Lincoln University
Te Whare Wānaka o Aoraki
CHRISTCHURCH•NEW ZEALAND

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.

The Potential of Beef Lungs as a Protein Ingredient and Peptide Source

---

A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Doctor of Philosophy

at

Lincoln University

by

Sasika Reshan Jayawardena

Lincoln University

2020

To my beloved Mom & Dad for raising me to believe that all good things are possible…!

Declaration

Parts of this thesis are submitted or accepted for publication and/or presented in advance of submission of the thesis:

Publications

- Jayawardena, S. R., Morton, J. D., Brennan, C. S., & Bekhit, A. E. A. (2018). Utilisation of beef lung protein powder as a functional ingredient to enhance protein and iron content of fresh pasta. *International Journal of Food Science & Technology, 54*(3), 610-618. doi:10.1111/ijfs.13927
- 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.Proceeding of International Congress of Meat Science Technology (ICoMST). http://icomst-proceedings.helsinki.fi/papers/2019_19_24.pdf (Extended Abstract)
- 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. Proceeding of 64th International Congress of Meat Science and Technology (ICoMST) 2018, 04-09th August 2018, Melbourne, Australia. (Extended abstract)
- Jayawardena, R., Morton, J. D., Brennan, C., Bhat, Z. F., & Bekhit, A. E. D. A. (2019). Effect of drying temperature on the quality and in vitro protein digestion of beef-lung protein powder. Proceedings of 5th International Conference on Food Structures, Digestion and Health 2019, 30th September to 3rd October 2019, Rotorua, New Zealand (Abstract)

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. 65th International Congress of Meat Science and Technology (ICoMST) 2019, 4-9th August 2019, Berlin, Germany (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, 2-4th July 2019, Christchurch, New Zealand (Poster presentation)

- Jayawardena, R., Morton, J. D., Brennan, C., Bhat, Z. F., & Bekhit, A. E. D. A. (2019). Effect of drying temperature on the quality and in vitro protein digestion of beef-lung protein powder 5th International Conference on Food Structures, Digestion and Health 2019, 30th September to 3rd October 2019, Rotorua, New Zealand (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 to enhance protein and iron content of plant-based diets. 64th International Congress of Meat Science and Technology (ICoMST) 2018, 12-17th 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, 20-21st & 24th September 2018, Lincoln University, New Zealand (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 to enhance protein and iron content of plant-based diets. Lincoln University Post-graduate Conference-2018, 20-21st & 24th September 2018, Lincoln University, New Zealand (Oral presentation).

Awards

- Best poster presentation award, NZIFST conference 2019, 02-04th July 2019, Christchurch, New Zealand.

- Lincoln University travel grant 2018 to attend ICOMST-2018 at Melbourne, Australia.

- Best poster presentation award in Lincoln University Postgraduate Conference-2018, 20-21st & 24th September 2018, Lincoln University, New Zealand.

Supervisory team

Associate Professor James David Morton was the principal supervisor and Professor Charles Brennan was the associate supervisor and Associate Professor 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.

The Potential of Beef Lungs as a Protein Ingredient and Peptide Source

by

Reshan Jayawardena

Beef lungs are an underutilised co-product of the meat industry that could be used as an ingredient to supplement the protein content of cereal foods. Incorporating beef lung into widely consumed food can improve the protein quality of the food chain. Air oven drying was used to convert raw beef lungs to beef lung powder (BLP). Beef lungs were dried at 60 ˚C for 32 hours and ground into beef lung powder (BLP). BLP was incorporated into the pasta as a model food for delivery of macro and micronutrients. BLP had 87% protein content (dry weight basis) with a rich essential amino acid profile and contained 1 mg/g iron. Fresh semolina pasta was used as a model food, and BLP could be incorporated at up to 20% based on preliminary trials. Incorporation of 10% BLP improved the indispensable amino acid score (IAAS) of the pasta from 0.48 to 0.91, Pasta with 10% BLP showed better textural, colour and cooking characteristics compared to those where BLP was incorporated at 15% and 20% ($P < 0.05$). Incorporation of BLP into the pasta also significantly ($P < 0.05$) lowered the predicted glycaemic response. Therefore, the inclusion of BLP improved the nutritional value of a starchy food and is a potential ingredient in the development of new food products.

Further research was focused on reducing drying time and temperature to lower the cost of production and increase the nutrient content. Raw beef lungs were minced and dried for 23 hours at 50 ˚C, 11 hours at 70 ˚C or 6 hours at 100 ˚C. The resulting BLP powders were analysed for physicochemical and nutritional properties. All of the powders were able to absorb three times their weight in water. The BLP dried at 50 °C preserved 40% of the total iron as haem iron, which has a higher bioavailability, but this significantly decreased to 29% when the lungs were dried at 100°C. All the powders dried at50, 70

and 100 °C were within microbial safety limits for total viable bacterial count, yeast and mould and there was no evidence of coliform bacteria after six months of storage. The higher quality of BLP dried at 50 °C was confirmed by low levels of lipid (0.51 MDA mg/Kg sample) and protein (8.6 nmol carbonyl /mg protein) oxidation after six months of storage.

The bioaccessibility of the nutrients within BLP dried at different temperatures was assessed using a simulation of human digestion. The dried beef lungs at 50°C had a 96.3% *in vitro* protein digestibility (IVPD). This was significantly decreased 95.8% with drying at 70 °C. *In vitro* bioaccessibility of copper (Cu), iron (Fe) and sulphur(S) also significantly decreased as drying temperature increased. Bovine lung dried at 50°C could provide highly bioaccessible proteins and minerals for the human body.

Local meat processing plants were unable to provide edible beef lungs while meeting stringent export requirements. This prevented conducting any sensory analysis and would make commercialisation of a food supplement from bovine lungs difficult. As a result, the potential for using lungs for the production of bioactive peptides was investigated. The protein in raw beef lungs was digested and the resulting peptides analysed for angiotensin-1 converting enzyme (ACE) inhibitory activity. Kiwifruit extract and the commercial fungal proteases, FP31K and FP60K were used to produce beef lung hydrolysates. Active fractions were purified, and the amino acid sequence of the peptides were analysed by mass spectrometry. The highest ACE inhibitory activity was identified in FP31K enzymatic hydrolysates, and the peptides in this fraction had an IC_{50} of 24 µg/mL for ACE inhibition. The novel peptide sequence "Val-Ser-Pro-Gly-Met-Pro" was identified as a possible ACE inhibitory peptide. Peptide analysis revealed collagen and elastin as the dominant proteins in beef lung hydrolysates.

This study produced economical and nutritious protein powder from beef lungs. This nutritious BLP could be used to supplement starchy food protein and possibly help eliminate world malnutrition, if the regulatory obstacles could be overcome. Meanwhile, the raw beef lungs are a good source for producing ACE inhibitory peptides. If the meat industry could use lungs in pharmaceutical production of ACE inhibitors and other peptides, it would increase profit margins from this underused coproduct.

Keywords: Beef lung, air oven drying, protein nutrition, indispensable amino acid score (IAAS), iron content, glycaemic response, microbial food safety, lipid oxidation, protein oxidation, bioaccessibility, bioactive peptide, angiotensin-1 converting enzymes (ACE), inhibitors, amino acid, peptide sequence.

Acknowledgements

I express my sincere appreciation to those who have contributed to this thesis and supported me in one way or another during this amazing journey for, without any of them, this research work would not have been possible.

I would like to begin by thanking my big supervisory panel! It was a privilege to work with the three best and most renowned food scientists in the world. Firstly, I would like to express my sincere gratitude to main supervisor Associate Professor James D. Morton for his patience, time, guidance, knowledge and advice. I am fortunate to have him as my major supervisor and words are inadequate to express my sincere and deepest feelings. Thank you Sir, for your valuable time, co-operation, and generosity which made this work possible. I am extremely grateful to my co-supervisors, Professor Charles Brennan and Associate Professor Alaa El-Din A. Bekhit for their invaluable guidance, encouragement and academic stimulus. Both of you provided valuable feedback on the development of manuscripts and other publications and directed me through research-related questions and ideas. Further, I would like to express my sincere thanks to Professor Charles Brennan, for accepting me as a PhD student at Lincoln University, New Zealand, in first place.

I duly acknowledge Dr. Susan L. Mason for assisting and guiding me through research progress evaluation.

A special note of thanks should be given to Dr. Santanu Deb-choudhury for all technical assistance and expertise of amino acid sequencing works in AgResearch Lincoln.

I owe a great deal of thanks to the commercialisation manager Jonathan Cox for helping this research on behalf of ANZCO foods, New Zealand

I would like to give my warm gratitude to all the technical staff at the Lincoln University for timely and sincere help during the entire study. I specially thank laboratory manager Karl Gately for continued assistance and patience throughout my laboratory work. Further I would like to thank Leo Vanhanen, Letitia Stipkovits, Richard Hider for sharing their technical experience.

I express my appreciation to Dr. Luca Serventi for sharing his expert knowledge of food processing and at the same time, I express my gratitude to Dr. Venkata Chelikani and Omega Amoafo for helping me with food microbiology work and sharing their expert knowledge.

I want to express my gratitude to Professor Don Kulasiri and Professor Ravi Goonaratne for their psychological and moral support during the times when I required it the most. Additionally, I really want to acknowledge Caitriona Cameron (Head of Department: Academic and Career Skills) for personal guidance and advice to structure the thesis at the final stage of my PhD.

All former and current fellow students of Lincoln University have played an important role in my journey as a PhD fellow and I would like to thank them personally. I cannot find words to express my emotions towards my mature friend Dr. Nadir Saleeb for your immense kindness and support during

the life in New Zealand. I express my whole-hearted endearment to my friend Dr. Zuhaib Bhat for being a best friend at Lincoln University. I was really lucky to have you as my lab partner and my words fail to describe your psychological and moral support. Further, I record my sincere thanks to my colleagues Tirthankar Ghosh, Ajey, Sofnil, Klunklin, Meweal, Olivia, Ade, Nadeesha, Pradeep, Claire, Lokesh, Titon, Emily, Madhuri and all friends.

I would like to particularly express my sincere gratitude to the Sri Lankan community in New Zealand. I would like to extend my deepest gratitude to Aunty Celin, Uncle Rexi and their loving son and daughter for great care, hospitality and support in numerous ways. I express my overwhelming thanks to Thilak Aiya, Thiloka Akka and their loving sons for providing me accommodation and great caring. A special note of thanks should be given to my friend Samadhi Suraweera for endless support and hosting me in my very first days in New Zealand.

I would also like to acknowledge all my lecturers from the University of Peradeniya, Sri Lanka and Postgraduate Institute of Agriculture (PGIA), Sri Lanka who have supported and encouraged me ever since. Further, I am indebted to all my teachers in Sri Lanka who nurtured good attitudes, knowledge and skills.

I am extremely grateful to all my good old Sri Lankan friends, BFST group (especially Dimuthu Gunasekara, Prasad Wijekoon, Amila Gunasekara, Ravindu Mohindra, Sunanda Bandara and Amani Prasandika). My heartful thank goes to Dr.Geeshani Somarathne for inspiring me to start my PhD in New Zealand and helping me in numerous ways.

I would like to express my immense gratitude to my own loving elder brother (Thenuka Jayawardena) for playing the role of second dad in my life. Words fail to describe your support during my entire education journey. At the same time, I want to express my affection to sister-in-law (Ayeshani Abeywardena) and my nephew (Hiruka Jayawardena).

My deepest thanks also go to my Aunty (Nayana Ekanayake) and Uncle (Lakshman Jayawardena) in Canada who helped me in numerous ways during various stages of my PhD.

No words or phrases can convey my exact feelings towards my life partner (Harshani Withana) who took all responsibilities on her shoulders to support my PhD. At the same time, I want to acknowledge my father-in-law and mother-in-law for their endless affection and support throughout my study. Further, I cannot forget the support of two sisters (Sithumini Withana, Chinthanee Withana) and their families.

Finally, my whole-hearted gratitude goes to my adorable parents for their sacrifice, prayers and blessings. They have been living every single minute for my brother and me. With the heartiest reverence, I admire the confidence bestowed on me by my beloved Amma (Mom) and Appachchi (Dad). Thank you for teaching me that all good things are possible. Whatever I am today, I owe it to you.

Reshan Jayawardena

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Meat plays a major role in the human diet to fulfil essential nutritional requirements by providing quality protein. Based on the amino acid profile, animal protein can supply complete protein with high biological value and bioavailability. The global demand for meat is growing continuously, and expansion of production is required to meet this demand.

The expansion of meat production, and in particular beef, has been associated with environmental concerns. For instance, ruminants used 3.7 billion tonnes of the 4.7 billion tonnes feed biomass consumed by livestock globally in 2000 (Herrero, Havlík, Valin, Notenbaert, Rufino, Thornton, et al., 2013). Further, it is estimated that they are responsible for a large portion of the total livestock non-CO_2 greenhouse gas emission (Herrero, Havlík, Valin, Notenbaert, Rufino, & Thornton, 2013). Beef is 82 % of the meat produced from ruminants (FAOSTAT, 2018) and the beef industry is held responsible for the greatest environmental impact (Leonard, 2011). It is critical that the beef industry tries to increase the efficiency of production rather than just increase the number of animals.

The beef carcass is about 45% to 60% of live animal weight (Albertí et al., 2008) and the rest is either considered as waste or co-products. Better utilisation of these co-products could significantly increase the production efficiency (Lynch, Álvarez, O'Neill, Keenan, & Mullen, 2018; Mullen et al., 2017) and preserve a greater portion of the animal protein within the human food chain. However, co-product consumption is a culturally bound food habit in certain communities and much less popular than muscle meat. Beef lung is an underutilised co-product due to the poor aesthetic characteristics. Processing of beef lungs could modify the textural characteristics and possibly allow it to be incorporated into widely consumed foods to deliver protein globally. The lungs in adult cattle weigh around 3 kg and a considerable amount of protein could be preserved by bringing them on to the consumer's food plate. High meat-producing countries like New Zealand do not use this nutritious organ as edible food and it is used as pet food or a component of low value meat and bone meal.

The major meat producing countries typically have a low demand for co-products, while other parts of the world suffer from protein deficiency. These latter countries always struggle to fulfil their energy requirement rather than focusing on nutrition requirements. The World Food Program's hunger map (WFP, 2019) illustrated severely malnourished countries, where more than 35% of their population is undernourished. Therefore, they are more prone to have protein-energy malnutrition. The income

level of these hunger-stricken countries is at the bottom level of developing countries and they are unable to purchase expensive animal meat. Globally, the price of meat is higher than plant commodities, and there is an obvious purchasing problem for low income countries to obtain high quality protein. Having low price animal protein affordable could help fulfil their protein requirement. Beef lungs are underutilised co-product of the meat industry that has enormous potential as a protein supplement. So, there is an opportunity to bridge the research gap by utilising the accumulating beef lungs in higher meat producing countries and at the same time, fulfil the nutrition requirement of malnourished communities. This research focuses on producing a low cost, nutritious and safe protein supplement from lungs, with a long shelf life without requiring refrigeration.

There are a number of studies and reviews on beef co-products and beef lungs (Darine, Christophe, & Gholamreza, 2010, 2011; Lynch et al., 2018; O'Sullivan, Lafarga, Hayes, & O'Brien, 2017; Tridente, De Martino, & De Luca, 2019) and a few on processing of beef lungs for human consumption (Cardoso-Santiago & Arêas, 2001a; Cardoso-Santiago & Arêas, 2001b; Chávez-Jáuregui, Cardoso-Santiago, Silva, & Arêas, 2003). To the author's knowledge there are no reported research on converting beef lung into a food ingredient or for the production of ACE inhibitory peptides.

This PhD thesis is aimed to achieve four objectives and the thesis is structured accordingly

- To convert beef lung into a food ingredient and incorporate it into a starchy food (pasta) as a nutrition vehicle.
- To improve the processing conditions of this food ingredient to produce an economical and nutritious product with a long shelf life
- To determine the accessibility of nutrients from beef lung powder to the human body using an *in vitro* digestion product
- To determine the possibility of developing bioactive products as an alternative use of beef lung.

1.1 Thesis structure

Including this introduction, the thesis contains seven chapters. Chapter 2 is the literature review which gives background information upon which this project is based. Chapters 3 to 6 are experimental chapters prepared in journal paper format. Chapter 7 provides overall conclusions perceived from the project, followed by future work and a complete list of references. There is no separate methodology chapter to limit repetition of methods throughout the thesis, and methods are appropriately included in each experimental chapter. Figure 1.1 details the main idea of the experimental chapters and how they relate to each other.

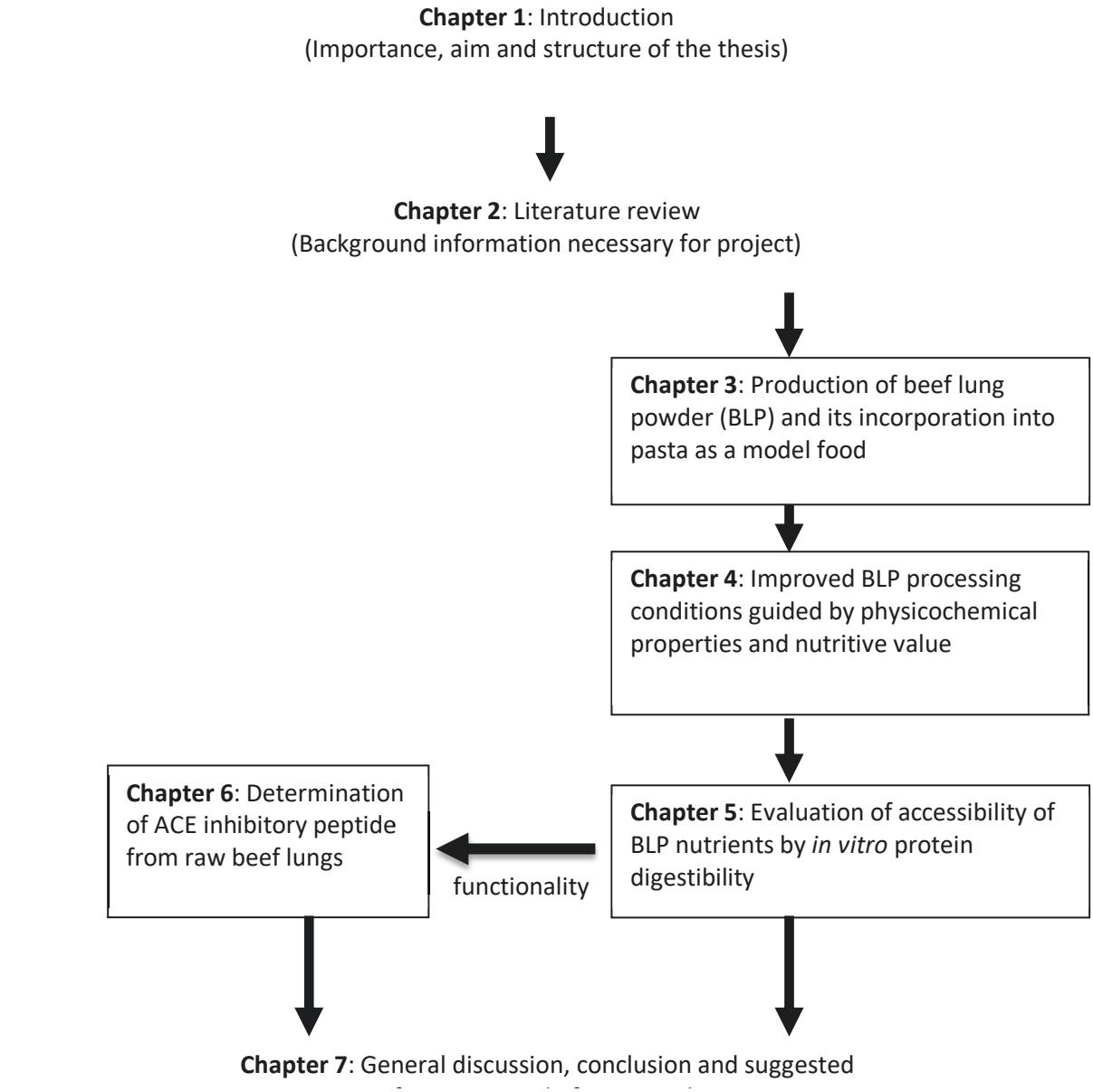


Figure 1.1 Flow chart of the thesis structure

Chapter 2

Literature Review

The global beef industry has grown due to increased demand for high quality protein and its sensory attributes. However, beef production is under pressure globally due to environmental concerns. Generally, 60% of live weight is recovered as beef carcass, and the remaining 40% is edible or inedible coproducts. It is vital to utilise this fraction efficiently to maximize profitability, utilize resources efficiently and reduce pressure on environment. Among the edible coproducts, beef lungs are a commonly underutilised organ, especially in meat-producing countries, with an average weight of nearly 3 Kg in adult cattle. Edible offal consumption depends on cultural behaviour (Toldrá, Aristoy, Mora, & Reig, 2012) and bovine lung consumption is not popular in western countries. Due to regulatory concerns and low demand for lungs, most of them are eliminated from the human food chain and used to produce meat and bone meals (MBM) by the rendering process.

Even though, affluent individuals in some countries eat offal due to cultural reasons and pay premium value, most Asian and African communities are accustomed to and prefer offal since they are cheaper and more accessible than expensive animal meat. This is especially true in hunger-stricken countries that suffer from nutritional deficiencies which can be eliminated by serving nutritious cheap food options such as offal. Beef offal, including lungs, possibly can be converted into protein ingredients and incorporated in food products. This approach can provide extra economical value for beef producing countries while serving animal proteins in a functional way with affordable price for hunger-stricken countries. Alternatively, the bioactive properties of offal could be used for the pharmaceutical industry to gain high profit margins.

2.1 Global and local meat production

Globally pork, chicken and beef are the dominant meat industries and global production reached 113 million tonnes of pork, 95 million tonnes of chicken and 63 million tonnes of beef & veal in 2018 (USDA-FAS, 2018)**.** Focusing on beef production, in 2018 the United States produced the highest volume of beef in the world at 12.3 million tonnes but its domestic consumption in the years from 2014 to 2016 was slightly higher (USDA-FAS, 2017, 2018). Thus, the United States is the largest beef importer in the world, followed by China, Japan, Hong Kong and Korea. The major beef exporters are Brazil, India, Australia, United States and New Zealand. For New Zealand, meat exports are second only to dairy. New Zealand produced 1.39 million tonnes of meat in 2017 and exported 0.99 million tonnes of that

(FAOSTAT, 2018). Beef is the largest meat production in New Zealand by weight and 0.64 million tonnes of beef was produced in 2017 (MIA, 2018). New Zealand domestic beef consumption was less than 0.1 million tonnes in 2017, due to its small population (5.0 million) and it exported 0.56 million tonnes of beef (FAOSTAT, 2018). New Zealand exported beef to 76 countries and its largest export market was the USA.

World meat consumption has increased continuously during the last two decades. Global meat consumption increased from 204 million tonnes in 1997 to 313 million tonnes in 2017 with the increasing population and increased individual consumption from 35.33 to 42.57 kg/ capita/year (FAOSTAT, 2018). Meat industries have continuously increased production to meet the rising demand. Using a larger proportion of each animal is a possible alternative to slaughtering more animals. The beef carcass weight is only 45% to 60% of animal live weight (Albertí et al., 2008) and the rest is either considered as edible coproducts or inedible portions. These are a potential source of products in either the food or pharmaceutical industry (García & Manrique, 2018).

2.2 Global coproduct market

The increase in meat production means that there is also a parallel increase in potential coproducts. Identification and quantification of global coproducts and their wastage are beneficial for the purpose of evaluating their potential and applications. The current literature does not distinguish the animal source of coproducts and wastage clearly. FAO statistics in 2019 provides the world production of coproducts but it does not differentiate particular coproducts or species. USDA data provided livestock statistics until mid of 2019 and forecast for 2020 but offal statistics were not available. Local meat production in New Zealand was reported by Meat Industry Association (MIA) annual report but it does not differentiate the edible offal figures according to animal species.

The importation and export of edible offal increased rapidly from 1993 to 2013. Both import and export quantities of edible offal were around 1 M tonnes in 1993, and had grown to 4.9 M tonnes exported and 3.8 M tonnes imported in 2013. World total edible offal exports for different regional markets were as follows; 52.8%- Europe, 30.7%-America, 10.7%-Asia, 5.4%-Oceania and 0.4%-Africa; importation was reported as 48%-Asia, 31.3% Europe, 10.3%-Africa, 10.2%-America and 0.1% Oceania in 2013. In 2017, Edible offal Import-export market, values and quantities were derived by region from the FAOSTAT (2018) database (Table 2.1). Global total edible offal export quantity was reported as 5,798,582 tonnes with market value of 9,957,040 thousand (*1000) US$ in 2017 and average offal value was 0.6 US$/Kg with including edible offal of cattle, sheep, pig, goat and liver of chicken, duck and gees. According to FAOSTAT (2016) data base, edible pig offal is the most highly demanded edible offal market in the world. Germany exported the largest quantity of pig offal in 2016 (668632 tonnes)

and highest total quantity was imported by China-Mainland (1.35 million tonnes). The top five importers of beef and offal were Hong Kong China, Egypt, Vietnam, Russian Federation and Japan. Hong Kong imported 272502 tonnes of cattle offal and was the largest importer in 2016 (FAOSTAT, 2016) and USA supplied 280805 tonnes of cattle edible offal as largest supplier followed by Australia, Brazil, Ireland and Argentina.

Table 2.1 World edible offal market importation and exportation by region (2017)

Export quantity of edible offal (tonnes)							
Region	**Cattle**	**Chicken**	**Duck**	**Geese**	**Pigs**	**Sheep**	**Total (%)**
Africa	9,700	7,172	72	33	462	1,331	0.3
Americas	646,167	1,023	30	58	777,091	720	24.6
Asia	135,656	4,023	57	185	558,692	6,183	12.2
Europe	528,540	738,665	14,092	2,114	2,090,960	22,187	58.6
Oceania	192,485	NA	91	NA	6,641	54,151	4.4
World	1,512,548	750,883	14,343	2,389	3,433,847	84,572	100
Export value of edible offal (1000 US$)							
Africa	16,441	6,301	113	48	754	2,942	0.3
Americas	1,682,359	1,264	371	212	1,274,769	1,832	29.7
Asia	322,449	10,800	182	412	1,386,496	16,006	17.4
Europe	1,026,788	640,543	133,865	19,100	2,632,030	40,590	45.1
Oceania	614,512	NA	112	NA	8,842	116,905	7.4
World	3,662,550	658,907	134,644	19,774	5,302,890	178,275	100
Import quantity of edible offal (tonnes)							
Africa	506,929	60,650	93	134	97,920	9,313	13.4
Americas	164,841	32,110	20	94	263,385	5,184	9.2
Asia	584,618	54,048	320	1,122	2,043,856	43,608	54.0
Europe	262,533	298,395	8,963	1,559	578,756	19,238	23.2
Oceania	7,253	30	30	0	1,619	2,914	0.2
World	1,526,174	445,232	9,426	2,908	2,985,536	80,259	100
Import value of edible offal (1000 US$)							
Africa	530,739	54,089	301	229	83,234	10,665	7.6
Americas	510,140	38,622	295	431	310,764	10,687	9.7
Asia	2,312,777	61,212	7,713	18,425	3,526,791	105,649	67.5
Europe	541,894	188,248	125,300	18,832	412,578	41,715	14.9
Oceania	13,814	59	809	2	2,522	7,044	0.3
World	3,909,364	342,230	134,418	37,919	4,335,888	175,760	100

*NA= Data not available

Edible offal can also include material traded for meat and bone meal (MBM) or animal feed. These underutilised coproducts produce a very low-price margin. The identification of underutilised edible coproducts from edible coproducts is important to increase production efficiency. The general categorising of underutilising coproducts and popular coproducts depends on the preference of the communities. The preference of the coproducts and their usage are entirely affected by the culture and country (Tenrisanna, 2015). However, liver, heart, spleen, pancreas, thymus coproducts are accepted by most communities. Liver is the most popular coproduct and UN FAO separately categorised liver in FAOSTAT (2018). Thymus and pancreas are used as sweetbread in culinary dishes (Awan et al., 2015).

Bovine lungs are large organs and generally rejected by consumers from Oceania and American region, hence they are regarded as an underutilised coproduct. The USA bans bovine lungs from entering the human consumption food chain (FSIS, 2015) due to possible contamination from the slaughterhouse. The USA is a major meat importer and these restrictions influences global meat industries who follow USA regulations. But the European Union (EC) has clearly established rules to obtain lungs and allowed their use for human consumption (EC 854/2004). Following well established EC regulations and redesign of processing flow may help to process offal without any clash between regulations.

2.3 Local coproducts market in New Zealand

Coproducts increase the returns from animal processing and contribute nearly 11% of the value of a slaughtered animal (Meat and Livestock Australia, 2014). Edible and inedible offal together contributed $1.4 billion in exports to the New Zealand economy in 2015 (MIA, 2015). New Zealand edible offal export market provided $211 million for 66401 tonnes in 2015. This was an average value of $ 3.10 per kilogram and was mainly exported to China, Japan, United Kingdom, Korea and Russia. Among coproducts, MBM has the highest production because industries usually underutilised coproducts like lungs put into rendering process to produce MBM. In 2015, the export value of MBM was twice as high as that of other edible offal, but edible offal export economic value higher than MBM (Figure 2.1). Beef lungs belong to edible offal according to MIA categorisation. But the USA is a key export market for New Zealand beef and they prohibit the sale of beef lung. This makes it very difficult for New Zealand meat processing premises to provide edible grade lung while meeting the strict USA importing requirements. If, however, it was able to remain in an edible form, it could provide better economic value. Locally, beef is a key meat industry in New Zealand and nearly 2.6 million cattle were slaughtered in 2015 (BLN, 2017). This high production of beef also leads to a large production of

offal including beef lungs. If 3kg is assumed to be the average weight of lung per animal (Cardoso-Santiago, Moreira-Araújo, Pinto e Silva, & Arêas, 2001) then the new Zealand beef industry would provide about 7800 tonnes of bovine lungs per annum. With this large supply of raw material, there is scope to add value and expand the export income.

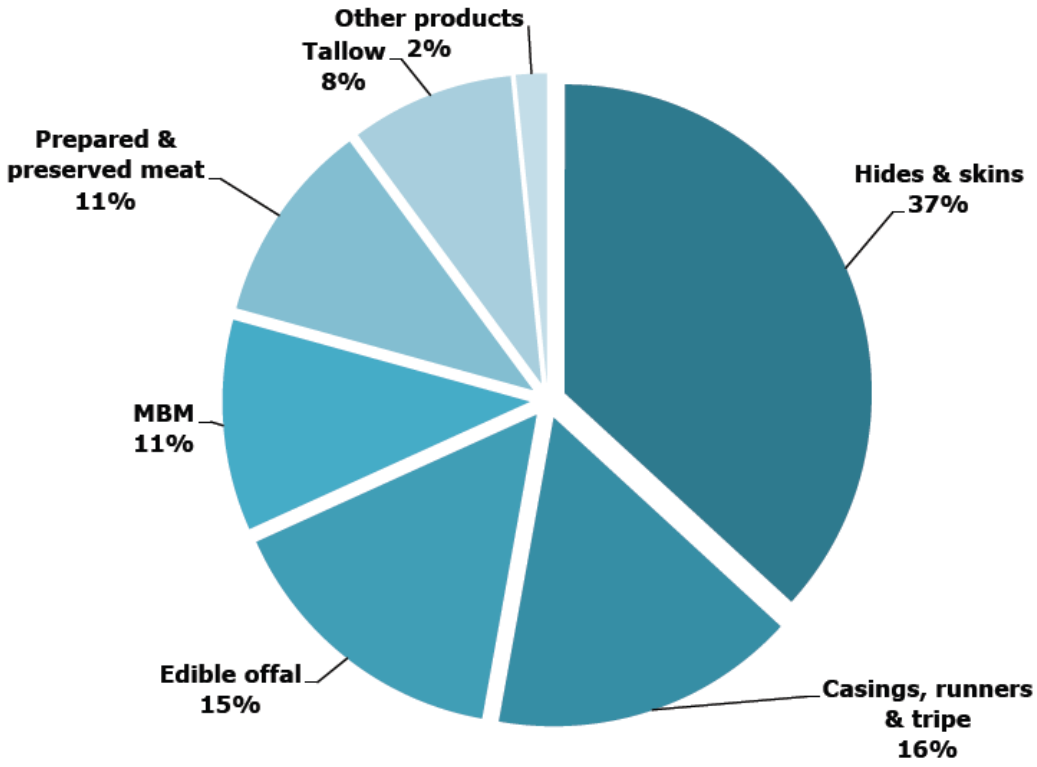


Figure 2.1 Animal coproducts exported by New Zealand in 2015 (Source: MIA annual report 2015)

2.4 Beef lungs

Bovine lungs have the structure of the general mammalian pulmonary system (Prohl, Ostermann, Lohr, & Reinhold, 2014). There are two compartments in the left lung and four compartments in the right lung (Prohl et al., 2014). These two lungs are connected by a windpipe or trachea and together they make up the bovine pulmonary system. Bovine lungs are a large internal organ with an average weight around 3Kg and are rich in essential nutrients and functional proteins (Cardoso-Santiago & Arêas, 2001b; Cardoso-Santiago et al., 2001).

The composition of fresh bovine lung is 16.2 % protein, 2.5 % fat, 0.98% ash and 79.4% water (USDA, 2020). Protein is the dominant macronutrient in bovine lungs and comprises 25% collagen

(Lynch et al., 2018) and 13.5% to 28% elastin (w/w defatted bovine lung) (Francis & Thomas, 1975a). Essential amino acids exceed 40% of the total amino acids in bovine lung protein, according to Fornias (1996). Our recent research demonstrated that dried beef lung powder has the maximum indispensable amino acid score (1) as a complete protein which matches the requirement for optimal nutrition status of older-children, adolescents and adults (Jayawardena, Morton, Brennan, & Bekhit, 2019a).

2.4.1 Legislations towards bovine lungs

Clear legislation for edible offal, and especially for the bovine lungs, are observed in the European Union. Bovine lungs are subject to current legal requirements, Europe food regulations (EC 854/2004) covers bovine lungs with the antemortem and postmortem inspections. An official veterinarian carries out an antemortem inspection of all animals before 24 hours post slaughter, and particular attention is to be paid to identify zoonotic diseases and listed diseases of World Organisation for Animal Health-OIE (OIE, 2020). The trachea and the main branches of the bronchi must be opened lengthwise, and the lungs must be incised in their posterior third, perpendicular to their main axes. Further, bronchial lymph nodes are incised and examined by the veterinarian. These incisions of lungs are necessary for the inspection of lungs for human consumption according to the Europe food regulations (EC 854/2004). Apart from this, all hygienic rules of offal obtaining process must meet the Europe food regulations (EC 853/2004).

New Zealand and Australia together have launched the food standards under the act of Food Standards Australia New Zealand in 1991. Food standard section 1.1.2-3 defined the meat offal as rest of the carcass excluding the flesh, bone and bone marrow. According to the definition, bovine lung belongs to the edible offal. However, the definition has included popular coproducts, blood, brain, heart, kidney, liver, pancreas, spleen, thymus, tongue and tripe as examples. Under the food standard schedule-22, edible tissues and organs other than muscles and animal fat from slaughtered animals belongs to the category of mammalian edible offal which can be prepared for wholesale or retail distribution. There is no specific regulation for bovine lungs in New Zealand and Australian legislation, and it should be developed more extensively and specifically for the edible offal that are destined to local markets.

There is no cultural practice among New Zealand local communities to eat beef lungs, and they are added to low-value MBM or pet-food. Establishing new regulations and smoothing the processing flow is important to produce beef lungs while producing meat for the US market without shaking the food regulations. New Zealand and other higher beef production countries have the ability to produce high quantities of protein rich beef lungs, while other parts of the world suffer from protein-energy malnutrition due to animal protein deficit (FAO, 2019b).

2.5 Low income countries have low meat consumption

World Food Program's hunger map (WFP, 2019) illustrated the world hunger-stricken countries, and they are Zambia, Malawi, Zimbabwe, Republic of Congo, Central African Republic, Chad, Uganda, Rwanda, Liberia, Yemen, Madagascar, Democratic People's Republic of Korea and Haiti. Unfortunately, world hunger increased three years in a row from 2016, and the rise in food prices has caused this hunger shock (FAO, 2019b). According to the world bank explanation, these hunger-stricken countries belong to the bottom of the developing countries. The hunger in the developing world results from an economic crisis and they need for any affordable food. The high price of animal protein causes this problem and the average per capita consumption of meat was reported around 25 Kg/year in developing countries. In contrast, in developed countries, per capita consumption was greater than 80 Kg/year (FAO, 2019a). The hunger-stricken countries are mostly in the African region and they have the least purchasing ability. Table 2.2 illustrate the income level and meat consumption of the highest meat consuming countries and hunger-stricken countries. The lowest income level of the high meat consuming countries was reported as 9812 USD/capita/year in Brazil, and the highest income level of hunger-stricken countries reported as 1513 USD/capita/year in Zambia. All other hunger-stricken countries remain below than 1500 USD/capita/year.

So, the excess production of beef lungs in New Zealand could easily be absorbed by these hunger-stricken countries after processing into a food ingredient. The processing of beef lung is important to convert it into an aesthetically appealing food because generally beef lungs are rejected from the consumer due to poor texture qualities (Chávez-Jáuregui et al., 2003). The processing of beef lungs into powders could modify this texture modification and has the potential to penetrate the high value protein market. The processing of beef lungs should modify the texture characteristics, preserve the nutritional value, have cost-effective process to deliver economical product and have a long shelf life without requiring a cold chain. Our project focused on preparing dried beef lung powder to achieve all these aspects to bridge the gap of a real-world problem. Further beef lung powder was used as a food ingredient for pasta as model food which is widely consumed and could possibly act as a vehicle of nutrients. This project creates an opportunity to sell bovine lungs for large scale beef producing countries and the same time fill the nutrient requirement of hunger-stricken countries

Local industries need to redesign their processing flow to obtaining edible beef lungs for these achievements. Due to the obstacles to producing edible beef lungs within current regulations, the project also focused on the production of a bioactive peptide, which could have a high economic value in the pharmaceutical industry. Peptides with ACE inhibitory activity were successfully obtained during the final project and may be an alternative pathway for meat industries to use beef lungs.

Table 2.2 GDP (PPP) comparison with highly meat consuming countries and hunger-stricken countries

Highest meat consuming countries		
Country	**GDP per capita-2017 (USD)**	**Meat consumption-2017 (Kg/capita/year)**
Hongkong	46394	137
USA	60160	124
Australia	57917	122
Argentina	14517	109
Macao	81211	103
New Zealand	43651	101
Spain	28316	100
Brazil	9812	100
Israel	42450	97
Soma	NA	97
Hunger-stricken countries		
Country	**GDP per capita-2017 (USD)**	**Meat consumption-2017 (Kg/capita/year)**
Central African Republic	428	35.9
Chad	698	26.4
Malawi	341	20.3
Liberia	453	18.27
Zimbabwe	1333	17.63
Haiti	776	17.16
Yemen	855	16.86
North Korea	NA	13.67
Zambia	1513	13.12
Madagascar	515	12.34
Uganda	634	9.69
Rwanda	748	9.08
Republic of Congo	462	NA

NA=data is not available
Data derived by FAOSTAT (2018)

2.6 Coproducts for world malnutrition

Half of the world children's deaths are contributed to Protein Energy Malnutrition (PEM) in developing countries (Haddad et al., 2015). According to the estimation of the World Health Organisation (WHO) in 2000, 32% of under-five children are malnourished in developing countries. The latest report of FAO (2019b) reported that one in nine people faces hunger in the world, and Africa is the highest hunger prevalence region as 1 in 5 people are hungry. World Food Program's hunger map (WFP, 2019) illustrated severely malnourished countries, where more than 35% of their population is

undernourished. Macro and micronutrient deficiencies are apparent in those groups, and they are more prone to have protein-energy malnutrition.

Two severe malnutrition syndromes in children are marasmus and kwashiorkor. According to Walton and Allen (2011), those suffering from marasmus have severe muscle wasting and minimal adipose tissues. A key symptom of kwashiorkor is nutritional oedema which swelling of body tissues in legs and arms by accumulating body fluid. Lack of protein in the diet causes this problem by lowering the plasma albumin concentration and reducing the osmotic pressure leads to fluid accumulation in tissues. A lack of antioxidants also contributes to oedema as damage to the cell membranes from free radicals causes an increase in vascular permeability (Scallan, Huxley, & Korthuis, 2010).

Treatment for severely malnourished children begins with ready to use therapeutic food (RUTF). These RUTFs are high energy and protein-enriched food with other micronutrients. Severely malnourished children are not given iron (Fe) rich foods to prevent the production of free radicals. Macronutrients, including iron, can be provided from 2 to 6 weeks during the rehabilitation period (WHO, 2013). Coproducts like lung, heart, kidney are rich in highly bio-available proteins, and lungs and spleen contain a high amount of iron (Bester, Schonfeldt, Pretorius, & Hall, 2018; Perignon, Barré, Gazan, Amiot, & Darmon, 2018). So these coproducts could provide the macronutrients and protein at low cost to use in rehabilitation period (Walton & Allen, 2011).

2.6.1 Iron deficiency anaemia

Anaemia is a Greek word which means "without blood", describing the low levels of red blood cells containing haemoglobin. Haemoglobin is a protein with a tightly bound iron-containing haem group. The haemoglobin in red blood cells is responsible for transporting oxygen from the lungs and return carbon dioxide to the lungs.

All mammals, including humans, have advanced mechanisms to regulate blood iron concentration. Body iron regulation proceeds with the help of iron stores in the body, mostly in the liver, bone marrow and spleen. A low protein diet decreases the formation of red blood cells and depletes iron reserves Borelli et al. (2007). This phenomenon shows the relationship between protein-energy malnutrition and anaemia.

Anaemia is the most abundant nutrition deficiency amongst the malnourished and highly related to low protein intake (Bernát, 1983; Fitzsimons & Brock, 2001). The World Health Organization (WHO) definition of anaemia is based on haemoglobin (Hb) concentration in the blood, and limits are established as <13 g Hb/dL for men and <12 g Hb/dL for women. Levels above 10.0 g Hb/dL are classified as mild grade anaemia (Wilson, Reyes, & Ofman, 2004).

A WHO survey from 1993 to 2005 reported that 25% of the world population was anaemic, and anaemia was the most abundant global nutritional disorder (De Benoist, Cogswell, Egli, & McLean, 2008). Further, preschool children, pregnant women and non-pregnant women were reported as highly anaemic prevalence groups, and 47.4%, 41.8% and 30.2% were anaemic respectively. Anaemia is mainly centred in Africa and South-East Asia and higher in all the countries which are suffering from malnutrition.

Many coproducts contain significant levels of iron, many other micronutrients and low level of fat content compared with muscle tissues (Table 2.3). The recent report of Biel, Czerniawska-Piątkowska, and Kowalczyk (2019) also showed lung, liver, heart and kidney meat coproducts that contained a low amount of fat, and a high amount of iron content compared with the semitendinosus muscle. According to Chung, Kim, and Han (2011), mice intervention studies revealed that high-fat diet impaired iron metabolism and diminished the intestinal iron intake. High-fat diet elevates the hepcidin level in the body, which regulates iron metabolism and restricts the absorption of iron. There is a possibility to produce healthy low-fat food with high micronutrient availability from underutilised coproducts.

2.6.2 Other minerals in coproducts

In addition to iron, other micronutrients play a vital role in human nutrition. The composition of coproducts makes them potentially useful use as mineral supplements. The macro and micronutrient compositions of different offal are shown in Table 2.3 compared with the premium semitendinosus muscle.

Calcium is an essential element in human nutrition and high demand during the growth period, pregnancy and breastfeeding. Calcium is necessary for muscle contraction, building strong bones and teeth, blood clotting, nerve impulse transmission and fluid balance (Piste, Sayaji, & Avinash, 2012). Calcium is highly available in the beef brain, muscle, kidney and lungs (Table 2.3).

Magnesium is essential in aerobic and anaerobic energy generation, and more than 300 enzymes use it as a cofactor (Jahnen-Dechent & Ketteler, 2012). Magnesium deficiency causes electrolyte disturbance, muscle spasms, poor coordination and loss of appetite (Soar et al., 2010). The highest magnesium content of offal was recorded as 22 mg/100g in the bovine spleen while beef lung contains 14 mg/g, and the lowest amount was reported in the bovine brain as 13 mg/g. Magnesium upper intake is 400 mg/day according to Baghurst (2006), and beef offal is unlikely to exceed the safer level.

Zinc and copper metal ions are highly reported in beef liver than other offal. Zinc was reported 4 mg/100g, and copper was available 9.6 mg/100g and bovine lung reached to 1.61 mg/g zinc and 0.26 mg/g copper. Zinc is very important metal iron and deficiencies affect nervous, skeletal, immune and reproduction system. These zinc deficiencies are vastly prevalence in high cereal and low animal food consuming areas (Roohani, Hurrell, Kelishadi, & Schulin, 2013). Copper is responsible for many physiological functions in the human body, such as angiogenesis, regulation of gene expression, brain development and immune system. More than 95% of copper in body plasma interact with ceruloplasmin copper-enzyme, which is involved in iron metabolism (Hellman & Gitlin, 2002).

The functions of selenium mineral overlaps with vitamin E (Strain & Cashman, 2009) and lower intake leads to Keshan disease, which cause bone deformations of children (Hartikainen, 2005). This vital mineral is abundant in the beef kidney, spleen, lung and liver meat than the muscle meat.

Beef lung and beef brain have poor texture, and are the most underutilised coproducts in New Zealand compared with the liver, heart, kidney and spleen. Beef lungs contain magnesium, zinc, copper, selenium like minerals and proteins higher than the bovine brain.

Table 2.3 Comparison of minerals and main macronutrients of beef offal and muscle (for 100g wet tissue)

Elements	**Lung**	**Liver**	**Heart**	**Kidney**	**Tongue**	**Brain**	**Spleen**	**Muscle (semitendinosus)**
Calcium, Ca	10	5	8	13	6.39	43	9	16
Phosphorus, P	224	387	218	255	133	362	296	195
Sodium, Na	198	68	97	185	69	126	85	53
Potassium, K	340	312	285	263	315	274	429	319
Magnesium, Mg	14	18	20	17	16	13	22	24
Iron, Fe	7.95	4.8	4.3	4.6	2.15	2.55	44.55	1.8
Zinc, Zn	1.61	4	1.7	1.95	2.32	1.02	2.11	3.5
Copper, Cu	0.26	9.66	0.395	0.43	0.07	0.2	0.168	0.091
Manganese, Mn	0.019	0.29	0.29	0.14	0.015	0.04	0.073	0.012
Selenium, Se (µg)	44.3	39	21	139	12	7.55	62.2	24
Protein (g)	16.2	20.3	17.25	17.5	19	10.86	18.3	20.7
Fat (g)	2.5	3.6	3.95	3.1	5.75	10.3	3	12.14
Moisture (g)	79.3	70	77	77	77	76	77.2	66

*Data derived by Biel et al. (2019) and USDA Food composition table(USDA, 2019); unit-mg unless stated otherwise

2.6.3 Health effect of protein bio-active protein

Proteins are the source of amino acids, and the amino acid composition varies from protein to protein. Essential amino acids are not synthesised in the human body and taken only from the protein of diet. The human body requires these essential amino acids with different ratios and different quantities for different life stages. The amino acid reference pattern represents these required amino acid ratios for different life stages (FAO, 2011), and this pattern helps to rate the quality of the protein. For instance, older child, adolescent and adult reference pattern consist of tryptophan-6.6, threonine- 25, isoleucine-30, leucine- 61, lysine-48, valine-40, histidine-16, sulphur amino acids-23 and aromatic amino acids-41 mg/g protein (FAO, 2011). The amino acid composition of inferior quality proteins partially matches with these ratios and complete proteins align with this. Proteins are rated using the indispensable amino acid score (IAAS) using reference patterns, and higher amino acid scores refer to complete proteins. In addition to the IAAS, protein digestibility is considered to contribute to the quality of protein, and it is crucial to evaluate the accessibility of amino acids to the human body. Animal-based proteins are rich in essential amino acids than plant-based protein and digestibility of animal protein is more than 90% while plant-based protein digestion remains around 80% (Berrazaga, Micard, Gueugneau, & Walrand, 2019). Animal coproducts, including beef lungs are rich in essential amino acids (Mullen et al., 2017). Our previous work, dried beef lung digestion reported more than 90% digestibility Jayawardena et al. (2019a). So, beef lungs have high potential to provide essential amino acids with high digestibility level as a nutritious food.

In addition to the nutrition supply of protein, recently recognised dietary peptide exerts various other functionalities which play a beneficial modulatory role in human body systems. The different sequence of peptides engages with different bioactive functions in the human body, and these are identified as bioactive peptides. Bioactive substances have been defined as "food component that can affect biological processes or substrates and, hence, have an impact on body function or condition and ultimately health" (Schrezenmeir, Korhonen, Williams, Gill, & Shah, 2000). This definition further refined by the Möller, Scholz-Ahrens, Roos, and Schrezenmeir (2008), the "bioactive" should be a dietary substance which has a measurable biological effect with physiologically feasible level. Further, "bioactivity" measured the health beneficial effect, not the damaging effect. Following the definition, animal protein-derived bioactive peptides are capable of influencing on the human physiological role by working on the positive health condition. In recent years, a number of bioactive peptides has been studied which was originated from animal proteins, but there is a lack of studies for the bioactivity of coproducts.

The most abundant animal protein is collagen and it is the primary constituent of many coproducts, including bone, cartilage and skin (Gómez-Guillén, Giménez, López-Caballero, & Montero,

2011). Collagen contains a poor amino acid profile due to it being composed of non-essential amino acids (Gly, Pro, and Hyp) (Gómez-Guillén et al., 2011). However, researchers have focused on bioactive peptides from the collagen-rich coproducts rather than nutrition supplements. Porcine skin collagen has been hydrolysed by Li, Chen, Wang, Ji, and Wu (2007) with different protease enzymes to derive Gln-Gly-Ala-Arg bioactive peptide with proven antioxidant activity. Chicken bone collagen hydrolysate have been produced by Zhang, Xiao, Samaraweera, Lee, and Ahn (2010) and were shown to exhibit cholesterol-lowering effect which might act as prevention substance of atherosclerosis as well as reported as an anti-inflammatory agent. Collagen of chicken legs (yellowish keratinised parts with nails) was hydrolysed by Aspergillus fungal protease to produce antihypertensive peptide with angiotensin 1 converting enzyme (ACE) inhibitory assay (Saiga et al., 2008). This study obtained the octapeptide sequence (Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro) with strong ACE inhibitory activity, and results were confirmed by rat bioassay. The peptide was administered to spontaneously hypertensive rats and significantly lowered their blood pressure. Around 25% of the protein in bovine lung is collagen (Lynch et al., 2018) and there is a high possibility to produce bioactive peptides by enzymatic digestion.

ACE inhibitory peptides are most extensively studied bioactive peptides which were derived from different protein sources. Most of these bioactive peptides were derived from milk proteins and the antihypertensive effect was confirmed by the in vivo rat studies and human trials as well (Aihara, Kajimoto, Hirata, Takahashi, & Nakamura, 2005; Hata et al., 1996; Jauhiainen et al., 2005; Korhonen, 2009; Masuda, Nakamura, & Takano, 1996; Mizushima et al., 2004; Nakamura, Yamamoto, Sakai, Okubo, et al., 1995; Nakamura, Yamamoto, Sakai, & Takano, 1995; Seppo, Jauhiainen, Poussa, & Korpela, 2003; Sipola, Finckenberg, Korpela, Vapaatalo, & Nurminen, 2002). But studies of bioactive peptides from animal coproducts are comparatively limited and no human intervention studies were reported. Produced peptides need to go through the intestinal lumen to show the bioactive effect and these in vivo studies are very important.

Some researchers argue that only di- and tri-peptides are absorbed through the intestinal lumen to the bloodstream (Miner-Williams, Stevens, & Moughan, 2014). In contrast, Mirdhayati, Hermanianto, Wijaya, Sajuthi, and Arihara (2016); Saiga et al. (2008) showed that even longer than tri-peptide have ability to show bioactivity in vivo study. Further, the bioactive compounds derived from coproducts are listed on Table 2.4

Table 2.4 Publications on the production of bioactive peptides from offal

Coproduct	**Processing method**	**Bioactive compound**	**References**
Bovine lungs	Protein extraction by extraction buffer and column chromatography	Purified bio-Active compound (Soluble guanylate cyclase)	(Mathis, Emmons, Curran, Day, & Tomasselli, 2008)
Bovine lungs	Enzymatic hydrolysis with high pressure pre-treatment	Hydrolysates with DPP-IV, PEP inhibitors	(Lafarga & Hayes, 2017)
Bovine lung	Enzymatic hydrolysis	Anti-inflammatory agent	(O'Sullivan et al., 2017)
Bovine, porcine hearts	Ammonium sulphate precipitation, Cation-exchange and affinity chromatography	Isolation of heparin-binding growth factors	(Quinkler et al., 1989)
Porcine Intestinal mucosa	Hydrolysate of intestinal mucosa, anion exchange	Production of heparin	(Griffin et al., 1995)
Duck skin	Enzymatic hydrolysis and RP-HPLC purification	Anti-oxidative peptide	(Lee et al., 2012)
Chicken bone	Enzymatic hydrolysis	angiotensin I-converting enzyme inhibitory peptide	(Cheng, Liu, Wan, Lin, & Sakata, 2008)
chicken legs (yellowish keratinised parts with nails)	Enzymatic hydrolysis with fungal proteases and membrane filtration	angiotensin I-converting enzyme inhibitory peptide	Saiga et al. (2008)
Bovine blood plasma	Enzymatic hydrolysis and RP-HPLC purification	angiotensin I-converting enzyme inhibitory peptide	(Wanasundara et al., 2002)
Porcine aorta	Solubilisation in salt solution and membrane filtration	Anti-cholesterol peptide	(Chernukha, Fedulova, & Kotenkova, 2015)
Bovine tendon	Acid extraction and salt precipitation. Purified by ion-exchange and gel filtration chromatography	angiotensin I-converting enzyme inhibitory peptide	(Banerjee & Shanthi, 2012)
Sheep brain	Buffer extraction and purified by ion-exchange and gel-filtration chromatography	Calmodulin Methyltransferase	(Han, Richardson, Oh, & Roberts, 1993)
Porcine brain	Acid extraction with pre-heat treatment and purified by series of chromatographic methods	Galanin-like Peptide	(Ohtaki et al., 1999)

2.7 Coproduct incorporation with starchy food

2.7.1 Extrusion cooking

Extrusion is a versatile and very efficient technology that is broadly used in food and feed processing. Extrusion utilises 100% input materials from feeding end of the extruder barrel to other end of forming products as output. Processing steps of, mixing, shearing, temperature and pressure building, metering, forming, expansion and cooling take place inside of the barrel to produce the extruded products (Brennan, Derbyshire, Tiwari, & Brennan, 2013; Cheftel, Kitagawa, & Queguiner, 1992). According to Ilo, Schoenlechner, and Berghofe (2000), ingredients with lower fat enhance the extrusion cooking behaviour and overall expansion of the direct expanded products. Thus, coproducts that are low in fat (Table 2.3) would have potential for extruded products.

The limiting factor of the utilisation of many coproducts in the human food chain is the poor quality of texture and low aesthetic appeal (Chávez-Jáuregui et al., 2003). Extruded products have altered organoleptic properties and have been reported as enhancing nutritional accessibility and availability of protein (Bastos & Arêas, 1990; Jayawardena, Morton, Brennan, & Bekhit, 2019b; Santiago, Moreira-Araújo, e Silva, & Arêas, 2001).

In 2011 Meat and Livestock Australia (MLA) initiated a project to produce extruded meat by using low-cost materials with high moisture extruded cooking (HMEC) (MIA 2011). Extruded product with better texture was obtained by using 36% trimmed red meat with standard HMEC, and further possibility indicated to produce extruded products with 67% meat (MIA 2011). Coproducts should be able to be substituted to produce retextured meat using high moisture extruded cooking process. Although the literature on extrusion is dominated by cereal-based products, high moisture extruded cooking has excellent potential to process high moisture (>40%) materials like meat (Akdogan, 1999; Cheftel et al., 1992; Osen, 2017) and higher potential to convert offal to retextured meat. Mechanically deboned chicken was used to prepare meat analog by Megard, Kitabatake, and Cheftel (1985) using high moisture extrusion. Low moisture extrusion was reported to produce fortified snacks with beef lungs successfully in Brazil (Moreira-Araújo, Araújo, & Arêas, 2008). These beef lung fortified snacks significantly reduced the prevalence of iron deficiency anaemia in preschool children. Series of beef lung protein extrusion projects were conducted with de-fatted beef lungs and successfully produced nutritious snacks with better textural characteristics (Arêas & Lawrie, 1984; Campos & Arêas, 1993; Cardoso-Santiago & Arêas, 2001a; Cardoso-Santiago & Arêas, 2001b; Moreira-Araújo et al., 2008; Santiago et al., 2001).

Conti-Silva, Pinto e Silva, and Arêas (2011) replaced soy protein with extruded rumen protein in different food products and the sensory acceptability was evaluated by consumer panellists. Extruded rumen protein improved the flavour of pork sausage and incorporation of rumen protein

was feasible based on the sensory results. Bovine and porcine lungs and bovine tripe proteins were mixed together by Mittal and Lawrie (1984) and extruded with soy grits to make 20% to 30% highly incorporated snacks. Garcia Zepeda et al. (1997) prepared beef snacks using extruded beef cardiac muscles and improved binding ability was demonstrated with non-binding ingredients. Further microbiologically safer snacks were prepared by extrusion coupling with electron beam irradiation and obtained safer beef snacks with lower screw speed and low steam injection levels (Garcia Zepeda et al., 1997). Extrusion is therefore a viable unit operation to produce snacks by converting unfavourable textural characteristics to a favourable state.

Further, cold extrusion takes place in pasta processing (Le Roux, Vergnes, Chaurand, & Abécassis, 1995) and this mechanism can be used to incorporate coproducts into starchy food. Meat emulsion was prepared by calf meat and incorporated into durum wheat pasta (Liu et al., 2016) and Jayawardena et al. (2019b) produced dried bovine lungs incorporated pasta with durum wheat. coproduct incorporations significantly lowered the glycaemic response like calf meat incorporation in durum wheat pasta.

2.7.2 Pasta as a model food

Pasta is a widely consuming food and its global popularity has shown continuous increase (Verardo, Ferioli, Riciputi, & Iafelice, 2009). Pasta can be produced by a simple extrusion process which has very low production cost and high production efficiency (Lemes, Takeuchi, Carvalho, & Danesi, 2012). These economical features of pasta manufacturing make it suitable as a nutrition vehicle for hunger-stricken countries.

Ordinary durum wheat pasta contains 77% average carbohydrate, and less than 10% protein. This small protein percentage also lacks lysine and threonine amino acids (Filip & Vidrih, 2015) which reduces the quality of the protein. Protein substitution could be the best way to increase the nutrition status of the pasta. Incorporation of different food ingredients into pasta to increase the nutrition content is extensively reported in the literature. Animal and plant proteins were incorporated into pasta successfully and improved the nutritional value. For instance, incorporation of legumes (Teterycz, Sobota, Zarzycki, & Latoch, 2020), mushroom(Kim, Lee, Heo, & Moon, 2016; Lu, Brennan, Serventi, Mason, & Brennan, 2016), fruits (Sant'Anna, Christiano, Marczak, Tessaro, & Thys, 2014), vegetables (Minarovičová, Lauková, Kohajdová, Karovičová, & Kuchtová, 2017) like plant-based incorporations and animal proteins like meat (Liu et al., 2016), muscles (Holovko, Helikh, Holovko, Prymenko, & Zherebkin, 2020), fish (Desai, Brennan, & Brennan, 2018) like incorporations were reported. Animal protein incorporation could increase the price margin of pasta and could not be an affordable price. But underutilised offal ingredient incorporation could lead to cheap quality pasta.

Pasta can be dried by air oven drying leading to internal moisture content below 12.5% (Ogawa & Adachi, 2014). The shelf life of the pasta increased by 1 to 2 years after the drying process and it could be stored at room temperature without maintaining a cold chain. Solar drying is a possible drying method for pasta manufacturing (Bolognese, Viesi, Bartali, & Crema, 2020) and an economical approach for low-income countries in the Sub-Saharan African region. All these facts are important to select pasta as a model food to incorporate protein and use as a nutrition vehicle for malnourished countries.

2.7.3 Drying of coproducts

Drying is an ancient method of food preservation and processing (Ayanwale, Ocheme, & OO, 2007; Potter, 1986). The water is removed from materials by evaporation or sublimation (Lewicki, 2004) and this restricts the growth of micro-organisms (Morgan, Herman, White, & Vesey, 2006). Dried products have increased shelf life, require less storage space, are easier to transport and most importantly, have the potential to be stored without refrigeration in developing countries with hot and humid conditions (Bradford et al., 2018; Mishra, Mishra, Pati, & Rath, 2017). Meat coproducts are highly perishable foods and prone to microbial contaminations with increased safety issues (Jeong et al., 2017), so it is more suitable to preserve these using drying technologies. Different ethnic groups in the world mostly use meat coproducts for their traditional food plate (Edwards, 2013) and it is challenging to maintain the cold chain in remote areas to reach them. Further, meat coproducts like beef lungs exhibit poor textural characteristics (Chávez-Jáuregui et al., 2003) and drying also positively affected the textural modification of coproducts. Currently beef jerky and biltong are popular meat dried products with premium meat cuts, and ample research is available on these products (Burfoot, Everis, Mulvey, Wood, & Campden, 2010; Calicioglu, Sofos, & Kendall, 2003; Calicioglu, Sofos, Samelis, Kendall, & Smith, 2002; Konieczny, Stangierski, & Kijowski, 2007; Nortjé, Buys, & Minnaar, 2005a); Nortjé, Buys, and Minnaar (2005b); (Park & Lee, 2005; Petit, Caro, Petit, Santchurn, & Collignan, 2014). However, there are few studies are available on offal drying. In a recent study, bovine lungs were minced and air oven dried by Jayawardena et al. (2019b) and produced protein powder with a high amino acid score. This lung powder was successfully incorporated into pasta to enrich the protein quality and lower the glycaemic response. Similarly, bovine lungs were air oven dried by Pinto, Colli, and Areas (1997) and produced extruded snacks with high iron content. Though there is a lack of studies in coproduct drying in the human food chain, drying of animal coproducts is popular in the pet food industry and US patents are available with different drying conditions for pet food (Patent US4020187A, US5045339A).

2.7.4 Coproduct hydrolysates

Protein hydrolysates are produced from hydrolysing of protein sources and used for several applications such as ingredients in nutritional and health products, infant formulae, medical and dietary supplements and flavouring agents (Philipps-Wiemann, 2018).

Traditionally, hydrolysates were produced using concentrated acids (6N HCl), generally used for amino acid analysis (Walker & Sweeney, 2009). This method has a list of drawbacks such as destroying tryptophan, partial destruction of serine and threonine, conversion of asparagine and glutamine to their acids (Walker & Sweeney, 2009). Alteration of these amino acids affects the nutritional quality of hydrolysates. Enzyme digestion prevents these drawbacks and produces protein hydrolysates. Enzymatic digestions of protein sources have been extensively studied in the food industry due to advantages of low energy consumption, lower enzymes to substrates ratio and ease of control the reaction. Further, changing enzyme type and hydrolysing time produce varieties of hydrolysates which creates a wide range of product diversity from the same protein source.

Different enzymes behaviours are explained in the study of hydrolysing bovine lung tissues with papain, pepsin and Alcalase enzymes to produce bovine lung hydrolysate (O'Sullivan et al., 2017). Among the proteases, Alcalase enzyme hydrolysates had anti-inflammatory activity by significant suppression of cytokine production in RAW264.7 cells while other hydrolysates had no significant effect. Meat coproducts can be used to produce food ingredients. The safety of the food ingredients should be ensured before applying to the human food chain. Safe flavour enhancer was produced from the chicken bone extract using enzymatic hydrolysis (Wang et al., 2016). In this study, proteins were extracted by hot pressure extraction method and proteins were hydrolysed with the Flavourzyme enzyme. Safety measures were studied by in-*vivo* rat study and there was no significant toxicity observed with 13-week administration.

Sheep visceral mass, including lungs, was hydrolysed using fungal proteases to produce nutritious hydrolysate (Bhaskar, Modi, Govindaraju, Radha, & Lalitha, 2007). The visceral mass protein digestibility corrected amino acid score (PDCAAS) reached to the higher level (0.93) corresponding to adult nutrition status. These studies suggest that slaughterhouse coproducts, like lungs, have the potential to produce nutritional supplements through protein hydrolysis.

The purpose of most of the studies of hydrolysates produced from coproducts was to isolate the bioactive peptides, and these isolations are listed on Table 2.4 Preparation of antihypertensive peptides with angiotensin 1 converting enzymes (ACE) inhibitory activity has been widely studied with meat and milk but few studies are available on coproducts. This is lack of studied area and there is a clear space to initiate more projects on hydrolysates with meat coproducts to prepare bioactive peptides including ACE inhibitory peptides. Animal parts like chicken bone, bovine tendons were

hydrolysed with different enzymes and the ACE inhibitory peptides were isolated (Banerjee & Shanthi, 2012; Cheng et al., 2008). This showed the higher potential to utilise meat co products than meat itself?.The studies of beef lungs were not reported to analyse ACE inhibitory peptides but Darine et al. (2010) reported that beef lung concentrates are rich in low molecular weight proteins. Low molecular weight protein is the characteristics of ACE inhibitory peptides (Pihlanto-Leppälä, 2000) and there is a high potential to produce ACE inhibitory peptide from beef lungs.

Clearly, there is a gap in knowledge regarding potential ways to utilize lungs in food products. The present research project aimed to investigate potential utilization of beef lungs as a food ingredient and peptide source under the beef coproducts regulations in NZ.

Table 2.5 Publications on the processing of coproducts

Coproduct	Processing method	Advantage/end product	Literature
Bovine Lungs	Oven-dried, defatted and extrusion cooking	High iron bioavailable snacks	(Pinto et al., 1997)
Bovine Lungs	Freeze-dried, defatted, extrusion with bacon flavour	High consumer acceptability	(Chávez-Jáuregui et al., 2003)
Bovine Lungs	Freeze-dried defatted extrusion with chickpea and corn	snacks to prevent anaemia in preschool children	(Moreira-Araujo, Araujo, & Areas, 2008)
Bovine lungs	Oven-dried and incorporated into pasta	Protein-rich pasta with low glycaemic response	(Jayawardena et al., 2019a)
Bovine lungs	alkaline solubilisation and PI precipitation	Protein extract with high emulsifying activity and forming property	(Darine et al., 2010) (Darine et al., 2011)
Bovine and porcine lungs	Alkali solubilisation and PI precipitation	High protein yield	(Lynch et al., 2018)
Bovine lung, liver, spleen and blade-bone	All coproducts steamed with cassava and wheat flour, slicing, air oven drying	Snack with high consumer acceptability	(Subba, 2002)
Bovine lung, spleen, heart, porcine lung, liver	Protein extraction with low ionic buffer	30% to 75% of extracted protein	(Nuckles, Smith, & Merkel, 1990)
Bovine & porcine lung and bovine tripe	Protein extracted and extruded with soy grits	Extruded snacks with 20% to 30% of coproducts	(Mittal & Lawrie, 1984)
bovine rumen	Defatted, air oven dried and extrusion	Protein ingredient with high consumer acceptability	(Conti-Silva et al., 2011)
Bovine heart	Protein extraction by acid solubilisation and salt precipitate	Low fat protein	(DeWitt, Gomez, & James, 2002)
Bovine heart and lips	Protein extraction by Salt solubilisation	High water holding capacity and emulsion stability	(Krasnowska, Górska, & Gergont, 1995)
Bovine liver	Enzymatic hydrolysis and membrane filtration	Hydrolysate with antioxidant activity	(Di Bernardini et al., 2011)
Calf head	Meat emulsion incorporated into pasta	Protein rich pasta with low glycaemic response	(Liu et al., 2016)
Porcine liver	Protein extraction by salt solubilisation	Low cost protein	(Steen et al., 2016)
Sheep intestine and stomach	Sterilising, enzymatic hydrolysis (fungal protease)	Protein hydrolysate	(Bhaskar et al., 2007)
Chicken bone	Hot pressure extraction, concentration with vacuum condenser, enzymatic hydrolysis	Safe meaty flavour enhancer	(Wang et al., 2016)
Buffalo rumen and heart	Emulsion preparation and incorporate into sausages.	High consumer acceptable sausages	(Krishnan & Sharma, 1990)

Chapter 3

Utilisation of Beef Lung Protein Powder as A Functional Ingredient to Enhance Protein and Iron Content of Fresh Pasta

3.1 Introduction

Meat consumption increases every year and efficient production and novel strategies for 'nose to tail' uses of the animal are needed to meet the ever increasing demand for protein. Global meat production reached 257 million tonnes in 2015 with pork, chicken and beef being the most important meats (USDA, 2016). Beef has a high economic and environmental impact (Pelletier & Tyedmers, 2010), and thus, it is important to maximise the efficiency of beef production and use. The beef carcass weight is only 45–60% of the animal live weight, the remainder of the weight represent generated co-products or waste (Muir & Thomson, 2008). Unfortunately, there is a low market demand for edible co-products in the countries which have high beef production and most of these co-products are rendered. The main product of rendering is animal feed which has a very low economic value (MIA, 2015). Based on their amino acid profile, meat co-products are a potential source of low cost and high quality protein (Mullen et al., 2017) for malnourished people. More than half of the deaths among children in developing world by infectious diseases have been reported to be associated with protein energy malnutrition (Cerqueira & Cardoso, 2017; WHO, 2017). information highlights a significant gap where the high production of meat in developed countries is typically associated with low demand for co-products from human food; while many developing countries are suffering from protein energy malnutrition due to the unaffordable cost of animal protein. There is a potential for novel uses of co-products to bridge this gap.

Beef lungs are large organs with an average weight near 3 kg and contain high levels of protein and bioavailable iron (Cardoso-Santiago & Arêas, 2001b; Cardoso-Santiago et al., 2001). Beef lung protein is rich in essential amino acids which comprise more than 40% of the total amino acids (Fornias, 1996). In western countries, fresh beef lungs have a low consumer demand due to strong aesthetic rejection and poor intrinsic textural quality (Chavez-Jauregui et al., 2003). Beef lungs are subject to current legal requirements, for example Europe food regulations (EC 854/2004) covers beef lungs for human consumption with the postmortem inspections and (EC)No 853/2004 covers the hygienic regulations during processing. In many Eastern (China, Korea, Japan and many other Asian countries) as well as Middle Eastern (Egypt and Turkey), African and South American countries, consumption of slaughtered animals' lungs is quite common. Beef lungs show a high level of processing potential as a

food ingredient (Darine et al., 2010). Extrusion and other processing methods can improve their textural and quality characteristics (Ar^eas, 1992). Beef lungs have successfully been incorporated into snacks to reduce the prevalence of anaemia in school children, illustrating their potential for improving the diet of malnourished populations (Moreira-Araujo et al., 2008).

Previous research has focused on the beef lung formulations and in vivo bio-availability but there is a need to understand the changes in physical and chemical characteristics after incorporation into food products. In this research, semolina fresh pasta was selected as a staple food for beef lung incorporation. The aim was to address the limiting amino acids in the semolina and to determine the optimal beef lung incorporation into pasta with consideration of the changes in physical, chemical and nutritional parameters. Starchy food with a high glycaemic index has been associated with type II diabetes (Willett et al., 2002) and although pasta is generally regarded as a medium to low glycaemic index food, any further reduction in glycaemic values of pasta may be of nutritional value. This research will investigate whether incorporating beef lung powder (BLP) into fresh pasta lowers the glycaemic index further.

3.2 Materials and method

3.2.1 Source of lungs

Fresh lungs derived from healthy bovine carcasses were obtained from ANZCO Limited, New Zealand.

3.2.2 Preparation of beef lung powder

Fresh lungs, without trachea derived from healthy animals, were obtained from ANZCO Limited, New Zealand and immediately transported on ice to AgResearch Limited (New Zealand) in insulated boxes and dried for 32 h at 60 °C using air oven dryer until reach to constant weight. Dried lungs were cut into small pieces and were packaged in airtight bags and transported to the food processing laboratory at Lincoln University. The dried lungs were ground to powder (BCG200 grinder, Breville, Sydney, Australia) and sieved using a standard 500 µm mesh. This BLP was used to prepare fresh pasta for further analysis.

3.2.3 Pasta preparation with beef lung powder

Control pasta dough was prepared using commercially available durum semolina powder (Sun Valley Foods, Christchurch, New Zealand). BLP was combined with semolina powder at 10%, 15% and 20% (w/w basis). Fresh pasta was prepared using a commercial pasta machine fitted with a 2.25-mm thick cylindrical spaghetti die (model: MPF15N235M; Fimar, Villa Verucchio, Ravenna, Italy) with 32.5 mL

of warm water (41 °C) was added to 100 g of the mixture (semolina plus BLP) and mixed for 20 min within the machine before extrusion (Lu et al., 2016).

3.2.4 Chemical composition analysis

Beef lung powder and freeze-dried control fresh pasta samples were analysed for chemical composition. Protein (%) was calculated by the summation of total amino acids. Moisture (%) was analysed as per AACC (2001) method No.44-16.01 by desiccation at 105 °C using air oven dryer. Ash (%) was determined by calcination at 550 °C, and fat (%) was determined by the Soxhlet extraction method (AOAC, 2005). Carbohydrate was calculated by the weight difference method.

3.2.5 Mineral profile analysis

Samples (0.2 g each) of dried BLP were mixed with 2.0 mL of nitric acid (69%) and 2.0 mL of hydrogen peroxide (30%) and were digested in a microwave (CEM MARS Xpress, Matthews, NC, USA) using a temperature programme of a linear increase from ambient to 90 °C over 15 min and then held at 90 °C for further 5 min. Digested samples were analysed by Inductively Coupled Plasma Optical Emission Spectrophotometer (Varian 720 ICP-OES, Melbourne, Australia). Settings were Plasma gas flow-15.0 L min^{-1}, Aux-1.5 L min^{-1}, Nebuliser 0.9 L min^{-1} with SeaSpray nebuliser and cyclonic spray chamber. Calibration standards and internal standards were serially diluted from (Merck, Darmstadt, Germany) ICP standard solutions using MilliQ water. Calibration curves were generated using four standards and standard blank.

3.2.6 Amino acid analysis

Amino acids were determined using the methods adopted by Fountoulakis & Lahm (1998) and Weiss et al. (1998). Samples were freeze-dried and ground through a 0.5-mm sieve into culture tubes. The samples were resuspended in 5.0 mL of 6 M HCl, and 10.0 µL 0.5 M amino-butyric acid was added as a standard. Samples were mixed by vortexing and sonicated for 5.0 min. Each tube was purged with nitrogen and immediately made airtight by closing the top. Then, samples were digested at 110 °C for 20 h. After cooling to room temperature, the hydrolysate was obtained and the tube was washed with 5 mL water two times and added to the hydrolysate. Then, the hydrolysate was dried in a rotary vacuum evaporator at 45 °C. The hydrolysate residue was resuspended in nanopure water, transferred to a volumetric flask and the volume was made to 50 ml. The samples were filtered through a 0.45-lm syringe into a 2 mL HPLC vial for the HPLC analysis.

All amino acids were analysed by HPLC 1100 series (Agilent Technologies, Waldrbom, Germany) provided with an autosampler, and fluorescence detector was used for analysis. HPLC column was

ACE, 3 µm C-18 (150 mm × 4.6 mm) with 40 °C column temperature. Indispensable amino acid ratio (IAAR), indispensable amino acid score (IAAS) and amino acid ratio were calculated for all amino acids according to the following equation, and amino acid score was the least amino acid ratio.

$$IAAR = \frac{\text{Indispensable amino acid amount } (mg\ /\ g\ protein)}{Standard\ reference\ patern}$$

3.2.7 Cooking quality

Optimal cooking time (OCT) was determined according to the method of Foschia et al. (2014). Raw pasta was cut into 4-cm-long strands (20 g) and cooked in 600 mL boiling water. Samples were removed every 30 s to check the white core disappearance by squeezing them in between two transparent plastic pieces. Time was taken for the disappearance of white cores from the pasta strands was taken as OCT.

Cooking loss

Cooking loss was determined according to the standard method 66-65 (AACC, 2000). Fresh pasta was cut into 4 cm strands, and 20 g samples were taken from undamaged strands. Samples obtained from all the developed products were cooked in boiling water (600 mL) until their OCT. Boiling water was decanted into a pre-weighed aluminium vessel. Cooked pasta and boiling pan were washed with 200 mL of water, and the wash water was also added to the aluminium vessel which was then dried in air oven dryer at 105 °C until constant weight. After drying, residue weight was measured and reported as a percentage of starting spaghetti weight. All analyses were made in triplicate.

Water absorption index and swelling index

Water absorption index (WAI), the measure of pasta hydration, was determined according to standard method 66-65 (AACC, 2000). Fresh pasta cooked until OCT was strained for 10 min and weighted to determine the WAI using the formula:

$$\text{WAI} = \frac{(\text{weight of cooked pasta} - \text{weight of fresh pasta})}{\text{weight of fresh pasta}} \times 100$$

Dried pasta weight was used to determine the swelling index (SI) according to the method described by Fardet *et al*. (1998). Cooked pasta was dried in air oven at 105 °C until it reached a constant weight. The analyses were made in triplicate.

$$\mathrm{SI} = \frac{(\text{weight of cooked pasta} - \text{weight of dried pasta})}{\text{weight of dried pasta}} \times 100$$

3.2.8 Textural characteristics

Fresh pasta was cooked in boiling water to reach the OCT. Then drained and left for 10 min until surface water was evaporated before measuring textural characteristics.

Elasticity and firmness

Cooked pasta elasticity was measured on single pasta strands, and firmness was measured based on the force required to cut five strands of pasta placed parallel to each other. A Texture Analyser (TA.XT2; Stable Micro System, Godalming, UK) equipped with a 5-kg load cell was used to analyse the pasta firmness and elasticity with equipment settings as in Foschia et al. (2014).

Elongation

The elongation of the cooked pasta was measured by the Texture Analyser (TA.XT2; Stable Micro System) equipped with a 5-kg load cell. Length difference of the pasta was measured simultaneously while measuring elasticity. The ability of pasta to be elongated was determined as extending pasta length until breakage and was calculated according to the following equation (Laleg et al., 2016).

$$\text{Elongation } (\%) = \frac{FinalLength - OriginalLength}{Originallength} \times 100$$

The original length of pasta was equal to the initial distance (10 mm) of elasticity settings in Texture Analyser. All textural characteristics were analysed in ten replicates.

3.2.9 Colour analysis

Pasta colour readings were taken immediately after processing and cooking. Two layers of pasta were mounted on black paper without gaps and ‘L’, ‘a’ and ‘b’ values were measured using a colorimeter (Minolta Chroma Meter CR210; Minolta Camera Co., Osaka, Japan) with the illuminant C (CIE, standard, 6774 K). Measurements were made at ten random places on the surface of uncooked and cooked pasta layers. Colour reading ‘L’ value represents darkness to lightness, ‘a’ value represents green to red and ‘b’ value represent blue to yellow when values change from negative to positive. The

colorimeter was calibrated to L = 98.03, a = 0.23 and b = 0.25 with standard white tile. All analyses were replicated six times.

3.2.10 *In vitro* digestion for predictive glycaemic impact

This method predicts the glycaemic response by measuring how rapidly free reducing sugars are liberated during *in vitro* hydrolysis. Cooked pasta was cut into small pieces, placed in a mortar and squeezed ten times with a pestle to simulate oral chewing (Foschia, Peressini, Sensidoni, Brennan, & Brennan, 2014). *In vitro* digestibility trials were described from Brennan, Derbyshire, Tiwari, and Brennan (2012). The 2.5 g weighed samples with 30 mL of RO water were added to digestion pots on a pre-heated IKAAG RT 15 multi stirred block (IKAWERKE Gmbit & Co., Staufen, Germany) and constantly stirred until reach 37 °C. Gastric digestion was started by adding 0.8 mL 1M HCl and 1 mL of 10 % pepsin (Sigma Aldrich, USA) solution in 0.05 M HCl while continuously stirring and incubated at 37 °C for 30 min. A 1 mL aliquot was taken from each digestion pot at the end of gastric digestion (start counting time as "0 min") and added to 4 mL ethanol to terminate the reaction. Amyloglucosidase (0.1 mL) added to the digestion pot, soon after taking 0 min aliquot to prevent the end-product inhibition of pancreatic α-amylase. Intestinal digestion was stimulated by adding 5 mL of 2.5% Pancreatin (Sigma Aldrich, USA) solution in 0.1 M sodium maleate buffer pH 6 for 120 minutes. Aliquots were taken during 20, 60 and 120 min and each was added to 4 mL ethanol. The samples were stored at 4 °C until analysis of reducing sugar using the 3.5-dinitrosalicylic acid (DNS) method.

All aliquots of test tubes were centrifuged at 1000 g for 10 minutes, and then 0.05 mL of the supernatant was transferred into individual glass vials. A 0.05 mL of reagent blank (RO water), 0.05 mL of glucose standards (5 mg/mL and 10 mg/mL) were transferred to separate tubes. Then, 0.25 mL of enzyme solution (1 % Invertase and 1% amyloglucosidase) added to each glass tube. All the tubes were incubated 20 min at room temperature before adding 0.75 mL of the DNS (reagent) to each tube. All tubes were covered with aluminium foil and heated in a boiling water bath for 10 min. The tubes were cooled and 4 mL RO water was added to each tube, and the absorbance was measured at 530 nm by using a spectrophotometer with 1cm path length. Reducing sugar released was calculated as mg /g sample and plotted the curve against the time and area under the curve (AUC). AUC was determined by dividing the graph into trapezoids (Matthews, Altman, Campbell, & Royston, 1990). Glucose released over the time and area under the curve (AUC) was calculated according to Matthews et al. (1990).

3.2.11 Statistical analysis

The data generated by repeating the experiments for different quality parameters were compiled and analysed using SPSS (IBM SPSS Statistics V22.0, Armonk, NY, USA). All experiments were performed in

triplicate unless otherwise stated. All data were reported as means ± standard deviation. A one-way analysis of variance, at the 0.05 level of significance, was carried out to investigate the significant differences between the treatments. When the ANOVA was significant ($P < 0.05$), means were separated by a pairwise comparison using Tukey's comparison test.

3.3 Results and discussion

3.3.1 Chemical analysis

Cereals are a staple food and characterised by a high proportion of carbohydrate and relatively low protein content as seen in the control pasta which is made up of semolina wheat (Table 3.1). BLP provides a useful supplement as it had a higher protein concentration, mineral (ash) and fat content than the pasta (Table 3.1). BLP had no detectable carbohydrate while the control pasta had 87% carbohydrate.

Fresh control pasta had a moisture content of 26.19± 2.17% (w/w) and BLP showed a low moisture (6.6 ± 0.02% w/w). The protein, fat and ash content of control fresh pasta (Table 3.1) were consistent with the results reported in previous research of OvandoMartinez et al. (2009), and the protein content (12%DM) was similar to Mercier et al. (2016), and similar to the semolina powder composition reported by USDA (2017). BLP protein and fat content results (Table 3.1) were consistent with Fornias (1996), and ash content results were similar to Cardoso-Santiago and Arêas (2001b). In the present study, the highest protein content was 27% in 20% BLP pasta and the lowest protein content was 20% in 10% BLP pasta (all content on a dry weight basis). Kadam & Prabhasankar (2012) added 30% shrimp meat into pasta which increased the protein content to 15.7% and adding 45% beef emulsion to pasta raised the protein to 15.5% on dry matter basis (Liu et al., 2016). The protein content of the fortified pasta in this research was higher than these studies and above the average protein content of 17.3% in a meta-analysis of fortified pasta (Mercier et al., 2016).

Mineral profile of beef lung powder

The high ash content of the BLP represents the high mineral content as identified by inductively coupled plasma mass spectrometry (ICP). The mineral profile is dominated by potassium, phosphorus, sulphur and sodium. Iron (Fe) was the next highest mineral with 1.0 mg/ g dry matter.

Essential minerals are elements required for the normal physiological functions of the body and the requirement of the specific minerals varies with the life stage of the person and gender. Lower and higher RDI/AI values were sourced from NHMRC (2006) for older child, adolescent or adult. Different amounts would be required for infants, and during pregnancy or lactation. For instance, iron

requirement during lactation is within the range stated in Table 3.2, but for pregnant woman, a higher amount of iron (27 mg/ day) is needed.

Iron plays an important role in the function of the body and is essential for the normal functioning of several enzymes and in haem pigments. The iron content of the body is highly conserved (Bothwell et al., 1979) but lack of iron in the diet can cause a varying degree of deficiencies ranging from low iron stores to iron deficiency anaemia (NHMRC, 2006). BLP is a rich and cheap source of iron (1 mg/ g dry matter basis). About 17 g of BLP is adequate to meet the highest daily requirement (Table 3.2) of adult women (19–50 years), whereas 8 g can easily meet the lower daily requirement (Table 3.2) of adult men and older women (>50 years). Further, the low levels of zinc and manganese in BLP may enhance the absorption of iron by reducing competitive inhibition (Rossander-Hulten et al., 1991). Iron from beef lungs is expected to be highly bioavailable as found with other animal sources (Hurrell & Egli, 2010). An intervention study (Cardoso Santiago et al., 2001) showed that incorporating 8% beef lung into snacks successfully reduced the prevalence of anaemia in the children from 61.5% to 11.5% in preschool children of a poor Brazilian region. In the current study, the minimum BLP incorporation was 10% and has a similar potential to reduce anaemia prevalence. Semolina flour contains only 0.03–0.04 mg/ g of iron on dry matter basis (USDA, 2018). After incorporating 10% BLP to semolina fresh pasta iron content increased up to 0.143 mg/ g dry matter.

Table 3.1 Proximate analysis of beef lung powder and control pasta.

Component (w/w, dry basis)	**Beef lung powder**	**Control pasta**
Protein	87.01 ± 2.85%	12.44 ± 0.36%
Fat	8.76 ± 0.07 %	0.44 ± 0.04%
Carbohydrate	0.0%	86.73 ± 0.43%
Ash	4.93 ± 0.08%	0.46 ± 0.03%

Results are the means (± SD) of triplicate samples (n=3)

Table 3.2 Comparison of beef lung powder with RDI/AI values.

Element	Amount / g (beef lung powder)	RDI/ AI value (lowest to highest)	BLP required to provide lowest and highest value (g)*
Ca (mg)	0.48 ± 0.002^{de}	500-1300	1042-2709
Cr (µg)	0.34 ± 0.03^{e}	11-35	33-103
Cu (mg)	0.00627 ± 0.0003^{e}	0.7-1.7	112-272
Fe (mg)	1.07 ± 0.007^{d}	8-18	8-17
K (mg)	10.92 ± 0.77^{a}	2000-3800	184-348
Mg (mg)	0.63 ± 0.008^{de}	80-420	127-667
Mn (mg)	0.00059 ± 0.0001^{e}	2-5.5	3390-9322
Na (mg)	7.30 ± 0.08^{c}	200-920	28-126
P (mg)	9.26 ± 0.03^{b}	460-1250	50-135
Zn (mg)	0.092 ± 0.005^{e}	8-14	87-153

Source of RDI /AI values: National Health and Medical Research Council, Australia;
RDI, recommended daily intake (Ca, Cr, Cu, Fe, Mg, P, Zn); AI, adequate intake (K, Mn, Na)
Different superscripts are significant ($p<0.05$)
Results are the means (± SD) of triplicate samples (n = 3)
*Denotes calculated values

3.3.2 Cooking characteristics and texture properties

Optimal cooking time, cooking loss, SI and WAI are important parameters of cooked pasta quality. OCT refers to the minimum boiling time to gelatinise the pasta core. Control fresh pasta OCT (6 min and 30 s) was similar to the results of Foschia et al. (2014) and Lu et al. (2016) that used the same processing procedures. Adding of BLP to the fresh pasta caused a significant ($P < 0.05$) increase in the OCT. Kadam & Prabhasankar (2012) also reported a similar increase in the OCT of the pasta incorporated with shrimp meat, what is of note in that the 10% BLP showed a higher OCT than the 15% and 20% BPL pasta. Kadam & Prabhasankar (2012) suggested that increasing protein content in pasta initially disrupts the starch–protein matrix and that high protein addition may lead to protein–protein association which forms cohesive structure and may result in a more homogeneous starch–protein complex. This could partially explain our observation. A clear pattern could not be identified with the WAI and SI. However, the WAI and SI were higher in BLP pasta compared to the control pasta (Table 3.3). WAI and SI increased significantly in the 10% pasta and could probably be associated with high OCT of the 10% pasta. The cooking loss of BLP incorporated pasta was significantly higher than the control pasta but did not significantly increase with the increasing percentage of BLP which is in agreement with the findings of Pasqualone et al. (2016). According to Cleary & Brennan (2006), higher cooking losses by exudates and leaching of the solid mass into the water was due to a discontinuous protein coat and weaker binding proteins of the fortified pasta. However, the maximum cooking loss

was 6.8% and conventionally a cooking loss below 7–8% is regarded as acceptable in pasta processing (Dick & Youngs, 1988; Doxastakis et al., 2007).

Elastic limit, firmness and elongation represent the textural properties of pasta (Table 3.3). Firmness was increased significantly ($P < 0.05$) with increasing BLP similar to Liu et al. (2016). An increasing pattern was recorded in the elastic limit of the pasta up to 15%, whereas a decreasing ($P < 0.05$) trend was observed in the elongation with increasing levels of the addition of the BLP.

Table 3.3 Cooking characteristics and textural properties.

Cooking characteristics	**Control pasta**	**10% BLP**	**15% BLP**	**20% BLP**
Optimal cooking time (OCT) (min)	6.5 ± 0.41^{c}	11 ± 0.52^{a}	8 ± 0.38^{b}	8.5 ± 0.55^{b}
Water absorption Index (g /100g)	64.91 ± 2.82^{d}	89.01 ± 2.48^{a}	72.01 ± 1.29^{c}	77.16 ± 1.40^{b}
Swelling index (water (g)/dry pasta (g)	1.63 ± 0.06^{b}	2.01 ± 0.06^{a}	1.73 ± 0.02^{b}	1.70 ± 0.06^{b}
Cooking loss (g / 100g)	3.57 ± 0.30^{b}	5.84 ± 1.53^{a}	6.84 ± 0.22^{a}	6.09 ± 0.82^{a}
Textural properties				
Elastic limit (g)	40.79 ± 3.98^{b}	39.88 ± 4.16^{b}	49.56 ± 5.69^{a}	43.03 ± 5.04^{b}
Firmness (g)	215.04 ± 9.10^{c}	323.69 ± 17.11^{b}	409.42 ± 16.25^{a}	418.85 ± 8.52^{a}
Elongation	45.77 ± 4.53^{a}	29.59 ± 4.96^{b}	24.29 ± 3.57^{c}	17.17 ± 4.04^{d}

Different superscripts are significant ($p<0.05$) along the row.
Results for cooking characteristics are the means (± SD) of triplicate samples (n=3)
Results for textural properties are the means (± SD) of 10 replicate samples (n= 10)

3.3.3 Cooked and fresh pasta colour

All colour values of the fortified pasta (Fig. 3.1) were within the range of an earlier pasta meta-analysis (Mercier et al., 2016). Both cooked and fresh control pasta colour values were in the range previously published by Liu et al. (2016) and Foschia et al. (2014).

Raw fresh pasta samples with BLP were significantly darker (low L* value) and redder (high a* value). The redness of cooked fortified pasta was significantly higher in 15% and 20% supplementation rate than 10% treatment (Table 3.4), and these results were consistent with the meat emulsion added pasta of Liu et al. (2016). The yellowness (b* value) of all pasta types increased after cooking and but yellowness decreased with the increasing in the concentration of BLP.

Table 3.4 Main colour values (L*a*b*) of raw and cooked pasta contained 10%, 15% and 20% beef lung powder or non-treated (control) samples.

Raw pasta colour values			
Product	L* value	a* value	b* value
Control pasta	75.43 ± 1.99^{a}	1.02 ± 0.46^{c}	12.51 ± 2.02^{a}
10% beef lung pasta	50.61 ± 1.49^{b}	3.62 ± 0.21^{b}	11.42 ± 0.38^{b}
15% beef lung pasta	40.73 ±0.71^{c}	6.77 ±0.17^{a}	-2.93 ± 0.09^{d}
20% beef lung pasta	38.28 ± 1.06^{d}	6.51 ± 0.08^{a}	-1.62 ± 0.21^{c}
Cooked pasta colour values			
Control pasta	69.19 ± 2.67^{a}	-0.11 ± 0.09^{c}	17.49 ± 0.48^{a}
10% beef lung pasta	39.66 ± 1.40^{b}	5.73 ± 0.30 ^{a}	14.54 ± 0.53^{b}
15% beef lung pasta	34.01 ± 0.99^{c}	5.55 ± 0.37^{ab}	12.74 ± 0.43^{c}
20% beef lung pasta	31.95 ± 0.97^{d}	5.30 ± 0.26^{b}	12.25 ± 0.46^{c}

Different superscripts are significant (p<0.05) along the column
Results are the means (± SD) of six samples (n = 6)

Figure 3.1 Images indicating the colour and consistency of control and beef lung powder containing pasta at 10%, 15% and 20% supplementation levels.

3.3.4 In vitro digestion for predictive glycaemic impact

Pasta is a high carbohydrate food and a reduction of its glycaemic impact is important for improving consumer health. The release of reducing sugar during in vitro digestion of all cooked pasta combinations over 120 min is shown in Table 3.5. It can be seen that there was rapid starch degradation during the first 20 min and slow digestion from 20 min to 120 min. The value of the area under the glycaemic response curve (AUC) shown in Table 3.5 demonstrated that incorporation of BLP significantly reduced ($P < 0.05$) the glycaemic response of pasta. So that at 20% inclusion rate the standardised AUC value was significantly lower than the control sample. This indicated the potential

to increase the slowly digestible fraction of carbohydrate in the sample by reducing starch degradation. Higher protein content can lower the glycaemic response by starch granules embedded in the protein matrix and may limit the access of enzymes to starch granules (Liu et al., 2016). But BLP 20% pasta increased the glycaemic response at initial 20 min. This can happen by creating ruptures in pasta structure due to high concentration of protein (Kadam & Prabhasankar, 2012) and expose starch granules to the enzymes. Control pasta AUC results were similar to the in vitro pasta digestion study of Foschia et al. (2014).

Table 3.5 Pasta reducing sugar release with *In vitro* digestion.

Minutes	**Reducing sugar (mg)/ pasta(g)**			
	Control Pasta	**BLP 10% pasta**	**BLP 15% pasta**	**BLP 20% pasta**
0	4.09 ± 15.13^{a3}	1.05 ± 8.85^{a3}	12.83 ± 5.28^{a3}	9.73 ± 2.69^{a3}
20	211.27 ± 7.49^{a2}	158.85 ± 15.46^{a2}	118.21 ± 27.81^{ab2}	195.36 ± 24.55^{b2}
60	282.85 ± 11.77^{a1}	233.14 ± 16.86^{b2}	230.46 ± 10.77^{b1}	230.51 ± 14.76^{b1}
120	305.33 ± 15.31^{a1}	276.81 ± 14.92^{ab1}	252.49 ± 26.47^{ab1}	263.05 ± 11.70^{b1}
Standard AUC	222.61 ± 4.21^{a}	185.53 ± 6.47^{bc}	170.79 ± 13.25^{c}	190.31 ± 8.18^{b}

AUC, area under the curve
Different superscripts in a row wise (lower case alphabet) and column wise (numbers) differ significantly ($P<0.05$)
Results are the means (± SD) of triplicate samples (n = 3)

3.3.5 Indispensable amino acid score

Indispensable amino acids are a critical component of protein quality as they cannot be synthesised in the human body. On the basis of colour, cooking characteristics and elongation of the texture results, the IAAS of control and 10% BLP pasta were compared (Table 3.6).

The BLP protein had 45% indispensable amino acids (IAA) compared to the 31% of control pasta. Incorporating 10% of BLP into the control pasta increased the IAA value to 37%. However, IAAS is the proper method to analyse the quality of the protein and in this experiment, IAAS was calculated using the reference pattern for adults and older child (FAO, 2011). Table 3.6 illustrates the level of histidine, tryptophan and sulphur amino acids and follows values reported earlier (Fornias, 1996; Mullen et al., 2017). However, their concentrations were higher than that recommended by the FAO (2011) an observed in the AAR value.

In this study, BLP was a balanced source of amino acids and achieved the highest IAAS (1) for protein quality according to the FAO expert consultation 2011 reference pattern (Table 3.6). Histidine and

lysine were the most limiting amino acids in control pasta according to the amino acid ratio (AAR). After incorporation of BLP at 10%, lysine was no longer limiting with an AAR >1 while the histidine ratio improved from 0.56 to 0.91 (Table 3.6). This illustrates that BLP has capacity to enrich the quality of plant protein.

Table 3.6 Amino acid ratios and indispensable amino acid scores (IAAS) of BLP and 10% BLP pasta.

Amino acid	**Beef lung powder (mg/g protein)**	**control pasta (mg/g protein)**	***10% Beef lung powder pasta (mg/g protein)**	**Older child, adolescent & adult reference pattern**	***Beef Lung powder AAR**	***control pasta AAR**	***10% pasta AAR**
Tryptophan	24.77 ± 3.34	15.51 ± 0.02	19.69	6.6	3.75	2.35	2.98
Threonine	43.51 ± 2.16	28.70 ± 0.03	35.17	25	1.74	1.15	1.41
Isoleucine	34.71 ± 1.13	36.50 ± 0.04	35.71	30	1.16	1.22	1.19
Leucine	88.72 ± 4.21	70.90 ± 0.06	78.68	61	1.45	1.16	1.29
Lysine	81.06 ± 4.95	22.99 ± 0.01	48.38	48	1.69	0.48	1.01
SAA	30.08 ± 0.97	23.71 ± 0.12	26.49	23	1.31	1.03	1.15
AAA	69.47 ± 3.02	66.72 ± 0.24	67.91	41	1.69	1.63	1.66
Valine	59.90 ± 3.01	38.42 ± 0.08	47.81	40	1.50	0.96	1.20
Histidine	21.59 ± 2.08	9.00 ± 0.14	14.51	16	1.35	0.56	0.91
IAAS					**1**	**0.48**	**0.91**

Reference pattern adopted from FAO Expert consultation, 2011; AAR, amino acid ratios; SAA, sulphur amino acid (methionine + cysteine); AAA, aromatic amino acid (phenylalanine + tyrosine)
Results are the means (± SD) of triplicate samples (n=3)
*Denotes calculated values

3.4 Conclusion

The incorporation of BLP into fresh pasta illustrated that BLP is a valuable under-utilised by-product in terms of its potential to improve the nutritional quality of protein-poor, carbohydrate-rich cereal foods, such as pasta. The incorporation of 10% BLP into fresh pasta was determined to be optimal based on the colour, cooking characteristics and elongation of textural properties. The inclusion of 10% BLP into pasta improved the iron content fivefold, increased the protein content by 60% and the IAAS from 0.48 to 0.91. BLP also decreased the glycaemic response of pasta. The opportunity exists to

improve the functional value of food products by the incorporation of lung material; however, further research is required to determine the sensory profile, consumer preference and microbiological content to establish the shelf life compared to conventional pasta. Pasteurisation may be required for industrial scale-up to meet the regulations of particular country.

Chapter 4

Effect of Different Drying Temperatures on the Physicochemical Properties of Beef Lung Powder

4.1 Introduction

Protein supplements play a crucial role in improving the nutrition status of various groups of people in society such as elderly people, pregnant women, malnourished people and those people recovery from wounds or surgery. The high demand for protein supplements and the cost of the protein have increased the price of protein supplements globally. Thus, scientists and food industry have started searching for underutilised protein sources for production of low-cost high-quality protein products. Given the fact that meat carcases constitute only 45% to 60% of animal live weight (Muir, Thomson, & Askin, 2008) and much of the remaining of the animal is protein-rich co-products (Lungs, kidney, heart, tongue etc..), there is an ample opportunity to utilise these co-products as a source of protein and its products. Making use of various underutilised co-products is a good strategy to produce nutritious and safe protein supplements since co-products have been used and still are consumed by many people around the world and have long history of safety. Furthermore, using the co-products will help to reduce environmental impact caused by disposing the material and improve profit margin since the raw material is cheap. The beef industry has a high cost production, and it is important to focus on underutilised co-products such as beef lungs, to increase production efficiency. Beef lungs has a high protein content and has been used as food in many cultures and sometimes used for pet food, but mostly rendered or considered as low value material.

Co-products are generally more prone to contamination than meat and are highly perishable and susceptible to microbial growth. Thus, it requires preservative techniques to stabilise them during storage, such as drying or freezing. The drying process suppresses the growth and multiplication of spoilage microorganisms by controlling the availability of water and is one of the best unit operations to preserve animal products (Traffano-Schiffo, Castro-Giráldez, Fito, & Balaguer, 2014). Dried protein powders have been successfully developed from different protein sources (Bishnoi et al., 2015; Ohkuma et al., 2008) including the protein powder from beef lungs developed in the previous study (Chapter 3). The beef lung powder had useful nutritional properties, did not require special storage conditions and could conveniently be used as an ingredient in fortified foods. Thorough characterisation of this newly developed protein powder, including its physical parameters, would be necessary before it could be proposed as an ingredient for development of protein supplements.

Different physical properties of food ingredients are important to the food industry at different processing stages, and these parameters are interconnected with each other. For instance, moisture content, particle size and shape (morphology) all affect the bulk density and powder flowability and physical properties of emulsions such as viscosity and solids concentrations (O'Hagan, Hasapidis, & Coder, 2015; Walton & Mumford, 1999). Both physical and chemical properties can change due to use of different processing conditions and these can directly affect the quality of the developed products. Physical parameters like hygroscopicity give an idea about moisture absorption to powder and are important to determine the storage conditions and packaging materials in industrial scale. Flowability of the powder is important to determine the unit operations and relevant machines to maintain the powder without flowing in processing premises. Bulk densities and absolute densities give an idea about relationship of volume and weight of the product and important parameter for distribution channels after processing. While our previous study (Chapter 3) reported the nutritional potential of beef lung protein powder, no information is available about the effect of different processing conditions on its quality and physical properties. In the present study, the impact of different processing temperatures during the preparation of beef lung protein powder on its physicochemical properties has been determined.

4.2 Methodology

4.2.1 Processing of beef lung powder

Hygienically derived frozen beef lungs (10 kgs) without trachea processed into beef lung powder using the facilities in the food-processing laboratory at Lincoln University (Lincoln, New Zealand). Beef lungs were thawed overnight at 4˚C and discoloured areas trimmed to obtain clean lungs. The trimmed lungs were separated into nine samples (970g ±122) and minced individually (TS-102aAL, Tasin, Taichung, Taiwan). Three of these minced samples were air oven-dried (E32M, Bakbar, Christchurch, NZ) at each temperatures until they reached a constant weight. This took 23 h and 20 minutes at 50 ˚C, 10 h and 55 minutes at 70 ˚C and 6 h at 100 ˚C. The weight of the dried samples was measured frequently, and the surface crust was broken for steady moisture evaporation. The dried beef lungs were ground into powder using an FP920 grinder (Kenwood, China) for 15 minutes, vacuum-packed in LDPE packaging and stored at room temperature for further analysis. The BLP tested for storage stability by measuring protein oxidation, lipid oxidation and microbiology growth was stored for six months

4.2.2 Particle size distribution, bulk density, absolute density, porosity & flowability

Dried beef lung powder samples were sieved using a series of standard sieves (0.5, 1.18 and 2.26 mm) to determine the particle size distribution of each sample. Other physical properties were measured after sieving the samples through the 500 µm sieve.

Bbulk density (þbulk)was measured according to Mahdavi, Jafari, Assadpoor, and Dehnad (2016) with 1 g of beef lung powder placed in a 10 mL plastic measuring cylinder. The measuring cylinder was tapped a constant volume was reached. The bulk density of the sample was calculated as the ratio between the weight of powder in the cylinder and the filled volume. The absolute density (þabs) was measured with a pycnometer using RO water as a standard.

The porosity (ε) was calculated according to equation 1 using bulk density to absolute density ratio (Rahman, Perera, Chen, Driscoll, & Potluri, 1996)

ε = 1 –(þbulk/þabs) ……. (Equation-1)

Flowability of the beef lung powder was analysed using Hausner ratio (HR), which is a number correlated to flowing ability of powders (Hausner, 1967). The BLP powder (10 g) was placed in a 25 mL measuring cylinder and the initial volume (Vb) was recorded. The filled cylinder was tapped 10 times by hammer and the final volume (Vf) was recorded. HR was calculated by using the formula mentioned in equation 2 based on the ratio of Vb and Vf. The flowability was classified into four categories (Hausner, 1967). (1) HR value 1.0 -1.1, free-flowing powder; (2) HR value 1.1-1.25, medium flowing powder; (3) HR value 1.25 - 1.4, difficult flowing powder and (4) HR value > 1.4, very difficult flowing powder.

Hausner ratio (HR) = (Vb / Vf)× 100…… (Equation-2)

4.2.3 Hygroscopicity of beef lung powder

Hygroscopicity of beef lung powder was measured according to the method of Cai and Corke (2000) with minor modifications. Samples (2 g) were taken in pre-weighed Petri dishes which were placed in an airtight plastic container with saturated sodium chloride solution. The airtight box was placed in an incubator at 30 ˚C to obtain constant relative humidity (75.09% RH). After 7 days, the hygroscopic moisture was weighed gravimetrically. Samples were analysed in triplicate. Results were expressed as absorbed moisture per 100 g of dry matter.

4.2.4 Water solubility index (WSI) and Water binding capacity (WBC)

The water solubility index was measured according to the method described by Mahdavi et al. (2016). The BLP power (12.5 g) was vortexed thoroughly with 30 mL RO water in a 50 mL centrifuge tube for

2 min. Then, the sample was incubated at 37 ˚C in a water bath for half an hour and subsequently was centrifuged at 17,640 x g for 20 min at 4 ˚C. The supernatant was collected into a pre-weighed beaker and oven-dried at 105 ˚C overnight. The weight difference was determined after drying and WSI was calculated by equation-3. The remaining pellet in the centrifuge tube was weighed to determine the water-binding capacity (WBC) of the sample using equation-4.

$$WSI = \frac{(weight\ difference\ of\ the\ supernatant\ after\ drying)}{Initial\ sample\ weight} \times 100 \quad \text{..... (Equation-3)}$$

$$WBC = \frac{(weight\ of\ pellet - Initial\ sample\ weight)}{Initial\ sample\ weight} \times 100 \quad \text{..... (Equation-4)}$$

4.2.5 Colour measurements

Colour values 'L*', 'a*' and 'b*' parameters were determined using a Chroma CR 400 colourimeter (Konica Minolta INC., Tokyo, Japan) with the illuminant C (CIE, standard, 6774 K). The colour values were measured at six random places on beef lung powder placed on black coloured paper. The L* value represents lightness, negative and positive a* values, represent the green to red colour spectrum, negative and positive b* represent blue and yellow colour components. The colorimeter was calibrated using a standard white tile (L = 98.03, a = -0.23 and b = 0.25). All values were mean of six replicate samples.

4.2.6 Pasting properties of BLP added semolina dough

Samples were prepared using three different levels of beef lung powder viz. 10%, 15% and 20% along with semolina flour. The semolina without BLP was used as a control. The apparent viscosity of the samples was measured by Rapid Visco Analyzer RVA S4 (Perten Instruments Pty. Ltd., Warriewood, Australia). Sample (3 g) was transferred into a canister and 25 mL of distilled water was added to it before operating the analyser. Standard general pasting method No. 1 heating and cooling cycle program was used where the samples were held at 50 °C and heated to 95 °C at 12 °C/min, a holding phase at 95 °C for 3.5 min, a cooling step from 95 to 50 °C at 10 °C/min, and a holding phase at 50 °C for 2 min were followed. Measurements of peak viscosity, trough viscosity, breakdown, final viscosity, setback and pasting temperature were directly recorded. Derived parameters were determined from direct measurements as described by Kumar, Brennan, Zheng, and Brennan (2018).

Stability ratio = trough viscosity / peak viscosity

Setback ratio = final viscosity / trough viscosity and

Relative breakdown = breakdown viscosity / setback viscosity

4.2.7 Protein (N) content of beef lung powder

Protein content of beef lung powder was determined using the AACC Micro-Kjeldahl method 113 976.05 (2000). Protein content was calculated by multiplying the nitrogen content with the factor of 6.25 (Lynch et al., 2018)

4.2.8 SDS-PAGE analysis of beef lung powder and raw beef lung

The protein profile of the BLP and raw lung samples was studied by using SDS-PAGE following the procedure described by Bhat, Morton, Mason, and Bekhit (2018)

Protein extraction from beef lung powder

Dried beef lung powder (0.5 g) or sample from fresh lungs (1 g) was homogenised for 60 s at 10,000 rpm with a Polytron homogeniser using 10 mL extraction buffer (0.1 M KCl, 1 mM EDTA, 1 mM Sodium azide (NaN_3), 25 mM potassium phosphate buffer at pH 7.0). The homogenate was centrifuged at 1000 g for 15 minutes at 4 $^{\circ}$C and the pellet was resuspended in buffer after supernatant was separated. The procedure was repeated three times for maximum extraction.

SDS-PAGE gel casting (Bio-Rad Mini-PROTEAN® 3)

12.0% Bis-Tris (0.75 mm × 10 well) gel (acrylamide: bisacrylamide = 37.5:1 [w/w], 0.1% [w/v] SDS, 0.05% [w/v] ammonium persulfate, and 0.384 M Tris–HCl, pH 8.8) was used as resolving gel. Stacking gel was a 4.0% polyacrylamide gel (acrylamide: bisacrylamide = 37.5:1 [w/w], 0.1% [w/v] SDS, 0.07% [w/v] ammonium persulfate, and 0.125 M Tris–HCl, pH 6.8). The polymerisation of both resolving gel and stacking gel was initiated by adding 0.1% [v/v] TEMED. The resolving gel was first added to Bio-Rad Mini-PROTEAN® 3 Casting Frame and immediately smoothing the gel surface by adding isobutanol. Isobutanol was washed out by RO water and blotted dry after 30 minutes of solidifying. Then stacking gel was added, and the teeth-comb was inserted to produce loading wells. The comb was unplugged after solidifying (30 minutes) the stacking gel and the trapped air in wells was flushed out using RO water.

SDS-PAGE running

Standardised 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 β-mercaptoethanol) and incubated 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 prepared in a Bio-Rad Mini-PROTEAN® 3 Casting Frame. The electrophoresis was performed in a Mini-Protean III electrophoresis (Bio-Rad Laboratories, Hercules, CA) tank using SDS (1×) running buffer (25

mM Tris-HCl, pH 8.6; 192 mM glycine; 0.1% SDS) at 150 V for 90 min at room temperature (21 °C ± 2.0). Fixing solution (50% Methanol: 7% Acetic acid) was used (20 mL) to prevent leaching out of proteins by shaking with gels for 15 minutes. Gels were washed three times in RO water for 5 min each time and stained in 20 mL GelCode® Blue Stain Reagent (Pierce) for one hour with gentle shaking on the rocker. Gels were destained in distilled water overnight to reveal the bands. The bands in the gels were analysed by Gel Doc Imaging System (XR+, Bio-Rad Laboratories Inc, Redmond, USA) using Image lab TM software (Version 6.0; Bio-Rad Laboratories).

4.2.9 Amino acid profile and mineral profile of beef lung powder

Amino acid and mineral profiles were determined following the same protocol of Chapter 3.2.5. Amino acid profile was measured using a HPLC 1100 series (Agilent Technologies, Waldrbom Germany). Minerals profile was measured using an Inductively Coupled Plasma Optical Emission Spectrophotometer (Varian 720 ICP-OES, Melbourne, Australia). (refer Chapter 3.2.6)

4.2.10 Haem iron analysis

Haem iron was analysed as described by Hornsey (1956). Beef lung powder (0.5 g) was transferred into a 15 mL Falcon tube and 7 mL of acidified acetone (40 ml of 32% acetone, 1 mL of concentrate HCl, 9 mL of distilled water) was added. The sample was homogenised for 15 s by vortex and 7 mL of acidified acetone was added again to the falcon tube. The sample tube was caped tightly and kept for 1 hour in dark. The samples were centrifuged at 2200 x g for 10 min and the supernatant was filtered using Whatman 3 filter paper with accelerating vacuum filtration unit. The filtrate absorbance was measured at 640 nm against the reagent blank. For raw beef lung, 2g sample was used for the experiment and the moisture content of the sample was considered. Falcon tubes were covered by aluminium foil throughout the analysis to avoid sunlight. Haematin was calculated by using a factor 136 µg haematin/g meat/mL (Hornsey, 1956) and the experiment was validated by using a standard haematin sample (product No: H3281, Sigma Aldrich, MO, USA). Haematin and haem iron were calculated according to the following equations (5 and 6):

Haematin µg /g sample = (AB× 136×final volume (mL)) / sample weight (g) …… (Equation-5)

Iron µg /g sample = (AB× 136×final volume(mL)× 55.847) / (sample weight (g) ×633.49) …… (Equation-6)

*Final volume: Added acidified acetone (15mL), All weights were in dry matter basis

4.2.11 Determination of protein oxidation using the DNPH method

The extent of protein oxidation was determined by evaluating the total carbonyl content using dinitrophenylhydrazine (DNPH) as described by Oliver, Ahn, Moerman, Goldstein, and Stadtman (1987) with slight modification. Dried meat powder sample (1 g) was weighed in 50 mL falcon tube and was homogenised using 1:10 (w/v) 20mM of sodium phosphate buffer (10 mL) containing 0.6 M NaCl (pH 6.5) for 30 s at 10000 rpm. Homogenate (0.2 mL) was transferred into separate Eppendorf tubes for protein and carbonyl measurements. Then, cold TCA (10%) was added and centrifuged at 5000 rpm for 5 minutes to precipitate the protein. After discarding the supernatant, 1 mL of 2 M HCl was added to one pellet for protein measurement and 1 mL of 0.2% (w/v) DNPH in 2 M HCl was added to other pellet for carbonyl measurement. Samples were incubated at room temperature for 1 hour with vortexing every 15 minutes. TCA (10%) was added and the samples were centrifuged (Centra GP6R, Thermo IEC, Needham Heights, MA, USA) at 5000 rpm for 5 minutes to separate the pellet. All pellets were washed 3 times with ethanol: ethyl acetate (1:1, v/v) to remove excess DNPH. Then the pellet was dissolved in 1.5 mL of 20 mM sodium phosphate buffer containing 6 M guanidine HCl (pH 6.5). Samples were stirred and centrifuged (Centra GP6R, Thermo IEC, Needham Heights, MA, USA) at 10000 rpm for 5 minutes to take the supernatant. Protein concentration of the supernatant was measured by using BCA protein assay kit at 562 nm. The absorbance of the samples was measured at 370 nm using 1 cm path length cuvettes. The carbonyl content was measured using OD and coefficient (21 $nM^{-1}cm^{-1}$) for protein hydrazones (Oliver et al., 1987). The carbonyl content expressed as nmol per milligram of protein using equation 5.

Carbonyl concentration= [Absorbance (370 nm)/21 nM-1cm-1]/protein concentration (mg/mL)…… (Equation-5)

4.2.12 TBARS assay for lipid oxidation

Lipid oxidation was assessed by measuring the TBARS in the samples using the method described by Pearson, Love, and Shorland (1977). A 5 g of minced raw sample or beef lung powder was homogenised in 25 mL distilled water for 1 min using a Polytron homogeniser 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 milligram malondialdehyde per kilogram sample.

4.2.13 Microbiology analysis

Total viable count, coliform and yeast and mould were determined using ready to use media plates (For Richard Laboratories, Auckland, New Zealand). Ten grams were obtained from each dried sample under complete aseptic conditions. Samples were homogenised with 90-mL sterile peptone water (Marks, Darmstadt, Germany) in sterile bag for 2 min using stomacher. Tenfold serial dilutions were prepared from the original homogenate (Swanson, Petran, & Hanlin, 2001). The samples were prepared near flame under laminar flow (Serial No. 8801/88, Gelman Sciences PTY. LTD., Australia).

4.2.14 Analysis for total aerobic bacteria, coliforms and fungi

Total viable counts (TVC)

Total viable counts were enumerated by spreading 100 µl from each dilution on the surface of plate count Agar and incubated at 35 °C for 48 h (Morton, 2001). The dilution of plates showing 30 to 300 colonies were selected for enumeration and expressed as colony forming units per gram (cfu/g) of sample

Coliform count

Coliform and other gram-negative pathogens were enumerated by inoculation of plate of MacConkey agar. The plates were incubated at 35 °C±2^{0}C for 48 h and counts were determined as colony forming units per gram (cfu/g) of sample.

Yeast and moulds

Yeast and moulds were enumerated by inoculation of plates of Sabaroud dextrose agar followed by incubation at 25±2^{0}C for 5 days (Beuchat & Cousin, 2001). Counts were determined as colony-forming units per gram (cfu/g) of sample.

4.2.15 Statistical analysis

The data generated by repeating the experiments for different parameters were compiled and analysed using SPSS (IBM SPSS Statistics V22.0, Armonk, NY, USA). All experiments were performed in

triplicate unless otherwise stated. All data were reported as means ± standard deviation. Differences between the treatments were analysed by one-way analysis of variance, at the 0.05 level of significance. When the ANOVA was significant ($P < 0.05$), means were separated by a pairwise comparison using Tukey's comparison test.

4.3 Results and discussion

4.3.1 Drying pattern of beef lungs with different drying temperatures

The bovine lungs were dried at 50 °C for 1400 minutes, 70 °C for 665 minutes and 100 °C for 360 minutes to reach constant weight (Figure 4.1). Different drying temperatures reached different moisture levels of final products, at 50 °C to 4.78%, 70 °C to 1% and 100 °C to 0.4% (average moisture content). Figure 4.1 illustrates that the drying curves were straighter than typically seen in the literature. Generally drying curves are more curved due to the decline in evaporation rate with time (Trujillo, Wiangkaew, & Pham, 2007). In our study, regular breaking of the surface crust facilitated evaporation while drying the minced bovine lungs which may have reduced the curvature.

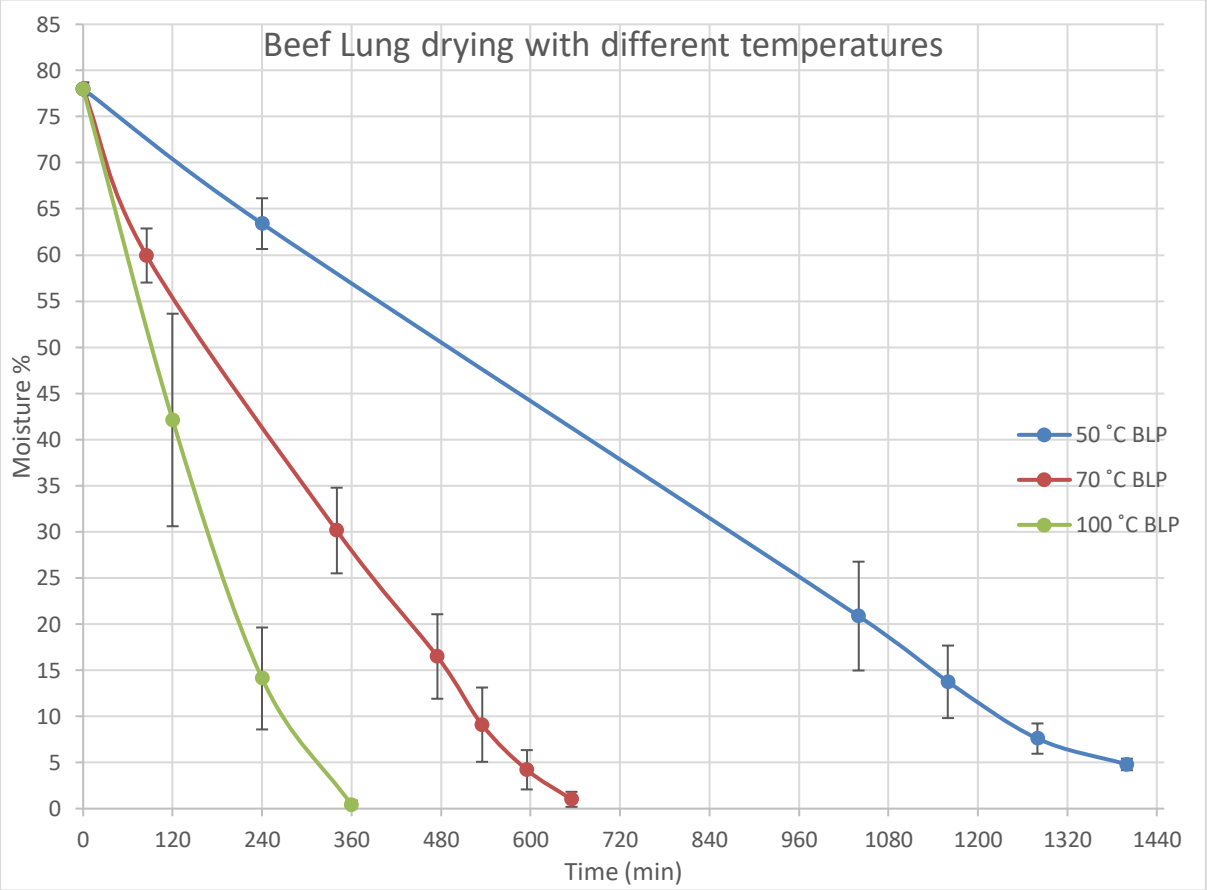


Figure 4.1 Beef lung drying with different temperatures

4.3.2 Particle size distribution of bovine lung powder

A considerable amount (45% to 70%, w/w) of fine beef lung powder (<0.5mm) was obtained at all drying temperatures (Figure 4.2). The increase in temperature of drying led to high percentage of fine particles (<0.5 mm). Drying at lower temperatures caused lower percentage of fine particles due to a greater caking effect resulted from a high moisture content

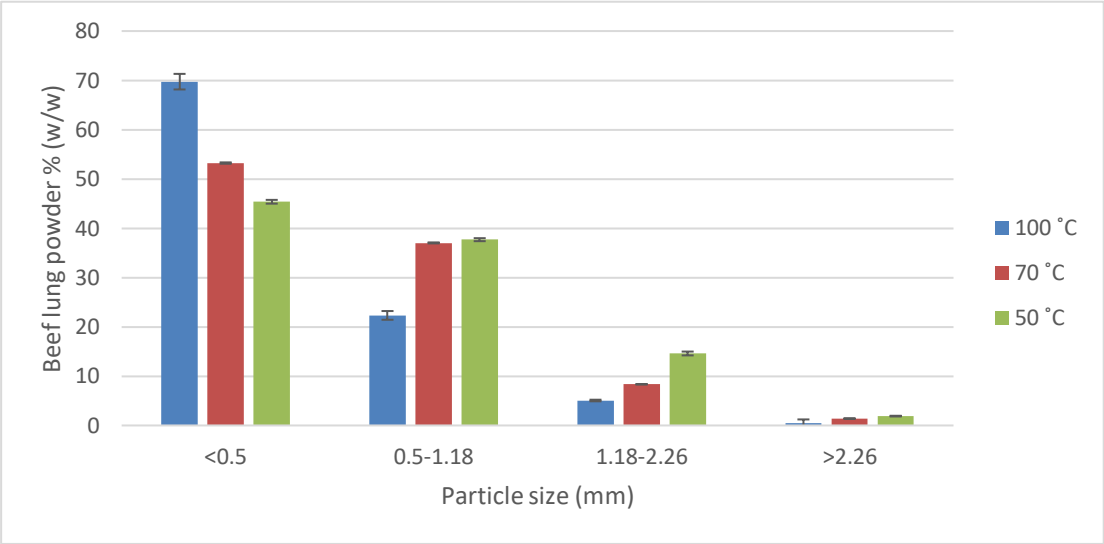


Figure 4.2 Beef lung powder particle size distribution with different drying temperatures

4.3.3 Bulk density, Absolute density, Porosity and Flowability

There was no statistically significant difference in bulk density among the different drying temperatures, but a pattern of increasing average bulk density was observed between 50 ˚C to 100 ˚C. The increment of bulk density with temperature may be due to closer packing of the powder with finer particles (Table 4.1). Higher bulk density lowers the occupying space and is a commercially important property to reduce the cost of storing and transportation. Absolute density corresponds to the solid density and spaces between particles are not taken into account (Tonon, Brabet, & Hubinger, 2010). Absolute density was significantly increased ($p<0.05$) with higher drying temperature (Table 4.1), and this may be a result of the reduced moisture content of the particles as the drying temperature is increased (Table 4.1). Porosity was significantly decreased by increasing of drying temperature from 50 ˚C to 100 ˚C drying and these results were similar to refractance window drying of meat powder reported by Rostami, Dehnad, Jafari, and Tavakoli (2018). Porosity indicates the air space inside the materials and intragranular spaces (López-Córdoba & Goyanes, 2017). High porous granules could lead to granular cavities and high surface area which can potentially change biochemical reactions (Sujka & Jamroz, 2007) such as digestion(Chapter 5). The Hausner ratio (HR) is an indication of powder flowability, and the HR for all beef lung powders was around 1.3 in the range of difficult flowing according to Hausner (1967).

4.3.4 Water binding capacity, water-soluble index and hygroscopicity

The affinity of water or moisture towards the dried bovine lung powder is important to determine the processing and storage conditions. Table 4.1 shows the water binding capacity (WBC), water soluble index (WSI), Hygroscopicity, and moisture content of the beef lung powder.

Significant differences in water binding capacity (WBC) were not observed with increasing drying temperature. All dried powder absorbed threefold the sample weight moisture (Table 4.1). A high-water binding capacity is an important feature in food processing industry. For instance, high WBC ingredients can be possibly used in the baking industry to bind water in dough (Southward, 2003).

The Water Soluble Index (WSI) is a measurement of the solubility of biomolecules like protein, carbohydrate, vitamins, minerals (Sharma, Singh, Hussain, & Sharma, 2017). The WSI of the BLP decreased significantly with the higher drying temperatures (Table 4.1). Hygroscopicity percentage also significantly decreased from 50 ˚C to 100˚C temperature BLP drying.

The moisture contents of the BLP powders were significantly reduced as drying temperature increased from 50 ˚C to 100 ˚C drying. Tonon et al. (2010) explained that low moisture powder has a higher

capacity to absorb moisture as a result of the water concentration gradient between the product and its surrounding. In contrast to this mechanism, water binding capacity did not increase significantly and WSI, hygroscopicity decreased with rising temperature. This may be a result of protein denaturation and exposure of the hydrophobic regions of the protein's core at high temperature which would generate repulsive force towards water molecules.

Considering these physical parameters, BLP should be stored in a dry and cold place in a moisture impermeable package, such as an aluminium pouch, soon after processing due to the high WBC and hygroscopicity.

4.3.5 Colour of beef lung powder

All colour parameters (L*, a*, and b*) of bovine lung powder were decreased with drying at higher temperatures. Beef lung powder dried at 50 ˚C were lighter (L* value) and yellower (b* value) and would be suitable for incorporating with light coloured food products. The redness value (a* value) of beef lung powder dried at 100 ˚C was significantly reduced compared to that of the BLP powder dried at 50 ˚C and 70 ˚C temperatures. At higher temperatures (70 ˚C to 100 ˚C), the red coloured myoglobin was possibly oxidised into the brown coloured metmyoglobin and metmyoglobin further converted into denatured metmyoglobin, which formed a dark brown colour (Shimokomaki, Youssef Youssef, & Terra, 2003). Similar results where redness was in the range from 2 to 5 have been reported in beef jerky (Kučerová, Marek, & Banout, 2018).

Table 4.1 Beef lung powder characteristics, colour values and nitrogen content with different temperature drying

Powder characteristics	**50 ˚C dried BLP**	**70 ˚C dried BLP**	**100 ˚C dried BLP**
Bulk Density(g/mL)	0.56±0.03	0.59±0.04	0.65±0.02
Absolute Density(g/mL)	1.18±0.02^{c}	1.27±0.03^{b}	1.48±0.03^{a}
Porosity	0.62±0.02^{a}	0.54±0.03^{b}	0.45±0.02^{c}
Hausner Ratio	1.29±0.04	1.28±0.03	1.32±0.01
WBC%	314±4.3	316±1.8	317±1.8
WSI %	16.69±0.91^{a}	11.76±0.19^{b}	10.36±0.15^{c}
Hygroscopicity %	15.97±1.03^{a}	13.22±0.55^{b}	13.48±0.81^{b}
Moisture content %	4.79±0.63^{a}	1.01±0.82^{b}	0.40±0.49^{c}
Colour values			
L*	62.72±1.60^{a}	55.20±1.59^{b}	53.61±1.41^{b}
a*	4.81±0.90^{a}	5.12±0.15^{a}	3.89±0.17^{b}
b*	18.26±0.60^{a}	13.07±0.96^{b}	12.35±0.50^{b}
Nitrogen content			
N% DM	13.67±0.26	13.58±0.07	13.54±0.06

* Mean ± Standard deviation in the same row followed by different superscript differ significantly at p<.05
BLP-beef lung powder, WBC-water binding capacity, WSI- water soluble index, DM- dry matter basis, N%-nitrogen content

4.3.6 The viscosity parameters of semolina flour with beef lung powder

Adding beef lung powder to the semolina flour resulted in a decrease in all viscosity parameters of the dough (including peak viscosity, through viscosity, final viscosity as well as break down and setback values) (Table 4.2). This situation is clearly illustrated in Figure 4.3 where the viscosity graphs are lower with increased beef lung powder percentage. A similar viscosity trend was seen after adding whey proteins to either taro flour (Onwulata and Konstance (2002) or rice flour (Shin, Gang, & Song, 2010).

Drying temperatures can affect the viscosity of the flour mixture and this was clearly identified with the different temperature curves at 10% substitution (Figure 4.4). Figure 4.4 illustrates that the control semolina had the highest viscosity, followed by 50 ˚C BLP substitutions, 70 ˚C BLP substitutions and 100 ˚C substitutions.

High peak viscosity curves generally lead to high breakdown viscosity (Tsakama, Mwangwela, Manani, & Mahungu, 2010) and high breakdown viscosity leads to unstable gel structures. Kumar et al. (2018) described that lower breakdown and higher stability ratio indicated low hydration, low swelling power, and high shear resistance, which lead to more stable gels. In agreement of these findings, the results of 50 ˚C BLP substitutions showed an inverse relationship between the breakdown viscosity and stability ratio (Table 4.2). High substitution of 50 ˚C BLP significantly increased the stability ratio (Table 4.2) which means that the addition of bovine lung powder leads to stable gel structure. The stable gels eventually could lead to increase the firmness of the product. The study of Brabet et al. (2013) showed the strong correlation between the stability ratio and firmness of noodles. The overall idea is that a higher percentage of 50 ˚C BLP incorporation would lead to a higher stability ratio and consequently would increase the firmness of the product. Previous work with BLP incorporated pasta (Chapter 3) confirmed this scenario. The firmness of pasta increased with high level of beef lung powder incorporation.

Setback ratio is defined as the ratio of viscosity at the completion of cooling (final viscosity) to the viscosity at the onset of cooling (trough viscosity) as described by Kim, Wiesenborn, Lorenzen, and Berglund (1996). Setback ratio is a predictive parameter of retrogradation (Kim et al., 1996) which is directly affect the storage stability. In the present study, both BLP increasing percentage and drying temperature increment decreased the setback ratio (Table 4.2). Further, Increment of protein percentage reduced the retrogradation due to reduction of starch concentration (Chen, Schols, and Voragen (2003). High level of BLP incorporation and high drying temperature of BLP appear to favour the BLP supplemented product from retrogradation point of view.

The relative break down pattern of semolina dough is shown in Table 4.2. At the breakdown, swollen starch granules break further, and amylose leaches to the solution (Zaidul, Norulaini, Omar, Yamauchi, & Noda, 2007). Table 4.2 illustrates that the relative breakdown decreased with the BLP

percentage corresponding to increasing substitution results reported by Kumar et al. (2018). However, no clear pattern related to drying temperature could be identified. The pasting temperature was increased by 4 degrees by adding 50 ˚C beef lung powder similar to adding whey protein and caseinate in Shin et al. (2010), but 70 ˚C and 100 ˚C beef lung powder did not show these temperature increments. Altogether, to maintain the viscosity characteristics similar to control semolina flour, 50 ˚C dried BLP with 10% incorporation level should be appropriated for semolina dough.

Table 4.2 RVA viscosity parameters of beef lung powder with semolina flour

Test	Peak viscosity (cP)	Trough viscosity (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak Time (min)	Pasting Temp (˚C)	Stability ratio	Setback ratio	Relative breakdown
Control semolina	2570±233^{a}	1940±105^{a}	631±127^{a}	3587±202^{a}	1641±101^{a}	5.51±0.03^{b}	87±0.51^{f}	0.76±0.029^{c}	1.84±0.02^{e}	0.38±0.06^{a}
50-10%-BLP	2034±81^{b}	1610±92^{b}	451±5^{b}	3153±134^{b}	1574±47^{ab}	5.47±0.00^{bc}	89±0.03^{bcd}	0.79±0.024^{c}	1.96±0.06^{cd}	0.29±0.01^{b}
50-15%-BLP	1496±87de	1270±49de	227±38^{cd}	2664±101^{cd}	1394±53^{cd}	5.42±0.04^{bcd}	90±0.46^{ab}	0.85±0.017^{b}	2.10±0.005^{ab}	0.16±0.02^{cd}
50-20%-BLP	1155±81^{f}	1062±66^{f}	93±14de	2332±129^{e}	1270±62de	5.40±0.07^{cd}	91±0.46^{a}	0.92±0.007^{a}	2.20±0.02^{a}	0.07±0.01^{e}
70-10%-BLP	1781±71^{bc}	1536±23^{b}	245±52^{c}	2970±9^{bc}	1434±16^{bc}	5.35±0.04^{d}	89±0.40^{cde}	0.86±0.024^{b}	1.93±0.02^{cde}	0.17±0.04^{c}
70-15%-BLP	1409±24^{def}	1332±37^{cde}	76±15^{e}	2686±53^{cd}	1354±67^{cde}	7.00±0.00^{a}	89±0.05^{bcd}	0.95±0.012^{a}	2.02±0.07^{bc}	0.06±0.01^{e}
70-20%-BLP	1235±89^{ef}	1158±82^{ef}	77±8^{e}	2418±135de	1260±53de	7.00±0.00^{a}	89±0.45^{bc}	0.94±0.003^{a}	2.09±0.03^{b}	0.06±0.005^{e}
100-10%-BLP	1650±22^{cd}	1515±45^{bc}	135±32^{cde}	2904±76^{bc}	1389±31^{cd}	5.22±0.04^{e}	87±0.49^{ef}	0.92±0.020^{a}	1.92±0.01^{cde}	0.10±0.03^{de}
100-15%-BLP	1499±44^{cde}	1438±37^{bcd}	61±8^{e}	2714±58^{cd}	1276±23de	7.00±0.00^{a}	88±0.83^{de}	0.96±0.005^{a}	1.89±0.01^{de}	0.05±0.01^{e}
100-20%-BLP	1298±70^{ef}	1208±76^{ef}	90±6^{e}	2411±103de	1203±29^{e}	7.00±0.00^{a}	86±0.06^{f}	0.93±0.008^{a}	2.00±0.04^{bc}	0.07±0.01^{e}

* Mean ± Standard deviation in the same column followed by different superscript differ significantly at p<.05

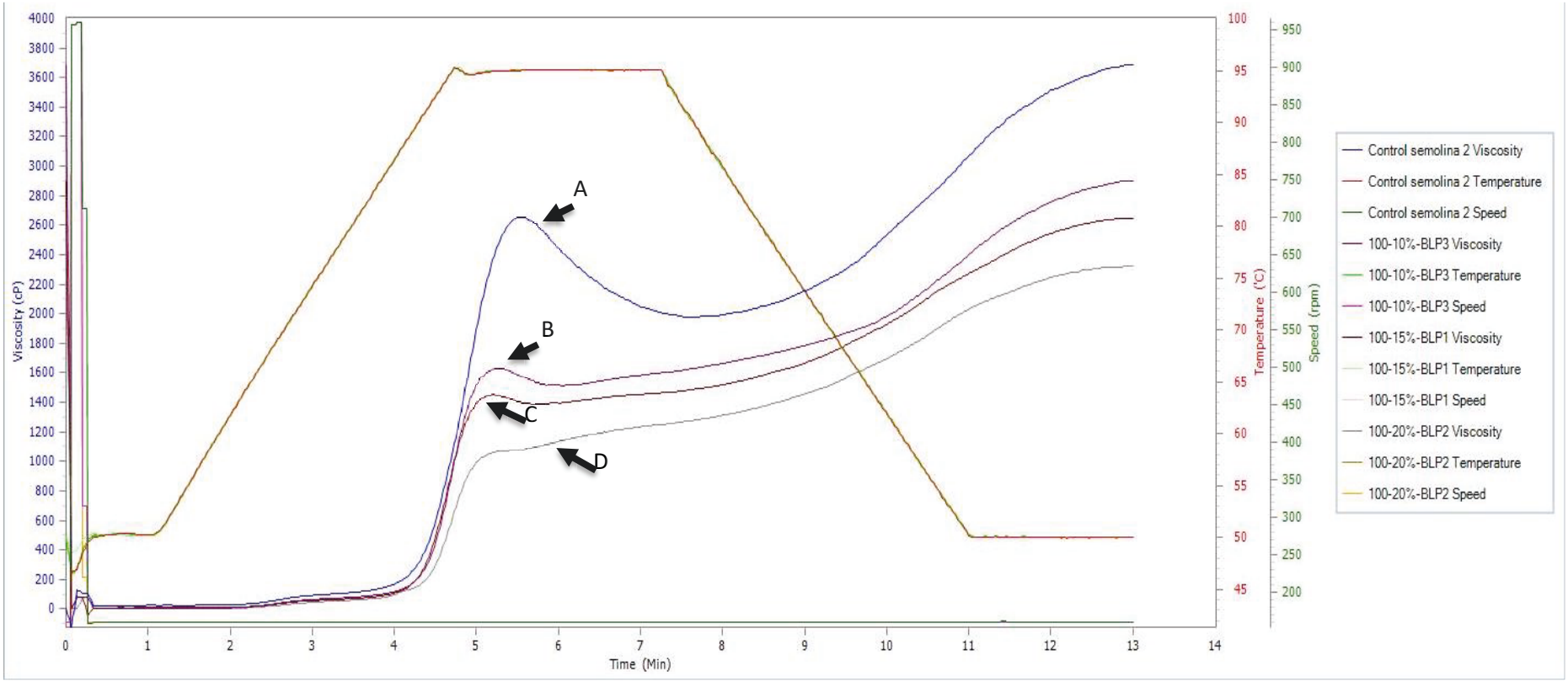


Figure 4.3 RVA viscosity changes with BLP ratio; A-semolina only(Control), B- 10% BLP(100 ˚C) with semolina (90%), C-15% BLP(100 ˚C) with semolina(85%), D-20% BLP(100 ˚C) with semolina (80%)

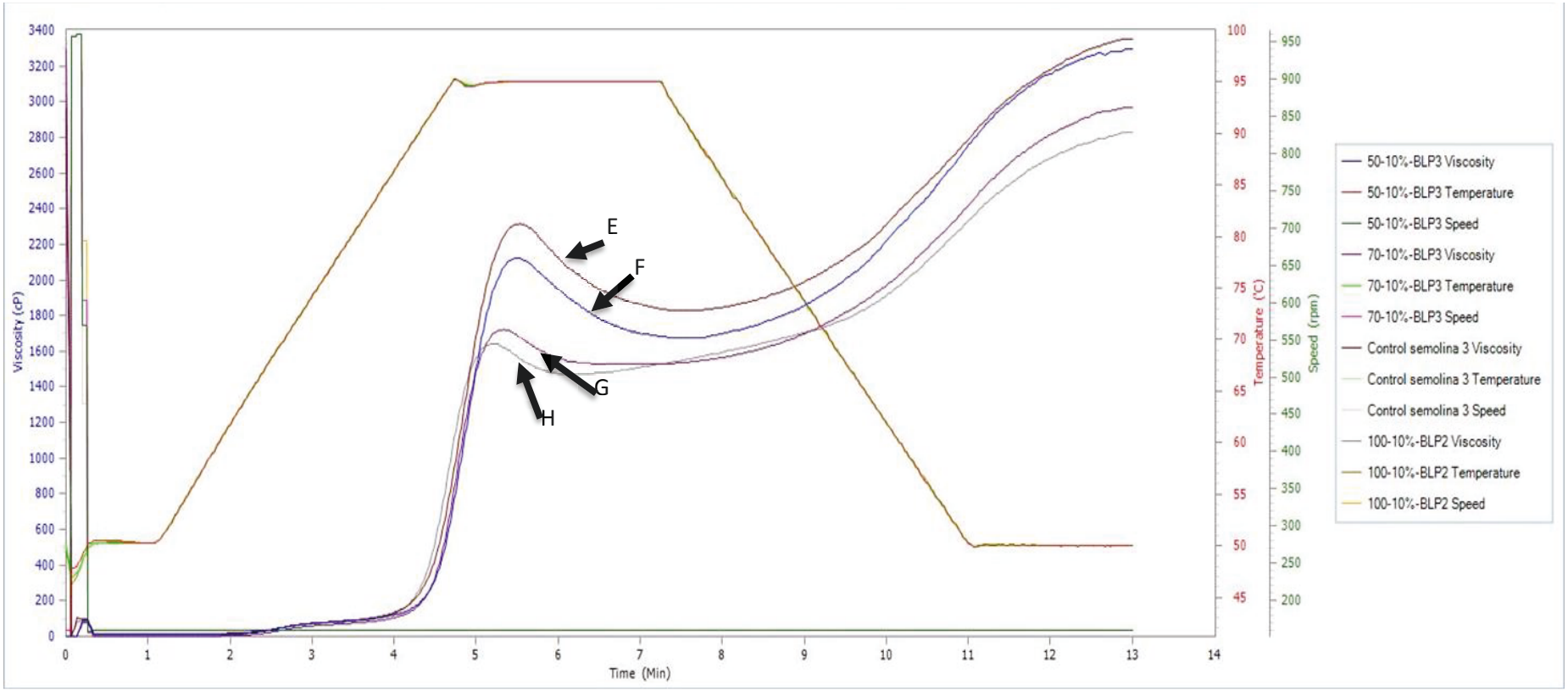


Figure 4.4 RVA viscosity changes with different drying temperature of BLP, E- semolina only (control), F- 50 ˚C BLP (10%) with semolina (90%), G-70 ˚C BLP (10%) with semolina (90%), H-100 ˚C BLP (10%) with semolina (90%)

4.3.7 SDS-PAGE analysis of beef lung powder and raw beef lung

The protein profile of dried beef lung powder was compared with the fresh beef lung protein extract using SDS-PAGE (Figure 4.5). The two thickest bands in fresh lung were at 67 and 10kDa. Band at 67 kDa was visible with decreasing intensity until the 70 ˚C drying temperature. A faint 67 kDa band was presented after Darine et al. (2010) extracted protein from beef lungs through alkaline solubilisation. The 44 kDa band in Figure 4.5 was also seen in the beef lung concentrate of Darine et al. (2010) as a thick band. The heat labile band at 27 kDa had completely disappeared after drying at 70 ˚C. The 17 kDa band was not seen in the raw beef lung and may have been produced by degrading larger molecules with heat. Band intensity analysis revealed that the (Table 4.3), 17 kDa peptide appeared in the dried powder and increased in intensity with temperature. The thickest band of our study was the 10 KDa band in raw meat and appeared as a weak band in 50 ˚C beef lung powder and completely disappeared from 70 ˚C to 100 ˚C. High temperatures could lead to protein aggregation and cause these low molecular protein bands to disappear. Overall, the lowest temperature of 50 ˚C drying beef lung powder preserved more bands compared with 70 ˚C and 100 ˚C beef lung powder (Figure 4.5).

Lower weight peptides have the potential for bioactive properties (Pihlanto-Leppälä, 2000) and raw beef lungs are possibly a good source for bioactive peptides due to the high intensity of bands around 10KDa.

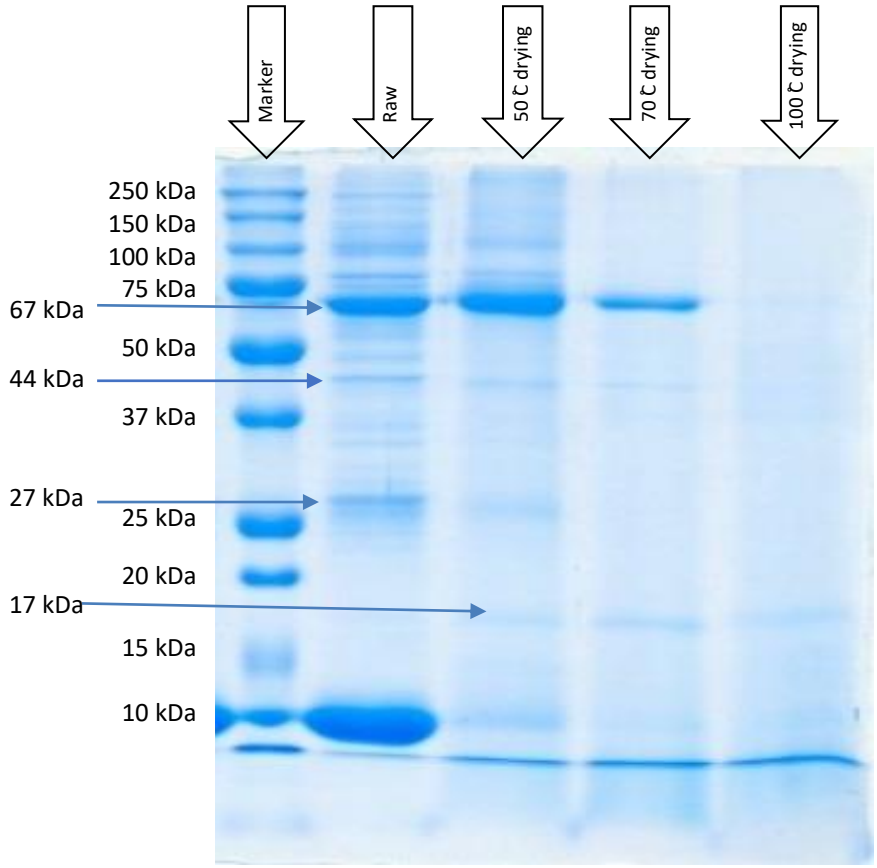


Figure 4.5 SDS-PAGE of raw beef lung and beef lung powders extraction

Table 4.3 SDS-PAGE relative band intensities of beef lung protein extract

Molecular Weight (KDa)	Relative band intensity (%)			
	Raw Beef lung	50 ˚C BLP	70 ˚C BLP	100 ˚C BLP
236	1.3±0.1	ND	ND	ND
131	1.9±0.2	ND	ND	ND
101	4.9±0.1a	4.3±0.1b	ND	ND
81	2.8±0.1b	4.8±a	ND	ND
67	41.5±0.5a	41±1.6a	35.2±3.4b	8.8±0.4c
55	1.4±0.1	ND	ND	ND
49	1.3±0.1	ND	ND	ND
44	3.1±0.3a	3.3±0.1a	2.4±0.1b	ND
34	1.2±0.4	ND	ND	ND
27	8.7±1.2a	3.6±1.7b	ND	ND
26	5.8±0.5	ND	ND	ND
17	ND	3.2±0.2c	5.5±0.5b	8.2±1.3a
10	36.1±0.9a	10.4±1.1b	ND	ND

Results are the means (±SD) of triplicate samples (n = 3).
Different superscripts are significant ($P < 0.05$) along the row.

4.3.8 Amino acid profile of beef lung powder

Table 4.4 shows the amino acid molar percentage in beef lung powder. Glycine was the most abundant as a molar percentage (16% to 18%) in beef lung powder, but it becomes the second highest on a weight basis because glycine is the simplest amino acid and has the lowest molar mass. Glycine was significantly increased by drying at 100 ˚C temperature, which may be due to cleaving the side chains of other amino acids. Glycine is the primary amino acid in collagen (Bhagavan & Ha, 2015), and the results reflected the fact that beef lung is a collagen-rich tissue. It has been reported that 25 to 30% of the beef lung protein is collagen (Lynch et al., 2018). The temperature did not have a significant effect on the other individual amino acids, but the total percentage of essential amino acids was significantly decreased after drying at 100 ˚C.

Glutamic acid made up 9% of the amino acids and was the highest amino acid by weight in the BLP due to its higher molar mass. These results corresponded with our previous work in Chapter 3.

Essential amino acids are nutritionally crucial elements. Among the essential amino acids, leucine, valine, lysine, threonine, and arginine were highly available on molar basis and most abundant essential amino acids in weight basis were leucine, lysine, arginine and valine (Table 4.4). These results were consistent with the findings of Cardoso-Santiago and Arêas (2001b); Schweigert, Bennett, and Guthneck (1954) in beef lungs. Phenylalanine and tyrosine are aromatic amino acids which consider together in nutritional parameters. These aromatic amino acids exceeded 5% of total amino acids together and contained around 70 mg/protein(g) in beef lung powder according to weight basis (Table 4.4). Beef lung powder compositions of USDA food composition tables (USDA, 2018) gave similar results for phenylalanine and tyrosine after calculating as dry matter basis.

Table 4.4 Essential and non-essential amino acids of beef lung powder with molar basis and weight basis

Essential AA						
Amino acid	**Molar basis**			**Weight basis**		
	50 ˚C BLP mol %	**70 ˚C BLP mol %**	**100 ˚C BLP mol%**	**50 ˚C BLP mg/g protein**	**70 ˚C BL mg/g protein**	**100 ˚C BLP mg/g protein**
Arginine	4.4±0.1	4.2±0.1	4.4±0.3	64.5±1.3	62.9±0.8	66.7±4.8
Histidine	1.6±0.1	1.8±0.1	1.7±0.1	24.3±1.4	27.2±1.1	26.3±2.2
Isoleucine	3.5±0.1	3.4±0.1	3.3±0.1	32.7±0.9	32.2±0.8	31.3±0.6
Leucine	8.6±0.3	8.7±0.3	8.1±0.2	79.5±2.5	81.4±2.9	77.1±1.6
Lysine	5.3±0.1	5.4±0.1	5.0±0.2	68.2±1.6	70.3±1.8	67.0±2.8
Methionine	1.7±0.0	1.7±0.07	1.6±0.1	17.9±0.4	18.4±0.7	17.3±0.8
Phenylalanine	3.5±0.1	3.5±0.1	3.3±0.1	40.1±1.4	41.6±1.2	39.8±0.8
Threonine	4.5±0.2	4.7±0.1	4.4±0.15	37.3±1.9	40.1±0.1	38.4±1.3
Tryptophan	1.8±0.6	1.8±0.04	1.7±0.1	25.5±9.2	26.3±0.6	25.6±1.2
Valine	6.6±0.3	6.4±0.2	6.1±0.1	54.0±2.2	54.1±2	51.9±0.8
Total EAA %	**41.4±2.0**	**41.6±1.2**	**39.6±1.4**	**49.9±1.3**	**49.9±1.1**	**48.6±1.4**
Non-essential AA						
Amino acid	**50BLP mol%**	**70BLP mol %**	**100BLP mol %**	**50 ˚C BLP mg/g protein**	**70 ˚C BLP mg/g protein**	**100 ˚C BLP mg/g protein**
Alanine	10.9±0.2	10.5±0.1	10.8±0.1	68.2±1.4	67.2±0.5	70.3±0.6
Asparagine	0.6±0.03	0.6±0.02	0.7±0.02	5.9±0.2	5.6±0.2	6.7±0.2
Aspartic	6.3±0.1	6.4±0.2	5.9±0.2	59.3±1.2	61.0±1.8	57.4±2.1
cysteine	1.4±0.1	1.5±0.1	1.4±0.1	11.8±0.9	12.6±0.7	12.2±0.8
Glutamic acid	9.3±0.4	9.4±0.1	9.0±0.2	96.5±4.3	99.1±0.7	96.7±2.5
Glutamine	0.05±0.01	0.04±0.01	0.04±0.001	0.5±1.2	0.5±0.1	0.5±0.01
Glycine	16.5±1.0^{d}	16.0±1.0^{d}	18.6±0.8^{c}	87.2±5.2^{b}	85.9±5.5^{b}	101.8±4.6^{a}
Proline	6.7±0.3	6.7±0.4	7.1±0.3	54.0±2.6	55.1±3.0	59.7±2.8
Serine	4.5±0.5	5.0±0.1	4.7±0.1	33.0±3.7	37.5±0.7	35.7±0.8
Tyrosine	2.3±0.1	2.3±0.1	2.2±0.05	28.7±0.6	30.0±1.2	28.6±0.7
Total non-EAA %	**58.5±2.8**	**58.4±2.0**	**60.4±1.9**	**50.0±1.7**	**50.0±1.2**	**51.5±1.3**

* Mean ± Standard deviation in the same column followed by different superscript differ significantly at p<.05

4.3.9 Mineral profile of beef lung powder

Animal protein sources provide a wide range of bioavailable micronutrients for human well-being. The content of various minerals such as Al, B, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, S, Se, and Zn were determined in the dried beef lung powder but the heavy metals Cd and Pb were not detected. Drying temperatures did not have a significant effect on mineral content (Table 4.5).

Table 4.5 Mineral content of beef lung powder (dry basis) at different drying temperatures

Mineral	BLP 50 ˚C drying (µg/g)	BLP 70 ˚C drying (µg/g)	BLP 100 ˚C drying (µg/g)
Al	6.06±0.34	4.98±0.31	4.04±0.39
B	0.52±0.02	1.11±0.04	0.49±0.08
Ca	480±20	486±35	520±25
Cd	ND	ND	ND
Cr	0.14±0.01	0.15±0.04	0.24±0.09
Cu	6.17±0.24	6.00±0.16	5.93±0.06
Fe	617±17	605±13	593±19
K	9108±151	8371±271	8349±44
Mg	509±5	496±3	498±3
Mn	0.59±0.02	0.51±0.01	0.58±0.03
Mo	1.33±0.06	1.08±0.04	0.97±0.08
Na	6367±49	6298±152	6293±49
Ni	0.62±0.04	1.84±0.91	1.60±0.3
P	9227±168	9011±55	9102±125
Pb	ND	ND	ND
S	13049±133	12987±89	13238±169
Zn	82±2	83.73±0.9	83.38±0.91

Food iron content is a vital micronutrient to prevent iron deficiency anaemia. Iron deficiency anaemia is the primary deficiency in worldwide, and it was identified as one of the six priorities of World Health Organisation (WHO) (McGuire, 2015). Chapter 3 illustrated that the iron content for dried beef lung was 1 mg/g, and it was lower at 0.6 mg/g in this study (Table 4.5). This decrease in iron could be due to washing out the blood from beef lungs during the mincing process.

The meat iron content comprises haem and non-haem iron (Table 4.6). The higher portion of iron is contributed from the blood as haem iron form and lower portion of iron is presented as non-haem form in animal live tissue. Haem iron is efficiently absorbed in the range of 15% to 25% while non-haem iron is absorbed in the range of 5% to 12% (Hallberg, 1983; Hurrell & Egli, 2010). High-temperature drying may cause oxidative cleavage of the iron-porphyrin structure of haem iron to convert haem to non-haem form and facilitate the release of the non-haem iron (Schricker & Miller, 1983).

Copper is an essential micronutrient for metabolically active tissues like brain, heart and liver (Trumbo, Yates, Schlicker, & Poos, 2001). Recommended dietary allowance (RDA) of copper for adult men and women is 1.2 to 1.7 (mg/day) and recommended upper level is 10mg/mL (Baghurst, 2006). According to the results shown in Table 4.5, beef lung powder can supply a considerable percentage of RDA value, but it is unlikely to reach toxic levels. High amounts of copper intake can impair iron

absorption. Further, a school children intervention study (Turgut, Polat, Inan, Turgut, & Emmungil, 2007) reported a high level of blood copper level could be lead to iron deficiency anaemia.

Magnesium plays an essential role in the human body. Magnesium involves in aerobic and anaerobic energy generation and more than 300 enzymes use it as a cofactor (Jahnen-Dechent & Ketteler, 2012). Beef lung powder contained around 500 µg/g of magnesium and it is the most abundant metal after iron. Magnesium upper intake is 400mg/day, and BLP is far unlikely to exceed the safer level. Other all minerals stay in safer level, and absence of heavy metals (Cd, Pb) give extra assurance about the safety of air oven-dried beef lung powder.

Table 4.6 The effect of drying on the haem iron % of BLP

BLP drying temperature	haem %	non-haem %
50 ˚C BLP	40.65±2.08^{a}	59.35±2.08^{b}
70 ˚C BLP	31.23±1.64^{b}	68.77±1.64^{a}
100 ˚C BLP	29.13±2.04^{b}	70.87±2.04^{a}

4.3.10 Safety of microbial, lipid oxidation and protein oxidation of the BLP

Microbial safety

Low microbial levels are key safety features of processed co-products. The safe limit of total viable count (TVC) for manufactured meat is 5×10^5 CFU according to the microbial reference guide New Zealand (MPI, 1995). Raw minced beef lungs had a 1.7×10^4 CFU of TVC in Table 4.7 by ensuring the safety of raw materials. Ready to eat meat products aerobic microbial limit is 1×10^3 and all drying powders were in safe range as ready to eat products after six months. Beef lung powder dried at 100 ˚C was in a sterile condition and no trace of TVC, coliform or yeast and mould was found. Yeast and moulds were found only in 50 ˚C beef lung powder and did not increase during storage. Yeast and mould safe level is 100 CFU for other foods but not specified for meat in New Zealand guidelines (MPI, 1995).

Lipid oxidation

Thiobarbituric acid reactive substances (TBARS) was used to determine the amount of lipid oxidation of products during storage. TBARS determined the aldehydes and ketones which are produced from the secondary auto-oxidation of lipids (Kaczmarek, Cegielska-Radziejewska, Szablewski, & Zabielski, 2015). Beef lung powder TBARS values significantly increased from 0.25 to 0.55 mg/Kg with 50 ˚C to 100 ˚C of drying temperature and also TBARS values increased significantly over the six months storage in LDPE airtight bags (Table 4.7). Our project BLP TBARS values are below than beef jerky production at 70 ˚C drying (Lim et al., 2014) and biltong production with γ irradiation

(Nortjé et al., 2005b). This low level of BLP lipid oxidation may be a result of not adding salts or salt-phosphate because adding salts to dried beef products like jerky and biltong catalysed the lipid oxidation (Devatkal & Mendiratta, 2001; Rhee & Ziprin, 2001; Torres, Pearson, Gray, Booren, & Shimokomaki, 1988). Lipid oxidation is the primary cause of quality deterioration. It is known to affect the flavour, odour, taste, colour, texture, and appearance of meat and meat products (Aalhus & Dugan, 2004). The relationship between the off flavour of lipid oxidation and TBARS value was established Greene and Cumuze (1982) with an untrained sensory panel and reported 0.6 to 2 mg MDA/1kg minimally required to identify the off flavours in beef. However, (Campo et al., 2006) later reported that 2 mg MDA/kg is the limiting threshold of lipid oxidation for off-flavour sensory attributes. However, BLP 50 ˚C and 70 ˚C drying contained 0.51 to 0.54 mg MDA/1kg and convenient for consumption even after six months storage, but BLP drying at 100 ˚C was not suitable after six months for consumption by considering the 0.6 mg MDA /Kg as the threshold limit.

Protein oxidation

The level of protein carbonyl measured by DNPH method is widely accepted as an indicator of protein oxidation. Protein oxidation of beef lung powder significantly increased with the drying temperature (Table 4.7). Mean carbonyl values non-significantly increased after six months storage for each drying temperature and increased with elevated drying temperature as well. Similarly, Astruc, Marinova, Labas, Gatellier, and Santé-Lhoutellier (2007); Santé-Lhoutellier, Astruc, Marinova, Greve, and Gatellier (2008) reported a significantly high level of carbonyl content with the high-temperature heat treatments on beef muscles. Further, Hu et al. (2017) reported that temperature and cooking methods are directly responsible for the carbonyl production of protein oxidation. Carbonyl group can react with the non-oxidised free amino acids to form amide bonds (Liu & Xiong, 2000b). This may lead to protein aggregation and may cause a reduction of digestibility and negatively impact on the nutritional status as well (Gatellier, Kondjoyan, Portanguen, & Santé-Lhoutellier, 2010). The protein oxidation results confirm, 50 ˚C drying is most suitable for beef lung powder production.

Table 4.7 Microbial count, lipid oxidation and protein oxidation

Microbial count (CFU)							
Test	**Initial value**				**After 6 months of storage**		
	Raw beef lungs	**50 ˚C BLP**	**70 ˚C BLP**	**100 ˚C BLP**	**50 ˚C BLP**	**70 ˚C BLP**	**100 ˚C BLP**
Total Viable count	17×10^3	2×10^2	1×10^2	0	3×10^2	1×10^2	0
Coliform	0	0	0	0	0	0	0
Yeast and Mould	8×10^2	1×10^2	0	0	1×10^2	0	0
Lipid oxidation							
TBARS (MDA mg/ Kg sample)	0.058 ± 0.006^{e}	0.25 ± 0.01^{d}	0.38 ± 0.02^{c}	0.55 ± 0.01^{b}	0.51 ± 0.03^{b}	0.54 ± 0.01^{b}	0.69 ± 0.07^{a}
Protein oxidation							
carbonyl (nmol/mg protein)	1.6 ± 0.38^{d}	7.1 ± 0.4^{c}	8.1 ± 0.42^{bc}	11.3 ± 0.97^{a}	8.6 ± 0.92^{bc}	10 ± 0.4^{ab}	12 ± 1.58^{a}

4.4 Conclusion

The present study was designed to determine the physicochemical characteristics of beef lung powder with the effect of 50 °C, 70 °C and 100 °C drying temperatures. This study has shown that all BLP powder in the difficult flowing state according to the Hausner ratio. All beef lung powder samples (50, 70 and 100 °C) presented threefold higher water-binding capacity compare to the sample weight. BLP dried at 50 °C preserved 40% of haem iron compare with total iron content and significantly decreased to 29% at 100°C dried BLP powder. According to the safety aspects, beef lung powder samples (50, 70 and 100 °C) were within the safety limits of total viable count, yeast & mould and negative from Coliform bacteria after six months of storage as well. BLP dried at 50 °C ensured the quality by generating the maximum 0.51 MDA mg/Kg sample in lipid oxidation and 8.6 carbonyls (nmol/mg protein) in protein oxidation after six months of storage.

Chapter 5

In Vitro Protein Digestion of Beef Lung Powder and Mineral Released

5.1 Introduction

Meat is a primary protein source with a high concentration of key micronutrients (iron, selenium, zinc and vitamins B6 and B12), and also provides essential amino acids in the human diet (Higgs, 2000). Protein demand is increasing with the rising human population, and food scientists and processors are looking for alternative animal protein sources and processing methods to meet the increasing demand. The utilisation of meat co-products increases the efficiency of the meat industry and has the potential of delivering high-quality proteins for human nutrition. There have been numbers of studies of the protein and micronutrient availability of several meat co-products (Chávez-Jáuregui et al., 2003; Santiago et al., 2001; Van-Heerden & Morey, 2014). However, processing condition effects on the nutritional quality, and the digestibility of these co-products is still unknown.

Beef lungs are an underutilised meat co-product in western countries (Chávez-Jáuregui et al., 2003) due to poor textural characteristics and strong aesthetic rejection. Bovine lungs have been reported to have quality protein, a high micronutrient bioavailability (Cardoso-Santiago & Arêas, 2001b; Santiago et al., 2001) and a large edible portion with an average weight of 3 Kg (Fornias, 1996) and are accepted by the European regulatory bodies (EC 854/2004). Beef lungs have the potential to provide a source of protein rich ingredients for protein-based food supplements.

Although meat and meat co-products contain all the essential amino acids, other factors, such as protein digestibility, amino acid bioavailability and digestion rate in the intestine, determine the actual nutritional quality and can affect the absorption of the end products (Mosoni & Mirand, 2003). Processing steps such as drying, cooking, and salting, can oxidise and denature proteins (Liu & Xiong, 2000a),which will change the physicochemical characteristics and release of amino acids (Chizzolini, Novelli, & Zanardi, 1998; Liu & Xiong, 2000b; Santé-Lhoutellier et al., 2008) and other micronutrients from a food matrix during digestion. Due to extra safety concerns associated with meat co-products, processing is critical to ensure food safety. In our previous study, beef lungs were thermally processed to protein powder as a proposed ingredient for protein-food supplements (Chapter 3). There is no information about how this thermal processing affects the protein digestion and release of minerals during *in vitro* protein digestion. This research evaluated the effect of different drying conditions on *in vitro* protein digestion of beef lung powder.

5.2 Methodology

5.2.1 Sample preparation

Hygienically derived frozen beef lungs without trachea were obtained from ANZCO Limited, New Zealand. Beef lungs were minced and dried in different temperatures (50 ˚C, 70 ˚C and 100 ˚C) on-air oven drier until getting constant weight. Powdered dried samples were used for this experiment. Please refer (4.2.1) for sample preparation in detail.

5.2.2 Protein content (%)

Total protein content (%) was determined by nitrogen analyser "Rapid MAX N exceed" (Serial No. 29154045, Elementar Analysensysteme GmbH, Donaustrasse, Hanau, Germany) using the Dumas method. All samples were produced in triplicate and individually loaded into the machine. This machine combusts samples at high temperature with oxygen to determine the protein percentage using nitrogen content.

5.2.3 Soluble protein (%)

Soluble proteins of digesta were measured using bicinchoninic acid (BCA) assay method (No: 23225, Pierce™ BCA Protein Assay Kit, Pierce Chemical Company, Illinois, USA). Absorption was measured at 570 nm in 96 well microplates using a microplate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany) and bovine serum albumin standard curve was used.

5.2.4 *In vitro* protein digestion

In vitro protein digestion was performed according to Kaur et al. (2016) with minor modifications and digestibility calculated according to Almeida, Monteiro, da Costa-Lima, Alvares, and Conte-Junior (2015). For each sample, BLP powder equal to 70 mg nitrogen and 17 mL of 0.1M HCl added to each polyvinyl digestion pots. These were stirred using magnetic fleas (500 rpm) on magnetic multi-stirrer (RT 15 Power, IKA®-Werke) at 37 °C until homogenous solutions were formed. Freshly prepared 2.5 mL pepsin (1031 U/mg, enzyme: substrate ratio = 1: 100 w/w, Sigma Aldrich, St Louis USA) in 0.1M HCl was added to each digestion pot, and the samples were incubated for 1 hour at 37 ˚C to simulate gastric digestion. The pepsin was inactivated by adding 2.5 mL of sodium phosphate buffer (0.1 M, pH 8.0) containing pancreatin (enzyme: substrate ratio = 1: 100 w/w, 350 U/mg, Sigma Aldrich, St Louis USA) and continuously incubated for 2 hours at 37 ˚C to simulate the intestinal digestion. Aliquots (1.0 mL) were taken at 0, 30 and 60 min from gastric digestion and

60 and 120 min from small intestinal digestion. These aliquots were collected to 2 mL sample tubes and placed on ice. Immediately after collection of aliquots, the pH level was changed to 8.0 for gastric digestion and 2.0 for intestinal digestion. Thereafter sample tubes were centrifuged at 4000 g for 15 min and the clear supernatants were collected and stored at -20 ˚C as digestion supernatant for further analysis.

The protein concentrations of the aliquots were measured using a microplate BCA assay (refer 5.2.3). *In vitro* Protein digestibility was analysed according to the following equation

In Vitro Protein Digestibility (IVPD)= (Ps–Pc)/P × 100

* Ps and Pc represent protein content of the sample digesta and control digesta after the digestion and P represents the protein content of the added BLP solid sample before the digestion.

5.2.5 Analysis of the free amino acids release in the digesta

A 5 mL aliquots of digestion supernatant were used to analyse the free amino acids. The thawed working samples were filtered through a 0.45 µm syringe into 2 mL HPLC vials for the HPLC analysis. All amino acids were analysed by HPLC 1100 series (Agilent Technologies, Waldrbom Germany) provided with an autosampler and fluorescence detector. HPLC column C-18, 3 µm (ACE-111-1546, Winlab, Scotland) with 150 mm × 4.6 mm dimension was used for amino acid separation. The solvent A (0.01 M, sodium phosphate buffer, pH 7.5 with 0.8% THF) and solvent B (50% methanol, 50% acetonitrile) was used at 40 ˚C column temperature. Column flow-rate was 0.7 mL/min and pump gradient settings were 0 to 14 min- 0% B to 40% B, at 20 min 50% B, *24 min to 29 min*-100% B, at 30 min- 0% B and kept equilibrating until 36 min. O-phthaldialdehyde (OPA) was used as a fluorescence derivative reagent for primary amino acids, with an excitation of 335 nm and emission of 440 nm using a fluorescence detector. At 22 min, secondary amino acids were detected utilising 9-fluorenylmethyl chloroformate (FMOC) by switching detector excitation 260 nm with emission 315 nm. Standard amino acid mix was run to produce a standard curve for calibration purposes.

5.2.6 Analysis of the mineral release in digesta

The digestion supernatant was thawed and used to analyse the mineral release. Samples were analysed by Inductively Coupled Plasma Optical Emission Spectrophotometer (Varian 720 ICP-OES, Melbourne, Australia). Settings used were: Plasma gas flow-15.0 L min $^{-1}$, Aux-1.5 L min $^{-1}$, Nebuliser 0.9 L min $^{-1}$ with SeaSpray nebuliser and cyclonic spray chamber. ICP calibration standards (Merck, Darmstadt, Germany) and internal standards were serially diluted using MilliQ water. Calibration curves were generated using four standards and standard blank.

5.2.7 Statistical analysis

The data generated by repeating the experiments 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 ± standard error of the means. The measured variables were set as dependent variables. The model included fixed effects for treatment, time (digestion time) and their interactions. 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 digestion time (Snedecor & Cochran, 1994).

5.3 Results and discussion

5.3.1 Effect of drying temperature on BLP *in vitro* digestion

An *in vitro* gastrointestinal digestion model was used simulating one hour of gastric digestion and two hours of intestinal digestion (Table 5. 1, figure 5.1). Soluble protein increases with time for all the samples with the highest values observed at the end of digestion. These results were in agreement with the findings of Bhat et al. (2018) and (Bhat, Morton, Mason, Jayawardena, & Bekhit, 2019) who also observed a similar increasing trend in soluble protein released with time during simulated gastrointestinal digestion of beef. Similar results were also reported by Kaur et al. (2016) for free amino nitrogen released during *in vitro* gastrointestinal digestion of beef.

Higher temperature significantly lowered the digestibility. The protein release of samples dried at 50 ˚C were significantly higher than the release from 70 ˚C and 100 ˚C dried beef lung powders throughout the digestion. The mean values for soluble protein (%) for 50 ˚C, 70 ˚C and 100 ˚C dried samples reached 21.8%, 18.4% and 17.4%, respectively at the end of the digestion (Table 5.1).

Although processing conditions have been reported to affect the protein released from meat samples (Kondjoyan, Daudin, & Santé-Lhoutellier, 2015), muscle composition has a minor effect on the digestion (Bax et al., 2013). Thermal processing has been reported to affect the digestion of proteins by affecting the number of hydrolysable sites available for digestion (Kondjoyan et al., 2015). Three-dimensional structure of proteins can be altered by heating which affects the protease active sites and influences the protein digestibility (Simonetti, Gambacorta, & Perna, 2016). Exposure to the high temperature of 100 ˚C promotes protein aggregation extensively and causes heavy unfolding which reduces the protease vulnerability whereas minor unfolding of structure facilitates the digestion (Gatellier & Santé-Lhoutellier, 2009; Promeyrat et al., 2010; Simonetti et al., 2016).

Table 5.1 Soluble protein (%) with the time

Digestion time(min)	**Soluble protein percentage (%)**		
	50 ˚C drying	**70 ˚C drying**	**100 ˚C drying**
0	10.4± 0.21^{a}	4.8± 0.33^{b}	4.7± 0.04^{b}
30	10.6± 0.19^{a}	6.0± 0.05^{b}	5.8± 0.2^{b}
60	11.1± 2.67^{a}	6.5± 0.04^{c}	7.3± 0.06^{b}
120	17.1± 0.29^{a}	16.6± 0.07^{b}	14.5± 0.30^{c}
180	21.8± 0.08^{a}	18.4± 0.67^{b}	17.4± 0.30^{c}

* Mean ± Standard deviation in the same row followed by different superscript differ significantly at $p<0.05$

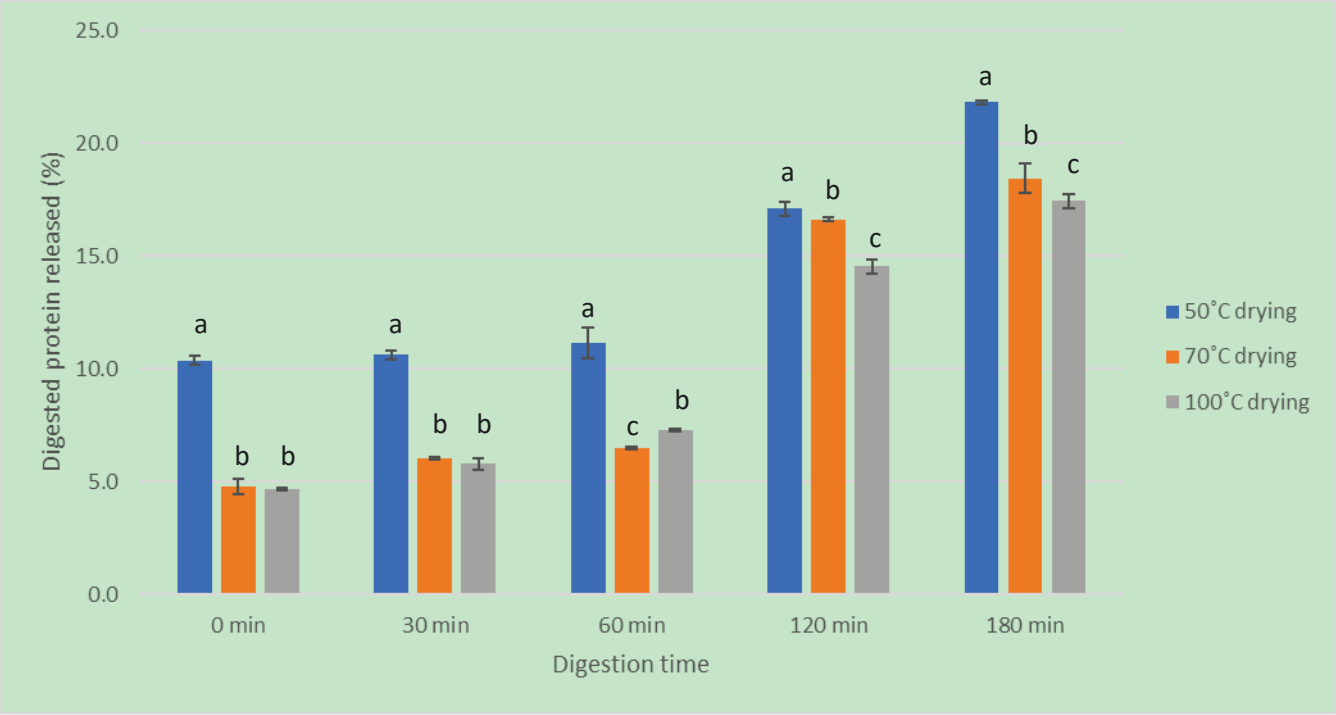


Figure 5.1 Digested protein in the digestion pot with the time

Protein digestibility percentage is a crucial factor to estimate the protein quality and represents the percent protein accessible to the body. This is the first study that focuses on the protein digestibility of dried beef lungs. The mean values for *in vitro* protein digestibility (%) of beef lung powder dried at 50 ˚C temperature was significantly higher ($P < 0.05$) than samples dried at 100 ˚C (Table 5.2). All three samples were highly digestible with values above 90%. This level of digestibility was similar to those reported by (Tavares, Dong, Yang, Zeng, & Zhao, 2018) for boiled hairtail (*Thichiurus lepturus*) fillets and Faber et al. (2010) for total digestibility of beef, pork, chicken and fish using an *in vivo* dog assay. Like other premium meat sources, beef lung powder showed a high digestibility and could possibly be used as a protein substitute. Farouk, Wu, Frost, Staincliffe, and Knowles (2019) also reported a digestibility of more than 90% for various beef coproducts (heart, kidney, spleen and liver). Beef lungs are abundant in low molecular weight proteins (Darine et al., 2010) which can undergo rapid hydrolysis during gastrointestinal digestion similar to the kidney and liver proteins reported by Farouk et al. (2019).

Table 5.2 Digestibility percentage of different temperature drying BLP

Beef lung powder	**Protein digestibility %**
50 ˚C BLP	96.35± 0.08[a]
70 ˚C BLP	95.87± 0.07[ab]
100 ˚C BLP	95.59±0.4[b]

Results are the means (SD) of six samples (n = 6).
Different superscripts are significant (P < 0.05) along the column.

5.3.2 Protein digestion of dried beef lung powder

Heat treatments can cause protein degradation. SDS-PAGE analysis was used to illustrate the changes in the profile of bovine lung protein produced at different drying temperatures. Figures 5.2 and 5.3 show the effects of different drying temperatures (50 ˚C, 70 ˚C, and 100 ˚C) on the protein profile of beef lung powder subjected to gastrointestinal digestion. Several bands appeared in all the samples during initial stages of gastric digestion. A higher number of intense bands appeared in the samples dried at 50 ˚C in comparison to 70 ˚C and 100 ˚C (Figure 5.2 and 5.3). These proteins in these bands which were present between 25 kDa to 100 kDa were gradually denatured with the increment of drying temperature (50 ˚C to 100 ˚C) and some bands completely disappeared. These patterns clearly indicate the effect of processing temperature on the digestion of proteins during the gastric phase. Protein aggregation with high temperature could be the reason for disappearing protein bands in SDS-PAGE

Most of the bands that were present during the gastric phase either disappeared or became less intense in the samples dried at 50 ˚C and 100 ˚C during the intestinal phase of digestion. There were few peptides in the samples dried at 70 ˚C which did not disappear even at the end of the digestion. Bhat et al. (2019) during gastrointestinal digestion of beef proteins and (Kaur et al., 2016) reported some limited peptides which did not disappear until the end of the digestion. However, after 3 hours digestion, all protein bands of 100 ˚C are disappeared completely (Figure 5.3) indication a high level of digestibility.

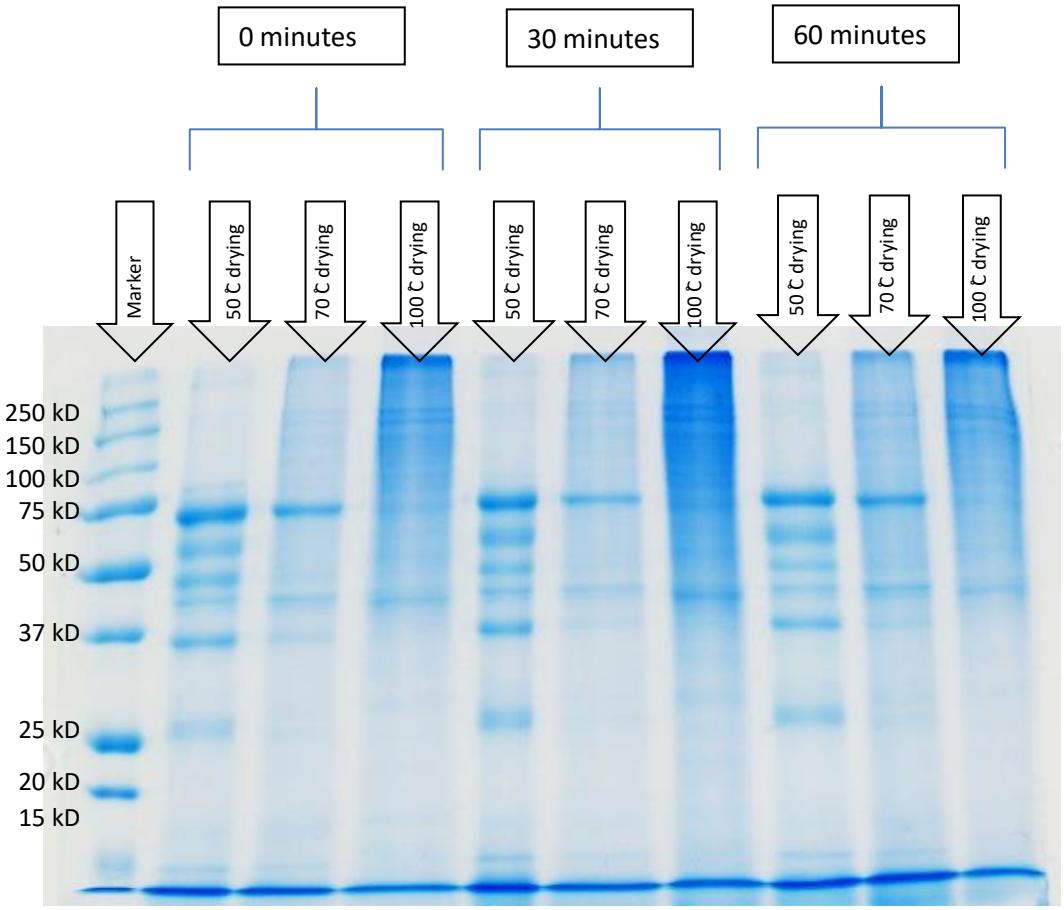


Figure 5.2 Gastric digestion of beef lung powder dried at different temperatures

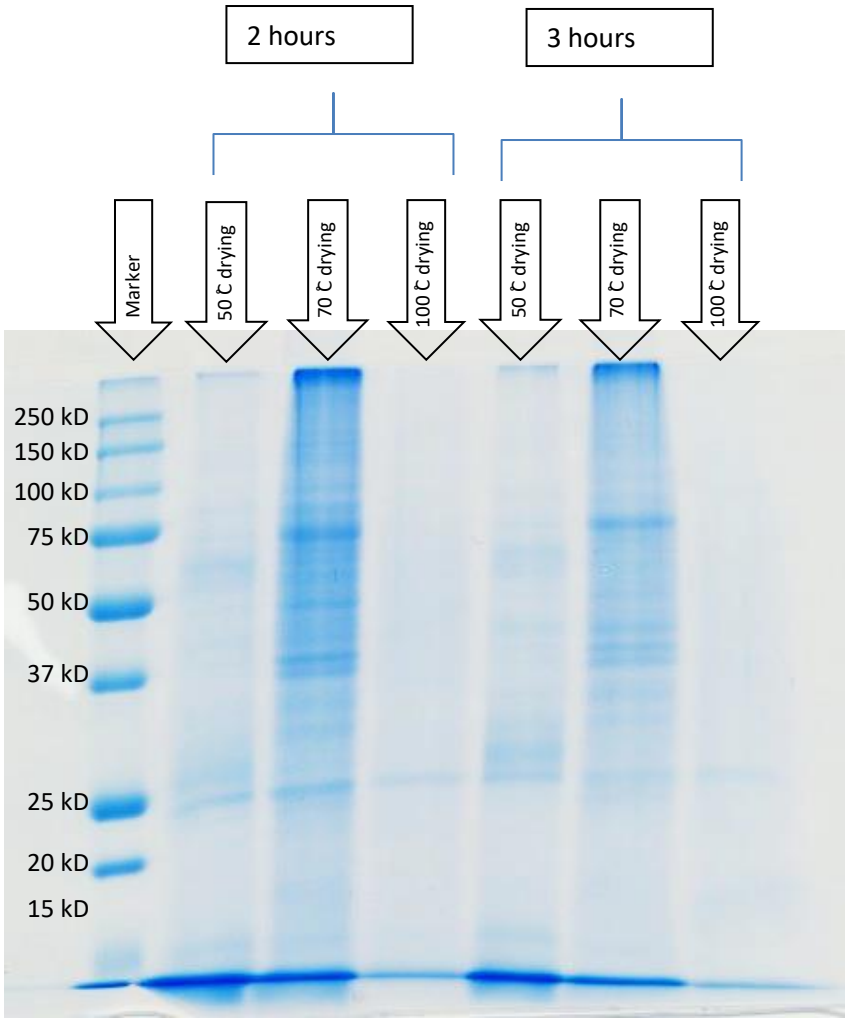


Figure 5.3 Protein profile of simulated intestinal digestion of beef lung powders which had been dried at different temperatures

5.3.3 Free amino acids released during digestion

The amino acids released from dried beef lung powders were compared with that of raw beef lungs (Table 5.3). Estimation of free amino acids provides a better estimate of protein availability because only free amino acids or shorter peptides can pass the intestinal epithelium to the bloodstream (Bhutia & Ganapathy, 2018).

The concentration of almost all free amino acids in digesta, whether essential or non-essential were significantly higher for samples dried at 50 ˚C in comparison to other samples, including the non-heat-treated raw beef lungs. Low temperature of 50 ˚C may have slightly unfolded the proteins to aid the protein digestion while high temperature (>50 ˚C) presumably caused severe aggregation of protein molecules that may have reduced the accessibility of digestive enzymes as explained by several publications (Gatellier & Santé-Lhoutellier, 2009; Promeyrat et al., 2010; Simonetti et al., 2016). A slight unfolding of proteins exhibits the hydrophobic peptides which are hidden in the core of protein structure to facilitate the accessibility of digestive enzymes (Djikaev & Ruckenstein, 2008; Zou, Zhou, Yu, Bai, & Wang, 2018). The most abundant amino acid in the digesta was leucine followed by glutamic acid, glycine and alanine for the raw extract and all the drying temperatures. The essential hydrophobic amino acids leucine, lysine and phenylalanine were released in greater amounts in the digesta for all drying conditions. Availability of essential amino acids is important as these can only be obtained from the diet (Reeds, 2000).

Protein oxidation could negatively impact on the protein digestion. This could be due to the oxidation of the protein's side chains to carbonyls and which interferes with the identification of cleavage sequences by proteases (Santé-Lhoutellier et al., 2008). In chapter 4, BLP protein oxidation increased with high-temperature drying. This protein oxidation may impair the protein digestion of BLP and reduce the release of free amino acids with increasing temperature. Sante-Lhoutellier, Aubry, and Gatellier (2007) prepared myofibrillar proteins from pig M. longissimus dorsi and oxidised these proteins with hydroxyl radicals. This study established a relationship between protein oxidation and loss of protein digestibility. Santé-Lhoutellier et al. (2008) confirmed a significant and negative correlation between pepsin activity and carbonyl group formation. Cystine and methionine are the most susceptible to oxidation due to their sulphur atom (Garrison, 1987)**.** Table 5.3 results showed a large reduction in cystine release with the digestion, but no reduction in methionine release. The thiol group of cysteine readily oxidise to produce thiyl radicals and these thiyl radicals can make disulphide bonds with another thiol group (Schöneich, 2016; Turell et al., 2008).

The essential branched chained amino acids, leucine and valine (Perelman & Lu, 2000) made up a large portion of the free amino acids (Table 5.3). Branched chained amino acids play an essential

role in muscle building particularly leucine which has an anabolic effect on muscle protein metabolism by increasing protein synthesis and reducing degradation (Blomstrand, Eliasson, Karlsson, & Köhnke, 2006). They also have a role in reducing the free fatty acid concentration in the bloodstream (Hormoznejad, Javid, & Mansoori, 2019) and a reduction in the sense of fatigue (Rahimi, Shab-Bidar, Mollahosseini, & Djafarian, 2017; Watson, Shirreffs, & Maughan, 2004). The free amino acid profile released following digestion of BLP powder is nutritionally and commercially attractive and has a strong potential for utilisation as an ingredient for the protein supplement industry.

Table 5.3 Free amino acid release with digestion

Essential AA				
Amino acid	**50BLP**	**70BLP**	**100BLP**	**Raw beef lung**
	µM	**µM**	**µM**	**µM**
Arginine	708±61^{bI}	357±40^{cGH}	1102±178^{aB}	639±57^{bFG}
Histidine	230±11^{aK}	117±21^{bKL}	63±14^{bcHI}	188±42^{aJK}
Isoleucine	610±30^{aJ}	391±58^{bFG}	294±41^{bF}	341±61^{bHI}
Leucine	3069±49^{aA}	1688±81^{cA}	1243±29^{dA}	2005±22^{bA}
Lysine	1823±78^{aD}	990±13^{bcC}	1126±205^{bB}	763±52^{cEF}
Methionine	280±16^{abK}	198±29^{bcJK}	133±23^{cGH}	313±60^{aHIJ}
Phenylalanine	1200±68^{aE}	675±61^{cD}	619±14^{cD}	857±45^{bDE}
Threonine	591±7^{aJ}	388±76^{bFG}	181±33^{cG}	442±73^{bH}
Tryptophan	138±24^{bL}	205±9^{aIJK}	134±14^{bGH}	157±21^{bK}
Valine	1463±71^{aE}	589±50^{bDE}	296±41^{cF}	647±4^{bFG}
Total EAA	**10114±417^{a}**	**5599±439^{bc}**	**5191±593^{c}**	**6353±437^{b}**
Non-essential AA				
Alanine	3039±74^{aA}	963±3^{cC}	504±8^{dE}	1123±52^{bC}
Asparagine	276.±19^{bI}	254±39^{bHIJ}	193±26^{bFG}	451±85^{aH}
Aspartic	625±16^{aJ}	323±45^{bGHI}	156±15^{cGH}	272±47^{bIJK}
Cysteine	24±6^{bM}	8±3^{bL}	7±4^{bI}	137±26^{aK}
Glutamic acid	2227±19^{aB}	1040±28^{bC}	744±28^{cC}	964±77^{bD}
Glutamine	248±26^{bK}	158±29^{cJK}	137±27^{cGH}	343±57^{aHI}
Glycine	2057±49^{aC}	1396±258^{bB}	1097±47^{bB}	1299±319^{bB}
Proline	810±61^{aH}	327±63^{bcGH}	185±24^{cFG}	414±96^{bHI}
Serine	1077±50^{aG}	485±64^{cEF}	178±49^{dG}	683±38^{bFG}
Tyrosine	823±56^{aH}	595±42^{bDE}	671±46^{bCD}	612±11^{bG}
Total non-EAA	**11206±379^{a}**	**5551±576^{b}**	**3873±277^{c}**	**6301±811^{b}**

Results are the means (SD) of three samples (n = 3).
Different superscripts in a row-wise (lower case alphabet) and column-wise (upper case alphabet) differ significantly ($P < 0.05$).

5.3.4 Mineral release with digestion

Effect of drying temperature on the release of various minerals from BLP during *in vitro* gastrointestinal simulation presents in Table 5.4. Mineral bioavailability is known as the proportion of mineral trapped in the food matrix that is available for absorption (Fairweather-Tait et al., 2005). Gastric acidic pH and peristaltic movements facilitate the food matrix disruption and release of minerals to digesta (Alminger et al., 2014). These released minerals can be absorbed in intestine or colon through epithelium by active or passive transportation (Etcheverry, Grusak, & Fleige, 2012; Gropper & Smith, 2012).

We have measured the release of several minerals of dietary importance from BLP during an *in vitro* gastrointestinal digestive simulation. Increasing the drying temperature significantly ($p<0.05$) decreased the release of copper (Cu), iron (Fe) and sulphur(S) and their maximum concentration was seen in the supernatant from raw and 50 ˚C dried samples. Similar results were observed by Garcia et al. (1996) for iron content and they reported a reduction of more than 70% in iron solubility in beef and rabbit meat upon cooking as soluble iron was converted to an insoluble form due to precipitation within the proteins during digestion.

The soluble iron state is in the ferrous form, and it can be oxidised by high-temperature air oven drying to the ferric ion, which is insoluble (Emerson, Roden, & Twining, 2012), Some amino acids, di-peptides, tri-peptides and polypeptides have iron-chelating ability (Li, Jiang, & Huang, 2017). While amino acids such as histidine, cystine, lysine chelate iron, glycine can form complexes with iron and enhance its absorption Kwiecień, Samolińska, and Bujanowicz (2015); Van (1973).

Heat-induced denaturation of proteins can diminish the release of metal ions. Our results are in agreement with the findings of another study on mineral bioavailability in pork, beef and chicken after baking on the conventional oven by Menezes, Oliveira, França, Souza, and Nogueira (2018) who also reported a significant decline in bio-accessibility of metal ions like copper and zinc. A significant reduction in sulphur-containing amino acids such as methionine and cystine with increasing drying temperature (Table 5.3) may have resulted in a significant decline in the sulphur concentration of the digesta.

Table 5.4 Mineral release during in vitro digestion

Element mg/l	Raw BL	50 ˚C drying	70 ˚C drying	100 ˚C drying
Ca	7.47±0.44e	8.97±0.19e	8.79±1.13d	8.45±1.09 d
Cr	0.02±0.00	0.02± 0.001	0.01±0.00	0.01±0.00
Cu	0.12±0.01^{A}	0.10± 0.001 ^{A}	0.04±0.01 ^{B}	0.01±0.00 ^{C}
Fe	2.60±0.49^{A}	1.73±0.09 ^{B}	0.73±0.26 ^{C}	0.53±0.10 ^{C}
K	247±8 ^{c}	294±3 ^{c}	263±29 ^{c}	238±37 ^{c}
Mg	10±0.51^{e}	12±0.14^{e}	11±1.34^{d}	10±1.35 ^{d}
Mn	0.01±0.001	0.02±0.001	0.01±0.001	0.01±0.001
Mo	0.02±0.001	0.02±0.001	0.01±0.001	0.01±0.001
Na	2054±76 ^{a}	2141±18 ^{a}	2085±214 ^{a}	2068±194 ^{a}
Ni	0.03±0.00	0.06±0.05	0.03±0.01	0.01±0.00
P	527±31 ^{b}	603±4^{b}	522±67 ^{b}	521±84^{b}
S	123±4 ^{Ad}	98±1.28 ^{Bd}	77±10 ^{BC}	68±13 $^{C cd}$
Se	0.04±0.01	0.03±0.01	0.02±0.00	0.03±0.01
Zn	1.45±0.15 ^{A}	1.32±0.03 ^{A}	1.36±0.14 ^{A}	0.91±0.07 ^{B}

Results are the means (±SD) of triplicate samples (n = 3).
Different superscripts in a row-wise (upper case alphabet) and column-wise (lower case alphabet) differ significantly ($P < 0.05$).

Conclusion

The temperature of drying for the bovine lung to form powder caused changes in the bioaccessibility of proteins and minerals. The beef lung dried at 50°C had a high *in vitro* protein digestibility (IVPD) as 96.3%. This was significantly decreased with drying at 70 °C and 100 °C. Similarly, the release of soluble proteins and free amino acid during *in vitro* digesta was significantly greater with low-temperature drying (50°C) and gradually decreased with the 70 °C and 100 °C drying. *In vitro* bioaccessibility of copper (Cu), iron (Fe) and sulphur(S) elements significantly decreased with the increasing temperature. Based on the results, drying bovine lung at 50°C will produce a powder with the highest bioaccessibility of proteins and minerals.

Constraints for sensory evaluation

This Chapter 5 confirmed the high digestibility of the bovine lung powder, and Chapter 3 showed the ability for BLP incorporation into starchy food as a food ingredient. The intention was to conduct sensory analysis to ensure palatability and consumer satisfaction for this beef lung incorporated pasta. We prepared an application for the sensory evaluation and found evidence of previous sensory analysis of beef lung incorporated products (Cardoso-Santiago & Arêas, 2001b; Cardoso-Santiago et al., 2001; Santiago et al., 2001). As a result of this, we gained ethical approval for conducting the sensory evaluation for beef lung incorporated pasta (Appendix A). We needed edible beef lungs processed according to hygienic requirements to perform a sensory evaluation, and we consulted with ANZCO Limited about providing these. Two major obstacles appeared at that point.

According to MPI information, lung processing as a human consumption was not permitted for companies who are exporting beef to the United States. The United States is a significant market for all the major meat exporters. Another major obstacle was that the meat industries would need to change their carcass inspection procedure with a veterinary surgeon to obtain the edible lungs. Procedure changes were very difficult due to the busy industrial environment. Though the collection of beef lung from home killed animals' was an alternative, the safety of the process couldn't be ensured. For these reasons, the sensory evaluation component was omitted. Because the inability of the meat export industry to process lungs to an edible grade, the focus of the PhD changed to consider alternative uses for lungs. Research moved to the production of the bioactive peptide from beef lungs in Chapter 6.

Chapter 6

Production of ACE Inhibitory Peptides from Beef Lung using Plant and Microbial Proteases

6.1 Introduction

Processing of animal co-products helps in sustaining the meat industry by providing economic and environmental advantages through the sale of these product for direct consumption (e.g. liver, kidney, heart and so on) or used in further processing (intestines for small goods and collagenous materials for gelatine production). However, some co-products, such as beef lungs, are restricted in their direct human consumption due to poor aesthetic qualities, safety issues and underdeveloped regulations. Beef lung is a co-product that has limited current economic value but possesses a high amount of low molecular mass proteins (Darine et al., 2010). These proteins may have the potential to produce anti-hypertensive peptides. Production of peptide-based products from animal sources for the treatment and prevention of hypertension provides enormous opportunities in biopharmaceutical manufacturing industries (Bhat, Kumar, & Bhat, 2017). However, information on the utilisation of beef lungs for production of anti-hypertensive peptides is generally lacking in the literature and appears to be a neglected research area.

Hypertension is a significant modern public health problem. Being responsible for 10.4 million deaths per year worldwide (Campbell et al., 2015), hypertension is a leading cause of mortality and morbidity among human adults globally (Bhat, Mason, Morton, Bekhit, & Bhat, 2017; Gąsowski & Piotrowicz, 2017) and has become a major health problem in children during last few decades (Karatzi et al., 2017). Around 40% of adults of the age of 25 and above suffer from hypertension globally and the number of people suffering from hypertension has increased from 600 million to 1 billion between 1980 and 2008 (Organization, 2010). Several synthetic ACE inhibitor drugs (such as benazepril, captopril, enalapril, perindopril, etc.) are available and can successfully manage human blood pressure. However, these are often associated with side effects such as hypotension, cough, skin rashes, headaches and fatigue (Bhat, Mason, et al., 2017; FitzGerald & Meisel, 2000). Food-derived peptides with anti-hypertensive properties are believed to be safer than ACE inhibitory drugs and less likely to cause side effects (Lee & Hur, 2017). Since the production of ACE inhibitory peptides from beef lungs is an unexplored area and needs immediate scientific attention, the present study was designed to generate enzymatic hydrolysates from beef lungs using kiwifruit extract and commercially available fungal proteases and investigate their ACE inhibitory activities.

New Zealand is one of the top kiwi fruit producing countries with 30% of the world total production (Skallerud & Olsen, 2011). Kiwifruit contains actinidin, a cysteine protease, which has selective digestion properties on proteins (Ha, Bekhit, & Carne, 2014) and has proven meat hydrolysing ability (Nieuwenhuizen, Beuning, Sutherland, Sharma, & Cooney, 2007). Actinidin has been reported to produce ACE inhibitory peptides in few plant-derived proteins (Zhang, Sun, Liu, Li, & Jiang, 2017) and may have a potential to produce peptides with ACE inhibitory properties from beef lungs.

Fungal protease 31,000 (FP31k) and 60000 (FP60k) (Enzyme Solutions, Victoria, Australia), which are obtained from *Aspergillus oryzae* along with a combination of acid, neutral and alkaline proteases, have proven exopeptidase and endo-peptidase activity (LiHui & Silva, 2009). Commercially available fungal proteases obtained from *Aspergillus oryzae* are active over an extensive pH range and are stable up to 70 ˚C (Tarté, 2009). The manufacturers (Enzyme Solutions, Victoria, Australia) information manuals have reported that FP31k and FP60k are produced by using different fermentation processes using maltodextrin diluent. These guidelines have reported several food industry applications for both these fungal proteases which include but are not limited *to production of protein hydrolysates in fish and soya, protein hydrolysis in fermentation media, modification of cheese, modification of gluten in the bakery industry and protein hydrolysis in brewing industry.* Studies regarding the meat tenderising properties of FP60K and FP31K for their application in the meat industry have been reported (Ha, Bekhit, Carne, & Hopkins, 2013). Proteases from *Aspergillus oryzae* are included in FDA's GRAS (Generally recognized as safe) list *(GRAS, 2018) that gives researchers and manufactures confidence to directly use them in the food industry.* Despite their role in protein hydrolysis and peptide generation, no study has evaluated the potential of FP31k and FP60k in the production of ACE inhibitory peptide from bovine lungs.

Therefore, the aim of this study was to evaluate the potential of FP31K, FP60K and kiwi fruit extract in the production of ACE inhibitory peptides from beef lung proteins.

6.2 Methodology

6.2.1 Research plan

The research plan is described in the flowchart presented in Figure 6.1. The protease enzymes were prepared by hydrating the enzyme powder in a 50 mM sodium phosphate buffer, pH 6, and their activity was determined using a peptide assay. The protein profile and enzyme activity of all proteases were determined by SDS-PAGE and casein zymography, respectively. Beef lung hydrolysates were prepared using standardised enzymes. Degree of hydrolysis, protein profile (SDS-PAGE) and ACE inhibitory activity of hydrolysates was determined at different time intervals during the digestion. The fractions with high activities were determined for each enzyme and passed through a 10KDa ultrafiltration membrane before loading on a gel filtration chromatography column. Eluted fractions were pooled, and the highest ACE inhibitory active fractions were selected. Selected fractions were passed through a 3KDa molecular cut-off membrane to remove larger peptides, and ACE inhibitory activities were determined. Purified highly active samples were desalted and subjected to mass spectrophotometry for protein sequencing. Identified amino acid sequences were analysed using amino acid databases.

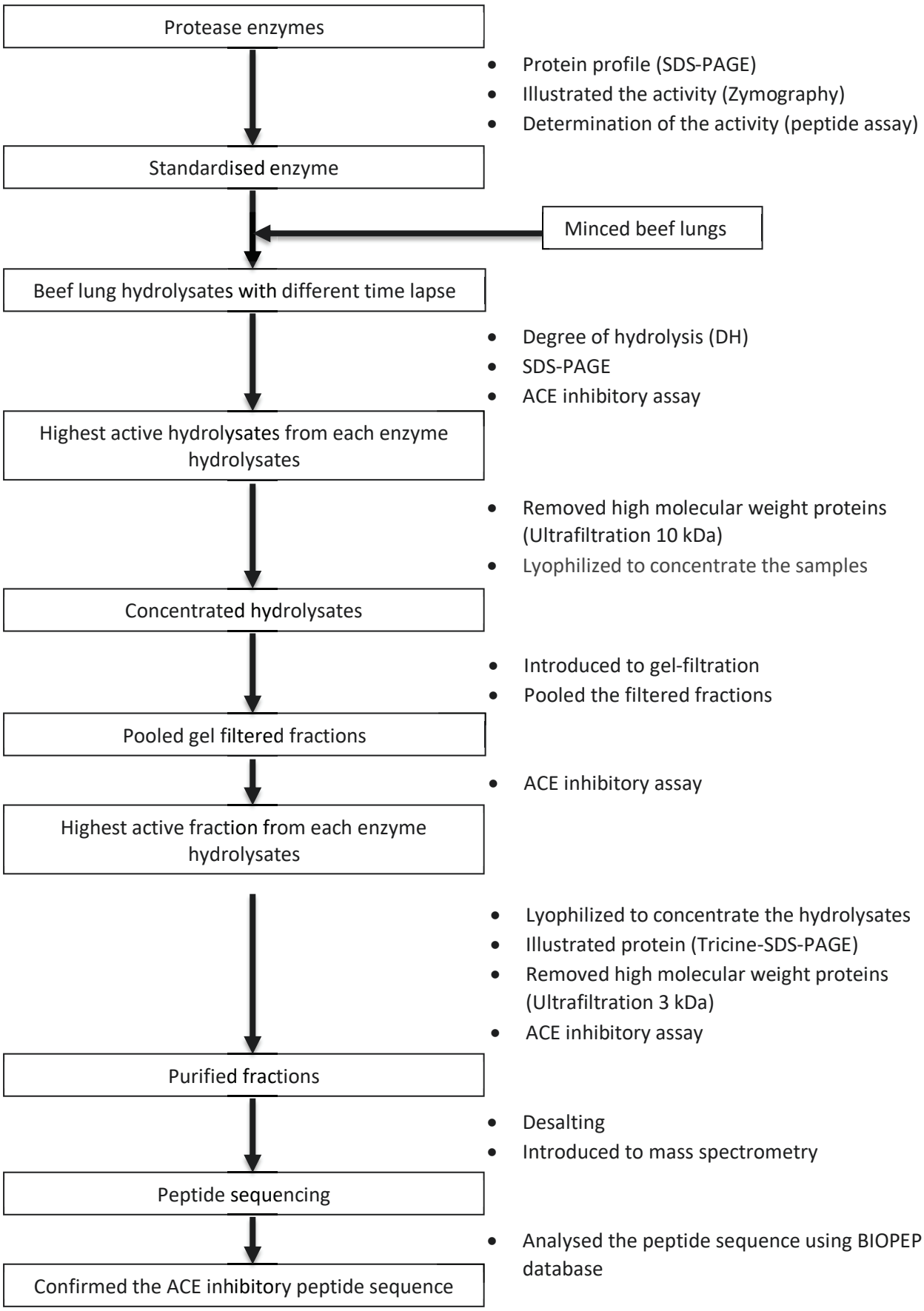


Figure 6.1 Flow chart of the research plan

6.2.2 Determination of the activity and composition of protease enzymes

Z-Lys pNP assay

Sample preparation

Samples were prepared by suspending 200 mg of fungal protease powder (FP31K/ FP60K) in 10 mL buffer (50 mM sodium phosphate, pH 6). Samples were centrifuged at 4000 g using Centra GP6R centrifuge (Thermo IEC, Needham Heights, MA, USA) for 10 minutes and supernatants were separated.

Fresh green kiwi fruits (*Acinidia deliciosa*) were purchased from the New World supermarket (Lincoln, New Zealand). Kiwifruits were squeezed using cheesecloth and collected extracts were centrifuged at 4000 g using Centra GP6R centrifuge for 10 minutes to separate the supernatants.

To prepare the enzyme samples, 1.386 mL of each supernatant were mixed with 7 µL of 500 mM DDT and 7 µL of 50 mM EDTA.

Assay

Proteolytic activity was measured as described in Choi and Laursen (2000). A reaction mixture was prepared from 50 µL of enzyme and 900 µL of phosphate buffer (50 mM, pH 6.0) in a cuvette (1 cm path length) and the reaction was initiated by adding 50 µL of the substrate (4 mM, Z-Lys-pNP). The absorbance was measured at 348 nm at 5-second intervals using a spectrophotometer at 25 °C. All samples were measured in triplicate. Phosphate buffer (50 mM, pH 6.0) replaced the 50 µL of enzyme in the controls and the rest of procedure was same.

The reaction rate was determined from the linear curve of the graph. The blank rate was subtracted from the sample rate and divided by the molar extinction coefficient for nitrophenol (5400 M-1.cm-1)to determine the activity as production of molarity of p-nitrophenol per minute ($M.min^{-1}$) (Equation 1The result of Equation 1 was converted to moles per minute ($mol.min^{-1}$) using 0.001 L assay volume and then converted to micro mole per minute (µ mol. min^{-1}) to express the activity in units (Equation 2).). A unit was defined as the amount of enzyme required to produce 1 µmol nitrophenol per minute (Boland & Hardman, 1972). The enzyme activities were expressed by dividing Equation 2 by the enzyme weight for Units. g^{-1}, enzyme volume for Units. mL^{-1}, and by the enzyme protein weight for specific activity, Units. protein g^{-1} (Equation 3 to 5).

Activity = $(\text{Sample rate} - \text{blank rate})/\Delta ß = M.min - 1 \quad (\Delta ß = 5400)$…… Equation (1)

Activity in Units = Equation (1) × 0.001L ×10^6 = µmol. min^{-1} …… Equation (2)

Enzyme activity in Units. g^{-1}= $(Equation\ (2))/(Added\ sample\ weight\ (g))$ ……..Equation (3)

Enzyme activity in Units. mL^{-1}= $(Equation\ (2))/(Added\ sample\ volume\ (mL))$ ……..Equation (4)

Specific activity in Units. protein g^{-1} = $(Equation\ (2))/(Added\ protein\ weight\ of\ sample\ (g))$ ……Equation (5)

6.2.3 Preparation of protein hydrolysates from beef lung extract

Frozen beef lungs without trachea were thawed for 24 hours at 4 ˚C and minced. Minced bovine lungs were packed in aluminium pouches and heat-treated by immersing in boiling water for 10 minutes. Beef lungs were cooled to room temperature and precisely measured (20 g ± 0.01) in digestion pots. The 200 mL of buffer (0.05 M sodium phosphate, pH 6) was added to all digestion pots and were placed on magnetic multi-stirrer. The digestion mixtures were stirred using a magnetic flea during incubation at 45 ˚C (monitoring with a digital thermometer). Incubation temperature was selected from Ha et al. (2013) which illustrated the maximum activity for both fungal proteases and Zhang et al. (2017) which illustrated the activity for actinidin. After attaining a temperature of 45 ˚C, 2 mL of kiwi fruit extract or 4.5 g of FP31k or 4 g of FP60k were added to digestion pots to reach an enzyme concentration of 0.4 Units. mL^{-1}. Aliquots (5 mL) were taken at five-time intervals (0, 2, 4, 6, and 8 hours) during digestion (in 15 mL Falcon tubes) and immediately incubated for 20 min in an 80 ˚C water bath to inactivate the enzymes (Mirdhayati et al., 2016) and were placed on ice. The cooled digesta were centrifuged at 15,770 g for 20 min at 4 ˚C with Centra GP6R centrifuge (Thermo IEC, Needham Heights, MA, USA) and the supernatants were collected as hydrolysates. The pH of the hydrolysates was modified to pH 7 using 1M NaOH and stored at -20 ˚C.

6.2.4 Determination of protein concentration and degree of hydrolysis

The total protein concentration of hydrolysates was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, USA) with bovine serum albumin as a standard. The degree of hydrolysis (DH) was determined by measuring the soluble protein content in 10% trichloroacetic acid (TCA) using the method of Edwards and Shipe (1978) with minor modifications. The 500 µL aliquot of each enzyme-treated hydrolysate was mixed with 500 µL of 20% (w/w) TCA. The mixture was centrifuged at 15,770 g for 15 min at 4 ˚C with Centra GP6R centrifuge (Thermo IEC, Needham Heights, MA, USA). The TCA soluble total protein in the supernatant was assayed by Pierce BCA Protein Assay Kit (Thermo scientific). The percentage of the degree of hydrolysis was expressed as follows:

% DH = (10% TCA-soluble protein/total protein) × 100

6.2.5 Protein profile with SDS–PAGE

The digestion pattern of the BLP proteins and protein profile of proteases were determined using 4-12% SDS-polyacrylamide gel. This electrophoresis was performed by following the method described in chapter 4 (refer 4.2.8- SDS-PAGE running)

6.2.6 Enzyme activity with zymography PAGE analysis

The zymography PAGE was adapted from Ilian et al. (2004) and designed for gelatine zymography. The 10% separating gel (0.75 mm × 10 well, pH 7.5) contained 0.05% gelatine, 10% [w/w] acrylamide: bisacrylamide, 0.224 M Tris–HCl (pH 7.5), 0.05% gelatine, 0.06% TEMED, 0.06% [w/v] ammonium persulfate. Stacking gel contained 3.85% polyacrylamide gel (3.85% [w/w] bisacrylamide, 0.1% [v/v] TEMED, 0.1% [w/v] ammonium persulfate, and 0.125 M Tris–HCl, pH 6.8). The resolving gel was first added to Bio-Rad Mini-PROTEAN® 3 Casting Frame and immediately smoothing the gel surface by adding isobutanol. Isobutanol was washed out by RO water and blotted dry after 30 minutes of solidifying. Then stacking gel was added, and the teeth-comb was inserted to produce loading wells. The comb was unplugged after solidifying (30 minutes) the stacking gel and the trapped air in wells was flushed out using RO water.

Mini-Protean III electrophoresis (Bio-Rad Laboratories, Hercules, CA) tank was set inside the insulated icebox with a magnetic stirrer to maintain the chilling temperature. Pre-electrophoresis was performed for 30 minutes using a running buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine, 1mM EGTA, 1 mM DDT) at 150 V before loading the samples. Loading samples were prepared by mixing 6X concentrated loading buffer (1X = 0.04 M Tris-HCl (pH 6.8), 6.5% Glycerol, 0.005% bromophenol blue) with standardised protein sample 1:6 (v/v) ratio. Prepared samples were loaded onto the gels in zymography system and run at 125 V for 3 hours at chilling temperature. The gels were incubated overnight using 20 mM Tris-HCl buffer (pH 7.4). Then gels were stained in 20 mL GelCode® Blue Stain Reagent (Thermo Fishaer Scientific, Illinois, US) for one hour and washed in distilled water overnight.

6.2.7 Tricine SDS–PAGE electrophoresis

Novex™ Tricine 10-20% mini Pre-Cast Gel (Life Technologies, California, US) was used to identify the low molecular protein bands using loading samples containing 20 µg purified proteins obtained from gel filtration. Purified protein fractions were lyophilized before using in tricine gels. The 4 µg.µL-1 of sample protein was mixed in 3:1 (v/v) with Novex™ Tricine SDS 4X Sample Buffer (No: LC1676, Thermo Scientific) and incubated at 70 ˚C for 15 minutes in the water bath. The gel cassette was rinsed with ionised water and the tape was peeled off from the bottom of the cassette. The Gel combs were gently pulled off in one smooth motion. Sample wells were rinsed with 1X Novex™ Tricine SDS Running Buffer (No: LC1675, Thermo Scientific). Gels were set up in the Mini-Cell tank and locked into place according

to the XCell SureLock™ Mini-Cell manual (IM-9003). Inner and outer chambers were filled with the 1X running buffer. Cathode chamber buffer level exceeded the level of the wells. The loading samples containing 20 µg protein were added to tricine gel wells including 7 µl of Spectra™ Low Range Protein Ladder (No: 26628, Thermo Scientific).

The gels were allowed to run for 70 minutes at a constant voltage of 125 V. After completing the electrophoresis, the gels were removed from the XCell SureLock™ Mini-Cell and the gels were taken out from cassettes for staining. The gels were fixed for 15 minutes on rocker using 20 mL fixing solution (50% methanol: 7% acetic acid) to prevent leaching out of proteins. After fixing each gel was washed three times in RO water (5 min each) and stained in 20 mL GelCode® Blue Stain Reagent (Pierce, Thermo Fisher Scientific) for one hour with gentle shaking on the rocker. Stained gels were washed in distilled water overnight to reveal the bands.

6.2.8 Peptide purification

Ultrafiltration using 10 kDa membrane

Frozen hydrolysates were thawed overnight and filtered through 45 µM filter paper under vacuum, then hydrolysates were filtered through Amicon® ultra-4 10 K membrane (MWCO, 10,000; Amicon Co., Beverly, MA) filter units with 7500 g centrifugal force for 20 minutes and filtrates were freeze-dried. Freeze-dried filtrate sample (1 g) was dissolved in 4 mL buffer (20 mM phosphate buffer, pH 7) and centrifuged at 15770 g for 10 minutes at 4 ˚C using Centra GP6R centrifuge (Thermo IEC, Needham Heights, MA, USA) to separate the clear supernatant. This supernatant was introduced to the gel-filtration column for further experiments.

Ultrafiltration using 3 kDa membrane

After fractionation (refer 6.2.8-Fractionation), concentrated fractions were passed through 3 kDa membrane Amicon® ultra-0.5 (MWCO, 3000; Amicon Co., Beverly, MA) using 14000 g centrifugal force for 20 minutes at 4 ˚C using Centra GP6R centrifuge (Thermo IEC, Needham Heights, MA, USA). Filtrate was used for peptide sequencing.

Preparation of gel filtration column

The gel media (Sephadex™ G-25, Medium, 100 g) was allowed to swell in excess (1 L) buffer (20 mM sodium phosphate buffer, pH 7.2) including 0.05% (W/V) sodium aside in cold room (4 ± 2 ˚C) for overnight. Media slurry was degassed under vacuum for 20 minutes before pouring into the column. The column XK 26/100 (Amersham Pharmacia Biotech) was used for this experiment. First, the empty column was rinsed with 0.01 M Sodium Hydroxide and RO water. Then cleaned column was levelled and mounted to stand vertically using sprit level. The column end piece was connected to a syringe and filled the same buffer using the syringe to ensure no air bubbles were trapped under the

net. The column was flushed with buffer leaving few mL at the bottom. Then pre-prepared media slurry was re-suspended and poured into the column through the wall with one continues motion with the help of a glass rod. The buffer was filled to form a meniscus at the top of the column. From the adaptor (upper-end piece) air bubbles were removed by drawing the buffer using a syringe. The adaptor was Inserted at an angle into the column, ensuring that no air was trapped under the net. Adaptor O-ring was adjusted to give a sliding seal on the column wall. The adaptor was pushed slowly down the column so that any air in the tubing was displaced by eluent. All tubing connections were made as bubble-free eluent connection between the column and FPLC machine (BIOLOGIC DUOFLOW ™, Bio-Rad, California). The adaptor was Locked in position on the gel surface and the column was packed at 10 mL/min until the gel bed had reached a constant height. Packing was continued until the gel bed was stable and re-positioned the adaptor on the gel surface as necessary. The column was calibrated using potassium chromate and blue dextran to determine the void volume.

Fractionation

After ultra-filtration, the peptide supernatant (4 mL) was filtered through a 0.45 µm sterile syringe filter and was introduced onto the pre-prepared Sephadex G-25 column (2.6×100cm). The column was eluted as a flow rate of 5 mL min^{-1} buffer (20 mM sodium phosphate buffer, pH 7.2) with collection of 8 mL fractions and the protein of the column eluent measured at 280 nm. The fractions were pooled according to UV peaks at 280 nm. The time-lapse of 32-41, 50-66, 77-93 min kiwifruit extract fractions, 35-43, 50-67, 73-93 min FP31K fractions and 2-13, 23-35, 37-42, 43-53, 63-75, 77-95 min FP60k fractions were pooled separately. The pooled fractions were freeze-dried and re-dissolved with 2 mL of buffer (50 mM borate buffer, pH8.2). The fractions were passed through 3 kDa membrane (refer 6.2.8- Ultrafiltration using 3KDa membrane) and used for peptide sequencing. Meanwhile, ACE inhibitory activity of each pooled fractions was determined using ACE inhibitory assay (refer 6.2.10.)

6.2.9 Peptide sequencing

Sample desalting

The highest active (ACE inhibitory activity) pooled fraction from each enzyme digestion was used for peptide sequencing. The samples were desalted by binding the peptides to a C18 Empore™ disk and eluted with 70% acetonitrile (ACN) in 0.1% formic acid (FA). The eluted peptides were then dried down in a vacuum centrifuge and resuspended in 50 µl 0.1% FA prior to injection on the mass spectrometer.

Mass spectrometric analysis

The mass spectrometric analysis was carried out on a nanoflow Ultimate 3000 UHPLC (Dionex Softron GmbH, Germering, Germany) coupled to Impact II mass spectrometer equipped with a CaptiveSpray source (Bruker Daltonik, Bremen, Germany). For each sample, 1 µL of the sample was loaded on a C18 PepMap100 nano-Trap column (300 µm ID x 5 mm, 5 micron 100Å) at a flow rate of 3000 nl/min. The trap column was then switched in line with the analytical column ProntoSIL C18AQ (100 µm ID x 150 mm 3-micron 200Å). The reverse-phase elution gradient was from 2% to 20% to 45% B over 60 min, total 88 min at a flow rate of 600 nL/min. Solvent A was LCMS-grade water with 0.1% Formic acid; solvent B was LCMS-grade ACN with 0.1% FA.

Amino acid sequences were identified in data-dependent MS/MS mode, where the acquisition speed was 2 Hz in MS and 1-32 Hz in MS/MS mode depending on precursor intensity. Ten precursors were selected in the m/z 150-2200 range, with 1-8 charged peptides selected. The analysis was performed in positive ionization mode with a dynamic exclusion of 60 sec.

The BIOPEP data base was used to identify amino acid sequences which matched known ACE inhibitory peptides (Minkiewicz, Iwaniak, & Darewicz, 2019). The bovine bioactive peptide data was used to identify the source of proteins and cryptides.

6.2.10 Inhibition of angiotensin 1-converting enzyme (ACE) assay

Inhibition of angiotensin 1-converting enzyme (ACE) was measured using the colourimetric assay described by Jimsheena and Gowda (2009). Hydrolysate samples (32 µL) were mixed with 5 µL of angiotensin-converting enzyme (1 UmL^{-1}) (#A6778, Sigma-Aldrich Corporation, Missouri, USA) in 600 µL microcentrifuge tubes. Sample tubes were Incubated at 37°C for 10 minutes in water bath. Then 13 µL of substrate solution (5 mM hippuryl-L-histidyl-L-leucine, 50 mM sodium borate buffer, 0.3 M NaCl, pH 8.2) was added to each of the samples to initiate the reaction. Samples were further incubated for one hour at 37°C and 25 µL of 1 M HCl was added to quench the reaction. For colour development, 100 µL of pyridine and 50 µL of benzene sulfonyl chloride were added to each tube and tubes were shaken vigorously for 10 seconds to form yellow colour. The tubes were left to cool on ice for five minutes and 200 µl was transferred to a clear 96-well microplate and absorbance were measured at 410 nm using a plate reader (415-2080-FLUOstar Omega, Ortenberg, Germany) within one hour. The negative control contained 32 µL of buffer (50 mM sodium borate buffer, 0.3 M NaCl, pH8.2) and the positive control contained 32 µL of 10 µM captopril instead of sample hydrolysate. The standard curve was constructed using hippuric acid at various concentrations from 100 µM to 1 mM. The percentage of ACE inhibition was calculated based on equation 6.

$$ACE\ inhibition\ \% = 100 - \frac{T_{HA}}{C_{HA}} \times 100 \text{..... Equation 6}$$

Where T_{HA} is the mean concentration of hippuric acid in test wells and C_{HA} is the average concentration of hippuric acid in the negative control wells.

Half maximal inhibitory concentration (IC_{50} value) was determined for the samples. Each peptide sample was assayed with different protein concentrations to generate the Inhibition curve. The IC50 value was taken from the protein concentration of the inhibition curve, which corresponded to half of the maximal inhibition.

6.2.11 Statistical analysis

The data generated by repeating the experiments for different parameters were compiled and analysed using SPSS (IBM SPSS Statistics V22.0, Armonk, NY, USA). All experiments were performed in triplicate (n=3) and all data were reported as means and standard deviation. Differences between the treatments were analysed by one-way analysis of variance, at the 0.05 level of significance. When the ANOVA was significant ($P < 0.05$), means were separated by a pairwise comparison using Tukey's comparison test.

6.3 Results and discussion

6.3.1 Protein profile of proteases

Analysis of enzyme protein profile is important to determine the purity of proteases. Figure 6.2 presents the analysis of soluble protein content of fungal proteases (FP31k, FP60k) and kiwifruit extract on SDS-PAGE. Fungal protease FP31k showed protein bands from 10 kDa to 50 kDa and FP60k protease showed protein bands above 25 kDa. Ha et al. (2013) reported similar patterns for FP31k and FP60k with most of the bands present below 50 kDa and above 20KDa, respectively. There were four bands in the kiwifruit extract which putatively correspond to actinidin, thaumatin-like protein, KiTH and Kirola proteins (Gong, Morton, Bhat, Mason, & Bekhit, 2019). Actinidin, which is an active protease in kiwifruit extract, has been reported with a molecular mass within the range of 25.2 to 30 KDa (Boland & Hardman, 1973; Miraghaee, Mostafaie, Kiani, & Kahrizi, 2011; Richardson, Ansell, & Drummond, 2018).

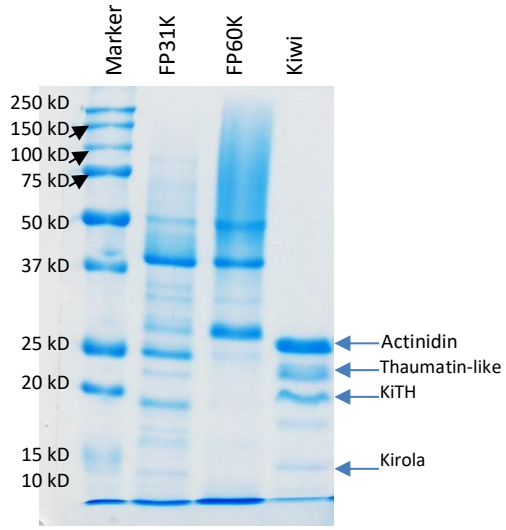


Figure 6.2 SDS-PAGE protein profile of protease enzymes (kiwi, kiwi fruit extract; FP31k, 31k fungal protease; FP 60k, 60k fungal protease; protein 20 µg were loaded in each sample lane)

The protein hydrolysing ability was evaluated by using gelatine zymography for all the enzymes (Figure 6.3). Both FP31k and FP60k proteases showed a major band as a gelatine hydrolysing patch on the upper half of the gel. Actinidin, which is the main digestive protease in kiwifruit (EC 3.4.22.14) (Boland, 2013), showed a band in the lower half of the gel. Results suggest that FP31K and FP60K proteases have high molecular weight in comparison to the actinidin. All enzymes were active and had the ability to digest gelatine.

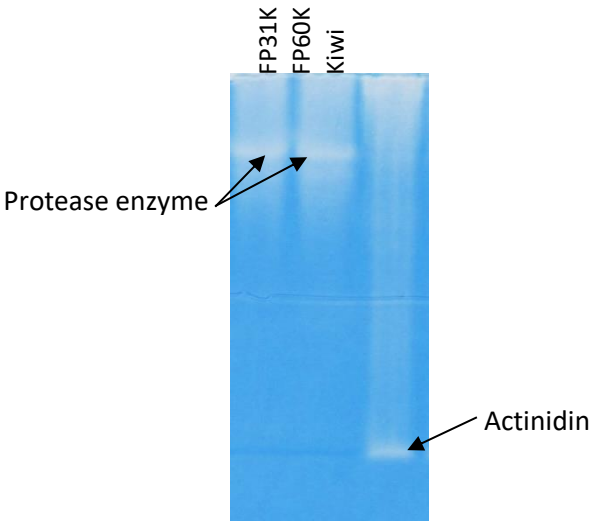


Figure 6.3 Gelatine zymography; FP31K, 31K fungal proteas; FP60K, 60K fungal protease; Kiwi, fresh kiwi fruit extract. (2×10^{-4} units were loaded in each sample lane

6.3.2 Activity and specific activity of the proteases (FP31 K, FP60 and kiwi fruit extract)

It is important to standardise the activity of the enzymes in these digestions. Table 6.1 presents the activity and specific activity (Unit/mg protein) of the enzymes as assessed by Z-Lys-pNP peptide assay. Kiwifruit extract had the highest specific activity (1 U/mg protein) followed by FP31K and FP60k proteases. The proteases FP31K and FP60K were present in solid powder form and their activity was measured as 17.8 U/g and 20 U/g, respectively. Kiwifruit extract was present in liquid form and activity was 39.6 U/mL

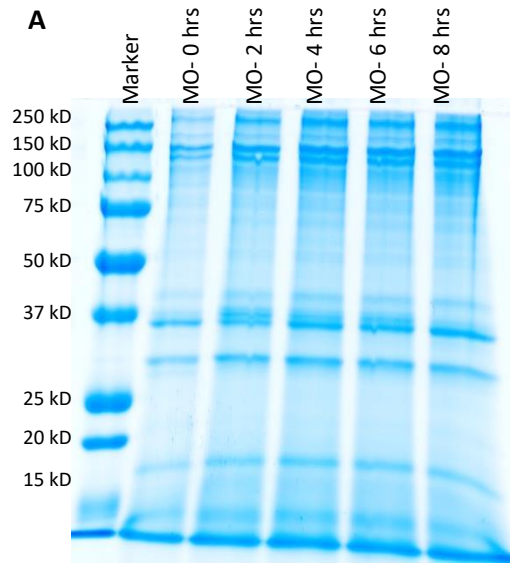
Table 6.1 Activity of kiwi fruit extract, FP31k and FP60K fungal protease

Enzyme	Specific activity (U/mg protein)	Activity	
		U/g sample	U/mL
FP31K	0.26±0.02 [b]	17.78±0.01[b]	-
FP60K	0.07±0.01 [c]	20.00±0.13[a]	-
Kiwifruit extract	1.09±0.06 [a]	-	39.56±2.04

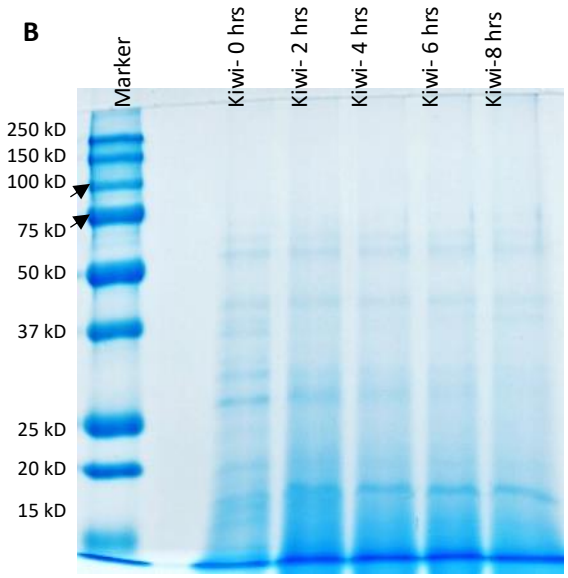
* U=Unit = µmol/min
* Mean ± Standard deviation in the same column followed by different superscript differ significantly at p<.05

6.3.3 Proteolysis of minced beef lung

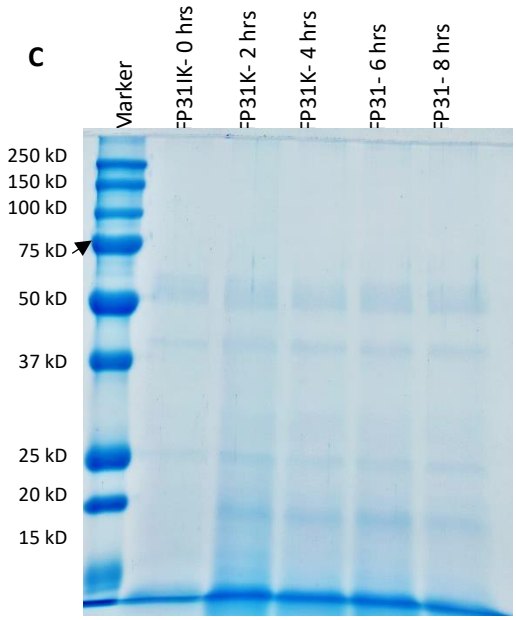
The hydrolytic patterns of beef lung subjected to protease incubation for 8 hours are presented from gel 'A' to 'D' in Figure 6.4. Gel 'A' presents the hydrolysis of blank sample without addition of any enzymes. Intensity of all bands did not change with the time of hydrolysis indicating that there was limited or no protein degradation without added protease during the 8 hours. Beef lung hydrolysate produced by the hydrolysis with kiwifruit extract (Figure 6.4B) showed reduced intensity of most of protein bands above 25kDa with time and most of the bands had totally disappeared by 8 hours of digestion. It shows a strong hydrolysing ability of kiwi fruit extract for beef lung proteins. Hydrolysis with fungal proteases FP31k and FP60k protein band hydrolysing is illustrated in C and D respectively. There was more protein degradation in FP31k than in FP60K. Similar results were recorded by Ha et al. (2013) who observed greater hydrolysis of connective tissue by FP31k in comparison to FP60k during first 30 minutes.



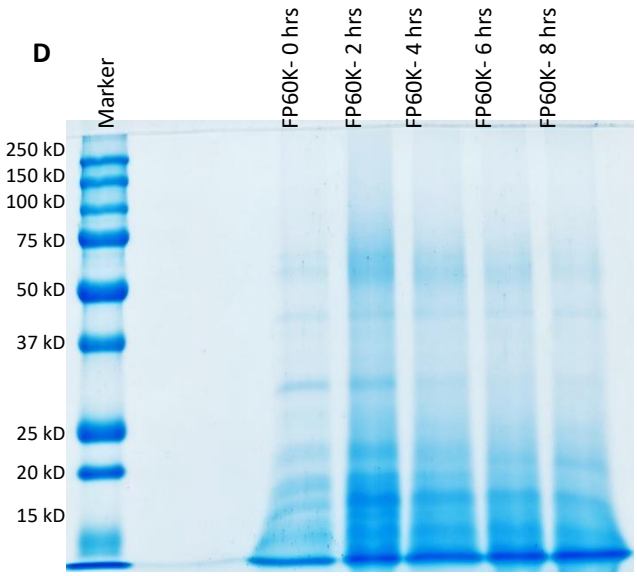
A: SDS-PAGE protein profile of beef lung protein with meat only blank digestion. (Protein 20 µg were loaded in each sample lane



B: SDS-PAGE protein profile of beef lung protein digestion with Kiwifruit extract (Kiwi). (Protein 20 µg were loaded in each sample lane.



C: SDS-PAGE protein profile of beef lung protein digestion with FP 31K fungal protease. (Protein 20 µg were loaded in each sample lane



D: SDS-PAGE protein profile of beef lung protein digestion with FP 60K fungal protease. (Protein 20 µg were loaded in each sample lane

Figure 6.4 Protein profile (SDS-PAGE) of beef lung digestion for 0 to 8 hours with FP31K, FP60K and Kiwifrut extract

6.3.4 Degree of hydrolysis of minced beef lung with incubation time

Degree of hydrolysis of beef lung protein during incubation time of 0 to 8 hours was also measured as TCA-soluble protein and is presented in Figure 6.5. Fungal protease FP31k showed the highest degree of hydrolysis (98%) after 8 hours followed by FP60K and kiwi fruit extract. Jang and Lee (2005) prepared beef rump hydrolysate with different protease enzymes and the degree of hydrolysis was measured using a similar method. It took them more than 12 hours to achieve 50% of hydrolysis. In our study, beef lungs were boiled which may have increased the hydrolysis by gelatinising the collagen of beef lungs, reported to contain high percentage of collagen (Francis & Thomas, 1975b), making it more vulnerable to hydrolysis (Zhang, Olsen, Grossi, & Otte, 2013). By cleaving the hydrogen bonds of adjacent polypeptide collagen chains (helix-coil transition) and by exposing the inner sites in the triple-helix structure of collagen, boiling increases the susceptibility of collagen to enzymatic hydrolysis (Zhang, Li, & Shi, 2006). Kiwifruit enzymes are not able to access the crosslinked triple helix of collagen but once this has been opened up with heat treatment, it shows the protease activity (Sugiyama et al., 2005). Some *Aspergillus oryzae* strains like Mi153 do have collagenase activity (Wanderley, Neto, Filho, Lima, & Teixeira, 2017) but there is no reported activity with either the FP31K or FP60K proteases.

Hydrolysis of the blank sample (without enzymes) was lowest throughout the incubation period which showed the significance of proteases during protein hydrolysis.

The TCA protein solubilisation method is a well-documented and widely used method in many publications. But non-hydrolysed small peptides can be found in the soluble phase instead of the hydrolysed free amino acids (Moraise et al., 2013). Rutherfurd (2010) argued that accuracy of the method depended on the type of hydrolytic enzymes and the size of the hydrolysed peptides. These possible factors may have had an influence on the very high degree of hydrolysis rate (98%) in our results (Figure 6.5).

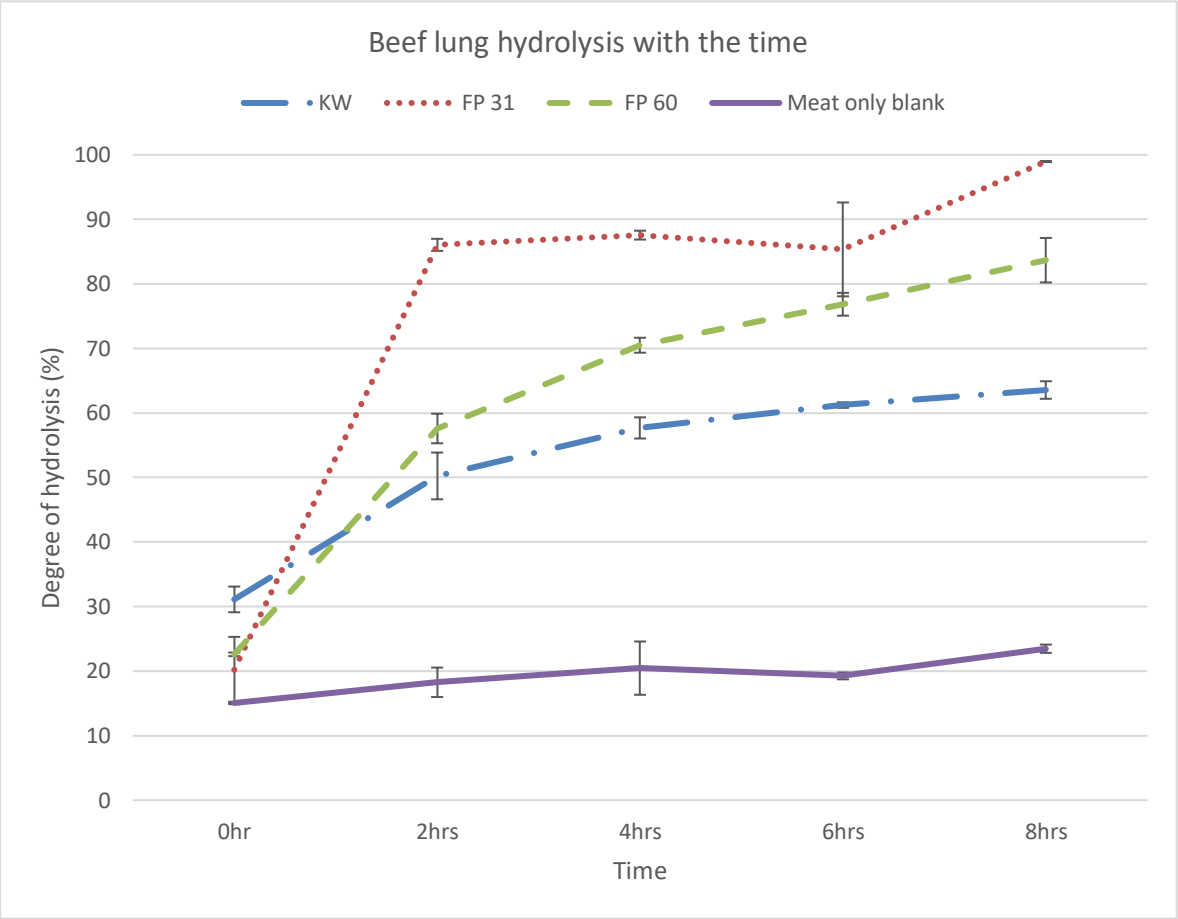


Figure 6.5 Degree of hydrolysis of beef lung mince during 0, 2, 4, 6 and 8 hours incubation with different enzymes (KW, Kiwifruit extract; FP 31, FP31 fungal protease; FP 60, FP60 fungal protease)

6.3.5 ACE inhibitory activity of beef lung hydrolysates with different enzymes and incubation time

All hydrolysed samples were analysed for ACE inhibitory activity at different times and are presented in Table 6.2. The highest ACE inhibitory activity was recorded for Kiwi extract at 2 hours, FP31k extract at 6 hours and FP60k extract at 8 hours of incubation (67.4%, 71.1% and 67.3%, respectively). Mirdhayati et al. (2016) and Jang and Lee (2005) also found no relationship between the ACE inhibitory activity and hydrolysis time during beef rump and goat meat digestion, respectively. Maximum ACE inhibitory activities of our hydrolysates were higher than the crude hydrolysate reported by Mirdhayati et al. (2016) and Jang and Lee (2005).

Table 6.2 ACE inhibitory activity (%) of enzymatic hydrolysates from beef lung mince at different incubation hours

Enzymes	Digestion hours				
	0	2	4	6	8
Kiwi	39.8±1.1^{d}	**67.4±1.1^{a}**	46.1±4.9^{c}	44.4±2.9^{c}	60.7±1.9^{b}
FP 31K	38.7±2.3^{d}	49.2±1.3^{c}	57.9±1.9^{b}	**71.1±0.4^{a}**	50.7±0.8^{c}
FP 60K	40.7±2.9^{d}	54.1±5.2^{c}	64.9±1.6^{b}	47.0±1.8^{c}	**67.3±0.8^{a}**

* Mean ± Standard deviation in the same row followed by different superscript differ significantly at p<.05
Kiwi, kiwifruit extract; FP31, FP31 fungal protease; FP60, FP60 fungal protease

The hydrolysates with highest ACE-inhibitory activity (Kiwi extract-2h, FP31k-6h and FP60k-8h) were further compared to the control without enzymes (meat-only-blank), control without beef lung (enzyme-only-blank) and 10 nM captopril as a positive control (Figure 6.6). All enzymes were able to cause 20% to 30% ACE inhibition in the absence of beef lung. Duttaroy (2015) also reported the ACE inhibition potential of Kiwifruit extract, but there are no reports of FP 31K and FP 60K ACE inhibitory activity in the literature. Non –hydrolysed beef also caused 30-40% inhibition of ACE activity. The FP31K protease showed 27% and 35% ACE inhibition in enzyme-only-blank and beef lung-only-blank respectively. Captopril at 10nM caused a 77% inhibition of ACE activity. A similar range of results were also presented by Ryder, Bekhit, McConnell, and Carne (2016).

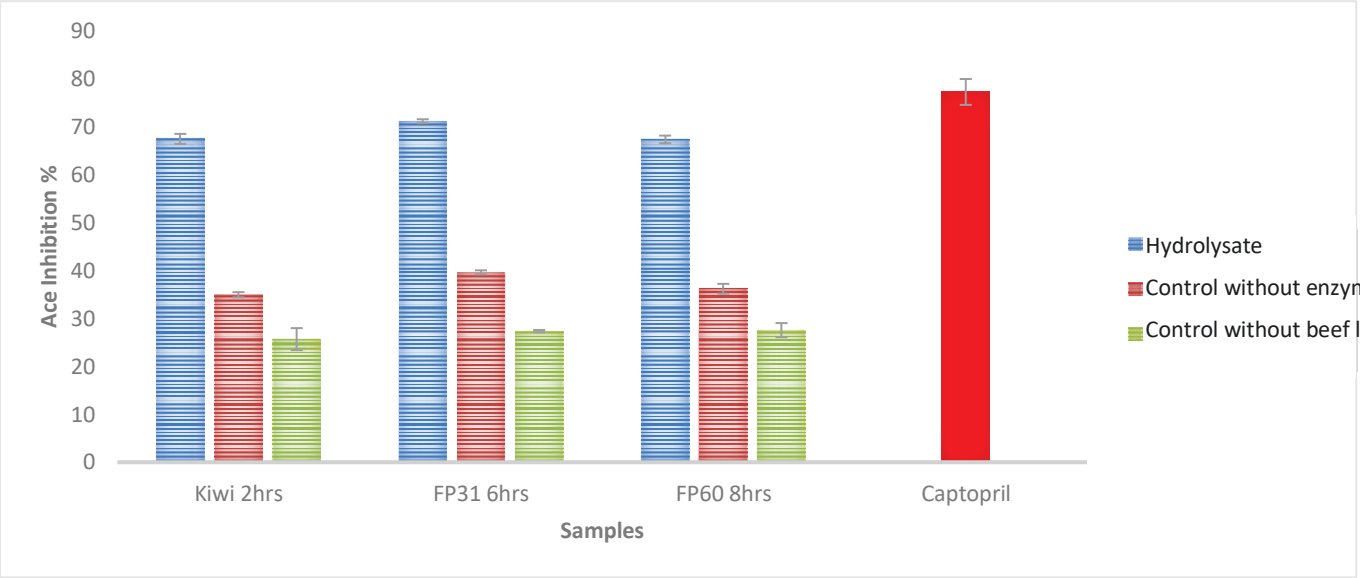


Figure 6.6 ACE inhibitory activity percentage of selected KW 2hrs, FP31 6hrs, FP60 8hrs samples and captopril positive standard with their meat only blank and enzyme only blank

6.3.6 Peptide purification and identification

Selected fractions of crude proteins (FP31k-6h, Kiwi extract-2h and FP60k-8h) were purified using a Sephadex™ G-25 100×26 cm gel filtration column. Sephadex G-25 gel matrix is generally used for separation of low molecular proteins (Hagel & Janson, 1992) and yields low fractionation molecular range (1 kDa to 5 KDa). This technique was employed in our study because very low molecular weight peptides (2-12 amino acyl residues) are responsible for the ACE inhibitory activity (Byun & Kim, 2002; Gu, Li, Liu, Yi, & Cai, 2011; Ryder et al., 2016). The longer column was important for better resolution of protein peaks, however, it produced broad peaks with greater volume (Healthcare, 2010). The protein fractions had low concentrations and were concentrated before further analysis. Figure 6.7,6. 8 and 6.9 presents the well-separated peaks of proteins using 280 nm UV detector of fast protein liquid chromatography (FPLC) System-(BIOLOGIC DUOFLOW ™ 10 system, Bio-Rad, California, USA). Standardised ACE inhibitory percentages have been mentioned corresponding to each protein peak in Figure 6.7 to 6.9.

Figure 6.7 illustrates that the kiwi fruit extract produced three main peaks and the ACE inhibitory activity increased with the elution time. Similarly, for fungal protease 31K and 60K, the ACE inhibitory activity also increased with the time (Figures 6.8, 6.9). All enzyme hydrolysates exhibited the highest ACE inhibitory activity at the last peak of eluted protein between 80 to 90 minutes (Figures 6.7-6.9). During Gel filtration chromatography larger molecules are eluted first and smallest peptides are eluted last from the column. These results further confirm that ACE inhibition is associated with

low molecular weight peptides. These results are in agreement with previously reported studies (Gu et al., 2011; Halim, Yusof, & Sarbon, 2016; Ryder et al., 2016).

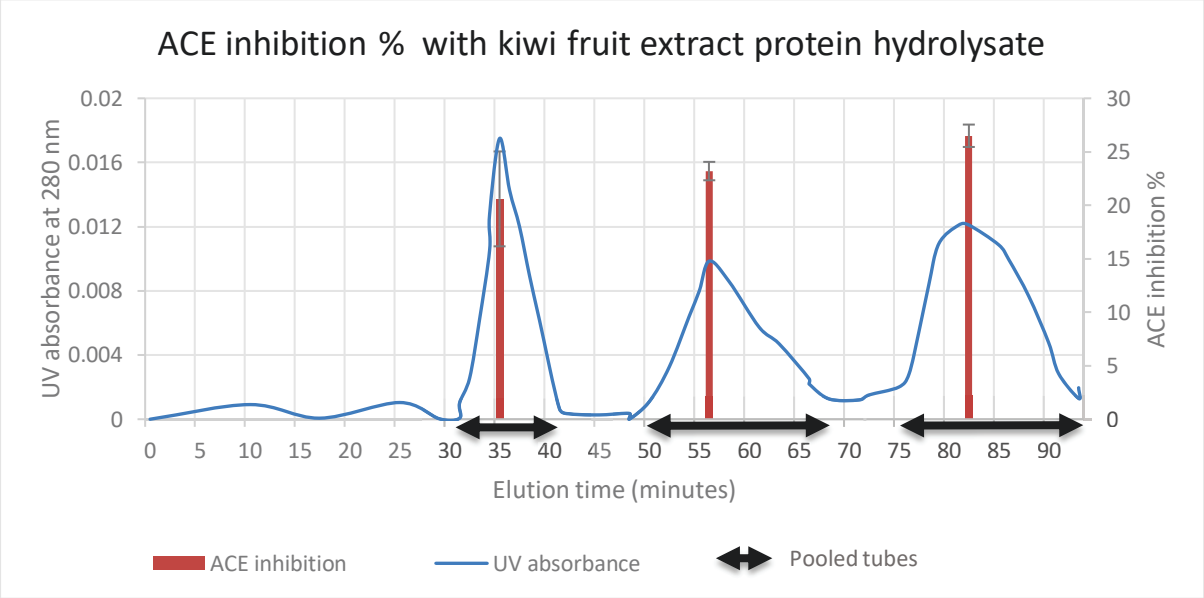


Figure 6.7 Fractionation of protein hydrolysates of beef lungs with Kiwifruit extract on a Sephadex G-25 gel filtration column and ACE inhibitory activity of pooled tubes

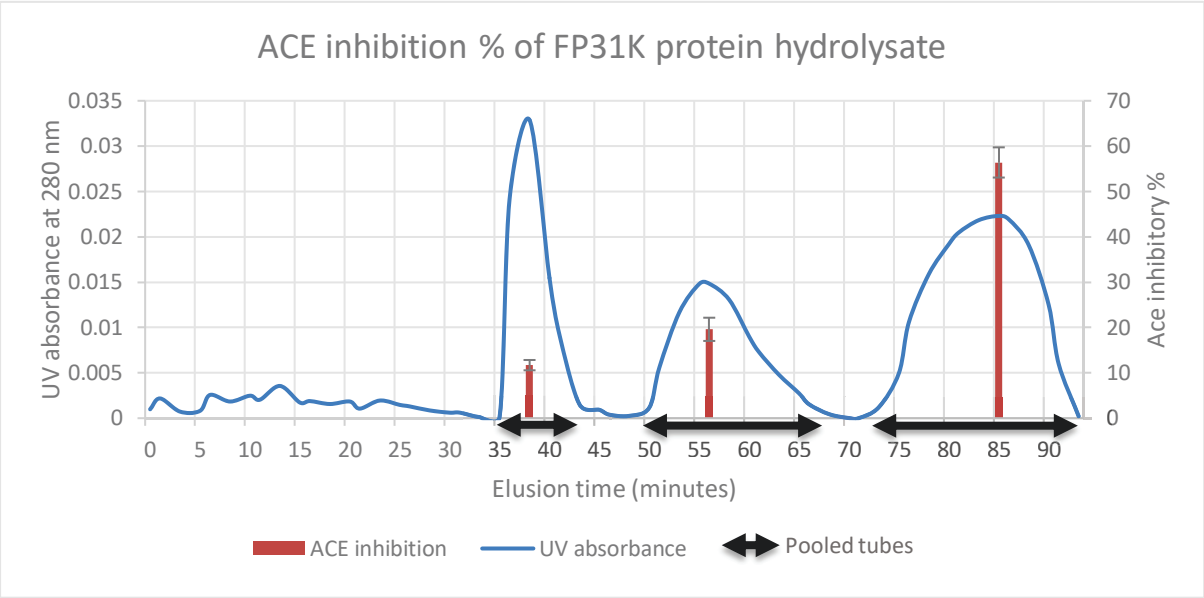


Figure 6.8 Fractionation of protein hydrolysates of beef lungs with FP31k protease on a Sephadex G-25 gel filtration column and ACE inhibitory activity of pooled tubes

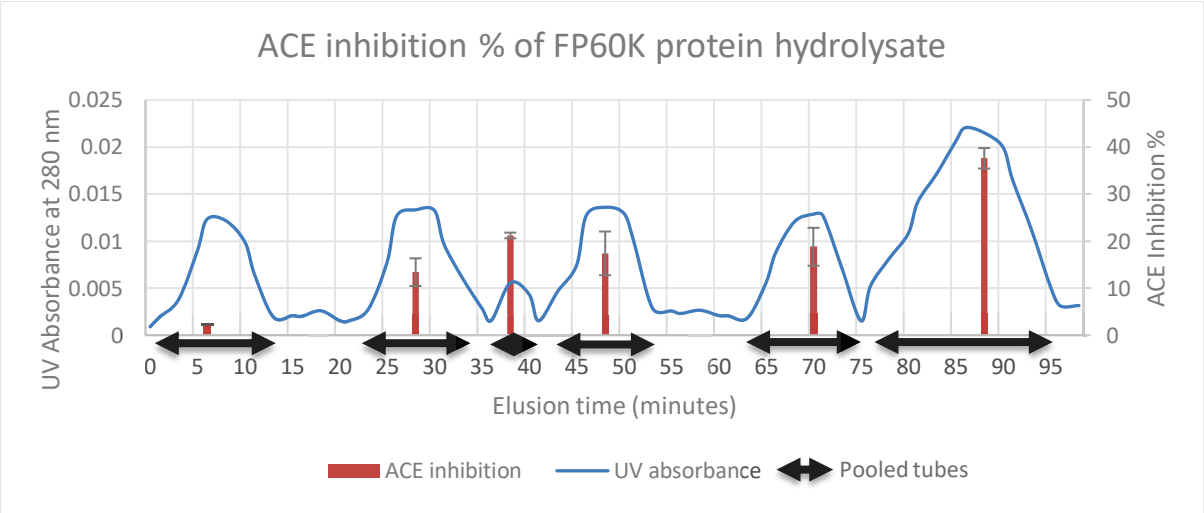
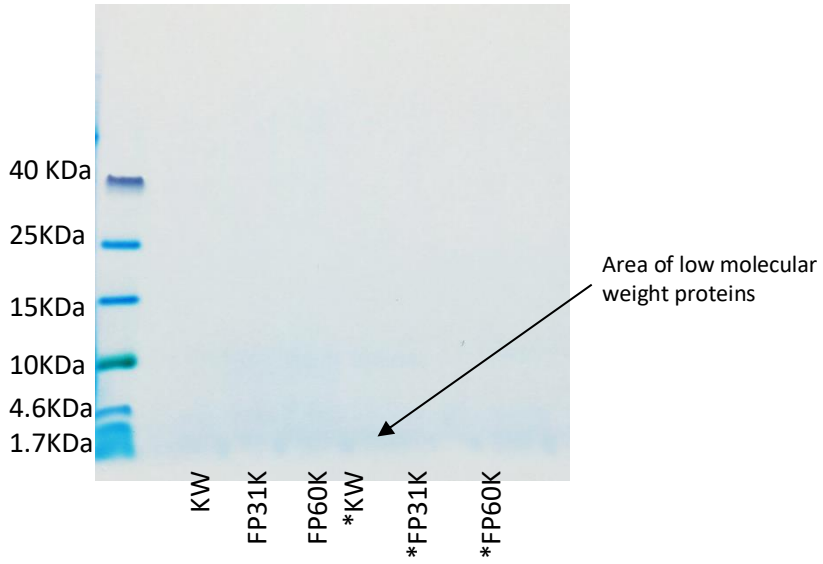


Figure 6.9 Fractionation of protein hydrolysates of beef lungs with FP60k protease on a Sephadex G-25 gel filtration column and ACE inhibitory activity of pooled tubes.

6.3.7 Tricine SDS-PAGE

Tricine SDS-PAGE gels are used to illustrate low molecular weight proteins which cannot be identified through Bis-Tris SDS-PAGE gels (Schagger & Jagow, 1987). Figure 6.10 presents the tricine gel with a molecular weight range of below 40 KDa. Proteins of selected high active eluted fractions appeared below 1.7 KDa confirming the lower weight of highly active peptides. Ultrafiltration with 3 KDa membranes for these fractions enriched them for low molecular weight peptides. ACE inhibition of the fractions increased after 3 KDa filtration (Table 6. 3), however, no difference was observed in the protein bands on the tricine gel (Figure 6.10)



*Filtrate of 3 KDa membrane

Figure 6.10 Highest active fractions with 10%-20% tricine gel

6.3.8 Changes of IC50 values throughout the process

Table 6.3 illustrates the IC_{50} value for ACE-Inhibitory activity of protein hydrolysates produced from beef lungs using different enzymes during the purification steps. IC_{50} value is defined as a minimum protein concentration (mg/mL) required to inhibit 50% of ACE activity. IC_{50} value of enzyme hydrolysate produced by kiwi fruit extract reduced more than half to 0.67±0.007 mg/mL by only filtering the fractions through a 10 KDa membrane. Membrane filtration is an economic way of producing cheap bioactive peptides which requires low technical skills compare to other technologies and can be easily achieved in industrial processes (Yang et al., 2019). Passing the fractions of kiwi fruit extract, FP31K and FP60K hydrolysates through the gel filtration and 3 KDa ultrafiltration purified the proteins which changed their IC_{50} values to 52 ± 3, 24 ± 1 and 29 ± 5 (µg/mL), respectively. IC_{50} values of beef lung hydrolysates recorded in our study are lower than IC_{50} values reported for goat meat

hydrolysate by Jamhari et al. (2013); Mirdhayati et al. (2016). However, the IC_{50} value in our study for captopril (positive control) was 1.4 ng/mL which was very high inhibition activity in comparison to our purified protein fractions. Our results are within the range reported for beef hydrolysates (IC_{50} values of 23 to 24 µg/mL) by Jang and Lee (2005).

Table 6.3 IC_{50} values of ACE inhibitory activity of protein hydrolysates during purification (mg/mL)

Protein hydrolysates	Purification steps			
	Enzymatic hydrolysate	UF* 10 KD extract	Gel filtered fraction (highest activity peaks)	UF* 3 KD extract
Kiwi	1.41±0.02[b]	0.67±0.007[c]	0.098±0.002[b]	0.052±0.003[a]
FP31K	1.81±0.01[a]	1.53±0.02[a]	0.0423±0.011[a]	0.024± 0.001[c]
FP60K	1.76±0.02[a]	1.05±0.004[b]	0.044±0.003[c]	0.029±0.005[b]

*UF, Ultrafiltration (Through 10KD/ 3KD membrane)

* Mean ± Standard deviation in the same column followed by different superscript differ significantly at p<.05

6.3.9 Peptide identification

The fractions with highest ACE inhibitory activity were analysed using mass spectrometry for amino acid sequences for peptides. Table 6.4 presents the peptide sequences found in the in different beef lung fractions. Many ACE inhibitory peptides have been reported to contain 2 to 12 amino acid residues (Byun & Kim, 2002) and high proportion of our results were also within the same range. A total of 193 peptides were identified from FP31k bovine lung hydrolysates. Six tetrapeptides were identified and only of the identified 18 peptides had more than 12 amino acids. Ninety percent of the peptides were 4 and 12 amino acids. A hexa-peptide, Val-Ser-Pro-Gly-Met-Pro, was identified as a likely ACE inhibitory peptide even though it was not in in the BIOPEP database. Peptides which initiate with valine (V), leucine (L) or isoleucine (I) and terminate with proline (P) have been reported to have a strong ACE inhibitory activity by several studies (Matsumura, Fujii, Takeda, Sugita, and Shimizu (1993); Hrynkiewicz, Iwaniak, Bucholska, Minkiewicz, and Darewicz (2019). Based on this evidence Val-Ser-Pro-Gly-Met-Pro peptide sequence may have strong ACE inhibitory activity

The second highest activity was reported from FP60K digested fractions (Table 6.3) which showed peptide sequences in Table 6.4. A total of 55 peptides were identified from FP60K hydrolysates. This hydrolysate contained 93% of its peptides between 4 to 12 amino acids. The percentage of smaller peptide pool corresponds to the ACE inhibitory activity according to the results of Ryder et al. (2016). None of the peptides had characteristics which would suggest ACE inhibitory activity.

A total of 378 different peptide sequences were found in the Kiwifruit extract hydrolysate (Table 6.4). The lowest ACE inhibitory activity was observed in Kiwifruit extract hydrolysate, and it had

the lowest (81%) smaller peptide pool in between 4 to 12 amino acids compared to the FP31K and FP60K hydrolysates.

Parent proteins of amino acid sequences were determined from the bovine protein database, and many of the peptides did not have corresponding proteins (Table 6.5). FP31K and FP60K fungal protease derived peptides mainly from bovine serum albumin (BSA) and structural proteins like collagen, actin, myosin and filamin. BSA is the most abundant blood serum protein in bovine (Assadpour & Jafari, 2019) and bovine lungs may contain a high amount of BSA due to lung containing a significant amount of blood. Further, Table 6.4 showed the presence of globin A1 like blood-related proteins. The peptides of kiwifruit extract hydrolysate mainly originated from structural proteins with collagens, elastin, and filamins prominent (Table 6.4). The highest number of (94) peptides were derived from Collagen chains with 48 peptides from collagen α-2(I) chain and 17 peptides from collagen α-1(I) chain. Elastin produced 79 peptides the next most abundant proteins, globin C1, filamin A, haemoglobin β, histone H2A type 2-C and heat shock protein β-1 produced 20, 8,7,7 and 7 peptides respectively (Table 6.4). These results confirmed high proportion of collagen in bovine lungs (Francis & Thomas, 1975b). Other sarcoplasmic proteins and membrane proteins were also represented in the peptide pool.

Peptide sequencing had a limited ability to identify di-peptides and tri-peptides and identified tetrapeptides as the lowest amino acid sequences. Due to this limitation, cryptids were analysed to reveal amino acid sequences buried within the peptides (Pimenta & Lebrun, 2007) with known ACE inhibitory activity. These cryptid results were presented in Table 6.6 for each enzyme hydrolysates. The ACE inhibitory activities of hidden amino acid sequences were revealed through this process, and there is a high possibility to produce these smaller ACE inhibitory peptides which may be unidentified due to limited technical ability.

Identified ACE inhibitory peptides should have the ability to reach the bloodstream through the gastrointestinal tract. Generally, ACE inhibitory peptides are shorter peptides which less accessible to gastrointestinal enzyme degradation (Matthews & Payne, 1980) and could be absorbed without further hydrolysis. Roberts, Burney, Black, and Zaloga (1999) has reported the evidence of potential absorbance of peptides larger than di- and tri- peptides using a rat bioassay. Further ACE inhibitory peptide with 8 amino acids was derived by the Saiga et al. (2008) with strong ACE inhibitory effect. The anti-hypertensive effect of this peptide was confirmed with the in *vivo* rat-bioassay by showing the significantly low blood pressure level.

Table 6.4 Summary table of the number of peptides with parent proteins following digestion of bovine lungs with different proteases

Parent Protein	**FP31K digestion (number of peptides)**	**FP60K digestion (number of peptides)**	**Kiwi fruit extract digestion (number of peptides)**
Actin- aortic smooth muscle	3	3	0
Actin, cytoplasmic 2	0	0	4
AGER, splice variant 8	1	0	0
Annexin A6	1	0	0
Collagen α-1(I) chain	0	0	17
Collagen α-1(II) chain	0	0	2
Collagen α-1(III) chain	0	0	3
Collagen α-1(XV) chain	0	0	1
Collagen α-1(XVII) chain	0	0	3
Collagen α-2(I) chain	3	2	48
Collagen α-2(IV) chain	0	0	7
Collagen α-2(V) chain	0	0	1
Collagen α-2(VI) chain	0	0	3
Collagen α-2(VIII) chain	0	0	1
Collagen α-3(IV) chain	0	0	1
Collagen α-3(V) chain	0	0	2
Collagen α-3(VI) chain	0	0	4
Collagen α-5(IV) chain	0	0	2
Decorin	0	0	2
Elastin	0	0	79
Epsin 1	2	0	0
Ezrin	0	0	2
Fibrinogen α chain	0	1	0
Filamin A	1	1	8
Gelsolin	0	0	1
Globin A1	3	0	1
Globin C1	0	0	20
Heat shock cognate 71 (HSC71)	1	0	0
Hemoglobin subunit α	0	0	4
Hemoglobin β	2	0	7
Histone H2A type 2-C	0	0	7
Histone H4	1	0	0
HNRP-H2	1	0	0
HNRP-L	1	0	0
HSP 70	0	0	1
HSPB1	0	0	7
HSPG2	2	0	0
LAMC1	0	0	1
LIMD 1	0	0	1
M130	1	0	0
MAPKISS	0	0	2
Myosin light chain 3	1	0	0
Myosin light chain 3	1	0	0

Myosin regulatory light chain 2	2	0	0
Myosin-1	0	1	0
Myosin-7	2	0	0
Niban apoptosis regulator 2	1	0	0
PDIA6	1	0	0
Peroxiredoxin-5, mitochondrial	1	0	0
PGRMC	1	3	0
PGRMC 2	0	1	0
Reticulon	2	1	4
Serum albumin	8	3	0
Tensin-1	4	0	0
Tensin-2	1	0	0
Tight junction protein 1	1	0	0
TtRLC	1	0	0
Vimentin	1	0	0
zyxin	1	0	0
β-lactoglobulin	1	0	0
Total sequenced peptides	193	54	378

* M130=Scavenger receptor cysteine-rich type 1 protein M130, PGRMC= Progesterone receptor membrane component, EFEMP1=EGF containing fibulin extracellular matrix protein 1, HSP70= Heat Shock Protein70, HSPB1=Heat shock protein β-1, ASM=Aortic smooth muscle, HSPG2= Heparan sulfate proteoglycan 2, AHSG=alpha-2-HS-glycoprotein, Hsc71=Heat shock cognate 71 kDa protein, Adenylyl CAP=Adenylyl cyclase-associated protein, SDC2=Sushi domain containing 2, TtRLC=Tryptophan--tRNA ligase- cytoplasmic, AGER=Advanced glycosylation end product-specific receptor-splice variant 8, SPTAN1=Spectrin alpha non-erythrocytic 1, MAPKISS= MAPK-interacting and spindle-stabilizing protein-like, PDIA6= Protein disulfide isomerase family A member 6, LAMC1= Laminin subunit gamma 1, HNRP-L= Heterogeneous nuclear ribonucleoprotein L-like, MLC-2= Myosin regulatory light chain 2, LIMD1=LIM domain-containing protein 1, HNRP-H2=Heterogeneous nuclear ribonucleoprotein H2

Table 6.5 Bovine lung peptide digestion with different proteases (A- FP31K protease B- FP60K protease C-Kiwifruit extract)

A

Bovine lung peptide digestion with FP31K protease					
Parent protein	**peptides**	**Peptide Length**	**Parent protein**	**peptides**	**Peptide Length**
Actin, ASM	TERGYSF	7	Myosin light chain 3	IKIEFTPEQIEEFKEAFTLFDR	22
Actin, ASM	VPIYEGY	7	Myosin regulatory light chain 2	EAPGPINFTVFLQMFGEK	18
Actin, ASM	YELPDGQVIT	10	Myosin regulatory light chain 2	PEETILNAFKVFD	13
AGER, splice variant 8	SPQGDPWDS	9	Myosin-7	QLEAEKLELQSALEEAEASLEHEEGK	26
Annexin A6	DFPDFNPS	8	Myosin-7	QLEAEKLELQSALEEAEASLEHEEGKILR	29
Collagen α-2(I) chain	FDGDFY	6			
Collagen α-2(I) chain	FGFDGDF	7	Reticulon	WDPSPVS	7
Collagen α-2(I) chain	GFDGDFY	7	Reticulon	WDPSPVSS	8
Complement factor B	DFENGEYWP	9	M130	GEGSGPIW	8
Epsin 1	TDPWGAPV	8	Serum albumin	AFDEKLF	7
Epsin 1	TDPWGAPVS	9	Serum albumin	AWSVARL	7
Filamin A	DLGDGVY	7	Serum albumin	ELLYY	5
Globin A1	DSFSNGM	7	Serum albumin	ELLYYA	6
Globin A1	LLGNVLVVVLAR	12	Serum albumin	ELLYYAN	7
Globin A1	PENFKLLGNVLVVVLAR	17	Serum albumin	GEYGFQN	7
HSC71	DLGGGTF	7	Serum albumin	NKYNGVFQ	8
Hemoglobin β	FESFGDL	7	Serum albumin	RHPYFYAPELLYYANK	16
Hemoglobin β	FESFGDLS	8	Vimentin	YTSSPGGVY	9
HNRP-H2	YGGGYGGYDD	10		FP31K	5
HNRP-L	EFGPISY	7		AAPSAPWVGA	10
Histone H4	TLYGFGG	7		AGGPASAQG	9
Major allergen β-lactoglobulin	VAGTWYSLAMAASDISLLDAQSAPLR	26		APGSYDFG	8

PGRMC	YLGDDY	6		ASFSNF	6
Myosin light chain 3	IEFTPEQIEEFKEAFTLFDR	20		ASSGGGGGGLM	11
	AVVEPYN	7		FDGDF	5
	AWGPGLE	7		FDISSFADL	9
	DEFPF	5		FDLL	4
	DFGFLD	6		FDNRGGGGF	9
	DFGPNGEV	8		FDSDWGEF	8
	DFPGF	5		FELF	4
	DFSGAVY	7		FELVN	5
	DFSSCQA	7		FETF	4
	DLRFQ	5		FGLGTPE	7
	DPAGPPEASV	10		FHSADARGG	9
	DPVNF	5		FPHFD	5
	DQGGAGLE	8		FPVGF	5
	DSGGPGGAGGGA	12		GEFPGGLM	8
	DSWDGSDATVF	11		GFDGDF	6
	DYLDVGF	7		GFFDPN	6
	EAASGTPSP	9		GGAGVGAGGQGP	12
	EDKAGFRS	8		GGGGAAGGPQ	10
	EDSGDYPLTM	10		GGGGGGPGGGGGG	13
	EFMDGQP	7		GGGGGGQAP	9
	ELMF	4		GGGGGLGGGLGN	12
	ELSYGY	6		GGGGGSGGGGGGGA	14
	EMWLSK	6		GGGGGSGPPSV	11
	ERGYSFTTT	9		GGVCGGGAGG	10
	ESGGPANGTP	10		GPIGPVG	7
	ETFNTPAMY	9		GPLGPPGPS	9
	ETGAPGVAVAPG	12		GPPADPWGGT	10
	EVQIGGPGS	9		GPPPSADG	8

	EWPLL	5		GPQGPPGSIGPQ	12
	FDGASGLP	8		GSEPIQY	7
	GTWNPGRP	8		PFGSVPF	7
	GYSSGSPVY	9		PGFGGEPGPQ	10
	HGGTTADM	8		PGPINFTVFLQMFGEK	16
	IDSPGPE	7		PMMPGMLM	8
	KAFPGML	7		PSKEPEFDPSKIKIEFTPEQIEEFKEAFTLFDR	33
	LDLL	4		PSSPDWGTF	9
	LDSPGPE	7		QALSYRE	7
	LELYYA	6		QDSLGGNA	8
	LPEEWSQ	7		QDWTGGK	7
	LPGGPGPSP	9		QDWTGGKE	8
	MAGGGPEP	8		QDWTGGKEF	9
	MDPDLLGSKGEKGDPGLPGIPGVAGP KGYQ	30		QIDDGWMY	8
	MLGDAIM	7		QKVVAGVAN	9
	MLGPSLSPGQD	11		QLLY	4
	MMSSTSN	7		QLSYGYD	7
	MPASPNP	7		QLSYGYDE	8
	MPGDF	5		QMNALGP	7
	MSGLLGPGGM	10		QNDPWTPT	8
	NDPWTPT	7		QNGGTCTHG	9
	NFENPFY	7		QPIDDGWM	8
	NFGTPEFL	8		QPNAAGSEC	9
	NFVAF	5		QTALVELLK	9
	NFVAFV	6		QYEAY	5
	NGGGSIN	7		SAFSNF	6
	NQNDPWTPT	9		SAGGGSGGGI	10
	NTMLTF	6		SEGPIQY	7

	NVPIYEGY	8		SFGDL	5
	PATGAANGLN	10		VSPGMP	6
	SGYPGAGGYP	10		VYSSTSN	7
	SLPGACAANP	10		WDTANNPL	8
	SPTQGAGPAGL	11		WDTANNPLY	9
	SPVPSATE	8		WGDPSVE	7
	SVSLDTGFP	9		WLGSSSATN	9
	SWQEGDT	7		WNGPVGV	7
	TDYALF	6		WNPPEGASPN	10
	TEPWDPL	7		WVSGLSGYTD	10
	TSAPGFP	7		YDLYDTSAGEGTY	13
	TTGSGGAGG	9		YEGFDF	6
	TTNGPTES	8		YGPEGPY	7
	VDPVNF	6		YPASTVCP	8
	VEPIPWN	7		YQPEYLY	7
	VGTHGGSGGGAGP	13		YSGVGSGP	8
	VIQYFAVIAAIGDR	14		YVGDF	5
	VNPTVFF	7		YYDSPSVN	8

B

Bovine lung peptide digestion with FP60K protease					
Parent protein	**Peptides**	**Peptide Length**	**Parent protein**	**peptides**	**Peptide Length**
Actin, ASM	NVPIYEGY	8		ELSYGY	6
Actin, ASM	NWDDMEK	7		EYEAY	5
Actin, ASM	VPIYEGY	7		FASPDDR	7
Collagen α-2(I) chain	GFDGDFY	7		FDGDF	5
Collagen α-2(I) chain	GPSGGGYEF	9		FDGDFY	6
Fibrinogen α chain	PSSPDWGTF	9		FDGDYF	6
Filamin A	AWGPGLE	7		FDLF	4
PGRMC	YGPEGPY	7		FDVF	4
Myosin-1	QVEQEKSEIQAALEEAEASLEHEEGKILR	29		FETF	4
ND	WNDPSVQ	7		FGASPP	6
PGRMC	YLGDDY	6		FSTF	4
PGRMC 2	YGPAGPY	7		LQEGW	5
Reticulon	WDPSPVSS	8		LQEWGY	6
Serum albumin	DAFLGSFLYEYSR	13		LQEWGY	6
Serum albumin	GLVLIAFSQYLQQCPFDEHVK	21		LQEWGYA	7
Serum albumin	LGEYGFQNALIVR	13		LYGYE	5
	ACVVDQ	6		NFGNEF	6
	ADGSYF	6		QDWTGGKEF	9
	AWGGPL	6		QFHL	4
	DFVF	4		QLSYGY	6
	DWTGGKEF	8		QLSYGYD	7
	EFHL	4		QYEAY	5
	ELLYY	5		SFGDL	5

	SYGGSL	6		SFSNGM	6
	SYDF	4		SSGLY	5
	TYFPH	5		YSCFLF	6
	WAGAPP	6		YSVY	4

C

Bovine lung peptide digestion with kiwifruit extract					
Parent protein	**peptides**	**Peptide Length**	**Parent protein**	**peptides**	**Peptide Length**
Actin, cytoplasmic 2	DDDIAALVVDN	11	Collagen α-1(I) chain	GPAGPPGFPGAVG	13
Actin, cytoplasmic 2	DSYVGDEAQSKRGILT	16	Collagen α-1(I) chain	GPIGPVG	7
Actin, cytoplasmic 2	VAPEEHPVLL	10	Collagen α-1(I) chain	GPPGFPGAVG	10
Actin, cytoplasmic 2	VFPSIVG	7	Collagen α-1(I) chain	IAGQRGVVG	9
Adenylyl CAP	SGSDDSASRSALF	13	Collagen α-1(I) chain	ISVPGPMG	8
AHNAK nucleoprotein	FPDVEFDIK	9	Collagen α-1(I) chain	ISVPGPMGPSGPR	13
AHNAK nucleoprotein	GPQITGPSVE	10	Collagen α-1(I) chain	KSGDRGETGPAGPAGPIGPVG	21
AHNAK nucleoprotein	GPQITGPSVEG	11	Collagen α-1(I) chain	LSVPGPM	7
AHNAK nucleoprotein	LPSLEGGLN	9	Collagen α-1(I) chain	QLSYGYDEK	9
AHNAK nucleoprotein	VDINFPKVE	9	Collagen α-1(I) chain	QLSYGYDEKSTG	12
Aldehyde oxidase 1	YPQAPVVMG	9	Collagen α-1(II) chain	GLPGPPGPSG	10
α-1B-glycoprotein	SPAGPEAQFELR	12	Collagen α-1(II) chain	SPGPAGPIG	9
α-2-HS-glycoprotein	SVVVGPSVVA	10	Collagen α-1(III) chain	ERGAPGPQGPPGAPG	15
Calponin-1	LTPEYPELG	9	Collagen α-1(III) chain	EYEAYDVK	8
Carbonic anhydrase 3	ASYDPGSAKTILN	13	Collagen α-1(III) chain	NVGLAGLT	8
Carbonic anhydrase 3	EPPVPLVR	8	Collagen α-1(XVII) chain	GPQGPPGSIGPQG	13
Carbonic anhydrase 3	SSAENEPPVPLVR	13	Collagen α-1(XVII) chain	GVPGAPGIPG	10
Collagen α-1(V) chain	GPAGPMGLT	9	Collagen α-1(XVII) chain	PVGPAGLPG	9
Collagen α-1(I) chain	AGPAGPIGPVG	11	Collagen α-2(I) chain	AAGAPGPQGPVGPVG	15
Collagen α-1(I) chain	AGPIGPVG	8	Collagen α-2(I) chain	AAGATGARGLVG	12
Collagen α-1(I) chain	GERGFPGLPGPSG	13	Collagen α-2(I) chain	AAGPTGPIG	9
Collagen α-1(I) chain	GFPGADGVA	9	Collagen α-2(I) chain	AGAPGPQGPVGPVG	14
Collagen α-1(I) chain	GPAGPAGPIG	10	Collagen α-2(I) chain	APGPAGARGSDGSVGPVGPAGPIG	24
Collagen α-1(I) chain	GPAGPAGPIGPVG	13	Collagen α-2(I) chain	APGPQGPVGPVG	12

Collagen α-1(I) chain	GPAGPIGPVG	10	Collagen α-2(I) chain	ARGSDGSVGPVGPAGPIG	18
Collagen α-2(I) chain	ASGPPGFVG	9	Collagen α-2(I) chain	LPGIDGRPGPIGPAG	15
Collagen α-2(I) chain	AVGPRGPSGPQ	11	Collagen α-2(I) chain	PVGPAGPIG	9
Collagen α-2(I) chain	AVGPRGPSGPQG	12	Collagen α-2(I) chain	QFDAKGGGPGPMG	13
Collagen α-2(I) chain	EIGPAGPPGPPGL	13	Collagen α-2(I) chain	QPGAVGPA	8
Collagen α-2(I) chain	EIGPAGPPGPPGLR	14	Collagen α-2(I) chain	QPGAVGPAG	9
Collagen α-2(I) chain	ENGPVGPTGPVG	12	Collagen α-2(I) chain	QPGAVGPAGIR	11
Collagen α-2(I) chain	GAAGLPGVA	9	Collagen α-2(I) chain	RSGETGASGPPGFVG	15
Collagen α-2(I) chain	GAAGLPGVAG	10	Collagen α-2(I) chain	SAGPPGFPG	9
Collagen α-2(I) chain	GAAGPTGPIG	10	Collagen α-2(I) chain	SGETGASGPPGFVG	14
Collagen α-2(I) chain	GAPGPQGPVGPVG	13	Collagen α-2(I) chain	SVGPVGPAGPIG	12
Collagen α-2(I) chain	GENGPVGPTGPVG	13	Collagen α-2(I) chain	TPGPQGLLG	9
Collagen α-2(I) chain	GETGASGPPGFVG	13	Collagen α-2(I) chain	VGLPGLL	7
Collagen α-2(I) chain	GEVGLPGLS	9	Collagen α-2(IV) chain	EMGPQGPLGSHGGYT	15
Collagen α-2(I) chain	GEVGPAGPNGFA	12	Collagen α-2(IV) chain	EVGPTGDFG	9
Collagen α-2(I) chain	GFPGSPGNIGPAG	13	Collagen α-2(IV) chain	EVGPTGDFGDIG	12
Collagen α-2(I) chain	GGPGPMGLM	9	Collagen α-2(IV) chain	GLPGAPGAVGTPG	13
Collagen α-2(I) chain	GIDGRPGPIGPA	12	Collagen α-2(IV) chain	IAIERGPVGPQG	12
Collagen α-2(I) chain	GIDGRPGPIGPAG	13	Collagen α-2(IV) chain	TPGITGIPQ	9
Collagen α-2(I) chain	GLPGVAGSVG	10	Collagen α-2(IV) chain	YTGPPGLQG	9
Collagen α-2(I) chain	GPAGARGSDGSVGPVGPAGPIG	22	Collagen α-3(IV) chain	EMGKPGLPG	9
Collagen α-2(I) chain	GPAGPAGPRGEVG	13	Collagen α-5(IV) chain	GPPGSPGLPG	10
Collagen α-2(I) chain	GPPGESGAAGPTGPIG	16	Collagen α-5(IV) chain	GQPGIPGLPGDPGYP	15
Collagen α-2(I) chain	GPQGPVGPVG	10	Collagen α-2(V) chain	GVQGPEGKLGPLG	13
Collagen α-2(I) chain	GPRGFPGSPGNIGPAG	16	Collagen α-3(V) chain	EPGPRGLIGPR	11
Collagen α-2(I) chain	GPRGFPGSPGNIGPAGKEGPVG	22	Collagen α-3(V) chain	GEPGPRGLIGPR	12
Collagen α-2(I) chain	GPVGPTGPVG	10	Collagen α-2(VI) chain	DIANTPHELY	10
Collagen α-2(I) chain	GSDGSVGPVGPAGPIG	16	Collagen α-2(VI) chain	DPGIEGPIG	9
Collagen α-3(VI) chain	LSDAGITPLF	10	Collagen α-2(VI) chain	NVVPTVVAVG	10

Collagen α-3(VI) chain	QSGVVPFILQ	10	Collagen α-3(VI) chain	DAGITPLF	8
Collagen α-3(VI) chain	TPLTTLT	7	Elastin	GAGIPGAPGAIPGIG	15
Collagen α-2(VIII) chain	GLPGAPGQGGAPGP	14	Elastin	GAGIPGGVA	9
Collagen α-1(XV) chain	TTYSGNSELITF	12	Elastin	GAGIPGGVAG	10
Decorin	GLNNLAKLG	9	Elastin	GAGVLPGVG	9
Decorin	SSGIENGAFQ	10	Elastin	GAGVLPGVGVG	11
EFEMP1	ASGVVPGGGFVA	12	Elastin	GAIPGVPGVG	10
Elastin	AAGGFPGVG	9	Elastin	GAVPGAVGLG	10
Elastin	AAQFGLGPGVG	11	Elastin	GGGAFAGIPGVGPFG	15
Elastin	AFPGALVPGGPAG	13	Elastin	GIGGVGGLG	9
Elastin	AGGAGVLPGVG	11	Elastin	GIPGVGPFG	9
Elastin	AGGAGVLPGVGVG	13	Elastin	GLAGPGLG	8
Elastin	AGIPGVGPFG	10	Elastin	GLPAGVPGLG	10
Elastin	AGLPAGVPGLG	11	Elastin	GLVPGAPGAIPGVPGVG	17
Elastin	AGQPFPIG	8	Elastin	GQQPGVPLG	9
Elastin	AGQPFPIGGGAGGLG	15	Elastin	GVFFPGAGLG	10
Elastin	AGQPFPIGGV	10	Elastin	GVGDLGGAGIPG	12
Elastin	AGQPFPIGGVA	11	Elastin	GVGDLGGAGIPGGVA	15
Elastin	AGVPGLGVG	9	Elastin	GVGGLGVGGLG	11
Elastin	AIPGVPGVG	9	Elastin	GVGPGGFPGIG	11
Elastin	ALPGAFPGAL	10	Elastin	GVPGGVFFPGAGLG	14
Elastin	APGAIPGVPGVG	12	Elastin	GVVPGVG	7
Elastin	APKLPGVGPQA	11	Elastin	IGLGPGGVIG	10
Elastin	AVPGAVGLG	9	Elastin	IPGVGPFG	8
Elastin	DLGGAGIPGGVA	12	Elastin	LGPGGVIG	8
Elastin	GAAGGFPGVG	10	Elastin	LPAGVPGLG	9
Elastin	GAGGAGVLPGVG	12	Elastin	LPGVGPQA	8
Elastin	GAGGAGVLPGVGVG	14	Elastin	LPGVYPGGVLPGAG	14
Elastin	PAVMGAGGVGAGPAGGASVSH	21	Elastin	LSPIFPGGAG	10

Elastin	PGVGVPG	7	Elastin	LSPIFPGGAGGLG	13
Elastin	PGVGVPGVGVPG	12	ELM2 and Myb/SANT domain containing 1	GLQPAGPLGQSHLA	14
Elastin	QPFPIGGGAGGLG	13	Ezrin	APPPPPVY	8
Elastin	QPGYPGGVGAPGAGGASVSH	20	Ezrin	TAPPPPPVY	9
Elastin	SPIFPGGAGGLG	12	Filamin A	DGSPVPSSPFQ	11
Elastin	TGAVVPQLG	9	Filamin A	DGVPVPGSPF	10
Elastin	VAPGIGLGPG	10	Filamin A	DGVPVPGSPFS	11
Elastin	VAPGIGLGPGG	11	Filamin A	DGVPVPGSPFSLE	13
Elastin	VAPGIGLGPGGVIG	14	Filamin A	FSVEGPSQ	8
Elastin	VAPGVGVVPGVG	12	Filamin A	SPAEFIVN	8
Elastin	VGAGVPGLG	9	Filamin A	VPYEAGTYSLN	11
Elastin	VGGIGGVGGLG	11	Filamin A	YTPVQQGPVG	10
Elastin	VGGIPTF	7	Gelsolin	VPFDAATLH	9
Elastin	VGGIPTFG	8	Globin A1	KEFTPVLQ	8
Elastin	VGPGGFPGIG	10	Globin C1	AEALERMF	8
Elastin	VGVAPGIG	8	Globin C1	AEALERMF	8
Elastin	VGVGVPGLG	9	Globin C1	AEYGAEALERMF	12
Elastin	VGVPGLG	7	Globin C1	ASHLPSDFTPAVH	13
Elastin	VGVPGLGVG	9	Globin C1	ASLDKFL	7
Elastin	VGVPGVG	7	Globin C1	ASLDKFLA	8
Elastin	VGVPGVGVPGVG	12	Globin C1	ASLDKFLAN	9
Elastin	VGVVPGVG	8	Globin C1	ASLDKFLANVS	11
Elastin	VPGLGVG	7	Globin C1	AVEHLDDLPGALS	13
Elastin	VPGLGVGVGVPG	12	Globin C1	GAEALERMF	9
Elastin	VPGVGVPG	8	Globin C1	GHAAEYGAEALE	12
Elastin	VPGVGVPGVG	10	Globin C1	GHAAEYGAEALER	13
Elastin	VSTGAVVPQL	10	Globin C1	GHAAEYGAEALERM	14
Elastin	VSTGAVVPQLG	11	Globin C1	GHAAEYGAEALERMF	15

Globin C1	PSDFTPAVH	9	Globin C1	LRVDPVNF	8
Globin C1	SHLPSDFTPAVH	12	Globin C1	NVSTVLT	7
Globin C1	VDPVNFKLL	9	Histone H2A type 2-C	GVLPNIQA	8
Globin C1	VDPVNFKLLS	10	Histone H2A type 2-C	GVLPNIQAV	9
HSP70	QGAGGPGAGGFG	12	Histone H2A type 2-C	IAQGGVLPNIQ	11
HSPB1	AERRVPFSLL	10	Histone H2A type 2-C	SSRAGLQFPVG	11
HSPB1	AIEGPAYNRALS	12	LAMC1	EANDILNNLK	10
HSPB1	ALPAAAIEGPAY	12	LIMD 1	SGWSGTPGSDPLL	13
HSPB1	ALPAAAIEGPAYN	13	MAPKISS	APTDPAAAGPLGPWG	15
HSPB1	IEGPAYNRALS	11	MAPKISS	TPSPVPFGPTPTG	13
HSPB1	QSAEITIPVTF	11	Niban apoptosis regulator 2	QLPSEKLVG	9
HSPB1	SSLSPEGTLT	10	Peroxiredoxin-5, mitochondrial	DAIPSVEVF	9
Hemoglobin β	ADFQKVVA	8	PDIA6	GGAFPTIS	8
Hemoglobin β	DLSTADAVMN	10	Reticulon	APLVDFG	7
Hemoglobin β	NEFTPVL	7	Reticulon	ATPASAPLVDFG	12
Hemoglobin β	NEFTPVLQ	8	Reticulon	GPLPAAPLA	9
Hemoglobin β	QADFQKVVA	9	Reticulon	NDFVPPAPR	9
Hemoglobin β	QADFQKVVAG	10	SPTAN1	QWINEKEAALT	11
Hemoglobin β	QADFQKVVAGVA	12	SDC2	TFNNDPADDFTLR	13
Hemoglobin subunit α	LSFPTT	6	Tensin 2	SPTPAFPLA	9
Hemoglobin subunit α	LSFPTTK	7	Tensin-1	DQEPGAFIIR	10
Hemoglobin subunit α	VDPVNF	6	Tensin-1	GSFVSPSPLS	10
Hemoglobin subunit α	VDPVNFKL	8	Tensin-1	SPSGGSTVSFS	11
HSPG2	ASISGVSLE	9	Tensin-1	TNTPPSPGFG	10
HSPG2	SPSPGELVF	9	Tight junction protein 1	EPVPLSY	7
Histone H2A type 2-C	AGLQFPVG	8	TtRLC	STFFPALQ	8
Histone H2A type 2-C	GGVLPNIQ	8	Zyxin	SGYVPPPVT	9

Histone H2A type 2-C	GVLPNIQ	7		AAQPFLF	7
	AGVIFPVG	8		AEALEALSY	9
	ALVELL	6		AFAPVLK	7
	AMLVAGLE	8		FLAAWP	6
	ANPLLE	6		FLLRT	5
	APAPLL	6		FPLPPPPVA	9
	APDLNLHL	8		FPTTK	5
	APRTAVVPA	9		GAPSFPLG	8
	APTPLL	6		GDVGTPGPPGPAG	13
	AQLPVLL	7		GGAPSFPLG	9
	ASLFGSVL	8		GPGALQGSGLAPGP	14
	ASLFGSVL	8		GPIPVAPIG	9
	ATLNELL	7		GPVGPGLMT	9
	ATMAAPGGGGAGPPGVGGG	19		GQAVAGSPSP	10
	CKLVGVGHG	9		GSGHGPR	7
	DGSAFPFDIEG	11		GVSDPGRR	8
	DLLALL	6		HPLPTT	6
	DPFTIKPLD	9		IITADDGSGGPL	12
	DPLLEL	6		KNPGALL	7
	DPPANIQLF	9		KPGAEGGAGGGAGAAGGASVSH	22
	DTPSCPPALL	10		LAGLTL	6
	EGLPGPQGAPGLMGQ	15		LAGQAGALR	9
	EIPASVF	7		LAVELL	6
	ELLL	4		LELPFVK	7
	ENKYVDSQGHLY	12		LKLPGVL	7
	EQIPLLVQ	8		LLMPTTK	7
	ETLLEL	6		LPALL	5
	EVGGGPGVGYNVN	13		LPAPLVR	7
	LSTAALE	7		LPGVGVPG	8

	LSVPGMP	7		LPSLL	5
	LTAAPLV	7		SGVHTFPAVLQ	11
	LTLPVL	6		SGVPGVRLL	9
	LTPLL	5		SLDAYPVLN	9
	MMPDGTLG	8		SPLPVLT	7
	MPPLT	5		SQVPPGPTPPLQF	13
	NEGLPAPIVR	10		SSFAEGLSE	9
	NPPVGPIG	8		SSYMPEPVT	9
	PAGGPYGGVGAPAGGGASVSH	21		STALYGESDL	10
	PFPSGGGGVGAGGGASVGAYT	21		SYMPEPVT	8
	PGGLLL	6		TAPLGAL	7
	PPSAVGSPAAAP	12		TAPLQL	6
	QALPVLL	7		TFPAVL	6
	QALSYREAVLR	11		TFPAVLQ	7
	QGLLPVL	7		TLLL	4
	QNLNDRLA	8		TPFITNPGYDT	11
	QTLLEL	6		TVFPTT	6
	RDGGPRGGPA	10		VAPEEHPTLL	10
	SEGALSPGGLA	11		VAVAGPLG	8
	SFPLPTLT	8		VDLDVLKSR	9
	SGVHTFPAVL	10		VEAGALL	7
				VSPLT	5

Table 6.6 Cryptides for ACE inhibitory activity

BLP digested with FP31K enzyme		BLP digested with FP60K enzyme		BLP digested with kiwi fruit extract	
Sequence	**BIOPEP data base ID**	**Sequence**	**BIOPEP data base ID**	**Sequence**	**BIOPEP data base ID**
AA	7590	AA	7590	AA	7590
AF	7583	AF	7583	AF	7583
AGP	9058	AGP	9058	AGP	9058
AP	7584	AP	7584	AGSS	8096
AR	7742	DA	7606	AP	7584
AV	8951	DG	7681	AR	7742
DA	7606	DY	9072	AV	8951
DAQSAPLRVY	9112	EA	7623	DA	7606
DG	7681	EG	7622	DG	7681
DY	9072	EI	7826	EA	7623
EA	7623	EK	7840	EG	7622
EG	7622	EY	7752	EI	7826
EK	7840	FG	7605	EK	7840
ESAGIH	9002	FGASTRGA	7747	ESAGIH	9002
EV	7828	FP	3502	EV	7828
EY	7752	FQ	9076	EY	7752
FG	7605	GE	7615	FG	7605
FGASTRGA	7747	GF	7591	FGASTRGA	7747
FGG	9195	GGY	3515	FP	3502
FP	3502	GL	7599	FPFEVFGK	3394
FPFEVFGK	3394	GLY	9033	FQ	9076
FQ	9076	GP	7512	FQKVVAG	3365
GE	7615	GPA	3342	FQKVVAK	3366
GEP	7817	GY	3532	GE	7615
GF	7591	GYALPHA	3357	GEP	7817
GGY	3515	HL	7602	GF	7591
GI	7596	IL	9079	GGY	3515
GL	7599	LF	3551	GI	7596
GP	7512	LG	7619	GL	7599
GPA	3342	PG	7625	GP	7512
GQ	7610	PL	7513	GPA	3342
GR	7603	PP	7836	GQ	7610
GRP	3378	VE	7829	GR	7603
GV	7608	VF	3384	GRP	3378
GY	3532	VP	7587	GV	7608
GYALPHA	3357	VPAAPPK	8544	GY	3532
IG	7595	VR	7628	GYALPHA	3357
IL	9079	VY	3492	HL	7602
IPA	3507	WA	9089	IF	7593
IW	7544	YAAAT	8699	IG	7595
KA	7743	YE	9078	IL	9079

KG	7604	YG	3553	IPA	3507
LA	7585	YGG	7647	IRA	3547
LAA	3539			KF	7692
LF	3551			KG	7604
LG	7619			KP	7810
LKA	7569			LA	7585
MM	9085			LAA	3539
MPACGS	9105			LF	3551
MW	9091			LG	7619
PG	7625			LKA	7569
PL	7513			LSPA	3564
PLG	7510			MM	9085
PP	7836			MPACGS	9105
RF	3489			PFPE	3374
RP	7582			PG	7625
TE	7830			PKAIP	3742
TP	9073			PL	7513
VAA	3518			PLG	7510
VAGTWY	3509			PP	7836
VE	7829			PQAFP	3585
VF	3384			PR	3537
VG	7594			RA	7588
VP	7587			RP	7582
VPAAPPK	8544			RR	7741
VY	3492			TP	9073
WL	9107			VAA	3518
WM	9090			VE	7829
YAAAT	8699			VF	3384
YE	9078			VG	7594
YG	3553			VGVPGGV	8363
YGG	7647			VP	7587
YN	9185			VPAAPPK	8544
YP	3666			VR	7628
YV	9077			VY	3492
YVA	9036			VYP	3505
YYAKPAAVR	8397			YE	9078
				YG	3553
				YGG	7647
				YN	9185
				YP	3666
				YV	9077
				YVA	9036

Beef lung hydrolysates have also been reported to produce varieties of bioactive peptides with a number of other activities. Webster, Ledward, and Lawrie (1982) produced beef lung hydrolysates using heat-treated beef lungs with Neutrase and Alcalase enzymes. The heat-treated beef lungs showed above 90% of degree of hydrolysates similar to our results of FP31K hydrolysate in 6.3.4. The study of (O'Sullivan et al., 2017) produced beef lung hydrolysates using papain, pepsin, and Alcalase. Only the Alcalase beef lung hydrolysates showed significant inhibition of cytokine production in RAW264.7 cells. Further, Alcalase bovine lung hydrolysate showed antiproliferative activity above 0.005% W/V sample concentration. In a separate study, bovine lung hydrolysates produced the dipeptidyl peptidase-IV- (DPP-IV; EC 3.4.14.5) and prolyl endopeptidase- (PEP; EC 3.4.21.26) inhibitory peptides using Alalase and collagenase enzymes (Lafarga & Hayes, 2017). Pressure-treated bovine lung with Alcalase produced bioactive inhibitory peptides with the greatest activity. These had between 2 and 9 amino acids. These studies indicate the potential of utilising beef lungs as a source of bioactive compounds.

6.3.10 Implications

The main implication is identifying large number of peptides in purified fractions unexpectedly. More purification steps should be involved to achieve this target and reverse-phase HPLC and ion exchange chromatography could be a suitable approached for future researches. Di-peptides and tri-peptides were not identified in results and this could be a limitation of the mass spectrometry.

6.4 Conclusion

Anti-hypertensive peptides can be produced from the beef lung hydrolysates of Kiwifruit extract, fungal protease 31K and fungal protease 60K. Beef lung hydrolysis of fungal protease 31K produced the highest active ACE inhibitory fractions and purified through the ultrafiltration and gel filtration. Purified ACE inhibitory peptide of fungal protease 31k exhibited the IC_{50} value of 24 µg/mL. New peptide sequence “Val-Ser-Pro-Gly-Met-Pro” was identified as a possible ACE inhibitory peptide.

Chapter 7

General Discussion

Beef lungs are an underutilised co-product which has a significant nutritional value. In western countries, beef lungs have a low consumer demand due to strong aesthetic rejection and poor intrinsic textural quality (Chávez-Jáuregui et al., 2003). Processing of beef lung as a food ingredient and incorporation into widely consumed foods could act as a vehicle to deliver nutrition to communities with high levels of nutrition deficit. In the first experiment (Chapter 3), durum wheat pasta was used as a model food for beef lung supplementation. Beef lungs that had been dried at 60°C for 32 hours were received from ANZCO Foods, New Zealand. These lungs were then ground to prepare beef lung powder (BLP).

BLP in Chapter 3 was reported to have 87% of protein content which is suitable as a protein supplement. However, protein sources have different quality levels which depends on their amino acid composition (Shaheen et al., 2016). The indispensable amino acid score (IAAS) of BLP was the maximum of "1". This means that BLP contained all the necessary amino acids with required ratios and quantities to deliver the nutrition reference pattern of the older child, adolescent or adult. This reference pattern (Chapter 3) covers the amino acid requirement of all ages except infants and 0.5 to 3 years toddlers (FAO, 2011). However, infants are supposed to have exclusive breastfeeding (Cernadas, Noceda, Barrera, Martinez, & Garsd, 2003), and toddlers are more comfortable with semisolid foods than protein powder.

In Chapter 3, durum wheat pasta was selected as a model food to incorporate BLP. Because pasta is a widely consumed food around the world, BLP supplementation of pasta would be a suitable vehicle to introduce beef lungs into the human food chain. BLP 10% incorporation level exhibited more positive factors like lowest cooking loss, lighter and yellowish colour values (Chapter 3). Further, control pasta IAAS shifted from 0.48 to 0.91 and iron content increased 0.03 to 0.143 mg g^{-1}, after supplementation of 10% BLP. The glycaemic response also decreased with the BLP supplementation as healthy food.

The branched-chain amino acids (leucine, isoleucine and valine) have anabolic properties (Holeček, 2018) to build muscles. Leucine is a highly available essential amino acid in BLP followed by valine and isoleucine (Chapter 3 and 4), So there is a possibility of using BLP as a muscle-building protein supplement. The demand for protein supplements continuously growing and it has had a large profit margin for some industries. Using currently undervalued beef lungs to produce protein supplement would underpin profitability. Further, processing of beef lungs involved in simple and low-cost processing techniques (mincing, air-oven drying, grinding) in Chapter 3 and 4 to produce an economical end product. These low-cost animal proteins are essential for world hunger countries

which have less purchasing ability for costly animal proteins. Further, this beef lung powder could prevent the protein and micronutrient deficiencies in malnourished communities. These hunger countries are centred in the African region, and there is potential to establish processing facilities their as well.

Most of the African countries belong to the tropical region, and solar power is a major free energy source. So, there is a possibility of establishing solar driers for beef lung drying in African region. Further, solar units can easily reach the 50 °C temperature (Chauhan & Rathod, 2020) which is recommended temperature for beef lung drying in Chapter 4. Chapter 3 described that beef lungs were dried for a long time (32 hours) which was an economic disadvantage. Meat producing temperate countries like New Zealand cannot use solar power consistently and have to use electricity or fuel. Reducing the BLP drying temperature is identified as a key point in Chapter 4. Then Chapter 4 outlined the mincing of beef lungs to reduce the drying time and temperature. Mincing of beef lungs increased the drying surface area and successfully dried at 50 °C, 70 °C and 100 °C temperatures for 23, 11 and 6 hours respectively.

Physicochemical properties of BLP were measured and all BLP powders showed threefold higher water-binding capacity (WBC) than sample weight. These high WBC values positively affect baking doughs to absorb water (Southward, 2003). In Chapter 4, beef lung powder was mixed with semolina dough and the RVA viscosity curve was determined. Chapter 4 described the lowering of retrogradation in baking doughs with BLP incorporation. This lowering effect may give additional advantage to the baking products like bread and biscuits. Viscosity curves in Chapter 4, showed the increasing hardness of semolina dough with the BLP and these results are consistent with the increasing hardness of pasta in Chapter 3. So, firm foods like biscuits and cookies may suitable for BLP incorporation without changing textural characteristics. Further, increasing percentage of BLP reduced the lightness “L” colour values and darker the product; the BLP incorporation could be suitable with dark coloured biscuits and cookies without affect the product colour. Beef lungs have been incorporated into hot extruded snacks by Chávez-Jáuregui et al. (2003); Moreira-Araujo et al. (2008). So, there is a possibility of producing hot extruded snacks like puffed snacks with dried beef lung powder as well.

Viscosity curves of 50 °C drying were close to the control (semolina only) curve and other higher drying temperatures curves were at lower levels. These viscosity differences could occur due to protein denaturation at higher temperatures. Further, BLP protein degradation at higher temperatures (70 °C and 100 °C) were obvious on the SDS-PAGE protein profile when compared with the 50 °C temperature (Chapter 4). Altogether, protein degradation at higher temperature could decrease dough viscosity and low temperature drying increase the viscosity. Further, low temperature dried (50 °C) BLP had pasting properties similar to adding undenatured whey protein and caseinate in Shin et al. (2010).

Although high temperature drying denature the protein, amino acid profile of BLP was not altered except the glycine amino acid. The glycine content significantly increased at 100 °C drying which may due to the side chain cleavage of other amino acids at high temperatures. Glycine is not an essential amino acid and does not affect the indispensable amino acid score (IAAS) (Rutherfurd, Fanning, Miller, & Moughan, 2015). Further, IAAS of BLP cannot be altered with BLP drying temperatures due to the consistent behaviour of essential amino acid profile during the drying process.

BLP powder was further evaluated for the mineral content in Chapter 4, and these results correspond with the initially produced BLP in Chapter 3 except the iron content. The whole lung was dried in Chapter 3 to produce BLP, but minced beef lungs were used in Chapter 4. The mincing process washed off the blood from beef lungs, and this could cause decreasing the 1 mg/g iron content in Chapter 3 to 0.6mg/g iron content in Chapter 4. In addition to the physicochemical parameters, it is crucial to ensure the safety aspect of the BLP. Total viable count, coliform and yeast & mould were counted and all parameters were in safe range after 6 months of storage according to the microbial reference guide New Zealand (MPI, 1995). These findings suggest the possibility of safely obtaining raw beef lungs from New Zealand slaughterhouses and safely processing of BLP.

Lipid oxidation is the primary cause of quality deterioration (Ahmed et al., 2016). The relationship between the off flavour of lipid oxidation and TBARS value has established the Greene and Cumuze (1982). They have reported that the threshold for determining off flavours in beef is 0.6 to 2 mg MDA in beef. According to that reference, Chapter 4 BLP dried at 50 ˚C to 70˚C temperature is convenient for consumption even after six months storage, but BLP dried at 100 ˚C was not suitable for consumption after six months of storage by considering the threshold limit as 0.6 mg MDA /Kg. But unfortunately, a sensory analysis was not conducted to identify the off flavours due to the constrains of obtaining food grade beef lungs which are described in end of Chapter 5.

Protein oxidation was also limited at 50 ˚C drying temperature, and 50 ˚C was identified as the most suitable drying temperature for beef lungs in Chapter 4. The protein oxidation could lead to protein aggregation and may cause a reduction of digestibility and negatively impact on the nutritional status (Gatellier et al., 2010) as described in Chapter 5.

Storage conditions of Beef lung powder is crucial in industrial level and Chapter 4 described the possibility of storing at room temperature. So, BLP can easily distribute to low income countries at low delivery cost without maintaining a cold chain. However, the higher water affinity of BLP can be a problem of industrial level storing and moisture impermeable aluminium pouches can be recommended for long term storage. The 50 °C dried beef lung powder showed significantly higher water solubility index (WSI) and significantly higher porosity, which could affect the digestion of the BLP (Schweigert et al., 1954). Chapter 5 confirmed the higher-level protein digestibility of BLP dried at 50 °C, and there could be an association with WSI and porosity.

Chapter 5 showed the digestion of BLP dried at 50 ˚C, 70 ˚C and 100 ˚C by simulating human gastrointestinal digestion. All three samples were highly digested above the 90% digestibility. The digestion level was similar to the boiled hairtail (*Thichiurus lepturus*) fillets digestion Tavares (Tavares et al., 2018) and beef, pork, chicken and fish digestion with *in vivo* dog assay (Faber et al., 2010). The 50 ˚C dried BLP showed significantly higher (96%) protein digestibility compare with other drying temperatures. Further, free amino acid release was also significantly higher in BLP dried at 50 ˚C than higher temperatures. Decrease of releasing free amino acid could occur due to the elevated protein oxidation of BLP with higher temperatures according to the results of Chapter 4.

The mineral release was observed during the digestion In Chapter 5 to ensure the nutritive value. Higher drying temperatures (70 ˚C to 100 ˚C) significantly ($p<0.05$) reduced the release of copper (Cu), iron (Fe) and sulphur(S) and their maximum concentration was seen in raw and 50 ˚C dried samples. The soluble iron could convert to an insoluble form due to precipitate with the proteins (Garcia et al., 1996; O'Loughlin, Kelly, Murray, FitzGerald, & Brodkorb, 2015). Another meat roasting study showed that meat roasting on the conventional oven was reported a significant decline in bio-accessibility of metal ions like copper and zinc (Menezes et al., 2018) similar to Chapter 5 results of BLP. In overall, beef lungs should be processed in low temperature (50 ˚C) as a mineral supplement for better mineral bio-accessibility.

SDS-PAGE analysis of Chapter 4 illustrated the low molecular proteins in raw beef lungs and described the possibility of having bioactive peptides. Chapter 6 focused on producing ACE inhibitory peptides from raw beef lungs. Bovine lung hydrolysates were prepared by two fungal proteases (FP31K, FP60K) and kiwi fruit extract. In this study IC_{50} values of beef lung hydrolysates are lower than IC_{50} of goat meat hydrolysates (Jamhari et al., 2013; Mirdhayati et al., 2016). So, beef lungs are more suitable to prepare ACE inhibitory peptides by enzymatic digestion. Then, amino acid sequence of ACE inhibitory peptide was identified as Val-Ser-Pro-Gly-Met-Pro. Newly identified sequence was not recorded previously in the BIOPEP database. Chapter 6 described the possibility of having strong ACE inhibitory activity in Val-Ser-Pro-Gly-Met-Pro peptide sequence based on literature evidences (Matsumura et al. (1993); Hrynkiewicz et al. (2019). Further research should be carried out to confirm the ACE inhibitory activity of the identified amino acid sequence. There is possibility of synthesising this amino acid sequence and administrate into live subject to observe the blood pressure lowering effect. Chapter 6 confirmed collagen, elastin and bovine serum albumin as dominant proteins in bovine lungs by matching sequences with their parental proteins.

Altogether, beef lung powder (BLP) is a protein and mineral enriched food ingredient which compatible with incorporating to starchy foods like semolina pasta. Beef lung powder contains highly digestive protein and rich in essential amino acids. Safety of the BLP was assured for the total viable count, coliform and yeast & mould with six months of storage. Further, it is advisable to store in a cool and dry place within a moisture impermeable aluminium pouch due to high moisture affinity. These

characteristics of bovine lung powder make it suitable for low-income hunger countries to provide quality proteins for an affordable price. This project identified the accumulation of underutilised beef lungs in major meat-producing countries like New Zealand, and at the same time identified the higher prevalence of protein energy malnutrition in low-income hunger countries. This project successfully bridged the gap by producing low-cost beef lung protein powder and incorporating into widely consumed food. Until establishing a better regulatory system to obtain edible beef lungs, raw beef lungs can use to produce ACE inhibitory peptides for the pharmaceutical industries to increase the economic value of the bovine lungs.

7.1.1 **Future research**

1) Sensory attributes of beef lung powder need to be validated, which was not done in our first experimental chapter. So, future researchers should focus on obtaining edible bovine lungs by complying with their local food standards.

2) Chapter 5 determined the nutrition release with in *vitro* digestion, and it will be interesting to validate the bioavailability of essential nutrients. Bioavailability of nutrients can be validated using the *in vitro* Caco 2 cell line as a model of the intestinal barrier.

3) A lot of peptides were identified In Chapter 6 from the purified fractions. The number of peptides could be reduced by further purifying the fractions through reverse phase HPLC and ion-exchange chromatography.

4) The ACE inhibitory activity of the newly identified peptide sequence (Val-Ser-Pro-Gly-Met-Pro) needs to be further validated for future pharmaceutical uses. For instance, chemically synthesised peptide sequence can be administrated into the diet of spontaneously hypertensive rats to demonstrate the lowering blood pressure.

Appendix A

Ethical approval for sensory evaluation of beef lung powder incorporated semolina pasta

Research Management Office

T 64 3 423 0817
PO Box 85084, Lincoln University
Lincoln 7647, Christchurch
New Zealand
www.lincoln.ac.nz

11 October 2018

Application No: 2018- 34

Title: Incorporation of beef lung powder into semolina pasta

Applicant: R Jayawardena

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.

References

Aalhus, J. L., & Dugan, M. E. R. (2004). Spoilage, Factors Affecting | Oxidative and Enzymatic. In W. K. Jensen (Ed.), *Encyclopedia of Meat Sciences* (pp. 1330-1336). Oxford: Elsevier. Retrieved from http://www.sciencedirect.com/science/article/pii/B012464970X001227. doi:https://doi.org/10.1016/B0-12-464970-X/00122-7

Ahmed, M., Pickova, J., Ahmad, T., Liaquat, M., Farid, A., & Jahangir, M. (2016). Oxidation of Lipids in Foods. *Sarhad Journal of Agriculture, 32*(3).

Aihara, K., Kajimoto, O., Hirata, H., Takahashi, R., & Nakamura, Y. (2005). Effect of powdered fermented milk with Lactobacillus helveticus on subjects with high-normal blood pressure or mild hypertension. *Journal of the American College of Nutrition, 24*(4), 257-265.

Akdogan, H. (1999). High moisture food extrusion. *International Journal of Food Science & Technology, 34*(3), 195-207.

Albertí, P., Panea, B., Sañudo, C., Olleta, J. L., Ripoll, G., Ertbjerg, P., & Christensen, M. (2008). Live weight, body size and carcass characteristics of young bulls of fifteen European breeds. *Livestock Science, 114*(1), 19-30. doi:https://doi.org/10.1016/j.livsci.2007.04.010

Almeida, C. C., Monteiro, M. L. G., da Costa-Lima, B. R. C., Alvares, T. S., & Conte-Junior, C. A. (2015). In vitro digestibility of commercial whey protein supplements. *LWT-Food Science and Technology, 61*(1), 7-11.

Alminger, M., Aura, A. M., Bohn, T., Dufour, C., El, S., & Gomes, A. (2014). In vitro models for studying secondary plant metabolite digestion and bioaccessibility. *Comprehensive Reviews in Food Science and Food Safety, 13*(4), 413-436.

Arêas, J., & Lawrie, R. (1984). Effect of lipid-protein interactions on extrusion of offal protein isolates. *Meat Science, 11*(4), 275-299.

Assadpour, E., & Jafari, S. M. (2019). An overview of biopolymer nanostructures for encapsulation of food ingredients. In S. M. Jafari (Ed.), *Biopolymer Nanostructures for Food Encapsulation Purposes* (pp. 1-35): Academic Press. Retrieved from http://www.sciencedirect.com/science/article/pii/B978012815663600001X. doi:https://doi.org/10.1016/B978-0-12-815663-6.00001-X

Astruc, T., Marinova, P., Labas, R., Gatellier, P., & Santé-Lhoutellier, V. (2007). Detection and Localization of Oxidized Proteins in Muscle Cells by Fluorescence Microscopy. *Journal of Agricultural and Food Chemistry, 55*(23), 9554-9558. doi:10.1021/jf0717586

Awan, Z., Tariq, M., Muhammad, A., Satti, N., Mukhtar, T., Akram, W., & Yasin, M. F. (2015). Edible by-products of meat. *Veterinaria, 3*(1), 33-36.

Ayanwale, B., Ocheme, O., & OO, O. (2007). The Effect of Sun-Drying and Oven-Drying on the. *Pakistan Journal of Nutrition, 6*(4), 370-374.

Baghurst, K. (2006). *Nutrient reference values for Australia and New Zealand: including recommended dietary intakes*. Canberra: National Health and Medical Research Council.

Banerjee, P., & Shanthi, C. (2012). Isolation of novel bioactive regions from bovine Achilles tendon collagen having angiotensin I-converting enzyme-inhibitory properties. *Process Biochemistry, 47*(12), 2335-2346.

Bastos, D., & Arêas, J. (1990). Lung proteins: effect of defatting with several solvents and extrusion cooking on some functional properties. *Meat Science, 28*(3), 223-235.

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*(3-4), 1249-1262.

Bernát, I. (1983). Protein-Deficiency Anemia. In I. Bernát (Ed.), *Iron Metabolism* (pp. 299-300). Boston, MA: Springer US. Retrieved from https://doi.org/10.1007/978-1-4615-7308-1_21. doi:10.1007/978-1-4615-7308-1_21

Berrazaga, I., Micard, V., Gueugneau, M., & Walrand, S. (2019). The Role of the Anabolic Properties of Plant- versus Animal-Based Protein Sources in Supporting Muscle Mass Maintenance: A Critical Review. *Nutrients, 11*(8), 1825. doi:10.3390/nu11081825

Bester, M., Schonfeldt, H. C., Pretorius, B., & Hall, N. G. (2018). The nutrient content of selected South African lamb and mutton organ meats (offal).

Beuchat, L., & Cousin, M. (2001). Yeasts and molds. *Compendium of methods for the microbiological examination of foods, 4*, 209-215.

Bhagavan, N. V., & Ha, C.-E. (2015). Chapter 23 - RNA and Protein Synthesis. In N. V. Bhagavan & C.-E. Ha (Eds.), *Essentials of Medical Biochemistry (Second Edition)* (pp. 419-446). San Diego: Academic Press. Retrieved from http://www.sciencedirect.com/science/article/pii/B9780124166875000233. doi:https://doi.org/10.1016/B978-0-12-416687-5.00023-3

Bhaskar, N., Modi, V. K., Govindaraju, K., Radha, C., & Lalitha, R. G. (2007). Utilization of meat industry by products: Protein hydrolysate from sheep visceral mass. *Bioresource Technology, 98*(2), 388-394. doi:https://doi.org/10.1016/j.biortech.2005.12.017

Bhat, Z. F., Kumar, S., & Bhat, H. F. (2017). Antihypertensive peptides of animal origin: A review. *Critical Reviews in Food Science and Nutrition, 57*(3), 566-578.

Bhat, Z. F., Mason, S., Morton, J. D., Bekhit, A. E.-D. A., & Bhat, H. F. (Eds.). (2017). *Antihypertensive Peptides from Animal Proteins*: Springer International Publishing

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 & Emerging Technologies, 50*, 132-138. doi:https://doi.org/10.1016/j.ifset.2018.09.006

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

Bhutia, Y. D., & Ganapathy, V. (2018). Chapter 47 - Protein Digestion and Absorption. In H. M. Said (Ed.), *Physiology of the Gastrointestinal Tract (Sixth Edition)* (pp. 1063-1086): Academic Press. Retrieved from http://www.sciencedirect.com/science/article/pii/B9780128099544000475. doi:https://doi.org/10.1016/B978-0-12-809954-4.00047-5

Biel, W., Czerniawska-Piątkowska, E., & Kowalczyk, A. (2019). Offal Chemical Composition from Veal, Beef, and Lamb Maintained in Organic Production Systems. *Animals, 9*(8), 489.

Bishnoi, S., Khanna, N., Bishnoi, N., Tewari, A., Ghadwal, S., & Ahlawat, S. S. (2015). Development of Chicken Meat Powder Incorporated Instant Idli Mixes. *Journal of Animal Research, 5*(3), 527.

BLN. (2017). *Export cattle slaughter New Zealand year* Beef + Lamb New Zealand Economic Service. Retrieved from https://beeflambnz.com/sites/default/files/data/files/Cattle%20Slaughter%20Trend.pdf

Blomstrand, E., Eliasson, J., Karlsson, H. K., & Köhnke, R. (2006). Branched-chain amino acids activate key enzymes in protein synthesis after physical exercise. *The Journal of Nutrition, 136*(1), 269S-273S.

Boland, M. (2013). Chapter Four - Kiwifruit Proteins and Enzymes: Actinidin and Other Significant Proteins. In M. Boland & P. J. Moughan (Eds.), *Advances in Food and Nutrition Research* (Vol. 68, pp. 59-80): Academic Press. Retrieved from http://www.sciencedirect.com/science/article/pii/B9780123942944000043. doi:https://doi.org/10.1016/B978-0-12-394294-4.00004-3

Boland, M. J., & Hardman, M. J. (1972). Kinetic studies on the thiol protease from *Actinidia chinensis*. *FEBS Letters, 27*(2), 282-284. doi:https://doi.org/10.1016/0014-5793(72)80641-0

Boland, M. J., & Hardman, M. J. (1973). The Actinidin-Catalysed Hydrolysis of N-α-Benzyloxycarbonyl-l-lysine p-Nitrophenyl Ester: pH Dependence and Mechanism. *European Journal of Biochemistry, 36*(2), 575-582.

Bolognese, M., Viesi, D., Bartali, R., & Crema, L. (2020). Modeling study for low-carbon industrial processes integrating solar thermal technologies. A case study in the Italian Alps: The Felicetti Pasta Factory. *Solar Energy, 208*, 548-558.

Borelli, P., Blatt, S., Pereira, J., de Maurino, B. B., Tsujita, M., & de Souza, A. C. (2007). Reduction of erythroid progenitors in protein-energy malnutrition. *British Journal of Nutrition, 97*(2), 307-314. doi:10.1017/s0007114507172731

Brabet, C., Reynoso, D., Dufour, D., Mestres, C., Arredondo, J., & Scott, G. (2013). Starch content and properties of 106 sweetpotato clones from the world germplasm collection held at CIP, Peru, Korea. *2*(1), 1.

Bradford, K. J., Dahal, P., Van Asbrouck, J., Kunusoth, K., Bello, P., Thompson, J., & Wu, F. (2018). The dry chain: Reducing postharvest losses and improving food safety in humid climates. *Trends in Food Science & Technology, 71*, 84-93.

Brennan, M. A., Derbyshire, E., Tiwari, B. K., & Brennan, C. S. (2012). Enrichment of extruded snack products with coproducts from chestnut mushroom (Agrocybe aegerita) production: interactions between dietary fiber, physicochemical characteristics, and glycemic load. *Journal of Agricultural and Food Chemistry, 60*(17), 4396-4401.

Brennan, M. A., Derbyshire, E., Tiwari, B. K., & Brennan, C. S. (2013). Ready-to-eat snack products: the role of extrusion technology in developing consumer acceptable and nutritious snacks. *International Journal of Food Science & Technology, 48*(5), 893-902. doi:10.1111/ijfs.12055

Burfoot, D., Everis, L., Mulvey, L., Wood, A., & Campden, R. (2010). Literature review on microbiological hazards associated with biltong and similar dried meat products. *Food Standard Agency, London, 87*.

Byun, H.-G., & Kim, S.-K. (2002). Structure and activity of angiotensin I converting enzyme inhibitory peptides derived from Alaskan pollack skin. *BMB Reports, 35*(2), 239-243.

Cai, Y., & Corke, H. (2000). Production and properties of spray-dried amaranthus betacyanin pigments. *Journal of Food Science, 65*(7), 1248-1252.

Calicioglu, M., Sofos, J. N., & Kendall, P. A. (2003). Influence of marinades on survival during storage of acid-adapted and nonadapted Listeria monocytogenes inoculated post-drying on beef jerky. *International Journal of Food Microbiology, 86*(3), 283-292.

Calicioglu, M., Sofos, J. N., Samelis, J., Kendall, P. A., & Smith, G. C. (2002). Destruction of acid-and non-adapted Listeria monocytogenes during drying and storage of beef jerky. *Food Microbiology, 19*(6), 545-559.

Campbell, N. R., Lackland, D. T., Lisheng, L., Niebylski, M. L., Nilsson, P. M., & Zhang, X. H. (2015). Using the Global Burden of Disease study to assist development of nation-specific fact sheets to promote prevention and control of hypertension and reduction in dietary salt: a resource from the World Hypertension League. *The Journal of Clinical Hypertension, 17*(3), 165-167.

Campo, M., Nute, G., Hughes, S., Enser, M., Wood, J., & Richardson, R. (2006). Flavour perception of oxidation in beef. *Meat Science, 72*(2), 303-311.

Campos, M. A., & Arêas, J. G. (1993). Protein nutritional value of extrusion-cooking defatted lung flour. *Food Chemistry, 47*(1), 61-66. doi:https://doi.org/10.1016/0308-8146(93)90303-W

Cardoso-Santiago, R., & Arêas, J. A. G. (2001a). Nutritional evaluation of snacks obtained from chickpea and bovine lung blends. *Food Chemistry, 74*(1), 35-40.

Cardoso-Santiago, R. A., & Arêas, J. A. G. (2001b). Nutritional evaluation of snacks obtained from chickpea and bovine lung blends. *Food Chemistry, 74*(1), 35-40. doi:http://dx.doi.org/10.1016/S0308-8146(00)00335-6

Cardoso-Santiago, R. A., Moreira-Araújo, R. S. R., Pinto e Silva, M. E. M., & Arêas, J. A. G. (2001). The potential of extruded chickpea, corn and bovine lung for malnutrition programs. *Innovative Food Science & Emerging Technologies, 2*(3), 203-209. doi:https://doi.org/10.1016/S1466-8564(01)00038-8

Cernadas, J. M. C., Noceda, G., Barrera, L., Martinez, A. M., & Garsd, A. (2003). Maternal and perinatal factors influencing the duration of exclusive breastfeeding during the first 6 months of life. *Journal of Human Lactation, 19*(2), 136-144.

Cerqueira, A. M. M. D., & Cardoso, F. D. S. H. (Eds.). (2017). *Nutritional diseases* (Second ed.). Amsterdam, Netherlands: Elsevier.

Chauhan, Y. B., & Rathod, P. P. (2020). A comprehensive review of the solar dryer. *International Journal of Ambient Energy, 41*(3), 348-367. doi:10.1080/01430750.2018.1456960

Chávez-Jáuregui, R. N., Cardoso-Santiago, R. A., Silva, M. E. P. e., & Arêas, J. A. (2003). Acceptability of snacks produced by the extrusion of amaranth and blends of chickpea and bovine lung. *International Journal of Food Science & Technology, 38*(7), 795-798.

Cheftel, J., Kitagawa, M., & Queguiner, C. (1992). New protein texturization processes by extrusion cooking at high moisture levels. *Food Reviews International, 8*(2), 235-275.

Chen, Z., Schols, H., & Voragen, A. (2003). Physicochemical properties of starches obtained from three varieties of Chinese sweet potatoes. *Journal of Food Science, 68*(2), 431-437.

Cheng, F.-Y., Liu, Y.-T., Wan, T.-C., Lin, L.-C., & Sakata, R. (2008). The development of angiotensin I-converting enzyme inhibitor derived from chicken bone protein. *Animal Science Journal, 79*(1), 122-128. doi:10.1111/j.1740-0929.2007.00507.x

Chernukha, I. M., Fedulova, L. V., & Kotenkova, E. A. (2015). Meat by-product is a Source of Tissue-specific Bioactive Proteins and Peptides against Cardio-vascular Diseases. *Procedia Food Science, 5*, 50-53. doi:https://doi.org/10.1016/j.profoo.2015.09.013

Chizzolini, R., Novelli, E., & Zanardi, E. (1998). Oxidation in traditional Mediterranean meat products. *Meat Science, 49*, S87-S99.

Choi, K. H., & Laursen, R. A. (2000). Amino-acid sequence and glycan structures of cysteine proteases with proline specificity from ginger rhizome Zingiber officinale. *European Journal of Biochemistry, 267*(5), 1516-1526. doi:10.1046/j.1432-1327.2000.01152.x

Chung, J., Kim, M. S., & Han, S. N. (2011). Diet-induced obesity leads to decreased hepatic iron storage in mice. *Nutrition Research, 31*(12), 915-921. doi:10.1016/j.nutres.2011.09.014

Conti-Silva, A. C., Pinto e Silva, M. E. M., & Arêas, J. A. G. (2011). Sensory acceptability of raw and extruded bovine rumen protein in processed meat products. *Meat Science, 88*(4), 652-656. doi:https://doi.org/10.1016/j.meatsci.2011.02.024

Darine, S., Christophe, V., & Gholamreza, D. (2010). Production and functional properties of beef lung protein concentrates. *Meat Science, 84*(3), 315-322.

Darine, S., Christophe, V., & Gholamreza, D. (2011). Emulsification properties of proteins extracted from beef lungs in the presence of xanthan gum using a continuous rotor/stator system. *LWT-Food Science and Technology, 44*(4), 1179-1188.

De Benoist, B., Cogswell, M., Egli, I., & McLean, E. (2008). Worldwide prevalence of anaemia 1993-2005; WHO Global Database of anaemia.

Desai, A., Brennan, M. A., & Brennan, C. S. (2018). The effect of semolina replacement with protein powder from fish (Pseudophycis bachus) on the physicochemical characteristics of pasta. *LWT, 89*, 52-57.

Devatkal, S., & Mendiratta, S. (2001). Use of calcium lactate with salt-phosphate and alginate-calcium gels in restructured pork rolls. *Meat Science, 58*(4), 371-379.

DeWitt, C. M., Gomez, G., & James, J. (2002). Protein extraction from beef heart using acid solubilization. *Journal of Food Science, 67*(9), 3335-3341.

Di Bernardini, R., Rai, D. K., Bolton, D., Kerry, J., O'Neill, E., & Mullen, A. M. (2011). Isolation, purification and characterization of antioxidant peptidic fractions from a bovine liver sarcoplasmic protein thermolysin hydrolyzate. *Peptides, 32*(2), 388-400. doi:https://doi.org/10.1016/j.peptides.2010.11.024

Djikaev, Y., & Ruckenstein, E. (2008). Thermal denaturation of a native protein via spinodal decomposition in the framework of first-passage-time analysis. *Physical Review E, 78*(1), 011909.

Duttaroy, A. k. (2015). Kiwifruit Juice. In S. Fereidoon & Alasalvar (Eds.), *Handbook of Functional Beverages and Human Health* (pp. 331-337). Boca Raton: CRC Press. doi:10.1201/b19490-30

Edwards, J. H., & Shipe, W. F. (1978). Characterization of plastein reaction products formed by pepsin, α-chymotrypsin, and papain treatment of egg albumin hydrolysates. *Journal of Food Science, 43*(4), 1215-1218.

Edwards, N. (2013). *Offal: A Global History*: Reaktion Books.

Emerson, D., Roden, E., & Twining, B. (2012). The microbial ferrous wheel: iron cycling in terrestrial, freshwater, and marine environments. *Frontiers in Microbiology, 3*, 383.

Etcheverry, P., Grusak, M. A., & Fleige, L. E. (2012). Application of in vitro bioaccessibility and bioavailability methods for calcium, carotenoids, folate, iron, magnesium, polyphenols, zinc, and vitamins B6, B12, D, and E. *Frontiers in Physiology, 3*, 317.

Faber, T., Bechtel, P., Hernot, D., Parsons, C. M., Swanson, K. S., Smiley, S., & Fahey Jr, G. (2010). Protein digestibility evaluations of meat and fish substrates using laboratory, avian, and ileally cannulated dog assays. *Journal of Animal Science, 88*(4), 1421-1432.

Fairweather-Tait, S., Lynch, S., Hotz, C., Hurrell, R., Abrahamse, L., & Beebe, S. (2005). The usefulness of in vitro models to predict the bioavailability of iron and zinc: a consensus statement from the HarvestPlus expert consultation. *International Journal for Vitamin and Nutrition Research, 75*(6), 371-374.

FAO. (2011). *Dietary protein quality evaluation in human nutrition: report of an fao expert consultation*. Auckland, New Zealand. Retrieved from http://www.fao.org/ag/humannutrition/35978-02317b979a686a57aa4593304ffc17f06.pdf

FAO. (2019a). *OECD-FAO Agricultural Outlook 2019-2028*. OECD Publishing, Paris. Retrieved from https://www.oecd-ilibrary.org/content/publication/agr_outlook-2019-en. doi:doi:https://doi.org/10.1787/agr_outlook-2019-en

FAO, &. (2019b). *The State of Food Security and Nutrition in the World 2019. Safeguarding against economic slowdowns and downturns* Rome,FAO.

FAOSTAT. (2016). Food and Agriculture Organization of the United Nations, FAOSTAT Database. Retrieved January 30, 2017

FAOSTAT. (2018). Food and Agriculture Organization of the United Nations. FAOSTAT Database. Retrieved January 02, 2020

Fardet, A., Hoebler, C., Baldwin, P. M., Bouchet, B., Gallant, D. J., & Barry, J. L. (1998). Involvement of the protein network in thein vitro degradation of starch from spaghetti and lasagne: a microscopic and enzymic study. *Journal of Cereal Science, 27*(2), 133-145. doi:http://dx.doi.org/10.1006/jcrs.1997.0157

Farouk, M. M., Wu, G., Frost, D. A., Staincliffe, M., & Knowles, S. O. (2019). Factors Affecting the Digestibility of Beef and Consequences for Designing Meat-Centric Meals. *Journal of Food Quality, 2019*.

Filip, S., & Vidrih, R. (2015). Amino acid composition of protein-enriched dried pasta: is it suitable for a low-carbohydrate diet? *Food Technology and Biotechnology, 53*(3), 298-306.

FitzGerald, R. J., & Meisel, H. (2000). Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *British Journal of Nutrition, 84*(S1), 33-37.

Fitzsimons, E., & Brock, J., &. (2001). *The anaemia of chronic disease: Remains hard to distinguish from iron deficiency anaemia in some cases*: British Medical Journal Publishing Group.

Fornias, O. V. (1996). Edible by-products of slaughter animals. In *FAO Animal Production and Health Paper 123.* Rome: FAO.

Foschia, M., Peressini, D., Sensidoni, A., Brennan, M. A., & Brennan, C. S. (2014). Mastication or masceration: Does the preparation of sample affect the predictive in vitro glycemic response of pasta? *Starch - Stärke, 66*(11-12), 1096-1102. doi:10.1002/star.201300156

Francis, G., & Thomas, J. (1975a). Isolation and chemical characterization of collagen in bovine pulmonary tissues. *Biochemical Journal, 145*(2), 287-297.

Francis, G., & Thomas, J. (1975b). Isolation and chemical characterization of collagen in bovine pulmonary tissues. *The Biochemical journal, 145*(2), 287-297. doi:10.1042/bj1450287

FSIS. (2015). *Slaughter inspection training - livestock postmortem inspection*. Retrieved from https://www.fsis.usda.gov/wps/wcm/connect/ad2cab87-9bf9-4ead-969a-cec2d4753c30/LSIT_PostMortem.pdf?MOD=AJPERES

García, C. Á., & Manrique, I. M. (2018). Meat Proteins as a Potential Source of Bioactive Ingredients for Food and Pharmaceutical Use. In M. Hayes (Ed.), *Novel Proteins for Food, Pharmaceuticals and Agriculture* (pp. 29-49). Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1002/9781119385332.ch2. doi:10.1002/9781119385332.ch2

Garcia, M. N., Martinez-Torres, C., Leets, I., Tropper, E., Ramirez, J., & Layrisse, M. (1996). Heat treatment on heme iron and iron-containing proteins in meat: Iron absorption in humans from diets containing cooked meat fractions. *The Journal of Nutritional Biochemistry, 7*(1), 49-54. doi:https://doi.org/10.1016/0955-2863(95)00166-2

Garcia Zepeda, C. M., Kastner, C. L., Wolf, J. R., Boyer, J. E., Kropf, D. H., Hunt, M. C., & Setser, C. S. (1997). Extrusion and low-dose irradiation effects on destruction of Clostridium sporogenes spores in a beef-based product. *Journal of Food Protection, 60*(7), 777-785.

Garrison, W. M. (1987). Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins. *Chemical Reviews, 87*(2), 381-398. doi:10.1021/cr00078a006

Gąsowski, J., & Piotrowicz, K. (2017). Hypertension in the elderly: Change of, or new implications within the existing, paradigm? *European Geriatric Medicine, 8*(4), 289-292.

Gatellier, P., Kondjoyan, A., Portanguen, S., & Santé-Lhoutellier, V. (2010). Effect of cooking on protein oxidation in n-3 polyunsaturated fatty acids enriched beef. Implication on nutritional quality. *Meat Science, 85*(4), 645-650. doi:https://doi.org/10.1016/j.meatsci.2010.03.018

Gatellier, P., & Santé-Lhoutellier, V. (2009). Digestion study of proteins from cooked meat using an enzymatic microreactor. *Meat Science, 81*(2), 405-409.

Gómez-Guillén, M. C., Giménez, B., López-Caballero, M. E., & Montero, M. P. (2011). Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids, 25*(8), 1813-1827. doi:https://doi.org/10.1016/j.foodhyd.2011.02.007

Gong, X., Morton, J. D., Bhat, Z. F., Mason, S. L., & Bekhit, A. E. D. A. (2019). 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 & Technology, 55*(2), 742-750.

GRAS. (2018). Microorganisms & microbial-derived ingredients used in food (partial list). Retrieved 1/02/2020, from FDA https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list

Greene, B., & Cumuze, T. (1982). Relationship between TBA numbers and inexperienced panelists' assessments of oxidized flavor in cooked beef. *Journal of Food Science, 47*(1), 52-54.

Griffin, C. C., Linhardt, R. J., Van Gorp, C. L., Toida, T., Hileman, R. E., Schubert, R. L., & Brown, S. E. (1995). Isolation and characterization of heparan sulfate from crude porcine intestinal mucosal peptidoglycan heparin. *Carbohydrate Research, 276*(1), 183-197. doi:https://doi.org/10.1016/0008-6215(95)00166-Q

Gropper, S. S., & Smith, J. L. (2012). *Advanced nutrition and human metabolism*: Cengage Learning.

Gu, R.-Z., Li, C.-Y., Liu, W.-Y., Yi, W.-X., & Cai, M.-Y. (2011). Angiotensin I-converting enzyme inhibitory activity of low-molecular-weight peptides from Atlantic salmon (Salmo salar L.) skin. *Food Research International, 44*(5), 1536-1540. doi:https://doi.org/10.1016/j.foodres.2011.04.006

Ha, M., Bekhit, A. E.-D., & Carne, A. (2014). Effects of l- and iso-ascorbic acid on meat protein hydrolyzing activity of four commercial plant and three microbial protease preparations. *Food Chemistry, 149*, 1-9. doi:https://doi.org/10.1016/j.foodchem.2013.10.082

Ha, M., Bekhit, A. E. D., Carne, A., & Hopkins, D. L. (2013). Comparison of the proteolytic activities of new commercially available bacterial and fungal proteases toward meat proteins. *Journal of Food Science, 78*(2), C170-C177.

Haddad, L. J., Hawkes, C., Achadi, E., Ahuja, A., Ag Bendech, M., & Bhatia, K. (2015). *Global Nutrition Report 2015: Actions and accountability to advance nutrition and sustainable development*: Intl Food Policy Res Inst.

Hagel, L., & Janson, J. (Eds.). (1992). *Size-exclusion chromatography* (5th Edition ed. Vol. 51). Amsterdam: Elsevier.

Halim, N., Yusof, H., & Sarbon, N. (2016). Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology, 51*, 24-33.

Hallberg, L. (1983). Iron requirements and bioavailability of dietary iron. In *Nutritional Adequacy, Nutrient Availability and Needs* (pp. 223-244): Springer.

Han, C. H., Richardson, J., Oh, S. H., & Roberts, D. M. (1993). Isolation and kinetic characterization of the calmodulin methyltransferase from sheep brain. *Biochemistry, 32*(50), 13974-13980. doi:10.1021/bi00213a030

Hartikainen, H. (2005). Biogeochemistry of selenium and its impact on food chain quality and human health. *Journal of Trace elements in Medicine and Biology, 18*(4), 309-318.

Hata, Y., Yamamoto, M., Ohni, M., Nakajima, K., Nakamura, Y., & Takano, T. (1996). A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *The American Journal of Clinical Nutrition, 64*(5), 767-771.

Hausner, H. H. (1967). *Friction conditions in a mass of metal powder*: Polytechnic Inst. of Brooklyn. Univ. of California, Los Angeles.

Healthcare, G., &. (2010). *Gel filtration: principles and methods*: General Electric Company.

Hellman, N. E., & Gitlin, J. D. (2002). Ceruloplasmin metabolism and function. *Annual Review of Nutrition, 22*(1), 439-458.

Herrero, M., Havlík, P., Valin, H., Notenbaert, A., Rufino, M. C., & Thornton, P. K. (2013). Biomass use, production, feed efficiencies, and greenhouse gas emissions from global livestock systems. *Proceedings of the National Academy of Sciences, 110*(52), 20888-20893.

Herrero, M., Havlík, P., Valin, H., Notenbaert, A., Rufino, M. C., Thornton, P. K., . . . Obersteiner, M. (2013). Biomass use, production, feed efficiencies, and greenhouse gas emissions from global livestock systems. *Proceedings of the National Academy of Sciences, 110*(52), 20888-20893.

Higgs, J. D. (2000). The changing nature of red meat: 20 years of improving nutritional quality. *Trends in Food Science & Technology, 11*(3), 85-95.

Holeček, M. (2018). Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. *Nutrition & Metabolism, 15*(1), 33.

Holovko, T., Helikh, A., Holovko, M., Prymenko, V., & Zherebkin, M. (2020). Scientific substantiation of technology of pasta based on freshwater mussels enriched with selenium.

Hormoznejad, R., Javid, A. Z., & Mansoori, A. (2019). Effect of BCAA supplementation on central fatigue, energy metabolism substrate and muscle damage to the exercise: a systematic review with meta-analysis. *Sport Sciences for Health, 15*(2), 265-279.

Hornsey, H. (1956). The colour of cooked cured pork. I.—Estimation of the Nitric oxide-Haem Pigments. *Journal of the Science of Food and Agriculture, 7*(8), 534-540.

Hrynkiewicz, M., Iwaniak, A., Bucholska, J., Minkiewicz, P., & Darewicz, M. (2019). Structure¯Activity Prediction of ACE Inhibitory/Bitter Dipeptides-A Chemometric Approach Based on Stepwise Regression. *Molecules (Basel, Switzerland), 24*(5), 950. doi:10.3390/molecules24050950

Hu, L., Ren, S., Shen, Q., Chen, J., Ye, X., & Ling, J. (2017). Proteomic study of the effect of different cooking methods on protein oxidation in fish fillets. *RSC Advances, 7*(44), 27496-27505.

Hurrell, R., & Egli, I. (2010). Iron bioavailability and dietary reference values. *The American Journal of Clinical Nutrition, 91*(5), 1461S-1467S.

Ilian, M. A., Bekhit, A. E.-D. A., Stevenson, B., Morton, J. D., Isherwood, P., & Bickerstaffe, R. (2004). Up-and down-regulation of longissimus tenderness parallels changes in the myofibril-bound calpain 3 protein. *Meat Science, 67*(3), 433-445.

Ilo, S., Schoenlechner, R., & Berghofe, E. (2000). Role of lipids in the extrusion cooking processes. *Grasas y Aceites, 51*(1-2), 97-110.

Jahnen-Dechent, W., & Ketteler, M. (2012). Magnesium basics. *Clinical Kidney Journal, 5*(Suppl 1), i3-i14. doi:10.1093/ndtplus/sfr163

Jamhari, J., Yusiati, L., Suryanto, E., Cahyanto, M., Erwanto, Y., & Muguruma, M. (2013). Comparative study on angiotensin converting enzyme inhibitory activity of hydrolysate of meat protein of Indonesian local livestocks. *Journal of the Indonesian Tropical Animal Agriculture, 38*(1), 27-33.

Jang, A., & Lee, M. (2005). Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates. *Meat Science, 69*(4), 653-661. doi:10.1016/j.meatsci.2004.10.014

Jauhiainen, T., Vapaatalo, H., Poussa, T., Kyrönpalo, S., Rasmussen, M., & Korpela, R. (2005). Lactobacillus helveticus fermented milk reduces blood pressure in 24-h ambulatory blood pressure measurement. *American Journal of Hypertension, 18*, 1600-1605.

Jayawardena, S. R., Morton, J. D., Brennan, C. S., & Bekhit, A. E. A. (2019a). Utilisation of beef lung protein powder as a functional ingredient to enhance protein and iron content of fresh pasta. *International Journal of Food Science & Technology, 54*(3), 610-618. doi:10.1111/ijfs.13927

Jayawardena, S. R., Morton, J. D., Brennan, C. S., & Bekhit, A. E. D. A. (2019b). Utilisation of beef lung protein powder as a functional ingredient to enhance protein and iron content of fresh pasta. *International Journal of Food Science & Technology, 54*(3), 610-618. doi:10.1111/ijfs.13927

Jeong, J., Lee, J., Lee, H., Lee, S., Kim, S., & Ha, J. (2017). Quantitative microbial risk assessment for Campylobacter foodborne illness in raw beef offal consumption in South Korea. *Journal of Food Protection, 80*(4), 609-618.

Jimsheena, V. K., & Gowda, L. R. (2009). Colorimetric, high-throughput assay for screening angiotensin I-converting enzyme inhibitors. *Analytical Chemistry, 81*(22), 9388-9394.

Kaczmarek, A., Cegielska-Radziejewska, R., Szablewski, T., & Zabielski, J. (2015). TBARS and microbial growth predicative models of pork sausage stored at different temperatures. *Czech Journal of Food Sciences, 33*(4), 320-325.

Karatzi, K., Protogerou, A. D., Moschonis, G., Tsirimiagou, C., Androutsos, O., Chrousos, G. P., . . . Manios, Y. (2017). Prevalence of hypertension and hypertension phenotypes by age and gender among schoolchildren in Greece: The Healthy Growth Study. *Atherosclerosis, 259*, 128-133.

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*(5), 2389-2397.

Kim, S., Lee, J. W., Heo, Y., & Moon, B. (2016). Effect of Pleurotus eryngii mushroom β-glucan on quality characteristics of common wheat pasta. *Journal of Food Science, 81*(4), C835-C840.

Kim, Y. S., Wiesenborn, D. P., Lorenzen, J. H., & Berglund, P. (1996). Suitability of edible bean and potato starches for starch noodles. *Cereal Chemistry, 73*(3), 302-308.

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.

Konieczny, P., Stangierski, J., & Kijowski, J. (2007). Physical and chemical characteristics and acceptability of home style beef jerky. *Meat science, 76*(2), 253-257.

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. *Journal of Functional Foods, 1*(2), 177-187.

Krasnowska, G., Górska, I., & Gergont, J. (1995). Evaluation of functional properties of offal proteins. *Food / Nahrung, 39*(2), 149-155. doi:10.1002/food.19950390208

Krishnan, K. R., & Sharma, N. (1990). Studies on emulsion-type buffalo meat sausages incorporating skeletal and offal meat with different levels of pork fat. *Meat Science, 28*(1), 51-60. doi:https://doi.org/10.1016/0309-1740(90)90019-3

Kučerová, I., Marek, Š., & Banout, J. (2018). Solar Drying and Sensory Attributes of Eland (Taurotragus oryx) Jerky. *Journal of Food Quality, 2018*.

Kumar, L., Brennan, M., Zheng, H., & Brennan, C. (2018). The effects of dairy ingredients on the pasting, textural, rheological, freeze-thaw properties and swelling behaviour of oat starch. *Food Chemistry, 245*, 518-524.

Kwiecień, M., Samolińska, W., & Bujanowicz, B. (2015). Effects of iron–glycine chelate on growth, carcass characteristic, liver mineral concentrations and haematological and biochemical blood parameters in broilers. *Journal of Animal Physiology and Animal Nutrition, 99*(6), 1184-1196.

Lafarga, T., & Hayes, M. (2017). Effect of pre-treatment on the generation of dipeptidyl peptidase-IV- and prolyl endopeptidase-inhibitory hydrolysates from bovine lung. *Irish Journal of Agricultural and Food Research, 56*(1), 12-24.

Le Roux, D., Vergnes, B., Chaurand, M., & Abécassis, J. (1995). A thermomechanical approach to pasta extrusion. *Journal of Food Engineering, 26*(3), 351-368. doi:https://doi.org/10.1016/0260-8774(94)00060-M

Lee, S. J., Kim, Y.-S., Hwang, J.-W., Kim, E.-K., Moon, S.-H., Jeon, B.-T., & Jeon, Y.-J. (2012). Purification and characterization of a novel antioxidative peptide from duck skin by-products that protects liver against oxidative damage. *Food Research International, 49*(1), 285-295. doi:https://doi.org/10.1016/j.foodres.2012.08.017

Lee, S. Y., & Hur, S. J. (2017). Antihypertensive peptides from animal products, marine organisms, and plants. *Food Chemistry, 228*, 506-517.

Lemes, A. C., Takeuchi, K. P., Carvalho, J. C. M. d., & Danesi, E. D. G. (2012). Fresh pasta production enriched with spirulina platensis biomass. *Brazilian Archives of Biology and Technology, 55*(5), 741-750.

Leonard, B. (2011). *USDA Agricultural Projections To 2017*: DIANE Publishing.

Lewicki, P. P. (Ed.). (2004). *Drying* (Vol. 1): Elsevier Ltd.

Li, B., Chen, F., Wang, X., Ji, B., & Wu, Y. (2007). Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. *Food Chemistry, 102*(4), 1135-1143. doi:https://doi.org/10.1016/j.foodchem.2006.07.002

Li, Y., Jiang, H., & Huang, G. (2017). Protein hydrolysates as promoters of non-haem Iron absorption. *Nutrients, 9*(6), 609. doi:10.3390/nu9060609

LiHui, L., & Silva, K. D. (2009). *Development of novel technology to manufacture blood derived functional ingredients: stage II report – enzyme modulated blood protein flavour*. North Sydney, NSW: Meat & Livestock Australia Limited

Lim, H., Kim, G., Jung, E., Seo, H., Joo, S., Jin, S., & Yang, H. (2014). Effect of curing time on the physicochemical and sensory properties of beef jerky replaced salt with soy sauce, red pepper paste and soybean paste. *Asian-Australasian Journal of Animal Sciences, 27*(8), 1174.

Liu, G., & Xiong, Y. L. (2000a). Electrophoretic pattern, thermal denaturation, and in vitro digestibility of oxidized myosin. *Journal of Agricultural and Food Chemistry, 48*(3), 624-630.

Liu, G., & Xiong, Y. L. (2000b). Oxidatively induced chemical changes and interactions of mixed myosin, β-lactoglobulin and soy 7S globulin. *Journal of the Science of Food and Agriculture, 80*(11), 1601-1607.

Liu, T., Hamid, N., Kantono, K., Pereira, L., Farouk, M. M., & Knowles, S. O. (2016). Effects of meat addition on pasta structure, nutrition and in vitro digestibility. *Food Chemistry, 213*, 108-114. doi:https://doi.org/10.1016/j.foodchem.2016.06.058

López-Córdoba, A., & Goyanes, S. (2017). Food Powder Properties. In *Reference Module in Food Science*: Elsevier. Retrieved from http://www.sciencedirect.com/science/article/pii/B9780081005965211980. doi:https://doi.org/10.1016/B978-0-08-100596-5.21198-0

Lu, X., Brennan, M. A., Serventi, L., Mason, S., & Brennan, C. S. (2016). How the inclusion of mushroom powder can affect the physicochemical characteristics of pasta. *International Journal of Food Science & Technology, 51*(11), 2433-2439.

Lynch, S. A., Álvarez, C., O'Neill, E. E., Keenan, D. F., & Mullen, A. M. (2018). Optimization of protein recovery from bovine lung by pH shift process using response surface methodology. *Journal of the Science of Food and Agriculture, 98*(5), 1951-1960.

Mahdavi, S. A., Jafari, S. M., Assadpoor, E., & Dehnad, D. (2016). Microencapsulation optimization of natural anthocyanins with maltodextrin, gum arabic and gelatin. *International Journal of Biological Macromolecules, 85*, 379-385.

Masuda, O., Nakamura, Y., & Takano, T. (1996). Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *The Journal of Nutrition, 126*(12), 3063-3068.

Mathis, K. J., Emmons, T. L., Curran, D. F., Day, J. E., & Tomasselli, A. G. (2008). High yield purification of soluble guanylate cyclase from bovine lung. *Protein Expression and Purification, 60*(1), 58-63.

Matsumura, N., Fujii, M., Takeda, Y., Sugita, K., & Shimizu, T. (1993). Angiotensin I-converting enzyme inhibitory peptides derived from bonito bowels autolysate. *Bioscience, Biotechnology, and Biochemistry, 57*(5), 695-697.

Matthews, D., & Payne, J. (1980). Transmembrane Transport of Small Peptides. In *Current Topics in Membranes and Transport* (Vol. 14, pp. 331-425): Elsevier.

Matthews, J. N., Altman, D. G., Campbell, M. J., & Royston, P. (1990). Analysis of serial measurements in medical research. *British Medical Journal, 300*(6719), 230-235. doi:10.1136/bmj.300.6719.230

McGuire, S. (2015). World Health Organization. Comprehensive implementation plan on maternal, infant, and young child nutrition. Geneva, Switzerland, 2014. *Advances in Nutrition, 6*(1), 134-135.

Megard, D., Kitabatake, N., & Cheftel, J. (1985). Continuous restructuring of mechanically deboned chicken meat by HTST extrusion-cooking. *Journal of Food Science, 50*(5), 1364-1369.

Menezes, E. A., Oliveira, A. F., França, C. J., Souza, G. B., & Nogueira, A. R. A. (2018). Bioaccessibility of Ca, Cu, Fe, Mg, Zn, and crude protein in beef, pork and chicken after thermal processing. *Food Chemistry, 240*, 75-83. doi:https://doi.org/10.1016/j.foodchem.2017.07.090

MIA. (2015). *Meat industry association annual report*. Retrieved from http://www.mia.co.nz/docs/annual_reports/MIA_Annual_Report_2015.pdf

MIA. (2018). *Meat industry association annual report*. Retrieved from file:///C:/Users/resha/Downloads/MIA-Annual-report-2018-9-Sep.pdf

Minarovičová, L., Lauková, M., Kohajdová, Z., Karovičová, J., & Kuchtová, V. (2017). Effect of pumpkin powder incorporation on cooking and sensory parameters of pasta. *Potravinárstvo: Slovak Journal of Food Sciences, 11*(1), 373-379.

Miner-Williams, W. M., Stevens, B. R., & Moughan, P. J. (2014). Are intact peptides absorbed from the healthy gut in the adult human? *Nutrition Research Reviews, 27*(2), 308-329.

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM Database of Bioactive Peptides: Current Opportunities. *International Journal of Molecular Sciences, 20*(23), 5978.

Miraghaee, S. S., Mostafaie, A., Kiani, S., & Kahrizi, D. (2011). Investigation on protein pattern in kiwifruit (Actinidia deliciosa). *World Applied Sciences Journal, 15*(10), 1398-1402.

Mirdhayati, I., Hermanianto, J., Wijaya, C. H., Sajuthi, D., & Arihara, K. (2016). Angiotensin converting enzyme (ACE) inhibitory and antihypertensive activities of protein hydrolysate from meat of Kacang goat (Capra aegagrus hircus). *Journal of the Science of Food and Agriculture, 96*(10), 3536-3542.

Mishra, B., Mishra, J., Pati, P., & Rath, P. (2017). Dehydrated Meat Products—A Review. *Int. J. Livest. Res, 7*, 10-22.

Mittal, P., & Lawrie, R. A. (1984). Extrusion studies of mixtures containing certain meat offals: Part 1—Objective properties. *Meat Science, 10*(2), 101-116. doi:https://doi.org/10.1016/0309-1740(84)90063-9

Mizushima, S., Ohshige, K., Watanabe, J., Kimura, M., Kadowaki, T., Nakamura, Y., & Tochikubo, O. (2004). Randomized controlled trial of sour milk on blood pressure in borderline hypertensive men. *American Journal of Hypertension, 17*(8), 701-706.

Möller, N. P., Scholz-Ahrens, K. E., Roos, N., & Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: indication for health effects. *European Journal of Nutrition, 47*(4), 171-182.

Moraise, H. A., Silvestre, M. P. C., Silva, V. D. M., e Silva, M. R., Silva, A. C. S., & Silveira, J. N. (2013). Correlation between the degree of hydrolysis and the peptide profile of whey protein concentrate hydrolysates: effect of the enzyme type and reaction time. *American Journal of Food Technology, 8*(1), 1-16.

Moreira-Araújo, R. S., Araújo, M. A., & Arêas, J. A. (2008). Fortified food made by the extrusion of a mixture of chickpea, corn and bovine lung controls iron-deficiency anaemia in preschool children. *Food Chemistry, 107*(1), 158-164.

Moreira-Araujo, R. S. R., Araujo, M. A. M., & Areas, J. A. G. (2008). Fortified food made by the extrusion of a mixture of chickpea, corn and bovine lung controls iron-deficiency anaemia in preschool children. *Food Chemistry, 107*(1), 158-164. doi:10.1016/j.foodchem.2007.07.074

Morgan, C. A., Herman, N., White, P., & Vesey, G. (2006). Preservation of micro-organisms by drying; a review. *Journal of Microbiological Methods, 66*(2), 183-193.

Morton, R. D. (2001). Aerobic plate count. *Compendium of Methods for The microbiological examination of foods, 4*, 63-67.

Mosoni, L., & Mirand, P. P. (2003). Type and timing of protein feeding to optimize anabolism. *Current Opinion in Clinical Nutrition & Metabolic Care, 6*(3), 301-306.

MPI. (1995). *Microbiological reference criteria for food*. New Zealand. Retrieved from https://www.mpi.govt.nz/dmsdocument/21185-microbiological-reference-criteria-for-food

Muir, P., Thomson, B., & Askin, D. (2008). A review of dressing out percentage in New Zealand livestock. *Wellington: Ministry of Agriculture and Fisheries*.

Muir, P. D., & Thomson, B. C. (2008). *A review of dressing out percentage in New Zealand livestock*. Retrieved from https://www.mpi.govt.nz/dmsdocument/2963-review-dressing-out-percentage-in-nz-livestock

Mullen, A. M., Álvarez, C., Zeugolis, D. I., Henchion, M., O'Neill, E., & Drummond, L. (2017). Alternative uses for co-products: Harnessing the potential of valuable compounds from meat processing chains. *Meat Science*.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science, 78*(4), 777-783.

Nakamura, Y., Yamamoto, N., Sakai, K., & Takano, T. (1995). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *Journal of Dairy Science, 78*(6), 1253-1257.

Nieuwenhuizen, N. J., Beuning, L. L., Sutherland, P. W., Sharma, N. N., & Cooney, J. M. (2007). Identification and characterisation of acidic and novel basic forms of actinidin, the highly abundant cysteine protease from kiwifruit. *Functional Plant Biology, 34*(10), 946-961.

Nortjé, K., Buys, E., & Minnaar, A. (2005a). Effect of γ-irradiation on the sensory quality of moist beef biltong. *Meat Science, 71*(4), 603-611.

Nortjé, K., Buys, E. M., & Minnaar, A. (2005b). Effect of γ-irradiation on the sensory quality of moist beef biltong. *Meat Science, 71*(4), 603-611. doi:https://doi.org/10.1016/j.meatsci.2005.05.004

Nuckles, R., Smith, D., & Merkel, R. (1990). Meat by-product protein composition and functional properties in model systems. *Journal of Food Science, 55*(3), 640-643.

O'Sullivan, S. M., Lafarga, T., Hayes, M., & O'Brien, N. M. (2017). Bioactivity of bovine lung hydrolysates prepared using papain, pepsin, and Alcalase. *Journal of Food Biochemistry, 41*(6), e12406.

O'Hagan, P., Hasapidis, K., & Coder, A. (2015). Particle size analysis of food powders. In C. Onwulata (Ed.), *Encapsulated and Powdered Foods* (pp. 215–245). Boca Raton, FL: CRC Press.

O'Loughlin, I. B., Kelly, P. M., Murray, B. A., FitzGerald, R. J., & Brodkorb, A. (2015). Molecular characterization of whey protein hydrolysate fractions with ferrous chelating and enhanced iron solubility capabilities. *Journal of Agricultural and Food Chemistry, 63*(10), 2708-2714.

Ogawa, T., & Adachi, S. (2014). Measurement of moisture profiles in pasta during rehydration based on image processing. *Food and Bioprocess Technology, 7*(5), 1465-1471.

Ohkuma, C., Kawai, K., Viriyarattanasak, C., Mahawanich, T., Tantratian, S., Takai, R., & Suzuki, T. (2008). Glass transition properties of frozen and freeze-dried surimi products: Effects of sugar and moisture on the glass transition temperature. *Food Hydrocolloids, 22*(2), 255-262.

Ohtaki, T., Kumano, S., Ishibashi, Y., Ogi, K., Matsui, H., Harada, M., . . . Fujino, M. (1999). Isolation and cDNA cloning of a novel galanin-like peptide (GALP) from porcine hypothalamus. *Journal of Biological Chemistry, 274*(52), 37041-37045.

OIE. (2020). *World organisation for animal health*. Retrieved 11/04, 2020,from https://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2020/.

Oliver, C. N., Ahn, B.-W., Moerman, E. J., Goldstein, S., & Stadtman, E. R. (1987). Age-related changes in oxidized proteins. *Journal of Biological Chemistry, 262*(12), 5488-5491.

Onwulata, C., & Konstance, R. (2002). Viscous properties of taro flour extruded with whey proteins to simulate weaning foods 1. *Journal of food processing and preservation, 26*(3), 179-194.

Organization, W. H. (2010). *World health statistics 2010*: World Health Organization.

Osen, R. (2017). *Texturization of pea protein isolates using high moisture extrusion cooking*. Technische Universität München.

Park, J. H., & Lee, K.-H. (2005). Quality characteristics of beef jerky made with beef meat of various places of origin. *Korean Journal of Food and Cookery Science, 21*(4), 528-535.

Pearson, A. M., Love, J. D., & Shorland, F. B. (1977). "Warmed-Over" Flavor in Meat, Poultry, and Fish. In C. O. Chichester, E. M. Mrak & G. F. Stewart (Eds.), *Advances in Food Research* (Vol. 23, pp. 1-74): Academic Press. Retrieved from

http://www.sciencedirect.com/science/article/pii/S0065262808603262. doi:https://doi.org/10.1016/S0065-2628(08)60326-2
Pelletier, N., & Tyedmers, P. (2010). Forecasting potential global environmental costs of livestock production 2000-2050. *Proceedings of the National Academy of Sciences of the United States of America, 107*(43), 18371-18374. doi:10.1073/pnas.1004659107
Perelman, D., & Lu, N. (2000). Requirements for branched chain amino acids and their interactions in the nematode Caenorhabditis elegans. *Nematology, 2*(5), 501-506.
Perignon, M., Barré, T., Gazan, R., Amiot, M.-J., & Darmon, N. (2018). The bioavailability of iron, zinc, protein and vitamin A is highly variable in French individual diets: Impact on nutrient inadequacy assessment and relation with the animal-to-plant ratio of diets. *Food Chemistry, 238*, 73-81. doi:https://doi.org/10.1016/j.foodchem.2016.12.070
Petit, T., Caro, Y., Petit, A.-S., Santchurn, S. J., & Collignan, A. (2014). Physicochemical and microbiological characteristics of biltong, a traditional salted dried meat of South Africa. *Meat Science, 96*(3), 1313-1317.
Philipps-Wiemann, P. (2018). Proteases—Human Food. In C. S. Nunes & V. Kumar (Eds.), *Enzymes in Human and Animal Nutrition* (pp. 267-277): Academic Press. Retrieved from http://www.sciencedirect.com/science/article/pii/B9780128054192000137. doi:https://doi.org/10.1016/B978-0-12-805419-2.00013-7
Pihlanto-Leppälä, A. (2000). Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends in food science & technology, 11*(9-10), 347-356.
Pimenta, D. C., & Lebrun, I. (2007). Cryptides: Buried secrets in proteins. *Peptides, 28*(12), 2403-2410. doi:https://doi.org/10.1016/j.peptides.2007.10.005
Pinto, T., Colli, C., & Areas, J. A. G. (1997). Effect of processing on iron bioavailability of extruded bovine lung. *Food chemistry, 60*(4), 459-463.
Piste, P., Sayaji, D., & Avinash, M. (2012). Calcium and its Role in Human Body. *Int J Res Pharm Biomed Sci, 4*, 2229-3701.
Potter, N. N. (1986). Heat preservation and processing. In *Food Science* (pp. 169-200). New York: Springer.
Prohl, A., Ostermann, C., Lohr, M., & Reinhold, P. (2014). The bovine lung in biomedical research: visually guided bronchoscopy, intrabronchial inoculation and in vivo sampling techniques. *Journal of Visualized Experiments : JoVE*(89), 51557. doi:10.3791/51557
Promeyrat, A., Gatellier, P., Lebret, B., Kajak-Siemaszko, K., Aubry, L., & Santé-Lhoutellier, V. (2010). Evaluation of protein aggregation in cooked meat. *Food Chemistry, 121*(2), 412-417.
Quinkler, W., Maasberg, M., Bernotat-Danielowski, S., Lüthe, N., Sharma, H. S., & Schaper, W. (1989). Isolation of heparin-binding growth factors from bovine, porcine and canine hearts. *European Journal of Biochemistry, 181*(1), 67-73. doi:10.1111/j.1432-1033.1989.tb14694.x
Rahimi, M. H., Shab-Bidar, S., Mollahosseini, M., & Djafarian, K. (2017). Branched-chain amino acid supplementation and exercise-induced muscle damage in exercise recovery: A meta-analysis of randomized clinical trials. *Nutrition, 42*, 30-36.
Rahman, M. S., Perera, C. O., Chen, X. D., Driscoll, R. H., & Potluri, P. L. (1996). Density, shrinkage and porosity of calamari mantle meat during air drying in a cabinet dryer as a function of water content. *Journal of Food Engineering, 30*(1), 135-145. doi:https://doi.org/10.1016/S0260-8774(96)00013-1
Reeds, P. J. (2000). Dispensable and indispensable amino acids for humans. *The Journal of Nutrition, 130*(7), 1835S-1840S.
Rhee, K., & Ziprin, Y. (2001). Pro-oxidative effects of NaCl in microbial growth-controlled and uncontrolled beef and chicken. *Meat Science, 57*(1), 105-112.
Richardson, D. P., Ansell, J., & Drummond, L. N. (2018). The nutritional and health attributes of kiwifruit: a review. *European Journal of Nutrition, 57*(8), 2659-2676. doi:10.1007/s00394-018-1627-z
Roberts, P. R., Burney, J., Black, K. W., & Zaloga, G. P. (1999). Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. *Digestion, 60*(4), 332-337.
Roohani, N., Hurrell, R., Kelishadi, R., & Schulin, R. (2013). Zinc and its importance for human health: An integrative review. *Journal of Research in Medical Sciences, 18*(2), 144-157.

Rostami, H., Dehnad, D., Jafari, S. M., & Tavakoli, H. R. (2018). Evaluation of physical, rheological, microbial, and organoleptic properties of meat powder produced by Refractance Window drying. *Drying technology, 36*(9), 1076-1085.

Rutherfurd, S. (2010). Methodology for Determining Degree of Hydrolysis of Proteins in Hydrolysates: A Review. *Journal of AOAC International, 93*, 1515-1522. doi:10.1093/jaoac/93.5.1515

Rutherfurd, S. M., Fanning, A. C., Miller, B. J., & Moughan, P. J. (2015). Protein digestibility-corrected amino acid scores and digestible indispensable amino acid scores differentially describe protein quality in growing male rats. *The Journal of Nutrition, 145*(2), 372-379.

Ryder, K., Bekhit, A. E.-D., McConnell, M., & Carne, A. (2016). Towards generation of bioactive peptides from meat industry waste proteins: Generation of peptides using commercial microbial proteases. *Food chemistry, 208*, 42-50.

Saiga, A., Iwai, K., Hayakawa, T., Takahata, Y., Kitamura, S., Nishimura, T., & Morimatsu, F. (2008). Angiotensin I-Converting Enzyme-Inhibitory Peptides Obtained from Chicken Collagen Hydrolysate. *Journal of Agricultural and Food Chemistry, 56*(20), 9586-9591. doi:10.1021/jf072669w

Sant'Anna, V., Christiano, F. D. P., Marczak, L. D. F., Tessaro, I. C., & Thys, R. C. S. (2014). The effect of the incorporation of grape marc powder in fettuccini pasta properties. *LWT-Food Science and Technology, 58*(2), 497-501.

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*(4), 1488-1494. doi:10.1021/jf072999g

Sante-Lhoutellier, V., Aubry, L., & Gatellier, P. (2007). Effect of Oxidation on In Vitro Digestibility of Skeletal Muscle Myofibrillar Proteins. *Journal of Agricultural and Food Chemistry, 55*(13), 5343-5348. doi:10.1021/jf070252k

Santiago, R. C., Moreira-Araújo, R. S. d. R., e Silva, M. P., & Arêas, J. (2001). The potential of extruded chickpea, corn and bovine lung for malnutrition programs. *Innovative Food Science & Emerging Technologies, 2*(3), 203-209.

Scallan, J., Huxley, V. H., & Korthuis, R. J. (2010). Capillary fluid exchange: regulation, functions, and pathology. *Morgan & Claypool Publishers.* Symposium conducted at the meeting of the Colloquium Lectures on Integrated Systems Physiology- From Molecules to Function

Schagger, H., & Jagow, G. V. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry, 166*, 368-379.

Schöneich, C. (2016). Thiyl radicals and induction of protein degradation. *Free radical research, 50*(2), 143-149. doi:10.3109/10715762.2015.1077385

Schrezenmeir, J., Korhonen, H., Williams, M., Gill, H., & Shah, N. (2000). Foreword. *The British Journal of Nutrition, (S1):1*, 84.

Schricker, B., & Miller, D. (1983). Effects of cooking and chemical treatment on heme and nonheme iron in meat. *Journal of Food Science, 48*(4), 1340-1343.

Schweigert, B., Bennett, B. A., & Guthneck, B. T. (1954). Amino acid composition of organ meats *Journal of Food Science, 19*(1-6), 219-223.

Seppo, L., Jauhiainen, T., Poussa, T., & Korpela, R. (2003). A fermented milk high in bioactive peptides has a blood pressure–lowering effect in hypertensive subjects. *The American Journal of Clinical Nutrition, 77*(2), 326-330.

Shaheen, N., Isman, s., Munmun, S., Mohiduzzaman, M., thingnganing, & Longvah, T. (2016). Amino acid profiles and digestible indispensable amino acid scores of proteins from the prioritized key foods in Bangladesh. *Food Chemistry, 213*, 83-89.

Sharma, C., Singh, B., Hussain, S. Z., & Sharma, S. (2017). Investigation of process and product parameters for physicochemical properties of rice and mung bean (Vigna radiata) flour based extruded snacks. *Journal of food science and technology, 54*(6), 1711-1720. doi:10.1007/s13197-017-2606-8

Shimokomaki, M., Youssef Youssef, E., & Terra, N. N. (2003). CURING. In B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition* (2 ed., pp. 1702-1708). Oxford: Academic Press.

Retrieved from http://www.sciencedirect.com/science/article/pii/B012227055X003163. doi:https://doi.org/10.1016/B0-12-227055-X/00316-3

Shin, M., Gang, D.-O., & Song, J.-Y. (2010). Effects of protein and transglutaminase on the preparation of gluten-free rice bread. *Food Science and Biotechnology, 19*(4), 951-956.

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.

Sipola, M., Finckenberg, P., Korpela, R., Vapaatalo, H., & Nurminen, M.-L. (2002). Effect of long-term intake of milk products on blood pressure in hypertensive rats. *Journal of Dairy Research, 69*(1), 103-111.

Skallerud, K., & Olsen, S. O. (2011). Export Marketing Arrangements in Four New Zealand Agriculture Industries: An Institutional Perspective. *Journal of International Food & Agribusiness Marketing, 23*(4), 310-329. doi:10.1080/08974438.2011.621841

Snedecor, G. W., & Cochran, W. G. (1994). Statistical Methods. 8th Edn IOWA State University Press. *Ames, Iowa, USA*.

Soar, J., Perkins, G. D., Abbas, G., Alfonzo, A., Barelli, A., Bierens, J. J. L. M., & Brugger, H. (2010). European Resuscitation Council Guidelines for Resuscitation 2010 Section 8. Cardiac arrest in special circumstances: Electrolyte abnormalities, poisoning, drowning, accidental hypothermia, hyperthermia, asthma, anaphylaxis, cardiac surgery, trauma, pregnancy, electrocution. *Resuscitation, 81*(10), 1400-1433. doi:10.1016/j.resuscitation.2010.08.015

Southward, C. R. (2003). CASEIN AND CASEINATES | Uses in the Food Industry. In B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition (Second Edition)* (pp. 948-958). Oxford: Academic Press. Retrieved from http://www.sciencedirect.com/science/article/pii/B012227055X001796. doi:https://doi.org/10.1016/B0-12-227055-X/00179-6

Steen, L., Glorieux, S., Goemaere, O., Brijs, K., Paelinck, H., Foubert, I., & Fraeye, I. (2016). Functional properties of pork liver protein fractions. *Food and Bioprocess Technology, 9*(6), 970-980.

Strain, J. S., & Cashman, K. D. (2009). Minerals and trace elements. *Introduction to Human Nutrition*, 188.

Subba, D. (2002). Acceptability and nutritive value of keropok-like snack containing meat offal. *International Journal of Food Science & Technology, 37*(6), 681-685. doi:10.1046/j.1365-2621.2002.00600.x

Sugiyama, S., Hirota, A., Okada, C., Yorita, T., Sato, K., & Ohtsuki, K. (2005). Effect of kiwifruit juice on beef collagen. *Journal of Nutritional Science and Vitaminology, 51*(1), 27-33.

Sujka, M., & Jamroz, J. (2007). Starch granule porosity and its changes by means of amylolysis. *International agrophysics, 21*(1), 107.

Swanson, K., Petran, R., & Hanlin, J. (2001). Culture methods for enumeration of microorganisms. *Compendium of methods for the microbiological examination of foods, 4*, 53-62.

Tarté, R. (2009). Meat-derived protein ingredients. In *Ingredients in meat products* (pp. 145-171): Springer.

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, 96*, 476-481.

Tenrisanna. (2015). *Offal and beef demand in indonesia and Australia's trade prospects*. University of Southern Queensland.

Teterycz, D., Sobota, A., Zarzycki, P., & Latoch, A. (2020). Legume flour as a natural colouring component in pasta production. *Journal of Food Science and Technology, 57*(1), 301-309.

Toldrá, F., Aristoy, M.-C., Mora, L., & Reig, M. (2012). Innovations in value-addition of edible meat by-products. *Meat Science, 92*(3), 290-296.

Tonon, R. V., Brabet, C., & Hubinger, M. D. (2010). Anthocyanin stability and antioxidant activity of spray-dried açai (Euterpe oleracea Mart.) juice produced with different carrier agents. *Food Research International, 43*(3), 907-914.

Torres, E., Pearson, A. M., Gray, J. I., Booren, A. M., & Shimokomaki, M. (1988). Effect of salt on oxidative changes in pre-and post-rigor ground beef. *Meat science, 23*(3), 151-163.
Traffano-Schiffo, M. V., Castro-Giráldez, M., Fito, P., & Balaguer, N. (2014). Thermodynamic model of meat drying by infrarred thermography. *Journal of Food engineering, 128*, 103-110.
Tridente, A., De Martino, L., & De Luca, D. (2019). Porcine vs bovine surfactant therapy for preterm neonates with RDS: systematic review with biological plausibility and pragmatic meta-analysis of respiratory outcomes. *Respiratory research, 20*(1), 28.
Trujillo, F. J., Wiangkaew, C., & Pham, Q. T. (2007). Drying modeling and water diffusivity in beef meat. *Journal of Food Engineering, 78*(1), 74-85.
Trumbo, P., Yates, A. A., Schlicker, S., & Poos, M. (2001). Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *Journal of the Academy of Nutrition and Dietetics, 101*(3), 294.
Tsakama, M., Mwangwela, A., Manani, T., & Mahungu, N. (2010). Physicochemical and pasting properties of starch extracted from eleven sweet potato varieties. *African Journal of Food Science and Technology, 1*(4), 090-098.
Turell, L., Botti, H., Carballal, S., Ferrer-Sueta, G., Souza, J. M., & Durán, R. (2008). Reactivity of Sulfenic Acid in Human Serum Albumin. *Biochemistry, 47*(1), 358-367. doi:10.1021/bi701520y
Turgut, S., Polat, A., Inan, M., Turgut, G., & Emmungil, G. (2007). Interaction between anemia and blood levels of iron, zinc, copper, cadmium and lead in children. *The Indian Journal of Pediatrics, 74*(9), 827-830.
USDA-FAS. (2017). Livestock and poultry: world markets and trade. *United States Department of Agriculture, Foreign Agricultural Service*.
USDA-FAS. (2018). Livestock and poultry: world markets and trade. *United States Department of Agriculture, Foreign Agricultural Service*.
USDA, &. (2016). *Livestock and poultry: world markets and trade*: USDA foreign agriculture service.
USDA. (2019). National Nutrient Database for Standard Reference. Retrieved 2020/01/19 https://ndb.nal.usda.gov/ndb/foods/show/13328?n1=%7BQv%3D1%7D&fgcd=&man=&lface t=&count=&max=25&sort=default&qlookup=Beef%2C+variety+meats+and+by-products%2C+lungs%2C+raw&offset=&format=Full&new=&measureby=&Qv=1&ds=&qt=&q p=&qa=&qn=&q=&ing=
USDA. (2020). USDA food data central. Available from USDA Agricultural Research Service Retrieved 2020/10/01 https://fdc.nal.usda.gov/
Van-Heerden, S. M., & Morey, L. (2014). Nutrient content of South African C2 beef offal. *Journal of Food Measurement and Characterization, 8*, 249-258.
Van, C. D. (1973). Enhancement of iron absorption from ligated segments of rat intestine by histidine, cysteine, and lysine: effects of removing ionizing groups and of stereoisomerism. *The Journal of nutrition, 103*(1), 139-142.
Verardo, V., Ferioli, F., Riciputi, Y., & Iafelice, G. (2009). Evaluation of lipid oxidation in spaghetti pasta enriched with long chain n− 3 polyunsaturated fatty acids under different storage conditions. *Food Chemistry, 114*(2), 472-477.
Walker, J. M., & Sweeney, P. J. (2009). Production of protein hydrolysates using enzymes. In *The Protein Protocols Handbook* (pp. 989-993): Springer.
Walton, D., & Mumford, C. (1999). Spray dried products—characterization of particle morphology. *Chemical Engineering Research and Design, 77*(1), 21-38.
Walton, E., & Allen, S. (2011). Malnutrition in developing countries. *Paediatrics and Child Health, 21*(9), 418-424. doi:https://doi.org/10.1016/j.paed.2011.04.004
Wanasundara, P. K. J. P. D., Ross, A. R. S., Amarowicz, R., Ambrose, S. J., Pegg, R. B., & Shand, P. J. (2002). Peptides with Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity from Defibrinated, Hydrolyzed Bovine Plasma. *Journal of Agricultural and Food Chemistry, 50*(24), 6981-6988. doi:10.1021/jf025592e
Wanderley, M. C. d. A., Neto, J. M. W. D., Filho, J. L. d. L., Lima, C. d. A., & Teixeira, J. A. C. (2017). Collagenolytic enzymes produced by fungi: a systematic review. *Brazilian Journal of Microbiology : [publication of the Brazilian Society for Microbiology], 48*(1), 13-24. doi:10.1016/j.bjm.2016.08.001

Wang, J.-Z., Sun, H.-M., Zhang, C.-H., Hu, L., Li, X., & Wu, X.-W. (2016). Safety assessment of Maillard reaction products of chicken bone hydrolysate using Sprague-Dawley rats. *Food & Nutrition Research, 60*(1), 27827. doi:10.3402/fnr.v60.27827

Watson, P., Shirreffs, S. M., & Maughan, R. J. (2004). The effect of acute branched-chain amino acid supplementation on prolonged exercise capacity in a warm environment. *European Journal of Applied Physiology, 93*(3), 306-314.

Webster, J. D., Ledward, D. A., & Lawrie, R. A. (1982). Protein hydrolysates from meat industry by-products. *Meat Science, 7*(2), 147-157. doi:10.1016/0309-1740(82)90080-8

WFP, &. (2019). *Hunger map 2019* Rome, Italy: World Food Programe.

WHO. (2013). *Pocket book of hospital care for children: guidelines for the management of common childhood illnesses*: World Health Organization.

WHO. (2017). Children: reducing mortality. Retrieved 2018/02/08 http://www.who.int/mediacentre/factsheets/fs178/en/

Wilson, A., Reyes, E., & Ofman, J. (2004). Prevalence and outcomes of anemia in inflammatory bowel disease: a systematic review of the literature. *Am J Med, 116*(7), 44-49. doi:10.1016/j.amjmed.2003.12.011

Yang, Z., Zhou, Y., Feng, Z., Rui, X., Zhang, T., & Zhang, Z. (2019). A review on reverse osmosis and nanofiltration membranes for water purification. *Polymers, 11*(8), 1252.

Zaidul, I., Norulaini, N. N., Omar, A. M., Yamauchi, H., & Noda, T. (2007). RVA analysis of mixtures of wheat flour and potato, sweet potato, yam, and cassava starches. *Carbohydrate Polymers, 69*(4), 784-791.

Zhang, B., Sun, Q., Liu, H.-J., Li, S.-Z., & Jiang, Z.-Q. (2017). Characterization of actinidin from Chinese kiwifruit cultivars and its applications in meat tenderization and production of angiotensin I-converting enzyme (ACE) inhibitory peptides. *LWT, 78*, 1-7. doi:https://doi.org/10.1016/j.lwt.2016.12.012

Zhang, W., Xiao, S., Samaraweera, H., Lee, E. J., & Ahn, D. U. (2010). Improving functional value of meat products. *Meat Science, 86*(1), 15-31.

Zhang, Y., Olsen, K., Grossi, A., & Otte, J. (2013). Effect of pretreatment on enzymatic hydrolysis of bovine collagen and formation of ACE-inhibitory peptides. *Food Chemistry, 141*(3), 2343-2354.

Zhang, Z., Li, G., & Shi, B. (2006). Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. *Journal of the Society of Leather Technologists and Chemists, 90*(1), 23.

Zou, X., Zhou, G., Yu, X., Bai, Y., & Wang, C. (2018). In vitro protein digestion of pork cuts differ with muscle type. *Food Research International, 106*, 344-353. doi:https://doi.org/10.1016/j.foodres.2017.12.070