

## Lincoln University Digital Thesis

### Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- you will use the copy only for the purposes of research or private study
- you will recognise the author's right to be identified as the author of the thesis and due acknowledgement will be made to the author where appropriate
- you will obtain the author's permission before publishing any material from the thesis.

# **Determining the Mechanism of Pulsed Electric Field for Improving Meat Quality**

---

A thesis  
submitted in partial fulfilment  
of the requirements for the Degree of  
Doctor of Philosophy of Livestock Products Technology

at  
Lincoln University  
by  
Zuhaib Fayaz Bhat

---

Lincoln University  
2019

## Declaration

Some aspects of this thesis have been published and submitted for publication in international peer review journals and presented at conferences.

### Publications

#### Research papers

- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2020). The application of pulsed electric field as a sodium reducing strategy for meat products. *Food Chemistry*, 306, <https://doi.org/10.1016/j.foodchem.2019.125622>.
- Gong, X., Morton, J. D., Bhat, Z. F., Mason, S. L., & Bekhit, A. E. D. A. (2020). Comparative efficacy of actinidin from green and gold kiwi fruit extract on *in vitro* simulated protein digestion of beef *Semitendinosus* and its myofibrillar protein fraction. *International Journal of Food Science and Technology*, 55(02), 742-750.
- Bhat, Z. F., Morton, J. D., Zhang, X., Mason, S. L., & Bekhit, A. E. D. A. (2020). Sous-vide cooking improves the quality and *in vitro* digestibility of *Semitendinosus* from culled dairy cows. *Food Research International*, 127, <https://doi.org/10.1016/j.foodres.2019.108708>.
- Bhat, Z. F., Morton, J. D., Mason, S. L., Jayawardena, R., & Bekhit, A. E. D. A. (2019). Pulsed electric field: A new way to improve digestibility of cooked meat. *Meat Science*, 155, 79-84.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2019). Does pulsed electric field have a potential to improve the quality of beef from older animals and how? *Innovative Food Science and Emerging Technologies*, 56, <https://doi.org/10.1016/j.ifset.2019.102194>.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2019). Pulsed electric field operates enzymatically by causing early activation of calpains in beef during ageing. *Meat Science*, 153, 144-151.
- Bhat, Z. F., Morton, J. D., Mason, S. L., Mungure, T., & Bekhit, A. E. D. A. (2019). Pulsed electric field: Effect on *in vitro* simulated gastrointestinal protein digestion of deer *Longissimus dorsi*. *Food Research International*, 120, 793-799.

- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2019). Pulsed electric field improved protein digestion of beef during *in vitro* gastrointestinal simulation. *LWT-Food Science and Technology*, *102*, 45-51.
- Bhat, Z. F., Morton, J. D., Mason, S. L., Mungure, T., Jayawardena, R., & Bekhit, A. E. D. A. (2019). Effect of pulsed electric field on calpain activity and proteolysis of venison. *Innovative Food Science and Emerging Technologies*, *52*, 131-135.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Pulsed electric field: Role in protein digestion of beef *Biceps femoris*. *Innovative Food Science and Emerging Technologies*, *50*, 132-138.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Calpain activity, myofibrillar protein profile and physicochemical properties of beef *Semimembranosus* and *Biceps femoris* from culled dairy cows during ageing. *Journal of Food Processing and Preservation*, *42*(12), e-13835.

## Review articles

- Mir, S. S., Bhat, H. F., & Bhat Z. F. (2020). Dynamic actin remodeling in response to lysophosphatidic acid. *Journal of Biomolecular Structure and Dynamics*, DOI: 10.1080/07391102.2019.1696230.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2019). Current and future prospects for the use of pulsed electric field in the meat industry. *Critical Reviews in Food Science and Nutrition*, *59*, 1660-1674.
- Bhat, Z. F., Morton, J. D., Mason, S. L., Bekhit, A. E. D. A. & Bhat, H. F. (2019). Technological, regulatory and ethical aspects of *in vitro* meat: A future slaughter-free harvest. *Comprehensive Reviews in Food Science and Food Safety*, Doi: 10.1111/1541-4337.12473.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Applied and emerging methods for meat tenderization: A comparative perspective. *Comprehensive Reviews in Food Science and Food Safety*, *17*, 841-859.

- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Role of calpain system in meat tenderness. *Food Science and Human Wellness*, 7, 196-204.
- Bhat, Z. F., Mason, S. L., Morton, J. D., Bekhit, A. E. D. A., & Bhat, H. F. (2017). Obesity and neurological disorders: Dietary perspective of a global menace. *Critical Reviews in Food Science and Nutrition*, 59, 1294-1310.

## Book chapters

- Bekhit, A. E. D. A., Morton, J. D., Bhat, Z. F., & Kong, L. (2019). Meat colour: Factors affecting colour stability. In: *Encyclopaedia of Food Chemistry*, Melton, L., Shahidi, F., & Varelis, P. (eds.), Reference Module in Food Science, Elsevier Publications, pp. 202-210.
- Bekhit, A. E. D. A., Morton, J. D., Bhat, Z. F., & Zequan, X. (2019). Meat colour: Chemistry and measurement systems. In: *Encyclopaedia of Food Chemistry*, Melton, L., Shahidi, F., & Varelis, P. (eds.), Reference Module in Food Science, Elsevier Publications, pp. 211-217.
- Morton, J. D., Bhat, Z. F., & Bekhit, A. E. D. A. (2019). Proteases and Meat Tenderization. In: *Encyclopaedia of Food Chemistry*, Melton, L., Shahidi, F., & Varelis, P. (eds.), Reference Module in Food Science, Elsevier Publications, pp. 309-313.
- Bhat, Z. F., Mason, S. L., Morton, J. D., Bekhit, A. E. D. A., & Bhat, H. F. (2017). Antihypertensive peptides from animal proteins. In: *Bioactive Molecules in Food*, Mérillon, J. M., & Ramawat, K. G. (eds.), Reference Series in Phytochemistry, Springer International Publishing, pp. 1-36.

## Presentations

- Bhat, Z. F., Morton, J. D., Mason, S. L., Jayawardena, R., & Bekhit, A. E. D. A. (2019). Pulsed electric field improves the protein digestion of venison (*Cervus elaphus*) during *in vitro* gastrointestinal digestion. 65<sup>th</sup> International Congress of Meat Science Technology (ICoMST) 2019, 04-09<sup>th</sup> August 2019, Berlin, Germany (Poster presentation).
- Bhat, Z. F., Morton, J. D., Mason, S. L., Jayawardena, R., & Bekhit, A. E. D. A. (2019). Does pulsed electric field have a role in the sodium reduction strategy for meat products? NZIFST conference 2019, 02-04<sup>th</sup> July 2019, Christchurch, New Zealand (Poster presentation).

- Bhat, Z. F., Morton, J. D., Mason, S. L., Jayawardena, R., & Bekhit, A. E. D. A. (2019). Sous-vide processing improves the quality and *in vitro* protein digestion of beef from culled dairy cows. 5<sup>th</sup> International Conference on Food Structures, Digestion and Health 2019, 30<sup>th</sup> September to 3<sup>rd</sup> October 2019, Rotorua, New Zealand (Poster presentation).
- Jayawardena, R., Morton, J. D., Brennan, C., Bhat, Z. F., & Bekhit, A. E. D. A. (2019). Optimizing the processing of beef lung protein powder to provide a nutritious ingredient rich in proteins and minerals. NZIFST conference 2019, 02-04<sup>th</sup> July 2019, Christchurch, New Zealand (Poster presentation).
- Jayawardena, R., Morton, J. D., Brennan, C., Bhat, Z. F., & Bekhit, A. E. D. A. (2019). Processing of beef lungs as a nutritious protein supplement and evaluate the processing effect on *in vitro* digestibility. 65<sup>th</sup> International Congress of Meat Science Technology (ICoMST) 2019, 04-09<sup>th</sup> August 2019, Berlin, Germany (Poster presentation).
- Bhat, Z. F., Morton, J. D., Mason, S. L., Bekhit, A. E. D. A., & Jayawardena, R. (2018). Effect of pulsed electric field on the calpain activity and proteolysis of beef *Semimembranosus* muscle. 64<sup>th</sup> International Congress of Meat Science Technology (ICoMST) 2018, 12-17<sup>th</sup> August 2018 Melbourne, Australia (Oral presentation).
- Bhat, Z. F., Morton, J. D., Mason, S. L., Bekhit, A. E. D. A., & Jayawardena, R. (2018). Effect of pulsed electric field on the calpain activity and proteolysis of beef *Biceps femoris* muscle. Lincoln University Post-graduate Conference-2018, 20-21<sup>st</sup> & 24<sup>th</sup> September 2018, Lincoln University, New Zealand (Oral presentation).
- Jayawardena, R., Morton, J. D., Brennan, C., Bhat, Z. F., & Bekhit, A. E. D. A. (2018). Beef lung protein powder as a functional ingredient to enhance protein and iron content of plant-based diets. 64<sup>th</sup> International Congress of Meat Science Technology (ICoMST) 2018, 12-17<sup>th</sup> August 2018 Melbourne, Australia (Poster presentation).
- Jayawardena, R., Morton, J. D., Brennan, C., Bhat, Z. F., & Bekhit, A. E. D. A. (2018). Beef lung protein powder as a functional ingredient for plant-based diets. Lincoln University Post-graduate Conference-2018, 20-21<sup>st</sup> & 24<sup>th</sup> September 2018, Lincoln University, New Zealand (Poster presentation).

## Awards

- Best poster presentation award, NZIFST conference 2019, 02-04<sup>th</sup> July 2019, Christchurch, New Zealand.
  
- Travel award 2019 by New Zealand Society for Biochemistry and Molecular Biology for ICOMST-2019, Berlin, Germany.
  
- MacMillan Brown 2018, Lincoln University, New Zealand.
  
- Lincoln University travel grant 2018 to attend ICOMST-2018 at Melbourne, Australia.
  
- Faculty Research Award 2018 in Nutrition and Nursing. Hosted by news magazine Careers360 and awarded by Human Resource Development Minister of India.
  
- Best poster presentation award in Lincoln University Post-graduate Conference-2018, 20-21<sup>st</sup> & 24<sup>th</sup> September 2018, Lincoln University, New Zealand.

## **Supervisory team**

Associate Professor Dr. James David Morton was the principal supervisor and Dr. Susan L. Mason was the associate supervisor and Dr. Alaa El-Din A. Bekhit was the external supervisor for this PhD programme.



Abstract of a thesis submitted in partial fulfilment of the requirements for the  
Degree of Doctor of Philosophy of Livestock Products Technology

Determining the Mechanism of Pulsed Electric Field for Improving Meat Quality

by

Zuhaib Fayaz Bhat

Pulsed electric field (PEF) is a novel non-thermal technology that has recently attracted the attention of meat scientists and technologists due to its ability to modify membrane structure and enhance mass transfer. Several studies have confirmed the potential of PEF for improving meat tenderness in both pre-rigor and post-rigor muscles during ageing. However, there is a high degree of variability between studies and the underlying mechanisms for the reported outcomes are not clearly understood. While some studies have suggested physical disruption as the main cause of PEF induced tenderness, enzymatic nature of the tenderization seems to be the most plausible mechanism. Several studies have suggested the potential of PEF to mediate the tenderization process due to its membrane altering properties causing early release of calcium ions and early activation of the calpain proteases. However, experimental research is yet to confirm this postulation.

The changes in the calpain activity and protein profile of beef from older animals during ageing is poorly explored and further understanding of biochemical processes is needed to design useful approaches for tenderization. Relatively few experiments have examined the effects of ageing on the quality of beef from older animals. To elucidate the changes in the calpain activity and protein profile of beef from culled dairy cows during ageing, cold-boned *Semimembranosus* (n=6) and *Biceps femoris* (n=6) were vacuum packaged and stored for 14 days at  $4\pm 1$  °C. A significant ( $P<0.05$ ) effect of ageing was observed on the pH, shear force and myofibrillar fragmentation index of both the muscles. Casein zymography results indicated the presence of intact and autolyzed forms of calpain 1 and calpain 2. An increase in proteolysis was observed in both the muscles during ageing. Ageing for two weeks resulted in reduction ( $P<0.05$ ) of the shear force of both muscles by 30%, however, the aged muscles were still excessively tough.

To evaluate the impact of PEF on the quality of beef from older animals, cold-boned beef *Semimembranosus* (n=6) and *Biceps femoris* (n=6) were processed with two different PEF treatments viz. T1 (5 kV, 90 Hz, 20  $\mu$ s) and T2 (10 kV, 20 Hz, 20  $\mu$ s) and were vacuum packaged and stored for 14 days at  $4\pm 1$  °C along with a non-treated control. Samples from venison *Longissimus dorsi* (n=6) were also treated with PEF [T1 (2.5 kV, 50 Hz, 20  $\mu$ s) and T2 (10 kV, 90 Hz, 20  $\mu$ s)] and were subjected to 21

days of ageing at  $4\pm 1$  °C. An improvement was recorded in calpain activity of all the samples treated with PEF along with an early activation of calpain 2 in beef. Increased proteolysis of troponin-T and desmin was also recorded, however, no significant ( $P>0.05$ ) impact was observed on the shear force and myofibrillar fragmentation index of any of the muscles. These results provided an experimental evidence for the enzymatic nature of PEF.

PEF has been reported to affect the structural and functional properties of proteins, which suggests that it may influence the digestion of meat proteins. To evaluate the impact of PEF on the protein digestion kinetics, PEF-treated beef *Semimembranosus* and *Biceps femoris* [T1 (5 kV, 90 Hz, 20  $\mu$ s) and T2 (10 kV, 20 Hz, 20  $\mu$ s)] and venison *Longissimus dorsi* [T1 (2.5 kV, 50 Hz, 20  $\mu$ s) and T2 (10 kV, 90 Hz, 20  $\mu$ s)] were subjected to *in vitro* simulated gastrointestinal digestion. Both raw ( $n=3$ ) and cooked ( $n=3$ ) samples were used separately. Samples were collected at 0, 30, and 60 minutes of gastric digestion and 120 and 180 minutes of intestinal digestion. PEF processing affected the digestion kinetics of all the muscles by modifying the protein profile (SDS-PAGE) of the meat digests and significantly ( $P<0.05$ ) increasing the protein digestibility (%) and soluble protein (%). Concentration of almost all the free amino acids in all muscles were numerically higher ( $P>0.05$ ) for the PEF treated samples whereas no significant ( $P>0.05$ ) impact was observed on the release of minerals such as Fe, Zn, Cr, Cu, Mg, Ni, Na or K. PEF processing improved the digestion kinetics of the beef and venison during gastrointestinal digestion simulation in both raw and cooked samples.

PEF has been shown to accelerate salt diffusion and enhance mass transfer processes in meat indicating a possibility for its application in sodium reduction management. By influencing the cellular and membrane permeability and by affecting structural and functional properties of proteins, PEF is expected to improve the salt diffusion and possibly sodium perception during chewing. The potential use of PEF to reduce sodium in processed meat was investigated using beef jerky as a model system ( $n=6$ ). Beef jerky was prepared using different levels of NaCl viz. 2.0% (control), 1.2% (T1) and 1.2% along with PEF-processing (T2, 0.52 kV/cm, 10 kV, 20 Hz, 20  $\mu$ s). A significant ( $P<0.05$ ) effect of PEF was observed on shear force (N), toughness (N/mm.sec) and firmness (N/mm) of the products, which was also reflected in the sensory scores. PEF-treated samples (T2) had significantly ( $P<0.05$ ) lower sodium content than the control, however, the sensory scores were comparable ( $P>0.05$ ) with control and more than 84% of the panellists preferred T2 samples over T1 for saltiness. No undesirable effects of PEF were observed on colour, yield (%) and oxidative and microbial stability. These results suggest that PEF has a sodium-reduction potential that can play a role in public health.

The general scope of the present project was to elucidate how PEF affects the meat quality during ageing and to explore the possible application of PEF in protein digestion and sodium reduction. It was expected that this project will decipher the mechanisms of how PEF affects meat quality and protein digestion and elucidate a possible role of PEF in sodium reduction. Research on PEF-assisted sodium reduction and its role in protein digestion is unavailable in the literature.

**Keywords:** Pulsed electric field, *Semimembranosus*, *Biceps femoris*, *Longissimus dorsi*, ageing, calpains, casein zymography, shear force, proteolysis, western blotting, *in vitro* gastrointestinal digestion, protein digestibility, free amino acids, SDS-PAGE, mineral profile, sodium reduction, lipid oxidation, protein oxidation, microbial stability, sensory analysis.

## Acknowledgements

With unending humility, at every outset, I would like to thank, “The almighty God”, who blessed me with limitless strengths and favourable circumstances, to face and pass through all odds successfully at this juncture.

At the outset of this epistle, I consider myself fortunate and greatly privileged to have worked under the supervision and guidance of Associate Professor Dr. James D. Morton. Words are inadequate to express my sincere and deepest feelings of gratitude for his kind affection, benevolent guidance, meticulous supervision, wholehearted encouragement, critical appreciation in execution of my work and for all the trust he had in my ability primarily responsible for the present accomplishment.

It is my sole prerogative to place on record my indebtedness and everlasting gratitude to the members of my advisory committee Dr. Susan L. Mason and Dr. Alaa El-Din A. Bekhit for their help, innovative guidance and invaluable suggestions during my PhD programme.

I avail myself of this rare opportunity to express my ecstatic thanks to Dr. Hannah Lee for her technical assistance and impeccable guidance.

I duly acknowledge thanks to the official staff at Lincoln University for timely and sincere help during the course of my entire study. I am particularly thankful to laboratory technicians Karl Gately and Leo Vanhanen for their continued assistance throughout my research.

I owe a great deal of thanks to the staff of department of Food Science, Otago University for being kind enough to provide all the necessary facilities and guidance.

I owe my gratitude towards Jennifer Kwan, PhD student and staff of Silver-fern Farms, Belfast for her help and support.

I record with immense pleasure, my sincere thanks to my colleagues Claire, Olivia, Nadia and friends Venkata, Brenda, Emily, Fatima, Maduri, Maina and all Residential Assistants (Georgia, Ashleigh, Kate, James Berry, Madison, Charan, Rebecca, Darshil, James Lee, Hannah, Josh, Tom) and staff of accommodation office (Jamie, Trevor, Rachel, Kiersten) for their wholehearted support and for sharing the moments of laughter and sorrow during this investigation.

Words fail to describe the love and moral support I received during stay at Lincoln University. In this perspective, I wish to exert my eulogy and whole-hearted endearment to my friends Reshan and Rouaf for their psychological and moral support during the times when I required the most.

All the words in the lexicon will be futile and meaningless, if I fail to divulge my extreme sense of regards to adorable parents for their sacrifice, prayers and blessings. Where emotions are involved, words cease to mean, and this happens when I think of my father who sacrificed all pleasure of life for cause of his children. With heartiest reverence, I admire the confidence bestowed on me by my mom (Mrs Dilshada Fayaz) and dad (Mr. Fayaz A. Bhat). Whatever I am today, I owe it to them.

Words sound hollow and phrases lose their meaning, as I find myself unable to put forth my warmest love and thanks to my sisters, Mrs Huma and Dr. Hina and my brother in laws, Dr. Mushtaq and Dr. Rais for their tender attachment and constant encouragement which helped me the most during crisis ridden phase of this mammoth task.

I have no words to express my love and affection for my nieces (Milhaan and Khirad) and my nephew.

A very special gratitude goes to Lincoln University and Indian Council of Agricultural Research (ICAR), New Delhi for all help and financial support during my PhD.

No words or phrases can convey my exact feelings towards that unknown soul behind the screen that will become my life partner one day.

# Short Table of Contents

Chapter 1 Introduction and Literature Review.....	1-39
Chapter 2 Materials and Methods.....	40-69
Chapter 3 Calpain activity, myofibrillar protein profile and physicochemical properties of beef <i>Semimembranosus</i> and <i>Biceps femoris</i> from culled dairy cows during ageing.....	70-80
Chapter 4 Does pulsed electric field have a potential to improve the quality of beef <i>Semimembranosus</i> from older animals and how? .....	81-93
Chapter 5 Pulsed electric field operates enzymatically by causing early activation of calpains in beef <i>Biceps femoris</i> during ageing .....	94-106
Chapter 6 Effect of pulsed electric field on calpain activity and proteolysis of venison .....	107-114
Chapter 7 Pulsed electric field improved protein digestion of beef <i>Semimembranosus</i> during <i>in vitro</i> gastrointestinal simulation .....	115-126
Chapter 8 Pulsed electric field: Role in protein digestion of beef <i>Biceps femoris</i> .....	127-137
Chapter 9 Pulsed electric field: Effect on <i>in vitro</i> simulated gastrointestinal protein digestion of deer <i>Longissimus dorsi</i> .....	138-149
Chapter 10 Pulsed electric field: A new way to improve digestibility of cooked beef .....	150-160
Chapter 11 Cooking does not impair the impact of pulsed electric field on the protein digestion of venison during <i>in vitro</i> gastrointestinal simulation .....	161-171
Chapter 12 Does pulsed electric field have a role in the sodium reduction strategy for meat products? .....	172-185
Chapter 13 General Discussion and Conclusion .....	186-191
Appendix.....	193-194
References .....	195-235

# Table of Contents

<b>Abstract .....</b>	<b>viii</b>
<b>Acknowledgements .....</b>	<b>xi</b>
<b>Short Table of Contents .....</b>	<b>xiii</b>
<b>Table of Contents .....</b>	<b>xiv</b>
<b>List of Tables .....</b>	<b>xxiii</b>
<b>List of Figures .....</b>	<b>xxvi</b>
<b>Abbreviations and Symbols.....</b>	<b>xxxi</b>
<b>Chapter 1 Introduction and Literature Review .....</b>	<b>1</b>
1.1 Introduction .....	1
1.2 Applications in food industry .....	2
1.3 Mechanism of action .....	2
1.4 PEF effect on meat quality .....	4
1.4.1 Meat tenderness.....	9
1.4.1.1 Mechanism of action for PEF on meat texture .....	10
1.4.1.2 Effect of PEF on post-rigor (cold-boned) muscles.....	10
1.4.1.3 Effect of PEF on pre-rigor (hot-boned) muscles.....	14
1.4.2 Proteolysis.....	15
1.4.2.1 Calpain system .....	16
1.4.2.2 Proteolysis of Desmin and Troponin-T.....	23
1.4.2.3 Effect of PEF on Proteolysis of Desmin and Troponin-T.....	25
1.4.3 Digestibility of meat proteins.....	28
1.4.4 Mineral content .....	29
1.4.5 Meat safety .....	31
1.4.6 Processed meats .....	33
1.4.6.1 Accelerated diffusion processes.....	33
1.4.6.2 Sodium reduction.....	34
1.4.6.3 Restructured meat products .....	35
1.4.6.4 Lipid and protein oxidation .....	35
1.4.6.5 Sensory quality .....	35
1.4.7 Supercooling .....	36
1.5 Limitations of PEF.....	36
1.6 Research gap.....	37
1.7 Aim of Research .....	38
1.8 Objectives .....	38
1.9 Hypothesis.....	38

1.10	Thesis structure.....	39
<b>Chapter 2 Materials and Methods .....</b>		<b>40</b>
2.1	Overview .....	40
2.2	Pulsed electric field and meat samples .....	41
2.2.1	PEF machine.....	41
2.2.2	Beef muscles used for ageing (for chapter 3) .....	41
2.2.3	Beef muscles used for PEF processing (for chapter 4, 5, 7, 8, 10) .....	41
2.2.3.1	PEF processing conditions for beef muscles (for chapter 4, 5, 7, 8, 10) .....	43
2.2.4	Beef muscles used for PEF processing for beef jerky (for chapter 12) .....	43
2.2.4.1	PEF processing conditions for beef muscles for beef jerky (for chapter 12) .....	44
2.2.5	Venison muscles used for PEF processing (for chapter 6, 9, 11) .....	44
2.2.5.1	PEF processing conditions for venison muscles (for chapter 6, 9, 11).....	44
2.2.6	Electrical input .....	48
2.2.7	Change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ ) .....	48
2.3	Meat Quality .....	48
2.3.1	Shear force .....	48
2.3.2	Purge loss (%) and cooking loss (%) .....	50
2.3.3	Myofibrillar fragmentation index (MFI) .....	50
2.4	Protein Analysis.....	51
2.4.1	Preparation of sarcoplasmic and myofibrillar fractions.....	51
2.4.2	Protein concentration .....	51
2.4.3	Casein zymography .....	52
2.4.4	SDS-PAGE .....	52
2.4.5	Immunoblotting (Western blot analysis) .....	54
2.4.6	Equipment used for biochemical analysis.....	55
2.5	<i>In vitro</i> gastrointestinal protein digestion simulation .....	57
2.5.1	<i>In vitro</i> protein digestion of raw meat samples.....	57
2.5.2	<i>In vitro</i> protein digestion of cooked meat samples .....	57
2.5.3	<i>In vitro</i> protein digestibility (%).....	58
2.5.4	Protein content (%) .....	59
2.5.5	Soluble protein (%).....	59
2.5.6	Free amino acid analysis .....	59
2.5.7	Mineral profile analysis.....	60
2.6	Preparation of beef jerky .....	62
2.6.1	Yield (%).....	62
2.6.2	Water activity ( $a_w$ ).....	62
2.6.3	pH .....	62



2.6.4	Instrumental texture analysis .....	63
2.6.5	Colour measurement .....	64
2.6.6	Sensory evaluation .....	64
2.6.7	Thiobarbituric acid reacting substances (TBARS).....	65
2.6.8	Protein oxidation by DNPH (dinitrophenylhydrazine) method.....	66
2.6.9	Microbiological characteristics .....	66
2.6.9.1	Sample preparation.....	66
2.6.9.2	Total plate count .....	67
2.6.9.3	Coliform count.....	68
2.6.9.4	Yeast and mould count .....	68
2.7	Statistical analysis .....	68
2.7.1	Chapter 3, 4, 5, 6, 12 .....	68
2.7.2	Chapter 7, 8, 9.....	68
2.7.3	Chapter 10, 11.....	69

<b>Chapter 3 Calpain activity, myofibrillar protein profile and physicochemical properties of beef <i>Semimembranosus</i> and <i>Biceps femoris</i> from culled dairy cows during ageing .....</b>		<b>70</b>
3.1	ABSTRACT.....	70
3.2	INTRODUCTION.....	70
3.3	MATERIALS AND METHODS.....	71
3.4	RESULTS AND DISCUSSION.....	72
3.4.1	pH.....	72
3.4.2	Shear force (N) .....	73
3.4.3	Myofibrillar fragmentation index (MFI) .....	75
3.4.4	Calpain activity .....	76
3.4.5	Myofibrillar protein profile (SDS-PAGE).....	77
3.5	CONCLUSIONS.....	80

<b>Chapter 4 Does pulsed electric field have a potential to improve the quality of beef <i>Semimembranosus</i> from older animals and how?.....</b>		<b>81</b>
4.1	Abstract.....	81
4.2	Introduction .....	81
4.3	Materials and methods.....	83
4.4	Results and discussion .....	83
4.4.1	Electrical input .....	83
4.4.2	Change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ ).....	83
4.4.3	Purge loss (%).....	85
4.4.4	Cooking loss (%) .....	85
4.4.5	Shear force (N) .....	86
4.4.6	Myofibrillar fragmentation index (MFI) .....	88

4.4.7	Calpain activity .....	89
4.4.8	Proteolysis of desmin and troponin-T.....	90
4.4.9	Myofibrillar protein profile (SDS-PAGE).....	92
4.5	Conclusions .....	93
<b>Chapter 5 Pulsed electric field operates enzymatically by causing early activation of calpains in beef <i>Biceps femoris</i> during ageing .....</b>		<b>94</b>
5.1	Abstract.....	94
5.2	Introduction .....	94
5.3	Materials and methods.....	96
5.4	Results and discussion .....	96
5.4.1	Electrical input .....	96
5.4.2	Change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ ) .....	97
5.4.3	Purge loss (%).....	98
5.4.4	Cooking loss (%) .....	99
5.4.5	Shear force (N) .....	100
5.4.6	Myofibrillar fragmentation index (MFI) .....	101
5.4.7	Calpain activity.....	102
5.4.8	Proteolysis of desmin and troponin-T.....	103
5.4.9	SDS-PAGE (Myofibrillar protein profile).....	105
5.5	Conclusions .....	106
<b>Chapter 6 Effect of pulsed electric field on calpain activity and proteolysis of venison .....</b>		<b>107</b>
6.1	Abstract.....	107
6.2	Introduction .....	107
6.3	Materials and methods.....	109
6.4	Results and discussion .....	109
6.4.1	Shear force .....	109
6.4.2	Myofibrillar fragmentation index.....	110
6.4.3	Calpain activity .....	111
6.4.4	Myofibrillar protein profile (SDS-PAGE).....	112
6.4.5	Proteolysis of desmin and troponin-T.....	113
6.5	Conclusions .....	114
<b>Chapter 7 Pulsed electric field improved protein digestion of beef <i>Semimembranosus</i> during <i>in vitro</i> gastrointestinal simulation .....</b>		<b>115</b>
7.1	Abstract.....	115
7.2	Introduction .....	115
7.3	Materials and methods.....	117
7.4	Results and discussion .....	117
7.4.1	Protein digestibility (%).....	117

7.4.2	Soluble protein (%).....	119
7.4.3	Protein profile (SDS-PAGE).....	120
7.4.4	Free amino acid analysis .....	122
7.4.5	Mineral profile analysis.....	124
7.5	Conclusion.....	126
<b>Chapter 8 Pulsed electric field: Role in protein digestion of beef <i>Biceps femoris</i> .....</b>		<b>127</b>
8.1	Abstract.....	127
8.1.1	Industrial relevance.....	127
8.2	Introduction .....	127
8.3	Materials and methods.....	129
8.4	Results and discussion .....	129
8.4.1	Protein digestibility (%).....	129
8.4.2	Soluble protein (%).....	130
8.4.3	Protein profile (SDS-PAGE).....	131
8.4.4	Free amino acids .....	134
8.4.5	Mineral profile .....	135
8.5	Conclusions .....	137
<b>Chapter 9 Pulsed electric field: Effect on <i>in vitro</i> simulated gastrointestinal protein digestion of deer <i>Longissimus dorsi</i>.....</b>		<b>138</b>
9.1	Abstract.....	138
9.2	Introduction .....	138
9.3	Materials and methods.....	140
9.4	Results and discussion .....	140
9.4.1	<i>In vitro</i> protein digestibility (%).....	140
9.4.2	Soluble protein (%).....	142
9.4.3	Protein profile (SDS-PAGE).....	143
9.4.4	Free amino acid analysis .....	146
9.4.5	Mineral profile analysis.....	148
9.5	Conclusions .....	149
<b>Chapter 10 Pulsed electric field: A new way to improve digestibility of cooked beef.....</b>		<b>150</b>
10.1	Abstract.....	150
10.2	Introduction .....	150
10.3	Materials and methods.....	152
10.4	Results and discussion .....	152
10.4.1	Protein digestibility (%).....	152
10.4.2	Soluble protein (%).....	154
10.4.3	Protein profile (SDS-PAGE).....	155
10.4.4	Free amino acid analysis .....	157

10.4.5 Mineral profile analysis.....	158
10.5 Conclusions .....	160
<b>Chapter 11 Cooking does not impair the impact of pulsed electric field on the protein digestion of venison during <i>in vitro</i> gastrointestinal simulation .....</b>	<b>161</b>
11.1 Abstract.....	161
11.2 Introduction .....	161
11.3 Materials and methods.....	163
11.4 Results and discussion .....	163
11.4.1 In vitro protein digestibility (%) .....	163
11.4.2 Soluble protein (%).....	165
11.4.3 Protein profile (SDS-PAGE).....	166
11.4.4 Free amino acid analysis .....	168
11.4.5 Mineral profile analysis.....	169
11.5 Conclusions .....	171
<b>Chapter 12 Does pulsed electric field have a role in the sodium reduction strategy for meat products? .....</b>	<b>172</b>
12.1 Abstract.....	172
12.2 Introduction .....	172
12.3 Materials and methods.....	174
12.4 Results and discussion .....	174
12.4.1 Electrical input .....	174
12.4.2 Physicochemical parameters and colour .....	175
12.4.2.5 Mineral profile .....	177
12.4.3 Textural characteristics .....	178
12.4.4 Sensory evaluation.....	180
12.4.5 Microbial characteristics .....	182
12.4.6 Lipid and protein oxidation.....	183
12.5 Conclusions .....	185
<b>Chapter 13 General Discussion and Conclusion .....</b>	<b>186</b>
<b>Appendix.....</b>	<b>193</b>
A.1 Approval letter for sensory evaluation from Human Ethics Committee .....	193
A.2 Hedonic scale used for sensory evaluation.....	194
<b>References .....</b>	<b>195</b>

## List of Tables

Table 1.1 Findings of different studies elucidating the effect of pulsed electric field on the tenderness of different muscles.....	5
Table 1.2 Findings of different studies elucidating the effect of pulsed electric field on the quality characteristics of different muscles.....	7
Table 1.3 Findings of different studies elucidating the effect of pulsed electric field on proteolysis of different muscles.....	26
Table 4.1 Pulsed electric field processing parameters used for beef <i>Semimembranosus</i> and effect on the average temperature (°C) and conductivity (mS/cm).....	84
Table 4.2 Effect of pulsed electric field on the physicochemical properties of beef <i>Semimembranosus</i> during aging.....	86
Table 5.1 Pulsed electric field processing parameters used for beef <i>Biceps femoris</i> and effect on the average temperature (°C) and conductivity (mS/cm).....	98
Table 5.2 Effect of pulsed electric field and ageing on the purge loss and cooking loss of beef <i>Biceps femoris</i> .....	99
Table 7.1 Effect of pulsed electric field on the soluble protein (%) of beef <i>Semimembranosus</i> during <i>in vitro</i> simulated gastrointestinal digestion.....	119
Table 7.2 Effect of pulsed electric field on the release of free amino acids during <i>in vitro</i> simulated gastrointestinal digestion of beef <i>Semimembranosus</i> .....	123
Table 7.3 Effect of pulsed electric field on the release of minerals during <i>in vitro</i> simulated gastrointestinal digestion of beef <i>Semimembranosus</i> .....	125
Table 8.1 Effect of pulsed electric field on the soluble protein (%) of beef <i>Biceps femoris</i> during <i>in vitro</i> simulated gastrointestinal digestion.....	131

Table 8.2 Effect of pulsed electric field on the release of free amino acids during <i>in vitro</i> simulated gastrointestinal digestion of beef <i>Biceps femoris</i> .....	135
Table 8.3 Effect of pulsed electric field on the release of minerals during <i>in vitro</i> simulated gastrointestinal digestion of beef <i>Biceps femoris</i> .....	137
Table 9.1 Effect of pulsed electric field on protein digestibility of deer <i>Longissimus dorsi</i> during <i>in vitro</i> simulated gastrointestinal protein digestion.....	141
Table 9.2 Effect of pulsed electric field on the soluble protein (%) of deer <i>Longissimus dorsi</i> during <i>in vitro</i> simulated gastrointestinal protein digestion.....	142
Table 9.3 Effect of pulsed electric field on the release of free amino acids during <i>in vitro</i> simulated gastrointestinal digestion of deer <i>Longissimus dorsi</i> .....	147
Table 9.4 Effect of pulsed electric field on the release of minerals during <i>in vitro</i> simulated gastrointestinal digestion of deer <i>Longissimus dorsi</i> .....	148
Table 10.1 Effect of pulsed electric field on the soluble protein (%) of cooked beef <i>Semimembranosus</i> during <i>in vitro</i> simulated gastrointestinal digestion.....	154
Table 10.2 Effect of pulsed electric field on the release of free amino acids during <i>in vitro</i> simulated gastrointestinal digestion of cooked beef <i>Semimembranosus</i> .....	158
Table 10.3 Effect of pulsed electric field on the release of minerals during <i>in vitro</i> simulated gastrointestinal digestion of cooked beef <i>Semimembranosus</i> .....	159
Table 11.1 Effect of pulsed electric field on the soluble protein (%) of cooked venison <i>Longissimus dorsi</i> during <i>in vitro</i> simulated gastrointestinal digestion.....	165
Table 11.2 Effect of pulsed electric field on the release of free amino acids during <i>in vitro</i> simulated gastrointestinal digestion of cooked venison <i>Longissimus dorsi</i> .....	169
Table 11.3 Effect of pulsed electric field on the release of minerals during <i>in vitro</i> simulated gastrointestinal digestion of cooked venison <i>Longissimus dorsi</i> .....	170

Table 12.1 Pulsed electric field processing parameters used and average temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ ) for beef <i>Semimembranosus</i> .....	175
Table 12.2 Effect of pulsed electric field processing on the colour, shear force and physicochemical properties of beef jerky.....	177
Table 12.3 Effect of pulsed electric field on the mineral content of beef jerky. .....	178
Table 12.4 Effect of pulsed electric field processing on the sensory characteristics of beef jerky.....	182
Table 12.5 Effect of pulsed electric field processing on the microbiological characteristics and oxidative stability of beef jerky.....	184

## List of Figures

Figure 1.1 PEF-induced electroporation in a biological membrane.....	3
Figure 1.2 Possible mechanism for how PEF treatment can affect the tenderness of meat.....	11
Figure 1.3 Domain structures of $\mu$ -calpain (calpain 1), m-calpain (calpain 2) and calpastatin .....	18
Figure 1.4 How PEF treatment may affect the digestion of meat proteins during gastrointestinal digestion.....	30
Figure 2.1 An overview of the experimental design .....	40
Figure 2.2 Pulsed electric field system at the University of Otago.....	42
Figure 2.3 Chilly bin used to transport muscles .....	45
Figure 2.4 Vacuum packaged muscles.....	45
Figure 2.5 Splitting muscles into six blocks .....	45
Figure 2.6 Adjusting the dimensions of each block .....	45
Figure 2.7 PEF treatment chamber .....	46
Figure 2.8 PEF treatment chamber with muscle block.....	46
Figure 2.9 Measuring the conductivity of muscle .....	46
Figure 2.10 Measuring the temperature of muscle .....	46
Figure 2.11 PEF treatment chamber in PEF machine.....	47
Figure 2.12 PEF processing of the muscle block.....	47



Figure 2.13 Samples for ageing .....	47
Figure 2.14 Samples for molecular analysis (snap frozen).....	47
Figure 2.15 MIRINZ tenderometer and accessories used for measuring shear force.....	49
Figure 2.16 SDS-PAGE (Mini-Protean III electrophoresis tank).....	53
Figure 2.17 Western Blot Analysis (Immunoblotting) .....	55
Figure 2.18 Equipments used for biochemical analysis (Casein zymography, SDS PAGE and Immunoblotting) .....	56
Figure 2.19 <i>In vitro</i> gastrointestinal digestion simulation.....	58
Figure 2.20 HPLC used for the analysis of free amino acids.....	60
Figure 2.21 Inductively coupled plasma optical emission spectrophotometer.....	61
Figure 2.22 Texture analyser used for measuring shear force, toughness and firmness of beef jerky.....	63
Figure 2.23 Sensory laboratory at Lincoln University.....	65
Figure 2.24 Laminar flow used for microbiology.....	67
Figure 3.1 Effect of ageing on the pH of beef <i>Biceps femoris</i> and <i>Semimembranosus</i> .....	72
Figure 3.2 Effect of ageing on the shear force (Newtons) of beef <i>Biceps femoris</i> and <i>Semimembranosus</i> .....	74
Figure 3.3 Effect of ageing on the myofibrillar fragmentation index (MFI) of beef <i>Biceps femoris</i> and <i>Semimembranosus</i> .....	75
Figure 3.4 Effect of ageing on the calpain activity of beef <i>Semimembranosus</i> .....	76

Figure 3.5 Effect of ageing on the calpain activity of beef <i>Biceps femoris</i> .....	77
Figure 3.6 Effect of ageing on the myofibrillar protein profile (SDS-PAGE) of beef <i>Semimembranosus</i> .....	78
Figure 3.7 Effect of ageing on the myofibrillar protein profile (SDS-PAGE) of beef <i>Biceps femoris</i> .....	79
Figure 4.1 Effect of pulsed electric field on the shear force (Newtons) of beef <i>Semimembranosus</i> .....	87
Figure 4.2 Effect of pulsed electric field on the myofibrillar fragmentation index (MFI) of beef <i>Semimembranosus</i> .....	89
Figure 4.3 Effect of pulsed electric field on the calpain activity of beef <i>Semimembranosus</i> .....	90
Figure 4.4 Effect of pulsed electric field on the proteolytic pattern of troponin-T of beef <i>Semimembranosus</i> .....	91
Figure 4.5 Effect of pulsed electric field on the proteolytic pattern of desmin of beef <i>Semimembranosus</i> .....	92
Figure 4.6 Effect of pulsed electric field on the myofibrillar protein profile (SDS-PAGE) of beef <i>Semimembranosus</i> .....	92
Figure 5.1. Effect of pulsed electric field on the shear force (Newtons) of beef <i>Biceps femoris</i> .....	101
Figure 5.2 Effect of pulsed electric field on the myofibrillar fragmentation index (MFI) of beef <i>Biceps femoris</i> .....	101
Figure 5.3. Effect of pulsed electric field on the calpain activity of beef <i>Biceps femoris</i> .....	102
Figure 5.4 Effect of pulsed electric field on the proteolytic pattern of desmin of beef <i>Biceps femoris</i> .....	104
Figure 5.5 Effect of pulsed electric field on the proteolytic pattern of troponin-T of beef <i>Biceps femoris</i> .....	104

Figure 5.6 Effect of pulsed electric field on the myofibrillar protein profile (SDS-PAGE) of beef <i>Biceps femoris</i> .....	105
Figure 6.1 Effect of pulsed electric field on the shear force (Newtons) of venison <i>Longissimus dorsi</i> aged for 21 days.....	110
Figure 6.2 Effect of pulsed electric field on the myofibrillar fragmentation index (MFI) of venison <i>Longissimus dorsi</i> aged for 21 days.....	111
Figure 6.3 Effect of pulsed electric field on the calpain activity of venison <i>Longissimus dorsi</i> aged for 21 days.....	112
Figure 6.4 Effect of pulsed electric field on the myofibrillar protein profile (SDS-PAGE) of venison <i>Longissimus dorsi</i> .....	112
Figure 6.5 Effect of pulsed electric field on the pattern of desmin of venison <i>Longissimus dorsi</i> aged for 21 days.....	113
Figure 6.6 Effect of pulsed electric field on the pattern of troponin-T of venison <i>Longissimus dorsi</i> aged for 21 days.....	114
Figure 7.1 Effect of pulsed electric field on the protein digestibility of beef <i>Semimembranosus</i> during <i>in vitro</i> simulated gastrointestinal digestion.....	117
Figure 7.2 Effect of pulsed electric field on the protein profile (SDS-PAGE) of beef <i>Semimembranosus</i> subjected to <i>in vitro</i> simulated gastrointestinal digestion.....	121
Figure 8.1 Effect of pulsed electric field on the protein digestibility of beef <i>Biceps femoris</i> during <i>in vitro</i> simulated gastrointestinal digestion.....	130
Figure 8.2 Effect of pulsed electric field on the protein profile (SDS-PAGE) of beef <i>Biceps femoris</i> subjected to <i>in vitro</i> simulated gastrointestinal digestion.....	133
Figure 9.1 Effect of pulsed electric field on the protein profile (SDS-PAGE) of deer <i>Longissimus dorsi</i> subjected to <i>in vitro</i> simulated gastrointestinal digestion.....	145

Figure 10.1 Effect of pulsed electric field on the protein digestibility of cooked beef <i>Semimembranosus</i> during <i>in vitro</i> simulated gastrointestinal digestion.....	153
Figure 10.2 Effect of pulsed electric field on the protein profile (SDS-PAGE) of cooked beef <i>Semimembranosus</i> subjected to <i>in vitro</i> simulated gastrointestinal digestion.....	156
Figure 11.1 Effect of pulsed electric field on the protein digestibility of cooked venison <i>Longissimus dorsi</i> subjected to <i>in vitro</i> simulated gastrointestinal digestion.....	164
Figure 11.2 Effect of pulsed electric field on the protein profile (SDS-PAGE) of cooked venison <i>Longissimus dorsi</i> subjected to <i>in vitro</i> simulated gastrointestinal digestion.....	167
Figure 12.1 Effect of pulsed electric field on the texture properties of beef jerky.....	179

## Abbreviations and Symbols

%	Percent
°C	Celsius
µg	Microgram
µl	Microliter
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BF	<i>Biceps femoris</i>
Ca	Calcium
dH <sub>2</sub> O	Distilled water
D	Depth
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FAO	Food and Agricultural Organization
g	Gram
h	Hour
H	Height
HCl	Hydrogen chloride
HPLC	High performance liquid chromatography
KCl	Potassium chloride
kDa	Kilodalton
KH <sub>2</sub> PO <sub>4</sub>	Potassium dihydrogen phosphate
kPa	Kilopascal

L	Length
LD	<i>Longissimus dorsi</i>
MFI	Myofibril fragmentation index
MgCl <sub>2</sub>	Magnesium chloride
min	Minutes
MIRINZ	Meat Industry Research Institute of New Zealand
mL	Millilitre
mM	Millimolar
ms	Millisecond
NaCl	Sodium chloride
pH	Potential hydrogen
rpm	Rotations per minute
sec	Seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SM	<i>Semimembranosus</i>
TBS	Tris buffered saline
TBARS	Thiobarbituric acid reacting substances
TEMED	Tetramethylethylenediamine
TTBS	Tris buffered saline with tween
Tween20	Polyoxyethylene (20)sorbitan monolaurate
v/v	Volume by volume
W	Width
w/v	Weight by volume
w/w	Weight by weight

# Chapter 1

## Introduction and Literature Review

Parts of this chapter have been published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Applied and emerging methods for meat tenderization: A comparative perspective. *Comprehensive Reviews in Food Science and Food Safety*, 17, 841-859.

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Current and future prospects for the use of pulsed electric field in the meat industry. *Critical Reviews in Food Science and Nutrition*, Doi:10.1080/10408398.2018.1425825.

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Role of calpain system in meat tenderness. *Food Science and Human Wellness*, 7, 196-204.

### 1.1 Introduction

Pulsed electric field (PEF) is considered as a very promising non-thermal technique of preserving foods and improving food quality. The technology involves the use of electric field pulses of short duration (several nanoseconds to several milliseconds) with electric field strength of 0.1-80 kV/cm applied to a food placed between or passed through two electrodes (Barba et al. 2018; Puértolas and Barba 2016; Koubaa et al. 2015; Buckow et al. 2014; Buckow et al. 2013). Electric field strength, treatment temperature and energy delivery are the three most important parameters identified for PEF processing (Toepfl et al. 2014b; Amiali et al. 2007; Lebovka et al. 2005; Heinz et al. 2003). As a non-thermal technology, pulsed electric field processing causes less degradation of nutritional and sensory characteristics of foods than traditional thermal processing technologies (Buckow et al. 2013; Walkling-Ribeiro et al. 2010; Rivas et al. 2006). It exhibits many advantages such as lower treatment temperature, shorter processing time and potential continuous flow in comparison to traditional processing technologies (Walkling-Ribeiro et al. 2011; Puértolas et al. 2010), making it a very appealing technology for food manufacturers. Although, it has been widely investigated for its industrial pasteurization and sterilization potential for liquid foods like milk and other dairy products, liquid eggs, fruit juice, wine, beer and other alcoholic beverages (Milani et al. 2015; Delsart et al. 2014; Buckow et al. 2014; Timmermans et al. 2014; Monfort et al. 2010), the use of PEF as a food processing technology in solid foods (Liu et al. 2017a; Aguiló-Aguayo et al. 2017; Ignat et al. 2015), particularly meat, has only recently emerged (Khan et al. 2017a; Ma et al. 2016; Bekhit et al. 2016; Suwandy et al. 2015a; 2015b; 2015c; 2015d).

## **1.2 Applications in food industry**

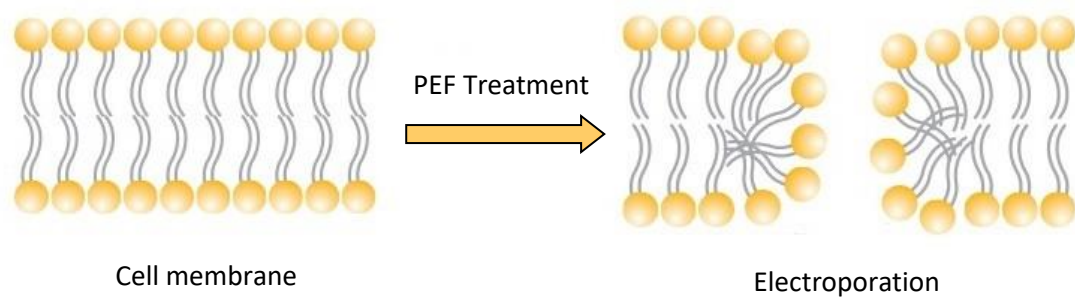
Electric pulses of very short duration and high intensity can induce irreversible structural changes in cell membranes resulting in increased membrane permeability (Zimmermann et al. 1976), enhanced mass transfer (Janositz et al. 2011) and disruption or breakdown of cellular tissue (Toepfl et al. 2014a). Thus, PEF can be used to replace or improve existing processes for food preservation, food modification and tissue disintegration (Bekhit et al. 2017; Toepfl et al. 2014a). The first commercial application of PEF was the installation of a PEF system in USA in 2006 for fruit juice preservation (Clark 2006) which was followed with the installation of the first commercial PEF juice line in 2009 in Europe (Toepfl et al. 2014b). The first commercial PEF preservation system for vegetable processing was installed in 2010. Systems with capacities up to 5000 litres/h are currently installed and available for fruit juice preservation in Europe (Toepfl et al. 2014b; Toepfl 2012). PEF technology has potential application in several food processes, like cold pasteurization and sterilization (Barba et al. 2015; Uchida et al. 2008), inactivation of enzymes (Zhao et al. 2010), promotion of extraction and recovery of bioactive compounds (Rodríguez-Roque et al. 2015; Abenoza et al. 2013), reduction of the allergenicity of certain food products (Johnson et al. 2010), potential reduction of food contaminants and pesticide residues (Zhang et al. 2012), food dehydration (Wiktor et al. 2014), and freezing processes (Wiktor et al. 2013). While the technology has been recognized as a non-thermal technology, as its effects do not require heat addition, ohmic heating is generated during high treatment intensity. While this can be a useful synergistic effect for microbial inactivation and controlled by rapid cooling post-treatment, it can also have negative effects on the quality and appearance of solid food materials such as a cooking effect in fresh meat (Bekhit et al. 2014c).

## **1.3 Mechanism of action**

In the absence of an external electric field, there is a naturally occurring perpendicular transmembrane potential of about 10 mV in a cell due to the accumulation of charges of opposite polarity on each side of the membrane (Toepfl et al. 2014b). When exposed to an external electric field, an additional potential is induced which depends on the strength of the applied field surrounding the cells. During PEF processing, the food is placed between two electrodes and an external electric field is applied which induces the movement of ions along the direction of lines of force of the applied electric field inside as well as outside the cells. This accumulate ions on the membranes causing the polarisation of the cell (Teissie et al. 1985) which results in a reduction in the thickness of the membranes due to the forces of attraction between oppositely charged ions on either side of the membrane (electro-compressive forces). When electric field strength exceeds the critical threshold value of transmembrane potential of approximately 1 volt (Weaver 2000; Zimmerman 1996; Hamilton and Sale 1967), these electro-compressive forces cause electrical breakdown or viscoelastic deformation of the



cell membrane which can be observed as pores. This electrical breakdown or pore formation, also known as electroporation, increases the permeability of membranes (Zimmermann et al. 1976). Figure 1.1 is a pictorial representation of electroporation in a biological membrane.



**Figure 1.1 PEF-induced electroporation in a biological membrane**

In addition to this electromechanical explanation of electroporation, another theory explains the electroporation based on formation of hydrophilic pores from hydrophobic pores in the membrane. The hydrophobic pores, which are naturally formed due to thermal fluctuations, exceed a critical size and become hydrophilic because these require less energy to maintain the structure and are more stable under the conditions (Joshi et al. 2002; Weaver and Chizmadzhev 1996). When PEF is applied, energy needed for the formation of pores gets reduced due to the increased transmembrane potential and Joule heating effect; this increases the number and size of pores and reduces the critical size. This expansion and accumulation of hydrophilic pores is believed to be responsible for electroporation (Joshi et al. 2002; Weaver and Chizmadzhev 1996; Barnett and Weaver 1991).

The intensity of electric field must exceed a critical strength for electroporation to occur (Barbosa-Canovas and Sepulveda 2005). The electric field strength above which the permeability increases is known as critical electric field and assuming a biological membrane of 5 nm thickness, this translates to a dielectric strength of 2 kV/cm (Glaser et al. 1998). The electric field strength to achieve the electroporation depends on several factors like food properties (whether a food is liquid, viscoelastic or solid, and its dielectric characteristics), process parameters (temperature, pulse duration, amplitude, and number of pulses), cell parameters (type, size, shape, and orientation) and membrane characteristics (ionic strength, thickness, and structure) (Toepfl et al. 2014b; Saulis 2010). At low PEF intensity treatment, electroporation is often reversible; however, membrane breakdown will occur when the ratio of pore size to membrane surface area becomes too large (Buckow et al. 2014). For irreversible pore formation, a critical electric field strength of 1-2 kV/cm is required for plant cells and 10-14 kV/cm for microbial cells (1-10  $\mu\text{m}$ ) (Toepfl et al. 2005). Electroporation of microbial cell membranes (microbial inactivation) such as those of *Listeria innocua* or *Escherichia coli* can be achieved by using high electric field strengths of 10-40 kV/cm and energy input of more than 40 kJ/kg (Toepfl et al. 2014a). A lower field strength and energy input is required for the disintegration of plant

and animal tissue due to their larger cell size. Pore formation in animal tissue, such as meat, can be achieved by using electric field strengths of 1-10 kV/cm and energy input of 0.5-10 kJ/kg (Toepfl et al. 2014a) whereas an electric field strength of 0.7-3 kV/cm and energy input of 1-20 kJ/kg are sufficient for the treatment of plant tissues (Corrales et al. 2008; Zimmermann et al. 1976). Studies on fresh meat indicated that at 10 kV/cm intensity, cooking effects start to appear on the meat edges (Bekhit et al. 2014c). This information collectively suggests that PEF could be used to create structural changes in fresh meat, i.e. tenderization, but is unlikely to be useful for microbial inactivation as that requires more intensive treatment.

#### **1.4 PEF effect on meat quality**

Studies on the utilization of PEF in solid foods like meat are generally lacking (O'Dowd et al. 2013; Gudmundsson and Hafsteinsson 2001), however, Topfl (2006) identified meat as one of the most promising applications to achieve a broad industrial exploitation of PEF in a study comparing the energy requirements to induce pore formation in different biological membrane systems. PEF treatment of meat was reported to enhance mass transfer during drying as well as brining of the meat products. Further, an improvement in water binding was noticed during cooking due to improved micro-diffusion of brine and water binding agents. Several papers have been published since then on the applications of PEF processing in meat technology highlighting several areas like meat safety, tenderization, supercooling and accelerated brining (Mok et al. 2017; Khan et al. 2017a; Ma et al. 2016; Bekhit et al. 2016; Suwandy et al. 2015a; 2015b; 2015c; 2015d; McDonnell et al. 2014; Faridnia et al. 2015; Haughton et al. 2012). Table 1.1 and 1.2 summarizes findings of different studies on effect of PEF on tenderness and other quality parameters of different muscles.

Because of its potential for cell membrane permeabilization, PEF is able to modify several quality traits of meat like texture, colour, and water-holding capacity and enhance mass transfer processes like curing and brining (McDonnell et al. 2014; Gudmundsson and Hafsteinsson 2001). Further, the moderate temperature rise (5-30°C) observed during PEF processing due to mild ohmic heating (Lindgren et al. 2002) could affect the meat quality (O'Dowd et al. 2013) and the combination of the two could have a thermo-electric effect on the muscle cell membranes (Ortega-Rivas 2011) and consequently on meat quality attributes. While studying the effect of PEF processing on volatile profile and sensory attributes of cooked lamb meats, Ma et al. (2016) reported that PEF treatment affected the temporal flavour profiles of meaty and oxidized flavour attributes. All PEF treated samples were associated with browned, juicy, livery, and meaty flavour attributes. Meaty, roast beef, juicy, browned, fatty, and salty are some of the terms associated with "positive" attributes (Ma et al. 2016). The following sections of the review will discuss the possibilities and opportunities that PEF can provide to the meat industry.

**Table 1.1 Findings of different studies elucidating the effect of pulsed electric field on the tenderness of different muscles**

<b>Authors</b>	<b>Muscle Studied</b>	<b>Muscle status</b>	<b>PEF Treatment</b>	<b>Findings</b>
Suwandy et al. (2015b)	Beef <i>Longissimus lumborum</i>	Pre-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Toughness increased with treatment intensity
Bekhit et al. (2014c)	Beef <i>Longissimus lumborum</i>	Post-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Tenderness increased regardless of intensity, Shear force reduced by 19.5 %
Suwandy et al. (2015b)	Beef <i>Semimembranosus</i>	Pre-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Tenderness increased regardless of intensity, Shear force reduced by 21.6 %
Bekhit et al. (2014c)	Beef <i>Semimembranosus</i>	Post-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Tenderness increased with treatment intensity, Shear force reduced by 4.1, 10.4, 19.1% for 20, 50, 90 Hz
Bekhit et al. (2016)	Beef <i>Longissimus lumborum</i>	Pre-rigor	Repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 μs)	Tenderness reduced with 3× PEF treatment, 1× and 2× treatment had no effect
Suwandy et al. (2015d)	Beef <i>Longissimus lumborum</i>	Post-rigor	Repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 μs)	Tenderness increased with repeats, Shear force decreased by 2.5 N for each additional treatment
Bekhit et al. (2016)	Beef <i>Semimembranosus</i>	Pre-rigor	Repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 μs)	Tenderness increased with lowest shear force in 3× PEF treatment
Suwandy et al. (2015d)	Beef <i>Semimembranosus</i>	Post-rigor	Repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 μs)	Tenderness was not affected by PEF, Shear force not affected by PEF
Suwandy et al. (2015c)	Beef <i>Longissimus lumborum</i>	Post-rigor	(10 kV, 90 Hz, 20 μs)	No effect on shear force due to fibre direction or initial muscle pH
Suwandy et al. (2015a)	Beef <i>Longissimus lumborum</i>	Post-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Tenderness increased regardless of intensity, Shear force reduced by 19.0 %
Suwandy et al. (2015a)	Beef <i>Semimembranosus</i>	Post-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Tenderness increased with treatment intensity,

				Shear force reduced by 19.0%
O'Dowd et al. (2013)	Beef <i>Semitendinosus</i>	Post-rigor	1.9 kV/cm, 65 Hz, 20 $\mu$ s	Tenderness was not affected by PEF, Shear force not affected by PEF
McDonnell et al. (2014)	Pork <i>Longissimus thoracis et lumborum</i>	Post-rigor	1.2 or 2.3 kV/cm x100 or 200 Hz x 150 or 300 pulses	No significant effect of PEF was observed on the texture profile analysis
Arroyo et al. (2015a)	Turkey breast meat	Post-rigor	7.5, 10, 12.5 kV (fresh meat), 14, 20, 25 kV (frozen meat) x 10, 55, 110 Hz	Tenderness was not affected by PEF in both fresh and frozen samples, Shear force not affected by PEF
Arroyo et al. (2015b)	Beef <i>Longissimus thoracis et lumborum</i>	Post-rigor	1.4 kV/cm, 10 Hz, 20 $\mu$ s, 300 and 600 pulses	Samples showed tendency towards reducing toughness, Shear force not affected by PEF
Faridnia et al. (2014)	Beef <i>Longissimus thoracis</i>	Post-rigor	0.2-0.6 kV/cm, 1-50 Hz, 20 $\mu$ s	Tenderness was not affected by PEF, Shear force was not affected by PEF
Faridnia et al. (2015)	Beef <i>Semitendinosus</i>	Post-rigor	1.4 kV/cm, pulse width 20 $\mu$ s, 50 Hz	Freezing-thawing + PEF improved tenderness, Tenderness was not improved by PEF alone
Faridnia et al. (2016)	<i>Biceps femoris</i>	Post-rigor	1.7kV/cm, 50 Hz, 20 $\mu$ s	Tenderness increased in treated samples, Shear force reduced in PEF samples
Khan et al. (2017)	Beef <i>Longissimus et lumborum</i>	Post-rigor	2.5 kV, 200 Hz, 20 $\mu$ s-LPEF 10 kV, 200 Hz, 20 $\mu$ s-HPEF	Tenderness was not affected by PEF, Shear force of HPEF samples was higher than LPEF and untreated samples
Toepfl (2006)	Pork haunches and Shoulder, Beef meat	Post-rigor	0.5-5 kV/cm, 50-1000 pulses, 1-25 kJ/kg	Tenderness increased in treated samples

**Table 1.2 Findings of different studies elucidating the effect of pulsed electric field on the quality characteristics of different muscles**

<b>Authors</b>	<b>Muscle studied</b>	<b>Muscle status</b>	<b>PEF Treatment</b>	<b>Findings</b>
Toepfl (2006)	Pork shoulder, haunches, sausage products	Post-rigor	0.5-5 kV/cm, 50-1000 pulses, 1-25 kJ/kg	Mass transfer enhancement in meat, Enhanced drying rates of raw ham and sausage products, Accelerated the curing process, Improved water binding capacity
Suwandy et al. (2015b)	Beef <i>Longissimus lumborum</i>	Pre-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Temperature rise of 0.6 - 4.4 °C, Purge loss (%) was not affected by PEF, Cooking loss (%) tended to increase
Bekhit et al. (2014c)	Beef <i>Longissimus lumborum</i>	Post-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Temperature rise of 0.4 and 7.7 °C, Purge loss (%) increased linearly with voltage and frequency, Cooking loss (%) lowered in PEF treated samples
Suwandy et al. (2015b)	Beef <i>Semimembranosus</i>	Pre-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Temperature rise of 0.7 - 8.1 °C, Purge loss (%) increased regardless of intensity, Cooking loss (%) was not affected by PEF
Bekhit et al. (2014c)	Beef <i>Semimembranosus</i>	Post-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Temperature rise of 0.4 and 8.0 °C, Purge loss (%) increased linearly with voltage, Cooking loss (%) was not affected by PEF
Bekhit et al. (2016)	Beef <i>Longissimus lumborum</i>	Pre-rigor	Repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 μs)	Temperature rise of 6.5 - 13.4 °C, Purge loss % increased by 1.38% for every extra treatment, Cooking loss (%) was not affected by PEF
Bekhit et al. (2016)	Beef <i>Semimembranosus</i>	Pre-rigor	Repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 μs)	Temperature rise of 1.8 - 6.7 °C, Purge loss (%) affected linearly by number of PEF repeats, Cooking loss (%) was not affected by PEF
Suwandy et al. (2015d)	Beef <i>Longissimus lumborum</i>	Post-rigor	Repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 μs)	Temperature rise of 7.7 - 16.2 °C, pH and purge loss (%) were not affected by PEF, Cooking loss (%) increased regardless of PEF repeats Redness (a values) decreased with PEF repeats
Suwandy et al. (2015d)	Beef <i>Semimembranosus</i>	Post-rigor	Repeated (1×, 2×, 3×) PEF treatment	Temperature rise of 7.7 - 15.6 °C, pH and cooking loss (%) were not affected by PEF,

			(10 kV, 90 Hz, 20 $\mu$ s)	Purge loss (%) increased regardless of PEF repeats, Redness (a values) decreased with PEF repeats
Suwandy et al. (2015c)	Beef <i>Longissimus lumborum</i>	Post-rigor	10 kV, 90 Hz, 20 $\mu$ s	No significant effect of PEF was found on total water loss, meat colour and lipid stability
O'Dowd et al. (2013)	Beef <i>Semitendinosus</i>	Post-rigor	1.9 kV/cm, 65 Hz, 20 $\mu$ s	Temperature rise of 5-22 °C, Weight loss (%) increased linearly with temperature of PEF, No adverse effect on drip loss (%), moisture (%), water activity, total expressible moisture and solid (%), Muscle fibre bundles appeared smaller in diameter and Hunter L values were significantly lower for PEF samples
Arroyo et al. (2015a)	Turkey breast meat	Post-rigor	7.5, 10, 12.5 kV (fresh meat), 14, 20, 25 kV (frozen meat) x 10, 55, 110 Hz	No significant effect of PEF was observed on weight loss (%), cook loss (%), lipid oxidation, texture and colour of fresh and frozen meat samples
Arroyo et al. (2015b)	Beef <i>Longissimus thoracis et lumborum</i>	Post-rigor	1.4 kV/cm, 10 Hz, 20 $\mu$ s, 300 and 600 pulses	Temperature rise of 7.7 °C (300 pulses), 14.5 °C (600 pulses), No significant effect of PEF on weight loss (%), cook loss (%) or storage loss (%), Colour L, a, and b values were not affected by PEF
Faridnia et al. (2014)	Beef <i>Longissimus thoracis</i>	Post-rigor	0.2-0.6 kV/cm, 1-50 Hz, 20 $\mu$ s	No effect of PEF on pH, cooking loss and colour stability, Moisture (%) decreased significantly by 0.7-3.6%
Faridnia et al. (2015)	Beef <i>Semitendinosus</i>	Post-rigor	1.4 kV/cm, pulse width 20 $\mu$ s, 50 Hz	Increased purge loss (%), No effect of PEF on free fatty acid profiles, omega6/omega 3, polyunsaturated/saturated fatty acids ratios, A two log-unit increase in aerobic microbial counts during log phase of frozen-thawed PEF-treated samples
Faridnia et al. (2016)	<i>Biceps femoris</i>	Post-rigor	1.7 kV/cm, 50 Hz, 20 $\mu$ s	Significant increase in conductivity, purge loss (%) and temperature (Mean temperature of PEF sample - 26.53 °C), Significant decrease in pH, Cooking loss was not affected, Dramatic increase in the number of ruptured myofibrils along the z-

				lines making muscle more porous structure
McDonnell et al. (2014)	Pork <i>Longissimus thoracis et lumborum</i>	Post-rigor	1.2 or 2.3 kV/cm, 100 or 200 Hz, 150 or 300 pulses	No significant effect of PEF on cook loss (%), water holding capacity, total viable count and lipid oxidation (TBARS), Potential may exist for reduced curing time through PEF
Ma et al. (2016)	Lamb shoulder ( <i>Infraspinatus</i> ), rib ( <i>Longissimus</i> ), loin ( <i>Longissimus, Psoas major</i> )	Post-rigor	1-1.4 kV/cm, 90 Hz, 20µs	PEF induced significant changes in volatile compounds, PEF affected temporal flavour of meaty and oxidized flavour attributes, Significant effect on lipid oxidation in chilled meat but led no off flavour
Khan et al. (2017)	Beef <i>Longissimus et lumborum</i>	Post-rigor	2.5 kV, 200 Hz, 20 µs - LPEF 10 kV, 200 Hz, 20 µs - HPEF	Significantly higher L* values and lower a* values in HPEF samples, Significantly lower P, K and Fe concentrations in HPEF samples, Higher lipid oxidation in HPEF than LPEF

#### 1.4.1 Meat tenderness

Given that the tenderness of meat largely depends on the overall integrity of muscle cells (Hughes et al. 2014), PEF processing, which has the potential to enhance cell disruption, presents an environmentally friendly and energy efficient tenderization technique that could be applied to muscles for cost effective alterations to the muscle cell structure (Toepfl et al. 2006). Unlike some other methods of tenderization (Bekhit et al. 2014a; Bekhit et al. 2014b), PEF processing does not cause undesirable effects like severe structural and oxidative changes, and off-flavour development. Further, it does not generate environmental hazards and there is no evidence of toxicity (Pal 2017; Kumar et al. 2015). It is a well-established low-cost operational method for irreversible permeabilization of cell membranes without a significant rise in temperature within certain processing conditions (Toepfl et al. 2006). PEF is a stand-alone technology that can be applied to different muscles either pre-rigor or post-rigor (Suwandy et al. 2015a). This technology could be used to upgrade the less tender meat cuts by optimizing the technological inputs to different meat cuts and thereby optimizing the product quality (Bekhit et al. 2016).

#### **1.4.1.1 Mechanism of action for PEF on meat texture**

Given the fact that electroporation in animal tissues, such as meat, could be achieved by using electric field strengths of 1-10 kV/cm and energy input of 0.5-10 kJ/kg (Toepfl et al. 2014a), PEF treatment of muscles before aging could help in the tenderization process through early activation of calpains by release of calcium ions from cell organelles due to enhanced membrane permeability. This probable mechanism is further supported by evidence that a post-mortem aging period is required for gaining the tenderization benefit of PEF through increased proteolysis (Warner et al. 2017; Bekhit et al. 2016; Suwandy et al. 2015a, b, c; Bekhit et al. 2014c). However, other factors like release of cathepsins from lysosomes, accelerated glycolysis due to calcium release (pre-rigor muscles) and physical disruption of muscles may contribute. Figure 1.2 is a pictorial representation of how PEF treatment may affect the tenderness of meat.

#### **1.4.1.2 Effect of PEF on post-rigor (cold-boned) muscles**

In general, it appears that PEF has an influence on the tenderization process of meat during aging as several workers have reported a positive effect of the technology on the tenderization process (Ma et al. 2016; Suwandy et al. 2015a; 2015c; 2015d; Faridnia et al. 2015, Bekhit et al. 2014c), however, there is no agreement in the literature on whether it significantly enhances tenderness. The potential of PEF to improve meat tenderization in cold-boned muscles seems to depend on several factors including electric field strength, muscles under study, and the use of an aging period. Although, it is independent of initial muscle pH and fibre direction (orientation in the field) (Suwandy et al. 2015c), there appears to be a minimum electric field intensity above which PEF induces an improvement in the tenderization process in each muscle during a proper aging period.

No significant effect of PEF treatment was observed by O'Dowd et al. (2013) on beef tenderization process, however, Bekhit et al. (2014b) attributed these results to the absence of an aging period. Likewise, Arroyo et al. (2015b) also observed no significant gain of the PEF processing on the tenderness following a short aging period of 2 days. Although, both O'Dowd et al. (2013) and Arroyo et al. (2015b) used PEF treatments of high electric field intensity [(1.9 kV/cm, 65 Hz, 20  $\mu$ s); (7.5, 10, 12.5 kV for fresh meat; 14, 20, 25 kV for frozen meat; 10, 55, 110 Hz), respectively], absence of a proper aging period following the PEF treatment could be the possible reason for the non-significant response, since it is generally agreed that meat tenderness is a biochemical process (Koohmaraie and Geesink 2006). Physical disruption may also be a contributing factor since mechanical tenderization is a well-documented technology to improve meat tenderness (Bekhit et al. 2014a), however, the amount of energy required is enormous. PEF is mostly believed to mediate tenderness through proteolysis (Suwandy et al. 2015a, b) which is not an instantaneous process and requires time (aging) to achieve



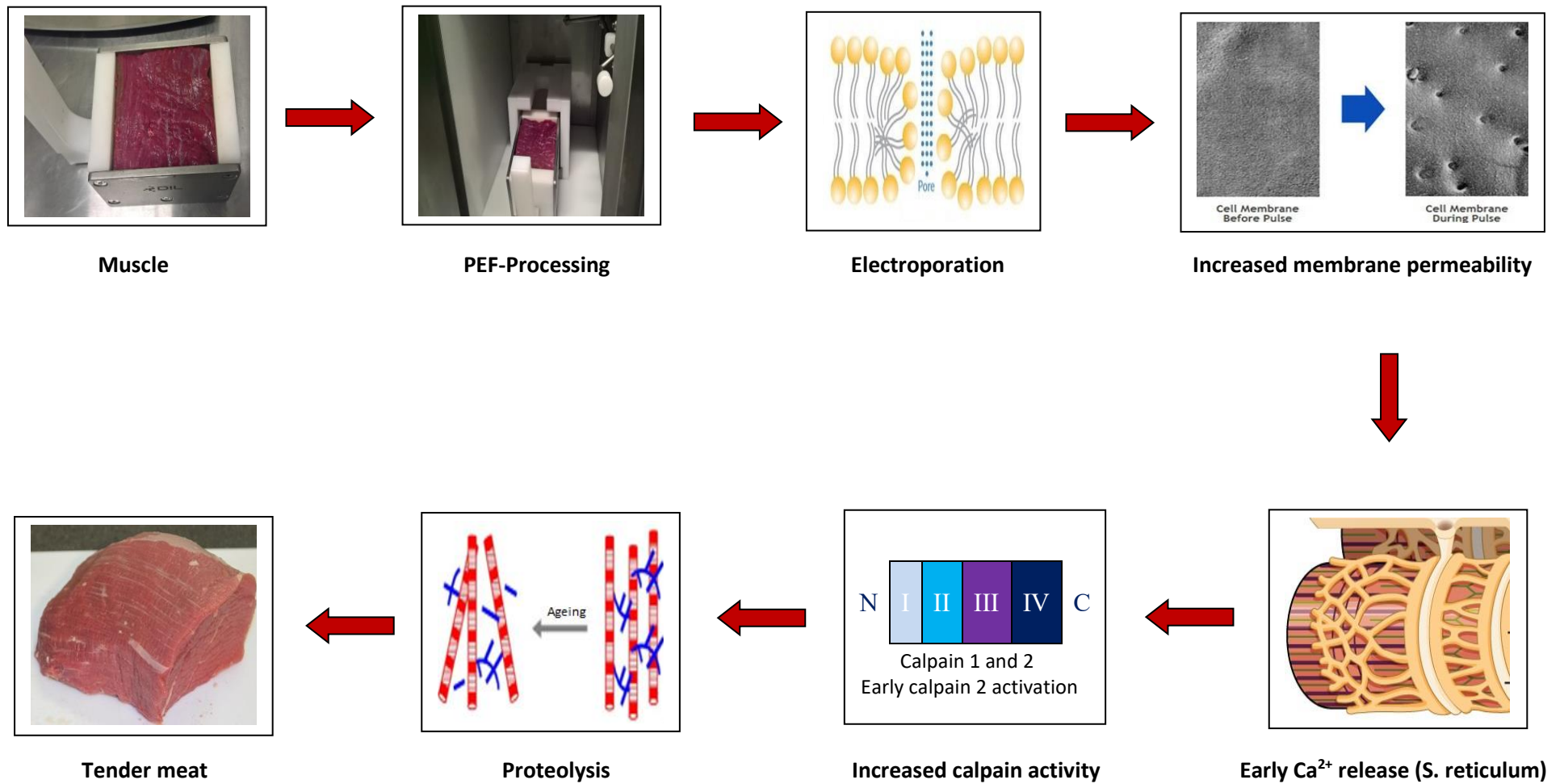


Figure 1.2 Possible mechanism for how PEF treatment can affect the tenderness of meat

improvement in tenderness. This may be supported by the finding of O'Dowd et al. (2013) who observed some additional myofibril breakdown in the PEF treated samples that resulted in smaller particle sizes as determined by laser diffraction particle size analysis. Although, no significant improvement was observed in tenderness by O'Dowd et al. (2013) and Arroyo et al. (2015b), it is worth mentioning that both of these studies had observed a tendency towards reducing toughness for PEF treated samples. Where no significant effects were observed on the tenderization in the above studies, a significant improvement was observed in tenderness of cold-boned beef *Biceps femoris* treated with PEF of strength of 1.7-2.0 kV/cm, 50 Hz, 185 kJ/kg followed by an aging period of 21 days at 4 °C (Faridnia et al. 2016).

The importance of a post-treatment aging period for PEF-induced tenderness was, however, rejected by a study which included an aging period of 26 days and no significant improvement was observed in the tenderness of cold-boned beef *Longissimus thoracis et lumborum* subjected to PEF treatment (Arroyo et al. 2015a). Similarly, Faridnia et al. (2014) reported no effect of PEF on the tenderness of beef *Longissimus thoracis* followed by vacuum aging. Unlike other studies, which have reported a significant effect of PEF on tenderness, one thing common among these two studies was the use of low intensity PEF. Faridnia et al. (2014) used PEF treatment of 0.2 to 0.6 kV/cm, 1-50 Hz, 20 µs for the cold-boned beef *Longissimus thoracis* and Arroyo et al. (2015a) used PEF treatment of 1.4 kV/cm, 10 Hz, 20 µs for the *Longissimus thoracis et lumborum* muscle. Although, no significant improvement was observed in tenderness by Arroyo et al. (2015a) and Faridnia et al. (2014), both these studies reported a tendency towards reducing toughness for PEF treated samples. Further, these studies have suggested that the PEF treatment used may not have been strong enough to induce physical disruption of muscle fibres, suggesting physical disruption may play an important role in the tenderization process. There is also, however, a possibility that low intensity PEF used in these studies may not have induced an irreversible membrane permeabilization required to cause the release of calcium ions and enzymes from the cell organelles and result in the significant tenderization observed in other studies. Thus, treatment intensity as well as aging period appears to play an important role in determining the effect of PEF on tenderization.

The conflicting results of different studies regarding the tenderization effect of PEF were explained by Bekhit et al. (2017) on the grounds of sample size, origin, statistical validity and inaccurate design and reporting of studies. Variations and contradictions in the results obtained in some of the studies were attributed to various reasons like use of insufficient number of experimental units or small size of meat samples and use of meat of unknown background. Due to inevitable variation in tenderness observed within groups of animals raised together (Devine et al. 2006), use of statistically valid and sufficient number of experimental units (animals or carcasses) is required to generate meaningful results. Further, variation in tenderness among and within the muscles makes it necessary to include blocking and randomization in the experiment design to avoid the effects of carcass sides and location within

muscles. Small size samples, used in some studies, makes it impossible to involve blocking and randomization in the experiment design. Studies involving use of samples of unknown origin makes it difficult to understand and replicate the work and ignores the biochemical background of the materials used (Bekhit et al. 2017).

While PEF of low electric field intensity has mostly failed to produce significant effects on tenderization, PEF with similar electric field intensity has generated some significant and interesting results in other studies. Several studies have been conducted on evaluating the effect of PEF on cold-boned beef *Semimembranosus* and *Longissimus lumborum* muscles using electric field intensity of 5, 10 kV (equivalent to 0.625 and 1.25 kV/cm) at 20, 50, or 90 Hz. Both Suwandy et al. (2015a) and Bekhit et al. (2014c) reported a decrease of 19% in the shear force of *Semimembranosus*. This decrease in the shear force of *Semimembranosus* was dependent on the PEF frequency (20, 50, or 90 Hz) and showed a decreasing trend with increasing level of frequency (4.1, 10.4 and 19.1% reduction at 20, 50 and 90 Hz, respectively). Yet another study (Suwandy et al. 2015d) showed no significant effect of repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 µs) on the shear force of beef *Semimembranosus*. The *Longissimus lumborum* muscle appeared to behave differently with PEF treatment but like *Semimembranosus* showed a decrease in shear force with PEF treatment by up to 19.0% (Suwandy et al. 2015a). Bekhit et al. (2014c) also reported a similar decrease in shear force in *Longissimus* by up to 19.5%, however, unlike *Semimembranosus* the decline in shear force was observed to be independent of frequency (20, 50, or 90 Hz) of the PEF treatment (Suwandy et al. 2015a; Bekhit et al. 2014c). A significant effect of repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 µs) was also observed on the shear force of beef *Longissimus lumborum* which showed a decrease of 2.5 N with every extra application of PEF treatment (Suwandy et al. 2015d). The differences in the behaviour of two muscles with PEF treatment may be attributed to the anatomical and physiological differences between the two muscles which influence the factors like heat generation, conductivity, fibre-type composition and membrane properties.

No significant effect of PEF was observed by Khan et al. (2017a) in cold-boned beef loins (*M. Longissimus et lumborum*) treated with high intensity of 2.5 kV, 200 Hz (LPEF) and 10 kV, 200 Hz (HPEF) at 1 and 14 days of aging who rather observed a toughening effect of the PEF as the shear-force of the muscles increased with PEF treatment compared to untreated control. They suggested that this toughening, which was generally observed more in HPEF treated samples than LPEF samples, was due to the denaturation of the proteins and enzymes involved in the tenderization process by the heat produced due to the Joule effect (Ohmic heating) which supports the enzymatic nature of the PEF induced tenderization. A moderate rise in temperature (5-30°C) has already been reported during PEF processing due to mild ohmic heating (Lindgren et al. 2002). Further, high intensity PEF treatments have also been reported to produce heat and increase the temperature of beef muscles (Khan et al. 2017a; Bekhit et al. 2014c; Suwandy et al. 2015c; 2015d).

### 1.4.1.3 Effect of PEF on pre-rigor (hot-boned) muscles

PEF could be of importance in improving the quality of hot-boned muscles that are separated from the hot carcasses in pre-rigor state and are generally less tender than cold-boned muscles (Troy and Kerry 2010; White et al. 2006). Hot-boning offers several benefits for the meat industry, such as less space required, less handling of carcasses and faster production. Hot-boned muscles could be treated separately with different process parameters to obtain optimal results (Bekhit et al. 2016). Two recent studies (Bekhit et al. 2016; Suwandy et al. 2015b) were reported on the effect of PEF treatment on the tenderization of pre-rigor beef muscles during aging. Both used beef topsides (*M. Semimembranosus*) and beef loins (*M. Longissimus lumborum*) as the study model and evaluated the effect of different electric field intensities (5, 10 kV at 20, 50, or 90 Hz) and repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20  $\mu$ s) on the tenderization during aging. The PEF treatments (5, 10 kV at 20, 50, or 90 Hz) affected the tenderness of hot-boned beef topsides with up to 21.6% reduction observed in the average shear force. The reduction in shear force was independent of the treatment intensity, though the shear force tended to decrease with increasing frequency (20, 50, or 90 Hz) of the PEF treatment. The same pattern was observed with the repeated PEF treatment as the shear force kept on decreasing with repeated PEF treatment (10 kV, 90 Hz, 20  $\mu$ s) and the lowest shear force was observed with 3× PEF treatment at 3 days post-treatment time, however, this beneficial effect was reported to disappear with longer aging periods. A negative effect of the PEF treatments (5, 10 kV at 20, 50, or 90 Hz) was reported by the same workers on the tenderness of hot-boned loins as average shear force tended to increase, indicating increased toughness of muscle with an increase in the PEF frequency (20, 50, or 90 Hz). Further, repeated 3× PEF treatment was also observed to reduce the tenderness, although no effect was seen on the tenderization with 1× and 2× PEF treatments. This result was different from what was reported for cold boned loins where an improvement in tenderness was observed regardless of the treatment intensity (Bekhit et al. 2014c). This suggests that the effect of PEF treatment will also depend on the post-mortem status of the muscle. The differences in the tenderization behaviour of these two muscles with PEF treatment may be attributed to the differential effect of PEF on the water holding capacity of these muscles (Bekhit et al. 2016; Suwandy et al. 2015b).

These results demonstrated that the tenderizing effects of PEF will be dependent on the biochemistry of the treated meat as evident by the muscle type and rigor time. Thus, complete profiling for PEF effects on various muscles will be required. Furthermore, these results suggest that meat cuts composed of various muscles will be affected differently; therefore, application of the technology to small animals such as lamb may not be practical.

### 1.4.2 Proteolysis

Tenderness of meat largely depends on the overall integrity of muscle cells which can be disrupted by the degradation of key myofibrillar and cytoskeletal proteins (Hughes et al. 2014). Weakening of the myofibrillar structures by proteolytic enzymes due to the degradation of muscle proteins like titin, nebulin and desmin has been reported during ageing by several workers (Huff-Lonergan et al. 1996; Hopkins and Thompson 2002). Thus, the meat tenderizing mechanisms during ageing are primarily enzymatic in nature and involve several intracellular proteolytic systems capable of post-mortem proteolysis (Ouali et al. 2006). Several endogenous proteolytic systems present in meat like calpains, lysosomal proteases, and cathepsins have the capability of degradation of myofibrillar and cytoskeletal proteins (Dransfield 1994), but the calpain system has been shown to cause the proteolysis of cytoskeletal proteins (titin and nebulin) and intermediate filaments (desmin) during ageing (Huff-Lonergan et al. 1996). There is a great body of research that links the calpain system to the tenderization of muscles and considers it responsible for majority of post-mortem proteolysis and a significant driver of meat tenderization during aging (Geesink et al. 2006; Koohmaraie and Geesink 2006; Huff-Lonergan and Lonergan 1999; Huff-Lonergan et al. 1996; Koohmaraie 1992).

The calpain system, responsible for postmortem proteolysis in pre- and postrigor muscle (Huff-Lonergan and Lonergan 1999; Huff-Lonergan et al. 1996; Koohmaraie 1992), comprises endogenous proteases (calpains) which are considered as the primary candidates for muscle protein degradation initiated during the first 24 h postmortem (Huff-Lonergan and Lonergan 1999; Huff-Lonergan et al. 1996; Koohmaraie 1992) and their endogenous inhibitor, calpastatin. Considerable evidence suggests that calpains are responsible for proteolysis and tenderization in lamb, beef and pork. Tenderization rates in different species are inversely correlated to the calpastatin:calpain ratio (Koohmaraie et al. 1991). These enzymes cleave the same muscle proteins that are degraded during post-mortem ageing (Huff-Lonergan et al. 1996; Kendall et al. 1993) and have the capability to degrade the muscle proteins including intermediate filament proteins, like desmin and the structural proteins, like titin (Huff-Lonergan and Lonergan 1999; Huff-Lonergan et al. 1996) with minimal effects on myosin and actin (Dayton et al. 1976). A strong decline was observed in the post-mortem proteolysis in terms of loss of intact desmin and troponin-T in transgenic mice over-expressing calpastatin (Kent et al. 2004). These results were later confirmed by a similar study in  $\mu$ -calpain knockout mice (Geesink et al. 2006). Other evidence that support the role of calpains comes from analysing the effects of  $\beta$ -adrenergic agonists which are involved in muscle protein turnover and have been reported to reduce the muscle protein degradation (Bohorov et al. 1987) by elevating the calpastatin activity resulting in tougher meat (Dunshea et al. 2005). Likewise, the high levels of calpastatin found in the hypertrophied muscles of callipyge lambs significantly reduced post-mortem proteolysis and meat tenderness (Geesink and Koohmaraie 1999b). A simple injection of post-mortem muscle with calcium accelerates post-mortem

proteolysis and tenderization (Wheeler et al. 1997; Koohmaraie et al. 1989), suggesting the involvement of calpains. Although, all these evidences indicate role of calpain system in post-mortem proteolysis and tenderization during ageing, however, they do not indicate which of the calpain isoforms are responsible for the protein degradation.

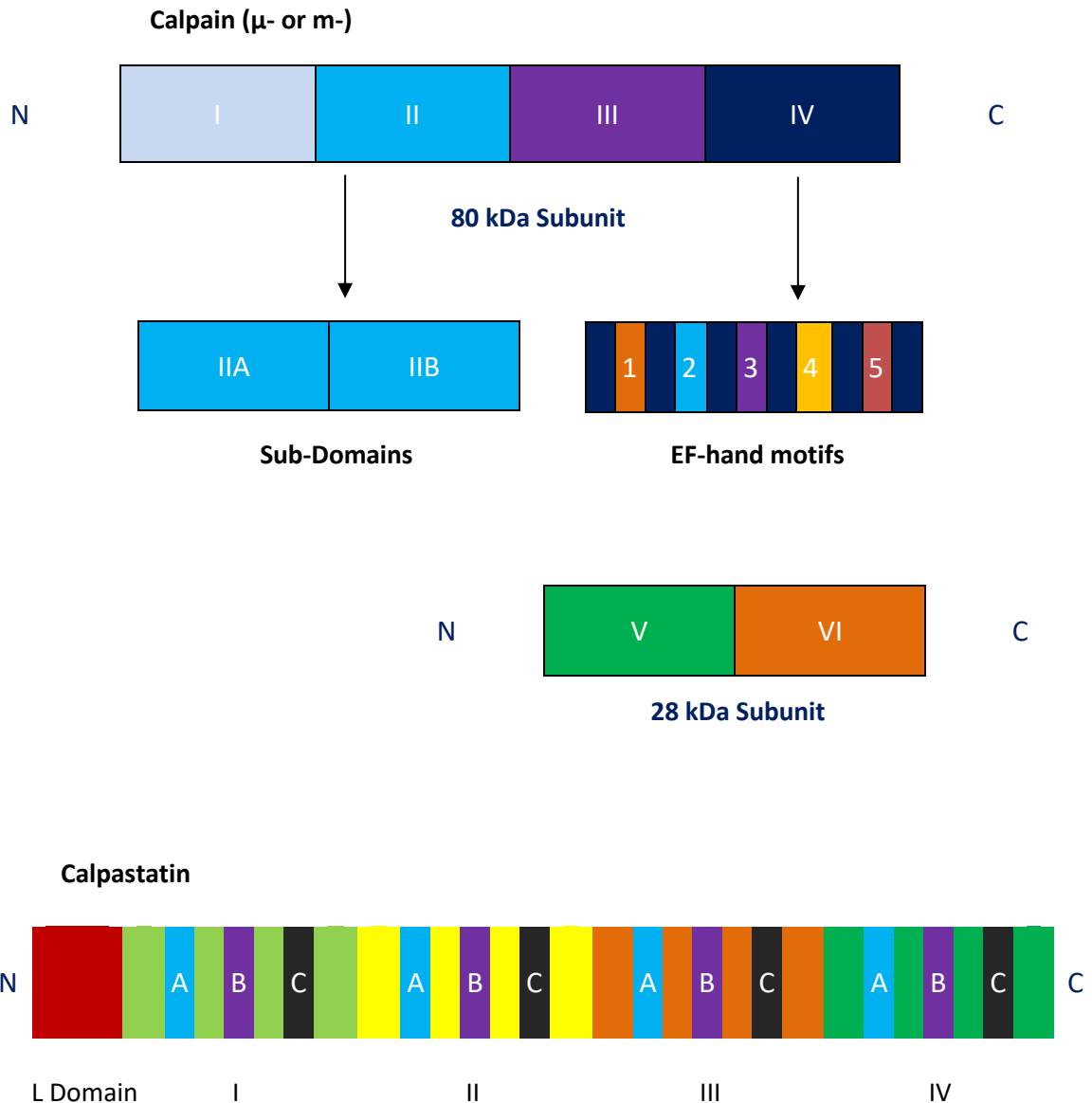
#### **1.4.2.1 Calpain system**

The calpain system is the most extensively studied enzyme system in the meat science that contributes to meat tenderization (Koohmaraie and Geesink 2006; Sentandreu et al. 2002). Present in almost all eukaryotes and a few bacteria, it is a large family of intracellular calcium dependent cysteine neutral proteinases which comprises several isoforms of the proteolytic enzyme calpain and their endogenous inhibitor, calpastatin (Croall and Demartino 1991; Goll et al. 2003). The  $\mu$ -calpain and m-calpain are the two best-characterized isoforms out of 15 isoforms identified [calpain 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, calpain small subunit 1 (also known as calpain 4)] so far which are classified on the basis of tissue distribution into ubiquitous and tissue-specific forms (Cruzen 2013; Goll et al. 2003). The calpain system in the skeletal muscles consists of two ubiquitously expressed proteases viz.  $\mu$ -calpain (calpain 1) and m-calpain (calpain 2) in addition to p94 (tissue specific calpain 3) and calpastatin (Moudilou et al. 2010; Koohmaraie and Geesink 2006; Goll et al. 2003). The  $\mu$ -calpain is mostly bound to myofibrils (70%) and most of the m-calpain is located in the cytosol (Ilian et al. 2004a; Xu et al. 2009) along with their inhibitor, calpastatin (Tullio et al. 1999). The  $\mu$ -calpain and m-calpain require different  $\text{Ca}^{2+}$  levels for their activation. While m-calpain needs 400 to 800  $\mu\text{M}$   $\text{Ca}^{2+}$  for half-maximal activity,  $\mu$ -calpain needs only 3 to 50  $\mu\text{M}$   $\text{Ca}^{2+}$  for half-maximal activity (Goll et al. 2003).

##### **1.4.2.1.1 Structure of calpains**

Figure 1.3 shows the domain structures of  $\mu$ -calpain, m-calpain and calpastatin. The calpains have highly homologous structure and both  $\mu$ -calpain and m-calpain are heterodimers composed of a similar large 80 kDa catalytic subunit and share an identical small 28 kDa regulatory subunit (Suzuki 1990). The large 80 kDa subunit is composed of four (I, II, III, IV) domains (Goll et al. 2003). Both the calpains have two domains (V, VI) in the small subunit from the N-terminus and C-terminus, respectively. Domain V is rich in glycine and is the site for phospholipid binding. Domain VI contains five  $\text{Ca}^{2+}$ -binding sites also known as EF-hand motifs (Toldra and Reig 2015). The N-terminal domain of the 80 kDa subunit, domain I, has no sequence homology to any known polypeptide and its removal modulates the proteolytic activity. The catalytic domain, domain II, contains a cysteine residue (sub-domain IIA) as well as a histidine residue (sub-domain IIB) and their relative positions are conserved in all cysteine proteinases (Suzuki 1990). The activity of calpains may depend on other regions of the molecule in

addition to the active site, as the studies have confirmed that the fragments containing the catalytic domain (domain II) may not have catalytic activity by themselves (Nishimura and Goll 1991) showing the importance of conformational state on the activity of these enzymes (Strobl et al. 2000). A  $\text{Ca}^{2+}$ -binding domain, domain III, is not homologous to any other known protein and is linked to the catalytic domain II. It has sequences that predict EF hand calcium binding sites (Goll et al. 2003) and may regulate the activity of calpain through binding of phospholipids and critical electrostatic interactions (Tompa et al. 2001; Strobl et al. 2000). A calmodulin-like domain, domain IV, is also known as penta-EF domain and has five EF-hands. The first four EF-hands in domain IV are  $\text{Ca}^{2+}$ -binding sites. The fifth EF-hand motifs in the carboxyl end of domains IV and VI do not bind calcium but interact with each other to form a heterodimer of the two calpain subunits (Huff Lonergan et al. 2010; Suzuki et al. 2004). Calpastatin, endogenous inhibitor of  $\mu$ - and m-calpain, is a 70-80 kDa protein and has an N-terminal L domain and four repeating domains (I, II, III, IV). Each of the four repeating domains is able to inhibit one calpain molecule (Campbell and Davies 2012; Raynaud et al. 2005). Calpastatin in the erythrocytes is a small 46 kDa version of the protein and some isoforms, particularly in cardiac muscle, also contain a 68 amino acid XL N-terminal domain (Raynaud et al. 2005; Wendt et al. 2004). Originally referred to as p94, calpain 3 is a 94 kDa calpain isoform bearing sequence homology of approximately 50% with the large domain of  $\mu$ - and m-calpain (Sorimachi et al. 1989). It may form homodimers *in vivo* as it lacks the 28 kDa small subunit (Ravulapalli et al. 2005). This calpain protease is specific to skeletal muscle and is also found in an 82 kDa form in the retina and lens (Cruzen 2013).



**Figure 1.3 Domain structures of  $\mu$ -calpain (calpain 1), m-calpain (calpain 2) and calpastatin (Not to scale)**

#### 1.4.2.1.2 Mechanisms of action

Both of the calpains, i.e.  $\mu$ -calpain and m-calpain, bind calcium ions at various locations during activation. Although, m-calpain has the potential to bind 11 to 20 calcium ions and  $\mu$ -calpain may bind 5 to 8 calcium ions, the crystallized structural study of m-calpain in the presence of 5 mM calcium level has proven that it only binds 10 calcium ions (Hanna et al. 2008). Depending on the calcium level, the EF-hand motifs of IV and VI can bind three to four calcium ions which cause a small conformational shift that result in the dissociation of small subunit (Elce et al. 1997). However, this conformational change caused by the binding of calcium ions to the EF hands is probably not enough to result in



activation of m-calpain (Hosfield et al. 1999). The protease core domains, IIA and IIB, have no protease activity by themselves and it is only the presence of calcium ions on key binding sites that causes a conformational shift which allows protease activity (Moldoveanu et al. 2002). Thus, proteolysis is dependent on the binding of calcium ions at the two protease core sites which disrupts the salt bridges between the Glu333 and Arg104 residues in domain II, allowing the catalytic triad (cysteine (Cys105) residue in domain IIA and asparagines (Asp262) and histidine (His286) residues in domain IIB) to come together, resulting in activation (Cruzen 2013; Benyamin 2006). Several factors, like phospholipid binding, autolysis, or binding to membrane activator proteins, have been reported to reduce the calcium requirement (Salamino et al. 1993; Saido et al. 1992).

Unlike other endogenous proteases, like cathepsins,  $\mu$  and m-calpain are quite specific for their substrates. They have many protein substrates and cleave at many different protein sequences at very specific sites and do not completely degrade the substrates to their constituent amino acids. These proteases mostly cleave the substrates having coils and loops and their specificity is determined more by protein conformation than amino acid sequence (Fan et al. 2013; duVerle et al. 2011). However, studies involving computer modelling have identified many substrate sequence specificities (Sorimachi et al. 2012).

The inhibition of the activity of  $\mu$ -calpain and m-calpain by calpastatin is also a calcium dependent event as calpain-calpastatin binding requires calcium. The mechanism involved is through preventing the calpain proteolytic activation, membrane binding and the expression of catalytic activity (Huff Lonergan et al. 2010). Conformational changes are induced in the calpains due to the binding of calcium ions, specifically in domains I-IV and VI. The region A and region C of the calpastatin molecule binds to domain IV and VI of calpain molecule, respectively. This causes the region B of the calpastatin inhibitory domain to make various contacts with regions of domains I to III of calpains, blocking the active site of calpains and thereby inhibiting its activity (Huff Lonergan et al. 2010; Kawasaki and Kawashima 1996). TIPPLYR is the central inhibitory sequence in the calpastatin molecule (Kawasaki and Kawashima 1996) and most of the isoforms of calpastatin vary in the XL or L domain, and thus do not show much change in the calpain inhibitory ability (De Tullio et al. 2007; Goll et al. 2003).

### **1.4.2.1.3 Properties of calpains and role in proteolysis**

#### **1.4.2.1.3.1 $\mu$ -calpain and m-calpain**

Several studies provide compelling evidence that tenderization of meat during post-mortem ageing is predominantly modulated by the calpain system (Mohrhauser et al. 2011; Geesink et al. 2006; Koohmaraie and Geesink 2006; Ji and Takahashi 2006; Hopkins and Thompson 2002; Koohmaraie et al. 1988). Although, both isoforms ( $\mu$ -calpain and m-calpain) target and cleave the same myofibrillar proteins, the calcium requirements for activation of m-calpain is far higher than the calcium

concentrations attained post-mortem (maximal 210-230  $\mu\text{M}$ ) and will never reach that level unless exogenous calcium is added to the meat (Geesink et al. 2006; Koohmaraie and Geesink 2006). Therefore,  $\mu$ -calpain is considered to be of much greater significance for tenderness development through post-mortem proteolysis (Ji and Takahashi 2006; Koohmaraie et al. 1987). Further, the activation of  $\mu$ -calpain coincides with the post-mortem period (within 3 days of slaughter) when proteolysis of key myofibrillar proteins takes place whereas m-calpain is not activated early post-mortem (Sensky et al. 1996). The autolysis of  $\mu$ -calpain and subsequent loss of most of its activity has been reported to occur within 7 days post-mortem (Camou et al. 2007) whereas m-calpain activity did not decrease in lamb biceps femoris up to 56 days post-mortem (Geesink and Koohmaraie 1999b). Moreover, the degradation patterns similar to those observed in post-mortem muscle are produced when myofibrils are directly incubated with  $\mu$ -calpain (Huff-Lonergan et al. 1996). Very little post-mortem proteolysis is observed in the muscles of  $\mu$ -calpain knockout mice (Geesink et al. 2006), suggesting its predominant effect on catalysis of proteins during post-mortem ageing (Zhang et al. 2006).

Although, calcium availability is required for their activation, both  $\mu$ -calpain and m-calpain will autolyze when exposed to calcium levels necessary for their activation, thus proteolytic ability is accompanied with autolysis (Huff-Lonergan et al. 2010). The autolysis results in progressive degradation of 80 kDa subunit of  $\mu$ -calpain to 78 kDa and eventually to 76 kDa whereas the mass of the 80 kDa subunit of m-calpain reduces to 78 kDa (Zimmerman and Schlaepfer 1991). The 28 kDa subunit of both  $\mu$ -calpain and m-calpain is reduced to 18 kDa after losing its glycine-rich domain (Parkes et al. 1985). The calcium requirement of  $\mu$ -calpain and m-calpain for their activity is reduced by brief autolysis whereas extended autolysis results in inactivation of the enzymes (Edmunds et al. 1991). This reduction of the calcium requirement explains how these enzymes are active in cellular conditions which rarely, if ever, acquire the calcium levels needed for their activity. The autolyzed forms of both  $\mu$ -calpain (76 kDa) and m-calpain (78 kDa) requires 92 percent and 82 percent less calcium, respectively, than their unautolyzed (80 kDa) forms (Edmunds et al. 1991). The autolyzed form of  $\mu$ -calpain binds tightly to the myofibrils and other subcellular organelles due to its increased hydrophobic nature (Boehm et al. 1998). Since  $\mu$ - and m-calpain activity and proteolysis is always accompanied by autolysis, presence of the autolyzed form of calpain in post-mortem tissue means it has been active and measurement of calpain autolysis gives an estimate of the activity of these enzymes up to the analysed point of time.

#### **1.4.2.1.3.2 Calpain 3**

Discovered as the third calpain and named so, calpain 3 is not inhibited by calpastatin (Ono et al. 2004) and requires only nanomolar levels of calcium for activation (García Díaz et al. 2006). In addition to its proteolytic activity, it also seems to play a structural role in skeletal muscle fibers in the triad regions

composed of one T-tubule and two terminal cisternae (Kramerova et al. 2008). This structure regulates calcium release during contraction and lack of calpain 3 was reported to decrease the calcium release in the fibers due to an impairment of the triad-associated protein complex (Kramerova et al. 2008). Expressed almost exclusively in skeletal muscle (Sorimachi et al. 1989), this calpain caught the attention of researchers due to its association with the giant myofibrillar protein titin, also known as connectin. Calpain 3 has also been reported to degrade calpastatin *in vitro* and may possibly regulate the activity of  $\mu$ -calpain and m-calpain if the same were true *in vivo* (Ono et al. 2004). Calpain 3 binds with titin at N2 or M-line region (Kramerova et al. 2004; Sorimachi et al. 1995) which is a known site linked with proteolysis during meat tenderization (Taylor et al. 1995a). The binding of calpain 3 with titin protects it from auto-degradation and its removal from titin results in rapid autolysis. Due to this reason early investigations involving its purification were met with difficulty. Calpain 3 is also susceptible to sodium dependent autolysis and has been reported to have sodium dependent activity with different substrate specificity compared to its calcium dependent activity (Ono et al. 2010). Although, calpain 3 was initially thought to have a major role in post-mortem proteolysis and tenderization process due to its association with titin, studies over the years have nullified its importance and the role that calpain 3 may play in the process, if any, appears to be minor (Geesink et al. 2005). Given the fact that calpain 3 is not inhibited by calpastatin (Ono et al. 2004), animals with very high calpastatin would not produce tough meat had it have any substantial role in meat tenderization process. Further, no association was found between calpain 3 expression and the tenderness level in porcine *Longissimus dorsi* muscle (Parr et al. 1999). However, variations in tenderness in sheep have been reported to correlate strongly with the variations in calpain 3 mRNA and protein levels (Ilian et al. 2001). In order to see the role of calpain 3(p94) in meat tenderization, Geesink et al. (2005) compared the post-mortem proteolysis patterns of p94 knockout mice with wild type mice and reported no detectable difference in desmin, nebulin, troponin-T or vinculin degradation, suggesting the non-involvement of calpain 3 in meat tenderisation.

#### **1.4.2.1.4 Factors affecting the activity of calpains**

In addition to the sarcoplasmic  $Ca^{2+}$  concentration, several environmental factors like temperature, pH, and oxidation have been reported to influence the activity of calpains (Geesink et al. 2006; Koohmaraie 1992; Dayton et al. 1976).

##### **1.4.2.1.4.1 Temperature**

Influence of elevated temperature during early post-mortem on the proteolysis and calpain activity has been reported by several studies (Thomson et al. 2008; White et al. 2006; Hwang et al. 2004;

Geesink et al. 2000). The  $\mu$ -calpain is very sensitive to temperature (Manting et al. 2017) and its activity has been observed to be primarily regulated by muscle temperature in post-mortem bovine muscle (Mohrhauser et al. 2014). Accelerated  $\mu$ -calpain activation has been reported in lamb as well as beef muscles when incubated at elevated temperatures (Pomponio and Ertbjerg 2012; Hwang et al. 2004; Geesink et al. 2000). Faster  $\mu$ -calpain activation was observed by Geesink et al. (2000) at temperatures of 25 to 35 °C in lamb and by Hwang et al. (2004) at 36 °C in beef by using immunoblot analysis of muscle incubated for 24 h. Likewise faster proteolysis was observed in pieces of beef muscle incubated at 25 °C for 8 h post-mortem (White et al. 2006). Although, a faster proteolysis was also observed in beef muscles that entered rigor at 15 °C up to 3 days post-mortem (Thomson et al. 2008), less total proteolysis has been reported during prolonged ageing (Thomson et al. 2008; Geesink et al. 2000) due to lower stability of calpain at higher temperature causing early loss of  $\mu$ -calpain activity (Thompson et al. 1990). Elevated temperatures (above 25 °C) during rigor have also been reported to decrease the activity of calpastatin (Geesink et al. 2000).

The activity of m-calpain was also significantly faster at elevated temperatures (Pomponio and Ertbjerg 2012; Dayton et al. 1976) and this was attributed to both the increased temperature and increased free calcium concentrations (Cruzen 2013). The sarcoplasmic calcium level has been reported to increase in porcine muscle under certain time-temperature combinations that rises to the level required for activation of  $\mu$ -calpain and later also for m-calpain (Pomponio et al. 2008). An increase in extractable free calcium was also observed during the onset of rigor in beef muscles incubated at a temperature of 36 °C (Hwang et al. 2004).

#### **1.4.2.1.4.2 pH**

The pH is another factor that may affect the activity of calpains and proteolysis of their myofibrillar protein substrates. At pH values and ionic strengths physiologically found in post-mortem muscle, both  $\mu$ -calpain as well as m-calpain show less activity against myofibrillar protein substrates (Huff-Lonergan and Lonergan 1999; Geesink and Koohmaraie 1999a). Change in pH and ionic strengths may cause the conformational changes in enzymes as well as in their substrates affecting the activity of enzymes and the susceptibility of substrates. These changes may render the substrates less vulnerable to cleavage by  $\mu$ -calpain (Huff-Lonergan and Lonergan 1999). The rate of decline of early post-mortem pH is an important factor that influences the autolysis and activation of  $\mu$ -calpain, with accelerated decline considered favourable for accelerated proteolysis of known calpain substrates (Carlin et al. 2006; Melody et al. 2004). Slightly accelerated pH decline in the muscles also end up having an accelerated rate of tenderization and, sometimes may also have an advantage in water holding capacity (Simmons et al. 2008; Melody et al. 2004). Too rapid pH decline is not considered favourable as it will reduce the autolysis of calpains and proteolysis of their substrates. In pork, a rapid pH decline has been reported

to almost completely arrest autolysis of  $\mu$ -calpain (Barbut et al. 2008) and degradation of desmin and talin (Bee et al. 2007). Increased protein denaturation due to low pH and high temperature combinations may be held responsible for this condition.

#### **1.4.2.1.4.3 Oxidation**

Oxidation is another factor that can affect the activity of  $\mu$ -calpain and influence post-mortem tenderization. Incubation with  $H_2O_2$  (200 mM) results in loss of activity of  $\mu$ -calpain, reversible under reducing conditions, due to the formation of a disulfide bond between the active site Cys 115 and Cys 108 (Lametsch et al. 2008). It has also been reported to decrease the degradation of desmin *in vitro* (Carlin et al. 2006). Chen et al. (2014) studied the effect of oxidation on the susceptibility of purified desmin to degradation by  $\mu$ -calpain and reported that oxygen radicals changed the secondary structure and proteolytic susceptibility of desmin and decreased its degradation rate after incubation with  $\mu$ -calpain for 1 h. Xue et al. (2012) while studying the effect of oxidation on degradation of myofibrillar proteins by  $\mu$ -calpain, found that oxidative modification of myofibrillar proteins change their susceptibility. The oxygen radicals increased the proteolytic susceptibility of MHC and  $\alpha$ -actinin and reduced the degradation of troponin-T. Irradiation, which induces protein oxidation, has also been reported to decrease  $\mu$ -calpain autolysis and degradation of troponin-T and desmin in beef longissimus (Rowe et al. 2004).

#### **1.4.2.1.4.4 Pulsed electric field**

Some emerging technologies, like PEF and high-pressure processing, have the potential to affect the activity of calpains and thus influence the rate and extend of tenderization during ageing (Warner et al. 2017). PEF has the ability to alter the membrane properties (Barba et al. 2015; Deng et al. 2014) and may affect the activity of the calpains by causing early release of calcium ions from sarcoplasmic reticulum (Warner et al. 2017; Bekhit et al. 2016). Several studies have confirmed the potential of PEF in improving the tenderization process of both pre-rigor and post-rigor muscles during ageing (Khan et al. 2017a; Qianli et al. 2016; Bekhit et al. 2014, 2016; Suwandy et al. 2015a; 2015b; 2015c; 2015d; Faridnia et al. 2015).

### **1.4.2.2 Proteolysis of Desmin and Troponin-T**

There is a particularly well-defined organisation of intermediate filaments in the skeletal muscles which builds a network that interconnects the myofibrils to each other at the Z-disk level. In mature muscle fibers, this intermediate filament network is mainly composed of the protein desmin (Lazarides

and Hubbard 1976) and provides mechanical strength to the muscle fibers by maintaining structural and functional integrity. These intermediate fibers extend and encircle myofibrils constituting a three-dimensional scaffold around the Z-disk and connect the entire contractile apparatus to the sarcolemma, the nuclei, mitochondria, and other organelles (Paulin and Li 2004). Desmin is one of the earliest marker proteins for mammalian skeletal muscle during ageing as it participates in the early establishment of sarcomere structure. Due to its role in structure-maintaining functions, desmin degradation is believed to be essential for the tenderization of meat (Taylor et al. 1995b) and is believed to have a significant impact on meat quality during post-mortem ageing (Huff-Lonergan et al. 1996; Taylor et al. 1995b).

Several workers have reported the degradation of desmin during meat ageing (Christensen et al. 2004; Huff-Lonergan et al. 1996; Hwan and Bandman 1989; Kristensen and Purslow 2001; Morrisson et al. 1998; Verrez-Bagnis et al. 1999). Degradation of desmin has been directly linked to calpain activity by using *in vitro* models of post-mortem muscles (Kitamura et al. 2005; Baron et al. 2004; Lametsch et al. 2004; Huff-Lonergan et al. 1996). Both m- and  $\mu$ -calpain have been reported to effectively degrade desmin at identical sites (Baron et al. 2004; Croall and De-Martino 1991). Degradation of desmin during early post-mortem ageing was reported to generate fragments of approx. 50 to 39 kDa in pork (Christensen et al. 2004; Schafer et al. 2002). Degradation of desmin was evident at 3 days post-mortem ageing and after 7 days, it was almost completely degraded (Kemp and Parr 2012; Muroya et al. 2010) and thus it is believed to have a significant influence on the shear force and water-holding capacity of meat which consequently affect meat tenderization (Huff-Lonergan et al. 2010; Kristensen and Purslow 2001; Taylor et al. 1995b).

Involved in the calcium-dependent regulation of skeletal muscle contraction, troponin-T subunit is the tropomyosin-binding component which makes up the elongated portion of the troponin complex. Degradation of troponin-T, especially the 30 kDa fragment, is considered to be an indicator of meat tenderization (Harris et al. 2001). There is a good correlation between the two events as the degradation of troponin-T progresses simultaneously with the tenderization of meat during post-mortem ageing (Penny and Dransfield 1979).

Degradation of troponin-T possibly leads to the fragmentation of the myofibrils by disrupting its interaction with other actin filament components. Like desmin, degradation of troponin-T has also been directly linked to calpain activity by using *in vitro* models (Kitamura et al. 2005; Baron et al. 2004; Lametsch et al. 2004; Huff-Lonergan et al. 1996) and is used in proteomics as a marker for the ongoing proteolysis in beef (Iwanowska et al. 2010) and in pig meat (Lana and Zolla 2016). Polypeptide segments of 28 and 30 kDa are obtained from the degradation of intact troponin-T by  $\mu$ -calpain. The magnitude of these concomitant polypeptides strongly correlated with meat tenderness during post-mortem ageing (Rowe et al. 2003). Thus, troponin-T degradation has been used in pioneering studies aimed at meat tenderness prediction (Sun et al. 2014).

### 1.4.2.3 Effect of PEF on Proteolysis of Desmin and Troponin-T

Troponin-T and desmin are important structural proteins of the muscle and their post-mortem degradation has been associated with the loss of myofibrillar integrity (Sun et al. 2014; Kitamura et al. 2005; Geesink et al. 2000; Ho et al. 1996). Their proteolysis (Han et al. 2009; Kitamura et al. 2005; Claeys et al. 2004; Geesink et al. 2000; Ho et al. 1996; Ho et al. 1994) has been significantly correlated to shear force values (Marino et al. 2013). Therefore, both troponin-T and desmin have been used as markers of myofibrillar protein degradation and meat tenderization (Sun et al. 2014; Wheeler and Koohmaraie 1999; O'Halloran et al. 1997; Koohmaraie and Shackelford 1991). Several studies have reported changes in the proteolysis patterns of troponin-T and desmin in pre-rigor and post-rigor beef muscles treated with PEF during aging (Bekhit et al. 2016; Suwandy et al. 2015a; b; c; d). Table 1.3 shows findings of different studies on effect of PEF on proteolysis of different muscles.

Suwandy et al. (2015a) used troponin-T and desmin as markers in a study on the effect of PEF treatments (5, 10 kV at 20, 50, or 90 Hz) on the shear force and proteolysis of cold-boned beef *Longissimus lumborum* muscle for an aging period of 21 days. Where a significant reduction was observed in the shear force of the muscle (up to 19%) unaffected by the treatment intensity, a significant proteolysis of troponin-T was observed in 5 kV–90 Hz and 10 kV–20 Hz treated samples at day 3 and day 7 post-treatment in addition to 10 kV–50 Hz in subsequent post-treatment times (day 14 and 21). The desmin degradation also followed a similar pattern with higher proteolysis observed in 5 kV–50 Hz and 10 kV–20 Hz treated samples at day 3 and day 7 post-treatment. Increased degradation of desmin was observed in all PEF treated samples, except 10 kV–90 Hz treatment, at 14 and 21 days. Collectively, these degradation patterns of troponin-T and desmin provided evidence for improved proteolysis in PEF treated meat, both early post-mortem and during subsequent post-mortem storage. These proteolysis results agreed with the shear force results of the muscle and support the enzymatic basis of PEF induced tenderization. However, the tenderization effect observed in PEF 10 kV–90 Hz treated samples indicates some other mechanism, like physical disruption, also operates.

**Table 1.3 Findings of different studies elucidating the effect of pulsed electric field on proteolysis of different muscles**

Authors	Muscle Studied	Muscle Status	PEF Treatment	Findings
Suwandy et al. (2015b)	Beef Longissimus lumborum	Pre-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Significant proteolysis of troponin-T and Desmin, Extensive proteolysis was observed in 5 kV-20 Hz and 10 kV-20 Hz samples
Bekhit et al. (2016)	Beef Longissimus lumborum	Pre-rigor	Repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 μs)	Increased proteolysis of troponin T in 1× PEF treatment, Decreased proteolysis of troponin T in 2× and 3× PEF samples
Suwandy et al. (2015a)	Beef Longissimus lumborum	Post-rigor	5 kV, 10 kV × 20, 50, 90 Hz	Increased proteolysis of troponin-T and Desmin both early and subsequent post-mortem, Prominent proteolysis was observed in 5 kV-90 Hz, 10 kV-20 Hz and 10 kV-50 Hz samples
Suwandy et al. (2015d)	Beef Longissimus lumborum	Post-rigor	Repeated (1×, 2×, 3×) PEF treatment (10 kV, 90 Hz, 20 μs)	Increased proteolysis of troponin T and Desmin in 1× PEF treatment, Decreased proteolysis of troponin T and Desmin in 2× and 3× PEF samples
Suwandy et al. (2015c)	Beef Longissimus lumborum	Post-rigor	10 kV, 90 Hz, 20 μs	Increased proteolysis of troponin-T and Desmin in PEF treated samples, More increase in proteolysis in low-pH (5.5-5.8) than high-pH (>6.1) samples

In a similar study, Suwandy et al. (2015b) studied the effect of PEF treatments (5, 10 kV at 20, 50, or 90 Hz) on the proteolysis patterns of hot-boned beef *Longissimus lumborum* muscle. While shear force tended to increase with treatment frequency, a significant proteolysis of troponin-T was observed in 5 kV–20 Hz and 10 kV–20 Hz treated samples at 3, 7, and 14 days post-treatment with prominent bands on day 21. Degradation of troponin-T was observed to be higher in all PEF treated samples compared to control on all days' post-treatment except in 5 kV–50 Hz treated sample on day 7 and 14. The desmin degradation showed a similar pattern with most prominent proteolysis in 5 kV–20 Hz and 10 kV–20 Hz treated samples at 3, 14 and 21 days post-treatment. Desmin proteolysis was also prominent in 10 kV–90 Hz treated sample at 3 days post-treatment. Increased degradation of desmin was observed in all PEF treated samples compared to controls at 7- and 21-days post-treatment except 5 kV–90 Hz sample at day 7. The difference in the degradation pattern of cold-boned and hot-boned beef *Longissimus*



*lumborum* muscle was attributed to the higher final temperature of the hot-boned muscles after PEF-treatment (Suwandy et al. 2015b).

The effect of repeated (1×, 2× or 3×) PEF treatment (10 kV, 90 Hz, 20 μs) on the proteolysis of cold-boned beef *Longissimus lumborum* muscle has also been evaluated (Suwandy et al. 2015d). While shear force of the muscle was found to decrease by 2.5 N with every extra application, increased proteolysis of desmin and troponin-T was reported only in muscles subjected to 1× PEF treatment. Less degradation of desmin and troponin-T observed with increasing number of PEF treatments suggest another operational mechanism, like physical disruption, for PEF induced tenderisation in beef. In a similar study evaluating the effect of repeated (1×, 2× or 3×) PEF treatment (10 kV, 90 Hz, 20 μs) on the proteolysis of hot-boned beef *Longissimus lumborum* (Bekhit et al. 2016), increased proteolysis was observed in muscles subjected to 1× PEF treatment in terms of troponin-T degradation and the pattern observed was 1× PEF samples > 2× PEF samples > untreated samples > 3× PEF samples. Increased proteolysis was observed in 1× PEF treated samples over the entire 21 days of aging period compared to control samples whereas 2× and 3× PEF treated samples showed less degradation than the control. Since 3× PEF treatment was reported to produce toughest hot-boned meat and 1× and 2× PEF treatments didn't affect the tenderization in *Longissimus lumborum*, therefore this proteolysis pattern supported the suggestion that high intensity PEF treatment produced unfavourable conditions for post-mortem proteolysis, likely by inactivation of proteases as a result of heat generated at high treatment intensity. The samples subjected to 3× PEF treatments resulted in a temperature increase of  $13.4 \pm 5.2$  °C with a final temperature of  $38.3 \pm 5.23$  °C (Bekhit et al. 2016). The rate and the extent of tenderisation in pre-rigour meat is highly influenced by the temperature of the storage. Several studies have reported that the tenderness of red meat decreases when exposed to temperatures above 25 °C or below 10 °C (Devine et al. 2002; Geesink et al. 2000) due to the negative effect of high temperature on post-mortem proteolysis (Kim et al. 2012; Thomson et al. 2008). Pre-rigour incubation of beef loins at 38 °C has been reported to induce protein denaturation and limit the extent of μ-calpain autolysis and degradation of desmin. The net outcome of the incubation process was an increased shear force and a decrease in the water holding capacity (Kim et al. 2012).

The effect of initial pH and meat structural arrangement (fibre orientation) on the proteolysis pattern of PEF treated (10 kV, 90 Hz, 20 μs) cold-boned beef *Longissimus lumborum* muscle has also been studied (Suwandy et al. 2015c). Although, no significant change was observed in the shear force of PEF treated samples in comparison to untreated samples, increased proteolysis of troponin-T and desmin was observed in the PEF treated samples. Increased degradation of troponin-T and desmin was also observed in the low-pH muscle samples (5.5–5.8) in comparison to the high-pH (>6.1) samples which agreed with the results of the tenderness measurements as the low-pH muscles tended to have lower shear force. No effect of fibre orientation was reported on the proteolysis of PEF treated muscle samples.

Based on the available literature about the various aspects of meat tenderness, it is evident that PEF has a potential to influence the tenderization process of muscle and could be utilized as a post-mortem intervention to maximize the tenderness gain. However, a great deal of research is required before it becomes a commercial reality in the meat industry.

### **1.4.3 Digestibility of meat proteins**

Meat proteins are susceptible to aggregation during high-temperature thermal processing which causes unfolding of the tightly coiled polypeptide chains and they form large aggregates due to the formation of intermolecular cross-links (Santé-Lhoutellier et al. 2008; Morzel et al. 2006; Stadtman 1993). Accordingly, the use of non-thermal processing technologies, including pulsed electric field, for processing of meat is of interest. Unlike liquid foods (milk, wine, juices), severe PEF conditions are required to achieve sufficient microbial reduction in protein-fat based matrices, like meat, due to protective effects of food components like proteins and lipids (Monfort et al. 2011; Martín-Belloso et al. 1997). Use of high intensity electric fields, however, could disrupt the electric interactions of protein peptide chains (Park and Boxer 2002) which may initiate subsequent macroscopic changes such as interactions with other proteins through exposure of susceptible regions. PEF has been reported to induce partial unfolding of the proteins. This depends on the total energy input and on electric field strength and plays a key role in the subsequent intermolecular interactions that are responsible for formation of intermolecular cross-links and protein aggregates (Liu et al. 2017b).

The digestion process for meat starts with proteolysis of meat proteins into small peptides by enzymes such as pepsin, trypsin and  $\alpha$ -chymotrypsin. These peptides are further broken down into amino acids by amino- and carboxypeptidases and absorbed in the intestine. However, the digestibility of PEF treated meat by digestive tract enzymes has not been elucidated. Further, PEF treatment has already been reported to reduce the availability of some protein-bound minerals in meat (Khan et al. 2017a) and increase the temperature of the muscles during processing (Bekhit et al. 2014c; O'Dowd et al. 2013), indicating potential denaturation at high treatment intensities. Studies have confirmed that PEF treatment can cause denaturation and aggregation of egg proteins and both pulse intensity and energy input are decisive factors in determining PEF-induced denaturation and aggregation (Liu et al. 2017b; Wua et al. 2014; Zhao et al. 2009). Wua et al. (2014) observed partial protein unfolding in PEF treated egg proteins and reported insoluble aggregates resulting from covalent and non-covalent binding between heterogeneous proteins. PEF is known to cause the inactivation of endogenous food enzymes (Li et al. 2008; Riener et al. 2008) and protein denaturation and aggregation in soybean protein isolates (Li et al. 2007). Sharma et al. (2016) also reported an increase in surface hydrophobicity of milk proteins with increase in intensity of PEF treatment. Thus, PEF may induce intermolecular cross-links and aggregation of meat proteins. This intermolecular cross-linking, aggregation and denaturation of meat

proteins can influence their degradation by digestive enzymes (Gatellier and Santé-Lhoutellier 2009; Santé-Lhoutellier et al. 2008; Santé-Lhoutellier et al. 2007) as digestive process largely depends on the structure and physicochemical state of proteins (Kaur et al. 2014; Kong and Singh 2008). Recently, Chian et al. (2019) studied the effect of PEF processing (1.0 to 1.25 kV/cm, pulse number 500 and 2000) on the ultrastructure and *in vitro* protein digestibility of beef *Longissimus thoracis*. The PEF processing improved *in vitro* protein digestibility by at least 18% and digestive profiles (SDS-PAGE) of treated samples were different from control samples. Information is not generally available in the literature about the effect of PEF processing on the digestibility of meat proteins and needs further comprehension and scientific attention. Figure 1.4 is a pictorial representation of how PEF treatment can affect the digestion of meat proteins.

#### **1.4.4 Mineral content**

Given the fact that PEF causes electroporation in meat and has potential to enhance cellular permeability and mass transfer processes, it could affect the mineral content of meat during processing and storage. Meat is generally regarded as an excellent source of minerals like iron, zinc and phosphorus and any change in the mineral content would be of commercial interest. Khan et al. (2017a) studied the effect of low (2.5 kV, 200 Hz and 20  $\mu$ s) and high PEF (10 kV, 200 Hz and 20  $\mu$ s) on the concentration of four nutritionally important minerals (Fe, Zn, K, and P) of raw and cooked cold-boned beef *M. Longissimus et lumborum* at 1 and 14 days post-treatment. The concentrations of K and P were significantly decreased by PEF treatment and cooking as well as by post-treatment aging period. The concentration of Fe was significantly decreased by PEF treatment only with more loss observed in high PEF than low PEF treated samples. The concentration of Zn was not affected by PEF treatment. These results suggest that low and high PEF treatments can lead to changes in beef muscles with different outcomes on quality. In addition to lowered levels of mineral (P, K and Fe) concentrations, high PEF has also been reported to negatively affect the shear force and colour stability of beef *Longissimus et lumborum* (Khan et al. 2017a). Thus, there is an optimal PEF treatment for beef cuts within a range of processing parameters. Khan et al. (2017b) studied the effect of low (2.5 kV, 200 Hz and 20  $\mu$ s) and high PEF (10 kV, 200 Hz and 20  $\mu$ s) on the mineral content of raw and cooked chicken breast muscles. No significant effect of PEF was reported on the mineral content of the muscles; however, a significant effect of cooking was observed as the concentration of minerals viz. P, K and Zn decreased upon cooking. The concentration of Fe was not affected by PEF treatment, storage or cooking. These results suggest that PEF technology has no effect on concentrations of P, K, Fe and Zn in chicken breast muscles and will not affect the nutritional value of these minerals.

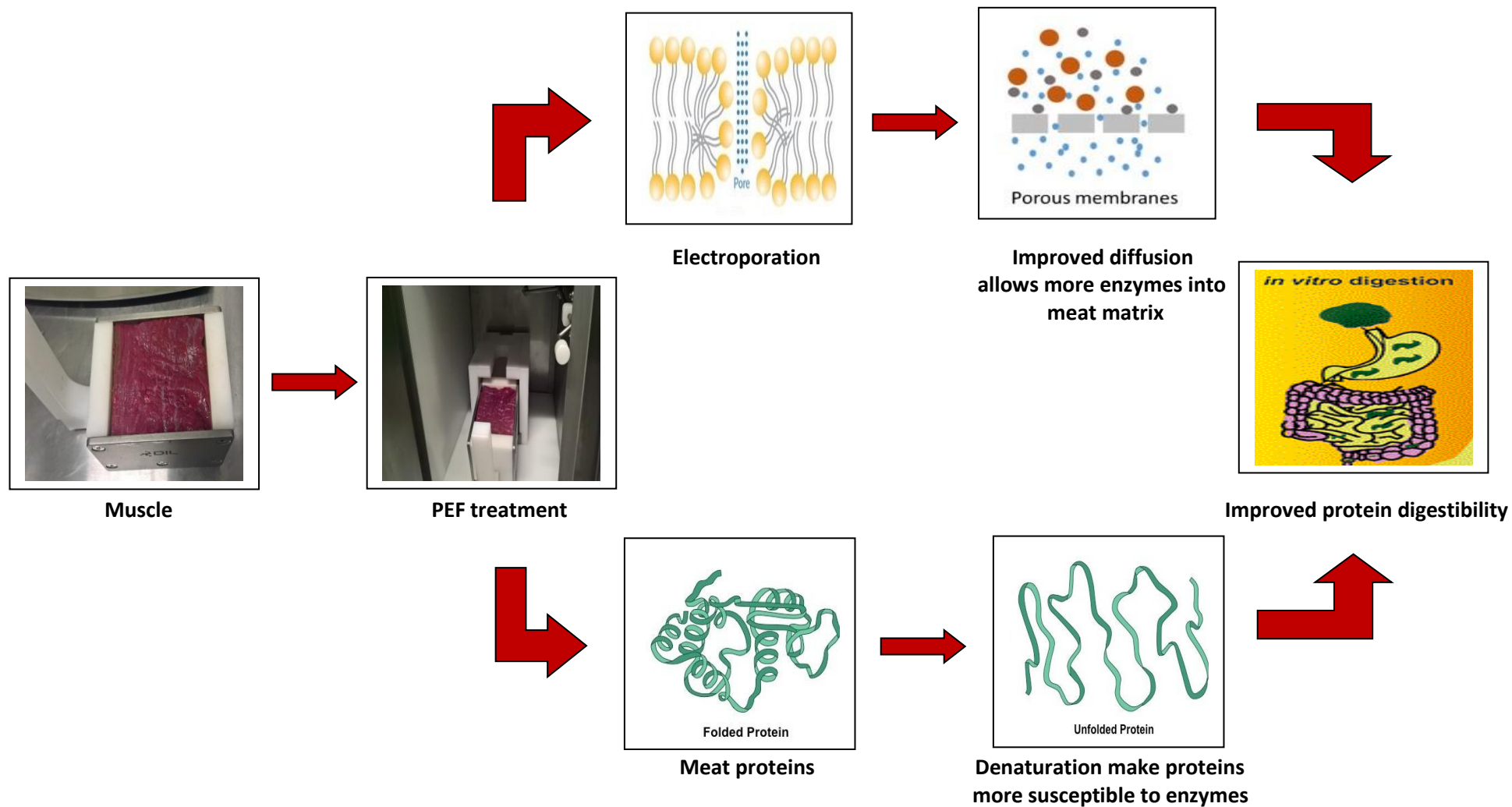


Figure 1.4 How PEF treatment may affect the digestion of meat proteins during gastrointestinal digestion

Khan et al. (2018) studied the effect of low (2.5 kV, 200 Hz and 20  $\mu$ s) and high PEF (10 kV, 200 Hz and 20  $\mu$ s) on the levels of 40 macro- and micro-minerals in raw and cooked cold-boned beef loins at 1 and 14 days of post-treatment and in chicken breasts at 1 and 4 days. PEF treatment was reported to reduce the concentration of Ca (calcium), Na (sodium) and Mg (magnesium) and increase the concentration of Cr (chromium) in beef compared to control. Chicken breast treated with high PEF showed significantly higher Ni (nickel) concentration than control and samples treated with low PEF. This increase in Cr and Ni concentration of the meat is within the safety limits and there are no chances of toxicity (Meditext 2005; EPA 2016). Both high and low PEF treated samples had higher Cu (copper) concentrations than control samples. These results suggest a differential effect of PEF on mineral content according to the type of meat and the possible release of elements from the PEF electrodes to meat samples. Pataro et al. (2014) also studied the release of Fe, Cr, Ni and Mg from stainless steel electrodes in buffers and reported an increased metal release with an increase in total specific energy input and in the presence of halides in the treated material. The above studies document the migration of minerals from the electrodes of standard PEF systems to the treated food. The electrodes used in these systems were of high quality. However, higher levels of sample contamination may occur if PEF systems are made in-house or engineered by independent labs where the quality of the electrodes is unknown.

#### **1.4.5 Meat safety**

At high electric field strengths (>20 kV/cm), PEF has been shown to be lethal to many spoilage and pathogenic bacteria at or near ambient temperature (Zhao et al. 2013; Haughton et al. 2012; Moritz et al. 2012; Rodríguez-González et al. 2011) and can be used as an alternative to conventional thermal pasteurization processes to inactivate food microbes and quality related enzymes while retaining the nutritional, sensory and health-promoting characteristics of the products (Sánchez-Vega et al. 2014). Sufficient microbial inactivation depends on the properties of food matrices and intensity of the pulses in terms of energy, field strength and number of pulses applied on the bacterial strain under study (Toepfl et al. 2007a). It is one of the most validated non-thermal food preservation techniques (Sanz-Puig et al. 2016) and is mostly used as an alternative to thermal pasteurization for liquid and semi-solid stuffs like milk, egg liquid, juices and potato dextrose agar (Zhao et al. 2013, Haughton et al. 2012; Zhang et al. 1994). Treatment at 25-40 kV/cm has been reported to reduce number of pathogenic and spoilage bacteria by 3-6 logs and extend the shelf-life to 3-4 weeks for milk and 5-8 weeks for milk beverages under refrigerated storage (Zhao et al. 2012).

PEF causes inactivation of enzymes as well as destruction of spoilage and pathogenic microorganisms through formation of hydrophilic pores as well as the forced opening of protein channels in the membrane (Buckow et al. 2014; Sharma et al. 2014). The preservative potential of the technology has

been studied under commercial conditions on the microorganisms of public health importance, such as *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes*, achieving log reductions of 2.0-4.2, 0.5-4.0, 1.5-3.3, and 0.6-1.5, respectively, depending on processing conditions and food type (Saldaña et al. 2014). Due to their low conductivity and high protein-fat content, PEF seems to have limited applicability on solid foods like meat and meat products. PEF was reported to be ineffective at controlling *E. coli* O157:H7 in beef burgers or on beef trimmings (Bolton et al. 2002). Application of PEF resulted in 2-log reduction of *E. coli* K12 suspended in a meat injection solution (Rojas et al. 2007) but a high electric field strength of  $\geq 7$  kV/cm was required which resulted in arcing. A reduction of 8-log in *E. coli* O157:H7 was observed on goat meat immersed in a brine solution using a pulsed DC square wave electric signal for a treatment time of 32 minutes (Saif et al. 2006). Test meat pieces of 25 x 25 x 30 mm sizes weighing approximately  $20.0 \pm 2.0$  g were cut from thawed goat meat (frozen at  $-20$  °C for up to 4 weeks) inoculated with *Escherichia coli* O157:H7 on the surface and covered with a thin film of 0.15 M sodium chloride solution. Stachelska et al. (2012) investigated the efficacy of PEF for inactivation of *Y. enterocolitica* ( $6.7 \log_{10}$  CFU/g of meat) in minced beef meat using pulse frequency of 28 to 2800 MHz and electric field strength of 300 V/m. PEF treatment with the pulse frequency of 28 MHz was reported to be ineffective for inactivating *Y. Enterocolitica* in beef samples; however, the pulse frequency of 280 MHz was effective for inactivating *Y. Enterocolitica* in beef samples stored at  $-20$  °C for 30 days. PEF treatment with 2800 MHz pulse frequency was highly effective in controlling the bacteria in meat stored both at  $+4$  °C and at  $-20$  °C. The authors concluded that PEF is a safe and effective method of meat decontamination and can be successfully carried out on frozen meat for enhancing the meat safety. Recently, a two log-unit increase in aerobic microbial counts was reported by Faridnia et al. (2015) during log phase of frozen-thawed PEF-treated beef samples in comparison to untreated control. Significantly higher microbial counts for the treated samples were explained by the increased purge loss observed in these samples. Haughton et al. (2012) studied the efficacy of PEF to inactivate a range of microorganisms (*Campylobacter isolates*, *Escherichia coli* and *Salmonella enteritidis*) on raw chicken meat and in liquid media. No significant reductions were observed in total viable counts of *Enterobacteriaceae*, *C. jejuni*, *E. coli* or *S. Enteritidis* in inoculated samples of raw chicken treated with PEF (3.75 and 15 kV/cm, 5 Hz, 10 ms). Isolates of *Campylobacter* in liquid were susceptible to PEF treatment (65 kV/cm, 500 Hz, 5 ms) with reductions of between 4.33 and 7.22  $\log_{10}$  CFU/mL. The authors concluded that PEF technology may have potential to reduce contamination of process water; however, it is not suitable as an intervention measure for food safety for the control of microbial contaminants on broilers during processing. To achieve better results in terms of food safety and quality, novel combinations of PEF with other hurdle technologies were recently proposed which included the addition of antimicrobial agents (Clemente et al. 2019; Bermúdez-Aguirre et al. 2012; Smith et al. 2002) and other emerging physical hurdles such as high intensity light pulses and manothermosonication (Palgan et al. 2012). A

sequential combination approach of PEF processing (0.25-1 kV/cm) followed by re-suspension in oregano essential oil ( $\frac{1}{4}$  MIC) showed a synergistic behaviour and achieved an inactivation in the same range as those acquired using conventional thermal treatments in chicken (Clemente et al. 2019). The overall conclusion is that PEF treatment of intact meat cuts does not cause a significant impact on the microbial load and better effects can be found in meat immersed or suspended in solutions. The technology is unlikely to be useful for the meat industry for improving the safety of fresh meat cuts.

## **1.4.6 Processed meats**

### **1.4.6.1 Accelerated diffusion processes**

Cell membranes play an important role in the curing and brining process of the cured meats as they resist the free diffusion and equalisation of salt (NaCl) in the muscle tissue (Janositz et al. 2011). PEF is reported to enhance mass transfer processes because of its membrane permeabilization potential (Toepfl et al. 2014a; Siemer et al. 2012) and this property finds it an application in the meat industry in the curing process. Several techniques have been used to accelerate the curing process and reduce processing time but have not considered the resistance offered by the membranes. Toepfl and Heinz (2007a) were first to show the potential of PEF in the brine acceleration process when they observed that prior treatment with PEF (3 kV/cm) could improve the diffusion of salt and nitrite in pork. Recently, McDonnell et al. (2014) confirmed the potential of PEF in accelerating the brining process in pork. PEF treatments of varying energy densities (22.6-181.1 kJ/kg) were used as pre-treatments and evaluated for rate of saline diffusion. Two treatments viz. 1.2 kV/cm 100 Hz for 300 pulses and 2.3 kV/cm 100 Hz for 300 pulses were reported to increase the salt content (NaCl) significantly in comparison to control. These findings confirm the potential of PEF in accelerating the salting process and reducing the curing time. Although, the exact mechanism of how PEF improved the salt diffusion process was not deciphered, it was suggested that PEF could fragment the myofibrils (O'Dowd et al. 2013) and create gaps within the muscle structure (Gudmundsson and Hafsteinsson 2001) which could aid in the diffusion process (McDonnell et al. 2014). A similar mechanism was suggested by Gudmundsson and Hafsteinsson (2001) while studying the effects of PEF on the texture of salmon, demonstrating that gaps in the microstructure of salmon caused collagen leak into extracellular space. PEF has been reported to cause a porous structure in ham that holds brine through capillary forces (Klonowski et al. 2006). Faridnia et al. (2016) reported a dramatic increase in the number of ruptured myofibrils along the Z-lines in PEF treated beef samples which resulted in a muscle with a more porous structure that accounted for the observed increase in electrical conductivity and purge loss. PEF has also been reported to accelerate the fermentation process of raw sausages (salami type) by improving the availability of intracellular liquid for bacterial cultures in minced beef (Raso and Heinz 2006).

Due to its potential to accelerate diffusion processes, one of the positive effects of PEF treatment is the reduction of the processing time during dry ageing of meat through increased water movement. It has been reported to facilitate a faster rate of moisture loss and accelerate the dry ageing process in venison without any detrimental effect on the total weight loss (Mungure et al. 2017).

#### **1.4.6.2 Sodium reduction**

Low sodium meat products are suitable for persons with high blood pressure disorders and help to improve the health of the population (Kunnath et al. 2015; Cardoso et al. 2014). Strategies to reduce salt in processed meat products have been especially focused around restructured meat products and many low sodium ingredient options have been successfully produced in restructured meat products like sausages (Inguglia et al. 2017). The structural functions of salt-soluble proteins in these products have been replaced by the addition of other ingredients like milk or soy proteins, starches and gums (Fellendorf et al. 2016). In addition, the use of the microbial enzyme transglutaminase, which improves the quality parameters like textural and emulsifying properties, heat stability, and gelation (Gaspar and De Góes-Favoni 2015; Moreno et al. 2010), also allows a reduced salt content of restructured meat products (Cardoso et al. 2014).

One of the current approaches to reduce the sodium content of processed foods and meat products is by improving the salt diffusion using novel processing technologies like high pressure processing or ultrasound (Inguglia et al. 2017). A natural increase in the saltiness was reported in dry-cured pork loin treated with high pressure in a study due to a change in interaction between sodium ions and protein structures which may have caused a higher release of sodium to the taste receptors on the tongue (Clariana et al. 2011). Salt reduction using ultrasound is based on the understanding that it can modify cell membranes and affect mass transfer processes like curing and brining. Use of ultrasound leads to a better distribution of salt during brining which delivers a higher salt perception even with lower overall salt content (Alarcon-Rojo et al. 2015). Thus, a rapid curing technology along with the increased salt gain rate could allow benefits like salt reduction (Tao and Sun 2015). No such attempt has been made using pulsed electric field which also has the potential to enhance mass transfer and has been recently reported to accelerate the curing process by improving the salt diffusion in the pork (McDonnell et al. 2014). The PEF processing of meat may result in a better salt distribution which could deliver a higher salt perception. Thus, PEF is expected to aid in protein extraction during restructuring process and may also help to reduce sodium content of the restructured meat products. Future research needs to focus on these unexplored areas.



#### **1.4.6.3 Restructured meat products**

PEF has been reported to enhance the recovery of compounds, food additives and nutraceuticals from different matrices by electrically piercing the cell membrane which loses its semi-permeability temporarily or permanently under its influence (Rodríguez-Roque et al. 2015; Deng et al. 2014; Barba et al. 2015; Abenoza et al. 2013). The semi-permeable membrane which separates the cell cytoplasm from its surroundings is considered as the main barrier for mass transport processes aiming for diffusion of soluble products out of the food tissues. Techniques like PEF, which cause the physical disintegration of the membranes, facilitate diffusion and improve mass transport processes (Toepfl et al. 2014a). Permeabilization of cellular tissue has been reported to improve all mass transfer processes, such as distillation or extraction (Siemer et al. 2012). PEF has been investigated as a cold process (non-thermal) for extraction of intracellular compounds (Barba et al. 2012; Gachovska et al. 2010) and has recently been reported to be very effective for improving extraction from borage leaves (Segovia et al. 2015), sugar beet (Lebovka et al. 2007; Vorobiev and Lebovka 2008) and betalains (red purple pigments) from red beets (Loginova 2011). It has been proposed to be a promising future industrial application for extraction of soluble matter from chicory roots (Loginova et al. 2010). Given the fact that the restructuring process involves extraction of proteins from the membrane bound muscle fibres, PEF could probably find an application in restructured meat products as pre-treatment of meat is expected to improve the release of myofibrillar and sarcoplasmic proteins.

#### **1.4.6.4 Lipid and protein oxidation**

Oxidative reactions, with lipids and proteins as main targets in meats, have been considered as one of the leading causes of deterioration of quality in meat and meat products (Silva et al., 2018). While most studies have reported no impact of PEF treatment on the lipid and protein oxidation of beef muscles (Alahakoon et al. 2019; Suwandy et al. 2015c; Faridnia et al. 2015), Kantono et al. (2019) recently reported a significant increase in lipid oxidation (TBARS) of PEF processed beef samples. Arroyo et al. (2015b) also observed no change in the lipid oxidation of PEF treated turkey breast compared to control.

#### **1.4.6.5 Sensory quality**

A very limited number of studies have evaluated the impact of PEF on the sensory characteristics of meat and meat products. While several workers have reported a significant impact of PEF on the tenderness in beef muscles in terms of instrumental texture analysis, such as shear force measurement (Bekhit et al. 2016; Bekhit et al. 2014c), Arroyo et al. (2015b) observed a significant ( $P < 0.05$ ) decrease in the scores for tenderness for PEF treated turkey breast in comparison to untreated samples. While

evaluating the sensory properties of PEF treated beef muscles, Arroyo et al. (2015a) recorded that 60% of the panellists scored PEF treated samples as tender in comparison to 27.5% who scored untreated samples as tender.

While Arroyo et al. (2015a) observed no effect of PEF processing on the odour scores of beef muscles, other studies involving the temporal dominance of sensations showed that PEF treated samples were associated with juicy, browned, livery, brothy and meaty flavour attributes (Kantono et al. 2019; Ma et al. 2016). Arroyo et al. (2015b) observed a significant ( $P < 0.05$ ) decrease in odour scores of PEF treated turkey breast in comparison to untreated samples, however, no effect of PEF treatment was observed on the colour scores of the turkey breast. Impact of PEF on sensorial properties of meat and meat products is inconclusive and further experimentation is required before arriving at a logical conclusion.

#### **1.4.7 Supercooling**

Defined as the process of lowering the temperature below the usual freezing point of a product without the formation of ice crystals, supercooling is a metastable state of water (Stonehouse and Evans 2015) that could be utilized to prevent the quality loss in meat due to the formation of ice crystals during freezing. Supercooled meat products could be processed or consumed at freezing storage conditions without quality deterioration by ice crystallization. Recently, a combination of electric field and magnetic field treatments was reported to affect the mobility of water molecules (Mok et al. 2015; Wowk 2012) and the combination of both these technologies was explored to achieve an extension of a supercooled state in chicken breast (Mok et al. 2017). PEF, a promising technology to improve the freezing and drying processes (Carbonell-Capella et al. 2016; Puértolas and Barba 2016; Koubaa et al. 2015), was applied in combination with oscillating magnetic field to vibrate water molecules and inhibit sudden ice nucleation in chicken breast (Mok et al. 2017). Temperature of chicken breast samples decreased to  $-6.5\text{ }^{\circ}\text{C}$  in a supercooling state during the whole testing period of 12 hours at the freezer temperature of  $-7\text{ }^{\circ}\text{C}$  ( $\pm 0.5$ ). The samples were also analysed for various quality parameters like microstructure, drip loss, colour, texture, pH and lipid oxidation to evaluate the impact of supercooling on the quality of meat. PEF-assisted supercooling was reported to be highly effective in maintaining the original meat quality without significant physical damage or chemical changes.

#### **1.5 Limitations of PEF**

Continuous PEF systems are considered as efficient, high-speed and low maintenance, however, high initial capital investments and cost involved in changing the plant design are viewed as highly prohibitive (Jeyamkondan et al. 1999). The reliability and cost effectiveness of the equipment are being

constantly improved and the technology is heading for wider industrial application (Toepfl and Heinz 2007b). However, nonthermal processing technologies, like PEF and HPP, have been reported to have higher environmental impact (in terms of CO<sub>2</sub> production) than traditional thermal pasteurization (Sampedro et al. 2014). While PEF is a novel non-thermal technology for production of microbiologically safe liquid foods under high-speed continuous process without affecting their sensorial and nutritional quality, it appears to be an ineffective technology for microbial decontamination of fresh meat. The high intensity PEF treatments required to inactivate the microbial load in meat have an adverse impact on sensorial and nutritional quality of meat. Further, heat produced during such intense treatments in batch mode could denature the endogenous proteolytic enzymes and affect the tenderization process during aging. This technology also fails to inactivate bacterial spores, and this prevents its use for food and water sterilization (Pillet et al. 2016). The primary applications of this technology in food preservation are thus focused on pasteurization (Saldaña et al. 2014). A potential solution to this limitation may be the combined use of other hurdle technologies, such as natural preservatives (lactic acid or essential oils) or antimicrobial agents, to improve the safety of PEF treated fresh meat. Another limitation of PEF is its uneven treatment distribution in non-uniform and complex food matrices in continuous PEF systems. This will be particularly challenging under the factory settings where bone-in meat cuts with variations in fat content will provide a matrix of non-uniform tissue density resulting in unevenly treated product with variations in effective electro-permeabilization. The non-homogenous distribution of the electric field was demonstrated by Golberg et al. (2015) who observed less than 40% of the PEF strength in the cells near vascular structures in a rat liver due to the formation of “electric fields sinks”. The implementation of PEF technology in meat industry requires guaranteeing that all parts of the meat cuts receive the established treatment during the process to achieve a permeabilization level that assures tenderization. Currently, all published reports available on application of PEF used almost homogeneous meat samples devoid of fat and bones. The available information can only be applicable to the lean muscles stated in the reported studies and therefore, a great deal of research is still needed before a commercial system becomes a reality. Moreover, different muscles and cuts in different species would need different PEF treatments for optimum results. Research is required to standardize the optimum treatment parameters for different cuts and muscles for different species.

## **1.6 Research gap**

Several studies have evaluated the effect of PEF on the quality characteristics of meat, however, no information is available in the literature, to the best of our knowledge, whether PEF affects the post-mortem calpain activity in meat. The results of different studies are varied and inconclusive and need further comprehension. Moreover, the underlying mechanisms are not clear, and no information is

available whether PEF processing can be effective in meat from deer and older animals such as culled dairy cows. Other applications of PEF such as its role in meat protein digestion and sodium reduction are completely unknown and unexplored. There is a dire need for a comprehensive study that will involve a wide range of models, such as different species, different age, and muscles of different tenderness potential, to elucidate the effect of PEF on the quality of meat.

## **1.7 Aim of Research**

The overall aim of this research was to determine how PEF affects meat quality and identify possible roles of PEF in meat protein digestion and sodium reduction. The effect of PEF on calpain activity, proteolysis and physicochemical properties of beef and venison muscles was evaluated during ageing. The influence of PEF processing on digestion kinetics of beef and venison during *in vitro* gastrointestinal protein digestion was elucidated. A reduced-sodium beef jerky was developed by using PEF processing and direct salt (NaCl) reduction to evaluate the potential of PEF-mediated sodium reduction for meat products.

## **1.8 Objectives**

To achieve the aim of the research, following objectives were designed:

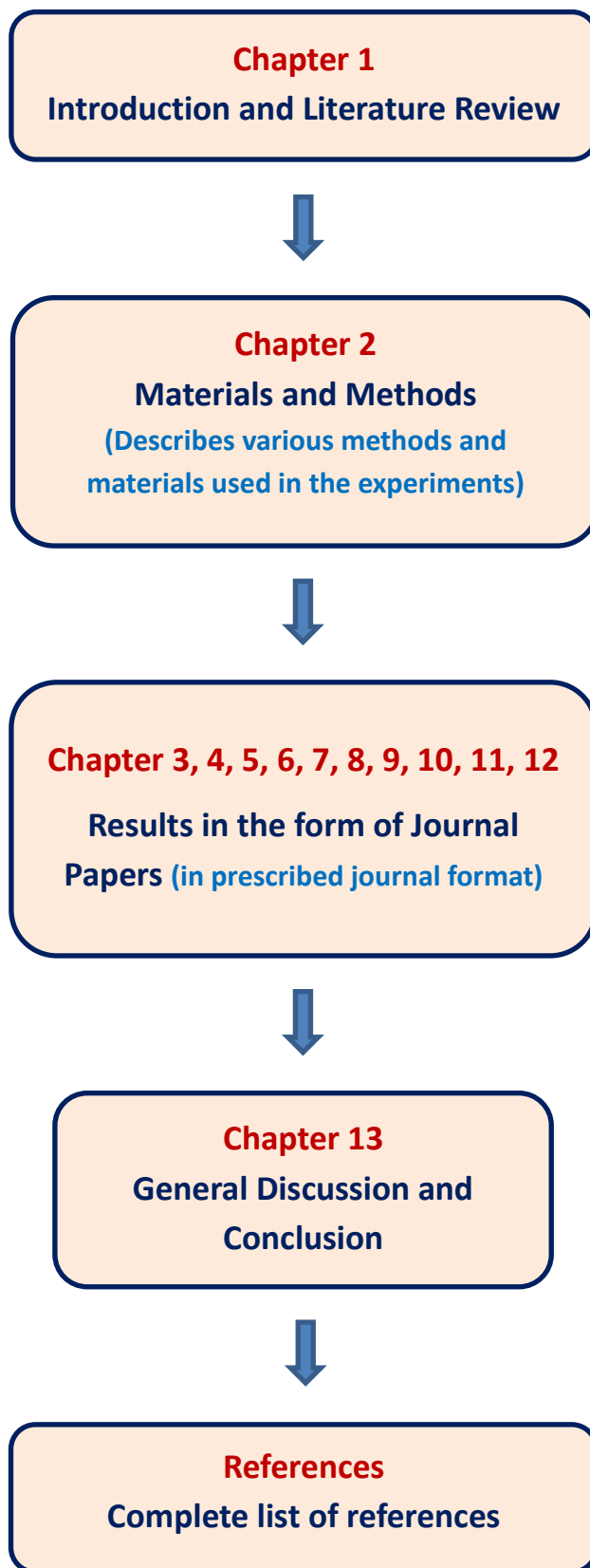
1. To evaluate the effect of pulsed electric field on the calpain activity and proteolysis of beef and venison muscle during ageing
2. To evaluate the effect of pulsed electric field on the *in vitro* protein digestibility of the beef and venison muscle
3. To evaluate the effect of pulsed electric field on the development of reduced-sodium beef jerky

## **1.9 Hypothesis**

The experiments were based on the hypotheses that:

1. By inducing electroporation, PEF has a potential to increase membrane permeability and cause early release of calcium from sarcoplasmic reticulum that can induce early activation of calpains and increase the calpain activity.
2. PEF can affect the structural and functional properties of meat proteins and can influence their susceptibility to enzymatic hydrolysis during gastrointestinal digestion.
3. PEF has a potential to improve the diffusion and distribution of salt in a meat matrix. It can influence the sensory perception for saltiness by affecting the interaction between sodium ions and proteins and the release of sodium from the meat matrix during chewing.

## 1.10 Thesis structure



# Chapter 2

## Materials and Methods

### 2.1 Overview

This chapter covers details about methods used and information about the materials used in all the experiments. An overview of the experimental design is given in Figure 2.1.

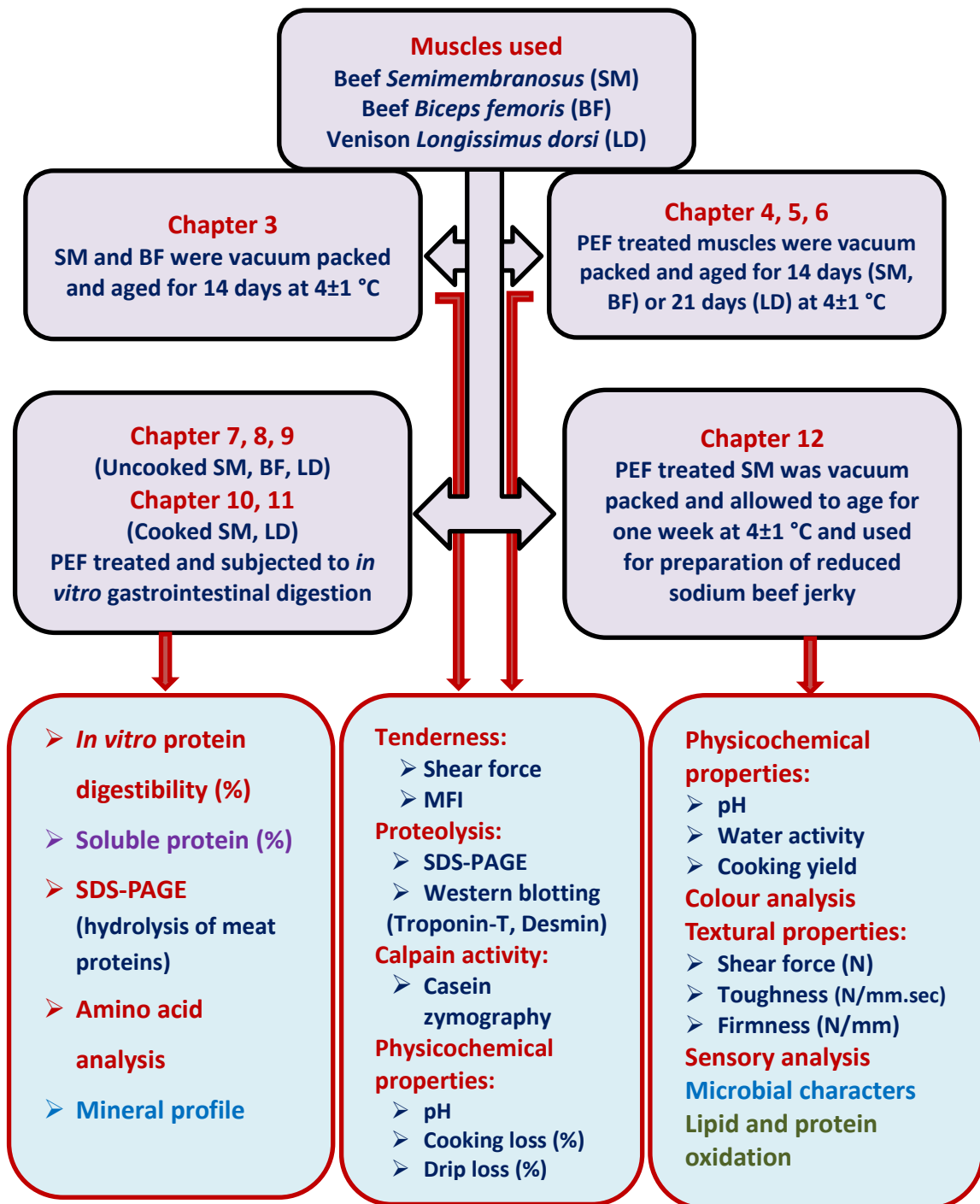


Figure 2.1 An overview of the experimental design

## **2.2 Pulsed electric field and meat samples**

### **2.2.1 PEF machine**

The PEF system (Elcrack-HPV5, DIL, Quakenbruck, Germany) available at the University of Otago was used for processing the meat samples for all the experiments. The machine (Figure 2.2) was operated in a batch mode using a bipolar square wave form. An oscilloscope (Model UT2025C, Uni-Trend Group Ltd, Hong Kong, China) was used to monitor the pulse shape used. The PEF treatment chamber (L x W x D = 13 × 8 × 5 cm) had two parallel stainless-steel electrodes positioned (8 cm) apart by a Teflon insulating material (Figure 2.7).

### **2.2.2 Beef muscles used for ageing (for chapter 3)**

Beef *Semimembranosus* (topside) and *Biceps femoris* (silverside) were obtained from six carcasses (six topsides and six silversides from six animals) of culled dairy cows (average age of nine years) from Silver Fern Farms Ltd, Christchurch, New Zealand. The average hot carcass weight was  $188.00 \pm 20.17$  kg. The carcasses were processed according to the standard industry practice [electrically stimulated (120 V, 15 Hz, 2.5 ms pulse, 60 ms interval, for a total time of 40 seconds) to avoid cold shortening and the temperature of the chilling room was maintained above 15 °C till completion of rigor]. The muscles were excised at 12 h post-mortem (which is industry practice for culled dairy animals in meat plants in New Zealand), vacuum packaged (polyethylene-aluminium-polyethylene laminate) and transferred to the laboratory under refrigerated conditions ( $4 \pm 1$  °C). Upon receiving, all visible fat and connective tissues were removed, and the trimmed meat samples were processed into blocks and were randomly assigned to 1, 2, 7 or 14 days of storage at  $4 \pm 1$  °C. On the day of the designated storage time, a subsample was snap frozen, vacuum packed and stored at  $-80$  °C for further biochemical analyses. The remaining sections of the samples were vacuum packed and stored frozen at  $-20$  °C for measurement of shear force and myofibrillar fragmentation index (MFI) which occurred within 1 week of freezing.

### **2.2.3 Beef muscles used for PEF processing (for chapter 4, 5, 7, 8, 10)**

Beef *Semimembranosus* (topside) and *Biceps femoris* (silverside) were obtained from six carcasses (six topsides and six silversides from six animals) of culled dairy cows (average age of nine years) from Silver Fern Farms Ltd, Christchurch, New Zealand. The carcasses used were different from the ones used in section 2.2.2 above. The average hot carcass weight was  $202.8 \pm 7.1$  kg. The muscles were excised at 12 h post-mortem, vacuum packaged and transferred to the laboratory under refrigerated conditions ( $4 \pm 1$  °C). The six muscles (topside and silverside processed separately) were sliced into 18



Oscilloscope



PEF digital console



Panel of PEF machine



Figure 2.2 Pulsed electric field system at the University of Otago



blocks (06 x 03 = 18 blocks, average weight of  $414.9 \pm 11.3$  g, L x W x H =  $13 \times 8 \times 5$  cm) after removing the separable fat and connective tissues and were randomly allocated for PEF treatments.

### **2.2.3.1 PEF processing conditions for beef muscles (for chapter 4, 5, 7, 8, 10)**

Beef *Semimembranosus* and *Biceps femoris* obtained from six carcasses of culled dairy cows were excised, vacuum packaged, stored at  $4 \pm 1$  °C and were treated with PEF at 30 h post-mortem. Previous studies (Bekhit et al. 2014c) on *Semimembranosus* clearly indicated that PEF technology is flexible, and the level of tenderness improvement was not dependent on the meat post-mortem time (within 3 days post-mortem) which allows greater flexibility in the use of this technology without any post-mortem time constraints. The six muscles (*Semimembranosus* and *Biceps femoris* separately) sliced into 18 blocks were randomly allocated to PEF treatments i.e. 5 kV, 90 Hz, 20  $\mu$ s ( $T_1$ ) and 10 kV, 20 Hz, 20  $\mu$ s ( $T_2$ ) and a non-treated control. The samples were treated for a total treatment time of 30 seconds. The PEF conditions used in this study were based on the findings of the previous studies (Bekhit et al. 2016; Suwandy et al. 2015a, b; Bekhit et al. 2014c) which have reported a significant effect of PEF on tenderness of beef muscles. A lower frequency (20 Hz) was used with the higher voltage (10 kV) to keep a balance of effectiveness and avoid excessive heat generation (Bekhit et al. 2014c) to prevent the denaturation of calpain proteases. The muscle blocks were in close contact with the electrodes of PEF treatment chamber and direction of the meat fibres was kept parallel to the electrodes of the PEF system. The PEF treated samples were processed into three slices that were assigned randomly to day 1, 7 or 14 of ageing at  $4 \pm 1$  °C. On the day of the designated storage time, the samples for shear force and MFI were vacuum packed and stored at  $-20$  °C for analysis within 1 week of freezing. A subsample was snap frozen, vacuum packed and stored at  $-80$  °C for further biochemical analysis. Samples (n=3) were collected randomly from *Semimembranosus* and *Biceps femoris* separately on day 1 of ageing for *in vitro* simulated gastrointestinal digestion (chapter 7, 8, 10) and were vacuum packed and stored frozen at  $-20$  °C. Figure 2.3 to 2.14 shows a pictorial presentation of whole PEF processing operation that ended up at the samples for ageing and analysis.

### **2.2.4 Beef muscles used for PEF processing for beef jerky (for chapter 12)**

Six beef topsides (*Semimembranosus*, premium quality) were obtained from six carcasses (different from the ones used in section 2.2.2 or 2.2.3) of average hot carcass weight of  $289.5 \pm 5.64$  kg (Silver Fern Farms Ltd, Christchurch, New Zealand). The muscles were excised, vacuum packaged, stored at  $4 \pm 1$  °C and were treated with PEF at 24 h post-mortem. The meat samples were removed of any visible fat and connective tissues and were processed into 18 blocks (L x W x H =  $13 \times 8 \times 5$  cm, average weight of  $441.0 \pm 13.12$  g) for PEF treatment.

#### **2.2.4.1 PEF processing conditions for beef muscles for beef jerky (for chapter 12)**

Each of the six beef *Semimembranosus* muscles were sliced into three sections to give 18 blocks which were randomly allocated into three groups viz. control (2% NaCl), reduced salt (1.2% NaCl) and reduced salt with PEF treatment (1.2% NaCl-PEF). Samples were treated with PEF (0.52 kV/cm, 10 kV, 20 Hz, 20  $\mu$ s) at 24 h post-mortem and the meat fibre direction was kept parallel to the electrodes. The samples were treated for a total treatment time of 30 seconds. A lower frequency (20 Hz) was used with a higher voltage (10 kV) to minimize the heat generation. The PEF conditions used in this study were based on the results of the previous studies (Bekhit et al. 2016; Suwandy et al. 2015a, b; Bekhit et al. 2014c) which have reported a significant impact of PEF on tenderness of beef *Semimembranosus*. The meat samples were vacuum packed, allowed to age for one week and used for the development of beef jerky.

#### **2.2.5 Venison muscles used for PEF processing (for chapter 6, 9, 11)**

*Longissimus dorsi* (loin) was obtained from six carcasses of red deer (*Cervus elaphus*) from the Lorneville plant (Alliance Group, Invercargill, New Zealand). The average hot carcass weight was  $108 \pm 9.8$  kg. The muscles were excised at 24 h post-mortem, vacuum packaged and stored at  $4 \pm 1$  °C. After removal of all the visible fat and connective tissues, the trimmed meat samples were processed into 18 blocks (L x W x H =  $13 \times 8 \times 5$  cm, average weight of  $318 \pm 11.6$  g) and randomly allocated for PEF treatments.

##### **2.2.5.1 PEF processing conditions for venison muscles (for chapter 6, 9, 11)**

Six venison *Longissimus dorsi* were excised at 24 h post-mortem and were treated with PEF within 6 h. The muscle blocks (06 x 03 = 18) were randomly allocated to PEF treatment combinations i.e. 2.5 kV, 50 Hz, 20  $\mu$ s ( $T_1$ ) and 10 kV, 90 Hz, 20  $\mu$ s ( $T_2$ ) plus a non-treated control. The samples were treated for a total treatment time of 30 seconds. The PEF conditions used were based on the findings of the previous studies (Bekhit et al. 2016; Suwandy et al. 2015a, b; Bekhit et al. 2014c) which have reported a significant effect of PEF on tenderness and proteolytic patterns of beef muscles. The PEF treated samples were aged for 21 days at  $4 \pm 1$  °C. The samples for shear force and MFI were vacuum packed and stored at  $-20$  °C for analysis within 1 week of freezing. A subsample was snap frozen, vacuum packed and stored at  $-80$  °C for further biochemical analysis. Samples (n=3) for *in vitro* simulated gastrointestinal digestion were collected randomly and were vacuum packed and stored frozen at  $-20$  °C.



**Figure 2.3 Chilly bin used to transport muscles**



**Figure 2.4 Vacuum packaged muscles**



**Figure 2.5 Splitting muscles into six blocks**



**Figure 2.6 Adjusting the dimensions of each block**



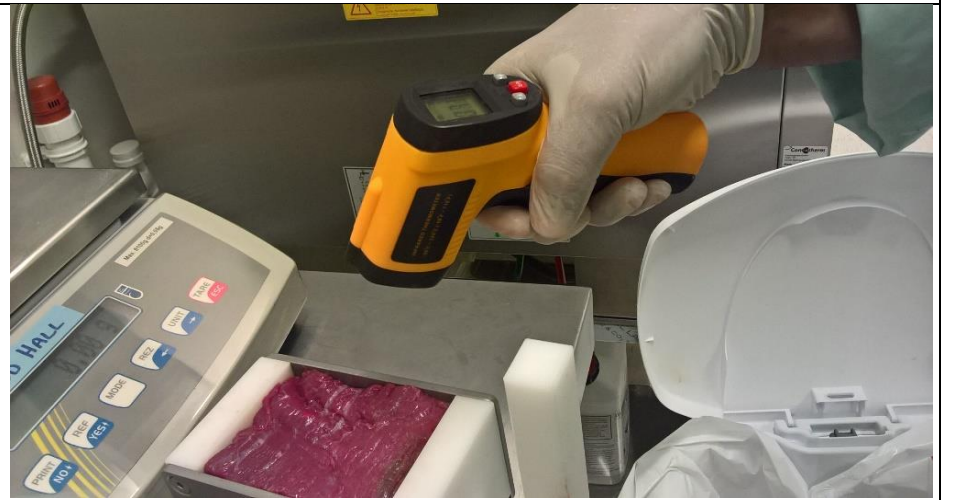
**Figure 2.7 PEF treatment chamber**



**Figure 2.8 PEF treatment chamber with muscle block**



**Figure 2.9 Measuring the conductivity of muscle**



**Figure 2.10 Measuring the temperature of muscle**



**Figure 2.11 PEF treatment chamber in PEF machine**



**Figure 2.12 PEF processing of the muscle block**



**Figure 2.13 Samples for ageing**



**Figure 2.14 Samples for molecular analysis (snap frozen)**

## 2.2.6 Electrical input

The PEF processing parameters recorded from the PEF machine during the treatment were pulse count, pulse peak power, pulse peak current, pulse electric field strength, pulse peak voltage, resistance and pulse energy. The energy density (kJ/kg) was determined by the method described by Bekhit et al. (2014c) using the equation:

$$\text{Energy density (Q)} = \frac{V^2t}{Rv}$$

where V is the voltage in volts, t is the treatment time (pulse duration in  $\mu\text{S}$  x number of pulses), R the resistance in ohms and v is the weight of the sample in g.

## 2.2.7 Change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ )

The temperature ( $^{\circ}\text{C}$ ) and electrical conductivity (mS/cm) of each meat block was measured at four locations per block (close to the electrodes and in the middle of the blocks) before and immediately after PEF processing using an infrared thermometer (Tech imports, Auckland, New Zealand) and an electrical conductivity meter (LF-Star, Matth us, Germany), respectively, and the results were reported as the change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ ).

## 2.3 Meat Quality

### 2.3.1 Shear force

Shear force was determined as per the method described by Chrystall and Devine (1991) using a MIRINZ tenderometer (Figure 2.15) based on ten replicate measurements for each sample. Meat samples were weighed and cooked individually in plastic bags immersed in a water bath maintained at  $80^{\circ}\text{C}$  until they attained an internal temperature of  $75^{\circ}\text{C}$  as measured individually using Fluke type K temperature probes attached to Fluke 52 thermometer (Fluke Corp., Everett, WA). The cooked meat samples were cooled on ice and patted dry with paper towels. The cooked samples were cut into strips with a cross section area of  $10 \times 10 \times 25$  mm, parallel to the muscle fibre direction using a double-bladed scalpel with blades set 10 mm apart. The meat strips were placed into the metal wedge of a MIRINZ tenderometer (AgResearch MIRINZ, Hamilton, New Zealand). The tenderness values were obtained by measuring the peak force required to cut across the meat strip, and mean force values (reported in Newton, N) were used in the statistical analysis. The mean reading of the MIRINZ tenderometer (kPa) were converted to shear force (kgF) and then to Newton (N) using equation:

$$\text{Shear force (N)} = [(kPa \times 0.216) - 2.030] \times 9.8$$



**MIRINZ Tenderometer**



**Gas cylinder with gauge**



**Metal wedge for meat strips**



**Waterbath maintained at 80 °C**



**Fluke 52 thermometer**



**Double-bladed scalpel and forceps**

**Figure 2.15 MIRINZ tenderometer and accessories used for measuring shear force**

The percentage change in the shear force from day 1 to day 14 was determined by the following equation:

$$\text{Change in shear force (\%)} = \frac{SF_{1d} - SF_{14d}}{SF_{1d}} \times 100$$

where  $SF_{1d}$  is the shear force on day 1 (N) and  $SF_{14d}$  is the shear force on day 14 (N).

### 2.3.2 Purge loss (%) and cooking loss (%)

Purge and cooking loss of vacuum packaged meat samples was determined after 1, 7 and 14 days of ageing using the following formulae:

$$\text{Purge loss (\%)} = 100 \times \left[ 1 - \frac{\text{Weight of the sample after storage}}{\text{Initial weight of the sample before storage}} \right]$$

$$\text{Cooking loss (\%)} = 100 \times \left[ 1 - \frac{\text{Weight of the sample after cooking}}{\text{Weight of the sample before cooking}} \right]$$

### 2.3.3 Myofibrillar fragmentation index (MFI)

Originally described by Culler et al. (1978), the method was modified as described by Hopkins et al. (2004) and Silva et al. (2017). Duplicate 0.5 g samples of the muscle were taken and sliced along the fibre direction avoiding any connective tissue or visible fat. Muscle samples were homogenized twice (2 x 30 sec) by using Polytron homogenizer (Polytron® PT – MR 3100, Kinematica AG, Switzerland) with a 10 mm diameter shaft at 19,000 rpm in 50 mL Falcon tubes containing 30 mL of ice-cold buffer and held on ice between homogenizations. The buffer [0.1 M KCl, 1 mM EDTA (di-sodium), 1 mM sodium azide (NaN<sub>3</sub>) and 25 mM potassium phosphate (7 mM KH<sub>2</sub>PO<sub>4</sub> and 18 mM K<sub>2</sub>HPO<sub>4</sub>)] was adjusted to a pH of 7 using K<sub>2</sub>HPO<sub>4</sub> (base) or KH<sub>2</sub>PO<sub>4</sub> (acid) at a temperature of 5 °C. After homogenization, the myofibril suspensions were filtered into a 100 mL beaker using mesh strainers with 1 mm<sup>2</sup> holes to remove connective tissue. Washing the myofibrils with 5 mL of cold buffer facilitated filtration through the mesh. The filtrates were centrifuged (Centra GP6R, Thermo IEC, Needham Heights, MA, USA) at 2 °C at 1000 g for 10 min and the supernatant decanted. The pellets of myofibrils were re-suspended in 10 mL of buffer, shaken thoroughly and centrifuged again. This process was repeated two times, and the pellet finally re-suspended in 10 mL of cold buffer. The protein concentration of the suspensions was determined in triplicate using the bicinchoninic acid (BCA) method (Pierce Chemical Company, Illinois, USA). Absorption was measured at 560 nm in a micro-plate reader (FLUOstar Omega, BMG



Labtech GmbH, Ortenberg, Germany) in accordance with the specified protocol and a bovine serum albumin standard curve was used. Aliquots of the suspensions were diluted in buffer to a final protein concentration of 0.5 mg/mL in triplicate. The diluted protein suspensions were poured into a cuvette, mixed and the absorbance measured immediately at 540 nm using a spectrophotometer (V-1200 Spectrophotometer, Global Science, VWR International, Leuven). The mean of the triplicate absorbance readings was multiplied by 150 to give index values for myofibrillar fragmentation. The percentage change in the MFI from day 1 to day 14 was also determined.

## **2.4 Protein Analysis**

### **2.4.1 Preparation of sarcoplasmic and myofibrillar fractions**

Sarcoplasmic and myofibrillar protein fractions were separated according to the procedure described by Ilian et al. (2004b) with some minor modification. The beef samples frozen at  $-80^{\circ}\text{C}$  were trimmed of visible fat and connective tissue. A  $1.00 \pm 0.01$  g of meat was excised from each subsample and was finely diced and homogenized by Polytron homogenizer (Polytron<sup>®</sup> PT – MR 3100, Kinematica AG, Switzerland) in 10 volumes of ice-cold buffer [0.1 M KCl, 2 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1 mM  $\text{NaN}_3$ , 25 mM  $\text{KH}_2\text{PO}_4$  (7 mM  $\text{KH}_2\text{PO}_4$  and 18 mM  $\text{K}_2\text{HPO}_4$ ), pH 7.0] in 50 mL Falcon tubes at 10,000 rpm for 30 sec x 2 times with 30 sec cooling in between the homogenizations on the ice. The homogenate was centrifuged at  $1000 \times g$  at  $4^{\circ}\text{C}$  for 15 min and the resulting supernatant (sarcoplasmic fraction) was collected in clean 2.0 mL microcentrifuge tubes and stored at  $-80^{\circ}\text{C}$  until further processing. The pellet was suspended in the homogenization buffer and centrifuged as before. This washing step was repeated two times and the pellet was finally resuspended in 5 volumes of ice-cold buffer, vortexed, followed by addition of denaturing buffer (50.0 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 12.5 mM EDTA; 0.02% bromophenol blue; 1% (v/v) 2  $\beta$ -mercaptoethanol) and incubation at  $70^{\circ}\text{C}$  for 15 minutes (myofibrillar fraction).

### **2.4.2 Protein concentration**

The concentrations of the sarcoplasmic and myofibrillar extracts were determined in triplicate using the bicinchoninic acid (BCA) method (Pierce Chemical Company, Illinois, USA). Absorption was measured at 570 nm in a micro-plate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany) using bovine serum albumin as a standard and the extraction buffers as blanks.

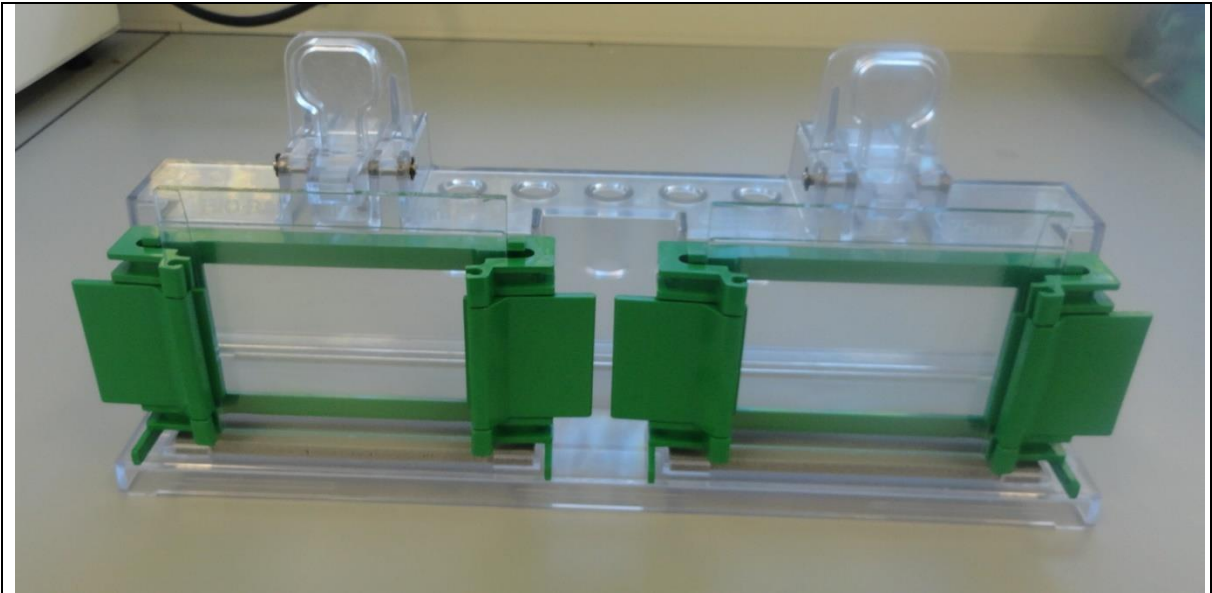
### 2.4.3 Casein zymography

The proteolytic activity of calpains was determined in the sarcoplasmic fractions by casein zymography (Raser et al. 1995) as described by Lee et al. (2008) with some modification (n=6). A mixture of calpains extracted from 6 muscle samples on day 0 was loaded on each gel as a standard. The quantification of the calpain activity was determined by expressing the density of the bands from samples relative to the density of the reference standard within each gel. A two-phase casein zymogram mini gel (a casein gel topped by a stacking gel) was prepared. A ten percent non-denaturing polyacrylamide mix (0.225 M Tris-HCl, pH 7.5; 10% acrylamide (37.5:1); 0.06% ammonium persulfate; 0.06% TEMED) was copolymerised with 0.05% casein (pH 7.5, Hammersten grade, BDH) in a BioRad Mini-PROTEAN® 3 Casting Frame at 0.75 mm thickness. Once the casein gel was set, a 4 percent non-denaturing stacking gel for sample loading was prepared (0.125 M Tris-HCl, pH 6.8; 3.85% acrylamide (37.5:1); 0.1% ammonium persulfate; 0.1% TEMED). Once the stacking gel was set, the gel was pre-run at 4 °C in zymography running buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; 1 mM EGTA; 1 mM DTT) in a Mini-Protean III (BioRad) electrophoresis tank at 125 V for 15 min. A known amount of soluble protein sample was mixed with zymogram loading buffer (0.04 M Tris-HCl, pH 6.8; 6.5% glycerol; 0.005% bromophenol blue; 0.8% 2-β-mercaptoethanol) and was loaded on to the pre-run casein gel. All gels were run with the zymogram running buffer at 150 V for 60 min at 4°C. After electrophoresis, the gel was removed from the tank and incubated with gentle shaking overnight at room temperature in calcium incubation buffer (20 mM Tris-HCl, pH 7.4; 20 mM Ca<sup>2+</sup>; 10 mM DTT). Gels were rinsed with distilled water for 15 min and then stained with Simply Blue™ Safe Stain (Invitrogen) and washed several times in dH<sub>2</sub>O. Transparent bands appeared on a blue stained background gel. The calpains separated by electrophoresis at 150 V for 60 min revealed four bands corresponding to native calpain 1, autolysed calpain 1, native calpain 2 and autolysed calpain 2. Calpain activity was indicated by clear zones in the stained gels. The extent of calpain activity in each band was determined by the density of the reversed image of the gel using the Gel Doc™ XR<sup>+</sup> with Image Lab™ Software (S. No. 721BR15168, Bio-Rad, USA).

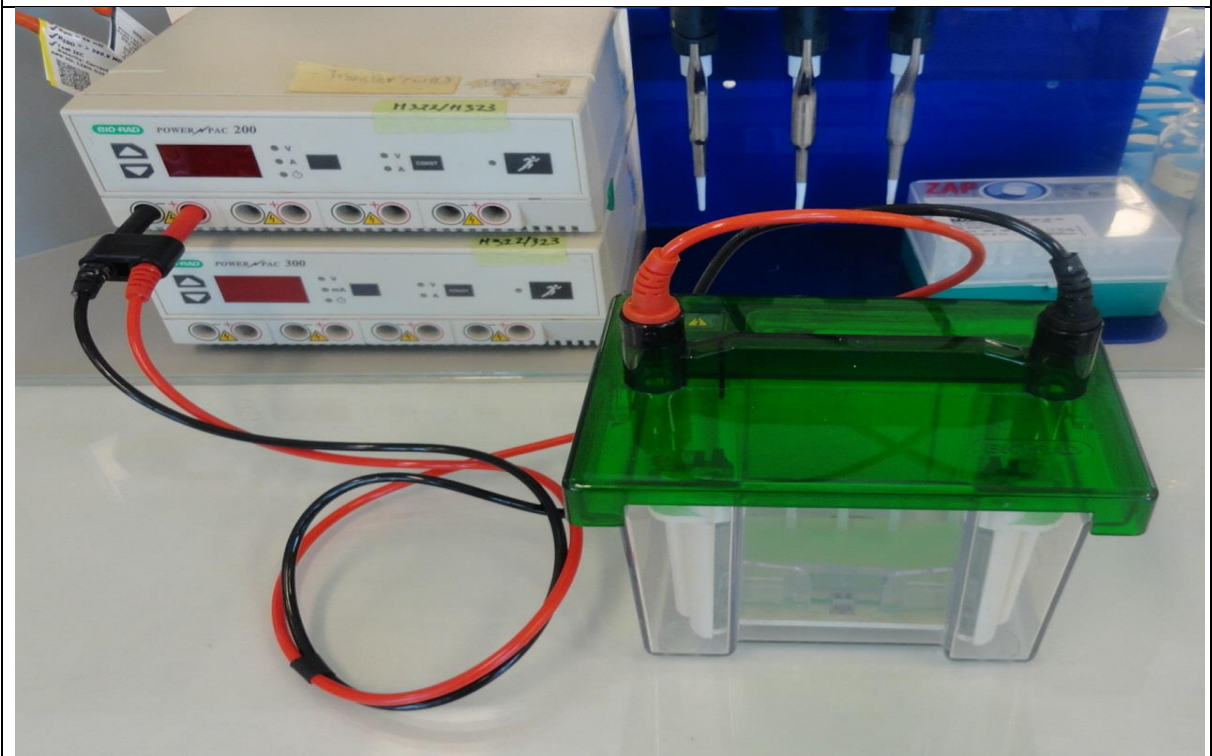
### 2.4.4 SDS-PAGE

SDS-PAGE was used to examine the myofibrillar protein profiles of the muscles following the method described by Lee et al. (2008). A 12.0% polyacrylamide gel (acrylamide: bisacrylamide = 37.5:1 [w/w], 0.10% [w/v] SDS, 0.05% [v/v] TEMED, 0.05% [w/v] ammonium persulfate, and 0.384 M Tris-HCl, pH 8.8) was used as the separating gel and 4.0% polyacrylamide gel (acrylamide: bisacrylamide = 37.5:1 [w/w], 0.10% [w/v] SDS, 0.10% [v/v] TEMED, 0.07% [w/v] ammonium persulfate, and 0.125 M Tris-HCl, pH 6.8) was used as the stacking gel. Known amounts of soluble protein samples from various

treatments were mixed with SDS sample loading buffer (50.0 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 12.5 mM EDTA; 0.02% bromophenol blue; 1% (v/v) 2  $\beta$ -mercaptoethanol) and denatured at 70 °C for 15 min. After cooling, the stock samples and molecular markers (Precision Plus Protein™ Standards, All Blue) were loaded onto Bis-Tris gels (0.75 mm x 10 well) prepared in a BioRad Mini-PROTEAN® 3 Casting Frame (Figure 2.16).



**Preparation of gels**

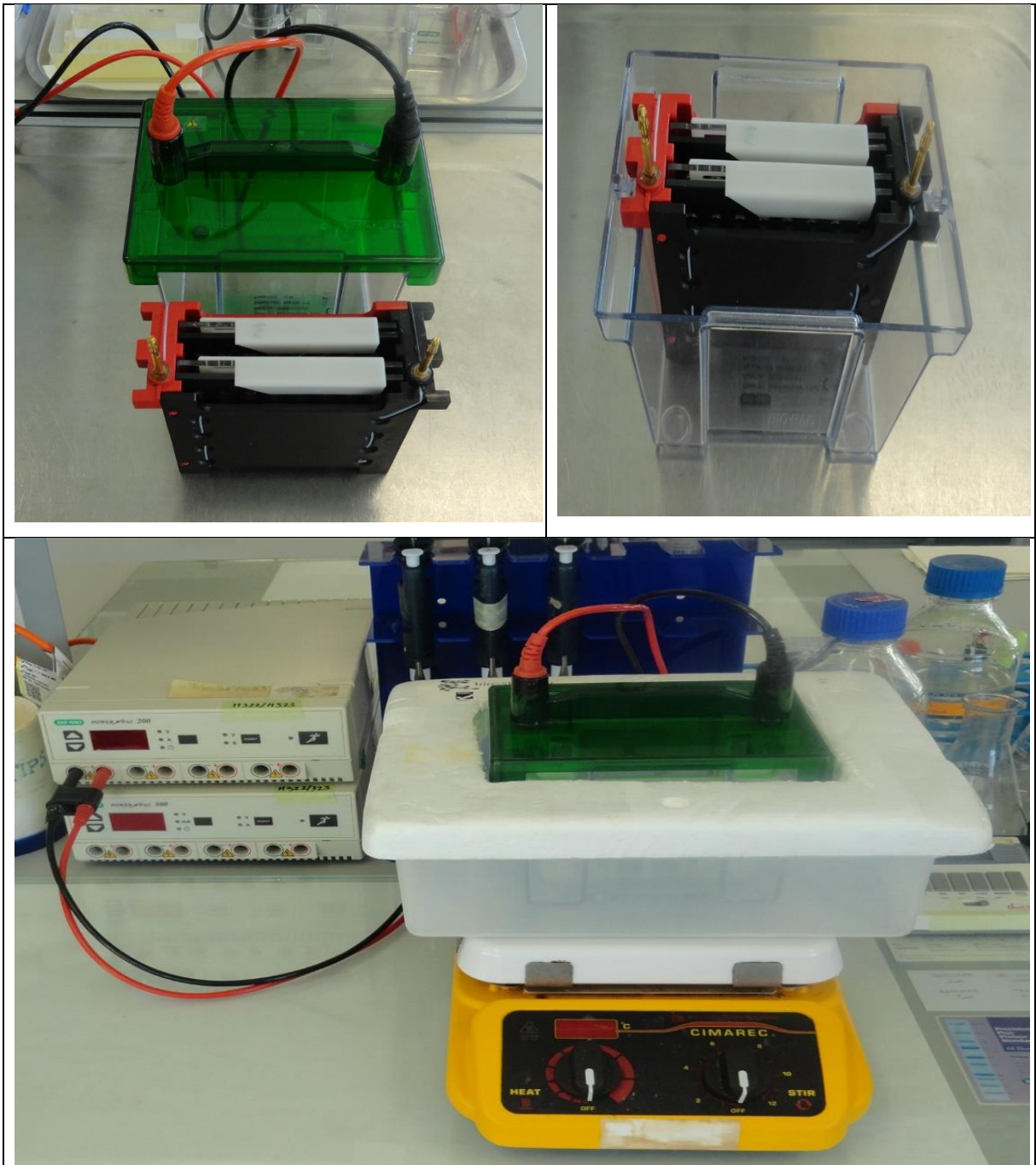


**Figure 2.16 SDS-PAGE (Mini-Protean III electrophoresis tank)**

The electrophoresis was performed in a Mini-Protean III electrophoresis (Bio-Rad Laboratories, Hercules, CA, Figure 2.16) tank using SDS (1x) running buffer (25 mM Tris-HCl, pH 8.6; 192 mM glycine; 0.1% SDS) at 150 V for 1 h at room temperature (21 °C ± 2.0). The gels were washed three times in dH<sub>2</sub>O for 5 min each time and stained in 20 ml GelCode® Blue Stain Reagent (Pierce) for 1 h with gentle shaking on the rocker or were further processed for Western blotting. Stained gels were washed with dH<sub>2</sub>O and were analysed using the Gel Doc™ XR<sup>+</sup> with Image Lab™ Software (S. No. 721BR15168, Bio-Rad, USA).

#### **2.4.5 Immunoblotting (Western blot analysis)**

The levels of troponin-T and desmin were determined by Western blot analysis (n=6) of the myofibrillar proteins (Figure 2.17). After electrophoresis, the gel was removed and rinsed with transfer buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine). The gel and polyvinylidene difluoride (PVDF) membrane (Milipore Corporation, Bedford, MA, USA) were assembled together, and proteins from the gel were electroblotted on to the PVDF over 1 h at constant 0.7 A in cold (4°C) transfer buffer. Once the separated proteins on the gel were transferred onto the PVDF, the membrane was incubated with 5% blocking buffer [5% non-fat dry milk in TTBS (Tris-Tween buffered saline, 20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.05% Tween 20)] for 1 h at room temperature. The membrane was washed three times with TTBS and probed with monoclonal antibody to desmin (D1033, Sigma) and anti-troponin T (T6277, Sigma) at 1: 1000 in 3% blocking buffer by incubating for 16 h at cold temperature (4±1 °C). The blots were then washed three times with TTBS and incubated with a mixture of secondary antibodies [goat-anti mouse IgG A3562, 1 :2000 and Precision StrepTactin-AP conjugate, 1 :5000 (from BioRad, for labelling unstained molecular marker)] in 3% blocking buffer for 1 h at room temperature. The blots were washed three times with TTBS and followed by washing with TBS (20 mM Tris-HCl, pH 7.5; 500 mM NaCl) for three times to remove the Tween20. The detection of proteins was visualised with alkaline phosphatase conjugate kit (BioRad) and were analysed using the Gel Doc™ XR<sup>+</sup> with Image Lab™ Software (S. No. 721BR15168, Bio-Rad, USA).



**Figure 2.17 Western Blot Analysis (Immunoblotting)**

#### **2.4.6 Equipment used for biochemical analysis**

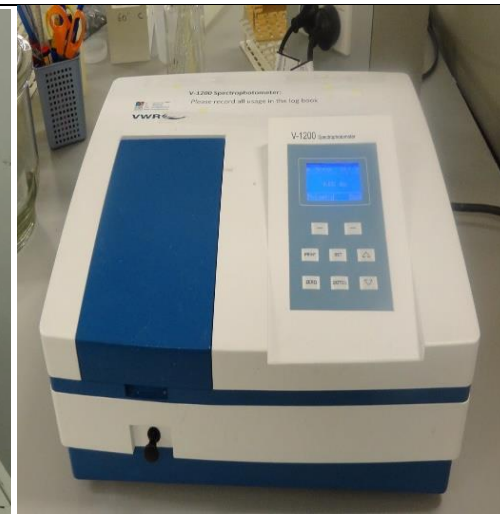
Some of the equipment's used for biochemical analysis such as casein zymography, electrophoresis and immunoblotting are presented in Figure 2.18.



**FLUOstar Omega Microplate Reader**



**Multifuge X3R Centrifuge**



**Spectrophotometer (V-1200)**



**Gel Doc™ XR+**



**Centra GP6R, Thermo IEC Centrifuge**



**Polytron Homogenizer**

**Figure 2.18 Equipments used for biochemical analysis (Casein zymography, SDS PAGE and Immunoblotting)**

## **2.5 *In vitro* gastrointestinal protein digestion simulation**

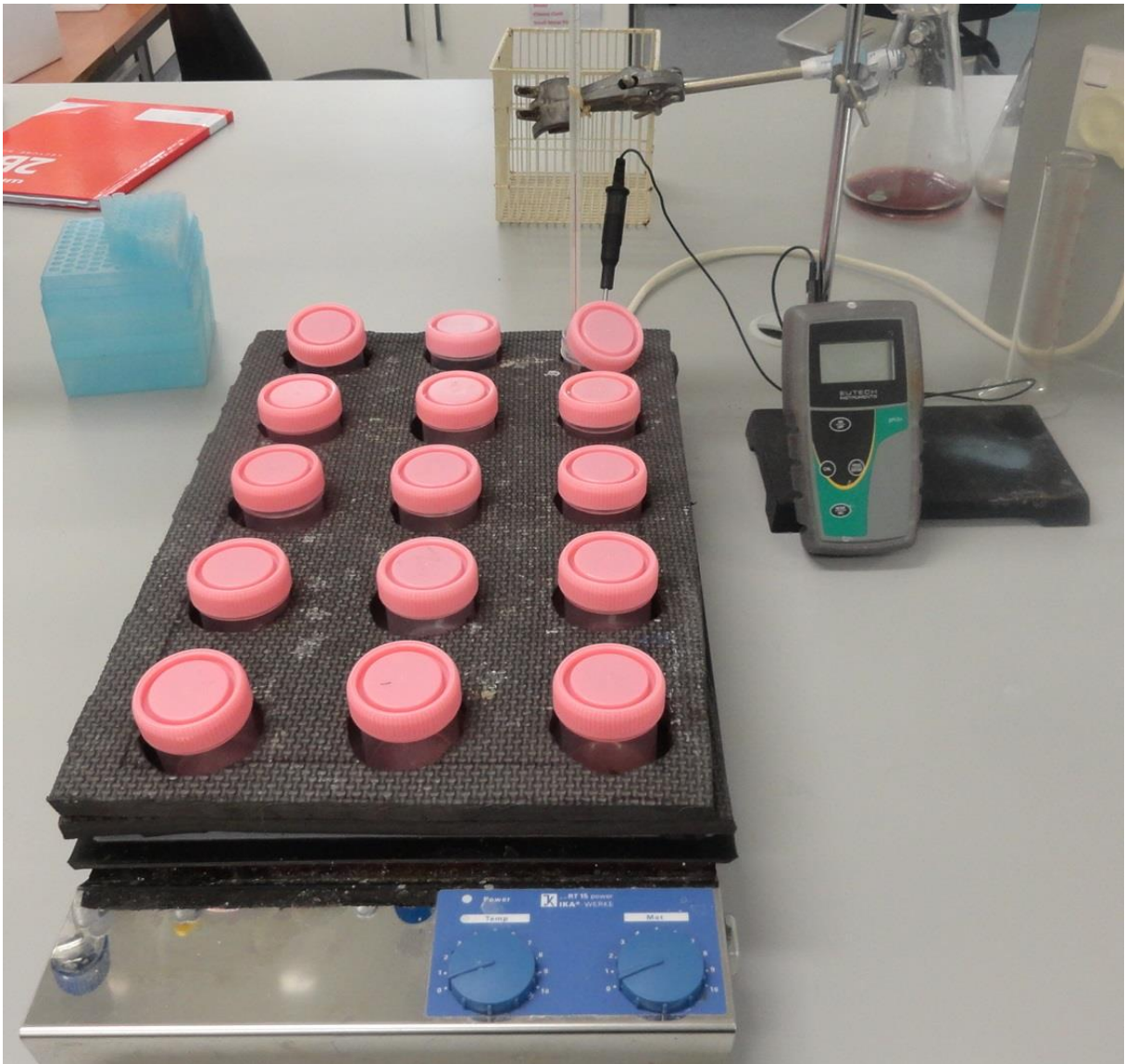
### **2.5.1 *In vitro* protein digestion of raw meat samples**

The PEF treated samples along with control were subjected to *in vitro* simulated gastrointestinal digestion according to the method used by Kaur et al. (2016) with some modification. Since heating has been reported to affect the microstructure and digestibility of meat (Qi et al. 2018), the present study evaluated the effect of PEF processing on raw meat to elucidate the exact quantum of effect that was induced by PEF treatment alone. Cooking of meat would certainly have an influence on the structural changes induced by PEF and would either boost or counter the effect of PEF.

Samples equivalent to 437.50 mg protein (~ 70 mg N) were weighed after finely chopping the muscles into small pieces (~ 0.5 mm). Samples were stirred (using magnetic flea @500 rpm) in 17 mL of 0.1 M HCl (pH 1.9 ± 0.1) in polyvinyl containers on magnetic multi-stirrer (RT 15 Power, IKA®-Werke) at 37 °C. A freshly prepared solution of pepsin (2.5 mL, enzyme: substrate ratio = 1: 100 w/w, 1031 U/mg, Sigma Aldrich, St Louis USA) in HCl (0.1 M) was added to start the hydrolysis to mimic the gastric phase of digestion. The samples were digested for 1 h at 37 °C and thereafter the reaction was stopped by inactivating the pepsin by adding sodium phosphate buffer (pH 8.0, 0.1 M) along with pancreatin (enzyme: substrate ratio = 1: 100 w/w in phosphate buffer, 350 U/mg, Sigma Aldrich, St Louis USA) to mimic the intestinal phase of digestion for 2 h at 37 °C. Aliquots were taken at 0, 30 and 60 minutes of gastric digestion and 60 and 120 minutes of small intestinal digestion. The pH of the aliquots was adjusted to 8.0 for gastric digestion and 2.0 for small intestinal digestion. The aliquots were centrifuged at 4000 x g for 15 minutes and a clear supernatant was obtained and stored at -20 °C for further analysis.

### **2.5.2 *In vitro* protein digestion of cooked meat samples**

Both control and PEF treated samples were cooked individually in plastic bags immersed in a water bath maintained at 80 °C to a core temperature of 75 °C as measured individually using Fluke type K temperature probes attached to Fluke 52 thermometer (Fluke Corp., Everett, WA). The digestion of cooked meat samples was done as described in section 2.6.1 above.



**Figure 2.19 *In vitro* gastrointestinal digestion simulation**

### **2.5.3 *In vitro* protein digestibility (%)**

The *in vitro* protein digestibility (%) values were calculated according to the method of Almeida et al. (2015) using an equation:

$$\% \text{ Digestibility} = (P_s - P_b) / P \times 100$$

where  $P_s$  and  $P_b$  represent the protein content in the sample and in the blank, respectively after the digestion.  $P$  represents the protein content of the sample before the digestion.



#### **2.5.4 Protein content (%)**

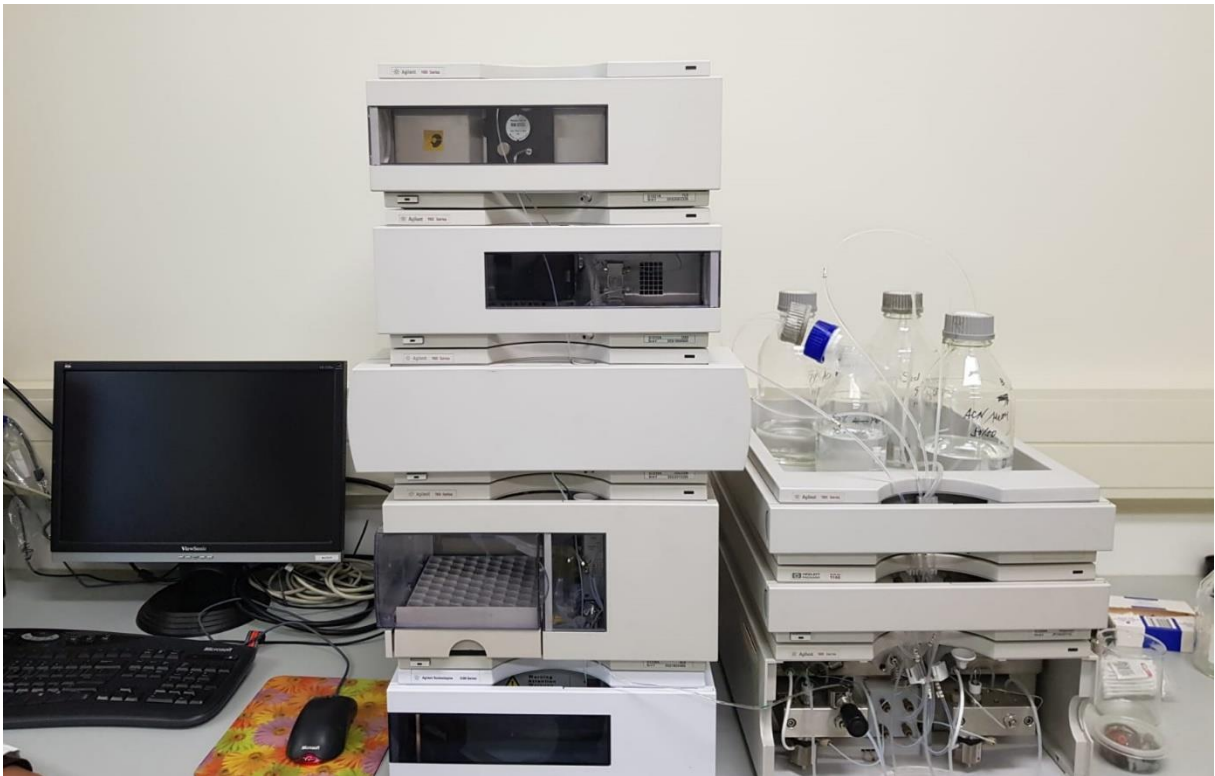
The protein content of the samples was determined by Dumas method by using a N/protein analyzer "Rapid MAX N exceed" (Serial No. 29154045, Elementar Analysensysteme GmbH, Donaustrasse 7, 63452 Hanau, Germany). This instrument utilizes the EAS REGAINER® technology and works according to the principle of catalytic tube combustion under oxygen at a high temperature. Samples were taken in triplicate and loaded individually into the machine and the protein content (nitrogen content x 6.25) was determined.

#### **2.5.5 Soluble protein (%)**

The soluble protein of the digested samples was determined in triplicate using the bicinchoninic acid (BCA) method (Pierce Chemical Company, Illinois, USA). Absorption was measured at 570 nm in a microplate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany) in accordance with the manufacturer guidelines and a bovine serum albumin was used to construct a standard curve for the quantitation of the protein. Absorption was measured at 570 nm and the respective extraction buffers were used as blanks.

#### **2.5.6 Free amino acid analysis**

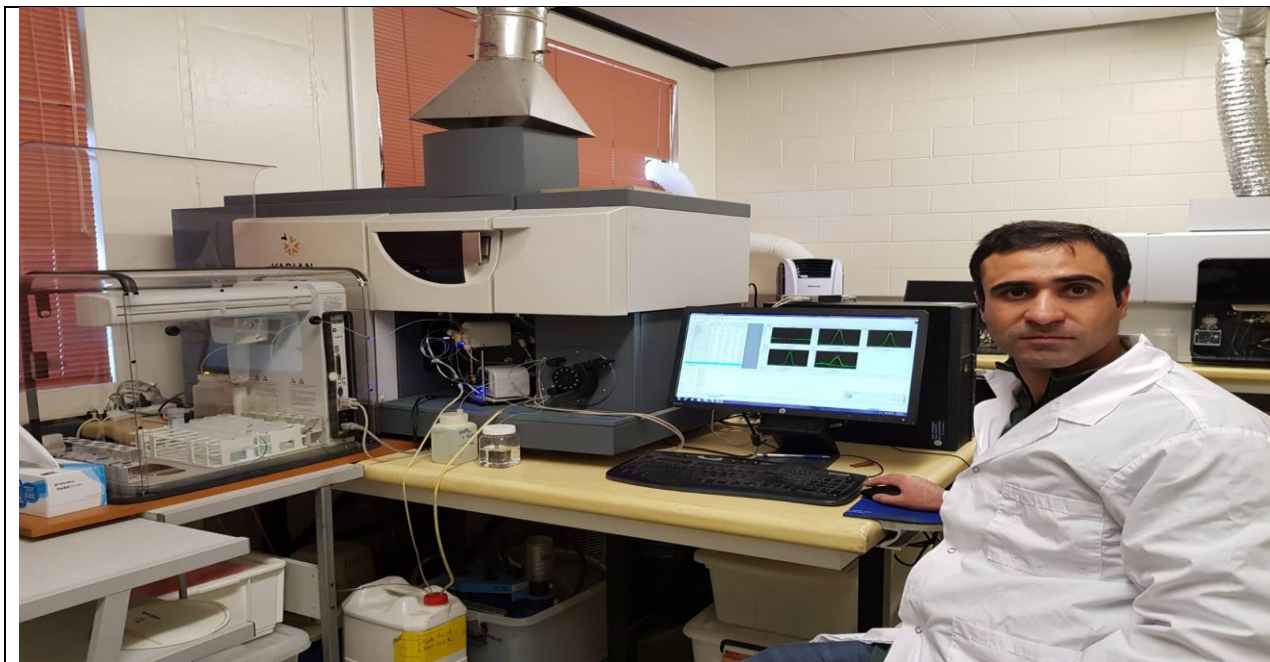
Free amino acids of the digested samples were determined using the methods adopted by Fountoulakis and Lahm (1998) and Weiss et al. (1998). The hydrolysate residue obtained after drying the digested samples in a rotary vacuum evaporator (45 °C) was resuspended in nanopure water and the volume was made to 50 mL in a volumetric flask. For the HPLC analysis, the samples were filtered into a 2 mL HPLC vial using a 0.45 µm syringe. The samples were derivatised with o-phthalaldehyde-3-mercaptopropionic acid for the primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for proline and separated by a modified version of the method of Heems et al (1998). An HPLC 1100 series (Agilent Technologies, Waldbronn, Germany) was used for the analysis of all the amino acids and was provided with a fluorescence detector and an autosampler (Figure 2.20). ACE 3 µm C-18 (150 mm × 4.6 mm) HPLC column with 40 °C column temperature was used for the analysis. The HPLC mobile phase A was 10 mM sodium phosphate buffer, pH 7.5 with 0.8% tetrahydrofuran (THF) and mobile phase B was 50% methanol, 50% acetonitrile. The gradient elution was 0-40% B in 14 minutes, then to 50% B over 6 minutes and up to 100% B over 4 minutes and maintained at 1-00% B for 5 minutes. The detector was started at excitation 335 nm, emission 440 nm and changed to 260 nm excitation, 315 nm emission at 26 minutes to detect proline.



**Figure 2.20 HPLC used for the analysis of free amino acids**

### **2.5.7 Mineral profile analysis**

Digested samples were analysed by Inductively Coupled Plasma Optical Emission Spectrophotometer (Varian 720 ICP-OES, Melbourne, Australia; Figure 2.21). Settings were Plasma gas flow- 15.0 L/min, Aux- 1.5 L/min, Nebulizer- 0.9 L/min with SeaSpray nebulizer and cyclonic spray chamber. Merck ICP standard solutions were serially diluted using MilliQ water for calibration standards and internal standards. Four standards and a standard blank were used for generation of the calibration curves.



**Digestion unit**



**Plasma chamber**



**Automatic sampling unit**



**Digestion unit**

**Figure 2.21 Inductively coupled plasma optical emission spectrophotometer**

## 2.6 Preparation of beef jerky

The method described by Ojha et al. (2018) and Luckose et al. (2017) for preparation of jerky products was followed with suitable modifications. The meat was sliced into 0.6 cm thick slices using a meat slicer (Bizerba, Balingen, Germany). The sliced meat samples were cut parallel in direction to muscle fibers and used for development of beef jerky viz. control (2% NaCl), reduced salt (1.2% NaCl, T<sub>1</sub>) and reduced salt with PEF treatment (1.2% NaCl-PEF, T<sub>2</sub>). For preparation of control samples, the beef slices were cured for 18 h at 4 °C in a curing solution containing 70% water, 2.0% salt (NaCl), 2.0% sugar and 0.05% sodium nitrite (w/w, based on raw meat weight). The curing of the T<sub>1</sub> and T<sub>2</sub> samples differed only in that the salt concentration was reduced to 1.2% salt (NaCl). The PEF-treated meat was used for preparation of T<sub>2</sub> samples. The products were cooked in a pre-heated oven (Clayson Laboratory Apparatus Ltd, New Zealand) at 75 °C (high fan speed) for initial 60 min and then dried at 60 °C for a time till the water activity level reached ≤0.80 (~7 h). Samples were taken from oven at regular intervals (half hourly) and analysed for water activity. The products were removed from oven, cooled to ambient temperature, packaged (polyethylene-aluminium-polyethylene laminates) and stored at room temperature (25±1 °C) for 30 days.

### 2.6.1 Yield (%)

The yield (%) of the product (beef jerky) was determined by using the following equation:

$$\text{Yield (\%)} = 100 \times \frac{\text{Weight of samples after drying}}{\text{Initial weight before drying}}$$

### 2.6.2 Water activity (a<sub>w</sub>)

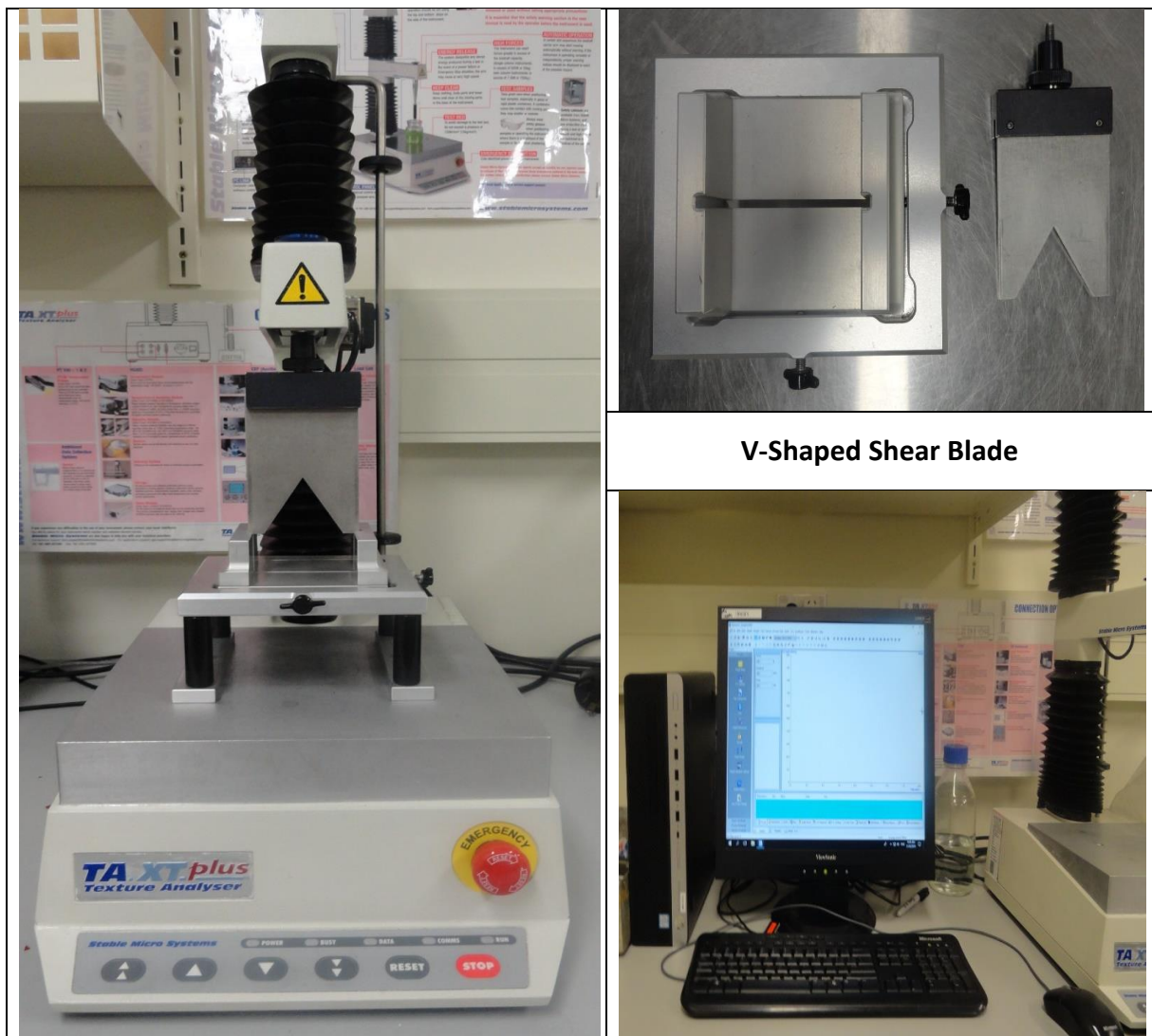
A water activity meter (Serial No. 07079689B, Aqualab, Series 3TE, U.S.A.) was used to determine the water activity of three different jerky strips from each sample. The average of the three measurements was used for data analysis.

### 2.6.3 pH

To determine the pH, 1 g of muscle was mixed with 10 mL deionised water and homogenised for 20 s using a Polytron homogenizer (Polytron® PT – MR 3100, Kinematica AG, Switzerland) at 15000 rpm. The pH of homogenate was measured using a glass electrode connected to a calibrated pH meter at 25 °C (Faridnia et al. 2014).

## 2.6.4 Instrumental texture analysis

The method described by Ojha et al. (2018) (firmness and toughness) and Luckose et al. (2017) (shear force) for jerky products was followed with suitable modifications. Beef jerky samples were cut as close as practicable to 3.0 × 2.0 × 0.4 cm (L x W x H) pieces and their shear force, toughness and firmness values were measured on TA.XT-plus Texture Analyser (Serial No. 12835, Stable Micro Systems, Surrey, UK, Figure 2.22) by using a Warner-Bratzler V-shaped shear blade (5.0 mm/s test speed) or a WB reversible blade (5.0 mm/s test speed) and a 50 kg load cell calibrated with 1 kg weight according to the manufacturer instructions (Stable Micro Systems). Cross-sections of the samples were placed midway to the blade and the samples were cut perpendicular to the fiber direction. The analyses were performed on ten replicates for each treatment and the results were averaged. The results obtained were expressed as shear force (N), toughness (N/mm.sec) and firmness (N/sec) of the samples.



**Figure 2.22 Texture analyser used for measuring shear force, toughness and firmness of beef jerky**

### 2.6.5 Colour measurement

Surface colour (lightness,  $L^*$ ; redness,  $a^*$ ; yellowness,  $b^*$ ) of dried beef jerky were evaluated using a CR400 Minolta Chromameter (Konica Minolta, Japan). Colour determinations were performed 20 min after the packages were opened. Determinations were performed on six jerky strips per treatment and there were six determinations per jerky sample (three on each surface of jerky strip). The chroma ( $C^* = [a^{*2} + b^{*2}]^{1/2}$ ) and hue angle ( $H^* = \tan^{-1} [b^*/a^*]$ ) were calculated.

### 2.6.6 Sensory evaluation

The products were evaluated for colour, flavour, saltiness, tenderness and overall acceptability. A mixed gender untrained panel of 65 members participated in the sensory evaluation. The sensory evaluation was held in multiple sessions (06) in one day and evaluations took place in individual booths under white fluorescence light in sensory analysis laboratory (Figure 2.23). Three samples were presented to the panellists and the serving order of the samples was randomized. Three-digit coded samples (4.0 cm x 3.0 cm, two strips per sample) were served at room temperature (25°C) and water was given for oral rinsing between the samples to avoid carry-over effect. Panellists were asked to evaluate each sample based on a 9-point hedonic scale where 9 = liked extremely and 1 = disliked extremely (Appendix A.2). The participants were also asked to rank the samples according to the intensity of saltiness and the preferred salt concentration based on liking. Out of 65 panellists, 70% were familiar with the product. The ethnic composition of the panel was 56% Asian, 29% Europeans and 15% other ethnicities. The sensory analysis was approved by the Lincoln University Human Ethics Committee (Application No. 2018-35, 12 Oct 2018).



**Figure 2.23 Sensory laboratory at Lincoln University**

### **2.6.7 Thiobarbituric acid reacting substances (TBARS)**

Lipid oxidation was assessed by measuring the TBARS in the samples using the method described by Pearson et al. (1977). A 5 g minced sample was homogenized in 25 mL distilled water for 1 min using a Polytron homogenizer at 9,000 rpm. A 3 mL aliquot of the homogenate was added to 3 mL

thiobarbituric acid/ trichloroacetic acid stock solution (0.032 M 2-thiobarbituric acid, 1.14 M trichloroacetic acid in 0.32 M HCl) and vortex-mixed. Samples were incubated at 94°C for 15 min in a water bath for colour development. Samples were centrifuged (Centra GP6R, Thermo IEC, Needham Heights, MA, USA) at 2,500 rpm for 15 min after a cooling period of 10 min and absorbance of the supernatant was measured at 535 nm (V-1200 Spectrophotometer, Global Science, VWR International, Leuven). A standard calibration curve was prepared using 1,1,3,3-tetraethoxypropane and the results were expressed in mg malondialdehyde per kilogram.

### **2.6.8 Protein oxidation by DNPH (dinitrophenylhydrazine) method**

Protein oxidation, as measured by the total carbonyl content, was evaluated by derivatisation with dinitrophenylhydrazine (DNPH) according to the method described by Roldan et al. (2014). One gram of sample was homogenised 1:10 (w/v) in 20 mM sodium phosphate buffer containing 0.6 M NaCl (pH 6.5) using a Polytron homogenizer (Polytron® PT – MR 3100, Kinematica AG, Switzerland) for 30 sec. Two equal aliquots of 0.2 mL were taken from the homogenates and dispensed in 2 mL Eppendorf tubes. Proteins were precipitated by cold 10% TCA (1 mL) and subsequently centrifuged (5 min at 9000 × g, 4 °C). One pellet was treated with 1 mL 2 M HCl (protein concentration measurement) and the other with an equal volume of the sample volume of 0.2% (w/v) DNPH in 2 M HCl (carbonyl concentration measurement). Both samples were incubated for 1 h at room temperature. Samples were then precipitated by 10% TCA (1 mL) and washed twice with 1 mL ethanol/ethyl acetate (1:1, v/v) to remove excess of DNPH. The pellets were dissolved in 1.5 mL of 20 mM sodium phosphate buffer containing 6 M guanidine HCl (pH 6.5), stirred and centrifuged for 2 min at 5031 × g to remove insoluble fragments. The protein concentration was calculated based on the absorption at 280 nm compared to a bovine serum albumen standard curve. The carbonyl content was expressed as nmol of carbonyl per milligram of protein using an absorption coefficient of 21.0 nM<sup>-1</sup>cm<sup>-1</sup> at 370 nm for protein hydrazones.

### **2.6.9 Microbiological characteristics**

Total plate, coliform and yeast and mould count (log cfu/g) were determined following the methods described by APHA (1984). Ready-to-use media plates (Fort Richard Laboratories, Auckland, New Zealand) were used for the analysis.

#### **2.6.9.1 Sample preparation**

10 g of the sample was taken aseptically and homogenized with 90 mL sterile peptone water (Merck, Darmstadt, Germany) in sterile bag for 2 min using stomacher and original homogenate (dilution of



$10^{-1}$ ) were prepared. From the original homogenate, tenfold serial dilutions were prepared. The sample preparation was done near flame under laminar flow (Serial No. 8801/88, Gelman Sciences PTY. LTD., Australia, Figure 2.24).



**Figure 2.24 Laminar flow used for microbiology**

### **2.6.9.2 Total plate count**

The pour plate technique was followed by spreading 100  $\mu\text{L}$  from each dilution on the surface of Plate Count Agar and incubated at  $35^{\circ}\pm 2^{\circ}\text{C}$  for 24 hrs. Following incubation plates showing 30-300 colonies were preferably counted and expressed as  $\log_{10}$  colony forming units per gram (cfu/g) of sample.

### **2.6.9.3 Coliform count**

Coliform and other gram-negative pathogens were enumerated by inoculation of plate of MacConkey agar. The plates were incubated at  $35^{\circ}\pm 2^{\circ}\text{C}$  for 24 hrs and the counts were determined as colony forming units per gram (cfu/g) of sample.

### **2.6.9.4 Yeast and mould count**

Yeast and mould count were enumerated by inoculation of plates of Sabaroud dextrose agar followed by incubation at  $25^{\circ}\text{C}$  for 5 days.

## **2.7 Statistical analysis**

All experiments were analysed by the General Linear Model using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). All data were reported as means  $\pm$  standard error of the means. For pairwise comparison of the means, Duncan's multiple range tests were used at 0.05 level of significance to find out the effects of the treatment and storage period (Snedecor and Cochran 1994).

### **2.7.1 Chapter 3, 4, 5, 6, 12**

The data generated by repeating the experiments ( $n=6$ ) for different parameters were compiled and analysed either by one-way ANOVA or a two-way ANOVA using General Linear Model using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). All data were reported as means  $\pm$  standard error ( $n=6$ ). The measured variables were set as dependent variables. The model included fixed effects for treatment (PEF), time (ageing) and the interaction between treatment and time. The random effects in the model were effects for carcasses, blocks within cuts, slices within blocks and their interactions with fixed effects. For sensory evaluation, treatment was considered as the main effect and consumers as random variable. For pairwise comparison of the means, Duncan's multiple range tests were used at 0.05 level of significance to find out the effects of the treatment and storage period (Snedecor and Cochran 1994).

### **2.7.2 Chapter 7, 8, 9**

The data generated by repeating the experiments ( $n=3$ ) for different parameters were compiled and analysed by one-way ANOVA except for soluble protein (%) that was analysed using a repeated measurements ANOVA to investigate the effect of treatments at the 5 incubation times by General

Linear Model using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). All data were reported as means  $\pm$  standard error of the means. The measured variables were set as dependent variables. The model included fixed effects for treatment (PEF), time and their interactions. The random effects in the model were effects for carcass and its interactions with fixed effects. Duncan's multiple range tests, at the 0.05 level of significance, were used for comparing the means to find out the effect of treatment and storage period (Snedecor and Cochran 1994).

### **2.7.3 Chapter 10, 11**

The data generated by repeating the experiments (n=3) for different parameters were compiled and analysed by Independent samples t-test to determine the effect of PEF treatment except for soluble protein (%) that was analysed by two-way ANOVA using a repeated measurements ANOVA to investigate the effect of treatments at the 5 incubation times by General Linear Model using Statistical Package for Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA). The measured variables were set as dependent variables and the results were reported as means  $\pm$  standard errors. The fixed effects included in the model were treatment (PEF), time (digestion time) and their interactions. The random effects in the model were effects for carcasses, blocks within cuts and their interactions with fixed effects. The effect of digestion time and treatment were analysed using Duncan's multiple range tests or Levene's test for equality of means, at the 0.05 level of significance (Snedecor and Cochran 1994).

## Chapter 3

# Calpain activity, myofibrillar protein profile and physicochemical properties of beef *Semimembranosus* and *Biceps femoris* from culled dairy cows during ageing

This chapter is published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Calpain activity, myofibrillar protein profile and physicochemical properties of beef *Semimembranosus* and *Biceps femoris* from culled dairy cows during ageing. *Journal of Food Processing and Preservation*, 42(12), e-13835.

### 3.1 ABSTRACT

The present study was conducted to elucidate the changes in the calpain activity, protein profile and physicochemical properties of cold-boned muscles from culled dairy cows during ageing. *Semimembranosus* and *Biceps femoris* were used as the study model and were vacuum packaged and stored for 14 days at  $4\pm 1$  °C. The samples were analysed for pH, shear force, myofibrillar fragmentation index (MFI), calpain activity and myofibrillar protein profile (SDS-PAGE). A significant ( $P < 0.05$ ) effect of ageing was observed on the pH, shear force and MFI of both the muscles. Casein zymography results indicated the presence of intact and autolyzed forms of calpain 1 and calpain 2. An increase in autolysis of the calpains and proteolysis was observed in both the muscles during ageing. Ageing for two weeks was effective ( $P < 0.05$ ) in reducing the shear force of both muscles by 30%, however, the aged muscles were still excessively tough.

**Keywords:** Ageing; *Semimembranosus*; *Biceps femoris*; calpain activity; myofibrillar protein profile; shear force

### 3.2 INTRODUCTION

Ageing has been reported to improve the tenderness of beef *Semimembranosus* and *Biceps femoris* by several studies (Colle, & Doumit, 2017; Colle et al., 2016). The calpain system, a family of endogenous calcium-dependent cysteine-proteases, is associated with meat tenderness during ageing and is believed to regulate the rate and the extent of the post-mortem degradation of certain myofibrillar proteins including troponin-T, desmin, nebulin, titin, and filamin (Bhat, Morton, Mason, & Bekhit, 2018c; Huff-Lonergan, Zhang, & Lonergan, 2010; Koohmaraie, & Geesink, 2006). Considered as the key enzyme for meat tenderization, calpain 1 is believed to be largely responsible for early post-mortem

proteolysis and thus plays a significant role in tenderization during ageing (Bhat, Morton, Mason, & Bekhit, 2018c; Pomponio, & Erbjerg, 2012; Kemp, Sensky, Bardsley, Buttery, & Parr, 2010; Geesink, Kuchay, Chishti, & Koohmaraie, 2006). Calpain 2 has also been reported to be active and make a significant contribution to proteolysis during an extended post-mortem ageing (Colle et al., 2018; Colle, & Doumit, 2017).

The activity of calpains depends on several post-mortem factors such as pH, temperature and intracellular calcium concentration (Bhat, Morton, Mason, & Bekhit, 2018c; Mohrhauser, Lonergan, Huff-Lonergan, Underwood, & Weaver, 2014). The age of an animal has also been reported to influence the calpain activity (Gheisari, Shekarforoush, & Aminlari, 2007) and may have a profound impact on the proteolytic activities of the enzymes and subsequent tenderization of muscles from mature animals during ageing. However, information is generally lacking in the literature about the effect of ageing on the calpain activity of the muscles from older animals, such as culled dairy cows, and the subsequent tenderization of such muscles during ageing. Meat obtained from culled dairy animals is often excessively tough and thus it has been generally used to produce comminuted meat products.

Although the impact of post-mortem ageing on beef tenderness is well established (Bhat, Morton, Mason, & Bekhit, 2018b; Colle et al., 2015; Dixon et al., 2012; Gruber et al., 2006; Bratcher, Johnson, Littell, & Gwartney, 2005), relatively few experiments have examined the effects of ageing on the quality of beef from older animals. The changes in the calpain activity and protein profile of muscles from culled dairy cows during ageing is poorly explored and further understanding of biochemical processes is needed to design useful approaches for tenderization. The objective of this study was to evaluate the physicochemical, proteolytic and enzymatic changes that occur during the ageing of *Semimembranosus* and *Biceps femoris* from culled dairy cows. Myofibrillar fragmentation index (MFI), as a marker of proteolysis, the myofibrillar protein profile (SDS-PAGE) and activities of the calpain proteases (calpain 1 and calpain 2) were investigated over two weeks of ageing to determine whether the change in shear force, if any, was associated with enzymatic changes.

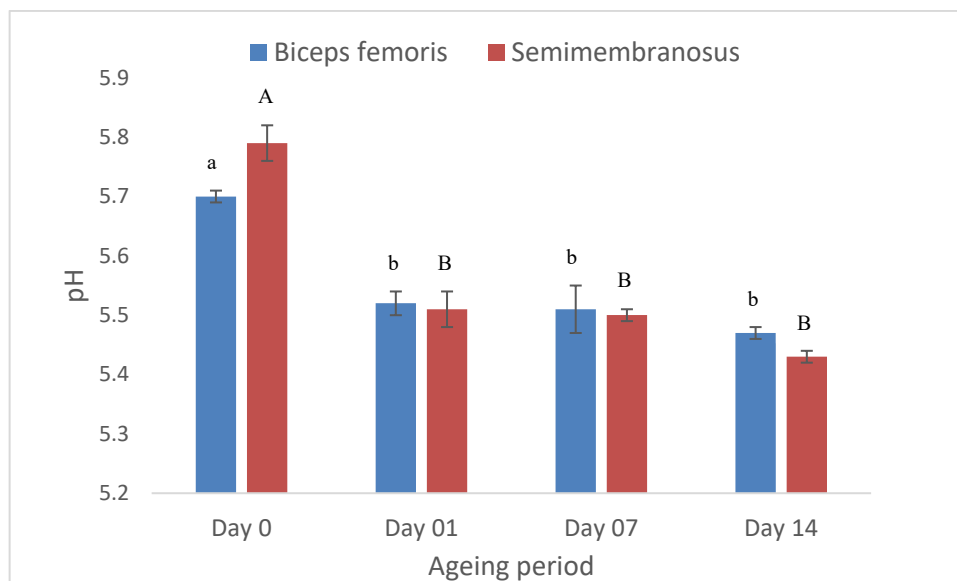
### **3.3 MATERIALS AND METHODS**

Meat samples were prepared as per the method described in section 2.2.2. Shear force and myofibrillar fragmentation index (MFI) of the samples was measured according to the methods described in section 2.3.1 and 2.3.3, respectively. Sarcoplasmic and myofibrillar fractions were separated by following the method described in section 2.4.1. Protein concentration, casein zymography, SDS-PAGE and analysis of the protein bands was done by following the methods described from section 2.4.2 to 2.4.4. Statistical analysis of the data was performed according to the method described in section 2.7.1.

### 3.4 RESULTS AND DISCUSSION

#### 3.4.1 pH

A significant effect of ageing ( $P < 0.05$ ) was observed and the pH of both the muscles showed a significant decrease on day 1 of the ageing (Figure 3.1). No significant difference was observed in the pH after day 1 throughout the period of ageing. The ultimate pH values observed for the muscles were within the normal range (5.4 to 5.8) for beef (Fiorentini et al., 2018). Similar results were observed by Shange, Makasi, Gouws, & Hoffman (2018) who also observed a similar decline in the pH of *Biceps femoris* aged for 12 days at refrigeration temperature. This decline in pH may be due to the anaerobic glycolysis of the residual glycogen available in the meat producing lactic acid, which causes a fall in the pH (Po"so", & Puolanne, 2005). In addition, vacuum packaged meats are dominated by lactic acid bacteria, which produce lactic acid in their metabolism.



**Figure 3.1** Effect of ageing on the pH of beef *Biceps femoris* and *Semimembranosus*

Different superscripts on columns (lower case for *Biceps femoris* and upper case for *Semimembranosus*) differ significantly ( $P < 0.05$ ),  $n = 6$ , Mean  $\pm$  SE

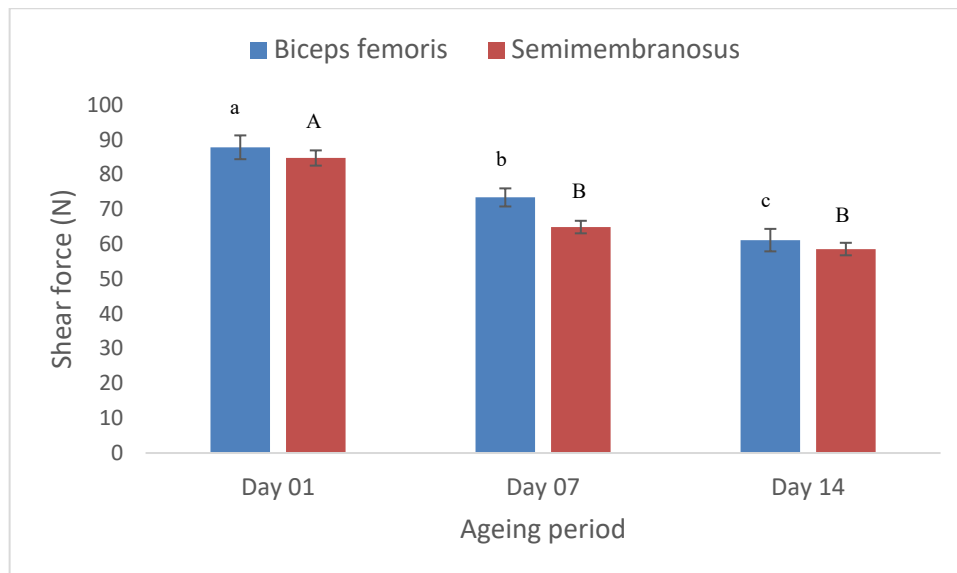
The rate and extent of the pH decline under different temperatures has a substantial impact on the ageing potential and degree of shortening (Kim, Warner, & Rosenvold, 2014; Hwang, & Thompson, 2001) and can subsequently affect the tenderization by affecting the proteolytic enzyme activity, particularly of calpain 1 (Contreras-Castillo, Lomiwes, Wu, Frost, & Farouk, 2016; Lomiwes, Farouk, Wiklund, & Young, 2014; Lomiwes, Farouk, Frost, Dobbie, & Young, 2013; Koohmaraie, 1996). The

ultimate pH (pHu) of muscle plays a crucial role in the post-mortem proteolysis of myofibrillar proteins and has been reported to have a role in the activation of effector caspase 3/7 that impact the tenderization of meat (Huang et al., 2018; Lomiwes, Farouk, Wiklund, & Young, 2014; Lomiwes, Farouk, Frost, Dobbie, & Young, 2013). Meat with low (5.4 to 5.8) or high pHu (> 6.2) reaches better tenderness than intermediate pHu (5.8 to 6.2) meat (Pulford et al., 2008), however, high pHu meat has an unpleasant taste and is dark (Viljoen, de Kock, & Webb, 2002). Inconsistent tenderness, delayed meat tenderization or reduced ageing potential have been reported in intermediate pHu beef as due to delayed activation of effector caspase 3/7, slowing the process of programmed cell death (Lomiwes, Farouk, Wiklund, & Young, 2014; Lomiwes, Farouk, Frost, Dobbie, & Young, 2013; Pulford et al., 2009).

### 3.4.2 Shear force (N)

A significant ( $P < 0.05$ ) impact of ageing was observed and the shear force of both the muscles decreased significantly with ageing (Figure 3.2). A total of 30% and 31% decrease was observed in the average shear force value of *Biceps femoris* and *Semimembranosus*, respectively, during entire period of ageing. These results are in agreement with the findings of Colle, & Doumit (2017) who also observed a significant decrease in the shear force of beef muscles during ageing. A total decline of 17% was observed in the shear force of *Longissimus dorsi* of culled dairy cows subjected to 7 days of ageing (Bunmee, Jaturasitha, Kreuzer, & Wicke, 2014). Smith, Bush, van de Ven, & Hopkins (2016) also observed a significant decrease in the shear force of the muscles of alpaca during ageing. The authors also observed a significant impact of the animal's age on the shear force of *Semimembranosus*, such that the shear force of the muscle increased by  $5.7 \pm 1.6$  N for each yearly increase in animal age.

The quantity of connective tissue, particularly that of collagen, influences beef tenderness and high positive correlations have been reported between shear force and total collagen in beef (Dubost et al., 2013; Dransfield et al., 2003). Further, among the three main contributors (sarcomere length, connective tissue content and proteolysis of myofibrillar proteins) of beef tenderness (Gheisari, Shekarforoush, & Aminlari, 2007), connective tissue content is a major contributor to the tenderness of muscles such as *Biceps femoris* and *Semimembranosus* (Koochmaraie, Kent, Shackelford, Veiseth, & Wheeler, 2002). The positive ageing impact on eating quality attributes of such muscles could be further maximized through establishing specific ageing strategies for individual muscles and for individual age groups. Some studies have suggested a beneficial effect of extended ageing on beef muscles. Muscles like *Biceps femoris* and *Semimembranosus* have been demonstrated to exhibit moderate to high ageing responses, respectively, with potential to continue tenderizing beyond 28 days of ageing (Gruber et al., 2006). In a study investigating the effect of long-period ageing on beef tenderness (Colle et al., 2016), the consumer perception of *Semimembranosus* tenderness was greater on 42<sup>nd</sup> day than on 14<sup>th</sup> day of ageing. These results were further supported by the findings of Colle, & Doumit (2017).



**Figure 3.2 Effect of ageing on the shear force (Newtons) of beef *Biceps femoris* and *Semimembranosus* (Mean  $\pm$  SE)**

Different superscripts on columns (lower case for *Biceps femoris* and upper case for *Semimembranosus*) differ significantly ( $P < 0.05$ ),  $n = 6$

Development of best ageing strategies for the muscles of mature animals should encompass identifying and understanding the optimal biochemical and biophysical modification conditions to maximize the impact of ageing process on such muscles. Significant opportunities still exist to scientifically address how post-mortem ageing process can be further capitalized to positively impact profitability and palatability of fresh meat (Kim et al., 2018). Developing age-specific ageing strategies that specify optimal ageing conditions for different age groups will be beneficial for the meat industry to maximise the positive impacts on meat palatability. Further, meeting the increasing meat demand of increasing human population in future would mean utilizing the limited animal resources as efficiently as possible, adding newer sources of quality meat to the value chain by adding value to the already existing sources of inferior quality meats, such as from culled dairy animals.

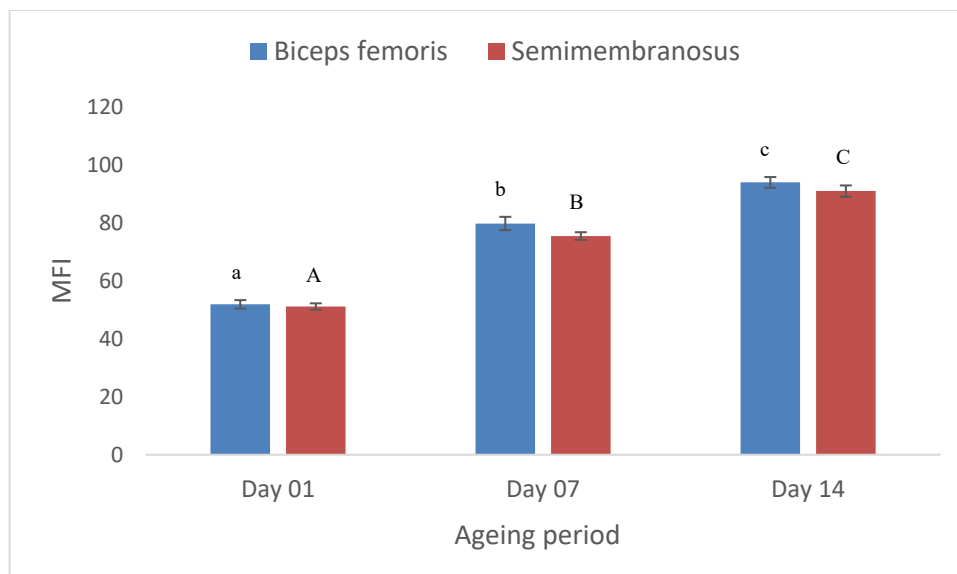
Among the various possibilities, another effective strategy for maximizing tenderization benefit of ageing in muscles from culled animals, that is yet to be explored, is the prior use of a novel non-thermal technology such as pulsed electric field (PEF). Recently, prior treatment of PEF has been reported to significantly reduce the shear force of beef muscles during ageing (Bekhit, Suwandy, Carne, van de Ven, & Hopkins, 2016; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015a, b, c; Bekhit, van de Ven, Suwandy, Fahri, & Hopkins, 2014c; Faridnia, Bekhit, Niven, & Oey, 2014). Some workers have reported physical disruption as the main cause behind the PEF-induced tenderness (Arroyo et al., 2015a, b; O'Dowd, Arimi, Noci, Cronin, & Lyng, 2013) whereas others have suggested enhanced proteolysis



through early release of calcium ions and early activation of the calpain proteases as the main mechanism (Bhat, Morton, Mason, & Bekhit, 2018a,d; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015a, b, c; Bekhit, van de Ven, Suwandy, Fahri, & Hopkins, 2014c). Prior use of PEF may open a new possibility for age-specific or muscle-specific ageing for effective reduction of toughness associated with the muscles of culled dairy animals.

### 3.4.3 Myofibrillar fragmentation index (MFI)

A significant ( $P < 0.05$ ) effect of ageing was observed and the MFI values for both the muscles increased significantly with ageing period (Figure 3.3). A total of 45% and 44% increase was observed in the average MFI value of *Biceps femoris* and *Semimembranosus*, respectively, during the whole ageing period. Bunmee, Jaturasitha, Kreuzer, & Wicke (2014) also observed a significant ( $P < 0.05$ ) increase in the MFI values of *Longissimus lumborum* from culled dairy cows during a post-mortem ageing period. Similar results were also presented by Biswas, Tandon, & Sharma (2016), Naveena et al., (2015) and Li et al., (2012) who also observed a significant increasing trend in the MFI of different muscles during ageing.



**Figure 3.3 Effect of ageing on the myofibrillar fragmentation index (MFI) of beef *Biceps femoris* and *Semimembranosus* (Mean  $\pm$  SE)**

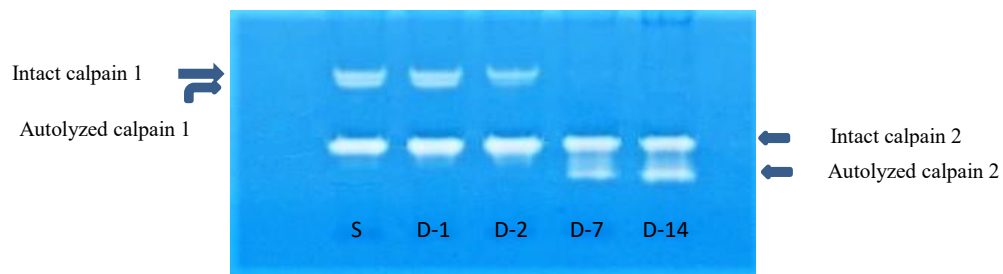
Different superscripts on columns (lower case for *Biceps femoris* and upper case for *Semimembranosus*) differ significantly ( $P < 0.05$ ),  $n = 6$

The MFI, which is related to the post-mortem proteolysis of myofibrillar proteins, correlates positively with meat tenderness (Silva et al., 2017). It is believed to be a good indicator of the extent of the post-

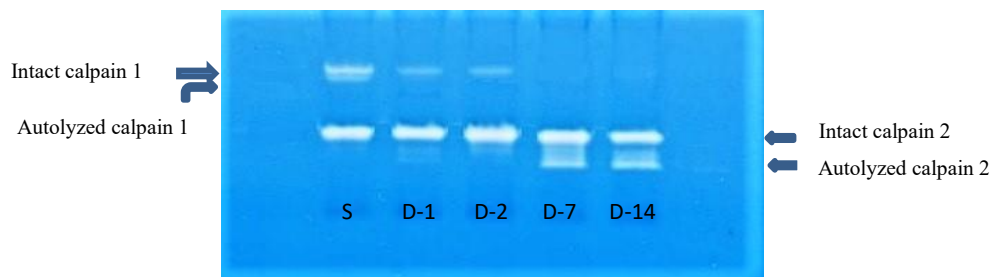
mortem proteolysis in meat (Ngapo, & Vachon, 2017) and is strongly correlated with shear force and post-mortem sensory tenderness (Rajagopal, & Oommen, 2015; Li et al., 2014). The increasing trend of MFI with ageing has been reported to be due to the increasing proteolysis of key structural proteins in the I-band of the sarcomere and due to the weakening of the myofibril linkages (Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). The direct comparison of MFI with literature is often difficult as it is greatly influenced by several factors such as ageing time, breed and the process of homogenization (Onopiuk, Pottorak, & Wierzbicka, 2018).

### 3.4.4 Calpain activity

The tenderization effect of ageing, which is mostly attributed to the proteolysis of key structural and associated muscle proteins responsible for the integrity of myofibrils, is regulated by several intracellular proteolytic systems including the calpains, cathepsins, and caspases (Wang et al., 2018a; Huff-Loneragan, Zhang, & Lonergan, 2010; Koohmaraie, & Geesink, 2006). Numerous studies have reported a significant role of the calpain system, principally calpain 1, in the tenderization process that occur during ageing (Huff-Loneragan, Zhang, & Lonergan, 2010; Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Taylor et al., 1995). In the present study, calpain 1 activity, both intact and autolyzed, decreased with ageing time and was detected until day 2 of ageing in both muscles (Figures 3.4 and 3.5).



**Figure 3.4 Effect of ageing on the calpain activity of beef *Semimembranosus* [S = standard, D-1 = day 1 of aging, D-2 = day 2 of aging, D-7 = day 7 of aging, D-14 = day 14 of aging, 35 µg samples were loaded in each lane]**



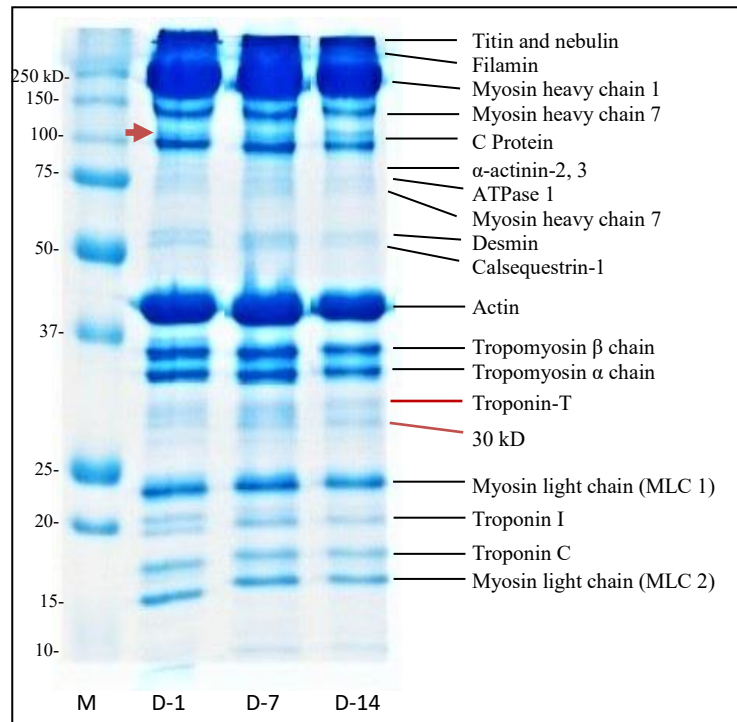
**Figure 3.5 Effect of ageing on the calpain activity of beef *Biceps femoris* [S = standard, D-1 = day 1 of aging, D-2 = day 2 of aging, D-7 = day 7 of aging, D-14 = day 14 of aging, 35 µg samples were loaded in each lane]**

On day 7 of ageing, the band for calpain 1 was not detectable in either of the muscles. Similar results were reported by Biswas, Tandon, & Sharma (2016) who also observed the bands for calpain 1 only up to 72 h post-mortem. Calpain 1 is autolysed in the early stages of ageing and is believed to be responsible for most, if not all, of the post-mortem proteolysis of key myofibrillar proteins (Du et al., 2018; Geesink, Kuchay, Chishti, & Koochmaraie, 2006; Koochmaraie, & Geesink, 2006). Only 5.4% of the calpain activity present in the zero-hour reference standard was detected by Colle, & Doumit (2017) in the beef *Semimembranosus* by day 2 of ageing. The calpain 1 activity was reported to be minimal (< 4%) in both beef *Semimembranosus* and *Longissimus lumborum* by Camou, Marchello, Thompson, Mares, & Goll (2007) by day 2 of ageing.

Native calpain 2 activity decreased whereas the autolysed calpain 2 activity increased with the ageing time, with highest activity detected on day 14 in both the muscles. An increase in the autolysed calpain 2 activity with ageing time was also recorded by Colle, & Doumit (2017); Pomponio et al., (2008) and Camou, Marchello, Thompson, Mares, & Goll (2007) in beef *Semimembranosus*. Calpain 2 requires a higher level of calcium for its activation and gets autolysed at later stages of ageing when intracellular calcium concentration increases to a level that would activate calpain 2 (Colle, & Doumit, 2017; Morton et al., 1999).

### 3.4.5 Myofibrillar protein profile (SDS-PAGE)

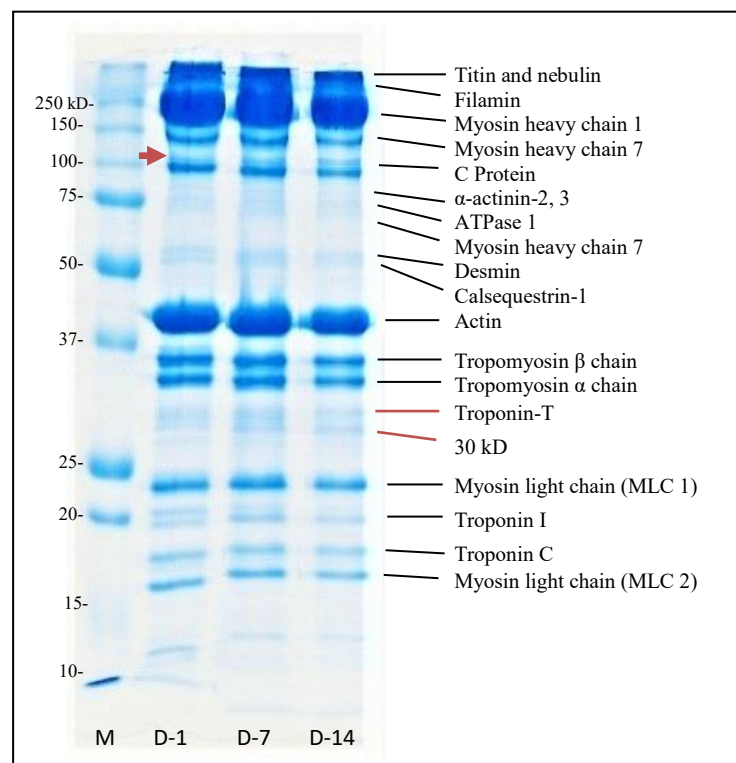
The myofibrillar protein profile of beef *Semimembranosus* and *Biceps femoris* samples obtained at different intervals of ageing are presented in SDS-PAGE gel electrophoretograms in Figures 3.6 and 3.7, respectively.



**Figure 3.6 Effect of ageing on the myofibrillar protein profile (SDS-PAGE) of beef *Semimembranosus* [M = marker, D-1 = day 1 of aging, D-7 = day 7 of aging, D-14 = day 14 of aging, 15 µg samples were loaded in each lane, Protein identification was derived from that reported by Ha (2012) and Bekhit et al., (2016)]**

The intensity of the 110 kD protein band increased with ageing time and the band was most intense on day 14 of ageing in both the muscles. Normally seen in aged beef (Stoeva, Byrne, Mullen, Troy, & Voelter, 2000; O'Halloran, Troy, Buckley, & Reville, 1997), the intensity of the 110 kDa and 90 kD protein band is believed to be an indicator of proteolysis of myosin heavy chain (Yates, Dutson, Caldwell, & Carpenter, 1983). The 30 and 32 kD were more intense on day 7 and day 14 in comparison to day 1 of ageing in both the muscles. Known to be a part of 27–32 kDa group, 30 and 32 kDa protein bands are produced during post-mortem degradation of muscle proteins (Marino et al., 2013; Han, Morton, Bekhit, & Sedcole, 2009). Obtained from the proteolysis of intact troponin-T, the magnitude of the concomitant 28 and 30 kDa polypeptide segments strongly correlates with the post-mortem proteolysis and meat tenderness during ageing (Rowe, Maddock, Trenkle, Lonergan, & Huff-Lonergan, 2003). Troponin-T, a subunit that coordinates the interaction between actin and myosin and regulates the striated muscle contraction, is believed to be one of the sensitive substrates of calpain 1 (Geesink,

Kuchay, Chishti, & Koohmaraie, 2006). The proteolysis of troponin-T results in the loss of integrity of thin filaments causing the degradation of myofibrils and thus contributes to the tenderness during ageing (Taylor et al., 1995). Likewise, the band for desmin was more intense on day 7 than day 1 in both the muscles. Desmin, which is an important cytoskeletal protein located around the Z-disk in muscle cells, has a significant role in maintaining the structural integrity of myofibrils and its degradation would result into loss of integrity and weaken the contraction among the muscle cells (Pearce, Rosenvold, Andersen, & Hopkins, 2011; Huff-Lonergan, Zhang, & Lonergan, 2010). Like troponin-T, proteolysis of desmin is predominantly believed to be caused by calpain 1 during post-mortem ageing.



**Figure 3.7 Effect of ageing on the myofibrillar protein profile (SDS-PAGE) of beef *Biceps femoris* [M = marker, D-1 = day 1 of aging, D-7 = day 7 of aging, D-14 = day 14 of aging, 15 µg samples were loaded in each lane, Protein identification was derived from that reported by Ha (2012) and Bekhit et al., (2016)]**

### **3.5 CONCLUSIONS**

By reducing the toughness of the muscles by a margin of 30%, it was concluded that post-mortem ageing could play a significant role in improving the quality of the muscles obtained from culled dairy animals. Since the aged muscles were still excessively tough (as indicated by their shear force values), future research may explore other possibilities such as exploit the extra benefit of an extended ageing or may focus on the prior use of non-thermal technologies, such as pulsed electric field, to provide a new age-specific strategy for ageing to reduce the extra toughness associated with the muscles of culled dairy animals.

## Chapter 4

### Does pulsed electric field have a potential to improve the quality of beef *Semimembranosus* from older animals and how?

This chapter is published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2019). Does pulsed electric field has a potential to improve the quality of beef from older animals and how? *Innovative Food Science and Emerging Technologies*, 56, <https://doi.org/10.1016/j.ifset.2019.102194>.

#### 4.1 Abstract

A study was designed to investigate whether pulsed electric field (PEF) has any potential to improve the quality of beef from older animals and to elucidate the mechanism involved. PEF treated beef *Semimembranosus* samples viz. T<sub>1</sub> (5 kV, 0.36 kV/cm, 90 Hz), T<sub>2</sub> (10 kV, 0.60 kV/cm, 20 Hz) and a non-treated control were vacuum packaged and stored for 14 days at 4±1 °C. Samples were analysed for casein zymography, western blotting, SDS-Page and various physicochemical properties. PEF treated samples showed higher calpain activity in comparison to control along with an early activation of calpain 2. Increased proteolysis of troponin-T and desmin was also recorded, however, no significant ( $P > 0.05$ ) impact was observed on the shear force and myofibrillar fragmentation index. By influencing the calpain activity of the muscle, this study confirmed the enzymatic nature of PEF and concludes that PEF has a limited potential to improve the quality of excessively tough muscles from culled dairy animals.

**Keywords:** Pulsed electric field; *Semimembranosus*; calpain activity; western blotting; shear force; physicochemical properties

#### 4.2 Introduction

Pulsed electric field (PEF) is regarded as a promising mild-processing technology that finds its application in several areas of food processing, preservation and quality (Koubaa et al., 2018; Gabrić et al., 2017). This novel non-thermal technology has attracted the attention of the meat industry due to its ability to induce electroporation of cell membranes that affects the permeability, mass transfer and diffusion processes (Bhat, Morton, Mason, & Bekhit, 2018a, d). Several studies have been conducted over the last five years to evaluate the potential of PEF processing on the tenderization of meat during ageing. These studies report varying results with no scientific evidence about the

underlying mechanisms. While some authors believe physical disruption as one of the contributing factors for PEF induced tenderness, enhanced proteolysis, mediated by the calpain system, seems to be the most plausible and widely accepted mechanism (Bhat, Morton, Mason, & Bekhit, 2018a, d). PEF-induced electroporation has the potential to cause an early activation of calpains by affecting the membrane permeability of sarcoplasmic reticulum causing an early release of calcium. However, no study has evaluated the effect of PEF processing on the calpain activity of beef during ageing and there is no scientific evidence to support the enzymatic mechanism for PEF processing.

There is also a high degree of variability among the studies about the efficacy of PEF to induce tenderization. Some studies demonstrate a significant impact of PEF treatment on the shear force of muscles studied (Bekhit, Suwandy, Carne, van de Ven, & Hopkins, 2016; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015a, b, d; Bekhit, van de Ven, Suwandy, Fahri, & Hopkins, 2014c), while others only suggest a tendency of PEF to reduce shear force (Arroyo et al., 2015a; Arroyo et al., 2015b; Faridnia, Bekhit, Niven, & Oey, 2014; O'Dowd, Arimi, Noci, Cronin, & Lyng, 2013). These studies are not conclusive and there seems to be no consensus among different authors about the mechanisms involved. While some studies have suggested physical disruption as the main cause of PEF induced tenderness, enzymatic nature of the tenderization seems to be the most plausible mechanism. Several studies have suggested the potential of PEF to mediate the tenderization process due to its membrane altering properties causing early release of calcium ions and early activation of the calpain proteases (Bekhit et al. 2016; Suwandy et al. 2015a, b, c; Bekhit et al. 2014c). However, there is dearth of knowledge and lack of experimental proof on whether PEF treatment affects the enzyme activity of calpains and how different PEF processing conditions would affect this system.

The application of PEF to the meat for its tenderization effect is an innovative strategy, however, considerable research is required before this technology could be applied in the meat industry (Bhat et al., 2018a). The objective of this study was to evaluate the physicochemical, enzymatic and proteolytic changes that occur after PEF treatment of post-rigor *Semimembranosus*. Myofibrillar fragmentation index (MFI) was utilized as a proteolytic marker and the activities of the calpain proteases (calpain 1 and calpain 2) were determined to analyse whether the change in meat tenderness, if any, was linked with enzymatic changes. Proteolytic pattern of desmin and troponin-T and myofibrillar protein profile (SDS-PAGE) was evaluated to analyse if post-mortem proteolysis was a contributing factor to the PEF-induced tenderness. The present study involved the use of muscles from culled dairy animals which were excessively tough and a greater reduction in shear force would generally be required to obtain a significant change, providing a more challenging model to validate the potential of PEF in inducing a tenderization effect during the ageing process.

Meat from older animals, such as culled dairy animals, is an excellent source of amino acids and minerals and could serve as a nutritional dietary source for those who are on limited budgets. However, meat from older animals is excessively tough and needs some post-mortem interventions



for subsequent tenderization (Bhat et al., 2018b). Some studies have examined the effect of traditional post-mortem treatments, such as ageing, on the quality of beef from older animals, however, no study has evaluated the potential of PEF processing on the quality of beef from older animals and needs scientific attention.

### **4.3 Materials and methods**

Meat samples were prepared as per the method described in section 2.2.3. The meat samples were treated with PEF as per the method described in section 2.2.3.1. Electrical input and change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ ) were measured according to the methods described in section 2.2.6 and 2.2.7, respectively. Shear force (N), purge loss (%), cooking loss (%) and myofibrillar fragmentation index (MFI) of the samples were measured according to the methods described in section 2.3.1 to 2.3.3. Sarcoplasmic and myofibrillar fractions were separated by following the method described in section 2.4.1. Protein concentration, casein zymography, SDS-PAGE, immunoblotting and analysis of the protein bands was done by following the methods described from section 2.4.2 to 2.4.5. Statistical analysis of the data was performed according to the method described in section 2.7.1.

### **4.4 Results and discussion**

#### **4.4.1 Electrical input**

The mean values for various electrical parameters recorded during PEF treatment of beef *Semimembranosus* viz.  $T_1$  (5.0 kV) and  $T_2$  (10.0 kV) are presented in Table 4.1. The actual electric field strength recorded during the PEF treatment of beef *Semimembranosus* was 0.36 kV/cm for  $T_1$  (5.0 kV) and 0.60 kV/cm for  $T_2$  (10.0 kV). The electric field strength has been reported to be directly affected by the voltage and independent of pulse frequency (Suwandy et al., 2015b; Bekhit et al., 2014c). By affecting the transmembrane potential of the sarcolemma, the strength of the electric field is most crucial in causing the irreversible electroporation that can lead to faster rates of cellular reactions by increasing the interactions between enzymes and their substrates at the cellular level. Similar electric field strength values were observed by Suwandy et al. (2015b) and Bekhit et al. (2014c) for similar PEF treatments (5 kV, 10 kV  $\times$  20, 50, 90 Hz) in beef *Semimembranosus* and *Longissimus lumborum*.

#### **4.4.2 Change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ )**

The mean values for temperature ( $^{\circ}\text{C}$ ) and conductivity change recorded in beef *Semimembranosus* during PEF treatment viz.  $T_1$  (5.0 kV) and  $T_2$  (10.0 kV) are presented in Table 4.1. The average change

in temperature in the muscle samples in the present study was  $12.76 \pm 1.39$  °C for T<sub>2</sub> (10.0 kV) samples which was significantly ( $P < 0.05$ ) higher than  $4.83 \pm 1.21$  °C observed for T<sub>1</sub> (5.0 kV) samples. The higher energy density and pulse peak power values associated with higher voltage were translated into larger temperature change of the T<sub>2</sub> (10.0 kV) samples. Similar findings were also recorded by Suwandy et al. (2015b) and Bekhit et al. (2014c) for PEF treatments in beef *Semimembranosus* and *Longissimus lumborum* who also observed similar increase in temperature of the said muscles. The average temperature increase observed after PEF treatment in our study was, however, smaller than that observed by O'Dowd et al. (2013) in beef *Semitendinosus* who observed an increase of 5 to 30 °C depending on the electric field and the frequency used (1.1–2.8 kV/cm; 5–200 Hz).

**Table 4.1 Pulsed electric field processing parameters used for beef *Semimembranosus* and effect on the average temperature (°C) and conductivity (mS/cm) (Mean ± SE)\***

PEF Treatment	T <sub>1</sub> (5 kV)	T <sub>2</sub> (10 kV)
Input voltage (kV)	5.0	10.0
Pulse frequency (Hz)	90.0	20.0
Pulse number	2725	2723
Pulse width (µs)	20.0	20.0
Pulse peak power (kW)	$142 \pm 1.48^a$	$546 \pm 4.51^b$
Pulse peak current (A)	$49.3 \pm 1.67^a$	$112.6 \pm 1.02^b$
Electric field strength (kV/cm)	$0.36 \pm 0.02^a$	$0.60 \pm 0.00^b$
Energy density (kJ/kg)	$18.63 \pm 0.94^a$	$73.28 \pm 2.04^b$
Temperature change ( $\Delta T$ , °C)	$4.83 \pm 1.21^a$	$12.76 \pm 1.39^b$
Conductivity change ( $\Delta\sigma$ , mS/cm)	$1.26 \pm 0.69$	$1.98 \pm 0.42$

\*Mean ± SE with different superscripts in a row differ significantly ( $P < 0.05$ )

Initial temperature of the samples =  $4.22 \pm 0.92$  °C (T<sub>1</sub>),  $4.35 \pm 1.12$  °C (T<sub>2</sub>)

Initial conductivity (mS/cm) of the samples =  $9.91 \pm 1.62$  (T<sub>1</sub>),  $9.81 \pm 1.27$  (T<sub>2</sub>)

n = 6

No significant ( $P > 0.05$ ) effect of PEF treatment was observed on the conductivity of the muscle. Similar findings were also recorded by Suwandy et al. (2015b) and Bekhit et al. (2014c) in beef *Semimembranosus* and *Longissimus lumborum* who also recorded no significant effect for PEF treatments on conductivity of the said muscles. Electrical conductivity represents the muscle tissue's capability to conduct electricity and can be used as an indicator of muscle quality and structure (Ramkumar, 2017). Determined by the water characteristics, the electrical properties of meat are

complex because of its anisotropic nature that results in varying electrical characteristics. Relatively higher electrical conductivity in raw meat is characterized by high amount of free water whereas relatively low electrical conductivity indicates that product would be drier (Martinez, 2017; Ramkumar, 2017). The importance of electrical conductivity in PEF-treated meat becomes particularly important because it can be used to analyse the integrity of cell membranes and characteristics of muscle tissue (Martinez, 2017; Põldvere, Tänavots, Saar, Sild, & Lepasalu, 2016). A temporary effect of PEF treatment was observed on the conductivity of *Semitendinosus* by O'Dowd et al. (2013). This increase in conductivity was associated with the rise in temperature caused by PEF processing and the samples returned to similar conductivity as that of control samples after cooling.

#### **4.4.3 Purge loss (%)**

Purge loss is one of the significant quality attributes of fresh meat packaged under vacuum and reflects the weight loss from meat during storage due to escape of the liquid from meat into the packaging (Pearce, Rosenvold, Andersen, & Hopkins, 2011). It is of economic significance to meat processors since high purge losses can impact the functionality of the meat and contribute to significant financial losses. No effect ( $P > 0.05$ ) of PEF treatment was observed on the purge of the muscle samples during entire period (14 days) of ageing (Table 4.2). However, a significant ( $P < 0.05$ ) effect of ageing was observed as purge loss of all the samples including control increased significantly with ageing time. Suwandy et al. (2015b) and Bekhit et al. (2014c) also observed no significant effect of PEF treatments on purge loss of beef *Semimembranosus* and *Longissimus lumborum* during entire period of ageing.

#### **4.4.4 Cooking loss (%)**

No effect ( $P > 0.05$ ) of PEF treatment was observed on the cooking loss of the muscle samples during entire period of ageing (Table 4.2). Cooking has been reported to denature the muscle proteins which directly affects their water holding characteristics by influencing the structural characteristics of muscle proteins (Tornberg, 2005). Such structural changes have been reported to lead to substantial cooking losses in the range of 15 to 35% of which 90% is generally water (Pearce, Rosenvold, Andersen, & Hopkins, 2011). Our results suggest that PEF treatment under our experimental conditions did not alter the myofibrillar proteins thermally nor damaged muscle fibres and connective tissue that might enhance the movement of water out of the muscle. Faridnia et al. (2014) and Bekhit et al. (2014c) also observed no significant effect of PEF treatments on cooking loss of beef *Longissimus thoracis* and *Longissimus lumborum*, respectively. A significant ( $P < 0.05$ ) effect of ageing was observed in case of control samples only and the cooking losses decreased significantly on day 14 of the ageing. This decreasing trend with storage time may be attributed to the lower availability of free moisture due to

increasing purge losses of control samples with ageing time. The increased permeability of the PEF treated samples may also have contributed a little towards this variation in cooking loss between control and the treated samples. Ageing has been reported to affect the water holding capacity (has positive correlation with pH) of beef and has the potential to affect the cooking loss of the muscles (Kim et al. 2017). Bekhit et al. (2014c) also observed a significant ( $P < 0.05$ ) effect of ageing on the cooking loss of control samples only.

**Table 4.2 Effect of pulsed electric field on the physicochemical properties of beef *Semimembranosus* during aging (Mean  $\pm$  SE)\***

PEF Treatment	Period of aging		
	Day 01	Day 07	Day 14
<b>Purge loss (%)</b>			
Control	2.90 $\pm$ 0.34 <sup>a</sup>	5.55 $\pm$ 0.67 <sup>b</sup>	5.98 $\pm$ 0.39 <sup>b</sup>
T <sub>1</sub> (5 kV)	2.74 $\pm$ 0.10 <sup>a</sup>	6.30 $\pm$ 0.90 <sup>b</sup>	6.39 $\pm$ 0.61 <sup>b</sup>
T <sub>2</sub> (10 kV)	3.03 $\pm$ 0.29 <sup>a</sup>	5.47 $\pm$ 0.24 <sup>b</sup>	6.25 $\pm$ 0.40 <sup>b</sup>
<b>Cooking loss (%)</b>			
Control	31.44 $\pm$ 2.09 <sup>a</sup>	26.21 $\pm$ 1.46 <sup>ab</sup>	24.96 $\pm$ 2.07 <sup>b</sup>
T <sub>1</sub> (5 kV)	27.13 $\pm$ 2.98 <sup>a</sup>	27.88 $\pm$ 0.54 <sup>a</sup>	28.27 $\pm$ 1.83 <sup>a</sup>
T <sub>2</sub> (10 kV)	26.01 $\pm$ 2.58 <sup>a</sup>	29.71 $\pm$ 2.33 <sup>a</sup>	28.53 $\pm$ 1.30 <sup>a</sup>

\*Mean  $\pm$  SE with different superscripts in a row differ significantly ( $P < 0.05$ )

T<sub>1</sub> = Samples treated with PEF at 5 kV, 90 Hz, 20  $\mu$ s

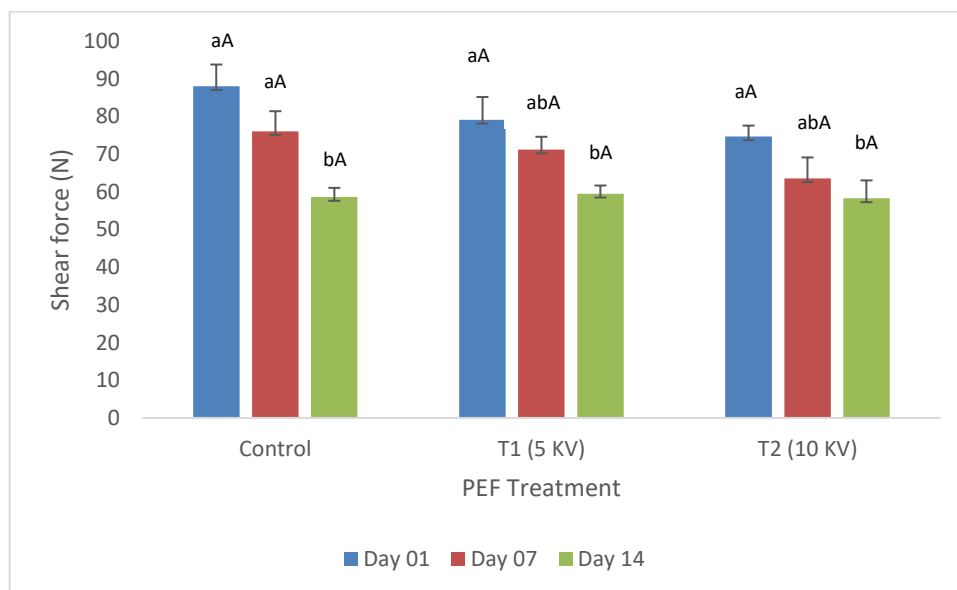
T<sub>2</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu$ s

n = 6

#### 4.4.5 Shear force (N)

The mean shear force values for PEF treated meat samples were lower, however, no significant ( $P > 0.05$ ) differences were observed between PEF treated and control samples during entire period of ageing (Figure 4.1). These results were in agreement with the findings of Arroyo et al. (2015a) who reported a tendency towards reducing toughness in PEF (1.4 kV/cm, 10 Hz, 20  $\mu$ s, 300 and 600 pulses) treated beef *Longissimus thoracis et lumborum*, however, no significant effect of PEF treatment was reported on the shear force of the muscle. In our study we also observed a tendency of PEF towards reducing shear force as the mean shear force for T<sub>1</sub> (5.0 kV) samples was 8.9 N (10.11%) lower and T<sub>2</sub> (10.0 kV) samples was 13.31 N (15.12%) lower than mean shear force value of control samples on day

1 of ageing. A similar trend was also observed on day 7 of ageing as the mean shear force for T<sub>1</sub> (5.0 kV) samples was 4.86 N (6.39%) lower and T<sub>2</sub> (10.0 kV) samples was 12.48 N (16.41%) lower than mean shear force value of control samples. Another study conducted on turkey breast (Arroyo et al., 2015b) demonstrated that PEF treatment had no significant effect on shear force either in frozen (2.1 kV/cm; 300 pulses of 20 µs; 10 Hz) or fresh (1.2 kV/cm; 300 pulses of 20 µs; 10 Hz) meat. The authors suggested that the PEF treatment used in the study was not sufficient to induce physical disruption of meat fibres to a point where it could have affected tenderness, suggesting physical disruption as the main mechanism for PEF-induced tenderness. O'Dowd et al. (2013) also reported similar findings in PEF (1.9 kV/cm, 65 Hz, 20 µs) treated beef *Semimembranosus* and came up with similar suggestion of physical disruption being the mechanism behind PEF-induced tenderness. Faridnia et al. (2014) also presented similar results of PEF treatment (0.2-0.6 kV/cm, 1-50 Hz, 20 µs) in beef *Longissimus thoracis*. The high shear force values observed in our study are typical of *Semimembranosus*, which is an intermediate tough muscle (Sullivan, & Calkins, 2011), and will tend to be tougher as the meat came from culled dairy cows (Roberts et al., 2018).



**Figure 4.1 Effect of pulsed electric field on the shear force (Newtons) of beef *Semimembranosus* (n = 6, Mean ± SE)**

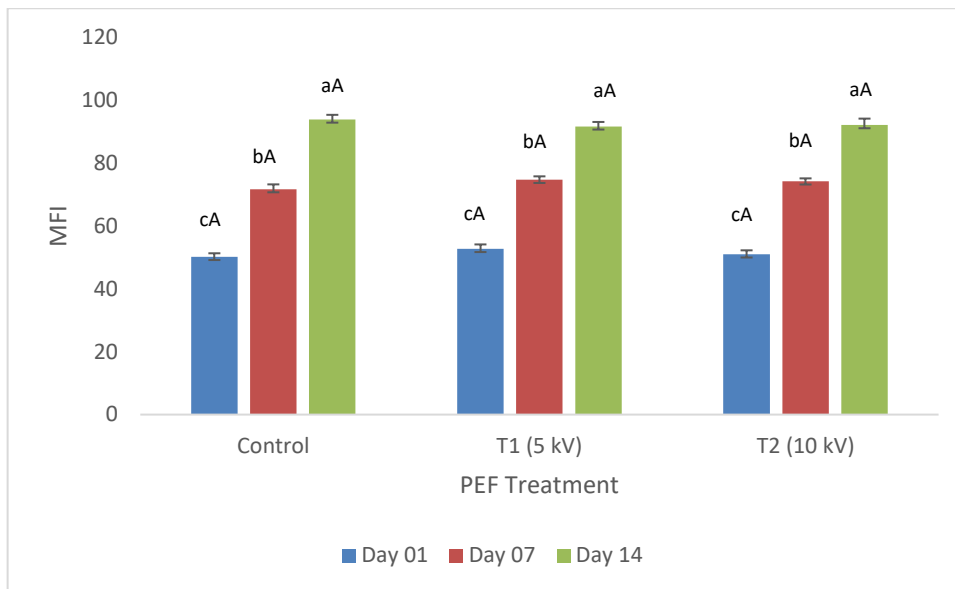
Different superscripts on columns (lower case alphabets within a group and upper case between groups) differ significantly ( $P < 0.05$ )

In contrast to our findings, Suwandy et al. (2015a, b, d) and Bekhit et al. (2014c) reported a significant effect of PEF treatments on shear force of beef *Semimembranosus* and *Longissimus lumborum* during ageing period. Similar treatment conditions and the same PEF machine was used in our experiment;

however, the effect of PEF was non-significant that might be attributed to the origin of muscle samples since the variation in connective tissues, particularly collagen, has been reported to affect the meat texture (Dransfield, 1977). Proteolysis of myofibrillar proteins, sarcomere length, and connective tissue content are the three main components that account for most, if not all, of the explainable variation seen in tenderness of aged meat (Gheisari, Shekarforoush, & Aminlari, 2007). The relative contribution of each of the above factors to tenderness is, however, dependent on the individual muscle under study. While proteolysis is the major determinant of tenderness of *Longissimus* muscle, connective tissue content is a major contributor to the tenderness of muscles such as *Semimembranosus* and *Biceps femoris* (Koochmaraie, Kent, Shackelford, Veiseth, & Wheeler, 2002). The potential of PEF to significantly reduce shear force in cold-boned muscles depends on various factors including muscles under study, electric field strength, and the use of an ageing period (Bhat et al., 2018a). A minimum electric field intensity is required above which PEF induces a substantial effect on tenderization process during a proper ageing period (Bhat et al., 2018a). The applied electric field needs to exceed a critical limit of 0.5 kV/cm for an animal cell to observe an electroporation effect (Töpfl, 2006), therefore, the electric field strength applied in this study (0.6 kV/cm, T<sub>2</sub>) should deliver an effective electric field and lead to cell permeabilization.

#### **4.4.6 Myofibrillar fragmentation index (MFI)**

No significant ( $P > 0.05$ ) effect of PEF was observed on MFI of muscles during whole ageing period (Figure 4.2). A significant ( $P < 0.05$ ) effect of ageing was observed and the mean MFI values for all the samples showed a significant ( $P < 0.05$ ) increase with increasing ageing time. Similar findings were also observed by O'Dowd et al. (2013) in PEF (1.9 kV/cm, 65 Hz, 20  $\mu$ s) treated beef *Semitendinosus* who also reported a non-significant ( $P > 0.05$ ) effect of PEF treatment on MFI values. However, the study demonstrated that some additional myofibril breakdown had occurred in PEF treated samples which also agreed with the findings for particle sizes analysed by laser diffraction. Several studies have demonstrated that degradation of myofibrillar proteins is the principal mechanism of meat tenderization (Huff-Lonergan, Zhang, & Lonergan, 2010). MFI is strongly correlated with shear force and post-mortem sensory tenderness. Correlation coefficients of 0.72 or higher have been reported between MFI and sensory tenderness (Moller, Vestergaard, & Wismer-Pedersen, 1973) or inversely to shear force (Culler, Parrish, Smith, & Cross, 1978). The MFI has been demonstrated to be a good indicator of the extent of proteolysis of muscle myofibrillar proteins during post-mortem storage and the increase in the value of MFI during post-mortem ageing period has been reported to be the result of rupture of myofibrils in the I-band of the sarcomere (Taylor et al., 1995).

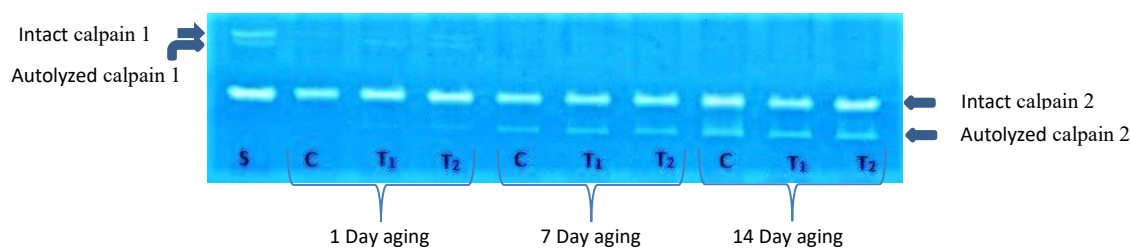


**Figure 4.2 Effect of pulsed electric field on the myofibrillar fragmentation index (MFI) of beef *Semimembranosus* (n = 6, Mean ± SE)**

Different superscripts on columns (lower case alphabets within a group and upper case between groups) differ significantly ( $P < 0.05$ )

#### 4.4.7 Calpain activity

Calpain activity, which involves autolysis and subsequent inactivation, was determined by casein zymography (Figure 4.3). The proteolytic breakdown of key myofibrillar and associated proteins by the calcium dependent calpains, particularly calpain 1, is known to play a central role in the tenderisation process during post-mortem ageing (Bhat, Morton, Mason, & Bekhit, 2018c; Huff-Lonergan et al., 2010; Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). Calpain 1, which is considered responsible for most of the post-mortem tenderization of beef (Geesink et al., 2006), is autolysed in the early stages of ageing. In our study calpain 1 activity, both intact and autolysed, decreased with the ageing time and was detected only on day 1 of ageing. Calpain 1 activity was not detected in any of the samples at day 7. Slightly higher levels of autolysed active calpain 1 were visible in the sarcoplasmic fraction of PEF treated samples in comparison to control, however, no significant ( $P > 0.05$ ) effect of PEF was observed on the calpain activity of the muscles. Colle, & Doumit (2017) reported a similar decrease in calpain-1 activity of the *Semimembranosus* and by day 2 of ageing, only 5.4% of the initial calpain activity was present.



**Figure 4.3 Effect of pulsed electric field on the calpain activity of beef *Semimembranosus* [S = standard, C = control, T<sub>1</sub> = 5 kV (90 Hz), T<sub>2</sub> = 10 kV (20 Hz), 35 µg samples were loaded in each lane]**

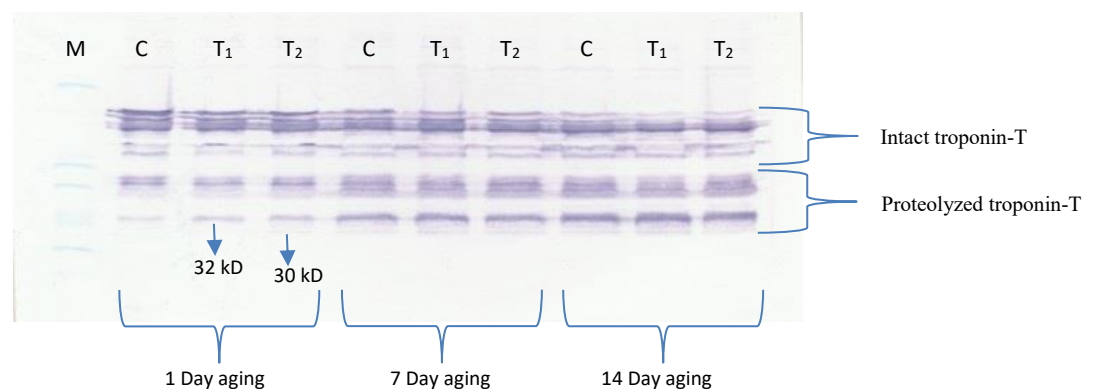
On the other hand, autolysed calpain 2 activity increased with the ageing time with highest activity detected on day 14. The autolysed calpain 2 activity was higher in PEF treated samples in comparison to control on day 1 of the ageing and the bands could be visualized only in the PEF treated samples. Calpain 2, which necessitates high levels of calcium for activation, is not normally autolysed in the early stages of ageing as post-mortem free calcium levels are too low to activate calpain-2 (Ji, & Takahashi, 2006). However, one proposed mechanism for PEF for aiding in the tenderization process is through early activation of calpains by release of calcium ions from cell organelles due to enhanced membrane permeability (Bhat et al., 2018a; Bekhit et al., 2014c). This suggested mechanism is further confirmed by evidence that a post-mortem ageing period is necessary for gaining the tenderization benefit of PEF through increased proteolysis (Bekhit et al., 2016; Suwandy et al., 2015a, b; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015c; Bekhit et al., 2014c). Colle, & Doumit (2017) also reported an increase in autolysed calpain-2 activity of the *Semimembranosus* with ageing.

#### 4.4.8 Proteolysis of desmin and troponin-T

Troponin-T is one of three components of the troponin complex that binds with tropomyosin and plays a significant role in the regulation of skeletal muscle contraction. Being a major component of the intermediate filaments, desmin interlink myofibrils at the level of the Z disks and connect them to the nuclear and plasma membranes (Goldfarb, Vicart, Goebel, & Dalakas, 2004). Because of their significant roles in preserving the structural integrity of myofibrils, the post-mortem degradation of desmin and troponin-T has been implicated for the loss of integrity and weakening of myofibrils, thereby improving meat tenderness (Huff-Lonergan et al., 2010). Degradation of both desmin and troponin-T has been directly linked to calpain activity using *in vitro* models of post-mortem muscles (Kitamura et al., 2005; Baron, Jacobsen, & Purslow, 2004).

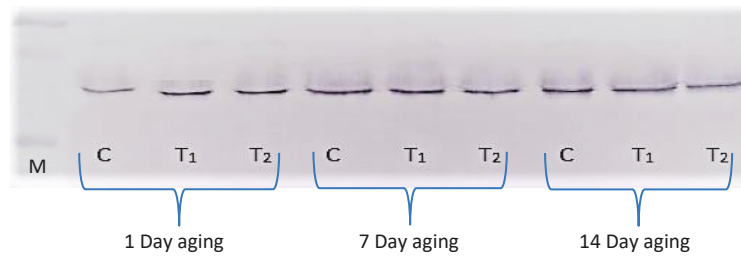


In the present study, calpain-mediated proteolysis was analysed through the detection of a 30-kD fragment of troponin-T known to result from calpain activity. The 30 kD fragment derived from troponin-T has been demonstrated to be an indicator of tenderization during meat ageing (Huang, Huang, Ma, Xu, & Zhou, 2012). The Western blot profiles presented in our study (Figure 4) exhibited differences in stain intensity of the 30 kD protein bands between PEF treated ( $T_1$ ,  $T_2$ ) and control samples (Figure 4.4). The 30 kD band could be readily visualized only in the  $T_2$  (10.0 kV) samples on day 1 of ageing. The PEF-treated samples showed increased degradation of troponin-T during subsequent post-mortem ageing, however, no significant ( $P > 0.05$ ) difference was observed among the samples. Used as a marker for myofibrillar protein degradation in beef, troponin-T degradation has been proven to be promoted under various tenderization treatments (Han, Morton, Bekhit, & Sedcole, 2009; Claeys, Smet, Balcaen, Raes, & Demeyer, 2004) and has been significantly correlated to shear force and MFI (Marino et al., 2013). Bekhit et al. (2016) and Suwandy et al. (2015a) also reported an increase in the proteolysis of troponin-T in PEF treated beef *Semimembranosus*.



**Figure 4.4 Effect of pulsed electric field on the proteolytic pattern of troponin-T of beef *Semimembranosus* [M = marker, C = control,  $T_1$  = 5 kV (90 Hz),  $T_2$  = 10 kV (20 Hz), 15  $\mu$ g samples were loaded in each lane]**

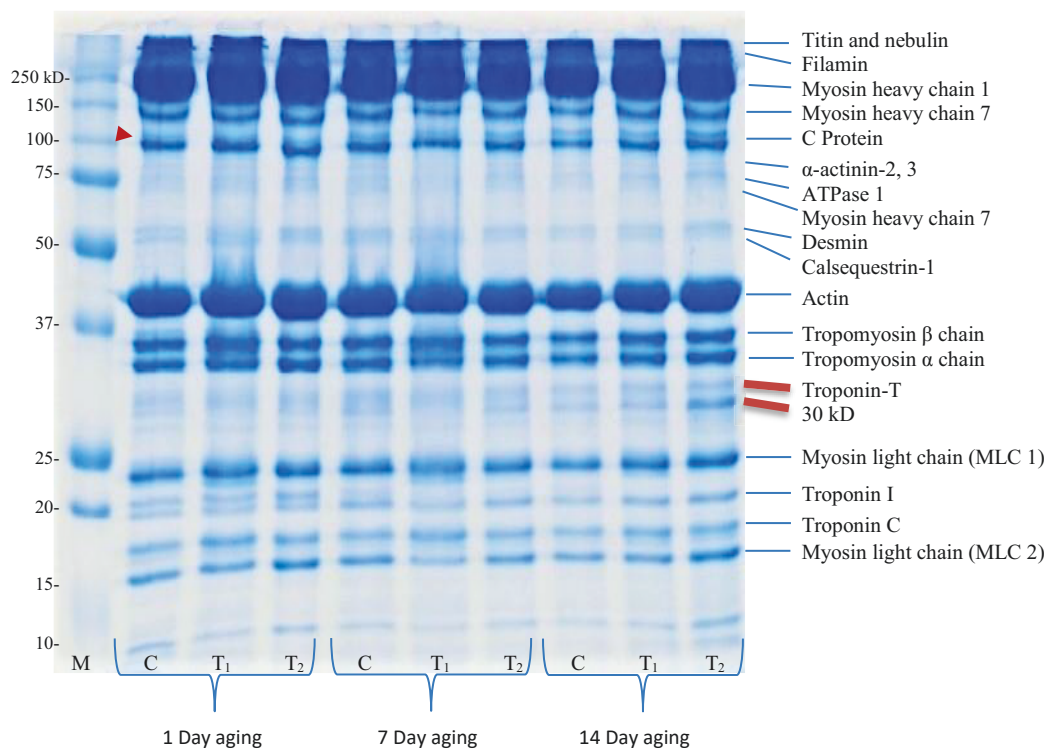
The Western blot profiles for desmin (Figure 4.6) also showed differences in intensity of the bands between PEF treated ( $T_1$ ,  $T_2$ ) and control samples on day 1 of ageing (Figure 4.5). Desmin proteolysis was recorded higher for PEF treated ( $T_1$ ,  $T_2$ ) samples in comparison to control on day 1 of ageing. These results agreed with the findings of calpain activity that was also observed higher in PEF treated samples on day 1 of the ageing. Post-mortem degradation of desmin is used as a marker for proteolysis and tenderization (Koochmariaie, & Shackelford, 1991). Bekhit et al. (2016) and Suwandy et al. (2015a) also reported an increased proteolysis of desmin in PEF treated samples in beef *Semimembranosus* during early post-mortem as well as subsequent post-mortem storage.



**Figure 4.5** Effect of pulsed electric field on the proteolytic pattern of desmin of beef *Semimembranosus* [M = marker, C = control, T<sub>1</sub> = 5 kV (90 Hz), T<sub>2</sub> = 10 kV (20 Hz), 20 µg samples were loaded in each lane]

#### 4.4.9 Myofibrillar protein profile (SDS-PAGE)

The effect of PEF treatment on the myofibrillar protein profile of beef *Semimembranosus* at different intervals of ageing is presented in Figure 4.6.



**Figure 4.6** Effect of pulsed electric field on the myofibrillar protein profile (SDS-PAGE) of beef *Semimembranosus* [M = marker, C = control, T<sub>1</sub> = 5 kV (90 Hz), T<sub>2</sub> = 10 kV (20 Hz), 20 µg samples were loaded in each lane, Protein identification was derived from that reported by Suwandy et al., (2015d) and Bekhit et al., (2016)]

The intensity of the 90 kD protein band was higher in PEF treated samples in comparison to control on day 14 of ageing. A similar trend was observed for 110 kD band, which was not visible on day 1 of ageing, showed intense band for PEF treated samples in comparison to control on day 14 of ageing. The intensity of the 110 and 90 kD protein bands, which can normally be seen in aged beef (Stoeva, Byrne, Mullen, Troy, & Voelter, 2000), are believed to be an indicator of proteolysis of myosin heavy chain (Yates, Dutson, Caldwell, & Carpenter, 1983). The 30 and 32 kD, which were barely visible in the samples during early post-mortem storage, became more intense in the PEF treated samples on day 14 of ageing. The 30 and 32 kD protein bands are produced during post-mortem proteolysis of meat and is known as 27–32 kD group (Han, Morton, Bekhit, & Sedcole, 2009; Marino et al., 2013). Polypeptide segments of 28 and 30 kD are obtained from the degradation of intact troponin-T by calpain 1. The magnitude of these concomitant polypeptides strongly correlates with the meat tenderness during post-mortem ageing (Rowe, Maddock, Trenkle, Lonergan, & Huff-Lonergan, 2003).

#### **4.5 Conclusions**

Collectively, the results from casein zymography, Western blots for desmin and troponin-T and myofibrillar protein profile clearly demonstrated that PEF processing increased the proteolysis in treated samples through improved calpain activity during ageing. Increase in the calpain activity was also accompanied with an early activation of calpain 2, however, no significant impact of PEF treatment was recorded on the shear force of the samples. The PEF processing induced electroporation and thereby increased the calpain activity and proteolysis, however, the magnitude of the effect seemed insufficient to produce a significant impact on the tenderization process in excessively tough muscles from older animals. A faster rate of tenderization observed in PEF treated samples during first week of ageing may be helpful in reducing the total time of ageing. This study reports for the first time the effect of PEF on the calpain activity of beef and expands our understanding of how PEF mediates the tenderization during ageing. It may be concluded that PEF processing has limited application in the muscles from older animals. Future studies may focus on using stronger PEF conditions for the muscles from culled dairy animals and comparing their response with muscles from younger animals.

## Chapter 5

### **Pulsed electric field operates enzymatically by causing early activation of calpains in beef *Biceps femoris* during ageing**

This chapter is published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2019). Pulsed electric field operates enzymatically by causing early activation of calpains in beef during ageing. *Meat Science*, 153, 144-151.

#### **5.1 Abstract**

A study was conducted to elucidate the impact of pulsed electric field (PEF) on the activity of calpains, proteolytic activity of desmin and troponin-T and physicochemical properties of beef *Biceps femoris* during ageing. The meat samples (meat blocks) were subjected to two PEF treatments; T<sub>1</sub> (5 kV, 90 Hz, 0.38 kV/cm) and T<sub>2</sub> (10 kV, 20 Hz, 0.61 kV/cm) and a non-treated control was run in parallel. The samples were vacuum packaged and aged for 1, 7 and 14 days at 4±1 °C. This study reports for the first time the impact of PEF-processing on the calpain activity in beef. Early post-mortem activation of calpain 2 was observed in PEF-treated samples. An increase in the calpain activity and proteolysis of desmin and troponin-T was observed. No significant effect of PEF was observed on the shear force of tough muscles from culled dairy animals during the entire ageing period.

**Keywords:** Beef; shear force; casein zymography; Western blotting; physicochemical properties

#### **5.2 Introduction**

Tenderness is the predominant determinant for the quality of beef and is a complex physical property that strongly influences the consumers' decision for repeat purchase of a product (Mennecke, Townsend, Hayes, & Lonergan, 2007; Miller, Carr, Ramsey, Crockett, & Hoover, 2001; Dransfield, 1994). Defined by the ease of mastication (Jiang et al., 2018), consumers perceive tenderness as an indicator for the eating quality of beef and they are willing to pay a premium price for a product that is more likely to be tender (Morton, Bhat, & Bekhit, 2017; Miller, Carr, Ramsey, Crockett, & Hoover, 2001). Satisfying customers with a consistent high-quality product is of prime importance for continued success of the meat industry (Kim et al., 2018). Several post-mortem interventions such as electrical stimulation, suspension methods, blade tenderization and ageing are effective in improving the tenderness of the beef and are often used in combination by the meat industry to maximise the tenderness benefit (Bhat et al., 2018a, d; Lang et al., 2016; Hopkins, 2014; Koohmaraie, & Geesink, 2006). Several emerging non-thermal technologies such as ultrasound and high-pressure processing

have also been reported to have a positive influence on the tenderness of beef (Warner et al., 2017; Bhat, Morton, Mason, & Bekhit, 2018a, d). Recently, a number of studies (Bekhit, Suwandy, Carne, van de Ven, & Hopkins, 2016; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015a, b, d; Bekhit, van de Ven, Suwandy, Fahri, & Hopkins, 2014c) have revealed the potential of pulsed electric field (PEF) in improving the tenderness of beef during ageing. All these studies have suggested the possibility of an enzymatic mechanism for the tenderization effect of PEF, however, there is no experimental evidence to prove this postulation. To harvest the full potential of this technology, the underlying mechanisms that operate at the cellular level and are responsible for the tenderization effect need to be elucidated.

PEF is a novel non-thermal technology that has been applied to produce high quality food products with a natural flavour and fresh appearance and is gaining recognition due to the increasing demands of consumers for fresh and natural foods (Wang et al., 2018b; Bhat, Morton, Mason, & Bekhit, 2018a). PEF has already proven its potential for improving or modifying several commercially important food processes such as pasteurization and sterilization of heat sensitive liquids (Buchmann, Bloch, & Mathys, 2018), inactivation of enzymes (García-Parra et al., 2018), freezing processes (Velickova et al., 2018) and dehydration processes (Liu, Grimi, Lebovka, & Vorobiev, 2018a). This technology involves the use of electric field pulses of short duration (ranging from nanoseconds to milliseconds) with electric field strength of 0.1-80 kV/cm applied to a food passed through or placed between two electrodes (Bhat et al., 2018a). Although the technology is used commercially in the food industry in some countries (Bhat et al., 2018a), there is limited understanding of the impact of PEF processing on solid food matrices (Liu, Burritt, Eyres, & Oey, 2018), particularly high-fat and high-protein foods such as meat (Bhat et al., 2018a). PEF involves the application of an external electric field to induce electroporation, a phenomenon that involves the formation of pores and increases the permeability of biological membranes (Ostermeier, Giersemehl, Siemer, Töpfl, & Jäger, 2018). This increased membrane permeability facilitates the exchange of intracellular components with the cell's surroundings and can induce certain useful effects that have not been fully explored such as improved salt diffusion and supercooling (Bhat et al., 2018a). One of these unexplored areas is to elucidate the role that this electro-permeabilization can play activating the calpain system, and hence the potential for PEF to increase tenderization during the process of ageing. The calpain system in muscle comprises two calcium-dependant cysteine proteases, calpain 1 and 2 (Koochmaraie, & Geesink, 2006; Goll, Thompson, Li, Wei, & Cong, 2003). The calpain system is associated with degradation of myofibrillar proteins and higher calpain activity, particularly calpain-1, is related with increased tenderness (Bhat, Morton, Mason, & Bekhit, 2018d; Koochmaraie, & Geesink, 2006). By precisely controlling the processing parameters, PEF could be exploited to facilitate an early release of calcium across the electroporated sarcoplasmic membranes causing early activation of calpains. No information is available in the literature about the effect of PEF on calpain activity of muscles during ageing.

Several studies have evaluated the effect of PEF on the tenderization of beef (Bekhit et al., 2016; Suwandy et al., 2015a, b, d; Arroyo et al., 2015; Bekhit et al., 2014c; O'Dowd et al., 2013), however, the results of the studies have been highly variable. Several factors including muscles under investigation, electric field strength, and the use of an aging period have been reported to have an influence on the potential of PEF to improve meat tenderization (Bhat et al., 2018a). Further research is required in this area to validate the potential of PEF and its consistent efficacy in inducing a tenderization effect during the ageing process. We decided on a more challenging model, using muscles from culled dairy animals, to determine the magnitude of the benefit that could be induced by the prior treatment of PEF during ageing. The objective of this study was to treat *Biceps femoris* with PEF at two levels and elucidate the changes in the calpain proteases (calpain 1 and calpain 2), proteolysis of two calpain substrates (desmin and troponin-T) and associated physicochemical properties during subsequent ageing.

### **5.3 Materials and methods**

Meat samples were prepared as per the method described in section 2.2.3. The meat samples were treated with PEF as per the method described in section 2.2.3.1. Electrical input and change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ ) were measured according to the methods described in section 2.2.6 and 2.2.7, respectively. Shear force (N), purge loss (%), cooking loss (%) and myofibrillar fragmentation index (MFI) of the samples were measured according to the methods described in section 2.3.1 to 2.3.3. Sarcoplasmic and myofibrillar fractions were separated by following the method described in section 2.4.1. Protein concentration, casein zymography, SDS-PAGE, immunoblotting and analysis of the protein bands was done by following the methods described from section 2.4.2 to 2.4.5. Statistical analysis of the data was performed according to the method described in section 2.7.1.

### **5.4 Results and discussion**

#### **5.4.1 Electrical input**

The average values for various electrical parameters namely input voltage, pulse frequency, pulse number, pulse peak voltage, pulse peak current, electric field strength and energy density of the PEF treatments of beef *Biceps femoris* muscle are presented in Table 5.1. The mean electric field strength was 0.38 kV/cm for  $T_1$  (5.0 kV) and 0.61 kV/cm for  $T_2$  (10.0 kV) treatment. Electric field strength is the most crucial parameter for inducing irreversible electroporation in biological membranes during PEF processing of meat (Suwandy et al., 2015b; Bekhit et al., 2014c). The electroporation of sarcoplasmic reticulum is suggested to cause an early release of calcium ions which should lead to the early

activation of calpains and increased tenderization during ageing after PEF processing (Bhat et al., 2018b; Warner et al., 2017). The electric field strength in the present study was within a range (0.27 to 0.56 kV/cm) reported in beef *Longissimus lumborum* treated at 5 kV or 10 kV × 20, 50, 90 Hz, 20 μs and beef *Semimembranosus* (0.28 to 0.51 kV/cm) under similar PEF treatment (Bekhit et al., 2014c; Suwandy et al., 2015b). A slightly higher range (0.58 to 0.73 kV/cm) was reported under similar PEF treatment (10 kV, 90 Hz, 20 μs) in beef *Longissimus lumborum* (Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015c).

#### 5.4.2 Change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ )

The average values for the change in temperature ( $^{\circ}\text{C}$ ) and conductivity ( $\sigma$ ) observed during PEF treatment of beef *Biceps femoris* are presented in Table 5.1. Although PEF is recognized as a non-thermal technology, ohmic heat can be generated during the process (O'Dowd, Arimi, Noci, Cronin, & Lyng, 2013). The average rise in the temperature observed during  $T_2$  (10.0 kV) treatment of the muscle was 11.08  $^{\circ}\text{C}$  and was significantly ( $P < 0.05$ ) higher than the average rise of 8.18  $^{\circ}\text{C}$  observed during  $T_1$  (5.0 kV) treatment. The higher rise in the temperature of the samples treated with  $T_2$  (10.0 kV) can be attributed to the higher energy density associated with the treatment. The average temperature rise observed in our study was far lower than the 30  $^{\circ}\text{C}$  rise observed by O'Dowd et al. (2013) in some of their samples of beef *Semitendinosus* (1.9 kV/cm, 65 Hz, 20 μs). Several studies have reported the influence of temperature on calpain activity and proteolysis during post-mortem ageing (reviewed by Bhat et al. 2018c, d; Thomson, Gardner, Simmons, & Thompson, 2008). Any substantial rise in temperature during PEF processing will have direct consequences on the tenderness of meat due to its direct influence on the activity of calpains and subsequent effect on the proteolysis of muscle proteins. Calpains, particularly calpain 1, have been reported to be highly sensitive to any temperature change. Primarily regulated by muscle temperature in post-mortem bovine muscle (Mohrhauser, Lonergan, Huff-Lonergan, Underwood, & Weaver, 2014; Du et al., 2017), calpain 1 has been reported to lose most of its activity within three hours at 25  $^{\circ}\text{C}$  whereas calpain 2 has been observed to be more stable (Ertbjerg, Christiansen, Pedersen, & Kristensen, 2012).

Generally expressed in microsiemens ( $\mu\text{s}$ ) or siemens (s, previously known as mho) in meat research, electrical conductivity represents the muscle tissue's capability to conduct electricity and can be used as an indicator of muscle quality and structure (Ramkumar, 2017). Determined by the water characteristics, the electrical properties of meat are complex because of its anisotropic nature that results in varying electrical characteristics. Relatively higher electrical conductivity in raw meat is characterized by high amount of free water whereas relatively low electrical conductivity indicates that product would be drier (Martinez, 2017; Ramkumar, 2017). The importance of electrical conductivity in PEF-treated meat becomes particularly important because it can be used to analyse the integrity of

cell membranes and characteristics of muscle tissue (Martinez, 2017; Pöldvere, Tänavots, Saar, Sild, & Lepasalu, 2016). PEF processing had no significant ( $P > 0.05$ ) effect on the conductivity of the beef *Biceps femoris*. Similar findings were reported by Suwandy et al. (2015b) and Bekhit et al. (2014c) who also observed no significant effect of PEF processing on the conductivity of the beef muscles. A temporary effect of PEF treatment on the conductivity of beef *Semitendinosus* was reported by O'Dowd et al. (2013) who observed a significant increase in the conductivity of the muscle that was temperature dependent and lasted only until muscles cooled down.

**Table 5.1 Pulsed electric field processing parameters used for beef *Biceps femoris* and effect on the average temperature (°C) and conductivity (mS/cm) (Mean ± SE)\***

PEF Treatment	T <sub>1</sub> (5 kV)	T <sub>2</sub> (10 kV)
Input voltage (kV)	5.0	10.0
Pulse frequency (Hz)	90.0	20.0
Pulse number	2725	2723
Pulse width (µs)	20.0	20.0
Pulse peak power (kW)	144.8 ± 1.10 <sup>a</sup>	553.8 ± 9.68 <sup>b</sup>
Pulse peak current (A)	46.8 ± 1.38 <sup>a</sup>	111.3 ± 1.68 <sup>b</sup>
Electric field strength (kV/cm)	0.38 ± 0.01 <sup>a</sup>	0.61 ± 0.01 <sup>b</sup>
Energy density (kJ/kg)	21.17 ± 0.72 <sup>a</sup>	74.24 ± 2.97 <sup>b</sup>
Temperature change (ΔT, °C)	8.18 ± 0.83 <sup>a</sup>	11.08 ± 0.64 <sup>b</sup>
Conductivity change (Δσ, mS/cm)	1.23 ± 0.21	1.91 ± 0.28

\*Mean ± SE with different superscripts in a row differ significantly ( $P < 0.05$ )

Initial temperature of the samples = 4.39 ± 0.85 °C (T<sub>1</sub>), 4.78 ± 1.20 °C (T<sub>2</sub>)

Initial conductivity (mS/cm) of the samples = 5.92 ± 1.59 (T<sub>1</sub>), 5.22 ± 1.18 (T<sub>2</sub>)

n = 6

### 5.4.3 Purge loss (%)

Mean values for purge loss (%) for the PEF treated *Biceps femoris* muscle observed during ageing are presented in Table 5.2. Purge loss, measured by the weight of the liquid found in the package, has a bearing on the quality of fresh meat and is of economic significance as it reflects the weight loss from meat during ageing. A significant ( $P < 0.05$ ) effect of ageing was observed and the mean values for purge loss (%) showed a significant increasing trend with storage period. The PEF treatment had no significant ( $P > 0.05$ ) influence on the purge loss of the muscle at any point during ageing that might be attributed to the lower temperature change observed during the treatment. Since water holding



capacity of meat is greatly influenced by the state of the muscle proteins, the level of denaturation of proteins, associated with a temperature rise, has direct bearing on the drip loss of meat during storage (Filho, Cazedey, Fontes, Ramos, & Ramos, 2017). Degradation of proteins during ageing can largely influence the water holding capacity and affect the purge loss of fresh meat during post-mortem storage. The PEF processing has been previously reported to have no effect on the purge loss of beef muscles during ageing (Suwandy et al., 2015b; Bekhit et al., 2014c).

#### 5.4.4 Cooking loss (%)

The mean values for cooking loss (%) of the PEF treated muscle during ageing are presented in Table 5.2. No significant effect ( $P > 0.05$ ) of PEF processing or ageing was observed on the cooking loss of the *Biceps femoris* during the entire period of ageing. By altering the structural properties of the muscle proteins (Tornberg, 2005), cooking has been reported to affect the water characteristics of the meat by causing denaturation of the proteins (Bertram, Kohler, Böcker, Ofstad, & Andersen, 2006). Significant cooking losses ranging from 15 to 35% have been reported from such structural changes (Pearce, Rosenvold, Andersen, & Hopkins, 2011).

**Table 5.2 Effect of pulsed electric field and ageing on the purge loss and cooking loss of beef *Biceps femoris* (Mean  $\pm$  SE)\***

PEF Treatment	Period of aging		
	Day 01	Day 07	Day 14
<b>Purge loss (%)</b>			
Control	1.74 $\pm$ 0.24 <sup>a</sup>	3.05 $\pm$ 0.29 <sup>b</sup>	4.17 $\pm$ 0.60 <sup>b</sup>
T <sub>1</sub> (5 kV)	1.90 $\pm$ 0.19 <sup>a</sup>	3.17 $\pm$ 0.15 <sup>b</sup>	5.05 $\pm$ 0.49 <sup>c</sup>
T <sub>2</sub> (10 kV)	2.44 $\pm$ 0.27 <sup>a</sup>	3.56 $\pm$ 0.46 <sup>ab</sup>	5.08 $\pm$ 0.84 <sup>b</sup>
<b>Cooking loss (%)</b>			
Control	29.08 $\pm$ 2.36	29.14 $\pm$ 1.70	31.37 $\pm$ 1.97
T <sub>1</sub> (5 kV)	30.72 $\pm$ 0.32	29.00 $\pm$ 3.02	29.26 $\pm$ 0.80
T <sub>2</sub> (10 kV)	26.99 $\pm$ 4.95	29.72 $\pm$ 1.05	29.54 $\pm$ 1.26

\*Mean  $\pm$  SE with different superscripts in a row (lower case alphabet) differ significantly ( $P < 0.05$ )

No significant difference was observed in the means  $\pm$  SE along the column

T<sub>1</sub> = Samples treated with PEF at 5 kV, 90 Hz, 20  $\mu$ s

T<sub>2</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu$ s

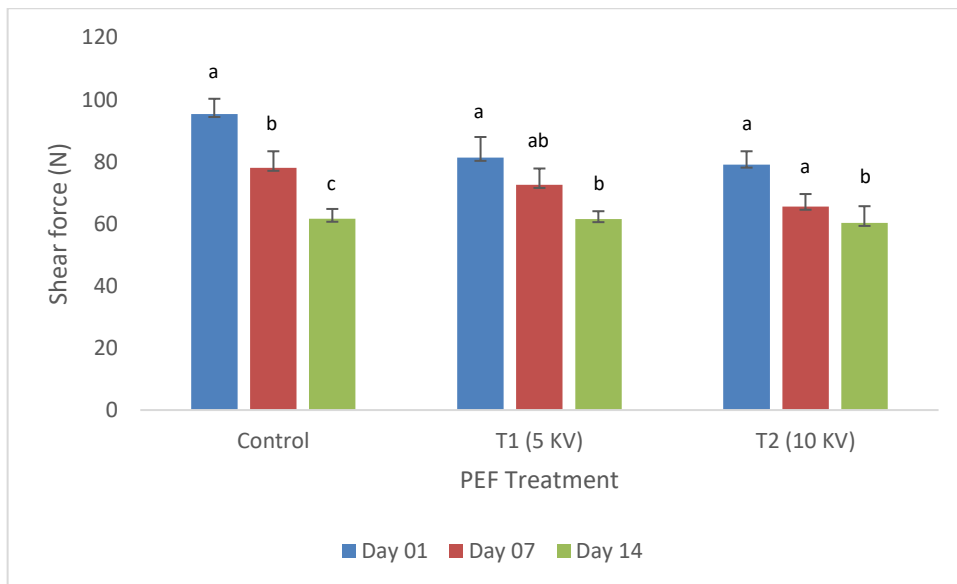
n = 6

PEF processing in the current research did not increase the cooking loss of the muscle which suggests no or little damage to the muscle fibres and thermal alterations of the proteins. This might be attributed to the electric field intensity (0.38 and 0.61 kV/cm) used in the study that did not result in a substantial increase in the temperature and appears not to have caused much damage to the muscle structure. Suwandy et al. (2015b) also reported no significant impact of PEF treatments on cooking loss of beef muscles.

#### **5.4.5 Shear force (N)**

The mean shear force values for PEF treated meat samples are presented in Figure 5.1. The high shear force values observed for all the samples in our study are typical of *Biceps femoris*, which is a tough muscle (Sullivan, & Calkins, 2011), and will tend to be tougher as the meat came from culled dairy cows (Roberts et al., 2018). As expected, ageing caused a significant ( $P < 0.05$ ) decline in all treatments. However, the PEF treatments used in our study did not induce a significant impact on the shear force of muscles obtained from culled dairy animals. Lower arithmetical values were observed for the samples treated with PEF throughout the period of storage, however, no significant ( $P > 0.05$ ) differences were observed between control and PEF treated samples. Arroyo et al., (2015) reported similar results in PEF-treated beef *Longissimus thoracis et lumborum* and the samples treated with PEF had mathematically lower shear force values compared to control indicating the tendency of PEF processing towards reducing the shear force. However, like our study no significant differences were observed by the authors between PEF treated and control samples. The authors suggested physical disruption as the mechanism for PEF-induced tenderness and reported that PEF treatments used might not have been sufficient to induce enough physical disruption to generate a significant response. Faridnia, Bekhit, Niven, & Oey (2014) and O'Dowd et al. (2013) also presented similar findings in beef *Longissimus thoracis* and *Semitendinosus*, respectively and observed no significant difference between PEF treated samples and control.

Several studies, however, have reported a significant effect of PEF treatments on the shear force of beef muscles during ageing and have suggested an enzymatic mechanism for PEF-induced tenderness (Suwandy et al., 2015a, b, d; Bekhit et al., 2014c). It is worth mentioning here that these studies neither used muscles from culled dairy animals nor have provided any experimental evidence of enzymatic mechanisms involved. Several factors seem to affect the ability of PEF to reduce shear force in muscles (Bhat et al., 2018a; Bekhit, Suwandy, Carne, Ahmed, & Zirong, 2017). Although, a tendency of PEF to reduce the shear force was recorded, the processing conditions used in our study appears not to be sufficient to induce a significant response on the shear force of muscles from culled dairy animals.

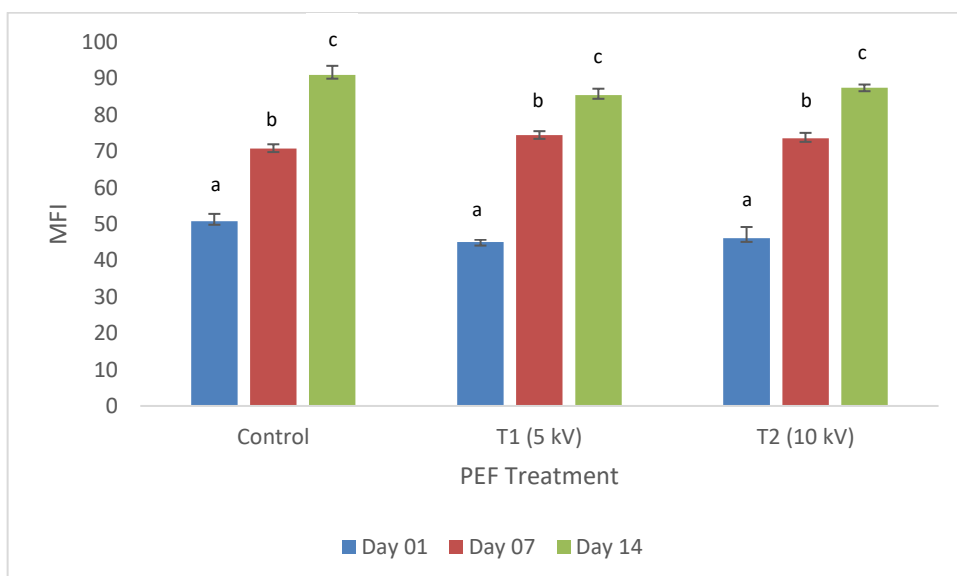


**Figure 5.1. Effect of pulsed electric field on the shear force (Newtons) of beef *Biceps femoris* (Mean ± SE)**

Different superscripts (within a treatment) on columns differ significantly ( $P < 0.05$ ),  $n = 6$

#### 5.4.6 Myofibrillar fragmentation index (MFI)

The PEF treatment did not produce a significant ( $P > 0.05$ ) effect on MFI of the muscle during ageing (Figure 5.2).



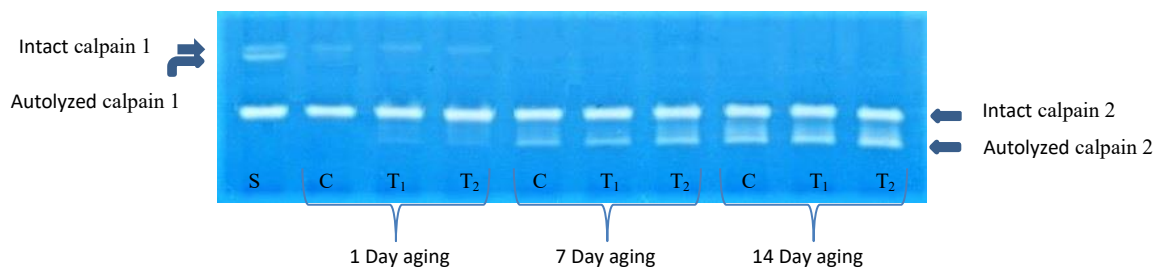
**Figure 5.2 Effect of pulsed electric field on the myofibrillar fragmentation index (MFI) of beef *Biceps femoris* (Mean ± SE)**

Different superscripts (within a treatment) on columns differ significantly ( $P < 0.05$ ),  $n = 6$

Ageing on the other hand showed a significant ( $P < 0.05$ ) effect on MFI of the muscle and the mean values for all the samples showed an increasing trend with storage time. O'Dowd et al. (2013) observed a similar non-significant ( $P > 0.05$ ) effect of PEF (1.9 kV/cm, 65 Hz, 20  $\mu$ s) treatment on MFI of beef *Semitendinosus*. An additional myofibril breakdown was noticed in PEF treated samples by the authors which also agreed with their findings for particle sizes analysed by laser diffraction (O'Dowd et al., 2013). MFI is strongly correlated with shear force and post-mortem sensory tenderness (Li et al., 2014) and is a good indicator of the extent of proteolysis of muscle myofibrillar proteins during post-mortem ageing (Ngapo, & Vachon, 2017). The direct comparison of MFI with literature is often difficult as it is greatly influenced by several factors such as breed, ageing time, and the process of homogenization (Onopiuk, Poltorak, & Wierzbicka, 2018).

#### 5.4.7 Calpain activity

Calpain activity was determined by casein zymography (Figure 5.3). Calpain 1 activity was detected only on day 1 of ageing and no bands were detected after 7 days of ageing. No difference was observed in calpain 1 activity between control and the PEF-treated samples. Calpain 1 is believed to have a central role in the post-mortem tenderisation process and is autolysed in the early stages of ageing (Bhat et al., 2018d; Huff-Loneragan, Zhang, & Lonergan, 2010; Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Koohmaraie, & Geesink, 2006). A similar decrease in calpain 1 activity during ageing has been reported by Colle, & Doumit (2017) in beef *Longissimus lumborum* and *Semimembranosus*.



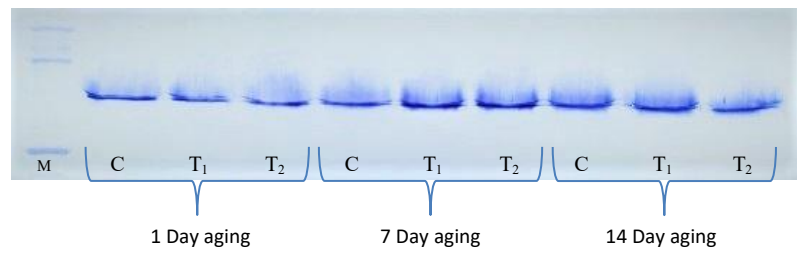
**Figure 5.3. Effect of pulsed electric field on the calpain activity of beef *Biceps femoris* [S = standard, C = control, T<sub>1</sub> = 5 kV (90 Hz), T<sub>2</sub> = 10 kV (20 Hz), 35  $\mu$ g samples were loaded in each lane]**

The autolysed calpain 2 activity was higher for PEF-treated samples in comparison to the control samples and was detected only in PEF-treated samples on day 1 of the ageing. The activity of calpains depends on several post-mortem factors such as pH, temperature and intracellular calcium

concentration (Bhat, Morton, Mason, & Bekhit, 2018c, d). Calpain 2 is not normally activated and autolysed in the early stages of ageing as it requires higher levels of calcium for activation (Morton, Bickerstaffe, Kent, Dransfield, & Keeley, 1999). The free calcium requirements for half maximal activity of calpain 2 are 400-800  $\mu\text{M}$  (Goll, Thompson, Li, Wei, & Cong, 2003) and these concentrations are not usually available during early post-mortem (Ji, & Takahashi, 2006; Geesink, & Koohmaraie, 1999a). The activation of calpain 2 at one day post-mortem in PEF-treated samples could be explained by early release of calcium ions from sarcoplasmic reticulum due to enhanced membrane permeability (Bhat et al., 2018a; Bekhit et al., 2014c). This mechanism is supported by the evidence that a post-mortem ageing period is required for gaining the tenderization benefit of PEF through increased proteolysis (Bekhit et al., 2016; Suwandy et al., 2015a, b, c; Bekhit et al., 2014c). The autolysed calpain 2 activity was also higher for PEF-treated samples in comparison to control on day 7 and 14 of the ageing and increased with the storage time with highest activity detected on day 14 of the ageing. An increase in the autolysed calpain-2 activity with ageing has been reported in beef muscles (Colle, & Doumit, 2017). The present study used muscles from culled dairy animals that can also influence the outcome. The age of an animal has also been reported to influence the calpain activity and may have profound impact on the proteolytic activities of the enzymes and subsequent tenderization of muscles (Bhat et al., 2018c, d).

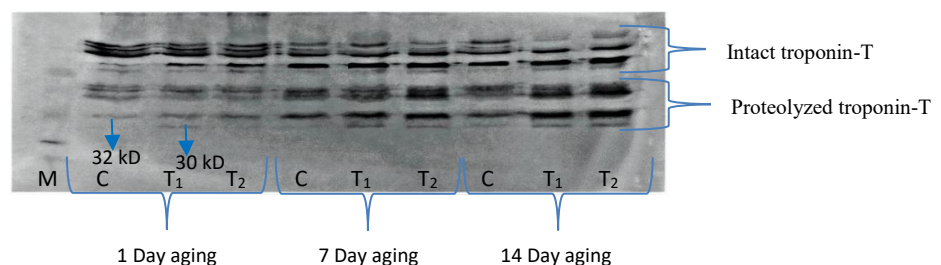
#### **5.4.8 Proteolysis of desmin and troponin-T**

The Western blot profiles for desmin (Figure 5.4) showed differences in intensity of the bands between PEF treated ( $T_1$ ,  $T_2$ ) and control samples on day 7 of the ageing. The proteolysis for desmin was greater for PEF treated ( $T_1$ ,  $T_2$ ) samples in comparison to control on day 7 of the ageing. These results were in agreement with the findings of calpain activity that was also observed higher in PEF treated samples on day 7 of the ageing. Desmin has structural functions and participates in the early establishment of sarcomere structure. Its proteolysis is one of the earliest markers for skeletal muscle degradation and is believed to have a significant impact on the quality of meat during aging (Bhat et al., 2018d). Its degradation is believed to have an important role by influencing the water-holding capacity and shear force of meat (Bhat et al., 2018d; Melody et al., 2004). Desmin is a calpain substrate and its degradation has been demonstrated to be directly related to calpain activity in several *in vitro* models and is used as a marker for post-mortem proteolysis and tenderization (Bhat et al., 2018d; Wheeler, & Koohmaraie, 1999; O'Halloran, Troy, Buckley, & Reville, 1997).



**Figure 5.4 Effect of pulsed electric field on the proteolytic pattern of desmin of beef *Biceps femoris* [M = marker, C = control, T<sub>1</sub> = 5 kV (90 Hz), T<sub>2</sub> = 10 kV (20 Hz), 20 µg samples were loaded in each lane]**

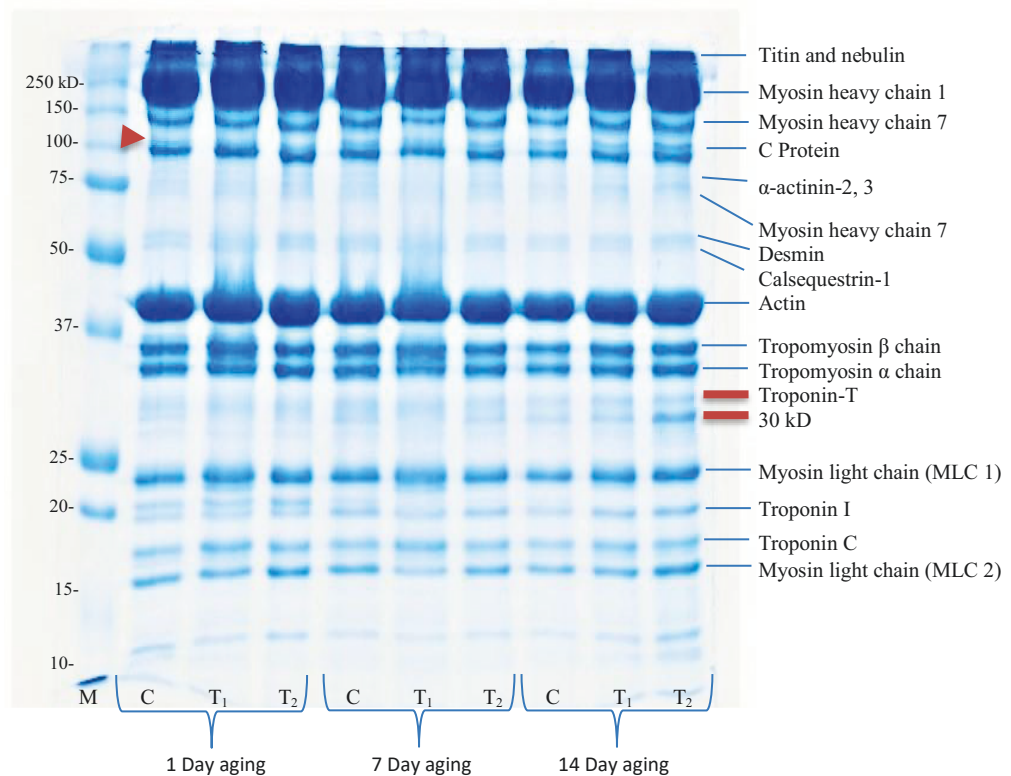
Troponin-T is a substrate of calpain 1 and produces a 30 kDa fragment that has been reported as a marker of proteolysis during meat ageing (Huang, Huang, Ma, Xu, & Zhou, 2012; Prates, Ribeiro, & Correia, 2001; Penny, & Dransfield, 1979). Post-mortem proteolysis of troponin-T has been directly associated to calpain activity using *in vitro* models of post-mortem muscles (Kitamura et al., 2005; Huff-Lonergan et al., 1996). The detection of this 30 kDa fragment of troponin-T was used to analyse the calpain-mediated proteolysis in this study. The Western blot profiles for troponin-T (Figure 5.5) showed differences in the stain intensity of the 30 kDa protein bands between PEF treated (T<sub>1</sub>, T<sub>2</sub>) and control samples on all days of storage. The 30 kDa band was fainter for control samples on all days of storage indicating increased degradation of troponin-T in PEF-treated samples during early and subsequent post-mortem ageing. Troponin-T degradation is promoted under various tenderization treatments (Han, Morton, Bekhit, & Sedcole, 2009; Claeys, Smet, Balcaen, Raes, & Demeyer, 2004; Ho, Stromer, & Robson, 1994) and is significantly correlated to shear force and MFI (Marino et al., 2013). It has been used as a marker for proteolysis of myofibrillar proteins in beef (Sun et al., 2014; Hopkins, & Thompson, 2002). Increased proteolysis of troponin-T has been reported in PEF treated samples in beef muscles (Bekhit et al., 2016; Suwandy et al., 2015a).



**Figure 5.5 Effect of pulsed electric field on the proteolytic pattern of troponin-T of beef *Biceps femoris* [M = marker, C = control, T<sub>1</sub> = 5 kV (90 Hz), T<sub>2</sub> = 10 kV (20 Hz), 15 µg samples were loaded in each lane]**

#### 5.4.9 SDS-PAGE (Myofibrillar protein profile)

The impact of PEF treatment on the myofibrillar protein profile of beef *Biceps femoris* muscle at different intervals of ageing is presented in Figure 5.6. The intensity of the 110 kD protein band was higher in PEF treated samples in comparison to control on day 14 of ageing. The intensity of the 110 kD protein band, which can normally be seen in aged beef (Stoeva, Byrne, Mullen, Troy, & Voelter, 2000; O'Halloran et al., 1997), is believed to be an indicator of proteolysis of myosin heavy chain (Yates, Dutson, Caldwell, & Carpenter, 1983). The 28, 30 and 32 kDa protein bands are produced by the degradation of intact troponin-T by calpain 1 during post-mortem proteolysis of beef and are known as the 27-32 kDa group (Marino et al., 2013; Han, Morton, Bekhit, & Sedcole, 2009).



**Figure 5.6 Effect of pulsed electric field on the myofibrillar protein profile (SDS-PAGE) of beef *Biceps femoris* [M = marker, C = control, T<sub>1</sub> = 5 kV (90 Hz), T<sub>2</sub> = 10 kV (20 Hz), 20 µg samples were loaded in each lane, Protein identification was derived from that reported by Ha, (2012); Suwandy et al. (2015d) and Bekhit et al. (2016)]**

The 30 and 32 kD which were barely visible in the samples during early post-mortem storage, became more intense in the PEF treated samples on day 14 of ageing. The magnitude of these concomitant

polypeptides strongly correlates with meat tenderness during post-mortem ageing (Rowe, Maddock, Trenkle, Lonergan, & Huff-Lonergan, 2003).

## **5.5 Conclusions**

This study provides an experimental evidence for the enzymatic nature of PEF-induced tenderness and expands our understanding of how PEF mediates the tenderization during ageing. Collectively the results from Western blotting, casein zymography, and myofibrillar protein profile provided evidence for increased calpain activity and the subsequent proteolysis in PEF treated samples during post-mortem ageing. Early post-mortem activation of calpain 2 was recorded in the PEF treated samples. However, the increase in calpain activity and proteolysis did not result in a significant decline in the shear force compared to control. The PEF treatments used in the present study seemed insufficient to induce permeabilization in muscles from culled dairy animals to a point where it could have produced a significant impact on the tenderization process. A faster rate of tenderization observed in PEF treated samples during first week of ageing may be helpful in reducing the total time of ageing. This technology may be helpful to New Zealand meat industry in reducing the time of ageing for export quality beef and lamb that is currently aged for 6 to 12 weeks. Future studies may use stronger PEF treatments to induce irreversible electroporation effect in the muscles from culled dairy animals.



## Chapter 6

# Effect of pulsed electric field on calpain activity and proteolysis of venison

This chapter is published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., Mungure, T., Jayawardena, R., & Bekhit, A. E. D. A. (2019). Effect of pulsed electric field on calpain activity and proteolysis of venison. *Innovative Food Science and Emerging Technologies*, 52, 131-135.

### 6.1 Abstract

The present study evaluated the effect of pulsed electric field (PEF) on the calpain activity, proteolytic pattern and myofibrillar protein profile of cold-boned *Longissimus dorsi* obtained from red deer (*Cervus elaphus*). PEF treated samples, T<sub>1</sub> (2.5 kV, 50 Hz) and T<sub>2</sub> (10 kV, 90 Hz) and a non-treated control were aged for 21 days at 4±1 °C. The samples were analysed for shear force (N), myofibrillar fragmentation index (MFI), calpain activity, proteolysis of desmin and troponin-T and myofibrillar protein profile (SDS-PAGE). No significant impact of PEF was observed on the shear force and MFI. A slight increase was recorded in the calpain activity and proteolysis of troponin-T, suggesting a tendency of PEF to improve the tenderization process. Collectively, the results from casein zymography, Western blotting and myofibrillar protein profile suggest that PEF did not produce any significant impact on the tenderization process of venison.

**Keywords:** Pulsed electric field; venison; shear force; myofibrillar fragmentation index; calpain activity; Western blotting; myofibrillar protein profile

### 6.2 Introduction

With ever increasing demand for meat, particularly red meat, interest in venison has increased over the decades with annual world production increasing from one million tonnes in 1970s (FAOSTAT, 2017) to the present figure of around two million tonnes (Costa, Mafra, Oliveira, & Amaral, 2016). With a low triglyceride content and a high proportion of proteins and polar lipids (Bartoň, Bureš, Kotrba, & Sales, 2014), venison has a positive image as a naturally low-fat and healthy food product (Ludwiczak, Stanisz, Bykowska, Skadanowska, & Slósarz, 2016). Australia, New Zealand, Canada and China are countries with well-established deer farming industries, however, interest in deer farming is increasing quickly in European countries and the USA (Kudrnáčová, Bartoň, Bureš, & Hoffman, 2018). The tenderness and collagen content of venison are considered comparable to that of beef (Bureš et al., 2015; Dominik et al., 2012) and like beef, post-mortem interventions, such as ageing, electrical

stimulation or pulsed electric field (PEF), are required to maximise tenderness. Information on ageing and electrical stimulation of venison is available in the literature (Piaskowska, Daszkiewicz, Kubiak, & Zapotoczny, 2016; Tešanović et al., 2011; Wiklund et al., 2009), however, no information is available, to the best of our knowledge, on the effect of PEF on the quality parameters of venison and this needs immediate scientific attention.

PEF is currently used in several biotechnological, medical and food applications. It can induce cell permeabilization, the formation of pores on the cell membranes, a phenomenon known as electroporation (Bot et al., 2018) without provoking undesirable sensory or biochemical changes (Kantar et al., 2018; Bhat, Morton, Mason, & Bekhit, 2018a, d). Because of the minimal thermal changes observed during the process, PEF has a significant advantage over conventional thermal processing methods that permits the use of this technology for temperature-sensitive processes (Giteru, Oey, & Ali, 2018) preserving a natural flavour, robust taste, fresh appearance and producing high quality food products (Wang et al., 2018b). PEF treatment of muscles before ageing has been reported to improve the tenderization process in beef (Bekhit, Suwandy, Carne, van de Ven, & Hopkins, 2016; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015a, b, c; Bekhit, van de Ven, Suwandy, Fahri, & Hopkins, 2014c) and the most widely suggested mechanism is through early activation of calpains by release of calcium ions from cell organelles due to electroporation. However, no studies have been reported for the effects of PEF on venison and no information is available whether PEF treatment will be effective in maximizing the tenderization benefit of ageing in venison or on the calpain activity of venison treated with PEF. Previous studies on beef muscles have indicated that an ageing period is required to gain the PEF-induced tenderization benefit (Bekhit et al., 2016; Suwandy et al., 2015a; Bekhit et al., 2014c). Studies have also shown that PEF processing increased the proteolysis of myofibrillar proteins during ageing in beef muscles (Suwandy et al., 2015a, b, c; Bekhit et al., 2014c), however, no such study has been conducted on venison. No information is available as to whether PEF processing will increase the proteolysis of proteins after an extended ageing.

The objective of this study was to evaluate the effect of PEF processing on the proteolytic, enzymatic and physicochemical changes of venison *Longissimus dorsi* subjected to an extended post-mortem ageing. Myofibrillar fragmentation index (MFI) was used as a marker of proteolysis and the activities of the calpain proteases (calpain 1 and calpain 2) were investigated to determine whether change in meat tenderness, if any, was associated with enzymatic changes. This study reports for the first time the effect of PEF on the calpain activity of venison. Proteolytic pattern of desmin and troponin-T and the myofibrillar protein profile (SDS-PAGE) were analysed to determine if post-mortem proteolysis was a contributing factor to any PEF-induced tenderness.

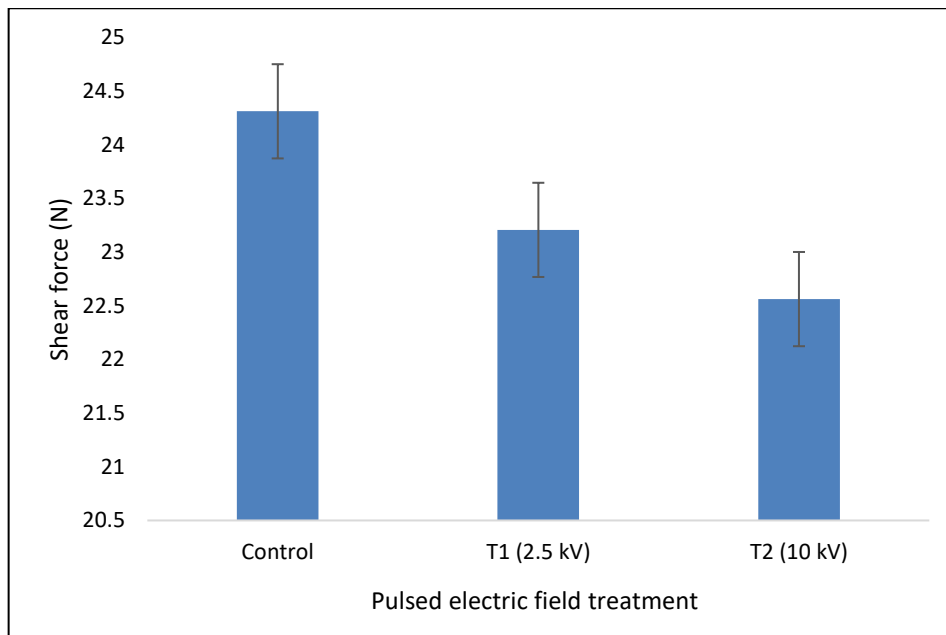
## 6.3 Materials and methods

Meat samples were prepared as per the method described in section 2.2.5. The meat samples were treated with PEF as per the method described in section 2.2.5.1. Electrical input and change in temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ ) were measured according to the methods described in section 2.2.6 and 2.2.7, respectively. Shear force (N), purge loss (%), cooking loss (%) and myofibrillar fragmentation index (MFI) of the samples were measured according to the methods described in section 2.3.1 to 2.3.3. Sarcoplasmic and myofibrillar fractions were separated by following the method described in section 2.4.1. Protein concentration, casein zymography, SDS-PAGE, immunoblotting and analysis of the protein bands was done by following the methods described from section 2.4.2 to 2.4.5. Statistical analysis of the data was performed according to the method described in section 2.7.1.

## 6.4 Results and discussion

### 6.4.1 Shear force

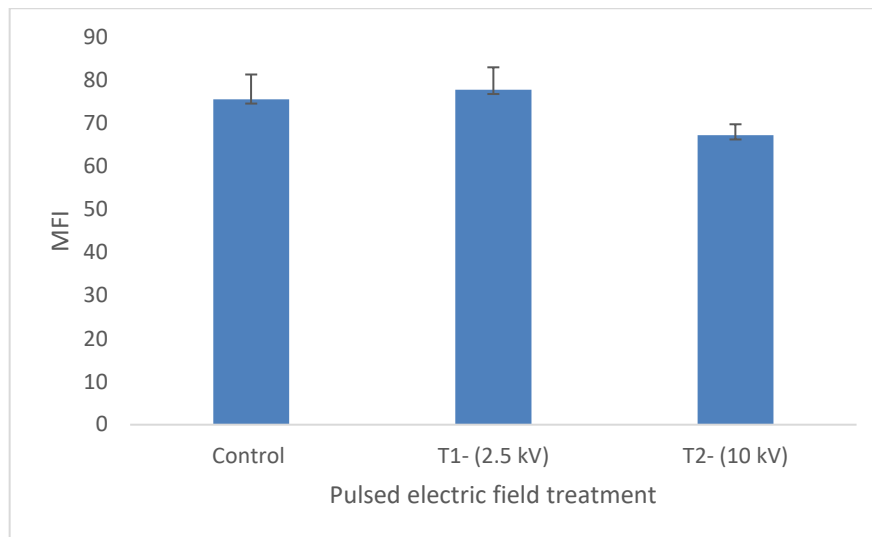
No significant ( $P > 0.05$ ) difference was observed in the shear force between control and PEF treated samples after 21 days of ageing (Figure 6.1). Similar findings have been reported in the muscles of other species such as turkey and beef. Arroyo et al. (2015a) found no significant impact of PEF processing (1.4 kV/cm, 10 Hz, 20  $\mu$ s, 300 and 600 pulses) on shear force of beef *Longissimus thoracis et lumborum*. No significant effect of PEF treatment was reported on the shear force of turkey breast either in frozen (2.1 kV/cm, 10 Hz) or fresh (1.2 kV/cm, 10 Hz) form (Arroyo et al., 2015b). In contrast to our study, several workers (Bekhit et al., 2016; Suwandy et al., 2015a, b; Bekhit et al., 2014c) have reported a significant impact of PEF processing on the shear force of beef muscles. Several factors, such as electric field strength, muscles under study, and the use of an ageing period, are required to induce a significant response in the shear force of cold-boned muscles (Bhat et al., 2018a). A minimum electric field intensity is required above which PEF induces irreversible electroporation in animal tissues during an ageing period (Bhat et al., 2018a).



**Figure 6.1 Effect of pulsed electric field on the shear force (Newtons) of venison *Longissimus dorsi* (n = 6) aged for 21 days (Mean ± SE)**

#### 6.4.2 Myofibrillar fragmentation index

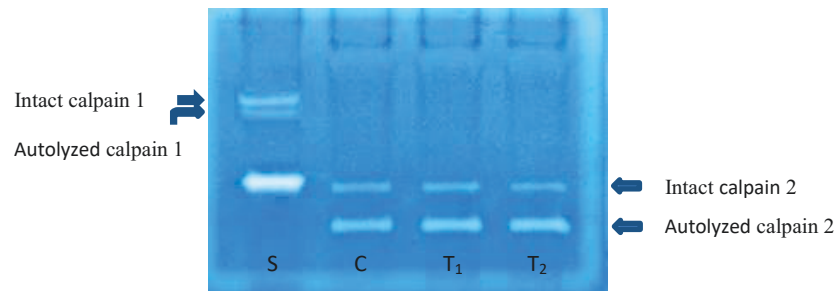
No significant ( $P > 0.05$ ) impact of PEF processing was observed on MFI of the muscles and the mean values of PEF-treated samples were comparable with that of control (Figure 6.2). O'Dowd, Arimi, Noci, Cronin, & Lyng (2013) also observed no significant impact of PEF processing on the MFI of beef *Semitendinosus*. However, those authors reported some additional myofibril breakdown in the PEF treated samples which was in agreement with the results for particle sizes obtained by laser diffraction. Several studies have confirmed that MFI is a good indicator of tenderness and the extent of post-mortem proteolysis of muscle cytoskeletal and associated proteins during ageing (Ngapo, & Vachon, 2017; Taylor, Geesink, Thompson, Koochmaraie, & Goll, 1995). Strong correlations have been demonstrated between shear force, post-mortem sensory tenderness and the MFI (Li et al., 2014).



**Figure 6.2 Effect of pulsed electric field on the myofibrillar fragmentation index (MFI) of venison *Longissimus dorsi* (n = 6) aged for 21 days (Mean  $\pm$  SE)**

### 6.4.3 Calpain activity

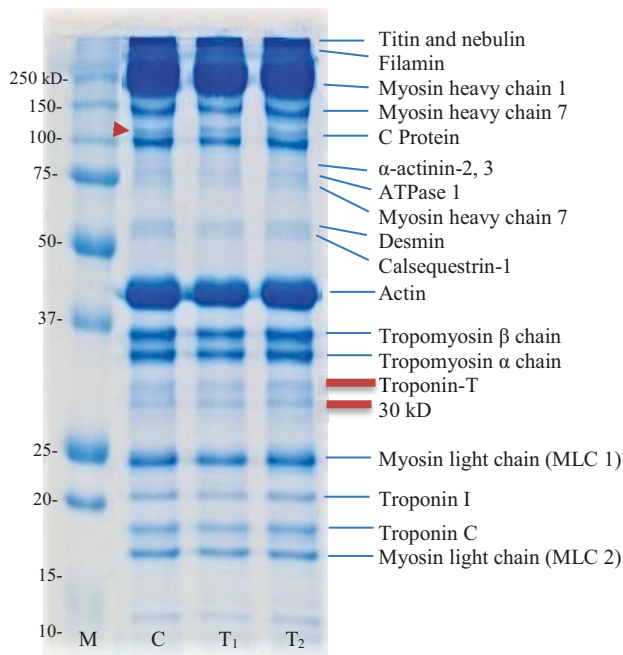
No calpain 1 activity, either intact or autolysed, was detected in any of the samples (n=6) after 21 days of ageing (Figure 6.3). The calpain 1 is believed to be active and rapidly autolysed in the early stages of post-mortem ageing and is recognized as the principle enzyme responsible for most of the post-mortem tenderization (Bhat, Morton, Mason, & Bekhit, 2018c, d; Geesink, Kuchay, Chishti, & Koohmaraie, 2006). Autolysed calpain 2 activity was higher in PEF treated samples as more intense bands were present in T<sub>1</sub> (2.5 kV, 50 Hz) and T<sub>2</sub> (10 kV, 90 Hz) samples on day 21 of ageing. The native calpain 2 activity was lower in T<sub>2</sub> (10 kV, 90 Hz) samples in comparison to control. Calpain 2 requires higher concentration of calcium for activation and is not normally autolysed in the early stages of ageing (Morton, Bickerstaffe, Kent, Dransfield, & Keeley, 1999). The calpains are believed to be the predominant proteases responsible for most of the post-mortem proteolysis and tenderization benefit of ageing (Huff-Lonergan, Zhang, & Lonergan, 2010; Geesink et al., 2006; Taylor et al., 1995). Although, the differences observed in calpain activities between control and PEF treated samples were not significant ( $P > 0.05$ ), calpain activities of PEF-treated samples were numerically higher. Several studies have suggested that the tenderizing potential of PEF processing is through activation of calpains by release of calcium ions due to electro-permeabilization (Bhat et al., 2018a; Bekhit et al., 2014c). Since activity of calpain 1 is not detected after few initial days of ageing, proteolysis of myofibrillar proteins during extended ageing has been attributed to the activation of calpain 2 during late post-mortem (Colle, & Doumit, 2017).



**Figure 6.3 Effect of pulsed electric field on the calpain activity of venison *Longissimus dorsi* (n=6) aged for 21 days [S = standard, C = control, T<sub>1</sub> = 2.5 kV (50 Hz), T<sub>2</sub> = 10 kV (90 Hz), 35 µg samples were loaded in each lane]**

#### 6.4.4 Myofibrillar protein profile (SDS-PAGE)

The effect of the PEF processing on the myofibrillar protein profile of venison *Longissimus dorsi* is presented in Figure 6.

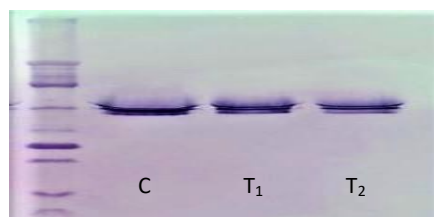


**Figure 6.4 Effect of pulsed electric field on the myofibrillar protein profile (SDS-PAGE) of venison *Longissimus dorsi* [M = marker, C = control, T<sub>1</sub> = 2.5 kV (50 Hz), T<sub>2</sub> = 10 kV (90 Hz), 20 µg samples were loaded in each lane, Protein identification was derived from that reported by Ha, (2012); Suwandy et al., (2015d) and Bekhit et al., (2016)]**

Normally seen in aged beef (Stoeva, Byrne, Mullen, Troy, & Voelter, 2000), the intensity of the 90 kDa and 110 protein bands are believed to be an indicator of proteolysis of myosin heavy chain (Yates, Dutson, Caldwell, & Carpenter, 1983). No difference was observed in the intensity of either of the bands between PEF treated samples and control after 21 days of ageing. Degradation products of troponin-T, the 28, 30 and 32 kDa protein bands are produced during post-mortem proteolysis by calpains and are known as the 27–32 kDa group (Marino et al., 2013). The intensity of these bands strongly correlates with meat tenderness during post-mortem ageing (Rowe, Maddock, Trenkle, Lonergan, & Huff-Lonergan, 2003). The 30 and 32 kD bands which were slightly visible in the control samples, became noticeably more intense in the PEF treated samples, particularly in T<sub>2</sub> (10 kV, 90 Hz). The results were concomitant with the calpain activity as no significant ( $P > 0.05$ ) differences were observed. The higher calpain activity of PEF-treated samples may have translated into higher post-mortem proteolysis of troponin-T. There is, however, a remote possibility that the PEF processing parameters used in this study might not have been sufficient to produce an irreversible electroporation necessary to induce a significant response.

#### 6.4.5 Proteolysis of desmin and troponin-T

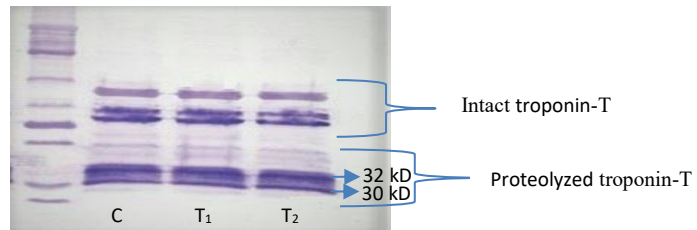
The Western blot profiles for desmin and troponin-T (Figures 6.5 and 6.6) showed no significant differences ( $P > 0.05$ ) in the intensity of the bands between PEF treated (T<sub>1</sub>, T<sub>2</sub>) and control samples (n=6) on day 21 of the ageing.



**Figure 6.5 Effect of pulsed electric field on the pattern of desmin (immunoblot) of venison *Longissimus dorsi* aged for 21 days [C = control, T<sub>1</sub> = 2.5 kV (50 Hz), T<sub>2</sub> = 10 kV (90 Hz), 20 µg samples were loaded in each lane]**

Known to result from the action of the calpains, the present study analysed the calpain-mediated proteolysis through the detection of a 30-kDa fragment of troponin-T, believed to be an indicator of the post-mortem proteolysis during meat ageing (Huang, Huang, Ma, Xu, & Zhou, 2012). The Western blot profiles presented in our study showed no differences in stain intensity of the 30 kDa protein

bands between PEF treated ( $T_1$ ,  $T_2$ ) and control samples subjected to three weeks of ageing. These results are in agreement with the findings of the calpain activity where no significant impact of PEF-processing was observed. Due to their indispensable role in maintaining the structural integrity of the myofibrils (Huff-Lonergan et al., 2010; Geesink, Bekhit, & Bickerstaffe, 2000), post-mortem degradation of desmin and troponin-T is used as a marker for myofibrillar protein degradation and tenderization during ageing (Sun et al., 2014; Hopkins, & Thompson, 2002).



**Figure 6.6 Effect of pulsed electric field on the pattern of troponin-T of venison *Longissimus dorsi* aged for 21 days [C = control,  $T_1$  = 2.5 kV (50 Hz),  $T_2$  = 10 kV (90 Hz), 15  $\mu$ g samples were loaded in each lane]**

Degradation of both desmin and troponin-T has been directly linked to calpain activity using *in vitro* models of post-mortem muscles (Kitamura et al., 2005; Baron, Jacobsen, & Purslow, 2004). In contrast to our results in venison, Bekhit et al. (2016) and Suwandy et al. (2015a) reported an increased proteolysis of both desmin and troponin-T in PEF treated beef muscles during ageing. It seems that this technology works differently with muscles from different species and clearly the PEF processing parameters used in this study were not enough to produce an irreversible electroporation necessary to cause early calcium release to induce a significant response on the activity of calpains. Studies in future may focus on exploring suitable PEF processing conditions to induce an irreversible permeabilization effect to harvest a sizable and a significant tenderization response in venison.

## 6.5 Conclusions

No significant effect was observed on the shear force after an ageing of 21 days; however, PEF-processing showed a mild tendency towards increasing the calpain activity and proteolysis of venison. Given the fact that similar PEF conditions have reported a significant tenderization effect in beef, different processing conditions might be required to induce a significant response in muscles from different species.



## Chapter 7

### Pulsed electric field improved protein digestion of beef

#### *Semimembranosus* during *in vitro* gastrointestinal simulation

This chapter is published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2019). Pulsed electric field improved protein digestion of beef during *in vitro* gastrointestinal simulation. *LWT-Food Science and Technology*, 102, 45-51.

#### 7.1 Abstract

Effect of pulsed electric field (PEF) on *in vitro* simulated gastrointestinal protein digestion of cold-boned beef *Semimembranosus* was elucidated. PEF treated [T<sub>1</sub>, 5.0 kV (90 Hz); T<sub>2</sub>, 10 kV (20 Hz)] samples along with control were subjected to *in vitro* simulated gastrointestinal protein digestion. Samples collected at 0, 30 and 60 min of gastric digestion and 120 and 180 min of intestinal digestion were analysed for protein digestibility (%), soluble protein (%), protein profile (SDS-PAGE), free amino acid analysis and mineral profile. Significantly higher ( $P < 0.05$ ) protein digestibility (%) and soluble protein (%) values were observed for PEF treated samples. No negative impact ( $P > 0.05$ ) of PEF was observed on the release of various minerals from the muscle during the digestion. By modifying the protein profile of the meat digests, PEF processing had a positive influence on *in vitro* digestion kinetics that led to a faster and greater digestion of proteins during simulated gastrointestinal digestion.

**Keywords:** Pulsed electric field; *in vitro* protein digestion; protein profile; free amino acid analysis; mineral profile

#### 7.2 Introduction

One of the main food security threats in the 21<sup>st</sup> century is that many people do not have enough food and the problem is escalating (Hovhannisyan, & Grigoryan, 2016). With an annual growth rate of 1.10%, about 83 million people are added yearly to the world population (United Nations, 2017) and more than two thirds of the already existing 7.2 billion world population are currently malnourished or starving (Pimentel, & Burgess, 2018). There is very limited potential to increase the available food resources due to environmental challenges and limited scope for expansion in arable land area. Therefore, the available food resources are destined to be shared by more and more people and several strategies to cope with the increased food demand have been investigated. These new strategies include reduction of food waste and efficient utilization of the available resources (e.g.

recovery of protein from old seed cakes), prospecting new food sources (e.g. insects) and improving the digestibility of already existing foods. As significant amounts of protein can reach the colon undigested in typical Western diets (Roager, & Licht, 2018; Birkett, Muir, Phillips, Jones, & O'Dea, 1996), much could be done to mitigate the malnourishment and suffering by reducing the wastage of proteins by producing foods with improved digestibility and nutritive value. Further, as the fermentation of non-hydrolysed proteins by colonic flora can result in production of mutagenic products, such as phenol and p-cresol, improving protein digestion rate would have a positive impact on human health by decreasing the risk of colon cancer (Simonetti, Gambacorta, & Perna, 2016).

Processes, such as high pressure or high-temperature cooking, which have potential to affect the structural and functional properties of the proteins by inducing modifications such as cross-linking, unfolding, and aggregation, can influence the susceptibility of proteins to gastrointestinal enzymatic hydrolysis and affect the release of amino acids and polypeptides and their bioavailability during digestion (Gharibzahedi et al., 2019; Horitan et al., 2018; Kaur et al., 2016; Kaur, Maudens, Haisman, Boland, & Singh, 2014; Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008). Non-thermal processing technologies, such as ultrasound and high-pressure processing, have been reported to have a positive influence on the digestion kinetics of protein-rich foods during *in vitro* simulations (Kaur et al., 2016; Barba, Terefe, Buckow, Knorr, & Orlien, 2015), however, such information is not generally available for pulsed electric field (PEF) which has the potential to influence the protein digestive kinetics by affecting the protein interactions and by inducing structural changes (Bhat, Morton, Mason, & Bekhit, 2018d; Han, Cai, Cheng, & Sun, 2018; Liu, Oey, Bremer, Silcock, & Carne, 2018). Affected by the targeted nutrient and the food matrix, nonthermal processing technologies, such as PEF, can be utilized as useful tools to influence the release of micronutrients from the foods during *in vitro* digestion trials (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014).

PEF is a novel non-thermal technology that has several applications in food processing (Barba, Koubaa, Prado-Silva, Orlien, & Sant'Ana, 2017a; Barba, Parniakov et al., 2015) with a promising potential in meat and meat products in several areas such as supercooling, tenderization and curing (Bhat et al., 2018a, d). However, the effects of PEF processing on the digestibility of meat proteins are generally unknown. Insights on the basic mechanisms of how PEF processing may affect meat protein digestibility will be invaluable as this knowledge could be utilized for the development of novel protein structures and muscle foods with improved digestibility. Thus, the objective of this study was to evaluate the effect of PEF processing on the digestive kinetics of beef *Semimembranosus* subjected to *in vitro* gastrointestinal digestive simulation. The samples were analysed for protein digestibility (%), soluble protein (%), protein profile (SDS-PAGE), free amino acid analysis and mineral profile to investigate if PEF processing has any impact on meat digestibility and the release of nutrients during *in vitro* gastrointestinal digestion.

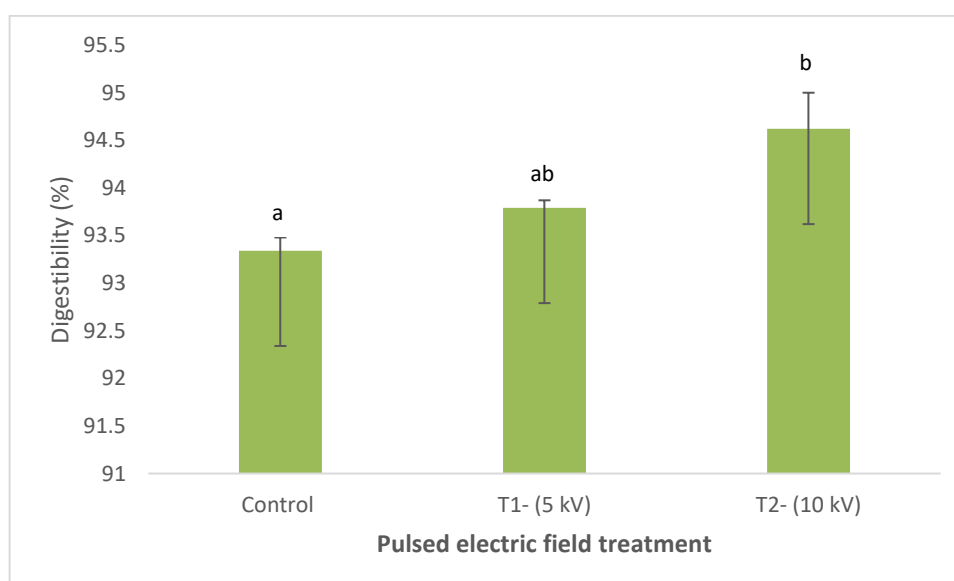
### 7.3 Materials and methods

Meat samples were prepared as per the method described in section 2.2.3. The meat samples were treated with PEF as per the method described in section 2.2.3.1. *In vitro* protein digestion, *in vitro* protein digestibility (%), protein content (%), soluble protein (%), free amino acid analysis and mineral profile analysis were performed according to the methods described from section 2.5.1 to 2.5.7. Protein concentration, SDS-PAGE and analysis of the protein bands was done by following the methods described in section 2.4.2 and 2.4.4. Statistical analysis of the data was performed according to the method described in section 2.7.2.

### 7.4 Results and discussion

#### 7.4.1 Protein digestibility (%)

The mean digestibility (%) for PEF processed beef *Semimembranosus* viz. T<sub>1</sub> (5.0 kV, 90 Hz) and T<sub>2</sub> (10 kV, 20 Hz) subjected to *in vitro* simulated gastrointestinal protein digestion are presented in Figure 7.1.



Different superscripts on columns differ significantly ( $P < 0.05$ )

T<sub>1</sub> = Samples treated with PEF at 5 kV, 90 Hz, 20  $\mu$ s

T<sub>2</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu$ s

Data was analysed by 1-way ANOVA using SPSS-21

n = 3 for each treatment

**Figure 7.1 Effect of pulsed electric field on the protein digestibility of beef *Semimembranosus* during *in vitro* simulated gastrointestinal digestion (Mean  $\pm$  SE)**

A significant effect for PEF processing was observed, and the beef treated with PEF (10kV, 20Hz) had significantly ( $P < 0.05$ ) higher *in vitro* digestibility (%) than the untreated beef. Similar values have been found for pork, beef, chicken and fish samples subjected to *in vitro* simulated gastrointestinal digestion (Tavares, Dong, Yang, Zeng, & Zhao, 2018; Wen et al., 2015). The extent of digestibility of the proteins after hydrolysis with gastrointestinal proteases has been used to evaluate the bioavailability of dietary proteins (Wen et al., 2015; Pennings et al., 2013).

Structural changes which induce partial unfolding of proteins have been reported to increase the susceptibility of proteins to hydrolysis by gastrointestinal proteases (Simonetti, Gambacorta, & Perna, 2016; Promeyrat et al., 2010). Modifications in the structure of the proteins have been reported to increase surface activity by exposure of hydrophobic domains and improve protein solubility by lowering the molecular weight (Primozic, Duchek, Nickerson, & Ghosh, 2018). Minimal processing technologies, such as high-hydrostatic pressure, have been reported to modify the secondary and tertiary structure of bovine serum albumin and bovine lactoferrin resulting in a more unfolded conformation (De Maria, Ferrari, & Maresca, 2016). This effect induced by high pressure has been reported to influence the digestive kinetics of meat proteins during *in vitro* simulated gastrointestinal digestion (Kaur et al., 2016). PEF processing can affect secondary, tertiary and quaternary protein structures by interfering with the electrostatic or hydrophobic interactions and disulfide or hydrogen bonds and can thus positively influence the digestion of the proteins (Giteru, Oey, & Ali, 2018; Liu, Oey, Bremer, Carne, & Silcock, 2017b).

The non-homogenous charge distribution along the protein backbone has been reported to cause a gradual stretching or deformation of the protein molecule along the electric field during PEF processing (Freedman, Haq, Edel, Jemth, & Kim, 2013; Freedman et al., 2011). By destabilizing the protein molecules, PEF processing induces protein unfolding by disrupting the electrostatic interactions of individual polypeptide chains and local electrostatic fields, thereby destabilizing the secondary and tertiary structure of the proteins (Zhao, Tang, Lu, Chen, & Li, 2014). Further, the ohmic heat produced during the process, if it is generated at significant level near a protein denaturation region, could play a central role in the denaturation and aggregation of heat-sensitive proteins (Han, Cai, Cheng, & Sun, 2018; Hermawan, Evrendilek, Dantzer, Zhang, & Richter, 2004). Thus, by inducing partial unfolding of the meat proteins (Liu, Oey, Bremer, Carne, & Silcock, 2017b), PEF processing has the potential to enhance the hydrolysis of proteins by gastrointestinal enzymes (Liu, Oey, Bremer, Silcock, & Carne, 2018). The total energy input and electric field strength play a major role in influencing the intermolecular interactions and are decisive for PEF-induced protein unfolding and denaturation (Liu, Oey, Bremer, Carne, & Silcock, 2017b). The actual electric field strength recorded during the PEF treatment of beef *Semimembranosus* was 0.36 kV/cm for T<sub>1</sub> and 0.60 kV/cm for T<sub>2</sub>. The average change in temperature in the muscle samples was 4.83 °C for T<sub>1</sub> (5.0 kV) samples and 12.76 °C for T<sub>2</sub> (10.0 kV) samples. No significant ( $P > 0.05$ ) change was observed in the conductivity of the muscle.

### 7.4.2 Soluble protein (%)

The mean values for soluble protein (%) for PEF processed beef *Semimembranosus* viz. T<sub>1</sub> (5.0 kV, 90 Hz) and T<sub>2</sub> (10 kV, 20 Hz) subjected to *in vitro* simulated gastrointestinal protein digestion are presented in Table 7.1. PEF-treated samples collected during gastric digestion were not significantly different from untreated control. However, significantly ( $P < 0.05$ ) higher soluble protein (%) values were recorded for PEF-treated samples collected at 120 and 180 min of intestinal digestion. These results together with the results for protein digestibility (%) indicate that more digestion had occurred in PEF-treated samples which can be attributed to the electroporation effect caused by PEF processing in the muscle cells. The increased membrane permeability might have allowed greater diffusion of proteases into the muscle mass resulting in more proteolysis of the muscle proteins in PEF processed samples. PEF processing has been reported to enhance the mass transfer processes (Bhat, Morton, Mason, & Bekhit, 2018a, d) and accelerate the diffusion of salt in pork during curing (McDonnell, Allen, Chardonnerau, Arimi, & Lyng, 2014). Further, PEF processing might also have improved the susceptibility of proteins to hydrolysis by gastrointestinal enzymes by inducing structural changes in the proteins, such as partial unfolding and denaturation, which are believed to enhance the enzymatic hydrolysis of proteins (Giteru, Oey, & Ali, 2018; Liu, Oey, Bremer, Silcock, & Carne, 2018; Liu, Oey, Bremer, Carne, & Silcock, 2017b). The digestive enzymes, such as pepsin and trypsin, have been reported to act more effectively on unfolded and denatured proteins (Kaur et al., 2016). By inducing structural changes, PEF processing has been reported to enhance the susceptibility of egg white proteins to gastrointestinal digestion (Liu, Oey, Bremer, Silcock, & Carne, 2018).

**Table 7.1 Effect of pulsed electric field on the soluble protein (%) of beef *Semimembranosus* during *in vitro* simulated gastrointestinal digestion (Mean ± SE)\***

Treatments	<i>In vitro</i> gastric digestion			<i>In vitro</i> intestinal digestion	
	0 Minutes	30 Minutes	60 Minutes	120 Minutes	180 Minutes
Control	6.52 ± 0.45 <sup>a</sup>	8.58 ± 0.45 <sup>ab</sup>	9.48 ± 0.74 <sup>b</sup>	26.79 ± 1.11 <sup>CA</sup>	28.23 ± 0.91 <sup>CA</sup>
T <sub>1</sub> (5 kV)	8.15 ± 0.92 <sup>a</sup>	9.67 ± 0.77 <sup>a</sup>	10.67 ± 1.02 <sup>a</sup>	28.12 ± 1.24 <sup>bAB</sup>	30.36 ± 1.29 <sup>bAB</sup>
T <sub>2</sub> (10 kV)	9.50 ± 1.02 <sup>a</sup>	11.18 ± 1.31 <sup>a</sup>	12.13 ± 1.50 <sup>a</sup>	31.64 ± 0.70 <sup>bB</sup>	32.61 ± 1.04 <sup>bB</sup>

\*Mean ± SE with different superscripts in a row wise (lower case alphabet) and column wise (upper case alphabet) differ significantly ( $P < 0.05$ )

T<sub>1</sub> = Samples treated with PEF at 5 kV, 90 Hz, 20 μs

T<sub>2</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20 μs

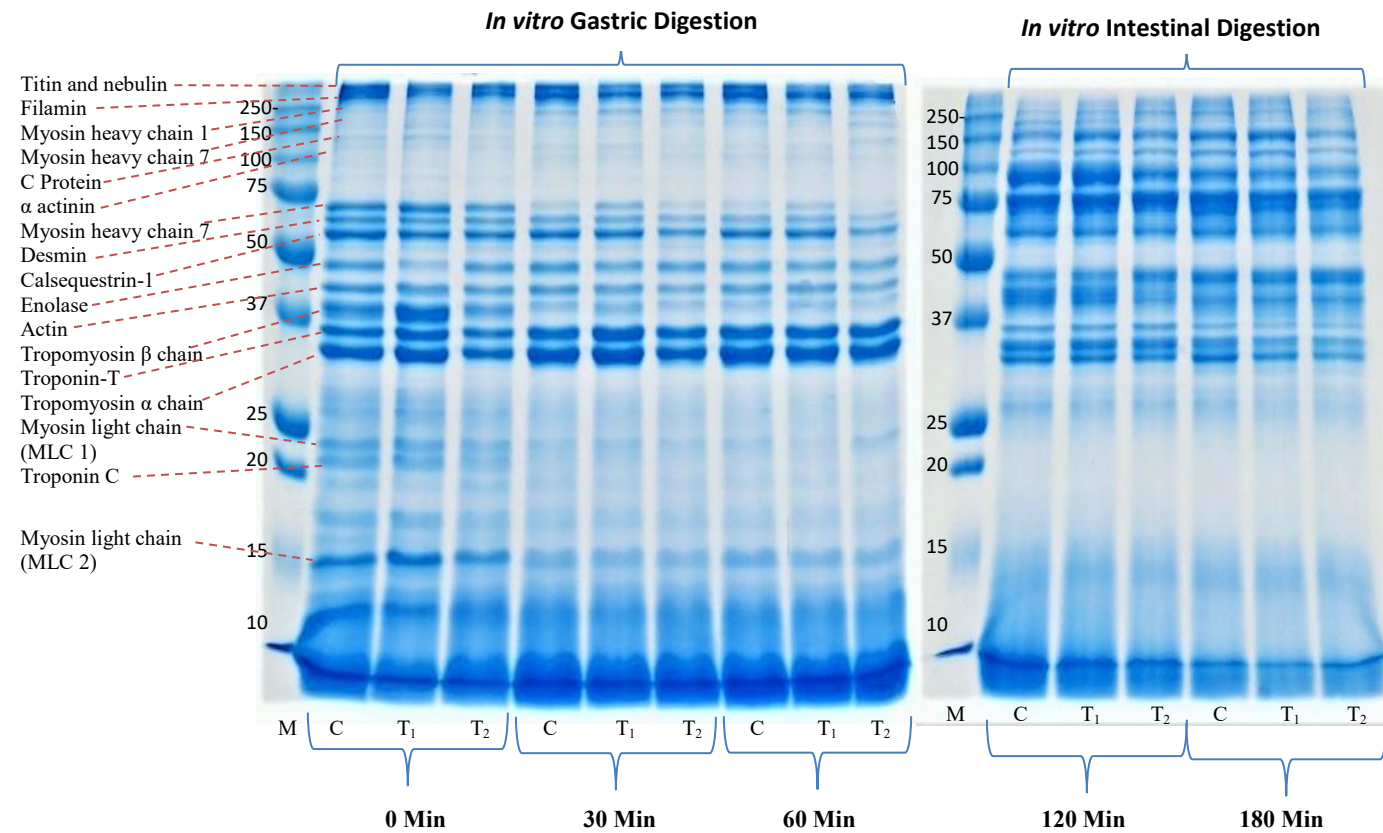
Data was analysed by 2-way ANOVA using SPSS-21

n = 3 for each treatment

### 7.4.3 Protein profile (SDS-PAGE)

The effect of PEF treatment on the protein profile of beef *Semimembranosus* digests obtained at different intervals of simulated gastrointestinal protein digestion is presented in SDS-PAGE gel electrophoretogram in Figure 7.2. The major identified bands included titin and nebulin (>250 kD), filamin (>250 kD), myosin heavy chain MHC 1 (>220 kD), myosin heavy chain MHC 7 (>220 kD), C protein (135 kD),  $\alpha$ -actinin (95 kD), desmin (53 kD), calsequestrin 1 (52 44-55 kD), enolase (46 kD), actin (42 kD), tropomyosin  $\beta$ -chain (37 kD), troponin-T (35 kDa), tropomyosin- $\alpha$ -chain (33 kDa), myosin light chain MLC<sub>1</sub> (22 kDa), troponin-C (19 kDa) and myosin light chain MLC<sub>2</sub> (16 kDa). A range of bands is visible in the electrophoretogram for gastric and intestinal phase of digestion for both control and PEF-treated samples with some minor differences. The peptides from the range of 10 to 25 kD which were released during the gastric phase of digestion disappeared during the intestinal phase of digestion suggesting that further digestion of the peptides had occurred during the intestinal phase by the pancreatin enzymes hydrolysing them into free amino acids or low molecular weight peptides. A range of bands from the molecular weight of 75 to 250 kD appeared during the intestinal phase which were not present during the gastric phase of digestion, indicating that pancreatin enzyme also caused a greater hydrolysis of muscle proteins releasing further peptides of higher molecular weight.

As evident from the changes in intensity of various bands, PEF processing influenced the proteolytic pattern and digestion kinetics of the muscle during *in vitro* digestion process. The intensity of some bands during the gastric phase of digestion, corresponding to the proteins myosin heavy chain MHC-7, desmin, calsequestrin 1, enolase, actin, tropomyosin  $\beta$ -chain, troponin-T, and tropomyosin- $\alpha$ -chain, was lower in case of PEF treated samples, particularly for T<sub>2</sub> (10 kV) samples. This difference in the gastric digestion products between PEF-treated samples and control, which became more obvious with increasing digestion time, indicates that more hydrolysis has occurred in PEF processed samples. The intensity of the bands for T<sub>2</sub> (10 kV) samples was more affected than the intensity of T<sub>1</sub> (5 kV) samples suggesting more digestion in T<sub>2</sub> (10 kV) samples. This was supported by a similar trend of increased protein digestibility (%) and soluble protein (%) for PEF treated samples. PEF processing induces electroporation in biological membranes and increases the permeability and mass transfer processes in animal tissues such as meat (Bhat, Morton, Mason, & Bekhit, 2018a, d). This increased membrane permeability can increase the diffusion of proteases into the membrane bound muscle fibres resulting in more hydrolysis during gastrointestinal digestion. Further, PEF processing has been reported to induce various structural changes, such as denaturation and unfolding, which are believed to improve the susceptibility of proteins for hydrolysis by gastrointestinal proteases. PEF processing has been reported to enhance the susceptibility of proteins to gastrointestinal digestion (Liu, Oey, Bremer, Silcock, & Carne, 2018).



**Figure 7.2 Effect of pulsed electric field on the protein profile (SDS-PAGE) of beef *Semimembranosus* subjected to *in vitro* simulated gastrointestinal digestion [M = marker, C = control, T<sub>1</sub> = 5.0 kV (90 Hz), T<sub>2</sub> = 10 kV (20 Hz), 15 µg samples were loaded in each lane, Protein identification was derived from that reported by Kaur et al., (2016) and Bhat et al., (2018d)]**

As found in the gastric phase of digestion, the intensity of most of the bands corresponding to major meat proteins which appeared during the intestinal phase was lower for PEF-treated samples, suggesting more enzymatic hydrolysis of these proteins and peptides with PEF processing. The lowest intensities were observed for T<sub>2</sub> (10 kV) samples after completion of 180 minutes of intestinal phase of digestion. These results clearly indicate greater hydrolysis of PEF treated proteins occurred during the intestinal phase of digestion. Like many other food processes which can induce favourable protein structural changes, PEF processing has the potential to positively influence the gastrointestinal digestion of proteins (Bhat, Morton, Mason, & Bekhit, 2018a, f). Application of both non-thermal and thermal processes, which induce favourable structural changes such as partial protein unfolding, have been reported to enhance the susceptibility of proteins to gastrointestinal digestion (Stefanović et al., 2017; Hoppe, Jung, Patnaik, & Zeece, 2013).

#### **7.4.4 Free amino acid analysis**

The mean values for various free amino acids released during *in vitro* simulated gastrointestinal protein digestion of PEF-processed beef *Semimembranosus* [T<sub>1</sub> (5.0 kV, 90 Hz) and T<sub>2</sub> (10 kV, 20 Hz)] are presented in Table 7.2. Numerically higher values were observed for almost all the amino acids for the samples processed with PEF, although no significant ( $P > 0.05$ ) difference were observed between control and PEF treated samples. Mediated by gastric and pancreatic proteases, the digestion and subsequent absorption of dietary proteins primarily occurs in the stomach and the small intestine and breaks down the proteins into an assimilable form for anabolic purposes. *In vitro* simulated gastrointestinal digestion trials are extensively used for evaluation of bioaccessibility of nutrients from food matrices because of certain advantages such as safe, inexpensive, fast, relatively simple, possibility of screening numerous samples and no ethical objections as associated with *in-vivo* methods (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014). The model that we used in our study for *in vitro* gastrointestinal digestion did not include an oral phase (chewing and salivary enzymes) or a brush-border enzymatic phase, it only simulated the enzymatic conditions and pH of gastrointestinal digestion and included a gastric and an intestinal phase. Due to its balanced composition of essential amino acids and its high digestibility (Oberli et al., 2015), meat is considered as a good quality protein source for humans (Sayd et al., 2018). The end products of the protein digestion could make a difference and affect the absorption of amino acids in the small intestine (Wen et al., 2015) because only single amino acids or short polypeptides (i.e. dipeptides and tripeptides) can be transported across the small intestine (MacFarlane, 2018). There are multiple amino acid transporters and proton-coupled peptide transporter for the transport and absorption of amino acids and small peptides in the intestines of animals (Liu et al., 2012; Bröer, 2008). While peptide transporter 1 is the major small peptide transporter present in the intestine of animals, amino acid transporters are classified into



several types such as the anionic amino acid transporter, the cationic amino acid transporter, the neutral amino acid transporter and the neutral and cationic transporter (Xiao et al., 2015; Lu, & Klaassen, 2006).

**Table 7.2 Effect of pulsed electric field on the release of free amino acids during *in vitro* simulated gastrointestinal digestion of beef *Semimembranosus* (Mean  $\pm$  SE)\***

Free amino acids ( $\mu$ M/litre)	PEF Treatment		
	Control	T <sub>1</sub> (5 kV)	T <sub>2</sub> (10 kV)
Aspartic acid	42.82 $\pm$ 1.92	44.43 $\pm$ 2.41	48.29 $\pm$ 2.09
Glutamic acid	87.85 $\pm$ 8.10	94.22 $\pm$ 8.40	98.71 $\pm$ 5.26
Cysteine	121.96 $\pm$ 10.49	128.08 $\pm$ 15.00	145.89 $\pm$ 9.39
Asparagine	141.11 $\pm$ 6.68	147.74 $\pm$ 9.48	156.34 $\pm$ 5.82
Serine	65.98 $\pm$ 4.94	69.70 $\pm$ 7.11	76.19 $\pm$ 7.47
Glutamine	776.82 $\pm$ 93.26	800.49 $\pm$ 94.61	844.57 $\pm$ 106.98
Histidine	62.30 $\pm$ 5.25	66.63 $\pm$ 5.06	70.36 $\pm$ 2.82
Glycine	131.58 $\pm$ 11.59	140.62 $\pm$ 9.80	137.87 $\pm$ 12.93
Threonine	150.50 $\pm$ 9.86	159.60 $\pm$ 11.07	177.46 $\pm$ 5.53
Arginine	443.23 $\pm$ 36.26	468.29 $\pm$ 46.30	504.48 $\pm$ 16.03
Alanine	447.09 $\pm$ 52.63	430.66 $\pm$ 46.70	439.59 $\pm$ 34.29
Taurine	158.72 $\pm$ 8.11	158.78 $\pm$ 22.57	176.84 $\pm$ 12.28
Tyrosine	831.38 $\pm$ 38.19	869.96 $\pm$ 50.00	930.24 $\pm$ 25.81
Valine	393.53 $\pm$ 18.37	407.07 $\pm$ 22.78	443.93 $\pm$ 10.76
Methionine	250.02 $\pm$ 12.99	263.46 $\pm$ 17.36	287.03 $\pm$ 11.74
Tryptophan	313.41 $\pm$ 22.26	322.18 $\pm$ 24.97	359.77 $\pm$ 19.79
Phenylalanine	862.24 $\pm$ 35.47	894.71 $\pm$ 46.98	950.46 $\pm$ 30.12
Isoleucine	459.34 $\pm$ 20.22	484.74 $\pm$ 26.95	518.26 $\pm$ 15.91
Lysine	551.93 $\pm$ 40.64	611.93 $\pm$ 63.88	663.59 $\pm$ 35.55
Leucine	1624.8 $\pm$ 67.69	1704.8 $\pm$ 94.18	1825.1 $\pm$ 47.69
Proline	70.11 $\pm$ 6.17	75.65 $\pm$ 7.11	79.32 $\pm$ 3.11

T<sub>1</sub> = Samples treated with PEF at 5 kV, 90 Hz, 20  $\mu$ s

T<sub>2</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu$ s

Treatments did not show any significant effect ( $P > 0.05$ )

Data was analysed by 1-way ANOVA using SPSS-21

n = 3 for each treatment

The exact mechanism how PEF increases the bioaccessibility of nutrients during the digestion process is not clear, however, PEF-induced electroporation is suggested to be the main reason (Barba et al., 2017b). This phenomenon, which is a direct output of electrical breakdown, allows a greater exchange of constituents between cell and its surroundings and may allow more diffusion of digestive proteases into the muscle mass during gastrointestinal digestion enhancing enzymatic hydrolysis in the PEF-treated samples (Bhat et al., 2018d). PEF processing has been reported to influence the microstructure and functional properties of proteins by modifying the interactions between amino acids (Yan-Yan et al., 2014). By causing the ionization of the chemical groups, such as carboxylic and amino groups, PEF treatment influences electrostatic interactions and promotes unfolding and aggregation of proteins, disrupting their secondary structure (Giteru, Oey, & Ali, 2018). By using different PEF processing combinations, favourable structural changes could be induced by regulating the microstructural changes to tailor specific functional properties of proteins (Giteru, Oey, & Ali, 2018; Mirmoghtadaie, Aliabadi, & Hosseini, 2016). Thus, by inducing favourable changes in protein structure that can increase the exposure of hydrolysis sites to digestive proteases, the release of amino acids could be enhanced. Prior treatment of PEF has already been reported to increase susceptibility of proteins to gastrointestinal digestion (Bhat et al., 2018f; Liu, Oey, Bremer, Silcock, & Carne, 2018).

#### **7.4.5 Mineral profile analysis**

The mean values for various minerals released during *in vitro* simulated gastrointestinal protein digestion of PEF-processed beef *Semimembranosus* [T<sub>1</sub> (5.0 kV, 90 Hz) and T<sub>2</sub> (10 kV, 20 Hz)] are presented in Table 7.3. PEF processing has been reported to significantly affect the concentration of some minerals in beef and chicken (Khan et al., 2018; Khan et al., 2017a, b). A significant decrease has been reported in the concentration of Fe, K and P in PEF treated beef muscles and the loss was greater in high PEF (10 kV, 200 Hz and 20 µs) than low PEF (2.5 kV, 200 Hz and 20 µs) treated samples (Khan et al., 2017a). A significant decrease was also reported in the concentration of Ca, Na and Mg in beef muscles whereas a significant increase was observed in the concentration of Cr (Khan et al., 2018). Significantly higher Ni concentration was also reported in chicken breast samples treated with high PEF, suggesting the possible migration of minerals to meat from the electrodes of the PEF systems. In our study, no significant ( $P > 0.05$ ) decrease was observed in the release of minerals such as Fe, K, P, Ca, Na and Mg from the muscle during gastrointestinal digestion. On the contrary, the concentration of these minerals was mathematically higher ( $P > 0.05$ ) for PEF treated samples in comparison to control which might be attributed to the higher membrane permeability induced by PEF processing that might have resulted in more release of minerals. Further, no significant ( $P > 0.05$ ) impact of PEF processing was observed on the concentration of Cr and Ni. It is important to mention here that the studies referred above measured the concentration of various minerals in the fresh meat itself and

employed a different method of detection whereas in our study we measured the concentration of these minerals in the liquid digesta only after they leached out from muscle mass during gastrointestinal enzymatic hydrolysis. Meat is considered as a rich source of minerals, such as Fe and Zn, and any change in the availability of minerals will surely have a commercial importance.

**Table 7.3 Effect of pulsed electric field on the release of minerals during *in vitro* simulated gastrointestinal digestion of beef *Semimembranosus* (Mean  $\pm$  SE)**

Minerals ( $\mu\text{g/mL}$ )	PEF Treatment		
	Control	T <sub>1</sub> (5 kV)	T <sub>2</sub> (10 kV)
Aluminium (Al)	0.321 $\pm$ 0.006	0.329 $\pm$ 0.002	0.330 $\pm$ 0.004
Arsenic (As)	0.01 $\pm$ 0.001	0.01 $\pm$ 0.002	0.01 $\pm$ 0.001
Boron (B)	0.583 $\pm$ 0.01	0.565 $\pm$ 0.02	0.574 $\pm$ 0.01
Calcium (Ca)	2.38 $\pm$ 0.13	2.51 $\pm$ 0.05	2.45 $\pm$ 0.08
Chromium (Cr)	0.020 $\pm$ 0.0001	0.021 $\pm$ 0.0001	0.021 $\pm$ 0.0004
Copper (Cu)	0.033 $\pm$ 0.003	0.045 $\pm$ 0.011	0.037 $\pm$ 0.003
Iron (Fe)	0.908 $\pm$ 0.15	1.20 $\pm$ 0.31	1.19 $\pm$ 0.15
Potassium (K)	252.81 $\pm$ 2.38	257.50 $\pm$ 5.82	261.82 $\pm$ 1.73
Magnesium (Mg)	13.87 $\pm$ 0.34	14.04 $\pm$ 0.26	14.13 $\pm$ 0.44
Manganese (Mn)	0.011 $\pm$ 0.001	0.011 $\pm$ 0.0005	0.011 $\pm$ 0.001
Nickle (Ni)	< 1.17 ppb	< 1.17 ppb	< 1.17 ppb
Sodium (Na)	1746.7 $\pm$ 26.57	1758.4 $\pm$ 20.08	1762.8 $\pm$ 14.14
Phosphorus (P)	448.83 $\pm$ 2.90	453.91 $\pm$ 4.54	452.20 $\pm$ 4.41
Sulphur (S)	101.32 $\pm$ 7.63	108.44 $\pm$ 9.29	108.76 $\pm$ 5.45
Zinc (Zn)	2.27 $\pm$ 0.13	2.91 $\pm$ 0.41	2.61 $\pm$ 0.06

Ni, Pb, Cd and Se were below detection levels

T<sub>1</sub> = Samples treated with PEF at 5 kV, 90 Hz, 20  $\mu\text{s}$

T<sub>2</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu\text{s}$

Data was analysed by 1-way ANOVA using SPSS-21

n = 3 for each treatment

## 7.5 Conclusion

PEF processing showed a significant ( $P < 0.05$ ) influence on the protein digestibility (%) and soluble protein (%) of beef *Semimembranosus* during *in vitro* simulated gastrointestinal digestion. This was also reflected in the protein profile of the PEF processed samples. Similar effects of PEF processing were also reflected in free amino acids as mathematically higher ( $P > 0.05$ ) values were recorded for PEF processed samples. In contrast to the previous reports, which have observed a significant impact of PEF treatment on the mineral concentration of fresh meat, no significant ( $P > 0.05$ ) impact of PEF processing was observed on the release of various minerals during the digestion process. By increasing the membrane permeability and inducing structural changes in proteins, PEF seems to have a positive influence on enzymatic hydrolysis of meat proteins during *in vitro* simulated gastrointestinal digestion. The PEF technology could be explored for the development of novel protein structures and meat products with enhanced digestibility and nutritional value.

## Chapter 8

### Pulsed electric field: Role in protein digestion of beef *Biceps femoris*

This chapter is published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Pulsed electric field: Role in protein digestion of beef *Biceps femoris*. *Innovative Food Science and Emerging Technologies*, 50, 132-138.

#### 8.1 Abstract

The present study was conducted to evaluate the effect of pulsed electric field (PEF) on the protein digestion kinetics of cold-boned beef *Biceps femoris* during *in vitro* simulated gastrointestinal digestion. Muscle was treated with PEF at 5 kV, 90 Hz, 20  $\mu$ s ( $T_1$ ) and 10 kV, 20 Hz, 20  $\mu$ s ( $T_2$ ) and subjected to *in vitro* gastrointestinal digestion along with an untreated control. Samples were collected at 0, 30, and 60 minutes of gastric digestion and 120 and 180 minutes of intestinal digestion. PEF processing affected the digestion kinetics by modifying the protein profile (SDS-PAGE) of the meat digests and significantly ( $P < 0.05$ ) increasing the protein digestibility (%) and soluble protein (%). Concentration of almost all the free amino acids was recorded higher for the PEF treated samples whereas no significant ( $P > 0.05$ ) impact was observed on the release of minerals such as Fe, Zn, Cr, Cu, Mg, Ni, Na or K. PEF processing improved the digestion kinetics of the beef muscle during gastrointestinal digestion simulation.

##### 8.1.1 Industrial relevance

PEF processing has already been reported to improve the tenderization of beef muscles during ageing process. The simulations and experiments conducted in this work showed that PEF processing has the potential to improve the protein digestion kinetics of beef muscle during *in vitro* gastrointestinal digestion and could be utilized for the development of novel muscle foods and protein structures with improved digestibility and nutritive value.

**Keywords:** Pulsed electric field; *Biceps femoris*; *in vitro* protein digestion; Protein digestibility; Free amino acid analysis; Mineral profile

#### 8.2 Introduction

Pulsed electric field, that uses electric field pulses of short duration to induce electroporation in cell membranes (Barba et al., 2015; Toepfl, Siemer, & Heinz, 2014a), is an emerging nonthermal technology

with promising applications in meat processing and technology (Bhat, Morton, Mason, & Bekhit, 2018a). This technology has already proven its potential for improving or modifying several food processes, such as extraction and valorization of bioactive compounds (Kantar, Boussetta, Lebovka, Foucart, & Vorobiev, 2018; Rocha et al., 2018), pasteurization and sterilization of heat sensitive liquids (Buchmann, Bloch, & Mathys, 2018; Wang et al., 2018b), inactivation of enzymes (García-Parra et al., 2018), enhancing properties of bioactive peptides (Liang, Cheng, & Wang, 2018), reduction of the allergenicity of certain food products (Ekezie, Cheng, & Sun, 2018), potential reduction of pesticide residues and food contaminants (Misra, 2015), freezing processes (Velickova et al., 2018), and dehydration processes including freeze drying (Liu, Grimi, Lebovka, & Vorobiev, 2018a). In addition, PEF processing has also been reported to have certain applications in the area of meat, such as enhancing the tenderization process (Bekhit, Suwandy, Carne, van de Ven, & Hopkins, 2016; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015a, b, c; Bekhit, van de Ven, Suwandy, Fahri, & Hopkins, 2014c), supercooling (Mok, Her, Kang, Hoptowit, & Jun, 2017), and accelerated brining (McDonnell, Allen, Chardonnerau, Arimi, & Lyng, 2014).

While PEF is widely recognized in many areas, evidence of its application and effect on the digestibility of foods, particularly high-protein food matrices, is almost lacking. Recently, PEF treatment was reported to promote the glycemic digestibility and emulsification stability of esterified starch molecules (Hong, Zeng, Han, & Brennan, 2018). This effect was explained on the basis of changes induced by PEF treatment at nanostructural level. PEF processing has also been reported to induce structural changes in waxy rice starch that significantly affected its digestibility (Zeng, Gao, Han, Zeng, & Yu, 2016). PEF processing is able to induce various structural changes in proteins and has been reported to do so in several non-meat proteins (Liu, Oey, Bremer, Silcock, & Carne, 2018; Han, Cai, Cheng, & Sun, 2018). It has the potential to induce such changes in protein-rich matrices such as meat and thus may affect their digestibility (Bhat et al., 2018a). PEF has been reported to induce certain modifications in proteins such as unfolding and aggregation and altering their structural and functional properties (Han et al., 2018; Liu et al., 2018b; Zhang, Wang, Jiang, & Qian, 2017). However, no information is available in the literature, to the best of our knowledge, on the influence that PEF can have on the protein digestibility of foods in general or meat in particular.

Recently, PEF processing was also reported to affect the concentration of certain minerals in meat (Khan et al., 2018; Khan et al., 2017a, b) which can affect the release of such minerals during the gastrointestinal digestion. Meat is generally regarded as a good source of minerals and any significant change in the release of minerals during the digestion process will have an impact on the commercial value of the meat. Taking into consideration all these facts, the objective of this study was to evaluate the effect of PEF processing on digestion kinetics of beef *Biceps femoris* subjected to *in vitro* simulated gastrointestinal digestion. Protein digestibility (%), soluble protein (%), protein profile, mineral profile and free amino acid analysis of the digesta was done to determine if PEF processing has any influence

on the digestion pattern and release of various nutrients during simulated digestion process. The study will expand our understanding of meat protein digestion and how PEF affects the process *in vitro*.

### **8.3 Materials and methods**

Meat samples were prepared as per the method described in section 2.2.3. The meat samples were treated with PEF as per the method described in section 2.3.3.1. *In vitro* protein digestion, *in vitro* protein digestibility (%), protein content (%), soluble protein (%), free amino acid analysis and mineral profile analysis were performed according to the methods described from section 2.5.1 to 2.5.7. Protein concentration, SDS-PAGE and analysis of the protein bands was done by following the methods described in section 2.4.2 and 2.4.4. Statistical analysis of the data was performed according to the method described in section 2.7.2.

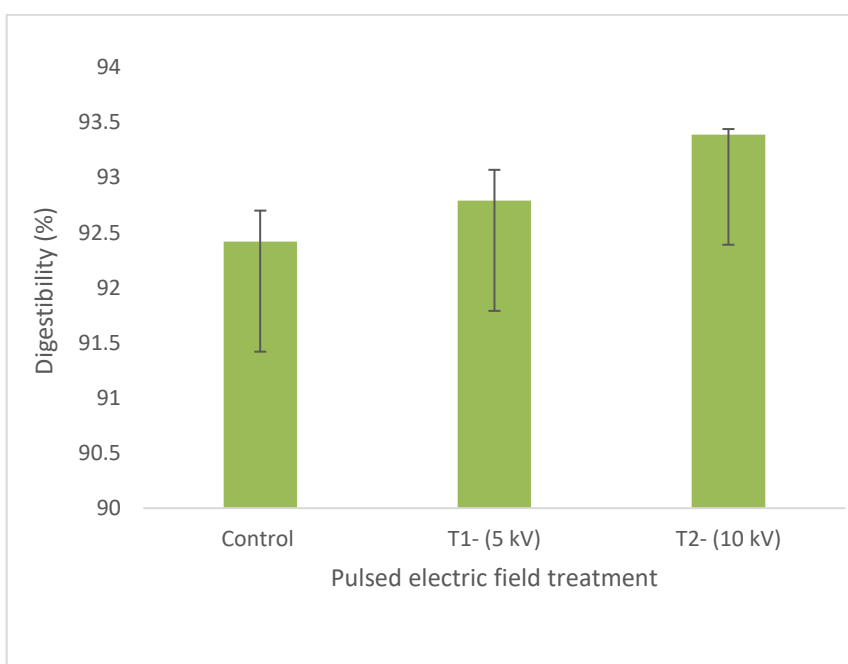
### **8.4 Results and discussion**

#### **8.4.1 Protein digestibility (%)**

The mean values for *in vitro* protein digestibility (%) for PEF treated beef *Biceps femoris* viz. T<sub>1</sub> (5.0 kV, 90 Hz) and T<sub>2</sub> (10 kV, 20 Hz) subjected to *in vitro* simulated gastrointestinal digestion are presented in Figure 8.1. PEF processing showed a significant ( $P < 0.05$ ) effect on the protein digestibility (%) as the mean values for samples treated with PEF (10 kV, 20 Hz) were significantly higher than control (untreated samples). Similar findings were also reported by Tavares, Dong, Yang, Zeng, & Zhao (2018) and Wen et al. (2015) who presented similar values for protein digestibility (%) for hairtail (*Thichiurus lepturus*) fillets and meat samples (pork, beef, chicken and fish), respectively, subjected to *in vitro* simulated gastrointestinal protein digestion. Like our study, Kondjoyan, Daudin, & Santé-Lhoutellier (2015) also obtained highest digestibility value at the end of the *in vitro* digestion. The true ileal digestibility of meat proteins has been measured to reach 95% (Silvester, & Cumings, 1995). While the kinetics and mechanisms of protein changes that occur during meat processing are well characterized, changes that PEF processing could induce on the digestion of meat proteins in the human body are not known.

The rate of digestion of proteins is the main factor that determines the assimilation of proteins in the diet (Kondjoyan et al., 2015). Kinetics of meat protein digestion is influenced by several factors including the denaturation of proteins, oxidation, and change in the number of hydrolysable sites available for the enzymatic cleavage which depends on time-temperature heating conditions (Kondjoyan et al., 2015). The composition of meat plays a limited role on its digestion (Bax et al., 2013), however, processing treatments, such as salting and cooking, can alter the rate of digestion of proteins (Bax et al., 2012; Hassoun, Sante-Lhoutellier, Lebert, Kondjoyan, & Daudin, 2011). Changes in the

structure of the proteins which have the potential to influence the protease active sites can also affect the susceptibility and digestion of meat proteins (Simonetti, Gambacorta, & Perna, 2016). A massive aggregation and greater protein-unfolding will cause a reduced proteolytic susceptibility whereas a partial unfolding of protein structure improves its susceptibility for enzymatic hydrolysis (Simonetti et al., 2016; Promeyrat et al., 2010; Gatellier, & Santé-Lhoutellier, 2009). Treatment of food proteins with PEF can induce structural changes in the protein molecules (Liang et al., 2018) by inducing unfolding which is followed by the disruption of the secondary structure (Giteru, Oey, & Ali, 2018). By increasing the membrane permeability for protease diffusion and by inducing partial unfolding of the proteins (Liu, Oey, Bremer, Carne, & Silcock, 2017b), prior treatment of the muscle with PEF can affect the digestion of meat proteins in a positive way by increasing the digestion rate and digested output product (Liu et al., 2018b).



**Figure 8.1 Effect of pulsed electric field on the protein digestibility of beef *Biceps femoris* during *in vitro* simulated gastrointestinal digestion (Mean  $\pm$  SE)**

#### 8.4.2 Soluble protein (%)

The average values for soluble protein (%) for PEF treated samples of beef *Biceps femoris* viz. T<sub>1</sub> (5 kV, 90 Hz) and T<sub>2</sub> (10 kV, 20 Hz) subjected to *in vitro* simulated gastrointestinal protein digestion are presented in Table 8.1. The mean soluble protein (%) of the samples treated with PEF during intestinal digestion both at 120 and 180 minutes were significantly ( $P < 0.05$ ) higher than control. The soluble



protein (%) of the PEF treated samples was also higher than control samples during gastric phase of digestion, however, no significant ( $P > 0.05$ ) difference was observed between PEF processed and control samples. Kaur et al. (2016) and Jung, de Lamballerie-Anton, & Ghoul (2000) also observed a similar increase in the soluble protein (%) for high-pressure processed beef muscles during *in vitro* simulated gastrointestinal digestion. The myofibrillar proteins are not directly in contact with proteases in the gastrointestinal tract, rather the proteases have to diffuse through the bolus to reach to the proteins (Kondjoyan et al., 2015). PEF processing increases the membrane permeability by inducing the electroporation that can increase the diffusion of proteases into the membrane bound muscle mass and allow greater digestion of the myofibrils resulting in the release of more proteins. Further, the susceptibility of meat proteins, such as myosin, to enzymatic hydrolysis by gastrointestinal proteases, such as pepsin, is highly affected by treatments, such as high-pressure processing, which have the potential to affect the protein structure and the availability of the hydrolysis sites on proteins (Liu, & Xiong, 2000). Like high-pressure processing, PEF-processing can also affect the structure of proteins by causing ionization of chemical groups and altering electrostatic interactions (Liu et al., 2018b; Liu et al., 2017b) and thus may affect the digestion process (Bhat et al., 2018a).

**Table 8.1 Effect of pulsed electric field on the soluble protein (%) of beef *Biceps femoris* during *in vitro* simulated gastrointestinal digestion (Mean  $\pm$  SE)\***

Treatments	<i>In vitro</i> gastric digestion			<i>In vitro</i> intestinal digestion	
	0 Minutes	30 Minutes	60 Minutes	120 Minutes	180 Minutes
Control	7.41 $\pm$ 0.90 <sup>a</sup>	8.69 $\pm$ 0.77 <sup>a</sup>	9.09 $\pm$ 1.01 <sup>a</sup>	24.62 $\pm$ 0.90 <sup>bA</sup>	25.62 $\pm$ 0.54 <sup>bA</sup>
T <sub>1</sub> (5 kV)	7.73 $\pm$ 0.34 <sup>a</sup>	8.80 $\pm$ 0.75 <sup>a</sup>	9.67 $\pm$ 0.37 <sup>a</sup>	25.88 $\pm$ 0.98 <sup>bAB</sup>	26.22 $\pm$ 0.22 <sup>bA</sup>
T <sub>2</sub> (10 kV)	7.80 $\pm$ 0.50 <sup>a</sup>	9.43 $\pm$ 0.42 <sup>ab</sup>	10.10 $\pm$ 0.89 <sup>b</sup>	28.16 $\pm$ 0.23 <sup>cB</sup>	29.14 $\pm$ 0.82 <sup>cB</sup>

\*Mean  $\pm$  SE with different superscripts in a row wise (lower case alphabet) and column wise (upper case alphabet) differ significantly ( $P < 0.05$ )

T<sub>1</sub> = Samples treated with PEF at 5 kV, 90 Hz, 20  $\mu$ s

T<sub>2</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu$ s

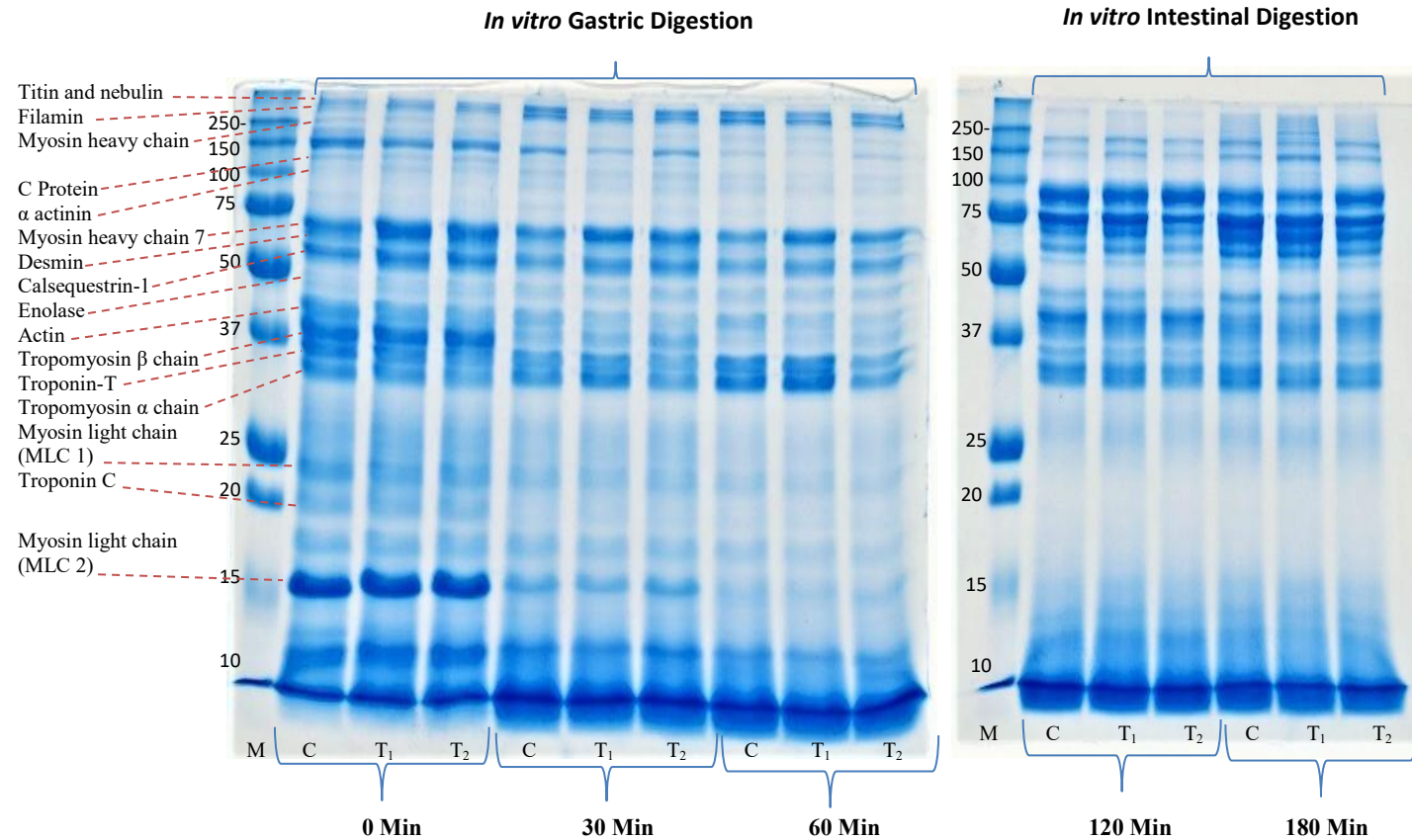
n = 3 for each treatment

### 8.4.3 Protein profile (SDS-PAGE)

The protein profile (SDS-PAGE gel electrophoretogram) for PEF treated beef *Biceps femoris* viz. T<sub>1</sub> (5.0 kV, 90 Hz) and T<sub>2</sub> (10 kV, 20 Hz) subjected to *in vitro* simulated gastrointestinal digestion are presented in Figure 8.2. A range of bands appeared in gastric and intestinal phase of digestion for both control and PEF-treated samples with some differences. The major identified bands include myosin light chain MLC<sub>2</sub> (16 kDa), troponin-C (19 kDa), myosin light chain MLC<sub>1</sub> (22 kDa), tropomyosin- $\alpha$ -chain (33 kDa),

troponin-T (35 kDa), tropomyosin  $\beta$ -chain (37 kD), actin (42 kD), enolase (46 kD), calsequestrin 1 (52-55 kD), desmin (53 kD),  $\alpha$ -actinin (95 kD), C protein (135 kD), myosin heavy chain MHC 7 (>220 kD), myosin heavy chain MHC 1 (>220 kD), filamin (>250 kD) and titin and nebulin (>250 kD). Several bands which were present during gastric phase of the digestion from molecular weight of 10 kD to 37 kD for both control as well as PEF treated samples either disappeared or became less intense during intestinal phase of digestion. These polypeptides which were produced during gastric phase of digestion by the hydrolysis induced by pepsin at an acidic pH might have been further hydrolysed by intestinal proteases resulting into free amino acids or polypeptides of lower molecular weight. The effect was more pronounced with increasing time of digestion. Similarly, new higher molecular weight peptides ranging from 75 kD to 250 kD appeared during the intestinal phase of digestion whereas as comparatively fewer peptides were present in the same range in the gastric phase of digestion. More hydrolysis of muscle proteins might have occurred during intestinal phase of digestion resulting in the release of more polypeptides of higher molecular weight.

Differences in the bands were observed not only between gastric and intestinal phases but also between control and PEF processed samples during the whole digestion process. The intensity of various bands, corresponding to proteins troponin-C (19 kDa), myosin light chain MLC<sub>1</sub> (22 kDa), tropomyosin- $\alpha$ -chain (33 kDa), troponin-T (35 kDa), tropomyosin  $\beta$ -chain (37 kD), actin (42 kD) and enolase (46 kD) which appeared during gastric phase of digestion, was lower in case of PEF treated samples, particularly for T<sub>2</sub> samples, in comparison to control. This effect became more prevalent with increasing time of digestion. This indicates that more digestion might have occurred in PEF processed samples in comparison to control. A similar trend could be seen during the intestinal phase of digestion as the intensity of several bands corresponding to T<sub>2</sub> samples were less intense in comparison to control or T<sub>1</sub> samples. The increased membrane permeability and protein structural changes induced by PEF processing might have increased the diffusion of gastrointestinal proteases into the muscle mass and enhanced the protein susceptibility by increasing the availability of hydrolysis sites for digestion. Several non-thermal technologies including high-pressure and PEF processing have been reported to enhance the susceptibility of proteins to gastrointestinal digestion (Liu et al., 2018b; Kaur et al., 2016).



**Figure 8.2** Effect of pulsed electric field on the protein profile (SDS-PAGE) of beef *Biceps femoris* subjected to *in vitro* simulated gastrointestinal digestion [M = marker, C = control, T<sub>1</sub> = 5 kV (90 Hz), T<sub>2</sub> = 10 kV (20 Hz), 20 μg samples were loaded in each lane, Protein identification was derived from that reported by Kaur et al., (2016); Kaur et al., (2014) and Ha (2012)]

#### 8.4.4 Free amino acids

Having a well-balanced amino acid profile, meat and meat products are an excellent source of proteins and contain all the essential amino acids which humans cannot synthesize (Pomélie, Santé-Lhoutellier, Sayd, & Gatellier, 2018). The amounts of the amino acids tyrosine, phenylalanine, tryptophan, arginine and lysine determine the extent of protein digestion in the gastrointestinal tract as they are the target cleavage sites of pepsin or trypsin (Zou et al., 2018; Savoie, Agudelo, Gauthier, Marin, & Pouliot, 2005; Erickson, & Kim, 1990). Pre-treatments, such as cooking and high-pressure processing, which can cause protein denaturation and formation of intermolecular cross links, resulting in aggregation, can affect the efficacy of digestive proteases and the release and bioavailability of amino acids (Luo, Taylor, Nebl, Ng, & Bennett, 2018; Morzel, Gatellier, Sayd, Renerre, & Laville, 2006). Several pre-treatments have been reported to affect the digestibility of meat proteins during *in vitro* gastrointestinal digestion. Cooking has been reported to decrease *in vitro* digestibility of beef and pork (Wen et al., 2015; Kaur et al., 2014) whereas ageing has been reported to increase the beef protein digestibility by increasing the pH (Farouk, Wu, Frost, Clerens, & Knowles, 2014). However, no information is available in the literature about the effect of PEF processing on the digestibility of meat proteins and the release of amino acids.

The active site of an enzyme must adapt and fit to the specific stereochemistry of the polypeptide chain of the protein substrate during the enzymatic hydrolysis for effective proteolysis and release of free amino acids (Fontana, Polverino de Laureto, De Filippis, Scaramella, & Zambonin, 1997). During denaturation, proteins are unfolded and become more flexible and open structures exposing the hydrophobic groups buried in their core and making them more susceptible to the binding and degradation of digestive enzymes (Zou et al., 2018; Djikaev, & Ruckenstein, 2008). PEF processing has been reported to cause several structural modifications in proteins including unfolding and denaturation (Liu et al., 2018b; Liu et al., 2017b) and can thus influence the hydrolysis of proteins and the release of free amino acids during gastrointestinal digestion (Liu et al., 2018b). In our study, the mean values for almost all the amino acids were higher for the PEF treated samples in comparison to control (Table 8.2). These results were also reflected in the protein digestibility (%) and soluble protein (%) of the PEF treated samples.

**Table 8.2 Effect of pulsed electric field on the release of free amino acids during *in vitro* simulated gastrointestinal digestion of beef *Biceps femoris* (Mean  $\pm$  SE)**

Free amino acids ( $\mu$ M/litre)	PEF Treatment		
	Control	T <sub>1</sub> (5 kV)	T <sub>2</sub> (10 kV)
Aspartic acid	41.28 $\pm$ 1.24	43.14 $\pm$ 0.94	46.66 $\pm$ 3.40
Glutamic acid	80.88 $\pm$ 6.55	87.07 $\pm$ 3.65	95.01 $\pm$ 5.89
Cysteine	107.80 $\pm$ 8.80	109.93 $\pm$ 3.57	132.60 $\pm$ 10.70
Asparagine	150.10 $\pm$ 3.05	158.21 $\pm$ 5.38	163.62 $\pm$ 5.18
Serine	88.57 $\pm$ 3.83	89.75 $\pm$ 9.85	95.60 $\pm$ 5.12
Glutamine	1155.9 $\pm$ 43.0	1172.2 $\pm$ 50.62	1258.0 $\pm$ 60.0
Histidine	58.38 $\pm$ 0.93	58.27 $\pm$ 6.44	66.92 $\pm$ 3.56
Glycine	173.61 $\pm$ 16.6	169.69 $\pm$ 18.56	188.72 $\pm$ 22.79
Threonine	146.82 $\pm$ 6.80	148.43 $\pm$ 6.38	162.11 $\pm$ 6.39
Arginine	395.53 $\pm$ 22.66	394.52 $\pm$ 13.03	442.73 $\pm$ 31.62
Alanine	429.24 $\pm$ 4.48	448.35 $\pm$ 35.87	452.44 $\pm$ 9.50
Taurine	392.80 $\pm$ 27.99	327.56 $\pm$ 32.15	414.73 $\pm$ 52.17
Tyrosine	788.63 $\pm$ 24.00	796.14 $\pm$ 22.67	857.57 $\pm$ 35.78
Valine	370.15 $\pm$ 14.22	382.50 $\pm$ 12.81	402.34 $\pm$ 16.44
Methionine	240.22 $\pm$ 8.98	247.77 $\pm$ 7.50	262.86 $\pm$ 12.47
Tryptophan	288.66 $\pm$ 14.82	296.63 $\pm$ 21.64	331.03 $\pm$ 31.35
Phenylalanine	831.38 $\pm$ 29.32	838.16 $\pm$ 17.71	897.89 $\pm$ 43.26
Isoleucine	441.17 $\pm$ 14.73	445.02 $\pm$ 16.92	475.27 $\pm$ 18.49
Lysine	564.91 $\pm$ 17.29	563.42 $\pm$ 35.94	627.79 $\pm$ 25.63
Leucine	1573.7 $\pm$ 43.66	1590.3 $\pm$ 58.46	1702.6 $\pm$ 67.81
Proline	69.51 $\pm$ 1.57	71.98 $\pm$ 1.64	77.87 $\pm$ 0.82

T<sub>1</sub> = Samples treated with PEF at 5 kV, 90 Hz, 20  $\mu$ s

T<sub>2</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu$ s

n = 3 for each treatment

#### 8.4.5 Mineral profile

A fraction of mineral that becomes available for intestinal absorption, known as mineral bioaccessibility, is released from the food matrix into the gastrointestinal tract (Fairweather-Tait et al., 2005). This release of dietary minerals begins when gastric enzymes and peristaltic movements disrupt

the meat matrix and the acidic pH aids in the release of minerals from their bound forms (Alminger et al., 2014). These solubilized minerals, which are present in the ionic form, are absorbed in the small intestine by active as well as passive transport (Corte-Real, & Bohn, 2018). The present study evaluated the impact of PEF processing on the release of minerals such as iron, zinc, calcium, potassium and magnesium which are minerals of great public health importance (FAO, & WHO, 2004) during *in vitro* digestion of meat. Previous studies (Khan et al., 2018; Khan et al., 2017a, b) which have evaluated the effect of PEF processing on the quality characteristics of the meat have reported a significant impact of the treatment on the mineral content. A significant decrease was reported in the concentration of minerals such as Fe, K, P, Ca, Na and Mg (Khan et al., 2018; Khan et al., 2017a). A significant increase was also observed in the concentration of Cr and Ni (Khan et al., 2018). Not only was a different method used for determining the concentration of minerals in comparison to the above studies, our study determined the concentration of these minerals in the liquid digesta obtained after gastrointestinal digestion whereas the above studies estimated the levels of these minerals in fresh meat only. In our study, we did not observe any significant ( $P > 0.05$ ) impact of PEF processing on the release of minerals during *in vitro* simulated gastrointestinal digestion process (Table 8.3). PEF processing had no significant ( $P > 0.05$ ) effect on the release of minerals such as Fe, K, P, Ca, Na, Mg, Cr, and Ni from beef *Biceps femoris* during *in vitro* digestion process.

**Table 8.3 Effect of pulsed electric field on the release of minerals during *in vitro* simulated gastrointestinal digestion of beef *Biceps femoris* (Mean  $\pm$  SE)**

Minerals ( $\mu\text{g/mL}$ )	PEF Treatment		
	Control	T <sub>1</sub> (5 kV)	T <sub>2</sub> (10 kV)
Aluminium (Al)	0.339 $\pm$ 0.005	0.331 $\pm$ 0.004	0.331 $\pm$ 0.005
Arsenic (As)	0.010 $\pm$ 0.003	0.010 $\pm$ 0.002	0.012 $\pm$ 0.001
Boron (B)	0.733 $\pm$ 0.06	0.712 $\pm$ 0.03	0.683 $\pm$ 0.02
Calcium (Ca)	3.21 $\pm$ 0.21	3.04 $\pm$ 0.21	3.00 $\pm$ 0.06
Chromium (Cr)	0.021 $\pm$ 0.0008	0.021 $\pm$ 0.0003	0.020 $\pm$ 0.0003
Copper (Cu)	0.048 $\pm$ 0.013	0.037 $\pm$ 0.001	0.039 $\pm$ 0.002
Iron (Fe)	1.33 $\pm$ 0.31	1.11 $\pm$ 0.22	1.22 $\pm$ 0.17
Potassium (K)	278.18 $\pm$ 4.94	277.29 $\pm$ 1.62	278.16 $\pm$ 7.47
Magnesium (Mg)	14.70 $\pm$ 0.35	14.56 $\pm$ 0.43	14.68 $\pm$ 0.84
Manganese (Mn)	0.013 $\pm$ 0.0007	0.011 $\pm$ 0.0010	0.011 $\pm$ 0.0011
Nickle (Ni)	< 1.17 ppb	< 1.17 ppb	< 1.17 ppb
Sodium (Na)	1821.8 $\pm$ 4.36	1797.4 $\pm$ 12.21	1801.7 $\pm$ 6.20
Phosphorus (P)	453.00 $\pm$ 7.12	451.02 $\pm$ 4.23	441.98 $\pm$ 3.92
Sulphur (S)	102.89 $\pm$ 5.09	100.72 $\pm$ 2.02	103.36 $\pm$ 5.37
Zinc (Zn)	2.39 $\pm$ 0.13	2.34 $\pm$ 0.07	2.51 $\pm$ 0.14

Ni, Pb, Cd, and Se were below detection levels

T<sub>1</sub> = Samples treated with PEF at 5 kV, 90 Hz, 20  $\mu\text{s}$

T<sub>2</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu\text{s}$

n = 3 for each treatment

## 8.5 Conclusions

Prior processing of meat samples with PEF had a significant effect on the digestion kinetics during *in vitro* simulated gastrointestinal digestion. PEF-induced membrane permeability and protein structural changes resulted in a significant ( $P < 0.05$ ) increase in protein digestibility (%) and soluble protein (%). Mean values for free amino acids were also higher for almost all amino acids for PEF treated samples. These results were further supported by the protein profile analysis of the digesta. PEF processing did not result in any significant ( $P > 0.05$ ) adverse effect on the release of minerals from the samples during the digestion process. PEF processing has the potential and could find applications in the development of meat products with better nutritive value and digestion properties.

## Chapter 9

### Pulsed electric field: Effect on *in vitro* simulated gastrointestinal protein digestion of deer *Longissimus dorsi*

This chapter is published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., Mungure, T., & Bekhit, A. E. D. A. (2019). Pulsed electric field: Effect on *in vitro* simulated gastrointestinal protein digestion of deer *Longissimus dorsi*. *Food Research International*, 120, 793-799.

#### 9.1 Abstract

The effect of pulsed electric field (PEF) on *in vitro* simulated gastrointestinal protein digestion of cold-boned deer *Longissimus dorsi* was elucidated. PEF treated samples viz. T<sub>1</sub> (2.5 kV, 50 Hz) and T<sub>2</sub> (10 kV, 90 Hz) along with a control were subjected to *in vitro* simulated gastrointestinal protein digestion. Samples were collected after 0, 30, and 60 minutes of gastric digestion and 120 and 180 minutes of intestinal digestion. A significant ( $P < 0.05$ ) increase was observed in the protein digestibility and soluble protein (%) of PEF treated samples, highlighting a positive influence of PEF processing on the digestion process. Higher concentrations ( $P > 0.05$ ) of almost all the free amino acids were observed for the PEF treated samples. No significant effect of PEF was observed on the release of various minerals. PEF may be utilized for the development of novel muscle foods with improved protein digestibility.

**Keywords:** Pulsed electric field; *Longissimus dorsi*; venison; *in vitro* protein digestion; digestibility (%); soluble protein (%); free amino acid analysis; mineral profile

#### 9.2 Introduction

The demand of consumers for high quality and convenient foods with natural flavour has driven the food industry to develop novel food processing technologies which cause minimum impairment of nutritional and sensory qualities (Hati, Patel, & Yadav, 2018; Guimarães, Silva, Freitas, Meireles, & Cruz, 2018). Among these minimal processing technologies, pulsed electric field (PEF) is widely recognized as a non-thermal technology and has received much attention over the last two decades (Kantar, Boussetta, Lebovka, Foucart, & Vorobiev, 2018; Wang et al., 2018b; Suchanek, & Olejniczak, 2018). It is an energy efficient green technology that maintains the nutritional and sensory quality of foods (Han, Cai, Cheng, & Sun, 2018; Bhat, Morton, Mason, & Bekhit, 2018a). While PEF processing has been widely investigated for its effects on liquid foods and beverages (Griffiths, & Walkling-Ribeiro, 2014), its application in solid foods, particularly meat, has only recently emerged (Bhat et al., 2018a; Bhat,



Morton, Mason, & Bekhit, 2018d; Khan et al., 2017a, b; Bekhit, Suwandy, Carne, van de Ven, & Hopkins, 2016; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015a, b, c, d). Tenderization, accelerated curing, and improved meat safety appear to be the areas where PEF could provide attractive options in meat processing (Bhat et al., 2018a). However, extensive research is required to improve our understanding of how PEF could be utilized to improve the quality attributes of meat (Bhat et al., 2018a, d).

PEF processing has the potential to change the microstructure of meat and the interactions of biomacromolecules including proteins (Giteru, Oey, & Ali, 2018; Hong, Chen, Zeng, & Han, 2016). PEF processing has been reported to induce changes in secondary and tertiary structure of proteins due to the ionization of some chemical groups or breaking of electrostatic interactions (Wei, Zeng, Tang, Jiang, & Liu, 2018). It can affect the secondary structure of proteins resulting in the loss of  $\beta$ -sheet and  $\alpha$ -helix (Zhao, Tang, Lu, Chen, & Li, 2014). As the susceptibility of proteins to enzymatic hydrolysis is largely determined by their structural features (Liu, Oey, Bremer, Silcock, & Carne, 2018), prior treatment with PEF may alter the digestibility of proteins by gastrointestinal proteases. PEF processing may expose hydrolysis sites previously in-accessible to digestive proteases. Recently, PEF processing has been reported to enhance the susceptibility of egg white proteins to gastrointestinal digestion without causing severe protein aggregation (Liu, Oey, Bremer, Silcock, & Carne, 2018). While PEF-induced changes in the structure of the proteins in foods have been extensively studied, no information is available in the literature regarding the susceptibility of meat proteins to subsequent gastrointestinal enzymatic hydrolysis. Further, no information is available about the effect of PEF processing on the release of free amino acids and minerals during gastrointestinal digestion.

PEF processing has also been reported to affect the concentration of minerals of the meat. The concentration of Fe, K and P was significantly decreased by PEF treatment of meat with greater loss observed in high PEF (10 kV, 200 Hz and 20  $\mu$ s) than low PEF (2.5 kV, 200 Hz and 20  $\mu$ s) treatment (Khan et al., 2017a). The concentration of Ca, Na and Mg was also reported to decrease whereas that of Cr increased in beef muscles treated with PEF. Higher Ni and Cu concentrations were reported in PEF-treated chicken breast (Khan et al., 2018). In contrast to above studies, Khan et al. (2017b) observed no effect on the concentration of P, K, Fe and Zn of PEF treated chicken breast. PEF processing has a differential effect on mineral content, which depends on the type of meat and the possible release of elements from the PEF electrodes (Bhat et al., 2018a). Several studies have suggested the migration of minerals from the electrodes of standard PEF systems to the treated food (Khan et al., 2017a, b; Bhat et al., 2018a). While studying the migration of metals such as Fe, Cr, Ni and Mg from stainless steel electrodes of PEF treatment chamber, Pataro et al. (2014) reported an increased metal release with an increase in total specific energy input. Further, given the fact that PEF causes electroporation in meat and has potential to enhance cellular permeability and mass transfer processes, it could have an influence on the release of minerals from meat during gastrointestinal digestion (Bhat, Morton,

Mason, & Bekhit, 2018f). The bio-accessibility of minerals, fraction that becomes available for absorption after getting released from meat matrix, depends on several factors such as degree of solubilization, ionization, chemical form, presence of other substances, thermal treatment, etc (da Silva et al., 2017).

While several reports show a texture modification in meat as a result of PEF treatment (Bhat et al., 2018a), the impact of such changes on the digestibility of meat proteins and availability of nutrients during digestion of meat is largely unknown and needs immediate scientific attention. Given the fact that PEF has been reported to affect the structure and quality of the proteins leading to modifications like unfolding, aggregation, and changes in their structural and functional properties (Han, Cai, Cheng, & Sun, 2018; Liu, Oey, Bremer, Silcock, & Carne, 2018; Zhang, Wang, Jiang, & Qian, 2017), it may influence the digestion of meat proteins. In the light of all the above facts, the objective of this study was to evaluate the effect of PEF treatment on *in vitro* simulated gastrointestinal digestion of venison *Longissimus dorsi*.

### 9.3 Materials and methods

Meat samples were prepared as per the method described in section 2.2.5. The meat samples were treated with PEF as per the method described in section 2.2.5.1. *In vitro* protein digestion, *in vitro* protein digestibility (%), protein content (%), soluble protein (%), free amino acid analysis and mineral profile analysis were performed according to the methods described from section 2.5.1 to 2.5.7. Protein concentration, SDS-PAGE and analysis of the protein bands was done by following the methods described in section 2.4.2 and 2.4.4. Statistical analysis of the data was performed according to the method described in section 2.7.2.

### 9.4 Results and discussion

#### 9.4.1 *In vitro* protein digestibility (%)

The mean values for digestibility (%) for PEF treated samples of deer *Longissimus dorsi* viz. T<sub>1</sub> (2.5 kV, 50 Hz) and T<sub>2</sub> (10 kV, 90 Hz) subjected to *in vitro* simulated gastrointestinal protein digestion are presented in Table 9.1. A significant ( $P < 0.05$ ) impact of PEF processing was observed and significantly higher values were observed for the PEF-treated samples subjected to gastrointestinal digestion. The mean digestibility (%) of T<sub>1</sub> and T<sub>2</sub> samples was 92.81% and 93.35%, respectively. Similar results were reported by Tavares, Dong, Yang, Zeng, & Zhao (2018) for the protein digestibility (%) of hairtail (*Thichiurus lepturus*) fillets subjected to *in vitro* protein digestion. Total amino acid digestibility of 89.1%, 89.1% and 90.4% have been reported for beef loin, pork loin and pollock fillet, respectively,

subjected to *in vitro* digestion (Faber et al., 2010). Digestibility of a protein and supply of essential amino acids are the two significant factors in protein evaluation (Tavano, Neves, & Júnior, 2016). Susceptibility of meat proteins to the action of enzymes during gastrointestinal digestion depends on structural changes and modifications that have potential to influence the protease active sites (Simonetti, Gambacorta, & Perna, 2016). A partial unfolding of protein structure enhances its protease susceptibility whereas greater protein-unfolding and massive aggregation will lead to a reduced proteolytic susceptibility (Simonetti, Gambacorta, & Perna, 2016; Promeprat et al., 2010; Gatellier, & Santé-Lhoutellier, 2009). PEF processing has the potential to induce structural changes in protein molecules (Liang, Cheng, & Wang, 2018) causing unfolding and aggregation of the proteins which leads to subsequent disruption of the secondary structure (Giteru, Oey, & Ali, 2018). While heating of meat has also been reported to increase the digestibility by inducing structural changes in meat proteins (Dominguez-Hernandez, Salasevicieneb, & Ertbjerg, 2018; Qi et al., 2018), the average temperature difference observed in our study during PEF treatment was 2.4 °C and 5.3 °C for treatment T<sub>1</sub> and T<sub>2</sub>, respectively. Given that the samples were incubated at 37 °C during gastrointestinal digestion, there is no possibility that the increase in digestibility observed in our study are due to thermal effects. By causing partial unfolding of the proteins (Liu, Oey, Bremer, Carne, & Silcock, 2017b), PEF processing has the potential to positively influence the hydrolysis of proteins by gastrointestinal enzymes and to increase protein digestion (Liu, Oey, Bremer, Silcock, & Carne, 2018). The electric field strength and total energy input, which determine the partial unfolding of the proteins, are the two processing parameters which play a central role in intermolecular interactions and are decisive factors for PEF-induced protein denaturation and aggregation (Liu, Oey, Bremer, Carne, & Silcock, 2017b).

**Table 9.1 Effect of pulsed electric field on protein digestibility of deer *Longissimus dorsi* during *in vitro* simulated gastrointestinal protein digestion (Mean ± SE)\***

Parameter	Control	T <sub>1</sub> (2.5 kV)	T <sub>2</sub> (10 kV)
<i>In vitro</i> protein digestibility (%)	91.69±0.35 <sup>a</sup>	92.81±0.40 <sup>ab</sup>	93.35±0.24 <sup>b</sup>

\*Mean ± SE with different superscripts in a row wise differ significantly ( $P < 0.05$ )

T<sub>1</sub> = Samples treated with PEF at 2.5 kV, 50 Hz, 20 μs

T<sub>2</sub> = Samples treated with PEF at 10 kV, 90 Hz, 20 μs

n = 3 for each treatment

By inducing protein structural changes, non-thermal processing technologies such as high-pressure and ultrasound have been reported to modify the susceptibility of proteins, such as egg white proteins, to subsequent enzymatic hydrolysis by gastrointestinal enzymes (Stefanović et al., 2017; Stefanović et al., 2014). Prior ultrasound treatment (high intensity) of egg white proteins has been reported to increase their hydrolysis during subsequent enzymatic hydrolysis by pepsin (Jovanović et al., 2016). A

similar effect has been demonstrated by prior treatment of egg white proteins with high pressure resulting in a greater degree of enzymatic hydrolysis (Hoppe, Jung, Patnaik, & Zeece, 2013). The authors of both the studies reported that the increase in the enzymatic hydrolysis was due to processing-induced unfolding of proteins which was different from that induced by thermal processing.

#### 9.4.2 Soluble protein (%)

The mean values for soluble protein (%) for PEF treated samples of deer *Longissimus dorsi* viz. T<sub>1</sub> (2.5 kV, 50 Hz) and T<sub>2</sub> (10 kV, 90 Hz) subjected to *in vitro* simulated gastrointestinal protein digestion are presented in Table 9.2. A significant ( $P < 0.05$ ) increasing trend was observed with the increasing digestion time in all the samples with highest values observed for samples collected on 180 minutes of intestinal phase of digestion. Significantly ( $P < 0.05$ ) higher values were observed for all the samples during intestinal phase of digestion in comparison to gastric phase, suggesting greater enzymatic hydrolysis of proteins has occurred during intestinal phase of digestion. Samples treated with PEF showed significantly ( $P < 0.05$ ) higher values for soluble protein (%) for the samples collected on 180 minutes of intestinal phase of digestion. Samples treated with PEF also showed higher values than control on all other intervals of gastrointestinal digestion, however, no significant ( $P > 0.05$ ) differences were observed. Similar increase in the soluble protein (%) was reported by Kaur et al. (2016) for the beef muscles treated with high pressure during simulated gastrointestinal digestion. Jung, de Lamballerie-Anton, & Ghoul (2000) also observed an increase in the soluble protein (%) for beef muscles treated with high pressure in comparison to untreated control.

**Table 9.2 Effect of pulsed electric field on the soluble protein (%) of deer *Longissimus dorsi* during *in vitro* simulated gastrointestinal protein digestion (Mean  $\pm$  SE)\***

Treatments	<i>In vitro</i> gastric digestion			<i>In vitro</i> intestinal digestion	
	0 Minutes	30 Minutes	60 Minutes	120 Minutes	180 Minutes
Control	8.14 $\pm$ 0.96 <sup>a</sup>	9.42 $\pm$ 0.32 <sup>a</sup>	10.59 $\pm$ 1.09 <sup>a</sup>	22.44 $\pm$ 0.35 <sup>b</sup>	22.49 $\pm$ 0.94 <sup>bA</sup>
T <sub>1</sub> (2.5 kV)	8.37 $\pm$ 0.64 <sup>a</sup>	10.46 $\pm$ 0.63 <sup>a</sup>	11.28 $\pm$ 0.44 <sup>a</sup>	25.37 $\pm$ 2.08 <sup>b</sup>	26.06 $\pm$ 1.56 <sup>bAB</sup>
T <sub>2</sub> (10 kV)	8.47 $\pm$ 0.83 <sup>a</sup>	10.65 $\pm$ 0.70 <sup>ab</sup>	11.97 $\pm$ 0.76 <sup>b</sup>	26.39 $\pm$ 0.71 <sup>c</sup>	28.04 $\pm$ 1.06 <sup>cB</sup>

\*Mean  $\pm$  SE with different superscripts in a row wise (lower case alphabet) and column wise (upper case alphabet) differ significantly ( $P < 0.05$ )

T<sub>1</sub> = Samples treated with PEF at 2.5 kV, 50 Hz, 20  $\mu$ s

T<sub>2</sub> = Samples treated with PEF at 10 kV, 90 Hz, 20  $\mu$ s

n = 3 for each treatment

PEF-treatment has been reported to induce protein denaturation (Liu, Oey, Bremer, Carne, & Silcock, 2017b) and digestive enzymes have been reported to act more effectively on denatured proteins (Kaur et al., 2016). Increased proteolysis of denatured proteins is well reported in non-meat proteins and has been attributed to the increased susceptibility of proteins by greater exposure of previously shielded peptide bonds to enzymatic attack (Kaur, Maudens, Haisman, Boland, & Singh, 2014; Grune, Reinheckel, Joshi, & Davies, 1995; Davies, Delsignore, & Lin, 1987). For instance, the susceptibility of egg white proteins to subsequent enzymatic hydrolysis has been reported to increase dramatically following application of either thermal or non-thermal processes, which induce structural changes such as protein unfolding and aggregation, enhancing their susceptibility to gastrointestinal enzymes, such as pepsin, trypsin and chymotrypsin (Stefanović et al., 2017; Hoppe, Jung, Patnaik, & Zeece, 2013; van der Plancken, Delattre, Indrawati, & Hendrickx, 2004). The availability of the hydrolysis sites on meat proteins, particularly myosin, for gastrointestinal enzymes could be highly influenced by structural modifications induced by pre-treatments like high pressure and cooking (Liu, & Xiong, 2000) and possibly by PEF-processing (Bhat et al., 2018a).

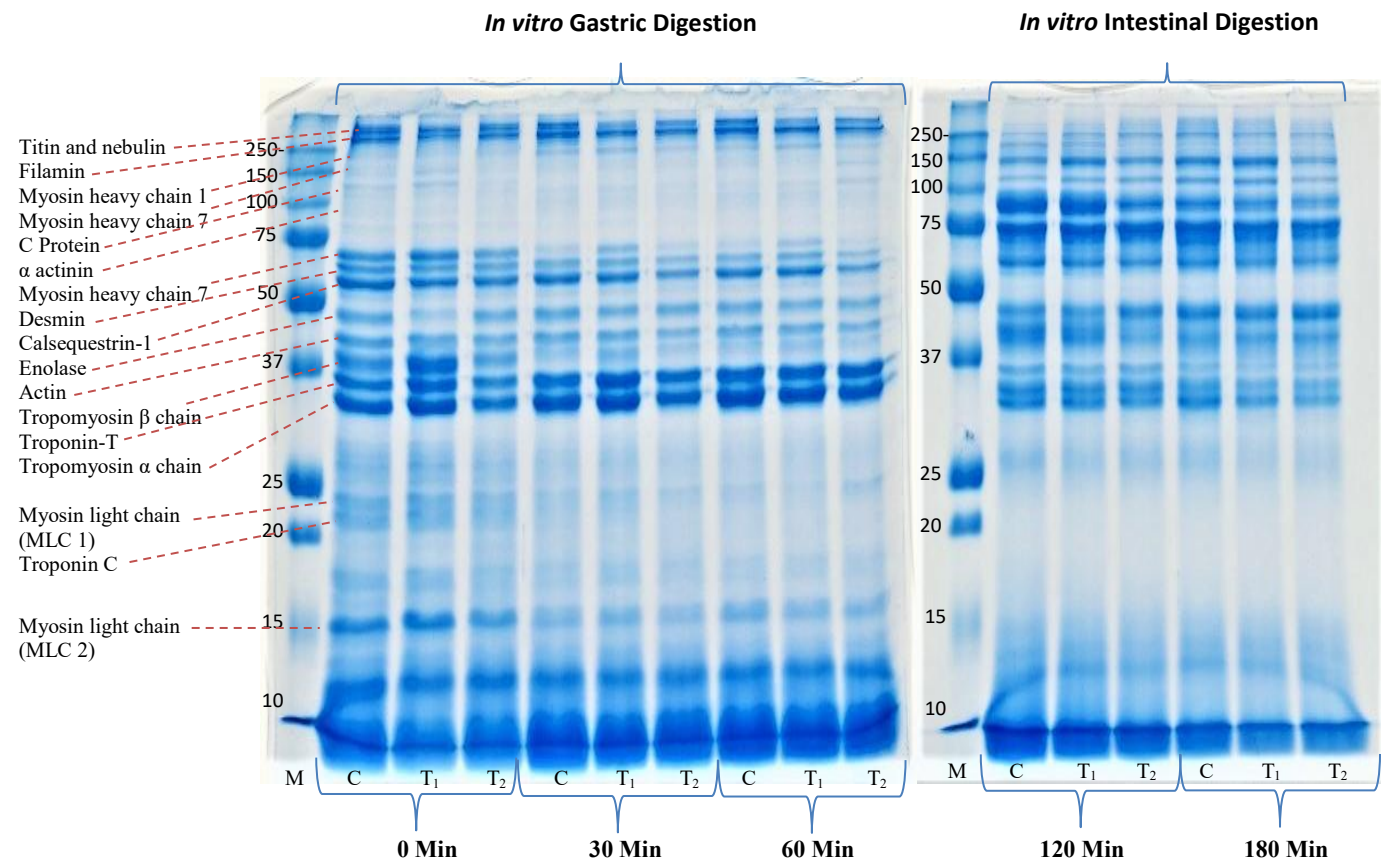
#### **9.4.3 Protein profile (SDS-PAGE)**

The effect of PEF treatment on the protein profile of deer *Longissimus dorsi* digests obtained at different intervals of simulated gastrointestinal protein digestion is presented in SDS-PAGE gel electrophoretogram in Figure 9.1. The major identified bands include titin and nebulin (>250 kD), filamin (>250 kD), myosin heavy chain MHC 1 (>220 kD), myosin heavy chain MHC 7 (>220 kD), C protein (135 kD),  $\alpha$ -actinin (95 kD), desmin (53 kD), calsequestrin 1 (52 44-55 kD), enolase (46 kD), actin (42 kD), tropomyosin  $\beta$ -chain (37 kD), troponin-T (35 kDa), tropomyosin- $\alpha$ -chain (33 kDa), myosin light chain MLC<sub>1</sub> (22 kDa), troponin-C (19 kDa) and myosin light chain MLC<sub>2</sub> (16 kDa). The electrophoretic patterns of both control and PEF-treated samples showed a range of bands in gastric and intestinal phase of digestion with some minor differences. In general, more bands of low molecular weight (25 to 10 kD) appeared in the gastric phase of digestion which disappeared during the intestinal phase of digestion. This indicates that more digestion has occurred during intestinal phase of digestion and the peptides produced by the pepsin during gastric digestion of the muscle were further hydrolysed by pancreatin into lower molecular weight polypeptides or into free amino acids. Some low molecular weight proteins probably may have solubilized at alkaline pH. In addition, a few bands of higher molecular weight (250 to 75 kD) appeared in the gastric phase of digestion whereas far more bands of higher molecular weight in the same range (250 to 75 kD) appeared during the intestinal phase of digestion. This indicates that more digestion of the muscle has occurred during intestinal phase of digestion by the pancreatin causing the release of more polypeptides of higher molecular weight from the samples.

The intensity of the various bands ranging from 53 kD to 33 kDa, corresponding to desmin, calsequestrin 1, myosin heavy chain MHC-7, enolase, actin, tropomyosin  $\beta$ -chain, troponin-T, and tropomyosin- $\alpha$ -chain, during gastric phase of digestion was lower in case of PEF treated samples, particularly for T<sub>2</sub> (10 kV) samples, in comparison to control, suggesting greater digestion has occurred in PEF processed samples. The differences in the gastric digestion products between control and PEF-treated samples became more obvious when the samples were digested for 60 minutes.

Intensities of all the bands with molecular weight of >25 kD, corresponding to the major meat proteins, decreased with an increase in PEF-treatment intensity, suggesting greater digestion of these proteins and peptides with an increase in electric field intensity. Recently, PEF processing of egg white proteins has been reported to enhance their susceptibility to gastrointestinal digestion (Liu, Oey, Bremer, Silcock, & Carne, 2018). The PEF processing may have increased the digestion of the proteins by inducing various protein structural changes, such as denaturation and unfolding, and by increasing the permeability of membrane bound muscle fibers thereby increasing the diffusion of gastrointestinal enzymes into the muscle mass.

The peptic hydrolysates produced during the gastric phase of digestion by the action of pepsin were further digested during intestinal phase. In addition, some new bands appeared in both control and PEF-treated samples, corresponding to peptides with molecular weight of 70 kD to 250 kD, which were not present before intestinal digestion. A similar pattern as that of gastric digestion was also observed for intestinal phase of digestion and the intensity of most of the bands with molecular weight of >25 kD, corresponding to the major meat proteins, decreased in PEF-treated samples with lowest intensities observed for T<sub>2</sub> (10 kV) samples after completion of 180 minutes of intestinal phase of digestion. These results clearly suggest greater digestion of the PEF treated proteins and peptides corresponding to these bands during intestinal phase of digestion. Other non-thermal technologies, such as high-pressure processing, have been reported to increase the digestion of meat proteins during *in vitro* gastrointestinal simulations (Kaur et al., 2016). Overall, analysis of SDS-PAGE electrophoretogram demonstrated that PEF processing affected digestion kinetics *in vitro* by modifying the protein profile of the meat digests. It led to faster and greater digestion of proteins and polypeptides during gastric and intestinal digestion, which may improve their bioavailability. These results suggest that PEF-induced protein denaturation and membrane permeabilization led to enhanced susceptibility of meat proteins towards pepsin and pancreatin action, resulting in faster digestion of these proteins.



**Figure 9.1** Effect of pulsed electric field on the protein profile (SDS-PAGE) of deer *Longissimus dorsi* subjected to *in vitro* simulated gastrointestinal digestion [M = marker, C = control, T<sub>1</sub> = 2.5 kV (50 Hz), T<sub>2</sub> = 10 kV (90 Hz), 15 µg samples were loaded in each lane, Protein identification was derived from that reported by Bhat et al., (2018c); Kaur et al., (2016) and Kaur et al., (2014)]

#### 9.4.4 Free amino acid analysis

The effect of PEF treatment on the release of free amino acids during simulated gastrointestinal digestion of deer *Longissimus dorsi* is presented in Table 9.3. A significant ( $P < 0.05$ ) impact of PEF processing was observed on the release of free amino acids during gastrointestinal digestion. Significant ( $P < 0.05$ ) influence of PEF-processing was observed on the release of free amino acid lysine and higher mean values were observed for the PEF treated samples in comparison to control. The mean values observed for almost all the free amino acids were higher in PEF treated samples than control.

*In vitro* simulated gastrointestinal digestive model is a widely recognized approach to obtain preliminary observations in determining bioavailability of the nutrients during gastrointestinal digestion. Digestibility of a protein and the bioavailability of its amino acids are the two most important factors that defines the food protein quality (Lorieau et al., 2018). Quantified by the amount of nitrogen that is absorbed by the organism, protein digestibility is analysed considering the ability of a protein to be absorbed in the intestinal tract after getting hydrolysed by gastrointestinal proteases. This process is influenced by the susceptibility of the proteins to enzymatic hydrolysis by gastrointestinal proteases as much as the ability to absorb the digested end products (Tavano, Neves, & Júnior, 2016). Absorption of peptides as large as octapeptides have been demonstrated (Pappenheimer, Dahl, Karnovsky, & Maggio, 1994), however, the absorption process usually occurs in the form of free amino acids or smaller peptides containing few amino acid residues, such as di-peptides, tri-peptides or tetra-peptides (Jahan-Mihan, Luhovyy, El Khoury, & Anderson, 2011; Pappenheimer, Dahl, Karnovsky, & Maggio, 1994). Several protein modifications that occur during thermal and non-thermal treatments, that can influence the susceptibility of proteins to enzymatic hydrolysis, have potential to affect the release of amino acids during gastrointestinal digestion (Kaur, Maudens, Haisman, Boland, & Singh, 2014; Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008; Morzel, Gatellier, Sayd, Renerre, & Laville, 2006; Stadtman, 1993). PEF processing, that is responsible for inducing several structural modifications in proteins (Liu, Oey, Bremer, Silcock, & Carne, 2018; Liu, Oey, Bremer, Carne, & Silcock, 2017b), has the potential to positively influence the hydrolysis of proteins and to increase the release of free amino acids during gastrointestinal digestion (Liu, Oey, Bremer, Silcock, & Carne, 2018).

By influencing the matrix structure through denaturation and unfolding of the proteins, the PEF processing can modify the digestion and absorption kinetics of the nutrients. The degree of denaturation determines the digestibility of the proteins (Lorieau et al., 2018; Jin et al., 2016). The enzymatic proteolysis of a protein substrate is only possible when the active site of a protease can adapt and bind to the specific stereochemistry of the polypeptide chain (Fontana, Poverino de Laureto, De Filippis, Scaramella, & Zambonin, 1997). This is easy to achieve when polypeptides have



very flexible and open structures which can be achieved during denaturation which induces unfolding of the proteins. Native or undenatured proteins have a more closed structure which inhibits the enzymes access to potential cleavage sites, making them more resistant to enzymatic hydrolysis by gastrointestinal proteases such as pepsin (Lorieau et al., 2018; Nguyen, Bhandari, Cichero, & Prakash, 2015; Lundin, Golding, & Wooster, 2008).

**Table 9.3 Effect of pulsed electric field on the release of free amino acids during *in vitro* simulated gastrointestinal digestion of deer *Longissimus dorsi* (Mean  $\pm$  SE)\***

Free amino acids ( $\mu$ M/litre)	PEF Treatment		
	Control	T <sub>1</sub> (2.5 kV)	T <sub>2</sub> (10 kV)
Alanine	830.49 $\pm$ 8.00	847.52 $\pm$ 34.42	889.98 $\pm$ 37.95
Arginine	1364.3 $\pm$ 65.37	1377.8 $\pm$ 32.24	1516.9 $\pm$ 40.84
Asparagine	358.61 $\pm$ 19.27	339.44 $\pm$ 18.74	370.55 $\pm$ 5.84
Aspartic acid	85.99 $\pm$ 6.87	87.88 $\pm$ 11.76	94.51 $\pm$ 3.43
Cysteine	179.23 $\pm$ 10.83	180.81 $\pm$ 2.80	201.61 $\pm$ 5.73
Glutamic acid	265.09 $\pm$ 16.22	249.03 $\pm$ 19.49	286.71 $\pm$ 2.07
Glutamine	892.77 $\pm$ 25.67	895.03 $\pm$ 51.00	1024.50 $\pm$ 42.16
Glycine	279.72 $\pm$ 16.64	264.74 $\pm$ 9.42	278.15 $\pm$ 5.68
Histidine	96.91 $\pm$ 5.26	97.66 $\pm$ 1.78	106.68 $\pm$ 1.47
Isoleucine	522.78 $\pm$ 22.11	526.27 $\pm$ 16.82	575.88 $\pm$ 10.29
Leucine	2036.5 $\pm$ 88.86	2078.9 $\pm$ 79.02	2244.4 $\pm$ 40.69
Lysine	1286.6 $\pm$ 65.90 <sup>a</sup>	1323.1 $\pm$ 5.83 <sup>ab</sup>	1450.4 $\pm$ 30.80 <sup>b</sup>
Methionine	370.45 $\pm$ 21.73	369.39 $\pm$ 24.61	407.37 $\pm$ 11.12
Phenylalanine	1085.1 $\pm$ 46.18	1102.9 $\pm$ 43.19	1180.1 $\pm$ 17.87
Proline	118.65 $\pm$ 11.10	118.07 $\pm$ 4.77	117.98 $\pm$ 7.03
Serine	286.19 $\pm$ 17.68	259.43 $\pm$ 23.03	293.30 $\pm$ 2.80
Taurine	77.66 $\pm$ 3.71	95.83 $\pm$ 12.26	90.25 $\pm$ 3.88
Threonine	285.63 $\pm$ 18.46	267.49 $\pm$ 22.54	302.44 $\pm$ 8.40
Tryptophan	547.26 $\pm$ 30.87	561.70 $\pm$ 32.34	627.29 $\pm$ 12.84
Tyrosine	1027.6 $\pm$ 55.57	1033.6 $\pm$ 27.86	1134.3 $\pm$ 3.31
Valine	640.62 $\pm$ 32.68	620.51 $\pm$ 43.80	691.39 $\pm$ 14.92

\*Mean  $\pm$  SE with different superscripts in a row differ significantly ( $P < 0.05$ )

T<sub>1</sub> = Samples treated with PEF at 2.5 kV, 50 Hz, 20  $\mu$ s

T<sub>2</sub> = Samples treated with PEF at 10 kV, 90 Hz, 20  $\mu$ s

n = 3 for each treatment

#### 9.4.5 Mineral profile analysis

The effect of PEF treatment on the release of various minerals during simulated gastrointestinal digestion of deer *Longissimus dorsi* is presented in Table 9.4. No significant ( $P > 0.05$ ) impact of PEF-processing was observed on the release of various minerals from the muscle samples during gastrointestinal digestion. Several studies have been reported elucidating the impact of PEF processing on the concentration of the minerals in beef muscles and chicken (Khan et al., 2018; Khan et al., 2017a, b). A significant decrease in the concentration of Fe, K and P was reported in beef muscles subjected to low (2.5 kV, 200 Hz and 20  $\mu$ s) and high PEF (10 kV, 200 Hz and 20  $\mu$ s) treatment with greater loss observed in these minerals in high PEF than low PEF treated samples (Khan et al., 2017a).

**Table 9.4 Effect of pulsed electric field on the release of minerals during *in vitro* simulated gastrointestinal digestion of deer *Longissimus dorsi* (Mean  $\pm$  SE)**

Minerals ( $\mu$ g/mL)	PEF Treatment		
	Control	T <sub>1</sub> (2.5 kV)	T <sub>2</sub> (10 kV)
Aluminium (Al)	0.327 $\pm$ 0.01	0.321 $\pm$ 0.004	0.318 $\pm$ 0.0005
Arsenic (As)	0.01 $\pm$ 0.002	0.01 $\pm$ 0.002	0.01 $\pm$ 0.001
Boron (B)	0.569 $\pm$ 0.04	0.561 $\pm$ 0.03	0.565 $\pm$ 0.04
Calcium (Ca)	2.78 $\pm$ 0.09	2.66 $\pm$ 0.15	2.68 $\pm$ 0.10
Chromium (Cr)	0.02 $\pm$ 0.0002	0.02 $\pm$ 0.0003	0.02 $\pm$ 0.0002
Copper (Cu)	0.045 $\pm$ 0.01	0.050 $\pm$ 0.004	0.043 $\pm$ 0.005
Iron (Fe)	0.59 $\pm$ 0.07	0.74 $\pm$ 0.09	0.68 $\pm$ 0.04
Potassium (K)	274.58 $\pm$ 7.27	273.07 $\pm$ 2.76	269.84 $\pm$ 4.85
Magnesium (Mg)	15.56 $\pm$ 0.64	16.15 $\pm$ 0.14	15.66 $\pm$ 0.24
Manganese (Mn)	0.016 $\pm$ 0.002	0.016 $\pm$ 0.001	0.017 $\pm$ 0.0004
Nickle (Ni)	< 1.17 ppb	< 1.17 ppb	< 1.17 ppb
Sodium (Na)	1847.9 $\pm$ 12.91	1836.3 $\pm$ 12.10	1831.8 $\pm$ 7.88
Phosphorus (P)	459.23 $\pm$ 7.96	457.19 $\pm$ 3.23	458.85 $\pm$ 4.06
Sulphur (S)	94.01 $\pm$ 2.12	94.90 $\pm$ 2.00	97.36 $\pm$ 0.92
Zinc (Zn)	2.88 $\pm$ 0.55	2.42 $\pm$ 0.19	2.67 $\pm$ 0.24

Ni, Pb, Cd, and Se were below detection levels

T<sub>1</sub> = Samples treated with PEF at 2.5 kV, 50 Hz, 20  $\mu$ s

T<sub>2</sub> = Samples treated with PEF at 10 kV, 90 Hz, 20  $\mu$ s

n = 3 for each treatment

While studying the effect of low (2.5 kV, 200 Hz and 20  $\mu$ s) and high PEF (10 kV, 200 Hz and 20  $\mu$ s) treatment on the concentration of 40 macro- and micro-minerals in beef muscles and chicken breast, Khan et al. (2018) reported a significant decrease in the concentration of Ca, Na and Mg whereas a significant increase was observed in the concentration of Cr in beef muscles. Significantly higher Ni concentration was also observed in chicken breast treated with high PEF in comparison to control. These studies have suggested the possible migration of minerals from the electrodes of PEF systems to the treated meat. However, the increase in Cr and Ni concentration reported in these studies were within the safety limits (Bhat et al., 2018a; EPA, 2016; Meditext, 2005). It is worth noting here that the above studies have used different method for estimation of minerals and have determined the concentration of various minerals in the fresh meat itself whereas in our study we have determined the concentration of these minerals in the liquid digesta obtained after the gastrointestinal digestion of the muscle. In our study, no significant ( $P > 0.05$ ) decrease was observed in the concentration of Fe, K, P, Ca, Na and Mg in the digesta obtained from PEF-treated samples subjected to gastrointestinal digestion. No significant ( $P > 0.05$ ) increase was observed in the Cr levels of PEF-treated samples either. The concentration of Ni was below the detection level in the digesta of all the samples. Based on our results, PEF processing has no negative consequences on the release of minerals during gastrointestinal digestion of deer meat, generally regarded as an excellent source of minerals such as Fe and Zn.

## 9.5 Conclusions

Pulsed electric field processing showed a positive influence on the *in vitro* simulated protein digestion process of deer *Longissimus dorsi*. Prior PEF-processing of meat caused a significant ( $P < 0.05$ ) increase in the *in vitro* protein digestibility (%), soluble protein (%), and availability of free amino acids, indicating the potential of this technology for the development of muscle foods with high nutritive value and digestibility. Unlike previous reports, which found a significant effect of PEF on mineral concentration of the fresh meat, no significant ( $P > 0.05$ ) impact of PEF processing was observed on the release of minerals, such as Fe, Zn, Cu, Cr, Mg, K, and P, during gastrointestinal digestion, suggesting that PEF processing does not impair the availability of the minerals from meat during digestion process. This study has opened a new area of research for PEF in meat processing and technology focused on the development of novel protein structures and futuristic foods with improved digestibility.

## Chapter 10

# Pulsed electric field: A new way to improve digestibility of cooked beef

This chapter is published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., Jayawardena, R., & Bekhit, A. E. D. A. (2019). Pulsed electric field: A new way to improve digestibility of cooked meat. *Meat Science*, 155, 79-84.

### 10.1 Abstract

The effect of pulsed electric field (PEF) on *in vitro* simulated gastrointestinal protein digestion of cooked beef *Semimembranosus* was elucidated. PEF treated ( $T_1$ , 10 kV, 20 Hz, 20  $\mu$ s) samples along with control were cooked (core temperature of 75 °C) and subjected to *in vitro* simulated gastrointestinal digestion. Samples were analysed for protein digestibility (%), protein profile (SDS-PAGE), soluble protein (%), free amino acid analysis and mineral profile. PEF treatment led to significantly higher ( $P < 0.05$ ) values for protein digestibility (%) and soluble protein (%). No significant ( $P > 0.05$ ) effect of PEF was recorded on the release of free amino acids and various minerals during gastrointestinal digestion. However, numerically higher values were observed for all the free amino acids for PEF treated samples. PEF treatment modified the protein profile of the meat digests and had a positive impact on *in vitro* digestion kinetics causing greater and faster digestion of proteins during *in vitro* gastrointestinal simulation.

**Keywords:** *In vitro* protein digestion; pulsed electric field; beef; digestibility (%); mineral profile; protein profile; free amino acid analysis

### 10.2 Introduction

One of the results of our longer average lifespan is an increased level of muscle wasting or sarcopenia and the subsequent inability of older people to maintain an active lifestyle (Lynch, & Koopman, 2018). Meat is an excellent source of amino acids and minerals that are required for muscle maintenance (Phillips, 2012) but is sometimes difficult to digest due to compact structure or high connective tissue content. This is particularly true for the cheaper meat available to those on limited budgets. This meat is often from non-prime animals, such as culled dairy cows, rich in connective tissues, tough to eat and difficult to digest. The result is a product with NPU (Net Protein Utilization) as low as 0.5 compared with a value of 0.75 - 0.8 for good quality meat (Bender, 1992). This research considers the use of a

novel technology, pulsed electric field (PEF), to improve the digestibility of *Semimembranosus* muscle from culled dairy cows.

Our knowledge and understanding about the digestion of high-protein solid food matrices, such as meat, is limited (Luo, Boom, & Janssen, 2015) and better understanding of this area would facilitate in maximising the benefits from proposed new food manipulations and processing technologies. Processes that modify the properties of the proteins are likely to affect the digestion of meat and influence the release and subsequent bioavailability of nutrients during the digestive process (Kaur et al., 2016; Kaur, Maudens, Haisman, Boland, & Singh, 2014). Food processing techniques such as high-pressure processing, ultrasound, PEF and several cooking methods have been observed to affect the digestion of proteins during *in vitro* simulated gastrointestinal digestion trials of meat (Zhang, Zhang, Chen, & Zhou, 2018; Bhat, Morton, Mason, & Bekhit, 2018a, f; Kaur et al., 2016; Kaur et al., 2014; Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008). These processes can affect the susceptibility of meat proteins to enzymatic hydrolysis by modifying the structural and functional properties of the proteins by inducing modifications such as unfolding, cross-linking and aggregation. Recent reports have shown pulsed electric field (PEF) causes structural and functional changes in food proteins (Bhat, Morton, Mason, & Bekhit, 2018a, d; Liu, Oey, Bremer, Silcock, & Carne, 2018; Han, Cai, Cheng, & Sun, 2018; Zhang, Wang, Jiang, & Qian, 2017). A suggested mechanism is that the ionization of various chemical groups or breaking of electrostatic interactions can modify the secondary and tertiary structure of proteins (Wei, Zeng, Tang, Jiang, & Liu, 2018) resulting in the loss of  $\alpha$ -helix and  $\beta$ -sheet (Zhao, Tang, Lu, Chen, & Li, 2014). Prior treatment with PEF can change the availability of the hydrolytic sites and alter the hydrolysis of meat proteins by gastrointestinal proteases during the digestion process. However, information is seldom available in the literature regarding the impact of PEF treatment on the digestion of meat proteins and subsequent release of peptides and amino acids. Information is also generally lacking on how PEF will affect the availability of minerals during the gastrointestinal digestion of cooked beef. PEF processing has been observed to change the concentration of various minerals within beef (Khan et al., 2018; Khan et al., 2017a, b) but the information is limited to the intact meat. How PEF will influence the release and bio-accessibility of such minerals during the gastrointestinal digestion of cooked beef remains unknown and warrants further research. Recently, the effect of PEF on *in vitro* digestibility of beef *Semimembranosus* was investigated and the results showed a significant impact of the treatment on the digestibility of the muscle (Bhat, Morton, Mason, & Bekhit, 2018e). The authors attributed the effect to the structural changes in proteins and electroporation induced by PEF treatment. However, that study used fresh uncooked meat and raised a serious question, what if cooking nullifies the effect of PEF? Cooking of meat has been reported to induce several structural changes in proteins (Kaur et al., 2014) and could either counter or enhance the mechanism by which PEF works and accordingly can nullify or enhance the effect of PEF treatment. Thus, the objective of the current research was to investigate the impact

of PEF treatment followed by cooking on the digestibility of the beef proteins and release of minerals using an *in vitro* gastrointestinal digestive simulation system. This knowledge will be helpful in evaluating the potential of PEF processing in the development of novel protein structures and muscle-based foods with improved digestibility.

### 10.3 Materials and methods

Meat samples were prepared as per the method described in section 2.2.3. The meat samples were treated with PEF as per the method described in section 2.2.3.1. Both control and PEF treated samples were cooked individually in plastic bags immersed in a water bath maintained at 80 °C to a core temperature of 75 °C as measured individually using Fluke type K temperature probes attached to Fluke 52 thermometer (Fluke Corp., Everett, WA). *In vitro* protein digestion, *in vitro* protein digestibility (%), protein content (%), soluble protein (%), free amino acid analysis and mineral profile analysis were performed according to the methods described from section 2.5.2 to 2.5.7. Protein concentration, SDS-PAGE and analysis of the protein bands was done by following the methods described in section 2.4.2 to 2.4.4. Statistical analysis of the data was performed according to the method described in section 2.7.3.

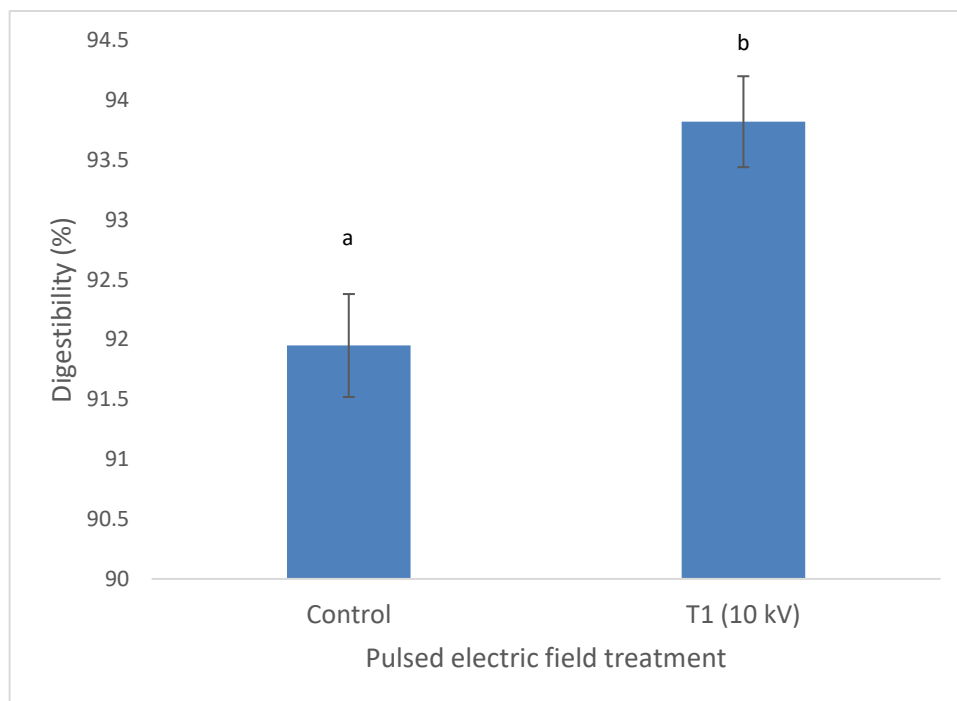
### 10.4 Results and discussion

#### 10.4.1 Protein digestibility (%)

Figure 10.1 presents the effect of PEF processing ( $T_1$ , 10 kV, 20 Hz, 20  $\mu$ s) on the digestibility (%) of cooked beef *Semimembranosus* subjected to *in vitro* simulated gastrointestinal protein digestion. Our model for *in vitro* gastrointestinal digestion was similar to the studies of Kaur et al. (2016); Kaur et al. (2014) and Bhat et al. (2018f) in that it did not include an oral phase and the pH and enzymatic conditions of the gastric and intestinal phases were simulated. PEF treatment of the *Semimembranosus* significantly ( $P < 0.05$ ) increased the digestibility (%) in comparison to control. Bioavailability of dietary proteins have been assessed by the extent of their digestibility during such *in vitro* gastrointestinal trials (Wen et al., 2015; Pennings et al., 2013). A similar range of values has been recorded for digestibility (%) of beef, pork, chicken and fish samples during comparable gastrointestinal digestion studies (Wen et al., 2015; Tavares, Dong, Yang, Zeng, & Zhao, 2018). The results of this study were in agreement with the findings of Bhat et al. (2018f) who observed a significant increase in the digestibility (%) of beef *Biceps femoris* treated with PEF during *in vitro* gastrointestinal digestion. The beef samples in that trial, however, were not cooked before digestive simulation. Cooking has been reported to have a significant impact on the digestibility of meat proteins

and bioavailability of various nutrients, such as amino acids and peptides, during gastrointestinal digestion (Kaur et al., 2014; Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008).

PEF processing causes breakdown of the cell membranes and local structural changes including conformational changes of protein structure (Töpfl, 2006). PEF treatment has also been observed to render muscle proteins more susceptible to denaturation (Bekhit, & Hopkins, 2014). One mechanism suggested for the effect of PEF processing is a gradual stretching or deformation of the protein molecules along the electric field due to non-homogenous charge distributions along the protein backbone (Freedman, Haq, Edel, Jemth, & Kim, 2013; Freedman et al., 2011). These changes destabilize the protein molecules and induce unfolding by disrupting the local electrostatic fields and electrostatic interactions of individual polypeptide chains (Zhao et al., 2014).



Different superscripts on columns differ significantly ( $P < 0.05$ )

**Figure 10.1 Effect of pulsed electric field on the protein digestibility of cooked beef *Semimembranosus* during *in vitro* simulated gastrointestinal digestion (Mean  $\pm$  SE)**

Processes which modify protein structure resulting in a more unfolded conformation can affect their susceptibility to hydrolysis by proteases during gastrointestinal digestion (Liu et al., 2018b; Liu et al., 2017b; Simonetti, Gambacorta, & Perna, 2016). These structural changes in proteins could result in a higher protein solubility and increased surface activity by exposure of hydrophobic domains (Primožic, Duchek, Nickerson, & Ghosh, 2018). PEF treatment has been reported to influence the digestive

kinetics of food proteins during gastrointestinal digestion (Bhat et al., 2018f; Giteru, Oey, & Ali, 2018; Liu, Oey, Bremer, Carne, & Silcock, 2017b).

The electric field strength and total energy input have a significant influence on the intermolecular interactions and determine the extent of denaturation and unfolding (Liu et al., 2017b). In our study, the electric field strength and energy density recorded during the PEF treatment was 0.60 kV/cm and 73.28 kJ/kg. Given that a critical field strength of 0.5 kV/cm has been reported for animal cells (Töpfl, 2006), the electric field strength used in our study might have delivered an effective electric field to induce the electroporation necessary to increase cell permeability. This may allow more diffusion of enzymes into the muscle cells during the digestion process (Bhat et al., 2018f). PEF treatment has been reported to accelerate the diffusion of salt in pork during curing (McDonnell, Allen, Chardonnerau, Arimi, & Lyng, 2014) and enhance mass transfer processes (Bhat, Morton, Mason, & Bekhit, 2018a). The PEF conditions used in these experiments were based on previous research and were intended to balance effectiveness with a limited increase in meat temperature.

#### 10.4.2 Soluble protein (%)

Table 10.1 presents the effect of PEF processing ( $T_1$ , 10 kV, 20 Hz, 20  $\mu$ s) on the soluble protein (%) of cooked beef *Semimembranosus* subjected to *in vitro* simulated gastrointestinal protein digestion.

**Table 10.1 Effect of pulsed electric field on the soluble protein (%) of cooked beef *Semimembranosus* during *in vitro* simulated gastrointestinal digestion (Mean  $\pm$  SE) \***

Digestion phase	Incubation time	Control	$T_1$ (10 kV)
<i>In vitro</i> gastric digestion	0 Minutes	11.75 $\pm$ 1.82 <sup>a</sup>	12.70 $\pm$ 0.61 <sup>a</sup>
	30 Minutes	15.91 $\pm$ 1.05 <sup>ab</sup>	17.54 $\pm$ 3.28 <sup>ab</sup>
	60 Minutes	18.62 $\pm$ 1.45 <sup>bc</sup>	20.86 $\pm$ 2.52 <sup>bc</sup>
<i>In vitro</i> intestinal digestion	120 Minutes	21.99 $\pm$ 1.12 <sup>cdA</sup>	26.38 $\pm$ 0.50 <sup>cdB</sup>
	180 Minutes	24.39 $\pm$ 1.72 <sup>dA</sup>	28.66 $\pm$ 0.56 <sup>dB</sup>

\*Mean  $\pm$  SE with different superscripts in a row wise (upper case alphabet) and column wise (lower case alphabet) differ significantly ( $P < 0.05$ )

$T_1$  = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu$ s

n = 3 for each treatment

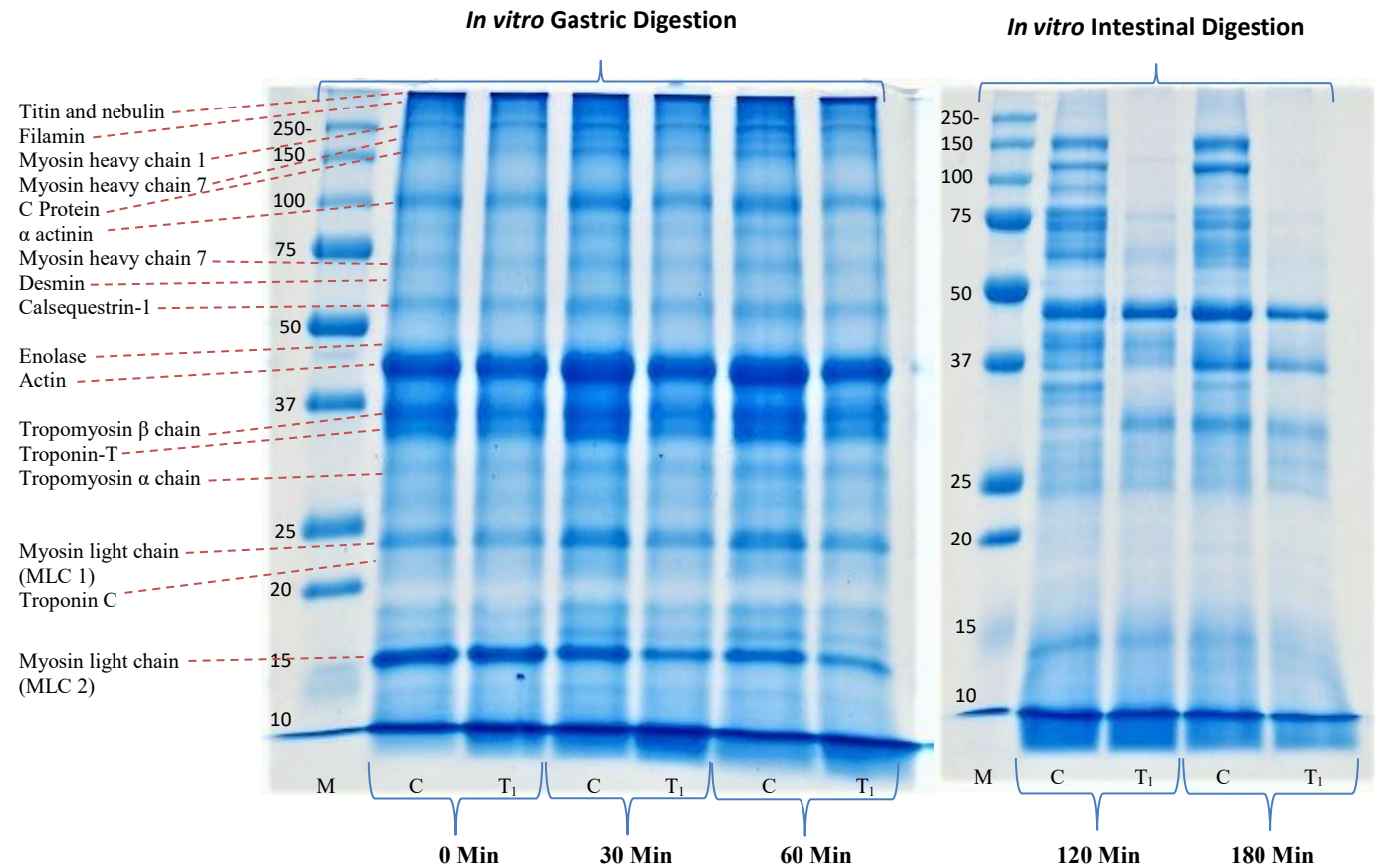
Soluble protein (%) values observed for the samples treated with PEF at 0, 30 and 60 min were not different from the control samples ( $P > 0.05$ ) during the gastric phase of digestion. However, a significant ( $P < 0.05$ ) effect was observed for PEF treatment at the end of gastrointestinal digestion and significantly higher values were recorded for PEF treated samples compared to the control samples.



These results clearly indicate that more enzymatic hydrolysis has occurred during the intestinal digestion phase in the PEF-treated samples causing more release of proteins during gastrointestinal digestion. Gastrointestinal proteases, such as pepsin and trypsin, have been observed to act more effectively on denatured and unfolded proteins (Kaur et al., 2016).

### 10.4.3 Protein profile (SDS-PAGE)

Figure 10.2 presents the effect of PEF processing ( $T_1$ , 10 kV-20 Hz-20  $\mu$ s) on the protein profile (SDS-PAGE gel electrophoretogram) of cooked beef *Semimembranosus* during *in vitro* protein digestion. As evident from the gel electrophoretogram, PEF treatment has an influence on the proteolytic pattern and digestion kinetics of the muscle during *in vitro* digestion process. There is a range of bands with visible differences between control and PEF treated samples throughout the digestion process. These differences between the bands, which became more prominent with increasing time of digestion, became more marked between the two phases of digestion. Throughout the gastric phase of digestion, one can see that most of the identifiable bands such as filamin (>250 kD), myosin heavy chain MHC 7 (>220 kD), myosin heavy chain MHC 1 (>220 kD), C protein (135 kD),  $\alpha$ -actinin (95 kD), desmin (53 kD), calsequestrin 1 (52 44-55 kD), enolase (46 kD), actin (42 kD), tropomyosin  $\beta$ -chain (37 kD), troponin-T (35 kDa), tropomyosin- $\alpha$ -chain (33 kDa), myosin light chain MLC<sub>1</sub> (22 kDa), troponin-C (19 kDa) and myosin light chain MLC<sub>2</sub> (16 kDa) are more intense for control samples in comparison to PEF treated samples. These differences clearly indicate that more enzymatic hydrolysis has occurred in PEF treated samples in comparison to control. These differences between control and PEF treated samples became more prominent during the intestinal phase of digestion. Most of the proteins which were present in the control samples during intestinal phase of digestion, including the large and abundant ones such as titin, nebulin, myosin and actin, either disappeared completely or became less intense in PEF treated samples. These findings are also in agreement with our results for digestibility (%) and soluble protein (%) as significant ( $P < 0.05$ ) differences were observed between control and PEF treated samples towards the end of gastrointestinal digestion. PEF treatment, which is believed to cause electroporation and increase membrane permeability in animal tissues, may have enhanced the diffusion of gastrointestinal enzymes into the membrane bound muscle fibres causing more hydrolysis of muscle proteins (Bhat et al., 2018a, b). Bhat et al. (2018f) also observed a greater and faster enzymatic hydrolysis for PEF treated samples in comparison to control during gastrointestinal digestion of raw beef *Biceps femoris*. The authors suggested that PEF-induced protein structural changes and improved membrane permeability led to a greater enzymatic hydrolysis by affecting the susceptibility of meat proteins by increasing the availability of hydrolytic sites for digestion.



**Figure 10.2** Effect of pulsed electric field on the protein profile (SDS-PAGE) of cooked beef *Semimembranosus* subjected to *in vitro* simulated gastro-intestinal digestion [M = marker, C = control, T<sub>1</sub> = 10 kV (20 Hz), 15 µg samples were loaded in each lane, Protein identification was derived from that reported by Bhat et al., (2018a); Kaur et al., (2016) and Kaur et al., (2014)]

Differences were also evident between the gastric and intestinal phase of digestion as several bands which were intense during gastric phase became less intense or disappeared during intestinal phase. Proteins which were hydrolysed during gastric phase by the enzyme pepsin were further hydrolysed into smaller peptides by pancreatin during the intestinal phase. In contrast, a few bands, particularly high molecular weight, appeared or became more intense during intestinal phase of digestion. These were presumably released by digestion from insoluble aggregates.

#### **10.4.4 Free amino acid analysis**

Table 10.2 presents the effect of PEF processing ( $T_1$ , 10 kV-20 Hz-20  $\mu$ s) on the release of free amino acids from cooked beef *Semimembranosus* during *in vitro* protein digestion. The concentration of various free amino acids viz. aspartic acid, glutamic acid, cysteine, asparagine, serine, glutamine, histidine, glycine, threonine, arginine, alanine, taurine, tyrosine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine, lysine and proline was determined in the liquid digesta after completion of gastrointestinal digestion. PEF treatment did not show any significant ( $P > 0.05$ ) impact on the release of free amino acids from the beef during gastrointestinal digestion, although mathematically higher values were observed for all amino acids for PEF processed samples suggesting a tendency of PEF to increase the hydrolysis of proteins. Digestion of proteins can lead to the generation of peptides of various sizes and not necessarily lead to free amino acids only.

Meat is regarded as a good source of quality protein for humans (Sayd et al., 2018) because of its high digestibility and a balanced composition of essential amino acids (Oberli et al., 2015). Since only free amino acids or short polypeptides (i.e. dipeptides and tripeptides) can be transported across the small intestine (MacFarlane, 2018), the end products of the digestion of proteins could make a difference and affect the absorption process in the small intestine (Wen et al., 2015). By modifying the interactions between amino acids (Yan-Yan et al., 2014), PEF treatment has potential to affect the microstructure and functional properties of proteins by ionizing the chemical groups, such as amino and carboxylic groups, affecting electrostatic interactions and causing unfolding of proteins by disrupting the secondary structure (Giteru et al., 2018). Favourable structural changes could be induced in the substrate proteins, such as meat proteins, to achieve full digestibility gains by using different PEF processing combinations.

**Table 10.2 Effect of pulsed electric field on the release of free amino acids during *in vitro* simulated gastrointestinal digestion of cooked beef *Semimembranosus* (Mean  $\pm$  SE)\***

Free amino acids ( $\mu\text{M}/\text{litre}$ )	PEF Treatment	
	Control	T <sub>1</sub> (10 kV)
Aspartic acid	61.31 $\pm$ 1.59	61.84 $\pm$ 4.08
Glutamic acid	144.46 $\pm$ 9.60	150.66 $\pm$ 8.81
Cysteine	108.32 $\pm$ 5.26	127.21 $\pm$ 8.27
Asparagine	75.28 $\pm$ 5.38	77.59 $\pm$ 1.75
Serine	94.81 $\pm$ 4.14	97.82 $\pm$ 2.39
Glutamine	527.23 $\pm$ 35.88	555.18 $\pm$ 40.94
Histidine	87.59 $\pm$ 7.81	87.62 $\pm$ 2.26
Glycine	130.28 $\pm$ 5.89	131.70 $\pm$ 8.61
Threonine	272.19 $\pm$ 10.46	272.42 $\pm$ 4.19
Arginine	675.33 $\pm$ 51.35	691.52 $\pm$ 23.29
Alanine	2186.8 $\pm$ 234	2229.2 $\pm$ 274
Taurine	417.42 $\pm$ 49.67	450.61 $\pm$ 71.32
Tyrosine	1104.8 $\pm$ 17.18	1115.0 $\pm$ 40.42
Valine	509.61 $\pm$ 7.73	523.91 $\pm$ 21.41
Methionine	304.49 $\pm$ 13.65	308.55 $\pm$ 5.37
Tryptophan	287.66 $\pm$ 20.89	288.76 $\pm$ 5.81
Phenylalanine	1084.3 $\pm$ 13.15	1092.4 $\pm$ 39.12
Isoleucine	583.43 $\pm$ 25.36	590.96 $\pm$ 9.71
Lysine	864.49 $\pm$ 40.12	864.79 $\pm$ 27.20
Leucine	2194.2 $\pm$ 94.43	2195.9 $\pm$ 55.94
Proline	61.35 $\pm$ 3.03	62.69 $\pm$ 0.71

T<sub>1</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu\text{s}$   
n = 3 for each treatment

#### 10.4.5 Mineral profile analysis

Table 10.3 presents the effect of PEF processing (T<sub>1</sub>, 10kV-20Hz-20 $\mu\text{s}$ ) on the release of various minerals from cooked beef *Semimembranosus* during *in vitro* protein digestion. Minerals, that are present in the meat matrix in the bound forms, start leaching out with the disruption of meat matrix by the peristaltic movements and the action of gastric enzymes at an acidic pH (Alminger et al., 2014).

Once released, these solubilized minerals which are present in ionic form, are assimilated in the small intestine by both passive and active transportation (Corte-Real, & Bohn, 2018). In general, meat is a good source of highly bioavailable iron, zinc and other minerals and any change in their composition or their release during digestion would be important both nutritionally and commercially (Bhat et al., 2018f).

**Table 10.3 Effect of pulsed electric field on the release of minerals during *in vitro* simulated gastrointestinal digestion of cooked beef *Semimembranosus* (Mean  $\pm$  SE)**

Minerals ( $\mu\text{g/mL}$ )	PEF Treatment	
	Control	T <sub>1</sub> (10 kV)
Aluminium (Al)	0.01 $\pm$ 0.0005	0.01 $\pm$ 0.001
Boron (B)	0.148 $\pm$ 0.0008	0.145 $\pm$ 0.003
Calcium (Ca)	3.62 $\pm$ 0.17	4.05 $\pm$ 0.37
Chromium (Cr)	0.011 $\pm$ 0.0007	0.012 $\pm$ 0.0004
Copper (Cu)	0.037 $\pm$ 0.001	0.038 $\pm$ 0.003
Iron (Fe)	0.278 $\pm$ 0.01	0.319 $\pm$ 0.02
Potassium (K)	240.67 $\pm$ 5.66	239.26 $\pm$ 0.84
Magnesium (Mg)	16.35 $\pm$ 0.24	16.45 $\pm$ 0.08
Manganese (Mn)	0.014 $\pm$ 0.002	0.014 $\pm$ 0.001
Sodium (Na)	2064.1 $\pm$ 24.35	2044.3 $\pm$ 27.05
Nickle (Ni)	0.002 $\pm$ 0.0001	0.001 $\pm$ 0.0001
Phosphorus (P)	476.8 $\pm$ 7.40	470.1 $\pm$ 4.18
Sulphur (S)	99.71 $\pm$ 1.39	99.81 $\pm$ 1.58
Selenium (Se)	0.021 $\pm$ 0.0003	0.017 $\pm$ 0.001
Zinc (Zn)	4.58 $\pm$ 0.20	4.49 $\pm$ 0.27

T<sub>1</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu\text{s}$   
n = 3 for each treatment

The concentration of various minerals such as Al, B, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, S, Se, and Zn was determined in the liquid digesta after completion of gastrointestinal digestion. PEF processing had no effect ( $P > 0.05$ ) on the release of various minerals from the muscle during *in vitro* protein digestion. Previous reports have suggested that PEF treatment had negatively affected the concentration of some minerals in fresh beef and chicken (Khan et al., 2018; Khan et al., 2017a, b). They found a reduction in the concentration of Fe, K, P, Ca, Na and Mg with PEF. They also reported increased concentrations of

Cr and Ni in fresh beef, possibly due to the migration of these minerals from the electrodes of their PEF system (Khan et al., 2018; Khan et al., 2017a). These findings created an apprehension that this may affect the amount of minerals released from PEF treated samples during digestion. We did not observe any negative effect of PEF treatment on the release of various minerals in our study, although it should be noted that a different analytical method was used to determine the concentration of various minerals in liquid digesta in comparison to above mentioned studies which have detected the concentration of various minerals in fresh and cooked meat.

## **10.5 Conclusions**

Results of the present study suggest that pulsed electric field caused a significant increase in the digestion of meat proteins during gastrointestinal simulation. PEF-treated samples showed higher protein digestibility (%) and soluble protein (%) suggesting greater rate and extent of enzymatic hydrolysis. This was reflected in the protein profile of the PEF treated samples. No negative impact of the technology was recorded on the release of various minerals during the digestion process. Electroporation and protein-structural changes induced by PEF seems to have a positive influence on digestion of cooked meat proteins during *in vitro* gastrointestinal simulation. PEF may be a useful technology to improve digestibility of meat for nutritionally vulnerable groups.

## Chapter 11

# Cooking does not impair the impact of pulsed electric field on the protein digestion of venison during *in vitro* gastrointestinal simulation

This chapter is submitted as:

Bhat, Z. F., Morton, J. D., Mason, S. L., Jayawardena, R., & Bekhit, A. E. D. A. (2018). Cooking does not impair the impact of pulsed electric field on the protein digestion of venison during *in vitro* gastrointestinal simulation. *Food Bioscience*, (Submitted).

### 11.1 Abstract

Cooking has potential to affect the mechanism through which pulsed electric field (PEF) operates and influence the protein digestion of meat. Previous studies have used fresh uncooked meat to validate the effect of PEF on protein digestibility neglecting the effect cooking could induce during the process. A study was conducted to elucidate the effect of PEF on protein digestion of *Longissimus dorsi* obtained from red deer (*Cervus elaphus*). PEF treated samples ( $T_1$ , 10 kV, 90 Hz, 20 $\mu$ s) were cooked (core temperature of 75 °C) and subjected to *in vitro* simulated gastrointestinal protein digestion along with a control. While a positive ( $P < 0.05$ ) impact of PEF processing was observed on overall protein digestion, no effect ( $P > 0.05$ ) was recorded on free amino acid analysis and mineral profile. Cooking had no negative influence on the mechanism through which PEF technology operates in improving the protein digestibility.

**Keywords:** *In vitro* protein digestion; cooking; pulsed electric field; venison; digestibility (%); mineral profile; free amino acid analysis

### 11.2 Introduction

Pulsed electric field (PEF) is a novel non-thermal technology that has been studied for several applications in meat and meat products (Bhat, Morton, Mason, & Bekhit, 2018a, d). This technology has been recorded to improve the tenderness of meat during ageing (Bekhit, Suwandy, Carne, van de Ven, & Hopkins, 2016; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015a, b; Bekhit et al., 2014c) and could provide an attractive option to the meat industry in future in effectively reducing the total time of ageing. Application of PEF has been demonstrated to affect the functional and structural properties of the proteins by affecting electrostatic interactions and causing several modifications

(Bhat, Morton, Mason, & Bekhit, 2018a, f; Han, Cai, Cheng, & Sun, 2018; Liu, Oey, Bremer, Silcock, & Carne, 2018). PEF processing can therefore have an influence on the hydrolysis of proteins during gastrointestinal digestion and can affect the release and bioavailability of amino acids and polypeptides from meat during digestion (Bhat et al., 2018f). Recently, a study reported a significant impact of PEF on *in vitro* protein digestibility of venison (Bhat et al., 2018g) and it was suggested that the effect was induced by PEF treatment by inducing structural changes in proteins and electroporation. However, the study involved the use of fresh uncooked venison neglecting the fact that cooking might have a potential to either nullify or enhance the mechanism through which PEF operates.

Cooking of meat not only modifies the sensory quality of meat (Dominguez-Hernandez, Salaseviciene, & Ertbjerg, 2018), it has also been reported to affect the digestibility of the meat (Qi et al., 2018). Cooking has been reported to induce several structural changes in meat proteins (Kaur et al., 2014). Protein denaturation induced by cooking causes muscle fibers to shrink both transversely and longitudinally, creating gaps between the fibers (Straadt, Rasmussen, Andersen, & Bertram, 2007). Cooking has been reported to marginally affect the bioavailability of amino acids from beef in an *in vivo* study on humans based on the real ileal digestibility and dietary N recovery in the blood (Oberli et al., 2015). Further, information is generally lacking in the literature about the effect of PEF processing on the digestibility of cooked venison. By positively influencing the hydrolysis of proteins during digestion, this technology has potential to improve the availability of nutrients and add value to meat and meat products (Bhat, Morton, Mason, & Bekhit, 2018f, g). Application of PEF for the improvement of digestibility of cooked venison is yet an unexplored area, thus a study was designed to evaluate the effect of prior treatment of PEF on *in vitro* protein digestion of cooked *Longissimus dorsi* obtained from red deer.

Farmed for its meat and antler products, red deer (*Cervus elaphus*) is one of the most important, abundant and widespread game species in the world and widely farmed in the countries of Europe, New Zealand, Australia, China, Canada and the USA (Kudrnáčová, Bartoň, Bureš, & Hoffman, 2018; Frank et al., 2017). Perceived as healthy and more organic than other types of meat and meat products (Tomasevic et al., 2018), consumption of wild game meat from large wild ungulates, such as red deer, is considered healthier than red meat due to its nutritional characteristics (Eugenio, Daniel, Tiziano, Anna, & Roberto, 2018). Having low-fat content with an optimal fatty-acid composition and high-quality protein (Eugenio, Daniel, Tiziano, Anna, & Roberto, 2018), venison has been reported to maintain its healthy poly-unsaturated fatty acid composition (n-6/n-3) even after cooking (Valencak, Gamsjäger, Ohrnberger, Culbert, & Ruf, 2015). The value of meat from red deer could be further added by improving its digestibility by prior treatment with PEF. Thus, the objective of this study was to evaluate the effect of PEF treatment on the protein digestion of venison subjected to *in vitro* gastrointestinal digestive simulation. The samples were analysed for protein digestibility (%), soluble



protein (%), free amino acid analysis, protein profile (SDS-PAGE) and mineral profile to evaluate if PEF treatment has any impact on protein digestibility and the release of amino acids and peptides during *in vitro* gastrointestinal digestion.

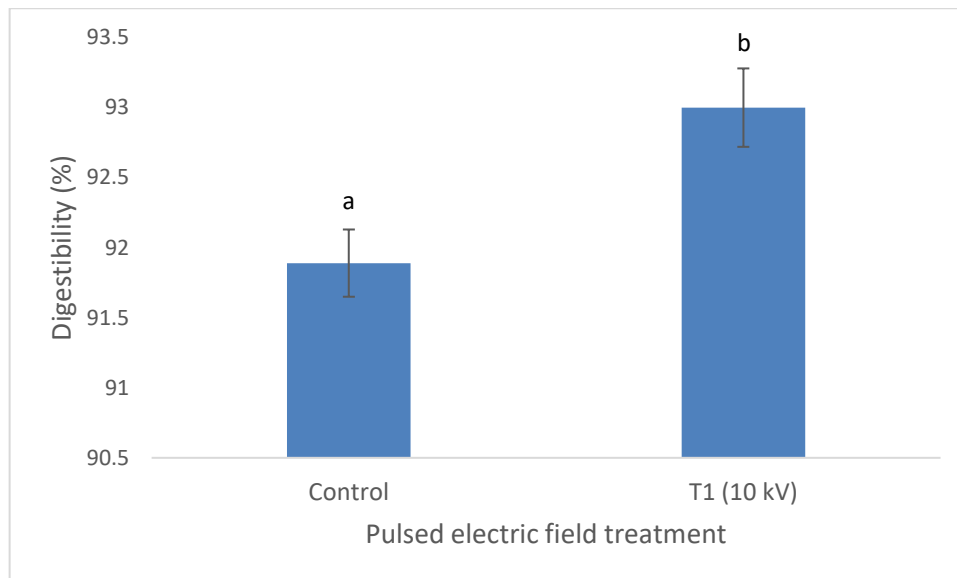
## 11.3 Materials and methods

Meat samples were prepared as per the method described in section 2.2.5. The meat samples were treated with PEF as per the method described in section 2.2.5.1. Both control and PEF treated samples were cooked individually in plastic bags immersed in a water bath maintained at 80 °C to a core temperature of 75 °C as measured individually using Fluke type K temperature probes attached to Fluke 52 thermometer (Fluke Corp., Everett, WA). *In vitro* protein digestion, *in vitro* protein digestibility (%), protein content (%), soluble protein (%), free amino acid analysis and mineral profile analysis were performed according to the methods described from section 2.5.2 to 2.5.7. Protein concentration, SDS-PAGE and analysis of the protein bands was done by following the methods described from section 2.4.2 to 2.4.4. Statistical analysis of the data was performed according to the method described in section 2.7.3.

## 11.4 Results and discussion

### 11.4.1 *In vitro* protein digestibility (%)

Figure 11.1 presents the effect of PEF processing ( $T_1$ , 10 kV-20 Hz-20  $\mu$ s) on the digestibility (%) of venison *Longissimus dorsi* subjected to *in vitro* simulated gastrointestinal protein digestion. An effect of PEF processing was recorded as the samples treated with PEF showed significantly higher values ( $P < 0.05$ ) at the end of the gastrointestinal digestion. A mean digestibility of 93.0 % was reported for PEF treated samples and was similar to the digestibility (%) of hairtail (*Thichiurus lepturus*) fillets observed by Tavares, Dong, Yang, Zeng, & Zhao (2018) during *in vitro* simulated protein digestion. Similar findings were also published by Bhat et al. (2018f) who observed a significant increase in the digestibility (%) of beef *Biceps femoris* treated with PEF during *in vitro* gastrointestinal digestion. Affected by various processing methods, digestibility of food proteins reflects their potential to meet the demands of indispensable amino acids (Boye, Wijesinha-Bettoni, & Burlingame, 2012) and is an important factor in protein evaluation in addition to the supply of essential amino acids (Tavano, Neves, & Júnior, 2016).



Different superscripts on columns differ significantly ( $P < 0.05$ )

**Figure 11.1 Effect of pulsed electric field on the protein digestibility (%) of cooked venison *Longissimus dorsi* subjected to *in vitro* simulated gastrointestinal digestion (Mean  $\pm$  SE)**

Novel food processing technologies such as high-pressure processing, PEF, sous-vide and ultrasound, which affect the structural and functional properties of proteins, have been reported to affect the digestibility of various food proteins *in vitro* by increasing their susceptibility to the enzymatic hydrolysis during gastrointestinal digestion (Bhat et al., 2018f; Zhu, Kaur, Staincliffe, & Boland, 2018; Stefanović et al., 2017; Stefanović et al., 2014). An increased susceptibility of egg white proteins to enzymatic hydrolysis was observed by Jovanović et al. (2016) on prior treatment with ultrasound. Treatment with ultrasound is believed to increase the accessibility of enzymes to hydrolytic sites by inducing the unfolding of proteins. Similar effect was reported by Hoppe, Jung, Patnaik, & Zeece (2013) who also observed a greater degree of enzymatic hydrolysis of egg white proteins treated with high pressure. This processing-induced unfolding of proteins was reported to be different from that induced by thermal processing in both the studies.

Like high-pressure and ultrasound, PEF processing has also been reported to affect the structural properties of food proteins (Liang, Cheng, & Wang, 2018) and by controlling the two important processing factors i.e. total energy input and electric field strength, PEF could be utilized to induce partial unfolding of meat proteins to increase their susceptibility to enzymatic hydrolysis by gastrointestinal proteases (Liu, Oey, Bremer, Silcock, & Carne, 2018). Denatured proteins have been reported to be more susceptible and are more effectively hydrolysed by gastrointestinal digestive proteases (Kaur et al., 2016) because structural changes play a significant role in influencing the protease active sites during gastrointestinal digestion (Simonetti, Gambacorta, & Perna, 2016).

### 11.4.2 Soluble protein (%)

Table 11.1 presents the effect of PEF processing ( $T_1$ , 10 kV-20 Hz-20  $\mu$ s) on the soluble protein (%) of venison *Longissimus dorsi* subjected to simulated *in vitro* gastrointestinal protein digestion. A significant ( $P < 0.05$ ) impact of PEF processing was recorded and higher values ( $P < 0.05$ ) were reported for PEF treated samples at the end of gastrointestinal digestion. No significant ( $P > 0.05$ ) difference was observed between control and PEF treated samples for soluble protein (%) during gastric phase of digestion, although higher values were recorded for PEF treated samples suggesting more enzymatic hydrolysis of the proteins. Kaur et al. (2016) reported a similar increase in soluble protein (%) for the beef samples treated with high-pressure suggesting more enzymatic hydrolysis of the treated proteins. Increased hydrolysis of the proteins in treated samples was suggested to be because of pressure-induced protein denaturation increasing the susceptibility of proteins by exposing inaccessible peptide bonds which were previously shielded to enzymatic attack (Kaur, Maudens, Haisman, Boland, & Singh, 2014; Grune, Reinheckel, Joshi, & Davies, 1995). Bhat et al. (2018f) also observed a similar increase in soluble protein (%) for beef samples treated with PEF during *in vitro* gastrointestinal digestion.

**Table 11.1 Effect of pulsed electric field on the soluble protein (%) of cooked venison *Longissimus dorsi* during *in vitro* simulated gastrointestinal digestion (Mean  $\pm$  SE)\***

Treatments	<i>In vitro</i> gastric digestion			<i>In vitro</i> intestinal digestion	
	0 Minutes	30 Minutes	60 Minutes	120 Minutes	180 Minutes
Control	12.76 $\pm$ 0.38 <sup>a</sup>	18.89 $\pm$ 2.42 <sup>b</sup>	19.06 $\pm$ 0.30 <sup>b</sup>	22.65 $\pm$ 0.58 <sup>bc</sup>	24.66 $\pm$ 0.99 <sup>CA</sup>
$T_1$ (10 kV)	13.03 $\pm$ 0.43 <sup>a</sup>	19.69 $\pm$ 2.07 <sup>b</sup>	21.17 $\pm$ 2.00 <sup>bc</sup>	24.98 $\pm$ 1.09 <sup>cd</sup>	27.73 $\pm$ 0.28 <sup>dB</sup>

\*Mean  $\pm$  SE with different superscripts in a row wise (lower case alphabet) and column wise (upper case alphabet) differ significantly ( $P < 0.05$ )

$T_1$  = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu$ s  
n = 3 for each treatment

Application of thermal and non-thermal processes have been observed to enhance the susceptibility of food proteins to subsequent hydrolysis by gastrointestinal proteases, such as trypsin, chymotrypsin or pepsin, by inducing structural changes such as protein unfolding and denaturation (Stefanović et al., 2017; Hoppe, Jung, Patnaik, & Zeece, 2013; van der Plancken, Delattre, Indrawati, & Hendrickx, 2004). Structural modifications capable of influencing the hydrolytic sites could be induced in meat proteins, such as myosin, by prior treatment with processes such as cooking or high pressure (Liu, & Xiong, 2000)

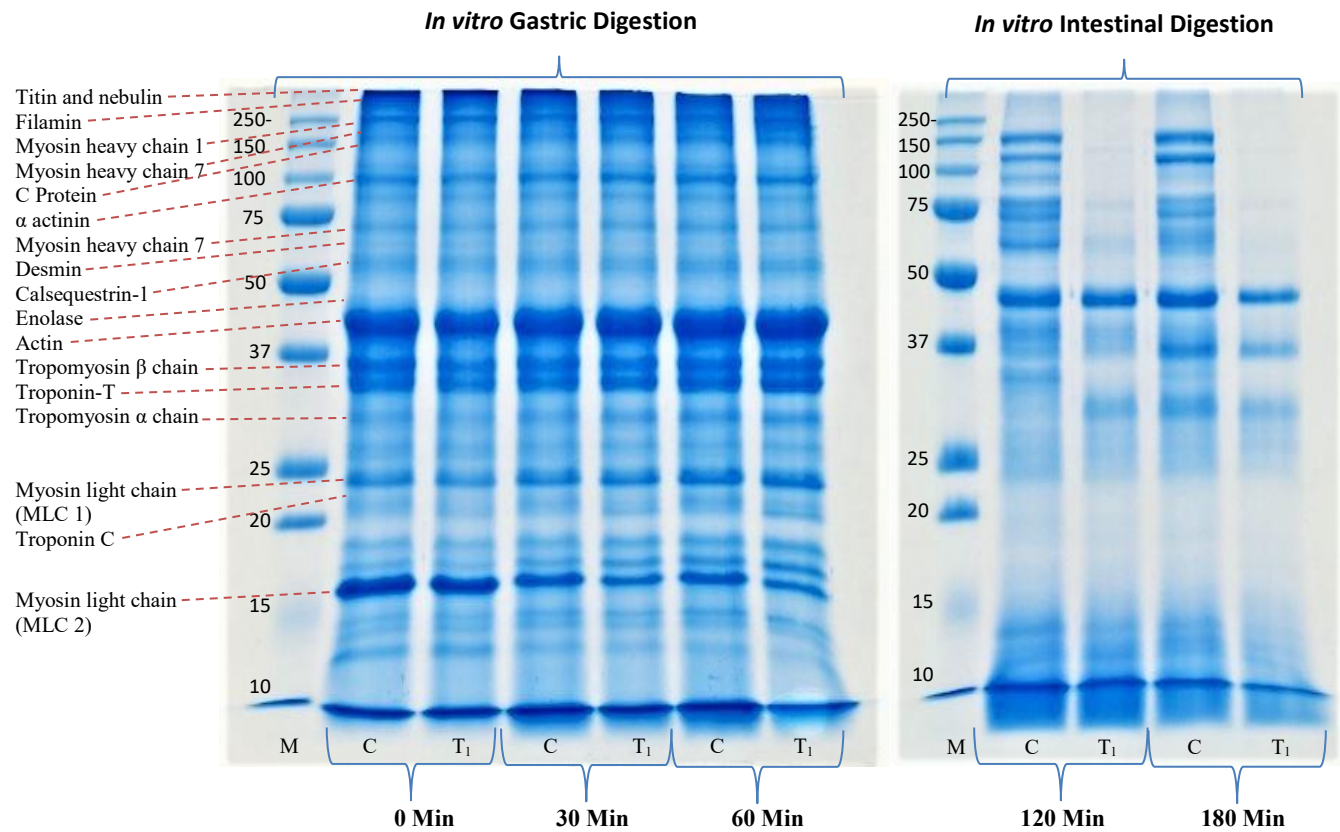
and possibly by PEF treatment (Bhat et al., 2018a, f). The effect of protein modifications on protein digestibility is well known (Thérona et al., 2018).

### 11.4.3 Protein profile (SDS-PAGE)

Figure 11.2 presents the effect of PEF processing ( $T_1$ , 10 kV-20 Hz-20  $\mu$ s) on the protein profile (SDS-PAGE gel electrophoretogram) of venison *Longissimus dorsi* subjected to simulated *in vitro* gastrointestinal digestion. A range of bands appeared in control and PEF treated samples during gastric and intestinal phase of digestion. The pattern of the bands suggest that more digestion has occurred during the intestinal phase of digestion and the peptides produced by the protease pepsin during gastric phase were further hydrolysed by proteases present in pancreatin into lower peptides and free amino acids. This caused several bands to disappear during the intestinal phase which otherwise were present during gastric phase of digestion.

Comparing the intestinal phase with the gastric phase of digestion, one can see that low molecular weight bands such as myosin light chain  $MLC_2$  (16 kDa), troponin-C (19 kDa), myosin light chain  $MLC_1$  (22 kDa), tropomyosin- $\alpha$ -chain (33 kDa), troponin-T (35 kDa) and tropomyosin  $\beta$ -chain (37 kD) which were prominent during gastric phase either disappeared or became less intense during intestinal phase of digestion. This difference in the intensity of the bands between two phases of the digestion could also be visualized for high molecular weight bands such as filamin (>250 kD), titin and nebulin (>250 kD), myosin heavy chain MHC 7 (>220 kD), myosin heavy chain MHC 1 (>220 kD), C protein (135 kD),  $\alpha$ -actinin (95 kD), desmin (53 kD), calsequestrin 1 (52 44-55 kD), enolase (46 kD) and actin (42 kD).

Looking at the gel electrophoretogram, one can also clearly visualize the differences in the bands between control and PEF treated samples. These differences, which become more prominent with the time of digestion and between gastric and intestinal phase of digestion, suggest a faster and greater rate of digestion for PEF treated samples. The intensity of almost all the bands, low and high molecular weight, was more for control samples in comparison to PEF treated samples. This difference becomes more and more visible during intestinal phase where most of the bands present in control samples disappear in PEF treated samples. This clearly indicates that greater and faster digestion has occurred in PEF treated samples in comparison to control. This is further confirmed by the fact that significantly higher digestibility (%) was recorded for PEF treated samples in comparison to control at the end of the digestion. These results suggest that PEF processing might have increased the susceptibility of meat proteins to gastrointestinal digestion by inducing various structural changes responsible for improving the enzymatic hydrolysis of the proteins. PEF-induced electroporation might also have added to the effect by increasing the penetration of the enzymes into the muscle matrix. PEF processing has already been demonstrated to increase the susceptibility of proteins from egg white to gastrointestinal digestion (Liu, Oey, Bremer, Silcock, & Carne, 2018).



**Figure 11.2** Effect of pulsed electric field on the protein profile (SDS-PAGE) of cooked venison *Longissimus dorsi* subjected to *in vitro* simulated gastrointestinal digestion [M = marker, C = control, T<sub>1</sub> = 10 kV (90 Hz), 20 µg samples were loaded in each lane, Protein identification was derived from that reported by Kaur et al., (2016); Kaur et al., (2014) and Ha (2012)]

Other non-thermal technologies such as high-pressure and ultrasound have also been reported to increase the susceptibility of food proteins, including meat proteins, to gastrointestinal digestion *in vitro* (Stefanović et al., 2017; Jovanović et al., 2016; Kaur et al., 2016; Stefanović et al., 2014; Hoppe, Jung, Patnaik, & Zeece, 2013). An increased enzymatic hydrolysis was observed by Bhat et al. (2018f) for PEF treated samples during gastrointestinal digestion of raw beef *Biceps femoris*. The increased enzymatic hydrolysis was attributed to PEF-induced protein structural changes and enhanced membrane permeability that might have increased the susceptibility of meat proteins.

#### **11.4.4 Free amino acid analysis**

Table 11.2 presents the effect of PEF processing ( $T_1$ , 10 kV, 20 Hz, 20  $\mu$ s) on the release of free amino acids from venison *Longissimus dorsi* subjected to simulated *in vitro* gastrointestinal digestion. The proteins are broken down to free amino acids or smaller peptides with few amino acid residues, such as tetra-peptides, tripeptides and di-peptides before absorption process starts (Pappenheimer, Dahl, Karnovsky, & Maggio, 1994; Jahan-Mihan, Luhovyy, El Khoury, & Anderson, 2011). Structure of a protein, that is influenced by several thermal and non-thermal processes, can affect the access of gastrointestinal enzymes to the cleavage sites on proteins and thus can affect the rate and extend of hydrolysis of proteins during digestion. Denaturation, which causes unfolding of proteins and makes them more flexible and open, can influence the hydrolysis of protein substrate by affecting the interaction between protein substrate and active site of proteases. Because of their more compact and closed structure, native proteins offer more resistance to the enzymatic hydrolysis by impeding the free access of enzymes to potential cleavage sites (Lorieau et al., 2018; Nguyen, Bhandari, Cichero, & Prakash, 2015). Several thermal and non-thermal processes, such as cooking, ultrasound, high-pressure and PEF processing, which have been reported to cause varying degree of denaturation and structural changes in meat proteins, have the potential to affect the rate of hydrolysis and the release of free amino acids from meat proteins during gastro-intestinal digestion. Many of these processes have already been demonstrated to have a positive influence on the digestion of meat proteins and release of free amino acids during gastrointestinal digestion trials (Stefanović et al., 2017; Jovanović et al., 2016; Kaur et al., 2016). Higher mathematical values were observed for all the free amino acids for PEF treated samples, however, no significant ( $P > 0.05$ ) difference was observed between control and treated samples.

**Table 11.2 Effect of pulsed electric field on the release of free amino acids during *in vitro* simulated gastrointestinal digestion of cooked venison *Longissimus dorsi* (Mean  $\pm$  SE)\***

Free amino acids ( $\mu$ M/litre)	PEF Treatment	
	Control	T <sub>1</sub> (10 kV)
Aspartic acid	113.90 $\pm$ 7.61	116.85 $\pm$ 2.67
Glutamic acid	338.18 $\pm$ 17.59	348.89 $\pm$ 9.61
Cysteine	101.89 $\pm$ 4.75	116.74 $\pm$ 7.65
Asparagine	150.45 $\pm$ 6.19	159.80 $\pm$ 4.69
Serine	264.05 $\pm$ 15.49	272.74 $\pm$ 8.24
Glutamine	540.97 $\pm$ 32.27	572.57 $\pm$ 22.69
Histidine	106.68 $\pm$ 2.21	117.19 $\pm$ 3.95
Glycine	291.32 $\pm$ 23.35	294.59 $\pm$ 10.47
Threonine	369.63 $\pm$ 8.74	387.42 $\pm$ 12.99
Arginine	1929.8 $\pm$ 126.51	2084.1 $\pm$ 67.50
Alanine	2351.3 $\pm$ 167.29	2422.6 $\pm$ 159.34
Taurine	673.06 $\pm$ 22.52	680.55 $\pm$ 16.11
Tyrosine	1215.1 $\pm$ 24.26	1296.9 $\pm$ 64.55
Valine	632.49 $\pm$ 10.69	669.71 $\pm$ 22.63
Methionine	362.93 $\pm$ 2.44	388.99 $\pm$ 19.24
Tryptophan	354.83 $\pm$ 10.75	381.81 $\pm$ 33.02
Phenylalanine	1068.5 $\pm$ 26.40	1144.6 $\pm$ 61.94
Isoleucine	571.23 $\pm$ 17.16	615.02 $\pm$ 21.50
Lysine	1644.5 $\pm$ 78.39	1731.7 $\pm$ 39.59
Leucine	2229.1 $\pm$ 105.16	2409.1 $\pm$ 107.28
Proline	91.37 $\pm$ 1.57	96.68 $\pm$ 8.48

T<sub>1</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu$ s

n = 3 for each treatment

#### 11.4.5 Mineral profile analysis

Table 11.3 presents the effect of PEF processing (T<sub>1</sub>, 10 kV-20 Hz-20  $\mu$ s) on the release of various minerals from venison *Longissimus dorsi* subjected to *in vitro* simulated gastrointestinal digestion. PEF treatment has been observed to affect the concentration of some minerals in fresh meat. While it has reduced the concentration of minerals such as Fe, K, P, Ca, Na and Mg in beef (Khan et al., 2018; Khan

et al., 2017), concentration of Cr and Ni has been reported to increase due to possible migration of these ions from electrodes to meat during processing (Khan et al., 2018). There is no information in the literature whether PEF processing will also affect the release of these minerals during gastrointestinal digestion and affect the commercial value of the meat. A different method of estimation was used for measuring the level of these minerals in the liquid digesta to see if PEF treatment has any influence on the release of these minerals during gastrointestinal digestion *in vitro*. No adverse effect of PEF processing was recorded on the release of various minerals as no significant ( $P > 0.05$ ) difference was reported between control and PEF treated samples for any of these minerals (Fe, K, P, Ca, Na, Mg, Cr and Ni). Although, PEF treatment affects the concentration of various minerals in fresh meat, there is no such effect on the release of these minerals during digestion process of venison. Bhat et al. (2018f) also observed no effect ( $P > 0.05$ ) of PEF processing on the release of various minerals during gastrointestinal digestion of raw beef *Biceps femoris*.

**Table 11.3 Effect of pulsed electric field on the release of minerals during *in vitro* simulated gastrointestinal digestion of cooked venison *Longissimus dorsi* (Mean  $\pm$  SE)**

Minerals ( $\mu\text{g/mL}$ )	PEF Treatment	
	Control	T <sub>1</sub> (10 kV)
Aluminium (Al)	0.016 $\pm$ 0.006	0.016 $\pm$ 0.001
Boron (B)	0.15 $\pm$ 0.004	0.15 $\pm$ 0.005
Calcium (Ca)	4.22 $\pm$ 0.82	4.29 $\pm$ 0.40
Chromium (Cr)	0.012 $\pm$ 0.0002	0.014 $\pm$ 0.001
Copper (Cu)	0.05 $\pm$ 0.004	0.05 $\pm$ 0.002
Iron (Fe)	0.30 $\pm$ 0.07	0.26 $\pm$ 0.007
Potassium (K)	265.64 $\pm$ 13.21	264.11 $\pm$ 4.42
Magnesium (Mg)	19.34 $\pm$ 0.11	19.07 $\pm$ 0.13
Manganese (Mn)	0.22 $\pm$ 0.01	0.23 $\pm$ 0.01
Sodium (Na)	2102.0 $\pm$ 4.28	2098.3 $\pm$ 30.94
Nickle (Ni)	0.0016 $\pm$ 0.0002	0.0019 $\pm$ 0.0004
Phosphorus (P)	500.09 $\pm$ 4.89	505.34 $\pm$ 9.86
Sulphur (S)	100.19 $\pm$ 2.00	103.31 $\pm$ 5.50
Selenium (Se)	0.02 $\pm$ 0.006	0.03 $\pm$ 0.004
Zinc (Zn)	2.68 $\pm$ 0.42	2.70 $\pm$ 0.46

T<sub>1</sub> = Samples treated with PEF at 10 kV, 20 Hz, 20  $\mu\text{s}$   
n = 3 for each treatment



## 11.5 Conclusions

A positive effect of PEF-processing was observed on the protein digestion of cooked venison during *in vitro* gastrointestinal simulation. Prior treatment of muscle with PEF resulted in a faster and greater digestion with a significant ( $P < 0.05$ ) increase in *in vitro* protein digestibility (%) and soluble protein (%). No negative impact of PEF processing was observed on the release of minerals, such as Fe, Zn, Cu, Cr, Mg, K, Ni and P, from venison during gastro-intestinal digestion. This study has expanded our understanding of digestion of meat proteins and how PEF technology could be utilized to improve the digestibility of meat, a step towards nutritional security.

## Chapter 12

### Does pulsed electric field have a role in the sodium reduction strategy for meat products?

This chapter is published as:

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2020). The application of pulsed electric field as a sodium reducing strategy for meat products. *Food Chemistry*, 306, <https://doi.org/10.1016/j.foodchem.2019.125622>.

#### 12.1 Abstract

This study investigated the potential of pulsed electric field (PEF) in the sodium-reduction strategy for processed meat. Beef jerky was used as a model and prepared using different levels of NaCl viz. 2.0% (control), 1.2% (T<sub>1</sub>) and 1.2% along with PEF-processing (T<sub>2</sub>, 0.52 kV/cm, 10 kV, 20 Hz, 20 μs). A significant (P<0.05) effect of PEF was observed on shear force (N) and toughness (N/mm.sec) of the products, which was also reflected in sensory scores. No effects for PEF were observed on colour, yield (%) and oxidative and microbial stability. PEF-treated samples (T<sub>2</sub>) had significantly (P<0.05) lower sodium content than the control, however, the sensory scores were comparable (P>0.05) with control and more than 84% of the panellists preferred T<sub>2</sub> samples over T<sub>1</sub> for saltiness. Results suggest that PEF-treatment improved saltiness naturally by influencing the salt-diffusion and sodium-delivery that led to better perception during chewing. PEF has a sodium-reduction potential that can play a role in public health.

**Keywords:** Pulsed electric field; sodium reduction; beef; jerky; storage stability; sensory analysis

#### 12.2 Introduction

Meat and meat products are an excellent source of proteins, minerals such as iron and zinc, and vitamins and are significant components of the balanced diet (Felisberto, Galvao, Picone, Cunha, & Pollonio, 2015; Brewer, 2012). Processed meat and meat products are also recognized for their high sodium content and excessive sodium intake is considered as a critical public health issue due to its correlation with hypertension and a subsequent increased risk for cardiovascular diseases (Bhat, Mason, Morton, Bekhit, & Bhat, 2017; WHO 2006). In the developed world, processed food products are responsible for around 75% of daily sodium intake (Aaslyng, Vestergaard, & Koch, 2014) with meat and meat products estimated to account for 20-30% of the sodium intake (Aaslyng et al., 2014).

Considering the great economic and health burden associated with excessive sodium intake by the population, several developed countries have launched programmes and strategies aiming at reducing the consumption of dietary sodium (Woodward, Eyles, & Mhurchu, 2012) and meat products are at the centre of attention (Kameníka, Saláková, Vyskočilová, Pechová, & Haruštiaková, 2017). The World Health Organization recommends a maximum daily intake of 5 g of salt (NaCl, equivalent to up to 2 g of sodium) for adults (Kloss, Meyer, Graeve, & Vetter, 2015).

Most of the sodium in meat preparations and meat products comes from table salt (NaCl), although it is not the only source of sodium during meat processing and other additives in the form of sodium salts, such as phosphates, glutamates, lactates, and nitrites, could add to the sodium burden (Kameníka et al., 2017). The addition of 2.0% NaCl, a common salt level for meat products, corresponds to 8 g Na/kg of raw product. A salt concentration of at least 2% is required in most meat formulations to ensure the ionic strength needed for solubilization and extraction of the salt-soluble proteins which are required for emulsion stability and the product texture (Verma, & Banerjee, 2012). A salt (NaCl) level of  $\geq 2\%$  has also been recommended for control of spoilage microorganisms and for flavour and texture of processed meat products such as sausages and bacon (Lucke, 2014; Pearson, & Tauber, 1984). Thus, sodium chloride plays a significant role in shelf life (microbial stability) and sensorial acceptance (flavour and texture) and is responsible for multiple functional properties of restructured and emulsified meat products (Felisberto et al., 2015; Dilnawaz, Kumar, & Bhat, 2017). This makes the direct reduction of salt in meat products difficult without observing adverse effects on the quality of the products.

Direct reduction of salt in meat products must be compensated by the addition of other ingredients or treatments which affect the functional properties. A successful strategy to develop reduced sodium meat products has been the use of alternative ingredients such as non-sodium salts and organic acids (Inguglia, Zhang, Tiwari, Kerry, & Burgess, 2017). Non-thermal technologies such as ultrasound and high-pressure have also proven their potential in sodium reduction application by influencing several factors such as diffusion and release of sodium from the meat matrix, interaction between proteins and salt ions, and sensory perceptions (Bhat, Morton, Mason, & Bekhit, 2018a) and are considered a novel approach to reduce sodium content in processed foods (Inguglia et al., 2017). However, no information is available in the literature whether pulsed electric field (PEF), which is known to influence the protein structure and function, can alter the distribution and perception of sodium from the meat matrix during chewing and allow sodium reduction in processed muscle foods. PEF can influence the diffusion, distribution and release of sodium from the meat matrix by inducing electroporation and increasing the membrane and cellular permeability. This may alter the interaction between proteins and salt ions and influence the release of sodium during chewing (Bhat et al., 2018a). PEF has been reported to significantly accelerate the diffusion of salt during the curing process in fresh pork (McDonnell, Allen, Chardonnerau, Arimi, & Lyng, 2014). Further, PEF is believed to have a limited role

in meat safety as well (Bhat et al., 2018a). Therefore, the present study aims to investigate if PEF treatment could be a useful approach towards salt reduction in meat products using beef jerky as a model system.

## **12.3 Materials and methods**

Meat samples were prepared as per the method described in section 2.2.4. The meat samples were treated with PEF as per the method described in section 2.2.4.1. Beef jerky was prepared according to the method described in section 2.6. Cooking yield (%), water activity ( $a_w$ ), pH, instrumental texture analysis, colour measurement and sensory evaluation was performed as per the methods described from section 2.6.1 to 2.6.6. Mineral profile analysis was performed according to the method described in section 2.5.7. Lipid oxidation (thiobarbituric acid reacting substances, TBARS), protein oxidation (dinitrophenylhydrazine) and microbiological characteristics were done by following the methods described from section 2.6.7 to 2.6.9. Statistical analysis of the data was performed according to the method described in section 2.7.1.

## **12.4 Results and discussion**

### **12.4.1 Electrical input**

The mean values for various PEF processing parameters (Table 12.1) such as electric field strength, pulse peak power, pulse peak current, total specific energy, conductivity increase and temperature increase were 0.52 kV/cm, 417.75 (kW), 100.15 (A), 13.79 kJ/kg, 1.23 and 4.0 °C, respectively. The initial temperature (°C) and conductivity (mS/cm) of the meat samples was 5.86 °C and 10.97, respectively. The applied electric field needs to exceed a critical limit of 0.5 kV/cm for an animal cell to observe an electroporation effect (Töpfl, 2006), therefore, the electric field strength applied in this study (0.52 kV/cm,  $T_2$ ) will deliver an effective electric field and lead to cell permeabilization.

**Table 12.1 Pulsed electric field processing parameters used and average temperature ( $\Delta T$ ) and conductivity ( $\Delta\sigma$ ) for beef *Semimembranosus***

PEF Treatment	Mean $\pm$ SE
Input voltage (kV)	10.0
Pulse frequency (Hz)	20.0
Pulse number	606.0
Pulse width ( $\mu$ s)	20.0
Pulse peak power (kW)	417.75 $\pm$ 3.34
Pulse peak current (A)	100.15 $\pm$ 0.90
Electric field strength (kV/cm)	0.52 $\pm$ 0.01
Energy density (kJ/kg)	13.79 $\pm$ 0.36
Temperature change ( $^{\circ}$ C)	4.0 $\pm$ 0.77
Conductivity change (mS/cm)	1.23 $\pm$ 0.12

Initial temperature of the samples was 5.86  $\pm$  0.46  $^{\circ}$ C

Initial conductivity (mS/cm) of the samples was 10.97  $\pm$  0.37

n = 6

#### 12.4.2 Physicochemical parameters and colour

The mean values for water activity ( $a_w$ ), pH, cooking yield (%), colour ( $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*$ ,  $H^*$ ) and mineral content for beef jerky treated with different levels of salt (NaCl) and PEF are presented in Tables 12.2 and 12.3.

##### 12.4.2.1 Water activity ( $a_w$ )

The water activity ( $a_w$ ) of all the samples was around 0.80 and was comparable ( $P > 0.05$ ) to control. Food Safety and Inspection Service (FSIS) of United States Department of Agriculture (USDA) recommends that jerky products should be dried to a water activity ( $a_w$ ) level of  $\leq 0.85$  under aerobic conditions or  $\leq 0.91$  under anaerobic conditions to ensure safety and control pathogens (USDA-FSIS, 2014).

#### **12.4.2.2 pH**

The pH of all the samples was comparable ( $P>0.05$ ) and no effect of PEF treatment or salt level was observed. All the samples had a mean pH of 5.8. Suwandy et al. (2015b) also observed no significant change in pH of PEF treated beef.

#### **12.4.2.3 Cooking yield (%)**

No effect of PEF treatment or salt level was observed ( $P>0.05$ ) on the cooking yield (%). Alahakoon, Oey, Bremer, & Silcock (2019); Bhat, Mason, Morton, & Bekhit (2019b) and Arroyo et al. (2015a) also observed no change in cooking losses of PEF-treated beef muscles.

#### **12.4.2.4 Colour analysis**

Colour is an important attribute for meat and meat products and has significant economic importance particularly for fresh meat. No effect of PEF processing or salt level was observed on the colour of beef jerky and similar ( $P>0.05$ ) values were observed for all the samples for all colour attributes viz.  $L^*$  (lightness),  $a^*$  (redness),  $b^*$  (yellowness),  $C^*$  (chroma) and  $H^*$  (hue angle). These results clearly suggest that PEF treatment does not affect the colour of beef and were in agreement with the findings of previous studies (Alahakoon et al., 2019; Arroyo et al., 2015a; Faridnia, Bekhit, Niven, & Oey, 2014). Recently, Kantono et al. (2019) reported an improvement in the colour of beef *Biceps femoris* with higher values for redness and chroma immediately after the PEF treatment in comparison to control. However, authors reported no such change in the colour of beef *Semitendinosus*. Our results are in agreement with the results of Yong et al. (2019) and Sindelar, Terns, Meyn, & Boles (2010) who also observed similar values for colour for jerky prepared from beef muscles.

**Table 12.2 Effect of pulsed electric field processing on the colour, shear force and physicochemical properties of beef jerky (Mean ± SE)\***

Parameters	Control (2% NaCl)	T <sub>1</sub> (1.2% NaCl)	T <sub>2</sub> (1.2% NaCl-PEF)
Water activity (a <sub>w</sub> )	0.79 ± 0.01	0.80 ± 0.002	0.80 ± 0.003
pH	5.82 ± 0.03	5.80 ± 0.04	5.82 ± 0.04
Yield (%)	38.54 ± 1.34	36.77 ± 0.96	37.88 ± 1.39
Lightness (L*)	24.03 ± 0.32	24.00 ± 0.47	23.01 ± 0.29
Redness (a*)	12.65 ± 0.64	13.22 ± 0.93	12.09 ± 1.30
Yellowness (b*)	4.00 ± 0.31	4.65 ± 0.54	4.20 ± 0.49
Chroma (C* = [a* <sup>2</sup> +b* <sup>2</sup> ] <sup>1/2</sup> )	13.27 ± 0.70	14.03 ± 1.05	12.80 ± 1.39
Hue angle (H* = tan <sup>-1</sup> [b*/a*])	17.46 ± 0.65	19.10 ± 0.85	19.14 ± 0.55
Shear force (N)	81.01 ± 3.99 <sup>A</sup>	78.70 ± 5.54 <sup>A</sup>	63.21 ± 3.90 <sup>B</sup>

\*Mean ± SE with different superscripts in a row differ significantly (P < 0.05)

Control = Samples treated with 2.0% NaCl

T<sub>1</sub> = Samples treated with 1.2% NaCl

T<sub>2</sub> = Samples treated with PEF (10 kV, 20 Hz, 20 μs, 0.52 kV/cm) and 1.2% NaCl

n = 6 for each treatment

#### 12.4.2.5 Mineral profile

No significant (P>0.05) effect of PEF processing was recorded on the minerals of beef jerky such as Fe, Zn, K, Na, P, Ca, Cr, and Mg. A few studies have investigated the effect of PEF treatment on the mineral content of fresh beef and have reported a significant decrease in the concentration of some minerals such as Fe, K, P, Ca, Na and Mg (Khan et al., 2018; Khan et al., 2017) and a significant increase in the concentration of some such as Cr and Ni (Khan et al., 2018) due to migration of the mineral ions from meat to the electrodes. It is important to mention here that a different method was used to determine the concentration of these minerals in our study in comparison to the above studies. A significant (P<0.05) effect of salt level was observed and the sodium content of PEF treated beef jerky (T<sub>2</sub>, 1.2% NaCl) was 34% lower in comparison to control (2% NaCl). A 40% reduction of salt level in the formulation of the marinade for beef jerky resulted a decrease of 34% of sodium content in the finished product.

A product could be labelled as reduced sodium if its sodium content is reduced by at least 25% than the regular product (U.S.FDA, 2018). Barretto et al. (2018) also reported a 28.50% reduction in sodium content of restructured cooked ham treated with ultrasound and a reduced salt level. According to

United States Food and Drug Administration (U.S.FDA, 2018), the recommended daily value for sodium is less than 2.3 g per day for adults and children of 4 years of age and older.

**Table 12.3 Effect of pulsed electric field on the mineral content of beef jerky (Mean  $\pm$  SE)\***

<b>Minerals (mg/kg)</b>	<b>Control (2% NaCl)</b>	<b>T<sub>1</sub> (1.2% NaCl)</b>	<b>T<sub>2</sub> (1.2% NaCl-PEF)</b>
Aluminium (Al)	18.21 $\pm$ 1.32	17.18 $\pm$ 0.71	18.67 $\pm$ 0.62
Boron (B)	0.51 $\pm$ 0.10	0.43 $\pm$ 0.08	0.43 $\pm$ 0.07
Calcium (Ca)	180.02 $\pm$ 5.40	186.14 $\pm$ 11.01	176.59 $\pm$ 7.59
Chromium (Cr)	0.04 $\pm$ 0.01	0.04 $\pm$ 0.003	0.06 $\pm$ 0.01
Copper (Cu)	2.65 $\pm$ 0.15	2.65 $\pm$ 0.11	2.76 $\pm$ 0.15
Iron (Fe)	77.03 $\pm$ 3.34	68.74 $\pm$ 2.74	75.66 $\pm$ 4.08
Potassium (K)	6285.38 $\pm$ 605	6255.91 $\pm$ 833	6204.90 $\pm$ 395
Magnesium (Mg)	630.58 $\pm$ 24.08	614.67 $\pm$ 50.0	622.57 $\pm$ 13.86
Manganese (Mn)	0.47 $\pm$ 0.03	0.43 $\pm$ 0.02	0.46 $\pm$ 0.02
Sodium (Na)	15044.0 $\pm$ 615 <sup>A</sup>	9216.5 $\pm$ 375 <sup>B</sup>	9886.0 $\pm$ 909 <sup>B</sup>
Phosphorus (P)	4383.16 $\pm$ 190	4379.22 $\pm$ 365	4377.84 $\pm$ 48.8
Sulphur (S)	6051.67 $\pm$ 99.26	6179.61 $\pm$ 159.7	6013.87 $\pm$ 236.4
Zinc (Zn)	114.46 $\pm$ 11.61	119.07 $\pm$ 12.09	110.07 $\pm$ 10.01

\*Mean  $\pm$  SE with different superscripts in a row differ significantly (P < 0.05)

Ni, Pb, Cd and Se were below detection levels

Control = Samples treated with 2.0% NaCl

T<sub>1</sub> = Samples treated with 1.2% NaCl

T<sub>2</sub> = Samples treated with PEF (10 kV, 20 Hz, 20  $\mu$ s, 0.52 kV/cm) and 1.2% NaCl

n = 6 for each treatment

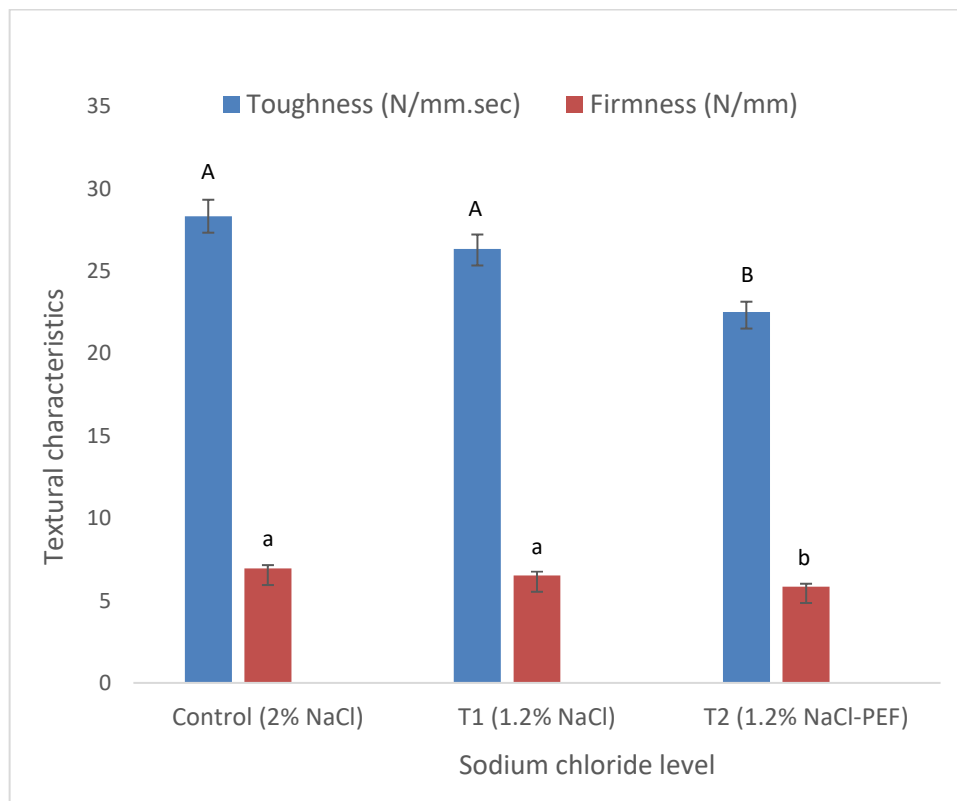
### 12.4.3 Textural characteristics

The mean values for various textural characteristics viz. shear force (N), toughness (N/mm.sec) and firmness (N/mm) for beef jerky treated with different levels of salt (NaCl) and PEF are presented in Table 12.2 and Figure 12.1. A significant impact of PEF processing was observed on all the textural characteristics and significantly (P<0.05) lower values were observed for shear force (N), toughness (N/mm.sec) and firmness (N/mm) for the PEF processed samples (1.2% NaCl-PEF) in comparison to control (2% NaCl) and T<sub>1</sub> (1.2% NaCl) samples. A reduction of 22% was recorded in shear force of PEF processed samples in comparison to control. PEF processing has been reported to decrease the shear force in beef muscles (Bekhit et al., 2016; Bekhit et al., 2014c) with up to 22% reduction in shear force



of beef *Semimembranosus* muscle (Suwandy et al., 2015a). Bhat et al. (2019b) also observed a reduction of 17% in the shear force of PEF-treated beef *Biceps femoris* but this result was not significant ( $P>0.05$ ). It is important to note that those muscles were obtained from culled dairy animals and were reported to be excessively tough.

Recently, Kantono et al. (2019) also reported a significant improvement in the tenderness of beef *Biceps femoris* and *Semitendinosus* in both fresh and frozen PEF treated samples. PEF processing has been reported to decrease the shear force by causing early post-mortem activation of calpain 2 and by improving the calpain activity in beef muscles by causing early release of calcium ions from sarcoplasmic reticulum through electroporation (Bhat et al., 2019b). Proteolysis of both desmin and troponin-T, which has been directly linked to calpain activity in muscles, has been reported to increase in PEF treated samples (Bhat et al., 2019b). The proteolytic degradation of key myofibrillar proteins is known to play a central role in the tenderisation process during ageing (Huff-Lonergan, Zhang, & Lonergan, 2010).



Mean  $\pm$  SE with different superscripts differ significantly ( $P < 0.05$ )

Control = Samples treated with 2.0% NaCl

T<sub>1</sub> = Samples treated with 1.2% NaCl

T<sub>2</sub> = Samples treated with PEF (10 kV, 20 Hz, 20  $\mu$ s, 0.52 kV/cm) and 1.2% NaCl

n = 6 for each treatment

**Figure 12.1 Effect of pulsed electric field on the texture properties of beef jerky (Mean  $\pm$  SE)**

#### **12.4.4 Sensory evaluation**

The mean values for various sensory attributes for beef jerky treated with different levels of salt (NaCl) and PEF are presented in Table 12.4. Out of 65 panellists, 70% were familiar with the product. The ethnic composition of the panel was 56% Asian, 29% Europeans and 15% other ethnicities.

##### **12.4.4.1 Flavour and colour**

Salt reduction significantly ( $P < 0.05$ ) decreased the flavour scores with the control (2% NaCl) samples higher than the  $T_1$  (1.2% NaCl) samples. However, no difference ( $P > 0.05$ ) was observed between the scores for  $T_2$  (1.2% NaCl-PEF) and control samples. This difference between control (2% NaCl) and  $T_1$  (1.2% NaCl) samples may be attributed to the salt reduction since it is an established fact that NaCl influences the flavour of the meat products and any direct reduction affects the quality and sensory scores (Pretorius, & Schönfeldt, 2018). Sodium not only binds with protein receptors to convey the familiar salty taste but also enhances some of the natural flavours, such as meaty and savoury notes, present in meat (Wilson, Komitopoulou, & Incles, 2012). It enhances the aroma by affecting the osmotic pressure that decreases the solubility of volatile aroma compounds in meat matrix and enhances their release. In fact, the main challenge for direct salt reduction in meat products in terms of flavour is not the reduction in saltiness itself but the loss of impact on enhancing the savoury and meaty flavours (Pretorius, & Schönfeldt, 2018; Wilson et al., 2012). While Arroyo et al. (2015a) reported no difference between the odour scores for PEF treated and untreated beef muscles, temporal dominance of sensations showed that PEF treated samples were associated with juicy, browned, livery, brothy and meaty flavour attributes (Kantono et al., 2019; Ma et al., 2016).

No effect of PEF or salt reduction was observed on the sensory scores for colour and no significant difference ( $P > 0.05$ ) was observed among the samples. This was consistent with the instrumental colour analysis where no difference was recorded among the samples. Arroyo et al. (2015b) also found no effect of PEF treatment on the colour scores for turkey breast.

##### **12.4.4.2 Saltiness**

A significant effect of salt reduction was recorded on the scores for saltiness of the products. Significantly ( $P < 0.05$ ) higher values were observed for the control (2% NaCl) samples in comparison to  $T_1$  (1.2% NaCl) samples whereas no difference ( $P > 0.05$ ) was observed between  $T_2$  (1.2% NaCl-PEF) and control samples. These effects were also visible on the results of the ranking tests where 53% of the panellists ranked control (2% NaCl) samples whereas 36% ranked  $T_2$  (1.2% NaCl-PEF) samples as the most preferred salt level. Overall, 84% of the panellists preferred  $T_2$  (1.2% NaCl-PEF) over  $T_1$  (1.2%

NaCl) samples for saltiness. In the ranking test for intensity of saltiness, 73% panellists ranked control (2% NaCl) samples whereas 16% ranked T<sub>2</sub> (1.2% NaCl-PEF) samples as most intense salt level. Overall, 71% ranked T<sub>2</sub> (1.2% NaCl-PEF) samples as more intense than T<sub>1</sub> (1.2% NaCl) samples.

Processing of meat by non-thermal technologies, such as high pressure and ultrasound, has been reported to be helpful in designing of reduced sodium meat products by influencing the salt diffusion and sensory perception (Inguglia et al. 2017). Treatment of dry-cured pork loin with high pressure resulted in a natural increase in the saltiness due to a change in interaction between protein structures and salt ions that possibly resulted into more leaching of sodium from meat matrix to the taste receptors on the tongue during chewing (Clariana et al. 2011). By contributing to the disruption of myofibrils and modifying various quality characteristics including the acceptability, Zheng et al. (2017) reported that high pressure-heat treatment allowed sodium reduction of chicken meat batters for production of low-salt meat products.

Ultrasound processing has also been examined for its salt reduction potential based on the premise that it can alter the mass transfer processes, such as brining and curing, by modifying the cell membranes. By improving the salt distribution during brining process, ultrasound has the potential to deliver higher salt perception even with lower overall salt content (Alarcon-Rojo et al. 2015). Ultrasound treatment caused the micro fissures on the myofibrils and improved the colour and sensory acceptance of reduced sodium restructured cooked ham (Barretto, Pollonio, Romero, & Barretto, 2018). Thus, salt reduction like benefits could be obtained by the rapid curing processes along with the improved salt gain rate (Tao, & Sun, 2015). In the light of these facts, the results observed in our study may be attributed to the PEF treatment which has potential to induce similar effects in meat such as changes in protein structure and function, modifications in microstructure, enhanced membrane permeability and mass transfer processes (Bhat et al., 2018).

#### **12.4.4.3 Tenderness and overall acceptability**

An effect of PEF was observed on tenderness scores and significantly ( $P < 0.05$ ) higher scores were recorded for T<sub>2</sub> (1.2% NaCl-PEF) samples in comparison to control (2% NaCl) and T<sub>1</sub> (1.2% NaCl) samples. These findings agreed with our instrumental textural results. Arroyo et al. (2015a) reported that 60% of the panellists scored PEF treated samples as tender in comparison to 27.5% who scored untreated samples as tender.

**Table 12.4 Effect of pulsed electric field processing on the sensory characteristics of beef jerky (Mean  $\pm$  SE)\***

Parameters	Control (2% NaCl)	T <sub>1</sub> (1.2% NaCl)	T <sub>2</sub> (1.2% NaCl-PEF)
Colour	7.10 $\pm$ 0.24	7.27 $\pm$ 0.20	7.22 $\pm$ 0.20
Flavour	6.36 $\pm$ 0.21 <sup>A</sup>	5.75 $\pm$ 0.17 <sup>B</sup>	6.13 $\pm$ 0.19 <sup>AB</sup>
Saltiness	6.66 $\pm$ 0.22 <sup>A</sup>	5.81 $\pm$ 0.21 <sup>B</sup>	6.17 $\pm$ 0.21 <sup>AB</sup>
Tenderness	4.75 $\pm$ 0.28 <sup>A</sup>	4.77 $\pm$ 0.27 <sup>A</sup>	5.60 $\pm$ 0.29 <sup>B</sup>
Overall acceptability	6.31 $\pm$ 0.21 <sup>A</sup>	5.70 $\pm$ 0.18 <sup>B</sup>	6.24 $\pm$ 0.20 <sup>AB</sup>

\*Means  $\pm$  SE with different superscripts in a row differ significantly ( $P < 0.05$ )

Control = Samples treated with 2.0% NaCl

T<sub>1</sub> = Samples treated with 1.2% NaCl

T<sub>2</sub> = Samples treated with PEF (10 kV, 20 Hz, 20  $\mu$ s, 0.52 kV/cm) and 1.2% NaCl

Hedonic scale of 1-9 was used for sensory evaluation (n = 65) where 1 = dislike extremely and 9 = like extremely

An effect of salt reduction and PEF was recorded on overall acceptability scores of the products. Significantly ( $P < 0.05$ ) higher values were observed for the control (2% NaCl) samples in comparison to T<sub>1</sub> (1.2% NaCl) samples whereas no difference ( $P > 0.05$ ) was observed between T<sub>2</sub> (1.2% NaCl-PEF) and control samples. This might be reflective of the changes in the scores of other sensory parameters such as flavour, tenderness and saltiness.

#### 12.4.5 Microbial characteristics

The mean values for the microbial characteristics during storage for beef jerky treated with different levels of salt (NaCl) and PEF are presented in Table 12.5.

##### 12.4.6.1 Total plate, coliform, and yeast and mould count (log cfu/g)

No effect ( $P > 0.05$ ) of PEF or salt level was observed on total plate count (TPC) of beef jerky during storage. A significant ( $P < 0.05$ ) effect of storage time was observed and the mean TPC values showed an increasing trend with increasing days of storage. Mean values of all the products were within the limit of 5.33 log cfu/g on 30<sup>th</sup> day of storage which is considered as limit of acceptability for cooked meat products (Cremer, & Chipley, 1977). Similar results were reported by Barretto et al. (2018) who also observed no impact of salt reduction and ultrasound treatment on microbial counts of restructured cooked ham. PEF processing has been reported to have a limited role in meat safety (Bhat et al., 2018) and novel combinations of PEF with other hurdle technologies, such as antimicrobial

agents, were recently proposed (Clemente, Condón-Abanto, Pedrós-Garrido, Whyte, & Lyng, 2019; Bermúdez-Aguirre et al., 2012). A sequential combination approach of PEF processing (0.25-1 kV/cm) followed by re-suspension in oregano essential oil ( $\frac{1}{4}$  MIC) showed a synergistic behaviour and achieved an inactivation in the same range as those acquired using conventional thermal treatments in chicken (Clemente et al., 2019).

The coliforms were not detected throughout the period of storage which could be due to their destruction during cooking at 75°C which is higher than their thermal death point (57°C). Further, the water activity of all the samples was around 0.80 which ensures food safety and prevents the growth of pathogens in jerky products (USDA-FSIS, 2014). Several workers have reported zero counts for cooked meat products (Kelam, Bhat, Kumar, Noor, & Desai, 2018; Noor, Bhat, Kumar, & Mudiyansele, 2018). The yeast and moulds were also not detected in any sample throughout the period of storage. Kim, Chun, Song, & Song (2010) also reported no detection of yeast and moulds in beef jerky during aerobic storage at 20 °C for two months.

#### **12.4.6 Lipid and protein oxidation**

Oxidative reactions, with lipids and proteins as main targets in meats, have been considered as one of the leading causes of deterioration of quality in meat and meat products (Silva et al., 2018). The mean values for lipid (TBARS) and protein (Total carbonyl content) oxidation during storage for beef jerky treated with different levels of salt (NaCl) and PEF are presented in Table 12.5.

##### **12.4.7.1 TBARS (mg malondialdehyde/kg)**

Lipid oxidation, a complex free radical chain reaction that affects unsaturated lipids and involves the consumption of molecular oxygen, leads to the development of rancidity that affects the sensorial and nutritional characteristics of muscle foods during storage. Several factors affect the extent of lipid oxidation in meat products such as metal catalysts, enzymes, fat composition, myoglobin, water activity, processing and storage (Chaijan, & Panpitat, 2017; Vieira, Zhang, & Decker, 2017). Measurement of lipid oxidation by TBARS assay, using malondialdehyde as an oxidation marker, is the most common method used for meat products (Reitznerová et al., 2017).

While a significant ( $P < 0.05$ ) effect of storage time was observed on the TBARS values of beef jerky, no impact of PEF treatment or salt reduction was observed on the lipid oxidation of the products. Similar results were reported by Barretto et al. (2018) who also observed no impact of salt reduction (at 25% and 50%) and ultrasound treatment on lipid oxidation of restructured cooked ham. They also found an increasing trend in the TBARS values with storage time. The release of iron, which is an active lipid oxidation catalyst, from sources such as haemoglobin, myoglobin and ferritin with storage catalyses

lipid oxidation (Monahan, 2000). TBARS values of up to 2.0 mg malondialdehyde/kg have been reported not to affect the sensory acceptance of the meat products (O'Neill, Galvin, Morrissey, & Buckley, 1998). Previous studies have also reported no impact of PEF treatment on lipid oxidation of beef muscles (Alahakoon et al., 2019; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015c; Faridnia et al., 2015). Arroyo et al. (2015b) observed no change in the lipid oxidation of PEF treated turkey breast compared to control.

**Table 12.5 Effect of pulsed electric field processing on the microbiological characteristics and oxidative stability of beef jerky (Mean ± SE)\***

Treatments	STORAGE PERIOD (DAYS)		
	0	15	30
<b>Total plate count (log cfu/g)</b>			
Control	1.56 ± 0.06 <sup>A</sup>	1.99 ± 0.03 <sup>B</sup>	2.52 ± 0.02 <sup>C</sup>
T <sub>1</sub> (1.2% NaCl)	1.60 ± 0.03 <sup>A</sup>	2.02 ± 0.02 <sup>B</sup>	2.60 ± 0.02 <sup>C</sup>
T <sub>2</sub> (1.2% NaCl-PEF)	1.58 ± 0.05 <sup>A</sup>	2.01 ± 0.03 <sup>B</sup>	2.54 ± 0.05 <sup>C</sup>
<b>Coliform count (log cfu/g)</b>			
Control	ND	ND	ND
T <sub>1</sub> (1.2% NaCl)	ND	ND	ND
T <sub>2</sub> (1.2% NaCl-PEF)	ND	ND	ND
<b>Yeast and mould count (log cfu/g)</b>			
Control	ND	ND	ND
T <sub>1</sub> (1.2% NaCl)	ND	ND	ND
T <sub>2</sub> (1.2% NaCl-PEF)	ND	ND	ND
<b>TBARS (mg malondialdehyde/kg)</b>			
Control	0.31 ± 0.01 <sup>A</sup>	0.40 ± 0.01 <sup>B</sup>	0.65 ± 0.01 <sup>C</sup>
T <sub>1</sub> (1.2% NaCl)	0.27 ± 0.02 <sup>A</sup>	0.36 ± 0.008 <sup>B</sup>	0.60 ± 0.02 <sup>C</sup>
T <sub>2</sub> (1.2% NaCl-PEF)	0.29 ± 0.01 <sup>A</sup>	0.37 ± 0.01 <sup>B</sup>	0.59 ± 0.02 <sup>C</sup>
<b>Total carbonyl content (nmol/mg of protein)</b>			
Control	1.68 ± 0.03 <sup>A</sup>	2.47 ± 0.02 <sup>B</sup>	2.86 ± 0.03 <sup>C</sup>
T <sub>1</sub> (1.2% NaCl)	1.69 ± 0.02 <sup>A</sup>	2.46 ± 0.02 <sup>B</sup>	2.86 ± 0.02 <sup>C</sup>
T <sub>2</sub> (1.2% NaCl-PEF)	1.75 ± 0.04 <sup>A</sup>	2.58 ± 0.06 <sup>B</sup>	2.92 ± 0.02 <sup>C</sup>

\*Mean ± SE with different superscripts in a row wise differ significantly (P < 0.05)

Control = Samples treated with 2.0% NaCl

T<sub>1</sub> = Samples treated with 1.2% NaCl

T<sub>2</sub> = Samples treated with PEF (10 kV, 20 Hz, 20 μs, 0.52 kV/cm) and 1.2% NaCl

n = 6 for each treatment, ND = Not detected

#### **12.4.7.2 Total carbonyl content (nmol/mg of protein)**

Oxidation of proteins in muscle foods, induced by reactive oxygen species (ROS) or by free radicals formed during oxidative stress, leads to the formation of several oxidation derivatives. Due to their mutagenic and cytotoxic potential, these protein oxidation products have been linked to various pathological conditions in humans (Estévez, & Luna, 2017). By attacking the peptide backbone and the side chain of amino acids, oxidation of proteins can result in several changes in meat, such as decrease in protein solubility, loss of water-holding capacity and essential amino acids, and degradation of colour and texture (Soladoye, Juarez, Aalhus, Shand, & Estévez, 2015; Estévez, 2015).

While a significant ( $P < 0.05$ ) effect of storage time was observed on the total carbonyl content of beef jerky, no impact of PEF treatment or salt reduction was observed on the protein oxidation of the products. An increasing trend was observed in the total carbonyl content of all the samples with increasing storage time and was in agreement with the findings reported by Alahakoon (2018) who also observed a similar increase in total carbonyl content in beef muscles treated with PEF during ageing. Silva et al. (2018) also reported an increase in total carbonyl content of chicken jerky with storage time.

### **12.5 Conclusions**

Prior treatment of beef with PEF allowed a direct reduction of salt (NaCl) without any detrimental effect on sensory quality and lipid-oxidative and microbial stability of the products. By influencing the salt diffusion and distribution in the meat matrix and possibly the release and perception of sodium during chewing, PEF treatment improved the saltiness naturally. PEF treatment did not affect the colour and cooking yield of the products and confirmed the superior tenderness of the PEF-treated products. This study reports for the first time a sodium reduction potential for PEF that can find a new application in meat industry.

## Chapter 13

### General Discussion and Conclusion

PEF is an emerging non-thermal technology that has been widely investigated for its effects on liquid foods and beverages (Griffiths, & Walkling-Ribeiro, 2014) and is used commercially in the food industry in some countries (Bhat et al. 2018a, d). However, there is a limited understanding of the impact of PEF processing on solid food matrices (Liu et al. 2018a), particularly high-protein foods such as meat (Bhat et al. 2018a, d). PEF involves the application of an external electric field to induce electroporation, formation of pores, thereby increasing the permeability of the cellular membranes (Ostermeier et al. 2018). This increased membrane permeability facilitates the exchange of intracellular components with the cell's surroundings and produces several effects (Vaessen et al. 2018) that has recently attracted the attention of meat scientists and technologists (Bhat et al. 2018a; Bekhit et al. 2016, 2014c).

Several studies have evaluated the potential of PEF for improving meat tenderness, however, there is a high degree of variability between studies and the underlying mechanisms are not clearly understood. While variability may affect the significance of results, several studies have reported an overall decrease in shear force in PEF-treated beef during ageing and in general a decline in the range of 16 to 21% is widely reported (Bekhit et al. 2016, 2014c; Suwandy et al. 2015a, b; Bhat et al. 2019a, b). While some studies have suggested physical disruption as the main cause of PEF induced tenderness, others suggest enhanced proteolysis as the plausible mechanism (Bekhit et al. 2016, 2014c; Bhat et al. 2018a, d). Physical disruption as the underlying mechanism for PEF-induced tenderness means that the technology should be effective for muscles from younger as well as older animals with more cross-linked collagen. However, none of the studies conducted on PEF have used muscles from older animals. While some studies have suggested the potential of PEF to mediate the tenderization process by causing early release of calcium ions and early activation of the calpain proteases (Bekhit et al. 2016, 2014c; Bhat et al. 2018a), experimental research is yet to confirm this postulation and no information is available in the literature about the effect of PEF processing on the calpain activity. There is a need for a comprehensive study that would involve different models to elucidate the underlying mechanisms and to confirm the tenderness potential of this technology. In lieu of these facts, a wide range of models were used in our study to elucidate the effect of PEF on the quality of meat which included different species (beef and venison), different age (meat from culled dairy animals and premium beef), and muscles of different tenderness potential (*Biceps femoris*-tough, *Semimembranosus*-intermediate, *Longissimus dorsi*-tender).

Little information is available in the literature about the effect of ageing on the calpain activity of the muscles from older animals and the subsequent tenderization of such muscles during ageing. Chapter



3 presents the changes in the calpain activity, protein profile and physicochemical properties of cold-boned *Semimembranosus* and *Biceps femoris* from culled dairy cows during ageing for two weeks ( $4\pm 1$  °C). Casein zymography results indicated the presence of intact and autolyzed forms of calpain 1 and calpain 2. Calpain 1 activity, both intact and autolysed, decreased with ageing time and was detected until day 2 of ageing in both muscles. Only 5.4% of the calpain activity present in the zero-hour reference standard was detected by Colle, & Doumit (2017) in the beef *Semimembranosus* by day 2 of ageing. Native calpain 2 activity decreased whereas the autolysed calpain 2 activity increased with the ageing time, with highest activity detected on day 14 in both the muscles. Calpain 2 requires a higher level of calcium for its activation and gets autolysed at later stages of ageing when intracellular calcium concentration increases to a level that would activate calpain 2 (Colle, & Doumit, 2017; Morton et al. 1999). An increase in proteolysis was also observed in both muscles during ageing. A similar increase in the calpain activity and proteolysis of beef muscles has been reported previously (Colle, & Doumit, 2017; Geesink et al. 2006; Taylor et al. 1995b). A significant ( $P<0.05$ ) effect of ageing was observed on the shear force and MFI of both the muscles. A similar decline has been reported by Bunmee et al. (2014) in the shear force of *Longissimus dorsi* of culled dairy cows subjected to one week of ageing. Ageing for two weeks was effective ( $P<0.05$ ) in reducing the shear force of both muscles by 30%, however, both of the aged muscles were still excessively tough (as indicated by their high shear force values) suggesting a need for supplementary post-mortem intervention, such as PEF, to reduce the extra toughness associated with the muscles of culled dairy animals.

Chapter 4 and 5 elucidated the effect of PEF on the calpain activity, proteolysis and physicochemical properties of beef *Semimembranosus* and *Biceps femoris* from culled dairy cows during ageing for two weeks. No effect of PEF was observed on the purge loss and cooking loss of the PEF treated samples [ $T_1$  (5 kV, 90 Hz, 20  $\mu$ s),  $T_2$  (10 kV, 20 Hz, 20  $\mu$ s)] and this was in agreement with the findings of previous studies (Suwandy et al. 2015b; Bekhit et al. 2014c). Our results suggest that PEF treatment under our experimental conditions did not alter the myofibrillar proteins thermally nor damaged muscle fibres and connective tissue that might enhance the movement of water out of the muscle. A tendency of PEF towards reducing shear force was observed, however, no significant difference was recorded in the shear force of PEF treated samples and the non-treated control. This might be attributed to the origin of muscle samples since the variation in connective tissues, particularly collagen, has been reported to affect the meat texture (Dransfield, 1977). While proteolysis is the major determinant of tenderness of *Longissimus* muscle, connective tissue content is a major contributor to the tenderness of muscles such as *Semimembranosus* and *Biceps femoris* (Koochmaraie et al. 2002). The high shear force values observed in our study are typical of *Semimembranosus* and *Biceps femoris* and will tend to be tougher as the meat came from culled dairy cows (Roberts et al. 2018). These results were in agreement with the findings of Arroyo et al. (2015b) and Arroyo et al. (2015a) who observed no effect of PEF treatment on the shear force of beef *Longissimus thoracis et lumborum* and turkey breast,

respectively. Proteolysis of myofibrillar proteins, sarcomere length, and connective tissue content are the three main components that account for most, if not all, of the explainable variation seen in tenderness of aged meat (Gheisari et al. 2007). The relative contribution of each of the above factors to tenderness is, however, dependent on the individual muscle under study. The potential of PEF to significantly reduce shear force in cold-boned muscles depends on various factors including muscles under study, electric field strength, and the use of an ageing period (Bhat et al., 2018a). A minimum electric field intensity is required above which PEF induces a substantial effect on tenderization process during a proper ageing period (Bhat et al. 2018a).

An early activation of calpain 2 was recorded in both the muscles along with an improvement in calpain activity of the samples treated with PEF during ageing. This study reports for the first time the effect of PEF treatment on the calpain activity of beef. These findings were consistent with the results for proteolysis as increased degradation of troponin-T and desmin was also recorded in PEF treated samples. Given that muscles from older animals are rich in connective tissues, particularly collagen, which predominantly contributes to their toughness, it seems that PEF operates enzymatically through activation of calpains and enhanced proteolysis. Physical disruption seems only to have a limited role and PEF appears to have a limited potential to improve the quality of collagen-rich tough muscles from culled dairy animals. This technology, however, can be used to reduce the total time of ageing to achieve a given quantum of tenderness.

Chapter 6 evaluated the effect of PEF on the calpain activity, proteolytic pattern and myofibrillar protein profile of venison (*Cervus elaphus*) *Longissimus dorsi* subjected to an ageing period of three weeks ( $4\pm 1$  °C). The activities of the calpain proteases (calpain 1 and calpain 2) were investigated to determine whether change in meat tenderness, if any, was associated with enzymatic changes. No significant effect of PEF was observed on the shear force of the muscle. No significant effect of PEF treatment was reported on the shear force of turkey breast either in frozen (2.1 kV/cm, 10 Hz) or fresh (1.2 kV/cm, 10 Hz) form (Arroyo et al. 2015b). In contrast to our study, several workers (Bekhit et al. 2016; Suwandy et al. 2015a, b; Bekhit et al. 2014c) have reported a significant impact of PEF processing on the shear force of beef muscles. Several factors, such as electric field strength, muscles under study, and the use of an ageing period, are required to induce a significant response in the shear force of cold-boned muscles (Bhat et al. 2018a). A slight increase was recorded in the calpain activity and proteolysis of troponin-T, suggesting a tendency of PEF to improve the tenderization process in venison. The calpains are believed to be the predominant proteases responsible for most of the post-mortem proteolysis and tenderization benefit of ageing (Huff-Lonergan et al. 2010; Geesink et al. 2006; Taylor et al. 1995). Since activity of calpain 1 is not detected after few initial days of ageing, proteolysis of myofibrillar proteins during extended ageing has been attributed to the activation of calpain 2 during late post-mortem (Colle, & Doumit, 2017).

Chapter 7, 8 and 9 studied the effect of PEF on the protein digestion of beef *Semimembranosus*, *Biceps femoris* and venison *Longissimus dorsi*, respectively, during *in vitro* gastrointestinal digestion simulation. Since cooking has the potential to alter the effect of PEF, because heating is known to affect the microstructure and digestibility of meat (Qi et al. 2018), the present research evaluated the effect of PEF processing on uncooked meat to elucidate the exact quantum of effect induced by PEF treatment alone. A significant effect of PEF processing was observed, and the samples treated with PEF (10kV, 20Hz, 20  $\mu$ s) had significantly ( $P<0.05$ ) higher *in vitro* protein digestibility (%) than the untreated samples for all the muscles. Similar values have been reported for pork, beef, chicken and fish samples subjected to *in vitro* simulated gastrointestinal digestion before (Tavares et al. 2018; Wen et al. 2015). Significantly ( $P<0.05$ ) higher soluble protein (%) values were also recorded for PEF-treated samples collected during intestinal phase of digestion for all the muscles. These results together with the results for protein digestibility (%) indicate that more digestion had occurred in PEF-treated samples for all the muscles. PEF processing can affect secondary, tertiary and quaternary protein structures by interfering with the electrostatic or hydrophobic interactions and disulfide or hydrogen bonds and can thus positively influence the digestion of the proteins (Giteru et al. 2018; Liu et al. 2017b). Structural changes which induce partial unfolding of proteins have been reported to increase the susceptibility of proteins to hydrolysis by gastrointestinal proteases (Simonetti et al. 2016; Promeprat et al. 2010). Modifications in the structure of the proteins have been reported to increase surface activity by exposure of hydrophobic domains and improve protein solubility by lowering the molecular weight (Primožic et al. 2018).

PEF processing influenced the proteolytic pattern and digestion kinetics of all the muscles during *in vitro* digestion process. The intensity of several bands during the gastric phase of digestion was lower in case of PEF treated samples, particularly for T<sub>2</sub> (10 kV) samples. This difference in the gastric digestion products between PEF-treated samples and control, which became more obvious with increasing digestion time, indicates that more hydrolysis has occurred in PEF processed samples. The intensity of the bands for T<sub>2</sub> (10 kV) samples was more affected than the intensity of T<sub>1</sub> (5 kV) samples for all muscles suggesting more digestion in T<sub>2</sub> (10 kV) samples. This was supported by a similar trend of increased protein digestibility (%) and soluble protein (%) for PEF treated samples. By destabilizing the protein molecules, PEF processing induces protein unfolding by disrupting the electrostatic interactions of individual polypeptide chains and local electrostatic fields, thereby destabilizing the secondary and tertiary structure of the proteins (Zhao et al. 2014). The total energy input and electric field strength play a major role in influencing the intermolecular interactions and are decisive for PEF-induced protein unfolding and denaturation (Liu et al. 2017b).

Mathematically higher values were observed for almost all the amino acids for the samples processed with PEF for all the muscles, although no significant ( $P>0.05$ ) difference were observed between control and PEF treated samples except for lysine in case of *Longissimus dorsi* which showed

significantly higher values for PEF treated samples. By increasing the membrane permeability and inducing structural changes in proteins, PEF seems to have a positive influence on enzymatic hydrolysis of meat proteins during *in vitro* simulated gastrointestinal digestion. Structural changes which induce partial unfolding of proteins have been reported to increase the susceptibility of proteins to hydrolysis by gastrointestinal proteases (Simonetti et al. 2016; Promeprat et al. 2010). The digestive enzymes, such as pepsin and trypsin, have been reported to act more effectively on unfolded and denatured proteins (Kaur et al. 2016). By inducing structural changes, PEF processing has been reported to enhance the susceptibility of egg white proteins to gastrointestinal digestion (Liu et al. 2018). Electroporation, which allows a greater exchange of constituents between the cell and its surroundings, may allow more diffusion of digestive proteases during gastrointestinal digestion leading to enhanced enzymatic hydrolysis in PEF-treated samples. Non-thermal technologies, such as high-hydrostatic pressure, have been reported to influence the digestive kinetics of meat proteins during *in vitro* simulated gastrointestinal digestion (Kaur et al. 2016).

In contrast to previous reports, which have observed a significant impact of PEF treatment on the mineral concentration of fresh meat (Khan et al. 2018; Khan et al. 2017a, b), no significant ( $P > 0.05$ ) impact of PEF processing was observed on the release of various minerals during the digestion process. A significant decrease has been reported in the concentration of Fe, K, P, Ca, Na and Mg in PEF treated beef muscles whereas a significant increase was observed in the concentration of Cr in beef and Ni in chicken breast (Khan et al. 2018; Khan et al. 2017a, b), suggesting the possible migration of minerals to meat from the electrodes of the PEF systems. Meat is considered as a rich source of minerals, such as Fe and Zn, and any change in the concentration of minerals will surely have a commercial importance.

While chapter 7, 8 and 9 concluded a positive impact of PEF on the protein digestion of uncooked muscles, it was not clear whether cooking will undo or influence this impact of PEF on protein digestion. To assess whether cooking will have any kind of influence, chapter 10 and 11 studied the impact of PEF on protein digestion of cooked beef *Semimembranosus* and venison *Longissimus dorsi*, respectively. PEF treated samples ( $T_1$ , 10 kV, 90 Hz, 20  $\mu$ s) were cooked (core temperature of 75 °C) and subjected to *in vitro* simulated gastrointestinal protein digestion along with a control. A significant effect of PEF processing was observed, and the samples treated with PEF (10 kV, 20 Hz, 20  $\mu$ s) had significantly ( $P < 0.05$ ) higher *in vitro* protein digestibility (%) and soluble protein (%) than the untreated samples for both the muscles. A positive influence of PEF processing was observed on the proteolytic pattern and digestion kinetics of both muscles during the *in vitro* digestion process and mathematically higher values were observed for free amino acids for PEF treated samples. PEF and high-temperature cooking, which have potential to affect the structural and functional properties of the proteins by inducing modifications such as cross-linking, unfolding, and aggregation, can influence the susceptibility of proteins to gastrointestinal enzymatic hydrolysis and affect the release of amino acids

and polypeptides and their bioavailability during digestion (Bhat et al. 2018d; Han et al. 2018; Liu et al. 2018). No significant ( $P>0.05$ ) impact of PEF processing was observed on the release of various minerals during the digestion process. This research clearly indicated that PEF processing has a positive influence on protein digestion of cooked and uncooked meat.

Chapter 12 evaluated the potential of PEF in the sodium-reduction strategy for processed-meat products. Beef jerky was used as a model of study and prepared using different levels of NaCl viz. 2.0% (control), 1.2% ( $T_1$ ) and 1.2% along with PEF-processing ( $T_2$ , 0.52 kV/cm, 10 kV, 20 Hz, 20  $\mu$ s). A significant impact of PEF processing was observed on all the textural characteristics and significantly ( $P<0.05$ ) lower values were observed for shear force (N), toughness (N/mm.sec) and firmness (N/mm) for the PEF processed samples (1.2% NaCl-PEF) in comparison to control (2% NaCl) and  $T_1$  (1.2% NaCl) samples. PEF processing has been reported to decrease the shear force in beef muscles before (Bekhit et al. 2016; Bekhit et al. 2014c). This study confirmed the superior tenderness of PEF processed products and a reduction of 22% was recorded in shear force of PEF processed samples in comparison to control. This effect was reflected on sensory scores of the products as significantly ( $P<0.05$ ) higher scores were recorded for tenderness for  $T_2$  (1.2% NaCl-PEF) samples in comparison to control (2% NaCl) and  $T_1$  (1.2% NaCl) samples. Arroyo et al. (2015b) reported that 60% of the panellists scored PEF treated samples as tender in comparison to 27.5% who scored untreated samples as tender. Given that PEF only showed a tendency to reduce shear force in muscles from culled dairy animals with high collagen content (in chapter 4 and 5) and showed a significant impact on shear force of muscles from younger animals, these results confirm the enzymatic nature of PEF induced tenderness. PEF processing did not affect ( $P>0.05$ ) the other quality parameters such as colour, cooking yield and storage stability (microbial and oxidative) of the products.

PEF-treated samples ( $T_2$ , 1.2% NaCl-PEF) had significantly ( $P<0.05$ ) lower sodium content than the control (2% NaCl), however, the sensory scores for colour, flavour, saltiness and overall acceptability were comparable ( $P>0.05$ ) with control. These effects were also visible on the results of the ranking tests where more than 84% of the panellists preferred  $T_2$  (1.2% NaCl-PEF) samples over  $T_1$  (1.2% NaCl) for saltiness. 53% of the panellists ranked control (2% NaCl) samples whereas 36% ranked  $T_2$  (1.2% NaCl-PEF) samples as the most preferred salt level. In the ranking test for intensity of saltiness, 71% ranked  $T_2$  (1.2% NaCl-PEF) samples as more intense than  $T_1$  (1.2% NaCl) samples. Our results suggest that PEF-treatment improved saltiness naturally by influencing the salt-diffusion and sodium-delivery that led to better perception during chewing. PEF has a sodium-reduction potential that can play a role in public health. Processing of meat by non-thermal technologies, such as high pressure and ultrasound, have been reported to be helpful in designing of reduced sodium meat products by influencing the salt diffusion and sensory perception (Barretto et al. 2018; Inguglia et al. 2017).

## **Future studies**

Future studies can compare the effect of PEF on the calpain activity of muscles from older animals with younger animals. The mechanisms on how PEF improves protein digestion are still unknown and need scientific attention. Future studies can evaluate how PEF affects the structure and function of meat proteins that affects their digestibility during gastrointestinal digestion. Insights on the basic mechanisms of how PEF affects meat protein digestibility will be invaluable as this knowledge could be utilized for the development of novel protein structures and muscle foods with improved digestibility. Efforts are required to elucidate the mechanisms how PEF affects the sodium perception, whether it is a simple diffusion process that increases the sodium content of the samples or does it affect the interaction between sodium ions and proteins and the release of sodium during chewing. A comprehensive study is required to examine how this technology will be applied in the meat industry on actual meat cuts of varying shapes and densities. Further, this technology can have a role in extraction and recovery of enzymes, hormones and other heat sensitive molecules from animal tissues.

# Appendix

## A.1 Approval letter for sensory evaluation from Human Ethics Committee

12 October 2018

Application No: 2018-35

Title: Development of reduced-sodium beef jerky using pulsed electric field

Applicant: Zuhaib F. Bhat

---

Research Management Office

T 64 3 423 0817  
PO Box 85084, Lincoln University  
Lincoln 7647, Christchurch  
New Zealand  
[www.lincoln.ac.nz](http://www.lincoln.ac.nz)

The Lincoln University Human Ethics Committee has reviewed the above noted application. Thank you for your response to the questions which were forwarded to you on the Committee's behalf.

I am satisfied on the Committee's behalf that the issues of concern have been satisfactorily addressed. I am pleased to give final approval to your project.

Please note that this approval is valid for three years from today's date at which time you will need to reapply for renewal.

Once your field work has finished can you please advise the Human Ethics Secretary, Alison Hind, and confirm that you have complied with the terms of the ethical approval.

May I, on behalf of the Committee, wish you success in your research.

Yours sincerely



Grant Tavinor  
Chair, Human Ethics Committee

**PLEASE NOTE:** The Human Ethics Committee has an audit process in place for applications. Please see 7.3 of the Human Ethics Committee Operating Procedures (ACHE) in the Lincoln University Policies and Procedures Manual for more information.

## A.2 Hedonic scale used for sensory evaluation

Sensory attribute	Sensory score								
	1	2	3	4	5	6	7	8	9
Colour	Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
Saltiness	Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
Flavour	Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
Tenderness	Extremely tough	Very much tough	Moderately tough	Slightly tough	Neither tough nor tender	Slightly tender	Moderately tender	Very much tender	Extremely tender
Overall acceptability	Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely



## References

- Aaslyng, M. D., Vestergaard, C., & Koch, A. G. (2014). The effect of salt reduction on sensory quality and microbial growth in hotdog sausages, bacon, ham and salami. *Meat Science*, *96*, 47-55.
- Abenoza, M., Benito, M., Saldana, G., Alvarez, I., Raso, J., & Sanchez-Gimeno, A. C. (2013). Effects of pulsed electric field on yield extraction and quality of olive oil. *Food Bioprocess Technology*, *6*, 1367-1373.
- Aguiló-Aguayo, I., Gangopadhyay, N., Lyng, J. G., Brunton, N., & Rai, D. K. (2017). Impact of pulsed light on colour, carotenoid, polyacetylene and sugar content of carrot slices. *Innovative Food Science and Emerging Technology*, *42*, 49-55.
- Alahakoon, M. A. U. (2018). The impact of pulsed electric field and sous vide processing on the quality of beef brisket. *Thesis*, Doctor of Philosophy, Department of Food Science, University of Otago, New Zealand. <http://hdl.handle.net/10523/8507>.
- Alahakoon, M. A. U., Oey, I., Bremer, P., & Silcock, P. (2019). Process optimisation of pulsed electric fields pre-treatment to reduce the sous vide processing time of beef briskets. *International Journal of Food Science and Technology*, *54*(3), 823-834.
- Alarcon-Rojo, A. D., Janacua, H., Rodriguez, J. C., Paniwnyk, L., & Mason, T. J. (2015). Power ultrasound in meat processing. *Meat Science*, *107*, 86-93.
- Almeida, C. C., Monteiro, M. L. G., Carneiro da Costa-Lima, B. R., Alvares, T. S., & Conte-Junior, C. A. (2015). *In vitro* digestibility of commercial whey protein supplements. *LWT - Food Science and Technology*, *61*, 7-11.
- Alminger, M., Aura, A. M., Bohn, T., Dufour, C., El, S. N., Gomes, A., & Santos, C. N. (2014). *In vitro* models for studying secondary plant metabolite digestion and bioaccessibility. *Comprehensive Reviews in Food Science and Food Safety*, *13*, 413-436.
- Amiali, M., Ngadi, M. O., Smith, J. P., & Raghavan, G. S. V. (2007). Synergistic effect of temperature and pulsed electric field on inactivation of *Escherichia coli* O157:H7 and *Salmonella enteritidis* in liquid egg yolk. *Journal of Food Engineering*, *79*, 689-694.
- APHA (1984). *Compendium of methods for the microbiological examination of foods* (2nd ed.). American Public Health Association, Washington DC.
- Arroyo, C., Eslami, S., Brunton, N. P., Arimi, J. M., Noci, F., & Lyng, J. G. (2015a). An assessment of the impact of pulsed electric fields processing factors on oxidation, colour, texture, and sensory attributes of turkey breast meat. *Poultry Science*, *94*, 1088-95.
- Arroyo, C., Lascorz, D., O'Dowd, L., Noci, F., Arimi, J., & Lyng, J. G. (2015b). Effect of pulsed electric field treatments at various stages during conditioning on quality attributes of beef *longissimus thoracis et lumborum* muscle. *Meat Science*, *99*, 52-59.
- Barba, F. J., Ahrné, L., Xanthakis, E., Landerslev, M. G., & Orlien, V. (2018). Innovative technologies for food preservation. In: Innovative technologies for food preservation- Inactivation of spoilage

- and pathogenic microorganisms. Barba, F. J., Sant'Ana, A. de S., Orlie, V., & Koubaa, M., (Editors), pp. 25-51, Academic Press, Elsevier Inc.
- Barba, F. J., Grimi, N., & Vorobiev, E. (2015). New approaches for the use of non-conventional cell disruption technologies to extract potential food additives and nutraceuticals from microalgae. *Food Engineering Reviews*, *7*, 45-62.
- Barba, F. J., Jäger, H., Meneses, N., Esteve, M. J., Frígola, A., & Knorr, D. (2012). Evaluation of quality changes of blueberry juice during refrigerated storage after high pressure and pulsed electric fields processing. *Innovative Food Science and Emerging Technologies*, *14*, 18-24.
- Barba, F. J., Koubaa, M., Prado-Silva, L., Orlie, V., & Sant'Ana, A. S. (2017a). Mild processing applied to the inactivation of the main foodborne bacterial pathogens: A review. *Trends in Food Science & Technology*, *66*, 20-35.
- Barba, F. J., Mariutti, L. R. B., Bragagnolo, N., Mercadante, A. Z., Barbosa-Cánovas, A. V., & Orlie, V. (2017b). Bioaccessibility of bioactive compounds from fruits and vegetables after thermal and nonthermal processing. *Trends in Food Science & Technology*, *67*, 195-206.
- Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N., Saraiva, J. A., Raso, J., Martin-Belloso, O., Witrowa-Rajchert, D., Lebovka, N., & Vorobiev, E. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. *Food Research International*, *77*, 773-798.
- Barba, F. J., Terefe, N. S., Buckow, R., Knorr, D., & Orlie, V. (2015). New opportunities and perspectives of high-pressure treatment to improve health and safety attributes of foods. A review. *Food Research International*, *77*, 725-742.
- Barbosa-Cánovas, G. V., & Sepulveda, D. R. (2005). Novel food processing technologies. New York, Marcel Dekker.
- Barbut, S., Sosnicki, A. A., Lonergan, S. M., Knapp, T., Ciobanu, D. C., Gatcliffe, L. J., Huff-Lonergan, E., & Wilson, E. W. (2008). Progress in reducing the pale, soft and exudative (PSE) problem in pork and poultry meat. *Meat Science*, *79*, 46-63.
- Barnett, A., & Weaver, J. C. (1991). A unified quantitative theory of reversible electrical breakdown and rupture. *Bioelectrochemistry and Bioenergetics*, *25*, 163-182.
- Baron, C. P., Jacobsen, S., & Purslow, P. P. (2004). Cleavage of desmin by cysteine proteases: Calpains and cathepsin B. *Meat Science*, *68*, 447-456.
- Barretto, T. L., Pollonio, M. A. R., Romero, J. T., & Barretto, A. C. S. (2018). Improving sensory acceptance and physicochemical properties by ultrasound application to restructured cooked ham with salt (NaCl) reduction. *Meat Science*, *145*, 55-62.
- Bartoň, L., Bureš, D., Kotrba, R., & Sales, J. (2014). Comparison of meat quality between eland (*Taurotragus oryx*) and cattle (*Bos taurus*) raised under similar conditions. *Meat Science*, *96*(1), 346-352.

- Bax, M. L., Aubry, L., Ferreira, C., Daudin, J. D., Gatellier, R., Rémond, D., & Santé-Lhoutellier, V. (2012). Cooking temperature is a key determinant of *in vitro* meat protein digestion rate: Investigation of underlying mechanisms. *Journal of Agricultural and Food Chemistry*, *60*, 2569-2576.
- Bax, M. L., Sayd, T., Aubry, L., Ferreira, C., Viala, D., & Chambon, C. (2013). Muscle composition slightly affects *in vitro* digestion of aged and cooked meat: Identification of associated proteomic markers. *Food Chemistry*, *136*, 1249-1262.
- Bee, G., Anderson, A. L., Lonergan, S. M., & Huff-Lonergan, E. (2007). Rate and extent of pH decline affect proteolysis of cytoskeletal proteins and water-holding capacity in pork. *Meat Science*, *76*, 359-365.
- Bekhit, A. E. A., Suwandy, V., Carne, A., Ahmed, I. A. M., & Zirong, W. (2017). Manipulation of meat quality: electrical stimulation and pulsed electric field. In: Bekhit, A. E. A. (Ed.), *Advances in meat processing technology*, CRC Press, ISBN 9781498700481, pp. 121-163.
- Bekhit, A. E. A., Suwandy, V., Carne, A., van de Ven, R., & Hopkins, D. L. (2016). Effect of repeated pulsed electric field treatment on the quality of hot-boned beef loins and topsides. *Meat Science*, *111*, 139-146.
- Bekhit, A. E. D., Carne, A., Ha, M., & Franks, P. (2014a). Physical interventions to manipulate texture and tenderness of fresh meat: a review. *International Journal of Food Properties*, *17*, 433-453.
- Bekhit, A. E. D., Hopkins, D. L., Geesink, G., Bekhit, A. E. D., & Franks, P. (2014b). Exogenous proteases for meat tenderization. *Critical Reviews in Food Science and Nutrition*, *54*, 1012-1031.
- Bekhit, A. E. D., van de Ven, R., Suwandy, V., Fahri, F., and Hopkins, D. L. (2014c). Effect of pulsed electric field treatment on cold-boned muscles on different potential tenderness. *Food Bioprocess Technology*, *7*, 3136-3146.
- Bender, A. (1992). Meat and meat products in human nutrition in developing countries. Food and Nutrition Paper 53, Food and Agriculture Organization of The United Nations, Rome, <http://www.fao.org/3/t0562e/T0562E05.htm> (accessed on 20.04.2019).
- Benyamin, Y. 2006. The structural basis of calpain behaviour. *The FEBS Journal*, *273*, 3413-3414.
- Bermúdez-Aguirre, D., Dunne, C. P., & Barbosa-Canovas, G. V. (2012). Effect of processing parameters on inactivation of *Bacillus cereus* spores in milk using pulsed electric fields. *International Dairy Journal*, *24*, 13-21.
- Bertram, H. C., Kohler, A., Böcker, U., Ofstad, R., & Andersen, H. J. (2006). Heat-induced changes in myofibrillar protein structures and myowater of two pork qualities: A combined FT-IR spectroscopy and low-field NMR relaxometry study. *Journal of Agricultural and Food Chemistry*, *54*, 1740-1746.
- Bhat, Z. F., Mason, S. L., Morton, J. D., Bekhit, A. E. A., & Hina F. Bhat (2017). Antihypertensive peptides from animal proteins. In: *Bioactive Molecules in Food*, Mérillon, J. M., Ramawat, K. G. (eds.),

Reference Series in Phytochemistry, pp. 1-36, Springer International Publishing, [https://doi.org/10.1007/978-3-319-54528-8\\_18-1](https://doi.org/10.1007/978-3-319-54528-8_18-1).

- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. A. (2018a). Current and future prospects for the use of pulsed electric field in the meat industry. *Critical Reviews in Food Science and Nutrition*, Doi: 10.1080/10408398.2018.1425825.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. A. (2018f). Pulsed electric field: Role in protein digestion of beef *Biceps femoris*. *Innovative Food Science and Emerging Technologies*, <https://doi.org/10.1016/j.ifset.2018.09.006>.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. A. (2018b). Calpain activity, myofibrillar protein profile and physicochemical properties of beef *Semimembranosus* and *Biceps femoris* from culled dairy cows during ageing. *Journal of Food Processing and Preservation*, 42(12), e13835.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. A. (2018d). Applied and emerging methods for meat tenderization: a comparative perspective. *Comprehensive Reviews in Food Science and Food Safety*, 17(04), 841-859.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. A. (2018c). Role of calpain system in meat tenderness. *Food Science and Human Wellness*, 7, 196-204.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. A. (2018e). Pulsed electric field improved protein digestion of beef during *in vitro* gastrointestinal simulation. *LWT-Food Science and Technology*, 102, 45-51.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. A. (2019a). Does pulsed electric field have a potential to improve the quality of beef from older animals and how? *Innovative Food Science and Emerging Technologies*, 56, <https://doi.org/10.1016/j.ifset.2019.102194>.
- Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. A. (2019b). Pulsed electric field operates enzymatically by causing early activation of calpains in beef during ageing. *Meat Science*, 153, 144-151.
- Bhat, Z. F., Morton, J. D., Mason, S. L., Bekhit, A. E. A. & Mungure, T. (2018g). Pulsed electric field: Effect on *in vitro* simulated gastrointestinal protein digestion of deer *Longissimus dorsi*. *Food Research International*, <https://doi.org/10.1016/j.foodres.2018.11.040>.
- Birkett, A., Muir, J., Phillips, J., Jones, G., & O'Dea, K. (1996). Resistant starch lowers faecal concentrations of ammonia and phenols in humans. *American Journal of Clinical Nutrition*, 63, 766-772.
- Biswas, A. K., Tandon, S., & Sharma, D. (2016). Identification of different domains of calpain from blood and goat skeletal muscle and their influence on quality during post-mortem ageing of meat during holding at 4±1 °C. *LWT - Food Science and Technology*, 71, 60-68.

- Boehm, M. L., Kendall, T. L., Thompson, V. F., & Goll, D. E. (1998). Changes in the calpains and calpastatin during post-mortem storage of bovine muscle. *Journal of Animal Science*, *76*, 2415-2434.
- Bohorov, O., Buttery, P. J., Correia, J. H. R. D., & Soar, J. B. (1987). The effect of the  $\beta$ -2-adrenergic agonist clenbuterol or implantation with oestradiol plus trenbolone acetate on protein metabolism in wether lambs. *British Journal of Nutrition*, *57*, 99-107.
- Bolton, D. J., Catarama, T., Byrne, C., Sheridan, J. J., McDowell, D. A., & Blair, I. S. (2002). The ineffectiveness of organic acids, freezing and pulsed electric fields to control *Escherichia coli* O157:H7 in beef burgers. *Letters in Applied Microbiology*, *34*, 139-43.
- Bot, F., Verkerk, R., Mastwijk, H., Anese, M., Fogliano, V., & Capuano, E. (2018). The effect of pulsed electric fields on carotenoids bioaccessibility: The role of tomato matrix. *Food Chemistry*, *240*, 415-421.
- Boye, J., Wijesinha-Bettoni, R., & Burlingame, B. (2012). Protein quality evaluation twenty years after the introduction of the protein digestibility corrected amino acid score method. *British Journal of Nutrition*, *108*, S183-211.
- Bratcher, C. L., Johnson, D. D., Littell, R. C., & Gwartney, B. L. (2005). The effects of quality grade, aging, and location within muscle on Warner-Bratzler shear force in beef muscles of locomotion. *Meat Science*, *70*, 279-284.
- Brewer, M. S. (2012). Reducing the fat content in ground beef without sacrificing quality: a review. *Meat Science*, *91*, 385-395.
- Bröer, S. (2008). Amino acid transport across mammalian intestinal and renal epithelia. *Physiological Reviews*, *88*, 249-286.
- Buchmann, L., Bloch, R., & Mathys, A. (2018). Comprehensive pulsed electric field (PEF) system analysis for microalgae processing. *Bioresource Technology*, *265*, 268-274.
- Buckow, R., Chandry, P. S., Ng, S. Y., McAuley, C. M., & Swanson, B. G. (2014). Opportunities and challenges in pulsed electric field processing of dairy products. *International Dairy Journal*, *34*, 199-212.
- Buckow, R., Ng, S., & Toepfl, S. (2013). Pulsed electric field processing of orange juice: a review on microbial, enzymatic, nutritional, and sensory quality and stability. *Comprehensive Reviews in Food Science and Food Safety*, *12*, 455-467.
- Bunmee, T., Jaturasitha, S., Kreuzer, M., & Wicke, M. (2014). Can calcium chloride injection facilitate the ageing-derived improvement in the quality of meat from culled dairy cows? *Meat Science*, *96*, 1440-1445.
- Bureš, D., Bartoň, L., Kotrba, R., & Hakl, J. (2015). Quality attributes and composition of meat from red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and Aberdeen Angus and Holstein cattle (*Bos taurus*). *Journal of the Science of Food and Agriculture*, *95*(11), 2299-2306.

- Camou, J. P., Marchello, J. A., Thompson, V. F., Mares, S. W., & Goll, D. E. (2007). Effect of post-mortem storage on activity of  $\mu$ - and m-calpain in five bovine muscles. *Journal of Animal Science*, *85*, 2670-2681.
- Campbell, R. L., & Davies, P. L. (2012). Structure-function relationships in calpains. *Biochemistry Journal*, *447*, 335-351.
- Carbonell-Capella, J. M., Buniowska, M., Barba, F. J., Esteve, M. J., & Frígola, A. (2014). Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. *Comprehensive Reviews in Food Science and Food Safety*, *13*, 155-171.
- Carbonell-Capella, J. M., Parniakov, O., Barba, F. J., Grimi, N., Bals, O., Lebovka, N., & Vorobiev, E. (2016). "Ice" juice from apples obtained by pressing at subzero temperatures of apples pretreated by pulsed electric fields. *Innovative Food Science and Emerging Technologies*, *33*, 187-194.
- Cardoso, C., Ribeiro, B., & Mendes, R. (2014). The influence of fish age, salt level, and MTGase addition on the quality of gels prepared from unwashed mince of farmed meagre (*Argyrosomus regius*). *Food Science and Technology International*, *20*, 253-263.
- Carlin, K. R. M., Huff-Loneragan, E., Rowe, L. J., & Lonergan, S. M. (2006). Effect of oxidation, pH, and ionic strength on calpastatin inhibition of  $\mu$ - and m-calpain. *Journal of Animal Science*, *84*, 925-937.
- Chaijan, M., & Panpitat, W. (2017). Mechanism of oxidation in foods of animal origin. In Banerjee, R., Verma, A. K., & Siddiqui, M. W. (Eds.). *Natural antioxidants: Applications in foods of animal origin*. Apple Academic Press, Canada.
- Chen, Q., Huang, J., Huang, F., Huang, M., & Zhou, G. (2014). Influence of oxidation on the susceptibility of purified desmin to degradation by  $\mu$ -calpain, caspase-3 and -6. *Food Chemistry*, *150*, 220-226.
- Chian, F. M., Kaur, L., Oey, I., Astruc, T., Hodgkinson, S., & Boland, M. (2019). Effect of pulsed electric fields (PEF) on the ultrastructure and *in vitro* protein digestibility of bovine longissimus thoracis. *LWT-Food Science and Technology*, *103*, 253-259.
- Christensen, M., Henckel, P., & Purslow, P. P. (2004). Effect of muscle type on the rate of post-mortem proteolysis in pigs. *Meat Science*, *66*, 595-601.
- Chrystall, B. B., & Devine, C. E. (1991). *Quality assurance for tenderness. Publication No. 872*. Meat Industries Research Institute of New Zealand.
- Claeys, E., Smet, S. D., Balcaen, A., Raes, K., & Demeyer, D. (2004). Quantification of fresh meat peptides by SDS-PAGE in relation to ageing time and taste intensity. *Meat Science*, *67*(2), 281-288.
- Clariana, M., Guerrero, L., Sarraga, C., Diaz, I., Valero, A., & Garcia-Regueiro, J. A. (2011). Influence of high-pressure application on the nutritional, sensory and microbiological characteristics of sliced

- vacuum packed dry-cured ham. Effects along the storage period. *Innovative Food Science and Emerging Technologies*, 12, 456-465.
- Clark, P. (2006). Pulsed electric field processing. *Food Technology*, 60, 66-67.
- Clemente, I., Condón-Abanto, S., Pedrós-Garrido, S., Whyte, P., & Lyng, J. G. (2019). Efficacy of pulsed electric fields and antimicrobial compounds used alone and in combination for the inactivation of in liquids and raw chicken *Campylobacter jejuni*. *Food Control*, DOI: 10.1016/j.foodcont.2019.01.017.
- Colle, M. J., & Doumit, M. E. (2017). Effect of extended aging on calpain-1 and -2 activity in beef *longissimus lumborum* and *semimembranosus* muscles. *Meat Science*, 131, 142-45.
- Colle, M. J., Nasados, J. A., Rogers, J. M., Kerby, D. M., Colle, M. M., Van Buren, J. B., Richard, R. P., Murdoch, G. K., Williams, C. J., & Doumit, M. E. (2018). Strategies to improve beef tenderness by activating calpain-2 earlier postmortem. *Meat Science*, 135, 36-41.
- Colle, M. J., Richard, R. P., Killinger, K. M., Bohscheid, J. C., Gray, A. R., Loucks, W. I., & Doumit, M. E. (2015). Influence of extended aging on beef quality characteristics and sensory perception of steaks from the *gluteus medius* and *longissimus lumborum*. *Meat Science*, 110, 32-39.
- Colle, M. J., Richard, R. P., Killinger, K. M., Bohscheid, J. C., Gray, A. R., Loucks, W. I., & Doumit, M. E. (2016). Influence of extended aging on beef quality characteristics and sensory perception of steaks from the *biceps femoris* and *semimembranosus*. *Meat Science*, 119, 110-117.
- Contreras-Castillo, C. J., Lomiwes, D., Wu, G., Frost, D., & Farouk, M. M. (2016). The effect of electrical stimulation on post-mortem myofibrillar protein degradation and small heat shock protein kinetics in bull beef. *Meat Science*, 113, 65-72.
- Corrales, M., Toepfl, S., Butz, P., Knorr, D., & Tauscher, B. (2008). Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: a comparison. *Innovative Food Science and Emerging Technologies*, 9, 85-91.
- Corte-Real, J., & Bohn, T. (2018). Interaction of divalent minerals with liposoluble nutrients and phytochemicals during digestion and influences on their bioavailability—a review. *Food Chemistry*, 252, 285-293.
- Costa, H., Mafra, I., Oliveira, M. B. P. P., & Amaral, J. S. (2016). Game: types and composition. In: Caballero, F. T. B., Finglas, P. M., & Toldrá F. (Eds.). *Encyclopedia of food and health* (pp. 177–183). Oxford: Academic Press.
- Cremer, M. L., & Chipley, J. R. (1977). Satellite food service system: Time and temperature and microbiological and sensory quality of precooked frozen hamburger patties. *Journal of Food Protection*, 40, 603-607.
- Croall, D., & DeMartino, G. (1991). Calcium-activated neutral protease (calpain) system: Structure, function, and regulation. *Physiological Reviews*, 71, 813-847.

- Cruzen, S. M. (2013). Characterization of the skeletal muscle calpain/calpastatin system in growth models in swine and cattle. *Graduate Theses and Dissertations*. Paper 13305, downloaded from <http://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=4312&context=etd>
- Culler, R. D., Parrish, F. C., Smith, G. C., & Cross, H. R. (1978). Relationship of myofibril fragmentation index to certain chemical, physical and sensory characteristics of bovine longissimus muscle. *Journal of Food Science*, *43*(4), 1177-1180.
- da Silva, F. L. F., de Lima, J. P. S., Melo, L. S., da Silva, Y. S. M., Gouveia, S. T., Lopes, G. S., & Matos, W. O. (2017). Comparison between boiling and vacuum cooking (sous-vide) in the bioaccessibility of minerals in bovine liver samples. *Food Research International*, *100*, 566–571.
- Davies, K. J. A., Delsignore, M. E., & Lin, S. W. (1987). Protein damage and degradation by oxygen radicals. II. Modification of amino acids. *The Journal of Biological Chemistry*, *262*, 9902-9907.
- Dayton, W. R., Reville, W. J., Goll, D. E., & Stromer, M. H. (1976). A Ca<sup>2+</sup>-activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. *Biochemistry*, *15*, 2159-2167.
- De Maria, S., Ferrari, G., & Maresca, P. (2016). Effects of high hydrostatic pressure on the conformational structure and the functional properties of bovine serum albumin. *Innovative Food Science & Emerging Technologies*, *33*, 67-75.
- De Tullio, R., Averna, M., Stifanese, R., Parr, T., Bardsley, R. G., Pontremoli, S., & Melloni, E. (2007). Multiple rat brain calpastatin forms are produced by distinct starting points and alternative splicing of the N-terminal exons. *Archives of Biochemistry and Biophysics*, *465*, 148-156.
- Delsart, C., Cholet, C., Ghidossi, R., Grimi, N., Gontier, E., Geny, L., Vorobiev, E., & Mietton-Peuchot, M. (2014). Effects of pulsed electric fields on Cabernet Sauvignon grape berries and on the characteristics of wines. *Food Bioprocess Technology*, *7*, 424-436.
- Deng, Q., Zinoviadou, K. G., Galanakis, C. M., Orlie, V., Grimi, N., Vorobiev, E., Lebovka, N., & Barba, F. J. (2014). The effects of conventional and non-conventional processing on glucosinolates and its derived forms, isothiocyanates: Extraction, degradation, and applications. *Food Engineering Reviews*, *7*, 357-381.
- Devine, C. E., Payne, S. R., Peachey, B. M., Lowe, T. E., Ingram, J. R., & Cook, C. J. (2002). High and low rigor temperature effects on sheep meat tenderness and ageing. *Meat Science*, *60*, 141–146.
- Dilnawaz, H. M., Kumar, S., & Bhat, Z. F. (2017). *Ipomoea batatas* as a novel binding agent for hot-set restructured binding systems and green coffee bean for improved lipid oxidative stability and storage quality. *Nutrition and Food Science*, *47*(5).
- Dixon, C. L., Woerner, D. R., Tokach, R. J., Chapman, P. L., Engle, T. E., Tatum, J. D., & Belk, K. E. (2012). Quantifying the “aging response” and nutrient composition for muscles of the beef round. *Journal of Animal Science*, *90*, 996-1007.



- Djikaev, Y. S., & Ruckenstein, E. (2008). Thermal denaturation of a native protein via spinodal decomposition in the framework of first-passage-time analysis. *Physical Review E*, 78, 011909.
- Dominguez-Hernandez, E., Salaseviciene, A., & Ertbjerg, P. (2018). Low-temperature long-time cooking of meat: Eating quality and underlying Mechanisms. *Meat Science* 143, 104-113.
- Dominik, P., Pavlík, Z., Steinhauserová, I., Saláková, A., Buchtová, H., & Steinhauser, L. (2012). The effect of soluble collagen on the texture of fallow deer meat. *Maso International*, 1(1), 57-61.
- Dransfield, E. (1977). Intramuscular composition and texture of beef muscles. *Journal of the Science of Food and Agriculture*, 28, 833-842.
- Dransfield, E. (1994). Optimization of tenderization, aging and tenderness. *Meat Science*, 36, 105-121.
- Dransfield, E., Martin, J. F., Bauchart, D., Abouelkaram, S., Lepetit, J., Culioli, J., Jurie, C., & Picard, B. (2003). Meat quality and composition of three muscles from French cull cows and young bulls. *Animal Science*, 76, 387-399.
- Du, M., Li, X., Li, Z., Li, M., Gao, L., & Zhang, D. (2017). Phosphorylation inhibits the activity of  $\mu$ -calpain at different incubation temperatures and  $\text{Ca}^{2+}$  concentrations *in vitro*. *Food Chemistry*, 228, 649-655.
- Du, M., Xin, Li, Zheng, Li, Shen, Q., Wang, Y., Li, G., & Zhang, D. (2018). Phosphorylation regulated by protein kinase A and alkaline phosphatase play positive roles in  $\mu$ -calpain activity. *Food Chemistry*, 252, 33-39.
- Dubost, A., Micol, D., Picard, B., Lethias, C., Andueza, D., Bauchart, D., & Listrat, A. (2013). Structural and biochemical characteristics of bovine intramuscular connective tissue and beef quality. *Meat Science*, 95, 555-561.
- Dunsha, F. R., D'Souza, D. N., Pethick, D. W., Harper, G. S., & Warner, R. D. (2005). Effects of dietary factors and other metabolic modifiers on quality and nutritional value of meat. *Meat Science*, 71, 8-38.
- duVerle, D. A., Ono, Y., Sorimachi, H., & Mamitsuka, H. (2011). Calpain cleavage prediction using multiple kernel learning. *PLoS ONE*, 6, e19035.
- Edmunds, T., Nagainis, P. A., Sathe, S. K., Thompson, V. F., & Goll, D. E. (1991). Comparison of the autolyzed and unautolyzed forms of  $\mu$ - and m-calpain from bovine skeletal muscle. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*, 1077, 197-208.
- Ekezie, F. G. C., Cheng, J. H., & Sun, D. W. (2018). Effects of nonthermal food processing technologies on food allergens: A review of recent research advances. *Trends in Food Science & Technology*, 74, 12-25.
- Elce, J. S., Davies, P. L., Hegadorn, C., Maurice, D. H., & Arthur, J. S. C. (1997). The effects of truncations of the small subunit on m-calpain activity and heterodimer formation. *Biochemistry Journal*, 326, 31-38.

- EPA (2016). Nickle compounds. Downloaded from: <https://www.epa.gov/sites/production/files/2016-09/documents/nickle-compounds.pdf>.
- Erickson, R. H., & Kim, Y. S. (1990). Digestion and absorption of dietary protein. *Annual Review of Medicine*, *41*, 133-139.
- Ertbjerg, P., Christiansen, L. S., Pedersen, A. B., & Kristensen, L. (2012). The effect of temperature and time on activity of calpain and lysosomal enzymes and degradation of desmin in porcine longissimus muscle. 58<sup>th</sup> International Congress of Meat Science and Technology, 12-17<sup>th</sup> August 2012, Montreal, Canada.
- Estévez, M. (2015). Oxidative damage to poultry: From farm to fork. *Poultry Science*, *94*, 1368-1378.
- Estévez, M., & Luna, C. (2017). Dietary protein oxidation: A silent threat to human health? *Critical Reviews in Food Science and Nutrition*, *57*, 3781-3793.
- Eugenio, D., Daniel, V., Tiziano, T., Anna, A.M.G., & Roberto, V. (2018). Consumer preferences for red deer meat: A discrete choice analysis considering attitudes towards wild game meat and hunting. *Meat Science*, 10.1016/j.meatsci.2018.07.031.
- Faber, T. A., Bechtel, P. J., Hernot D. C., Parsons C. M., Swanson, K. S., Smiley, S., & Fahey Jr., G. C. (2010). Protein digestibility evaluations of meat and fish substrates using laboratory, avian, and ileally cannulated dog assays. *Journal of Animal Science*, *88*, 1421-1432.
- Fairweather-Tait, S., Lynch, S., Hotz, C., Hurrell, R., Abrahamse, L., Beebe, S., & Trinidad, T. P. (2005). The usefulness of in vitro models to predict the bioavailability of iron and zinc: A consensus statement from the harvest plus expert consultation. *International Journal for Vitamin and Nutrition Research*, *75*, 371-374.
- Fan, Y.-X., Zhang, Y., & Shen, H.-B. (2013). LabCaS: Labeling calpain substrate cleavage sites from amino acid sequence using conditional random fields. *Proteins: Structure, Function, and Bioinformatics* *81*, 622-634.
- FAO, & WHO (2004). Vitamin and mineral requirements in human nutrition (Second edition). Italy: Rome: WHO.
- FAOSTAT (2017). Food and Agriculture Organization of the United Nations. Retrieved from: <http://www.fao.org/faostat/en/#data/QL/visualize>.
- Faridnia, F., Bekhit, A. E. A., Niven, B., & Oey, I. (2014). Impact of pulsed electric fields and post-mortem vacuum aging on beef longissimus thoracis muscles. *International Journal of Food Science and Technology*, *49*, 2339-2347.
- Faridnia, F., Bremer, P., Burritt, D.J., & Oey, I. (2016). Effects of pulsed electric fields on selected quality attributes of beef outside flat (*Biceps femoris*). Jarm, T., & Kramar, P. (eds.), 1<sup>st</sup> world congress on electroporation and pulsed electric fields in biology, Medicine and Food and Environmental Technologies (WC 2015), IFMBE Proceedings 53, 51, DOI: 10.1007/978-981-287-817-5\_12.

- Faridnia, F., Ma, Q. L., Bremer, P. J., Burritt, D. J., Hamid, N., & Oey, I. (2015). Effect of freezing as pre-treatment prior to pulsed electric field processing on quality traits of beef muscles. *Innovative Food Science and Emerging Technologies*, 29, 31-40.
- Farouk, M. M., Wu, G. J., Frost, D. A., Clerens, S., & Knowles, S. O. (2014). The in vitro digestibility of beef varies with its inherent ultimate pH. *Food & Function*, 5(11), 2759-2767.
- Felisberto, M. H. F., Galvao, M. T. E. L., Picone, C. S. F., Cunha, R. L., Pollonio, M. A. R. (2015). Effect of prebiotic ingredients on the rheological properties and microstructure of reduced-sodium and low-fat meat emulsions. *LWT - Food Science and Technology*, 60, 148-155.
- Fellendorf, S., O'Sullivan, M. G., & Kerry, J. P. (2016). Impact of ingredient replacers on the physicochemical properties and sensory quality of reduced salt and fat black puddings. *Meat Science*, 113, 17-25.
- Filho, R. A. T., Cazedey, H. P., Fontes, P. R., Ramos, A. L. S., & Ramos, E. M. (2017). Drip loss assessment by different analytical methods and their relationships with pork quality classification. *Journal of Food Quality*, <https://doi.org/10.1155/2017/9170768>.
- Fiorentini, G., Messana, J. D., José Neto, A., Sgobi, E. G., Castagnino, P. S., & Berchielli, T. T. (2018). Performance and meat quality of Nellore bulls fed crude glycerine combined with soybean oil. *Animal Feed Science Technology*, 241, 45-54.
- Fontana, A., Polverino de Laureto, P., De Filippis, V., Scaramella, E., & Zambonin, M. (1997). Probing the partly folded states of proteins by limited proteolysis. *Folding & Design*, 2(2), R17-R26.
- Fountoulakis, M., & Lahm, H. W. (1998). Hydrolysis and amino acid composition analysis of proteins. *Journal of Chromatography A*, 826(2), 109-134.
- Frank, K., Bleier, N., Tóth, B., Sugár, L., Horn, P., Barta, E., Orosz, L., & Stéger, V. (2017). The presence of Balkan and Iberian red deer (*Cervus elaphus*) mitochondrial DNA lineages in the Carpathian Basin. *Mammalian Biology*, 86, 48-55.
- Freedman, K. J., Haq, S. R., Edel, J. B., Jemth, P., & Kim, M. J. (2013). Single molecule unfolding and stretching of protein domains inside a solid-state nanopore by electric field. *Scientific Reports*, 3, 1638.
- Freedman, K. J., Jürgens, M., Prabhu, A., Ahn, C. W., Jemth, P., Edel, J. B., & Kim, M. J. (2011). Chemical, thermal, and electric field induced unfolding of single protein molecules studied using nanopores. *Analytical Chemistry*, 83(13), 5137-5144.
- Gabrić, D., Barba, F., Roohinejad, S., Gharibzahedi, S. M. T., Radojčin, M., Putnik, P., & Kovačević, D. B. (2017). Pulsed electric fields as an alternative to thermal processing for preservation of nutritive and physicochemical properties of beverages: A review. *Journal of Food Engineering*, 41(1), e12638.

- Gachovska, T., Cassada, D., Subbiah, J., Hanna, M., Thippareddi, H., & Snow, D. (2010). Enhanced anthocyanin extraction from red cabbage using pulsed electric field processing. *Journal of Food Science*, *75*, E323-E329.
- García Díaz, B. E., Gauthier, S., & Davies, P. L. (2006). Ca<sup>2+</sup> Dependency of calpain 3 (p94) activation. *Biochemistry*, *45*, 3714-3722.
- García-Parra, J., González-Cebrino, F., Delgado-Adámez, J., Cava, R., Martín-Belloso, O., Elez-Martínez, P., & Ramíre, R. (2018). Application of innovative technologies, moderate-intensity pulsed electric fields and high-pressure thermal treatment, to preserve and/or improve the bioactive compounds content of pumpkin. *Innovative Food Science and Emerging Technologies*, *45*, 53-61.
- Gaspar, A. L. C., & de Góes-Favoni, S. P. (2015). Action of microbial transglutaminase (MTGase) in the modification of food proteins: A review. *Food Chemistry*, *171*, 315-322.
- Gatellier, P., & Santé-Lhoutellier, V. (2009). Digestion study of proteins from cooked meat using an enzymatic microreactor. *Meat Science*, *81*, 405-409.
- Geesink, G. H., & Koohmaraie, M. (1999a). Effect of calpastatin on degradation of myofibrillar proteins by I-calpain under post-mortem conditions. *Journal of Animal Science*, *77*, 2685-2692.
- Geesink, G. H., & Koohmaraie, M. (1999b). Post-mortem proteolysis and calpain/calpastatin activity in callipyge and normal lamb biceps femoris during extended post-mortem storage. *Journal of Animal Science*, *77*, 1490-1501.
- Geesink, G. H., Bekhit, A. D., & Bickerstaffe, R. (2000). Rigor temperature and meat quality characteristics of lamb longissimus muscle. *Journal of Animal Science*, *78*, 2842-2848.
- Geesink, G. H., Kuchay, S., Chishti, A. H., & Koohmaraie, M. (2006).  $\mu$ -Calpain is essential for postmortem proteolysis of muscle proteins. *Journal of Animal Science*, *84*, 2834-2840.
- Geesink, G. H., Taylor, R. G., & Koohmaraie, M. (2005). Calpain 3/p94 is not involved in post-mortem proteolysis. *Journal of Animal Science*, *83*, 1646-1652.
- Gharibzahedi, S. M. T., Hernández-Ortega, C., Welte-Chanes, J., Putnik, P., Barba, F. J., Mallikarjunan, K., Escobedo-Avellaneda, Z., & Roohinejad, S. (2019). High pressure processing of food-grade emulsion systems: antimicrobial activity and effect on the physicochemical properties. *Food Hydrocolloids*, *87*, 307-320.
- Gheisari, H. R., Shekarforoush, S. S., & Aminlari, M. (2007). Comparative studies on calpain activity of different muscles of cattle, camel, sheep and goat. *Iranian Journal of Veterinary Research*, *8*, 225-230.
- Giteru, S. G., Oey, I., & Ali, M. A. (2018). Feasibility of using pulsed electric fields to modify biomacromolecules: A review. *Trends in Food Science & Technology*, *72*, 91-11.

- Glaser, R. W., Leikin, S. L., Chernomordik, L. V., Pastushenko, V. F., & Sokirko, A. I. (1998). Reversible electrical breakdown of lipid bilayers: formation and evolution of pores. *Biochimica et Biophysica Acta*, 940, 275-287.
- Goldfarb, L. G., Vicart, P., Goebel, H. H., & Dalakas, M. C. (2004). Desmin myopathy. *Brain*, 127(Pt 4), 723-734.
- Goll, D. E., Thompson, V. F., Li, H. Q., Wei, W., & Cong, J. Y. (2003). The calpain system. *Physiological Reviews*, 83, 731-801.
- Griffiths, M. W. & Walkling-Ribeiro, M. (2014). Pulsed electric field processing of liquid foods and beverages. In: *Emerging Technologies for Food Processing*, Chapter 7, Elsevier Ltd., <http://dx.doi.org/10.1016/B978-0-12-411479-1.00>.
- Gruber, S. L., Tatum, J. D., Scanga, J. A., Chapman, P. L., Smith, G. C., & Belk, K. E. (2006). Effects of postmortem aging and USDA quality grade on Warner-Bratzler shear force values of seventeen individual beef muscles. *Journal of Animal Science*, 84, 3387-3396.
- Grune, T., Reinheckel, T., Joshi, M., & Davies, K. J. A. (1995). Proteolysis in cultured liver epithelial cells during oxidative stress. *The Journal of Biological Chemistry*, 270, 2344-2351.
- Gudmundsson, M., & Hafsteinsson, H. (2001). Effect of electric field pulses on microstructure of muscle foods and roes. *Trends Food Science and Technology*, 12, 122-128.
- Guimarães, J. D. T., Silva, E. K., Freitas, M. Q. D., Meireles, M. A. D. A., & Cruz, A. G. D. (2018). *Non-thermal emerging technologies* and their effects on the functional properties of dairy products. *Current Opinion in Food Science*, 22, 62-66.
- Ha, M. H. (2012). *Characterisation of cysteine proteases and their catalytic impact on meat myofibril and meat connective tissue proteins*. Master of Science, University of Otago.
- Hamilton, W. A., & Sale, A. J. H. (1967). Effects of high electric fields on microorganisms: 2. Mechanism of action of the lethal effect. *Biochimica et Biophysica Acta*, 148, 789-800.
- Han, J., Morton, J. D., Bekhit, A. E. D., & Sedcole, J. R. (2009). Pre-rigor infusion with kiwifruit juice improves lamb tenderness. *Meat Science*, 82, 324-330.
- Han, Z., Cai, M. J., Cheng, J. H., & Sun, D. W. (2018). Effects of electric fields and electromagnetic wave on food protein structure and functionality: A review. *Trends in Food Science and Technology*, 75, 1-9.
- Hanna, R. A., Campbell, R. L., & Davies, P. L. (2008). Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. *Nature*, 456, 409-412.
- Harris, S. E., Huff-Lonergan, E., Lonergan, S. M., Jones, W. R., & Rankins, D. (2001). Antioxidant status affects color stability and tenderness of calcium chloride injected beef. *Journal of Animal Science*, 79(3), 666-677.
- Hassoun, A., Sante-Lhoutellier, V., Lebert, A., Kondjoyan, A., & Daudin, J. D. (2011). Incidence of various process parameters on in vitro protein digestibility of beef meat. Incidence of various process

- parameters on in vitro protein digestion of beef meat. In: 57<sup>th</sup> International Congress of Meat Science and Technology, Vol. Paper 868 (pp. 1–4), 7–12 August 2011, Ghent-Belgium.
- Hati, S., Patel, M., & Yadav, D. (2018). Food bioprocessing by *non-thermal plasma technology*. *Current Opinion in Food Science*, *19*, 85-91
- Haughton, P. N., Lyng, J. G., Cronin, D. A., Morgan, D. J., Fanning, S., & Whyte P. (2012). Efficacy of pulsed electric fields for the inactivation of indicator microorganisms and food borne pathogens in liquids and raw chicken. *Food Control*, *25*, 131-135.
- Heems, D., Luck, G., Fraudeau, C., & Verette, E. (1998). Fully automated precolumn derivatization, on-line dialysis and high-performance liquid chromatographic analysis of amino acids in food, beverages and feedstuff. *Journal of Chromatography A*, *798*, 9-17.
- Heinz, V., Toepfl, S., & Knorr, D. (2003). Impact of temperature on lethality and energy efficiency of apple juice pasteurization by pulsed electric fields treatment. *Innovative Food Science and Emerging Technologies*, *4*, 167-175.
- Hermawan, N., Evrendilek, G. A., Dantzer, W., Zhang, Q., & Richter, E. (2004). Pulsed electric field treatment of liquid whole egg inoculated with *Salmonella enteritidis*. *Journal of Food Safety*, *24*(1), 71-85.
- Ho, C. Y., Stromer, M. H., & Robson, R. M. (1994). Identification of the 30 kDa polypeptide in post-mortem skeletal muscle as a degradation product of troponin-T. *Biochimie*, *76*(5), 369-375.
- Hong, J., Chen, R., Zeng, X. A., & Han, Z. (2016). Effect of pulsed electric fields assisted acetylation on morphological, structural and functional characteristics of potato starch. *Food Chemistry*, *192*, 15-24.
- Hong, J., Zeng, X. A., Han, Z., & Brennan, C. S. (2018). Effect of pulsed electric fields treatment on the nanostructure of esterified potato starch and their potential glycemic digestibility. *Innovative Food Science and Emerging Technologies*, *45*, 438-446.
- Hopkins, D. L. (2014). Tenderising mechanisms: Mechanical. In: Dikeman, M., and Devine C. (Eds.), *Encyclopaedia of meat science*. Vol. 3. London, UK: Academic Press, Elsevier.
- Hopkins, D. L., & Thompson, J. M. (2002). The degradation of myofibrillar proteins in beef and lamb using denaturing electrophoresis – An overview. *Journal of Muscle Foods*, *13*, 81-102.
- Hopkins, D. L., Martin, L., & Gilmour, A. R. (2004). The impact of homogenizer type and speed on the determination of myofibrillar fragmentation. *Meat Science*, *67*, 705-710.
- Hoppe, A., Jung, S., Patnaik, A., & Zeece, M. G. (2013). Effect of high-pressure treatment on egg white protein digestibility and peptide products. *Innovative Food Science and Emerging Technologies*, *17*, 54-62.
- Horita, C. N., Baptista, R. C., Caturla, M. Y. R., Lorenzo, J. M., Barba, F. J., & Sant'Ana, A. S. (2018). Combining reformulation, active packaging and non-thermal post-packaging decontamination

- technologies to increase the microbiological quality and safety of cooked ready-to-eat meat products. *Trends in Food Science & Technology*, 72, 45-61.
- Hosfield, C. M., J. S. Elce, P. L. Davies, & Jia, Z. C. (1999). Crystal structure of calpain reveals the structural basis for Ca<sup>2+</sup>-dependent protease activity and a novel mode of enzyme activation. *EMBO Journal*, 18, 6880-6889.
- Hovhannisyan, S. V., & Grigoryan, K. A. (2016). The main problems and features of the global and local meat production. *Annals of Agrarian Science*, 14, 315-318.
- Huang, F., Ding, Z., Zhang, C., Hu, H., Zhang, L., & Zhang, H. (2018). Effects of calcium and zinc ions injection on caspase-3 activation and tenderness in post-mortem beef skeletal muscles. *International Journal of Food Science and Technology*, 53, 582-589.
- Huang, M., Huang, F., Ma, H., Xu, X., & Zhou, G. (2012). Preliminary study on the effect of caspase-6 and calpain inhibitors on postmortem proteolysis of myofibrillar proteins in chicken breast muscle. *Meat Science*, 90, 536-542.
- Huff-Lonergan, E., & Lonergan, S. M. (1999). Post-mortem mechanisms of meat tenderization: The roles of the structural proteins and the calpain system. In Xiong, Y. L., Ho, C. -T., & Shahidi, F. (Eds.), *Quality attributes of muscle foods* (pp. 229–251). New York: Kluwer Academic/Plenum Publishers.
- Huff-Lonergan, E., Mitsuhashi, T., Beekman, D. D., Parrish, F. C., Olson, D. G., & Robson, R. M. (1996). Proteolysis of specific muscle structural proteins by mu-calpain at low pH and temperature is similar to degradation in post-mortem bovine muscle. *Journal of Animal Science*, 74(5), 993-1008.
- Huff-Lonergan, E., Zhang, W., & Lonergan, S. M. (2010). Biochemistry of post-mortem muscle – Lessons on mechanisms of meat tenderization. *Meat Science*, 86(1), 184-195.
- Hughes, J. M., Oiseth, S. K., Purslow, P. P., & Warner, R. D. (2014). A structural approach to understanding the interactions between colour, water-holding capacity and tenderness. *Meat Science*, 98(3), 520-532.
- Hwan, S. F., & Bandman, E. (1989). Studies of desmin and  $\alpha$ -actinin degradation in bovine *Semitendinosus* muscle. *Journal of Food Science*, 54, 1426-1430.
- Hwang, I. H., & Thompson, J. M. (2001). The interaction between pH and temperature decline early postmortem on the calpain system and objective tenderness in electrically stimulated beef *longissimus dorsi* muscle. *Meat Science*, 58, 167-174.
- Hwang, I. H., Park, B. Y., Cho, S. H., & Lee, J. M. (2004). Effects of muscle shortening and proteolysis on Warner–Bratzler shear force in beef *longissimus* and *semitendinosus*. *Meat Science*, 68, 497-505.

- Ignat, A., Manzocco, L., Brunton, N. P., Nicoli, M. C., and Lyng, J. G. (2015). The effect of pulsed electric field pre-treatments prior to deep-fat frying on quality aspects of potato fries. *Innovative Food Science and Emerging Technologies*, 29, 65-69.
- Ilian, M. A., Bekhit, A. E. D., Stevenson, B., Morton, J. D., Isherwood, P., & Bickerstaffe, R. (2004b). Up- and down-regulation of *longissimus* tenderness parallels changes in the myofibril-bound calpain 3 protein. *Meat Science*, 67, 433-445.
- Ilian, M. A., Bickerstaffe, R., & Greaser, M. L. (2004a). Post-mortem changes in myofibrillar-bound calpain 3 revealed by immunofluorescence microscopy. *Meat Science*, 66, 231-240.
- Ilian, M. A., Morton, J. D., Kent, M. P., Le Couteur, C. E., Hickford, J., Cowley, R., & Bickerstaffe, R. (2001). Intermuscular variation in tenderness: Association with the ubiquitous and muscle-specific calpains. *Journal of Animal Science*, 79, 122-132.
- Inguglia, E. S., Zhang, Z., Tiwari, B. K., Kerry, J. P., & Burgess, C. M. (2017). Salt reduction strategies in processed meat products - A review. *Trends in Food Science and Technology*, 59, 70-78.
- Inguglia, E. S., Zhang, Z., Tiwari, B. K., Kerry, J. P., & Burgess, C. M. (2017). Salt reduction strategies in processed meat products - A review. *Trends Food Science and Technology*, 59, 70-78.
- Iwanowska, A., Iwanska, E., Grzes, B., Mikolajczak, B., Pospiech, E., Rosochacki, S., Juszcuk-Kubiak, E., & Łyczyński, A. (2010). Changes in proteins and tenderness of meat from young bulls of four breeds at three ages over 10 days of cold storage. *Animal Science Papers and Reports*, 28(1), 13-25.
- Jahan-Mihan, A., Luhovyy, B. L., El Khoury, D., & Anderson, G. H. (2011). Dietary proteins as determinants of metabolic and physiologic functions of the gastrointestinal tract. *Nutrients*, 3, 574-603.
- Janositz, A., Noack, A. K., & Knorr, D. (2011). Pulsed electric fields and their impact on the diffusion characteristics of potato slices. *LWT - Food Science and Technology*, 44, 1939-1945.
- Jeyamkondan, S., Jayas, D. S., & Holley, R. A. (1999). Pulsed electric field processing of foods: a review. *Journal of Food Protection*, 62, 1088-96.
- Ji, J. R., & Takahashi, K. (2006). Changes in concentration of sarcoplasmic free calcium during post-mortem ageing of meat. *Meat Science*, 73, 395-403.
- Jiang, H., Yoon, S. C., Zhuan, H., Wang, W., Lawrence, K. C., & Yang, Y. (2018). Tenderness classification of fresh broiler breast fillets using visible and near-infrared hyperspectral imaging. *Meat Science*, 139, 82-90.
- Jin, Y., Yu, Y., Qi, Y., Wang, F., Yan, J., & Zou, H. (2016). Peptide profiling and the bioactivity character of yogurt in the simulated gastrointestinal digestion. *Journal of Proteomics*, 141, 24-46.
- Johnson, P. E., Van der Plancken, I., Balasa, A., Husband, F. A., Grauwet, T., Hendrickx, M., Knorr, D., Mills, E., & Mackie, A. R. (2010). High pressure, thermal and pulsed electric-field-induced



- structural changes in selected food allergens. *Molecular Nutrition and Food Research*, *54*, 1701-1710.
- Joshi, R. P., Hu, Q., Schoenbach, K. H., & Hjalmanson, H. P. (2002). Improved energy model for membrane electroporation in biological cells subjected to electrical pulses. *Physics Review E*, *65*, 041920.
- Jovanović, J. R., Stefanović, A. B., Šekuljica, N. Ž., Tanasković, S. M. J., Dojčinović, M. B., Bugarski, B. M., & Knežević-Jugović, Z. D. (2016). Ultrasound pre-treatment as a useful tool to enhance egg white protein hydrolysis: Kinetics, reaction model, and thermodynamics. *Journal of Food Science*, *81*(11), C2664-C2675.
- Jung, S., de Lamballerie-Anton, M., & Ghou, M. (2000). Modifications of ultrastructure and myofibrillar proteins of *post-rigor* beef treated by high pressure. *LWT-Food Science and Technology*, *33*, 313-319.
- Kameníka, J., Saláková, A., Vyskočilová, V., Pechová, A., & Haruštiaková, D. (2017). Salt, sodium chloride or sodium? Content and relationship with chemical, instrumental and sensory attributes in cooked meat products. *Meat Science*, *131*, 196-202.
- Kantar, S. E., Boussetta, N., Lebovka, N., Foucart, F., & Vorobiev, E. (2018). Pulsed electric field treatment of citrus fruits: Improvement of juice and polyphenols extraction. *Innovative Food Science & Emerging Technologies*, *46*, 153-161.
- Kantono, K., Hamid, N., Oey, I., Wang, S., Xu, Y., Ma, Q., Faridnia, F., & Farouk, M. (2019). Physicochemical and sensory properties of beef muscles after pulsed electric field processing. *Food Research International*, *121*, 1-11.
- Kaur, L., Astruc, T., Vénien, A., Loison, O., Cui, J., Irastorza, M., & Boland, M. (2016). High pressure processing of meat: effects on ultrastructure and protein digestibility. *Food Function*, *7*, 2389-2397.
- Kaur, L., Maudens, E., Haisman, D. R., Boland, M. J., & Singh, H. (2014). Microstructure and protein digestibility of beef: the effect of cooking conditions as used in stews and curries. *LWT - Food Science and Technology*, *55*, 612-620.
- Kawasaki, H., & Kawashima, S. (1996). Regulation of the calpain calpastatin system by membranes. *Molecular Membrane Biology*, *13*, 217-224.
- Kelam, I. K., Bhat, Z. F., Kumar, S., Noor, S., & Desai, A. (2018). The effects of bioactive edible film containing *Terminalia arjuna* on the stability of some quality attributes of chevon sausage. *Meat Science*, *140*, 38-43.
- Kemp, C. M., & Parr, T. (2012). Advances in apoptotic mediated proteolysis in meat tenderisation. *Meat Science*, *92*(3), 252-259.
- Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., & Parr, T. (2010). Tenderness—an enzymatic view. *Meat Science*, *84*, 248-256.

- Kendall, T. L., Koohmaraie, M., Arbona, J. R., Williams, S. E., & Young, L. L. (1993). Effect of pH and ionic strength on bovine m-calpain and calpastatin activity. *Journal of Animal Science*, *71*, 96-104.
- Kent, M.P., M.J. Spencer, M. Koohmaraie, (2004). Post-mortem proteolysis is reduced in transgenic mice overexpressing calpastatin, *Journal of Animal Science*, *82*, 794-801.
- Khan, A. A., Randhawa, M. A., Carne, A., Ahmed, I. A. M., Barr, D., Reid, M., & Bekhit, A. E. A. (2017a). Effect of low and high pulsed electric field on the quality and nutritional minerals in cold boned beef *M. longissimus et lumborum*. *Innovative Food Science and Emerging Technology*, *41*, 135-143.
- Khan, A. A., Randhawa, M. A., Carne, A., Ahmed, I. A. M., Barr, D., Reid, M., & Bekhit, A. E. A. (2018). Effect of low and high pulsed electric field processing on macro- and micro-minerals in beef and chicken. *Innovative Food Science and Emerging Technology*, *5*, 273-279.
- Khan, A., Randhawa, M. A., Carne, A., Ahmed, I. A. M., Barr, D., Reid, M., & Bekhit, A. E. A (2017b). Quality and nutritional minerals in chicken breast muscle treated with low and high pulsed electric fields. *Food and Bioprocess Technology*, *11*, 122-131.
- Kim, H.-J., Chun, H.-H., Song, H.-J., & Song, K.-B. (2010). Effects of electron beam irradiation on the microbial growth and quality of beef jerky during storage. *Radiation Physics and Chemistry*, *79*, 1165-1168.
- Kim, J.-H., Kim, D.-H., Ji, D.-S., Lee, H.-J., Yoon, D.-K., & Lee, C.-H. (2017). Effect of aging process and time on physicochemical and sensory evaluation of raw beef top round and shank muscles using an electronic tongue. *Korean Journal of Food Sciences of Animal Resources*, *37*(6), 823-832.
- Kim, Y. H. B., Ma, D., Setyabrata, D., Farouk, M. M., Lonergan, S. M., Huff-Lonergan, E., & Hunt, M. C. (2018). Understanding postmortem biochemical processes and post-harvest aging factors to develop novel smart-aging strategies. *Meat Science*, <https://doi.org/10.1016/j.meatsci.2018.04.031>.
- Kim, Y. H. B., Stuart, A., Nygaard, G., & Rosenvold, K. (2012). High pre-rigor temperature limits the ageing potential of beef that is not completely overcome by electrical stimulation and muscle restraining. *Meat Science*, *91*, 62-98.
- Kim, Y. H. B., Warner, R. D., & Rosenvold, K. (2014). Influence of high pre-rigor temperature and fast pH fall on muscle proteins and meat quality: a review. *Animal Production Science*, *54*, 375-395.
- Kitamura, S., Muroya, S., Tanabe, S., Okumura, T., Chikuni, K., & Nishimura, T. (2005). Mechanism of production of troponin T fragments during post-mortem aging of porcine muscle. *Journal of Agricultural and Food Chemistry*, *53*, 4178-4181.
- Klonowski, I., Heinz, V., Toepfl, S., Gunnarsson, G., & Þorkelsson, G. (2006). Applications of pulsed electric field technology for the food industry, IFL report 06-06, project no. 1677. Down from: [http://www.matis.is/media/matis/utgafa/Skyrsla\\_06-06.pdf](http://www.matis.is/media/matis/utgafa/Skyrsla_06-06.pdf).

- Kloss, L., Meyer, J. D., Graeve, L., & Vetter, W. (2015). Sodium intake and its reduction by food reformulation in the European Union – A review. *NFS Journal*, *1*, 9-19.
- Kondjoyan, A., Daudin, J. D., & Santé-Lhoutellier, V. (2015). Modelling of pepsin digestibility of myofibrillar proteins and of variations due to heating. *Food Chemistry*, *172*, 265-271.
- Kong, F., & Singh, R. (2008). A model stomach system to investigate disintegration kinetics of solid foods during gastric digestion. *Journal of Food Science*, *73*, E202-E210.
- Koohmaraie, M. (1992). The role of Ca<sup>2+</sup>-dependent proteases (calpains) in post-mortem proteolysis and meat tenderness. *Biochimie*, *74*, 239-245.
- Koohmaraie, M. (1996). Biochemical factors regulating the toughening and tenderization processes of meat. *Meat Science*, *43*, 193-201.
- Koohmaraie, M., & Geesink, G. H. (2006). Contribution of post-mortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Science*, *74*, 34-43.
- Koohmaraie, M., & Shackelford, S. D. (1991). Effect of calcium chloride infusion on the tenderness of lamb fed a b-adrenergic agonist. *Journal of Animal Science*, *69*, 2463-2471.
- Koohmaraie, M., Babiker, A. S., Schroeder, A. L., Merkel, R. A., & Duston, T. R. (1988). Acceleration of post-mortem tenderization in ovine carcasses through activation of Ca<sup>2+</sup>-dependent proteases. *Journal of Food Science*, *53*, 1638-1641.
- Koohmaraie, M., Crouse, J. D., & Mersmann, H. J. (1989). Acceleration of post-mortem tenderization in ovine carcasses through infusion of calcium chloride: Effect of concentration and ionic strength. *Journal of Animal Science*, *67*, 934-942.
- Koohmaraie, M., Kent, M. P., Shackelford, S. D., Veiseth, E., & Wheeler, T. M. (2002). Meat tenderness and muscle growth: Is there a relationship? *Meat Science*, *62*, 345-352.
- Koohmaraie, M., Seidemann, S. C., Schollmeyer, J. E., Dutson, T. R., & Crouse, J. D. (1987). Effect of post-mortem storage on Ca<sup>++</sup>-dependent proteases, their inhibitor and myofibril fragmentation. *Meat Science*, *19*, 187-196.
- Koohmaraie, M., Whipple, G., Kretchmar, D. H., Crouse, J. D., & Mersmann, H. J. (1991). Post-mortem proteolysis in longissimus muscle from beef, lamb and pork carcasses. *Journal of Animal Science*, *69*, 617-624.
- Koubaa, M., Barba, F. J., Kovačević, D. B., Putnik, P., Santos, M. D., Queirós, R. P., Moreira, S. A., Inácio, R. S., Fidalgo, L. G., & Saraiva, J. A. (2018). Pulsed electric field processing of fruit juices. In: *Fruit juices - extraction, composition, quality and analysis*, Elsevier Inc., pp. 437-449.
- Koubaa, M., Rosello-Soto, E., Sic-Zlabur, J., Rezek-Jambrak, A., Brncic, M., Grimi, N., & Barba, F. J. (2015). Current and new insights in the sustainable and green recovery of nutritionally valuable compounds from *Stevia rebaudiana Bertoni*. *Journal of Agricultural and Food Chemistry*, *63*, 6835-6846.

- Kramerova, I., Kudryashova, E., Tidball, J. G., & Spencer, M. J. (2004). Null mutation of calpain 3 (p94) in mice causes abnormal sarcomere formation in vivo and in vitro. *Human Molecular Genetics*, *13*, 1373-1388.
- Kramerova, I., Kudryashova, E., Wu, B., Ottenheijm, C., Granzier, H., & Spencer, M. J. (2008). Novel role of calpain-3 in the triad-associated protein complex regulating calcium release in skeletal muscle. *Human Molecular Genetics*, *17*, 3271-3280.
- Kristensen, L., & Purslow, P. P. (2001). The effect of ageing on the water-holding capacity of pork: Role of cytoskeletal proteins. *Meat Science*, *58*, 17-23.
- Kudrnáčová, E., Bartoň, L., Bureš, D., & Hoffman, L. C. (2018). Carcass and meat characteristics from farm-raised and wild fallow deer (*Dama dama*) and red deer (*Cervus elaphus*): A review. *Meat Science*, *141*, 9–27.
- Kumar, Y., Patel, K. K., & Kumar, K. (2015) Pulse electric Field processing in food technology. *International Journal of Engineering Studies and Technical Approach*, *1*, 6-17.
- Kunnath, S., Lekshmi, M., Chouksey, M. K., Kannuchamy, N., & Gudipati, V. (2015). Textural quality and oxidative stability of restructured *Pangasius* mince: Effect of protein substrates mediated by transglutaminase. *Journal of Food Science and Technology*, *52*, 351-358.
- Lametsch, R., Lonergan, S., & Huff-Lonergan, E. (2008). Disulfide bond within mu-calpain active site inhibits activity and autolysis. *Biochimica et Biophysica Acta -Proteins and Proteomics*, *1784*, 1215-1221.
- Lametsch, R., Roepstorff, P., Møller, H. S., & Bendixen, E. (2004). Identification of myofibrillar substrates for  $\mu$ -calpain. *Meat Science*, *68*, 515-521.
- Lana, A. & Zolla, L. (2016). Proteolysis in meat tenderization from the point of view of each single protein: A proteomic perspective. *Journal of Proteomics*, *147*, 85-97.
- Lang, Y., Sha, K., Zhang, R., Xie, P., Luo, X., Sun, B., Li, H., Zhang, L., Zhang, S., & Liu, X. (2016). Effect of electrical stimulation and hot boning on the eating quality of Gannan yak longissimus lumborum. *Meat Science*, *112*, 3-8.
- Lazarides, E., & Hubbard, B. D. (1976). Immunological characterisation of the subunit of the 100 A filament from muscle cell. *Proceedings of the National Academy of Science of the United States of America*, *73*, 4344-4348.
- Lebovka, N. I., Praporscica, I., Ghnimia, S., & Vorobiev, E. (2005). Temperature enhanced electroporation under the pulsed electric treatment of food tissue. *Journal of Food Engineering*, *69*, 177-184.
- Lebovka, N. I., Shynkaryk, M. V., El-Belghiti, K., Benjelloun, H., & Vorobiev, E. (2007). Plasmolysis of sugarbeet: pulsed electric fields and thermal treatment. *Journal of Food Engineering*, *80*, 639-644.

- Lee, H. Y. (2001). Calcium homeostasis in lens transparency and the involvement of calpains in cataract. *PhD Thesis*, <https://researcharchive.lincoln.ac.nz/handle/10182/1897>.
- Lee, H. Y. Y., Morton, J. D., Sanderson, J., Bickerstaffe, R., & Robertson, L. J. G. (2008). The involvement of calpains in opacification induced by Ca<sup>2+</sup>-overload in ovine lens culture. *Veterinary Ophthalmology*, *11*, 347-355.
- Li, K., Zhang, Y., Mao, Y., Cornforth, D., Dong, P., Wang, R., Zhu, H., & Luo, X. (2012). Effect of very fast chilling and aging time on ultra-structure and meat quality characteristics of Chinese Yellow cattle M. *Longissimus lumborum*. *Meat Science*, *92*, 795-804.
- Li, P., Wang, T., Mao Y., Zhang Y., Niu L., Liang R., Zhu L., & Luo X. (2014). Effect of ultimate pH on postmortem myofibrillar protein degradation and meat quality characteristics of Chinese Yellow Crossbreed Cattle. *The Scientific World Journal*, Article ID 174253, <http://dx.doi.org/10.1155/2014/174253>.
- Li, Y. Q., Chen, Q., Liu, X. H., & Chen, Z. X. (2008). Inactivation of soybean lipoxygenase in soymilk by pulsed electric fields. *Food Chemistry*, *109*, 408-414.
- Li, Y. Q., Chen, Z. X., & Mo, H. Z. (2007). Effects of pulsed electric fields on physicochemical properties of soybean protein isolates. *LWT - Food Science and Technology*, *40*, 1167-1175.
- Liang, R., Cheng, S., & Wang, X. (2018). Secondary structure changes induced by pulsed electric field affect antioxidant activity of pentapeptides from pine nut (*Pinus koraiensis*) protein. *Food Chemistry*, *254*, 170-184.
- Lindgren, M., Aronsson, K., Galt, S., & Ohlsson, T. (2002). Simulation of the temperature increase in pulsed electric field (PEF) continuous flow treatment chambers. *Innovative Food Science and Emerging Technologies*, *3*, 233-245.
- Liu, C., Grimi, N., Lebovka, N., & Vorobiev, E. (2018a). Effects of pulsed electric fields treatment on vacuum drying of potato tissue. *LWT- Food Science and Technology*, *95*, 289-294.
- Liu, G., & Xiong, Y. I. (2000). Electrophoretic pattern, thermal denaturation on in vitro digestibility of oxidized myosin. *Journal of Agricultural and Food Chemistry*, *48*, 624-630.
- Liu, T., Burritt, D. J., Eyres, G. T., & Oey, I. (2018). Pulsed electric field processing reduces the oxalate content of oca (*Oxalis tuberosa*) tubers while retaining starch grains and the general structural integrity of tubers. *Food Chemistry*, *245*, 890–898.
- Liu, T., Dodds, E., Leong, S. Y., Eyres, G. T., Burritt, D. J., & Oey, I. (2017a). Effect of pulsed electric fields on the structure and frying quality of “kumara” sweet potato tubers. *Innovative Food Science and Emerging Technologies*, *39*, 197-208.
- Liu, Y. F., Oey, I., Bremer, P., Carne, A., & Silcock, P. (2017b). Effects of pH, temperature and pulsed electric fields on the turbidity and protein aggregation of ovomucin-depleted egg white. *Food Research International*, *91*, 161-170.

- Liu, Y. F., Oey, I., Bremer, P., Silcock, P., & Carne, A., (2018b). Proteolytic pattern, protein breakdown and peptide production of ovomucin-depleted egg white processed with heat or pulsed electric fields at different pH. *Food Research International*, 108, 465-474.
- Liu, Z., Zhou, Y., Feng, J., Lu, S., Zhao, Q., & Zhang, J. (2012). Characterization of oligopeptide transporter (PepT1) in grass carp (*Ctenopharyngodon idella*). *Comparative Biochemistry and Physiology - Part B: Biochemical and Molecular Biology*, 164, 194-200.
- Loginova, K. V., Lebovka, N. I., & Vorobiev, E. (2011). Pulsed electric field assisted aqueous extraction of colorants from red beet. *Journal of Food Engineering*, 106, 127-133.
- Loginova, K. V., Shynkaryk, M. V., Lebovka, N. I., & Vorobiev, E. (2010). Acceleration of soluble matter extraction from chicory with pulsed electric fields. *Journal of Food Engineering*, 96, 374-379.
- Lomiwes, D., Farouk, M. M., Frost, D. A., Dobbie, P. M., & Young, O. A. (2013). Small heat shock proteins and toughness in intermediate pHu beef. *Meat Science*, 95, 472-479.
- Lomiwes, D., Farouk, M. M., Wiklund, E., & Young, O. A. (2014). Small heat shock proteins and their role in meat tenderness: A review. *Meat Science*, 96, 26-40.
- Lonergan, E. H., Zhang, W. A., & Lonergan, S. M. (2010). Biochemistry of postmortem muscle - lessons on mechanisms of meat tenderization. *Meat Science*, 86(1), 184-195.
- Lorieau, L., Halabi, A., Ligneul, A., Hazart, E., Dupont, D., & Floury, J. (2018). Impact of the dairy product structure and protein nature on the proteolysis and amino acid bioaccessibility during in vitro digestion. *Food Hydrocolloids*, 82, 399-411.
- Lu, H., & Klaassen, C. (2006). Tissue distribution and thyroid hormone regulation of Pept1 and Pept2 mRNA in rodents. *Peptides*, 27, 850-857.
- Lucke, F. K. (2014). Fermented Meats. In: The Oxford Handbook of Food Fermentations, Bamforth, C. W., & Ward, R. E. (editors), Oxford University Press, pp. 494.
- Luckose, F., Pandey, M. C., & Harilal, P. T. (2017). Effect of sodium chloride reduction on drying kinetics of restructured chicken jerky. *Food Bioscience*, 19, 156-162.
- Ludwiczak, A., Stanisz, M., Bykowska, M., Składanowska, J., & Ślósarz, P. (2016). Effect of storage on quality traits of the semimembranosus muscle of farmed fallow deer (*Dama dama*) bucks and does. *Animal Science Journal*, <https://doi.org/10.1111/asj.12732>.
- Lundin, L., Golding, M., & Wooster, T. J. (2008). Understanding food structure and function in developing food for appetite control. *Nutrition and Dietetics*, 65, S79-S85.
- Luo, J., Taylor, C., Nebl, T., Ng, K., & Bennett, L. E. (2018). Effects of macro-nutrient, micro-nutrient composition and cooking conditions on in vitro digestibility of meat and aquatic dietary proteins. *Food Chemistry*, 254, 292-301.
- Luo, Q., Boom, R. M., & Janssen, A. E. M. (2015). Digestion of protein and protein gels in simulated gastric environment. *LWT - Food Science and Technology*, 63, 161-168.

- Lynch, G. S., & Koopman, R. (2018). Dietary meat and protection against sarcopenia. *Meat Science*, 144, 180-185.
- Ma, Q., Hamid, N., Oey, I., Kantono., K., Faridnia, F., Yoo, M., & Farouk, M. (2016). Effect of chilled and freezing pre-treatments prior to pulsed electric field processing on volatile profile and sensory attributes of cooked lamb meats *Innovative Food Science and Emerging Technologies*, 37, 359-374.
- MacFarlane, N. G. (2018). Digestion and absorption. In: *Physiology of the Gastrointestinal Tract* (Sixth Edition), Bhutia, Y. D., & Ganapathy, V. (Editors), pp. 1063-1086.
- Marino, R., Albenzio, M., Malva, A. D., Santillo, A., Loizzo, P., & Sevi, A. (2013). Proteolytic pattern of myofibrillar protein and meat tenderness as affected by breed and ageing time. *Meat Science*, 95, 281-287.
- Martín-Belloso, O., Vega-Mercado, H., Qin, B. L., Chang, F. J., Barbosa-Canovas, G. V., & Swanson, B. C. (1997). Inactivation of *Escherichia coli* suspended in liquid egg using pulsed electric fields. *Journal of Food Processing and Preservation*, 21, 193-208.
- Martinez, S. (2017). Predicting water holding capacity and forms of water loss in beef sources. Texas State University. *Master Thesis*, <https://digital.library.txstate.edu/bitstream/handle/10877/6563/MARTINEZ-THESIS-2017.pdf?sequence=1&isAllowed=y>.
- McDonnell, C. K., Allen, P., Chardonnerau, F. S., Arimi, J. M., & Lyng, J. G. (2014). The use of pulsed electric fields for accelerating the salting of pork. *LWT - Food Science and Technology*, 59, 1054-1060.
- Meditext-Medical Management (2005). Chromium Hexavalent Salts. TOMES Information System. Denver, CO: Micromedex, Inc.
- Melody, J. L., Lonergan, S. M., Rowe, L. J., Huiatt, T. W., Mayes, M. S., & Huff-Lonergan, E. (2004). Early postmortem biochemical factors influence tenderness and water holding capacity of three porcine muscles. *Journal of Animal Science*, 82, 1195-1205.
- Mennecke, B. E., Townsend, A. M., Hayes, D. J., & Lonergan, S. M. (2007). A study of the factors that influence consumer attitudes toward beef products using the conjoint market analysis tool. *Journal of Animal Science*, 85, 2639-59.
- Milani, E. A., Alkhafaji, S., & Silva, F. V. M. (2015). Pulsed electric field continuous pasteurization of different types of beers. *Food Control*, 50, 223-229.
- Miller, M. F., Carr, M. A., Ramsey, C. B., Crockett, K. L., & Hoover, L. C. (2001). Consumer thresholds for establishing the value of beef tenderness. *Journal of Animal Science*, 79, 3062-8.
- Mirmoghtadaie, L., Shojaee Aliabadi, S., & Hosseini, S. M. (2016). Recent approaches in physical modification of protein functionality. *Food Chemistry*, 199, 619-627.

- Misra, N. N. (2015). The contribution of non-thermal and advanced oxidation technologies towards dissipation of pesticide residues. *Trends in Food Science & Technology*, 45, 229-244.
- Mohrhauser, D. A., Underwood, K. R., & Weaver, A. D. (2011). In vitro degradation of bovine myofibrils is caused by  $\mu$ -calpain, not caspase-3. *Journal of Animal Science*, 89, 798-808.
- Mohrhauser, D. A., Lonergan, S. M., Huff-Lonergan, E., Underwood, K. R., & Weaver, A. D. (2014). Calpain-1 activity in bovine muscle is primarily influenced by temperature, not pH decline. *Journal of Animal Science*, 92 (3), 1261-1270.
- Mok, J. H., Choi, W., Park, S. H., Lee, S. H., & Jun, S. J. (2015). Emerging pulsed electric field (PEF) and static magnetic field (SMF) combination technology for food freezing. *International Journal of Refrigeration*, 50, 137-145.
- Mok, J. H., Her, J. Y., Kang, T., Hoptowitz, R., & Jun, S. (2017). Effects of pulsed electric field (PEF) and oscillating magnetic field (OMF) combination technology on the extension of supercooling for chicken breasts. *Journal of Food Engineering*, 196, 27-35.
- Moldoveanu, T., Hosfield, C. M., Lim, D., Elce, J. S., Jia, Z., & Davies, P. L. (2002). A  $\text{Ca}^{2+}$  switch aligns the active site of calpain. *Cell*, 108, 649-660.
- Moller, A. J., Vestergaard, T., & Wismer-Pedersen, J. (1973). Myofibril fragmentation in bovine longissimus dorsi as an index of tenderness. *Journal of Food Science*, 38(5), 824-825.
- Monahan, F. J. (2000). *Oxidation of lipids in muscle foods: fundamental and applied concerns*. NY, USA: John Wiley & Sons, Inc.
- Monfort, S., Gayán, E., Condón, S., Raso, J., & Álvarez, I. (2011). Design of a combined process for the inactivation of *Salmonella enteritidis* in liquid whole egg at 55 °C. *International Journal of Food Microbiology*, 145, 476-482.
- Monfort, S., Gayan, E., Saldana, G., Puértolas, E., Condon, S., Raso, J., & Alvarez, I. (2010). Inactivation of *Salmonella typhimurium* and *Staphylococcus aureus* by pulsed electric fields in liquid whole egg. *Innovative Food Science and Emerging Technologies*, 11, 306-313.
- Moreno, H. M., Carballo, J., & Borderías, A. J. (2010). Use of microbial transglutaminase and sodium alginate in the preparation of restructured fish models using cold gelation: Effect of frozen storage. *Innovative Food Science and Emerging Technologies*, 11, 394-400.
- Moritz, J., Balasa, A., Jaeger, H., Meneses, N., & Knorr, D. (2012). Investigating the potential of polyphenol oxidase as a temperature-time-indicator for pulsed electric field treatment. *Food Control*, 26, 1-5.
- Morrisson, E., Mielche, M., & Purslow, P. P. (1998). Immunolocalisation of intermediate filaments proteins in porcine meat. Fibre type and muscle-specificity variations during conditioning. *Meat Science*, 50, 91-104.



- Morton, J. D., Bhat, Z. F., & Bekhit, A. E. D. (2017). Proteases and meat tenderization. In: Encyclopedia of Food Chemistry, Elsevier Publications, <https://doi.org/10.1016/B978-0-12-814026-0.21663-6>.
- Morton, J., Bickerstaffe, R., Kent, M., Dransfield, E., & Keeley, G. (1999). Calpain-calpastatin and toughness in *M. longissimus* from electrically stimulated lamb and beef carcasses. *Meat Science*, 52, 71-79.
- Morzel, M., Gatellier, Ph., Sayd, T., Renerre, M., & Laville, E. (2006). Chemical oxidation decreases proteolytic susceptibility of skeletal muscle myofibrillar proteins. *Meat Science*, 73, 536-543.
- Moudilou, E. N., Mouterfi, N., Exbrayat, J. M., & Brun, C. (2010). Calpains expression during *Xenopus Laevis* development. *Tissue Cell*, 42, 275-281.
- Mungure, T. E., Bekhit, A. E. D., Birch, J., Kanokruangrong, S., Carne, A., & Farouk, M. F. (2017). Impact of ageing method and PEF treatment on the meat quality and stability of conjugated linoleic acid in venison. 63rd International congress of meat science and technology, 13<sup>th</sup> - 18<sup>th</sup> August 2017, Cork, Ireland.
- Muroya, S., Ertbjerg, P., Pomponio, L., & Christensen, M. (2010). Desmin and troponin T are degraded faster in type IIb muscle fibers than in type I fibers during post-mortem aging of porcine muscle. *Meat Science*, 86(3), 764-769.
- Naveena, B. M., Muthukumar, M., Kulkarni, V. V., Kumar, Y. P., Rani, K. U., & Kiran, M. (2015). Effect of aging on the physicochemical, textural, microbial and proteome changes in emu (*Dromaius novaehollandiae*) meat under different packaging conditions. *Journal of Food Processing and Preservation*, 39, 2497-2506.
- Ngapo, T. M., & Vachon, L. (2017). The impact of homogeniser speed, dispersing aggregate size and centrifugation on particle size analyses of pork as a measure of myofibrillar fragmentation. *Meat Science*, 133, 166-172.
- Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2015). Gastrointestinal digestion of dairy and soy proteins in infant formulas: An in vitro study. *Food Research International*, 76, 348-358.
- Nishimura, T., & Goll, D. E. (1991). Binding of calpain fragments to calpastatin. *The Journal of Biological Chemistry*, 266, 11842-11850.
- Noor, S., Bhat, Z. F., Kumar, S., & Mudiyansele, R. J. (2018). Preservative effect of *Asparagus racemosus*: A novel additive for bioactive edible films for improved lipid oxidative stability and storage quality of meat products. *Meat Science*, 139, 207-212.
- O'Neill, L. M., Galvin, K., Morrissey, P. A. & Buckley, D. J. (1998) Comparison of effects of dietary olive oil, tallow and vitamin E on the quality of broiler meat products. *British Poultry Science*, 39, 365-371.
- Oberli, M., Marsset-Baglieri, A., Airinei, G., Sante-Lhoutellier, V., Khodorova, N., Remond, D., Foucault-Simonin, A., Piedcoq, J., Tomé, D., Fromentin, G., Benamouzig, R., & Gaudichon, C. (2015). High

- true ileal digestibility but not post-prandial utilization of nitrogen from bovine meat protein in humans is moderately decreased by high-temperature long-duration cooking. *Journal of Nutrition*, 145, 2221-2228.
- O'Dowd, L. P., Arimi, J. M., Noci, F., Cronin, D. A., & Lyng, J. G. (2013). An assessment of the effect of pulsed electrical fields on tenderness and selected quality attributes of post rigour beef muscle. *Meat Science*, 93, 303-309.
- O'Halloran, G. R., Troy, D. J., Buckley, D. J., & Reville, W. J. (1997). The role of endogenous proteases in the tenderisation of fast glycolysing muscle. *Meat Science*, 47, 187-210.
- Ojha, K. S., Granato, D., Rajuria, G., Barba, F. J., Kerry, J. P., & Tiwari, B. K. (2018). Application of chemometrics to assess the influence of ultrasound frequency, *Lactobacillus sakei* culture and drying on beef jerky manufacture: Impact on amino acid profile, organic acids, texture and colour. *Food Chemistry*, 239, 544-550.
- Ono, Y., Kakinuma, K., Torii, F., Irie, A., Nakagawa, K., Labeit, S., Abe, K., Suzuki, K., & Sorimachi, H. (2004). Possible regulation of the conventional calpain system by skeletal muscle specific calpain, p94/calpain 3. *Journal of Biological Chemistry*, 279, 2761-2771.
- Ono, Y., Ojima, K., Torii, F., Takaya, E., Doi, N., Nakagawa, K., Hata, S., Abe, K., & Sorimachi, H. (2010). Skeletal muscle-specific calpain is an intracellular Na<sup>+</sup>-dependent protease. *Journal of Biological Chemistry*, 285, 22986-22998.
- Onopiuk, A., Połtorak, A., & Wierzbicka, A. (2018). The impact of muscle and aging time on meat tenderness in the carcasses of Limousin 3 Holstein-Friesian crossbred bulls. *Journal of Food Processing and Preservation*, e13619, DOI: 10.1111/jfpp.13619.
- Ortega-Rivas, E. (2011). Critical issues pertaining to application of pulsed electric fields in microbial control and quality of processed fruit juices. *Food Bioprocess Technology*, 4, 631-645.
- Ostermeier, R., Giersemehl, P., Siemer, C., Töpfl, S., & Jäger, H. (2018). Influence of pulsed electric field (PEF) pre-treatment on the convective drying kinetics of onions. *Journal of Food Engineering*, DOI:10.1016/j.jfoodeng.2018.05.010.
- Ouali, A., Hernan Herrera-Mendez, C., Coulis, G., Becila, S., Boudjellal, A., Aubry, L., & Sentandreu, M. A. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Science*, 74, 44-58.
- Pal, M. (2017). Pulsed electric field processing: An emerging technology for food preservation. *Journal of Experimental Food Chemistry*, 3, 2.
- Palgan, I., Muñoz, A., Noci, F., Whyte, P., Morgan, D. J., Cronin, D. A., & Lyng, J. G. (2012). Effectiveness of combined Pulsed Electric Field (PEF) and Manothermosonication (MTS) for the control of *Listeria innocua* in a smoothie type beverage. *Food Control*, 25, 621-625.

- Pappenheimer, J. R., Dahl, C. E., Karnovsky, M. L., & Maggio, J. E. (1994). Intestinal absorption and excretion of octapeptides composed of D amino acids. *Proceedings of the National Academy of Sciences of the United States of America* 91 (pp. 1942-1945).
- Park, E. S., & Boxer, S. G. (2002). Origins of the sensitivity of molecular vibrations to electric fields: carbonyl and nitrosyl stretches in model compounds and proteins. *Journal of Physical Chemistry B.*, 106, 5800-5806.
- Parkes, C., Kembhavi, A. A., & Barrett, A. J. (1985). Calpain inhibition by peptide epoxides. *The Journal of Biochemistry*, 230, 509-516.
- Parr, T., Sensky, P. L., Scothern, G. P., Bardsley, R. G., Buttery, P. J., Wood, J. D., & Warkup, C. (1999). Relationship between skeletal muscle-specific calpain and tenderness of conditioned porcine *longissimus* muscle. *Journal of Animal Science*, 77, 661-668.
- Pataro, G., Falcone, M., Donsì, G., & Ferrari, G. (2014). Metal release from stainless steel electrodes of a PEF treatment chamber: Effects of electrical parameters and food composition. *Innovative Food Science and Emerging Technology*, 21, 58-65.
- Paulin, D., & Li, Z. (2004). Desmin: A major intermediate filament protein essential for the structural integrity and function of muscle. *Experimental Cell Research*, 301, 1-7.
- Pearce, K. L., Rosenvold, K., Andersen, H. J., & Hopkins, D. L. (2011). Water distribution and mobility in meat during the conversion of muscle to meat and ageing and the impacts of fresh meat quality attributes - A review. *Meat Science*, 89, 111-124.
- Pearson A. M., Love J. D., & Shorland F. B. (1977). Warmed-over flavour in meat, poultry and fish. *Advances in Food Research*, 23, 1-74.
- Pearson, A. M., & Tauber, F. W. (1984). Curing. In: *Processed Meats* (2<sup>nd</sup> Edn.), Springer Science & Business Media, pp. 48.
- Pennings, B., Groen, B. B., van Dijk, J. W., de Lange, A. Kiskini, A., Kuklinski, M., Senden, J. M., & van Loon, L. J. (2013). Minced beef is more rapidly digested and absorbed than beef steak, resulting in greater postprandial protein retention in older men. *American Journal of Clinical Nutrition*, 98, 121-128.
- Penny, I. F., & Dransfield, E. (1979). Relationship between toughness and troponin T in conditioned beef. *Meat Science*, 3(2), 135-141.
- Phillips, S. M. (2012). Nutrient-rich meat proteins in offsetting age-related muscle loss. *Meat Science*, 92, 174-178.
- Piaskowska, N., Daszkiewicz, T., Kubiak, D., & Zapotoczny, P. (2016). Quality of meat (*longissimus dorsi*) from male fallow deer (*Dama dama*) packaged and stored under vacuum and modified atmosphere conditions. *Asian-Australasian Journal of Animal Sciences*, 29(12), 1782-1789.
- Pillet, F., Formosa-Dague, C., Baaziz, H., Dague, E., & Rols, M.-P. (2016). Cell wall as a target for bacteria inactivation by pulsed electric fields. *Scientific Reports*, 6, 19778.

- Pimentel, D., & Burgess, M. (2018). World human population problems. Reference module in earth systems and environmental sciences, *Encyclopaedia of the Anthropocene*, pp. 313-317.
- Pośoś, A. R., & Puolanne, E. (2005). Carbohydrate metabolism in meat animals. *Meat Science*, 70, 423-434.
- Põldvere, A., Tänavots, A., Saar, R., Sild, S., & Lepasalu, L. (2016). Effect of heat treatment at constant 120 °C temperature on the rheological and technological properties of pork. *Agronomy Research*, 14, 1434-1441.
- Pomélie, D. D. L., Santé-Lhoutellier, V., Sayd, T., & Gatellier, P. (2018). Oxidation and nitrosation of meat proteins under gastrointestinal conditions: Consequences in terms of nutritional and health values of meat. *Food Chemistry*, 243, 295-304.
- Pomponio, L., & Ertbjerg, P. (2012). The effect of temperature on the activity of  $\mu$ - and m-calpain and calpastatin during post-mortem storage of porcine *longissimus muscle*. *Meat Science*, 91, 50-55.
- Pomponio, L., Lametsch, R., Karlsson, A. H., Costa, L. N., Grossi, A., & Ertbjerg, P. (2008). Evidence for post-mortem m-calpain autolysis in porcine muscle. *Meat Science*, 80, 761-764.
- Prates, J. A. M., Ribeiro, A. M. R., & Correia, A. A. D. (2001). Role of cysteine endopeptidases (EC 3.4.22) in rabbit meat tenderisation and some related changes. *Meat Science*, 57(3), 283-290.
- Pretorius, B., & Schönfeldt, H. C. (2018). The contribution of processed pork meat products to total salt intake in the diet. *Food Chemistry*, 238, 139-145.
- Primozic, M., Duchek, A., Nickerson, M., & Ghosh, S. (2018). Formation, stability and in vitro digestibility of nanoemulsions stabilized by high-pressure homogenized lentil proteins isolate. *Food Hydrocolloids*, 77, 126-141.
- Promeyrat, A., Gatellier, P., Lebret, B., Kajak-Siemaszko, K., Aubry, L., & Sante-Lhoutellier, V. (2010). Evaluation of protein aggregation in cooked meat. *Food Chemistry*, 121, 412-417.
- Puértolas, E., & Barba, F. J. (2016). Electrotechnologies applied to valorization of by-products from food industry: Main findings, energy and economic cost of their industrialization. *Food Bioproducts Process*, 100, 172-184.
- Puértolas, E., Saldana, G., Condon, S., Alvarez, I., & Raso, J. (2010). Evolution of polyphenolic compounds in red wine from *Cabernet Sauvignon* grapes processed by pulsed electric fields during aging in bottle. *Food Chemistry*, 119, 1063-1070.
- Pulford, D. J., Dobbie, P., Fraga-Vazquez, S., Fraser-Smith, E., Frost, D. A., & Morris, C. A. (2009). Variation in bull beef quality due to ultimate muscle pH is correlated to endopeptidase and small heat shock protein levels. *Meat Science*, 83, 1-9.
- Pulford, D. J., Fraga Vazquez, S., Frost, D. F., Fraser-Smith, E., Dobbie, P., & Rosenvold, K. (2008). The intracellular distribution of small heat shock proteins in post-mortem beef is determined by ultimate pH. *Meat Science*, 79, 623-630.

- Qi, J., Li, X., Zhang, W., Wang, H., Zhou, G., & Xu, X. (2018). Influence of stewing time on the texture, ultrastructure and in vitro digestibility of meat from the yellow feathered chicken breed. *Animal Science Journal*, *89*, 474-482.
- Qianli, Ma., Hamid, N., Oey, I., Kantono., K., Faridnia, F., Yoo, M., & Farouk, M. (2016). Effect of chilled and freezing pre-treatments prior to pulsed electric field processing on volatile profile and sensory attributes of cooked lamb meats. *Innovative Food Science and Emerging Technologies*, *37*, 359-374.
- Rajagopal, K., & Oommen, G. T. (2015). Myofibril fragmentation index as an immediate postmortem predictor of buffalo meat tenderness. *Journal of Food Processing and Preservation*, *39*, 1166-1171.
- Ramkumar, M. S. (2017). The correlation between color and electrical conductivity in beef. *Master Thesis*, <https://digital.library.txstate.edu/bitstream/handle/10877/6953/RAMKUMAR-THESIS-2017.pdf?sequence=1&isAllowed=y>.
- Raser, K. J., Posner, A., & Wang, K. K. (1995). Casein zymography: A method to study mu-calpain, m-calpain, and their inhibitory agents. *Archives of Biochemistry and Biophysics*, *319*, 211-216.
- Raso, J., & Heinz, V. (2006). Pulsed electric fields technology for the food industry: Fundamentals and applications. New York, USA: Springer.
- Ravulapalli, R., Diaz, B. G., Campbell, R. L., & Davies, P. L. (2005). Homodimerization of calpain 3 penta-EF-hand domain. *Journal of Biochemistry*, *388*, 585-591.
- Raynaud, P., Gillard, M., Parr, T., Bardsley, R., Amarger, V., & Levéziel, H. (2005). Correlation between bovine calpastatin mRNA transcripts and protein isoforms. *Achieves of Biochemistry and Biophysics*, *440*, 46-53.
- Reitznerová, A., Šuleková, M., Nagy, J., Marcinčák, S., Semjon, B., Čertík, M., & Klempová, T. (2017). Lipid peroxidation process in meat and meat products: A comparison study of malondialdehyde determination between modified 2-thiobarbituric acid spectrophotometric method and reverse-phase high-performance liquid chromatography. *Molecules*, *22*, 1988, doi:10.3390/molecules22111988.
- Riener, J., Noci, F., Cronin, D. A., Morgan, D. J., & Lyng, J. G. (2008). Combined effect of temperature and pulsed electric fields on apple juice peroxidase and polyphenoloxidase inactivation. *Food Chemistry*, *109*, 402-407.
- Rivas, A., Rodrigo, D., Martínez, A., Barbosa-Canovas, G. V., & Rodrigo, M. (2006). Effect of PEF and heat pasteurization on the physical-chemical characteristics of blended orange and carrot juice. *LWT - Food Science and Technology*, *39*, 1163-1170.
- Roager, H. M., & Licht, T. R. (2018). Microbial tryptophan catabolites in health and disease. *Nature Communications*, *9*, 3294. DOI: 10.1038/s41467-018-0547.

- Roberts, J. C., Rodas-González, A., Juárez, M., Lo'pez-Campos, O., Larsen, I. L., & Aalhus, J. L. (2018). Tenderness and sensory attributes of 11 muscles from carcasses within the Canadian cull cow grades. *Canadian Journal of Animal Science*, *98*, 230-240.
- Rocha, C. M. R., Genisheva, Z., Ferreira-Santos, P., Rodrigues, R., Vicente, A. A., Teixeira, J. A., & Pereira, R. N. (2018). Electric field-based technologies for valorization of bioresources. *Bioresource Technology*, *254*, 325–339.
- Rodríguez-González, O., Walkling-Ribeiro, M., Jayaramb, S., & Griffiths, M. W. (2011). Cross-protective effects of temperature, pH, and osmotic and starvation stresses in *Escherichia coli* O157:H7 subjected to pulsed electric fields in milk. *International Dairy Journal*, *21*, 953-962.
- Rodriguez-Roque, M. J., De Ancos, B., Sanchez-Moreno, C., Cano, M. P., Elez-Martinez, P., & Martin-Belloso, O. (2015). Impact of food matrix and processing on the in vitro bioaccessibility of vitamin C, phenolic compounds, and hydrophilic antioxidant activity from fruit juice-based beverages. *J. Functional Foods*, *14*, 33-43.
- Rojas, M. C., Martin, S. E., Wicklund, R. A., Paulson, D. D., Desantos, F. A., & Brewer, M. S. (2007) Effect of high-intensity pulsed electric fields on survival of *Escherichia coli* K-12 suspended in meat injection solutions. *Journal of Food Safety*, *27*, 411-425.
- Roldan, M., Antequera, T., Armenteros, M., & Ruiz, J. (2014). Effect of different temperature–time combinations on lipid and protein oxidation of sous-vide cooked lamb loins. *Food Chemistry*, *149*, 129-136.
- Rowe, L. J., Maddock, K. R., Lonergan, S. M., & Huff-Lonergan, E. (2004). Oxidative environments decrease tenderization of beef steaks through inactivation of mu-calpain. *Journal of Animal Science*, *82*, 3254-3266.
- Rowe, L. J., Maddock, K. R., Trenkle, A., Lonergan, S. M., & Huff-Lonergan, E. (2003). Effects of oxidation on beef tenderness and calpain activity. *Journal of Animal Science*, *81*, 74.
- Saido, T. C., Shibata, M., Takenawa, T., Murofushi, H., & Suzuki, K. (1992). Positive regulation of mu-calpain action by polyphosphoinositides. *Journal of Biological Chemistry*, *267*, 24585-24590.
- Saif, S. M. H., Lan, Y., Williams, L. L., Joshee, L., & Wang, S. (2007). Reductions of *Escherichia coli* O157:H7 on goat meat surface with pulsed dc square wave signal. *Journal of Food Engineering*, *77*, 281-288.
- Salamino, F., De Tullio, R., Mengotti, P., Viotti, P. L., Melloni, E., & Pontremoli, S. (1993). Site-directed activation of calpain is promoted by a membrane-associated natural activator protein. *Journal of Biochemistry*, *290*, 191-197.
- Saldaña, G., Álvarez, I., Condón, S., & Raso, J. (2014). Microbiological aspects related to the feasibility of PEF technology for food pasteurization. *Critical Reviews in Food Science and Nutrition*, *54*, 1415-1426.

- Sampedro, F., McAloon, A., Yee W., Fan X., & Geveke D. J. (2014). Cost analysis and environmental impact of pulsed electric fields and high-pressure processing in comparison with thermal pasteurization. *Food Bioprocess Technology*, 7, 1928-1937.
- Sánchez-Vega, R., Elez-Martínez, P., & Martín-Belloso, O. (2014). Effects of high intensity pulsed electric fields processing parameters on the chlorophyll content and its degradation compounds in broccoli juice. *Food and Bioprocess Technology*, 7, 1137-1148.
- Santé-Lhoutellier, V., Astruc, T., Marinova, P., Greve, E., & Gatellier, P. (2008). Effect of meat cooking on physicochemical state and in vitro digestibility of myofibrillar proteins. *Journal of Agricultural and Food Chemistry*, 56, 1488-1494.
- Santé-Lhoutellier, V., Aubry, L., & Gatellier, P. (2007). Effect of oxidation on in vitro digestibility of skeletal muscle myofibrillar proteins. *Agricultural and Food Chemistry*, 55, 5343-5348.
- Sanz-Puig, M., Santos-Carvalho, L., Cunha, L. M., Pina-Pérez, M. C., Martínez, A., & Rodrigo, D. (2016). Effect of pulsed electric fields (PEF) combined with natural antimicrobial by-products against *S. typhimurium*. *Innovative Food Science and Emerging Technology*, 37, 322-328.
- Saulis, G. (2010). Electroporation of cell membranes: the fundamental effects of pulsed electric fields in food processing. *Food Engineering Reviews*, 2, 52-73.
- Savoie, L., Agudelo, R. A., Gauthier, S. F., Marin, J., & Pouliot, Y. (2005). In vitro determination of the release kinetics of peptides and free amino acids during the digestion of food proteins. *Journal of AOAC International*, 88, 935-948.
- Sayd T., Dufour C., Chambon C., Buffière C., Remond D., & Santé-Lhoutellier V. (2018). Combined *in vivo* and *in silico* approaches for predicting the release of bioactive peptides from meat digestion. *Food Chemistry*, 249, 111-118.
- Schafer, A., Rosenvold, K., Purslow, P. P., Andersen, H. J., & Henckel, P. (2002). Physiological and structural events post-mortem of importance for drip loss in pork. *Meat Science*, 61, 355-366.
- Segovia, F. J., Luengo, E., Corral-Pérez, J. J., Raso, J., & Almajano, M. P. (2015). Improvements in the aqueous extraction of polyphenols from borage (*Borago officinalis* L.) leaves by pulsed electric fields: Pulsed electric fields (PEF) applications. *Industrial Crops and Products*, 65, 390-396.
- Sensky, P. L., Parr, T., Bardsley, R. G., & Buttery, P. J. (1996). The relationship between plasma epinephrine concentration and the activity of the calpain enzyme system in porcine *longissimus* muscle. *Journal of Animal Science*, 74, 380-387.
- Sentandreu, M. A., Coulis, G., & Ouali, A. (2002). Role of muscle endopeptidases and their inhibitors in meat tenderness. *Trends in Food Science and Technology*, 13, 400-421.
- Shange, N., Makasi, T. N., Gouws, P. A., & Hoffman, L. C. (2018). The influence of normal and high ultimate muscle pH on the microbiology and colour stability of previously frozen black wildebeest (*Connochaetes gnou*) meat. *Meat Science*, 135, 14-19.

- Sharma, P., Oey, I., & Everett, D. W. (2016). Thermal properties of milk fat, xanthine oxidase, caseins and whey proteins in pulsed electric field-treated bovine whole milk. *Food Chemistry*, *207*, 34-42.
- Sharma, P., Oey, I., Bremer, P., & Everett, D. W. (2014). Reduction of bacterial counts and inactivation of enzymes in bovine whole milk using pulsed electric fields. *International Dairy Journal*, *39*, 146-156.
- Siemer, C., Toepfl, S., & Heinz, V. (2012). Mass transport improvement by PEF- applications in the area of extraction and distillation. In: Zereshki, S. (Ed.), *Distillation- Advances from modelling to applications*. In Tech, pp. 211-232.
- Silva, F. A. P., Estévez, M., Ferreira, V. C. S., Silva, S. A., Lemos, L. T. M., Ida, E. I., Shimokomaki, M., & Madruga, M. S. (2018). Protein and lipid oxidations in jerky chicken and consequences on sensory quality. *LWT - Food Science and Technology*, *97*, 341-348.
- Silva, L. H. P., Paulino, P. V. R., Assis, G. J. F., Assis, D. E. F., Estrada, M. M., Silva, M. C., Silva, J. C., Martins, T. S., Filho, S. C. V., Paulino, M. F., & Chizzotti, M. L. (2017). Effect of post-weaning growth rate on carcass traits and meat quality of Nellore cattle. *Meat Science*, *123*, 192-197.
- Silvester, K. R., & Cumings, J. H. (1995). Does digestibility of meat protein help explain large bowel cancer risk. *Nut Cancer*, *24*, 279-288.
- Simmons, N. J., Daly, C. C., Cummings, T. L., Morgan, S. K., Johnson, N. V., & Lombard, A. (2008). Reassessing the principles of electrical stimulation. *Meat Science*, *80*, 110-122.
- Simonetti, A., Gambacorta, E., & Perna, A. (2016). Antioxidative and antihypertensive activities of pig meat before and after cooking and *in vitro* gastrointestinal digestion: Comparison between Italian autochthonous pig *Suino Nero Lucano* and a modern crossbred pig. *Food Chemistry*, *212*, 590-595.
- Sindelar, J. F., Terns, M. J., Meyn, E., & Boles, J. A. (2010). Development of a method to manufacture uncured, no-nitrate/nitrite-added whole muscle jerky. *Meat Science*, *86*, 298-303.
- Smith, K., Mittal, G. S., & Griffiths, M. W. (2002). Pasteurisation of milk using pulsed electric field and antimicrobials. *Journal of Food Science*, *67*, 2304-2308.
- Smith, M. A., Bush, R. D., van de Ven, R. J., & Hopkins, D. L. (2016). Effect of electrical stimulation and ageing period on alpaca (*Vicugna pacos*) meat and eating quality. *Meat Science*, *111*, 38-46.
- Snedecor, G. W., & Cochran, W. G. (1994). *Statistical Methods*, 8th ed. Iowa State University Press, Iowa.
- Soladoye, O. P., Juarez, M. L., Aalhus, J. L., Shand, P., & Estévez, M. (2015). Protein oxidation in processed meat: Mechanisms and potential implications on human health. *Comprehensive Reviews in Food Science and Food Safety*, *14*, 106-122.
- Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., & Suzuki, K. (1989). Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and



- mu-types. Specific expression of the mRNA in skeletal muscle. *Journal of Biological Chemistry*, 264, 20106-20111.
- Sorimachi, H., Kinbara, K., Kimura, S., Takahashi, M., Ishiura, S., Sasagawa, N., Sorimachi, N., Shimada, H., Tagawa, K., Maruyama, K., & Suzuki, K. (1995). Muscle-specific calpain, p94, responsible for limb girdle muscular dystrophy type 2A, associates with connectin through IS2, a p94-specific sequence. *Journal of Biological Chemistry*, 270, 31158-31162.
- Sorimachi, H., Mamitsuka, H., & Ono, Y. (2012). Understanding the substrate specificity of conventional calpains. *Biological Chemistry*, 393, 853-871.
- Stachelska, M. A., Stankiewicz-Szymczak, W., Jakubczak, A., Świsłocka, R., & Lewandowski, W. (2012). Influence of pulsed electric field on the survival of *Yersinia enterocolitica* in minced beef meat: assessment of microbiological activity of selected cell lines of bacteria under influence of physical-chemical factors. *Aparatura Badawcza i Dydaktyczna*, 17, 13-17.
- Stadtman, E. R. (1993). Oxidation of free amino acids and amino acids residues in proteins by radiolysis and by metal-catalyzed reactions. *Annual Review of Biochemistry*, 62, 797-821.
- Stefanović, A. B., Jovanović, J. R., Dojčinović, M. B., Lević, S. M., Nedović, V. A., Bugarski, B. M., & Knežević-Jugović, Z. D. (2017). Effect of the controlled high-intensity ultrasound on improving functionality and structural changes of egg white proteins. *Food and Bioprocess Technology*, 10(7), 1-16.
- Stefanović, A. B., Jovanović, J. R., Grbavčić, S. Ž., Šekuljica, N. Ž., Manojlović, V. B., Bugarski, B. M., & Knežević-Jugović, Z. D. (2014). Impact of ultrasound on egg white proteins as a pretreatment for functional hydrolysates production. *European Food Research and Technology*, 239(6), 979-993.
- Stoeva, S., Byrne, C. E., Mullen, A. M., Troy, D. J., & Voelter, W. (2000). Isolation and identification of proteolytic fragments from TCA soluble extracts of bovine *M. longissimus dorsi*. *Food Chemistry*, 69, 365-370.
- Stonehouse, G. G., and Evans, J. A. (2015). The use of supercooling for fresh foods: a review. *Journal of Food Engineering*, 148, 74-79.
- Straadt, I. K., Rasmussen, M., Andersen, H. J., & Bertram, H. C. (2007). Aging-induced changes in microstructure and water distribution in fresh and cooked pork in relation to water-holding capacity and cooking loss—A combined confocal laser scanning microscopy (CLSM) and low-field nuclear magnetic resonance relaxation study. *Meat Science*, 75, 687-695.
- Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H., Suzuki, K., Bode, W. (2000). The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. *Proceedings of the National Academy of Science of USA*, 97, 588-592.

- Suchanek, M., & Olejniczak, Z. (2018). Low field MRI study of the potato cell membrane electroporation by pulsed electric field. *Journal of Food Engineering*, 231, 54-60.
- Sullivan, G. A., & Calkins, C. R. (2011). Ranking beef muscles for Warner-Bratzler shear force and trained sensory panel ratings from published literature. *Journal of Food Quality*, 34, 195-203.
- Sun, X., Chen, K. J., Berg, E. P., Newman, D. J., Schwartz, C. A., Keller, W. L., & Carlin, M. K. R. (2014). Prediction of troponin-T degradation using colour image texture features in 10 d aged beef longissimus steaks. *Meat Science*, 96, 837-842.
- Suwandy, V., Carne, A., van de Ven, R., Bekhit, A. E. A., & Hopkins, D. L. (2015a). Effect of pulsed electric field on the proteolysis of cold boned beef *M. Longissimus lumborum* and *M. Semimembranosus*. *Meat Science*, 100, 222-226.
- Suwandy, V., Carne, A., van de Ven, R., Bekhit, A. E. A., & Hopkins, D. L. (2015b). Effect of pulsed electric field treatment on hot-boned muscles of different potential tenderness. *Meat Science*, 105, 25-31.
- Suwandy, V., Carne, A., van de Ven, R., Bekhit, A. E. A., and Hopkins, D. L. (2015c). Effect of pulsed electric field treatment on the eating and keeping qualities of cold-boned beef loins: impact of initial pH and fibre orientation. *Food and Bioprocess Technology*, 8, 1355-1365.
- Suwandy, V., Carne, A., van de Ven, R., Bekhit, A. E. A., and Hopkins, D. L. (2015d). Effect of repeated pulsed electric field treatment on the quality of cold-boned beef loins and topsides. *Food and Bioprocess Technology*, 8, 1218-1228.
- Suzuki, K. (1990). The structure of calpains and the calpain gene. In: Mellgren, R. L., & Murachi, T. (Eds.), Intracellular calcium-dependent proteolysis, Intracellular calcium dependent proteolysis (pp. 25-35). Boca Raton, FL: CRC Press.
- Suzuki, K., Hata, S., Kawabata, Y., & Sorimachi, H. (2004). Structure, activation, and biology of calpain. *Diabetes*, 53, S12-S18.
- Tao, Y., & Sun, D. W. (2015). Enhancement of food processes by ultrasound: A review. *Critical Reviews in Food Science and Nutrition*, 55, 570-594.
- Tavano, O. L., Neves, V. A., & Júnior, S. I. D. S. (2016). In vitro versus in vivo protein digestibility techniques for calculating PDCAAS (protein digestibility-corrected amino acid score) applied to chickpea fractions. *Food Research International*, 89, 756-763.
- Tavares, W. P. S., Dong, S., Yang, Y., Zeng, M., & Zhao, Y. (2018). Influence of cooking methods on protein modification and *in vitro* digestibility of hairtail (*Thichiurus lepturus*) fillets. *LWT-Food Science and Technology*, DOI: 10.1016/j.lwt.2018.06.006.
- Taylor, R. G., Geesink, G. H., Thompson, V. F., Koohmaraie, M., & Goll, D. E. (1995b). Is Z-disk degradation responsible for postmortem tenderization? *Journal of Animal Science*, 73, 1351-1367.

- Taylor, R., Tassy, C., Briand, M., Robert, N., Briand, Y., & Ouali, A. (1995a). Proteolytic activity of proteasome on myofibrillar structures. *Molecular Biology Reports*, *21*, 71-73.
- Teissie, J., Prats, M., Soucaille, P., & Tocanne, J. F. (1985). Evidence for conduction of protons along the interface between water and a polar lipid monolayer. *Proceedings of National Academy of Science of USA*, *82*, 3217-3221.
- Tešanović, D., Kalenjuk, B., Tešanović, D., Psodorov, Đ., Ristić, Z., & Marković, V. (2011). Changes of biochemical and sensory characteristics in the musculus *longissimus dorsi* of the fallow deer in the early phase post-mortem and during maturation. *African Journal of Biotechnology*, *10*(55), 11668-11675.
- Thérona, L., Chambona, C., Sayda, T., Poméliea, D. D. I., Santé-Lhoutelliera, V., & Gatelliera, P. (2018). To what extent does the nitrosation of meat proteins influence their digestibility? *Food Research International*, *113*, 175-182.
- Thompson, V. F., Goll, D. E., & Kleese, W. C. (1990). Effects of autolysis on the catalytic properties of the calpains. *Biological Chemistry Hoppe-Seyler*, *371*, 177-185.
- Thomson, K. L., G. E. Gardner, N. Simmons, and J. M. Thompson. 2008. Length of exposure to high post-rigor temperatures affects the tenderisation of the beef *M. Longissimus dorsi*. *Australian Journal of Experimental Agriculture*, *48*, 1442-1450.
- Thomson, K. L., Gardner, G. E., Simmons, N., & Thompson, J. M. (2008). Length of exposure to high post-rigor temperatures affects the tenderisation of the beef *M. longissimus dorsi*. *Australian Journal of Experimental Agriculture*, *48*, 1442-1450.
- Timmermans, R. A. H., Nierop Groot, M. N., Nederhoff, A. L., van Boekel, M. A. J. S., Matser, A. M., & Mastwijk, H. C. (2014). Pulsed electric field processing of different fruit juices: Impact of pH and temperature on inactivation of spoilage and pathogenic micro-organisms. *International Journal of Food Microbiology*, *173*, 105-111.
- Toepfl, S. (2006). Pulsed electric fields (PEF) for permeabilization of cell membranes in food- and bioprocessing: Applications, process and equipment design and cost analysis. *Published doctoral thesis*. Germany: Berlin University of Technology. Download from: <https://depositonce.tu-berlin.de/handle/11303/1738>.
- Toepfl, S. (2012). Pulsed electric field food processing - industrial equipment design and commercial applications. *Stewart Postharvest Reviews*, *4*, 1-7.
- Toepfl, S., & Heinz, V. (2007a). Application of pulsed electric fields to improve mass transfer in dry cured meat products. *Fleischwirtschaft International*, *22*, 62-64.
- Toepfl, S., & Heinz, V. (2007b). Pulsed electric field processing of foods. *New Food*, *3*, 1594. <https://www.newfoodmagazine.com/article/1594/pulsed-electric-field-processing-of-foods/#respond>.

- Toepfl, S., Heinz, V., & Knorr, D. (2005). Overview of pulsed electric field processing for food. In: Sun, D. W. (Ed.), *Emerging technologies for food processing*. Elsevier Academic Press, London, pp. 69-97.
- Toepfl, S., Heinz, V., & Knorr, D. (2007). High intensity pulsed electric fields applied for food preservation. *Chemical Engineering and Processing: Process Intensification*, *46*, 537-546.
- Toepfl, S., Mathys, A., Heinz, V., & Knorr, D. (2006). Review: potential of high hydrostatic pressure and pulsed electric fields for energy efficient and environmentally friendly food processing. *Food Review International*, *22*, 405-423.
- Toepfl, S., Siemer, C., & Heinz, V. (2014a). Effect of high-intensity electric field pulses on solid foods. In: *Emerging technologies for food processing (2<sup>nd</sup> Edition)*, pp. 147-154, <http://dx.doi.org/10.1016/B978-0-12-411479-1.00008-5>.
- Toepfl, S., Siemer, C., Saldan~a-Navarro, G., & Heinz, V. (2014b). Overview of pulsed electric fields processing for food. In: *Emerging technologies for food processing*, pp. 93-114, <http://dx.doi.org/10.1016/B978-0-12-411479-1.00006-1>.
- Toldra, F., & Reig, M. (2015). Enzymes in meat and fish. In: *Improving and Tailoring Enzymes for Food Quality and Functionality* (pp. 199-212), (Ed. Yada, R.), Woodhead Publishing, ISBN: 978-1-78242-285-3.
- Tomasevic, I., Novakovic, S., Solowiej, B., Zdolec, N., Skunca, D., Krocko, M., & Djekic, I. (2018). Consumers' perceptions, attitudes and perceived quality of game meat in ten European countries. *Meat Science*, *142*, 5-13.
- Tompa, P., Emori, Y., Sorimachi, H., Suzuki, K., & Friedrich, P. (2001). Domain III of calpain is a Ca<sup>2+</sup>-regulated phospholipid-binding domain. *Biochemical and Biophysical Research Communications*, *280*, 1333-1339.
- Töpfl, S. (2006). Pulsed electric fields (PEF) for permeabilization of cell membranes in food and bioprocessing: Applications, process and equipment design and cost analysis (*PhD thesis*) Germany: Universität Berlin, <https://depositonce.tu-berlin.de/handle/11303/1738>.
- Tornberg, E. (2005). Effects of heat on meat proteins - Implications on structure and quality of meat products. *Meat Science*, *70*, 493-508.
- Troy, D. J., & Kerry, J. P. (2010). Consumer perception and the role of science in the meat industry. *Meat Science*, *86*, 214-226.
- Tullio, R. D., Passalacqua, M., Averna, M., Salamino, F., Melloni, E., & Pontremoli, S. (1999). Changes in intracellular localization of calpastatin during calpain activation. *Journal of Biochemistry*, *343*, 467-472.
- U.S.FDA, (2018). Use the nutrition facts label to reduce your intake of sodium in your diet. <https://www.fda.gov/food/resourcesforyou/consumers/ucm315393.htm> (accessed 26.04.2019).

- Uchida, S., Houjo, M., & Tochikubo, F. (2008). Efficient sterilization of bacteria by pulse electric field in micro-gap. *Journal of Electrostatics*, *66*, 427-431.
- United Nations (2017). World population prospects: The 2017 revision, key findings and advance tables. Department of Economic and Social Affairs, Population Division, Working Paper No. ESA/P/WP/248.
- USDA-FSIS. Compliance guideline for meat and poultry jerky produced by small and very small plants. <https://www.fsis.usda.gov/wps/wcm/connect/5fd4a01d-a381-4134-8b91-99617e56a90a/Compliance-Guideline-Jerky-2014.pdf?MOD=AJPERES> (accessed 26.04.2019).
- Valencak, T. G., Gamsjäger, L., Ohrnberger, S., Culbert, N. J., & Ruf, T. (2015). Healthy n-6/n-3 fatty acid composition from five European game meat species remains after cooking. *BMC research notes*, *8*, 273.
- van der Plancken, I., Delattre, M., Indrawati, V. L. A., & Hendrickx, M. E. G. (2004). Kinetic study on the changes in the susceptibility of egg white proteins to enzymatic hydrolysis induced by heat and high hydrostatic pressure pretreatment. *Journal of Agricultural and Food Chemistry*, *52*(18), 5621-5626.
- Velickova, E., Tylewicz, U., Rosa, M. D., Winkelhausen, E., Kuzmanova, S., & Romani, S. (2018). Effect of pulsed electric field coupled with vacuum infusion on quality parameters of frozen/thawed strawberries. *Journal of Food Engineering*, *233*, 57-64.
- Verma, A. K., & Banerjee, R., (2012) Low-sodium meat products: Retaining salty taste for sweet health. *Critical Reviews in Food Science and Nutrition*, *52*, 72-84.
- Verrez-Bagnis, V., Noel, J., Sautereau, C., & Fleurence, J. (1999). Desmin degradation in post-mortem fish muscle. *Journal of Food Science*, *64*, 240-242.
- Vieira, S. A., Zhang, G., & Decker, E. A. (2017). Biological implications of lipid oxidation products. *Journal of the American Oil Chemists' Society*, *94*, 339-351.
- Viljoen, H. F., de Kock H. L., & Webb, E. C. (2002). Consumer acceptability of dark, firm and dry (DFD) and normal pH beef steaks. *Meat Science*, *61*, 181-185.
- Vorobiev, E., & Lebovka, N. I. (2008). Pulsed electric fields induced effects in plant tissues: fundamental aspects and perspectives of application. In: Vorobiev, E., Lebovka, N. (Eds.), *Electro-technologies for extraction from food plants and biomaterials*. New York, Springer, pp. 39-82.
- Walkling-Ribeiro, M., Noci, F., Cronin, D. A., Lyng, J. G., & Morgan, D. J. (2010). Shelflife and sensory attributes of a fruit smoothie-type beverage processed with moderate heat and pulsed electric fields. *LWT - Food Science and Technology*, *43*, 1067-1073.
- Walkling-Ribeiro, M., Rodríguez-Gonzalez, O., Jayaram, S. H., & Griffiths, M. W. (2011). Processing temperature, alcohol and carbonation levels and their impact on pulsed electric fields (PEF) mitigation of selected characteristic microorganisms in beer. *Food Research International*, *44*, 2524-2533.

- Wang, A., Kang, D., Zhang, W., Zhang, C., Zou, Y., & Zhou, G. (2018a). Changes in calpain activity, protein degradation and microstructure of beef *M. semitendinosus* by the application of ultrasound. *Food Chemistry*, 245, 724-730.
- Wang, M. S., Wang, L. H., Bekhit, A. E. A., Yang, J., Hou, Z. P., Wang, Y. Z., Dai, Q. Z., & Zeng, X. A. (2018b). A review of sublethal effects of pulsed electric field on cells in food processing. *Journal of Food Engineering*, 223, 32-41.
- Warner, R. D., McDonnell, C. K., Bekhit, A. E. D., Claus, J., Vaskoska, R., Sikes, A., Dunshea, F.R., & Ha, M. (2017). Systematic review of emerging and innovative technologies for meat Tenderisation. *Meat Science*, 132, 72-89.
- Weaver, J. C. (2000). Electroporation of cells and tissues. *IEEE transactions on plasma science*, 28(1), 24-33.
- Weaver, J. C., & Chizmadzhev, Y. A. (1996). Theory of electroporation: a review. *Bioelectrochemistry and Bioenergetics*, 41, 135-160.
- Wei, J. N., Zeng, X. A., Tang, T., Jiang, Z., & Liu, Y. Y. (2018). Unfolding and nanotube formation of ovalbumin induced by pulsed electric field. *Innovative Food Science and Emerging Technologies*, 45, 249-254.
- Weiss, M., Manneberg, M., Juranville, J. F., Lahm, H. W., & Fountoulakis, M. (1998). Effect of the hydrolysis method on the determination of the amino acid composition of proteins. *Journal of Chromatography A*, 795(2), 263-275.
- Wen, S., Zhou, G., Song, S., Xu, X., Voglmeir, J., Liu, L., Zhao, F., Li, M., Li, L., Yu, X., Bai, Y., & Li, C. (2015). Discrimination of *in vitro* and *in vivo* digestion products of meat proteins from pork, beef, chicken, and fish. *Proteomics*, 15, 3688-3698.
- Wendt, A., Thompson, V. F., & Goll, D. E. (2004). Interaction of calpastatin with calpain: A review. *Journal of Biological Chemistry*, 385, 465-472.
- Wheeler, T. L., & Koohmaraie, M. (1999). The extent of proteolysis is independent of sarcomere length in lamb longissimus and psoas major. *Journal of Animal Science*, 77, 2444-2451.
- Wheeler, T. L., Koohmaraie, M., & Shackelford, S. D. (1997). Effect of post-mortem injection time and post injection aging time on the calcium-activated tenderization process in beef. *Journal of Animal Science*, 75, 2652-2660.
- White, A., O'Sullivan, A., Troy, D. J., & O'Neill, E. E. (2006). Manipulation of the pre-rigor glycolytic behaviour of bovine *M-longissimus dorsi* in order to identify causes of inconsistencies in tenderness. *Meat Science*, 73, 151-156.
- White, A., O'Sullivan, A., Troy, D. J., & O'Neill, E. E. (2006). Effects of electrical stimulation, chilling temperature and hot-boning on the tenderness of bovine muscles. *Meat Science*, 73, 196-203.
- WHO (2006). Reducing salt intake in populations. Paper presented at the Report of a WHO Forum and technical meeting, Paris, France.

- Wiklund, E., Farouk, M., Stuart, A., & Dobbie, P. (2009). Consumer evaluation of chilled-never-frozen versus chilled-frozen-thawed beef and venison. Proceedings: 55<sup>th</sup> International Congress of Meat Science and Technology, 16-21 August (pp. 1206–1209), Copenhagen, Denmark.
- Wiktor, A., Iwaniuk, M., Sledz, M., Nowacka, M., Chudoba, T., & Witrowa-Rajchert, D. (2013). Drying kinetics of apple tissue treated by pulsed electric field. *Drying Technology*, *31*, 112-119.
- Wiktor, A., Sledz, M., Nowacka, M., Chudoba, T., & Witrowa-Rajchert, D. (2014). Pulsed electric field pre-treatment for osmotic dehydration of apple tissue: experimental and mathematical modelling studies. *Drying Technology*, *32*, 408-417.
- Wilson, R., Komitopoulou, E., & Incles, M. (2012). Evaluation of technological approaches to salt reduction. Report prepared by Leatherhead Food Research, Leatherhead, Surrey, UK, [https://www.fdf.org.uk/resources/salt\\_reduction\\_2012.pdf](https://www.fdf.org.uk/resources/salt_reduction_2012.pdf).
- Woodward, E., Eyles, H., & Mhurchu, C. N. (2012). Key opportunities for sodium reduction in New Zealand processed foods. *Australian and New Zealand Journal of Public Health*, *36*(1), 84-9.
- Wowk, B. (2012). Electric and magnetic fields in cryopreservation. *Cryobiology*, *64*(3), 301-303.
- Wua, L., Zhao, W., Yang, R., & Chen, X. (2014). Effects of pulsed electric fields processing on stability of egg white Proteins. *Journal of Food Engineering*, *139*, 13-18.
- Xiao, D., Yin, J., Ren, W., He, J., Hu, X., Yin, Y., Luo, W., & Yu, X. (2015). Porcine circovirus type 2 affects the serum profile of amino acids and intestinal expression of amino acid transporters in mice. *RSC Advances*, *5*, 73651-73659.
- Xu, X. X., Shui, X., Chen, Z. H., Shan, C. Q., Hou, Y. N., & Cheng, Y. G. (2009). Development and application of a real-time PCR method for pharmacokinetic and biodistribution studies of recombinant adenovirus. *Molecular Biotechnology*, *43*, 130-137.
- Xue, M., Huang, F., Huang, M., & Zhou, G. (2012). Influence of oxidation on myofibrillar proteins degradation from bovine via  $\mu$ -calpain. *Food Chemistry*, *134*, 106-112.
- Yan-Yan, L., Ying, Z., Xin-An, Z., El-Mashad, H., Zhong-Li, P., & Qi-Jun, W. (2014). Effect of pulsed electric field on microstructure of some amino acid group of soy protein isolates. *International Journal of Food Engineering*, *10*(1), 113-120.
- Yates, L. D., Dutson, T. R., Caldwell, J., & Carpenter, Z. L. (1983). Effect of temperature and pH on the post-mortem degradation of myofibrillar proteins. *Meat Science*, *9*, 157-179.
- Yong, H. I., Lee, S. H., Kim, S. Y., Park, S., Park, J., Choe, W., & Jo, C. (2019). Color development, physicochemical properties, and microbiological safety of pork jerky processed with atmospheric pressure plasma. *Innovative Food Science & Emerging Technologies*, *53*, 78-84.
- Zeng, F., Gao, Q. Y., Han, Z., Zeng, X. A., & Yu, S. J. (2016). Structural properties and digestibility of pulsed electric field treated waxy rice starch. *Food Chemistry*, *194*, 1313-1319.

- Zhang, L., Wang, L. J., Jiang, W., & Qian, J. Y. (2017). Effect of pulsed electric field on functional and structural properties of canola protein by pre-treating seeds to elevate oil yield. *LWT - Food Science and Technology*, *84*, 73-81.
- Zhang, Q., Chang, F. J., Barbosa-Cánovas, G. V., & Swanson, B. G. (1994). Inactivation of microorganisms in a semisolid model food using high voltage pulsed electric fields. *Lebenson Wiss Technol*, *27*, 538-543.
- Zhang, W. G., Lonergan, S. M., Gardner, M. A., & Huff-Lonergan, E. (2006). Contribution of post-mortem changes of integrin, desmin and mu-calpain to variation in water holding capacity of pork. *Meat Science*, *74*, 578-585.
- Zhang, Y., Hou, Y., Chen, J., Chen, F., Liao, X., & Hu, X. (2012). Reduction of diazinon and dimethoate in apple juice by pulsed electric field treatment. *Journal of Science of Food and Agriculture*, *92*, 743-750.
- Zhang, Z., Zhang, X., Chen, W., Zhou, P. (2018). Conformation stability, in vitro digestibility and allergenicity of tropomyosin from shrimp (*Exopalaemon modestus*) as affected by high intensity ultrasound. *Food Chemistry*, *245*, 997-1009.
- Zhao, W., Tang, Y. L., Lu, L. X., Chen, X., & Li, C. Y. (2014). Review: Pulsed electric fields processing of protein-based foods. *Food and Bioprocess Technology*, *7*, 114-125.
- Zhao, W., Yang, R. J., Tang, Y. L., Zhang, W. B., & Hua, X. (2009). Investigation of the protein-protein aggregation of egg white proteins under pulsed electric fields. *Journal of Science of Food and Agriculture*, *57*, 3571-3577.
- Zhao, W., Yang, R., & Zhang, H. Q. (2012). Recent advances in the action of pulsed electric fields on enzymes and food component proteins. *Trends Food Science and Technology*, *27*, 83-96.
- Zhao, W., Yang, R., Hua, X., Zhang, W., Tang, Y., & Chen, T. (2010). Inactivation of polyphenoloxidase of pear by pulsed electric fields. *International Journal of Food Engineering*, *6*(3), <http://dx.doi.org/10.2202/1556-3758.1853>.
- Zhao, W., Yang, R., Shen, X., Zhang, S., & Chen, X. (2013). Lethal and sublethal injury and kinetics of *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* in milk by pulsed electric fields. *Food Control*, *32*, 6-12.
- Zheng, H. B., Han, M. Y., Yang, H. J., Tang, C. B., Xu, X. L., & Zhou, G. H. (2017). Application of high pressure to chicken meat batters during heating modifies physicochemical properties, enabling salt reduction for high-quality products. *LWT - Food Science and Technology*, *84*, 693-700.
- Zhu, X., Kaur, L., Staincliffe, M., & Boland, M. (2018). Actinidin pretreatment and sous vide cooking of beef brisket: Effects on meat microstructure, texture and in vitro protein digestibility. *Meat Science*, *145*, 256-265.



- Zimmerman, U. J. P., & Schlaepfer, W. W. (1991). Two-stage autolysis of the catalytic subunit initiates activation of calpain I. *Biochimica et Biophysica Acta -Protein Structure and Molecular Enzymology*, 1078, 192-198.
- Zimmermann, U. (1996). The effect of high intensity electric field pulses on eukaryotic cell membranes: fundamentals and applications. In: Zimmermann, U., Neil, G. A. (Eds.), *Electro-manipulation of cells*. CRC Press, Boca Raton, pp. 1-106.
- Zimmermann, U., Pilwat, G., Beckers, F., & Riemann, F. (1976). Effects of external electrical fields on cell membranes. *Bioelectrochemistry and Bioenergetics*, 3, 58-83.
- Zou, X., Zhou, G., Yu, X., Bai, Y., Wang, C., Xu, X., Dai, C., & Li, C. (2018). In vitro protein digestion of pork cuts differ with muscle type. *Food Research International* 106, 344-353.