017, Chauhan et al., 2019, Ijaz et al., 2020, Ijaz et al., 2022). DFD is usually associated with the animal experiencing stress prior to slaughter which results in a low concentration of glycogen in muscle at the time of death (Mahmood et al., 2019, Loredó-Osti et al., 2019, Steel et al., 2021). Pale, soft and exudative (PSE) meat occurs as a result of rapid glycolysis, where pH 5.8 or lower is reached within 45 min post-mortem while the muscle is still at a high temperature, leading to protein denaturation. PSE occurs predominantly in porcine muscle and is characterised by having a soft texture, high drip loss and pale colour (Chmiel et al., 2014, Álvarez et al., 2019, Wang et al., 2019). Conditions before slaughter that involves animals experiencing high levels of stress increase the glycolytic kinetics of the muscle, leading to a rapid pH decline (Hambrecht et al., 2005, Van de Perre et al., 2010, Vermeulen et al., 2015).

1.2.2 Glycolytic enzymes: regulation and pH effect

Three glycolytic enzymes: glycogen phosphorylase (GP), phosphofructokinase (PFK) and pyruvate kinase (PK); (Figure 1-1) have been identified as being responsible for limiting the rate of post-mortem glycolysis (Wang et al., 2019). As illustrated in Table

1-1, the activity of these rate limiting enzymes are impacted by pH and temperature, both of which change rapidly post-mortem.

1.2.2.1 Glycogen phosphorylase (GP)

GP is responsible for releasing glucose and glucose-1-phosphate from glycogen (glycogenolysis) (Ferguson and Gerrard, 2014). Once glucose-1-phosphate is liberated from glycogen, another enzyme, phosphoglucomutase, rapidly isomerises it to glucose-6-phosphate (Scheffler and Gerrard, 2007). Free glucose generated from glycogenolysis is either converted to glucose-6-phosphate by hexokinase or is allowed to accumulate in the post-mortem muscle (Matarneh et al., 2018). GP works by cleaving glycogen at the α -1, 4 glycosidic linkages and transferring an inorganic phosphate to the unit to form glucose-1-phosphate. This continues along the branched glucose chain until a specific point is reached, four residues from an α -1, 6 glycosidic linked branch (Ferguson and Gerrard, 2014), which is called the limit dextrin site. Here, GP works in collaboration with glycogen debranching enzyme which acts to cleave the remaining glycosyl branch to form free glucose and also works by transferring a maltotriosyl group to the next main glucose chain of the glycogen molecule. Therefore, the action of the glycogen debranching enzyme allows for continued hydrolysis and phosphorylation by GP (Kylä-Puhju et al., 2005). Without this action, it is estimated that only 34.6% of glucose would be released from glycogen (Ylä-Ajos and Puolanne, 2007).

GP is present in two forms, GP α , which is active and phosphorylated and GP β , an inactive, non-phosphorylated form. The inactive form is converted to the active form by the action of phosphorylase kinase, which phosphorylates a serine residue at position 14 in the protein sequence, allowing the catalytic site of the enzyme to access the substrate (Bai et al., 2020a). The phosphorylation of GP in muscle is controlled according to the energy requirements of the muscle (Schwägele et al., 1996a).

An interesting study by Bai et al. (2020a) indicated that in ovine *Longissimus thoracis* exhibiting slow ($\text{pH}_{6\text{h}} > 6.3$) and intermediate ($5.7 < \text{pH}_{6\text{h}} < 6.3$) pH decline, the level of phosphorylation from 6 to 24 h post-mortem is significantly lower than that of muscles showing a rapid ($\text{pH}_{6\text{h}} < 5.7$) pH decline; suggesting a relationship between

GP phosphorylation status and rate of pH decline. However, at 48 h post-mortem, no differences in the levels of phosphorylation of GP was observed between the three groups (Bai et al., 2020a). In addition, enzyme expression appeared to be related to the rate of glycogen degradation with muscles that exhibited a rapid pH decline ($\text{pH}_{6\text{h}} < 5.7$) having a greater level of expression of GP over a period of 48 h post-mortem compared to muscle with a slow ($\text{pH}_{6\text{h}} < 5.7$) or intermediate ($5.7 < \text{pH}_{6\text{h}} < 6.3$) pH decline. Furthermore, in fast pH declining ($\text{pH}_{6\text{h}} < 5.7$) ovine *Longissimus thoracis*, an increase in GP expression was seen between 0.5 h and 12 h post-mortem, before decreasing again. This greater expression early post-mortem coincides with a high glycogen content and its rapid depletion, for which GP is required. It appears that once glycogen levels decrease, so too does the expression of GP, as there is less glycogen to deplete and therefore, less GP was required. The expression of GP in intermediate ($5.7 < \text{pH}_{6\text{h}} < 6.3$) and slow ($\text{pH}_{6\text{h}} > 6.3$) pH decline groups increased from 0.5 h to 48 h post-mortem as there was greater glycogen content in the ovine muscles compared to that of the fast pH decline group during this period (Bai et al., 2020a).

GP is inhibited by ADP and glucose-6-phosphate, while it is activated by AMP and IMP (Ferguson and Gerrard, 2014). When the activity of GP, isolated from porcine *Longissimus dorsi thoracis et lumborum*, was measured *in vitro*, it was found that in the absence of AMP, GP was inactive, even though glycogen and phosphate were available (Schwägle et al., 1996a). However, when 0.5 mM AMP was added, GP activity increased due to phosphorylation. While ATP promotes the phosphorylation of GP, ATP can also inhibit the activity of GP when available in sufficient amounts. This occurs as ATP competes with AMP, a GP activator, for the allosteric binding site on GP, and therefore, inhibiting GP activity. Bai et al., (2020b) demonstrated that when extracted sarcoplasmic protein (from ovine *Longissimus thoracis*, immediately post-mortem) was incubated with ATP (0.5, 2 and 2.5 $\mu\text{M}/100 \mu\text{g}$ protein) for 72 h, the glycogen content did not change. This was in contrast to controls, where no ATP was added and glycogen content decreased over time, indicating glycogen degradation had taken place.

1.2.2.2 Phosphofructokinase (PFK)

PFK is considered to be one of the key enzymes involved in the regulation of glycolysis in the presence of excess glycogen and adequate levels of adenine nucleotides (Matarneh et al., 2018). PFK is present in a variety of oligomeric forms, which dictates its activity. When present in the tetrameric form the enzyme is active; however, when it is dissociated into dimeric and monomeric forms, it is inactive (Leite et al., 2007). There are two isoforms of the tetrameric structure with different functions. The first isoform, phosphofructokinase-1 (PFK), catalyses the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate consuming ATP and generating ADP. The second isoform, phosphofructokinase-2 (PFK-2), is involved in the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate, which functions as an activator of PFK by firstly decreasing the ability of ATP to inhibit PFK, and secondly by increasing the enzyme affinity for fructose-6-phosphate (Litwack, 2018).

While ATP is necessary for the phosphorylation of fructose-6-phosphate by PFK, it also has an inhibitory effect when present in concentrations over 1 mM (Zancan et al., 2008, Wang et al., 2024) while ADP, AMP and fructose-2,6-bisphosphate act as PFK activators (Chauhan et al., 2021). The activation and inactivation of PFK by fructose-2,6-bisphosphate and ATP, respectively, are primarily as a result of their ability to influence the quaternary structure of the enzyme and its oligomeric conformation, with ATP at concentrations above 1 mM dissociating PFK to inactive dimers, while ATP concentrations below 1 mM favour the active tetramer. The tetrameric structure of PFK is stabilised against the inhibitory effect of ATP by fructose-2,6-bisphosphate (Zancan et al., 2008). Another inhibitor of PFK is lactate, the final product of anaerobic glycolysis. This inhibition is dependent on the concentration of the lactate. In addition, it was found that fructose-2,6-bisphosphate protected PFK against dissociation by lactate (Leite et al., 2007, Leite et al., 2011). Post-mortem PFK activity decreases over time, and this could be linked to the decline in ATP concentration, which was postulated by Krschek et al., (2011), when investigating the concentration of adenine nucleotides and PFK activity between 45 min and 12 h post-mortem in porcine *Longissimus* muscle. However, PFK can be

phosphorylated at serine, tyrosine and threonine residues by a range of protein kinases, reducing the enzymes susceptibility to ATP inhibition and modifying the activation pattern by ATP (Sola-Penna et al., 2010). Chen et al. (2019) reported that the phosphorylation level of PFK and its activity, when measured *in vitro* in ovine *Longissimus thoracis*, were positively correlated.

PFK is sensitive to pH changes in post-mortem muscle (Table 1-1) and its inactivation has been associated with causing the cessation of glycolysis at pH 5.5 – 5.9 in bovine, porcine and poultry muscle (England et al., 2014, Matarneh et al., 2018). When the activity of PFK was investigated *in vitro* using pH buffer systems, it was found that at pH 8.2, 10 mM lactate did not affect the activity of PFK, but as the pH was reduced to pH 7.0 and 6.5, the enzyme became more susceptible to the effects of lactate. Lactate also affected PFK's affinity to ATP and fructose-6-phosphate as substrates (Leite et al., 2007). When GP and PK activities were measured in porcine *Longissimus thoracis et lumborum* using *in vitro* enzyme activity assays, England et al. (2014) found that GP and PK remained active for the first 24 h post-mortem, where the pH of the muscle reduced from pH 6.4 to pH 5.4. However, PFK activity, also measured using an *in vitro* enzyme activity assay, reduced significantly, especially in the first 60 min post-mortem, where its activity was decreased by 75% of its initial activity. During this time, the pH decreased to approximately pH 5.8 (England et al., 2014). The reduction in PFK activity resulted in the accumulation of intermediate products of glycolysis, such as glucose-6-phosphate and fructose-6-phosphate (Matarneh et al., 2018). Similar results were observed when chicken *Pectoralis major*, porcine *Longissimus thoracis et lumborum* and *Semitendinosus*, and bovine *Semimembranosus* at between 5 and 20 min post-mortem were homogenised in an *in vitro* buffer system designed to monitor changes in enzymatic activity over time under constant pH conditions of pH 6.5 and 5.6. For all muscles, little PFK activity was detected *in vitro* at pH 5.6 when compared to PFK activity at pH 6.5 (England et al., 2014). Interestingly, it is proposed that in porcine *Longissimus* muscle, which undergoes a rapid pH decline, PFK may become partially denatured within the first 20 min post-mortem as a result of the combination of low pH and high temperature

in the muscle experiencing rapid pH decline, which therefore leads to the inactivation of the enzyme (Allison et al., 2003).

1.2.2.3 Pyruvate Kinase (PK)

The final rate limiting enzyme is PK, which is responsible for the irreversible conversion of phosphoenolpyruvate and ADP to pyruvate and ATP (Scheffler and Gerrard, 2007). In normal porcine *Longissimus dorsi thoracis et lumborum* ($\text{pH}_{45 \text{ min}} > 6.2$), PK is present as two isoforms. In PSE porcine muscle ($\text{pH}_{45 \text{ min}} < 5.5$), PK occurs as three different isoforms. The additional isoform in PSE muscle results in a shift in the effect of pH on PK activity, where it was more active at acidic conditions. The presence of the third isoform in PSE muscle allows PK to maintain approximately 70% of its maximum activity at pH 5.5 compared to PK isolated from normal muscle, where PK activity was very low at pH 5.5 (Schwägele et al., 1996b).

As the pH drops post-mortem, the activity of PK decreases, with a >88% loss in activity at pH 5.5 compared to that at pH 7 (Allison et al., 2003). Shen et al. (2006) found that PK was most active early post-mortem in porcine *Longissimus* muscle, within the first 0.5 to 1 h post-mortem, and decreased thereafter with time post-mortem. Similarly to the other two rate limiting enzymes, GP and PFK, the activity of PK increases with increased degrees of phosphorylation (Chen et al., 2019). It has been shown, *in vitro*, that the phosphorylation of isoform 1 of PK extracted from porcine PSE muscle results in the generation of isoform 2 and 3, while the dephosphorylation of PK decreases the concentration of isoform 3, while isoforms 1 and 2 increase (Schwägele et al., 1996b).

A variety of compounds associated with glycolysis can have an effect on the activity of PK. Phosphoenolpyruvate (Wang et al., 2017), fructose-1,6-bisphosphate (Taber et al., 1998) and glucose-6-phosphate promote the activity of PK, while ATP has an inhibiting effect on the enzyme (Smart and Pritchard, 1982). The activity of PK is also reduced by acetyl CoA and fatty acids (Ferguson and Gerrard, 2014).

Table 1-1. The effect of pH and temperature on the activity of key glycolytic enzymes in muscle.

Enzyme	Sample details	Effect of temperature on enzyme activity	Effect of pH on enzyme activity	Method of analysis	Reference
GP, PFK, PK	Porcine <i>Longissimus thoracis</i> at 1 h post-mortem	N/A	Higher GP, PFK and PK activity in PSE muscle (pH < 6) compared to RFN muscle (pH > 6).	GP and PFK: ELISA; PK: commercial kit	(Wang et al., 2020)
GP	Ovine <i>Longissimus thoracis</i> at 0.5, 2, 6, 12, 24, 48 and 72 h post-mortem	N/A	Higher GP activity in fast pH decline (pH _{6h} < 5.7) muscle compare to slow pH decline (pH _{6h} > 6.30) muscle at 24 h post-mortem. No difference at 72 h post-mortem	Commercial kit	(Bai et al., 2020a)
GP	Bovine <i>Longissimus dorsi</i> at 0.5 and 24 h post-mortem	N/A	No differences in GP activity between normal (pH _{24h} < 5.8) and high pH muscle (pH _{24h} > 5.9). GP activity increased at 24 h post-mortem in normal pH muscle (pH _{24h} < 5.8).	Intensity of fluorescence signal after production of specific substrate	(Apaoblaza et al., 2015)
GP	Porcine <i>Longissimus dorsi thoracis et lumborum</i> at 45 min post-mortem	N/A	No differences in activity between PSE (pH _{45min} < 5.5) and normal muscle (pH _{45min} > 6.4). Optimum pH ~ pH 7	Intensity of fluorescence signal after production of specific substrate	(Schwägele et al., 1996a)
PFK	Porcine <i>Longissimus thoracis et lumborum</i> muscle (5 min post-	Lower activity at 42 °C compared to 33 °C after 240 min.	Up to 75% loss when pH reduced from pH 6.4 to pH 5.8; consistent reduction in activity	Intensity of fluorescence signal	(England et al., 2014)

	mortem); porcine <i>Semitendinosus</i> (20 min post-mortem); chicken <i>Pectoralis major</i> (5 min post-mortem); bovine <i>Semimembranosus</i> (15 min post-mortem)		from pH 6.5-5.6 in chicken, porcine and bovine muscle.	after production of specific substrate	
PFK	Crystalline form from rabbit muscle	Faster inactivation at 6 °C, compared to 12 °C	Activity decreases from pH 8 to pH 6; Reversible by increasing pH	Intensity of fluorescence signal after production of specific substrate	(Bock and Frieden, 1976)
PK	Porcine <i>Longissimus</i> muscle at 20, 45, 180 min and 24 h post-mortem	N/A	88% reduction in activity at pH 5.5 compared to pH 7	Intensity of fluorescence signal after production of specific substrate	(Allison et al., 2003)
PK	Porcine <i>Longissimus dorsi thoracis et lumborum</i> at 45 min post-mortem	N/A	Most active at pH 7. Isoforms 1 and 2 inactive at pH 5.5 (normal muscle (pH > 6.2)); Isoform 3 retained 40% activity at pH 5.5 (PSE muscle (pH < 5.5))	Intensity of fluorescence signal after production of specific substrate	(Schwägele et al., 1996b)

1.2.3 Other factors affecting post-mortem glycolytic kinetics

The rate and extent of post-mortem glycolysis may be affected by factors prior to the animal being slaughtered such as the nutritional status of the animal, stress prior to slaughter and seasonal variations (Frylinck et al., 2013, Apaoblaza et al., 2017, Chauhan and England, 2018). It is also influenced by the glycolytic capacity and glycolytic potential of the muscle as discussed previously (England et al., 2016). Characteristics of the animal being slaughtered, such as genetics, age, size and fat cover may also have a part to play in influencing the extent of glycolysis, and also the rate of both pH and temperature decline. In a study conducted by Cadavez et al. (2019), it was observed that heavier bovine carcasses with a better conformation achieved a faster pH decline (measured in the *Longissimus thoracis*) compared to carcasses that were lighter in weight. The heavier carcasses had higher glycogen reserves compared to animals with a lighter carcass weight, prompting a faster pH decline. Younger animals also achieved a lower pHu in this study. The authors highlighted that this was unexpected as younger animals usually have a higher pHu due to their higher energy demands and difficulty to adapt to stress; which may reduce the glycogen reserves. The amount of fat cover influenced the rate of temperature decline with carcasses having higher levels of fat taking longer to cool down compared to leaner carcasses. The temperature of the carcasses in turn influenced the rate of pH decline, with heavier carcasses with a higher fat cover, exhibiting a faster drop in pH, as the temperature of the carcass remained higher for longer. At 3 and 6 h, the temperature was 29.9 and 22.9 °C, respectively. This was in comparison to lighter carcasses, where the pH reduced at a slower rate, with an average temperature of 28.2 and 20.3 °C at 3 and 6 h, respectively. Genetics is another factor that has the potential to influence glycolysis and rate of pH decline, however, little research has been carried out on how genetic differences or individual genes effect these factors in beef.

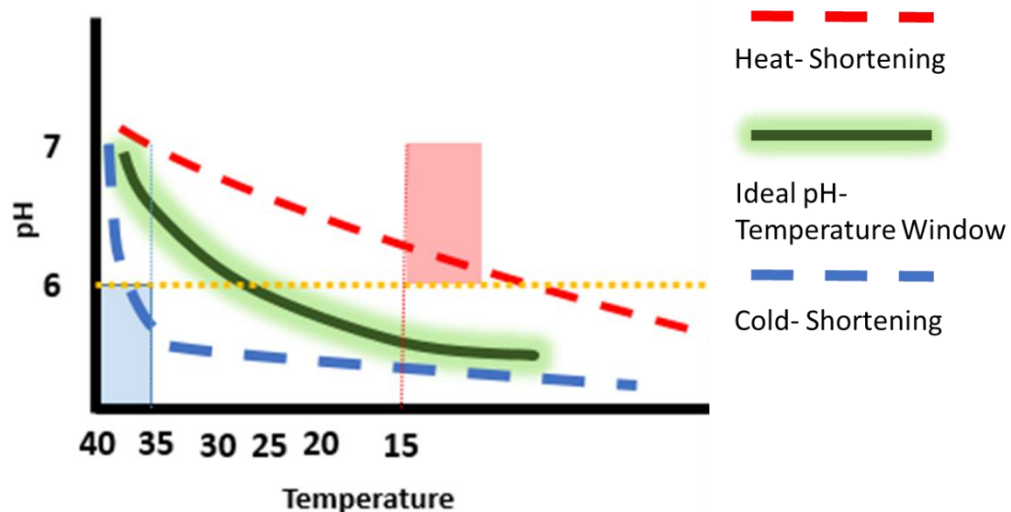


Figure 1-2. pH – temperature relationship illustrating the windows for hot and cold shortening, adapted from Gutzke et al. (2014).

The rate of chilling is important in relation to glycolysis and tenderness. The objective of chilling is to minimise evaporative loss and optimise microbial safety while maintaining quality parameters such as tenderness and colour (Zhang et al., 2019c, Álvarez et al., 2022). To achieve optimum meat eating quality, Meat Standards Australia (MSA) established the concept of an optimum pH – temperature window. When pH 6 is reached in muscle, the temperature should be between 15 and 35 °C (Figure 1-2). This guideline aimed to help meat processors maximise the eating quality of meat and avoid cold and hot shortening (Thompson, 2002). Strategies to allow for rapid chilling while preventing cold shortening in muscle include the use of electrical stimulation at early post-mortem stages, which accelerates the rate of pH decline by promoting muscle contraction (Zhang et al., 2019c).

1.3 Proteolysis

Proteolysis takes place during aging, which involves storing muscle for a period of time under controlled refrigerated conditions, allowing for the development of tenderness and other traits such as flavour and juiciness (Khan et al., 2016). Proteolysis involves the cleavage of key structural proteins in the muscle such as titin, nebulin, vinculin and desmin by endogenous proteolytic enzymes, most notably the calpains (Kemp et al., 2010). Titin and nebulin run parallel to the direction of the sarcomeres and are responsible for maintaining the longitudinal integrity of the

muscle, whilst desmin is thought to help align and attach adjacent myofibrils together (Robson et al., 1995). Desmin and vinculin are also involved in the structure of costameres (Taylor et al., 1995) (Figure 1-3). The costameres, located at the surface of the muscle cell attaching the sarcolemma to the myofibrils, are rapidly degraded within the first 24 – 72 h post-mortem, resulting in the sarcolemma being detached from the myofibrils. The degradation of the M-line and desmin, which connect M-lines and Z-disks together, results in the increased spatial separation of the myofibrils. Another structural change, which may contribute to the tenderness of meat, is the development of gaps between the Z-disk and I-band. This area is where titin and nebulin are located (Taylor et al., 1995, Feng et al., 2020). However, actin and myosin, the major myofibrillar proteins, are not significantly degraded post-mortem (Huff-Lonergan, 2014).

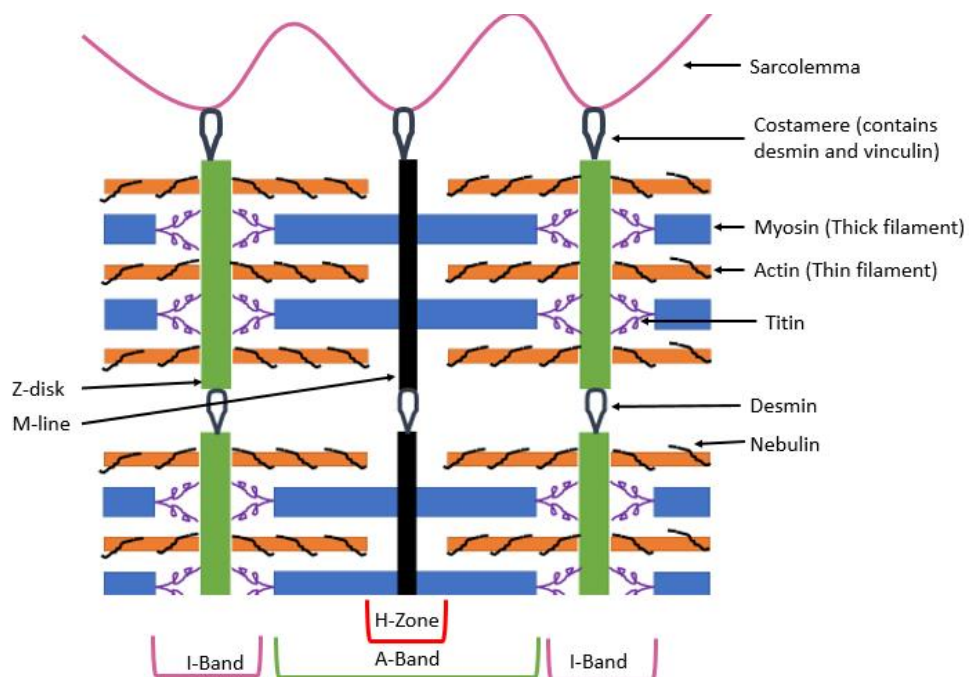


Figure 1-3. The myofibrillar structure of muscle with the main contractile and cytoskeletal fibres labelled, adapted from Kemp et al. (2010).

1.3.1 Main enzymes involved in proteolysis

For proteases to be considered key players in post-mortem proteolysis and tenderisation of muscle, they must meet three requirements: i) the enzyme must be naturally present in skeletal muscle; ii) they must have access to the proteins in the

costamere and myofibrils, and iii) be able to hydrolyse these same proteins during the tenderisation process to have an impact on the final tenderness of meat (Koochmaraie, 1996). A synergistic role between a number of different enzymes groups has been suggested, since all the structural changes that occur in post-mortem muscle cannot be explained by the action of one individual group of proteolytic enzyme (Lamare et al., 2002, Chéret et al., 2007, Ma and Kim, 2020, Ding et al., 2018, Houbak et al., 2008). This review will focus on the main enzyme systems thought to play a potential role in meat tenderisation: calpains, cathepsins, the proteasome complex, the caspase system and heat shock proteins. These systems will be discussed in detail including mechanisms of action, activators, inhibitors and the impact of pH and temperature on their activity. In Table 1-2, the impact of pH and temperature on the activity of these main proteolytic enzymes have been summarised.

1.3.1.1 Calpains and Calpastatin

μ - and m-calpain, also referred to as calpain-1 and calpain-2, respectively, can be found bound to myofibrils or in the cytosol (Bhat et al., 2018a) and are named according to their calcium requirements for activation, which are 3 – 50 $\mu\text{M Ca}^{2+}$ and 400 – 800 $\mu\text{M Ca}^{2+}$, respectively, for half maximal activity (Cruzen et al., 2014, Goll et al., 2003). Both calpain enzymes are comprised of two subunits, an 80 kDa subunit, which is responsible for the catalytic activity of the enzyme and a regulatory 28 kDa subunit (Cruzen et al., 2014). The 28 kDa subunit is identical in both calpains. While the 80 kDa subunits have some similarities, several differences can be observed and they are not encoded by the same genes (Huff Lonergan et al., 2010). The 80 kDa subunits are composed of four domains, each having different functions. Domain I is responsible for the activation of autolysis, which is described below; domain II contains cysteine and is the site of catalysis; domain III is a switch domain; and domain IV is the site for binding calcium (Sentandreu et al., 2002).

Calpains are usually inactive under normal physiological conditions. In the presence of activating levels of calcium, both μ - and m-calpain can bind calcium and experience a conformational change resulting in the activation of the catalytic site. This activation is concomitant with subsequent autolysis; which results in a consecutive

molecular weight reduction of both subunits. In μ -calpain, the 80 kDa subunit is reduced first to 78 kDa, and then to 76 kDa; in the m-calpain the 80 kDa subunit is reduced initially to 79 kDa and finally 78 kDa. In both enzymes, the 28 kDa is directly reduced to 18 kDa. This autolysis results in a decrease in the amount of calcium required for μ - and m-calpain enzymes activation (92% and 82% less, respectively, than unautolysed enzymes); however, if autolysis is extensive (dependent on pH and temperature conditions at the early post-mortem stage), the enzymes are inactivated (Li et al., 2004, Bhat et al., 2018a, Ji and Takahashi, 2006).

The main substrates of the calpains, during post-mortem storage, are titin, nebulin and desmin (Liu et al., 2016a). It has been proven that μ -calpain is largely responsible for proteolysis post-mortem, even in the presence of active m-calpain. Geesink et al. (2006) reported a significant reduction in the extent of proteolysis in the muscles of the μ -calpain knockout mice compared to those of control mice. In particular, there was a large reduction in the degradation of proteins such as nebulin, vinculin, troponin-T and desmin. It has also been shown that μ -calpain activity decreases post-mortem, while m-calpain remains stable (Colle and Doumit, 2017, Camou et al., 2007). All of this supports the conclusion that μ -calpain is a greater contributor to post-mortem tenderisation.

Calpains are neutral proteases, with both μ - and m-calpains being most active *in vitro*, at pH 6.5 and 7.5, respectively. Additionally, μ -calpain at pH 6.5 has a slower rate of autolytic degradation and so, the enzyme remains active after 60 min. In contrast, at pH 7.5, μ -calpain was almost completely autolysed after 20 min, and this resulted in the presence of less active μ -calpain in the *in vitro* assay after 60 min (Maddock et al., 2005). Very little proteolytic activity by μ - and m-calpain was observed when assayed at pH 5.8 (Camou et al., 2007). Several studies have examined the impact of the rate of pH decline on μ -calpain activity and myofibrillar degradation of muscle with apparently conflicting results. Mohrhauser et al. (2014) reported no significant differences in μ -calpain activity or troponin-T degradation following the *in vitro* degradation of extracted myofibrils between fast (1 h pH decline to 5.6 – 5.8) and slow (1 h pH decline to 6.2 – 6.4) pH decline. Pomponio et al. (2010) investigated μ -calpain activity in porcine *Longissimus dorsi* and reported that at 1 h

post-mortem, there were no differences in μ -calpain activity or autolysis among muscles with slow ($\text{pH} > 6.30$), intermediate ($6.00 < \text{pH} < 6.30$), or fast ($\text{pH} < 6.00$) pH declines. However, by 6 h post-mortem, muscles with a fast pH decline exhibited lower unautolysed μ -calpain activity and higher autolysed μ -calpain activity compared to muscles with intermediate or slow pH declines. By 24 h post-mortem, the fast pH decline group showed higher total μ -calpain activity and greater μ -calpain autolysis, resulting in increased myofibrillar fragmentation compared to slower pH decline groups. This difference in fragmentation diminished by 72 h and 144 h post-mortem, suggesting that pH decline rate influences the rate but not the extent of myofibrillar fragmentation over time. In a study by Rhee et al. (2006) on bovine *Longissimus thoracis et lumborum*, fast glycolytic muscle ($\text{pH} 6.2$ at 3 h post-mortem) exhibited increased tenderness at 7 days post-mortem due to earlier μ -calpain activation and accelerated proteolysis, while a slower pH decline ($\text{pH} 6.6$ at 3 h post-mortem) resulted in tougher meat despite higher calpain activity at 24 h post-mortem.

Pulford et al. (2009) reported that bovine *Longissimus dorsi* with low ultimate pH ($\text{pHu} < 5.7$) showed a rapid decline in μ -calpain activity by 1 day post-mortem, whereas muscles with a high ultimate pH ($\text{pHu} > 6.3$) maintained higher μ -calpain activity levels for up to 8 days post-mortem. This is in contrast to the findings of Lomiwes et al. (2013), who observed lower μ -calpain activity in high pHu ($\text{pHu} \geq 6.2$) muscle compared to low ($\text{pHu} \leq 5.79$) and intermediate ($\text{pHu} 5.8 - 6.19$) pHu muscle at every time point up to 7 days post-mortem. Lomiwes et al. (2013) and Lomiwes et al. (2014b) also found that autolysis of μ -calpain occurred immediately post-mortem in high pHu muscle, whereas it was delayed and first detected at 1 day post-mortem in low pHu muscle, suggesting delayed calpain activation. Intermediate pHu muscle showed even further delayed autolysis, with the active 78 kDa subunit still detectable at 21 (Zhao et al., 2022) and 28 days post-mortem (Lomiwes et al., 2013). On the other hand, the activity of m-calpain does not appear to be affected by the rate of pH decline or ultimate pH of the muscle (Pulford et al., 2009, Pomponio et al., 2010), possibly due to the high levels of calcium required for triggering its autolysis.

Temperature significantly affects calpain activity post-mortem, as reported by several authors, which is not surprising considering the role that temperature has as a determinant of the rate of enzymatic reactions. Camou et al. (2007) demonstrated that both μ -calpain and m-calpain purified from bovine *Diaphragm* are more active at 25 °C compared to 5 °C and 15 °C across a pH range of 5.8 to 7.5, *in vitro*. Mohrhauser et al. (2014) observed greater troponin-T degradation during *in vitro* digestion of purified myofibrils by μ -calpain at 22 °C compared to 4 °C across all time points up to 72 h. Hwang et al. (2004) investigated μ -calpain autolysis and troponin-T degradation in bovine *Longissimus thoracis et lumborum* and *Semitendinosus* incubated at 5 °C, 15 °C, and 36 °C for the first 24 h post-mortem, and found that higher temperatures led to faster μ -calpain autolysis and troponin-T degradation. White et al. (2006a) reported faster proteolysis in bovine *Longissimus dorsi* incubated at 25 °C for 8 h post-mortem compared to muscles incubated at 5 °C, following hot boning within 90 minutes post-mortem. Geesink et al. (2000) found that incubating ovine *Longissimus* muscle at 25 – 35 °C for the first 16 h post-mortem resulted in faster μ -calpain activation and proteolysis at 1 day post-mortem compared to samples incubated at lower temperatures (5 – 20 °C). However, after further aging at 2 °C for 14 days, samples incubated at temperatures above 25 °C showed less proteolysis and higher shear force values, suggesting a loss of μ -calpain activity over time due to excessive initial activation at higher temperatures. Pomponio and Erbjerg (2012) studied μ -calpain activity in porcine *Longissimus* muscle incubated at different temperatures (2 °C, 15 °C, 25 °C, and 30 °C) from 2 h to 120 h post-mortem. At lower temperatures (2 °C), μ -calpain activity persisted longer, with the unautolysed μ -calpain activity decreasing gradually over 120 h. In contrast, at higher temperatures (25 °C and 30 °C), μ -calpain activated faster, but unautolysed μ -calpain activity decreased rapidly and was undetectable after 24 h. Autolysed μ -calpain activity was highest at 25 °C and 30 °C, peaking at 48 h post-mortem and remaining active up to 120 h, albeit at reduced levels. Minimal autolysed μ -calpain activity was observed at 2 °C and 15 °C throughout the incubation period.

Calpastatin is a calcium dependent endogenous inhibitor of μ - and m-calpain found in skeletal muscle (Bhat et al., 2018a). It has a molecular weight of 70 – 80 kDa and

consists of five domains, a leader domain and 4 domains for inhibition, each one having the ability to inhibit one molecule of calpain (Cruzen et al., 2014). The mechanism of inhibition involves the binding of calpastatin, in the presence of calcium, to the calpains active site, hence blocking the binding of substrates by competitive inhibition, and preventing the conformational change required for the calpains to become active (Goll et al., 2003, Hanna et al., 2008). The amount of calcium required by calpastatin to reach half maximal capacity is typically below that required by μ - and m-calpain, either in the autolysed or unautolysed forms (Huff Lonergan et al., 2010). Calpastatin is also a substrate for the calpain enzymes themselves and the degradation of the inhibitor has been linked with the rate of proteolysis in muscle (Huff Lonergan et al., 2010). In addition, a relationship between the μ -calpain: calpastatin activity ratio and the rate of tenderisation of muscle has been observed (Cruzen et al., 2014); where higher ratios are indicative of greater post-mortem protein degradation.

1.3.1.2 Cathepsins and Cystatins

Several cathepsins have been studied in relation to meat tenderness, in particular cathepsins B, H and L, which are cysteine peptidases and cathepsin D, which is an aspartic peptidase (Lomiwes et al., 2014a). In order for cathepsins to gain access to the myofibrillar proteins, they must be released from the lysosomes. This occurs as time post-mortem increases and the pH of muscle decreases towards pH 5.8, leading to the destabilisation of the lysosome membrane, resulting in the release of the cathepsins into the sarcoplasm (Boya and Kroemer, 2008, Lomiwes et al., 2014a, Zhang et al., 2019a).

The activity of cathepsins in post-mortem bovine *Longissimus dorsi* within the first 24 h post-mortem is minimal, however, cathepsin activity increases significantly with time post-mortem (O'Halloran et al., 1997b, Tian et al., 2013, Lomiwes et al., 2014b), as the lysosomes lose their structural integrity throughout the aging process, allowing the release of cathepsins into the cytosol. After 14 days post-mortem, lysosomal breakdown is almost complete (Zeece et al., 1992). When evaluating the activity of cathepsins B, B + L, D and H in bovine *Longissimus dorsi* over time, post-mortem, Caballero et al. (2007) found that cathepsin activity increased within the

first 7 to 14 days post-mortem, before activity decreased again. The most active enzyme appeared to be cathepsin D, while cathepsin H was the least active. It was reported that cathepsins B and B + L were active at 3 to 14 days post-mortem, while cathepsin D became important later from 14 to 21 days post-mortem. It has been suggested that cathepsins B and B + L play a role in activating pro – cathepsin D, a precursor of cathepsin D, while it has also been suggested that cathepsin D may act as a cysteine protease regulator. Both suggestions may explain the low activity of cathepsins B, L and H between 14 and 21 days post-mortem, when cathepsin D appears to be more active (Caballero et al., 2007). Cathepsin H activity appears to be negligible and does not make a significant contribution to the tenderisation of meat throughout the aging process (3 – 21 days post-mortem) (Chéret et al., 2007, Caballero et al., 2007).

Cathepsins, like calpains, exhibit pH dependent activity. Lomiwes et al. (2014b) demonstrated significantly higher cathepsin B activity throughout a 28 days aging period in low ultimate pH ($pH_u \leq 5.79$) bovine *Longissimus dorsi* compared to higher pH_u muscle ($pH_u \geq 6.2$), where cathepsin B activity remained low. Pulford et al. (2009) found that cathepsin B levels doubled between 22 h and 2 days post-mortem, when measured in bovine *Longissimus dorsi* in samples of different pH_u values (high pH_u – $pH > 6.3$, intermediate pH_u – $5.7 < pH < 6.3$, low pH_u – $pH < 5.7$). Cathepsin B activity declined in high pH_u muscle ($pH_u > 6.3$) after 5 days post-mortem but continued to increase in low pH_u muscle ($pH_u < 5.7$) up to 8 days post-mortem. Cathepsin B activity in intermediate pH_u ($5.7 < pH_u < 6.3$) muscle remained constant up until 8 days post-mortem. O'Halloran et al. (1997b) reported that fast glycolytic muscle with rapid pH decline (pH 6 h = 5.36 – 5.70) released higher levels of free cathepsin B and B + L from lysosomes compared to slow glycolytic muscle (pH 6 h = 5.91 – 6.65) at early post-mortem time points, although differences decreased after 6 days post-mortem. This higher activity of cathepsin B and L in lower pH_u conditions aligns with their optimal pH range (pH 5.5 – 6.0) and activity observed in 24 h post-mortem muscle (Aranishi et al., 1997, Hu et al., 2023, Peng et al., 2023).

When the activity of cathepsin D was measured, *in vitro*, under different pH and temperature conditions by Krause et al. (2011), it was found that commercial bovine

cathepsin D was active over a pH range of pH 4 to 5, whereas cathepsin D, extracted and purified from ostrich *Iliofibularis* was active over a pH range of pH 4 to 6. In the same study, bovine and ostrich cathepsin D was active over a broad temperature range of between 30 and 55 °C, and maximum cathepsin D activity was observed at 45 °C.

Cystatins, which are present in the cytoplasm, act as specific inhibitors of the cathepsins, since these can bind reversibly to the active site of the cathepsins. While cystatins bind more specifically to cathepsin L (Nagaraj and Santhanam, 2006), they also regulate the activity of cathepsin B and H (Chéret et al., 2007). Little research has been conducted on cystatins and the potential effects of their influence on meat quality. This is an area of research that warrants further investigation.

1.3.1.3 Proteasome Complex

The proteasome (26S), also known as a multi – catalytic protease complex, has various roles in cellular processes such as controlling the cell cycle, signalling, transcription, apoptosis and metabolism and antigen processing (Houbak et al., 2008). The proteasome consists of two parts, the first is a 19S regulatory cap structure and this is attached to a 20S catalytic structure (Houbak et al., 2008). The 26S proteasome is reversibly dissociated into its subunits, the 19S domain and 20S proteasome when ATP levels are depleted (Peters et al., 1994). The 20S catalytic structure is barrel shaped and consists of 28 protein subunits. It has three proteolytic active sites, each with their own specificities: chymotrypsin activity, peptidase glutamyl-peptide hydrolysing activity and trypsin like activity (Dutaud et al., 2006b).

The 26S proteasome requires ubiquitin tagging of proteins, facilitated by ATP hydrolysis, to be recognised and degraded by the enzyme (Taillandier et al., 2004). In contrast, the 20S proteasome operates independently of ATP and ubiquitin (Houbak et al., 2008) and therefore, has the potential to contribute to the tenderisation of muscle. Following the injection of 2 h post-mortem ovine *Longissimus lumborum* with PRY – 41 (inhibitor of ubiquitination) or MG – 132 (inhibitor of proteasome), Liu et al. (2016b) demonstrated that inhibiting ubiquitination and proteasome activity reduced myofibrillar and ultrastructure disruption. Houbak et al. (2008) found that the inhibition of the proteasome in bovine *Longissimus dorsi*, using lactacystin,

reduced nebulin, actin, troponin-T, and fast twitch myosin light chain degradation, when examined *in vitro*, implicating the proteasome in post-mortem proteolysis. Robert et al. (1999) showed that bovine *Longissimus* myofibrils incubated with 20S proteasome (extracted from liver) at 37 °C, resulted in the *in vitro* hydrolysis of nebulin, heavy myosin chain, troponin-T, tropomyosin, and α -actinin over 24 h. Dutaud et al. (2006a) observed a sequence of structural changes in myofibrils treated with proteasome, *in vitro*, including Z-line spreading, I-band masking, and eventual disintegration of Z-lines and M-lines. These findings indicate the proteasome's significant role in myofibril degradation post-mortem.

The S20 proteasome has been shown to remain active post-mortem, with substantial activity detectable as the pH decreases to pH 5.7 within the first 24 h post-mortem, and remained active after 7 days post-mortem in bovine *Rectus abdominis* (Lamare et al., 2002). The activity of the S20 proteasome decreases slowly throughout post-mortem, potentially due to structural changes (Lamare et al., 2002). Changes in S20 proteasome concentration overtime post-mortem were found to be independent of the rate of pH decline well as the ultimate pH of muscle, but has been shown to vary across different muscle types (Dutaud et al., 2006b). Fast twitch muscles such as *Longissimus* and *Tensor fascia latae* exhibited a faster loss of 20S proteasome concentration when compared to slow twitch oxidative muscles such as the *Diaphragma pedialis*, when aged for 16 days post-mortem (Dutaud et al., 2006b).

In vitro assays of 20S proteasome activity reveal distinct pH optima: peptidase glutamyl-peptide hydrolysis peaks at pH 7.5, while trypsin and chymotrypsin activities peak around pH 8.5. However, proteasome purified from bovine *Diaphragma pedialis*, assayed at pH 5.5 (similar to the ultimate pH of muscle), exhibits reduced activity (approximately 15 – 30% of its optimal activity) depending on the specific proteolytic activity being measured (Dutaud et al., 2006b). Studies on the influence of temperature tended to focus on the activity of proteasomes at higher temperatures with deactivation occurring above 60 °C (Thomas et al., 2004, Dutaud et al., 2006b), however, an increase in activity at 60°C was reported by Yamamoto et al. (2005).

The 20S proteasome is thought to be involved in the degradation of proteins that are already damaged and altered structurally (Robert et al., 1999, Lamare et al., 2002, Ben-Nissan and Sharon, 2014). This has led to the hypothesis that there is a synergistic relationship between calpains and the proteasome, with calpains disrupting the myofibrillar structure and releasing proteins, allowing these proteins to be hydrolysed further by the proteasome (Robert et al., 1999). The proteasome may also be involved in the proteolysis of autolysed μ -calpains which are less stable as a result of structural changes caused by autolysis (Lamare et al., 2002).

1.3.1.4 Caspase Systems

Caspase enzymes are a family of cysteine aspartate – specific proteases (Kemp and Parr, 2012) that are characterised for their specificity of cleaving proteins at aspartic acid residues and have a role in apoptosis; which can be described as a programmed cell death (Sentandreu et al., 2002, Kumar, 2007). The activity of this particular category of enzymes is prompted by the loss of cell homeostasis following the slaughter of animals (Pulford et al., 2009). Fourteen members of the caspase family have so far been identified, and these have been divided into two main categories. Those responsible for inflammatory response in cell include caspases-1, -4, -5, -11 and -13; whereas those responsible for apoptosis include caspases-3, -6, -7, -8, -9, -10, and -12. Apoptosis caspases are also categorised as initiator caspases, which are caspases-8, -9, -10 and -12, and effector caspases -3, -6 and -7 (Kemp and Parr, 2012). Initiator caspases are activated via a number of different pathways, which enables them to activate the effector caspases downstream. Caspases-8 and -10 are activated via receptors on the surface of cells and caspase-9 is activated by environmental stresses such as hypoxia, which causes dysfunction of the mitochondria. While caspase-12 is activated in response to stress in the endoplasmic reticulum (Kemp et al., 2006). These initiator caspases in turn activate the effector caspases directly through the limited proteolytic cleavage of a linker unit which separates a small and large subunit of the catalytic domain of the effector caspase. This cleavage allows the caspase to reach its maximal activity. Substrates of these effector caspases include myofibrillar proteins such as desmin, myosin, actin and troponin-T (Kemp and Parr, 2012).

The activity of caspase-3/7 has been shown to vary between muscles. Caspase-3/7 activity in porcine *Longissimus dorsi*, *Psoas*, *Trapezius* and *Semitendinosus*, collected within 5 min post-mortem, was investigated by Kemp et al. (2006). It was found that caspase-3/7 activity was highest in *Semitendinosus* and lowest in *Longissimus dorsi*, while the activity of caspase-3/7 did not significantly differ between *Psoas* and *Trapezius*. The activity of caspase-9 in bovine *Longissimus thoracis*, *Semitendinosus* and *Psoas minor* was investigated by Cao et al. (2014), who observed an increase in caspase-9 activity, between 1 and 5 h post-mortem and activity decreases rapidly thereafter reflecting that the activation of caspase-9 by the hypoxic effect (Cao et al., 2014). In addition, Cao et al. (2013) reported that the activity of initiator caspases, caspase-8 and -9 was high early post-mortem, usually within the first 4 h and this was then followed by a decrease in activity from 4 to 24 h. Simultaneously, there was an increase in the activity of the effector caspase, caspase-3, whose activity increased between 0 and 24 h and then decreased from 24 to 48 h.

Caspase activity in muscle is influenced by the ultimate pH and the rate of pH decline post-mortem. Pulford et al. (2009) observed that bovine *Longissimus dorsi* with high pHu (pHu > 6.3) exhibited lower caspase activity compared to low pHu (pHu < 5.7) muscles at 22 h post-mortem. Intermediate pHu (5.7 < pHu < 6.3) muscles initially had similar caspase activity to high pHu muscles but subsequently the rate of reduction in activity was slower resulting in activity levels resembling those of low pHu levels by 22 h post-mortem, possibly due to delayed apoptosis activation signals. Pulford et al. (2009) also monitored caspases-8 and -9, which activate caspases-3/7. They found decreasing activity of caspases-8 and -9 within the first 3 h post-mortem, with caspase-9 activity higher than caspase-8 and no differences between different pHu groups. Ma et al. (2022) reported higher caspase-3 activity in bovine *Longissimus thoracis* with fast pH decline (pH < 5.8 at 6 h post-mortem) compared to slower pH decline muscles (pH > 6.2 at 6 h post-mortem). Caspase-3 activity peaked at 24 h post-mortem before declining. Li et al. (2023) found that in bovine *Longissimus lumborum*, the intensity of the 17 kDa band, which is the activated fragment of caspase-3, was significantly higher in normal pHu (pH 5.4 – 5.8) muscles compared to intermediate (pH 5.8 – 6.2) and high (pH ≥ 6.2) pHu muscles at 24 h post-mortem.

Conversely, the relative intensity of the band corresponding to caspase-9 via Western – blot was significantly lower in high pHu muscles ($\text{pH} \geq 6.2$) when measured at 10 h post-mortem. According to the authors, this indicates that caspase-9 activity was greater in high pHu muscles, when compared to intermediate and normal pHu muscles.

Purified caspase enzymes are active, *in vitro*, over a wide pH range, from pH 5 to 8.5 approximately. Optimum activity for caspases-6, -7 and -8 is approximately pH 7 with caspase-3 exhibiting optimum activity at a slightly higher pH at pH 7.4. There is limited information on caspase activity in relation to temperature. When the stability of caspase enzymes was measured *in vitro* by Stennicke and Salvesen (1997), caspases-3, -6, -7 and -8 were stable at 0 °C for 150 min; however, at 37 °C, caspases-7 and -8 saw a reduction in activity over a period of 150 min, while caspase-3 and -6 remained stable and maintained full activity (Stennicke and Salvesen, 1997).

The activity of caspase enzymes has also been linked with the availability of ATP in the cells. Caspases have been shown to have a reduced activity as time progresses post-mortem, coinciding with the depletion of ATP from the muscle. It is proposed that ATP may act as an activator for the caspase enzymes early post-mortem. Thus when ATP becomes depleted post-mortem, the activity of the caspase enzymes decreases (Ferrari et al., 1998, Cao et al., 2014, Huang et al., 2014, McKenzie et al., 2020). These studies collectively highlight that caspase activity in muscle, post-mortem is influenced by pHu levels and the rate of pH decline, with implications for the timing and extent of apoptosis-related proteolytic processes.

1.3.1.5 Heat Shock Proteins

Heat shock proteins (HSPs) act as chaperones in response to various stresses like heat, oxidation, toxins, or ischemia, which occur post-slaughter. Stress triggers the expression of heat shock factors, which translocate to the cell nucleus and form trimers. Phosphorylation enables the heat shock factors to bind to heat shock elements in the gene promoter regions, thereby initiating the synthesis of heat shock proteins (Balan et al., 2014, Lomiwes et al., 2014a). These proteins play a role as a defense mechanism by assisting in protein folding and preventing aggregation under stressful conditions (Lomiwes et al., 2014a, Hu et al., 2022). HSPs potentially form

associations with myofibrillar proteins, protecting proteins such as desmin and troponin-T from μ -calpain action as well as inhibiting caspase-3 activation (Ding et al., 2018). They are categorised into five groups according to their molecular weight, 60 kDa, 70 kDa, 90 kDa and 100 kDa, namely HSP60, HSP70, HSP90 and HSP100. Small heat shock proteins (sHSPs) are the final group and these can range in sizes from 12 to 43 kDa (Lomiwes et al., 2014a, Dubey et al., 2015). The sHSPs have been the focus of research in relation to meat tenderness, in particular $\alpha\beta$ -crystallin, HSP20 and HSP27. These three sHSPs are associated with refolding of denatured proteins and preventing the aggregation of proteins in living cells (Pulford et al., 2008).

sHSPs have the potential to delay the aging process in muscle and degradation of myofibrillar proteins. This is achieved by the binding of sHSP to myofibrillar proteins to maintain the integrity of the muscle as well as becoming an alternative substrate for the protease enzymes, in particular the μ -calpain, and reducing the degree of myofibrillar protein degradation (Lomiwes et al., 2014a). In addition, Voss et al. (2007) concluded that HSP27 interacted directly with amino-terminal pro-domain of caspase-3, inhibiting a proteolytic cleavage of a precursor for caspase-3, which is necessary for the activation of the enzyme. Ding et al. (2018) demonstrated that sHSP27 had an impact on the *in vitro* degradation of myofibrillar proteins. When caspase-3 and μ -calpain were incubated with myofibrillar proteins (extracted from 30 min post-mortem bovine *Longissimus thoracis*), for 2 h at 30 °C, with or without sHSP27, it was found that sHSP27 restricted the degradation of troponin-T and desmin, suggesting that sHSP27 has a role in regulating the activity of caspase-3 and μ -calpain, through competitive inhibition. However, the presence of sHSP27 had no impact on the degradation of titin and nebulin. Similarly, Lomiwes et al. (2014c), who investigated the effect of the addition of $\alpha\beta$ -crystallin on the *in vitro* degradation of desmin and titin (in myofibrils from bovine *Longissimus dorsi*) by μ -calpain, found that the addition of $\alpha\beta$ -crystallin reduced the degradation of desmin and titin and restricted μ -calpain activity. In addition, the findings of several studies support the hypothesis that the degradation of sHSPs post-mortem results in the loss of their anti-apoptotic function, which facilitates proteolysis of myofibrillar proteins

(Lomiwes et al., 2013, Lomiwes et al., 2014c, Balan et al., 2014, Ma and Kim, 2020, Ding et al., 2021).

It has been proposed that sHSPs modulate post-mortem proteolysis, leading to variation in meat tenderness (Lomiwes et al., 2014a, Oh et al., 2019). Some studies have demonstrated that there is a negative relationship between the concentration of sHSPs, such as HSP20, HSP27 and $\alpha\beta$ -crystallin, in muscle and meat tenderness (Lomiwes et al., 2014a, Gagaoua et al., 2020), while Ma and Kim (2020) reported that the presence of intact sHSPs reduces as the time post-mortem is extended, reducing their ability to protect the proteins from degradation. The reduction in pH, post-mortem, may also have an effect on the concentration sHSP in muscle, especially HSP27 and $\alpha\beta$ -crystallin. Pulford et al. (2008) evaluated changes in levels of sHSPs in bovine *Longissimus dorsi* throughout the first 22 h post-mortem. It was found that the levels of $\alpha\beta$ -crystallin decreased within the first 30 min post-mortem before increasing and reaching a maximum at 3 h post-mortem. A reduction in $\alpha\beta$ -crystallin levels was observed after 6 h and 22 h post-mortem. When the levels of HSP20 was investigated, it was found that the amount of HSP20 peaked at 30 min post-mortem. HSP20 levels then remained constant until 3 h post-mortem before declining up until 22 h post-mortem. At 22 h post-mortem, the levels of sHSP appear to be linked to the ultimate pH of muscle, where the lowest levels of sHSP were found in low pHu (pHu < 5.7) muscle, followed by muscle with an intermediate pHu (5.7 < pHu < 6.3) and the highest levels of sHSP were found in the high pHu (pHu > 6.3) group (Pulford et al., 2008). Similar trends were observed in 21 day post-mortem bovine *Longissimus lumborum* (Zhao et al., 2022) and 28 day post-mortem bovine *Longissimus dorsi* (Lomiwes et al., 2013), where high (pH > 6.2) and intermediate (pH 5.8 – 6.2) pHu muscle had higher relative intensity bands corresponding to HSP27 via Western – blot, at the end of the respective aging periods, when compared to low/normal pHu muscle (pH < 5.8).

Ma and Kim (2020) reported that the extent of degradation of sHSP post-mortem is muscle specific, with bovine *Longissimus lumborum* exhibiting a greater degradation of HSP27 when compared to *Semimembranosus* and *Psoas major*, regardless of aging time, up to 23 days post-mortem. In addition, these authors concluded that lower

levels of HSP27 degradation post-mortem leads to lower μ -calpain autolysis and lower or delayed cleavage of caspase-3, all of which decrease the extent of proteolysis. Interestingly, it has been found that *Longissimus thoracis et lumborum* from callipyge lamb exhibits less HSP27 degradation products throughout the aging process than those from control lambs. The former also had more intact desmin and troponin-T and higher Warner-Bratzler shear force values. The callipyge lamb also contained a higher abundance of intact μ -calpain for up to 9 days post-mortem, and had a lower level of 76 kDa subunit of μ -calpain, when compared to other normal genotypes (Cramer et al., 2018).

Table 1-2. The effect of pH and temperature on the activity of key proteolytic enzymes in muscle.

Enzyme	Sample details	Effect of temperature on enzyme activity	Effect of pH on enzyme activity	Method of analysis	Reference
Calpains	Enzymes extracted from porcine <i>Semimembranosus</i> at 25 min post-mortem	N/A	μ -calpain most active at pH 6.5; m-calpain most active at pH 7.5	Intensity of fluorescence signal after cleaving of specific substrate	(Carlin et al., 2006)
Calpains	Porcine <i>Longissimus dorsi</i> at 1, 6, 24, 72 h post-mortem	N/A	Rapid pH decline ($\text{pH}_{3\text{h}} < 6$) early post-mortem accelerates decay in native μ -calpain activity but increases activity of autolysed form at 72 h post-mortem; at 72 h post-mortem slow pH decline ($\text{pH}_{3\text{h}} > 6.3$) had greatest μ -calpain activity; rate of pH decline did not affect m-calpain activity over time	Casein zymography.	(Pomponio et al., 2010)
Calpains	Porcine <i>Longissimus dorsi</i> at 2, 6, 24, 48 and 120 h post-mortem; muscle incubated at different temperatures from 2 h post-mortem	High temperatures (30 °C) accelerates decline in native calpain activity, and increases activity of autolysed forms	N/A	Casein zymography.	(Pomponio and Ertbjerg, 2012)
Calpains	Purified μ - and m-calpain from bovine	μ - and m-calpain most active when incubated at 25 °C	μ - and m-calpain most active between pH 7 and 7.5, very	FITC – casein assay.	(Camou et al., 2007)

	<i>Diaphragm</i> muscle 30 – 45 min post-mortem		little proteolytic activity at pH 5.8		
Calpains	Bovine <i>Longissimus dorsi</i> at 0.5, 1, 3, 6, 22 h and 2, 3, 5, 8 days post-mortem	N/A	Low pHu (pH < 5.7) induced rapid decline in μ -calpain activity after 1 day post-mortem; μ -calpain activity higher in high (pH > 6.3) and intermediate (5.7 < pH < 6.3) pHu muscle after 22 h post-mortem; pHu did not affect m-calpain activity	Intensity of fluorescence signal after cleaving of specific substrate	(Pulford et al., 2009)
Calpains	Bovine <i>Longissimus dorsi</i> at 45 min, 1, 2, 7, 14 and 28 days post-mortem	N/A	Rapid autolysis of μ -calpain in high pHu muscle (pH > 6.2) when compared to low pHu (pH < 5.79) muscle	Immunoblots	(Lomiwes et al., 2014b)
Calpains	Bovine <i>Longissimus thoracis et lumborum</i> at 0.5, 4 and 24 h post-mortem	μ -calpain activity of slow chilled samples ($T_{1.5 \text{ hr}} = 33 \text{ }^{\circ}\text{C}$) lower than μ -calpain activity in rapidly chilled sample ($T_{1.5 \text{ hr}} = 23 \text{ }^{\circ}\text{C}$) at 4 h post-mortem; m-calpain activity not affected by temperature decline.	μ -calpain activity at 24 h post-mortem independent of pH decline; m-calpain activity not affected by pH decline.	Casein zymography	(Hwang and Thompson, 2001b)
Cathepsins	Bovine <i>Longissimus dorsi</i> at 45 min, 1, 2, 7, 14 and 28 days post-mortem	N/A	Cathepsin B activity higher in low pHu (pH < 5.79) muscle compared to high pHu (pH > 6.3) muscle	Intensity of fluorescence signal after cleaving of specific substrate	(Lomiwes et al., 2014b)

Cathepsins	Bovine <i>Longissimus dorsi</i> at 0.5, 1, 3, 6, 22 h and 2, 3, 5, 8 days post-mortem	N/A	Cathepsin B activity declined more rapidly in high pHu (pH > 6.3) muscle, compared to low pHu (pH < 5.7) muscle	Intensity of fluorescence signal after cleaving of specific substrate	(Pulford et al., 2009)
Proteasome 20S Complex	Purified from bovine <i>Diaphragm pedialis</i> within 1 h post-mortem	Optimum temperature 35 °C; Activity decreased sharply at temperatures greater than 35 °C; Inactive at 60 °C	Optimum activity between pH 7.5 – 8.5, depending on activity type.	Intensity of fluorescence signal after cleaving of specific substrate	(Dutaud et al., 2006b)
Proteasome 20S Complex	Crude extracts prepared using bovine <i>Rectus abdominis</i> muscle removed immediately post-mortem	N/A	Optimum activity between pH 6.5 and 8, depending on activity type.	Intensity of fluorescence signal after cleaving of specific substrate	(Lamare et al., 2002)
Proteasome 20S Complex	Purified from bovine shoulder muscle at 2 days post-mortem	Activity increased as the temperature increased from 37 °C to 60 °C.	Optimum activity measured at pH 8.	Intensity of fluorescence signal after cleaving of specific substrate	(Yamamoto et al., 2005)
Caspase System	Bovine <i>Longissimus dorsi</i> at 0.5, 1, 3, 6, 22 h, 2, 3, 5, 8 days post-mortem	N/A	Caspase 3/7 activity lower in high pHu (pH > 6.3) meat compared to low pHu (pH < 5.7) meat. Caspase 8 and 9 activity not affected by pHu	Intensity of fluorescence signal after cleaving of specific substrate	(Pulford et al., 2009)
Caspase System	Caspase-3, -6, -7 and -8 expressed and isolated from <i>E.coli</i>	Caspases-3, -6, -7 and -8 stable at 0 °C; Caspase-3 and -6 retained full activity at 37 °C;	Caspases-3, -6, -7 and -8 active over wide pH range (pH 5 – 9); Optimum activity of caspase-6, -7, and -8 measured at pH 7;	Intensity of fluorescence signal after cleaving of specific substrate	(Stennicke and Salvesen, 1997)

	Activity measured after incubated for 150 min	Caspase-7 and-8 activity reduced at 37 °C.	Optimum activity of caspase-3 measured at pH 7.5		
Heat Shock Proteins	Bovine <i>Longissimus dorsi</i> at 0.5, 1, 3, 6 and 22 h post-mortem	N/A	At 22 h post-mortem, high pHu (pH > 6.3) meat had the highest amount of sHSP (sHSP20 and $\alpha\beta$ -crystalline); Low pHu (pH < 5.7) meat containing the least amount of sHSP (sHSP20 and $\alpha\beta$ -crystalline).	Western Blotting and ELISA.	(Pulford et al., 2008)
Heat Shock Proteins	Bovine <i>Longissimus dorsi</i> at < 1 h, 1, 2, 7, 14 and 28 days post-mortem	N/A	Faster degradation of sHSP20, sHSP27 and $\alpha\beta$ -crystalline in low pHu (pH < 5.79) meat compared to high (pH > 6.2) and intermediate pHu (pH 5.8 – 6.19) meat; high pHu meat had highest concentration of sHSP (sHSP20, sHSP27 and $\alpha\beta$ -crystalline) at 28 days post-mortem	Western Blot and ELISA.	(Lomiwes et al., 2013)

1.4 Protein post-translational modifications of endogenous enzymes in post-mortem muscle

Post-translational modifications refer to any addition, removal or transformation of a proteins chemical structure following the biosynthesis of a particular protein within a living organism. These modifications play a role in the functionality and behaviour of the proteins, such as enzyme activity, protein-protein interactions and stability of the proteins (Cruz et al., 2019, Suskiewicz, 2024). Numerous different modifications that have been identified, (Li et al., 2021, Ramazi and Zahiri, 2021, Fan et al., 2024), however, for the purpose of the current review, phosphorylation and S-nitrosylation, both of which may influence glycolysis and proteolysis in post-mortem muscle, will be focused on. S-nitrosylation, induced by hypoxia and nitric oxide, inhibits enzymes such as GP and PK, thereby slowing glycolysis (Liu et al., 2018, Hou et al., 2019). In contrast, phosphorylation enhances the activity of key glycolytic enzymes, impacting lactic acid production and muscle pH (Li et al., 2017a, Chen et al., 2019).

1.4.1 Phosphorylation

Phosphorylation influences a wide variety of cellular functions and activities, including energy metabolism, apoptosis and the translocation of heat shock proteins, all of which have effects on post-mortem proteolysis and quality parameters such as water holding capacity and tenderisation (Ma and Kim, 2020). Protein phosphorylation occurs when protein kinase transfers a phosphate from ATP or GTP (guanine triphosphate) to the hydroxyl group of an amino acid, typically serine, tyrosine and threonine residues (Ubersax and Ferrell Jr, 2007, Zhang et al., 2020). This process is reversible, with the phosphate removed from a phosphoprotein by phosphatase via hydrolysis (Zhang et al., 2020, Ardito et al., 2017).

The influence of protein phosphorylation on the tenderness of ovine *Longissimus dorsi* was investigated by Chen et al. (2016). The results show that muscle classified as tough (having higher shear force values, 13.06 ± 2.42 kg v 7.57 ± 7.39 kg for tender muscle at 24 h post-mortem), had significantly higher levels of protein phosphorylation compared to tender muscle when measured at 4, 12 and 24 h post-mortem. In the tender group, it was found that the level of phosphorylation increased from 0.5 to 12 h post-mortem and then decreased. In contrast, in tough

muscle phosphorylation increased during the first 24 h post-mortem, where levels of phosphorylation increased more rapidly within the first 4 h post-mortem compared to between 4 and 24 h post-mortem. The results show that many of the proteins that were highly phosphorylated were involved in regulating the contraction of skeletal muscle as well as the sarcomeric functions of the muscle such as titin, heavy myosin chains and heat shock proteins, while others were involved in glycolysis such as GP and PFK (Chen et al., 2016).

The level of phosphorylation of myofibrillar proteins may also affect the extent of post-mortem proteolysis. Li et al. (2017b) reported that protein phosphorylation decreased the *in vitro* proteolysis of myofibrillar proteins (extracted from ovine *Longissimus dorsi* at 30 min post-mortem) by μ -calpain while dephosphorylation had the opposite effect, especially in relation to desmin and troponin-T. In contrast, phosphorylation did not appear to affect the degradation of myosin heavy chain and actin. Results from a study by Du et al. (2019) suggest that *in vitro* phosphorylation enhances the inhibitory effect of calpastatin on μ -calpain activity, while dephosphorylation has the opposite effect. Li et al. (2018) used protein kinase (staurosporine (M2066)) and phosphatase (PhosStop™) inhibitors to modulate the phosphorylation and dephosphorylation of myofibrillar proteins (extracted from ovine *Longissimus thoracis lumborum*) and reported that myofibrillar proteins treated with a protein kinase inhibitor had lower levels of phosphorylation compared to controls and phosphatase inhibitor group and had a higher myofibrillar fragmentation index at 2 and 24 h post-mortem. The lower levels of phosphorylation in the kinase inhibition group also had an impact on the degradation of individual proteins. The increased level of phosphorylation, as a result of the addition of the phosphatase inhibitor, appeared to inhibit the degradation of troponin-T and HSP27, while desmin degradation was not affected by phosphorylation levels when compared to controls.

1.4.2 S – Nitrosylation

Protein S-nitrosylation is a post-translational modification that occurs when nitrous oxide is covalently bonded to a sulfhydryl moiety of cysteines in protein, generating a S-nitrosothiol (Liu et al., 2018). Nitric oxide is a signalling molecule in the body and

can act as a modulator to regulate contraction and relaxation of muscles, myocyte differentiation as well as respiration and glucose uptake in the cell (Liu et al., 2019). Therefore, S-nitrosylation may have a role to play in regulating enzymatic activity and have an impact on the final meat quality in terms of colour, tenderness, or water holding capacity (Liu et al., 2019). Protein S-nitrosylation has been studied in relation to its potential effects on post-mortem proteolysis. The effect of S-nitrosylation on μ -calpain autolysis and the proteolysis of purified myofibrils was evaluated by Liu et al. (2016a), who reported that S-nitrosylation of μ -calpain (extracted from porcine *Longissimus lumborum*) was promoted by the addition of S-nitroglutathione (GSNO), a nitric oxide donor. Following the incubation of μ -calpain with GSNO (for 30 min at 37 °C), the relative intensity of the intact μ -calpain 80 kDa subunit increased and autolysed 76 kDa μ -calpain subunit decreased as the concentration of GSNO increased from 0 – 300 μ M, indicating that GSNO inhibited the autolysis of μ -calpain. Furthermore, when μ -calpain was treated with GSNO, particularly at concentrations greater than 300 μ M, and subsequently added to purified myofibrils (extracted from porcine *Longissimus lumborum*), a reduction in the degradation of titin, desmin, troponin-T and nebulin was observed. In samples containing μ -calpain incubated with 1,000 μ M GSNO, 87.9% of desmin was found to remain intact, in contrast to controls, where 97.4% of desmin was degraded. Zhang et al. (2018a) found that following the incubation of bovine *Semimembranosus* strips (1 x 1 x 3 cm) with N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME), an inhibitor of S-nitrosylation, for 24 h at 4 °C, there was an increase in the degradation of desmin and troponin-T when compared to muscle incubated in GSNO, following 1, 4 and 7 days storage at 4 °C. Similar results were reported by Hou et al. (2020b), where the incubation of bovine *Semimembranosus* strips (1.5 x 5 x 5 cm) in GSNO (for 24 h at 4 °C) inhibited the autolysis of μ -calpain and degradation of desmin at 1 day post-mortem, while L-NAME increased μ -calpain autolysis and desmin degradation, compared to controls. At 7 days post-mortem, no significant difference in intact desmin content was detected between control and L-NAME treated samples, however, the GSNO treated samples had a higher level of intact desmin, demonstrating that S-nitrosylation had an impact on post-mortem proteolysis. S-nitrosylation consequently had an effect on the shear force values of bovine *Semimembranosus*. No significant differences

between control and treated samples were evident at 1 day post-mortem. However, at 7 days post-mortem, GSNO treated samples had significantly higher shear force values when compared to controls, while L-NAME treated samples had significantly lower shear force values compared to controls (Hou et al., 2020b).

S-nitrosylation also appears to have the ability to impact the activity of caspases, where the incubation (24 h at 4 °C) of bovine *Semimembranosus* strips (1.5 x 5 x 5 cm) in GSNO inhibited the activity of caspase-3 and -9 and reduced the expression of caspase-3, while L-NAME promoted the activation of caspases, indicating that protein S-nitrosylation can inhibit cell apoptosis (Hou et al., 2020a). Interestingly, S-nitrosylation also has the potential to affect glycolysis (Wang et al., 2019). When investigating the differences in the levels of S-nitrosylation in pale, soft and exudative (PSE) (pH < 6.0) and red, firm and non-exudative (RFN) (pH > 6.0) porcine *Longissimus thoracis*, Wang et al. (2020) found that GP, PFK and PK exhibited higher S-nitrosylation levels in RFN samples compared to PSE samples, indicating that S-nitrosylation may have a role in regulating glycolytic enzyme activity. S-nitrosylation has been found to regulate the rate and extent of post-mortem glycolysis in porcine *Longissimus lumborum*, where it was found that muscle (10 x 10 x 1 cm) incubated in L-NAME, for 24 h at 4 °C, exhibited a greater rate and extent of pH reduction compared to controls and samples incubated in GSNO. It was also found that glycogen levels were significantly lower and lactate content was significantly higher than control and GSNO samples at 4 and 24 h. There was no difference between the rate and extent of pH decline or glycogen metabolism in control samples and samples treated with GSNO. When glycolytic enzyme activity was evaluated at 24 h post-mortem, GP and PK activity was greatest in samples treated with L-NAME and the lowest in the GSNO group (Zhang et al., 2019b). PFK activity has also reported to be effected by S-nitrosylation, where higher levels of S-nitrosylation in RFN porcine *Longissimus thoracis* decreased the activity of PFK, when measured at 1 h post-mortem (Wang et al., 2020).

Thus, protein S-nitrosylation significantly impacts post-mortem proteolysis by modulating the activity of μ -calpain and caspases, thereby affecting muscle protein degradation and tenderness. Additionally, S-nitrosylation influences glycolytic

enzyme activity, which in turn regulates the rate and extent of post-mortem glycolysis, ultimately affecting the rate of pH decline and final eating quality.

1.5 Future work and conclusions

Two major biochemical pathways, glycolysis and proteolysis, influence the process of the conversion of muscle to meat, and subsequent quality of the meat, particularly in terms of final tenderness. Post-mortem glycolysis is responsible for the reduction of pH within the first 24 h post-mortem. This reduction in muscle pH and its interaction with temperature has an overriding influence on many of the key enzymes involved in these two processes. The decrease in pH has an impact on the glycolytic enzymes themselves, in particular PFK, which has been identified as the principle rate-limiting enzyme for glycolysis. pH decline and temperature also has an impact on the proteolytic enzyme system in muscle. Post-mortem proteolysis is the result of the combined activity of various enzymes such as μ -calpains, cathepsins, proteasomes and caspases. Heat shock proteins also have an influence on proteolysis; although their mode of action still needs to be clarified.

While extensive work has been carried out in muscle and in *in vitro* systems on the effects of pH and temperature on glycolytic and proteolytic enzymes individually and collectively, it has proven difficult to control glycolysis and proteolysis in muscle, post-mortem in order to obtain optimal and uniform quality in meat. In addition, further work is needed to deepen our understanding on the effects of various technologies such as ultrasound, pulsed electrical fields and high hydrostatic pressure on glycolysis and proteolysis to maximise their potential to manipulate these processes and ultimately improve meat quality.

Chapter 2. Carcass interventions and their ability to impact endogenous enzymatic systems and quality of meat.

Overview

This literature review explores the application of traditional and innovative technologies as carcass interventions to influence post-mortem enzymatic activity, with an emphasis on improving tenderness. Traditional methods such as aging, allows endogenous enzymes to tenderise meat, while electrical stimulation is applied to manipulate glycolysis and proteolysis and physically disrupt the muscles. Hanging and hot boning have been modified to physically manipulate and stretch target muscles. Innovative processes such as pulsed electric fields, ultrasound and high pressure processing have been investigated for their ability to manipulate glycolysis and proteolysis, while also physically disrupting the structure of muscle. While variable effects have been observed, there is scope to further investigate the biochemical effects following the application of these technologies through the use of proteomics and metabolomics.

2.1 Introduction

Meat tenderness, as well as flavour, juiciness and colour, are important organoleptic qualities of meat that influence consumers' satisfaction, willingness to pay a premium and repeat purchases (Pethick et al., 2021, Liu et al., 2022a). The final tenderness of muscle is influenced by multiple pre- and post-slaughter factors such as diet, age, gender and size of the animal as well as the application of various carcass handling/interventions (Chauhan and England, 2018, Cadavez et al., 2019, Hwang et al., 2003, Hwang and Thompson, 2001b). The dominant biochemical events that occur early post-mortem and influence the final eating quality of muscle are glycolysis and proteolysis. Glycolysis involves a series of reactions involving the conversion of glycogen to lactic acid, resulting in the decline in pH in post-mortem muscle (Álvarez et al., 2019, Frylinck et al., 2013), while proteolysis is carried out predominantly by calpain enzymes and results in the degradation of proteins such as troponin-T, titin and nebulin (Huff Lonergan et al., 2010, Cruzen et al., 2014).

To achieve optimum tenderness and avoid hot or cold shortening, the concept of an optimum temperature/pH window was established by Meat Standards Australia (MSA), where it is recommended that the temperature of muscle post-mortem is between 15 and 35 °C at the time when pH 6 is reached (Thompson, 2002). Electrical stimulation operates by accelerating the rate of glycolysis and subsequent pH decline in muscle, enabling the rapid chilling of carcasses (Li et al., 2015, White et al., 2006a), while also altering the post-mortem proteolysis via modulating proteolytic enzyme activity and mechanical disruption of muscle (Li et al., 2012, Chen et al., 2020).

Traditional carcass interventions/handling procedures, such as hanging and aging have been used to enhance tenderness. Hanging (achilles and pelvic suspension) manipulates the position of the carcass, reducing muscle shortening (Moran et al., 2021), while aging (wet and dry) allows endogenous enzymes to naturally tenderise and enhance the flavour of meat over time (Khan et al., 2016). Hot boning in combination with electrical stimulation or wrapping methods such as Pi-Vac wrapping, can affect tenderness (White et al., 2006a, O'Sullivan et al., 2003). However, these methods can produce inconsistent results.

Innovative technologies such as pulsed electric fields, ultrasound, and high pressure processing have been investigated as potential tools to alter the tenderness of muscle, either mechanically by disrupting muscle structure and lysosomal membranes or enzymatically by altering calpain activity and facilitating the release of cathepsins (Table 2-1).

This review will explore the effects of traditional and innovative carcass interventions on post-mortem glycolysis and proteolysis, with a particular focus on beef, and examine their influence on meat quality.

Table 2-1. Traditional and innovative post-mortem interventions applied on muscle, categorised based on their mechanism of action.

Intervention	Mechanism of action		Novelty of intervention		Reference
	Mechanical	Biochemical	Traditional	Innovative	
Hanging	✓	✓	✓		(Ahnström et al., 2012a, 2012b, Baldassini et al., 2023, Hou et al., 2014, Moran et al., 2021)
Aging		✓	✓		(Kim et al., 2019, Kim et al., 2022, Ha et al., 2019, Lee et al., 2021, Khazzar et al., 2023, Sadowska et al., 2020, Shi et al., 2020)
Hot boning	✓		✓		(Taylor et al., 2013, Taylor and Toohey, 2011, Taylor et al., 2012, Toohey et al., 2012, O'Sullivan et al., 2003, Crownover et al., 2017, Neto et al., 2013).
Electrical Stimulation	✓	✓	✓		(Chen et al., 2020, Agbeniga and Webb, 2021, Webb and Agbeniga, 2020, Kim et al., 2013, Gursansky et al., 2010, White et al., 2006a)
Pulsed Electric Fields	✓	✓		✓	(Bhat et al., 2019a, Bhat et al., 2019b, Beño et al., 2023, Bhat et al., 2020, Guo et al., 2024c, He et al., 2024, Mungure et al., 2020, Zhang et al., 2022)
Ultrasound	✓	✓		✓	(Dang et al., 2022, Kent et al., 2024a, Kent et al., 2024b, de Lima et al., 2024, Gao et al., 2024, Marino et al., 2023, Wang et al., 2022)
High Pressure Processing	✓	✓		✓	(Morton et al., 2018, Lee et al., 2023, Ma et al., 2020, Utama et al., 2017, Utama et al., 2018, Neto et al., 2015)

2.2 Traditional Carcass Interventions/Handling Procedures

Aging, which is a traditional carcass intervention, allows endogenous enzymes, present naturally in muscle, to perform proteolysis, tenderising the meat. Electrical stimulation is a long established carcass intervention/handling procedure which manipulates the rate of pH decline by modifying the activity of glycolytic enzymes. In addition, electrical stimulation alters the activity of proteolytic enzymes, while also causing physical disruption to the muscle, aiding in the tenderisation of muscle. Finally, modifications to these traditional carcass interventions and hanging methods, such as the hanging position of carcasses (Achilles vs. Pelvis) and use of wrapping in conjunction with hot boning muscle, have been investigated as processes to improve meat tenderness and will be discussed in this section.

2.2.1 Hanging, hot boning and aging method

The conventional hanging method for carcasses or sides post-mortem involves suspension from the Achilles tendon (Figure 2-1A), however, other methods have been investigated for their impacts on tenderness. Pelvic suspension (Figure 2-1B), otherwise known as tender-stretching, is carried out by suspending the carcass by the aitch bone or pelvic ligament, leading to many of the muscles in the hindquarter and back of the animal being stretched and prevented from shortening (Thompson, 2002, Thompson et al., 2006).

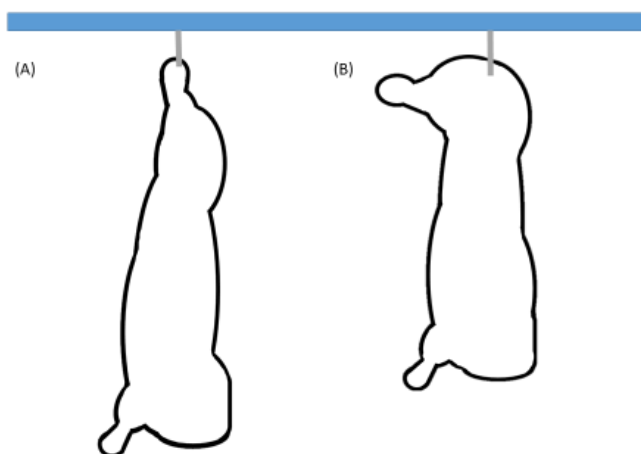


Figure 2-1. Different methods of hanging carcasses post-slaughter via A) the Achilles tendon and B) pelvic suspension, adapted from Ahnström (2008).

Pelvic suspension has been found to increase tenderness of muscle. Hou et al. (2014) found that at 1 and 7 days post-mortem, bovine *Longissimus dorsi* following pelvic suspension had significantly lower shear force values than the same muscle following Achilles suspension. Moreover, the shear force values of *Longissimus dorsi* muscles at 7 days post-mortem, where pelvic suspension was used, were equal to those at 14 days post-mortem using Achilles suspension. A similar trend was observed by Baldassini et al. (2023) when evaluating the impact of suspension method on bovine *Longissimus thoracis et lumborum*. The effect of suspension method on the shear force values is dependent on the muscle being examined. Pelvic suspension increased the sarcomere length and improved the instrumental tenderness of bovine *Longissimus dorsi*, *Semimembranosus* and *Gluteus medius*, while suspension method did not have an impact on the shear force values of *Psoas major*, *Adductor* and *Semitendinosus* (Ahnström et al., 2012b, Moran et al., 2021). Interestingly, it has been observed that pelvic suspension also significantly reduces purge loss during aging, which was attributed to the increase in sarcomere length (Ahnström et al., 2012a, 2012b, Hou et al., 2014).

Hot boning involves the removal of muscle from the carcass pre-rigor and prior to chilling and has been investigated as a strategy to allow for the subsequent optimal treatment of individual muscles (e.g. designing muscle specific chilling and electrical stimulation regimes) (Sørheim and Hildrum, 2002, White et al., 2006a). The main impact hot boning has on meat quality is increasing shear force values. White et al. (2006a) (hot boned bovine *Semimembranosus* and *Longissimus dorsi* within 90 min post-mortem) and Lang et al. (2016) (hot boned bovine *Longissimus lumborum* within 45 min post-mortem), found that hot boning leads to significantly higher shear force values when compared to the same muscles that were cold boned (removed following 48 or 72 h post-mortem, respectively). To combat the increase in shear force values that occurs following hot boning (White et al., 2006a, Neto et al., 2013, Lang et al., 2016), various methods have been developed to reduce the shortening, predominantly through the wrapping of muscle (Devine et al., 1999, Kim et al., 2012, Crownover et al., 2017) such as SmartStretch™/SmartShape™ (Taylor and Toohey, 2011, Taylor et al., 2013, Taylor et al., 2012, Toohey et al., 2012) or Pi-Vac Elasto-

Pack/Tenderbound (Wahlgren and Hildrum, 2001, O'Sullivan et al., 2003, Troy, 2006). These methods involve wrapping and restraining the muscle in various forms of plastic wrapping, preventing them from shortening. Wrapping the hot boned muscle mimics the restraint that occurs naturally when the muscle is on the skeleton during cold boning (Devine et al., 1999). While these methods have been found to be effective in reducing the shear force values and increasing the sarcomere length of bovine muscle, some studies have found the effect of wrapping hot boned muscle to be short term, where following aging for 14 day post-mortem, no significant differences appear between wrapped and non-wrapped muscle (Kim et al., 2012, Toohey et al., 2012, Taylor et al., 2013). However, other studies have observed a more sustained effect following wrapping (Wahlgren and Hildrum, 2001, O'Sullivan et al., 2003). The use of Pi-Vac Elasto-Pack wrapping, which involves inserting muscle into a flexible elastic material under vacuum and tightly wrapping muscle in film, was investigated by O'Sullivan et al. (2003), who hot boned 90 min post-mortem bovine *Longissimus dorsi* and found that Pi-Vac wrapping significantly reduced the shear force values of muscle at 7 and 14 days post-mortem when compared to controls, which were not Pi-Vac wrapped, and increased the tenderness scores of 14 day post-mortem muscle when assessed by a trained sensory panel. Pi-Vac wrapping also resulted in longer sarcomere lengths and reduced drip loss (O'Sullivan et al., 2003). When comparing the use of Pi-Vac wrapping to aging 1 h post-mortem bovine *Longissimus dorsi* in vacuum packaging, Wahlgren and Hildrum (2001) found that aging hot boned muscle in Pi-Vac wrapping for 2 and 9 days post-mortem significantly decreased the shear force values of muscle, when compared to muscle aged in vacuum packaging.

The hot boning of bovine muscle with other technologies such as electrical stimulation (White et al., 2006a, Lang et al., 2016), pulsed electric fields (Suwandy et al., 2015b, Bekhit et al., 2016), ultrasound (Got et al., 1999, Sikes et al., 2014, Lyng et al., 1998b) and high pressure processing (Morton et al., 2017, Morton et al., 2018) have also been investigated and are discussed in their respective sections.

Wet aging involves the aging of muscle in vacuum sealed bags under refrigerated conditions for 7 to 21 days (Dashdorj et al., 2016). In contrast, dry aging is a process

where unwrapped primals or subprimals are held under controlled conditions (temperature, relative humidity and air flow) for a number of weeks (Parrish Jr et al., 1991, Smith et al., 2008, Dashdorj et al., 2016, Vossen et al., 2022). While dry aging is not a new technology, there has been an increase over the past decade in research on this topic (Álvarez et al., 2023, Correa et al., 2024, Vossen et al., 2022) involving comparing quality characteristics of wet and dry aged muscle and investigating ways to reduce weight and trimming losses (Kim et al., 2016, Berger et al., 2018, Kim et al., 2022, Ha et al., 2019, Setyabrata et al., 2021, Setyabrata et al., 2022a, Lee et al., 2021, Kim et al., 2019, Bischof et al., 2023, Savas et al., 2024, Setyabrata et al., 2022b). Dry aging has been found to have similar shear force values, colour and cook loss (%) when compared to wet aged muscle, however, dry aged muscle has superior sensory scores for flavour, perceived tenderness and overall liking compared to wet aged counterparts (Kim et al., 2019, Kim et al., 2016, Ha et al., 2019, Berger et al., 2018). Dry aging of muscle leads to shrinkage and formation of a crust, due to the loss of water from the surface of the muscle, while moisture simultaneously migrates from the internal portion of the muscle to the surface (Ha et al., 2019, Li et al., 2014b, Álvarez et al., 2021). To combat this, a method of dry aging muscle in a water permeable bag has been developed and has been found to reduce shrinkage and trim loss, while having comparable quality characteristics to dry aged muscle (Berger et al., 2018, Dikeman et al., 2013).

2.2.2 Electrical Stimulation (ES)

Electrical stimulation was developed primarily in New Zealand and Australia to optimise meat quality, especially tenderness, by accelerating the rate of pH decline in early post-mortem muscle and enabling a rapid chilling regime (Adeyemi and Sazili, 2014). Electrical stimulation also physically disrupts the muscle fibres and alters the rate of proteolysis (Luo et al., 2008). Low or high voltage electrical stimulation, where voltages of less than 100 V or up to 1300 V, respectively, may be applied. Low voltage electrical stimulation (LVES) is applied shortly after stunning and bleeding, and involves the transfer of electrical current through the nervous system (Polidori and Vincenzetti, 2017) (Figure 2-2A). LVES is cheaper and safer to install and apply when compared to high voltage electrical stimulation (HVES), however, the electrode must

be manually applied making it labour intensive (Adeyemi and Sazili, 2014). HVES allows for application later post-mortem, as electrical current is delivered to the carcass directly (Polidori and Vincenzetti, 2017) (Figure 2-2B).

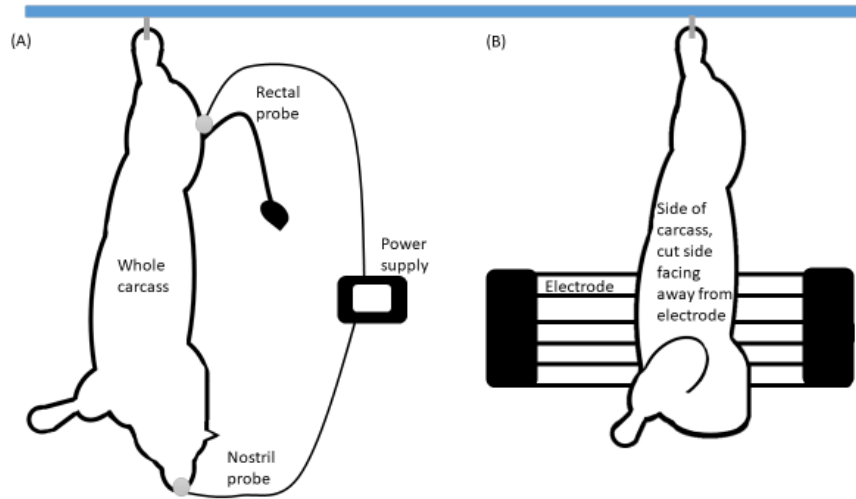


Figure 2-2. Schematic of the application of electrical stimulation to bovine carcasses, (A) via low voltage electrical stimulation (LVES), and (B) high voltage electrical stimulation (HVES), adapted from Polidori and Vincenzetti (2017) and AMPC (2015).

Factors affecting the efficacy of ES include the type, intensity and duration of the treatment (Abbasvali et al., 2012, Chen et al., 2020, Agbeniga and Webb, 2021, Cetin et al., 2012, Gursansky et al., 2010, Hwang and Thompson, 2001a), the muscle being treated (Hollung et al., 2007, Kim et al., 2013) and time post-mortem that ES is applied (Webb and Agbeniga, 2020, Agbeniga and Webb, 2021, Hwang and Thompson, 2001a).

The increase in the rate of pH decline following ES has been associated with an acceleration in post-mortem glycolysis (Rhee and Kim, 2001, Chen et al., 2020, Agbeniga and Webb, 2021, Webb and Agbeniga, 2020, Kim et al., 2013, Gursansky et al., 2010). In a recent study, Chen et al. (2020) investigated the impact of ES (2 V, 50 W, 72/90/108 s) on the concentration of glycogen in bovine *Longissimus* muscle and found that for all time points post-mortem (0 – 216 h), electrically stimulated muscle had significantly lower concentrations of glycogen when compared to controls with no differences being observed between ES treatments. Li et al. (2012, 2015) also

found that the ES (80 V, 35 s, 30 min post-mortem) of bovine *Longissimus lumborum* significantly increased the depletion of glycogen, ATP and creatine phosphate from muscle. The authors postulated that ES influenced the phosphorylation of glycolytic enzymes, such as glycogen debranching enzymes, glycogen phosphorylase and phosphofructokinase, increasing their activity and the metabolic flux in the ES muscle (Li et al., 2015).

ES can also influence the proteolysis of post-mortem muscle by enhancing the concentration of free calcium in muscle thus activating calpain enzymes; increasing cathepsin activity by disrupting the lysosomes and increasing the rate of pH decline; and physically disrupting the myofibrillar structure of muscle (Hwang et al., 2003). When investigating the effect of ES (80 V, 35 s, 30 min post-mortem *Longissimus lumborum*) on multiple proteolytic systems, Li et al. (2012) found that ES induced an earlier activation of calpain-1 activity, while calpain-2 activity was not affected by ES. An increase in the activity of cathepsin B and L in muscle up to 24 h post-mortem was also observed in the ES treated muscles (Li et al., 2012). However, the effect of ES on calpain-1 activity has been found to differ in different muscles from the same carcasses. Kim et al. (2013) found that ES (100 V, 60 Hz, 30 s, within 90 min post-mortem) significantly increased the activation and autolysis of calpain-1 activity in bovine *Longissimus dorsi*, when measured at 1 day post-mortem while having no significant impact on calpain activity in *Adductor*, *Gracilis* and *Semimembranosus* (deep and superficial) muscles (Kim et al., 2013). In addition, the effect of ES on the profile of calpain activity is influenced by time of application post-mortem. Webb and Agbeniga (2020) reported that ES (110 V, 17 pulses/s, 5 ms pulse width) applied at 7 min post-mortem resulted in a greater decline in calpain-1 activity in bovine *Longissimus lumborum* from 1 to 24 h post-mortem, when compared to that of muscle treated at 45 min.

Along with altering the activity of proteolytic enzyme activity, ES may physically disrupt muscle. Chen et al. (2020) (bovine *Longissimus* muscle, 21 V, 50 W, 30 s) and White et al. (2006a) (bovine *Longissimus dorsi* and *Semimembranosus*, 90 V, 14 Hz, 90 min post-mortem) reported that ES reduced the density of Z-disks, and resulted in the loss of the integrity of myofibrils. Sarcomere lengths were also altered. The

disruption of myofibrils was indicated by an increase in myofibril fragmentation following ES, when compared to non ES samples (Abbasvali et al., 2012, Chen et al., 2020).

ES has also been found to be advantageous when used on hot boned (removed within 90 min post-mortem) bovine *Semimembranosus* and *Longissimus dorsi*, where White et al. (2006a) found ES (90 V, 14 Hz) led to an increase in sarcomere lengths, and subsequent reduction in shear force values after aging for up to 14 days post-mortem, when compared to non ES controls. A similar effect to White et al. (2006a) was observed by Lang et al. (2016), who found that ES (21 V, 50 Hz, 0.25 A, 5 min post-mortem) of hot boned (removed within 45 min post-mortem) bovine *Longissimus lumborum* decreased the shear force values of muscle, when compared to the non ES treated samples.

The effects of electrical stimulation on the quality of meat has been recently reviewed by Gagaoua et al. (2025). The effects of electrical stimulation on shear force values, sensory tenderness, colour and water holding capacity of muscle were examined. In addition, proteomics was investigated as a tool to further explore how low voltage electrical stimulation induced changes in protein abundance and the mechanisms for these changes. From the data reviewed, 67 proteins changed following ES, with 14 proteins commonly identified across studies. The proteins identified included enzymes involved in glycolysis, structural and contractile proteins, heat shock proteins, and proteins related to oxidative stress and cell death/apoptosis. ES accelerated the breakdown of structural proteins and increased the content of heat shock proteins. It also promoted apoptosis and oxidative stress pathways. The findings of this review highlighted the ability ES has to improve meat quality which involves enhanced protein degradation and stress regulation, and accelerated pH decline (Gagaoua et al., 2025).

2.3 Innovative Technologies

The methods described in the previous sections have been demonstrated to improve tenderness as well as reduce variability in post-mortem muscle through mechanical and enzymatic means. However, the use of more innovative technologies, such as pulsed electric fields, ultrasound, and high pressure processing, have been

investigated for their potential use in the meat industry as interventions that improve tenderness.

2.3.1 Pulse electric fields (PEF)

Pulsed electric field (PEF) is a non-thermal process involving the placement of a material between two electrodes, where the material is subjected to short bursts of high voltage pulses (Gómez et al., 2019). Pulse durations can vary from nanoseconds to milliseconds, while the electric field strengths can vary from 100 – 300 V/cm to 20 – 80 kV/cm (Barba et al., 2015). The principal mode of action of PEF treatment is through electroporation, which increases the permeability of cell membranes (Bekhit et al., 2014). Electroporation involves applying an electric field that exceeds a critical threshold, to induce a dielectric breakdown of cell membranes through electro-compressive forces, creating or enlarging pores, and therefore, increasing the permeability of the cell membrane (Figure 2-3) (Demir et al., 2023, Gómez et al., 2019, Saulis, 2010). The critical electric strength for plant and animal cells is approximately 0.3 – 0.5 kV/cm, while for micro-organisms, it ranges from 10 – 15 kV/cm (Töpfl, 2006). Low intensity PEF treatments cause reversible damage, allowing cells to recover by resealing the pores. High intensity PEF treatments result in irreversible cell membrane damage and cell death (Figure 2-3) (Jaeger et al., 2008). While PEF is a non-thermal treatment (Bhat et al., 2018b), PEF treatment can raise sample temperatures from 1.9 °C with low intensity treatments, while high intensity treatments can cause temperature increases up to 40.5 °C (Khan et al., 2017).

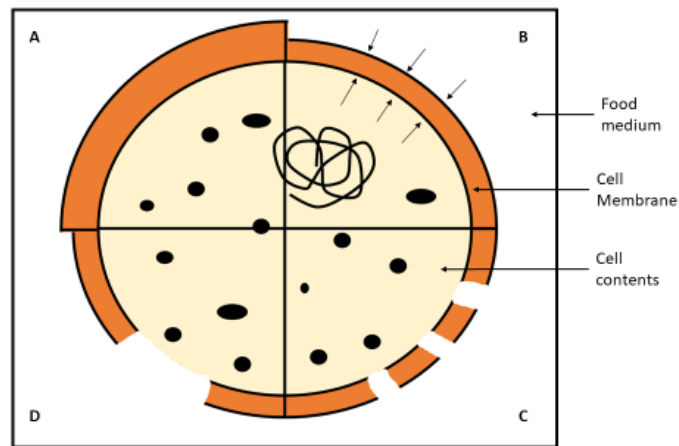


Figure 2-3. Effect of PEF on the structure of cell membranes, showing the A) intact cell membrane, B) compression during PEF, C) reversible and D) irreversible breakdown of the cell structure following treatment, adapted from Gómez et al. (2019).

Factors influencing the impact of PEF treatments include treatment frequency, voltage, duration, number of electrical pulses, cell properties and chamber design (Suwandy et al., 2015d, Gómez et al., 2019). The type and status of the muscle, whether it is pre- or post-rigor, can also influence the effect of PEF treatment on the tenderness of muscle. Bekhit et al. (2014) investigated the effects of PEF treatment (5/10 kV, 20/50/90 Hz) of 24 h post-mortem bovine *Longissimus lumborum* and *Semimembranosus*, and found that the muscles responded differently, where the reduction in the shear force values of the *Semimembranosus* was dependent on the frequency of the PEF treatment, whereas this was not the case for the *Longissimus lumborum* (Bekhit et al., 2014). Bekhit et al. (2016) speculated that variations in the response to PEF treatments by the different muscles could be due to differences in connective tissue content and collagen cross-linking.

PEF treatment has been found to enhance proteolysis in bovine muscle during subsequent aging. PEF treatment (5 kV, 90 Hz, 20 μ s/ 10 kV, 20 Hz, 20 μ s at 30 h post-mortem) increased calpain-2 activity in PEF treated bovine *Semimembranosus* (Bhat et al., 2019a) and *Biceps femoris* (Bhat et al., 2019b) after 1 day of aging, post-treatment, at 4 °C. Calpain-2 activity increased during aging and was higher in PEF

treated samples after 7 and 14 days post-treatment storage when compared to controls. In contrast, following one day of aging, no differences in calpain-1 activity were detected between control and PEF treated muscles (Bhat et al., 2019a, 2019b). It is proposed that PEF treatment may cause cellular damage and facilitate earlier calcium ion release facilitating early calpain activation. PEF treatment and electroporation may also enhance the activity of cathepsins, such as cathepsin B, L and D, by releasing them from the lysosomes, although this needs to be investigated further (Bekhit et al., 2014, Chian et al., 2019). Increased protein degradation was reported by Suwandy et al. (2015a) following PEF treatment (5/10 kV, 20/50/90 Hz) of 24 h post-mortem bovine *Longissimus lumborum* and *Semimembranosus*. This was illustrated by an intensification of 90 and 110 kDa bands, attributed to the breakdown of myosin heavy chains, and 30 kDa and 32 kDa fragments after 21 days of aging. The exception was the 10 kV-90 Hz treated samples, which did not show this increase (Suwandy et al., 2015a). These findings were in agreement with Bhat et al. (2019a, 2019b), who reported that after 14 days of aging, the intensity of the 90, 110, 30 and 32 kDa protein bands increased in PEF treated (5 kV, 90 Hz, 20 μ s and 10 kV, 20 Hz, 20 μ s at 30 h post-mortem) bovine *Semimembranosus* and *Biceps femoris*, respectively, compared to controls. Conversely, Faridnia et al. (2014) found that PEF treatment (0.2 – 0.6 kV cm⁻¹, 1 – 50 Hz, 20 μ s) did not change the protein profile of bovine *Longissimus thoracis* (treated at 48 h post-mortem) and stored for 1 – 3 days, when compared to controls. An increase in troponin-T and desmin breakdown in bovine *Longissimus lumborum* was observed by Suwandy et al. (2015a) for all PEF treatments (5/10 kV, 20/50/90 Hz) except 10kV-90Hz, when compared to control samples after aging for 14 and 21 days. Bhat et al. (2019a, 2019b) also found that PEF treatment (5 kV, 90 Hz, 20 μ s and 10 kV, 20 Hz, 20 μ s; treated at 30 h post-mortem) increased troponin-T and desmin degradation in bovine *Semimembranosus* and *Biceps femoris*, respectively. Repeated PEF treatments (10 kV, 90 Hz, 20 μ s, 1x/2x/3x) of cold boned bovine *Longissimus lumborum*, increased the intensity of the 90 kDa band at 3, 7 and 21 days post-mortem when compared to controls. The greatest intensity of the 90 kDa band was seen following 2x PEF treatments at all aging time points. Similar trends were observed for the 75 – 79 kDa and 110 kDa bands. The intensity of the 32 kDa band and degradation of troponin-T increased with PEF

treatment but decreased when the number of PEF treatments increased (Suwandy et al., 2015d). Multiple PEF treatments might negatively impact proteolysis, in particular the degradation of troponin-T, due to the extra heat produced (Suwandy et al., 2015d).

It has been proposed that PEF treatment can tenderise meat by inducing muscle contractions, disrupting sarcomeres and accelerating proteolysis via the release of calcium similar to ES (Bekhit et al., 2014). However, the effectiveness of PEF treatment in reducing shear force values varies. Suwandy et al. (2015a) found that PEF treatment (5/10 kV, 20/50/90 Hz) of 24 h post-mortem bovine *Longissimus lumborum* and *Semimembranosus* reduced the shear force values at all time points (3/7/14/21 days) during subsequent aging when compared to controls. The shear force values of the *Semimembranosus* decreased with increased treatment frequency while the *Longissimus lumborum* was not affected by treatment intensity. Similar results were observed by Bekhit et al. (2014) using the same muscles, *Longissimus lumborum* and *Semimembranosus*, and treatments (5/10 kV, 20/50/90 Hz). Repeated PEF treatments (10 kV, 90 Hz, 20 μ s, 1x/2x/3x) decreased the shear force values of cold boned bovine *Longissimus lumborum*, with the shear force values decreasing by approximately 2.5 N for each additional PEF application (Suwandy et al., 2015d). However, the shear force values of *Semimembranosus* was not affected by PEF treatment (Suwandy et al., 2015d). Faridnia et al. (2016) reported that PEF treatment (1.7 kV, 50 Hz, 20 μ s) during subsequent post-treatment aging (3/7/14/21 days) significantly reduced the shear force values of 24 h post-mortem bovine *Biceps femoris* when compared to controls. Studies by Arroyo et al. (2015) (48 h post-mortem bovine *Longissimus lumborum*, 1.4 kV/cm, 10 Hz, 20 μ s) and Bhat et al. (2019a) (30 h post-mortem bovine *Semimembranosus*, 5 kV, 90 Hz, 20 μ s) reported reductions in shear force values that were not statistically significant. Some studies (Suwandy et al., 2015c, Bhat et al., 2019b, Faridnia et al., 2014) found no significant differences in the shear force values of 24 – 48 h post-mortem bovine muscle (*Longissimus lumborum*, *Longissimus thoracis*, *Biceps femoris*) following PEF treatment (5 – 10 kV, 0.2 – 0.6 kV/cm, 1 – 90 Hz, 20 μ s), which may be due to insufficient treatment intensity.

PEF treatment has been found to affect the structure of muscle, making the muscle structure more porous (Faridnia et al., 2014, Zhang et al., 2022, Khan et al., 2017). Khan et al. (2017) observed gap formation between muscle bundles following low intensity PEF treatment (2.5 kV, 200 Hz, 20 μ s) of 24 h post-mortem bovine *Longissimus et lumborum*. This was in agreement with the findings of Zhang et al. (2022) following the PEF treatment (0.5 – 2.0 kV/cm, 0.78 – 12.50 kJ/kg, 1 Hz, 50 μ s) of 48 h post-mortem bovine *Longissimus lumborum*. High intensity PEF treatment (10 kV, 200 Hz, 20 μ s) of 24 h post-mortem bovine *Longissimus et lumborum* resulted in the shrinkage of the muscle fibre bundle, possibly due to additional water loss from the samples (Khan et al., 2017). O'Dowd et al. (2013) also noted a decreased diameter of muscle bundles following the PEF treatment (1.9 kV/cm, 65 Hz, 20 μ s) of 48 h post-mortem bovine *Semitendinosus* when compared to controls, and attributed this to significant water loss immediately after treatment. Chian et al. (2019) noted that PEF treatment (1 – 1.25 kV/cm, 50 Hz, 20 μ s) elongated the sarcomere lengths of 48 h post-mortem bovine *Longissimus thoracis*, and this effect increased with an increase in intensity (500 – 2000 pulses), possibly due to weakened I-band junctions and Z-disks (Chian et al., 2019). Changes were also observed following the PEF treatment (1.0 and 1.5 kV/cm, 50 Hz, 20 μ s) of collagen extracted from bovine deep *Pectoralis*, where electroporation weakened the structure of collagen, making it more soluble (Alahakoon et al., 2017).

Faridnia et al. (2014) suggested that the microstructural changes in muscle that arise from PEF treatment could enhance marinating and curing of meat and reduce processing times. Zhang et al. (2022) found that PEF treatment (0.5 – 2.0 kV/cm, 0.78 – 12.50 kJ/kg, 1 Hz, 50 μ s) accelerated the marinating of 48 h post-mortem bovine *Longissimus lumborum*, where all PEF treated samples had significantly higher total weight change compared to controls after 60 – 180 min of marinating. Weight change increased with increasing field strength, however, no significant differences were observed between 1.5 – 2 kV/cm treatments. PEF treatment (1 – 4 kV/cm, 125 Hz, 60/90 s) also has the potential to reduce the curing time of 24 h post-mortem bovine *Longissimus lumborum* by over 60%, as it was found to significantly increase the NaCl content and diffusion coefficient of muscle when brined for 150 min and this effect

increased with increased field strength (Guo et al., 2024c). Similar findings were reported for the curing (McDonnell et al., 2014a) and marinating (He et al., 2024) of porcine muscle and it has been suggested that PEF treatment could have applications in the production of reduced salt meat products through its ability to influence salt diffusion and delivery (Bhat et al., 2020). PEF treatment was also evaluated for its effects on the drying of meat products. Beňo et al. (2023) observed that PEF treatment (0.75 kV/cm, 100 Hz, 5 μ s, 50/100 pulses) significantly increased the evaporative rate of 96 h post-mortem bovine *Longissimus thoracis* and *Semimembranosus*, where samples treated with 100 pulses had an 18% and 12% lower water content, respectively, when compared to controls, following 2 h of drying. When PEF treatment (low intensity (LPEF) – 2.5 kV, 50 Hz, 20 μ s, high intensity (HPEF) – 10 kV, 50 Hz, 20 μ s) was used in combination with the dry aging (21 days aging, 4 °C, 80% relative humidity) of 24 h post-mortem venison *Longissimus dorsi*, PEF treatment increased dry aging weight loss, while not affecting purge loss (Mungure et al., 2020). These changes in drying rates were attributed to structural changes in the muscle following PEF treatment. Therefore, PEF has potential to accelerate a number of different processes used within the meat industry.

PEF treatment can influence the water holding capacity of muscle (Bekhit et al., 2014, Faridnia et al., 2014, 2015, 2016). Bekhit et al. (2014) found that PEF treatment of 24 h post-mortem bovine *Longissimus lumborum* (5/10 kV, 20/50/90 Hz) and storage time (3 – 21 days post-treatment) increased the purge loss. Purge loss increased with increased voltage and frequency of the treatment. The purge loss of 24 h post-mortem bovine *Semimembranosus* also increased with increased voltage of PEF treatment (5/10 kV, 20/50/90 Hz) (Bekhit et al., 2014). Similar observations were made by Khan et al. (2017), following the PEF treatment of 24 h post-mortem bovine *Longissimus et lumborum*, where high intensity PEF treated muscles (10 kV, 200 Hz and 20 μ s) exhibited higher purge losses than low intensity PEF treated (2.5 kV, 200 Hz, 20 μ s) or control muscles following 1 and 14 day post-treatment storage (4 °C). Faridnia et al., (2014, 2015, 2016) also reported significant increases in purge losses from 24 – 48 h post-mortem bovine *Longissimus thoracis* (0.2 – 0.6 kV/cm, 1 – 50 Hz, 20 μ s), *Semitendinosus* (1.4 kV/cm, 50 Hz, 20 μ s, 250 kJ/kg) and *Biceps femoris* (1.7

kJ/cm, 50 Hz, 20 μ s, 185 kJ/kg), respectively, following their respective PEF treatments. It was suggested by Bekhit et al. (2014) and Faridnia et al., (2014, 2015, 2016) that the ability of the muscle to retain moisture was reduced following PEF treatment due to structural changes in the myofibril (Faridnia et al., 2016), while Khan et al. (2017) attributed the increase in purge loss following high intensity PEF treatment to protein denaturation due to a high temperature increase. O'Dowd et al. (2013) observed that PEF treatment (1.1 – 2.8 kV/cm, 12.7 – 226 kJ/kg, 5 – 200 Hz, 152 – 300 pulse number) increased the weight loss of 48 h post-mortem bovine *Semitendinosus*. The increased weight loss was initially attributed to pore formation and enhanced free water movement. However, following subsequent NMR analysis, it was concluded that PEF treatment had no major effect on cell membranes and the increase in weight loss following PEF treatment was due to the loss of free water in the muscle (O'Dowd et al., 2013). Other measurements of water holding capacity, including drip loss, expressible moisture and cook loss were reported to be unaffected (O'Dowd et al., 2013, Faridnia et al., 2014, 2015, 2016) or reduced (O'Dowd et al., 2013) by PEF treatment, and this was attributed to less free water being available due to the higher initial purge or weight loss. In contrast, no significant changes in purge and cook loss of 24 – 30 h post-mortem bovine *Longissimus lumborum*, *Semimembranosus* and *Biceps femoris* were observed by Suwandy et al. (2015c) (10 kV, 90 Hz, 20 μ s) and Bhat et al. (2019a, 2019b) (5 kV-0.36 kV/cm-90 Hz/ 10 kV-0.6 kV/cm-20 Hz), respectively, following their respective PEF treatments. When analysing the effect of repeated PEF treatments (1x/2x/3x, 10 kV, 90 Hz, 20 μ s) on the purge and cook loss of cold boned bovine muscle, it was found that the purge loss of *Semimembranosus* increased significantly with PEF, regardless of the number of treatments, while PEF treatment did not significantly affect the cook loss of the same muscle. The opposite was found for *Longissimus lumborum*, where PEF treatment did not significantly affect purge loss, but significantly increased cook loss, irrespective of the number of treatments (Suwandy et al., 2015d).

Other quality parameters of PEF treated muscle have been investigated, including colour and lipid oxidation levels with varying results. The lightness (L^*) of 48 h post-

mortem bovine *Semitendinosus* (O'Dowd et al., 2013) (1.9 kV/cm, 65 Hz, 250 pulses, 20 μ s) and 24 h post-mortem bovine *Longissimus et lumborum* (Khan et al., 2017) (high intensity PEF - 10 kV, 200 Hz, 20 μ s) decreased following PEF treatment, which was attributed to heat denaturation and reduction in water holding capacity, increasing surface moisture content and amount of light reflected from the surface. Khan et al. (2017) found that high intensity PEF treatment (10 kV, 200 Hz and 20 μ s) decreased redness (a^*) and colour stability, and increased yellowness (b^*) 1 day post-treatment when compared to low intensity PEF treated muscles (2.5 kV, 200 Hz, 20 μ s) and controls. However, Suwandy et al. (2015c) (10 kV, 90 Hz, 20 μ s), Arroyo et al. (2015) (1.4 kV/cm, 10 Hz, 20 μ s, 300/600 pulses), Chian et al. (2019) (1 – 1.25 kV/cm, 500/2000 pulses, 50 Hz, 20 μ s) and Faridnia et al. (2016) (1.7 kV/cm, 50 Hz, 20 μ s) reported that PEF treatment did not significantly affect the L^* , a^* and b^* values of 24 – 48 h post-mortem bovine *Longissimus lumborum*, *Longissimus thoracis et lumborum*, *Longissimus thoracis* and *Biceps femoris*, respectively. Overall, it can be concluded that PEF can be used without causing detrimental effects to the instrumental colour parameters of muscle. When cold boned bovine *Longissimus lumborum* and *Semimembranosus* was subjected to repeated PEF treatments (1x/2x/3x, 10 kV, 90 Hz, 20 μ s), redness (a^*) decreased and hue (h^*) increased with an increased number of PEF treatments, due to the increased temperature generated following PEF treatment (Suwandy et al., 2015d).

PEF treatment (1.4 kV/cm, 50 Hz, 20 μ s/ 1x/2x/3x, 10 kV, 90 Hz, 20 μ s) did not significantly affect lipid oxidation (TBARS values) in 24 h post-mortem bovine *Semimembranosus* and *Longissimus lumborum* (Faridnia et al., 2015, Suwandy et al., 2015c, Suwandy et al., 2015d, Khan et al., 2017) and 24 h post-mortem venison *Longissimus dorsi* (LPEF – 2.5 kV, 50 Hz, 20 μ s, HPEF – 10.0 kV, 50 Hz and 20 μ s) (Mungure et al., 2020). Mungure et al. (2020) further investigated PEF treatment (LPEF – 2.5 kV, 50 Hz, 20 μ s, HPEF – 10.0 kV, 50 Hz and 20 μ s) for its potential to generate oxidising free radicals using NMR-based metabolomics. Metabolites detected included amino acids, sugars, alcohols and purine derivatives, however, PEF treatment had no effect on the metabolomic profile (Mungure et al., 2020).

PEF treatment and its effects on various quality parameters have also been investigated in hot boned muscle. When hot boned (4 h post-mortem) bovine *Longissimus lumborum* and *Semimembranosus* were treated with PEF (5/10 kV, 20/50/90 Hz), the shear force values of *Longissimus lumborum* increased with higher PEF frequency levels (Suwandy et al., 2015b). This result was unexpected as it was thought PEF treatment would induce early calpain activation, as observed in cold boned muscle (Bhat et al., 2019a, 2019b). PEF treatment decreased the shear force values of hot boned *Semimembranosus*, regardless of treatment intensity. Following 21 days storage post-treatment, PEF treated samples showed more degradation of troponin-T and desmin when compared to controls, with the degradation of these proteins being most prominent in samples treated with 5 kV-20 Hz and 10 kV-20 Hz (Suwandy et al., 2015b). Bekhit et al. (2016) investigated the impact of repeated PEF treatments (1x/2x/3x 10 kV, 90 Hz, 20 μ s) on hot boned (4 h post-mortem) bovine *Longissimus lumborum* and *Semimembranosus* and found that the 3x PEF treated *Longissimus lumborum* was the toughest at all aging time points (3/7/14/21 days post-mortem), possibly due to protein denaturation and reduced proteolysis, whereas the 1x/2x PEF treated and control were not significantly different.

2.3.2 Ultrasound (US)

Ultrasound (US) refers to sound waves with frequencies over 20 kHz, greater than that detectable by the human ear, and below microwave frequencies, up to 10 MHz (Tiwari, 2015). As an acoustic energy, US is a non-invasive and non-ionising mechanical form of energy, capable of accelerating, enhancing and controlling food processes without reducing its quality (Alarcon-Rojo et al., 2019). US is generated by acoustic waves, causing vibrations with wavelengths ranging from μ m to cm in water and biological tissues (Got et al., 1999) and it can be applied via an ultrasound water bath or probe (Alarcon-Rojo et al., 2019). US can be categorised as low or high intensity, depending on the frequency or intensity used. Low intensity US ($< 1 \text{ W/cm}^2$) can be used with high frequencies ($> 1 \text{ MHz}$) as a non-destructive method of analysis, process monitoring and assessing carcass fat thickness (Tiwari, 2015, Jayasooriya et al., 2007). High intensity US ($10 - 1000 \text{ W/cm}^2$), in combination with low frequencies

(20 – 100 kHz) can cause cell membrane disruption and accelerate chemical reactions (Bhat et al., 2018b).

The principle mode of action of US treatment is through acoustic cavitation, which involves the repeated compression, rarefaction and eventual collapse of microbubbles within the aqueous portion of the muscle. This can physically disrupt muscle structure through shear stress and local development of high pressure (Figure 2-4). US treatment can also have chemical effects such as the creation of free radicals (Tiwari, 2015, Alarcon-Rojo et al., 2019, Kang et al., 2016b, Dang et al., 2022). US treatment may alter enzymatic processes within muscle, by assisting the release of calcium, and activating and/or releasing enzymes, by altering the substrate, or enzyme-substrate interaction (Got et al., 1999, Huang et al., 2017). The impact of US treatment and its ability to cause cavitation and physical disruption is influenced by factors such as the frequency and intensity of the US treatment as well as treatment duration. Properties of the muscles being treated such as age post-mortem and type of muscle may also influence the outcome of US treatment (Jayasooriya et al., 2007, Gonzalez-Gonzalez et al., 2020, Wang et al., 2022, Chang et al., 2015, Diaz-Almanza et al., 2019, Kent et al., 2024b).

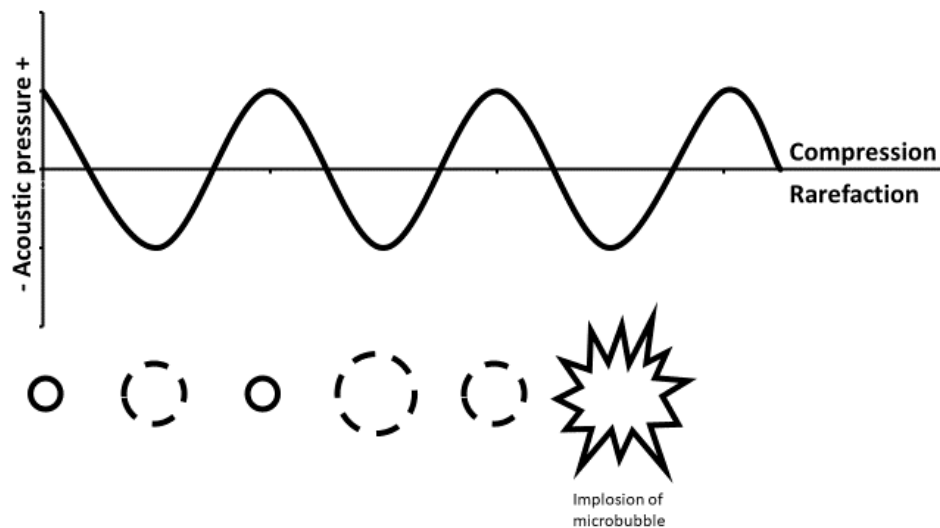


Figure 2-4. The compression and rarefaction of microbubbles, with eventual implosion as a result of US treatment, adapted from Astráin-Redín et al. (2021).

US treatment has the potential to alter post-mortem proteolysis of muscle, by affecting calpain, caspase-3 and cathepsin enzymes. Dang et al. (2022) found that the

US treatment (40 kHz, 12 W/cm², 40 min at 4 °C) of 24 h post-mortem bovine *Longissimus thoracis et lumborum* significantly increased caspase-3 activity immediately post-treatment when compared to controls. An increase in caspase-3 activity was also found by Chen et al. (2015) following the US treatment (40 kHz, 1500 W, 30/60 min) of chicken *Pectoralis superficialis*, immediately post-slaughter. The increase in caspase-3 activity was attributed to mitochondrial dysfunction following US treatment and increases in reactive oxygen species (Dang et al., 2022, Chen et al., 2014). US treatment can also promote calpain-1 activity and autolysis, as observed by Dang et al. (2022) and Wang et al. (2018a), who US treated 24 h post-mortem bovine *Longissimus thoracis et lumborum* (40 kHz, 12 W/cm², 40 min at 4 °C) and *Semimembranosus* (20 kHz, 25 W/cm², 20/40 min), respectively. It was found that when compared to controls, US treated muscles exhibited a more intense autolysed 76 kDa calpain subunit and lower intensity of the intact 80 kDa calpain subunit. Additionally, US treatment promoted desmin and troponin-T degradation when compared to controls (Wang et al., 2018a). The increase in calpain-1 activity following US treatment was attributed to an increase in free cytosolic calcium (Dang et al., 2022, Dolatowski and Stadnik, 2007, Got et al., 1999). Proteomic analysis has demonstrated that US treatment of bovine *Semitendinosus* (24 h post-mortem, 40 kHz, 100% amp, 460 W/cm², 15 min) (Marino et al., 2023) and *Longissimus lumborum* (2.5 h post-mortem, 40 kHz, 200 W, 40 min) (Gao et al., 2024), enhanced troponin-T degradation. US treatment (20 kHz, 300/600 W, 20 min) has also been reported to promote cathepsin B + L activity in 48 h post-mortem bovine *Semitendinosus* by Wang et al. (2022), when compared to controls at 0 and 4 days storage post-treatment, potentially due to the early release of cathepsins from lysosomes as a result of cavitation. Increasing the power from 300 to 600 W reduced the impact of US treatment on cathepsin activity. At 8 days post-treatment, no significant differences in cathepsin B + L was found between control and 300 W US treated muscle, while the activity in the 600 W US treated muscle was significantly lower (Wang et al., 2022). In contrast, other studies reported that US treatment (20 kHz for periods of 15 s) of 2 and 24 h post-mortem bovine (*Semimembranosus* and *Longissimus thoracis et lumborum*) and ovine (*Longissimus thoracis et lumborum*) muscle had no impact on proteolysis, as indicated by no differences in protein band

patterns on SDS-PAGE gels, compared to controls (Lyng et al., 1998b, Lyng et al., 1998a). The authors of these studies hypothesised that the matrix of the muscle presented a physical barrier to US, with the intensity of the US waves weakening as they travelled through the muscle.

Limited research has been carried out on the effect of US on post-mortem glycolysis in muscle, however, it has been investigated by Kent et al. (2024a, 2024b), both *in vivo* and *in vitro*, through the use of a glycolytic buffer system (England et al., 2014, England et al., 2016). When 2.5 h post-mortem bovine *Longissimus thoracis et lumborum* was treated with US (25/45 kHz, 15/30/45 min), it was found that US treatment (for 30 min) significantly increased the rate of pH decline in muscle when measured at different time points over a 24 h period, post-treatment. However, when the pH decline of the *in vitro* glycolytic buffer was monitored over a 24 h period, it was found that US did not have an effect on pH decline when compared to controls, indicating that US treatment did not have an impact on the inherent activity of glycolytic enzymes (Kent et al., 2024a). The authors concluded the limited effect of US treatment on the glycolytic system may be due to the matrix of the muscle dampening US waves, similar to the hypothesis of Lyng et al., (1998a, 1998b, 1997) and the accelerated decline in pH following the 30 min US treatment was not due to an impact of US on glycolytic enzyme activity, but due to a potential increase in calcium following US, as demonstrated by other authors (Got et al., 1999, Sikes et al., 2014). To further investigate the effect of US on inherent glycolytic enzyme activity, muscle homogenates were prepared from 2.5 h post-mortem bovine *Longissimus lumborum et thoracis* and treated with US (20 kHz, 60/100% amp (11/19 W/cm²), 15/30 min), by Kent et al. (2024b). It was found that only the most intense treatment (100% amp-30 min) had a significant effect on the glycolytic enzyme activity, when monitored *in vitro*, using the glycolytic buffer system. The 100% amp-30 min US treatment delayed the production of lactic acid and subsequent decline in pH decline, as well as leading to an accumulation in reducing sugars between 30 and 240 min of incubation. However, following 1440 min of *in vitro* incubation, there were no significant differences between the pH, lactic acid and reducing sugar content of glycolytic buffer systems containing treated and control homogenates. Overall, it was

concluded that US treatment had limited potential to manipulate the rate of glycolysis in bovine muscle.

US treatment has been investigated for its impact on shear force values and tenderness by several authors, with varying results found. When 24 h post-mortem bovine *Longissimus lumborum et thoracis* and *Semitendinosus* was treated with US (24 kHz, 12 W/cm², 30/60/120/240 s), Jayasooriya et al. (2007) reported that US treatment significantly reduced the shear force values of muscle following 0 and 1 days aging, post-treatment. However, the effect of US treatment was reduced after 3 to 8.5 days aging. US treatment (20 kHz, 25 W/cm², 20/40 min) of 24 h post-mortem bovine *Semitendinosus* significantly reduced the shear force values following 3 and 7 days aging, post-treatment, when compared to controls, while no significant differences were observed between the 20 and 40 min treatments (Wang et al., 2018a). Diaz-Almanza et al. (2019) found that the shear force values of 72 h post-mortem bovine *Longissimus lumborum* decreased as the US treatment (37 kHz, 90 W/cm², 0/10/20/40 min) duration increased, with the 40 min treatment reducing the peak force by 11.74 N, a reduction of 20.7%. When Chang et al. (2015) treated 48 h post-mortem bovine *Semitendinosus* with different US treatment durations (40 kHz, 1,500 W, 10/20/30/40/50/60 min), it was found that the 30 min treatment had significantly lower shear force values compared to controls. While these studies are not directly comparable due to the differences in treatment parameters and muscles, US treatments ranging from 20 to 40 min appear to be the most effective in reducing shear force values. The authors attributed the reduction in shear force values to acoustic cavitation and disruption to myofibrils. In addition, it has been suggested that the reduction in shear force values was due to US having an impact on connective tissue. Wang et al. (2022) demonstrated that US treatment (20 kHz, 300/600 W, 20 min) of 48 h post-mortem bovine *Semitendinosus* disrupted the structure of perimysial collagen, reducing the collagen denaturation temperature and increasing its heat solubility. This was in agreement with the results of Chang et al. (2015) who treated 48 h post-mortem bovine *Semitendinosus* with US (40 kHz, 1500 W, 10/20/30/40/50/60 min) and found that US, in particular the 50 min treatment, reduced the mechanical strength of connective tissue. The content of

permysial and endoimysial collagen and maximum thermal shrinkage temperature of the two components reduced with US treatment, which was attributed to the effects of US induced cavitation.

The type of muscle being treated with US influences the effect of US on tenderness. When 24 h post-mortem bovine *Longissimus lumborum*, *Infraspinatus* and *Cleidooccipitalis* were treated with US (40 kHz, 11 W/cm², 40/60/80 min) by Gonzalez-Gonzalez et al. (2020), it was observed that shear force values decreased significantly in all muscles with US treatment duration and aging time. While the *Infraspinatus* was the most tender muscle following US treatment and aging for 7 days, the *Longissimus lumborum* and *Cleidooccipitalis* benefitted the most from US treatment. In contrast, authors such as Lyng et al., (1997, 1998a, 1998b) (30 – 47 kHz, 0.29 – 0.62 W/cm², 5 – 90 min, 20 kHz, 62 W/cm²) and Carrillo-Lopez et al. (2019) (37 kHz, 7 W/cm², 60 min) found that US treatment did not have a significant effect on the peak load bite force or shear force values of 24 h post-mortem bovine and ovine muscles (*Longissimus thoracis et lumborum*, *Semimembranosus*, *Biceps femoris* and *Longissimus dorsi*). The authors attributed the lack of effect on the tenderness of muscle on insufficient treatment intensity.

US treatment can significantly alter the microstructure of muscle, potentially influencing tenderness. Following US treatment (40 kHz, 1500 W, 10/20/30/40/50/60 min) of 48 h post-mortem bovine *Semitendinosus*, significant changes to the microstructure were found in samples treated with US for 30 – 60 min, where shrinkage and separation of muscle fibres was observed. Other effects observed included the rupturing of muscle cells, sarcomere shrinkage, an increase in extracellular and intracellular cavities and canals, and formation of protein aggregates (Chang et al., 2015). US also disrupted the structure of connective tissue and collagen, where the endomysium became loosely arranged and collagen fibres were disorganised, whereas in controls, the perimysium and endomysium were clearly arranged with no gaps present between muscle fibres (Chang et al., 2015). Disruption and separation between the muscle fibres in 24 h post-mortem bovine *Longissimus lumborum*, *Infraspinatus* and *Cleidooccipitalis* was observed by Gonzalez-Gonzalez et al. (2020) at 0 and 14 days of aging following US treatment (40

kHz, 11 W/cm², 80 min). Further to this, it was found by Stadnik et al. (2008) and Wang et al. (2018a) that the US treatment of 24 h post-mortem bovine *Semimembranosus* (45 kHz, 2 W/cm², 120 s) and *Semitendinosus* (20 kHz, 25 W/cm², 20/40 min) led to swelling around the A-band region, particularly at the H-band and M-line. Gaps also appeared near the Z-line and between myofibrillar fibres (Kang et al., 2016a, Stadnik et al., 2008, Wang et al., 2018a).

The disruption US treatment causes to the microstructure of muscle may assist with processes such as curing and brining. This was investigated by Kang et al. (2016a), who US treated (20 kHz, 150 – 300 W, 30 – 120 min) 48 h post-mortem bovine *Longissimus dorsi*. They found that increasing US power (150 – 300 W) increased the rate of diffusion of water and salt into the muscle, due to the increase in space between myofibrils, and therefore, it was concluded that US has potential to accelerate the brining process. This was explored further by Kang et al. (2017), who found that US treatment (20 kHz, 150 – 300 W, 30/120 min) of 48 h post-mortem bovine *Longissimus dorsi*, in combination with brining, improved the water holding capacity and tenderness of muscle. This was possibly due to the combination of US and salt inducing the swelling of myofibrillar proteins and binding of protein side chains to water. US had a similar effect when used on porcine muscle, when investigated by Ozuna et al. (2013) (*Longissimus dorsi*, 40 kHz, 37.5 W/dm³, 15 – 120 min), McDonnell et al. (2014b) (120 h post-mortem porcine *Longissimus thoracis et lumborum*, 20 kHz, 4.2/11/19 W/cm², 10/25/40 min), Pan et al. (2022) (24 h post-mortem porcine *Biceps femoris*, 20 kHz, 350 W, 31.02 W/cm², 1 h) and Guo et al. (2024a, 2024b) (24 h post-mortem porcine loin, 23.6/26.8/32.3/40/55 kHz, 23.5 W, 25 min) who found that US increased the diffusivity of water and salt. US treatment also led to an increase in salt content, moisture content and the hardness of the muscles. Similar to PEF, US in combination with curing and brining has the potential to be used as a method to produce reduced salt meat products through its ability to influence salt diffusion and delivery (Inguglia et al., 2021).

US treatment can have an impact on the water holding capacity of muscle, however, results have been conflicting. When 24 h post-mortem bovine *Longissimus lumborum*, *Infraspinatus* and *Cleidooccipitalis* were treated with US (40 kHz, 11

W/cm², 40/60/80 min), it was found that the effect of US on the water holding capacity, measured via the press method, differed depending on the muscle. An immediate effect of US was observed in the *Cleidooccipitalis*, where US significantly increased the water holding capacity when compared to controls. US treatment significantly increased the water holding capacity of *Longissimus lumborum* and *Infraspinatus* after 7 and 14 days aging, following 40 and 80 min US treatment, respectively. When 24 h post-mortem porcine loin was US treated (23.6/26.8/32.3/40/55 kHz, 23.5 W, 25 min) and cured, it was found that cook loss decreased significantly with an increase in US frequency (Guo et al., 2024a). When the research group examined the effect of the US treatments on the states of water in the pork muscle, they found that US facilitated the conversion of free water to immobilised water, therefore improving the water holding capacity of muscle, by enhancing the water binding properties of myofibrils (Guo et al., 2024b). However, other authors such as Carrillo-Lopez et al. (2019) (bovine *Longissimus dorsi*, 37 kHz, 7 W/cm², 60 min) and Peña-Gonzalez et al. (2019) (24 h post-mortem bovine *Longissimus dorsi*, 40 kHz, 11 W/cm², 60 min) observed that US did not significantly affect the water holding capacity of muscle.

Other quality parameters of muscle potentially affected by US include colour and lipid oxidation. It was found that US treatment (37 kHz, 90 W/cm², 10/20/40 min) increased the lightness (L^*) of 72 h post-mortem *Longissimus lumborum*, while US did not have a significant effect yellowness (b^*) and chroma (C^*). Redness (a^*) showed a tendency to decrease with increased US treatment time, however, the differences to the controls were not significant (Diaz-Almanza et al., 2019). Similar results were reported by Peña-Gonzalez et al. (2019), where the US treatment (40 kHz, 11 W/cm², 60 min) of 24 h post-mortem bovine *Longissimus dorsi* significantly increased the lightness of muscle, and significantly reduced the redness of muscle, when compared to controls. The chroma value decreased and hue angle increased in US treated samples when compared to controls, while yellowness was not affected by US (Peña-Gonzalez et al., 2019). The increase in L^* values following US treatment is linked to an increase in surface free water, increasing the amount of light reflected (Diaz-Almanza et al., 2019). In contrast, Jayasooriya et al. (2007) found that US (24

kHz, 12 W/cm², 30/60/120/240 s) of 24 h post-mortem *Longissimus lumborum* et *thoracis* and *Semitendinosus* did not have a significant effect on the L^* , a^* , b^* , C^* values, or hue angle. Similarly, Stadnik et al. (2008, 2011) reported that US treatment (45 kHz, 2 W/cm², 120 s) of 24 h post-mortem bovine *Semimembranosus* did not have an effect on L^* , a^* and b^* values, however, the colour stability of muscle was reduced.

Several studies have shown that US treatment also has an impact on the extent of lipid and protein oxidation in muscle. Stadnik and Dolatowski (2008) found that US treatment (45 kHz, 2 W/cm², 120 s) of 24 h post-mortem bovine *Semimembranosus* led to an initial increase in lipid oxidation, however, as storage time increased up to 3 day post-treatment, the difference between control and treated samples reduced. No differences in the extent of protein oxidation, as indicated by carbonyl content, were found between control and treated samples. Peña-González et al. (2017) also reported that US treatment (40 kHz, 11 W/cm², 60 min) of 72 h post-mortem bovine *Longissimus dorsi* increased lipid oxidation, following 14 days aging, however, the levels remained within the threshold of consumer acceptability, and therefore, it was concluded that US did not compromise the oxidative stability of muscle (Peña-González et al., 2017). Increased US intensity led to increases in lipid oxidation in studies conducted by Kang et al. (2016b) (48 h post-mortem bovine *Longissimus dorsi*, 20 kHz, 2.39/6.23/11/32/20.96 W/cm², 30/60/90/120 min), de Lima et al. (2024) (24 h post-mortem bovine *Longissimus lumborum*, 40 kHz, 63.4 W, 276.36 W/cm² x 4.25 min, 422.67 W/cm² x 6.5 min, 747.79 W/cm² x 11.5 min) and Fallavena et al. (2020) (7 days post-mortem bovine *Biceps femoris*, 20 kHz, 22/35/46/65/84 W/cm², 15 min). Kang et al. (2016b) also noted an increase in protein oxidation following US, and this increased with increased treatment intensity and duration. The increase in lipid and protein oxidation was attributed to the breakdown of cell membranes, denaturation of protein and the generation of $\cdot\text{OH}$ free radicals due to US cavitation (Kang et al., 2016b, Peña-González et al., 2017).

Limited research has been carried out on the treatment of pre-rigor muscle with US and its impact on quality. Lyng et al. (1998b) investigated the use of US (20 kHz, 62 W/cm² for 15 s intervals) on 1 and 24 h post-mortem bovine *Longissimus thoracis* et

lumborum and *Semimembranosus* and found that US did not affect the tenderness and proteolysis of either muscles as monitored by changes in peak load bite-force values and SDS-PAGE protein profile. Similar results were found when US (20 kHz, 62 W/cm² for 15 s intervals) was applied to 2 and 24 h post-mortem ovine *Longissimus thoracis et lumborum* (Lyng et al., 1998a).

2.3.3 High Pressure Processing (HPP)

High pressure processing (HPP) is a non-thermal technology that is used primarily for the preservation of food by improving the quality and shelf life of a range of food products, including meat (Bhat et al., 2018b, Campus, 2010). For HPP to be applied to a product, it must be packaged and sealed in flexible material. The product is placed in a pressure vessel containing liquid to transmit the pressure instantaneously and isocratically, regardless of product shape or size (Figure 2-5). Pressure is generated directly or indirectly, using a piston or hydraulic pump, respectively (Campus, 2010, Simonin et al., 2012). HPP induces changes to the secondary, tertiary and quaternary structure of protein in muscle, altering conformational and molecular interactions within the protein structure, such as hydrogen and electrostatic bonding and hydrophobic interactions, which can impact the tenderness of meat and its functional properties such as water holding capacity (Troy et al., 2016). The effects of HPP on muscle is influenced by a range of treatment parameters such as the pressure (Lee et al., 2023, Zhang et al., 2018b, Neto et al., 2013, Utama et al., 2018, Marcos and Mullen, 2014), temperature (Marcos et al., 2010, McArdle et al., 2010, McArdle et al., 2011, McArdle et al., 2013, Ma and Ledward, 2004) and duration of treatment (Neto et al., 2013, Jung et al., 2003).

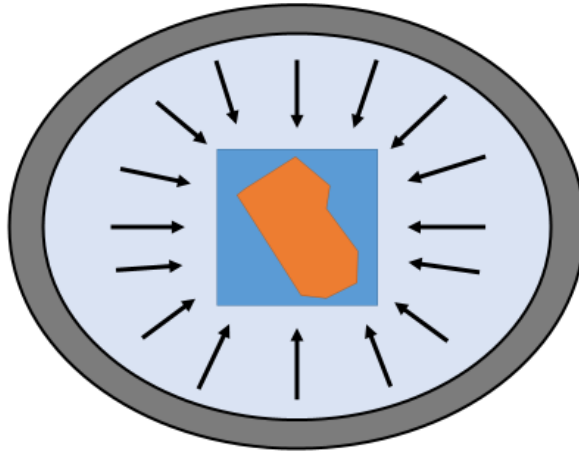


Figure 2-5. A schematic of applying HPP in a pressurised vessel with water as the medium for transmitting pressure to the product, adapted from Chacha et al. (2021).

HPP has an impact on the proteolytic enzymes in muscle. When HPP treatment (100/200/300 MPa, 3 min, 21 °C) was applied to 48 h post-mortem bovine *Semitendinosus*, 100 MPa did not significantly affect calpain activity, however, 200 and 300 MPa treatments led to a significant reduction in calpain activity to 72.51 and 46.02%, respectively, when compared to controls (Lee et al., 2023). In addition, Zhang et al. (2018b) treated calpain-1 and -2 in saline solution with HPP (100/200/300/400 MPa, 10 min, 20 °C) and observed that 200 MPa partially denatured calpain-1 and -2, while HPP treatments greater than 300 MPa almost completely eliminated calpain activity. Grossi et al. (2012) found that HPP treatment (50/100/200/300 MPa, 5 min, 5 °C) of 24 h post-mortem porcine *Longissimus dorsi* eliminated calpain activity, when 200 and 300 MPa was applied. When 50 min post-mortem bovine *Longissimus thoracis* was treated with HPP (175 MPa, 3 min), HPP reduced calpain-1 activation and at 24 h post-mortem, HPP treated samples had higher amounts of intact calpain-1 when compared to controls. HPP treatment did not appear to affect calpain-2 autolysis (Morton et al., 2018). However, HPP treatment had the opposite effect on cathepsin activity. When 48 h post-mortem bovine *Semitendinosus* was treated with HPP (100/200/300 MPa, 3 min, 21 °C), it was found that the 200 and 300 MPa treatments significantly increased cathepsin B activity, to 103.77 and 108.07%, respectively,

when compared to controls, while the 100 MPa treatment did not significantly affect cathepsin B activity (Lee et al., 2023). When 48 h post-mortem bovine *Biceps femoris* was treated with HPP (50 – 600 MPa, 20 – 300 s, 10 °C), it was found that cathepsin D activity increased with increased pressures, with the greatest activity found in samples treated with 500 – 600 MPa for less than 200 s (Jung et al., 2000b). Cathepsin B and L activity was also found to increase in porcine *Semitendinosus* following HPP treatment (600 MPa, 6 min, 5 °C, 6 days post-mortem) and throughout subsequent aging (Grossi et al., 2012). The increase in cathepsin activity following HPP could be explained by HPP disrupting the lysosomal membranes and accelerating the release of cathepsins (Jung et al., 2000b, Jung et al., 2000c, Grossi et al., 2012).

Limited research has been conducted on the effect of HPP on glycolysis in early post-mortem muscle. Morton et al. (2018) reported that the HPP treatment (175 MPa, 3 min) of 50 min post-mortem bovine *Longissimus thoracis* significantly increased the pH of muscle at 24 h post-mortem ($\text{pH } 5.9 \pm 0.02$) compared to controls ($\text{pH } 5.7 \pm 0.06$). They also found that HPP treatment significantly decreased the amount of glycogen phosphorylase, an enzyme responsible for glycogenolysis, in the sarcoplasmic fraction, and therefore, potentially reducing the rate of glycolysis (Morton et al., 2018). This was further investigated by Souza et al. (2011), who found that treating 45 min post-mortem porcine *Longissimus dorsi*, *Triceps brachii* and *Semembranosus* with HPP (215 MPa, 15 s, 33 °C) significantly increased the pH of muscle at 24 and 48 h post-mortem compared to controls. HPP significantly increased glucose content and significantly reduced lactate content in 48 h post-mortem porcine *Longissimus dorsi* compared to controls, which further indicates that HPP has the ability to inhibit glycolysis by inactivating the enzymes involved.

The impact HPP on the shear force values of muscle is dependent on many treatment related factors, in particular the pressure and temperature applied. In general, when HPP was applied to post-rigor muscle using ambient temperatures, the shear force values tended to increase, however, this was dependent on the pressure applied. When 24 h post-mortem bovine *Longissimus dorsi* was treated with HPP (100/200/300/400 MPa, 15 min, 18 °C), Neto et al. (2015) found that the effects of HPP varied depending on the pressure applied. Samples treated with 100 and 200

MPa resulted in a significant reduction in shear force values, with the 200 MPa treatment achieving a 21.12% decline in shear force values compared to controls. The 300 MPa treated samples had shear force values similar to controls, while the 400 MPa treatment significantly increased the shear force values. When 96 h post-mortem bovine *Longissimus lumborum* was HPP treated (300/500 MPa, 180 s, 15 °C), Utama et al. (2018) found that the shear force values significantly increased immediately post-treatment. However, following 28 days of aging, the shear force values of the 300 MPa treated muscles were similar to controls, while those of the 500 MPa treated muscle remained significantly higher (Utama et al., 2018). The shear force values of 48 h post-mortem *Longissimus dorsi* and *Biceps femoris* also increased significantly following HPP treatment (520 MPa, 260 s, 10 °C) and remained significantly higher following aging for 15 days post-treatment (Jung et al., 2000c). When similar HPP treatments were applied to muscle at higher temperatures (40 – 70 °C), it was found that HPP has some potential to reduce the shear force values of post-rigor muscle. When 8 day post-mortem bovine *Longissimus dorsi* was treated with a variety of HPP treatments (200/400/600/800 MPa, 20/40/60/70 °C, 20 min), Ma and Ledward (2004) observed that increasing the temperature of muscle to 60 and 70 °C without HPP treatment significantly increased the hardness of muscle. However, when 200 MPa was applied to muscle at 60 and 70 °C, it was found that the hardness of muscle was significantly reduced. The hardness of muscle increased at all temperatures investigated when pressure was increased to 400 – 600 MPa. In contrast, when McArdle et al. (2011) examined the effect of HPP treatment (400/600 MPa, 35/45/55 °C, 20 min) on the shear force values of 48 h post-mortem bovine *Pectoralis profundus*, they reported that the shear force values of the HPP treated samples did not differ significantly from the control, except for the 600 MPa – 35 °C treated samples, which had significantly higher shear force values. When considering the treatment parameters individually, it was found that independent of temperature, 600 MPa treated samples had significantly higher shear force values than the 400 MPa treated samples, while the 35 °C treated samples had significantly higher shear force values than the 45 and 55 °C samples. A similar effect was found in a later study by McArdle et al. (2013), where it was observed that HPP treatment (200/400/600 MPa, 20/40/60 °C, 20 min) of 48 h post-mortem ovine *Pectoralis*

profundus resulted in no significant differences in the shear force values of treated and control samples, except for the 200 MPa – 60 °C treatment, which had the lowest shear force values. When pressure and temperature were evaluated for their individual effects, it was found that 600 MPa had significantly higher shear force values when compared to 200 and 400 MPa treated samples, while the 60 °C treated samples had significantly lower shear force values than those treated with 20 and 40 °C. The toughening effect of increased pressures was attributed to the shortening and compression of sarcomeres (McArdle et al., 2011, 2013).

Morton et al. (2018) attributed an improvement in sensory tenderness following the HPP treatment of pre-rigor muscle, to the disruption HPP induced to the sarcomere structure. Morton et al. (2018) treated bovine *Longissimus thoracis* within 60 min of slaughter with HPP (175 MPa, 3 min) and found that HPP treatment led to increased myofibrillar fragmentation, and disruption to the structure of the sarcomere, with the Z-lines no longer in a regular pattern and the over lapping of thick and thin filaments in A-bands and H-zones no longer visible. Following the HPP treatment (100 – 600 MPa, 260 s, 10 °C) of 48 h post-mortem bovine *Biceps femoris*, it was found that following the 325 MPa treatment, sarcomeres contracted and thick and thin filaments aggregated and became disorganised. Other changes included increases in H-band width and loss of I-band integrity. Similar changes were found following 520 MPa treatment (Jung et al., 2000a).

HPP can also affect the water holding capacity of muscle. When Marcos et al. (2010, 2014) examined the impact of HPP treatment (200/400/600 MPa, 10/20/30 °C, 20 min) on the expressible moisture of 7 day post-mortem bovine *Longissimus dorsi* and *Longissimus thoracis et lumborum*, respectively, it was reported that pressures greater than 200 MPa significantly increased expressible moisture compared to controls, while treatment temperature did not have an effect. McArdle et al. (2010) investigated the impact of HPP treatment (200/300/400 MPa, 20/40 °C) on the cook loss of 48 h post-mortem bovine *Pectoralis profundus*, and observed that 300 and 400 MPa increased cook loss compared to 200 MPa, while HPP treatments at 40 °C reduced cook loss when compared to HPP treatments carried out 20 °C. However, when the cook loss of all treatment combinations were compared with the control,

the only HPP treatment that induced a cook loss greater than the control was the 400 MPa – 20 °C treatment combination (McArdle et al., 2010). Some researchers have attributed a reduction in water holding capacity following HPP treatment to a reduction in sarcoplasmic protein solubility, due to protein denaturation and aggregation (Marcos and Mullen, 2014). In addition, changes to the muscle structure may also be an important factor (Lowder and Mireles Dewitt, 2014).

HPP has been found to have a significant impact on the colour of muscle, in particular on the lightness (L^*) values, and this can have implications on consumer acceptability of HPP treated products. The L^* values of bovine muscle increases significantly following HPP treatment, as reported by Jung et al. (2000c) (48 h post-mortem bovine *Biceps femoris*, 520 MPa, 260 s, 10 °C), Marcos et al., (2010, 2014) (7 day post-mortem bovine *Longissimus* muscle, 200 – 400 MPa, 10 – 30 °C, 20 min) and McArdle et al. (2010) (48 h post-mortem bovine *Pectoralis profundus*, 200 – 400 MPa, 20 – 40 °C). The whitening effect observed following the HPP treatment of muscle has been attributed to the denaturation of globin and displacement of haem, while an increase in surface water may increase the reflection at the surface of muscle (Bolumar et al., 2021, Carlez et al., 1995). The same authors found that HPP did not have an impact on redness (a^*), while Marcos et al., (2010, 2014) found that HPP treatments using pressures greater than 200 MPa significantly increased yellowness (b^*). In contrast, Jung et al. (2003) observed that HPP treatment (50 – 600 MPa, 20 – 300 s, 10 °C) increased the a^* values of 48 h post-mortem bovine *Biceps femoris*, with increasing pressure, up until 350 MPa, where the a^* values were reduced. The increase in a^* values following HPP corresponded with a decrease in metmyoglobin content (Jung et al., 2003, Carlez et al., 1995).

HPP has the potential to increase lipid oxidation of muscle (Guyon et al., 2016) and this is influenced by HPP parameters such as pressures and temperature (Bolumar et al., 2012). Ma et al. (2007) carried out a range of HPP treatments (200 – 800 MPa, 20 – 70 °C, 20 min) on 8 day post-mortem bovine *Longissimus dorsi* and reported that TBARS values increased with increasing pressure, especially after 400 MPa, when HPP was performed at 20 and 40 °C. However, when HPP was applied at 60 and 70 °C, increasing pressures did not increase the lipid oxidation of muscle to the same

extent. Both McArdle et al. (2010) (48 h post-mortem bovine *Pectoralis profundus*, 200 – 400 MPa, 20 – 40 °C, 20 min) and Ma et al. (2020) (48 h post-mortem ovine *Biceps brachii*, *Longissimus* and *Infrapinatus*, 200 MPa, 60 s, 8 °C) found that lipid oxidation was not affected by 200 MPa treatments, however, TBARS values increased when pressure treatments were greater than 400 MPa. When investigating the mechanism of free radical formation in bovine muscle as a result of HPP treatment (800 MPa, 20 °C, 10 min), Bolumar et al. (2014) reported that radicals were formed in the sarcoplasmic and myofibrillar fractions of muscle, and these radicals were both protein and iron derived and act as catalysts for lipid oxidation post-HPP treatment.

Similar to PEF and US, limited studies have been carried out on the impact of HPP treatment of pre-rigor muscle. Overall, it appears that HPP has a positive impact on the quality of muscle, when applied pre-rigor. When Morton et al. (2017, 2018) treated bovine *Longissimus thoracis* within 60 min of slaughter with HPP (175 MPa – 3min/ 250 MPa – 2 min, followed by 1 day aging), it was found that the shear force values of the HPP treated samples were comparable to those of control samples that were aged for 28 days. HPP treatment (175 MPa for 3 min) of bovine *Gluteus medius*, within 60 min of slaughter, resulted in more tender muscle when compared to the untreated muscles aged for 28 days. The 175 MPa treatment had minimal impact on the colour of the muscles, and also resulted in a decrease in cook loss. A similar reduction in shear force values and increase in myofibrillar fragmentation was observed by Zhang et al. (2018b), when 2 h post-mortem porcine loin was treated with HPP (100/200/300/400 MPa, 20 °C, 10 min), however, 300 and 400 MPa had to be applied to achieve a significant decrease in shear force values when compared to controls. The decrease in shear force values following pre-rigor HPP treatment has been attributed to the physical effects HPP has on the structure of muscle, such as disruption to the sarcomeres and myofibrils, demonstrated by an increase in myofibrillar fragmentation (Morton et al., 2018).

2.4 Conclusion

Tenderness is an important attribute of meat quality and is influenced by post-mortem biochemical processes, in particular glycolysis and proteolysis. Traditional carcass interventions such as hanging, aging, and electrical stimulation have been

successfully applied to improve the tenderness of muscle. Similarly, innovative technologies such as pulsed electric fields, ultrasound and high pressure processing are potential methods to enhance the tenderness of meat, their effectiveness can be inconsistent, varying with muscle type and treatment parameters.

The variability in results following the application of innovative technologies on meat highlights the need for a deeper understanding of how the biochemical pathways are being altered. The emergence of new analytical technologies, such as proteomics and metabolomics offer the potential in elucidating how these interventions may alter the breakdown of protein, enzyme activity and metabolic pathways, hence clarifying the mechanisms responsible for tenderness improvements. Future research utilising these tools will enable us to understand the mechanisms at play following the various interventions.

Chapter 3. Assessing the impact of ultrasound on the rate and extent of early post-mortem glycolysis in bovine *Longissimus thoracis et lumborum*

Overview

This chapter explores the use of ultrasound as a potential intervention to manipulate the rate of glycolysis in early post-mortem bovine *Longissimus thoracis et lumborum*, *in vivo* and *in vitro*, through the use of a glycolytic buffer system. The study examined how ultrasound treatments of different intensities and durations impacted the rate of pH decline, both *in vivo* and *in vitro*, and by monitoring the *in vitro* depletion of glycogen and accumulation of lactic acid and reducing sugars. The results of this chapter have been published in the journal – Meat Science:

- **Kent, M. A.,** Mullen, A. M., O'Neill, E. & Álvarez, C. (2024). Assessing the impact of ultrasound on the rate and extent of early post-mortem glycolysis in bovine *Longissimus thoracis et lumborum*. Meat Science, 214, 109531, <https://doi.org/10.1016/j.meatsci.2024.109531>

3.1 Introduction

Tenderness is one of the main quality attributes that influences overall consumer satisfaction of meat, along with other organoleptic properties such as flavour and juiciness. Consistent tenderness is one of the main drivers that influences the consumers' willingness to pay a premium for beef products (Pethick et al., 2021) and to repeat a purchase. The rate of pH decline in post-mortem bovine muscle, has been found to have a significant effect on final tenderness (Ma et al., 2022, O'Halloran et al., 1997a, Veiseth-Kent et al., 2010). Furthermore, the interaction between pH and temperature post-mortem has been generally described as one of the main factors affecting tenderness (Hannula and Puolanne, 2004). To achieve optimum tenderness and avoid cold or hot shortening, it is recommended that muscle reaches pH 6 between temperatures of 12 and 35 °C (Thompson, 2002). A range of pre- and post-slaughter factors influence the rate of pH decline, including muscle type (oxidative or glycolytic), nutritional status, glycogen availability and pre-slaughter stress (Chauhan and England, 2018); as well as animal and carcass characteristics such as gender, age, size and fat cover (Cadavez et al., 2019). Post-mortem carcass interventions can also be applied to manipulate the rate of pH decline and temperature drop, such as controlled chilling regimes combined with electrical stimulation (Hwang et al., 2003, Hwang and Thompson, 2001b).

Ultrasound, a non-thermal technology, involves the use of sound waves that operate at frequencies greater than 20 kHz and these cannot be detected by the human ear. Ultrasound waves operate longitudinally, and results in the repeated compression and rarefaction of bubbles within the medium being treated with ultrasound. This leads to the eventual collapse of the bubble, resulting in acoustic or transient cavitation (Gallo et al., 2018), the local development of high pressures and shear stresses and may also contribute to the formation of free radicals (Alarcon-Rojo et al., 2019, Dang et al., 2022, Kang et al., 2016b). Ultrasound can alter enzymatic activity, by altering the substrate or modifying the interaction between the enzyme and substrate or changing the conditions in which the enzyme operates (Huang et al., 2017). Thus, it has the potential to modify the kinetics of pH decline in post-mortem muscle, since this is an enzymatic driven process. The use of ultrasound on

post-mortem bovine muscle has been investigated with varying results on meat quality traits such as tenderness, colour and water holding capacity reported (Fallavena et al., 2020, Gonzalez-Gonzalez et al., 2020, Jayasooriya et al., 2007). This technology may have potential to be developed as a new way to manipulate pH decline in early post-mortem muscle, however, the impact of ultrasound on this key metabolic pathway has not been investigated in depth to the best of our knowledge.

The aim of the present study was to investigate if ultrasound (15/30/45 min, 25/45 kHz) could be used as an alternative technology to change the rate of pH decline of early post-mortem bovine muscle, using *Longissimus thoracis et lumborum* as a model. The effect of the ultrasound treatments on the activity of glycolytic enzymes was also investigated. This involved preparing crude extracts from the muscles immediately post-treatment and assessing the activity of glycolytic enzymes present in the crude extracts on the basis of changes in pH and the concentration of various constituents of an *in vitro* buffer system (England et al., 2014, England et al., 2016).

3.2 Materials and methods

3.2.1 Chemicals and reagents

ADP, creatine, disodium hydrogen phosphate, NAD⁺, potassium sodium tartrate, sodium acetate (Sigma Aldrich, Ireland), ATP (Cytiva, United Kingdom), carnosine (Apollo Scientific, United Kingdom), 3,5-dinitrosalicylic acid, glycogen, hydrochloric acid, sodium hydroxide (Acrós Organic, ThermoFisher, United Kingdom), L-Lactic Acid assay kit (Megazyme, Co.Wicklow, Ireland), magnesium chloride, potassium chloride (VWR Chemicals, Ireland), perchloric acid (Alfa Aesar, United Kingdom), potassium hydroxide (Merck Emsure, Ireland), liquid nitrogen (AirProducts, Ireland). All chemicals were at least of reagent grade.

3.2.2 Muscle preparation and treatment

Bovine *Longissimus thoracis et lumborum* (LTL) muscles were collected from 4 crossbred animals of similar carcass classification (conformation score R, O=, fat score 2 to 3, based on the EUROP carcass classification system, aged 18 – 26 months, average side hot weight of 138.1 ± 1.63 kg) from a local commercial slaughterhouse (Kepak, Ireland). The muscle from the right side of the carcass was hot boned and

collected within 90 min of slaughter. The muscles were transported to the Teagasc Food Research Centre, Ashtown, under refrigeration, which took approximately 60 min. On arrival, the pH and temperature of the muscle were measured in three different locations, using a temperature/pH meter, with integrated temperature compensation (Hanna HI 98163 portable pH meter for meat and FC2323 meat pH electrode, Hanna Instruments Ltd., Leighton Buzzard, UK), which was calibrated prior to measurement using three pH buffers of 4.0, 7.0, and 10.0. The muscles were trimmed of visible fat and each muscle was cut into 13 steaks that were 1.5 cm thick. One steak from each animal was finely chopped and portions (5 g) were placed in cryovials, snap frozen in liquid nitrogen and stored at -80°C for metabolite analysis prior to treatment. Each steak was individually vacuum-packaged using a Henkelman Jumbo 42 (Original Henkelman Vacuum Systems, the Netherlands). Steaks were randomly assigned to a control group (one steak per each one of the treatment combinations) and treatment group, comprised of three ultrasound times (15/30/45 min) and two frequencies (25/45 kHz), giving a total of 6 different combinations. For each treatment (time and frequency combination), 1 steak was treated with the assigned treatment, while a second steak was used as a corresponding control (i.e. the steak was immersed in water at the same temperature, but with no ultrasound applied).

3.2.3 Ultrasound treatment

The steaks were treated with ultrasound for 15, 30 or 45 min at a frequency of either 25 or 45 kHz in an Elmasonic xtra ST 500H ultrasonic water bath (Elma Schmidbauer GmbH, Singen, Germany). The vacuum packed steaks were suspended and placed directly into the ultrasonic water bath. Corresponding control samples were placed in another water bath (LTD20G, Grant Instrument, UK). Both water baths were connected, so the water bath temperature in the ultrasound treated and control steaks were the same and maintained below 10°C (Figure 3-1). To eliminate the effect of time post-mortem on the effect of treatment, 25 kHz treatments were performed first on the muscles from animals 2 and 4, while 45 kHz treatment were performed first on muscles from animals 1 and 3.



Figure 3-1. The setup for ultrasound treatment using the water bath system.

On the completion of treatment, the steaks were removed from the vacuum packs and divided in two. One half of the steak was finely chopped and portions (5 g) were placed in cryovials, which were snap frozen in liquid nitrogen and stored in -80°C prior to freeze-milling and use in the *in vitro* buffer model system. The second half of the steak was stored at 4°C , unwrapped, where pH was measured at 0, 30, 90, 240 and 1440 min post-treatment using a calibrated portable pH meter.

3.2.4 *In vitro* buffer system

Experiments using the *in vitro* glycolytic buffer were carried out according to the method of England et al. (2016). Muscle samples from control and treatment groups were powdered in liquid nitrogen using a freezer mill (SPEX SamplePrep 6750 Freezer/Mill) and stored at -80°C . Powdered muscle samples (10 g) were homogenised using an Ultraturrax at 6,000 rpm for 30 s, at a ratio of 1:10 (w/v) into a reaction buffer containing 10 mM Na_2HPO_4 , 5 mM MgCl_2 , 60 mM KCl, 5 mM ATP, 0.5 mM ADP, 0.5 mM NAD^+ , 30 mM glycogen, 25 mM carnosine, 30 mM creatine and 10 mM sodium acetate (pH 7.4). The mixture was immediately transferred into a jacketed reaction vessel, where it was maintained at 25°C using a recirculating water bath (Lauda Dr. R. Wobser GmbH & Co. KG, Germany), with constant stirring by means of a magnetic stirrer. Aliquots for metabolite analysis were removed at 0, 30, 90, 240 and 1440 min. pH of the glycolytic buffer system at each time point was

measured using a calibrated pH meter, with built in temperature compensation (Mettler-Toledo AG, Analytical, CH-8603 Schwerzenbach, Switzerland). The pH meter was calibrated prior to measurement, using three pH buffers of 4.0, 7.0, and 10.0.

3.2.5 Analysis of metabolites in muscle prior to ultrasound treatment and the *in vitro* glycolytic buffer/muscle homogenate model system during incubation at 25 °C for 1440 min

Preparation of supernatants from muscle for analysis of reducing sugars and glycogen: To determine the concentration of reducing sugars and glycogen in muscle samples prior to ultrasound treatment, supernatants were prepared according to the method of Hammelman et al. (2003), with modifications. Briefly, 1.5 g of frozen muscle was homogenised in 10 ml of 0.5 M PCA using an Ultraturrax at 13,500 rpm for 30 s. For analysis of reducing sugars, supernatants were prepared by centrifuging the homogenates (2 ml) at 10,000 *g* for 10 min. For glycogen analysis, the PCA/muscle homogenates (1 ml, without centrifugation) were added to an equal amount of 2.5 M HCl and hydrolysed at 90 °C for 2 h in a water bath, centrifuged at 4,000 *g* for 10 min and the supernatant was neutralised with 2 ml of 1.25 M KOH.

Preparation of supernatants from muscle for lactic acid analysis: For lactic acid analysis, supernatants were prepared according to the method outlined in the enzymatic lactic acid kit (L-Lactic Acid (L-Lactate) Assay Kit, Megazyme, Wicklow, Ireland). Briefly, frozen muscle (5 g) was homogenised in 20 ml of 1 M PCA using an Ultraturrax at 13,500 rpm for 5 min (2 x 2.5 min with a 30 s break in between). Approximately 40 ml of distilled water was added to the homogenate and the pH was adjusted to pH 10 using 2 M KOH. The mixture was transferred to a 100 ml volumetric flask and brought to volume with distilled water. Aliquots (2 ml) were removed, centrifuged at 10,000 *g* for 10 min and the supernatants were subsequently analysed using the enzymatic kit.

Preparation of supernatants from the muscle/glycolytic buffer mixtures for reducing sugar, glycogen and lactic acid analysis: Supernatants were prepared according to the method of England et al., (2016, 2014). For reducing sugars and lactic acid analysis, aliquots (2 ml) of the homogenate/glycolytic buffer mixtures were added to an equal volume of ice cold 1 M PCA, the mixture was then centrifuged at 4,000 *g* for

10 min and the supernatants neutralised with 1 ml of 2 M KOH. Prior to analysis for lactic acid using the enzymatic kit, the neutralised supernatants were diluted with an equal volume of Milli Q water.

For glycogen analysis, aliquots (1 ml) of the homogenate/glycolytic buffer mixtures were removed and added to an equal amount of 2.5 M HCl. The mixtures were heated to 90 °C and centrifuged, and the supernatants were neutralised as described above. Prior to analysis for glycogen, the neutralised supernatants were diluted with an equal volume of Milli Q water.

Reducing sugars and glycogen content (as glucose equivalents) of the supernatants prepared from muscle samples or the homogenate/glycolytic buffer mixtures were analysed using the Dinitrosalicylic Acid (DNS) method (Ghose, 1987, Miller, 1959).

3.2.6 Statistical analysis

The number of biological replicates required for this study was determined following a power analysis, based on preliminary studies, where the variance of pH, glycogen, reducing sugars and lactic acid, and the analytical methods resolutions were evaluated. Based on an alpha value of 0.05 and power of 0.8, a pH difference of 0.2 required a sample size of at least 2 animals; thus, 4 animals were selected to randomise the post-mortem time that each treatment was applied (see section 3.2.3). Analysis of pH decline in the muscle, post-treatment, and all variables from the *in vitro* buffer was carried out using MIXED procedure of the Statistical Analysis System, version 9.4 (SAS Institute Inc., Cary, NC, USA) with 4 replicates and 3 factors. The impact of frequency (control, 25 kHz, 45 kHz), treatment duration (15, 30, 45 min) and the repeated measure factor, sample time (0, 30, 90, 240 and 1440 min) were tested for a three – way interaction. Animals, the random effect, were treated as blocks. Treatment means and interactions were compared using Tukey's test. In all cases, a *P* – value < 0.05 was considered as significant.

3.3 Results and discussion

3.3.1 Biochemical status of muscle pre-ultrasound treatment

When pH and temperature was measured at the time of arrival to the research centre, prior to ultrasound treatment, the average pH value of the muscles at the

beginning of the experiment (2.5 h post-mortem) was pH 6.08 ± 0.05 and the average temperature was $31.93\text{ }^{\circ}\text{C} \pm 0.41$. The average glycogen, lactic acid and reducing sugars content were $21.8 \pm 0.2\text{ mM}$, $61.2 \pm 0.5\text{ mM}$ and $8.3 \pm 0.4\text{ mM}$, respectively.

The pH values obtained in this study were similar to those reported by Onopiuk et al. (2016) and O'Halloran et al. (1997a) for bovine *Longissimus dorsi* between 2 and 4 h post-mortem. The glycogen content recorded in this study was above the range reported by Coombes et al. (2014) and Onopiuk et al. (2016), who reported quantities of between 20.6 mM and 9.3 mM of glycogen in bovine *Longissimus* muscle, measured at 1 h and 4 h post-mortem, respectively. The lactic acid content recorded in the current study was slightly higher than that reported in literature for *Longissimus dorsi* muscle at 2 and 4 h post-mortem, where concentrations of between 24.2 mM and 42.2 mM were found by Onopiuk et al. (2016). The concentration of reducing sugars recorded in the current study was similar to that reported by Zuo et al. (2022), where 9.4 mM and 8.3 mM of glucose was found in bovine *Longissimus lumborum* muscle at 45 min and 12 h post-mortem, respectively.

3.3.2 pH decline in muscle and *in vitro* buffer system model systems

3.3.2.1 Muscle

Ultrasound frequency had no significant effect ($P > 0.05$) on the rate of pH decline. Following the ultrasound treatment of bovine LTL, a significant ($P < 0.05$) interaction was found between ultrasound frequency (0/25/45 kHz) and treatment duration (15/30/45 min), when examined within a three – way analysis of variance (Table 3-1). Treatment duration had a significant ($P < 0.05$) effect on the rate of pH decline, where the 30 min treatments (with 25 and 45 kHz) appear to significantly accelerate the rate of pH decline, when compared to the 30 min controls (Table 3-1). The pH_u, measured at 24 h post-treatment, was not affected by any of the treatments applied. The impact of ultrasound on the rate of pH decline in pre-rigor muscle has not been investigated in detail, to the best of our knowledge. Furthermore, it is difficult to compare the results of our study with others due to variations in the muscles and ultrasound treatment parameters used.

Table 3-1. Average pH values over the course of 1440 min post-ultrasound treatment when measured in the muscle. N = 4. Data is mean $\pm$ SEM. The *P* – values from the *F* – Test, following a three way analysis of variance (ANOVA) are included.

	25 kHz 15 min T	25 kHz 15 min C	25 kHz 30 min T	25 kHz 30 min C	25 kHz 45 min T	25 kHz 45 min C	45 kHz 15 min T	45 kHz 15 min C	45 kHz 30 min T	45 kHz 30 min C	45 kHz 45 min T	45 kHz 45 min C
0	6.23 $\pm$ 0.06 ^a	6.04 $\pm$ 0.06 ^a	6.01 $\pm$ 0.07 ^a	6.19 $\pm$ 0.07 ^a	6.03 $\pm$ 0.07 ^a	6.13 $\pm$ 0.07 ^a	6.12 $\pm$ 0.08 ^a	6.24 $\pm$ 0.08 ^a	6.00 $\pm$ 0.07 ^a	6.10 $\pm$ 0.07 ^a	6.16 $\pm$ 0.07 ^a	6.13 $\pm$ 0.07 ^a
30	6.14 $\pm$ 0.06 ^{ab}	5.96 $\pm$ 0.06 ^a	5.86 $\pm$ 0.07 ^a	6.02 $\pm$ 0.07 ^a	5.96 $\pm$ 0.07 ^a	6.03 $\pm$ 0.07 ^a	6.01 $\pm$ 0.08 ^a	5.95 $\pm$ 0.08 ^{ab}	5.86 $\pm$ 0.07 ^{ab}	6.04 $\pm$ 0.07 ^a	6.04 $\pm$ 0.07 ^a	6.03 $\pm$ 0.07 ^a
90	6.00 $\pm$ 0.06 ^{abc}	5.90 $\pm$ 0.06 ^a	5.80 $\pm$ 0.07 ^{ab}	5.97 $\pm$ 0.07 ^a	5.92 $\pm$ 0.07 ^a	5.96 $\pm$ 0.07 ^a	5.97 $\pm$ 0.08 ^{ab}	5.87 $\pm$ 0.08 ^{ab}	5.80 $\pm$ 0.07 ^{ab}	5.95 $\pm$ 0.07 ^a	5.98 $\pm$ 0.07 ^a	5.95 $\pm$ 0.07 ^a
240	5.87 $\pm$ 0.06 ^{bc}	5.82 $\pm$ 0.06 ^{ab}	5.72 $\pm$ 0.07 ^{ab}	5.84 $\pm$ 0.07 ^{ab}	5.81 $\pm$ 0.07 ^{ab}	5.83 $\pm$ 0.07 ^{ab}	5.91 $\pm$ 0.08 ^{ab}	5.81 $\pm$ 0.08 ^{ab}	5.77 $\pm$ 0.07 ^{ab}	5.86 $\pm$ 0.07 ^{ab}	5.87 $\pm$ 0.07 ^{ab}	5.89 $\pm$ 0.07 ^{ab}
1440	5.52 $\pm$ 0.06 ^d	5.52 $\pm$ 0.06 ^b	5.53 $\pm$ 0.07 ^b	5.54 $\pm$ 0.07 ^b	5.53 $\pm$ 0.07 ^b	5.53 $\pm$ 0.07 ^b	5.54 $\pm$ 0.08 ^b	5.55 $\pm$ 0.08 ^b	5.53 $\pm$ 0.07 ^b	5.53 $\pm$ 0.07 ^b	5.54 $\pm$ 0.07 ^b	5.53 $\pm$ 0.07 ^b
<i>P</i> – values												
Frequency	0.5333											
Duration	0.0174											
Sampling Time	< 0.0001											
Frequency*Sampling Time	0.9993											
Duration*Sampling Time	0.9396											
Frequency*Duration	0.0004*											
Frequency*Duration*Sampling Time	0.9462											

C: control, T: treated. For each treatment, a corresponding C was immersed in water for the specified time, but without ultrasound treatment.

* Pairwise comparison between individual groups showed no significant difference, thus grouping is not possible.

Sikes et al. (2014), using a stronger, more intense ultrasound treatment than used in this study (2 MHz, 60% input power setting, 48 kPa acoustic pressure amplitude, 5 min), reported that ultrasound had no significant impact on the pH, immediately post-treatment, of bovine *Sternomandibularis* (treated 3 h post-mortem), however, they did not track pH decline over time post-treatment. When Got et al. (1999) applied high power ultrasound (2.6 MHz, 10 W/cm², for 2 x 15 s intervals) to pre-rigor bovine *Semimembranosus*, an immediate increase in pH, by 0.2 units, was observed. The pH of the treated muscle remained higher than controls for a further 2 to 4 h post-treatment before the differences levelled off. Similar to our findings, Got et al. (1999) found that ultrasound treatment had no significant impact on the ultimate pH of muscle when comparing the ultrasound treated and control samples.

3.3.2.2 *In vitro* buffer systems

The *in vitro* glycolytic buffer system used in this study was originally developed by Scopes (1973) and more recently has been re-established by Gerrard's research group to establish some key findings on the roles of phosphofructokinase (England et al., 2014), mitochondria (Matarneh et al., 2018, Matarneh et al., 2021, Scheffler et al., 2015), AMP deaminase (England et al., 2015) and glycogen (England et al., 2016) in determining the rate of pH decline and the ultimate pH of muscle. The *in vitro* glycolytic buffer system was used in the current study to establish whether or not the ultrasound treatment of intact muscle had a permanent impact on the activity of glycolytic enzymes.

When comparing *in vitro* buffer model systems containing control or ultrasound treated muscle as a source of glycolytic enzymes, ultrasound frequency ($P > 0.05$) and treatment duration ($P > 0.05$) had no significant effect on the rate of pH decline. The pH values of the *in vitro* buffer systems decreased from pH 7.4, prior to the addition of muscle, to an average of pH 5.64 ± 0.01 over the 24 h reaction period (Figure 3-2). No significant interaction ($P > 0.05$) was detected between treatment frequency and duration. No significant differences ($P > 0.05$) were observed in the pH at 1440 min (pHu) between control and treated samples or between different ultrasound treatments.

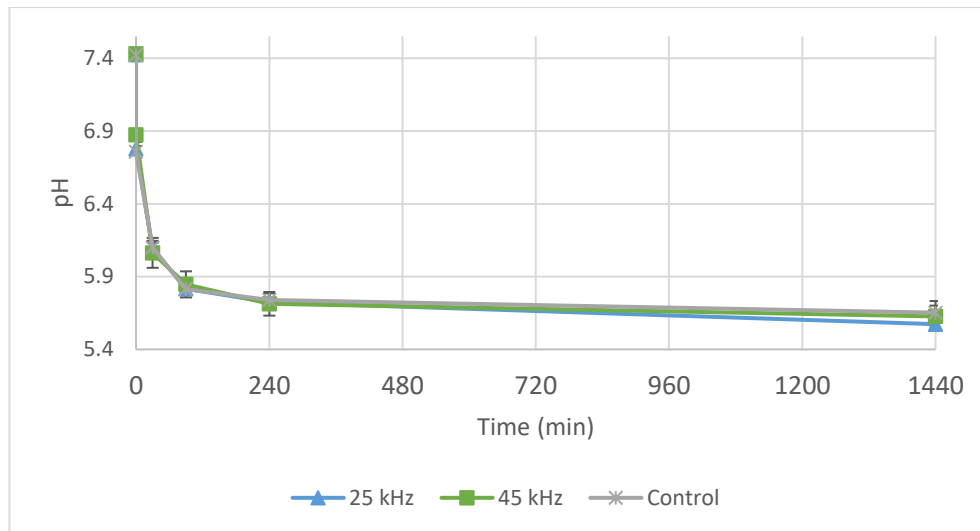


Figure 3-2. The average decline in pH over the course of 1440 min in the *in vitro* glycolytic buffer. N = 4. Data is mean $\pm$ SEM.

In this study, the rate of pH decline observed in control buffer systems were similar to that of England et al. (2015), who used porcine *Longissimus* muscle as a source of glycolytic enzymes in the *in vitro* buffer. The final pH values obtained using the *in vitro* glycolytic buffer in this study were within the range of values reported by Beline et al. (2021) and Chauhan et al. (2019), which were between pH 5.5 and 5.8, respectively.

3.3.3 Metabolites in the *in vitro* system

There were no significant differences ($P > 0.05$) in the rate of glycogen degradation between the *in vitro* buffer model systems containing ultrasound treated and control muscle at any time point, and no significant differences ($P > 0.05$) were observed between the different ultrasound treatments. Glycogen was included in the *in vitro* reaction buffer at 30 mM and its concentration decreased with time for controls and all treatments as a result of the activity of the enzymes present in the muscle added to the buffers, with the majority of glycogen degradation taking place within the first 240 min of the reaction (Figure 3-3). The rate of glycogen degradation decreased as the pH of the buffer approached pH 6. These results are consistent with those reported by England et al. (2016), England et al. (2015), Scheffler et al. (2015), Matarneh et al. (2017) and Chauhan et al. (2019), when using a similar *in vitro*

glycolytic buffer system to study post-mortem glycolysis in porcine, poultry and bovine muscles.

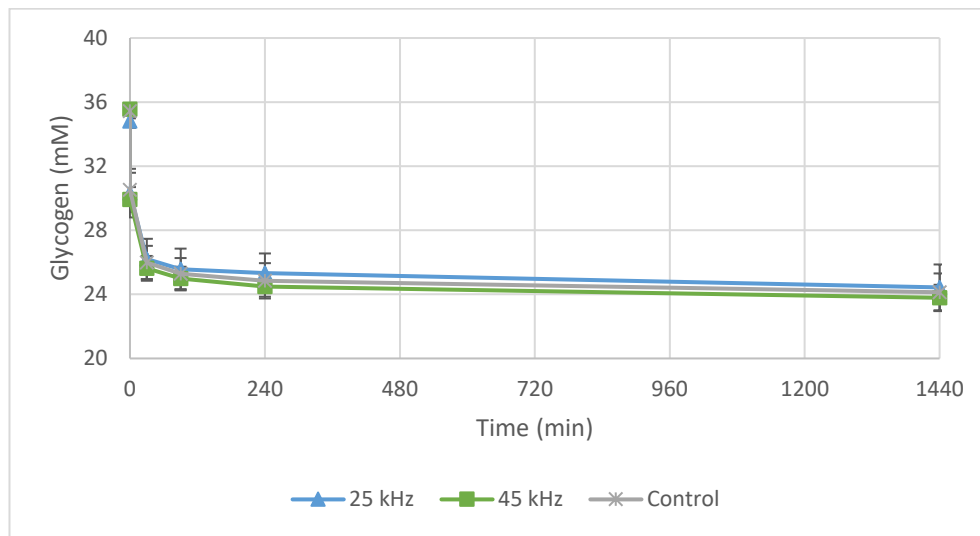


Figure 3-3. The average degradation of glycogen (mM) over the course of 1440 min in the *in vitro* glycolytic buffer. N = 4. Data is mean $\pm$ SEM.

It was found that there were no significant differences ($P > 0.05$) in the concentration of reducing sugars at the different sampling times when comparing *in vitro* buffer model systems containing treated (25/45 kHz, 15/30/45 min) and control muscle. An increase in reducing sugars in the first 30 min was observed, followed by a plateau in concentration between 30 and 90 min after the reaction was started, when the pH of the buffer approached pH 6. After this time point, the concentration of reducing sugars increased again from 240 to 1440 min (Figure 3-4). Glycogen is degraded by glycogen phosphorylase to release glucose-1-phosphate and debranching enzymes to release free glucose. Glucose-1-phosphate and glucose are then converted to glucose-6-phosphate by phosphoglucomutase and hexokinase, respectively, to be further utilised along the glycolytic pathway (Scheffler et al., 2015, Scheffler and Gerrard, 2007). Free glucose may also be allowed to accumulate in the muscle post-mortem (Matarneh et al., 2018). When Beline et al. (2021) investigated the progression of glucose in an *in vitro* glycolytic buffer system similar to that used in the current study, containing bovine *Longissimus thoracis* muscle, glucose content plateaued between 30 and 240 min of the reaction, before production resumed again from 240 min to 1440 min.

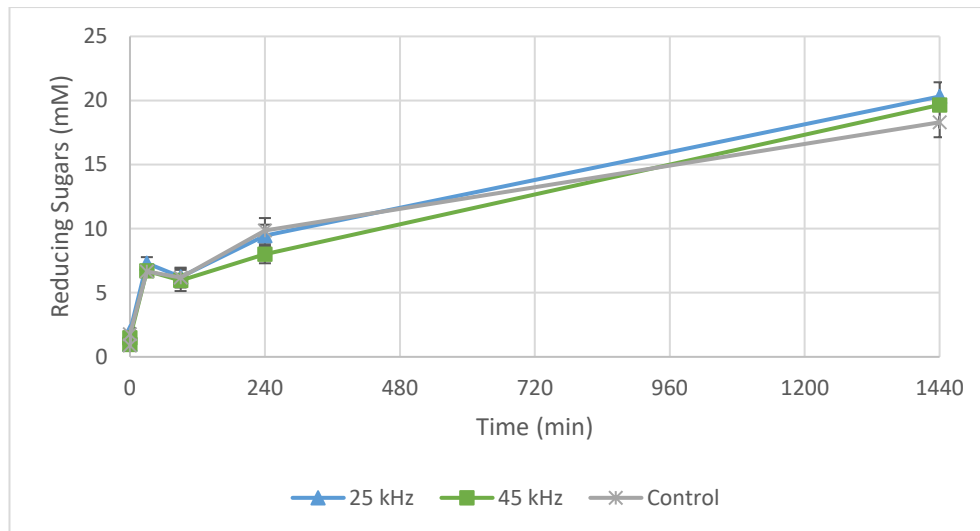


Figure 3-4. The average content of reducing sugars over the course of 1440 min in the *in vitro* glycolytic buffer. N = 4. Data is mean $\pm$ SEM.

The plateau in reducing sugars content between 30 and 90 min post-mortem observed in this study could be as a result of the efficient utilisation of reducing sugars by enzymes, such as glucose by hexokinase for glucose-6-phosphate production, at a rate equal to glucose production, while the subsequent increase in reducing sugar content between 90 and 1440 min could be as a result of the inhibition of enzymes such as hexokinase through negative feedback mechanisms, where there is sufficient glucose-6-phosphate in the system, making glucose unavailable for glycolysis and thus, allowing it to accumulate (Daly et al., 1999, Ferguson and Gerrard, 2014, Young et al., 1988).

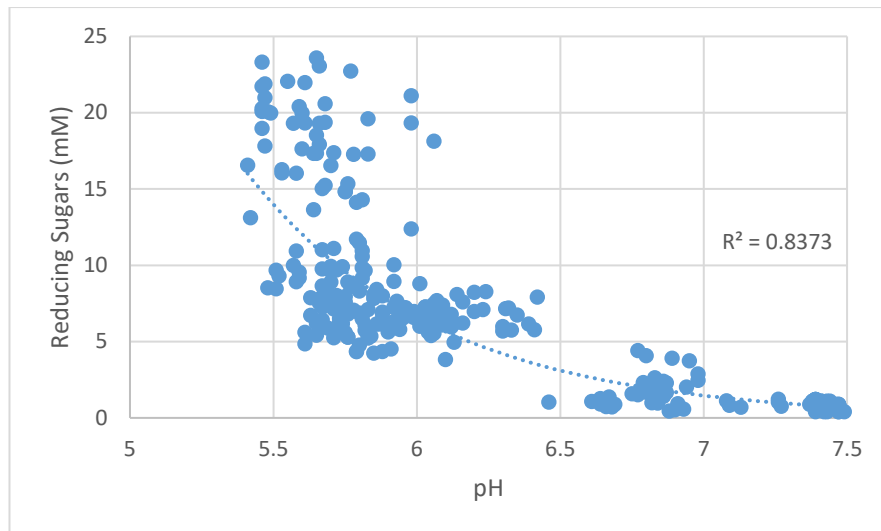


Figure 3-5. Reducing sugar content (mM) plotted against pH of the buffer, which appear to be exponentially related to each other. All samples points plotted. N = 4.

As the pH of the *in vitro* buffer decreased below pH 6 (90 min), there appears to be a change in the production/consumption balance of reducing sugars, as below this pH, the concentration of reducing sugars increases in the buffer system and this was observed for all treatments and controls (Figure 3-5). The accumulation of reducing sugars could also be related to the pH inactivation of phosphofructokinase, a key rate limiting enzyme, which decreases in activity as the pH of the reaction buffer and muscle reaches below pH 6 (England et al., 2014). Phosphofructokinase activity is also reduced as lactic acid accumulates (Leite et al., 2011), which happens simultaneously. The inactivation of phosphofructokinase, therefore, results in the subsequent accumulation of glucose, glucose-6-phosphate and fructose-6-phosphate (Matarneh et al., 2018).

As expected, the lactic acid content increased over time of the reaction in the *in vitro* buffer model systems containing treated (25/45 kHz, 15/30/45 min) and control muscle, with the majority of production taking place within the first 90 min of the reaction (Figure 3-6). There were no significant differences ($P > 0.05$) in lactic acid content when comparing treated and control samples at any time point and no significant differences ($P > 0.05$) were observed between the different ultrasound treatments. The profile of lactic acid production in the *in vitro* buffer model systems is in agreement with the results of England et al. (2016), England et al. (2015),

Scheffler et al. (2015) and Matarneh et al. (2017), when using an *in vitro* glycolytic buffer system similar to what was used in this study. Following 90 min of the reaction, where the pH of the buffer is below pH 6, there is a reduction in the rate of lactic acid production (Figure 3-6). The potential reason for this, as identified by England et al. (2014), is the inactivation of phosphofructokinase, which occurs when the pH of the reaction buffer decreases below pH 6. The inactivation of phosphofructokinase was also implicated by Matarneh et al. (2018) as the reason for the accumulation of glucose-6-phosphate during the later stages of incubation using a similar *in vitro* glycolytic buffer system. If phosphofructokinase is inactivated, there will be a decrease in the amount of substrate available to the reactions past this point of the glycolytic reaction, therefore, reducing the amount of substrate available for the production of lactic acid, halting its formation.

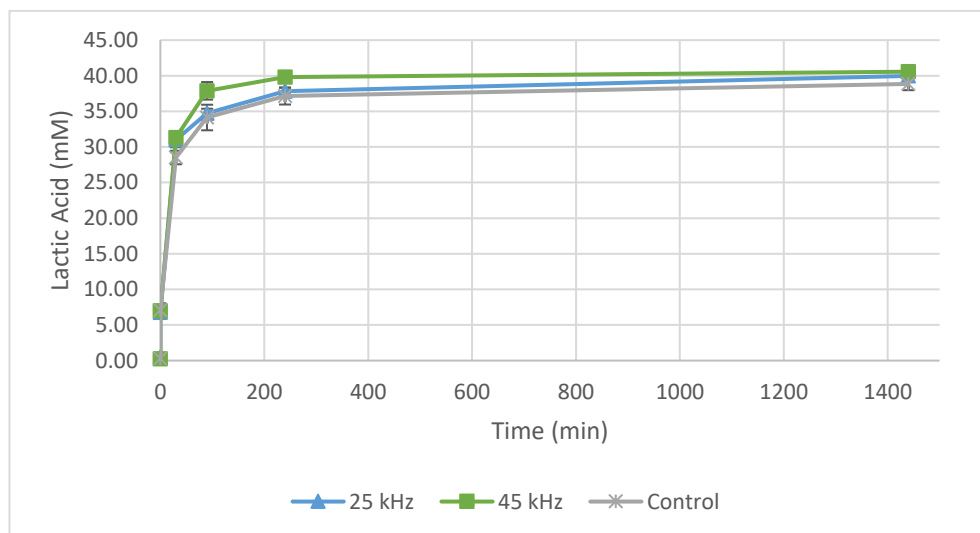


Figure 3-6. The average production of lactic acid (mM) over the course of 1440 min in the *in vitro* glycolytic buffer. N = 4. Data is mean $\pm$ SEM.

Ultrasound can alter enzymatic activity by either altering the substrate or the interaction between the enzyme and substrate (Huang et al., 2017). Low intensity treatments can be used to improve enzyme activity (that was one of the reasons to select low intensity treatments for this trial), whereas prolonged exposure to high intensity ultrasound treatment can result in the loss of enzyme activity (Huang et al., 2017, O'Donnell et al., 2010, Wang et al., 2018b). Our results show the ultrasound treatments used in this study did not permanently affect the activity of the enzymes

involved in glycolysis. The impact of ultrasound on the enzymes involved in post-mortem glycolysis has not been investigated, to the best of our knowledge, however, research has been carried out on the effect of ultrasound on the enzymes involved in post-mortem proteolysis. Chen et al. (2015) observed that ultrasound treatment (40 kHz for 30 and 60 min) of chicken *Pectoralis superficialis* significantly increased the activity of calpain and caspase-3, when measured *in vitro* using fluorometric assays. A similar increase in caspase-3 activity following the ultrasound treatment (40 kHz, 12 W/cm², 40 min at 4 °C) of 24 h post-mortem bovine *Longissimus thoracis et lumborum* was observed by Dang et al. (2022), however, in both of these studies the increase in enzyme activity as a result of ultrasound treatment was attributed to changes in the conditions experienced by the enzymes (e.g. cavitation-induced structural shocks to the ultrastructure resulting in membrane and mitochondrial damage) rather than a direct structural effect of ultrasound on the enzymes. Thus, the ability of ultrasound to increase the rate of pH decline in muscle post-mortem, as observed in this study, is likely due to ultrasound-induced changes within the muscle itself. Interestingly, Dang et al. (2022) demonstrated increased cytosolic calcium concentration in ultrasound treated beef steaks. It is well known that increases in calcium concentration play a key role in the ability of electrical stimulation to accelerate post-mortem glycolysis. Hence, it may also play a role in the mechanism whereby ultrasound increases the rate of post-mortem glycolysis.

3.4 Conclusion

The results show that the ultrasound treatments (specifically the 30 min treatments) used in this study do not permanently affect the activity of the enzymes involved in glycolysis, when analysed *in vitro*, but can accelerate the rate of pH decline in muscle post-mortem. This effect could be modulated by ultrasound induced shocks which cause changes to muscle ultrastructure and membranes which in turn change the conditions in which the glycolytic enzymes are operating. Further investigation is warranted to explore the mechanism involved.

Chapter 4. The impact of ultrasound treatment on glycolytic enzymes when applied to crude extracts from early post-mortem bovine muscle.

Overview

This chapter explores the effects of ultrasound treatment on the glycolytic enzyme activity of treated crude extracts, prepared from early post-mortem bovine *Longissimus thoracis et lumborum*. The aim of the study was to investigate if ultrasound affected the kinetics of glycolytic enzymes, following the removal of the muscle matrix. The effects of ultrasound on the crude extracts were monitored *in vitro*, through the use of a glycolytic buffer system, where the decline in pH and changes in concentration of glycogen, reducing sugars and lactic acid were monitored. The results of this chapter have been published in the journal – Ultrasonics Sonochemistry:

- **Kent, M. A.**, Mullen, A. M., O'Neill, E. & Álvarez, C. (2024). The impact of ultrasound treatment on glycolytic enzymes when applied to crude extracts from early post-mortem bovine muscle. *Ultrasonics Sonochemistry*, 104, 106842. <https://doi.org/10.1016/j.ultsonch.2024.106842>

4.1 Introduction

Tenderness, along with other organoleptic qualities, such as colour, flavour and juiciness, play an important role in the overall eating quality of meat (Liu et al., 2022a). The rate of early post-mortem glycolysis (O'Halloran et al., 1997a, Ma et al., 2022), as well as the interaction between pH and temperature decline in muscle (Hwang and Thompson, 2001b, White et al., 2006b), have been found to play an important role in determining meat tenderness. For optimum tenderness, it has been recommended by the Meat Standards Australia (MSA) that the muscle is between the temperatures of 15 and 35 °C when pH 6 is reached, since muscle is susceptible to hot and cold shortening outside of this pH/temperature window (Thompson, 2002). Factors that influence the rate of pH decline in muscle include the muscle type (oxidative or glycolytic), the nutritional and stress status of the animal pre-slaughter (Chauhan and England, 2018), as well as the age, gender and size of the animal (Cadavez et al., 2019). A number of post-mortem carcass interventions have been implemented in the meat industry to manipulate the rate of pH and temperature decline, such as chilling and electrical stimulation (Hwang et al., 2003, Hwang and Thompson, 2001a), aimed at avoiding the detrimental effects of heat and cold shortening and optimising tenderness.

Ultrasound (US) is a non-thermal technology that involves sound waves operating at frequencies of 20 kHz or greater. Ultrasound treatment results in continuous compression and rarefaction of bubbles within the medium being treated, with the eventual collapse of the bubbles causing acoustic or transient cavitation (Gallo et al., 2018), which can affect the macromolecules of enzymes and their substrates (Wang et al., 2018b). Ultrasound has been investigated for its ability to alter enzymatic activity in food systems. It can affect enzymes directly, the substrates or the interaction between the enzyme and substrate (Huang et al., 2017). Studies have been carried out to investigate the impact of ultrasound treatment on post-mortem (Jayasooriya et al., 2007, Fallavena et al., 2020, Gonzalez-Gonzalez et al., 2020), and pre-rigor bovine muscle (Lyng et al., 1997, 1998b, Got et al., 1999), primarily focused on the impact of ultrasound on the quality traits of muscle, such as tenderness, colour and water holding capacity, with varying results.

The impact of ultrasound treatment on the rate of glycolysis in pre-*rigor* muscle has not been studied in depth, to the best of our knowledge. Our group carried out a recent study (Kent et al., 2024a) investigating the impact of ultrasound treatments (25/45 kHz, 15/30/45 min) of pre-*rigor* bovine muscle on the inherent activities of glycolytic enzymes using an *in vitro* glycolytic buffer system. This glycolytic buffer system had previously been used by other researchers to study post-mortem glycolysis in porcine, chicken and bovine muscle (England et al., 2014, England et al., 2015, England et al., 2016, Matarneh et al., 2018, Chauhan et al., 2019, Beline et al., 2021). In a previous trial (Kent et al., 2024a), we found that the ultrasound treatment of intact bovine *Longissimus thoracis et lumborum* did not affect the inherent activity of glycolytic enzymes, when examined in an *in vitro* glycolytic buffer, post-treatment. Lyng, Allen and McKenna (1998a, 1998b) reported that ultrasound (20 kHz for periods of 15 s) had no significant impact on the proteolysis of pre- and post-*rigor* bovine and ovine muscle, and hypothesised that the structure of the muscle restricted the vibrations resulting from the ultrasound treatments, reducing the intensity of the ultrasound waves travelling through the muscle. Therefore, we have hypothesised that by removing the physical protection that the whole muscle matrix may exert, ultrasound may have the potential of affecting the kinetics of glycolytic enzymes. Thus, the aim of the current study is to investigate the effect of ultrasound treatment (20 kHz, 60% amp (11.4 W/cm²) and 100% amp (19 W/cm²) amplitude for 15 or 30 min) of muscle homogenates (muscle:water ratio of 1:5) on glycolysis, monitored *in vitro* using a glycolytic buffer system.

4.2 Materials and methods

4.2.1 Chemicals and reagents

ADP, creatine, disodium hydrogen phosphate, NAD⁺, potassium sodium tartrate, sodium acetate (Sigma Aldrich, Ireland), ATP (Cytiva), carnosine (Apollo Scientific, United Kingdom), 3,5-dinitrosalicylic acid, glycogen, hydrochloric acid, sodium hydroxide (Acrós Organic-ThermoFisher, UK), L-Lactic Acid assay kit (Megazyme, Ireland), magnesium chloride, potassium chloride (VWR Chemicals, Ireland), perchloric acid (Alfa Aesar, United Kingdom), potassium hydroxide (Merck Emsure,

Ireland), liquid nitrogen (AirProducts, Ireland). All chemicals were at least of reagent grade.

4.2.2 Animal and sample preparation

Bovine *Longissimus thoracis et lumborum* muscles were collected from 4 crossbred steers of similar carcass classification (conformation score O - to R +, aged 22 – 29 months, average side hot weight of 183.15 ± 28.11 kg) from a local commercial slaughterhouse (Kepak, Ireland). The muscle from the left hand side was hot boned from the carcass and collected within 90 min of slaughter. Under refrigeration, the muscles were transported to the Teagasc Food Research Centre, Ashtown, which took approximately 60 min. On arrival, the pH and temperature of the muscle were measured at three different locations, using a temperature/pH meter, with integrated temperature compensation (Hanna HI 98163 portable pH meter for meat and FC2323 meat pH electrode, Hanna Instruments Ltd., Leighton Buzzard, UK). Prior to measurement, the pH meter was calibrated using three pH buffers of 4.0, 7.0, and 10.0. Visible fat was trimmed from the muscles and each muscle was cut into 6 steaks of equal thickness (1.5 cm). The steaks were randomly assigned to a control group and treatment group, comprised of two ultrasound treatment durations (15/30 min) and two ultrasound intensities (100% or 60% amp (19 W/cm^2 and 11.4 W/cm^2 , respectively)), giving a total of 4 different combinations. A control sample was assigned to each treatment duration (15/30 min). The steaks were finely chopped and portions (5 g) were placed into cryovials, and snap frozen in liquid nitrogen. These portions of frozen muscle were pooled and powdered using liquid nitrogen and a freezer mill (SPEX SamplePrep 6750 Freezer/Mill). The powdered samples were stored at -80°C until the time of treatment. An additional steak from each animal was finely chopped and portions (5 g) were placed into cryovials, snap frozen in liquid nitrogen and stored at -80°C for determination of initial metabolite concentrations in muscles.

4.2.3 Preparation of supernatants for determination of initial metabolite concentrations in muscles

Samples for the analysis of glucose and glycogen were prepared according to Hammelman et al. (2003), with modifications. Briefly, for glucose analysis, 1.5 g of

frozen muscle was homogenised into 10 ml of 0.5 M perchloric acid (PCA) using an Ultraturrax (13,500 rpm for 30 s). Aliquots (2 ml) of homogenate were centrifuged, using a Sigma 1–15 Benchtop Centrifuge (SIGMA Laborzentrifugen GmbH, Germany) at 10,000 *g* for 10 min. For glycogen analysis, frozen muscle (1.5 g) was homogenised in 10 ml of 0.5 M PCA using an Ultraturrax (13,500 rpm for 30 s). Homogenate (1 ml) was added to an equal amount of 2.5 M HCl, hydrolysed at 90 °C for 2 h, centrifuged for 10 min at 4,000 *g* and the supernatant was neutralised with 1.25 M KOH (2 ml). Sample preparation for lactic acid analysis was carried out according to the method outlined in the L-Lactic Acid Kit Assay kit (Megazyme, Ireland), where 5 g of frozen muscle was homogenised in 20 ml of 1 M PCA using an Ultraturrax, for 5 min (2 x 2.5 min with a 30 s break in between) at 13,500 rpm. Approximately 40 ml of distilled water was added to the homogenate. The pH of the homogenate was adjusted to pH 10 using 2 M KOH, before being made to volume in a 100 ml volumetric flask. Aliquots (2 ml) of the homogenate were removed, centrifuged for 10 min at 10,000 *g* and subsequently analysed.

4.2.4 Ultrasound treatment

For ultrasound treatment, 20 g of powdered muscle (Section 4.2.2) was suspended in 100 ml of water using an Ultraturrax (6,000 rpm for 30 s). The homogenates were sonicated using a 20 kHz ultrasound probe (UIP500hdT, Hielscher ultrasound technology, Germany), where the probe, with a diameter of 1.8 cm, was submerged into the homogenate at a depth of 1.5 cm. The homogenates were treated with ultrasound for 15/30 min at amplitude levels of 60% or 100% amp (11.4 W/cm² and 19 W/cm², respectively), in a jacketed flask, where the temperature of the treated homogenate was maintained at 32 °C ± 2.5 °C with a circulating water bath (LTD20G, Grant Instrument, UK) (Figure 4-1). The mixtures were stirred constantly throughout the treatments. Controls were prepared where 20 g of powdered muscle was suspended in 100 ml of water using an Ultraturrax (6,000 rpm for 30 s) and the homogenate was stirred constantly for 15/30 min in a jacketed flask, without the application of US. The temperature of the control was maintained at 32 °C with a circulating water bath (Lauda ECO RE415S (Lauda Dr. R. Wobser GmbH & Co. KG, Germany)).



Figure 4-1. The setup for ultrasound treatment using the probe system.

4.2.5 *In vitro* glycolytic buffer model system

Experiments using the *in vitro* glycolytic buffer were carried out based on the method of England et al., (2016). Initially, the buffer was made up to contain 20 mM Na_2HPO_4 , 10 mM MgCl_2 , 120 mM KCl, 10 mM ATP, 1 mM ADP, 1 mM NAD^+ , 60 mM glycogen, 50 mM carnosine, 60 mM creatine and 20 mM sodium acetate (pH 7.4) (this is a double strength buffer which is subsequently diluted (1:1) on addition of the homogenate). On completion of the individual ultrasound treatments, half of the control or treated homogenate (50 ml) was added to 50 ml of the buffer and immediately transferred into a jacketed reaction buffer vessel. This resulted in an overall homogenate containing 10 mM Na_2HPO_4 , 5 mM MgCl_2 , 60 mM KCl, 5 mM ATP, 0.5 mM ADP, 0.5 mM NAD^+ , 30 mM glycogen, 25 mM carnosine, 30 mM creatine and 10 mM sodium acetate, with a 1:10 muscle (w/v) inclusion; resulting in the final concentrations as described by England et al. (2016). The temperature of the homogenate was maintained at 25 °C for the duration of the experiment, using a recirculating water bath (Lauda ECO RE415S (Lauda Dr. R. Wobser GmbH & Co. KG, Germany)), with constant stirring using a magnetic stirrer. Aliquots were removed at 0, 30, 90, 240 and 1440 min for subsequent metabolite analysis. The pH of the *in vitro* glycolytic buffer system was measured at each time point using a calibrated pH

meter, with built in temperature compensation (Mettler-Toledo AG, Analytical, CH-8603 Schwerzenbach, Switzerland).

4.2.6 Preparation of supernatants for metabolite analysis of the *in vitro* glycolytic buffer model system

For glycogen analysis, aliquots (1 ml) of homogenate or glycolytic buffer mixture were added to an equal amount of 2.5 M HCl, where the mixture was heated for 2 h at 90 °C in a water bath, centrifuged for 10 min at 4,000 *g* and neutralised with 1.25 M KOH (2 ml). The neutralised homogenate was subsequently diluted with an equal amount of Milli – Q water prior to analysis. For glucose (and other reducing sugars) and lactic acid analysis, aliquots (2 ml) of homogenate were removed and added to an equal amount of ice cold 1 M PCA, before being centrifuged at 4,000 *g* for 10 min and the supernatants were neutralised using 1 ml of 2 M KOH prior to analysis. For lactic acid quantification, a 50/50 dilution of the neutralised PCA extract was prepared with Milli – Q water to be within the detection limits of the kit.

4.2.7 Determination of concentration of metabolites in supernatants

The concentration of glucose (and other reducing sugars such as fructose and its phosphorylated conjugates) and glycogen (as glucose equivalents) in the supernatants was measured spectrophotometrically using the DNS method (Miller, 1959, Ghose, 1987). Lactic acid concentration in the supernatants was determined using an enzymatic kit (L-Lactic Acid (L-Lactate) Assay Kit, Megazyme, Wicklow, Ireland).

4.2.8 Statistical analysis

Data in this paper is presented as average $\pm$ SEM. To determine the number of replicates required for this study, a power analysis was performed, based on preliminary studies, where the resolutions of the analytical methods and variance of glucose (and other reducing sugars), glycogen and lactic acid were evaluated. To obtain a pH difference of 0.2, based on a power of 0.8 and alpha value of 0.05, a sample size of 2 was required. From this, a sample size of 4 was selected. Analysis of all variables from the *in vitro* buffer was carried out using MIXED procedure of the SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA) with 4 replicates and 3 factors: intensity (control, 100% or 60%), treatment duration (15 or 30 min) and the repeated

measure factor, sample time (0, 30, 90, 240 and 1440 min). Animals were treated as a random effect. Treatment means and interaction were compared using Tukey's test. In all cases, a P – value < 0.05 was considered as significant.

4.3 Results

4.3.1 Biochemical status of muscle prior to ultrasound treatment

The average pH value of the muscles, at 2.5 h post-mortem, was pH 6.42 (± 0.07) and the average temperature was 29.83 °C (± 0.60). The pH values obtained in this study were similar to those found by Onopiuk et al. (2016) and Byrne et al. (2000) in bovine *Longissimus dorsi*, at a similar time post-mortem.

The glycogen concentration was 1.28 ± 0.13 g/100 g, which is within the range of that reported by Coombes et al. (2014) and Onopiuk et al., (2016), who reported quantities of between 1.36 g/100 g and 0.62 g/100 g glycogen, measured at 1 and 4 h post-mortem, respectively, in bovine *Longissimus* muscle. The lactic acid content of muscle used in this study, 0.28 ± 0.03 g/100 g, was within the range reported in literature for bovine *Longissimus* and *Semitendinosus* muscle between 2 and 4 h post-mortem, where quantities of between 0.22 and 0.38 g/100g were found (Onopiuk et al., 2016, Bogdanowicz et al., 2022). The content of reducing sugars (mainly glucose at this stage) in the muscle used in this study was 0.068 ± 0.008 g/100 g, was within the range found in the literature, where levels of 0.036 g/100 g (Ramos et al., 2021) to 0.169 g/100 g (Zuo et al., 2022) were reported in bovine *Longissimus lumborum* within the first 3 h post-mortem. The results show that muscles employed for this study were a faithful representation of an average bovine *Longissimus* muscle at 2 or 3 h post-mortem.

4.3.2 pH decline

When muscle homogenates were added to the glycolytic buffer system (as sources of glycolytic enzymes), the pH declined over the 1440 min duration of incubation at 25 °C, with the pH decreasing, for controls and treatments, from an initial pH of 7.42 ± 0.01 , prior to the addition of muscle, to an average of pH 5.50 ± 0.04 , except for buffers containing muscle treated with 100% amp for 30 min, where the average pH after 1440 min was pH 5.78 ± 0.15 (Figure 4-2). While the average pH value of the

100% amp, 30 min treatment containing buffer system at 1440 min was numerically higher than the combined average pH of controls and other treatment containing buffers, no significant differences were observed between treatments at 1440 min. The majority of pH decline took place within the first 240 min. The rate of pH decline was significantly affected by ultrasound treatment duration ($P < 0.05$) and intensity ($P < 0.05$) and there was a significant interaction ($P < 0.05$) between these two parameters; where the treatment carried out at 100% amp for 30 min resulted in a slower rate of pH decline when compared to controls and other treatments. The slower rate of pH decline was observed for the first 240 min in particular, with the pH of the buffer system containing the 100% amp, 30 min treatment being significantly higher ($P < 0.05$) when compared to control and other treatment containing buffers between 30 and 240 min.

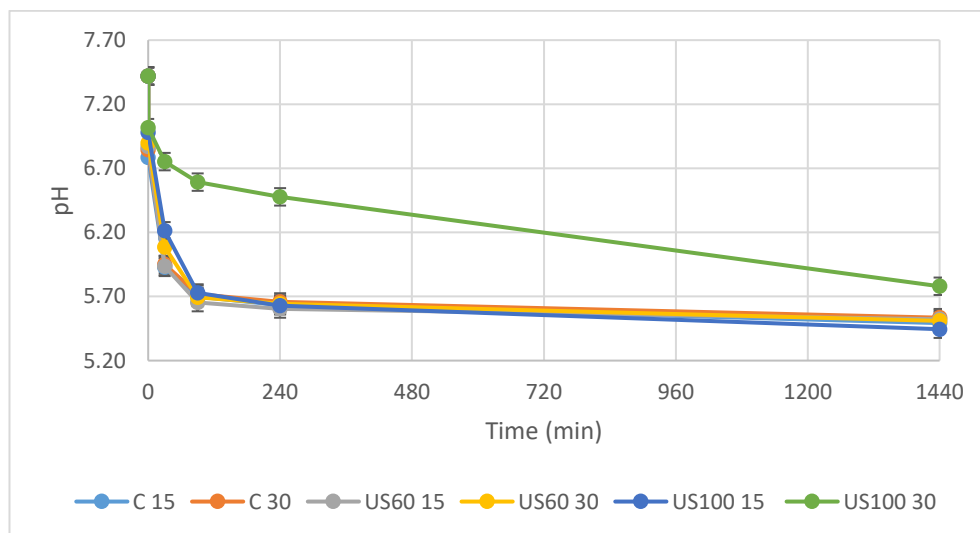


Figure 4-2. pH decline of the *in vitro* glycolytic reaction buffer over 1440 min (24 h) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60% amp for 15 min (US60 15), 60% amp for 30 min (US60 30), 100% amp for 15 min (US100 15), 100% amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SEM.

The rate of pH decline in the glycolytic buffer system, illustrated in Figure 4-2, for controls and all treatments except for that containing muscle homogenates treated at 100% amp ultrasound for 30 min, was similar to that reported by Beline et al. (2021), England et al. (2014, 2015, 2016) and Matarneh et al. (2018), who used

bovine, porcine and chicken muscle, respectively, as a source of glycolytic enzymes in a similar glycolytic buffer model system.

4.3.3 Concentration of metabolites in the *in vitro* glycolytic buffer model system

An *in vitro* glycolytic buffer system was employed in this study to assess the potential impact of the various ultrasound treatments on the inherent activities of glycolytic enzymes present in the muscle homogenates, and to monitor changes in glycogen, glucose (and other reducing sugars) and lactic acid content over a 24 h period of incubation. Overall, it was found that treatment time duration and intensity had a significant effect ($P < 0.05$) on the concentration of these metabolites, specifically following the 100% amp (19 W/cm²), 30 min treatment.

4.3.3.1 Glycogen

Glycogen was included in the *in vitro* glycolytic buffer at a concentration of 30 mM. During the incubation period, the concentration of glycogen decreased, as a result of the activity of glycolytic enzymes provided by the muscle homogenates. The majority of the glycogen degradation took place within the first 240 min, and this degradation pattern was in agreement with the findings of other studies using a similar buffer system (England et al., 2016, 2015, Matarneh et al., 2017, Scheffler et al., 2015) (Figure 4-3). No significant effect ($P > 0.05$) on glycogen degradation was observed with ultrasound intensity (control, 60% amp, 100% amp) or treatment duration (15 min, 30 min) at any given sampling point (Figure 4-3). These results indicate that ultrasound did not have an impact on the utilisation of glycogen, neither promoting its degradation nor impeding its depolymerisation.

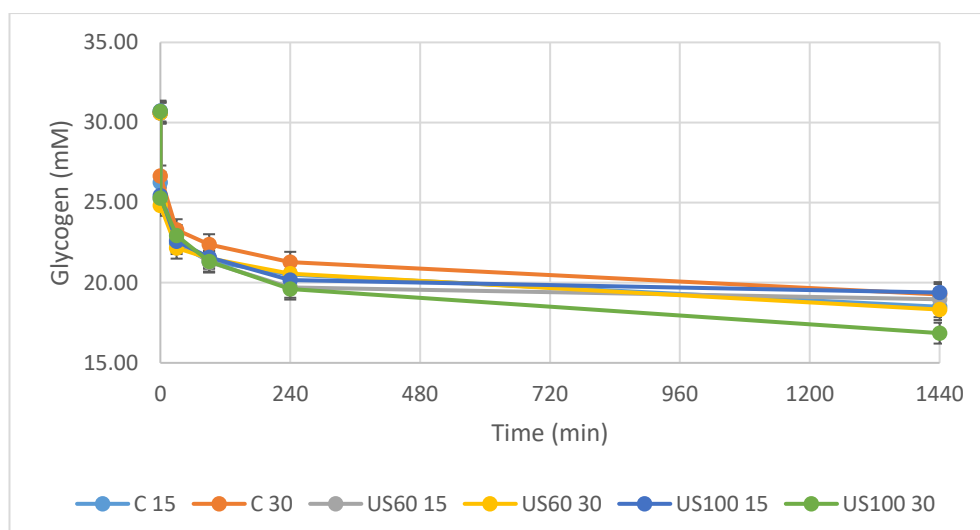


Figure 4-3. Glycogen concentration (mM) of the *in vitro* glycolytic reaction buffer over 1440 min (24 h) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60% amp for 15 min (US60 15), 60% amp for 30 min (US60 30), 100% amp for 15 min (US100 15), 100% amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SEM.

4.3.3.2 Glucose and other reducing sugars

In general, there was a rapid increase in the content of glucose and other reducing sugars within the first 240 min of the reaction buffer. The content of these monosaccharides continued to increase in the *in vitro* glycolytic buffer between 240 and 1440 min, but at a slower pace (Figure 4-4). Ultrasound intensity (control, 60% amp, 100% amp) and treatment duration (15 min, 30 min) had a significant ($P < 0.05$) impact on the levels of glucose (and other reducing sugars), while a significant interaction ($P < 0.05$) was also observed between ultrasound intensity and treatment duration. The 100% amp, 30 min ultrasound treatment resulted in a rapid increase in the levels of free sugars, with the content of glucose (and other reducing sugars) being significantly ($P < 0.05$) higher than in control and other treatment containing buffer systems at sampling points of 30 min, 90 min and 240 min. Their content in this buffer system peaked at 240 min before declining to levels similar to those of control and other treatment containing buffer systems at 1440 min. At 1440 min, no significant differences ($P > 0.05$) in glucose (and other reducing sugars) content was

observed between any of the buffer systems containing muscle of the various treatments (Figure 4-4).

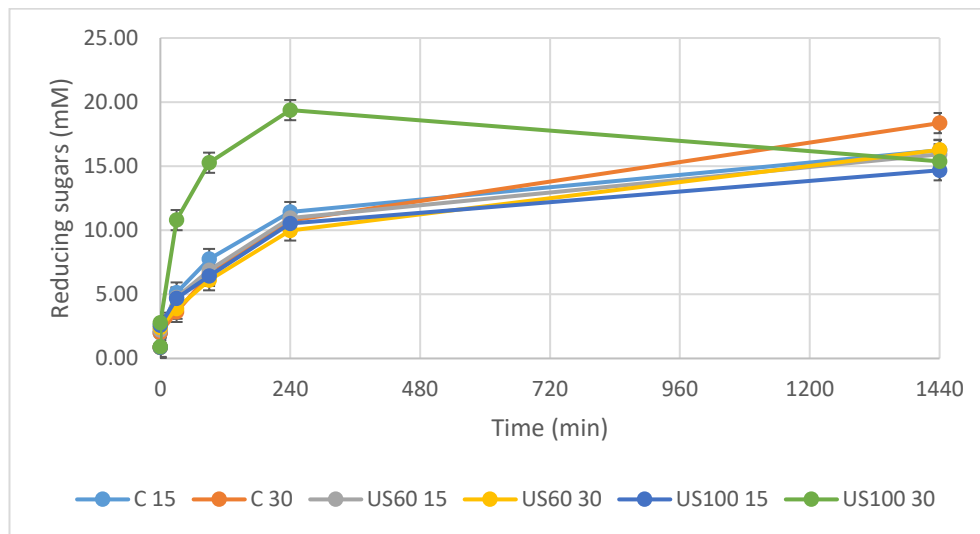


Figure 4-4. Glucose (and other reducing sugars) concentration (mM) of the *in vitro* glycolytic reaction buffer over 1440 min (24 h) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60% amp for 15 min (US60 15), 60% amp for 30 min (US60 30), 100% amp for 15 min (US100 15), 100% amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SEM.

4.3.3.3 Lactic acid

Lactic acid content in the glycolytic buffer systems, including all treatments and controls except for the 100% amp, 30 min treatment, increased rapidly in the first 90 min and then plateaued after 240 min (Figure 4-5); which is in agreement with the pattern of pH decline. Ultrasound intensity and duration had a significant impact ($P < 0.05$) on lactic acid in the buffers, and a significant interaction ($P < 0.05$) between the two treatment parameters was also observed. The 100% amp, 30 min ultrasound treatment resulted in a significant delay in the production of lactic acid from between 30 and 240 min, where the lactic acid content of this buffer system was significantly lower ($P < 0.05$) to that of control and other treatment containing buffers at the same time points (Figure 4-5). No significant differences ($P > 0.05$) were observed in the content of lactic acid at 1440 min in any of the buffer systems.

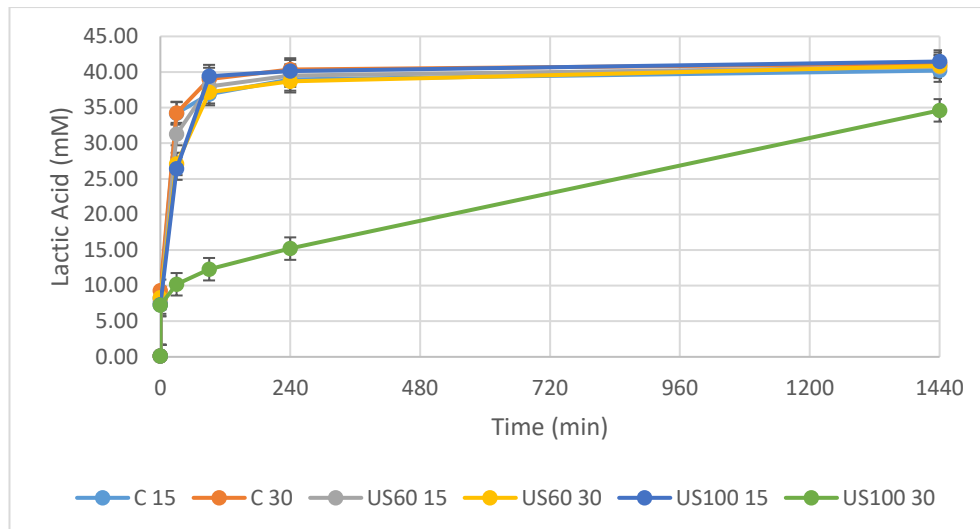


Figure 4-5. Lactic acid concentration (mM) of the *in vitro* glycolytic reaction buffer over 1440 min (24 h) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60% amp for 15 min (US60 15), 60% amp for 30 min (US60 30), 100% amp for 15 min (US100 15), 100% amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SEM.

4.4 Discussion

The initial step of glycolysis is the release of glucose from glycogen via the process of glycogenolysis and the enzyme responsible for this process is glycogen phosphorylase (GP). GP, together with glycogen debranching enzymes, release free glucose and glucose-1-phosphate to be utilised by the glycolytic pathway to produce ATP (Ferguson and Gerrard, 2014). The decline in the rate of glycogen degradation following 240 min reaction time (Figure 4-3) reflects a general decline in GP activity observed over time as described by England et al. (2014). The results of this study indicate that the ultrasound treatments of the muscle homogenates did not have a significant effect ($P > 0.05$) on the degradation or consumption of glycogen. This indicates that GP, nor the glycogen debranching enzymes, were not affected by subjecting muscle homogenates to any of the ultrasound treatments used.

The significant change in the concentration of glucose (and other reducing sugars) following the most aggressive ultrasound treatment used in this study (100% amp for 30 min), could potentially be due to ultrasound having an impact on the activity of hexokinase, an enzyme responsible for the conversion of glucose to glucose-6-

phosphate (Scheffler et al., 2015, Scheffler and Gerrard, 2007). Potential mechanisms that ultrasound could have an effect on the activity and structure of hexokinase includes the disruption of the enzyme's molecular structure, as well as having an impact on hexokinase's active site, reducing its ability to form an enzyme-substrate complex (Braginskaya et al., 1990). It is interesting to note that the optimum pH for hexokinase activity is pH 7.5 and activity reduces below pH 6.5 (Kumar et al., 2004). The pH of the *in vitro* glycolytic buffer system containing homogenate treated with 100% amp, 30 min ultrasound, reduced from pH 7.42 ± 0.01 to pH 6.48 ± 0.2 over the first 240 min (Figure 4-2) of the reaction. The pH of this buffer system remains closer to the optimum pH for hexokinase for longer and hence, if no other factor was coming into play, it could be expected that the enzyme would be more active for this treatment than controls or other treatments. However, the possible negative effect of the 100% amp, 30 min ultrasound treatment on hexokinase activity could explain the higher reducing sugars (mainly glucose) concentrations for the 100% amp, 30 min ultrasound treated group (compared to controls and other treatment groups) when pH is greater than pH 6.0 during the first 240 min (Figure 4-6); indicating that the inhibition originated in hexokinase activity by the 100% amp, 30 min ultrasound treatment is more relevant than the inhibition observed after a decrease in pH, as observed in control and other treatment groups.

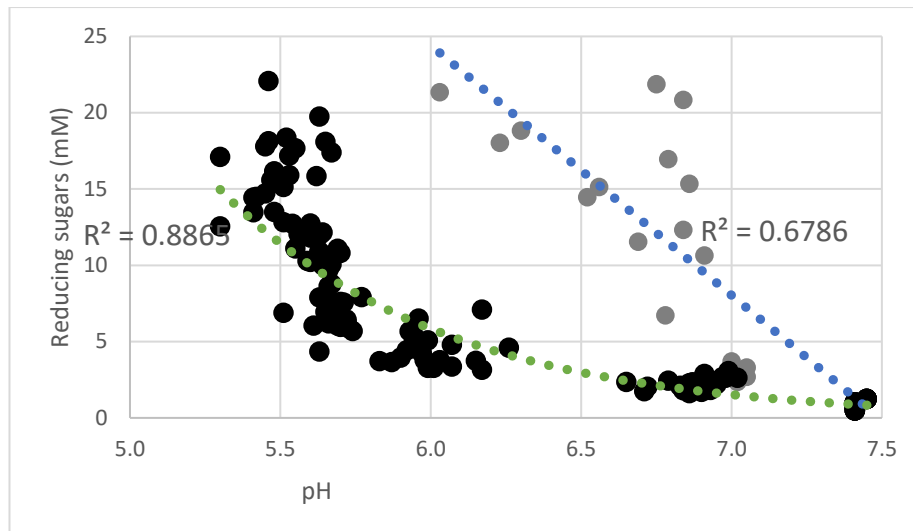


Figure 4-6. Glucose (and other reducing sugars) content (mM) plotted against pH of the buffer. All samples points plotted. N = 4. The grey • correspond to the points from buffer systems containing muscle treated with 100% amp US for 30 min for the first 240 min (where abnormal pH decline took place), while the black • corresponds to the buffers following a normal pH decline. The blue and green dotted lines are the respective lines of best fit.

While hexokinase is a possible candidate for modification by the most powerful ultrasound treatment used in this study, in theory the activity of any enzyme involved in glycolytic reactions subsequent to glucose formation and the step after fructose-1,6-bisphosphatase is degraded into glyceraldehyde-3-phosphate, could be affected and hence be responsible for the accumulation of glucose (and other reducing sugars) and the delay in lactic acid production observed. Such enzymes include phosphoglucose isomerase, already mentioned PFK and aldolase. As indicated by Matarneh et al. (2018), the loss of phosphofructokinase activity results in the prevention of further lactic acid production and the concomitant pH decline, as well as the accumulation of glucose and other reducing sugars such as glucose-6-phosphate and fructose-6-phosphate in post-mortem muscle. Interestingly, for the 100% amp 30 min ultrasound treatment, it was observed that the concentration of glucose (and other reducing sugars) concentration decreased between 240 and 1440 min, allowing the *in vitro* buffer system to achieve a similar pHu and lactic acid content to the buffer systems containing homogenates following controls and other

ultrasound treatments. Based on our experimental setup, it is not possible to determine if the inhibiting effect of ultrasound was partially reversible.

While there are few studies investigating the effects of ultrasound on enzymes linked to glycolysis, Braginskaya et al. (1990) reported that hexokinase, extracted from yeast, exhibits partial loss of catalytic activity, following exposure to ultrasound, with significantly greater effects observed following 1.5 W/cm² treatment compared to treatment of 1 W/cm². The authors of the paper attributed the loss of activity to the mechanical damage of acoustic streaming and cavitation bubbles, resulting in a reduced enzyme-substrate affinity of hexokinase (Braginskaya et al., 1990). This could potentially explain the accumulation of glucose and other reducing sugars, and delay in lactic acid production within the first 30 to 240 min in the glycolytic buffer containing muscle treated with 100% amp, 30 min ultrasound treatment.

4.5 Conclusion

In this study, it was observed that the ultrasound treatment of muscle homogenates using 100% amplitude for 30 min had a significant impact on the inherent activities of glycolytic enzymes measured *in vitro* using a glycolytic buffer system. These effects were manifested as changes in the rate of change of pH, and the profile of concentration of glucose (and other reducing sugars) and lactic acid during the incubation period, while the rate of glycogen consumption remained unchanged. Ultrasound intensity and duration had significant individual and interactive effects. The rate of pH decline, as well as glucose (and other reducing sugars) and lactic acid concentrations appeared to differ significantly between 30 min and 240 min of incubation in the *in vitro* buffer model system containing muscle homogenates treated with 100% amp (19 W/cm²) for 30 min, when compared to control and other treatment containing buffers. However, no differences were found after 1440 min of incubation. Thus it appears that the strongest ultrasound treatment had no significant effect on the subsequent activity of glycogen phosphorylase but appeared to adversely affect the activity of other glycolytic enzymes. Further investigation is warranted to identify the specific enzyme(s) that are impacted by ultrasound through further experimental work.

While a change in pH decline was observed in the current study following ultrasound treatment, it was only found after applying the most intensive treatment. Based on the results of the current study on the application of ultrasound on homogenates containing powdered muscle, the potential of using ultrasound as a tool commercially to manipulate the rate of glycolysis as a mechanism to optimise tenderness is limited.

Chapter 5. Evaluating high pressure processing as a strategy to accelerate water loss and its effect on eating quality in dry aged beef: A preliminary study.

Overview

This chapter examines the effect of a high pressure pre-treatment (200 MPa, 20 min, 8 °C) to dry aged bovine *Longissimus thoracis et lumborum*. The effect of the high pressure pre-treatment on weight loss, and quality parameters such as Warner-Bratzler shear force values, colour and lipid oxidation alongside calpain activity and myofibrillar fragmentation index, was investigated over a 28 day dry aging period. The results of this chapter have been published in the journal – Applied Food Research.

- **Kent, M. A.,** Cabral, E. M., Mullen, A. M., O'Neill, E. & Álvarez, C. (2025). Evaluating high pressure processing as a strategy to accelerate water loss and its effect on eating quality in dry aged beef: A preliminary study. *Applied Food Research*, 5(1), 100786. <https://doi.org/10.1016/j.afres.2025.100786>

5.1 Introduction

Aging of bovine muscle post-mortem improves texture and flavour characteristics through the degradation of key structural proteins and development of flavour compounds via enzymatic activity and lipid oxidation (Khan et al., 2016, Kim et al., 2016, Lee et al., 2021, Sadowska et al., 2020). Dry aging involves aging unwrapped primals or subprimals, under controlled temperature, air flow and relative humidity conditions for a number of weeks (Dashdorj et al., 2016). On the other hand, wet aging (which is more commonly used in the meat industry) involves aging primals or subprimals in water impermeable packaging, thus reducing evaporative weight loss and avoiding the formation of a crust which is characteristic of dry aged meat. Several studies have shown that dry aged beef has superior sensory traits such as flavour, when compared to wet aged beef, while having similar instrumental shear force values, bloom colour or % cook loss (Berger et al., 2018, Dikeman et al., 2013, Kim et al., 2016, Li et al., 2014b). However, dry aging significantly decreases the yield of saleable meat (Laster et al., 2008, Smith et al., 2008) due to weight losses associated with surface drying and the requirement to trim the outer crust prior to sale (Ha et al., 2019, Li et al., 2014b).

High pressure processing (HPP) is a non-thermal technology that involves the application of pressures between 100 and 900 MPa within a pressurised vessel, where a food product is treated instantaneously and uniformly. HPP has been implemented in the food industry to improve food safety without the use of additives or high temperatures (Campus, 2010). It has been found that HPP treatment of post-*rigor* bovine muscles at pressures over 200 MPa for a duration of 10-20 min has significant effects on quality parameters. In general, HPP accelerates lipid oxidation and negatively impacts colour (specifically on the lightness (L^*) parameter, where HPP appears to have a whitening effect). Water holding capacity has been reported to decrease, while the Warner-Bratzler shear force values of muscle remained unchanged or increased when compared to non-treated muscle (Marcos et al., 2010, Marcos and Mullen, 2014, McArdle et al., 2010, McArdle et al., 2011).

Since HPP has been shown to decrease the water holding capacity of muscle, there may be potential to use this technology as a pre-treatment to accelerate the dry

aging process, and therefore reduce the aging time, crust formation and associated production costs. To the best of our knowledge, the use of high pressure processing as a pre-treatment for beef that is subsequently dry aged has not yet been investigated. Therefore, the aim of the current study was to investigate if a high pressure pre-treatment (200 MPa for 20 min at 8 °C) impacts on the rate of weight loss, and the quality traits such as Warner-Bratzler shear force values, colour, cook loss and lipid oxidation of beef (*Longissimus thoracis et lumborum*) during dry aging. In addition, the effect of the HPP pre-treatment on proteolysis, was also monitored at different aging times (14, 21, 28 days).

The HPP treatment used in the current study was selected based on the research of McArdle et al. (2010, 2011) and Marcos et al. (2010, 2014), who found that 200 MPa for 20 min had minimal impact on quality traits of bovine muscle such as Warner-Bratzler shear force values and lipid oxidation, while decreasing the water holding capacity. Hence, this treatment may have potential to facilitate faster moisture loss from muscle during subsequent dry aging.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

EDTA, potassium chloride, potassium phosphate dibasic, trichloroacetic acid, 2-thiobarbituric acid (Sigma Aldrich, Ireland), magnesium chloride (VWR Chemicals, Ireland), Bradford reagent, Bovine serum albumin (BSA) standard (2 mg/ml), sodium azide (ThermoScientific, United Kingdom), CytoBuster™ protein extraction reagent, InnoZyme™ Calpain 1/2 Activity Assay Kit (Merck KGaA, Germany).

5.2.2 Animal and sample preparation

Longissimus thoracis et lumborum (LTL) muscles from the left and right side of four steer carcasses of similar conformations (age: 22-29 months; conformation score R-, O=, O+; fat score 2=, 3-; average weight: 170.5 ($\pm$ 16.33 kg)) were collected from a commercial slaughtering plant (Kepak, Ireland) at 72 h post-mortem. Each muscle was cut into 4 sections, approximately 20 cm x 12.5 cm x 6 cm in dimension, giving a total of 8 sections per animal. The average weight of the sections was 1361.68 $\pm$ 95.28 g. Subcutaneous fat was not removed from the sections. The sections were randomly

assigned to 8 different treatment combinations (dry aging (DA)/ dry aging with high pressure processing (DAHPP), combined with 0/14/21/28 days of dry aging). Each muscle section was individually vacuum packed in polyamide polyethylene bags and stored overnight at 4 °C.

5.2.3 High pressure processing and dry aging

Sections assigned for HPP were treated at 200 MPa for 20 min at 8 °C in an industrial pressurisation unit (420 Hiperbaric System (Burgos, Spain)). After HPP, all muscle sections were removed from the vacuum bags, weighed using a digital balance and randomly placed in a dry aging chamber (DRY AGER DX 1000®, DRY AGER®, Bad Saulgau, Germany), on racks, and aged for up to 28 days at 2 °C, 80% relative humidity and with an air flow ranging from 0.5 to 2.0 m/s. Throughout aging, the sections were moved every 3 – 4 days to remove any potential effect of location in the dry ager.

At each aging time (14/21/28 days), 8 sections were removed from the dry ager and weighed. The crust and subcutaneous fat was removed. Sections were divided into 4 steaks (approximately 2.5 cm thick) (numbered 1 to 4 from the anterior end) for further analysis (Figure 5-1).

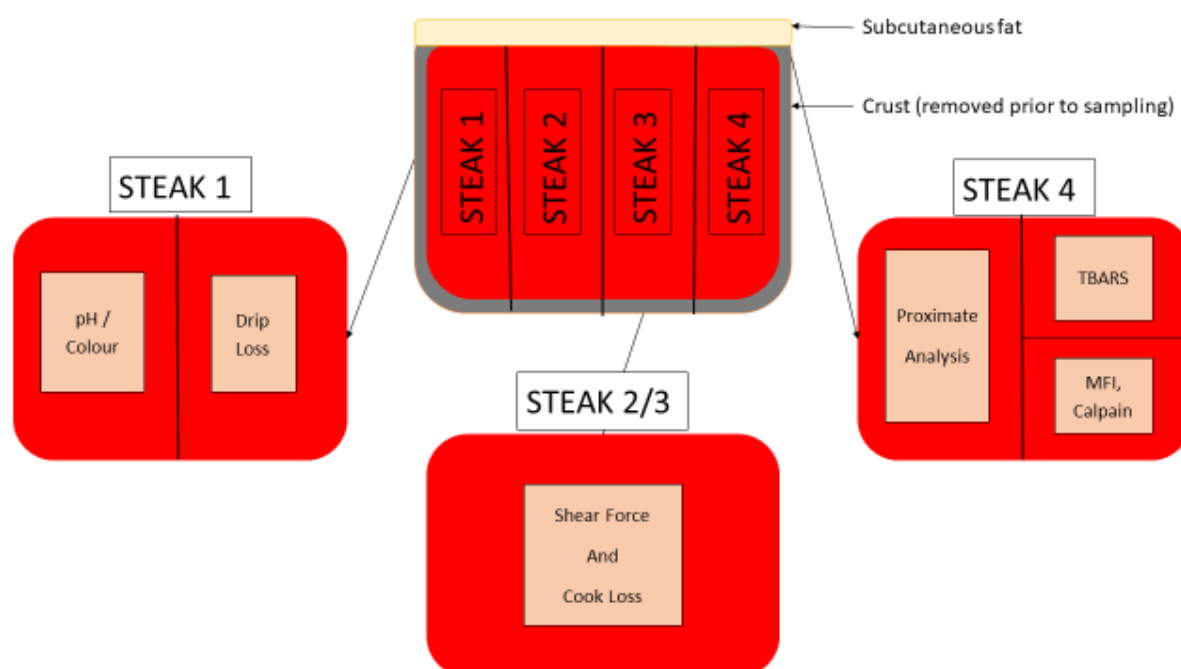


Figure 5-1. The sampling plan for sections following dry aging for 0, 14, 21 and 28 days.

5.2.4 pH and proximate analysis

pH was measured using steak 1 (Figure 5-1) of each section using a temperature/pH meter, with integrated temperature compensation (Hanna HI 98163 portable pH meter for meat and FC2323 meat pH electrode, Hanna Instruments Ltd., Leighton Buzzard, UK), by inserting the pH electrode directly into the muscle. An average of three measurements were taken per steak. The pH probe was calibrated prior to analysis using pH 4.00, 7.00 and 10.00 buffers.

Samples (approximately 100 g) for proximate analysis were taken from steak 4 (Figure 5-1) for all sections, vacuum packed and stored at -20°C until analysis. The samples were thawed overnight at 4°C and homogenised using a blender (Cookworks, Argos Limited, Milton Keynes, UK). Protein content was quantified using the Dumas method with a Leco Nitrogen Analyser (FP-628, Leco Corp., MI, USA), according to AOAC method 992.15 (AOAC, 1992). Protein content was calculated using a conversion factor of $\text{N} \times 6.25$. Moisture content was measured using Smart 6 (CEM Corporation, USA) and fat content was measured using Oracle NMR Smart Trac Rapid Fat Analyser (CEM Corporation USA), using pre-dried samples from moisture

content analysis, according to AOAC method 2008.06 (AOAC, 2008). Ash content was determined following the AOAC method 920.153 (AOAC, 1920), using a muffle furnace (Carbolite, Sheffield, England) at 550 °C. Analysis was carried out in duplicate for each sample.

5.2.5 Aging loss

The weight of the sections being aged for 28 days was measured and recorded at the same time each day throughout the dry aging period. Aging weight loss was calculated according to Eq. 5-1.

$$\text{Aging weight loss (\%)} = \frac{\text{Initial weight (g)} - \text{Weight after aging (g)}}{\text{Initial weight}} \times 100 \text{ (Eq. 5-1)}$$

5.2.6 Drip loss

Drip loss was measured according to the method of Honikel (1998). Briefly, samples of equal dimensions (2.54 x 5 x 7.5 cm) and weight (74 ± 2.5 g), were removed from steak 1 (Figure 5-1) of each section, avoiding any connective tissue. The samples were lightly blotted and weighed, before being suspended in a clear plastic bag, using string and a hook (unfolded paper clip), ensuring there was no contact between the sample and bag. The bag was loosely tied around the hook and suspended in a chill room for 72 h at 4 °C. Following this, the surface of the samples were blotted with tissue and re-weighed. Drip loss was expressed as a percentage of the weight of the original piece of muscle (Eq. 5-2).

$$\text{Drip loss (\%)} = \frac{\text{Initial weight (g)} - \text{Weight after 72hr suspension (g)}}{\text{Initial weight (g)}} \times 100 \text{ (Eq.5-2)}$$

5.2.7 Cook loss and Warner-Bratzler shear force

Steak 2 (Figure 5-1) from each section was vacuum packed and stored at –20 °C and subsequently used for the determination of cook loss (Shackelford et al., 1991) and Warner-Bratzler shear force values (Wheeler et al., 1996). The vacuum packed steaks were defrosted in circulating water at room temperature. External moisture was lightly blotted from the steak with tissue before being weighed. Steaks were placed into individual polyethylene bags and cooked in a water bath set at 72 °C (Grant Instruments Ltd., England). The internal temperature of the steaks was monitored using a probe (Eirelec Ltd., Ireland) until an internal core temperature of 70 °C was

reached. Excess water was removed from the bags and the steaks were allowed to cool to room temperature, blotted with a tissue paper and re-weighed. Cook loss was determined by calculating the difference between the initial and cooked weight of the steaks and was expressed as a percentage of the initial weight of the steak (Eq. 5-3).

$$\text{Cook loss (\%)} = \frac{\text{Initial weight (g)} - \text{Cooked weight (g)}}{\text{Initial weight (g)}} \times 100 \text{ (Eq. 5-3)}$$

The cooked steaks were packaged and stored overnight at 4 °C. Warner-Bratzler shear force measurements were carried out on 1.25 cm diameter cores taken from the steak, perpendicular to the direction of the muscle fibres, using an Instron 4464 Universal testing machine (Instron Ltd., Buckinghamshire, UK), with a 500 N loading cell and cross head speed of 50 mm/min. A total of 5 measurements were made for each steak.

5.2.8 Colour measurements

For each time point, a sample (4 x 4 x 2.5 cm) from the freshly cut steak 1 (Figure 5-1) was wrapped in cling film, allowed to bloom for 3 h at 4 °C. The colour of the bloomed surface was recorded using a HunterLab UltraScan Pro CIE $L^*a^*b^*$ system with a dual beam xenon flash spectrophotometer (Hunter Associates Laboratory, Inc., Reston, VA, USA) to measure lightness (L^*), redness (a^*) and yellowness (b^*). A light trap ($L^* = 0$) and standard white tile, covered with transparent cling film, was used for calibration. A standard illuminant D65 with a ten degrees observer angle and a 25 mm diameter port were used. The colour of each sample was measured at 5 random locations.

Colour measurements using the same parameters as above were also carried out on the cooked steaks from section 5.2.7.

Colour differences (ΔE) between control and HPP treated muscle were calculated according to the method of Jung et al. (2003), using Eq. 5-4., where the colour values for control samples at each sampling time point was used as the reference values in the calculation.

$$\Delta E = [(L^* - L_{ref}^*)^2 + (a^* - a_{ref}^*)^2 + (b^* - b_{ref}^*)^2]^{\frac{1}{2}} \text{ (Eq. 5-4)}$$

5.2.9 Lipid oxidation

Lipid oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS) values according to the method of Botsoglou et al. (1994). Samples (approximately 10 g) were removed at each sampling time point from steak 4 (Figure 5.1), vacuum packed and stored at $-20\text{ }^{\circ}\text{C}$ for further analysis. Prior to analysis, samples were thawed overnight at $4\text{ }^{\circ}\text{C}$. Briefly, 3.5 g of muscle was added to 20 ml MilliQ water and homogenised using an Ultraturrax (13,500 rpm for 30 s). Trichloroacetic acid (5 ml, 25% (w/v) TCA, ice cold) was added to the homogenate, vortexed and allowed to stand at $4\text{ }^{\circ}\text{C}$ for 15 min. The homogenate was centrifuged at $2,500\text{ g}$ for 30 min at $4\text{ }^{\circ}\text{C}$ and the supernatant was filtered using Whatman 52 filter paper. The supernatant (3.5 ml) was mixed with 1.5 ml of 0.6% (w/v) 2-thiobarbituric acid, heated in a water bath at $70\text{ }^{\circ}\text{C}$ for 30 min, and allowed to cool on ice before the absorbance at 532 nm was measured using a UV/Vis spectrophotometer. TBARS values were expressed as milligrams of malondialdehyde (MDA) per kilogram of sample (mg MDA/kg). For each steak, two independent extractions were prepared for analysis in duplicate.

5.2.10 Myofibrillar fragmentation index

Samples (approximately 3 g) were removed from steak 4 (Figure 5-1) of each section, placed in a cryovial, snap frozen and stored at $-80\text{ }^{\circ}\text{C}$ until time of analysis. Myofibrillar fragmentation index (MFI) values were determined according to the method of Culler et al. (1978), with modifications from Hopkins et al. (2000). Briefly, 1 g of muscle was homogenised in 10 ml ice cold MFI extraction buffer, consisting of 100 mM KCl, 20 mM K_2HPO_4 , 1 mM EDTA, 1 mM MgCl_2 and 1 mM NaN_3 , pH 7, with an Ultraturrax at 15,000 rpm, for 60 s (2 x 30 s, with a 30 s break between bursts). Homogenates were centrifuged at $1,000\text{ g}$ for 15 min at $4\text{ }^{\circ}\text{C}$. The supernatant was discarded. The pellets were gently inverted in 10 ml MFI extraction buffer and centrifuged again. The supernatant was removed and the pellets were re-suspended in 10 ml MFI extraction buffer using a vortex and the mixture was filtered through a filtration fabric (muslin cloth). The protein content of the filtrate was measured using

the Bradford method and a standard curve using BSA (Bradford, 1976). The protein content of the filtrate was adjusted to 0.5 ± 0.05 mg/ml. The absorbance of the solution was measured using a UV/Vis spectrophotometer at 540 nm. MFI values were obtained by multiplying the absorbance at 540 nm by 200. For each steak, two independent extractions were prepared for analysis in duplicate.

5.2.11 Calpain activity

Samples (approximately 1 g) were removed from steak 4 (Figure 5-1) for each section, placed in a cryovial, snap frozen and stored at -80°C for further analysis. Calpain activity was measured in duplicate according to the method outlined for using the InnoZyme™ Calpain 1/2 Activity Assay Kit (Merck KGaA, Darmstadt, Germany) (Preziosa et al., 2013, Salazar et al., 2009). Samples (0.1 g) were homogenised in 1 ml of CytoBuster™ Protein Extraction Reagent (Merck KGaA, Darmstadt, Germany), with an Ultraturrax (13,500 rpm for 30 s). The homogenates were centrifuged at 20,900 *g* for 5 min at 4°C and kept on ice during protein quantification and calpain activity analysis. The protein content of the supernatants was measured using the Bradford method and a standard curve prepared using BSA (Bradford, 1976). The protein content of the supernatants were adjusted to approximately 2 mg/ml and 50 μl aliquots were added to a 96 well plate. Activation buffer with reducing agent (100 μl) or activation buffer with reducing agent and calpain inhibitor (100 μl) was added to the wells. The 96 well plate was gently shaken (Stuart reciprocating shaker SSL2 (Cole-Parmer Instrument Co. Europe, PE19 8YX United Kingdom) for 3 min. Calpain substrate (50 μl) was added to the wells followed by incubation for 30 min in the dark at room temperature, with gentle shaking. Fluorescence was measured at an excitation wavelength of 320 nm and an emission wavelength of 480 nm using a Tecan SPARK® Multimode Microplate Reader and Tecan. SparkControl software (Tecan Group Ltd., 8708 Männedorf, Switzerland). Calpain activity was expressed in relative fluorescence units (RFU) and calculated by subtracting the fluorescence of samples containing the activation buffer and reducing agent from that of the corresponding samples containing the activation buffer, reducing agent and calpain inhibitor.

5.2.12 Statistical analysis

The data presented in this study is the average $\pm$ standard error mean (SEM). Analysis of all variables was performed using MIXED procedure of the SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA) with 4 replicates and 2 factors: treatment (dry aging (DA)/dry aging with high pressure processing (DAHPP)) and aging time, post-treatment (0/14/21/28 days). Animals were treated as a random effect. Treatment means and interactions were compared using Tukey's test. In all cases, a P – value < 0.05 was considered as significant.

5.3 Results and Discussion

5.3.1 pH

Aging time had a significant effect ($P < 0.05$) on pH of muscle. In addition, the pH of muscle increased significantly ($P < 0.05$) after HPP treatment and this was true for all time points (Table 5-1). No significant interaction ($P > 0.05$) was found between aging time and HPP. The increase in pH over aging time in control and HPP treated muscle may be due to proteolysis and the subsequent production of alkaline substances (Shi et al., 2020). The increase in pH following HPP treatment is in agreement with the results of Utama et al. (2017), who found that HPP (200 – 600 MPa for 180 s, 15 ± 2 °C) significantly increased the pH of bovine *Longissimus lumborum*. The increase in pH following HPP has been attributed to a decreased availability of acidic groups in muscle due to protein denaturation (Ma and Ledward, 2004, McArdle et al., 2010). Nevertheless, the pH values remained within the normal range of beef (5.5 – 5.8) (Holdstock et al., 2014, Li et al., 2023, Barón et al., 2021).

Table 5-1. The effect of aging time and HPP on the pH of dry aged bovine LTL. Results are the mean values of four biological replicates $\pm$ SEM.

	Aging time (days)				Treatment		Interaction	
	0	14	21	28	P – value	Mean	P – value	Aging x Treatment
DA	5.51 $\pm$ 0.03	5.55 $\pm$ 0.03	5.51 $\pm$ 0.03	5.58 $\pm$ 0.03	0.02	5.54 $\pm$ 0.02	< 0.001	0.8898
DAHPP	5.65 $\pm$ 0.03	5.72 $\pm$ 0.03	5.66 $\pm$ 0.03	5.73 $\pm$ 0.03		5.69 $\pm$ 0.02		

P – values from the F-Test, following MIXED procedure analysis.

DA – Dry aged, DAHPP – Dry aged and high pressure processing.

5.3.2 Weight loss and proximate composition

As expected, weight loss increased significantly ($P < 0.05$) with aging time (Figure 5-2) due to water loss from the surface of the muscle and simultaneous migration of water from the internal portion of the muscle to the surface (Ha et al., 2019, Santchurn et al., 2012). It was observed that HPP significantly ($P < 0.05$) increased the weight loss of muscle over the entire period of dry aging. However, no significant differences ($P > 0.05$) in weight loss between control and HPP treated sections were observed at any individual time points. No significant interaction ($P > 0.05$) was found between aging time and HPP (Figure 5-2). HPP may have induced protein denaturation, reducing the water holding capacity and facilitating moisture loss from the surface of the muscle (Marcos et al., 2010), as well as diffusion of water to the surface of the muscle. The results show that while HPP has the potential to accelerate moisture loss during dry aging, higher pressures may be required to have a more noticeable difference. However, this may be detrimental for other quality traits.

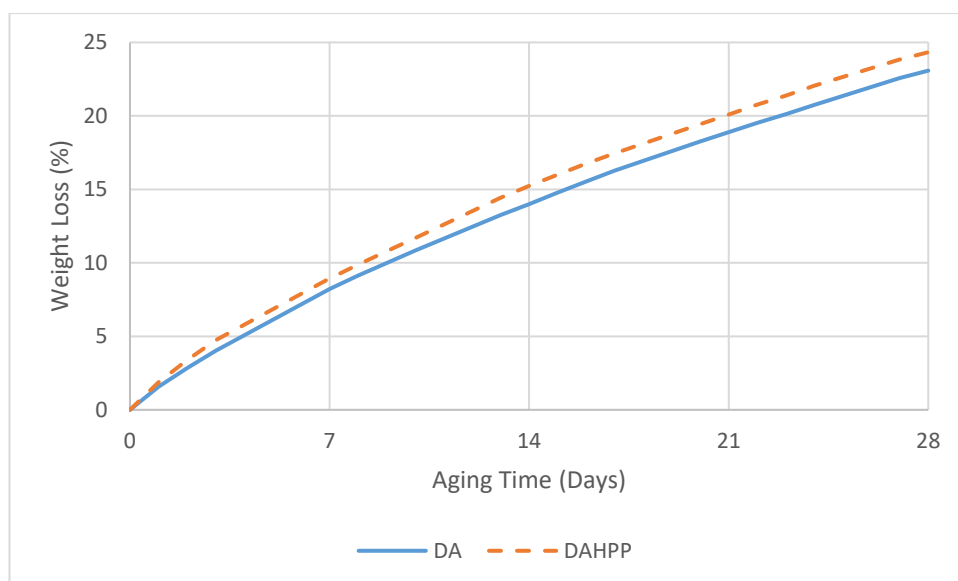


Figure 5-2. . Effect of aging time and HPP on the weight loss (%) of dry aged bovine LTL over a 28 day dry aging period. Results are mean values of four biological replicates.

DA – Dry aged, DAHPP – Dry aged and high pressure processing

Moisture content decreased significantly ($P < 0.05$) with aging time, while HPP did not have a significant effect ($P > 0.05$) on moisture content (Table 5-2) when compared to controls. Likewise, no interactive effect ($P < 0.05$) was observed between aging time and HPP. Moisture content was expected to decrease with dry aging time due to evaporative losses (Ha et al., 2019, Kim et al., 2019). Protein, fat and ash content increased significantly ($P < 0.05$) with aging time, while HPP did not have a significant effect ($P > 0.05$) (Table 5-2). No significant interactive effects ($P > 0.05$) between aging time and HPP was observed. An increase in protein, fat and ash content during dry aging was expected due to the reduction in moisture content and this is in agreement with the findings of other authors (Khazzar et al., 2023, Kim et al., 2019, Lee et al., 2017).

Table 5-2. The effect of aging time and HPP on the proximate composition of dry aged bovine LTL.

	Aging time (days)				<i>P</i> – value	Treatment		<i>P</i> – value	Interaction
	0	14	21	28		DA	DAHPP		Aging x Treatment
Moisture (%)	73.62 ± 1.08	71.86 ± 1.08	71.07 ± 1.08	69.53 ± 1.08	<0.0001	71.71 ± 1.03	71.33 ± 1.03	0.4541	0.5128
Protein (%)	23.89 ± 0.33	23.58 ± 0.33	24.43 ± 0.33	25.49 ± 0.33	0.0015	24.21 ± 0.25	24.48 ± 0.25	0.3908	0.6951
Fat (%)	1.99 ± 1.12	3.09 ± 1.12	3.61 ± 1.12	4.18 ± 1.12	0.0361	3.16 ± 1.06	3.28 ± 1.06	0.8102	0.8917
Ash (%)	1.06 ± 0.04	1.14 ± 0.04	1.25 ± 0.04	1.42 ± 0.04	<0.0001	1.20 ± 0.03	1.24 ± 1.06	0.3388	0.3557

Results are mean values of four biological replicates ± SEM. Each replicate was measured in duplicate.

P – values from the *F* – Test, following MIXED procedure analysis.

DA – Dry aged, DAHPP – Dry aged and high pressure processing

5.3.3 Drip loss and cook loss

Drip loss decreased significantly ($P < 0.05$) with aging time for control and HPP treated muscle, with the main reduction being observed between day 0 and 14 (Figure 5-3A). While HPP treated muscle had a significantly higher ($P < 0.05$) average drip loss over the four aging time points, when compared to controls, no significant differences were observed between the drip loss of DA and DAHPP samples at individual time points. There was no significant interaction ($P > 0.05$) between aging time and HPP. The reduction in drip loss over aging time is attributed to the reduction in moisture content as a result of dry aging, resulting in less free water being available (Álvarez et al., 2023). In addition, HPP (200 – 600 MPa, 10 – 30 °C, 20 min) has been found to reduce the water holding capacity (measured by expressible moisture) of bovine *Longissimus* muscle (Marcos et al., 2010, Marcos and Mullen, 2014). It was proposed by the authors that HPP negatively impacted the water holding capacity of muscle through the denaturation of sarcoplasmic proteins.

Aging time had a significant effect on cook loss, where the cook loss was significantly ($P < 0.05$) lower at day 28 when compared to day 0 (Figure 5-3B). HPP did not have a significant ($P > 0.05$) effect on cook loss (Figure 5-3B) and no significant interaction ($P > 0.05$) was found between aging time and HPP. The results of the current study are in agreement with the findings of Khazzar et al. (2023), Kim et al. (2022) and Lee et al. (2019), who reported that cook loss of bovine *Longissimus* muscle decreased significantly following 28 – 30 days of dry aging. This can be explained by the reduction in moisture content with dry aging time (Álvarez et al., 2023) and therefore, less water is available to be lost during cooking. Furthermore, when investigating the effects of HPP on bovine *Pectoralis profundus*, McArdle et al. (2010) found that HPP treatments of 200 MPa (for 20 min at 20 and 40 °C) did not significantly affect cook loss.

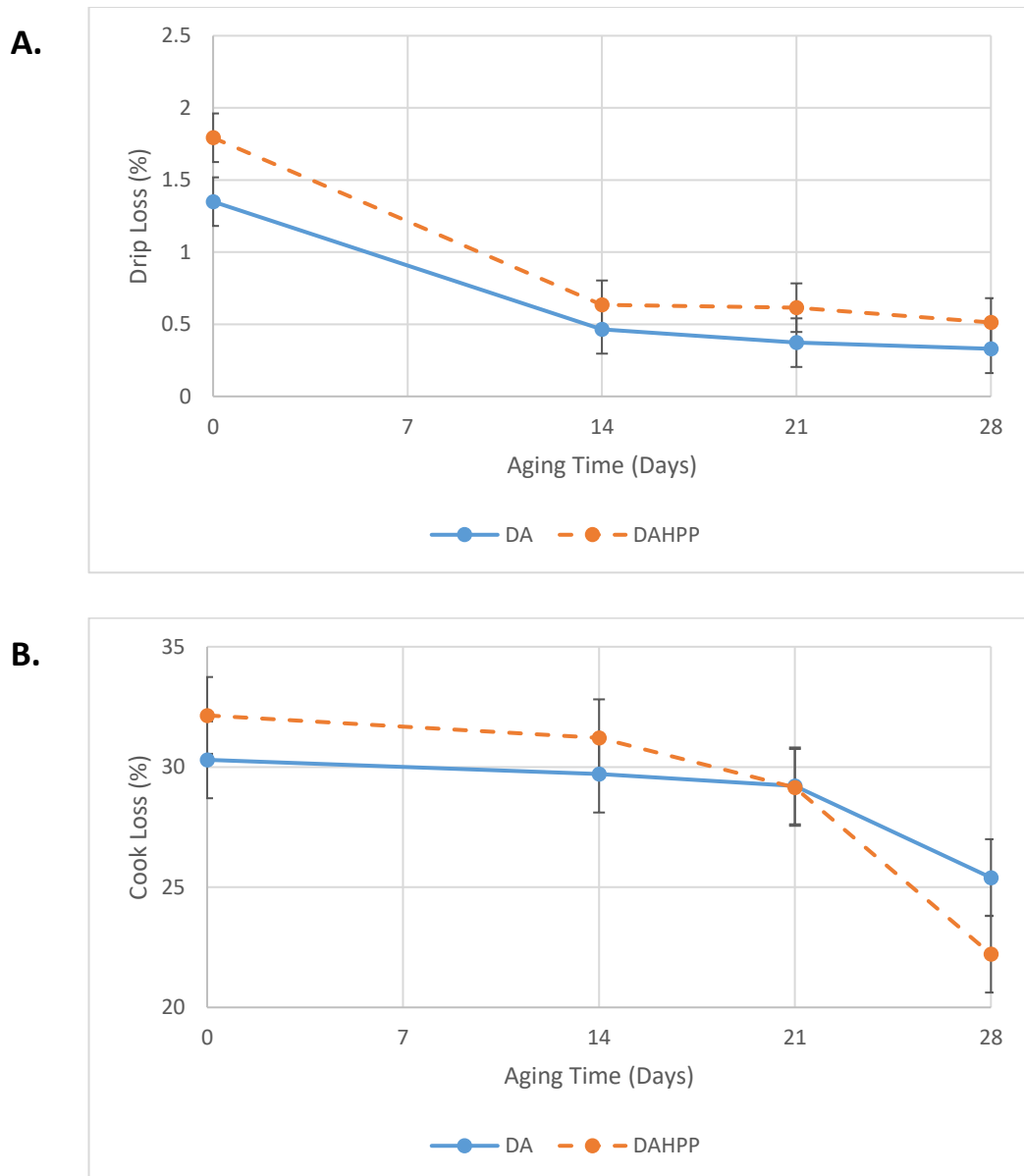


Figure 5-3. The effect of aging time and HPP on the A) drip loss (%) and B) cook loss (%) of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.

DA – Dry aged, DAHPP – Dry aged and high pressure processing

5.3.4 Warner-Bratzler Shear Force (WBSF) values

Aging time significantly ($P < 0.05$) reduced the WBSF values. HPP significantly ($P < 0.05$) increased the WBSF values of muscle immediately after treatment and after 21 days of aging (Figure 5-4). The final WBSF values of the HPP treated samples were not significantly different from controls after 28 days of aging ($P > 0.05$) (Figure 5-4).

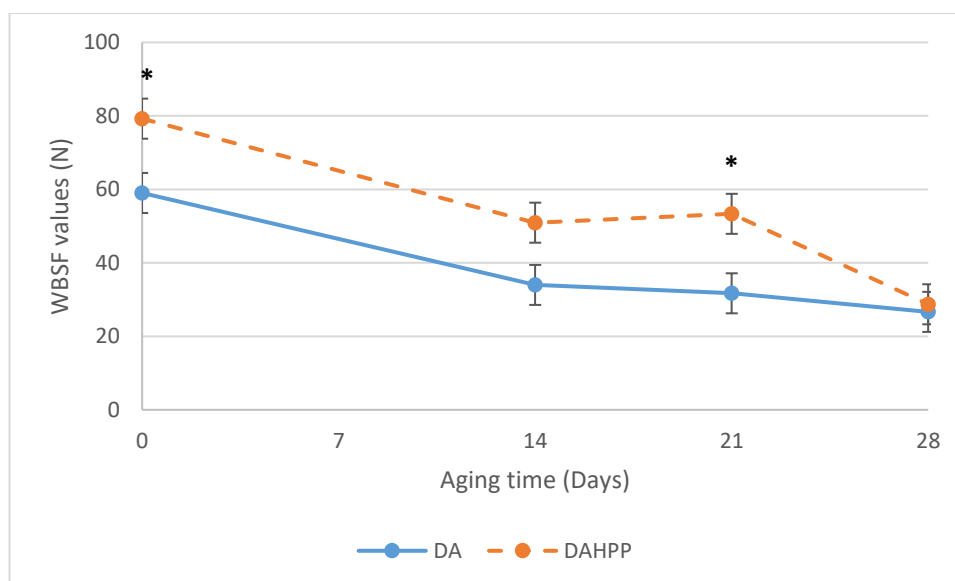


Figure 5-4. The effect of aging time and HPP on the WBSF values of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.

DA – Dry aged, DAHPP – Dry aged and high pressure processing.

* indicates significant differences between treatments

The observed decrease in WBSF values of beef with dry aging time is in agreement with the results of previous studies (Smith et al., 2008, Khazzar et al., 2023, Lee et al., 2017), and is due to the actions of proteolytic enzymes such as calpains, cathepsins and caspases (Huff Lonergan et al., 2010, Kemp et al., 2010). The results of this study are consistent with those of Ma and Ledward (2004), who reported that HPP up to 400 MPa (20 °C, 20 min), significantly increased the hardness of post-rigor bovine *Longissimus dorsi* immediately after treatment. The increase in WBSF values following HPP of post-rigor bovine muscle may be attributed to sarcomere shortening, and the aggregation and coagulation of proteins (Jung et al., 2000a). Furthermore, Utama et al. (2018) found that applying HPP (300 MPa, 180 s, 15 °C) to bovine *Longissimus lumborum* resulted in a significant increase in WBSF values immediately post-treatment, when compared to controls. However, following 28 days of wet aging, no significant differences in WBSF values were observed between control and 300 MPa treated muscle, similar to the findings of the current study using dry aging. From the results of this study, it can be concluded that the HPP treatment used in this study does not have a detrimental effect on WBSF values if sufficient

aging time is allowed. While HPP has the ability to increase weight loss over time, a compromise may need to be made in relation to eating quality if aging time was to be reduced.

5.3.5 Bloomed colour

Aging time did not have a significant effect ($P > 0.05$) on L^* values (lightness), however, HPP significantly increased ($P < 0.05$) the L^* values of bloomed muscle and this was observed for all aging time points (Table 5-3). No significant interaction ($P > 0.05$) between aging time and HPP was found.

The increase in L^* values following HPP observed in this study is in agreement with Marcos et al. (2010) and McArdle et al. (2010), who found that HPP treatment (200 – 600 MPa, 10 to 40 °C, 20 min) of bovine *Longissimus dorsi* and *Pectoralis profundus*, respectively, significantly increased the L^* values of freshly cut muscle when compared to controls. The whitening/bleaching effect of HPP has been attributed to protein denaturation and/or displacement or release of heme (Bolumar et al., 2021, Carlez et al., 1995), and may have a negative impact on consumers perception of the freshness of beef.

Aging time and HPP had a significant effect ($P < 0.05$) on the a^* values (redness) of freshly cut bloomed muscle. The a^* value at day 0 was significantly higher following HPP treatment when compared to controls. However, no significant differences ($P > 0.05$) were observed between HPP treated and control muscle after 14 days of aging and onwards (Table 5-3). A significant interaction ($P < 0.05$) between aging time and treatment was observed.

Aging time has been found to have no significant effect on the redness of freshly cut bloomed bovine *Longissimus* muscle dry aged for 8 and 19 days (Li et al., 2014b), while dry aging for periods greater than this led to a significant decrease in a^* values (Ha et al., 2019). A significant increase in the a^* values of bovine *Biceps femoris* was observed immediately post-HPP treatment (130 MPa for 260 s, 10 °C) by Jung et al. (2003), which was attributed to a decrease in metmyoglobin content. However, other studies have found that HPP treatments similar to that used in the current study, (200 MPa, 10 to 40 °C, 20 min) did not significantly affect the a^* values of the freshly

cut bloomed surface of bovine muscles such as *Pectoralis profundus* (McArdle et al., 2010), *Longissimus dorsi* (Marcos et al., 2010) and *Longissimus thoracis et lumborum* (Marcos and Mullen, 2014).

Aging time had a significant effect ($P < 0.05$) on b^* values (yellowness) of the freshly cut bloomed control bovine muscle, where it increased significantly between day 0 and 14 and then remained stable (Table 5-3). HPP significantly increased ($P < 0.05$) the yellowness of freshly cut bloomed muscle compared to control at day 0. While the b^* values of the HPP control muscle remained higher throughout the entire aging period, these differences were only significant ($P < 0.05$) at day 21. A significant interaction ($P < 0.05$) between aging time and HPP was found.

Table 5-3. Effect of aging time and high pressure processing on L^* , a^* , b^* values of dry aged bovine LTL. Results are the mean of four biological replicates $\pm$ SEM.

		Aging time (days)				P – value	Treatment		Interaction Aging x Treatment
		0	14	21	28		Mean	P – value	P – value
L^*	DA	40.25 $\pm$ 0.95 ^{Aa}	41.08 $\pm$ 0.95 ^{Aa}	41.59 $\pm$ 0.95 ^{Aa}	40.17 $\pm$ 0.95 ^{Aa}	0.2931	40.77 $\pm$ 0.58	<0.0001	0.9403
	DAHPP	47.08 $\pm$ 0.95 ^{Ba}	48.81 $\pm$ 0.95 ^{Ba}	48.71 $\pm$ 0.95 ^{Ba}	47.88 $\pm$ 0.95 ^{Ba}		48.12 $\pm$ 0.58		
a^*	DA	15.67 $\pm$ 0.54 ^{Aa}	20.06 $\pm$ 0.54 ^{Ab}	20.70 $\pm$ 0.54 ^{Ab}	20.79 $\pm$ 0.54 ^{Ab}	<0.0001	19.30 $\pm$ 0.35	0.0069	<0.0001
	DAHPP	20.39 $\pm$ 0.54 ^{Ba}	19.26 $\pm$ 0.54 ^{Aa}	21.25 $\pm$ 0.54 ^{Aa}	20.36 $\pm$ 0.54 ^{Aa}		20.31 $\pm$ 0.35		
b^*	DA	10.48 $\pm$ 0.51 ^{Aa}	14.00 $\pm$ 0.51 ^{Ab}	13.97 $\pm$ 0.51 ^{Ab}	13.62 $\pm$ 0.51 ^{Ab}	0.002	13.02 $\pm$ 0.26	<0.0001	0.0074
	DAHPP	16.27 $\pm$ 0.51 ^{Ba}	16.36 $\pm$ 0.51 ^{Aa}	17.05 $\pm$ 0.51 ^{Ba}	16.00 $\pm$ 0.51 ^{Aa}		16.42 $\pm$ 0.26		

Means with different letters (a – b) within rows indicate significant differences between time points ($P < 0.05$).

Means with different letters (A – B) within columns indicate significant differences between treatments ($P < 0.05$).

P – values from the F – Test, following MIXED procedure analysis.

DA – Dry aged, DAHPP – Dry aged and high pressure processing

Table 5-4. Effect of aging time and high pressure processing on L^* , a^* , b^* values of cooked dry aged bovine LTL. Results are the mean of four biological replicates $\pm$ SEM.

		Aging time (days)				Treatment		Interaction
		0	14	21	28	P – value	Mean	Aging x Treatment
L^*	DA	42.78 $\pm$ 0.83 ^{Aa}	43.86 $\pm$ 0.83 ^{Aa}	42.78 $\pm$ 0.83 ^{Aa}	44.13 $\pm$ 0.83 ^{Aa}	0.1896	43.39 $\pm$ 0.59	0.0074
	DAHPP	43.59 $\pm$ 0.83 ^{Aa}	44.96 $\pm$ 0.83 ^{Aa}	45.59 $\pm$ 0.83 ^{Aa}	44.99 $\pm$ 0.83 ^{Aa}		44.78 $\pm$ 0.59	
a^*	DA	6.40 $\pm$ 0.29 ^{Aa}	4.76 $\pm$ 0.29 ^{Abc}	4.90 $\pm$ 0.29 ^{Abc}	5.22 $\pm$ 0.29 ^{Aac}	0.0012	5.32 $\pm$ 0.16	0.1999
	DAHPP	5.84 $\pm$ 0.29 ^{Aa}	5.41 $\pm$ 0.29 ^{Aa}	4.87 $\pm$ 0.29 ^{Aa}	5.48 $\pm$ 0.29 ^{Aa}		5.40 $\pm$ 0.16	
b^*	DA	10.30 $\pm$ 0.72 ^{Aa}	8.56 $\pm$ 0.72 ^{Aa}	8.42 $\pm$ 0.72 ^{Aa}	9.52 $\pm$ 0.72 ^{Aa}	0.0158	9.20 $\pm$ 0.62	0.0520
	DAHPP	9.52 $\pm$ 0.72 ^{Aa}	10.00 $\pm$ 0.72 ^{Aa}	9.55 $\pm$ 0.72 ^{Aa}	10.91 $\pm$ 0.72 ^{Aa}		10.00 $\pm$ 0.62	

Means with different letters (a – b) within rows indicate significant differences between time points ($P < 0.05$).

Means with different letters (A – B) within columns indicate significant differences between treatments ($P < 0.05$).

P – values from the F – Test, following MIXED procedure analysis.

DA – Dry aged, DAHPP – Dry aged and high pressure processing

The effect of HPP on the colour of cooked muscle was also examined (Table 5-4). Overall, it was found that the lightness (L^*) and yellowness (b^*) values were significantly ($P < 0.05$) higher in cooked HPP steaks when compared to the cooked control steaks.

When evaluating the colour differences between the freshly cut bloomed surfaces of control and HPP treated samples, it was found that ΔE was approximately 8.5 following 28 days of aging, primarily due to differences in L^* values. Mokrzycki and Tatol (2011) proposed that a colour difference greater than 5 units allows observers to distinguish between samples. However, the colour differences (ΔE) between the cooked control and HPP treated samples were approximately 2 (at all aging times), indicating that the effect of HPP on the colour of cooked muscle was minimal and the colour change would only be noticeable to an experienced observer (Mokrzycki and Tatol, 2011). The results indicate that the bloom colour of dry aged beef is effected by HPP, most notably the lightness of the muscle, and this may affect the willingness of customers to buy the HPP treated fresh muscle, as colour is one of the primary factors considered at purchase. However, HPP could be applied to muscle that is presented to the consumer as a cooked product (e.g. in the catering industry) without having a detrimental effect on colour.

5.3.6 Lipid oxidation

TBARS values (mg MDA/kg) increased significantly ($P < 0.05$) with aging time (Figure 5-5), for both control and HPP treated samples. HPP significantly increased ($P < 0.05$) the TBARS values over the 28 day dry aging period when compared to control samples, however, no significant differences ($P > 0.05$) were observed between control and HPP samples at individual time points (Figure 5-5). No significant interaction ($P > 0.05$) was found between aging time and HPP.

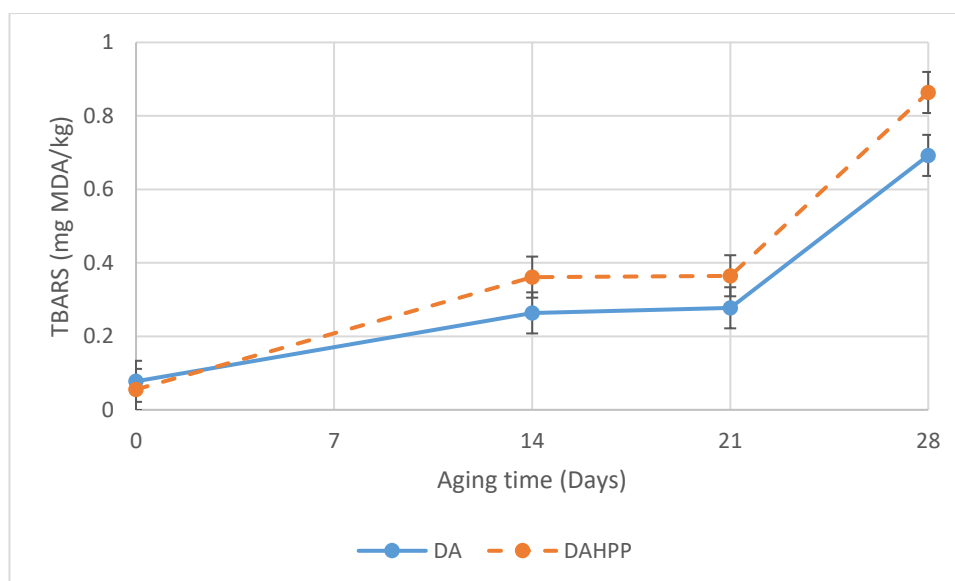


Figure 5-5. The effect of aging time and HPP on the TBARS values (mg MDA/kg) of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.

DA – Dry aged, DAHPP – Dry aged and high pressure processing

The increase in TBARS values with aging time has been attributed to cell membrane damage (Beltran et al., 2003) and an increase in the concentration of free iron, following the release from iron complexes, such as haemoglobin and myoglobin, in muscle. Free iron can act as a pro-oxidant and catalyse the lipid oxidation reaction (Liu et al., 2022b, Liu et al., 2023). Similar mechanisms have been attributed to the increase in lipid oxidation following HPP (Ma et al., 2007, Medina-Meza et al., 2014). An increase in TBARS values has been found following high pressure treatments (200 – 800 MPa, 20 to 70 °C, 20 min) of bovine *Pectoralis profundus* and *Longissimus dorsi* by McArdle et al. (2010) and Ma et al. (2007), respectively, however, pressures greater than 200 MPa (at room temperature) had to be applied for a significant change in TBARS values to occur. In this study, the HPP treated muscle had maximum TBARS values of 0.86 ± 0.06 mg MDA/ kg muscle which is below the threshold of 2.00 mg MDA/kg muscle, indicating that the level of lipid oxidation that occurred following HPP is within the limit for consumer acceptability (Campo et al., 2006).

5.3.7 Myofibrillar fragmentation index (MFI)

A significant increase ($P < 0.05$) in MFI values was observed with aging time. HPP significantly increased MFI values ($P < 0.05$) when compared to controls, and this was

the case for each time point (Figure 5-6). A significant interactive effect ($P < 0.05$) was found between aging time and HPP (Figure 5-6), where the increase in MFI values of HPP treated muscle was greater over the 28 day aging period compared to controls.

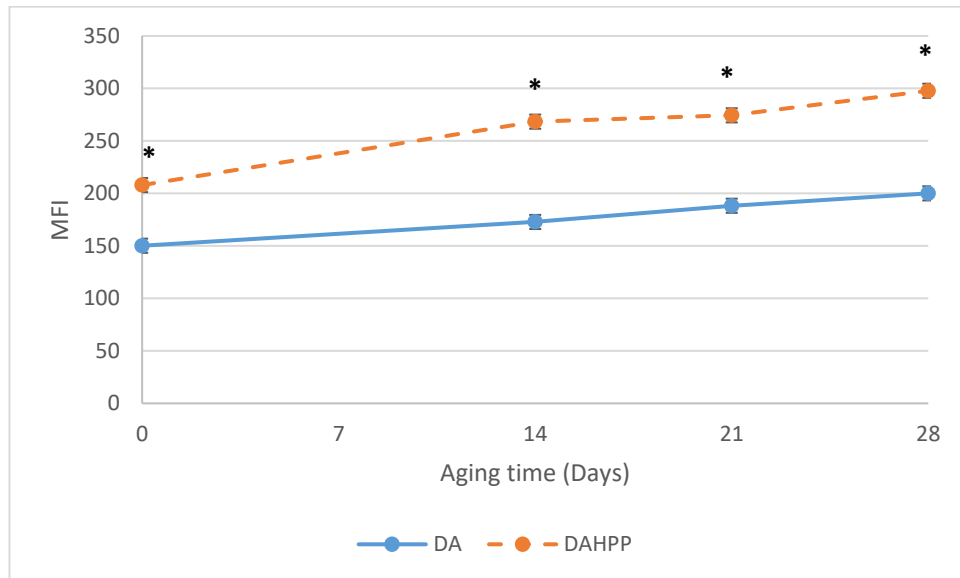


Figure 5-6. The effect of aging time and HPP on the MFI values of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.

DA – Dry aged, DAHPP – Dry aged and high pressure processing

* indicates a significant difference between treatments.

The increase in MFI values over aging time is in agreement with the findings of Li et al. (2014a), Ha et al. (2019) and Ramos et al. (2022) and may be attributed to protein degradation (Taylor et al., 1995, Hopkins et al., 2000). An increase in MFI values following HPP treatment was also reported by Zhang et al. (2018b), who found that HPP (200 MPa, 10 min) significantly increased the MFI values of pre-rigor porcine *Longissimus* muscle, when compared to control samples. MFI values increased further as the pressure was increased to 400 MPa (Zhang et al., 2018b). To the best of our knowledge, the effect of HPP on the MFI values of post-rigor bovine muscle has not been examined, however, it has been found that when post-rigor bovine muscle was treated with pressure levels of 200 MPa and above, for 4 – 5 min, the defined filamentous structure of muscle was lost and disruption was caused to the I-band, M-line and Z-line (Bolumar et al., 2021, Jung et al., 2000a, Suzuki et al., 1990).

These changes in the microstructure of the muscle may explain the increase in MFI values following HPP treatment.

5.3.8 Calpain activity

Aging time did not have a significant effect ($P > 0.05$) on calpain activity. HPP significantly decreased ($P < 0.05$) calpain activity (by 97.1 – 99.5%) (Figure 5-7) compared to control samples at all sampling times. No significant interaction ($P > 0.05$) was evident between aging time and HPP.

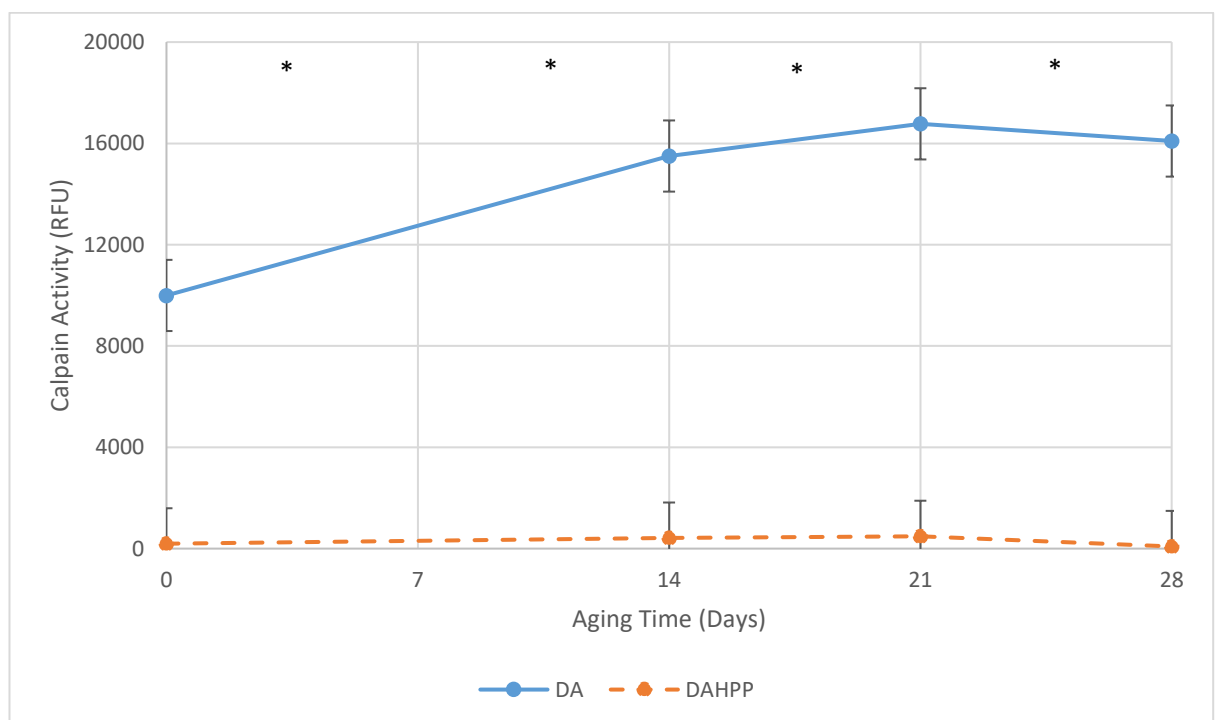


Figure 5-7. The effect of aging time and HPP on calpain activity of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.

* indicates significant differences between treatments for calpain activity.

At 4 days post-mortem (equivalent to day 0 of the current study), it has been documented that calpain-1 activity was already reduced to below 20% of its measured activity at 0 days post-mortem (Boehm et al., 1998, Camou et al., 2007, Colle and Doumit, 2017), in a range of bovine muscles (*Longissimus lumborum*, *Semimembranosus*, *Triceps brachii*, *Psoas major*). However, in the same muscles at 4 days post-mortem, between 82.4% and 77.9% unautolysed calpain-2 activity (Colle and Doumit, 2017) and 40% total calpain-2 activity (Camou et al., 2007) remained

when compared to the activity levels of day 0. Other authors have found that total calpain-2 activity remained unchanged (Geesink and Koohmaraie, 1999, Veiseth et al., 2001) or increased (Phelps et al., 2016), over time post-mortem, in ovine and bovine muscle.

The decrease in calpain activity observed in the current study following HPP is in agreement with Lee et al. (2023), who reported that the HPP treatment (200 MPa, 3 min, 21 °C) of 48 h post-mortem bovine *Semimembranosus*, significantly reduced calpain-1 activity, with 72.51% calpain-1 activity remaining when compared to controls. The higher remaining calpain activity found in the study by Lee et al. (2023) compared to the current study could be due to the shorter treatment time (3 min compared to 20 min).

Despite the decrease in calpain activity, MFI values increased at a faster rate in the treated samples than controls. The reasons for this remain unclear. It has been proposed that HPP may facilitate protein degradation via the denaturation and unfolding of proteins, and exposing the cleavage sites of proteolytic enzymes other than calpains, such as cathepsins (Lee et al., 2023, Jung et al., 2000c).

5.4 Conclusion

Based on the findings of the current study, HPP treatment (200 MPa for 20 min at 8 °C) increased the shear force values of muscle following 21 days of dry aging, however, no differences were observed following 28 days of dry aging. HPP significantly increased lipid oxidation, however, it remained within the acceptable threshold for lipid oxidation. HPP had a significant effect on the colour of freshly cut bloomed muscle. However, colour differences between control and HPP treated dry aged beef were reduced after cooking. It was found that HPP increased overall weight loss, during subsequent dry aging. However, higher pressures may be needed for a more significant effect in order for HPP pre-treatment to be considered as a useful strategy for industry, while considering the potential effects on quality attributes. Sensory analysis should be conducted to investigate what effect HPP has on the perceived texture and flavour profiles of dry aged bovine muscle.

Chapter 6. Evaluating the impact of a high pressure pre-treatment on dry aged bovine *Longissimus thoracis et lumborum* using SDS-PAGE and untargeted LC-MS metabolomics.

Overview

This chapter evaluates the impact that a high pressure pre-treatment (200 MPa, 20 min, 8 °C) and dry aging duration has on the protein and metabolomic profile of dry aged bovine *Longissimus thoracis et lumborum* using SDS-PAGE and untargeted UPLC-Q-TOF/MS. Peptides, phospholipids and nucleotides were identified as groups of metabolites that were affected by high pressure pre-treatment and aging time. The metabolomics profile changed with dry aging time and high pressure processing. The results of this chapter have been submitted to a peer review journal for publication.

- **Kent, M. A.**, Cabral, E. M., Brahmachari, R., Frías-Celayeta, J. M., O'Neill, E. & Álvarez, C. Evaluating the impact of a high pressure pre-treatment on dry aged bovine *Longissimus thoracis et lumborum* using SDS-PAGE and untargeted LC-MS metabolomics. Manuscript submitted.

6.1 Introduction

Aging improves the tenderness and flavour characteristics of beef through the degradation of structural proteins and development of flavour compounds via enzymatic activity and lipid oxidation. Dry aging involves storing primals or subprimals without packaging under controlled conditions (temperature, relative humidity, air flow) for an extended period of time (Dashdorj et al., 2016). In contrast, wet aged beef primals or subprimals are aged in water impermeable packaging. It has been shown in several studies that dry aged beef has superior sensory traits such as flavour compared to wet aged beef (Berger et al., 2018, Dikeman et al., 2013, Kim et al., 2016, Li et al., 2014b). The results of these studies also show that wet and dry aged meat exhibit similar instrumental shear force values, cook loss and bloom colour. However, dry aging results in greater weight loss due to surface drying and the need to trim the crust, significantly reducing the yield of saleable meat (Laster et al., 2008, Smith et al., 2008, Ha et al., 2019, Li et al., 2014b).

High pressure processing (HPP) involves the application of pressure (100 to 900 MPa), under controlled temperatures, to food products within a pressurised vessel, where pressure is applied instantaneously and uniformly (Campus, 2010). In general, when investigating the impact of HPP on the quality of bovine muscle, it has been found that HPP (200 – 600 MPa) accelerates lipid oxidation and negatively impacts colour (specifically increasing the lightness (L^*) parameter). Warner-Bratzler shear force (WBSF) values of HPP treated bovine muscles remain unchanged or increased compared to non-treated muscle while the water holding capacity of muscle has been reported to decrease with HPP (Marcos et al., 2010, Marcos and Mullen, 2014, McArdle et al., 2010, McArdle et al., 2011). The current research group demonstrated that HPP pre-treatment (200 MPa, 20 min, 8 °C) has the potential to accelerate the weight loss of dry aged bovine *Longissimus thoracis et lumborum*, without significantly affecting shear force values, cook loss (%) or lipid oxidation, when measured following 28 days dry aging. The same HPP pre-treatment (200 MPa, 20 min, 8 °C) significantly increased the myofibrillar fragmentation index (MFI) and reduced calpain activity (Chapter 5).

Metabolomics, using mass spectrometry (MS) (including tandem MS/MS, hyphenated LC-MS, GC-MS, and hyphenated/tandem LC-MS/MS, GC-MS/MS) and nuclear magnetic resonance (NMR), can be used to investigate the impact of processing factors on metabolites such as amino acids, peptides, nucleotides, sugars and organic acids, which are produced or changed due to biochemical reactions within food products such as meat (Muroya et al., 2020, Rubert et al., 2015). Metabolomic analysis can be categorised into two different types: untargeted metabolomics detects all metabolites within a sample, including unknown metabolites (Vinayavekhin and Saghatelian, 2010), whereas targeted metabolomics focuses on the measurement of specific identifiable metabolites of interest (Roberts et al., 2012). Metabolomics has been used to study the effect of different feeding regimes (Gómez et al., 2022), aging duration (Ma et al., 2017, Sun et al., 2024, Muroya et al., 2023), aging method (Kim et al., 2016, Bischof et al., 2021, Setyabrata et al., 2022a) and abnormal pH decline (Ijaz et al., 2022, Ramanathan et al., 2020) on the metabolomic profile of bovine muscle, and for the detection of meat adulteration (Trivedi et al., 2016, Windarsih et al., 2022).

To the best of our knowledge, SDS-PAGE and untargeted metabolomic analysis have not been used to investigate the impact of HPP pre-treatment on dry aged bovine muscle. Therefore, the aim of the current study was to evaluate the potential interactive effect of dry aging (14, 21, 28 days) and HPP pre-treatment (200 MPa for 20 min at 8 °C) on the protein and metabolomic profile of bovine *Longissimus thoracis et lumborum* through the use of 1D SDS-PAGE and untargeted metabolomics analysis using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS).

The HPP treatment used in the current study was chosen based on the findings of Marcos et al, (2010, 2014) and McArdle et al., (2010, 2011), who found that a 200 MPa, 20 min treatment decreased the water holding capacity of bovine muscle, while minimally impacting on quality parameters such as Warner-Bratzler shear force values and lipid oxidation.

6.2 Materials and Methods

6.2.1 Chemicals and reagents

Acetonitrile, CHAPS, DL – dithiothreitol, EZBlue™ gel staining reagent, formic acid (98% - 100%), Laemmli buffer, thiourea, urea (Sigma Aldrich, Ireland), Pharmalyte® 3-10 (Cytiva, Sweden), Bradford reagent, Bovine serum albumin (BSA) standard (2 mg/ml) (ThermoScientific, United Kingdom), methanol (Romil Pure Chemistry, Ireland)

6.2.2 Animal and sample preparation

Bovine *Longissimus thoracis et lumborum* (LTL) muscle from the left and right side of four carcasses of similar conformations (steers, age: 22 – 29 months; average weight: 170.5 ($\pm$ 16.33 kg); conformation score R-, O=, O+; fat score 2=, 3-) were collected from a commercial slaughtering plant (Kepak, Ireland) at 72 h post-mortem. Each muscle was cut into 4 sections approximately 20 cm x 12.5 cm x 6 cm in dimension (i.e. 8 sections per animal). The average weight of each section was 1361.68 $\pm$ 95.28 g. Subcutaneous fat was not removed from the sections before dry aging. The sections were individually vacuum packed in polyamide polyethylene bags and randomly assigned to 8 different treatment combinations (dry aging (DA)/ dry aging with high pressure processing (DAHPP), combined with 0/14/21/28 days of aging).

6.2.3 High pressure processing and dry aging

Sections assigned for DAHPP were treated at 200 MPa (20 min at 8 °C) in an industrial pressurisation unit (420 Hiperbaric System (Burgos, Spain)). After HPP, all muscle sections were removed from the vacuum bags, weighed using a digital balance and randomly placed in a dry aging chamber (DRY AGER DX 1000®, DRY AGER®, Bad Saulgau, Germany), on racks. The sections were dry aged for up to 28 days at 2 °C, 80% relative humidity and air flow ranging from 0.5 to 2.0 m/s. Sections were moved within the same unit every 3 – 4 days to remove any potential effect of location in the dry ager.

At each aging time (14/21/28 days), sections were removed from the dry ager and weighed. The crust and subcutaneous fat were removed and sections were divided into 4 steaks (approximately 2.5 cm thick). Samples for SDS-PAGE (approximately 2

g) and metabolomics analysis (approximately 5 g) were removed from steak 4, chopped and portioned into cryovials, snap frozen and stored at -80°C until further analysis.

6.2.4 One dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

6.2.4.1 Protein extraction and quantification

Frozen samples (approximately 150 mg) were homogenised in 3 ml of freshly prepared extraction buffer (8.3 M urea, 2 M thiourea, 1% (w/v) DL – dithiothreitol (DTT), 2% (w/v) CHAPS, 2% (v/v) Pharmalyte® 3 – 10), using an Ultraturrax (20,000 rpm for 2 x 30 s). The homogenates were incubated on ice for 30 min and centrifuged for 30 min at 10,000 *g* at 4°C (Bouley et al., 2004, Gagaoua et al., 2021). The protein concentration of the supernatants were measured using the Bradford method (Bradford, 1976) and a standard curve prepared using bovine serum albumin (BSA). The supernatants were stored at -80°C until further analysis.

6.2.4.2 Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970), with modification. The protein concentration of the supernatants were adjusted using MilliQ water and Laemmli buffer to ensure a final protein concentration of 40 μg in each well (Álvarez et al., 2023b). The samples were heated at 80°C for 15 min using a block heater (SBH130D/3, Bibby Scientific Limited, Staffordshire, UK). An aliquot of molecular standard (5 μl) (Precision Plus Protein Dual Color Standards, BioRad, Hercules, CA, USA) and each sample (30 μl) was loaded to a 4 – 20% Mini-PROTEAN® TGX™ precast protein gel (BioRad, Hercules, CA, USA) for 1D SDS-PAGE using a Mini-PROTEAN® Tetra Cell System (Bio-Rad, Hercules, CA, USA). A run time of approximately 1 h 30 min was used at 5 W to separate the proteins. When electrophoresis was complete, the gels were removed, washed with MilliQ water for 10 min with agitation, allowed to stain overnight in EZBlue™ Gel Staining Reagent, washed with MilliQ water and scanned using the GS-800 Calibrated Densitometer (BioRad, Hercules, CA, USA). The relative densities (%) of the bands were quantified using Quantity One software (Version 4.5.2) (Bio-Rad, Hercules, CA, USA).

6.2.5 Untargeted metabolomics

6.2.5.1 Sample preparation

Sample preparation for metabolomics analysis was carried out according to Windarsih et al. (2022), with modifications. Briefly, samples (1 g) were added to 5 ml of HPLC-grade methanol and vortexed (Heidolph™ Multi Reax, Heidolph Instruments GmbH & Co. KG, Germany) for 2 min at 2,000 rpm. The samples were homogenised using an Ultraturrax for 60 s (2 x 30 s) at 15,000 rpm and treated with ultrasound (25 kHz) in a water bath (Elma Schmidbauer GmbH, Singen, Germany), for 30 min at room temperature. Following ultrasonication, the homogenates were stored at –20 °C for 1 h to allow the proteins to precipitate. The supernatant (1 ml) was transferred to Eppendorf tubes and centrifuged at 2,000 *g* for 5 min, at room temperature and filtered through a 0.2 µm PTFE filter, before being transferred into HPLC vials. Pooled quality control (QC) samples were prepared by combining supernatant (50 µl) from each sample (N = 32).

6.2.5.2 Metabolomics analysis using UHPLC-Q-TOF/MS

Metabolomics analysis was carried out according to Windarsih et al. (2022), with modifications. Analysis was carried out using an Agilent 1290 Infinity II ultra high performance liquid chromatograph (UPLC) (Agilent Technologies, Santa-Clara, CA, USA), combined with an Agilent 6545 Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Agilent Technologies, Santa-Clara, CA, USA). An ACQUITY UPLC BEH C18 1.7 µm (2.1 mm x 150 mm) column (WATERS Corporation, Milford, Massachusetts, USA) was used for chromatographic separation for positive and negative ionisation modes. During analysis, the column temperature was set to 35 °C. The mobile phases used were A) water and 0.1% formic acid and B) acetonitrile and 0.1% formic acid. The gradient changes were as follows: 0 min 95% A, 20 – 35 min 10% A, 45 min 95% A. Dual AJS electrospray ionisation (ESI) in positive and negative modes were used to detect metabolites. QC samples were injected every 6 samples and were used to examine the stability of the system.

6.2.6 Data analysis

Relative density (%) of protein bands from 1D SDS-Polyacrylamide electrophoretogram: Analysis was carried out using ANOVA on RStudio, version 4.3.2

(Posit Software, Boston, MA, USA) with 4 replicates and 2 factors: treatment (dry aging (DA)/ dry aging with high pressure processing (DAHPP)) and aging time, post-treatment (0/ 14/ 21/ 28 days). Animals were treated as a random effect. Treatment means and interactions were compared using Tukey's test. In all cases, a P – value < 0.05 was considered as significant.

Metabolomics: The MSConvert software from ProteoWizard (version 3.0.2 (64 bit), Palo Alto, CA, USA) was used to convert the raw mass spectrometry data (.d) into the mzXML format. LC-MS data pre-processing (peak alignment, retention time correction and peak area extraction) was carried out on RStudio (version 4.3.2, Posit Software, Boston, MA, USA) using the XCMS package (version 4.0.2)(Smith et al., 2006). Statistical analysis was also carried out on RStudio (Appendix 1). Data was normalised using log transformation and centred before fold change and principal component analysis (PCA) analysis. Pareto scaling was applied prior to discriminant statistical analysis, including partial least square discriminant analysis (PLS-DA) and orthogonal partial least square discriminant analysis (OPLS-DA). PCA was used for initial data exploration, PLS-DA to evaluate the effect of treatments (DA/DAHPP) and aging (D0/D14/D21/D28), and OPLS-DA for more specific comparisons (D0 vs. D28, DA v DAHPP). Metabolites with a variable importance in projection (VIP) score > 1 from OPLS-DA and a P < 0.05 from a paired t-test were identified as significant differential metabolites. Volcano plot analysis was carried out to identify the metabolites that increased or decreased significantly with time (D0/D14/D21/D28) and treatment (DA/DAHPP). The fold-change (FC) was calculated by comparing the mean of each metabolite at different time points (D0/D14/D21/D28) between and within treatments (DA/DAHPP) and the overall effect of treatment (DA/DAHPP). Upregulated (increased with time/DAHPP) metabolites had a FC > 0.5 and P < 0.05. Downregulated (decreased with time/DAHPP) metabolites had a FC < -0.5 and P < 0.05. Metabolites were tentatively identified using the MassHunter software packages B.07.00 (Agilent Technologies, Santa Clara, CA, USA) where the observed accurate mass data and MS/MS spectra was compared with the accurate mass data and spectra of the METLIN Metabolomics and Tandem MS Database (Scripps Center for Metabolomics, La Jolla, CA, USA), which is licenced through MassHunter.

6.3 Results and discussion

6.3.1 SDS-PAGE profile

In the 1D SDS-polyacrylamide electrophoretogram the relative density (%) of band, corresponding to myosin heavy chain (MHC) (band 1) and the 160 kDa band (band 2) were significantly ($P < 0.05$) affected by aging time and HPP (Figure 6-1). A significant interactive effect ($P < 0.05$) was also present. The relative density (%) of band 1 increased significantly ($P < 0.05$) and that of band 2 did not change ($P > 0.05$) with dry aging time in the controls (DA). In contrast while following DAHPP, the relative density (%) of band 1 decreased significantly ($P < 0.05$) with dry aging time with a simultaneous increase ($P < 0.05$) in the relative density (%) of band 2 in the corresponding samples. These results suggest that DAHPP facilitated the degradation of MHC, illustrated by the increase ($P < 0.05$) in the relative density of the 160 kDa band. Polypeptides in the range of 170 – 138 kDa have previously been identified as proteolytic fragments of MHC by MALDI – TOF by Marino et al. (2013).

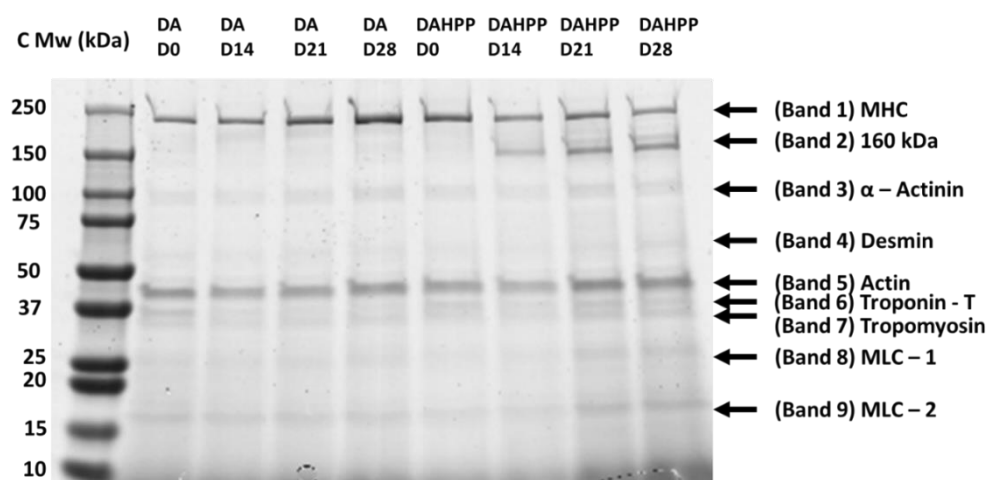


Figure 6-1. A representative 1D SDS-PAGE (4-20% gel) profile of bovine *Longissimus thoracis et lumborum* following dry aging (DA) and dry aging with high pressure processing (DAHPP) at four different aging times (D0, D14, D21, D28).

The relative density (%) of protein bands associated with α -actinin (band 3), desmin (band 4), troponin-T (band 6) and myosin light chain 2 (MLC2) (band 9) were not ($P > 0.05$) affected by dry aging time, however, they were significantly ($P < 0.05$) increased following DAHPP, when compared to controls at corresponding time points. The changes in band pattern observed between DA and DAHPP samples could be due to

changes in proteolysis or HPP affecting the extractability or solubility of the effected proteins (Marcos et al., 2010; Jung et al., 2000a).

6.3.2 Untargeted metabolomics

From the data obtained from the positive ionisation mode, 2,819 features were detected; 838 of these features were identifiable. The PCA score plot of the positive ionisation mode for the 8 treatment groups (DA/DAHPP, D0/D14/D21/D28) and QC samples is displayed in Figure 6-2A. PC1 and PC2 captured 66.5% and 10.2% of the dataset variance, respectively (while PC3, PC4 and PC5 captured 4.6%, 3% and 1.9%, respectively). PC1 is affected by aging (D0/D14/D21/D28), while PC2 is affected by treatment (DA/DAHPP).

At D0 (within 2 h of HPP treatment), the overlap of DA and DAHPP samples indicate that the initial metabolomic profiles were similar, suggesting that HPP did not have an immediate effect on the overall metabolomic profile. Following 14, 21 and 28 days of dry aging, differences were detected between DA and DAHPP samples, indicating that DA and DAHPP samples underwent different metabolomic changes over time. For both DA and DAHPP groups, metabolomic changes occurred progressively over time, with the majority of metabolomic changes taking place between D0 and D14.

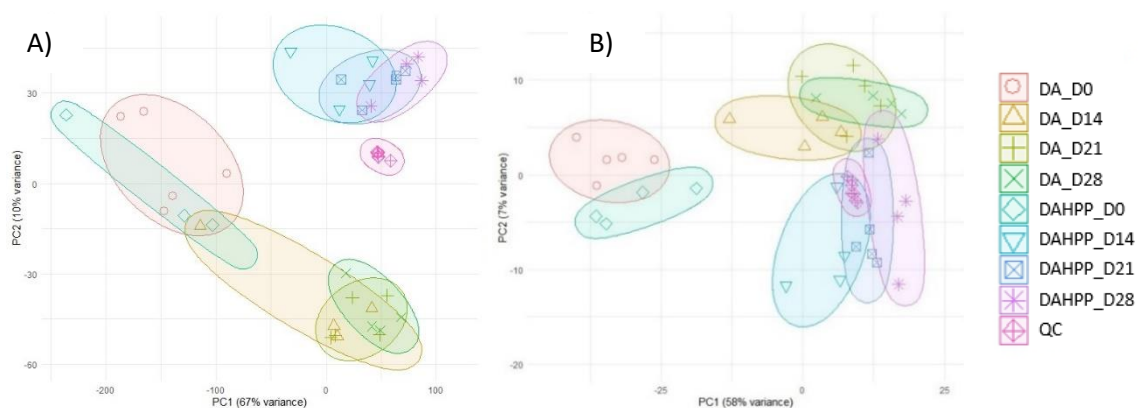


Figure 6-2. PCA score plot of treatment/time combinations for A) positive and B) negative ionisation mode.

From the data obtained using the negative ionisation mode, 434 features were detected; 179 of these features were identifiable. The PCA score plot of the negative ionisation mode for the 8 treatment groups (DA/DAHPP, D0/D14/D21/D28) and QC

samples (Figure 6-2B) present a similar trend to the data obtained from the positive ionisation mode. PC1 and PC2 captured 57.5% and 6.9% of the variance within the dataset, respectively (PC3, PC4 and PC5 captured 5.8%, 4.4% and 2.8%, respectively). PC1 and PC2 of the negative ionisation mode data account for less variation when compared to the positive ionisation mode data, potentially due to less features being present (2,819 features v 434 features). In the negative ionisation mode, D0 samples of DA and DAHPP samples are clustered together, suggesting a similar metabolomic baseline between samples; however, differences between the metabolomic profile of DA and DAHPP samples emerge following D14 of dry aging. The QC samples in the positive and negative ionisation modes were tightly clustered together, (Figure 6-2 A-B), indicating that the data was stable and reliable.

PLS-DA was performed on the positive ionisation mode features (Figure 6-3A) to determine the degree of class separation between the different groups (DA/DAHPP, D0/D14/D21/D28). Component 1 (X) accounts for 45% of the variance, while component 2 (Y) accounts for 10% of the variance. The PLS-DA grouped the samples into three main clusters. The first cluster contains the D0 samples, suggesting that these samples have a similar metabolite baseline and indicates that DAHPP does not have an immediate impact on the metabolomic profile of bovine LTL muscle. The second and third clusters group DA and DAHPP treatments separately at D14, D21 and D28, indicating differing changes over time for both treatments. A progressive change in the metabolomic profile is observed with time in the DA and DAHPP groups. The model assessing differences between DA and DAHPP samples over time (D0, D14, D21, and D28) indicates that a high proportion of the variance is explained by predictor X, while the model's overall predictive ability is modest, with a reasonable fit to the data ($R^2X = 0.543$, $R^2Y = 0.262$, $Q^2Y = 0.164$, RMSEE = 0.299). The ability to predict the response (Y = grouping) is limited when considering treatment and time effects together; likely due to the minimal evolution of the metabolome after D14. However, when considering the effect of treatment alone (DA/DAHPP) (Figure 6-3B), the models performance improves ($R^2X = 0.543$, $R^2Y = 0.864$, $Q^2Y = 0.733$, RMSEE = 0.194). This indicates that the model can clearly differentiate

between DA and DAHPP samples and that treatment alone has an effect on the metabolomic profile of bovine *Longissimus thoracis et lumborum*.

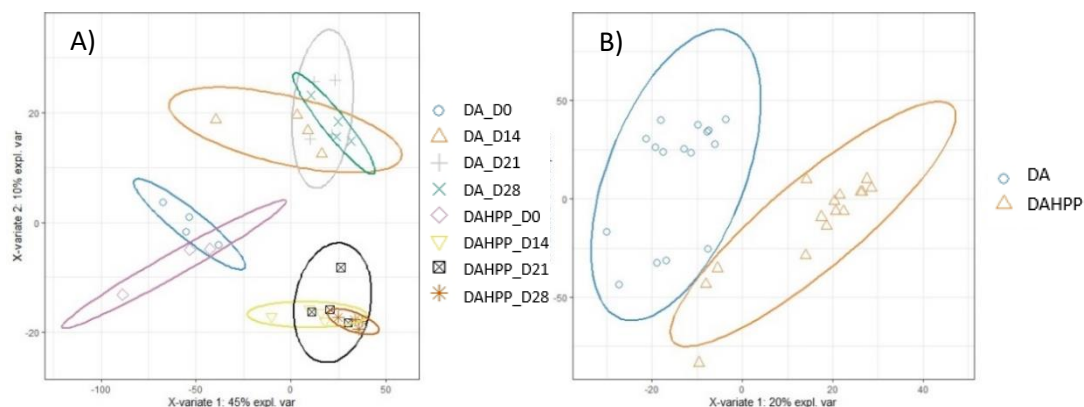


Figure 6-3. PLS-DA graph of A) all treatment/ time combinations and B) treatment groups of positive ionisation mode data.

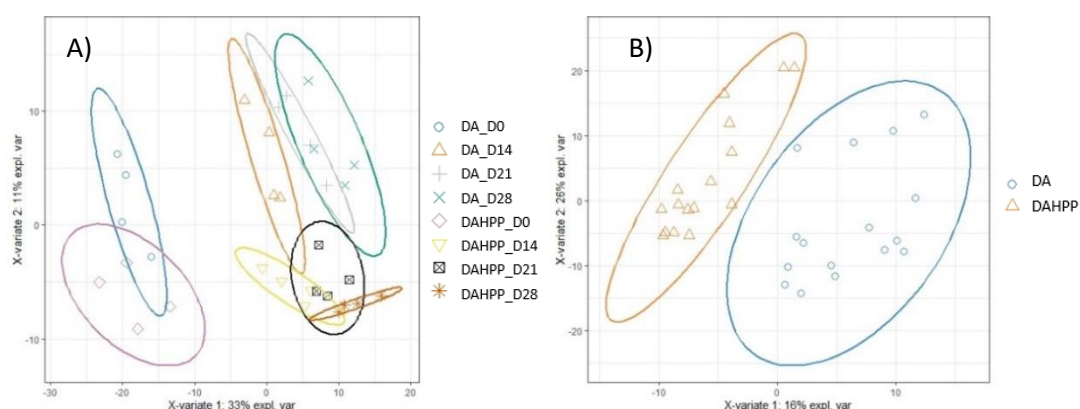


Figure 6-4. PLS-DA graph of A) all treatment/ time combinations and B) treatment groups of negative ionisation mode data.

The PLS-DA analysis of the negative ionisation mode features (Figure 6-4A) presents similar clustering of DA and DAHPP samples over dry aging time (D0/D14/D21/D28), when compared to the positive ionisation mode. In this case, component 1 (X) accounts for 30% of the variance of the negative ionisation mode, while component 2 (Y) accounts for 7% of the variance. Similar to the positive ionisation mode, progressive changes in the metabolome occur over time, however, very little differences occur between samples taken at D14, D21 and D28. When examining the differences between DA and DAHPP samples over dry aging time (D0/D14/D21/D28) using the negative ionisation mode the model's performance is modest when trying

to explain and predict the variance associated with dry aging time and treatment ($R^2X = 0.44$, $R^2Y = 0.256$, $Q^2Y = 0.129$, $RMSEE = 0.3$). However, similar to the positive ionisation mode the model performance is improved ($R^2X = 0.42$, $R^2Y = 0.864$, $Q^2Y = 0.629$, $RMSEE = 0.194$) when only the overall effect of DA and DAHPP is considered (Figure 6-4B). This again indicates that treatment type strongly influences metabolomic variation in the current study.

Further examination of the data obtained from the positive (Figure 6-5) and negative (Figure 6-6) ionisation modes was carried out using OPLS-DA to investigate the evolution of the metabolomic profiles between D0 and D28 of the DA and DAHPP treatments, and distinguish between DA and DAHPP treatments. OPLS-DA enhances the model's ability to focus on metabolomic differences driven by dry aging time and treatment, while minimising noise and unrelated variability. When examining the effect of dry aging time (D0 v D28) on the metabolomic profile of DA and DAHPP samples, using the data from the positive ionisation mode, a clear separation was observed when comparing D0 and D28 aging time points in DA and DAHPP samples (Figure 6-5 A-B). The model performance of predicting dry aging time (D0 and D28) in DA ($R^2X = 0.746$, $R^2Y = 0.999$, $Q^2Y = 0.959$, $RMSEE = 0.019$) and DAHPP ($R^2X = 0.843$, $R^2Y = 1$, $Q^2Y = 0.98$, $RMSEE = 0.003$) samples indicate that the models were able to predict aging time and differentiate between D0 and D28. DA and DAHPP treatments (Figure 6-5C) exhibited clear differences and the model ($R^2X = 0.602$, $R^2Y = 0.927$, $Q^2Y = 0.718$, $RMSEE = 0.145$) was able to emphasize that the two treatments had different metabolomic profiles. The data from the negative ionisation mode (Figure 6-6 A-C) also displayed similar separation between aging times (D0 and D28) following DA ($R^2X = 0.693$, $R^2Y = 1$, $Q^2Y = 0.963$, $RMSEE = 0.007$) and DAHPP ($R^2X = 0.657$, $R^2Y = 1$, $Q^2Y = 0.959$, $RMSEE = 0.011$) as well as between DA and DAHPP treatments overall ($R^2X = 0.507$, $R^2Y = 0.954$, $Q^2Y = 0.797$, $RMSEE = 0.115$). The model performances of the OPLS-DA for the negative ionisation mode indicated that the model could differentiate aging time points (D0 and D28) and treatment groups (DA and DAHPP). OPLS-DA analysis was able to differentiate all sample groups from each other, irrespective of aging time (D0/D14/D21/D28) or treatment (DA/DAHPP), highlighting its discriminative power.

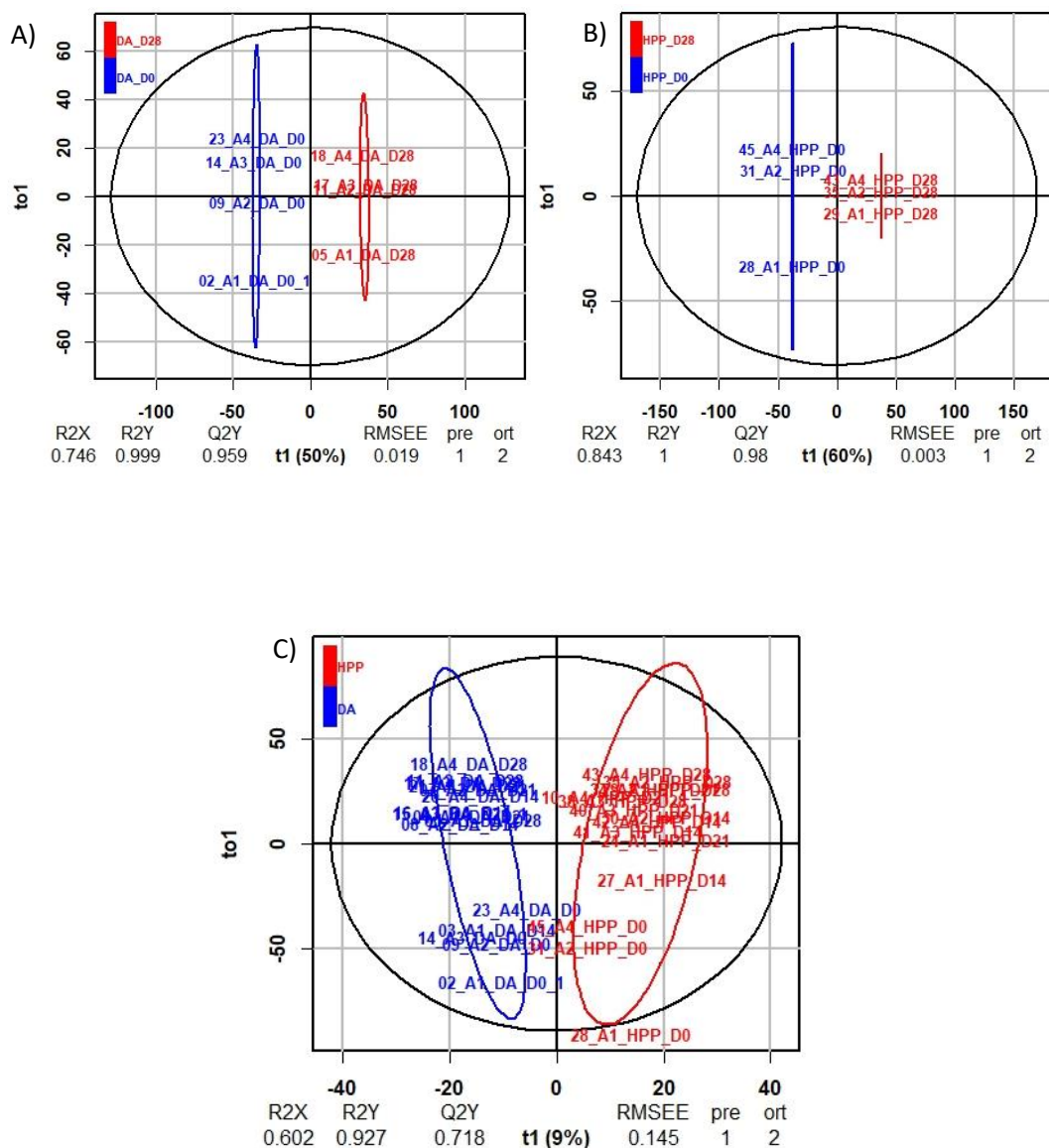


Figure 6-5. OPLS-DA model distinguishing D0 and D28 for A) DA and B) DAHPP treatments and C) overall effect of DA and DAHPP from the positive ionisation mode.

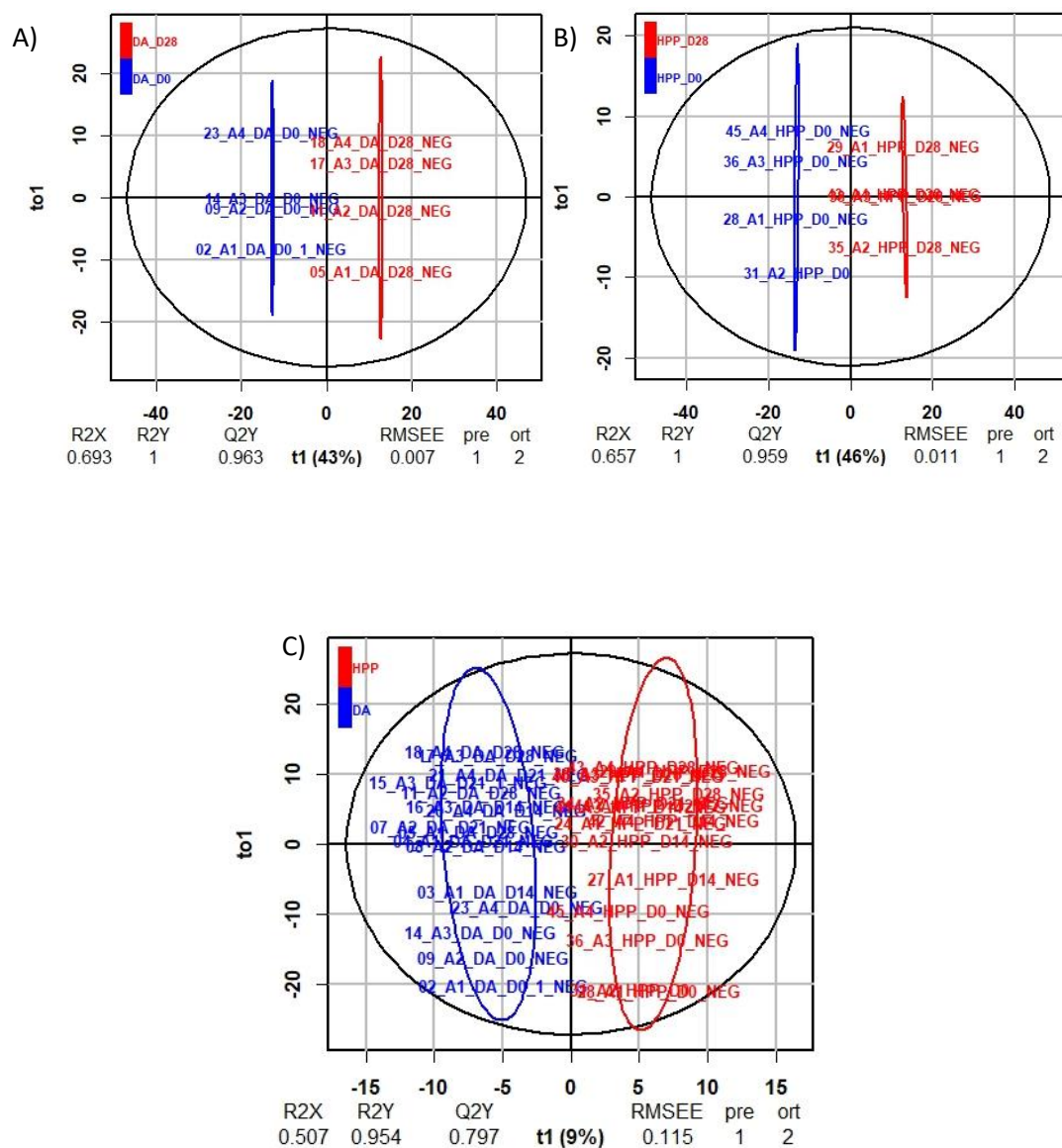


Figure 6-6. OPLS-DA model distinguishing D0 and D28 for A) DA and B) DAHPP treatments and C) overall effect of DA and DAHPP from the negative ionisation mode.

Volcano plots were used to analyse the differential abundant metabolites over aging time and as a result of HPP treatment in the positive ionisation mode. Within DA and DAHPP samples, the majority of metabolomic differences ($-0.5 > FC > 0.5$, $P < 0.05$) occurred within the first 14 days of dry aging, though changes continued to occur following 21 and 28 days of aging to a lesser extent (Figure 6-7A-B), in agreement with previous results. When examining the overall effect of DAHPP on the metabolomic profile of bovine LTL, it was found that DAHPP increased the concentration of 320 features ($FC > 0.5$, $P < 0.05$) and decreased the concentration of 122 features ($FC < -0.5$, $P < 0.05$) (Figure 6-7C). When comparing DA and DAHPP treated samples at corresponding aging times, little differences were observed at D0, where 37 features differed significantly ($-0.5 > FC > 0.5$, $P < 0.05$) in concentration. However, the number of differing metabolites increased with time, with 330 significantly ($-0.5 > FC > 0.5$, $P < 0.05$) differing metabolites at D14, 498 at D21 and 596 at D28 (Figure 6-7D). Similar trends were observed from the data obtained from the negative ionisation mode (Figure 6-8A-D).

The identifiable significant differential abundant metabolites ($VIP > 1$, $P < 0.05$) included peptides, phospholipids, nucleotides, and sugars, when examining the effect of dry aging time on both DA (Figure 6-9A-B) and DAHPP (Figure 6-10A-B) treated samples. The concentration of peptides, phospholipids and amino acids increased with aging time, while nucleotides and sugars either increased or decreased, in both DA and DAHPP samples, depending on the metabolite. When evaluating the overall effect of DAHPP (Figure 6-11), identifiable significant differential metabolites ($VIP > 1$, $P < 0.05$) included peptides and phospholipids, with the concentration of these metabolites increasing when muscle was subjected to HPP and subsequently dry aged

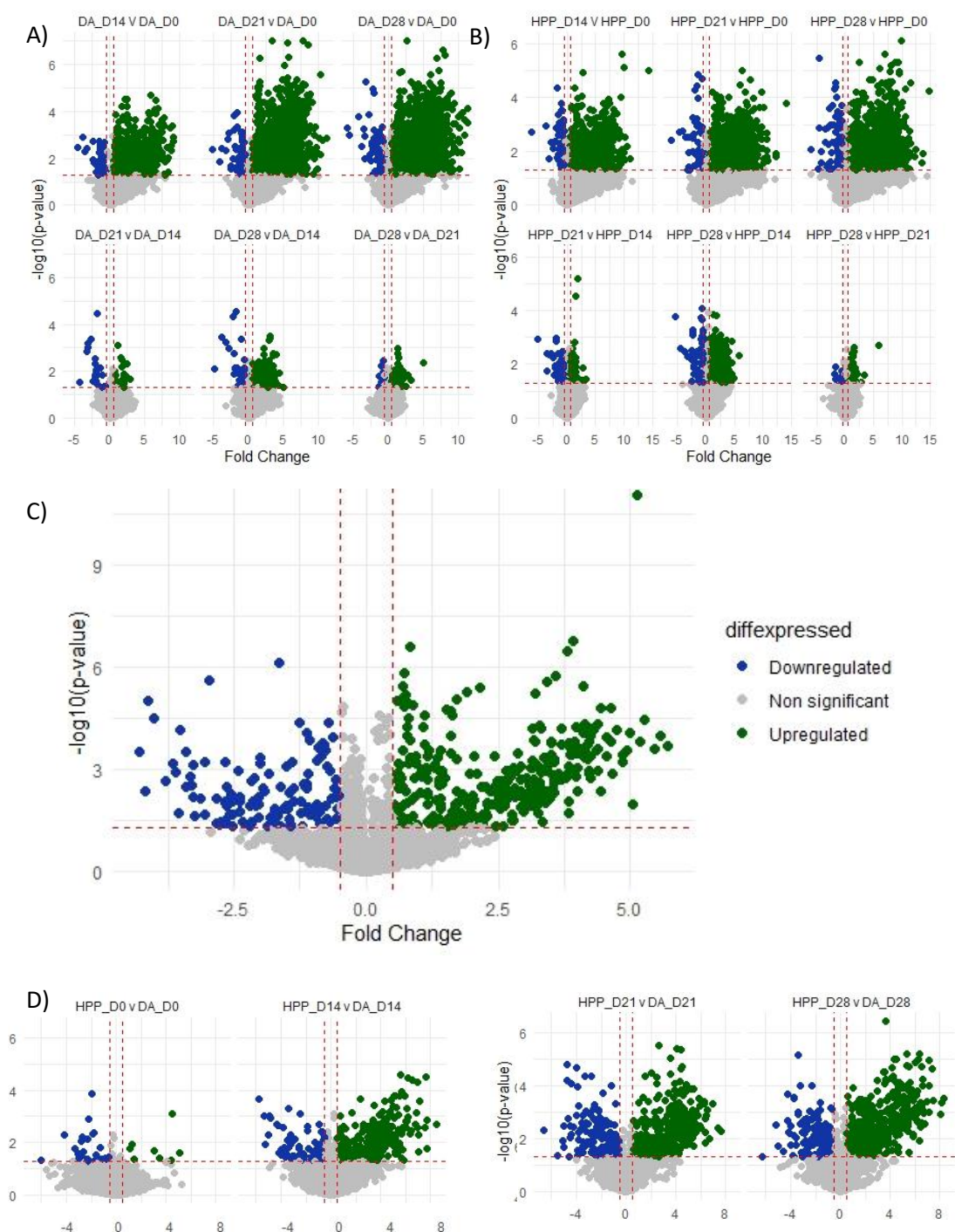


Figure 6-7. Volcano plots illustrating the effect of aging and DAHPP on metabolites. A) aging with DA, B) aging with DAHPP, C) overall effect of DAHPP compared to DA, and D) DAHPP v DA at individual times points. Data from the positive ionisation mode.

Upregulation in Figure 6-7A and 6-7B = concentration of detected metabolites increased with aging time. Upregulation in Figure 6-7C and 6-7D = DAHPP increased the concentration of detected metabolites.

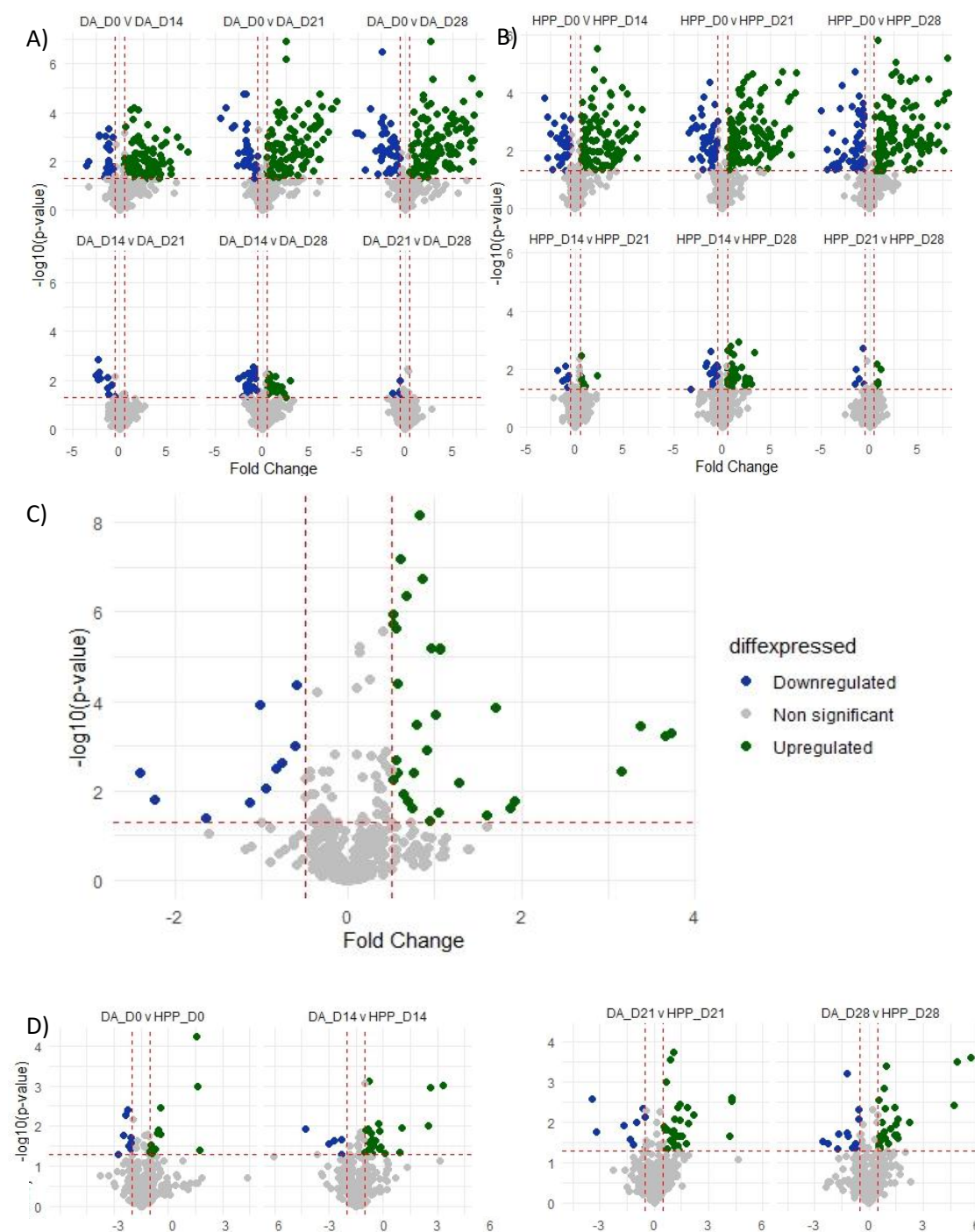


Figure 6-8. Volcano plots illustrating the effect of aging and DAHPP on metabolites
A) aging with DA, B) aging with DAHPP samples, C) overall effect of DAHPP
compared to DA, D) DAHPP v DA at individual time points. Data from the negative
ionisation mode.

Upregulation in Figure 6-8A and 6-8B = concentration of detected metabolites increased with aging time. Upregulation in Figure 6-8C and 6-8D = DAHPP increased the concentration of detected metabolites.

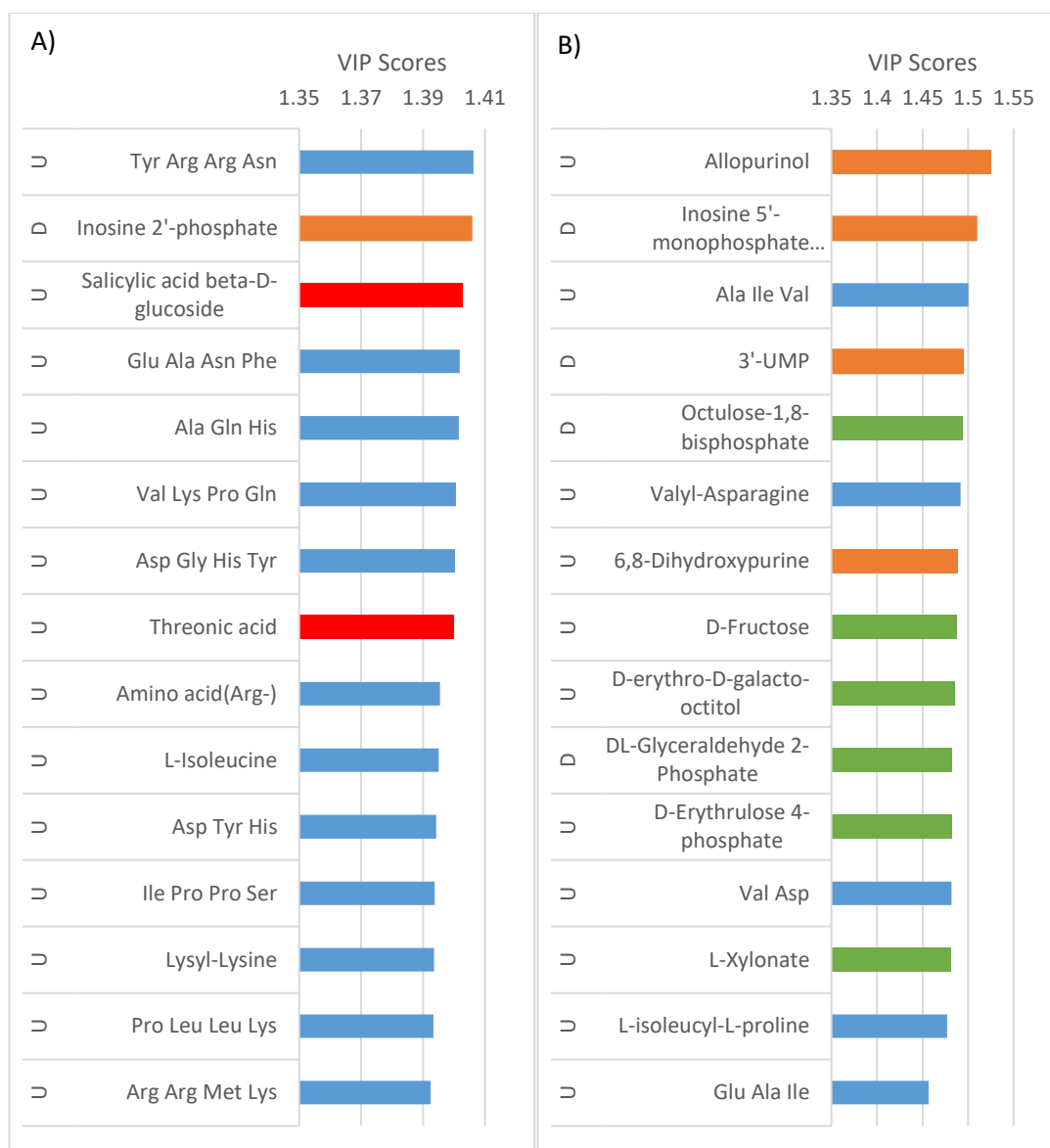


Figure 6-9. Top 15 identifiable significant differential metabolites (VIP > 1, $P < 0.05$) between DA – D0 and DA – D28, A) positive and B) negative ionisation modes.

VIP – Variable Importance in Projection; U – Concentration of metabolites increased ($FC > 0.5$, $P < 0.05$) with time; D – Concentration of metabolites decreased ($FC < -0.5$, $P < 0.05$) with time

Blue – Peptides; Yellow – Phospholipid; Green – Sugar; Red – Organic acid; Orange – Nucleotide

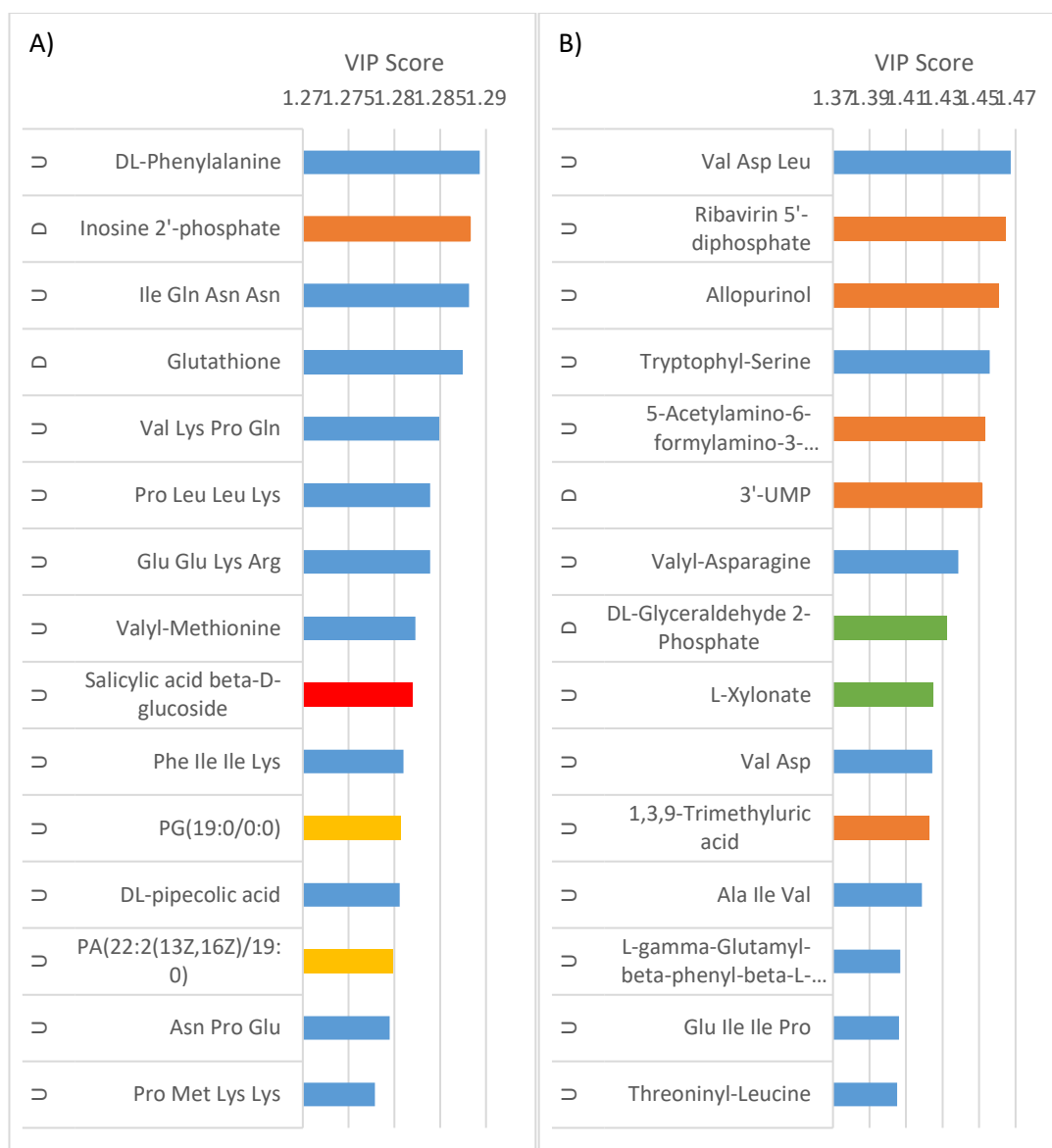


Figure 6-10. Top 15 identifiable significant differential metabolites (VIP > 1, $P < 0.05$) between HPP – D0 and HPP – D28, A) positive and B) negative ionisation modes.

VIP – Variable Importance in Projection; U – Concentration of metabolites increased ($FC > 0.5$, $P < 0.05$) with time. D – Concentration of metabolites decreased ($FC < - 0.5$, $P < 0.05$) with time.

Blue – Peptides; Yellow – Phospholipid; Green – Sugar; Red – Organic acid; Orange – Nucleotide

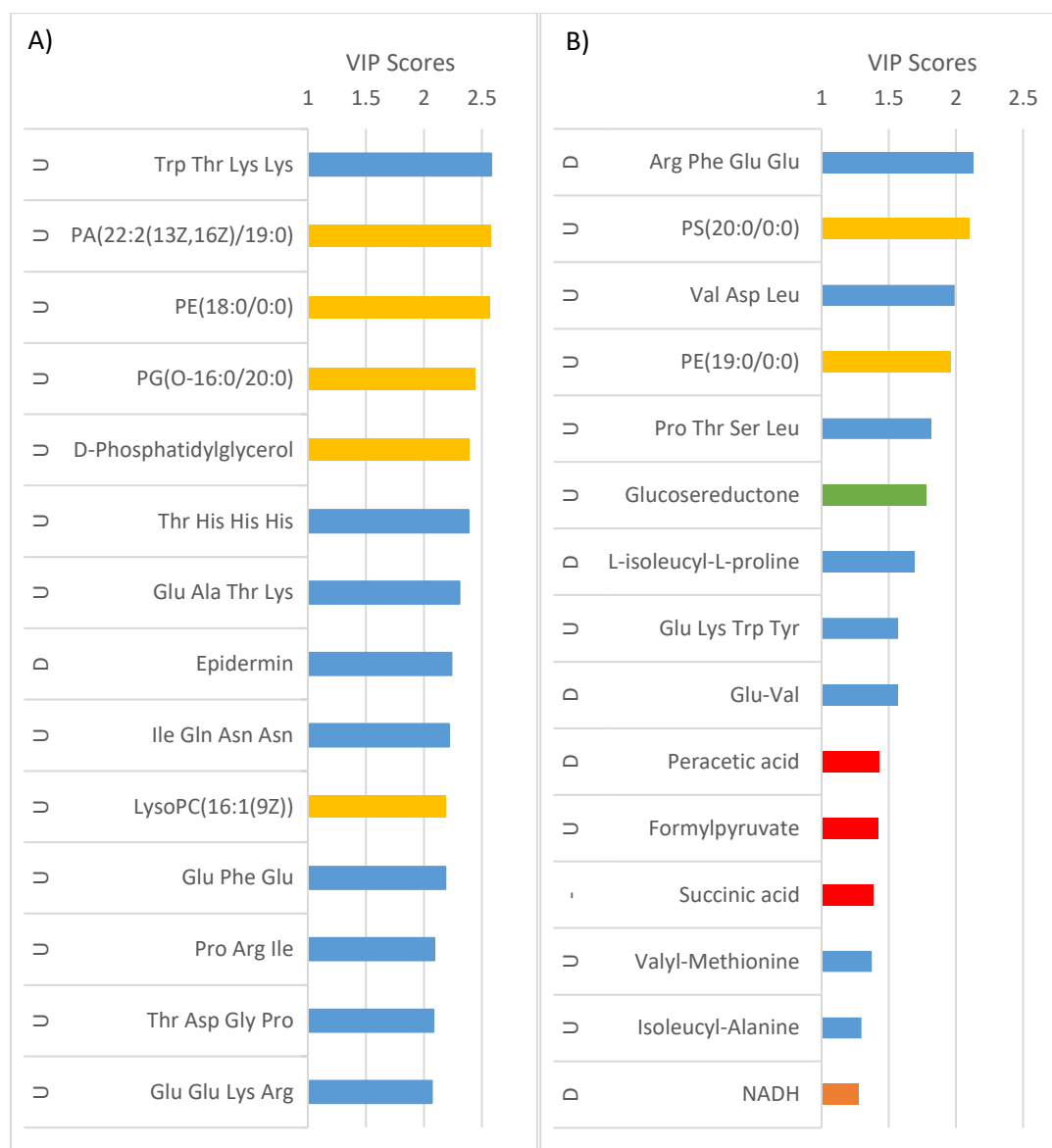


Figure 6-11. Top 15 identifiable significant differential metabolites (VIP > 1, $P < 0.05$) between DA and DAHPP treatments, A) positive and B) negative ionisation modes.

VIP – Variable Importance in Projection; U – DAHPP increased ($FC > 0.5$, $P < 0.05$) concentration of metabolites. D – DAHPP decreased ($FC < -0.5$, $P < 0.05$) concentration of metabolites. - - DAHPP did not affect ($-0.5 > FC > 0.5$, $P > 0.05$) the abundance of metabolites but was a significant differential metabolite (VIP > 1, $P < 0.05$).

Blue – Peptides; Yellow – Phospholipid; Green – Sugar; Red – Organic acid; Orange – Nucleotide

The combined PCA, PLS-DA, OPLS-DA and volcano plot analyses highlight how the metabolomic profile of bovine *Longissimus thoracis et lumborum* evolves over time, regardless of HPP pre – treatment. For both DA and DAHPP samples, aging increased the abundance of peptides and altered the abundance of various nucleotides (for instance, inosine 2'-phosphate decreased over time, while 3'-UMP accumulated as aging progresses, in both types of samples).

Similar PCA patterns to those described in this study (Figure 6-2A-B) have been reported by other authors when examining the metabolomic profile of early (0 – 9 days post-mortem) and later post-mortem (14 – 35 days post-mortem) muscle (Ma et al., 2017, Sun et al., 2024, Graham et al., 2010). The current findings align with these studies, as they show that the metabolomic profile of bovine muscle changed over time. Furthermore, the discriminative power of OPLS-DA between D0 and D28 in the current study (Figure 6-5A-B and Figure 6-6A-B) corresponds to the findings from other studies investigating the aging of bovine muscle over 15 days (Fu et al., 2024) and 28 days (Bischof et al., 2023) post-mortem. In these studies, OPLS-DA effectively discriminated between samples collected at day 0 and day 15 or 28, post-mortem. Similar to the findings of the current paper, the main compounds affected over time were amino acids and peptides. In addition, nucleotides such as hypoxanthine and inosine increased over time due to the breakdown of ATP, ADP and IMP (Graham et al., 2010, Sun et al., 2024).

The predominant changes observed with dry aging, in both DA and DAHPP samples, over time, was the increase in peptides. Peptides and amino acids are produced with aging time as a result of the degradation of proteins, post-mortem, by proteolytic enzymes such as calpain-1 and cathepsins (Song et al., 2022, Lomiwes et al., 2014b, Sun et al., 2024). Calpain activity significantly reduces following HPP treatments equal to or greater than 200 MPa (Chapter 5, Lee et al., 2023), however, HPP has been found to increase cathepsin activity and may also facilitate the breakdown of proteins by exposing the cleavage sites of proteolytic enzymes (Grossi et al., 2012, Jung et al., 2000c, Lee et al., 2023). When examining the identifiable significant differential metabolites of DA (Figure 6-9) and DAHPP (Figure 6-10) over time (D0 vs. D28), peptides are present in both groups. However, the specific peptides that

differentiate D0 and D28 in the DA and DAHPP treatment groups are different. This may reflect the differing proteolytic changes in the DAHPP samples, influenced by the enzymatic changes and structural modifications induced by the HPP pre-treatment.

After peptides, the content of phospholipids was the most impacted by HPP treatment, where HPP increased phospholipid content (Figure 6-10, Figure 6-11). Phospholipid content also increased with time in DA and DAHPP samples. Phospholipids are components of the cell membrane and play a role in cell metabolism (Bargui et al., 2021). Phospholipids can be either glycerophospholipids and sphingolipids and classed as phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA) or sphingomyeline (SM), depending on the composition of their polar head and back bone (Bargui et al., 2021, Bermingham et al., 2018). The phospholipids identified as differential metabolites in the current study include PC, PA, PE, PG and PS (Figure 6-10, Figure 6-11). In a recent study, Xu et al. (2024) found that the content of phospholipids in 96 h post-mortem bovine *Longissimus lumborum* increased between 7 and 14 days of refrigerated storage. de Oliveira Sartori et al. (2024) also observed an increase in the abundance of phospholipids between 3 and 21 days post-mortem in bovine *Longissimus lumborum*. In the case of the current study, the increase in the concentration of phospholipids following DAHPP (Figure 6-11) and over time (Figure 6-10) may be due to enhanced extractability, due to HPP disrupting the cell membranes and releasing cellular content (Medina-Meza et al., 2014).

The concentration of nucleotides such as inosine 5' monophosphate (IMP), hypoxanthine and inosine changed throughout aging in both the DA and DAHPP samples. IMP reduced significantly ($P < 0.05$) over time, while inosine and hypoxanthine increased significantly ($P < 0.05$) over time in both DA and DAHPP samples; which was expected since inosine and hypoxanthine arise from the hydrolysis of IMP (Tikk et al., 2006). DAHPP, however, did not ($P > 0.05$) have an impact on the abundance of these compounds. The impact of aging on the concentration of IMP, hypoxanthine and inosine in bovine muscle are in agreement

with the findings of Bischof et al. (2021), Kim et al. (2019) and Oh et al. (2018), who also reported that aging influenced the concentration of these metabolites.

The metabolites discussed, namely peptides, phospholipids and nucleotides, all play a role in flavour formation in beef (Dinh et al., 2021, Pavan et al., 2022, Setyabrata et al., 2021, Setyabrata et al., 2022a, Kim et al., 2016, Koutsidis et al., 2008). For example, inosine and hypoxanthine are associated with a bitter taste, while IMP is linked to umami flavour (Dashdorj et al., 2015, Muroya et al., 2019). Hence the changes in the metabolomic profile of dry aged beef resulting from the HPP pre-treatment highlighted in this study may result in changes to the flavour profile and further studies are warranted to investigate this effect.

6.4 Conclusion

The current study examines how HPP pre-treatment (200 MPa, 20 min, 8 °C) influences the protein and metabolomic profile of bovine *Longissimus thoracis et lumborum* following 28 days of dry aging (2 °C, 80% relative humidity, air flow ranging from 0.5 to 2.0 m/s). HPP pre-treatment induced changes to the protein profile during subsequent aging when compared to DA samples, where DAHPP samples exhibited decreases in the relative density (%) of MHC and increases in the relative density (%) of 160 kDa band over time. Over dry aging time, peptide concentrations increased and there were alterations in nucleotide concentrations in DA and DAHPP samples. DAHPP significantly altered the metabolomic profile of bovine LTL at all aging time points, resulting in an additional increase in the abundance of peptides and phospholipids. The majority of these metabolomic changes occurred within the first 14 days of dry aging, after which the profile of the metabolites evolved at a slower pace. However, different aging time points, with and without HPP treatment were distinguishable following OPLS-DA analysis. Changes in the concentration of metabolites such as peptides, phospholipids and nucleotides, which may act as precursors to flavour compounds, suggest that HPP pre-treatment has the potential to alter the flavour of dry aged beef. Therefore, further detailed investigations are warranted to determine the impact HPP pre-treatment on the flavour of dry aged bovine meat.

Chapter 7. General Discussion and Conclusions

7.1 General Discussion

Tenderness, along with flavour, colour and aroma, are important factors determining the final eating quality of meat and are influenced by a complex range of pre- and post-slaughter factors. Pre-slaughter factors that affect the quality of the meat include the breed, age and genetics, location of the muscle as well as rearing factors such as diet, exercise, health status and animal temperament (Hwang et al., 2010, Chriki et al., 2013, Gagaoua et al., 2018a, Gagaoua et al., 2018b, Soulat et al., 2018, Cafferky et al., 2019, Lebedová et al., 2021). Conditions immediately prior to slaughter, such as lairage, transport distance and pre-slaughter stress can also influence the final eating quality of meat (Reiche et al., 2019, Steel et al., 2021, Hultgren et al., 2022, Sullivan et al., 2024). Two enzyme driven processes, namely post-mortem glycolysis and proteolysis, play key roles in the conversion of muscle to meat and the rate and extent of these biochemical processes are greatly impacted by post-mortem conditions such as pH and temperature as discussed in Chapter 1 of this thesis.

Post-mortem glycolysis involves the degradation of glycogen under anaerobic conditions (triggered by exsanguination) to lactic acid and the concomitant reduction in pH (Ferguson and Gerrard, 2014, Chauhan and England, 2018). The rate limiting enzymes of glycolysis: glycogen phosphorylase, phosphofructokinase and pyruvate kinase, influence the rate of pH decline, and its kinetics are affected by pH and temperature. Phosphofructokinase has been identified as the primary rate limiting enzyme due to its susceptibility to pH, specifically as pH declines below pH 6, its activity is reduced significantly, arresting lactic acid production, even when glycogen and glucose are available as substrates (England et al., 2014, Wang et al., 2024).

Proteolytic enzymes, such as calpains and cathepsins, as well as other systems such as proteasomes, caspases and heat shock proteins are involved in the tenderisation of post-mortem muscle, later post-mortem, via the cleavage of key structural proteins such as titin, desmin and troponin-T (Kemp et al., 2010, Geesink et al., 2006). Calpain-1 has been identified as the key contributor to the proteolysis of bovine

muscle (Li et al., 2004, Bhat et al., 2018a, Ji and Takahashi, 2006). Proteolysis is impacted by external chilling conditions and the pH decline as a result of glycolysis. The interaction of pH and temperature influence the activity and efficacy of the proteolytic systems throughout aging. Thus, it is clear that both enzymatic system are interconnected and their interaction ultimately impacts meat quality, in particular tenderness. Due to the relevance of these enzymes in understanding the factors underpinning meat eating quality, extensive research has been carried out on the individual glycolytic and proteolytic enzymes in order to reveal how they function under different environmental conditions, in particular temperature and pH as outlined in Chapter 1.

As meat scientists strive to develop strategies to improve the level and consistency of meat quality, several carcass interventions and innovative technologies have been investigated for their potential to manipulate or control the enzymes involved in post-mortem glycolysis and proteolysis. These interventions can be categorised as traditional (electrical stimulation (ES), hanging, hot boning and wet or dry aging), or innovative (pulsed electric fields (PEF), ultrasound (US), or high pressure processing (HPP)) technologies. These interventions can operate mechanically and/or biochemically to alter the biochemical processes within muscle (driven by the endogenous enzymes), disrupting the muscle structure or a combinations of both; impacting various quality parameters, such as shear force values, colour and lipid oxidation, in different ways, as discussed in Chapter 2.

Extensive work has been carried out to understand the effects of pH and temperature on glycolytic and proteolytic enzymes; and although it has been proven difficult to control these processes in post-mortem muscle, as explored in Chapter 1 and Chapter 2, several carcass interventions have been already proposed and implemented, aiming for this goal. Nevertheless, additional research is required to fully elucidate the potential of various innovative technologies, such as ultrasound, pulsed electrical fields and high pressure processing, to modulate glycolysis and proteolysis in post- and pre-rigor muscle in order to ultimately improve meat quality.

Ultrasound was evaluated as a potential treatment to accelerate glycolysis in pre-rigor (2 – 4 h post-mortem) bovine *Longissimus thoracis et lumborum* (LTL) in

Chapters 3 and 4. Ultrasound is a non-thermal technology and involves the use of sound waves at frequencies greater than 20 kHz. Ultrasound involves the repeated compression and rarefaction of air bubbles within the medium being treated. An eventual collapse of the bubble results in acoustic or transient cavitation (Gallo et al., 2018), leading to the local development of high pressures and shear stresses (Alarcon-Rojo et al., 2019). Ultrasound can alter enzymatic activity, by modifying the substrate, the interaction between the enzyme and substrate or changing the conditions in which the enzyme is active in (Huang et al., 2017). To the best of our knowledge, the effect of ultrasound on the rate of glycolysis in *pre-rigor* bovine muscle has not been investigated. The effect of ultrasound on *pre-rigor* glycolysis was examined *in vivo*, by monitoring pH decline in muscle for 24 h post-treatment. In addition, the effect of ultrasound on the inherent activity of the glycolytic enzymes was assessed *in vitro*, adding homogenates of control and treated muscles to a glycolytic buffer and monitoring changes in pH and the concentration of glycogen, reducing sugars and lactic acid over time of incubation.

The glycolytic buffer system used in Chapter 3 (and again in Chapter 4) was re-established by Gerrard and his group as a method to mimic the post-mortem muscle environment in order to monitor the inherent activity of glycolytic enzymes in crude muscle extracts, via changes in pH, glycogen, reducing sugars and lactic acid (England et al., 2016, England et al., 2014). The glycolytic buffer provides an optimum environment for glycolytic enzymes. The glycolytic buffer system also ensures uniform substrate and pH conditions at the start of each reaction. This buffer system has previously been used to determine the roles of phosphofructokinase (England et al., 2014, Wang et al., 2024, Chauhan et al., 2021), mitochondria (Matarneh et al., 2018, Matarneh et al., 2021, Scheffler et al., 2015, Matarneh et al., 2017), AMP deaminase (England et al., 2015) and glycogen (England et al., 2016, Chauhan et al., 2019) in determining the rate of pH decline and the ultimate pH of muscle. The glycolytic buffer has been used in studies involving bovine, porcine and chicken muscle, reflecting the versatility of the system. By starting with a standard environment, the buffer enabled us to assess changes to the inherent activity of the glycolytic enzymes induced by ultrasound.

When examined *in vivo*, the rate of pH decline in the muscle was accelerated ($P < 0.05$) following the 30 min ultrasound treatments. No differences ($P > 0.05$) were found between any other treatments and controls. However, in the glycolytic buffer system, the ultrasound treatments investigated did not have a significant effect ($P > 0.05$) on pH decline or on the concentration of metabolites analysed during incubation. The results of Chapter 3 suggest that the change in the rate of pH decline observed *in vivo* as a result of the 30 min ultrasound treatments may be due to environmental changes within the muscle, such as increases in calcium concentration (Dang et al., 2022, Dolatowski and Stadnik, 2007, Got et al., 1999, Sikes et al., 2014) and not as a result of ultrasound directly impacting glycolytic enzyme activity. The lack of effect of ultrasound treatment used in this study on the apparent inherent activity of the glycolytic enzymes, when measured *in vitro*, may be due to insufficient ultrasound treatment intensity being applied to muscle, or the matrix of the muscle prevented ultrasound waves from impacting the glycolytic enzymes. This is in line with previous studies who found that the ultrasound treatment (20 kHz for periods of 15 s) of 2 and 24 h post-mortem bovine *Semimembranosus* and *Longissimus thoracis et lumborum* did not have an effect on proteolysis, which although is a different process, is also governed by enzymes, of intact pre- and post-rigor bovine and ovine muscle (Lyng et al., 1998a, Lyng et al., 1998b, Lyng et al., 1997).

To explore whether the matrix of intact bovine muscle acted as a protective barrier to prevent ultrasound from modifying the glycolytic enzymes, the experimental design in Chapter 4 involved applying ultrasound treatments to muscle homogenates rather than whole muscle. To the best of our knowledge, this had not been investigated before. Ultrasound treatment (60%/100% amp (11.4/19 W/cm²), for 15/30 min) was applied using an ultrasound probe. The effect of ultrasound on glycolysis was measured by monitoring pH decline and changes in the concentration of glycogen, reducing sugars and lactic acid over time in the same glycolytic buffer used in Chapter 3. No significant differences ($P > 0.05$) in glycogen degradation were observed between ultrasound treatments and controls. However, it was found that the 100% amp, 30 min treatment ultrasound treatment had a significant impact on glycolysis, resulting in a significant ($P < 0.05$) delay in lactic acid production and pH

decline between 30 and 240 min of incubation; at the same time an accumulation in reducing sugars was observed. Following 1440 min of incubation, no significant differences ($P > 0.05$) in pH or metabolite concentrations measured were present between buffer systems containing control and ultrasound treated homogenates.

These results indicate that one, or more, of the enzymes involved in converting reducing sugars, e.g. glucose, to lactic acid were affected by the 100% amp, 30 min ultrasound treatment; while the conversion of glycogen into reducing sugars was not impacted. While any enzyme involved in glycolytic reactions subsequent to glucose formation could be affected by ultrasound, hexokinase and phosphofructokinase were proposed as potential enzymes inhibited by the 100% amp, 30 min treatment. Hexokinase is responsible for the conversion of glucose to glucose-6-phosphate (Scheffler et al., 2015, Scheffler and Gerrard, 2007) and has been shown to be susceptible to ultrasound treatment (Braginskaya et al., 1990). Meanwhile, the loss of phosphofructokinase activity prevents the conversion of fructose-6-phosphate into fructose-1, 6-bisphosphate. An inhibition of both enzymes prevents lactic acid production and subsequent pH decline, as well as causing glucose and other reducing sugars such as glucose-6-phosphate and fructose-6-phosphate to accumulate (Matarneh et al., 2018). Regardless of what enzyme was responsible for the changes observed between 30 and 240 min of incubation, the effect of this ultrasound treatment did not result in the total cessation of enzymatic activity, as indicated by no significant differences being observed in pH and metabolites of interest following 1440 min of incubation. The limited effects observed using the *in vitro* glycolytic buffer system, suggested that ultrasound did not affect the inherent activity of glycolytic enzymes, unless these are exposed directly to high intensity ultrasound after removing the barrier that the meat matrix presents.

From the results of Chapters 3 and 4, it was concluded that ultrasound has limited potential as an alternative to electrical stimulation for altering the glycolysis in pre-rigor bovine muscle. Only the 30 min treatments impacted the rate of glycolysis in intact bovine muscle (Chapter 3).

The initial research plan for this work was to investigate the potential of ultrasound to manipulate proteolysis in bovine muscle. However, due to the limited effects of

ultrasound on glycolytic enzyme activity, (Chapter 3 and 4), the approach was reconsidered. Based on the findings of Chapter 2, high pressure processing (HPP) was identified as potentially a more effective alternative to influence the post-mortem proteolysis and subsequent meat quality. HPP has been investigated for its effects on meat quality prior to aging (McArdle et al., 2010, McArdle et al., 2011, Marcos et al., 2010, Marcos and Mullen, 2014). However, the interaction between HPP and dry aging has not yet been explored. Dry aging (DA) is a traditional method involving the storage of unwrapped carcasses or primals in controlled environmental conditions to produce tender meat with an enhanced and distinctive flavour, and is a topic of renewed interest in recent years (Álvarez et al., 2023, Correa et al., 2024, Vossen et al., 2022, Kim et al., 2022, Setyabrata et al., 2021, Setyabrata et al., 2022a, Lee et al., 2021, Bischof et al., 2023, Savas et al., 2024, Setyabrata et al., 2022b). Therefore, it was proposed to investigate the potential of a HPP pre-treatment to influence meat quality and alter the proteolysis of muscle during subsequent dry aging. HPP has been shown to alter the water holding capacity of muscle and disrupt the structure of proteins (McArdle et al., 2011, McArdle et al., 2010, Marcos et al., 2010, Marcos and Mullen, 2014). Thus, the potential of a HPP pre-treatment (200 MPa, 20 min, 8 °C) of bovine muscle (LTL at 96 h post-mortem) to accelerate weight loss and reduce the dry aging time, by facilitating water loss and therefore reducing processing cost and improving the economics of the process without having a detrimental impact on meat quality, was investigated in Chapter 5. In addition, the impact of a HPP pre-treatment on the proteolysis and metabolomic profile of dry aged bovine muscle was assessed in Chapters 5 and 6.

Dry aging with the high pressure pre-treatment (DAHPP) significantly ($P < 0.05$) increased the overall weight loss, indicating a potential reduction in dry aging time, however, other HPP pre-treatments might be more effective. DAHPP also increased the shear force values of meat during the first 21 days of aging, indicating that HPP reduced the tenderness of meat, which may be due to protein aggregation (Jung et al., 2000a). A dry aging period of 28 days was required for shear force values to be similar for control (DA) and DAHPP treatments. The results of Chapter 5 highlighted a potential dilemma in using HPP as a pre-treatment to dry aging, where increasing

process efficiency (shortened aging time required to reach same degree of moisture loss) negatively impacts eating quality (tenderness) which is not desirable commercially.

Another important consideration, based on the findings of the effects of HPP on the colour of muscle in Chapter 5, is whether the treated product is intended to be sold in a raw or cooked state. It was found that the colour difference (ΔE) between dry aged bovine muscle with (DAHPP) and without (DA) high pressure pre-treatment, following 28 days of aging, was significant ($\Delta E = 8.5$), primarily due to HPP increasing the lightness (L^*) of the meat, making DAHPP meat visibly distinguishable. This may affect the consumers' willingness to purchase raw HPP pre-treated meat, as colour is an important factor when influencing the purchase of a product. However, when comparing cooked dry aged muscle, with and without high pressure pre-treatment, the colour difference (ΔE) decreased to 2 indicating that only trained observers would notice a difference.

In addition, DAHPP was found to increase lipid oxidation, when compared to DA, as indicated by TBARS values, however, the increase was found to be within an acceptable threshold for consumer acceptability, with TBARS values remaining below 2 mg MDA/kg (Campo et al., 2006).

The results of Chapter 5 indicates the potential use of HPP as a method to accelerate the weight loss of dry aged bovine muscle. However, the effects of HPP on quality parameters such as shear force values and colour need to be taken into account when considering the importance of improving the efficiency of dry aging and eating quality. Other HPP pre-treatments, in terms of pressure, temperature and treatment duration, should be investigated for their effects on dry aging.

When considering the effect of HPP on post-mortem proteolysis in bovine LTL, changes in myofibrillar fragmentation index and calpain activity (Chapter 5), along with 1D SDS-PAGE and metabolomics (Chapter 6) were explored. DAHPP treatment significantly impacted the myofibrillar protein structure of dry aged bovine LTL, as noted by the higher values of the myofibrillar fragmentation index (MFI) ($P < 0.05$) at all aging time points in HPP treated samples, compared to control samples. However,

DAHPP significantly reduced ($P < 0.05$) calpain activity (by 97.1 – 99.5%) (Chapter 5), indicating that the initial increase in MFI was mainly due to physical disruption exerted by the HPP treatment. However, the MFI of DAHPP treated muscle continued to increase ($P < 0.05$) with dry aging time. It has been found that HPP facilitates the release of cathepsin enzymes from lysosomes and improves the accessibility of proteolytic enzymes to the cleavage sites of structural proteins to produce peptides (Grossi et al., 2012, Jung et al., 2000c, Lee et al., 2023). The results of this study suggest that HPP pre-treatment alters proteolysis in dry aged bovine muscle potentially by increasing the activity of enzymes other than calpains, such as cathepsins. DAHPP appears to delay tenderisation, as observed by higher shear force values in the first 21 days of aging. However, the shear force values of DA and DAHPP samples are the same after 28 days of dry aging. From these results, it is proposed that the HPP pre-treatment delays the tenderisation process by immediately inactivating calpain-1, while facilitating a gradual increase in cathepsin activity post-treatment as they are released from the lysosomes.

Analysis of the protein profile over aging time using 1D SDS-PAGE illustrated differing degradation patterns between DAHPP and DA samples. HPP and dry aging time significantly ($P < 0.05$) affected the relative density (%) of the bands corresponding to myosin heavy chain (MHC) and 160 kDa band, which has been identified within the range of MHC fragments (Marino et al., 2013). HPP treatment resulted in a decline ($P < 0.05$) in the relative density (%) of MHC, and an increase ($P < 0.05$) in the relative density (%) of the 160 kDa band, when compared to DA samples. In contrast, no significant ($P > 0.05$) differences were observed over time in the relative density (%) of the 160 kDa band, indicating that DAHPP facilitated the degradation of MHC, illustrated by the presence of the 160 kDa band. DAHPP increased ($P < 0.05$) the relative density (%) of bands corresponding to desmin, troponin-T, α -actinin and myosin light chain 2 (MLC2), when compared to DA samples. The increased relative density (%) of these proteins may be attributed to HPP disrupting the physical structure of muscle which is then further degraded during aging resulting in increased extractability of these proteins.

Metabolomics is a modern analytical tool with many potential uses in meat research as it offers insights into biochemical processes and provides a deeper understanding of changes in meat quality and the effects of different treatments. Within the field of meat science, metabolomics has been used to study the effect of aging duration (Ma et al., 2017, Sun et al., 2024, Muroya et al., 2023) and method (Kim et al., 2016, Bischof et al., 2021, Setyabrata et al., 2022a), abnormal pH decline (Ijaz et al., 2022, Ramanathan et al., 2020) and different feeding regimes (Gómez et al., 2022) on the metabolomic profile of meat. It has also been implemented in the detection of meat adulteration (Trivedi et al., 2016, Windarsih et al., 2022). Metabolomics in combination with multi-variant analysis (PCA, PLS-DA, OPLS-DA) can distinguish treatments and identify biomarkers associated with the specific treatments and aging times. Metabolomics could be used to discriminate between meat obtained from animals that followed grass and concentrate diets, which is important in the context of the Irish beef industry. It could also be used to authenticate meat from different aging methods (wet v dry aged meat) and aging durations.

Metabolomics was used as an additional tool in the current body of research to assess the effect of DAHPP on the metabolomic profile of dry aged bovine LTL over time and to compare the profile of DA and DAHPP treated samples. This offered an additional insight into the proteolysis of bovine muscle (Chapter 6) and the faith of other metabolites of relevance to meat eating quality (e.g. nucleotides). Analysis of the metabolomic data involved multi-variant analysis such as PCA, PLS-DA, OPLS-DA, and fold-changes ($-0.5 < FC < 0.5$, $P < 0.05$) represented in volcano plots. When examining the effect of dry aging time, it was found that the metabolomic profile of bovine LTL changed progressively over time. The main change over time was an increase in concentration of identifiable peptides, highlighting the importance of proteolysis during dry aging. PCA and PLS-DA suggested that HPP treatment did not have an immediate effect on the metabolomic profile of bovine muscle, as seen by the clustering of day 0 DA and DAHPP samples. As dry aging time increased, the metabolomic profile of DA and DAHPP samples differed, and these differences increased over time. When the identifiable significant differential metabolites ($VIP > 1$ (OPLS-DA), $P < 0.05$) of DA and DAHPP treated samples were examined over time,

the identified peptides differentiating day 0 and day 28 within the two treatment groups differed. This indicates differing patterns of peptide formation between the two treatments. It is proposed that in the DA samples, peptide formation was driven by the degradation of protein by calpains, which play a key role in proteolysis during the aging process. In contrast, proteolytic degradation in DAHPP samples was potentially due to a combination of factors: the physical disruption during HPP potentially facilitating the release of lysosomal cathepsin enzymes and enhancing protein degradation due to easier access of cleavage sites. In addition, when the overall effect of the HPP pre-treatment was examined, the primary effect that was observed was the increase in peptides. HPP also increased the concentration of phospholipids (VIP > 1, $P < 0.05$), potentially due to enhanced extractability resulting from the disruption of the muscle structure. In both DA and DAHPP samples, the concentration of nucleotides changed over time, where IMP decreased over time, while hypoxanthine and inosine increased over time. Changes in the concentration of metabolites such as peptides, phospholipids, and nucleotides, which may act as precursors to flavour compounds, suggest that HPP pre-treatment has the potential to alter the flavour profile of dry aged beef and potentially lead to the development of a premium dry aged flavour in a shorter dry aging period compared to traditional dry aging. Therefore, further investigations are warranted to investigate the impact of HPP pre-treatment on the flavour profile of dry aged bovine meat.

A considerable number of the metabolites detected in the current study remain unidentified. Combining metabolomics with other omics analysis such as proteomics, peptidomics and lipidomics, could offer a more comprehensive understanding of the biochemical changes that occur during aging and elucidate the potential of technologies such as HPP to modulate those processes in order to optimise meat quality and improve the efficiency of aging.

When considering the use of innovative technologies such as ultrasound and high pressure processing in commercial meat processing plants, many challenges must be addressed other than the various treatment parameters being used and their impact on meat quality. These include the scalability, integration with existing processing frameworks, efficiency and ability to manage animal to animal variation.

One key consideration is the size of the muscle that can be effectively treated, such as carcasses, primals, subprimals or small portions e.g. steaks. For ultrasound, the mode of delivery is an important consideration in this regard. Ultrasound water baths would be more practical for treating larger areas such as carcasses or various cuts of meat simultaneously. However, as demonstrated by the experimental conditions of Chapter 3, ultrasound water baths were unable to penetrate intact muscle, limiting their ability to modulate biochemical processes such as glycolysis. On the other hand, the ultrasound probe system is only able to deliver ultrasound to a localised area. It is therefore impractical for treating large areas of muscle. HPP has greater potential for commercial use in this sense, as it can uniformly treat meat of various shapes and sizes, making it a more efficient and scalable process compared to ultrasound. In addition to this, HPP is already widely used within the food industry as a method to improve food safety and extend the shelf life of various food products. HPP also requires no special preparation for meat products and could be easily integrated into existing processing lines, minimising disruption to workflow.

Another challenge that must be overcome when using ultrasound and HPP is the generation of heat, which requires additional measures to mitigate thermal effects. Both US water bath and probe systems can cause temperature increases in treated products, necessitating the incorporation of cooling systems to maintain optimal temperature conditions. On the other hand, while HPP can induce temperature changes during treatment, these are typically controlled within the system, reducing the risk of thermal effects on the product. However, in both cases, any increase in the operational costs associated with cooling or thermal management must be justified by the overall benefits of these technologies, such as improved meat quality, reduced aging times or extended shelf life.

Animal to animal variation presents another challenge for achieving consistent treatment outcomes with ultrasound and HPP. Differences in muscle size, composition and whether the muscle is pre- or post-*rigor* can influence the effectiveness of these treatments, as explored in Chapter 2. Tailoring treatment parameters such as pressure levels (HPP) or amplitude and frequency (US) to specific

muscle characteristics may help reduce variability and improve consistency of meat quality between animals.

7.2 Conclusion and future investigations

Meat quality, in particular, tenderness, as well as colour and flavour, are influenced by a wide range of pre- and post-mortem factors. However, biochemical processes such as glycolysis and proteolysis, play a pivotal role in the quality meat. Glycolysis is controlled by three key rate limiting enzymes: glycogen phosphorylase, phosphofructokinase and pyruvate kinase; and these contribute to the reduction in pH, post-mortem. Proteolysis plays a key role in the tenderisation of muscle. Calpain enzymes are key proteolytic enzymes, however, other systems that contribute to the proteolysis of bovine muscle include cathepsins, proteasomes, caspases and heat shock proteins. The glycolytic and proteolytic enzymes are affected by pH and temperature conditions within muscle. Meat researchers continue to strive to fully understand and control these two processes in order to optimise meat quality and meat processing efficiency.

The use of various technologies have been investigated for their potential ability to enhance the quality of meat, through mechanical or enzymatic means. Traditional technologies such as hanging and wrapping have been modified to enhance the stretching of muscles of interest, while electrical stimulation works enzymatically by altering glycolysis and rate of pH decline with chilling. Innovative technologies such as pulsed electric fields, ultrasound and high pressure processing alter meat quality through a combination of mechanical and biochemical methods. Studies investigating the use of innovative technologies to improve meat quality have yielded variable results reflecting the enormous challenge to identify a technology which can operate effectively in a commercial setting to improve meat quality.

This thesis has shown that the ultrasound treatments used had limited ability to manipulate the rate of glycolysis post-mortem. Little research has been carried out in this area. Further studies targeted at investigating the effects of ultrasound treatments of different intensities and durations on the action of the glycolytic system are warranted in order to fully elucidate if ultrasound can be exploited to manipulate post-mortem glycolysis.

This thesis has also shown that a HPP pre-treatment (200 MPa, 20 min, 8 °C) accelerated the weight loss of dry aged muscle over time, without having a detrimental effect on the quality (apart from colour) of bovine muscle following 28 days of dry aging. However, if it was to be used as a method to reduce the dry aging time, compromises in meat quality may have to be considered as at the shorter aging time of 21 days, the HPP pre-treated muscles had higher shear force values. Colour was another factor impacted by HPP, however, these effects could be mitigated if HPP pre-treated meat was sold as a cooked product. HPP altered calpain activity, protein degradation and the metabolomic profile of post-*rigor* bovine muscle. To the best of our knowledge the use of HPP as a pre-treatment for meat that is subsequently dry aged has not been previously investigated. The results presented in this thesis may act as a starting point for further studies that investigate the effect of different HPP treatment conditions (pressure, time and temperature) on the rate of drying and quality of dry aged bovine muscle in an attempt to optimise the use of HPP to accelerate dry aging without detrimentally affecting the quality of the muscle.

Metabolomics was applied to further investigate the impact of HPP pre-treatment on dry aging. It was found to distinguish dry aged bovine muscle, with and without HPP, and identify differences across aging times. The key differences in the pre-treated muscle was the profile of peptides, nucleotides and phospholipids. These metabolites can act as key precursors to flavour compounds in meat. Hence, further research is warranted to ascertain if HPP pre-treatments can be used to enhance the sensory characteristics of dry aged beef. This may provide opportunities to shorten the aging time while maintaining the characteristic flavour of dry aged beef.

Combining metabolomics with other omics analysis such as proteomics, peptidomics and lipidomics, could provide for a more comprehensive investigation of the potential of technologies such as HPP to modulate biochemical transformations that occur post-mortem with the target of optimising meat quality and improve the efficiency of aging.

Metabolomics has the potential of many applications in meat research such as a tool to track modifications in muscle biochemistry following various treatments and processing conditions including aging. However, to harness its full potential as an

analytical tool, standardisation and further technological developments are required. This includes sample preparation and data processing steps such as peak picking, alignment, and metabolite identification.

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Appendix 1 – RStudio Code for Section 6.2.6

#####Download libraries

```
library(MsExperiment)
```

```
library(stats)
```

```
library(dplyr)
```

```
library(tidyr)
```

```
library(ggplot2)
```

```
library(xcms)
```

```
library(BiocGenerics)
```

```
library(MSnbase)
```

```
library(SummarizedExperiment)
```

```
library(RColorBrewer)
```

```
library(pander)
```

```
library(MsFeatures)
```

```
library(imputeLCMD)
```

```
library(factoextra)
```

```
library(FactoMineR)
```

```
library(ggrepel)
```

```
library(parallel)
```

```
library(magrittr)
```

```
library(ggforce)
```

```
library(ropls)
```

```
library(mixOmics)
```

```
library(plotly)
```

```
library(ggfortify)
```

#####Path to folder containing files

```
setwd("E:/")
```

```
setwd
```

```
cds <-list.files("POS_Convert", recursive=T, full=T)
```

```

cdfs

#####Extract sample names and store in variable

pd <- data.frame(sample_name = sub(basename(cdfs), pattern = ".mzXML",
                                replacement = "", fixed = TRUE),
                stringsAsFactors = FALSE)

pd

pd$sample_type <- "study"
pd$sample_type[c(6,12,19,26,32,39,46)] <- "QC"
pd$sample_type[c(1,13,25,37,47)] <- "Blank"
pd$sample_group <- "DA_D0"
pd$sample_group[c(6,12,19,26,32,39,46)] <- "QC"
pd$sample_group[c(1,13,25,37,47)] <- "Blank"
pd$sample_group[c(3,8,16,20)] <- "DA_D14"
pd$sample_group[c(4,7,15,21,33)] <- "DA_D21"
pd$sample_group[c(5,11,17,18)] <- "DA_D28"
pd$sample_group[c(28,31,36,45)] <- "HPP_D0"
pd$sample_group[c(27,30,41,42)] <- "HPP_D14"
pd$sample_group[c(10,24,34,40,44)] <- "HPP_D21"
pd$sample_group[c(29,35,38,43)] <- "HPP_D28"
pd$sample_treatment <- "Blank"
pd$sample_treatment[c(6,12,19,26,32,39,46)] <- "QC"
pd$sample_treatment[c(2,9,14,22,23,3,8,16,20,4,7,15,21,33,5,11,17,18)] <- "DA"
pd$sample_treatment[c(28,31,36,45,27,30,41,42,10,24,34,40,44,29,35,38,43)] <-
"HPP"

pd$sample <- "ALL"

pd

##### Build dataframe that will store sample names/groups

raw_data <- readMSData(files = cdfs, pdata = new("NAnnotatedDataFrame", pd),
                      mode = "onDisk", msLevel. = 1)

raw_data

```

```

save(raw_data, file = "D:/ProjectName/Meat Proteomics/data/raw
data/MSRawData_ALL.RData")

load("D:/ProjectName/Meat Proteomics/data/raw data/MSRawData_ALL.RData")

group_colors <- paste0(brewer.pal(6, "Set1")[1:6])
names(group_colors) <- c("D0", "D14", "D21", "D28", "QC", "Blank")

#####Extract base peak chromatogram and total ion count of each sample

#####Plotting all chromatograms

bpis <- chromatogram(raw_data, aggregationFun = "max", BPPARAM =
SerialParam())

bpis

plot(bpis, col = group_colors[raw_data$sample_group], main = "All over time")

legend("topright", legend = c("D0", "D14", "D21", "D28", "QC", "Blank"), col =
c("#E41A1C99", "#377EB899", "#4DAF4A", "#984EA3", "#FF7F00", "#FFFF30"), cex =
0.6, lty = 1)

tc <- split(tic(raw_data), f = fromFile(raw_data))

boxplot(tc, col = group_colors[raw_data$sample_group], ylab = "intensity", main =
"Total ion current")

legend("topright", legend = c("D0", "D14", "D21", "D28", "QC", "Blank"), col =
c("#E41A1C99", "#377EB899", "#4DAF4A", "#984EA3", "#FF7F00", "#FFFF30"), cex =
0.6, lty = 1)

#####Initial peak checks and extraction of chromatograms

#####Define the rt and m/z range of the peak area

```

POSITIVE	NEGATIVE
###Leucine (POSITIVE) peak detection <pre>rtr <- c(0, 200) mzr <- c(132.075, 132.09)</pre> ###Tryptophan (POSITIVE) peak detection <pre>rtr2 <- c(100, 260) mzr2 <- c(205.09, 205.1)</pre>	###NEGATIVE peak detection <pre>rtr <- c(0, 150) mzr <- c(96.968, 96.974)</pre> ###NEGATIVE peak detection <pre>rtr2 <- c(100,130) mzr2 <- c(73.029, 73.035)</pre>

```
chr_raw <- chromatogram(raw_data, mz = mzr, rt = rtr)
```

```

par(mfrow = c(1,1))
plot(chr_raw, col = group_colors[chr_raw$sample_group])
raw_data %>%
  filterRt(rt = rtr) %>%
  filterMz(mz = mzs) %>%
  plot(type = "XIC") #helps find what the ppm parameter for CentWave should be
xchr <- findChromPeaks(chr_raw, param = CentWaveParam(snthresh = 1))
head(chromPeaks(xchr))
chromPeakData(xchr)

chr_raw2 <- chromatogram(raw_data, mz = mzs, rt = rtr)
plot(chr_raw2, col = group_colors[chr_raw2$sample_group])
raw_data %>%
  filterRt(rt = rtr) %>%
  filterMz(mz = mzs) %>%
  plot(type = "XIC")
xchr2 <- findChromPeaks(chr_raw2, param = CentWaveParam(snthresh = 1))
head(chromPeaks(xchr))
chromPeakData(xchr)

sys_name <- Sys.info()["sysname"]
bpparam <- switch(sys_name,
  Windows = {SnowParam(workers = max(1, detectCores() - 1), progressbar =
TRUE)},
  Linux = {MulticoreParam(workers = max(1, detectCores() - 1), progressbar =
TRUE)},
  MacOS = {MulticoreParam(workers = max(1, detectCores() - 1), progressbar =
TRUE)},
  stop("Unsupported operating system"))
bpparam

```

#####Set up CentWave parameters

POSITIVE	NEGATIVE
cwp <- CentWaveParam(peakwidth = c(20, 50), mzCenterFun = "mean", fitgauss = TRUE, snthresh = 6, noise = 3500, prefilter = c(5, 1000), ppm = 25, mzdiff = -0.005)	cwp <- CentWaveParam(peakwidth = c(10, 60), mzCenterFun = "mean", fitgauss = TRUE, snthresh = 5, noise = 3000, prefilter = c(5, 100), ppm = 20, mzdiff = -0.005)

Perform feature extraction

```
xdata <- findChromPeaks(raw_data, param = cwp, add = TRUE, BPPARAM =  
bpparam)
```

```
save(xdata, file = "D:/ProjectName/Meat Proteomics/xdata/MSXDataALL.RData")
```

```
load("D:/ProjectName/Meat Proteomics/xdata/MSXDataALL.RData")
```

Summarize the extracted features

```
summary(xdata)
```

```
chromPeakData(xdata)
```

```
mpp <- MergeNeighboringPeaksParam(expandRt = 1)
```

```
xdata_pp <- refineChromPeaks(xdata, mpp, BPPARAM = SerialParam())
```

```
summary_fun <- function(z)
```

```
  c(peak_count = nrow(z), rt = quantile(z[, "rtmax"] - z[, "rtmin"])))
```

```
T <- lapply(split.data.frame(  
  chromPeaks(xdata_pp), f = chromPeaks(xdata_pp)[, "sample"]), FUN =  
  summary_fun)
```

```
T <- do.call(rbind, T)
```

```
rownames(T) <- basename(fileNames(xdata_pp))
```

```
pandoc.table(T,
```

```
  caption = paste0("Summary statistics on identified chromatographic",  
    "peaks. Shown are numbers of identified peaks per",  
    "sample and widths/duration of chromatographic",  
    "peaks"))
```

#####ALIGNMENT

```
xdata_org <- dropAdjustedRtime(xdata_pp)
```

```
##### Initial peak grouping. Use sample_type as grouping variable
```

```
pdp_subs <- PeakDensityParam(sampleGroups = xdata_org$sample,
                             minFraction = 0.85)
```

```
xdata_org <- groupChromPeaks(xdata_org, param = pdp_subs)
```

```
##### Define subset-alignment options and perform the alignment
```

POSITIVE	NEGATIVE
<pre>pgp_subs <- PeakGroupsParam(minFraction = 0.15, subset = which(xdata_org\$sample_type == "QC"), subsetAdjust = "average", span = 0.5) xdata_adj <- adjustRtime(xdata_org, param = pgp_subs)</pre>	<pre>pgp_subs <- PeakGroupsParam(minFraction = 0.15, subset = which(xdata_org\$sample_type == "QC"), subsetAdjust = "average", span = 0.6) xdata_adj <- adjustRtime(xdata_org, param = pgp_subs)</pre>

```
#####Plot the differences of adjusted to raw retention time
```

```
clrs <- rep("#00000040", 8)
```

```
clrs[xdata_adj$sample_type == "QC"] <- c("#00ce0080")
```

```
par(mfrow = c(2, 1), mar = c(4, 4.5, 1, 0.5))
```

```
register(SerialParam())
```

```
plot(chromatogram(xdata_adj, aggregationFun = "max", chromPeaks = "none"),
```

```
  col = clrs)
```

```
grid()
```

```
plotAdjustedRtime(xdata_adj, col = clrs, peakGroupsPch = 1,
```

```
  peakGroupsCol = "#00ce0040")
```

```
grid()
```

```
plot(chr_raw, col = group_colors[chr_raw$sample_group])
```

```

chr_adj <- chromatogram(xdata_adj, rt = rtr, mz = mzt)
plot(chr_adj, col = group_colors[chr_adj$sample_group], peakType = "none")

#####Grouping chromatographic peaks based on time dimension peak densities
BWG <- seq(0.5, 10, by = 0.5)
feature_number <- c()
for (i in 1:length(BWG)) {
  pdp <- PeakDensityParam(sampleGroups = xdata_adj$sample_group,
                           minFraction = 0.4, bw = BWG[i], binSize = 0.015)
  xdata_BWG <- groupChromPeaks(xdata_adj, param = pdp)
  feature_number[i] = nrow(featureValues(xdata_BWG))}
feature_number
plot(BWG, feature_number)

```

POSITIVE	NEGATIVE
pdp <- PeakDensityParam(sampleGroups = xdata_adj\$sample_group, minFraction = 0.85, bw = 0.5, binSize = 0.5)	pdp <- PeakDensityParam(sampleGroups = xdata_adj\$sample_group, minFraction = 0.85, bw = .5, binSize = 0.5)
xdata <- groupChromPeaks(xdata_adj, param = pdp)	

```

featureData <- featureData(xdata)
featureData

res <- quantify(xdata, value = "into")
res

featureDef <-featureDefinitions(xdata)
featureDef

featureVal <-featureValues(xdata)

```

```
featureVal
table(is.na(featureVal))

xdata <- fillChromPeaks(xdata, param = ChromPeakAreaParam())

featureValaftrFilling <- featureValues(xdata)
featureValaftrFilling
table(is.na(featureValaftrFilling))

xdata <- fillChromPeaks(xdata,param = FillChromPeaksParam(expandMz = 1.5,
expandRt = 1.5))
featureValaftrSecndFilling <- featureValues(xdata)
featureValaftrSecndFilling
write.csv(featureValaftrSecndFilling, "ALL_FeatureValues.csv")
table(is.na(featureValaftrSecndFilling))
featureSummary(xdata, group = xdata$sample_group, perSampleCounts = TRUE)

##### Missing values before filling in peaks
apply(featureValues(xdata, filled = FALSE), MARGIN = 2,
      FUN = function(z) sum(is.na(z)))

## Missing values after filling in peaks
apply(featureValues(xdata), MARGIN = 2,
      FUN = function(z) sum(is.na(z)))

logTval <- log2(featureValaftrSecndFilling)
featureLog <- impute.QRILC(logTval, tune.sigma = 1)
hist(logTval)

extractFilledValues <- featureLog[[1]]
```

```
table(is.na(extractFilledValues))
hist(extractFilledValues)

centeringFactor <- apply(extractFilledValues, 1, mean)
l <- as.numeric(length(centeringFactor))
centeredValues <- matrix(,l, 47)
for(i in 1:l) {
  for(j in 1:47) {
    centeredValues[i,j] <- cbind(extractFilledValues[i,j] - centeringFactor[i])
  }
}
colnames(centeredValues) <- c(pd$sample_name)
centeredValues <- centeredValues[,sort(colnames(centeredValues))]
hist(centeredValues)

#####retrieving mz and rt details of detected peaks
rt <- featureDef@listData$rtmed
mz <- featureDef@listData$mzmed

details <- cbind(rt,mz)
colnames(details) <- c("retentionT", "m/z")
peakDetails <- as.data.frame(details)
peakDetails

###rounding up the decimals
peakDetails$'m/z' <- round(peakDetails$'m/z', digits = 4)
peakDetails$retentionT <- round(peakDetails$retentionT, digits = 4)

#### creating identifiers using mz_rt
peakDetails$Identifier <- paste(peakDetails$'m/z', "_", peakDetails$retentionT)
```

```
##### changing feature numbers as row names to identifiers
rownames(centeredValues) <- c(peakDetails$Identifier)
write.csv(centeredValues, "E:/ALL_CenteredValues.csv")

#####PCA
pc_positive <- prcomp(t(na.omit(centeredValues)))
pc_positive_Summary <- summary(pc_positive)
cumSum_p <- as.data.frame(pc_positive_Summary$importance[3,])
forRowNames <- pd$sample_group

####Scree plot
fviz_eig(pc_positive, addlabels = TRUE) +
  theme_minimal() +
  scale_x_discrete(breaks = c(1:ncol(pc_positive_Summary$importance)),
    labels = c(cumSum_p[,1]*100)) +
  ggtitle("ALL Screeplot")

####Score
score.data <- as.data.frame(pc_positive_Summary$x)
write.csv(score.data, "E:/pca_scores_ALL_POS.csv")

data <- read.csv("E:/pca_scores_ALL_POS.csv")
data <- cbind(data, forRowNames)

# Define shapes, fills, and colours for sample groups
unique_groups <- unique(pd$sample_group)
shapes <- seq_along(unique_groups) #Create a sequence of shapes
colors <- scales::hue_pal()(length(unique_groups)) #Generate distinct colours
fills <- colors #Use the same colours for fill
```

```
#####PCA plot

ggplot(data, aes(PC1, PC2, color = forRowNames, shape = forRowNames)) +
  geom_point(size = 4) +
  geom_text(aes(label = forRowNames), vjust = -1, color = "black") +
  geom_mark_ellipse(aes(fill = forRowNames), alpha = 0.1) + # Add filled ellipses
  labs(title = "PCA Plot with Ellipses",
        x = paste0("PC1 (", round(pc_positive_Summary$importance[2, 1] * 100), "%
variance)"),
        y = paste0("PC2 (", round(pc_positive_Summary$importance[2, 2] * 100), "%
variance)")) +
  theme_minimal() +
  scale_shape_manual(values = shapes) + # Explicitly define shapes
  scale_fill_manual(values = fills) + # Explicitly define fills
  scale_color_manual(values = colors) # Explicitly define colours

#####Filtering out Blank samples

pd_qc <- pd[-c(1,13,25,37,47,36),]
centeredValues_qc <-centeredValues[-c(1,13,25,37,47,36)]

#####Repeat PCA for quality check of data

#####Filtering out QC and Blank samples

centeredValues <- centeredValues[-c(1,6,10,12,13,19,22,25,26,32,33,37,39,46,47)]
pd <- pd[-c(1,6,10,12,13,19,22,25,26,32,33,37,39,46,47),]

#####DA V HPP

### function to run t test for fold change

ttest <- function(df, grp1, grp2) {
  x = df[grp1]
  y = df[grp2]
  x = as.numeric(x)
  y = as.numeric(y)
```

```
results = t.test(x,y)
results$p.value}

##### fold change HPP - DA
##### up - regulated components are higher in HPP than DA
##### down - regulated components are lower in HPP than DA
rawp.value.p = apply(centeredValues, 1, ttest, grp1 = c(1:7, 9, 11:17), grp2 = c(8,
18:32))
test.p = apply(centeredValues[,c(17:32)], 1, mean) ###DAHPP
control.p = apply(centeredValues[,c(1:16)], 1, mean) ###DA
class(test.p)
class(control.p)
foldChange.p <- test.p - control.p
par(mfrow = c(1,2))
hist(rawp.value.p)
hist(foldChange.p, xlab = "Fold Change (DA v HPP)")

results.p = cbind(foldChange.p, rawp.value.p)
results.p = as.data.frame(results.p)
results.p$probenname <- peakDetails$Identifier
results.p$diffexpressed <- "NO"
results.p$diffexpressed[results.p$foldChange.p > 0.5 & results.p$rawp.value.p <
0.05] <- "UP"
results.p$diffexpressed[results.p$foldChange.p < (-0.5) & results.p$rawp.value.p<
0.05] <- "DOWN"
results.p$difflabel <- NA
results.p$difflabel[results.p$diffexpressed != "NO"] <-
results.p$probenname[results.p$diffexpressed != "NO"]

# Generate volcano plot
volcano_plot <- ggplot(results.p, aes(x = foldChange.p, y = -log10(rawp.value.p))) +
```

```

geom_point(aes(color = diffexpressed), size = 2) +
scale_color_manual(values = c("#1134A6", "grey", "darkgreen"),
                    labels = c("Downregulated", "Non significant", "Upregulated")) +
geom_hline(yintercept = -log10(0.05), linetype = "dashed", color = "red") +
geom_vline(xintercept = c(-0.5, 0.5), linetype = "dashed", color = "red") +
labs(x = "Fold Change", y = "-log10(p-value)", title = "Volcano Plot") +
theme_minimal()
print(volcano_plot)

diffMetaboP <- data.frame(results.p$difflabel[results.p$diffexpressed != "NO"],
                        results.p$diffexpressed[results.p$diffexpressed != "NO"])
colnames(diffMetaboP) <- c('PosMetabo', 'PosExpression')
diffMetaboP <- diffMetaboP %>% separate(PosMetabo, c('m/z', 'rt'))

dim(diffMetaboP)
sum(diffMetaboP$PosExpression == "DOWN", na.rm = TRUE)
sum(diffMetaboP$PosExpression == "UP", na.rm = TRUE)
write.csv(diffMetaboP, "E:/POSexpressionDAvHPP.csv")

##### Create dataset to extract features that increase and decrease following HPP
labelsupdown <-
  subset(results.p, diffexpressed == "UP" | diffexpressed == "DOWN", select =
difflabel)$difflabel
labelsupdown
upanddown <- centeredValues[labelsupdown, c(1:32)]
upanddown

##### Scale the centered data (Pareto scaling) for PLS-DA and OPLS-DA
sd <- round(apply(centeredValues, 1, sd), 2)

```

sd

```
normValues <- matrix(,l,31)
for (i in 1:l) {
  for (j in 1:32) {
    normValues[i,j] <- cbind(centeredValues[i,j]/sd[i]) }}

colnames(normValues) <- colnames(centeredValues)
rownames(normValues) <- c(peakDetails$Identifier)
normValues
boxplot(centeredValues, main = "Before Pareto Scaling")
boxplot(normValues, main = "After Pareto Scaling")
```

#####PLS-DA

```
plsda.HPP <- opsls(t(normValues), pd$sample_treatment, predI = 2)
plsda_HPP <- plsda(t(normValues), pd$sample_treatment)
plotIndiv(plsda_HPP, ind.names = TRUE, ellipse = TRUE, legend = TRUE)
```

OPLS-DA - GIVES VIP SCORE

```
Treatment_oplsda <- opsls(t(na.omit(normValues)), pd$sample_treatment, orthol =
2)
plot(Treatment_oplsda, typeVc = "x-score", parAsColFcVn = pd$sample_treatment)
```

```
vipVn_DAvHPP <- Treatment_oplsda@vipVn
head(vipVn_DAvHPP)
```

```
vipVn_DAvHPP_abv1 <- as.data.frame(vipVn_DAvHPP[vipVn_DAvHPP>1])
rownames_VIP <- rownames(vipVn_DAvHPP_abv1)
a_DAvHPP <- as.data.frame(cbind(vipVn_DAvHPP_abv1, rownames_VIP))
```

#####T-Test

Perform t-test and store P-values

```

p_values_DAvHPP <- matrix(, l, 1)

for (i in 1:l) { p_values_DAvHPP[i] <- cbind(t.test(normValues[i, c(1:16)],
normValues[i, c(17:32)], alternative = "two.sided", paired = TRUE)$p.value)}

rowNAME_pVal_DAvHPP <- rownames(p_values_DAvHPP) %>%
c(peakDetails$Identifier)

p_value_significant_DAvHPP <-
as.data.frame(cbind(p_values_DAvHPP[p_values_DAvHPP<0.05],
rowNAME_pVal_DAvHPP[p_values_DAvHPP<0.05]))

colnames(p_value_significant_DAvHPP) <-c("p_Value", "DetectedFeature")

p_value_significant_DAvHPP

t_test_DAvHPP <- t.test(normValues[i, c(1:16)], normValues[i, c(17:32)], alternative
= "two.sided", paired = TRUE)

t_test_DAvHPP$p.value

#####Extract features with P – value < 0.05 and VIP > 1 and find common features -
significant differential metabolites

significantOnes_DAvHPP = NULL

len_vipScore_DAvHPP <- as.numeric(nrow(vipVn_DAvHPP_abv1))

len_pValue_DAvHPP <- as.numeric(nrow(p_value_significant_DAvHPP))

for (i in 1:len_pValue_DAvHPP) {
  for (j in 1:len_vipScore_DAvHPP) {
    if(p_value_significant_DAvHPP[i, 2] == a_DAvHPP[j, 2])
      {significantOnes_DAvHPP[j] <- cbind(p_value_significant_DAvHPP[i,2])}
  }
}

significantOnes_DAvHPP <- as.data.frame(na.omit(significantOnes_DAvHPP))

colnames(significantOnes_DAvHPP) <- c("DetectedFeature")

colnames(a_DAvHPP) <- c("VIPScore","DetectedFeature")

```

#####P values and VIP scores of significant differential metabolites into single dataset and order them according to VIP score

```

vipScore_pValSig_DAvHPP = matrix(nrow = dim(significantOnes_DAvHPP)[1], ncol =
3)

for (i in 1:dim(significantOnes_DAvHPP)[1]) {
  for(j in 1:dim(a_DAvHPP)[1]){
    if(significantOnes_DAvHPP$DetectedFeature[i] ==
a_DAvHPP$DetectedFeature[j])
      { vipScore_pValSig_DAvHPP[i,1]<-cbind(a_DAvHPP$DetectedFeature[j])
        vipScore_pValSig_DAvHPP[i,2]<-rbind(a_DAvHPP$VIPScore[j])} }
    for(k in 1:dim(p_value_significant_DAvHPP)[1]){
      if(significantOnes_DAvHPP$DetectedFeature[i] ==
p_value_significant_DAvHPP$DetectedFeature[k]){
        vipScore_pValSig_DAvHPP[i,3]<-rbind(p_value_significant_DAvHPP$p_Value[k])}
      }}

vipScore_pValSig_DAvHPP<- as.data.frame(vipScore_pValSig_DAvHPP)

colnames(vipScore_pValSig_DAvHPP) <- c("DetectedFeature", "VIPScore",
"p_value")

vipScore_pValSig_DAvHPP

vipScore_pValSig_DAvHPP$VIPScore<-
as.numeric(vipScore_pValSig_DAvHPP$VIPScore)

vipScore_pValSig_DAvHPP$p_value<-
as.numeric(vipScore_pValSig_DAvHPP$p_value)

vipScore_pValSig_DAvHPP<-
vipScore_pValSig_DAvHPP[order(vipScore_pValSig_DAvHPP[,2],decreasing = T),]

vipScore_pValSig_DAvHPP

```

Code presented for DAHPP v DA comparisons

Other comparisons:

DA v DAHPP at corresponding time points, DA over time, DAHPP over time

References consulted for conducting pre – processing of LC-MS data:

<https://training.galaxyproject.org/training-material/topics/metabolomics/tutorials/lcms-preprocessing/slides-plain.html>

<https://bioconductor.org/packages/devel/bioc/vignettes/xcms/inst/doc/xcms.html>

- accessed from July – September 2024

<https://master.bioconductor.org/help/course-materials/2019/CSAMA/materials/labs/lab-05-metabolomics/preprocessing-untargeted-metabolomics.html>



Assessing the impact of ultrasound on the rate and extent of early post-mortem glycolysis in bovine *Longissimus thoracis et lumborum*

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ABSTRACT

The rate of pH decline, early post-mortem, has been identified as a key factor that impacts the tenderness of meat, and manipulating this rate of pH decline is highly relevant to ensure consistent high quality meat. Ultrasound is a potential intervention in early post – mortem muscle that may have an impact on the rate of glycolysis through its ability to alter enzyme activity. Following a variety of different ultrasound treatments frequencies (25 and 45 kHz) and durations (15, 30 and 45 min), it was found, when analysed in muscle, that ultrasound treatment duration, specifically the 30 min treatment, and interaction between treatment duration and frequency, had a significant impact on the rate of pH decline, post – treatment. Frequency did not have a significant effect on the rate of pH decline, post – treatment, in muscle. Ultrasound did not have a significant permanent effect on the activity of glycolytic enzymes present in bovine *Longissimus lumborum et thoracis* muscle, where no significant differences were observed on the rate of pH decline and rate of change of reducing sugars, glycogen and lactic acid, when analysed in an *in vitro* glycolytic buffer. It seems that the impact observed in intact muscle is not as a consequence of a permanent change in enzymatic activity, instead indicating an impact on conditions in the muscle which enhanced enzyme activity.

1. Introduction

Tenderness is one of the main quality attributes that influences overall consumer satisfaction of meat, along with other organoleptic properties such as flavour and juiciness. Consistent tenderness is one of the main drivers that influences the consumer's willingness to pay a premium for beef products (Pethick, Hocquette, Scollan, & Dunshea, 2021) and to repeat a purchase. The rate of pH decline in post – mortem bovine muscle, have been found to have a significant effect on final tenderness (Ma, Yu, & Han, 2022; O'Halloran, Troy, & Buckley, 1997; Veiseth-Kent, Hollung, Ofstad, Aass, & Hildrum, 2010). Furthermore, the interaction between pH and temperature post – mortem has been generally described as one of the main factors affecting tenderness (Hannula & Puolanne, 2004). To achieve optimum tenderness and avoid cold or hot shortening, it is recommended that muscle reaches pH 6 between temperatures of 12 and 35 °C (Thompson, 2002). A range of pre – and post – mortem factors influence the rate of pH decline, including muscle type (oxidative or glycolytic), nutritional status, glycogen availability and pre-mortem stress (Chauhan & England, 2018); as well as animal and carcass characteristics such as gender, age, size and fat

cover (Cadavez, Xavier, & Gonzales-Barron, 2019). Post – mortem carcass interventions can also be applied to manipulate the rate of pH decline and temperature drop, such as controlled chilling regimes combined with electrical stimulation (Hwang, Devine, & Hopkins, 2003; Hwang & Thompson, 2001).

Ultrasound, a non – thermal technology, involves the use of sound waves that operate at frequencies >20 kHz and these cannot be detected by the human ear. Ultrasound waves operate longitudinally, and results in the repeated compression and rarefaction of bubbles within the medium being treated with ultrasound. This leads to the eventual collapse of the bubble, resulting in acoustic or transient cavitation (Gallo, Ferrara, & Naviglio, 2018), the local development of high pressures and shear stresses and may also contribute to the formation of free radicals (Alarcon-Rojo, Carrillo-Lopez, Reyes-Villagrana, Huerta-Jiménez, & Garcia-Galicia, 2019; Dang et al., 2022; Kang et al., 2016). Ultrasound can positively alter enzymatic activity, by altering the substrate or modifying the interaction between the enzyme and substrate or changing the conditions in which the enzyme operates (Huang et al., 2017). Thus, it has the potential to modify the kinetics of pH decline in post – mortem muscle, since this is an enzymatic driven process. The use of

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ultrasound on post – mortem bovine muscle has been investigated, with varying results on meat quality traits such as tenderness, colour and water holding capacity reported (Fallavena, Ferreira Marczak, & Mercali, 2020; Gonzalez-Gonzalez et al., 2020; Jayasooriya, Torley, D'Arcy, & Bhandari, 2007). This technology may have potential to be developed as a new way to manipulate pH decline in early post – mortem muscle, however, the impact of ultrasound on this key metabolic pathway has not been investigated in depth to the best of our knowledge.

The aim of the present study was to investigate if ultrasound (15/ 30/ 45 min, 25/ 45 kHz) could be used as an alternative technology to change the rate of pH decline of early post – mortem bovine muscle, using *Longissimus lumborum et thoracis* muscle as a model. The effect of the ultrasound treatments on the activity of glycolytic enzymes was also investigated. This involved preparing crude extracts from the muscles immediately post – treatment and assessing the activity of glycolytic enzymes present in the crude extracts on the basis of changes in the concentration of various constituents of an *in vitro* buffer system (England et al., 2016; England, Matarneh, Scheffler, Wachet, & Gerrard, 2014).

2. Materials and methods

2.1. Chemicals and reagents

ADP, creatine, disodium hydrogen phosphate, NAD⁺, potassium sodium tartrate, sodium acetate (Sigma Aldrich, Ireland), ATP (Cytiva, United Kingdom), carnosine (Apollo Scientific, United Kingdom), 3,5-dinitrosalicylic acid, glycogen, hydrochloric acid, sodium hydroxide (Acrós Organic, Thermofisher, United Kingdom), L-Lactic Acid assay kit (Megazyme, Co.Wicklow, Ireland), magnesium chloride, potassium chloride (VWR Chemicals, Ireland), perchloric acid (Alfa Aesar, United Kingdom), potassium hydroxide (Merck Emsure, Ireland), liquid nitrogen (AirProducts, Ireland). All chemicals were at least of reagent grade.

2.2. Muscle preparation and treatment

Bovine *Longissimus thoracis et lumborum* (LTL) muscles were collected from 4 crossbred animals of similar carcass classification (conformation score R, O=, fat score 2 to 3, based on the EUROP carcass classification system, aged 18–26 months, average side hot weight of 138.1 ± 1.63 kg) from a local commercial slaughterhouse (Kepak, Ireland). The muscle from the right side of the carcass was hot-boned and collected within 90 min of slaughter. The muscles were transported to Teagasc Research Centre, Ashtown, under refrigeration, which took approximately 60 min. On arrival, the pH and temperature of the muscle were measured in three different locations, using a temperature/pH meter, with integrated temperature compensation (Hanna HI 98163 portable pH meter for meat and FC2323 meat pH electrode, Hanna Instruments Ltd., Leighton Buzzard, UK), that was calibrated prior to measurement, using three pH buffers of 4.0, 7.0, and 10.0. The muscles were trimmed of visible fat and each muscle was cut into 13 steaks that were 1.5 cm thick. One steak from each animal was finely chopped and portions (5 g) were placed in cryovials, snap frozen in liquid nitrogen and stored at -80°C for metabolite analysis prior to treatment. Each steak was individually vacuum-packaged using a Henkelman Jumbo 42 (Original Henkelman Vacuum Systems, the Netherlands). Steaks were randomly assigned to a control group (one steak per each one of the treatment combinations) and treatment group, comprised of three ultrasound times (15, 30, 45 min) and two frequencies (25 and 45 kHz), giving a total of 6 different combinations. For each treatment (time and frequency combination), 1 steak was treated with the assigned treatment, while a second steak was used as a corresponding control (i.e. the steak was immersed in water at the same temperature, but with no ultrasound applied).

2.3. Ultrasound treatment

The steaks were treated with ultrasound for 15, 30 and 45 min at a frequency of either 25 or 45 kHz in an Elmasonic xtra ST 500H ultrasonic water bath (Elma Schmidbauer GmbH, Singen, Germany). The vacuum packed steaks were suspended and placed directly into the ultrasonic water bath. Corresponding control samples were placed in another water bath (LTD20G, Grant Instrument, UK). Both water baths were connected, so the water bath temperature in the ultrasound treated and control steaks were the same and maintained below 10°C . To eliminate the effect of time post-mortem on the effect of treatment, 25 kHz treatments were performed first on the muscles from animals 2 and 4, while 45 kHz treatment were performed first on muscles from animals 1 and 3.

On the completion of treatment, the steaks were removed from the vacuum packs and divided in two. One half of the steak was finely chopped and portions (5 g) were placed in cryovials, which were snap frozen in liquid nitrogen and stored in -80°C prior to freeze – milling and use in the *in vitro* buffer model system. The second half of the steak was stored at 4°C , unwrapped, where pH was measured at 0, 30, 90, 240 and 1440 min post-treatment using a calibrated portable pH meter.

2.4. In vitro buffer system

Experiments using the *in vitro* glycolytic buffer were carried out according to the method of England et al. (2016). Muscle samples from the control and treatment groups were powdered in liquid nitrogen using a freezer mill (SPEX SamplePrep 6750 Freezer/Mill) and stored at -80°C . Powdered muscle samples (10 g) were homogenised using an Ultraturrax at 6000 rpm for 30 s, at a ratio of 1:10 (w/v) into a reaction buffer containing 10 mM Na₂HPO₄, 5 mM MgCl₂, 60 mM KCl, 5 mM ATP, 0.5 mM ADP, 0.5 mM NAD⁺, 30 mM glycogen, 25 mM carnosine, 30 mM creatine and 10 mM sodium acetate (pH 7.4). The mixture was immediately transferred into a jacketed reaction vessel, where it was maintained at 25°C using a recirculating water bath (Lauda Dr. R. Wobser GmbH & Co. KG, Germany) with constant stirring by means of a magnetic stirrer. Aliquots for metabolite analysis were removed at 0, 30, 90, 240 and 1440 min. pH of the glycolytic buffer system at each time point was measured using a calibrated pH meter, with built in temperature compensation (Mettler-Toledo AG, Analytical, CH-8603 Schwerzenbach, Switzerland). The pH meter was calibrated prior to measurement, using three pH buffers of 4.0, 7.0, and 10.0.

2.5. Analysis of metabolites in muscle prior to ultrasound treatment and the in vitro glycolytic buffer/muscle homogenate model system during incubation at 25°C for 1440 min

Preparation of supernatants from muscle for analysis of reducing sugars and glycogen: To determine the concentration of reducing sugars and glycogen in muscle samples prior to ultrasound treatment supernatants were prepared according to the method of Hammelman et al. (2003), with modifications. Briefly, 1.5 g of frozen muscle was homogenised in 10 ml of 0.5 M PCA using an Ultraturrax at 13,500 rpm for 30 s. For analysis of reducing sugars, supernatants were prepared by centrifuging the homogenates (2 ml) at 10,000 g for 10 min. For glycogen analysis, the PCA/muscle homogenates (1 ml, without centrifugation) were added to an equal amount of 2.5 M HCl and hydrolysed at 90°C for 2 h in a water bath, centrifuged at 4000 g for 10 min and the supernatant was neutralised with 2 ml of 1.25 M KOH.

Preparation of supernatants from muscle for lactic acid analysis: For lactic acid analysis, supernatants were prepared according to the method outlined in the enzymatic lactic acid kit (L-Lactic Acid (L-Lactate) Assay Kit, Megazyme, Wicklow, Ireland). Briefly, frozen muscle (5 g) was homogenised in 20 ml of 1 M PCA using an Ultraturrax at 13,500 rpm for 5 min (2×2.5 min with a 30 s break in between). Approximately 40 ml of distilled water was added to the homogenate

and the pH was adjusted to pH 10 using 2 M KOH. The mixture was transferred to a 100 ml volumetric flask and brought to volume with distilled water. Aliquots (2 ml) were removed, centrifuged at 10,000 g for 10 min and the supernatants were subsequently analysed using the enzymatic kit.

Preparation of supernatants from the muscle/glycolytic buffer mixtures for reducing sugar, glycogen and lactic acid analysis: Supernatants were prepared according to the method of England et al. (2016, 2014). For reducing sugars and lactic acid analysis, aliquots (2 ml) of the homogenate/ glycolytic buffer mixtures were added to an equal volume of ice – cold 1 M PCA, the mixture was then centrifuged at 4000 g for 10 min and the supernatants neutralised with 1 ml of 2 M KOH prior to analysis. Prior to analysis for lactic acid using the enzymatic kit, the neutralised supernatants were diluted with an equal volume of Milli Q water.

For glycogen analysis, aliquots (1 ml) of the homogenate/ glycolytic buffer mixtures were removed and added to an equal amount of 2.5 M HCl. The mixtures were heated to 90 °C and centrifuged, and the supernatants were neutralised as described above. Prior to analysis for glycogen, the neutralised supernatants were diluted with an equal volume of Milli Q water.

Reducing sugars and glycogen content (as glucose equivalents) of the supernatants prepared from the muscle samples or the muscle/glycolytic buffer mixtures were analysed using the Dinitrosalicylic Acid (DNS) method (Ghose, 1987; Miller, 1959).

2.6. Statistical analysis

The number of biological replicates required for this study was determined following a power analysis, based on preliminary studies, where the variance of pH, glycogen, reducing sugars and lactic acid, and the analytical methods resolutions were evaluated. Based on an alpha value of 0.05 and power of 0.8, a pH difference of 0.2 required a sample size of at least 2 animals; thus, 4 animals were selected to randomize the post-mortem time that each treatment was applied (see section 2.3). Analysis of pH decline in the muscle, post – treatment, and all variables from the *in vitro* buffer was carried out using MIXED procedure of the Statistical Analysis System, version 9.4 (SAS Institute Inc., Cary, NC, USA) with 4 replicates and 3 factors. The impact of frequency (control, 25 kHz, 45 kHz), treatment duration (15, 30, 45 min) and the repeated measure factor, sample time (0, 30, 90, 240 and 1440 min) were tested for a three – way interaction. Animals, the random effect, were treated as blocks. Treatment means and interactions were compared using Tukey's test. In all cases, a *P* – value <0.05 was considered as significant.

3. Results and discussion

3.1. Biochemical status of muscle pre – ultrasound treatment

When pH and temperature was measured at the time of arrival to the research centre, prior to ultrasound treatment, the average pH value of the muscles at the beginning of the experiment (2.5 h post – mortem) was pH 6.08 ± 0.05 and the average temperature was $31.93 \text{ }^{\circ}\text{C} \pm 0.41$. The average glycogen, lactic acid and reducing sugars content were 21.8 ± 0.2 mM, 61.2 ± 0.5 mM and 8.3 ± 0.4 mM, respectively.

The pH values obtained in this study were similar to those reported by Onopiuk, Póltorak, and Wierzbicka (2016) and O'Halloran et al. (1997) for bovine *Longissimus dorsi* between 2 and 4 h post – mortem. The glycogen content recorded in this study was above the range reported by Coombes, Gardner, Pethick, and McGilchrist (2014) and Onopiuk et al. (2016), who reported quantities of between 20.6 mM and 9.3 mM of glycogen in bovine *Longissimus* muscle, measured at 1 h and 4 h post – mortem, respectively. The lactic acid content recorded in the current study was slightly higher than that reported in literature for *Longissimus dorsi* muscle at 2 and 4 h post – mortem, where concentrations of between 24.2 mM and 42.2 mM were found by Onopiuk et al. (2016). The concentration of reducing sugars recorded in the current

study was similar to that reported by Zuo et al. (2022), where 9.4 and 8.3 mM of glucose was found in bovine *Longissimus lumborum* muscle at 45 min and 12 h post-mortem, respectively.

3.2. pH decline in muscle and in vitro buffer system model systems

3.2.1. Muscle

Ultrasound frequency had no significant effect (*P* > 0.05) on the rate of pH decline. Following the ultrasound treatment of bovine LTL, a significant (*P* < 0.05) interaction was found between ultrasound frequency (0/25/45 kHz) and treatment duration (15/30/45 min), when examined within a three – way analysis of variance (Table 1). Treatment duration had a significant (*P* < 0.05) effect on the rate of pH decline, where the 30 min treatments (with 25 and 45 kHz) appear to significantly accelerate the rate of pH decline, when compared to the 30 min controls (Table 1). The pH_u, measured at 24 h post-mortem, was not affected by any of the treatments applied. The impact of ultrasound on the rate of pH decline in pre – rigor muscle has not been investigated in detail, to the best of our knowledge. Furthermore, it is difficult to compare the results of our studies with others due to variations in the muscles and ultrasound treatment parameters used.

Sikes, Mawson, Stark, and Warner (2014), using a stronger, more intense ultrasound treatment than used in this study (2 MHz, 60% input power setting, 48 kPa acoustic pressure amplitude, 5 min), reported that ultrasound had no significant impact on the pH immediately post – treatment of bovine *Sternomandibularis* (treated 3 h post – mortem), however they did not track pH decline over time post – treatment. When Got et al. (1999) applied high power ultrasound (2.6 MHz, 10 W/cm², for 2 × 15 s intervals) to pre – rigor bovine *Semimembranosus*, an immediate increase in pH, by 0.2 units, was observed. The pH of the treated muscle remained higher than the control for a further 2 to 4 h post-treatment before the differences levelled off. Similar to our findings, Got et al. (1999) found that ultrasound treatment had no significant impact on the ultimate pH of muscle when comparing the ultrasound treated and control samples.

3.2.2. In vitro buffer systems

The *in vitro* glycolytic buffer system used in this study was originally developed by Scopes (1973) and more recently has been re-established by Gerrard's research group to establish some key findings on the roles of phosphofructokinase (England et al., 2014), mitochondria (Matarneh et al., 2018; Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021; Scheffler, Matarneh, England, & Gerrard, 2015), AMP deaminase (England, Matarneh, Scheffler, Wachet, & Gerrard, 2015) and glycogen (England et al., 2016) in determining the rate of pH decline and the ultimate pH of muscle. The *in vitro* glycolytic buffer system was used in the current study to establish whether or not the ultrasound treatment of intact muscle had a permanent impact on the activity of glycolytic enzymes.

When comparing *in vitro* buffer model systems containing control or ultrasound treated muscle as a source of glycolytic enzymes, ultrasound frequency (*P* > 0.05) and treatment duration (*P* > 0.05) had no significant effect on the rate of pH decline. pH values of the *in vitro* buffer systems decreased from pH 7.4, prior to the addition of muscle, to an average of pH 5.64 ± 0.01 over the 24 h reaction period (Fig. 1). No significant interaction was detected between treatment frequency and duration (*P* > 0.05). No significant differences were observed in the pH at 1440 min (pH_u) between control and treated samples or between different ultrasound treatments.

In this study, the rate of pH decline observed in the control buffer systems was similar to that of England et al. (2015), who used porcine *Longissimus* muscle as a source of glycolytic enzymes in the *in vitro* buffer. The final pH values obtained using the *in vitro* glycolytic buffer in this study were within the range of the values reported by Beline et al. (2021) and Chauhan et al. (2019), which were between pH 5.5 and 5.8, respectively.

Table 1
Average pH values over the course of 1440 min post – ultrasound treatment when measured in the muscle. N = 4. Data is mean ± SE. The *p* – values from the F – Test, following a three way analysis of variance (ANOVA) are included.

	25 kHz 15 min T	25 kHz 15 min C	25 kHz 30 min T	25 kHz 30 min C	25 kHz 45 min T	25 kHz 45 min C	45 kHz 15 min T	45 kHz 15 min C	45 kHz 30 min T	45 kHz 30 min C	45 kHz 45 min T	45 kHz 45 min C
0	6.23 ± 0.06 ^a	6.04 ± 0.06 ^a	6.01 ± 0.07 ^a	6.19 ± 0.07 ^a	6.03 ± 0.07 ^a	6.13 ± 0.07 ^a	6.12 ± 0.08 ^a	6.24 ± 0.08 ^a	6.00 ± 0.07 ^a	6.10 ± 0.07 ^a	6.16 ± 0.07 ^a	6.13 ± 0.07 ^a
30	6.14 ± 0.06 ^{ab}	5.96 ± 0.06 ^a	5.86 ± 0.07 ^a	6.02 ± 0.07 ^a	5.96 ± 0.07 ^a	6.03 ± 0.07 ^a	6.01 ± 0.08 ^a	5.95 ± 0.08 ^{ab}	5.86 ± 0.07 ^{ab}	6.04 ± 0.07 ^a	6.04 ± 0.07 ^a	6.03 ± 0.07 ^a
90	6.00 ± 0.06 ^{abc}	5.90 ± 0.06 ^a	5.80 ± 0.07 ^{ab}	5.97 ± 0.07 ^a	5.92 ± 0.07 ^a	5.96 ± 0.07 ^a	5.97 ± 0.08 ^{ab}	5.87 ± 0.08 ^{ab}	5.80 ± 0.07 ^{ab}	5.95 ± 0.07 ^a	5.98 ± 0.07 ^a	5.95 ± 0.07 ^a
240	5.87 ± 0.06 ^{bc}	5.82 ± 0.06 ^{ab}	5.72 ± 0.07 ^{ab}	5.84 ± 0.07 ^{ab}	5.81 ± 0.07 ^{ab}	5.83 ± 0.07 ^{ab}	5.91 ± 0.08 ^{ab}	5.81 ± 0.08 ^{ab}	5.77 ± 0.07 ^{ab}	5.86 ± 0.07 ^{ab}	5.87 ± 0.07 ^{ab}	5.89 ± 0.07 ^{ab}
1440	5.52 ± 0.06 ^d	5.52 ± 0.06 ^b	5.53 ± 0.07 ^b	5.54 ± 0.07 ^b	5.53 ± 0.07 ^b	5.53 ± 0.07 ^b	5.54 ± 0.08 ^b	5.55 ± 0.08 ^b	5.53 ± 0.07 ^b	5.53 ± 0.07 ^b	5.54 ± 0.07 ^b	5.53 ± 0.07 ^b
P – values												
Frequency	0.5333											
Duration	0.0174											
Sampling Time	< 0.0001											
Frequency*Sampling Time	0.9993											
Duration*Sampling Time	0.9396											
Frequency*Duration	0.0004*											
Frequency*Duration*Sampling Time	0.9462											

C is control, T is treated. For each treatment, a corresponding control (C) was immersed in water for the specified time, but without ultrasound treatment.
* Pairwise comparison between individual groups showed no significant difference, thus grouping is not possible.

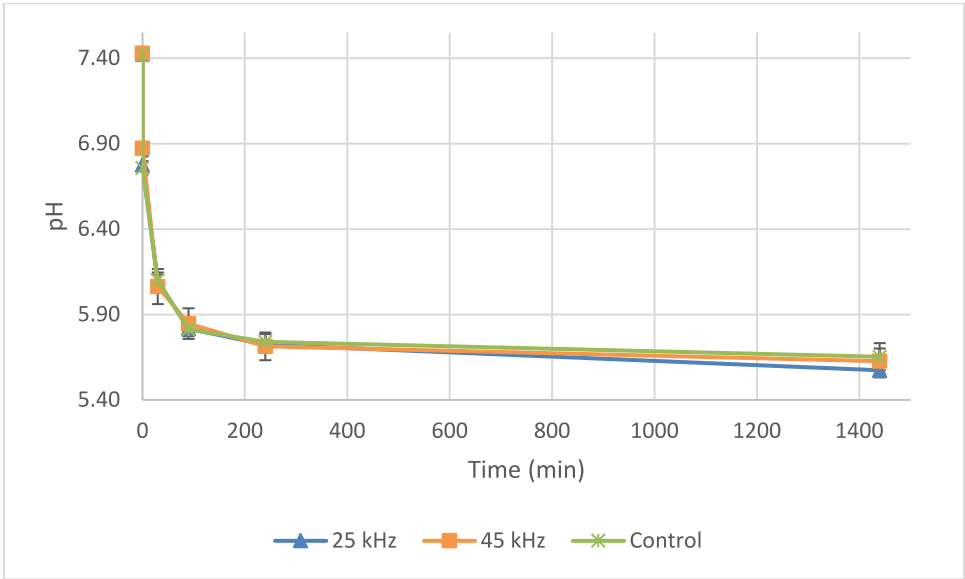


Fig. 1. The average decline in pH over the course of 1440 min in an *in vitro* glycolytic buffer. N = 4. Data is mean ± SE.

3.3. Metabolites in the *in vitro* system

There were no significant differences (*P* > 0.05) in the rate of glycogen degradation between the buffers model systems containing ultrasound treated and control muscle at any time point, and no significant differences (*P* > 0.05) were observed between the different ultrasound treatments. Glycogen was included in the *in vitro* reaction buffer at 30 mM and its concentration decreased with time for the control and all treatments as a result of the activity of the enzymes present in the muscles added to the buffers, with the majority of glycogen degradation taking place within the first 240 min of the reaction (Fig. 2). The rate of glycogen degradation decreased as the pH of the buffer approached pH 6. These results are consistent with those reported by England et al. (2016, 2015), Scheffler et al. (2015), Matarneh et al. (2017) and Chauhan et al. (2019), when using a similar glycolytic buffer system to study post – mortem glycolysis in porcine, poultry and bovine muscles.

It was found that there were no significant differences (*P* > 0.05) in the concentration of reducing sugars at the different sampling times when comparing buffer model systems containing treated (25/45 kHz, 15/30/45 min) and control muscle. An increase in reducing sugars in the first 30 min was observed, followed by a plateau in concentration between 30 and 90 min after the reaction was started, when the pH of the buffer approached pH 6. After this time point, the concentration of reducing sugars increased again from 240 to 1440 min (Fig. 3). Glycogen is degraded by glycogen phosphorylase to release glucose – 1 – phosphate and debranching enzymes to release free glucose. Glucose – 1 – phosphate and glucose are then converted to glucose – 6 – phosphate by phosphoglucosmutase and hexokinase, respectively, to be further utilized along the glycolytic pathway (Scheffler et al., 2015; Scheffler & Gerrard, 2007). Free glucose may also be allowed to accumulate in the muscle post – mortem (Matarneh et al., 2018). When Beline et al. (2021) investigated the progression of glucose in a glycolytic buffer similar to that used in the current study, containing bovine *Longissimus thoracis*

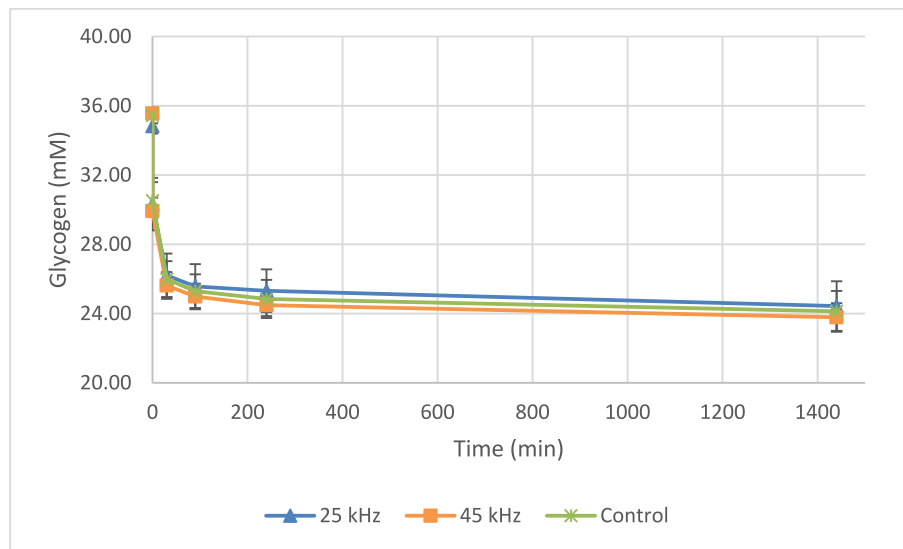


Fig. 2. The average degradation of glycogen (mM) over the course of 1440 min in an *in vitro* glycolytic buffer. N = 4. Data is mean $\pm$ SE.

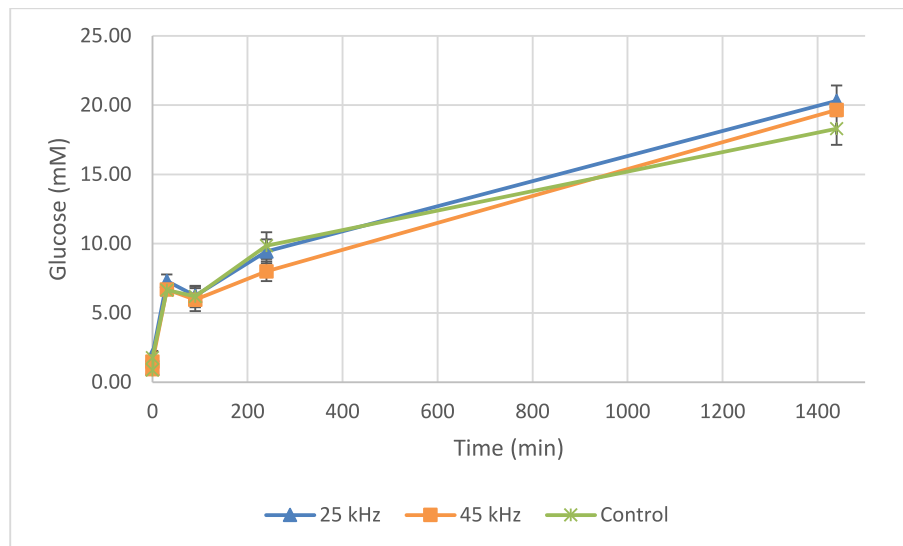


Fig. 3. The average content of reducing sugars over the course of 1440 min in an *in vitro* glycolytic buffer. N = 4. Data is mean $\pm$ SE.

muscle, glucose content plateaued between 30 and 240 min of the reaction, before production resumed again from 240 min to 1440 min.

The plateau in reducing sugars content between 30 and 90 min post-mortem observed in this study could be as a result of the efficient utilisation of reducing sugars by enzymes, such as glucose by hexokinase for glucose-6-phosphate production, at a rate equal to glucose production, while the subsequent increase in reducing sugar content between 90 and 1440 min could be as a result of the inhibition of enzymes such as hexokinase through negative feedback mechanisms, where there is sufficient glucose-6-phosphate in the system, making glucose unavailable for glycolysis and thus, allowing it to accumulate (Daly, Young, Graafhuis, Moorhead, & Easton, 1999; Ferguson & Gerrard, 2014; Young, Humphrey, & Wild, 1988).

As the pH of the *in vitro* buffer decreased below pH 6 (90 min), there appears to be a change in the production/consumption balance of reducing sugars, as below this pH, the concentration of reducing sugars increases in the buffer system and this was observed for all treatments and controls (Fig. S1, Supplementary material 1). The accumulation of reducing sugars could also be related to the pH inactivation of phosphofructokinase, a key rate limiting enzyme, which decreases in activity

as the pH of the reaction buffer and muscle reaches below pH 6 (England et al., 2014). Phosphofructokinase activity is also reduced as lactic acid accumulates (Leite et al., 2011), which happens simultaneously. The inactivation of phosphofructokinase, therefore, results in the subsequent accumulation of glucose, glucose-6-phosphate and fructose-6-phosphate (Matarneh et al., 2018).

As expected, the lactic acid content increased over time of the reaction in the buffer model systems containing treated (25/45 kHz, 15/30/45 min) and control muscle, with the majority of the production taking place within the first 90 min of the reaction (Fig. 4). There were no significant differences ($P > 0.05$) in lactic acid content when comparing treated and control samples at any time point and no significant differences ($P > 0.05$) were observed between the different ultrasound treatments. The profile of lactic acid production in the buffer model systems is in agreement with the results of England et al. (2016, 2015), Scheffler et al. (2015) and Matarneh et al. (2017) when using a glycolytic buffer system similar to what was used in this study. Following 90 min of the reaction, where the pH of the buffer is below pH 6, there is a reduction in the rate of lactic acid production (Fig. 4). The potential reason for this, as identified by England et al. (2014), is the

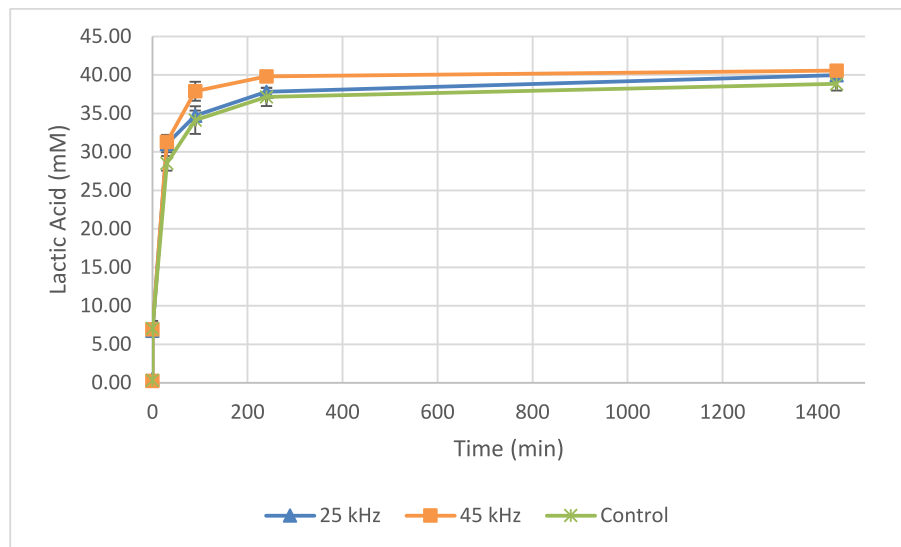


Fig. 4. The average production of lactic acid (mM) over the course of 1440 min in an *in vitro* glycolytic buffer. N = 4. Data is mean $\pm$ SE.

inactivation of phosphofructokinase, which occurs when the pH of the reaction buffer decreases below pH 6. The inactivation of phosphofructokinase was also implicated by Matarneh et al. (2018) as the reason for the accumulation of glucose-6-phosphate during the later stages of incubation using a similar *in vitro* glycolytic buffer system. If phosphofructokinase is inactivated, there will be a decrease in the amount of substrate available to the reactions past this point of the glycolytic reaction, therefore, reducing the amount of substrate available for the production of lactic acid, halting its formation.

Ultrasound can alter enzymatic activity by either altering the substrate or the interaction between the enzyme and substrate (Huang et al., 2017). Low intensity treatments can be used to improve enzyme activity (that was one of the reasons to select low intensity treatments for this trial), whereas prolonged exposure to high intensity ultrasound treatment can result in the loss of enzyme activity (Huang et al., 2017; O'Donnell, Tiwari, Bourke, & Cullen, 2010; Wang et al., 2018). Our results show the ultrasound treatments used in this study did not permanently affect the activity of the enzymes involved in glycolysis. The impact of ultrasound on the enzymes involved in post-mortem glycolysis has not been investigated, to the best of our knowledge, however, research has been carried out on the effect of ultrasound on the enzymes involved in post-mortem proteolysis. Chen et al. (2015) observed that ultrasound treatment (40 kHz for 30 and 60 min) of chicken *Pectoralis superficialis* significantly increased the activity of calpain and caspase-3, when measured *in vitro* using fluorometric assays. A similar increase in caspase-3 activity following the ultrasound treatment (40 kHz, 12 W/cm², 40 min at 4 °C) of 24 h post-mortem bovine *Longissimus thoracis et lumborum* was observed by Dang et al. (2022), however in both of these studies the increase in enzyme activity as a result of ultrasound treatment was attributed to changes in the conditions experienced by the enzymes (e.g. cavitation-induced structural shocks to the ultrastructure resulting in membrane and mitochondrial damage) rather than a direct structural effect of ultrasound on the enzymes. Thus, the ability of ultrasound to increase the rate of pH decline in muscle post-mortem, as observed in this study, is likely due to ultrasound-induced changes within the muscle itself. Interestingly, Dang et al. (2022) demonstrated increased cytosolic calcium concentration in ultrasound treated beef steaks. It is well known that increases in calcium concentration play a key role in the ability of electrical stimulation to accelerate post-mortem glycolysis. Hence, it may also play a role in the mechanism whereby ultrasound increases the rate of post-mortem glycolysis.

4. Conclusion

The results show that the ultrasound treatments (specifically the 30 min treatments) used in this study do not permanently affect the activity of the enzymes involved in glycolysis, when analysed *in vitro*, but can accelerate the rate of pH decline in muscle post-mortem. This effect could be modulated by ultrasound induced shocks which cause changes to muscle ultrastructure and membranes which in turn change the conditions in which the glycolytic enzymes are operating. Further investigation is warranted to explore the mechanism involved.

CRediT authorship contribution statement

Mary Ann Kent: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Anne Maria Mullen:** Writing – review & editing, Funding acquisition, Conceptualization. **Eileen O'Neill:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Carlos Álvarez:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

All the authors listed in this manuscript declare no conflict of interest.

Data availability

Data will be made available on request.

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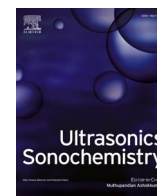
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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meatsci.2024.109531>.

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The impact of ultrasound treatment on glycolytic enzymes when applied to crude extracts from early post-mortem bovine muscle

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ABSTRACT

The rate of pH decline post – mortem and its interaction with temperature influences the final tenderness of meat, and therefore, the manipulation of the rate of pH decline is a strategy of interest in order to obtain consistent high quality meat. Ultrasound is a potential early post - mortem carcass intervention, which may alter the rate of glycolysis based on its ability to alter enzyme activity. In this study, homogenates (prepared from early post-mortem *Longissimus thoracis et lumborum* muscle) were subjected to different ultrasound intensities (0 %/60 %/100 % amp) and treatment durations (15/ 30 min). The effect of these treatments on the inherent activity of the glycolytic enzymes was investigated using an *in vitro* glycolytic buffer model system. It was found that ultrasound treatment intensity and duration had a significant interactive effect on the rate of pH decline, and on reducing sugars and lactic acid concentrations, specifically following the 100 % amp ultrasound for 30 min treatment and between 30 and 240 min incubation. No significant differences in pH or metabolites content were observed between treatments after 1440 min of incubation. No effect of ultrasound intensity or treatment duration was observed on the degradation of glycogen. Under the reported conditions of this trial, it can be concluded that the application of ultrasound has limited potential to have an impact on the glycolytic pathways in bovine muscle.

1. Introduction

Tenderness, along with other organoleptic qualities, such as colour, flavour and juiciness, plays an important role in the overall eating quality of meat [1]. The rate of early post-mortem glycolysis [2,3], as well as the interaction between pH and temperature decline in muscle [4,5], has been found to play an important role in determining meat tenderness. For optimum tenderness, it has been recommended by the Meat Standards Australia (MSA) that the muscle is between the temperatures of 15 and 35 °C when pH 6 is reached, since muscle is susceptible to hot- and cold-shortening outside of this pH – temperature window [6]. Factors that influence the rate of pH decline in muscle include the muscle type (oxidative or glycolytic), the nutritional and stress status of the animal pre-slaughter [7], as well as the age, gender and size of the animal [8]. A number of post-mortem carcass interventions have been implemented in the meat industry to manipulate the rate of pH and temperature decline, such as chilling and electrical stimulation [9,10], aimed at avoiding the detrimental effects of heat and cold shortening and optimising tenderness.

Ultrasound (US) is a non-thermal technology that involves sound waves operating at frequencies of 20 kHz or greater. Ultrasound treatment results in continuous compression and rarefaction of bubbles within the medium being treated, with the eventual collapse of the bubbles causing acoustic or transient cavitation [11] which can affect the macromolecules of enzymes and their substrates [12]. Ultrasound has been investigated for its ability to alter enzymatic activity in food systems. It can affect enzymes directly, the substrates or the interaction between the enzyme and substrate [13]. Studies have been carried out to investigate the impact of ultrasound treatment on post-mortem [14–16], and pre-rigor bovine muscle [17–19], primarily focused on the impact of ultrasound on the quality traits of the muscle, such as tenderness, colour and water-holding capacity, with varying results.

The impact of ultrasound treatment on the rate of glycolysis in pre-rigor muscle has not been studied in depth, to the best of our knowledge. Our group carried out a recent study (not yet published) investigating the impact of ultrasound treatments (25/45 kHz for 15/30/45 min) of pre-rigor bovine muscle on the inherent activities of glycolytic enzymes using an *in vitro* glycolytic buffer system. This glycolytic buffer

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system had previously been used by other researchers to study post-mortem glycolysis in porcine, chicken and bovine muscle [20–25]. In a previous trial (not published yet), we found that the ultrasound treatment of intact bovine *Longissimus thoracis et lumborum* did not affect the inherent activity of glycolytic enzymes, when examined in an *in vitro* glycolytic buffer, post-treatment. Lyng, Allen and McKenna [18,26] reported that ultrasound (20 kHz for periods of 15 sec) had no significant impact on the proteolysis of pre- and post-rigor bovine and ovine muscle, and hypothesised that the structure of the muscle restricted the vibrations resulting from the ultrasound treatments, reducing the intensity of the ultrasound waves travelling through the muscle. Therefore, we have hypothesised that by removing the physical protection that the whole muscle matrix may exert, US may have the potential of affecting the kinetics of glycolytic enzymes. Thus, the aim of the current study is to investigate the effect of ultrasound treatment (20 kHz, 60 % amp (11.4 W/cm²) and 100 % amp (19 W/cm²) amplitude for 15 or 30 min) of muscle homogenates (muscle:water ratio of 1:5) on glycolysis, monitored *in vitro* using a glycolytic buffer system.

2. Materials and methods

2.1. Chemicals and reagents

ADP, creatine, disodium hydrogen phosphate, NAD⁺, potassium sodium tartrate, sodium acetate (Sigma Aldrich, Ireland), ATP (Cytiva), carnosine (Apollo Scientific, United Kingdom), 3,5-dinitrosalicylic acid, glycogen, hydrochloric acid, sodium hydroxide (Acros Organic-ThermoFisher, UK), L-Lactic Acid assay kit (Megazyme, Ireland), magnesium chloride, potassium chloride (VWR Chemicals, Ireland), perchloric acid (Alfa Aesar, United Kingdom), potassium hydroxide (Merck Emsure, Ireland), liquid nitrogen (AirProducts, Ireland). All chemicals were at least of reagent grade.

2.2. Animal and sample preparation

Bovine *Longissimus thoracis et lumborum* muscles were collected from 4 crossbred steers of similar carcass classification (conformation score O - to R +, aged 22 – 29 months, average side hot weight of 183.15 ± 28.11 kg) from a local commercial slaughterhouse (Kepak, Ireland). The muscle from the left hand side was hot-boned from the carcass and collected within 90 min of slaughter. Under refrigeration, the muscles were transported to Teagasc Research Centre, Ashtown, which took approximately 60 min. On arrival, the pH and temperature of the muscle were measured at three different locations, using a temperature/pH meter, with integrated temperature compensation (Hanna HI 98163 portable pH meter for meat and FC2323 meat pH electrode, Hanna Instruments Ltd., Leighton Buzzard, UK). Prior to measurement, the pH meter was calibrated using three pH buffers of 4.0, 7.0, and 10.0. Visible fat was trimmed from the muscles and each muscle was cut into 6 steaks of equal thickness (1.5 cm). The steaks were randomly assigned to a control group and treatment group, comprised of two ultrasound treatment durations (15, 30 min) and two ultrasound powers (100 % or 60 % amp (19 W/cm² and 11.4 W/cm², respectively)), giving a total of 4 different combinations. A control sample was assigned to each treatment duration (15, 30 min). The steaks were finely chopped and portions (5 g), then were placed in cryovials, which were then snap frozen in liquid nitrogen. These portions of frozen muscle were pooled and powdered using liquid nitrogen and a freezer mill (SPEX SamplePrep 6750 Freezer/Mill). The powdered samples were stored at –80 °C until the time of treatment. An additional steak from each animal was finely chopped and portions (5 g) placed in cryovials, snap frozen in liquid nitrogen and stored at –80 °C for determination of initial metabolite concentrations in muscles.

2.3. Preparation of supernatants for determination of initial metabolite concentrations in muscles

Samples for the analysis of glucose and glycogen were prepared according to Hammelman *et al.* [27], with modifications. Briefly, for glucose analysis, 1.5 g of frozen muscle was homogenised in 10 ml of 0.5 M perchloric acid (PCA) using an Ultraturrax (13,500 rpm for 30 sec). Aliquots (2 ml) of homogenate were centrifuged, using Sigma 1–15 Benchtop Centrifuge (SIGMA Laborzentrifugen GmbH, Germany) at 10,000 g for 10 min. For glycogen analysis, frozen muscle (1.5 g) was homogenised in 10 ml of 0.5 M PCA using an Ultraturrax (13,500 rpm for 30 sec). Homogenate (1 ml) was added to an equal amount of 2.5 M HCl, hydrolysed at 90 °C for 2 hr, centrifuged for 10 min at 4,000 g and the supernatant was neutralised with 1.25 M KOH (2 ml). Sample preparation for lactic acid analysis was carried out according to the method outlined in the L-Lactic Acid Kit Assay kit (Megazyme, Ireland), where 5 g of frozen muscle was homogenised in 20 ml of 1 M PCA using an Ultraturrax, for 5 min (2 x 2.5 min with a 30 sec break in between) at 13,500 rpm. Approximately 40 ml of distilled water was added to the homogenate. The pH of the homogenate was adjusted to pH 10 using 2 M KOH, before being made to volume in a 100 ml volumetric flask. Aliquots (2 ml) of the homogenate were removed, centrifuged for 10 min at 10,000 g and subsequently analysed.

2.4. Ultrasound treatment

For ultrasound treatment, 20 g of powdered muscle (Section 2.2) was suspended in 100 ml of water using an Ultraturrax (6,000 rpm for 30 sec). The homogenates were sonicated using a 20 kHz ultrasound probe (UIP500hdT, Hielscher ultrasound technology, Germany), where the probe, with a diameter of 1.8 cm, was submerged into the homogenate at a depth of 1.5 cm. The homogenates were treated with ultrasound for 15 and 30 min at amplitude levels of 60 % or 100 % amp (11.4 W/cm² and 19 W/cm², respectively), in a jacketed flask, where the temperature of the treated homogenate was maintained at 32 °C ± 2.5 °C with a circulating water bath (LTD20G, Grant Instrument, UK). The mixtures were stirred constantly throughout the treatments. Controls were prepared where 20 g of powdered muscle was suspended in 100 ml of water using an Ultraturrax (6,000 rpm for 30 sec) and the homogenate was stirred constantly for 15 and 30 min in a jacketed flask, without the application of US. The temperature of the control was maintained at 32 °C with a circulating water bath (Lauda ECO RE415S (Lauda Dr. R. Wobser GmbH & Co. KG, Germany)).

2.5. *In vitro* glycolytic buffer system model system

Experiments using the *in vitro* glycolytic buffer were carried out based on the method of England *et al.* [22]. Initially, the buffer was made up to contain 20 mM Na₂HPO₄, 10 mM MgCl₂, 120 mM KCl, 10 mM ATP, 1 mM ADP, 1 mM NAD⁺, 60 mM glycogen, 50 mM carnosine, 60 mM creatine and 20 mM sodium acetate (pH 7.4) (this is a double strength buffer which is subsequently diluted (1:1) on addition of the homogenate). On completion of the individual ultrasound treatments, half of the control or treated homogenate (50 ml) was added to 50 ml of the buffer and immediately transferred into a jacketed reaction buffer vessel. This resulted in an overall homogenate containing 10 mM Na₂HPO₄, 5 mM MgCl₂, 60 mM KCl, 5 mM ATP, 0.5 mM ADP, 0.5 mM NAD⁺, 30 mM glycogen, 25 mM carnosine, 30 mM creatine and 10 mM sodium acetate, with a 1:10 muscle (w/v) inclusion; resulting in the final concentrations as described by England *et al.* [22]. The temperature of the homogenate was maintained at 25 °C for the duration of the experiment, using a recirculating water bath (Lauda ECO RE415S (Lauda Dr. R. Wobser GmbH & Co. KG, Germany)), with constant stirring using a magnetic stirrer. Aliquots were removed at 0, 30, 90, 240 and 1440 min for subsequent metabolite analysis. The pH of the glycolytic buffer system was measured at each time point using a calibrated pH meter, with built in

temperature compensation (Mettler-Toledo AG, Analytical, CH-8603 Schwerzenbach, Switzerland).

2.6. Preparation of supernatants for metabolite analysis of the *in vitro* glycolytic buffer model system

For glycogen analysis, aliquots (1 ml) of homogenate or glycolytic buffer mixture were added to an equal amount of 2.5 M HCl, where the mixture was heated for 2 hr at 90 °C in a water bath, centrifuged for 10 min at 4,000 g and neutralised with 1.25 M KOH (2 ml). The neutralised homogenate was subsequently diluted with an equal amount of Milli-Q water prior to analysis. For glucose (and other reducing sugars) and lactic acid analysis, aliquots (2 ml) of homogenate were removed and added to an equal amount of ice-cold 1 M PCA, before being centrifuged at 4,000 g for 10 min and the supernatants were neutralised using 1 ml of 2 M KOH prior to analysis. For lactic acid quantification, a 50/50 dilution of the neutralised PCA extract was prepared with Milli-Q water to be within the detection limits of the kit.

2.7. Determination of concentration of metabolites in supernatants

The concentration of glucose (and other reducing sugars such as fructose and its phosphorylated conjugates) and glycogen (as glucose equivalents) in the supernatants was measured spectrophotometrically using the DNS method [28,29]. Lactic acid concentration in the supernatants was determined using an enzymatic kit (L-Lactic Acid (L-Lactate) Assay Kit, Megazyme, Wicklow, Ireland).

2.8. Statistical analysis

Data in this paper is presented as average $\pm$ SEM. To determine the number of replicates required for this study, a power analysis was performed, based on preliminary studies, where the resolutions of the analytical methods and variance of glucose (and other reducing sugars), glycogen and lactic acid were evaluated. To obtain a pH difference of 0.2, based on a power of 0.8 and alpha value of 0.05, a sample size of 2 was required. From this, a sample size of 4 was selected. Analysis of all variables from the *in vitro* buffer was carried out using MIXED procedure of the SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA) with 4 replicates and 3 factors: intensity (control, 100 % or 0 %), treatment duration (15 or 30 min) and the repeated measure factor, sample time (0, 30, 90, 240 and 1440 min). Animals were treated as a random effect. Treatment means and interactions were compared using Tukey's test. In all cases, a P -value < 0.05 was considered as significant.

3. Results

3.1. Biochemical status of muscle prior to ultrasound treatment

The average pH value of the muscles, at 2.5 hr post-mortem, was pH 6.42 (± 0.07) and the average temperature was 29.83 °C (± 0.60). The pH values obtained in this study were similar to those found by Onopiuk *et al.* [30] and Byrne *et al.* [31] in bovine *Longissimus dorsi*, at a similar time post-mortem.

The glycogen concentration was 1.28 ± 0.13 g/100 g, which is within the range of that reported by Coombes *et al.* [32] and Onopiuk *et al.* [30], who reported quantities of between 1.36 g/100 g and 0.62 g/100 g glycogen, measured at 1 and 4 h post-mortem, respectively, in bovine *Longissimus* muscle. The lactic acid content of muscle used in this study, 0.28 ± 0.03 g/100 g, was within the range reported in literature for bovine *Longissimus* and *Semitendinosus* muscle between 2 and 4 h post-mortem, where quantities of between 0.22 and 0.38 g/100 g were found [30,33]. The content of reducing sugars (mainly glucose at this stage) in the muscle used in this study was 0.068 ± 0.008 g/100 g, which was within the range found in the literature, where levels of 0.036 g/100 g [34] to 0.169 g/100 g [35] were reported in bovine

Longissimus lumborum within the first 3 h post-mortem. The results show that muscles employed for this study were a faithful representation of an average bovine *Longissimus* muscle at 2 or 3 h post-mortem.

3.2. pH decline

When muscle homogenates were added to the glycolytic buffer system (as sources of glycolytic enzymes) the pH declined over the 1440 min duration of incubation at 25 °C, with the pH decreasing, for controls and treatments, from an initial pH of 7.42 ± 0.01 , prior to the addition of muscle, to an average of pH 5.50 ± 0.04 , except for buffers containing muscle treated with 100 % amp for 30 min, where the average pH after 1440 min was pH 5.78 ± 0.15 (Fig. 1). While the average pH value of the 100 % amp, 30 min treatment containing buffer system at 1440 min was numerically higher than the combined average pH of the control and other treatment containing buffers, no significant differences were observed between treatments at 1440 min. The majority of the decline in pH took place within the first 240 min. The rate of pH decline was significantly affected by ultrasound treatment duration ($P < 0.05$) and intensity ($P < 0.05$) and there was a significant interaction between these two parameters ($P < 0.05$); where the treatment carried out at 100 % amp for 30 min resulted in a slower rate of pH decline when compared to the controls and other treatments. The slower rate of pH decline was observed for the first 240 min in particular, with the pH of the buffer system containing the 100 %, 30 min treatment being significantly higher ($P < 0.05$) when compared to control and other treatment containing buffers between 30 and 240 min.

The rate of pH decline in the glycolytic buffer system, illustrated in Fig. 1, for controls and all treatments except for that containing muscle homogenates treated at 100 % amp ultrasound for 30 min, was similar to that reported by Beline *et al.* [25], England *et al.* [20–22] and Matarneh *et al.* [23], who used bovine, porcine and chicken muscle, respectively, as a source of glycolytic enzymes in a similar glycolytic buffer model system.

3.3. Concentration of metabolites in the *in vitro* glycolytic buffer model system

An *in vitro* glycolytic buffer system was employed in this study to assess the potential impact of the various ultrasound treatments on the inherent activities of glycolytic enzymes present in the muscle homogenates, and to monitor changes in glycogen, glucose (and other reducing sugars) and lactic acid content over a 24 h period of incubation. Overall, it was found that treatment time duration and intensity had a significant effect ($P < 0.05$) on the concentration of these metabolites, specifically following the 30 min, 100 % amp (19 W/cm²) treatment.

3.3.1. Glycogen

Glycogen was included in the *in vitro* glycolytic buffer at a concentration of 30 mM. During the incubation period, the concentration of glycogen decreased, as a result of the activity of glycolytic enzymes provided by the muscle homogenates. The majority of the glycogen degradation took place within the first 240 min, and this degradation pattern was in agreement with the findings of other studies using a similar buffer system [21,22,36,37] (Fig. 2). No significant effect ($P > 0.05$) on glycogen degradation was observed for ultrasound intensity (Control, 60 % amp, 100 % amp) or treatment duration (15 min, 30 min) at any given sampling point (Fig. 2). These results indicate that ultrasound did not have an impact on the utilisation of glycogen, neither promoting its degradation nor impeding its depolymerisation.

3.3.2. Glucose and other reducing sugars

In general, there was a rapid increase in the content of glucose and other reducing sugars within the first 240 min of the reaction buffer. The content of these monosaccharides continued to increase in the *in vitro* glycolytic buffer between 240 and 1440 min, but at a slower pace (Fig. 3). Ultrasound intensity (control, 60 % amp, 100 % amp) and

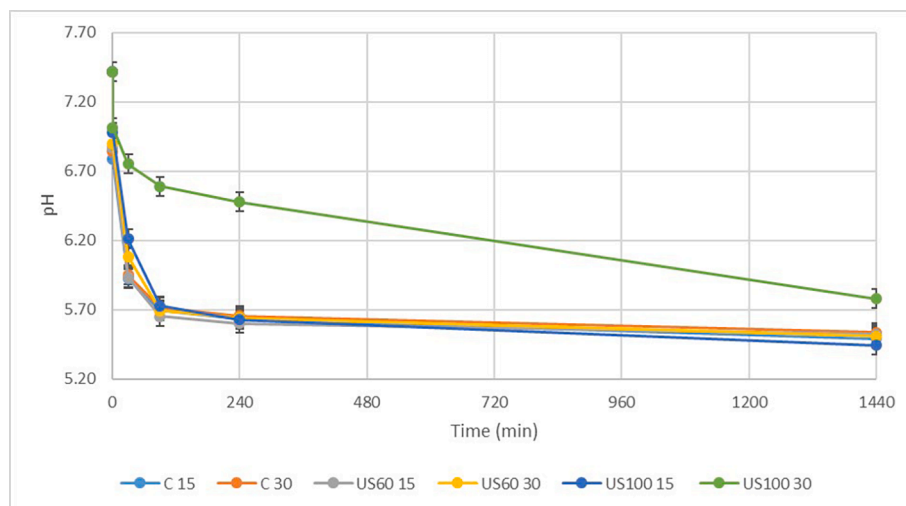


Fig. 1. pH decline of the *in vitro* glycolytic reaction buffer over 1440 min (24 hr) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60 % amp for 15 min (US60 15), 60 % amp for 30 min (US60 30), 100 % amp for 15 min (US100 15), 100 % amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SE.

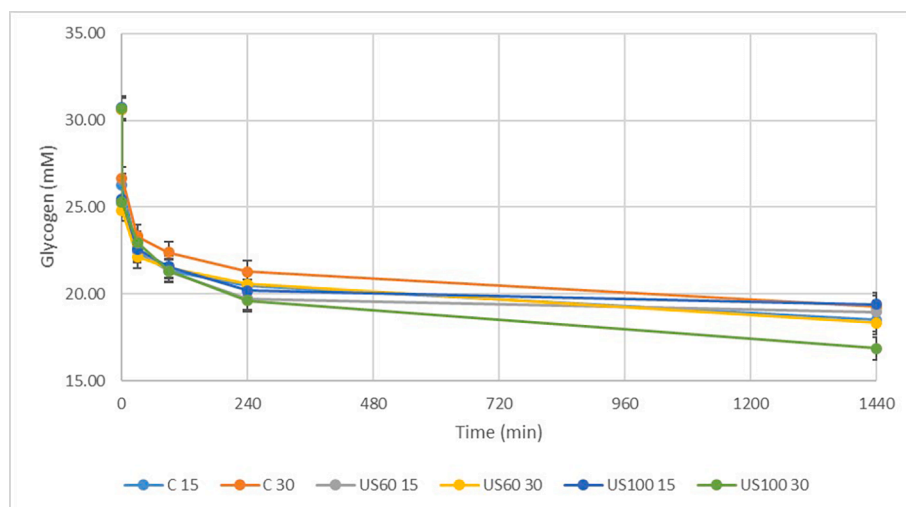


Fig. 2. Glycogen concentration (mM) of the *in vitro* glycolytic reaction buffer over 1440 min (24 hr) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60 % amp for 15 min (US60 15), 60 % amp for 30 min (US60 30), 100 % amp for 15 min (US100 15), 100 % amp for 30 min (US100 30). N = 4. Data is mean $\pm$ SE.

treatment duration (15 min, 30 min) had a significant impact on the levels of glucose (and other reducing sugars) ($P < 0.05$), while a significant interaction ($P < 0.05$) was also observed between ultrasound intensity and treatment duration. The 100 % amp, 30 min ultrasound treatment resulted in a rapid increase in the levels of free sugars, with the content of glucose (and other reducing sugars) being significantly higher than in the control and other treatment containing buffer systems at sampling points of 30 min, 90 min and 240 min ($P < 0.05$). Their content in this buffer system peaked at 240 min before declining to levels similar to those of the control and other treatment containing buffer systems at 1440 min. At 1440 min, no significant differences ($P > 0.05$) in glucose content was observed between any of the buffer systems containing muscle of the various treatments (Fig. 3).

3.3.3. Lactic acid

Lactic acid content in the glycolytic buffer systems, including all treatments and controls except for the 100 % amp and 30 min treatment, increased rapidly in the first 90 min and then plateaued after 240 min (Fig. 4); which is in agreement with the pattern of pH decline.

Ultrasound intensity and duration had a significant impact ($P < 0.05$) on lactic acid in the buffers, and a significant interaction ($P < 0.05$) between the two treatment parameters was also observed. The 100 % amp ultrasound treatment carried out for 30 min resulted in a significant delay in the production of lactic acid from between 30 and 240 min, where the lactic acid content of this buffer system was significantly lower ($P < 0.05$) to that of the control and other treatment containing buffers at the same time points (Fig. 4). No significant differences ($P > 0.05$) were observed in the content of lactic acid at 1440 min in any of the buffer systems.

4. Discussion

The initial step of glycolysis is the release of glucose from glycogen via the process of glycogenolysis and the enzyme responsible for this process is glycogen phosphorylase (GP). GP, together with glycogen debranching enzymes, release free glucose and glucose-1-phosphate to be utilised by the glycolytic pathway to produce ATP [38]. The decline in the rate of glycogen degradation following 240 min reaction time

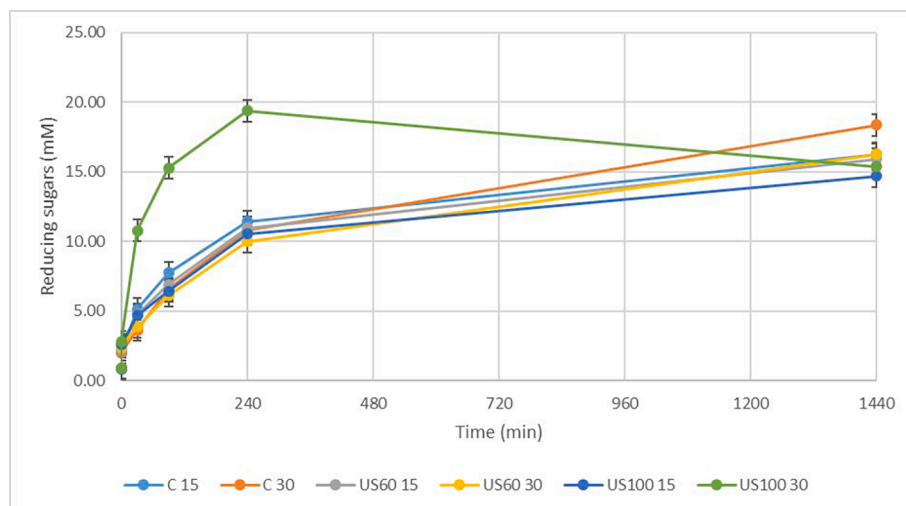


Fig. 3. Glucose (and other reducing sugars) concentration (mM) of the *in vitro* glycolytic reaction buffer over 1440 min (24 hr) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60 % amp for 15 min (US60 15), 60 % amp for 30 min (US60 30), 100 % amp for 15 min (US100 15), 100 % amp for 30 min (US100 30)). N = 4. Data is mean $\pm$ SE.

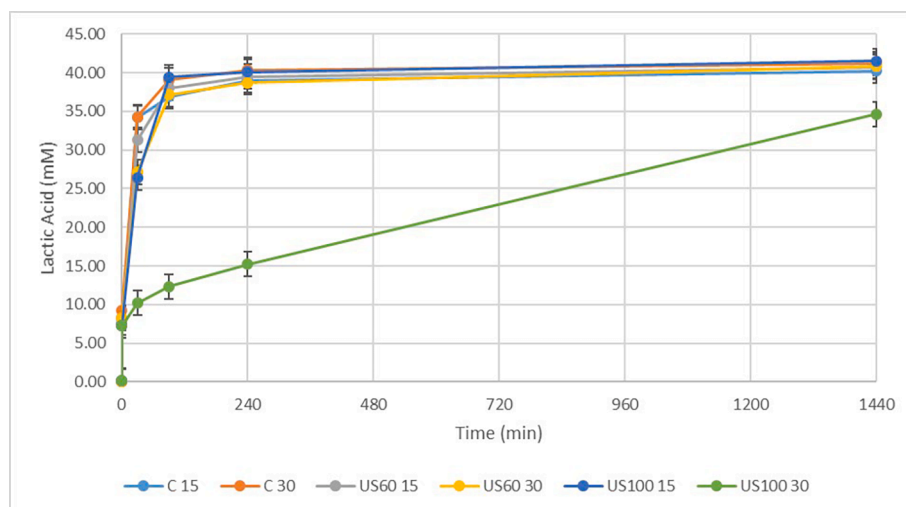


Fig. 4. Lactic acid concentration (mM) of the *in vitro* glycolytic reaction buffer over 1440 min (24 hr) at 25 °C following the addition of control or ultrasound treated muscle homogenates (Control for 15 min (C 15), Control for 30 min (C 30), 60 % amp for 15 min (US60 15), 60 % amp for 30 min (US60 30), 100 % amp for 15 min (US100 15), 100 % amp for 30 min (US100 30)). N = 4. Data is mean $\pm$ SE.

(Fig. 2) reflects a general decline in GP activity observed over time as described by England *et al.* [20]. The results of this study indicate that the ultrasound treatments of the muscle homogenates did not have a significant effect ($P > 0.05$) on the degradation or consumption of glycogen. This indicates that glycogen phosphorylase, nor the glycogen debranching enzymes, were not affected by subjecting muscle homogenates to any of the ultrasound treatments used.

The significant change in the concentration of glucose (and other reducing sugars) following the most aggressive ultrasound treatment used in this study (100 % amp for 30 min), could potentially be due to ultrasound having an impact on the activity of hexokinase, an enzyme responsible for the conversion of glucose to glucose-6-phosphate [37,39]. Potential mechanisms that ultrasound could have an effect on the activity and structure of hexokinase includes the disruption of the enzyme's molecular structure, as well as having an impact on the hexokinase's active site, reducing its ability to form an enzyme-substrate complex [40]. It is interesting to note that the optimum pH for hexokinase activity is pH 7.5 and activity reduces below pH 6.5 [41]. The pH of the *in vitro* buffer system containing homogenate treated with 100 %

amp, 30 min US, reduced from pH 7.42 ± 0.01 to pH 6.48 ± 0.20 over the first 240 min (Fig. 1) of the reaction. The pH of this buffer system remains closer to the optimum pH for hexokinase for longer and hence, if no other factor was coming into play, it could be expected that the enzyme would be more active for this treatment than controls or other treatments. However, the possible negative effect of the 100 % amp, 30 min ultrasound treatment on hexokinase activity could explain the higher reducing sugars (mainly glucose) concentrations for the 100 % amp, 30 min ultrasound treated group (compared to controls and other treatment groups) when pH is greater than pH 6.0 during the first 240 min (S Fig. 1); indicating that the inhibition originated in hexokinase activity by the 100 % amp, 30 min ultrasound treatment is more relevant than the inhibition observed after a decrease in pH, as observed in control and other treatment groups.

While hexokinase is a possible candidate for modification by the most powerful ultrasound treatment used in this study, in theory the activity of any enzyme involved in glycolytic reactions subsequent to glucose formation and the step after fructose-1,6-bisphosphatase is degraded into glyceraldehyde-3-phosphate, could be affected and hence

be responsible for the accumulation of glucose (and other reducing sugars) and the delay in lactic acid production observed. Such enzymes include phosphoglucose isomerase, already mentioned PFK and aldolase. As indicated by Matarneh *et al.* [23], the loss of phosphofructokinase activity results in the prevention of further lactic acid production and the concomitant pH decline, as well as the accumulation of glucose and other reducing sugars such as glucose-6-phosphate and fructose-6-phosphate in post – mortem muscle. Interestingly, for the 100 % amp 30 min ultrasound treatment, it was observed that the concentration of glucose (and other reducing sugars) concentration decreased between 240 and 1440 min, allowing the *in vitro* buffer system to achieve a similar pH and lactic acid content to the buffer systems containing homogenates following the control and other ultrasound treatments. Based on our experimental setup, it is not possible to determine if the inhibiting effect of ultrasound was partially reversible.

While there are few studies investigating the effects of ultrasound on enzymes linked to glycolysis, Braginskaya *et al.*, [40] reported that hexokinase, extracted from yeast, exhibits partial loss of catalytic activity, following exposure to ultrasound, with significantly greater effects observed following 1.5 W/cm² treatment compared to treatment of 1 W/cm². The authors of the paper attributed the loss of activity to the mechanical damage of acoustic streaming and cavitation bubbles, resulting in a reduced enzyme-substrate affinity of hexokinase [40]. This could potentially explain the accumulation of glucose and other reducing sugars, and delay in lactic acid production within the first 30 to 240 min in the glycolytic buffer containing muscle treated with 100 % amp, 30 min US.

5. Conclusion

In this study it was observed that the ultrasound treatment of muscle homogenates using 100 % amplitude for 30 min had a significant impact on the inherent activities of glycolytic enzymes measured *in vitro* using a glycolytic buffer system. These effects were manifested as in changes in the rate of change of pH, and the profile of concentration of glucose (and other reducing sugars) and lactic acid during the incubation period, while the rate of glycogen consumption remained unchanged. Ultrasound power and duration had a significant and interactive effect. The rate of pH decline, as well as glucose and lactic acid concentrations appeared to differ significantly between 30 min and 240 min of incubation in the *in vitro* buffer model system containing muscle homogenates treated with 100 % amplitude (19 W/cm²) for 30 min, when compared to the control and other treatment containing buffers. However, no differences were found after 1440 min of incubation. Thus it appears that the strongest ultrasound treatment had no significant effect on the subsequent activity of glycogen phosphorylase but appeared to adversely affect the activity of other glycolytic enzymes. Further investigation is warranted to identify the specific enzyme(s) that are impacted by ultrasound through further experimental work.

While a change in pH decline was observed in the current study following ultrasound treatment, it was only found after applying the most intensive treatment. Based on the results of the current study on the application of ultrasound on homogenates containing powdered muscle, the potential of using ultrasound as a tool commercially to manipulate the rate of glycolysis as a mechanism to optimise tenderness is limited.

CRedit authorship contribution statement

Mary Ann Kent: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anne Maria Mullen:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Eileen O'Neill:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Carlos Álvarez:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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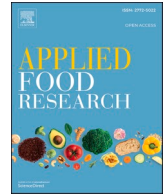
Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ultsonch.2024.106842>.

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Evaluating high pressure processing as a strategy to accelerate water loss and its effect on eating quality in dry aged beef: A preliminary study

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ABSTRACT

Dry aging (DA) of beef cuts is a process that requires a prolonged aging time (usually > 28 days) under controlled refrigerated conditions to obtain a distinctive product that has acceptable quality traits such as tenderness and colour, but with an enhanced signature flavour profile, when compared to wet aged meat. The objective of the current study was to investigate the potential of applying a high pressure pre-treatment (HPP; 200 MPa, 20 min at 8 °C) to bovine *Longissimus thoracis et lumborum*, to facilitate weight loss during dry aging (2 °C, 80 % relative humidity, air flow 0.5 – 2.0 m/s), thus reducing aging time by assessing its impact on weight loss, and meat quality parameters such as colour, Warner-Bratzler shear force values and lipid oxidation (TBARS), over a 28 day aging period. Under the conditions of the study, it was found that the high pressure pre-treatment significantly increased the overall weight loss of muscle. However, the pre-treatment increased Warner-Bratzler shear force values when compared to the control at 21 days of aging. In addition, the HPP pre-treatment significantly increased TBARS values, bloomed lightness (L^* values) and myofibrillar fragmentation index, while significantly decreasing calpain activity compared to controls.

1. Introduction

Aging of bovine muscle post – mortem improves texture and flavour characteristics through the degradation of key structural proteins and development of flavour compounds via enzymatic activity and lipid oxidation (Khan et al., 2016; Kim et al., 2016; Lee et al., 2021; Sadowska et al., 2020). Dry aging involves hanging carcasses, primals or subprimals, under controlled temperature, relative humidity conditions and increased air flow for a number of weeks (Dashdorj et al., 2016). On the other hand, wet aging (which is more commonly used in the meat industry) involves aging primals or subprimals in water impermeable vacuum packaging, thus reducing evaporative weight loss and avoiding the formation of a crust which is characteristic of dry aged meat. Several studies have shown that dry aged beef has superior sensory traits such as flavour, when compared to wet aged beef, while having similar instrumental shear force values, bloom colour or cooking loss (Berger et al., 2018; Dikeman et al., 2013; Kim et al., 2016; Li et al., 2014b). However, dry aging significantly decreases the yield of saleable meat (Laster et al.,

2008; Smith et al., 2008) due to weight losses associated with surface drying and the requirement to trim the outer crust prior to sale (Ha et al., 2019; Li et al., 2014b).

High pressure processing (HPP) is a non-thermal technology that involves the application of pressures between 100 and 900 MPa within a pressurised vessel, where a food product is treated instantaneously and uniformly. HPP has been implemented in the food industry to improve food safety without the use of additives or high temperatures (Campus, 2010). It has been found that HPP treatment of post – rigor bovine muscles at pressures over 200 MPa for a duration of 10–20 min has significant effects on quality parameters immediately or within 1 day post-treatment. In general, HPP accelerates lipid oxidation and negatively impacts colour (specifically on the lightness (L^*) parameter, where HPP appears to have a whitening effect on meat). Water holding capacity has been reported to decrease, while the Warner-Bratzler shear force values of muscle remained unchanged or increased when compared to non – treated muscle (Marcos et al., 2010; Marcos & Mullen, 2014; McArdle et al., 2010; McArdle et al., 2011).

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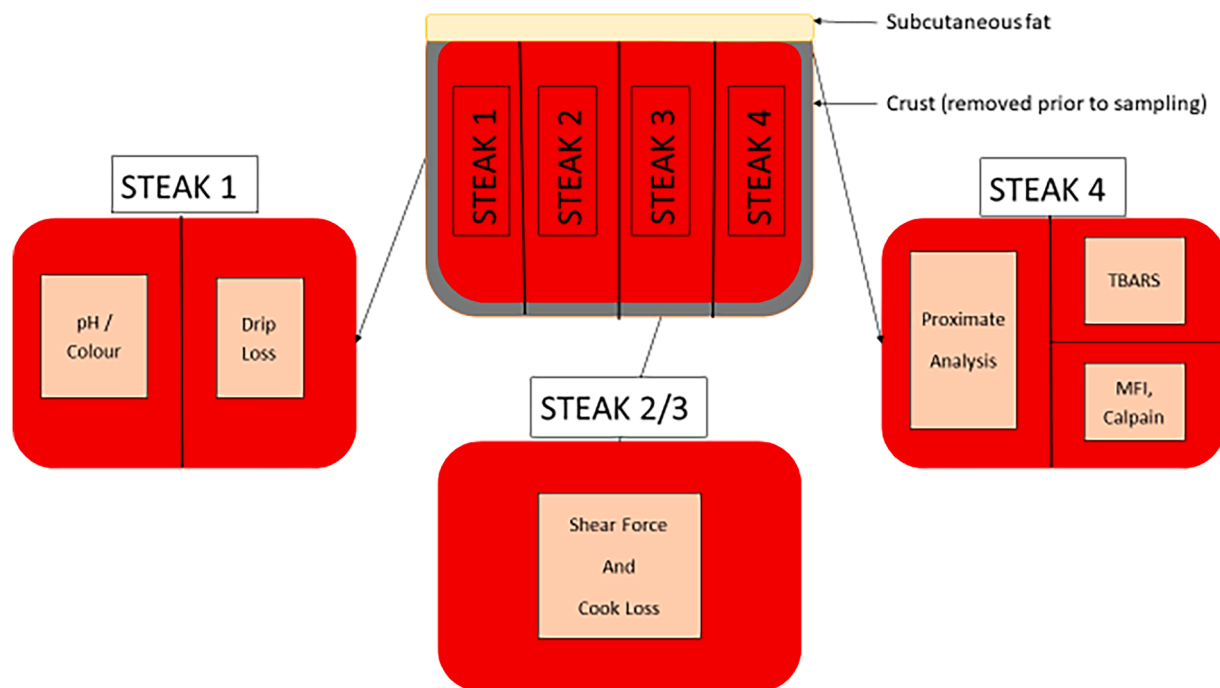


Fig. 1. The sampling plan for sections following dry aging for 0, 14, 21 and 28 days.

Since HPP has been shown to decrease water holding capacity of muscle, there may be potential to use this technology as a pre-treatment to accelerate the dry aging process, and therefore reduce the aging time, crust formation and associated production costs. To the best of our knowledge, the use of high pressure processing as a pre-treatment for beef that is subsequently dry aged has not yet been investigated. Therefore, the aim of the current study was to investigate if a high pressure pre-treatment (200 MPa for 20 min at 8 °C) impacts on the rate of weight loss, and meat quality traits such as Warner-Bratzler shear force values, colour, cook loss and lipid oxidation of beef (*Longissimus thoracis et lumborum*) during dry aging. In addition, the effect of the HPP pre-treatment on calpain activity and myofibrillar fragmentation was also monitored at different aging times (14, 21, 28 days).

The HPP treatment used in the current study was selected based on the research of McArdle et al. (2010, 2011) and Marcos et al. (2010, 2014), who found that 200 MPa for 20 min had minimal impact on meat quality traits of bovine muscle such as Warner-Bratzler shear force values and lipid oxidation while decreasing water holding capacity. Hence this treatment may have potential to facilitate faster moisture loss from muscle during subsequent dry aging.

2. Materials and Methods

2.1. Chemicals and reagents

EDTA, potassium chloride, potassium phosphate dibasic, trichloroacetic acid, 2 – thiobarbituric acid (Sigma Aldrich, Ireland), magnesium chloride (VWR Chemicals, Ireland), Bradford reagent, Bovine serum albumin (BSA) standard (2 mg/mL), sodium azide (ThermoScientific, United Kingdom), CytoBuster™ protein extraction reagent, InnoZyme™ Calpain 1/2 Activity Assay Kit (Merck KGaA, Germany).

2.2. Animal and sample preparation

Longissimus thoracis et lumborum (LTL) muscles from the left and right side of four steer carcasses of similar conformations (age: 22–29 months; conformation score R-, O=, O+; fat score 2=, 3-; average weight 170.5 ± 16.3 kg) were collected from a commercial slaughtering plant (Kepak,

Ireland) at 72 h post – mortem. Each muscle was cut into 4 sections approximately 20 cm x 12.5 cm x 6 cm in dimension, giving a total of 8 sections per animal. The average weight of the sections was 1361.7 ± 95.3 g. Subcutaneous fat was not removed from the sections. The sections were randomly assigned to 8 different treatment combinations (dry aging (DA)/ high pressure pre-treatment and dry aging (HPPDA), combined with 0/ 14/ 21/ 28 days of aging). Each muscle section was individually vacuum packed in polyamide polyethylene bags and stored overnight at 4 °C.

2.3. High pressure processing and dry aging

Sections assigned for HPPDA were treated at 200 MPa for 20 min at 8 °C in a 420 Hiperbaric system (Burgos, Spain). After the high pressure pre-treatment, samples assigned to both HPPDA and DA treatments were removed from the vacuum bags and weighed, using a digital balance and randomly placed in a dry aging chamber (DRY AGER DX 1000®, DRY AGER®, Bad Saulgau, Germany), on racks, and aged for up to 28 days at 2 °C, 80 % relative humidity and with an air flow ranging from 0.5 to 2.0 m/s. Throughout aging, the sections were moved every 3 – 4 days to remove any potential effect of location in the dry ager.

At each aging time (14/ 21/ 28 days), 8 sections were removed from the dry ager and weighed. The crust and subcutaneous fat was removed. Sections were divided into 4 steaks (approximately 2.5 cm thick, numbered 1 to 4 from the anterior end) for further analysis (Fig. 1).

2.4. pH and proximate analysis

pH was measured using steak 1 (Fig. 1) of each section using a temperature/pH meter, with integrated temperature compensation (Hanna HI 98,163 portable pH meter for meat and FC2323 meat pH electrode, Hanna Instruments Ltd., Leighton Buzzard, UK), by inserting the pH electrode directly into the muscle. An average of three measurements were taken per steak. The pH probe was calibrated at room temperature prior to analysis using pH 4, 7 and 10 buffers.

Samples (approximately 100 g) for proximate analysis were taken from steak 4 (Fig. 1) for all sections, vacuum packed and stored at – 20 °C until analysis. The samples were thawed overnight at 4 °C and

homogenised using a blender (Cookworks, Argos Limited, Milton Keynes, UK). Protein content was quantified using the Dumas method with a Leco Nitrogen Analyser (FP-628, Leco Corp., MI, USA), according to AOAC method 992.15 (AOAC, 1992). Protein content was calculated using a conversion factor of N x 6.25. Moisture content was measured using Smart 6 (CEM Corporation, USA) and fat content was measured using Oracle NMR Smart Trac Rapid Fat Analyser (CEM Corporation USA), using pre-dried samples from moisture content analysis, according to AOAC method 2008.06 (AOAC, 2008). Ash content was determined following the AOAC method 920.153 (AOAC, 1920), using a muffle furnace (Carbolite, Sheffield, England) at 550 °C. Analysis was carried out in duplicate for each sample.

2.5. Aging loss

The weight of the sections being aged for 28 days was measured and recorded at the same time each day throughout the dry aging period. Aging weight loss was calculated according to Eq. 1.

$$\text{Aging weight loss (\%)} = \frac{\text{Initial weight (g)} - \text{Weight after aging (g)}}{\text{Initial weight}} \times 100 \quad (1)$$

2.6. Drip loss

Drip loss was measured according to the method of Honikel (1998). Briefly, samples of equal dimensions (2.54 × 5 × 7.5 cm) and weight (74 ± 2.5 g), were removed from steak 1 (Fig. 1) of each section, avoiding any connective tissue. The samples were lightly blotted and weighed, before being suspended in a clear plastic bag, using string and a hook (unfolded paper clip), ensuring there was no contact between the sample and bag. The bag was loosely tied around the hook and suspended in a chill room for 72 h at 4 °C. Following this, the surface of the samples was blotted with tissue and re-weighed. Drip loss was expressed as a percentage of the weight of the original piece of muscle (Eq. 2).

$$\text{Drip loss (\%)} = \frac{\text{Initial weight (g)} - \text{Weight after 72hr suspension (g)}}{\text{Initial weight (g)}} \times 100 \quad (2)$$

2.7. Cooking loss and Warner-Bratzler shear force

Steak 2 (Fig. 1) from each section was vacuum packed and stored at –20 °C and subsequently used for determination of cooking loss (Shackelford et al., 1991) and Warner-Bratzler shear force values (WBSF) (Wheeler et al., 1996). The vacuum packed steaks were defrosted in circulating water at room temperature. External moisture was lightly blotted from the steak with tissue before being weighed. Steaks were placed into individual polyethylene bags and cooked in a water bath set at 72 °C (Grant Instruments Ltd., England). The internal temperature of the steaks was monitored using a probe (Eirelec Ltd., Ireland) until an internal core temperature of 70 °C was reached. Excess water was removed from the bags and the steaks were allowed to cool to room temperature, blotted with a tissue paper and re-weighed. Cooking loss was determined by calculating the difference between the initial and cooked weight of the steaks and was expressed as a percentage of the initial weight of the steak (Eq. 3).

$$\text{Cooking loss (\%)} = \frac{\text{Initial weight (g)} - \text{Cooked weight (g)}}{\text{Initial weight (g)}} \times 100 \quad (3)$$

The cooked steaks were packaged and stored overnight at 4 °C. Warner-Bratzler shear force measurements were carried out on 1.25 cm diameter cores taken from the steak, perpendicular to the direction of the muscle fibres, using an Instron 4464 Universal testing machine (Instron Ltd., Buckinghamshire, UK), with a 500 N loading cell and cross

head speed of 50 mm/min. The Warner-Bratzler shear force values were a mean of 5 shear measurements.

2.8. Colour measurements

For each time point, a sample (4 × 4 × 2.5 cm) from the freshly cut steak 1 (Fig. 1) was wrapped in cling film, allowed to bloom for 3 h at 4 °C. The colour of the bloomed surface was recorded using a HunterLab UltraScan Pro CIE L*a*b* system with a dual beam xenon flash spectrophotometer (Hunter Associates Laboratory, Inc., Reston, VA, USA) to measure lightness (L*), redness (a*) and yellowness (b*). A light trap (L* = 0) and standard white tile, covered with transparent cling film, was used for calibration. A standard illuminant D65 with a ten degrees observer angle and a 25 mm diameter port were used. The colour of each sample was measured at 5 random locations. Colour measurements using the same parameters as above were also carried out on the cooked steaks from section 2.7.

Colour differences (ΔE) between DA and HPPDA treated muscle were calculated according to the method of Jung et al., (2003) Eq. 4 where the colour values for the control samples at each sampling time point was used as the reference values in the calculation.

$$\Delta E = \left[(L^* - L_{ref}^*)^2 + (a^* - a_{ref}^*)^2 + (b^* - b_{ref}^*)^2 \right]^{\frac{1}{2}} \quad (4)$$

2.9. Lipid oxidation

Lipid oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS) values according to the method of Botso-glou et al., (1994). Samples (approximately 10 g) were removed at each sampling time point from steak 4 (Fig. 1), vacuum packed and stored at –20 °C for further analysis. Prior to analysis, samples were thawed overnight at 4 °C. Briefly, 3.5 g of muscle was added to 20 mL MilliQ water and homogenised using an Ultraturrax (13,500 rpm for 30 s). Trichloroacetic acid (5 mL, 25 % (w/v) TCA, ice cold) was added to the homogenate, vortexed and allowed to stand at 4 °C for 15 min. The homogenate was centrifuged at 2500 g for 30 min at 4 °C and the supernatant was filtered using Whatman 52 filter paper. The supernatant (3.5 mL) was mixed with 1.5 mL of 0.6 % (w/v) 2-thiobarbituric acid, heated in a water bath at 70 °C for 30 min, and allowed to cool on ice before the absorbance at 532 nm was measured using a UV – Vis spectrophotometer. TBARS values were expressed as milligrams of malondialdehyde (MDA) per kilogram of sample (mg MDA/kg). For each steak, two independent extractions were prepared for analysis in duplicate.

2.10. Myofibrillar fragmentation index

Samples (approximately 3 g) were removed from steak 4 (Fig. 1) of each section, placed in a cryovial, snap frozen and stored at –80 °C until time of analysis. Myofibrillar fragmentation index (MFI) values were determined according to the method of Culler et al., (1978), with modifications from Hopkins et al. (2000). Briefly, 1 g of muscle was homogenised in 10 mL ice cold MFI extraction buffer, consisting of 100 mM KCl, 20 mM K₂HPO₄, 1 mM EDTA, 1 mM MgCl₂ and 1 mM Na₃, pH 7, with an Ultraturrax at 15,000 rpm, for 60 s (2 × 30 s, with a 30 s break between bursts). Homogenates were centrifuged at 1000 g for 15 min at 4 °C. The supernatant was discarded. The pellets were gently inverted in 10 mL MFI extraction buffer and centrifuged again. The supernatant was removed and the pellets were re-suspended in 10 mL MFI extraction buffer using a vortex and the mixture was filtered through a filtration fabric (muslin cloth). The protein content of the filtrate was measured using the Bradford method and a standard curve using BSA (Bradford, 1976). The protein content of the filtrate was adjusted to 0.5 ± 0.05 mg/mL. The absorbance of the solution was measured using a UV/Vis spectrophotometer at 540 nm. MFI values were obtained by multiplying

Table 1

The effect of aging time and HPP on the pH of dry aged bovine LTL. Results are the mean values of four biological replicates $\pm$ SEM.

		Aging time (days)				P – value	Treatment		Interaction Aging x Treatment P – values
		0	14	21	28		Mean	P – value	
pH	DA	5.51 $\pm$ 0.03	5.55 $\pm$ 0.03	5.51 $\pm$ 0.03	5.58 $\pm$ 0.03	0.02	5.54 $\pm$ 0.02	< 0.001	0.8898
	HPPDA	5.65 $\pm$ 0.03	5.72 $\pm$ 0.03	5.66 $\pm$ 0.03	5.73 $\pm$ 0.03		5.69 $\pm$ 0.02		

P – values from the F – Test, following MIXED procedure analysis.

DA – Dry aged, HPPDA – High pressure pre-treatment and dry aged

the absorbance at 540 nm by 200. For each steak, two independent extractions were prepared for analysis in duplicate.

2.11. Calpain activity

Samples (approximately 1 g) were removed from steak 4 (Fig. 1) for each section, placed in a cryovial, snap frozen and stored at -80°C for further analysis. Calpain activity was measured in duplicate according to the method outlined for using the InnoZyme™ Calpain 1/2 Activity Assay Kit (Merck KGaA, Darmstadt, Germany) (Preziosa et al., 2013, Salazar et al., 2009). Samples (0.1 g) were homogenised in 1 mL of CytoBuster™ Protein Extraction Reagent (Merck KGaA, Darmstadt, Germany), with an Ultraturrax (13,500 rpm, 30 s). The homogenates were centrifuged at 20,900 g for 5 min at 4°C and kept on ice during protein quantification and calpain activity analysis. The protein content of the supernatants was measured using the Bradford method and a standard curve prepared using BSA (Bradford, 1976). The protein content of the supernatants were adjusted to approximately 2 mg/mL and 50 μL aliquots were added to a 96 well plate. Activation buffer with reducing agent (100 μL) or activation buffer with reducing agent and calpain inhibitor (100 μL) was added to the wells. The 96 well plate was gently shaken (Stuart reciprocating shaker SSL2 (Cole-Parmer Instrument Co. Europe, PE19 8YX United Kingdom) for 3 min. Calpain substrate (50 μL) was added to the wells followed by incubation for 30 min in the dark at room temperature, with gentle shaking. Fluorescence was measured at an excitation wavelength of 320 nm and an emission wavelength of 480 nm using a Tecan SPARK® Multimode Microplate Reader and Tecan. SparkControl software (Tecan Group Ltd., 8708 Männedorf, Switzerland). Calpain activity was expressed in relative fluorescence units (RFU) and calculated by subtracting the fluorescence

of samples containing the activation buffer and reducing agent from that of the corresponding samples containing the activation buffer, reducing agent and calpain inhibitor.

2.12. Statistical analysis

The data presented in this study is the average $\pm$ standard error mean (SEM). Analysis of all variables was performed using MIXED procedure of the SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA) with 4 replicates and 2 factors: treatment (dry aging (DA)/ high pressure pre-treatment and dry aging (HPPDA)) and aging time, post-treatment (0/ 14/ 21/ 28 days), along with their interactions. Animals were treated as a random effect. Treatment means and interactions were compared and in all cases a P – value < 0.05 was considered as significant. After this, a post-hoc Tukey's test was applied.

3. Results and Discussion

3.1. pH

Aging time had a significant effect ($P < 0.05$) on the pH of muscle. In addition, the pH of muscle increased significantly ($P < 0.05$) after HPPDA treatment and this was true for all time points (Table 1). No significant interaction ($P > 0.05$) was found between aging time and HPPDA. The increase in pH over aging time in DA and HPPDA treated muscle may be due to proteolysis and the subsequent production of alkaline substances (Shi et al., 2020). The increase in pH following HPPDA treatment is in agreement with the results of Utama et al., (2017), who found that HPP (200 – 600 MPa for 180 s, $15 \pm 2^{\circ}\text{C}$) significantly increased the pH of bovine *Longissimus lumborum*. The

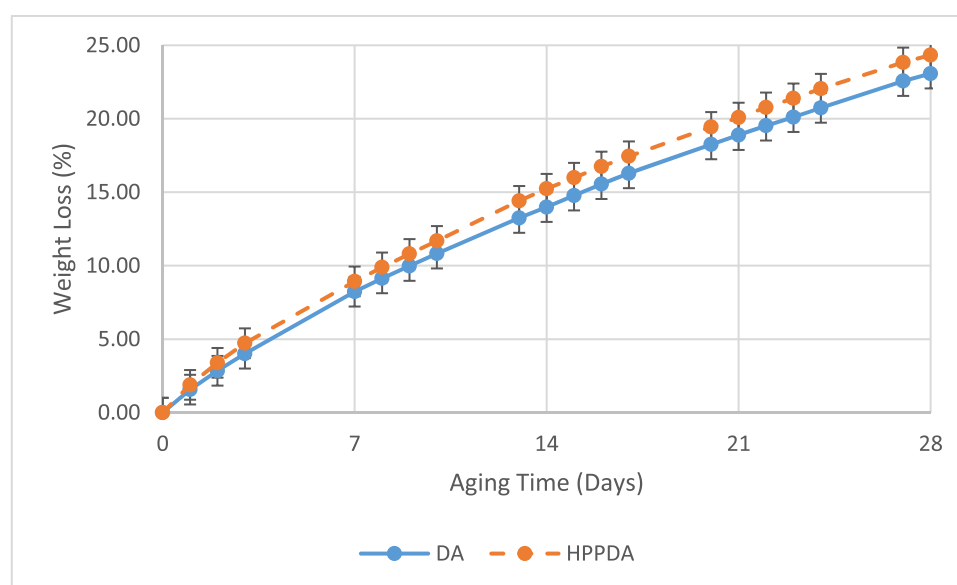


Fig. 2. Effect of aging time and HPP on the weight loss (%) of dry aged bovine LTL over a 28 day dry aging period. Results are mean values of four biological replicates.

DA – Dry aged, HPPDA – High pressure pre-treatment and dry aged.

Table 2
The effect of aging time and HPP on the proximate composition of dry aged bovine LTL. Results are mean values of four biological replicates ± SEM. Each replicate was measured in duplicate.

		Aging time (days)					Treatment		Interaction Aging x Treatment
		0	14	21	28	P – value	Mean	P – value	P – value
Moisture	DA	73.21 ± 1.19	71.98 ± 1.19	71.56 ± 1.19	70.08 ± 1.19	<0.0001	71.71 ± 1.03	0.4541	0.5128
	HPPDA	74.02 ± 1.19 ^a	71.75 ± 1.19 ^{ab}	70.58 ± 1.19 ^b	68.98 ± 1.19 ^b		71.33 ± 1.03		
Protein	DA	24.06 ± 0.46	23.34 ± 0.46	24.11 ± 0.46	25.35 ± 0.46	0.0015	24.21 ± 0.25	0.3908	0.6951
	HPPDA	23.72 ± 0.46	23.82 ± 0.46	24.64 ± 0.46	25.64 ± 0.46		24.48 ± 0.25		
Fat	DA	2.27 ± 1.23	2.94 ± 1.23	3.40 ± 1.23	4.02 ± 1.23	0.0361	3.16 ± 1.06	0.8102	0.8917
	HPPDA	1.71 ± 1.23	3.24 ± 1.23	3.82 ± 1.23	4.35 ± 1.23		3.28 ± 1.06		
Ash	DA	1.04 ± 0.05 ^a	1.18 ± 0.05 ^{ab}	1.19 ± 0.05 ^{ab}	1.39 ± 0.05 ^b	<0.0001	1.20 ± 0.03	0.3388	0.3557
	HPPDA	1.08 ± 0.05 ^a	1.11 ± 0.05 ^a	1.31 ± 0.05 ^{ab}	1.45 ± 0.05 ^b		1.24 ± 0.03		

Means with different letters (a – b) within rows indicate significant differences between time points ($P < 0.05$).
 P – values from the F – Test, following MIXED procedure analysis.
DA – Dry aged, HPPDA – High pressure pre-treatment and dry aged

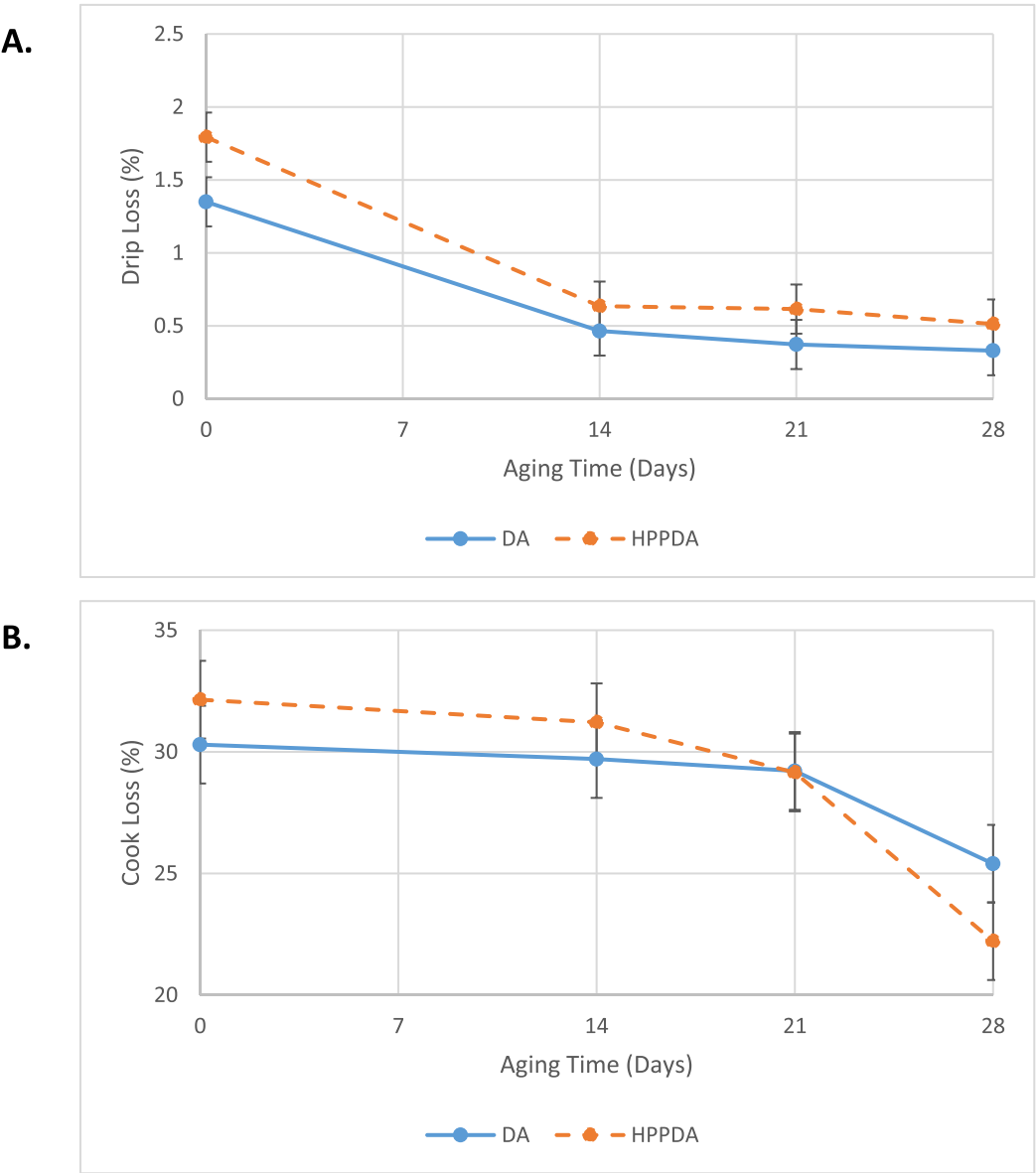


Fig. 3. The effect of aging time and HPP on the A) drip loss (%) and B) cook loss (%) of dry aged bovine LTL. Results are mean values of four biological replicates ± SEM.
DA – Dry aged, HPPDA – High pressure pre-treatment and dry aged.

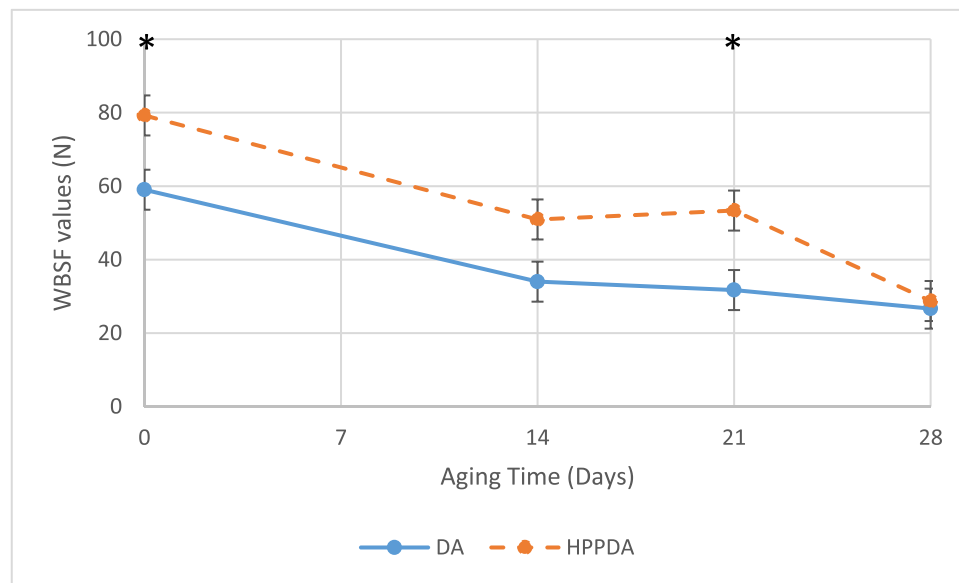


Fig. 4. The effect of aging time and HPP on the WBSF values of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.

* indicates significant differences between treatments.

DA – Dry aged, HPPDA – High pressure pre-treatment and dry aged.

increase in pH following HPP has been attributed to a decreased availability of acidic groups in muscle due to protein denaturation (Ma & Ledward, 2004; McArdle et al., 2010). Nevertheless, the pH values remained within the normal range of beef (pH 5.5 – 5.8) (Holdstock et al., 2014, Li et al., 2023, Barón et al., 2021).

3.2. Weight loss and proximate composition

As expected, weight loss increased significantly ($P < 0.05$) with aging time (Fig. 2) due to water loss from the surface of the muscle and simultaneous migration of water from the internal portion of the muscle to the surface (Ha et al., 2019, Santchurn et al., 2012). It was observed that HPPDA significantly ($P < 0.05$) increased the weight loss of muscle over the entire period of dry aging. However, no significant differences ($P > 0.05$) in weight loss between control and HPPDA treated sections were observed at any individual time points. No significant interaction ($P > 0.05$) was found between aging time and HPPDA. HPP may have induced protein denaturation, reducing the water holding capacity and facilitating moisture loss from the surface of the muscle (Marcos et al., 2010) as well as diffusion of water to the surface of the muscle. The results show that while HPP has the potential to accelerate moisture loss during dry aging, higher pressures may be required to have a more noticeable difference. However, this may be detrimental for other quality traits.

Moisture content decreased significantly ($P < 0.05$) with aging time, while HPPDA did not have a significant effect ($P > 0.05$) on moisture content (Table 2) when compared to the control (DA). Moisture content was expected to decrease with dry aging time due to evaporative losses (Ha et al., 2019; Kim et al., 2019). Protein, fat and ash content increased significantly ($P < 0.05$) with aging time, while HPPDA did not have a significant effect ($P > 0.05$) (Table 2). For all proximate components, no significant interactive effects ($P > 0.05$) between aging time and treatments were observed. An increase in protein, fat and ash content during dry aging was expected due to the reduction in moisture content and this is in agreement with the findings of other authors (Khazzar et al., 2023; Kim et al., 2019; Lee et al., 2017).

3.3. Drip loss and cooking loss

Drip loss decreased significantly ($P < 0.05$) with aging time for DA

and HPPDA treated muscle, with the main reduction being observed between day 0 and 14 (Fig. 3A). While HPPDA treated muscle had significantly higher ($P < 0.05$) average drip loss over the four aging time points, when compared to the controls, no significant differences were observed between the drip loss of DA and HPPDA samples at individual time points. There was no significant interaction ($P > 0.05$) between aging time and HPP. The reduction in drip loss over aging time is attributed to the reduction in moisture content as a result of dry aging, resulting in less free water being available (Álvarez et al., 2023). In addition, HPP (200 – 600 MPa, 10 – 30 °C, 20 min) has been found to reduce the water holding capacity (measured by expressible moisture) of bovine *Longissimus* muscle (Marcos et al., 2010; Marcos & Mullen, 2014). It was proposed by the authors that HPP negatively impacted the water holding capacity of muscle through the denaturation of sarco-plasmic proteins.

Aging time had a significant effect on cooking loss, where the cooking loss was significantly ($P < 0.05$) lower at day 28 when compared to day 0 (Fig. 3B). HPPDA did not have a significant ($P > 0.05$) effect on cooking loss (Fig. 3B) and no significant interaction ($P > 0.05$) was found between aging time and HPP. The results of the current study are in agreement with the findings of Khazzar et al., (2023), Kim et al., (2022) and Lee et al., (2019), who reported that cooking loss of bovine *Longissimus* muscle decreased significantly following 28 – 30 days of dry aging. This can be explained by the reduction in moisture content with dry aging time (Álvarez et al., 2023) and therefore, less water is available to be lost during cooking. Furthermore, when investigating the effects of HPP on bovine *Pectoralis profundus*, McArdle et al., (2010), found that HPP treatments of 200 MPa (for 20 min at 20 and 40 °C) did not significantly affect cooking loss.

3.4. Warner-Bratzler shear force (WBSF) values

Aging time significantly ($P < 0.05$) reduced the WBSF values. HPPDA significantly ($P < 0.05$) increased the WBSF values of muscle immediately after treatment and after 21 days of aging (Fig. 4). The final WBSF of the HPPDA treated samples were not significantly different from the controls after 28 days of aging ($P > 0.05$) (Fig. 4). The observed decrease in WBSF values of beef with dry aging time is in agreement with the results of previous studies (Smith et al., 2008; Khazzar et al., 2023 and Lee et al., 2017), and is due to the actions of proteolytic enzymes such as

Table 3Effect of aging time and high pressure processing on L^* , a^* , b^* values of dry aged bovine LTL. Results are the mean of four biological replicates $\pm$ SEM.

		Aging time (days)				P – value	Treatment		Interaction Aging x Treatment P – value
		0	14	21	28		Mean	P – value	
L^*	DA	40.25 $\pm$ 0.95 ^{Aa}	41.08 $\pm$ 0.95 ^{Aa}	41.59 $\pm$ 0.95 ^{Aa}	40.17 $\pm$ 0.95 ^{Aa}	0.2931	40.77 $\pm$ 0.58	<0.0001	0.9403
	HPPDA	47.08 $\pm$ 0.95 ^{Ba}	48.81 $\pm$ 0.95 ^{Ba}	48.71 $\pm$ 0.95 ^{Ba}	47.88 $\pm$ 0.95 ^{Ba}		48.12 $\pm$ 0.58		
a^*	DA	15.67 $\pm$ 0.54 ^{Aa}	20.06 $\pm$ 0.54 ^{Ab}	20.70 $\pm$ 0.54 ^{Ab}	20.79 $\pm$ 0.54 ^{Ab}	<0.0001	19.30 $\pm$ 0.35	0.0069	<0.0001
	HPPDA	20.39 $\pm$ 0.54 ^{Ba}	19.26 $\pm$ 0.54 ^{Aa}	21.25 $\pm$ 0.54 ^{Aa}	20.36 $\pm$ 0.54 ^{Aa}		20.31 $\pm$ 0.35		
b^*	DA	10.48 $\pm$ 0.51 ^{Aa}	14.00 $\pm$ 0.51 ^{Ab}	13.97 $\pm$ 0.51 ^{Ab}	13.62 $\pm$ 0.51 ^{Ab}	0.002	13.02 $\pm$ 0.26	<0.0001	0.0074
	HPPDA	16.27 $\pm$ 0.51 ^{Ba}	16.36 $\pm$ 0.51 ^{Aa}	17.05 $\pm$ 0.51 ^{Ba}	16.00 $\pm$ 0.51 ^{Aa}		16.42 $\pm$ 0.26		

Means with different letters (a – b) within rows indicate significant differences between time points ($P < 0.05$).Means with different letters (A – B) within columns indicate significant differences between treatments ($P < 0.05$).

P – values from the F – Test, following MIXED procedure analysis.

DA – Dry aged, HPPDA – High pressure pre-treatment and dry aged

calpains, cathepsins and caspases (Huff Lonergan et al., 2010; Kemp et al., 2010). The results of this study are consistent with those of Ma and Ledward (2004) who reported that HPP up to 400 MPa (20 °C, 20 min), significantly increased the hardness of post – rigor bovine *Longissimus dorsi* immediately after treatment. The increase in WBSF values following HPP of post – rigor bovine muscle may be attributed to sarcomere shortening, and the aggregation and coagulation of proteins (Jung et al., 2000a). Furthermore, Utama et al., (2018) found that applying HPP (300 MPa, 180 s, 15 °C) to bovine *Longissimus lumborum* resulted in a significant increase in WBSF values immediately post-treatment, when compared to the control. However, following 28 days of wet aging, no significant differences in WBSF values were observed between the control and 300 MPa treated muscle, similar to the findings of the current study using dry aging. From the results of this study, it can be concluded that the HPP treatment used in this study does not have a detrimental effect on WBSF values if sufficient aging time is allowed. While HPPDA has the ability to increase weight loss over time, a compromise may need to be made in relation to eating quality if aging time was to be reduced.

3.5. Bloomed colour

Aging time did not have a significant effect ($P > 0.05$) on L^* values (lightness), however, HPPDA significantly increased ($P < 0.05$) the L^* values of bloomed muscle, and this was observed for all aging time

points (Table 3). No significant interaction ($P > 0.05$) between aging time and HPP was found.

The increase in L^* values following HPPDA observed in this study is in agreement with Marcos et al., (2010) and McArdle et al., (2010), who found that HPP treatment (200 – 600 MPa, 10 to 40 °C, 20 min) of bovine *Longissimus dorsi* and *Pectoralis profundus*, respectively, significantly increased the L^* values of freshly cut muscle when compared to controls. The whitening/bleaching effect of HPP has been attributed to protein denaturation and/or displacement or release of heme (Bolumar et al., 2021; Carlez et al., 1995), and may have a negative impact on consumers perception of the freshness of beef.

Aging time and HPPDA had a significant effect ($P < 0.05$) on the a^* values (redness) of freshly cut bloomed muscle. The a^* value at day 0 was significantly higher following HPP treatment when compared to the control. However, no significant differences were observed between HPPDA treated and control muscle after 14 days of aging and onwards (Table 3). A significant interaction ($P < 0.05$) between aging time and treatment was observed.

Aging time has been found to have no significant effect on the redness of freshly cut bloomed bovine *Longissimus* muscle dry aged for 8 and 19 days (Li et al., 2014b), while dry aging for periods greater than this led to a significant decrease in a^* values (Ha et al., 2019). A significant increase in the a^* values of bovine *Biceps femoris* was observed immediately post HPP treatment (130 MPa for 260 s, 10 °C) by Jung et al., (2003), which was attributed to a decrease in metmyoglobin

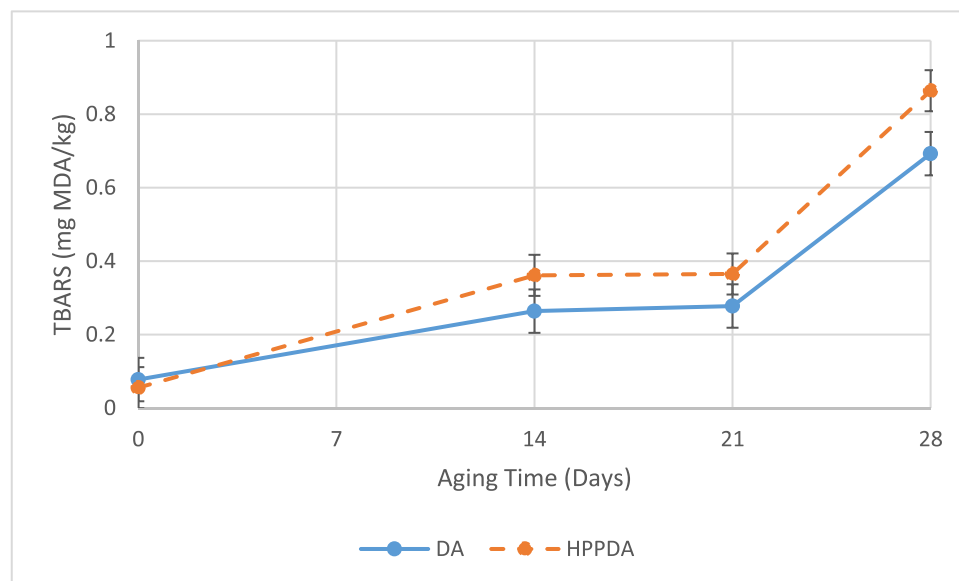


Fig. 5. The effect of aging time and HPP on the TBARS values (mg MDA/kg) of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM. DA – Dry aged, HPPDA – High pressure pre-treatment and dry aged.

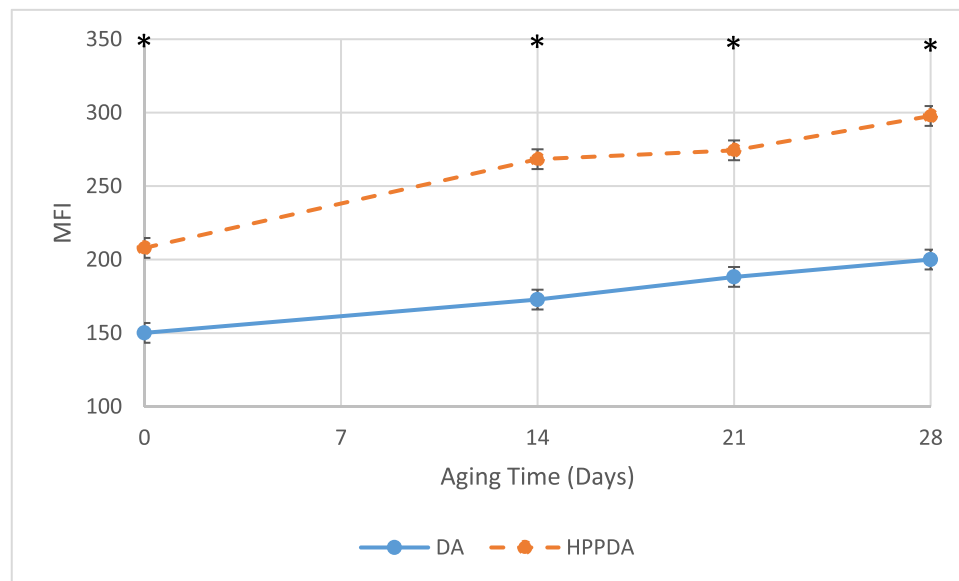


Fig. 6. The effect of aging time and HPP on the MFI values of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.

* indicates a significant difference between treatments.

DA – Dry aged, HPPDA – High pressure pre-treatment and dry aged.

content. However, other studies have found that HPP treatments similar to that used in the current study, (200 MPa, 10 to 40 °C, 20 min) did not significantly affect the a^* values of the freshly cut bloomed surface of bovine muscles such as *Pectoralis profundus* (McArdle et al., 2010), *Longissimus dorsi* (Marcos et al., 2010) and *Longissimus thoracis et lumborum* (Marcos & Mullen, 2014).

Aging time had a significant effect ($P < 0.05$) on b^* values (yellowness) of the freshly cut bloomed control bovine muscle, where it increased significantly between day 0 and 14 and then remained stable (Table 3). HPP significantly increased ($P < 0.05$) the yellowness of freshly cut bloomed muscle compared to control at day 0. While the b^* values of the HPPDA muscle remained higher throughout the entire aging period, when compared to the DA samples, these differences were only significant ($P < 0.05$) at day 21. A significant interaction ($P < 0.05$) between aging time and HPP was found.

The effect of HPPDA on the colour of cooked muscle was also examined (Supplementary Table 1). Overall, it was found that the lightness (L^*) and yellowness (b^*) values were significantly ($P < 0.05$) higher in cooked HPPDA steaks when compared to the cooked DA steaks.

When evaluating the colour differences between the freshly cut bloomed surfaces of the DA and HPPDA treated samples, it was found that ΔE was approximately 8.5 following 28 days of aging, primarily due to differences in L^* values. Mokrzycki and Tatol (2011) proposed that a colour difference greater than 5 units allows observers to distinguish between samples. However, the colour differences (ΔE) between the cooked DA and HPPDA treated samples was approximately 2 (at all aging times), indicating that the effect of HPPDA on the colour of cooked muscle was minimal and the colour change would only be noticeable to an experienced observer (Mokrzycki & Tatol, 2011). The results indicate that the bloom colour of dry aged beef is effected by HPP, most notably the lightness of the muscle, and this may affect the willingness of customers to buy the HPPDA treated fresh muscle, as colour is one of the primary factors considered at purchase. However, HPP could be applied to muscle that is presented to the consumer as a cooked product (e.g. in the catering industry) without having a detrimental effect on colour.

3.6. Lipid oxidation

TBARS values (mg MDA/ kg) increased significantly ($P < 0.05$) with

aging time (Fig. 5), for both control and treated samples. HPPDA significantly increased ($P < 0.05$) the TBARS values over the 28 day dry aging period when compared to the control samples, however, no significant differences ($P > 0.05$) were observed between control and HPPDA samples at individual time points (Fig. 5). No significant interaction ($P > 0.05$) was found between aging time and treatment.

The increase in TBARS values with aging time has been attributed to cell membrane damage (Beltran et al., 2003) and an increase in the concentration of free iron, following the release from iron complexes, such as haemoglobin and myoglobin, in muscle. Free iron can act as a pro-oxidant and catalyse the lipid oxidation reaction (Liu et al., 2022; Liu et al., 2023). Similar mechanisms have been attributed to the increase in lipid oxidation following HPP (Ma et al., 2007; Medina-Meza et al., 2014). An increase in TBARS values has been found following high pressure treatments (200 – 800 MPa, 20 to 70 °C, 20 min) of bovine *Pectoralis profundus* and *Longissimus dorsi* by McArdle et al., (2010) and Ma et al., (2007), respectively, however, pressures greater than 200 MPa (at room temperature) had to be applied for a significant change in TBARS values to occur. In this study, the HPPDA treated muscle had maximum TBARS values of 0.86 ± 0.06 mg MDA/ kg muscle which is below the threshold of 2.00 mg MDA/ kg muscle, indicating that the level of lipid oxidation that occurred following HPPDA is within the limit for consumer acceptability (Campo et al., 2006).

3.7. Myofibrillar fragmentation index (MFI)

A significant increase ($P < 0.05$) in MFI values was observed with aging time. HPPDA significantly increased MFI values ($P < 0.05$) when compared to the control samples, and this was the case for each time point (Fig. 6). A significant interactive effect ($P < 0.05$) was found between aging time and treatment (Fig. 6), where the increase in MFI values of HPPDA treated muscle was greater over the 28 day aging period when compared to the control.

The increase in MFI values over aging time is in agreement with the findings of Li et al., (2014a), Ha et al., (2019) and Ramos et al., (2022) and may be attributed to protein degradation (Taylor et al., 1995; Hopkins et al., 2000). An increase in MFI values following HPP treatment was also reported by Zhang et al., (2018) who found that HPP (200 MPa, 10 min) significantly increased the MFI values of pre-rigor porcine *Longissimus* muscle, when compared to control samples. MFI

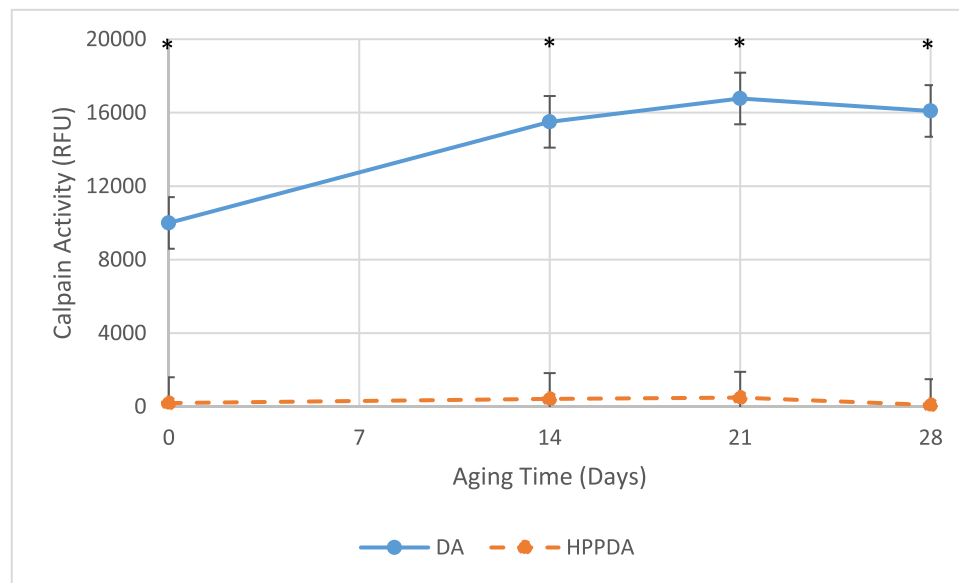


Fig. 7. The effect of aging time and HPP on the calpain activity of dry aged bovine LTL. Results are mean values of four biological replicates $\pm$ SEM.

* indicates significant differences between treatments for calpain activity.

DA – Dry aged, HPPDA – High pressure pre-treatment and dry aged.

values increased further as the pressure was increased to 400 MPa (Zhang et al., 2018). To the best of our knowledge, the effect of HPP on the MFI values of post – rigor bovine muscle has not been examined, however, it has been found that when post – rigor bovine muscle was treated with pressure levels of 200 MPa and above, for 4 to 5 min, the defined filamentous structure of muscle was lost and disruption was caused to the I – band, M – line and Z – line (Bolumar et al., 2021; Jung et al., 2000a; Suzuki et al., 1990). These changes in the microstructure of the muscle may explain the increase in MFI values following HPPDA treatment.

3.8. Calpain activity

Aging time did not have a significant effect ($P > 0.05$) on calpain activity. HPPDA significantly decreased ($P < 0.05$) calpain activity by 97.1 – 99.5 % (Fig. 7) compared to control samples at all aging time points. No significant interaction ($P > 0.05$) was evident between aging time and treatment.

At 4 days post – mortem (equivalent to day 0 of the current study), it has been documented that calpain – 1 activity was already reduced to below 20 % of its measured activity at 0 days post – mortem (Boehm et al., 1998; Camou et al., 2007; Colle & Doumit, 2017), in a range of bovine muscles (*Longissimus lumborum*, *Semimembranosus*, *Triceps brachii*, *Psoas major*). However, in the same muscles at 4 days post – mortem, between 82.4 % and 77.9 % unautolysed calpain – 2 activity (Colle & Doumit, 2017) and 40 % total calpain – 2 activity (Camou et al., 2007) remained when compared to the activity levels of day 0. Other authors have found that total calpain – 2 activity remained unchanged (Geesink & Koohmaraie, 1999; Veiseth et al., 2001) or increased (Phelps et al., 2016), over time, post – mortem, in ovine and bovine muscle.

The decrease in calpain activity observed in the current study following HPPDA is in agreement with Lee et al., (2023), who reported that the HPP treatment (200 MPa, 3 min, 21 °C) of 48 h post – mortem bovine *Semimembranosus*, significantly reduced calpain – 1 activity, with 72.51 % calpain – 1 activity remaining when compared to the control. The higher remaining calpain activity found in the study by Lee et al., (2023) compared to the current study could be due to the shorter HPP treatment time (3 min compared to 20 min).

Despite the decrease in calpain following HPPDA, MFI values increased at a faster rate in the treated samples than controls. The

reasons for this remain unclear. It has been proposed that HPP may facilitate protein degradation via the denaturation and unfolding of proteins, and exposing the cleavage sites of proteolytic enzymes other than calpains, such as cathepsins (Lee et al., 2023; Jung et al., 2000b).

4. Conclusion

Based on the findings of the current study, the HPP treatment (200 MPa for 20 min at 8 °C) increased the Warner-Bratzler shear force values of bovine LTL muscle following 21 days of dry aging, however, no differences were observed following 28 days of dry aging. HPPDA significantly increased lipid oxidation, however, it remained within the acceptable threshold for lipid oxidation. HPPDA had a significant effect on the colour of freshly cut bloomed muscle. However, colour differences between control and HPP treated dry aged beef, were reduced after cooking. It was found that HPPDA increased overall weight loss, during subsequent dry aging. However, higher pressures may be needed for a more significant effect in order for HPP pre-treatment to be considered as a useful strategy for industry, while considering the potential effects on quality attributes. In addition, sensory analysis should be conducted to investigate what effect HPPDA has on the perceived texture and flavour profiles of dry aged bovine muscle.

CRediT authorship contribution statement

Mary Ann Kent: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Eduarda M. Cabral:** Writing – review & editing, Investigation, Formal analysis. **Anne Maria Mullen:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Eileen O’Neill:** Conceptualization, Methodology, Project administration, Supervision, Validation, Writing – review & editing. **Carlos Álvarez:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical statement

This research did not involve studies with humans nor animals.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.afres.2025.100786](https://doi.org/10.1016/j.afres.2025.100786).

Data availability

Data will be made available on request.

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ICoMST, 2022, Kobe, Japan (Conference online) – Poster presentation

Investigating the effect of ultrasound on glycolysis in early post – mortem bovine muscle

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Objectives: Ultrasound (US) is a technology that has the ability to modify enzyme activity, and thus holds potential to manipulate/optimize glycolytic kinetics in early post-mortem muscle. This is of interest as it will allow a better understanding of how these metabolic processes can be manipulated to optimize final meat quality. While numerous studies have used US to modify proteolytic enzyme activity in post-rigor muscle, few have focused on the effect on pre-rigor glycolysis. Therefore, the objective of the study is to investigate the effect of US on the glycolytic related enzymes in early post-mortem muscle using an *in vitro* buffer system.

Materials and Methods: Bovine *Longissimus thoracis et lumborum* (LTL) was collected 1 hour postmortem from 4 heifers (18-26 months) from a commercial slaughtering plant (Kepak Group, Kilbeggan, Ireland) and transported to Teagasc Research Centre, Ashtown. The LTL was cut into 12 steaks (1.5 cm thickness), for controls (6) and treatments (6). The steaks were vacuum packed prior to US: 25 or 45 kHz for 15, 30 or 45 minutes, in a water bath < 10 °C. Controls underwent the same processing, with no US applied. After treatment, one half of the steak was used to monitor pH decline in the intact muscle for 24 h (1440 min); and the other half was snap frozen and processed for use in an *in vitro* glycolytic buffer according to England, Matarneh (1). Aliquots were removed at specific times to determine glycogen, glucose, lactic acid and pH values over time (0 to 1440mins). Glycogen and glucose

were determined using DNS method. A commercial kit (Megazyme, Ireland) was used for lactic acid. pH decline was modelled to the equation: $K = a - b \times e^{(-c \times t)}$ (2) to determine the kinetics of pH decline (c) using the mosaic package in RStudio (3). Data was analysed by ANOVA using MIXED procedure of SAS, considering intensity and duration of treatments and its interaction as a fixed effect, and individuals as random effect. pH and metabolites were evaluated as time-repeated measures using Tukey's test. When analysing kinetics of pH decline, Friedmans test was carried out in Genstat with Samples (Animals) as blocks, comparing 9 treatments.

Results: The *in vitro* buffer system was an effective tool to determine the effect of US on the inherent activity of enzymes within bovine muscle. Under the conditions of this experiment, US (25/45 kHz, 15/30/45 min) did not have a significant effect (p-value>0.05) on the rate of change for pH, glycogen, glucose or lactic acid when analysed over 1440 min in the glycolytic buffer. A similar outcome was observed in the pH profile of intact muscle. Interestingly, while glucose concentrations initially increased, in general a short lived decrease was observed around the 30 or 90 min time points. Glucose levels were highly correlated with pH over the reaction period ($R = -0.75156$, $P < 0.0001$), where glucose content increased exponentially. The inflexion point of the exponential curve was when pH declined below pH 6, indicating a change in the glucose production/consumption balance. This could be a result of a change in the enzymatic activity of phosphofructokinase or hexokinase, as the pH declines over time, where there appears to be a faster rate of glucose production compared to its consumption, allowing it to accumulate towards the later stages of the reaction.

Conclusion: Under our experimental conditions, US appeared to have no effect on the rate of glycolysis, as observed in the *in vitro* glycolytic buffer and intact muscle. However, a shift in the process of glycolysis was observed in the buffer system for all treatments as the pH of the buffer made the transition to below pH 6, where the degradation and accumulation of glycogen and lactic acid, respectively, slowed and glucose accumulated in the system

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Key words: Glycolysis, Ultrasound, pH, Post-mortem metabolism

Investigating the Effect of Ultrasound on Glycolysis Kinetics in Early Post-Mortem Bovine Muscle

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Objectives

- Alter post-mortem glycolysis – the process of converting glucose to ATP, H⁺ and lactate
- Why? – Has an impact on muscle quality and influences muscle shortening and causes other defects such as PSE and DFD

- Ultrasound – used to modify proteolytic enzyme activity in post-rigor muscle
- Principle mode of action - Acoustic Cavitation – Fluctuations in the pressure, continuous expansion and depression of air bubbles, eventually bursting and causing localized disruption in the muscle



- Few studies carried out in pre-rigor muscle and its effects on glycolysis, which is why it was decided to investigate this.



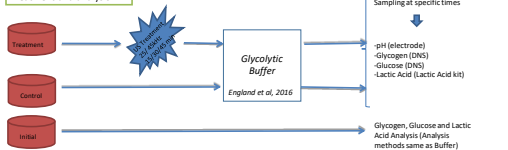
Materials and Methods

Sampling day

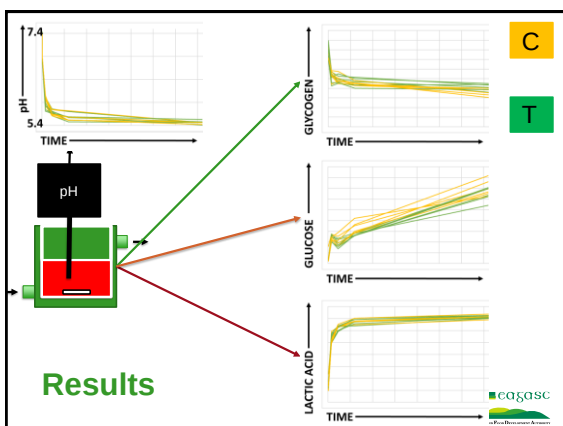
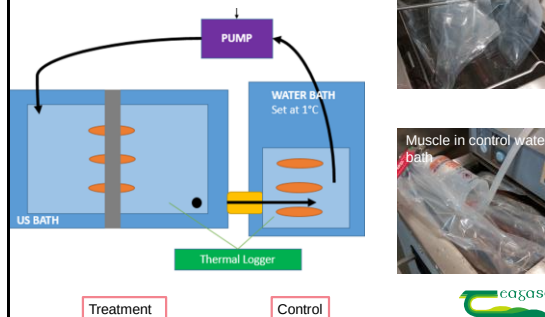
Animals were selected based on cross-breed, age range, gender and carcass conformation to have samples as homogeneous as possible. Muscle were hot-boned in Kepak, Kibbegan and transported to Teagasc, Ashtown via refrigerated van for sampling. Number of animals = 4.



Treatment and analysis



Ultrasound Treatment



Results

pH

- Model extracted from Gagaoua et al., (2021)
- Data modelled using RStudio
- Modelled pH decline of muscle and buffer to calculate rate of pH decline
- Ultrasound had no significant effect on the rate of pH decline in muscle or buffer

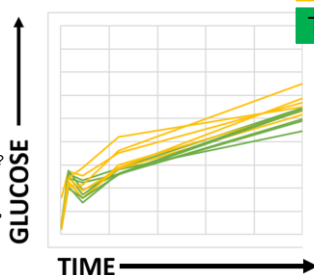
$$K = a - b \times e^{(-\alpha t)}$$

where K represents the parameter, a , its ultimate value, b , the extent of its decline, c , the rate of its decline, and t , the time (h) p.m.



Glucose

- Decline was seen between 30 min and 90 min of reaction before increasing again
- Requirement for glucose-6-phosphate?
- Inhibition of hexokinase/phosphofructokinase?
- Related to enzyme sensitivity to pH?



What happened?

- Matrix of muscle had a protective effect on the enzymes against the muscle?
- Enzymes are not effected by ultrasound!
- Higher ultrasound intensity needed?



- To determine if muscle structure has a protective role, further work is needed to ascertain if muscle structure has a protective role



Conclusions

Muscle

- Rate of pH change in muscle not significantly different (p-value > 0.05)

Buffer

- Experimental conditions did not appear to have a significant effect (p-value>0.05) on the rate of change in pH, glycogen, glucose and lactic acid when analysed over time in the *in vitro* buffer over 1440 min.
- As pH of the buffer made the transition to below pH 6, the degradation and accumulation of glycogen and lactic acid, respectively, slowed
- Glucose accumulation didn't slow down in the system with time



Acknowledgements

The funding for this research was kindly provided by Teagasc and the Walsh Scholarship Scheme.



ICoMST, 2022, Padua, Italy – Poster presentation

Does ultrasound have the capacity to modulate the glycolytic metabolism kinetics?

An *in vitro* study

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I. INTRODUCTION

Post-mortem glycolysis has an influencing effect on the tenderisation of muscle based on the interaction between rate of pH and temperature decline, during the onset of rigor. Ultrasound (US) is a non-thermal novel technology that has the ability to alter the rate of glycolysis though its ability to alter enzymatic activity [1]. The application of US to early post-mortem muscle is of interest as it may present a mechanism whereby the rate of post-mortem pH decline in muscle could be manipulated with the target of improving tenderness. Results of a previous study (not published) that our group carried out on the use of US on early post-mortem muscle showed that US had a significant effect on pH decline: but enzymatic activity was not affected when examined using a glycolytic buffer system. To understand if muscle structure was providing a physical protection to the glycolytic enzymes from the effect of US, the objective of the current study was to investigate the impact of US on the glycolytic activity when US is applied directly to muscle homogenates, prepared using early post-mortem muscle.

II. MATERIALS AND METHODS

Bovine *Longissimus thoracis et lumborum* (LTL) was collected from 4 steers, 1 h post-mortem, from a commercial slaughtering plant and transported to Teagasc Research Centre, Ashtown. The LTL was divided into 6 steaks and randomly assigned to

treatments described. Each steak was cut into small pieces, snap frozen in liquid nitrogen, powdered and stored at -80 °C. For US treatment, muscle was homogenised in water and placed in a jacketed vessel. During treatment, temperature was maintained at 30 °C, stirred constantly. The US treatments were 20 kHz: 60% amp (11.4 W/cm²) or 100% amp (19 W/cm²), for 15 or 30 min, using a US probe. Controls followed the same procedure, without US. Immediately after treatment, homogenates were added to an *in vitro* buffer system [2], where the solution was incubated for 1440 min at 25 °C with constant stirring. Aliquots were removed from the buffer at specific time points to determine glycogen, glucose and lactic acid content and pH. Glucose and glycogen content was determined using the DNS method. Lactic acid content was determined using a lactic acid kit (Megazyme, Ireland). Data was analysed as a repeated measures ANOVA using MIXED procedure of SAS 9.4. Treatment power and duration and their interaction were fixed effects and individual animals were considered random effects, sample time were repeated measures.

III. RESULTS AND DISCUSSION

The experimental conditions used (20 kHz US, 60/100% amp, 15/30 min) had a significant impact ($P < 0.05$) on the rate of pH decline, glucose and lactic acid content over 1440 min. US did not affect ($P > 0.05$) the rate of glycogen degradation. US treatment (100% amplitude (19 W/cm²), 30 min) had a significant effect on pH, and glucose and lactic acid content in the glycolytic buffer, when compared to the other treatments and controls, specifically between time points 30 min and 240 min. The final readings (at 1440 min) of pH and concentration of glucose and lactic acid did not differ significantly between any treatments or control. For controls and homogenates where US treatment (60% amp for 15 and 30 min, 100% amp for 15 min) had no effect, pH declined from 7.4 to 5.4 over 1440 min, with the majority of the decline taking place within the first 90 min. pH decline mirrored lactic acid production (Fig 1A). In these samples, glycogen content decreased rapidly in the first 90 min and then more slowly up to 1440 min. Glucose concentration increased rapidly in the first 240 min and at a slower rate thereafter (Fig 1B). However, following the use of 100% amp US for 30 min, pH decreased at a significantly slower rate with the pH of the glycolytic

buffer system not reaching pH 6 until after 240 min, compared to the control, where pH 6 was attained between 30 and 90 min. This corresponded with a delay in lactic acid production (Fig 1A). The accumulation of glucose accelerated, where the concentration of glucose was significantly greater ($P < 0.05$) than the controls between 30 and 240 min of the reaction. However, at 1440 min, there was no significant differences ($P > 0.05$) observed (Fig 1B). The results indicate that the 100% amp US for 30 min slowed down the consumption of glucose in the buffer system, and impacted reactions further down-stream in the glycolytic pathway, delaying the production of lactic acid, and subsequent pH reduction. Enzymes that may have been affected by the US treatment are hexokinase or phosphofructokinase. Since similar ultimate pH and metabolite concentrations were achieved after 1440 min when compared to controls, the results suggest that the effect of US may involve the significant reduction in enzyme activity.

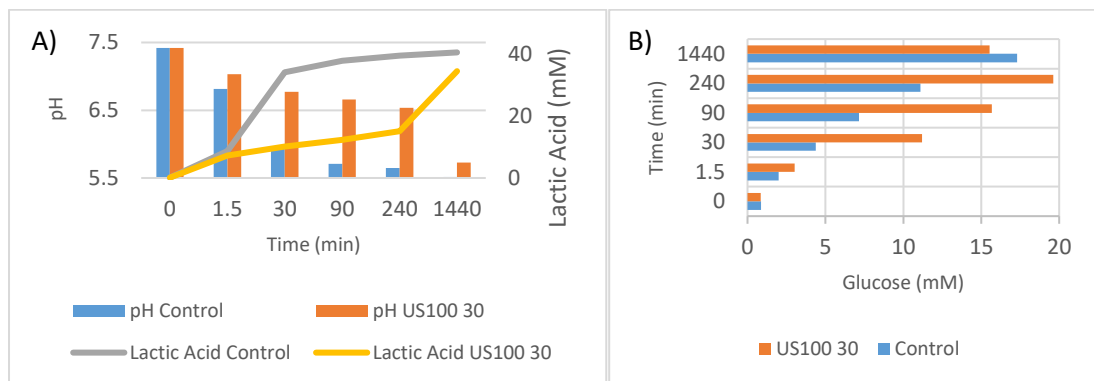


Figure 1. *In vitro* pH, lactic Acid (A) and glucose content (B) following 100% amp, 30 min US and control.

IV. CONCLUSION

Under the experimental conditions, US power and duration (100% amp (19 W/cm²) US for 30 min) had a significant interactive effect on the rate of pH decline, and glucose and lactic acid accumulation, when enzymatic activity of muscle homogenates was assessed *in vitro* using a glycolytic buffer system. This US treatment decreased the rate of pH decline and reduced lactic acid production in the first 240 minutes, while glucose was seen to accumulate. However, no significant differences were observed at 1440 min, indicating that US has the capacity of slow

down the rate of glycolysis but not completely arrest it. Further research is required to identify the specific enzymes affected by US treatment.

ACKNOWLEDGEMENTS

The funding for this research was kindly provided by Teagasc, under the Walsh Fellowship Scheme project 0802.

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DOES ULTRASOUND HAVE THE CAPACITY TO MODULATE THE GLYCOLYTIC METABOLISM KINETICS? AN *IN VITRO* STUDY

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Introduction

Post-mortem glycolysis and rate of pH/temperature decline in muscle has an impact on the tenderness of meat

Ultrasound (US) is a non-thermal technology with potential to alter the rate of glycolysis and pH decline via altering enzymatic activity

Previous study (not published) showed that following the US treatment of whole bovine muscle, no significant effect was observed on the inherent enzyme activity when investigated using an *in vitro* glycolytic buffer → **matrix of muscle protecting glycolytic enzymes?**

Objective

Investigate the impact of US on the inherent glycolytic enzyme activity when US is applied directly to crude enzyme extracts, prepared using early post-mortem bovine LTL muscle.

Materials and Methods



Bovine LTL (n = 4) hot-boned, divided, snap frozen at 2.5 hr post-mortem and powdered



Muscle homogenate from each animal treated with: 20 kHz, 0/11.4/19 W/cm² for 15/30 min, 30 °C



Treated muscle homogenate added to buffer (England et al, 2016), 25 °C, with stirring

pH, glucose, glycogen, lactic acid monitored at 0, 1.5, 30, 90, 240 and 1440 min

Results and Discussion

Under the experimental conditions of this study, US had a significant impact on the rate of pH decline, glucose and lactic acid content over 1440 min.

	NO EFFECT	EFFECT
Treatment	Control (C), 11.4 W/cm ² (60% amp) for 15/30 min, 19 W/cm ² (100% amp) for 15 min	19 W/cm ² (100% amp) for 30 min
pH	pH declined from pH 7.4 to 5.4 over 1440 min, majority of decline took place in first 90 min , where pH 6 was reached.	US reduced rate of pH decline. pH significantly (P > 0.05) higher between 30 and 240 min, when compared to C. pH 6 not reached until after 240 min.
Glycogen	US did not have a significant (P < 0.05) effect on glycogen degradation when compared to C	
Glucose	Content increased rapidly for the first 240 min, and at a slower rate thereafter.	Content significantly (P < 0.05) higher between 30 and 240 min compared to C.
Lactic Acid	Lactic acid content increased over 1440 min. The majority of the increase took place in first 90 min.	Lactic acid content significantly (P > 0.05) lower between 30 and 240 min, when compared to C. Majority of increase after 240 min

No significant differences (P > 0.05) in pH, glucose and lactic acid were observed at 1440 min

The reduced consumption of glucose impacted further reactions downstream such as the production of lactic acid and subsequent decline in pH.

Enzymes possibly impacted by US are phosphofructokinase or hexokinase.

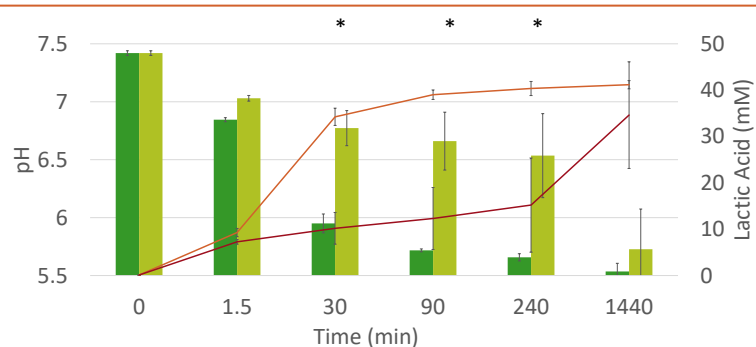


Fig 1. pH and lactic acid content (mM) over time following control and 100%, 30 min US

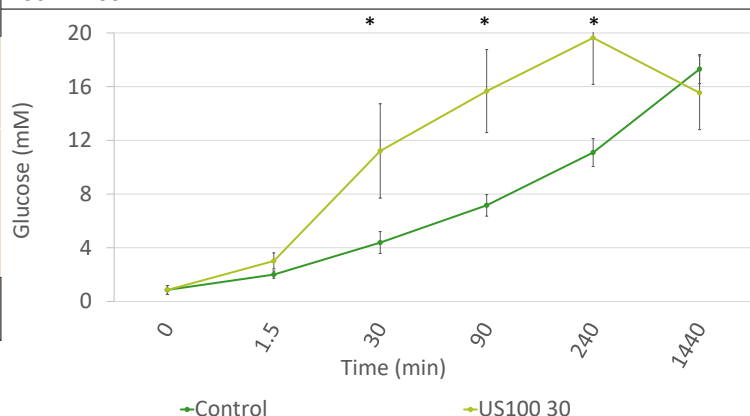


Fig 2. Glucose content (mM) over time following control and 100%, 30 min US

Conclusions

US power and duration (19 W/cm² (100% amp) for 30 min) had a significant interactive effect on pH, glucose and lactic acid content, when enzymatic activity of muscle homogenates was assessed *in vitro* using a glycolytic buffer system. Glycogen content was not effected by US. This US treatment decreased the rate of pH decline and lactic acid production in the first 240 minutes, while glucose was seen to accumulate. No significant differences were observed at 1440 min, indicating that US has the capacity of slow down the rate of glycolysis, *in vitro*, but not completely arrest it.

Further research is required to identify the specific enzymes affected by US treatment.

Acknowledgements and Funding: The funding for this research was kindly provided by Teagasc, under the Walsh Fellowship Scheme project 0802.

Reference: England, E. M., Matarneh, S. K., Oliver, E. M., Apaoblaza, A., Scheffler, T. L., Shi, H., Gerrard, D. E., 2016, Excess glycogen does not resolve high ultimate pH of oxidative muscle, *Meat Science*, 114, pp. 95-102

An investigation on the effect on high pressure processing on the water holding capacity of dry aged beef.

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Abstract: Dry aging involves storing cuts of meat, unwrapped, under controlled conditions (in terms of temperature, humidity and airflow) to produce a characteristic premium flavour and texture. High pressure processing (HPP) which involves the application of high pressure (up to 600 MPa) to muscle has been found to effect the water-holding capacity (WHC) of wet-aged muscle. Changes in the WHC of muscle has implications on cook loss and the juiciness of the cooked product. The aim of the current study was to examine the effects of HPP on the WHC of dry-aged meat by measuring drip loss, cook loss and myofibrillar fragmentation at different time points during dry aging. Bovine *Longissimus lumborum et thoracis* sections were subjected to 200 MPa for 20 min, at an ambient temperature. Treated and non-treated (control) meat was dry aged in refrigerated conditions (4 °C, 80% HR) for 28 days post-treatment. HPP significantly increased drip loss over the 28 days storage period, while drip loss reduced over time of storage. No significant differences were observed in cook loss between pressure treated and control samples at any aging time point; however, cook loss decreased significantly over time. The higher drip loss of muscle that underwent high pressure processing could be linked to higher protein denaturation and fragmentation. Myofibrillar fragmentation was found to be significantly higher at all time points following pressure treatment compared to control samples. Further sensory analysis should be carried out to determine if increased drip loss has an impact on flavour and juiciness of meat.

AN INVESTIGATION ON THE EFFECT OF HIGH PRESSURE PROCESSING ON THE WATER HOLDING CAPACITY OF DRY AGED BEEF.

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Introduction

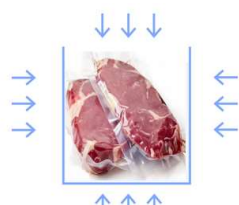
Dry ageing involves storing cuts of meat, unwrapped, under controlled conditions (in terms of temperature, humidity and airflow) to produce a characteristic premium flavour and texture.

High pressure processing (HPP) involves the application of high pressure (up to 600 MPa) to muscle and has been found to effect the water-holding capacity of wet-aged muscle. Changes in the water holding capacity of muscle has implications on cook loss and the juiciness of the cooked product.

Objective

The aim of the current study was to examine the effects of high pressure processing on the water holding capacity of dry-aged beef by measuring drip loss, cook loss and myofibrillar fragmentation at different time points throughout dry aging.

Materials and Methods



Bovine LTL (n = 4) was divided into 8 pieces and randomly assigned to treated/control and storage periods.

Muscle assigned to HPP treated with 200 MPa for 20 min, at an ambient temperature.



Muscle dry aged (4 °C, 80% relative humidity) for 0, 14, 21 and 28 days post-treatment.



Drip loss, cook loss and myofibrillar fragmentation index measured after 0, 14, 21 and 28 days dry ageing.

Results and Discussion

The water holding capacity of muscle was determined through the analysis of drip loss and cook loss of muscle.

High pressure treatment significantly ($P < 0.05$) increased drip loss over the 28 day storage period, while drip loss reduced significantly ($P < 0.05$) over time of storage. However, there was no significant interactive effect ($P > 0.05$) of treatment and storage time on drip loss.

No significant differences ($P > 0.05$) were observed in cook loss when high pressure treated and control samples were compared at any ageing time point. However, cook loss decreased significantly ($P > 0.05$) over storage time.

The higher drip loss observed in muscle that underwent high pressure processing could be as a result of higher protein denaturation and fragmentation. For this reason, the myofibrillar fragmentation index was analysed.

The myofibrillar fragmentation index was found to be significantly ($P < 0.05$) higher at all time points following the high pressure treatment in comparison to control samples. Myofibrillar fragmentation increased significantly ($P < 0.05$) with storage time and a significant interaction ($P < 0.05$) was found between treatment and storage time.

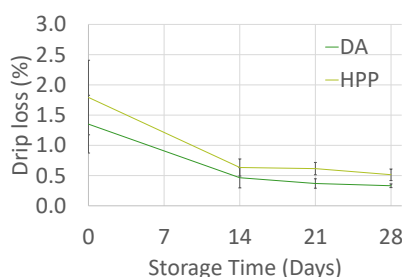


Fig 1. Drip loss (%) over storage time following dry ageing and dry ageing*HPP

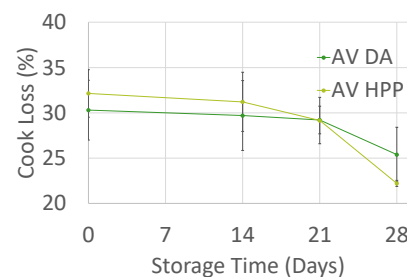


Fig 2. Cook loss (%) over storage time following dry ageing and dry ageing*HPP

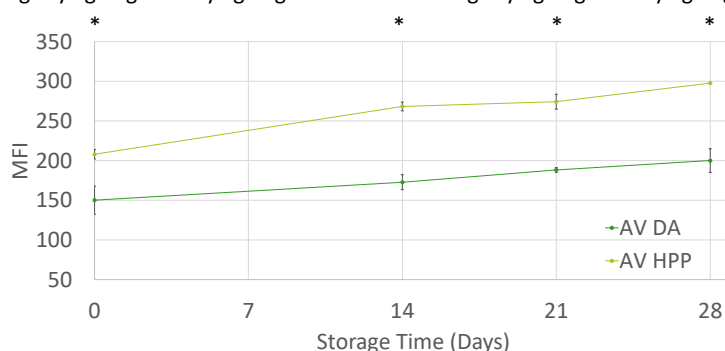


Fig 3. Myofibrillar fragmentation index (MFI), over storage time, following dry ageing and dry ageing*HPP

Conclusions

High pressure processing has been found to decrease the water holding capacity of muscle. This could imply that the application of pressure could be used to accelerate the dry ageing process to reach a specific moisture content. MFI could be correlated with drip loss as the structure of the meat is more disrupted following HPP treatment. Further sensory analysis should be carried out to determine if the increased drip loss has an impact on the flavour and juiciness of meat.

Acknowledgements and Funding: The funding for this research was kindly provided by Teagasc, under the Walsh Fellowship Scheme project 0802.

Under pressure: High pressure processing and quality attributes of dry aged beef

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Abstract: Dry aging involves hanging post rigor muscle under controlled temperature, relative humidity and airflow conditions generally for 14-28 days to produce premium beef with a characteristic texture and flavour. Since the water holding capacity of muscle has been found to decrease following the application of high pressure, high pressure processing (HPP) is a post-mortem intervention that could potentially increase the rate of water loss during dry aging and thus, potentially accelerate the dry aging process. The aim of the current study was to examine the effects of HPP of muscle prior to dry aging on the quality of dry aged beef, in particular tenderness, colour and lipid oxidation. Bovine *Longissimus lumborum* et *thoracis* sections were subjected to 200 MPa for 20 min, at an ambient temperature. Treated and non-treated (control) samples were dry aged in refrigerated conditions (4°C, 80% HR) for 28 days post-treatment. High pressure treatment significantly increased the shear force values of muscles compared to control muscles. When colour was measured following blooming, lightness (L^* values) and blueness (b^* values) were significantly higher in pressure treated muscles when compared to control muscles. Redness (a^* values) was significantly higher in pressure treated muscles at day 0. However, no significant differences in redness (a^* values) were observed between pressurised and control muscle after 14 days of storage. Pressure treatment promoted lipid oxidation, as TBARS values were significantly higher in pressure treated muscles, compared to control muscles, throughout the aging period. Overall, the HPP conditions used in this study had a negative impact on the quality parameters of dry aged beef.

UNDER PRESSURE: HIGH PRESSURE PROCESSING AND QUALITY ATTRIBUTES OF DRY AGED BEEF

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Introduction

Dry ageing (DA) is a process where post rigor muscle is kept under controlled temperature, relative humidity and airflow conditions, generally for 14-28 days to produce premium beef with a characteristic texture and flavour.

High pressure processing (HPP) is a post-mortem intervention that could potentially increase the rate of water loss during dry aging and thus, accelerate the dry ageing process. However, for this to be implemented commercially, the impact of HPP in combination with DA on meat quality must first be determined.

Objective

The aim of the current study was to examine the effects of HPP of muscle prior to dry aging, on the quality of dry aged beef, in particular tenderness, colour and lipid oxidation.

Materials and Methods



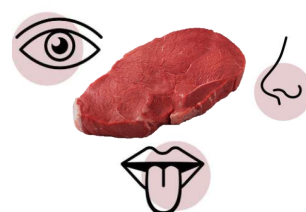
Bovine LTL (n = 4) was divided into 8 pieces and randomly assigned to treated/control and storage periods.



Muscle assigned to HPP treated with 200 MPa for 20 min, at an ambient temperature.



Muscle dry aged (4 °C, 80% relative humidity) for 0, 14, 21 and 28 days post-treatment.



Shear force (WBSF), blooming colour and TBARS measured following 0, 14, 21 and 28 days dry ageing.

Results and Discussion

High pressure treatment significantly ($P < 0.05$) increased the shear force values (WBSF) of muscles compared to control muscles. **No significant differences** ($P > 0.05$) were observed between the two treatments at day 14 and **day 28**. Shear force values decreased significantly ($P < 0.05$) with storage time post – treatment.

When colour was measured following blooming, lightness (L^* values) and yellowness (b^* values) were significantly higher ($P < 0.05$) in pressure treated muscles when compared to control muscles at all time points. Storage time post – treatment did not have a significant effect ($P > 0.05$) on L^* , however, it had a significant effect on b^* .

Redness (a^* values) was significantly higher ($P < 0.05$) in pressure treated muscles at day 0. However, no significant differences ($P > 0.05$) in redness (a^* values) were observed between pressurised and control muscle after 14 days of storage. The a^* values increased significantly ($P < 0.05$) with storage time post – treatment.

Pressure treatment promoted lipid oxidation. When lipid oxidation was analysed as TBARS values were significantly ($P < 0.05$) higher in pressure treated muscles, compared to control muscles, throughout the aging period.

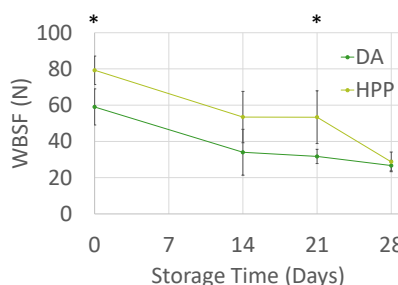


Fig 1. WBSF (N) over storage time following Dry Ageing and Dry Ageing*HPP

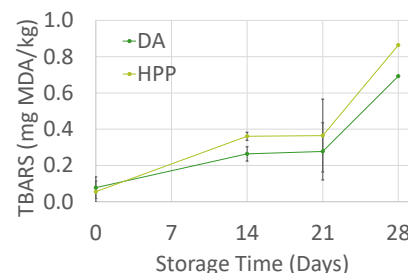


Fig 2. TBARS (mg MDA/kg) over storage time following Dry Ageing and Dry Ageing*HPP

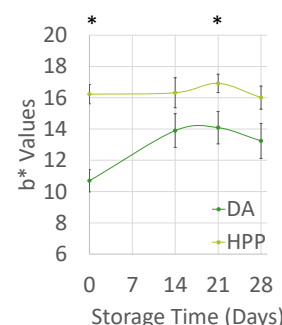
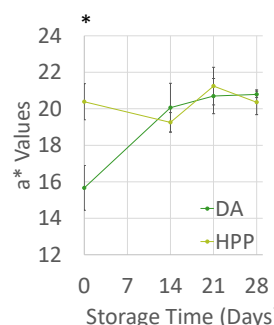
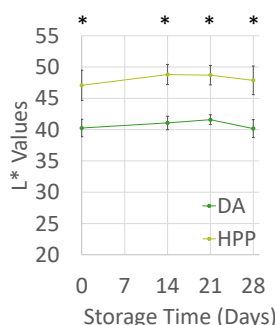


Fig 3. L^* values (lightness) (a), a^* values (redness) (b) and b^* values (yellowness) (c), following blooming, over storage time following dry ageing and dry ageing*HPP

Conclusions

Overall, while HPP resulted in an initial higher WBSF and colour change, after 28 days storage post-treatment, WBSF was not significantly different compared to control samples and TBARS values were within the threshold of acceptability. However, colour, especially L^* and b^* values, were significantly impacted by HPP over the 28 day storage period. An assessment could be carried out on consumer acceptance of the colour change that occurs due to HPP.

Acknowledgements and Funding: The funding for this research was kindly provided by Teagasc, under the Walsh Fellowship Scheme project 0802.

The effect of a high pressure pre-treatment on the metabolomics profile of bovine muscle following dry aging

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Abstract: Dry aging requires storing unwrapped muscle under controlled refrigerated conditions for prolonged periods of time (> 28 days), allowing moisture evaporation and development of beef with a signature flavour. High pressure processing (HPP) involves applying pressure (up to 600 MPa), and has been found to disrupt the structure of protein and enzymatic activity. The aim of the current study was to investigate the effect of HPP on the metabolomic profile of dry aged muscle. Bovine *Longissimus thoracis et lumborum* (N = 4) were treated with HPP (200 MPa, 20 min, 8 °C). Control and treated muscle were dry aged (2 °C, 80% RH, air flow 0.5 – 2.0 m/sec) for 28 days. Untargeted metabolomics analysis was carried out using UHPLC-Q-TOF/MS. PCA and PLS-DA statistical analysis indicated no significant differences between control and treated muscle at day 0. However, significant differences were evident between control and HPP treated samples following 28 days of dry aging. The predominant effect of HPP was an increase in identifiable peptides and phospholipids (VIP > 1, P < 0.05), warranting future sensory and pathway analysis. In summary, metabolomic analysis has identified potential flavour precursors of dry aged beef affected by HPP processing.



Impact of High Pressure Pre – Treatment on the Metabolomic Profile of Dry Aged Bovine Muscle

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Available at <https://doi.org/10.1016/j.meatsci.2024.109000>

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IFSTI Institute of Food Science & Technology of Ireland

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Overview Dry Aging

- Current topic of interest for researchers and industry
- Unwrapped muscle stored under controlled conditions (temperature, air flow and relative humidity) for prolonged periods of time (> 28 days)
- Development of signature meat flavour
- Weight loss - evaporative loss of moisture from surface
- Development of crust
- Yield loss = More expensive meat product

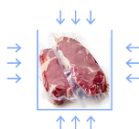


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Overview High Pressure Processing (HPP)

- Application of pressure to food
- Instantaneously and uniformly applied to food
- Meat – up to 600 MPa in literature
- Applied in food industry – increase food safety and shelf life
- Disruption to non – covalent bonds and protein denaturation, membrane permeabilisation, oxidation



3



Overview HPP and Proteolysis of bovine muscle

- HPP disrupts protein structure and enzymatic activity
- Previous study – HPP increased ($P < 0.05$) myofibrillar fragmentation index, decreased ($P < 0.05$) calpain activity (Kent et al, under peer – review)



- Calpain enzymes – primary enzymes responsible for post – mortem proteolysis and tenderisation of meat
- MFI increased with HPP – released lysosomal enzymes and increases accessibility of cleavage sites to enzymes

4



Metabolomics – Potential tool ?

- Metabolomics - study of metabolites (small molecules involved in or produced by biochemical processes) within a biological system e.g. amino acids, peptides, nucleotides, sugars and organic acids – potential flavour precursors in meat
- Methods: Mass spectroscopy (MS or MS/MS – separation with LC/ GC) and nuclear magnetic resonance (NMR)
- Untargeted metabolomics – all metabolites/ Targeted metabolomics – specific metabolites of interest
- Differentiate meat from animals of different feeding regimes/ aging methods/ aging times/adulteration



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Objective



Investigate the impact of high pressure pre-treatment on the metabolomic profile of dry aged bovine *Longissimus thoracis et lumborum*

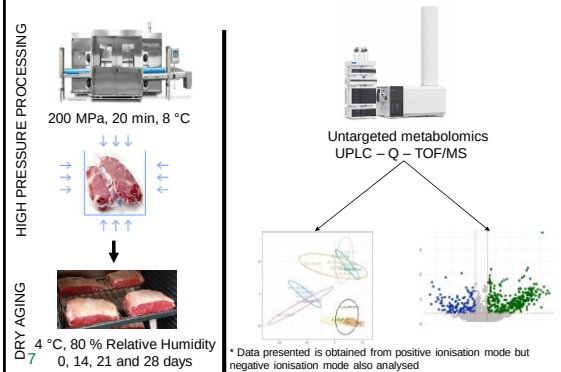


Changes in the metabolomic profile of bovine muscle may influence flavour of dry aged meat



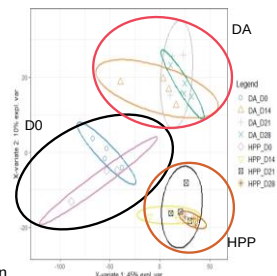
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Materials and Methods



Results

- Metabolomic profile evolved progressively over aging time
- DA and DAHPP samples at D0 not distinguishable using PLS-DA
- Differences emerge between treatments at 14, 21 and 28 days dry aging
- Majority of changes occur within first 14 days of dry aging

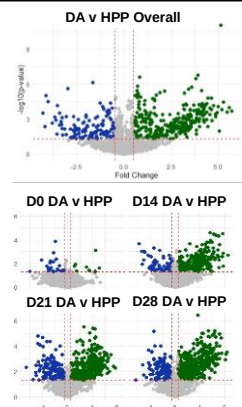


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8

Results cont.

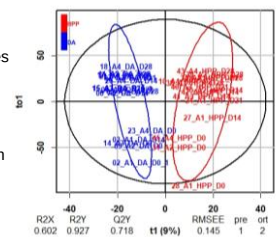
- HPP increased (green) and decreased (blue) many metabolites.
- Changes progressed over time D0 (37 metabolites affected) v D28 (596 metabolites affected) by HPP
- Reflects what is observed in PLS – DA



9

Results cont.

- OPLS-DA – distinguish DA and DAHPP groups
- Significant differential metabolites ($VIP > 1$, $P < 0.05$) between DA and DAHPP treatments
- Peptides and phospholipids main class of identifiable differential metabolites
- Proteolytic enzyme activity/increased extractability



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Conclusions and future work

- Metabolomics is a potential tool to distinguish treatments/aging time
- HPP altered metabolomic profile of dry aged bovine *Longissimus thoracis et lumborum*, compared to control
- Predominant effect of HPP – increase in peptides and phospholipids – proteolysis/enzymatic activity and proteolysis/extractability
- Further research warranted to explore how changes in metabolomic profile impacts on flavour of dry aged muscle

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11

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W
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12

ICoMST, 2025, Girona, Spain – Poster presentation

The effect of high-pressure pre-treatment and dry aging on the metabolomic profile of bovine muscle.

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INTRODUCTION

Dry aging involves the storage of unwrapped muscle under controlled refrigerated conditions for prolonged periods of time (> 28 days), allowing proteolysis and lipid oxidation to occur, along with moisture loss via evaporation of water from the surface of the muscle, leading to the development of a signature beef flavor [1]. The impact of high pressure processing (HPP) before dry aging has been found to disrupt the structure of muscle protein and alter proteolytic activity [2]. Therefore, the aim of the current study was to investigate the impact of HPP pre-treatment, followed by 28 days of dry aging, on the metabolomics profile of bovine *Longissimus thoracis et lumborum* muscle.

MATERIALS AND METHODS

Bovine *Longissimus thoracis et lumborum* muscle (LTL; left and right) was collected from 4 bulls, 72 h post-mortem. Each LTL was divided into 4 sections. Sections were

randomly assigned to two different treatments (dry aging (DA) or dry aging with a high pressure pre-treatment (HPPDA)), at four different dry aging times (0/ 14/ 21/ 28 days). Sections assigned for HPPDA were treated with 200 MPa (20 min, 8 °C). Dry aging was carried out in dry aging chambers for up to 28 days (2 °C, 80% relative humidity, air flow 0.5-2.0 m/sec). At each aging time point, samples were collected, snap frozen and stored at -80 °C until further analysis. One gram of sample was added to 5 ml of HPLC-grade methanol, vortexed for 2 min and homogenised (Ultraturrax for 2 x 30 sec) and treated in an ultrasound bath (25 kHz, 30 min, room temperature). Homogenates were stored at -20 °C for 1 h and transferred to Eppendorf tubes where they were centrifuged (2,000g x 5 min), filtered (0.2 µm PTFE filter) and transferred into HPLC vials. Pooled quality control (QC) samples were prepared. Untargeted metabolomic analysis was performed using UPLC-Q-TOF/MS (Agilent 1290 Infinity II UHPLC and Agilent 6545 Q-TOF/MS) with an ACQUITY UPLC BEH C18 1.7 µm (2.1 mm x 150 mm) column (WATERS Corporation). Mobile phases used were A) water and 0.1% formic acid and B) acetonitrile and 0.1% formic acid, using a gradient over 45 min. Dual AJS electrospray ionisation in positive mode was used to detect metabolites. QC samples were injected every six samples to examine the system stability. Raw mass spectrometry data (.d) was converted to .mzXML format using ProteoWizard (version 3.0.2 (64 bit)). The pre-processing of LC-MS data (peak alignment, retention time correction and peak extraction) was carried out using the XCMS package (version 4.0.2) in RStudio (version 4.3.2). Data was log transformed (normalisation) and centered for fold change and PCA analysis. Pareto scaling was applied prior to discriminant analysis (PLS-DA and OPLS-DA). Significant differential metabolites were those with a VIP score (variable importance in projection (OPLS-DA)) > 1 and *P* (paired t-test) < 0.05. Fold – change (FC) was calculated by comparing the mean of metabolites between treatments (DA/HPPDA) and different time points (D0/D14/D21/D28). Metabolites that increased with time or treatment had a FC > 0.5 and *P* < 0.05 while metabolites that decreased with time or treatment had a FC < - 0.5 and *P* < 0.05. Metabolites were tentatively identified using MassHunter software packages B.07.00 (Agilent Technologies) by comparing the observed accurate mass data and MS/MS spectra with the accurate mass data and spectra of the METLIN Metabolomics and Tandem MS Database (Scripps Center for Metabolomics).

RESULTS AND DISCUSSION

Principal component analysis (PCA) was performed as an initial quality control step, revealing that QC samples were clustered tightly together, indicating reliable and stable analysis (Figure 1). PLS-DA was conducted to assess class separation among sample groups, from which three distinct clusters emerged (Figure 2). DA and HPPDA treated samples overlapped at day 0, suggesting that HPP did not have an immediate effect on the metabolomic profile of bovine muscle. Over time, two clusters emerged, comprising of DA and HPPDA samples with the HPP pretreatment having a significant effect on the profiles of bovine muscle that were aged for 14, 21 and 28 days. HPP and dry aging time induced increases and decreases in metabolite abundance. When analyzing the effects of dry aging time on DA and HPPDA samples (Figure 3), the majority of changes occurred between 0 and 14 days of dry aging.



Figure 1. PCA of different sample groups Figure 2. PLS-DA of different sample groups

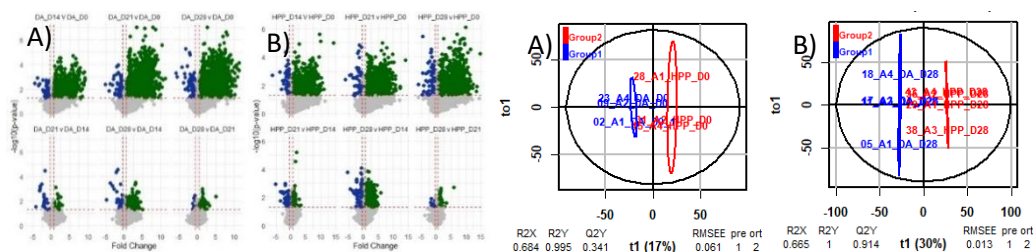


Figure 3. Volcano plots illustrating effect of aging in A) DA and B) HPPDA samples (increase=green, decrease=blue) Figure 4. OPLS-DA model distinguishing A) D0 and B) D28 between DA and HPPDA treatments

OPLS-DA enhanced group separation by removing unrelated variation and identifying metabolites responsible for group differentiation. Using OPLS-DA, it was possible to discriminate, with good predictability, DA and HPPDA samples at D0 and D28 (Figure 4), however, it was possible to differentiate between all sampling time points

between and within treatments (DA/HPPDA). Significant differential metabolites (VIP > 1, $P < 0.05$) distinguishing DA from HPPDA samples included peptides and phospholipids, where HPP appeared to increase their concentrations, potentially due to altered proteolytic enzyme activity, tissue disruption and increased extractability. Significant differential metabolites distinguishing aging time for both treatments included peptides, nucleotides (as well as phospholipids for HPPDA samples). Peptides and phospholipids increased over time, while nucleotides either increased or decreased (IMP decreased over time, conversely to inosine and hypoxanthine). These changes may occur due to proteolysis, energy metabolism and increased extractability. The metabolite groups found to be different between treatment and aging groups play a role in the flavor profile of bovine muscle.

CONCLUSION

Dry aging time and high pressure pre-treatment induced changes to the metabolomic profile of bovine muscle, especially on the concentration of peptides, phospholipids and nucleotides. These differential abundances allowed for the discrimination between DA and HPPDA samples and the four aging times, regardless the pre-treatment. These metabolites may act as precursors to flavor compounds in beef. Further investigations are warranted to determine the effect of HPP on the flavor of dry aged bovine meat.

ACKNOWLEDGEMENTS

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- Kent, M. A., Cabral, E. M., Mullen, A. M., O'Neill, E., Álvarez, C. (2025) Evaluating high pressure processing as a strategy to accelerate water loss and its effect on eating quality in dry aged beef: A preliminary study. *Applied Food Research*, 5: 100786

THE EFFECT OF HIGH PRESSURE PRE-TREATMENT AND DRY AGING ON THE METABOLOMIC PROFILE OF BOVINE MUSCLE

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Introduction

- ✓ Traditional dry aging (DA) involves storing unwrapped muscle under controlled refrigerated conditions for > 28 days to produce premium meat with characteristic flavour.
- ✓ High pressure processing (HPP) has been found to disrupt the structure of muscle protein and alter proteolytic activity in bovine muscle (1).
- ✓ Metabolomics is the study of small molecules/metabolites to understand biochemical processes and responses to treatments such as dry aging and HPP.

Objective

- ✓ The aim of this study was to investigate the impact of HPP pre-treatment on the metabolomic profile of bovine *Longissimus thoracis et lumborum* muscle, monitored over a period of 28 days.

Materials and Methods



Bovine LTL (N = 4, left and right) divided into 8 pieces. Randomly assigned to treated/control and storage times.



Muscle assigned to HPP (HPPDA) treated with 200 MPa for 20 min, at ambient temperature. Control samples also generated (DA).



Muscle dry aged (4 °C, 80% relative humidity) for 0, 14, 21 and 28 days post-treatment.



Muscle samples were prepared and untargeted metabolomic analysis was performed using UPLC-Q-TOF/MS.

Results and Discussion

PLS-DA (Fig. 1) → Assess class separation among sample groups. Three distinct clusters emerged: DA and HPPDA overlapped at day 0 - HPP did not have an immediate effect on the metabolomic profile of bovine muscle; Metabolomic profile changed over time in DA and HPPDA samples, following different evolution.

Volcano Plots (Fig. 2) → HPP and dry aging time induced increases (green) and decreases (blue) in metabolites abundance. The majority of changes occurred between 0 and 14 days of dry aging. Although further changes were detected until 28 d of aging for both groups

OPLS-DA (Fig. 3) → Group differentiation by removing unrelated variation and identifying metabolite features responsible for group differentiation; Possible to discriminate, with good predictability, DA and HPPDA samples at any aging time; HPPDA: Increased peptides and phospholipids; Aging: Increased peptides and dissimilar changes in nucleotides (decreased IMP, increased inosine & hypoxanthine).

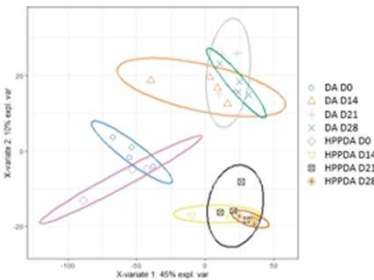


Fig 1. PLS-DA of different sample groups.

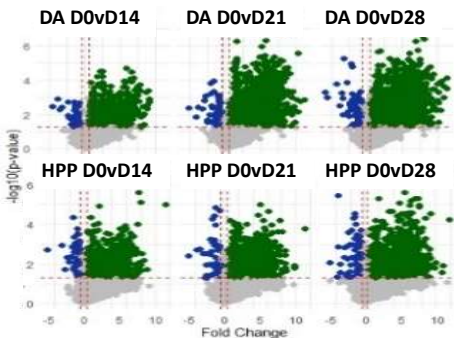


Fig 2. Volcano plots illustrating effects of aging in DA and HPPDA muscle samples (increased = green, decreased = blue).

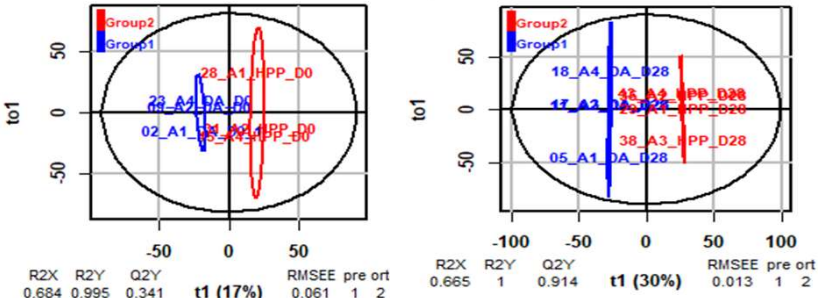


Fig 3. OPLS-DA model distinguishing A) D0 and B) D28 between DA and HPPDA treatments.

Conclusions

- ✓ Dry aging and high pressure pre-treatment induced changes to the metabolomic profile of bovine muscle (peptides, phospholipids and nucleotides). These changes allowed for the discrimination between DA and HPPDA samples and the four aging times, regardless the pre-treatment. These metabolites may act as precursors to flavour compounds in beef. Further investigations are warranted to determine the effect of HPP on the flavour of dry aged bovine meat.

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References: (1) - Kent, M. A., Cabral, E. M., Mullen, A. M., O'Neill, E., & Álvarez, C. (2025). Evaluating high pressure processing as a strategy to accelerate water loss and its effect on eating quality in dry aged beef: A preliminary study. *Applied Food Research*, 5(1), 100786.