Effect of High Pressure Processing (HPP) and Pulsed Electric Field (PEF) processing on digestibility of frozen lamb

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AA amino acid

AC&A accelerated conditioned and aged

ALA alanine

ANOVA Analysis of Variance

AMPK AMP-activated protein kinase

AOAC Association of Official Analytical Chemists

ARG arginine

ASN asparagine

ASP aspartic acid

BLNZ *Beef + Lamb New Zealand*

CFT chilled-frozen-thawed

CNF chilled-never-frozen

CYS cysteine

C&0 control and 0 day of storage

C&7 control and 7 days of storage

DGM dynamic gastric model

EAA essential amino acids

FAA free amino acid

FAO *Food and Agriculture Organization of the United Nations*

FID flame ionisation detector

FM formula

GC gas chromatography

GC-FID gas chromatography - flame ionisation detector

GLN glutamine

GLU glutamic acid

GLY glycine

HGS human gastric simulator

HIS histidine

HPLC High Performance Liquid Chromatography

HPP High pressure processing

ILE isoleucine

IMAC immobilized-metal affinity chromatography

IPUGS *In vitro* Physicochemical Upper Gastrointestinal System

LC-MS liquid chromatography-mass spectrometry

LEU leucine

LMW low-molecular-weight

LYS lysine

MALDI-ToF MS matrix-assisted laser desorption/ionization−time-of-flight mass spectrometry

MAP *Meat advisory panel*

MET methionine

MIA *Meat Industry Association*

mTOR mammalian target of rapamycin

nano LC-ESI-MS/MS liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer equipped with a nano electrospray ionization source

NCBA *National Cattlemen's Beef Association*.

N/A not available

ORN ornithine

PEA *Pearson Higher Education*

PEF Pulsed electric field

PHE phenylalanine

PRO proline

P&0 PEF and 0 day of storage

P&7 PEF and 7 days of storage

REML restricted maximum likelihood

SD standard deviation

SDS-PAGE sodium dodecyl sulfate−polyacrylamide gel electrophoresis

SER serine

SGF Simulated Gastric Fluid

SHIME Simulator of the Human Intestinal Microbial Ecosystem

SIF Simulated Intestinal Fluid

SSF Simulated Salivary Fluid

TBARS thiobarbituric acid-reactive substances

TIM TNO's Intestinal Microsystems'

THR threonine

TRP tryptophan

TYR tyrosine

VAL valine

Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, 'Effect of High Pressure Processing (HPP) and Pulsed Electric Field (PEF) processing on digestibility of frozen lamb', contains no material previously published or written by another person (except where explicitly defined in the acknowledgements) nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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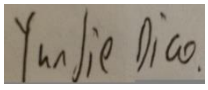
Signed:…  ……

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Abstract

Lamb is a popular meat source in New Zealand which contains of protein, fat and other micro-elements. The lamb meat is frozen after slaughter to increase its shelf life and to decrease the cost on transportation. However, the frozen lamb is considered as low quality meat because it has less stable tenderness, more drip loss and shorter colour stability than chilled lamb. High Pressure Processing (HPP) and Pulsed Electric Field (PEF) are both new technologies which are used for preserving food products. HPP treatment inactivate microorganisms and maintain microbial safety with effective cost, availability and efficiency without causing chemical reactions resulting in loss of nutrients and formation of off-flavours. And PEF is a food preservation technique where foods are exposed to a pulsed high voltage field so that the microorganisms are inactivated.

The aim of the present study was to characterize whether HPP and PEF technologies increase the nutritional values of frozen lamb in terms of the digestibility, measured as free amino acids (FAAs) released from meat proteins. Digestibility of different lamb cuts under HPP and PEF processing at three different simulated digestion phases (prior to digestion, oral (37 °C, 5 min), gastric (37 °C, 120 min), and intestinal (37 °C, 180 min)) were studied. The storage effects on the digestibility of PEF treated frozen lamb were also investigated. It was the first time to study the FAAs digestibility of HPP and PEF treated meat, particularly in lamb, by measuring the release of FAAs. In addition, the colour and moisture were also measured. A static *in vitro* digestion model was used to simulate the digestion process of HPP and PEF treated lamb. ANOVA and mixed-linear model analysis using R were applied for statistical analysis in this study.

Nineteen FAAs (nine essential and ten non-essential amino acids) were identified and quantified in both of HPP and PEF samples by using a commercial EZ-Faast kit and

gas chromatography. For HPP samples, the total FAAs in three digestion phases of two lamb cuts (flat and inside) were significantly different from one another ($P<0.0001$). The total FAA contents of flat and inside cuts increased from 0 MPa to 300 MPa and decreased from 300 MPa to 500 MPa throughout the digestion. For individual FAAs, similar trends were also detected. The FAAs' digestibility of different cuts were also affected which inside cut contained higher total FAA contents after 300 MPa pressure processing than the flat. The instrumental color of HPP lamb differed largely ($P<0.001$). PEF processing significantly ($P<0.001$) increased the total amount of FAAs at all three digestion phases. The storage period for 7 days had significant effects ($P<0.001$) on improving total FAA contents as well. The moisture content of lamb under 200 MPa treatment was the highest after cooking in both cuts. A combination of PEF processing and 7 days of storage contributed to reach the highest value of total FAA contents at the end of the intestinal phase. It suggests that PEF associated with frozen storage for 7 days had the best effects on improving total FAA contents in lamb compared to other processing conditions. The color and moisture content of PEF samples were significantly different ($P<0.01$) in different muscle cuts with respect to the storage times. Among seven PEF processed cuts, the full back contained the highest total FAAs at the end of the intestinal phase, while the bolar contained the least.

To sum up, both HPP and PEF technologies have shown satisfactory characteristics as meat preserving technologies, which not only maintain microbial safety of lamb, but also promote favorable nutritional values and digestibility of lamb meats.

Key words: lamb, high pressure processing, pulsed electric field, digestibility, FAAs

Chapter 1 Introduction

Lamb is a popular meat source which contains high nutritional value of protein, fat and other micro-elements (William, 2007). New Zealand lamb is famous for its high quality and is exported to 120 countries over the world (MIA(1), 2014). The lamb meat is frozen after slaughter to increase its shelf life and decrease the cost on transportation. The frozen lamb is considered as low quality meat because it has less stable tenderness, more drip loss and shorter colour stability than chilled lamb (Tanzi et al., 2004).

There are many processing treatments used to improve lamb quality and/or extend its shelf life. Currently, thermal processing is the most widely used in the meat industry. However, such inevitable disadvantages as the loss of nutrients and off-flavours limit the applicability of thermal processing on lamb products (Hugo, Auroram, & Torres, 2011). Therefore, two new non-thermal processing technologies, high pressure processing (HPP) and pulsed electric filed processing (PEF), have been developed to study whether they can extend shelf life of lamb without sacrificing its nutrition and sensory qualities. They are both environmentally friendly which can operate at room temperature so that they are regarded as good substitutions to the original technology of conventional heat pasteurization in meat preservation (Sonne et al., 2010).

Proteins in lamb contain hundreds to thousands of nitrogen-containing amino acids, which provides energy to our body (MAP, 2011). Researchers have assessed impacts of HPP and PEF on meat quality in many aspects, however, scarce information is available on the effect of these treatments on digestibility. The digestibility of FAA reflects the actual amount of amino acids our body can absorb, representing the nutritional value of lamb (Stuknytė et al., 2014).

The static *in vitro* digestion model was applied in the study. It has the advantages of

high reproducibility and fewer conditions need to be controlled, which make it appropriate for mechanistic studies and hypothesis building (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009; Minekus, Alminger, et al., 2014). The FAA contents from two important stages, gastric phase and small-intestinal phase, were analyzed to determine the differences with non-digested samples as pepsin in gastric phase and pancreatin in intestinal phase have strong ability to break down proteins into smaller peptides and amino acids.

The aim of the present study was to characterize whether HPP and PEF technologies increase the nutritional values of frozen lamb in terms of the digestibility of FAAs. The differences of FAA digestibility between frozen and non-frozen (refrigerated) lamb were also evaluated. In addition, two HPP treated cuts, flat and inside, and seven PEF processed cuts, bolar, cube roll, flat, full back, knuckle, rump and shank were studied to understand whether these treatments make certain lamb cuts more digestible or not. Positive effects were expected so that the frozen lamb would no longer be regarded as low quality although its price is cheap.

This thesis in composed of five parts. Chapter one introduces the study backgrounds and objectives. A literature review of the past studies of HPP and PEF on meat products is summarized in Chapter two. A brief review of human digestion system and *in vitro* digestion models in the literature can be also found in Chapter two. In Chapter three, materials and methods employed in the project as well as statistical analysis are presented. In Chapter four, the results obtained from the experiments are summarized and discussed. The final chapter concludes the study with main findings and suggestions for further research.

Chapter 2 Literature review

2.1 New Zealand lamb

Lamb is a kind of edible food composed of protein and water which is widely consumed in New Zealand (Lawrie & Ledward, 2006). Although lamb consumption occupies less than five percent of total global meat, it is still considered traditional meat food in New Zealand, North Africa and part of Asia (MIA(1), 2014). New Zealand is famous for its lamb quality and has become one of the world's largest exporter of lamb (Abdullah, 2011). The stock number of sheep in New Zealand has increased fast. In 2008, there was about 34.1 million heads which occupied 3.4 percent of total sheep population in the world (FAO(1), 2014). However, the population of sheep was expected to increase to 25 million from 2009 for the next four years, as predicted by Geoff (2009) which meant the sheep population per capita rose from 8 to 20 till the end of 2012. (Geoff, 2009). Such a huge stock number of sheep resulted in 98 percent of lamb in New Zealand being available for export (BLNZ(1), 2015). According to Meat Industry Association (MIA), 120 countries imported sheepmeat from New Zealand. The total volume of export was 402,661 tonnes in 2013/14, which increased in 1.13% when compared to 2012/13. Among these 120 countries, China was the biggest market for sheepmeat both in volume and the dollar value. The annual exports to China have grown from 27,000 tonnes to 160,000 tonnes in the last 10 years. Lamb meat occupied half of sheepmeat exports to China in 2013/14, which reached more than 80,000 tonnes (Figure 1) (BLNZ(2), 2015; MIA(1), 2014). The dominant exports of lamb are still the traditional cuts, which are legs and shoulder chops, and the primal cuts such as loins and ribs which are used for making lamb products. Nevertheless, there are still some shifts towards more chilled cuts which the proportion of chilled lamb export quantities rose from 9.6 to 19.8 percent at the end of September in 2008 (MIA(2), 2009).

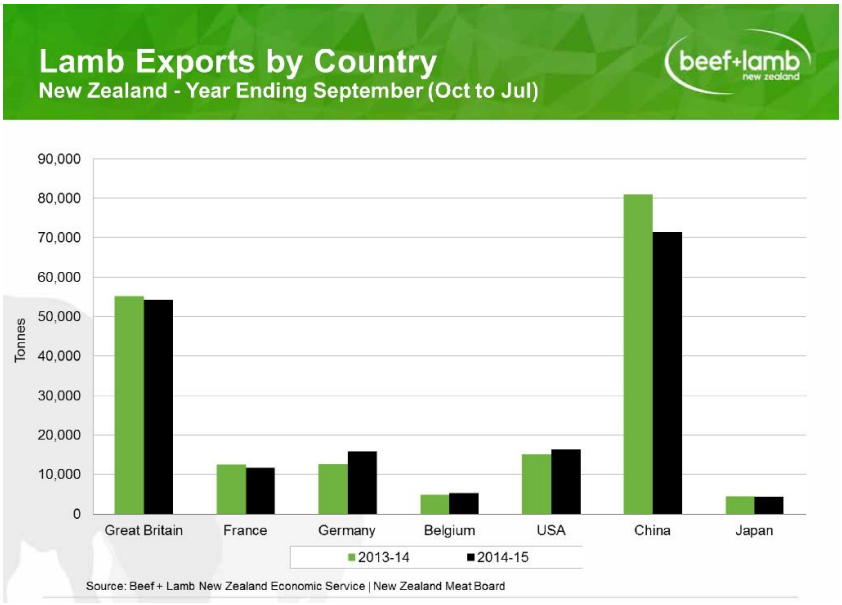


Figure 1 Countries importing lamb from New Zealand (BLNZ(2), 2015)

Because lamb is highly nutritious with high amounts of fatty acids, amino acids and else, it has become a necessary and unique food for the humans, in order to avoid nutritional deficiencies (Cabrera & Saadounb, 2014). The nutrition level of lamb depends on the condition of feeding regime (Lawrie & Ledward, 2006). Normally, the edible lamb contains a high level of protein and almost all the essential amino acids. It is also rich in those micro-elements that are essential to our body. These include zinc (4.5 mg/100 g lean lamb), phosphorus (197 mg/100 g lean lamb), riboflavin (0.23 mg/100 g lean lamb), Vitamin B_{12} (0.96 µg/100 g lean lamb), Vitamin B_6 (0.10 mg/100 g lean lamb) and iron (2.0 mg/100 g lean lamb) which provide 25 % of recommended dietary intake and contains no fiber. There are almost no carbohydrates in lamb (Anderson, Perryman, Young, & Prior, 2014; NCBA, 2007; William, 2007).

2.2. Lamb muscles (lamb cuts)

Similar to other types of red meats, there are large differences in the colour, tenderness, flavor and juiciness of various lamb carcasses arising from different sex, breed, age and feeding background (Berge et al., 2003). Within each lamb, variations

exist in tenderness, juiciness and flavour with different muscles (lamb cuts). For example, Christopher (1995) reported that the *longissimus muscle* of normal phenotype lamb was juicier and tender initially than the *supraspinatus* and *semitendinosus* muscle, and its juiciness and tenderness also sustained longer than the other two muscles. In addition, the flavour intensity of *longissimus muscle* did not differ from *semitendinosus* muscle but it was slightly higher than that of the *supraspinatus.* Similar research findings have been reported by Osman and Aldosari (2006) that the tenderness, flavour and juiciness of leg cut were significantly lower than loin cut on both Awassi and Najdi lamb meat, as well as the overall acceptability. Therefore each lamb cuts should be merchandised according to its palatability characteristics (Sheridan, Allen, Zieger, Marinkov, & Suvakov, 1991). Jung, Hwang, and Joo (2016) reported that most cuts are composed of different muscle fibre types, whose characteristics influence sensory traits, including flavour, juiciness and tenderness, and their overall palatability (described by a 9-point hedonic scale) is also affected by individual muscles or primal/retail cuts. For instance, the flavour had significant correlation with fat content and oleic acids, and the juiciness, tenderness and palatability were only contributed by fat. As a result, different prices are charged for different cuts from different lamb species which consumers are given many choices to select depend on their preferences (Sheridan et al., 1991). As what we can find in New Zealand supermarket, Countdown® lamb steaks rumps are sold the most expensive for $ 29.49 per kg and chop loins are also sold for a high price of $ 25.99 per kg. However, the price of lamb chop plain shoulders are set in a relatively low price for $15.99 per kg. In this project, eight cuts of lamb: knuckle, rump, flat, shank, full back, inside, cube roll and bolar will be analyzed (Figure 2). Referring to the lamb products available in Countdown supermarkets, shank, rump and cube roll are the most well-known and popular lamb cuts for consumers.

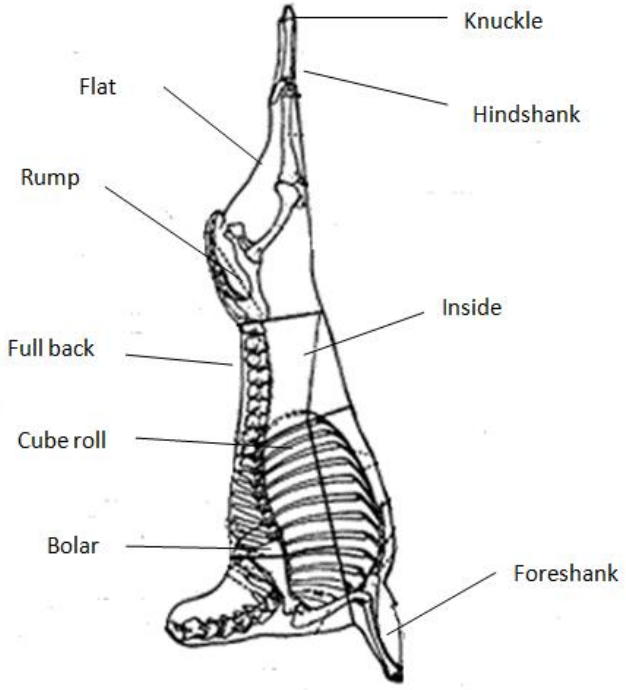


Figure 2 Illustration of the cuts to be analyzed in this project

2.3. Characteristics of frozen lamb meat

Freezing is a widely used technology for preservation of lamb. It increases the shelf life of lamb and decreases cost on transportation (e.g. ship), compared to chilled lamb (air transport) (Leygonie, Britz, & Hoffman, 2012b). However, the consequences of freezing on lamb quality remain as a significant problem. The price of New Zealand's traditional frozen accelerated conditioned and aged (AC&A) lamb is much cheaper than that of the chilled lamb. Freezing mainly influences the water fraction of lamb (Union, 1997). Ablikim et al. (2016) reported that the moisture content of Bashbay lamb and Xinjiang Merino lamb decreased from 75.47% and 74.88% to 68.10% and 69.12% after 1day freezing storage, and 74.90% and 73.96% after 15 days of storage. During freezing, ice crystals are formed between and within the fibres, physically damaging the micro-structure of lamb. As water freezes out, the concentration of the remaining solutes such as protein, lipids, vitamins and minerals increase, destroying the balance of the complex meat system (Leygonie, Britz, & Hoffman, 2012a; Leygonie et al., 2012b). The ice crystals draw water from intracellular space to

intercellular space which cause massive moisture loss during thawing, leading to a change of tenderness. The increase of solute concentration increases the susceptibility for protein denaturation, which a typical example is myoglobin (from bright red oxymyoglobin and dark red deoxymyoglobin to brown metmyoglobin). It is one of the proteins that denatures during freezing, and this results in a loss in colour stability (Jeong, Kim, Yang, & Joo, 2011; Leygonie et al., 2012b; Ngapo, Babare, Reynolds, & Mawson, 1999). Thus, the frozen lamb has less reliable tenderness, more drip loss and shorter colour stability than chilled-never-frozen (CNF) lamb. As a result, consumers consider the frozen lamb as low quality meat (Tanzi et al., 2004). However, Wiklund et al. (2009) investigated a research on the quality of CNF and chilled-frozen-thawed (CFT) lamb and reported that the eating qualities of both lamb loins were very similar. CFT had no significant effect on consumer preference for tenderness and overall liking of the meat with CNF from two different age groups of lamb loins. CNF was reported to be juicier than CFT for four month olds and CFT was tender than CNF in seven month olds. The frozen lamb showed brownness faster than CNF, and therefore Wiklund et al. (2009) suggested that CFT lamb could be best targeted to hotels or restaurants, which the unexpectable brown colour of frozen lamb would not be an issue as the consumers will not get to see the raw meat. Contradictory results were reported by Leygonie, Britz and Hoffman (2012a) that the quality of ostrich meat was negatively affected by freezing but some significant changes, including the increase in toughness, loss of colour, increase of protein oxidation and moisture loss, were still observed.

2.4. Proteins and amino acids in lamb

Lamb is a complete and a high source of proteins and amino acids (Lister, 2015; William, 2007). Proteins are made up of amino acids. They are broken down in our body during digestion into peptides and FAAs so that they can be absorbed to supply energy when carbohydrates or fats are scarce. They synthesize enzymes and hormones,

and build, repair and maintain body tissues. Proteins in food contain hundreds to thousands of nitrogen-containing amino acids, which part of them can be produced by our body. These are called nonessential amino acids, whilst others which we have to get from the diet are called essential amino acids. Lamb provides eight essential amino acids for adults including lysine, threonine, methionine, phenylalanine, tryptophan, leucine, isoleucine and valine. In addition, histidine is regarded as essential for children (MAP, 2011).

There are about 20-25 g of proteins in 100 g raw of red meat, while the cooked red meat contains up to 28-36 g protein/100 g. The difference is because the nutrients concentrate more during cooking with the loss of water in the meat. The average protein contents of Australian lamb meats were reported to be 21.9 g protein/100 g in lamb and 21.5 g protein/100 g in mutton (William, 2007). Compared to the true digestibility of 81.6% in beans (Dias, De Abreu, Silvestre, & Schwan, 2010) and 90.3% in dietary wheat (Bos et al., 2005), the digestibility of proteins in red meat has been reported to be about 94% (Table 1) (Bhutta, 2005).

Amino acids, as the molecular building blocks of proteins and polypeptides, are not only cell signaling molecules but also can act as regulators of gene expression and protein phosphorylation cascade. Wu (2009) mentioned that arginine, cysteine, glutamine, leucine, proline and tryptophan, which are called functional amino acids, regulate key metabolic pathways for maintenance, growth, reproduction and immunity. Among non-essential amino acids, glutamine, glutamate and arginine play important roles in regulating cell signaling, gene expression, immunity and antioxidative responses. Besides, they are also the major energy source for small intestine. Along with glycine, they regulate neurological function by participating in cell signaling via mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), extracellular signal-related kinase, Jun nuclear kinase, mitogen-activated protein kinase, and gases (NO, CO, and H_2S) (Kimura, 2010; Marc Rhoads & Wu, 2009). In

addition, leucine and tryptophan are the most emphasized among essential amino acids, in which leucine inhibits proteolysis and activates mammalian target of rapamycin to stimulate protein synthesis, and tryptophan modulates immunological and neurological functions via serotonin and melatonine (Ngapo et al., 1999). Apart from these, other amino acids own their individual functions in nutrition and metabolism. Dietary supplement with these amino acids were believed to be possibly beneficial for ameliorating health problem at different life stages and optimizing efficiency of metabolic transformations, for instance, enhancing muscle growth, preventing excess fat deposition and reducing adiposity (Wu, 2009).

Table 1 Digestibility of proteins from different food sources in humans (W. Wu et al., 1995)

Protein source	True protein digestibility (%)
Egg	97
Refined wheat	96
Milk and cheese	95
Peanut butter	95
Meat and fish	94
Soybean isolate	94
Soy flour	86
Whole wheat	86
Beans	78

*True protein digestibility (TDP) is calculated as the formula:

$$\mathrm{TDP} = \frac{\mathrm{Pi} - (\mathrm{Pf} - \mathrm{Pmf})}{\mathrm{Pi}} \mathrm{X100}$$

P_i: the protein intake of animals fed the test diet

$P_{f:}$ the protein in feces from animals fed the test diet

P_{mf}: the protein in feces from animals fed the protein-free diet

2.5 High Pressure Processing (HPP)

2.5.1 Principles of HPP

In recent years, food quality and safety have been the most important factors affecting consumers' acceptance, which have been leading a trend towards better tasting and lower or free in additives with a longer shelf life. Gamma irradiation or thermal

processing have been trialed and widely applied but they have met with some resistance from consumers in different extent. Thermal processing is still the most widely used and prevailing technology in the modern food industry to inactivate microorganisms and maintain microbial safety with effective cost, availability and efficiency. However, thermal processing has an inevitable disadvantage, in which the heat will cause chemical reactions resulting in loss of nutrients and formation of off-flavours (Hugo et al., 2011). Thus, it is meaningful to seek more effective methods continuously to minimize or eliminate undesirable changes in foods after thermal processing treatments (Kiera, Alan, & Gerald, 2008). High pressure processing (HPP), as one of the new preservative methods which has been researched for over a hundred years, has the potential to fulfill the requirements both from consumers and scientists (Campus, 2010; H. J. Ma & Ledward, 2013; Marcos, Aymerich, Guardia, & Garriga, 2007).

The principle of high pressure processing has originated from Le Chatelier's Principle that any decrease in volume is caused by an increase in pressure. It is connected to the Ideal Gas Law and easily described as the formula: PV=nRT (P: pressure applied, V: volume of the gas, n: the amount of substance of gas (in moles), R: gas constant, and T: the absolute temperature of the gas). The pressure applied shifts an equilibrium to the state in the smallest volume, progressing the reactions with a decrease in volume. Generally, the high pressure does not affect covalent bonds, so the primary structures of large molecules are unaffected. In addition, the pressure applied encourages the stabilization and the formation of hydrogen bonds, along with some destabilized weak linkages broken, which leads to a decrease in volume (Campus, 2010; Patterson, Ledward, & Rogers, 2005). A typical example given by Campus (2010) was transition from water to ice, the pressure opposing to the transition results in the lower freezing point with the increase of pressure. Abdul Ghani and Farid (2007) presented a formula to describe the relationship between ΔV, the difference between the partial volume of products and reactions, and P, the pressure applied to the products, with respect to T,

temperature:

$\Delta V = -RT\left(\frac{\delta \ln K}{\delta p}\right)_T$

ΔV: the difference between the partial volume of products and reactions;

K: the equilibrium constant of the chemical reaction of interest;

P: the pressure applied to the products;

R: the radius of cylinder;

T: temperature, °C.

Under high pressure, reactions with a negative activation volume are accelerated and by contrast, the pressure inhibits reactions with positive volume. ΔV is replaced by V_a^*, which is defined as the difference between the partial molar volume of the active state and the reactants. When V_a^* is zero, it means the rate is not affected (Campus, 2010).

According to the Isostatic principle, the transmittance of pressure is uniform and instantaneous, which means HPP is independent of product and equipment size and geometry because pressure transmission is not time nor mass dependent (Campus, 2010; H. J. Ma & Ledward, 2013; Marcos et al., 2007). Thus in contrast to the conventional thermal processing, HPP does not break covalent bonds, so it will not impair food quality and natural freshness, but extend microbiological shelf life (Balasubramaniam & Farkas, 2008). It is mild to preserve foods' natural characteristics to a satisfied extent but has destructive effects on pathogenic and spoilage microorganisms between pressures from 50 to 1000 MPa (Campus, 2010; Kiera et al., 2008). HPP foods are currently regarded as novel foods in Europe countries since they meet two of the factors in national regulations for new products (Regulation 258/97/EC), which are a new manufacturing processing employed to productions and a negligible history of human consumption (Hogan, Kelly, & Sun, 2005; Union, 1997).

2.5.2 Commercial applications of HPP to foods

HPP was first performed by Hites in West Virginia in 1899 with milk and fruits under 650 MPa which far exceeded the atmospheric pressure (Kiera et al., 2008). Japanese was the pioneer in developing the technology of HPP in food products. In the 1980s, food industries in Japan began to research the application of HPP in variety of commercial food products. It initially succeeded in fruit based products such as avocado paste and orange juice, inactivating bacteria and enzymes partially or completely at a pressure of 500 MPa, so that the products were able to be kept at the temperature of a fridge for several weeks without any apparent deterioration in quality (Andres, Adamsen, Moller, & Skibsted, 2004). In the early 1990s, in Japan, Medi-ya Ltd, a food company, launched a HPP food product, a high-acid jam, which was regarded as the first commercial food product with the use of HPP technology (Kiera et al., 2008). The commercial success of avocado paste was introduced by Avomex Inc in the USA in 1996, which set a technical standard of the quality of HPP (Torres & Velazquez, 2005). Based on the success of previous examples, by late 20th century, food industries in Japan, USA, and mainland Europe have started to produce and market HPP treated meat products. It was in 1998 in Spain that a cooked sliced ham and "tapas" (pork and poultry cuts) under HPP treatment (400 MPa, 10min at 8 °C) was first approved for sale validly whose shelf life was extended to 2 months with minimum colour and taste modifications. In 2002, the spicy sliced pre-cooked chicken and beef for fajitas processed with HPP were made available in the US market and it was able to be kept fresh for 21 days. In addition, cooked Serrano ham and Chorizo processed under 500MPa pressure for 4-10 min at 8 °C were made available in Spain in 2002 which their shelf lives were increased to 2 months and the required additives were reduced. (Andres et al., 2004; Campus, 2010; Kiera et al., 2008).

2.5.3 Effects of HPP on physiochemical properties of meat

As a technology which has been studied for more than one hundred years, HPP has

been widely applied in different food kinds, including meat and seafood, fruit, vegetable, and beverage to improve food quality or extend shelf life. The assessment of food quality is not only decided by microbial safety but also by other factors such as structural changes and biochemical and enzymatic reactions, concurrently affecting consumers' sensorial perception (Andres et al., 2004) . Beyond microbiological concerns, HPP induces considerable physicochemical changes in food products. For example, colour, texture, oxidation status, and so on (Duranton, Simonin, Guyon, Jung, & de Lamballerie, 2014). In this part, the effects of HPP on meat are reviewed.

Protein-rich foods, meat and seafoods, represent 45% of commercialized pressure-treated food products world-wide (Duranton et al., 2014). Their major modifications after HPP, including colour, texture, or oxidation are mainly contributed by the effects of pressure on the conformation of macromolecules and the way they interact with each other (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). Changes in meat colour and texture mainly come from the effects of high pressure on protein, while its oxidation involves proteins and lipids, affecting meat flavor significantly (Duranton et al., 2014).

Appearance, especially the colour, is the primary factor for consumers to identify the quality of meat. The colour of meat mainly depends on the amount and type of myoglobin which is a kind of protein containing a prosthetic group, a haem ring, with an iron atom in the central. Other proteins like haemoglobin and cytochrome C also play roles in the colour of beef, lamb and poultry (Campus, 2010). Rastogi et al. (2007) reported that the change in meat colour can be seen at the pressure above 300 MPa, even at low temperature, although 200 MPa was reported to induce a strong modification in the colour of beef *longissimus dorsi muscle by* Marcos et al. (2010). Table 2 summarizes the effects of pressure treatments on meat colour. Most of the studies have shown similar results that pressure high than 300 MPa changed meat colour significantly, except for Marcos et al. (2007) who reported that 400 MPa did

not change the colour of low acid fermented sausages significantly. And application of lower pressure of 80 to 100 MPa increased colour stability of beef muscles post-slaughter (Cheah & Ledward, 1997(a)).

Apart from colour, texture and water retention are regarded as the most important properties of meat (Duranton *et al*., 2014). Ma and Ledward (2004) and Del Olmo *et al*. (2010) reported that the tenderness of post-rigor beef and chicken breast fillet decreased at pressure of higher than 200 MPa at room temperature. The toughness of meat is related to the significant structural modification of myofibrils induced by pressure. As what Jung, De Lamballerie-Anton and Ghoul (2000) reported, pressure higher than 300 MPa increased post-rigor beef myofibril fragmentation significantly. Nevertheless, some studies reported opposite observations that tenderness was increased after HPP of post-rigor meat. Schenkova *et al.* (2007) observed an obvious improvement of beef muscle which was treated with pressure between 100 MPa and 300 MPa for 10 min. And the shear force of bovine muscle was reported to decrease significantly after being treated with a pressure of 500 MPa (10 °C, 8 min) (Ichinoseki, Nishiumi, & Suzuki, 2006). Accompanying with pressure-induced toughening of muscle meat, a decrease of water-binding capacity was reported (Duranton, Simonin, Cheret, Guillou, & de Lamballerie, 2012). Marcos, Kerry and Mullen (2010) investigated a study of high pressure on bovine *longissimus dorsi* that 400 MPa and 600 MPa (10-30°C, 20 min) weakened water-binding capacity of samples significantly, but there was no significant difference when treated at 200 MPa. More studies on meat texture and water binding capacity are summarized in Tables 3 and 4.

Lipid oxidation and protein oxidation are prevalent in meats. Lipid oxidation takes places in meat with high levels of poly-unsaturated fatty acids which are degraded to volatile short-chain products. These give rise to rancidity and off flavours of meat products and contribute towards deterioration on their sensory quality, such as colour/appearance, drip loss and texture (Europa, 2009; Min & Ahn, 2005). Addis

(1986) reported that the accumulation of by-product compounds caused by lipid oxidation may be even detrimental to the health of consumers. The impacts of high pressure on lipid oxidations have mostly been determined by measuring the secondary lipid oxidation products (thiobarbituric acid-reactive substances (TBARS) or hexanal content. For example, Dissing, Bruun-Jesen, and Skibsted (1997) reported that less formation of TBARS was found in turkey thigh muscle under pressure treatment at 400 MPa and lower pressures for 30 min and for 10 min, respectively, during 6 days of storage at 5 °C compared to thermal treatment at 100 °C for 10 min. In addition, higher pressure treatment to about 500 MPa for 30 min resulted in similar lipid oxidation extent as the thermo treatment. Ma et al. (2007) reported that HPP from 200 MPa increases the TBARS values of raw beef *longissimus dorsi* muscle after 7 days of storage at 4 °C. More marked increases were found under the pressures of higher than 400 MPa for at least fivefolds, while lower pressures only led to less than threefolds. The free fatty acids level or antioxidant enzyme activities have also been reported to indicate the status of lipid oxidation. Cliriana et al. (2011) studied the effect of HPP on lipid oxidation of dry-cured hams from the aspects of TBARS value, FAAs content and antioxidant enzyme activities. All of the three indices showed that the oxidative stability did not alter after 600 MPa pressure treatment at 15 °C for 6 min.

HPP accelerates lipid oxidation in meat, but does not increase its final oxidation level. The final oxidation level of the HPP treated meat is reported to be close to that of cooked meat products or of under high temperature treatment. For example, Wiggers, Kroger-Ohlsen, and Skibsted (2004) reported that the secondary lipid oxidation products were significantly increased in cooked chicken breast after being treated with 400 MPa and 600 MPa for 5 min at 10°C when compared to 200 MPa treatment and non-pressurized control. A similar result was published by Orlien, Hansen and Skibsted (2000) that the pressure treatment at 800 MPa for 10 min was found to cause the most damaging effect on lipid oxidation which gave the same level as high

temperature treatment. Pressure treatments at 600 MPa and 700 MPa (10 min) showed lower TBARS level in chicken breast compared to 800 MPa treatment but there was still some lipid oxidation observed. And no indication to rancidity was detected in chicken breast exposed to pressure between 300 MPa and 500 MPa (10 min). Table 6 is a summary of the threshold pressure of lipid oxidation on different origin of meat. Yet, there are not many research focus on lamb.

The oxidative reactions take place in muscle proteins as well that involve the loss of essential amino acids and decrease of protein digestibility, thereby affecting meat nutrition level (Duranton et al., 2014). The protein oxidation is possibly induced in three pathways, directly through reactive nitrogen species and reactive oxygen species which can either be free radical or non-free radical, and indirectly by secondary products of oxidative stress (Lund, Heinonen, Baron, & Estévez, 2011; Soladoye, Juaez, Aalhus, Shand, & Estevez, 2015). It results in the formation of protein carbonyl compounds, potentially impacting on the meat quality attributes, including water-holding capacity, texture and flavour (Estevez, 2011). Only a few studies have investigated protein oxidation after HPP. Jung et al. (2013) reported that the carbonyl content of chicken meat was significantly increased at 600 MPa (15 °C, 5 min), but no effect was detected at 300 MPa under the same condition. However, Cava et al. (2009) did not find any changes in protein oxidation in dry-cured Iberian ham under 200-300 MPa (14°C, 15-30 min). But interestingly, lipid oxidation appeared under the same conditions as reported by the authors. More studies HPP on lipid oxidation were summarized in Table 5.

As what mentioned above, most of the studies focused on the sensory qualities of HPP meat (pork, beef and chicken). More attentions are suggested to be paid on the effects of HPP on meat nutrition values such as protein, lipid, amino acids and fatty acids. These nutrients play crucial benefits to our health, and hence they should be considered be superior to the sensory attributes of meat. To date, only a few studies

have investigated to the effects of HPP on lamb. As lamb being one of the most popular meat products in New Zealand, more research should be encouraged to characterize the lamb.

Table 2 Effect of HPP on colour of meat

Meat source	Processing parameters	Results	References
Beef *Longissimus dorsi*	80-100 MPa for 20 min 2 days and 12 days post-slaughter Temperature: N/A	Colour stability[A] was improved that the level of surface metmyoglobin of pressure-treated samples corresponded to only 25 % metmyoglobin up to 12 days of storage, but the control achieved to 35 % after only 2-3 days. .	(Cheah & Ledward, 1997(b))
Beef *biceps femoris* muscle of dry green hams	400 MPa and 600 MPa Exposed to light for 0 hour and 24 hours Temperature: N/A	Lightness, redness and yellowness increased significantly on both pressures and under the light exposures.	(Serra et al., 2007)
Beef *M. longissimus dorsi*	200, 400 and 600 MPa 10, 20 and 30 °C	400 MPa with 30 °C increased lightness and yellowness the most from 24.04 to 55.34, and 9.50 to 18.10, respectively.	(Marcos, Kelly, & Mullen, 2010)
Low acid fermented sausages (fuet and chorizo) made by bellies and shoulders	400 MPa for 10 min at 17 °C	No significant difference in colour was found between fuet and chorizo.	(Marcos et al., 2007)
Parma ham slices	600 MPa for 3,6,9 min Temperature: N/A	Redness decreased over the treatment time with 9 min being the least red.	(Wiklund et al., 2009)
Dry-cured Iberian ham	200, 400, 600 and 800 MPa for 15 min Temperature: N/A	Lightness and redness decreased in all four pressure treatments. Redness decreases with pressure treatment increasing.	(Andres et al., 2004)

Note: [A] The colour stability was measured by the rate of formation of metmyoglobin in beef muscles

N/A: not available.

Table 3 Effect of HPP on texture and tenderness of meat

Meat source	Processing parameters	Results	References
Beef *biceps femoris muscles*	100 - 600 MPa 260 s 10 °C	Myofibril fragmentation increased significantly, indicating a decrease in tenderness.	(Jung, De Lamballerie-Anton, & Ghoul, 2000)
Beef bovine intramuscular connective tissue	0.1 to 500 MPa 8 °C 5 min	The shear force decresed significantly, which means the muscle was tenderized by pressure processing.	(Ichinoseki et al., 2006)
Beef post-rigor *longissimus dorsi muscle*	200, 400, 600 and 800 MPa 20-70 °C 20 min	The hardness increased with the increase of pressure at constant temperatures of 20 to 40 °C, which the highest was under 600 MPa at 40 °C for 11092.7±4852.6 g of hardness.	(H. J. Ma & Ledward, 2004)
Beef *semitendinosus* muscle	100, 300 and 500 MPa Time: N/A Temperature: N/A	The shear force and hardness decreased significantly at 300 MPa, but increased significantly at 100 and 500 MPa.	(Kim, Lee, Lee, Kim, & Yamamoto, 2007)
Pork *biceps femoris* muscle	500 MPa 6 min 20 °C	The shear force increased and the muscle fibres packed tightly, which means the tenderness decreased.	(Duranton et al., 2012)
Chicken breast	400 MPa Single-cycle treatments: 1, 3, 5, 10, 15 and 20 min Multiple-cycle treatments consist in two, three or four 1-min cycles, two or three 3-min cycles, and two 5-min cycles Temperature: 16 °C	Tenderness increased most in single-cycle treatment (49.81 N/g of shear force) in 10 min treatment and 15 min (43.96 N/g of shear force) treatments. In multiple-cycle treatments, tenderness increased with the number of 1-min cycles, but showed lower values in the more severe treatments.	(Del Olmo, Morales, Avila, Calzada, & Nunez, 2010)
Caiman tail meat	200, 300 and 400 MPa 20 °C	The cohesiveness, springiness and resistance of texture parameters increased.	(Canto et al., 2012)

	10 min 1, 15, 30 and 45 days of storage	The highest value of cohesiveness, springiness and resistance were 0.51±0.04 under 400 MPa for 15 days of storage, 0.79±0.05 under 300 MPa for 1 day of storage, and 0.31±0.05 under 400 MPa for 30 days of storage, respectively.	

N/A: not available.

Table 4 Effect of HPP on water-binding capacity of meat

Meat source	Processing parameters	Effects on water-binding capacity of meat	References
Beef *longissimus dorsi muscle*	400 and 600 MPa 10-30°C 20 min	Both pressure treatments increased the expressible moisture[A] from 21.75of non-treated sample to 30.58 and 33.35 at 10 °C, to 29.90 and 32.74 at 20 °C, and to 31.22 and 30.89 at 30 °C, indicating a reduction of water-binding capacity at these pressure levels.	(Marcos, Kerry, & Mullen, 2010)
Beef *Semitendinosus* muscle	>200 MPa 15 °C 5 min	The water-holding capacity was decreased by pressure treatment from 200 MPa	(Kim et al., 2007)
Pork *biceps femoris* muscle	500 MPa 6 min 20 °C	The pressure treatment reduced water-binding capacity compared to control cooked samples from around 68 % of pressurized sample to 63.5 % of the control.	(Duranton et al., 2012)
Pork sausage batter (made by barrwo pork meat)	0, 300 and 600 MPa, Time: N/A 10, 30 and 40 °C of initial temperature 2 %, 1 % and 0.5 % NaCl contents and 0.3 % phophase	The drip loss[B] in the sausage batter was significantly decreased at most from 21.16±0.5 to 8±0.36 under 600 MPa with increasing temperature to up to 40 °C of initial temperature in all of three salt contents, consequently the water-binding capacity was increased.	(Tintchev et al., 2013)

N/A: not available

Note[A]: The water-binding capacity was calculated by the expressible moisture

Note[B]: The water-binding capacity was characterized by the drip loss for the pressure levels of 300 MPa and 600 MPa and initial temperatures up to 40 °C.

Table 5 The threshold pressure of lipid oxidation on muscle foods

Meat source	Pressure parameter	Results	References
Post-rigor beef *longissimus dorsi*	>400 MPa 20-40 °C, 20 min 7 days of storage at 4 °C	Increases in TBARS values were found at ≥400 MPa for at least fivefolds than lower pressures of 200 MPa (less than threefolds). The highest increases was found under 400 MPa at 20 °C for 6.71±0.21 while the lowest was 1.23±0.09 under 200 MPa at 20 °C compared to 0.48±0.11 in the control.	(H. J. Ma, Ledward, Zamri, Frazier, & Zhou, 2007)
Post-rigor pork leg mince	≥300 MPa 20 °C, 20 min	Caused marked denaturation of the myofibrillar, which was possible to catalyze lipid oxidation.	(Cheah & Ledward, 1996)
Dry-cured Iberian ham and loin	300 MPa 30 min Refrigerated storage for 0, 60, and 90 days	300 MPa increased TBARS value in dry-cured Iberian ham for all of the three storage durations, but only the dry-cured loin stored for 90 days has shown an increase in TBARS from 0.26 of 0 day storage to 0.69.	(Cava, Ladero, Gonzalez, Carrasco, & Ramirez, 2009)
Chicken breast fillet	≥450 MPa 15 °C, 5 min 0, 3 and 7 days of storage	≥ 450 MPa had higher TBARS values over the storage times, which results in 0.62, 0.66 and 0.75of TBARS value under 450 MPa and 1.17, 1.75 and 2.32 under 600 MPa for 0, 3 and 7days, respectively, compared to 0.28, 0.48 and 0.47 of the control ones.	(Kruk et al., 2011)
Chicken breast	600 and 800 MPa 20-70 °C 20 min 7 days of storage	HPP accelerated the increasing rates of lipid oxidation at all temperatures in chicken breast, which increased most under 800 MPa at 40 °C for about 3.06 of TBARS value from 600 MPa, but still more stable than in beef *longissimus dorsi muscle* in general.	(H. J. Ma et al., 2007)
Turkey thigh	⩽500 MPa 10 °C, 30 min 6 days of storage	⩽500 MPa for 30 min resulted in lower TBARS values (highest for 15.51 under 500 MPa) than heat treatment at 100 °C for 10 min (22.73 of TBARS value).	(Dissing, Bruun-Jensen, & Skibsted, 1997)

2.6 Pulsed electric field (PEF)

2.6.1 Principles of PEF technology in foods

Pulsed electric field (PEF) processing is a food preservation technique where foods are exposed to a pulsed high voltage field. The principle of PEF technology is based on the application of short high voltage pulses with intensity of 20-80 kV/cm and duration of micro to milliseconds (Leadley & Williams, 2006). The actual processing time is calculated by multiplying the number of pulses with the effective pulse duration. Food to be treated is placed between two electrodes, where pulsed electric currents are delivered. The space between two electrodes is called the treatment gap (cm) of PEF chamber. The electric field strength, one of the important parameters of PEF processing, is calculated by dividing the peak discharge voltage (kv) by the length of treatment gap (cm), resulting in a unit of kv/cm. The high voltage applied from the electrodes forms an electric field that kills microorganisms (Zhang, Barbosa-Cánovas, & Swanson, 1995). Occasionally, liquid or semi-solid foods with no air bubbles and with low electrical conductivity, are treated with PEF. Electrical currents are transferred through the charged molecules present in the foods, in order to electrically breakdown the structure and materials within the foods (Mohamed & Amer Eissa, 2012). The electric field can be applied in a variety of forms, including exponential decays, square wave, bipolar or oscillatory pulse and so on. The most widely used one is the 'exponentially decaying pulse' due to its relatively simple generation and modification, which its waveforms are characterized by a rapid rise to the target voltage and followed by a slow decay toward zero (Barbosa-Cánovas, Pierson, Zhang, & Schaffner, 2000).

A typical PEF equipment is composed of (Figure 3):

- a high voltage pulse generator (a),
- an energy storage capacitor bank (b),

- a charging current limiting resistor (c),
- a switch to discharge energy from the capacitor across the food (d),
- a treatment chamber with two parallel plate electrodes (e),
- a monitor (f),
- a cooling system (g).

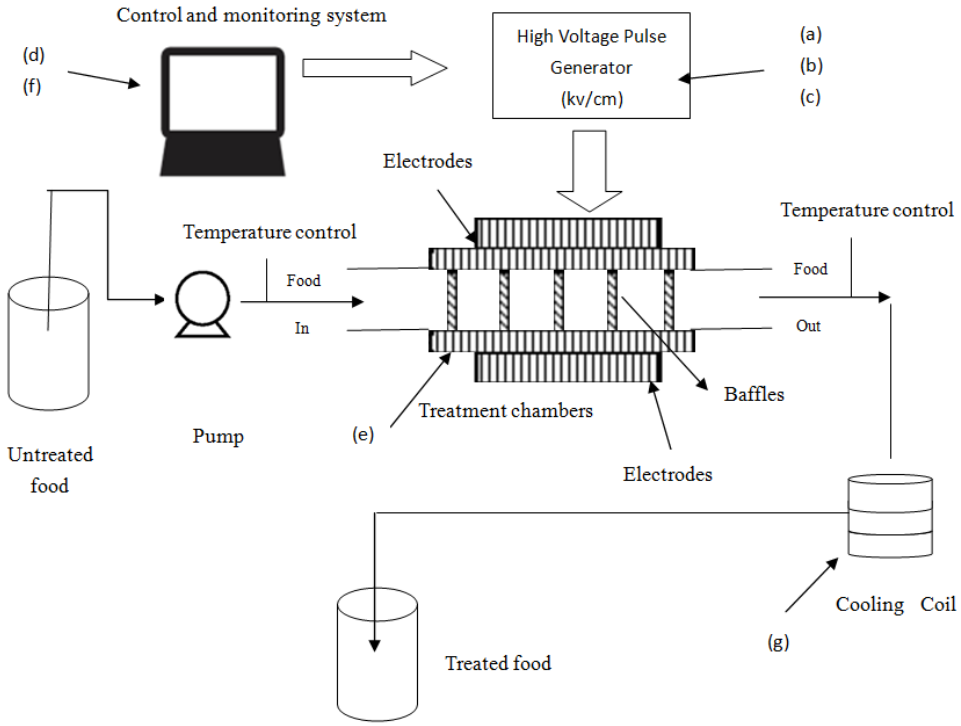


Figure 3 A schematic diagram of a PEF food processing system (modified from (Mohamed & Amer Eissa, 2012)

Using PEF, microbial inactivation is known to be achieved with minimal detrimental effects on food quality attributes. For food preservation applications, treatment times are usually less than 1 s with multiple short duration pulses of less than 5 μs. Similar to HPP, as a non-thermal technology, PEF is also believed to be superior to conventional thermal processing because it kills microorganisms while maintaining the original colour, texture, nutritional value of unprocessed food, and avoiding or causing gentle or undesirable changes in the sensory aspects of the food, which matches with the increasing consumer demand for "fresh-like" foods. (Leadley & Williams, 2006; Mohamed & Amer Eissa, 2012).

2.6.2 Effect of PEF on microorganisms in protein-based foods

Currently, for inactivation of microorganisms by PEF, very little of information is available on meat and meat products. What have been published to date, are contradictive. Bolton et al. (2002) investigated the efficiency of PEF on inactivating *Escherichia coli* O157:H7 in beef burgers and concluded that it was ineffective, without processing conditions. Another similar conclusion came from Haughton et al. (2012) that PEF treatments (3.75 and 15 kV/cm, 10 μs, 5Hz) to raw chicken did not result in any significant inactivation in total viable counts of *Enterobacteriaceae*, *C. jejuni*, *E. coli* or *S. Enteritidis*. Hence more studies are required to draw conclusions on the ability to inactivate commonly occurring microorganisms in meat and meat products.

2.6.3 Effect of PEF on the sensory quality of meat

PEF technology is usually used in liquid or semi-liquid foods. Recently, some studies have expressed interests in the use of PEF in solid foods in order to modify the structures for various reasons. For example extraction of bioactive compounds or change of physical properties of plant materials (O'Dowd, Arimi, Noci, Cronin, & Lying, 2013). Sufficient studies have investigated the effects of PEF on muscle foods. The potential of PEF to meat may enhance cell permeation by electroporation so that the tenderness could be improved consequently, particularly for those of tough muscles. Microbial cells exposed to an external electrical field for several microseconds processing respond by an electrical breakdown and local structural changes of the cell membrane, leading to a drastic increase in permeability, which the mobility of intracellular constituents are enhanced (Suwandy, Carne, Van De Ven, Bekhit, & Hopkins, 2015(b); Toephl, Heinz, & Knorr, 2007). It is estimated that PEF technology can possibly accelerate the release of enzymes and glycolysis process for early proteolysis, resulting in optimum conditions for meat tenderness. O'Dowd et al.

(2013) investigated the use of PEF to improve muscle quality of fresh beef *Musculus. semitendinosus*. As a result, the myofibrils structural changes took place with PEF treatment at 1.1-2.8 kV/cm with 5-200 Hz but the instrumental tenderness was unaffected. Bekhit et al. (2014) reported that PEF treatment (5 and 10kV/cm, 20, 50 and 90 Hz, 20 µs) enhanced benefit the tenderness of cold-boned beef loins *Musculus Longissimus lumborum* for 19.5 % reduction in the shear force regardless of the electrical input. The tenderness of beef topside (*Musculus semimbranosus*) muscle was increased by increasing the frequency to 20, 50 and 90 Hz, which has resulted in reduction in shear force by 4.1, 10.4 and 19.1 %, respectively. In 2015, a further study by Suwandy and Bekhit (2015a) showed a decrease in shear force of both beef muscles by up to 19 % after PEF processing under the same conditions. But the shear force reduction in *Musculus semimembranosus* was dependent on the PEF frequency while *Longissimus lumborum* was not affected by high voltage electric field. Moreover, the increasing degradation of troponin-T and desmin in PEF processed beef loins reflected an increase in proteolysis. Another similar study reported by the same authors in 2015 focused on the effect of PEF on the tenderness of hot-boned beef muscles. The results indicated a promising potential to tenderize beef *Musculus semimembranosus* muscle to have up to 21.6 % reduction in the shear force with PEF treatment. However, a reverse effect was found in beef *Musculus longissimus lumborum* muscle in which the muscle fibres were merged together for increasing shear force and lower tenderness with increasing treatment frequency (Suwandy, Carne, Van De Ven, Bekhit, & Hopkins, 2015(a)). From these studies, it is evident that the effect of PEF, and the parameters of PEF may have different impact(s) on the different types of muscles being treated.

Colour, pH, lipid oxidation and water loss in PEF-treated meat have been studied. Arroyo, *et al.* (2015) evaluated the impact of PEF processing (electric field strength of up to 3 kV/cm, distance between electrodes of 4 cm, pulse width of 20 μs, frequency of 5 Hz, and pulse number of 300 constant) on lipid oxidation, colour, texture and

sensory attributes of turkey breast meat. The results showed no significant differences in weight loss, cook loss, lipid oxidation, texture and colour (raw and cooked), neither on fresh nor frozen samples. But the sensory evaluation results indicated that there were slight differences between PEF-treated and non PEF-treated samples among panelists in the aspects of odor and texture. Faridnia et al. (2014) reported that the pH, colour stability, shear force and cooking loss of beef *Longissimus thoracis* were unaffected by PEF treatments (0.2–0.6 kV cm^{-1}, 1–50 Hz, 20 μs), while moisture content was significantly ($P < 0.05$) decreased by 0.7-3.6%. Another research conducted by Faridnia et al. (2016) indicated similar results on beef outside flat *Biceps femoris* muscles that there was no detrimental effect observed on beef cook loss and colour stability regardless of the length of ageing after PEF processing (electric field strength of 1.7 - 2.0 kV/cm, pulsed electrical energy of 185 kJ/kg, constant frequency of 50 Hz and constant pulse width of 20μs). However, significant microstructural changes took place. An increase in the number of myofibrils ruptured along the z-lones compared to non PEF-treated samples during ageing were reported, indicating that PEF treatment led to a more porus structure with beef *Biceps femoris* muscle and increases in electrical conductivity and purge loss. Considering the phenomenon of PEF inducing changes in microstructure of meat, the authors raised a suggestion that PEF could be potentially used to improve tenderness, decrease ageing and to alter functional properties.

Apart from these sensory attributes, there is no nutritional study available on PEF meat except Faridnia et al. (2015) reported that protein profiles of beef *Longissimus thoracis* muscles were not affected by PEF treatments. More studies of the effects of PEF on the nutritional value of meat should be done. Furthermore, in most of the current studies, the PEF parameters used are limited. For example, the electric field strength was limited in a lower intensity compared to 20-80 kV/cm of electric field intensity introduced in Section 2.6.1. Whether different parameters (higher electric field intensity, more pulse numbers or longer treatment time) improve the effects of

PEF processing on meat are still unknown. Further research warrants for this technology to be utilized with ideal parameters and outcomes in meat preservation.

2.7 Digestibility of proteins in foods

2.7.1 Introduction of human digestion system

The essence of protein digestion is divided into two main processes taking place simultaneously, which are mechanical transformations that breakdown foods into smaller and smaller particles, and enzymatic transformations that large structure of the foods or nutrients are hydrolysed into smaller constituents and absorbed into the bloodstream (Guerra et al., 2012). The mastication of foods by the teeth cuts the food particles into smaller sizes and increases the surface area of foods for better reaction with the digestive enzymes. This is a short but important step to influence the digestive rate significantly, especially the gastric empty rate. Small foods particles mix with secreted saliva to form a thin paste-like bolus which can be easily swallowed to pass down the oesophagus and into the stomach . The enzyme contained in saliva is α-amylase that reacts with carbohydrates (starch) and starts the first stage of digestion process which has no impact on protein digestion (Minekus, Alminger, et al., 2014; Wickham, Faulks, & Mills, 2009; Woda et al., 2010).

With the muscular action of peristalsis and the presence of gastric acid, the bolus travels to the stomach and continues to be digested with acid and enzymes to create chyme. The digestion in the stomach is regarded as the intermediary between eating behaviour in the mouth and physiological event of digestion and assimilation in the intestine. Proteins undergo enzymatic hydrolysis in the stomach and are broken down into peptides aided by pepsin in the gastric secretion. Sodium chloride and hydrochloric acid lead to decrease in pH from 5-6 to about 1.5 to promote protein hydrolysis (Guerra et al., 2012). A peptide hormone gastrin which is secreted by G cells in the gastric

gland, activates digestive enzymes. Hydrochloric acid is produced by gastric parietal cells and used to initiate the conversion of pepsinogen into pepsin to begin the digestion process and maintain the activity of pepsin within its optimum acidity (37 °C, pH 1.8-3.5) (Guerra et al., 2012; Krehbiel & Matthews, 2003). Pepsin is the most abundant gastric protease which is able to cleave proteins into small peptide fragments, producing mainly polypeptides with oligopeptides and FAAs in small quantity (Minekus, Almingr, & Others, 2014). Pepsin is mostly active in cleaving the peptide bonds formed between phenylalanine, tyrosine, leucine, valine and glutamic acid (Krehbiel & Matthews, 2003).

After the gastric digestion, the chyme enters the duodenum of small intestine through the opening of pyloric sphincter. Most digestion and absorption of nutrients occur in the small intestine, which is composed of duodenum, jejunum, and ileum. This stage of digestion is accomplished by intestinal enzymes which are exocrine secreted from pancreas and liver (PEA, 2014; Wickham et al., 2009).

In the small intestine, the extremely acidic chyme needs to be turned to alkaline for the digestive enzymes to work. This is accomplished by secretion of sodium bicarbonate from pancreatic duct combined with bile from the gall bladder in the duodenum (Guerra et al., 2012; Krehbiel & Matthews, 2003). Chyme is initially neutralized to about pH 6 in the duodenum rapidly followed with a gradually increase in pH from 6 to about 7.4 in the terminal ileum (Molly, Woestyne, & Verstaete, 1993). The protein and polypeptides in the chyme are broken down into peptides by pancreatic enzymes, including trypsin, chymotrypsin, elastase and carboxypeptidase A and B. These active hydrolysis of peptide bonds stimulates the secretion of proenzymes into duodenum by pancreas. The proenzymes are the synthesis and secretion of inactive precursors, which allow vertebrates to digest exogenous protein without destroying constituent protein in the pancreas (Krehbiel & Matthews, 2003). The pancreatic enzymes catalyse protein and polypeptides breaking down into small peptides and amino acids,

which each individual amino acid plays its unique roles. Trypsin is mainly in charge of catalysing the breakdown of carboxyl side of basic amino acids like lysine and arginine. For those linkages joining to aromatic acids, such as phenylalanine and tryptophan, are broken down by catalysis of chymotrypsin. And elastase is generally responsible for cleaving the peptide bonds containing aliphatic residues such as alanine, leucine, glycine, valine and isoleucine. Trypsin, chymotrypsin and elastase assist to release a large amount of terminal peptide bonds which are followed by aminopeptidases, carboxypeptidases and other special peptidase in the small intestine for further digestion. Carboxypeptidases A and B are zinc-containing metalloenzymes which are able to catalyse the hydrolysis of carboxyl-terminal bonds in polypeptide chains by cleaving single amino acids from the carboxyl-terminal ends of protein and peptides (Havenaar & Minekus, 1996; Krehbiel & Matthews, 2003). Restani and others (1992) reported that the peptic degradation liberates small peptides (2-6 amino acids residues) and FAAs. Similar and more detailed results have been reported later by Alpers (1994) that the products of pancreatic digestion can be divided into 60 % of oligopeptides made of up to 6 amino acids residues and 40 % of FAAs.

The indigestible substances travel to large intestine, which absorbs water and electrolytes, ferments polysaccharides with the colonic microbiota, and forms feces (Woda et al., 2010). No protein digestion takes place in the large intestine.

2.7.2 *In vitro* digestion models in the literature

There are usually three phases being simulated with *in vitro* digestion models: oral, gastric and small intestine phases, and large intestine and duodenal occasionally. These models simulate human physiological conditions, commonly taking the pH, temperature, presence of digestive enzymes, salt concentration and digestion time into account (Minekus, Alminger, et al., 2014). The most frequently used enzymes or biological molecules are amylase, pepsin, pancreatin, bile salts and lipase.

Analyzing the digestibility and absorption of specific nutrients is able to reflect the efficacy and availability of the ingested food (Vardakou et al., 2001; Yoo, 2009). Human nutrition studies, using *in vivo* feeding method, are considered the 'gold standard' providing the most accurate results. However, they are time consuming, costly with lengthy trials and bring ethical issues. Therefore, scientists are making efforts to develop *in vitro* digestion models (Minekus, Alminger, et al., 2014). The *in vitro* digestion models provides an alternative to *in vivo* testing methods. It allows a larger number of samples to be measured in parallel. The *in viro* digestion models have advantages as follow: high reproducibility, fewer conditions need to be controlled and easy sampling in the site of interest. These advantages make *in vitro* digestion models appropriate for mechanistic studies and hypothesis building (Fernández-García et al., 2009; Minekus, Almingr, et al., 2014).

Based on the research purpose, for example, if establishing a screening application or a confirmative study, static or dynamic *in vitro* models can be used to simulate different phases of digestion. Dynamic models can simulate the continuous changes of physiological conditions to study kinetic aspects of digestion in human gastrointestinal tract (Alminger et al., 2014; Minekus, Almingr, et al., 2014). They facilitate long-term studies, which are probably related to *in vivo* conditions and mostly computerized controlled. The 'Simulator of the Human Intestinal Microbial Ecosystem' (SHIME) developed by Molly *et al.* (1993) in Belgium and 'TNO's Intestinal Microsystems' (TIM) 1 and 2 created by Havenaar and Minekus (1996), are two representative dynamic *in vitro* digestion models. Recently, more dynamic models have been designed and developed for measurement of gastric biochemistry and mixing (Alminger et al., 2014). These include simulation of dynamic aspects of digestion by Menard French (Menard et al., 2014), *In vitro* Physicochemical Upper Gastrointestinal System (IPUGS) by Yoo (2009), the dynamic gastric model (DGM) developed at the Institute of Food Research in Norwich (Vardakou et al., 2001) and the human gastric simulator (HGS) developed in University of California-Davis and

Massey University (Kong & Singh, 2010).

Static models, also called biochemical methods, are simpler techniques compared to the dynamic models, which just include two or three digestion stages: gastric, intestinal phase, and oral phase occasionally. In these models, only a limited number of parameters are required to be controlled for physiological digestion and no physical processes occur during digestion such as shearing, hydration, changes in conditions over time or peristalsis (Alegria, Garcia-Liatas, & Cilla, 2015; Fernández-García et al., 2009; Wickham et al., 2009). Static models are predominantly utilized for digestion studies on simple foods and isolated or purified nutrients (Wickham et al., 2009). These models are able to evaluate the influence of digestion conditions on the effects of food structures, compositions, and food processing, either positive or negative (Alegria et al., 2015).

2.7.3 Application of *in vitro* digestion models to study protein digestion in meat and meat products

Many studies have assessed the digestibility or bioavailability of protein or protein related nutrients (peptides, amino acids) mainly from meat products using either static or dynamic *in vitro* digestion models. Many bioactive compounds have been found in meat and meat products, such as small peptides, carnosine, coenzyme Q10 and so on. Nutrient bioavailability, as another important index of digestion, is decided by the level of bioactive compounds, which are typically presented in a small quantity but with positive impact on human health (Marcolini et al., 2015). Ferranti et al. (2014) studied two Bresaola beef samples using a static *in vitro* digestion model. Their results showed that more than 170 liberated peptides were identified and the great part of peptides were reported to be released from the duodenal phase. More studies on meat digestion are presented in Table 6. Two studies from Table 6 reported by Gatellier and

Santé-Lhoutellier (2009) and Wen, Zhou, Li, et al. (2015) showed some interesting results in that meat cooking (100 °C) resulted significant decreases in protein digestibility during digestion, which indicated a negative effect of cooking on digestion efficiency. It was opposite to the common digestion knowledge that the proteins are denatured during thermal processing and their digestibilities should be increased after thermal cooking. Their results were in accordance to Promeyrat, Bax, et al. (2010) and Promeyrat, Gatellier, et al. (2010) that a decrease in protein particle number was observed during cooking in parallel with a reduction in particle size which was associated with protein aggregation. However, due to these conflicting results to current knowledge, more research is required to clarify this in further studies.

In addition, as new food processing technologies, the effects of HPP and PEF on digestibility of protein or protein related nutrients have aroused scientists' interests. Until now, only few studies have been reported. Kaur et al. (2016) reported that fewer proteins or peptides of high molecular weight were detected in high pressure processed meat (175 MPa and 600 MPa, at room temperature) after pepsin digestion, when compared with pepsin digested untreated meat. This indicates that more parent proteins were broken down in high pressure treated meats. However, whether more amino acids were broken down was not reported. Another research reported by Shirley and Parsons (2000) showed that significant decreases of amino acid digestibilities were found in most amino acids in meat and bone meals under various pressure treatments for poultry, and reductions were the largest for Cysteine and Lysine. Su, Li et al. (2010) reported that there was no significant difference on peptide profiles of low molecular weight from soy protein isolate hydrolysates after HPP.

As a conclusion, more research is required to study the digestibility of HPP and PEF processed meat in the form of various nutrients, such as the proteins, lipids, amino acids and fatty acids. The sources of these nutrients should not be confined to lamb

meat, but be expanded to other red meats including beef and pork. Their similarities and differences would be able to give numerous suggestions and ideas to the development and application of these new technologies.

Table 6 Summary of protein digestibility studies of meat and meat derived products using static *in vitro* digestion models

Samples being tested	Protein of interest	Digestive enzymes used	Transit time	Method used for quantification	Results	References
Beef bresaola	Bioactive peptide carnosine	α-amylase	5 min	Capillary zone electrophoresis (CZE) and ^{1}H nuclear magnetic resonance (NMR)	The gap between total carnosine content found as the bio-unaccessible soluble fraction was 11 % and 19 % for two independent samples, which represent the fraction of carnosine not accessible for intestinal absorption.	(Marcolini et al., 2015)
		Pepsin	60 min			
		Pancreatin and bile	180min and continue for another 120 min			
Bresaola made of lean bovine hindquarters	Muscle-derived proteins and peptides	α-amylase	5 min	Mass spectrometry-based proteomic and peptidomic strategies	173 proteolysis-stable peptides from the major structural (actin and myosin) of duodenal digestion phase were identified. This study was a first preliminary and would be an important inspiration to define the digestive degration of processed muscle proteins.	(Ferranti et al., 2014)
		Pepsin	60 min			
		Pancreatin and bile	180min and continue for another 120 min			
Cooked beef. *Rectus* abdominis	Proteins	Trypsin and chymotrypsin	130 min	A mathematical modeling for the determination of the digestive kinetic constants	Meat cooking decreased of protein digestibility by proteases of digestive tract. 30 min of cooking caused around 80 % decrease compared to 0 min of cooking.	(Gatellier & Santé-Lhoutellier, 2009)

Cooked beef, lamb. chicken, cod and pork myofibrillar or sacroplasmic protein extracts, casein and egg albumin	Low-molecular-weight (LMW) Iron-binding peptides	Pepsin	N/A	LC-MS[A] IMAC[B]	LMW peptides were generated by pepsin digestion and they were major facilitators of iron solubility. Iron-binding peptides had the enhancing effect of muscle tissue on iron absorption.	(Storckdieck, Bonsmann, & Hurrell, 2007)
		Pepsin or pancreatin	N/A			
Lamb *longissimus Dorsi*	Myofibrillar protein oxidation	Pepsin	1 h	Absorbance at 280 nm	The animal (lamb) diet did not affect the myofibrillar protein digestibility. The myofibrillar protein susceptibility was not influenced by storage time (0,2,4 and 7 days), but the increases were identified with the increase of storage time in the intestinal phase.	(Sante-Lhoutellier, Engel, Aubry, & Gatellier, 2008)
		Trypsin and chymotrypsin	30 min			
Pork *longissimus dorsi* muscles	Pork protein digestibility	Pepsin	120 min	SDS-PAGE MALDI−ToF MS LC-MS[A]	Cooking resulted in a significant decrease in digestibility of pork protein in both gastric and intestinal phase.	(Wen, Zhou, Li, et al., 2015)
		Trypsin	120 min			

Pork *longissimus dorsi*, beef *longissimus dorsi*, chicken *pectoralis,* fish muscle from silver carps	Meat proteins	Pepsin	120 min	MALDI−ToF MS LC-MS[A]	The protein *in vitro* digestibility was the greatest for pork (47.22 %) and lowest for beef (42.75 %), while they had similar amount of fragments from *in vivo* digestion. The *in vitro* digestibilities of fish and chicken were 46.98 % and 44.67%.	(Wen, Zhou, Song, et al., 2015)
		Trypsin	120 min		The *in vitro* digestibility was not significant different among four meat samples, neither was *in vivo* digestion. This study was able to interpret for the differences in physiological functions after the ingestion of different species of meat.	
Pork *longissimus dorsi*	Meat peptides	Stomacher	1 min	nano LC-ESI-MS/MS[C]	51 different peptides ranging from 6 to 16 amino acids in length were identified after digestion by using this technique, which are indicative of how pork proteins can be broken down into small fragments and absorbed more easily. This study presented the utilization of *in vitro* digestion and proteomic technology and filled the blank area to characterize the sequence of peptides generated after protein digestion.	(Escudero, Sentandreu, & Toldra, 2010)
		Pepsin	120 min			
		Pancreatin	120 min			

Note[A]: SDS-PAGE: sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MALDI-ToF MS: matrix-assisted laser desorption/ionization−time-of-flight mass spectrometry; LC-MS: liquid chromatography-mass spectrometry

Note[B]: IMAC: Immobilized-metal affinity chromatography, used to separate metal-binding peptides from a crude peptide mixture.

Note[C]: nano LC-ESI-MS/MS: liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer equipped with a nano electrospray ionization source.

N/A: not available.

Chapter 3 Materials and methods

3.1 Lamb

All lamb samples of different cuts were provided by AgResearch Ltd., New Zealand and further treated with HPP in Foodbowl Ltd., (an open facility operated by NZ Food Innovation Auckland) or pulsed electric field processing in University of Otago. Four animals with cold carcasses weight of 140.5-150.5 kg were used. All animals were stored at 4 °C for 48 hours after slaughter. Each cut was individually packed and hermetically sealed in high density polyethylene bags at 4 °C prior to HPP and PEF processing. (Q. L. Ma et al., 2016).

3.2 High Pressure Processing (HPP)

An industrial scale high pressure apparatus (HPP 055, Multivac, Multivac Sepp Haggenmüller GmbH & Co., Wolferschwenden, Germany) located in Foodbowl Ltd was used for HPP on two lamb muscles, inside and flat. The initial temperature was set around 7 to 8 °C. The temperature increased during the processing with the pressure being built up, but it was kept below 25 °C. 125 $MPa.min^{-1}$ of pressure was applied with water as a medium to transmit the pressure. Packaged lamb samples were placed in a cylindrical loading container and treated with four different pressure levels of 200 MPa, 300 MPa, 400 MPa and 600 MPa. The pressure was held for one minute. The pressure was released immediately once the processing was completed. After decompression, the samples were vacuum packed and stored at -20 °C for further analysis. Each treatment condition for lamb muscle was conducted in triplicates, independently. The samples were coded and weighed as shown in Table 7.

Table 7 Summary of HPP samples used in this study

	Pressure treatment									
Cuts	Control		200 MPa		300 MPa		400 MPa		500 MPa	
Flat	C-F4	24.52g	P600-F4	37.73g	H600-F4	30.21g	P400-F4	39.95g	P200-F4	37.13g
	C-F5	37.34g	P600-F5	39.32g	H600-F5	40.07g	P400-F5	35.47g	P200-F5	29.41g
	C-F6	32.58g	P600-F6	32.63g	H600-F6	32.77g	P400-F6	25.36g	P200-F6	29.27g
Inside	C-I4	80.5g	P600-I4	58.61g	H600-I4	72.89g	P400-I4	58.95g	P200-I4	76.45g
	C-I5	84.79g	P600-I5	81.47g	H600-I5	54.62g	P400-I5	93.99g	P200-I5	59.02g
	C-I6	83.22g	P600-I6	70.66g	H600-I6	63.10g	P400-I6	58.91g	P200-I6	70.57g

3.3 Pulsed Electric Field (PEF) processing

Seven frozen lamb muscles, knuckle, rump, flat, shanks, bolar, full back, and cube roll, were processed in a pilot plant scale PEF system (Elcrack-HVP 5, DIL Quakenbruck, Germany). Chilled and frozen-thawed lamb meat were used in this study. For chilled lamb, two animals (mean cold carcasses weight of 140.5-150.5 kg) were obtained from a local butchery in Dunedin, New Zealand. The chilled lamb was stored at 4 °C for 48 h after slaughter. For frozen-thawed meat, two animals were obtained at 48 h post-mortem from AgResearch Ltd., (mean cold carcasses weight of 140.5-150.5 kg) and then vacuum packed and stored at -20 °C for about 3 months until use. Prior to PEF processing, the frozen samples were thawed overnight at 4 °C (Q. L. Ma et al., 2016). The PEF processing was performed in a laboratory scale PEF system and lamb samples were placed parallel between two electrodes which were 40 cm apart. PEF processing of 1.0-1.4 $kV.cm^{-1}$, specific energy of 88-109 $kJ.kg^{-1}$, field strength with 90 Hz frequency and 20 μs pulse width for 10 seconds with pulse number of 964 was delivered through the lamb samples. After processing, the muscle samples were vacuum packed in polyethylene plastic bags and immediately stored at 4°C for 0 and 7 days of storage. Afterwards, the samples were stored at -20 °C before further analysis. Each treatment condition for lamb muscles was conducted in triplicates

independently (Q. L. Ma et al., 2016). The samples were coded and weighed as shown in Table 8.

Table 8 Summary of PEF samples used in this study

	Frozen			
Treatment	Control		PEF	
4°C storage	0 day	7 days	0 day	7 days
Bolar	25g	25g	25g	25g
Cube roll	25g	25g	25g	25g
Flat	25g	25g	25g	25g
Full back	25g	25g	25g	25g
Knuckle	25g	25g	25g	25g
Rump	25g	25g	25g	25g
Shank	25g	25g	25g	25g

3.4 Cooking of samples prior to *in vitro* digestion

The high pressure processed lamb samples were raw meat which needed to be cooked before doing the *in vitro* digestion experiments. To avoid or minimize the water loss of lamb, a low temperature vacuum cooking method called sous vide was applied. The samples were vacuum packed and cooked at 60 °C for 2 hours in water bath until the redness totally disappeared. All of the PEF and cooked HPP meats were homogenized with a coffee blender (Breville, BCG200, Australia) for 30 sec. The liquid left in the vacuum bags was poured into coffee blender for homogenization. After homogenization, the minced samples were vacuum packed and stored at 4 °C for the *in vitro* digestion experiment on the next day.

3.5 Static *in vitro* digestion

3.5.1 Preparation of simulated digestion fluids

Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared based on the human *in vivo* data with corresponding electrolyte stock solutions, enzymes and water, in accordance to Minekus et al. (2014). The purchased digestive enzymes were in powder form, which needed to be dissolved in water. With the addition of enzymes and water to the stock solution, dilution of the stock solution took place. In order to maintain the correct concentrations of the digestion solutions, the electrolyte stock solutions were prepared for ×1.25 concentrated. Three simulated digestion fluids, marked as SSF, SGF and SIF, were prepared to a final volume of 500 ml each, but made up to 400 ml with distilled water instead and stored at -20 °C. $CaCl_2(H_2O)_2$ was added to the electrolyte stock solutions at last to avoid precipitation during storage. The correct electrolyte concentrations (Table 9) for all of the digestion solution were achieved after adding digestive enzymes and water. 1 M of HCl, 6 M of HCl and 1 M of NaOH were prepared to adjust the pH during digestion. Before every stage starts, all of simulated fluids and enzyme solutions were pre-warmed in an oven to 37 °C so that the activities of enzymes would not be affected by the temperature of solutions used.

Table 9 Concentration of electrolytes in SSF, SGF and SIF (Minekus, Alminger, et al., 2014)

Constituent	SSF (mmol.L^{-1})	SGF (mmol.L^{-1})	SIF (mmol.L^{-1})
K^+	18.8	7.8	7.6
Na^+	13.6	72.2	123.4
Cl^-	19.5	70.2	55.5
H_2PO_4	3.7	0.9	0.8
HCO_3^-, CO_3^{2-}	13.7	25.5	85
Mg^{2+}	0.15	0.1	0.33
NH_4^+	0.15	1.0	-
Ca^{2+}	1.5	0.15	0.6

3.5.2 Static *in vitro* digestion protocol

In vitro digestion was divided into three phases, oral phase (Section 3.5.2.2), gastric phase (Section 3.5.2.3) and intestinal phase (Section 3.5.2.4). For each phase, the corresponding simulated fluids, enzymes and deionized water were added to a Schott bottle for continuous simulation. The whole digestion process was simulated in a shaking water bath (Gyrotory Water Bath Shaker, Model G76, New Brunswick Scientific Co., INC, USA) at 37 °C at 5 rpm for 5 min in oral phase, 120 min in gastric phase and 180 min in intestinal phase. The procedure is outlined in Figure 4. The pH values of oral phase, gastric phase and intestinal phase were adjusted to 7.0, 3.0 and 7.0, respectively, by HCl and NaOH, and monitored with a pH meter (HI 4221, HANA Instrument) before adding the enzymes. The volume of HCl and NaOH added for pH adjustment were recorded for further calculation.

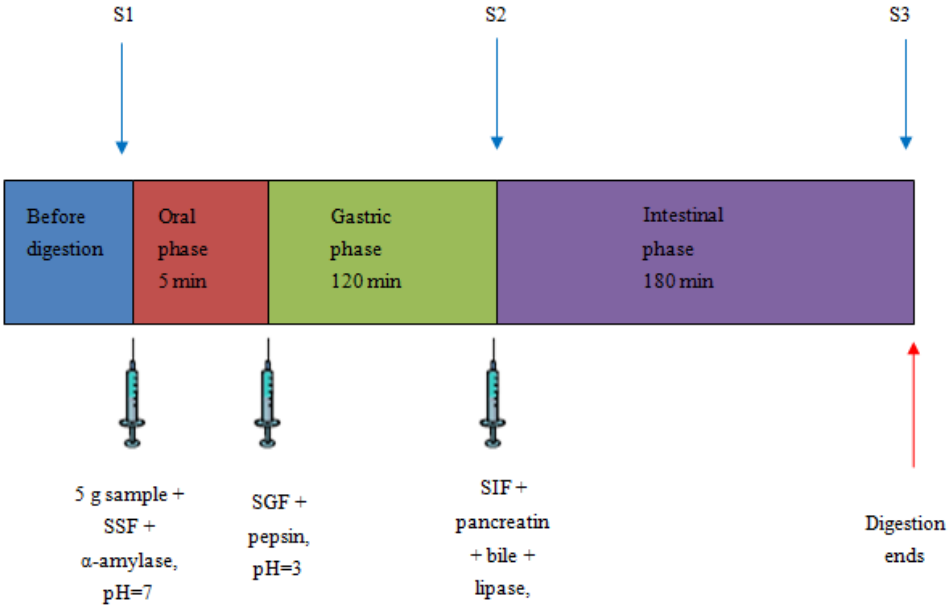


Figure 4 Flow chart outlining the static in vitro digestion protocol with corresponding simulated fluids and enzymes.

3.5.2.1 Extraction of FAAs prior to digestion (S1)

FAAs in lamb samples before digestion (S1) were also studied. The method was based on Mustafa, Aman, Anderson and Kamal-Eldin (2007) with modifications. 1 g of homogenized lamb sample was mixed with 7.5 ml methanol (Sigma-Aldrich®, HPLC grade) and 5.4 g glass beads (diameter of 0.2 cm)using a vortex-genie (Scientific Industries, Inc., Vortex-Genie 2)for 30 min at a high speed. The pH was adjusted between 2 to 5 to fit for FAAs analysis. The sample mixtures were centrifuged at 3000 rpm (Centrifuge 5810R, Eppendorf) for 10 min. The supernatant was further centrifuged at 8000 rpm (Z216 Mk, Hermle Labortechnlk Gmbh, 2007, Germany) for another 10 min to remove small particles and obtain clear supernatant. The supernatant was transferred to a 1.5 ml micro-centrifuge tube and stored in a -20 °C freezer for GC analysis.

3.5.2.2 Oral phase

5 g of homogenized lamb sample was mixed with 3.5 ml SSF, 25 µl of 0.3 M $CaCl_2$ and 975 µl of deionized water thoroughly with glass rod in a schott bottle. 0.5 ml of salivary α-amylase (Source from *Aspergillus oryzae,* ≥ 150 units/mg protein, biuret, Sigma-Aldrich®) of 1500 U/ml was added to the samples to achieve 75 $U.ml^{-1}$ in the final mixture after the pH of the mixture was adjusted to 7.0 with NaOH (Minekus, Alminger, et al., 2014). The bottle was capped tightly and incubated in shaking water bath (Gyrotory® water bath shaker, model G76, New Brunswick Scientific, Edison, N.J., USA) at 5 rpm, for 5 min at 37 °C. No sample was taken from the oral phase.

3.5.2.3 Gastric phase (S2)

In gastric phase, porcine pepsin (≥250 units/mg, Sigma-Aldrich®) was prepared with SGF electrolyte stock solution and added to achieve 2000 $U.ml^{-1}$ in the final mixture. 10 ml of oral bolus was mixed with 7.5 ml of SGF electrolyte stock solution, 5 µl of 0.3 M $CaCl_2$, 695 µl water and 0.2 ml of 1 M of HCl, followed by 1.6 ml of porcine pepsin solution of 2500 $U.ml^{-1}$ after the pH was adjusted to 3.0 with HCl (Minekus, Alminger, et al., 2014). The mixture was then incubated in a shaking water bath (5 rpm) at 37 °C for 2 hours.

3.5.2.4 Intestinal phase (S3)

Intestinal phase digestion was the last stage of whole *in vitro* digestion process. In this phase, pancreatin (3X, U.S.P., MP Biomedicals, LLC) (made up with SIF electrolyte stock solution), bile (bile extract porcine, Sigma-Aldrich®) (made up with deionized water) and lipase (Source from Thermomyces Lanugunosa, Novozymes, Lipozyme® TL 100L, 100 KLU/g) were added to achieve the following activities in the final mixture: pancreatin 100 $U.ml^{-1}$, bile extract porcine 38.4 $mg.ml^{-1}$, lipase 2000 $U.ml^{-1}$ (Minekus, Alminger, et al., 2014). Thus, 20 ml of gastric chyme was mixed with 11

ml SIF electrolyte stock solution, 40 µl $CaCl_2$, 1.31 ml water and 0.15 ml of 1 M NaOH. 5.0 ml of pancreatin solution of 800 $U.ml^{-1}$, 2.5 ml of 160 mM bile and 0.8 ml lipase were added after pH was adjusted to 7.0. The mixture was then incubated for 3 hours at 37 °C in a shaking water bath (5 rpm).

Two samples were taken during the whole process; one was from gastric phase (S2) (120 min) and one from intestinal phase (S3) (180 min). 2 ml of sample was pipetted from the reactor into a centrifuge tube at the end of each phase except for the oral phase. All of the digested samples were snap frozen in liquid nitrogen immediately upon collection to inactivate the digestive enzymes and stored at -20 °C for further analysis. All digestibility analyses were carried out in triplicate for each lamb sample.

3.6 Extraction of FAAs

Digested lamb samples were centrifuged at 3000 rpm (Centrifuge 5810R, Eppendorf) for 10 min. The supernatant was further centrifuged at 8000 rpm (Z216 Mk, Hermle Labortechnlk Gmbh, 2007, Germany) for another 10 min to remove small particles and obtain clear supernatant. The supernatant was transferred to a 1.5 ml micro-centrifuge tube and stored in a -20 °C freezer until use. The pH of the samples collected at the end of the intestinal phase (S3) were adjusted between 2 to 5 to fit for the requirements of FAAs analysis by EZ Faast™ Kit.

3.7 FAAs profile analysis

A commercial free (physiological) amino acids analysis kit (EZ Faast™, Phenomenex®, Torrance, U.S.A) was used to measure FAAs available in the samples. This consisted of solid phase extraction, FAAs elution, derivatization and liquid/liquid extraction steps (Appendix 1). Derivatized samples were analyzed by gas

chromatography (GC). All of these procedures were performed in accordance to the supplier's manual from the EZ Faast Kit's manual (Phenomenex). 0.2 mM Norvaline in 10% N-propanol was used as an internal standard. All GC analysis were carried out once for each extracted sample.

Separation and quantification of amino acid derivatives were achieved by a Shimadzu GC-2010 Gas Chromatograph with an auto injector (AOC-20i, Shimadzu), a Flame Ionisation Detector (FID) (Figure 5) and a split injector. Zeborn ZB-AAA 10 m × 0.25 mmx0.25 μm GC column provided from the commercial kit was used. The instrument settings were followed by the user manual with some modifications for better separation of individual amino acids. 1 μl of derivatized sample was injected into the column at a split ratio of 1:15 at 300 °C with a hot needle. Constant Nitrogen gas flow of 1.46 $mL.min^{-1}$ was used as carrier gas. The oven program was programmed as: initial temperature was set at 50 °C, 50 $°C.min^{-1}$, ramp to 120 °C, held at 120 °C for 30 seconds, increased to 165 °C at a rate of 5 $°C.min^{-1}$, 20 $°C.min^{-1}$ ramp to 320 °C, and held at 320 °C for 1 min. Amino acids were identified and quantified by comparing their retention times with 26 amino acids from the standard mixture which was included in the kit, with four calibration levels ranged from 50 to 400 $nmoles.ml^{-1}$. In order to accurately measure those amino acids with peak concentrations beyond the calibrated range provided by the standard mixture, individual standard curves with higher concentrations were prepared using analytical standard L-amino acids from Sigma®. All peak areas in the chromatography were unified with the area and concentration of internal standard prior to calculation. The concentration of FAAs in samples was presented as $mg.100g^{-1}$ of processed lamb.

Figure 5 Shimadzu GC-2010 Gas Chromatograph with AOC-20i Auto Injector

3.8 Moisture analysis

Moisture content of the processed lamb samples was determined by oven drying methods based on AOAC Official Method 950.46B (AOAC, 2012). All of processed lamb samples were vacuum packed and stored in a - 20 °C freezer to avoid moisture loss during the storage. Crucibles were used to place the samples. The weight of dry crucibles were recorded and placed in a desiccator before use. 3 g of the wet samples were homogenized and placed in crucibles. All samples were dried in an oven (SANYO Electric Biomedical Co. Ltd, Japan) at 105 °C for 12 hours until a constant weight was reached. Crucibles with dried samples were completely cooled down to ambient temperature in a desiccator and the weight was recorded. All samples were measured in triplicate. The moisture of both raw and cooked HPP samples were measured. The moisture of PEF samples were only available for cooked data because the samples were processed to be cooked directly during PEF treatment. The moisture content was calculated by using the formula below:

Moisture content (%) = $\frac{Ws1-Ws2}{Ws1}$ x 100%

W_{s1}: Weight of the wet sample;

W_{s2}: Weight of the dry sample.

3.9 Colour analysis

Colour parameters of processed lamb samples were measured with a Hunter Lab colourimeter (45/0, Colourflex, Chroma meter) (Figure 6). Before analysis, the color meter was calibrated with three colour standard plates (white (L^*=93.94, a^*=-0.95, b^*=0.94), green (L^*=51.67, a^*=-26.62, b^*=13.29) and black). Each sample was cut into small pieces (2mm*2mm*2mm) and well-distributed placed in a plastic petri dish. It was made sure that no light was emitted through the interspace. The following colour coordinates were measured: lightness (L*), redness (a*) and yellowness (b*).

Figure 6 Hunter Lab color meter.

3.10 Statistical analysis

The experimental data in this study was collated by Microsoft Office Excel 2007 and subjected to statistical analysis using XLSAT 2014 software (Addinsoft, USA).

Analysis of variance (ANOVA) was carried out at the 0.01 level of significance to analyze the effect of HPP and PEF processing on FAAs profiles, moisture and colour. R software 2016 (The R Foundation for Statistical Computing, version 3.3.1) were used for mixed-linear model analysis.

Chapter 4 Results and discussion

4.1 FAAs in HPP and PEF lamb cuts

HPP and PEF processing are both new technologies for meat preservation. The nutritional studies of processed meat are the frontiers in their application development, which are largely dependent on the protein digestibility of these meat. In order to pass through the small intestine wall and enter the bloodstream, the proteins must undergo a series of proteolysis and to be broken down into smaller peptides (tripeptides or dipeptides) or amino acids (Sante-Lhoutellier et al., 2008). Considering that the digestion and absorption of all protein sources are almost complete, the level of FAAs released from a certain amount of proteins after digestion process can be regarded as an indicator of protein digestibility (Cheng, 2016; Stuknytė et al., 2014). Therefore, in this research, the breakdown of protein aggregates upon *in vitro* digestion of HPP and PEF processed lamb meat was studied by monitoring the release of FAAs. Nineteen amino acids, including nine essential amino acids (valine (VAL), leucine (LEU), isoleucine (ILE), phenylalanine (PHE), methionine (MET), lysine (LYS), histidine (HIS), tyrosine (TYR) and tryptophan (TRP)), and ten non-essential amino acids (alanine (ALA), glycine (GLY), threonine (THR), serine (SER), asparagine (ASN), proline (PRO), glutamic acid (GLU), aspartic acid (ASP), glutamine (GLN) and ornithine (ORN)) were identified and quantified by EZ Faast^{TM} Kit, Phenomenex ®, Torrance, U.S.A and gas chromatography. Another two amino acids, cysteine (CYS) and arginine (ARG), were also reported to be present in lamb meat by Madruga et al. (2010), which were in negligible amount of 0.01 mg and 9.3 mg/ 100 g cooked lamb, respectively. However, they were not analysed in the current study because the peaks were unable to be quantified using the EZ Faast^{TM} Kit used.

4.1.1 Effect of HPP on digestibility of lamb cuts

4.1.1.1 Effect of pressure

The FAA contents of high pressure processed lamb flat and inside cuts after *in vitro* digestion, analyzed by GC are shown in Table 12. The results are given as Mean ± SD (mg/100 g lamb) of the amount of FAAs. The levels of total FAAs in three digestion stages have obviously differed in magnitudes of 200, 450, 2000 mg/100g lamb, respectively.

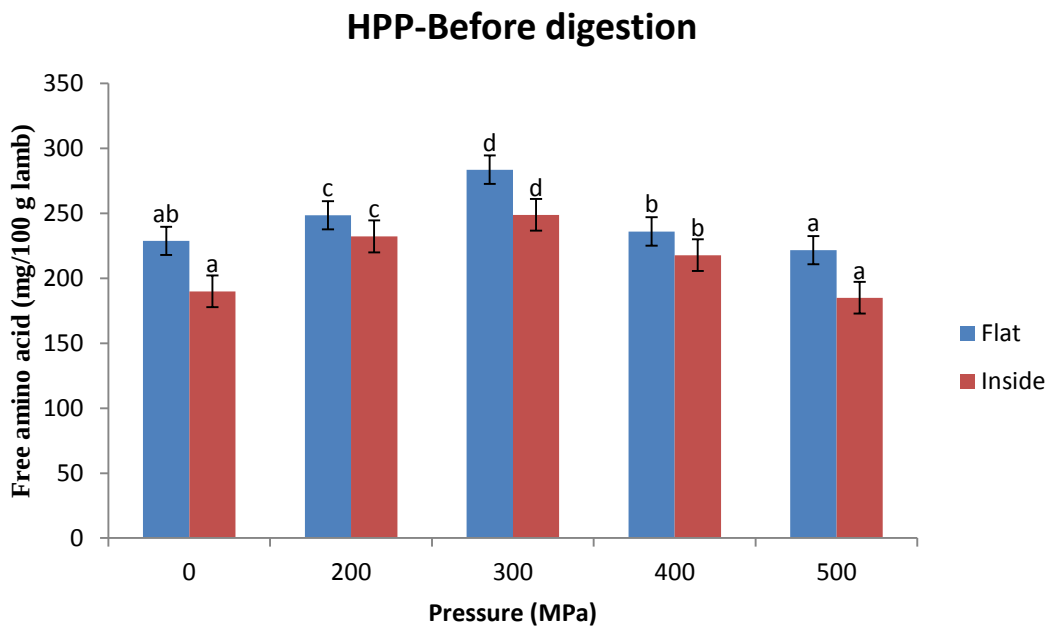
Significant differences ($P < 0.0001$) among the four HPP samples and the control were detected at each stage of *in vitro* digestion processing (initial stage, the end of gastric phase and intestinal phase), for both flat and inside cuts. Pressure treatment of 300 MPa resulted in the highest total FAAs content in three phases in flat and inside cuts, and the concentrations were 283.57±1.26 and 248.85 ±4.15 mg/100 g lamb before digestion, 612.19±2.61 and 430.63±3.46 mg/100 g lamb in gastric phase, and 2004.65±13.06 and 2020.91±7.73 mg/100 g lamb in intestinal phase, respectively, as shown in Table 12 and Figure 7. The total FAAs content has shown an increasing tendency from 200 MPa to 300 MPa and then it decreased after 300 MPa. From the results, it is evident that the higher the pressure applied, the lesser amount of total FAAs were present, indicating a decrease in digestibility of lamb. A reliable explanation of this phenomenon was given by Bajovic, Bolumar, and Heinz (2012) that muscle proteins were unfolded up to 300 MPa. Pressure over 300 MPa led to an enhanced denaturation, aggregation and gel formation. Thus, 300 MPa can be regarded as a threshold of high pressure processing on lamb proteins (Sun & Holley, 2010). Pressure at 300 MPa is able to completely unfold protein structures while the pressure higher than 300 MPa may partially unfold or fold protein structures, which leads to adverse effects or decrease the digestibility of proteins in lamb. It is noteworthy that the relationship between the degree of protein aggregation and its

digestibility plays important roles in the discussion. Galazka and Ledward (1998) reported a theoretical description about the effect of high pressure on protein structure that it is primarily related to the rupture of non-covalent interactions (electrostatic and hydrophobic) within protein molecules when the pressure is applied. The weak linkages stabilize protein's secondary, tertiary and quaternary structures responding to different pressure conditions. The high pressure compresses the internal cavity and decreases the volume of protein, destabilizing the non-covalent interaction which finally results in protein denaturation. Much of the secondary structure of proteins, like amino acid residues with peptide bonds, have been reported to be retained after pressurization. A certain degree of protein unfolding, depending on the specific protein and conditions applied to partial or total denaturation, that exposes hydrophobic region of protein for aggregation has been observed by Bajovic, Bolumar and Heinz (2012) and Sun and Holley (2010). Ruiz et al. (2016) and Tang, Chen and Ma (2009) reported that the digestibility of proteins are closely related to the extent of their aggregation, which the increasing level of aggregation led to lower amount of proteins extracted out and followed with a decreased protein digestibility, although the protein aggregations they reported were heat-induced aggregation. Moreover, unfolding intermediates or unfolded states are commonly used to describe the protein folding/unfolding process. It was reported by Wang, Nema, and Teagarden (2010) that completely folded or unfolded protein, in contrast, do not aggregate easily because the hydrophobic side chains are either randomly scattered or mostly buries out of contact with water. This sufficiently supports that the relationships among the digestibility of protein, effects of high pressure on unfolding proteins and protein aggregation. As a result, combining what was mentioned above, a pressure treatment of 300 MPa had the most ideal effect on protein digestibility, and pressures above this level decreased its digestibility, due to the enhanced protein aggregation, resulting in less FAAs broken down.

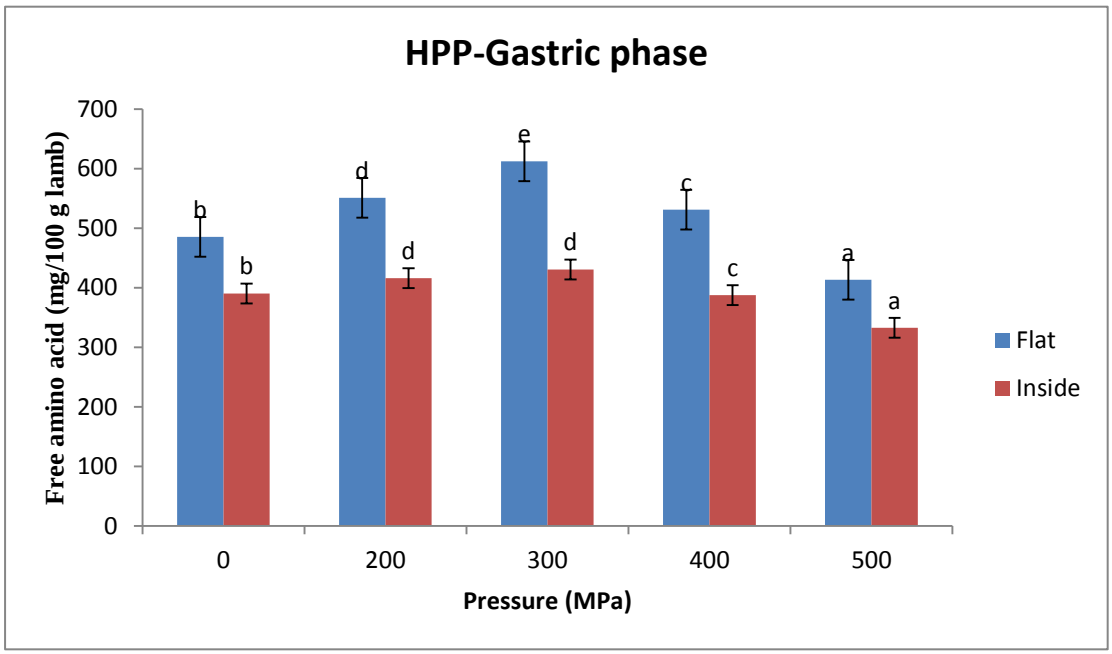
Both flat and inside samples under 300 MPa pressure treatment have shown the

highest level of the FAA contents after *in vitro* digestion. The values of the FAAs throughout three sampling time point were significantly higher than the samples under other pressure treatments and the control ($P < 0.0001$), indicating that 300 MPa high pressure treatment was the most effective condition to improve the breaking down from such big molecules as proteins or polypeptides to FAAs either prior to digestion or post-digestion. Among these individual amino acids in the 300 MPa sample, four essential amino acids, LEU, PHE, LYS and TRP contributed the most to increase the amount of total FAAs released at the end of the intestinal phase. These amino acids were released the most after peptic-acid hydrolysis and pancreatic proteolysis when compared with those prior to digestion. In addition, the amount of GLN, a non-essential amino acid, was also presented in a relatively high level, although its value had no significant difference with samples treated at other pressures. However, the results for 500 MPa was the opposite. The FAA contents in both digested flat and inside samples under 500 MPa pressure treatment had always the lowest amount of FAAs among the five different pressure levels during the whole digestion process, even lower than the control one, which suggest that excessively high pressure processing may have negative effects on the digestibility of proteins measured in the form of FAAs being released. Besides, 400 MPa pressure treatment did not change the level of total FAAs release obviously at the end of the intestinal phase which the value was very close to the untreated sample. The amount of the FAAs under 200 MPa pressure treatment in both cuts were higher than the untreated samples but far lower than that of the 300 MPa ones, indicating that 200 MPa pressure treatment has positive effects on FAA digestibility to some extent, but not as effective as 300 MPa. The range of positive effects from high pressure may extend from 0 MPa to 300 MPa. It would be desirable to repeat this study with smaller intervals of pressure, for example, an increment of 20 MPa rather than 100MPa, in order to validate this trend in the future.

(a)


HPP-Before digestion
Free amino acid (mg/100 g lamb)
350
300
250
200
150
100
50
0
ab
a
c
c
d
d
b
b
a
a
0
200
300
400
500
Pressure (MPa)
Flat
Inside

(b)


HPP-Gastric phase
Free amino acid (mg/100 g lamb)
700
600
500
400
300
200
100
0
b
b
d
d
e
d
c
c
a
a
0
200
300
400
500
Pressure (MPa)
Flat
Inside

(c)

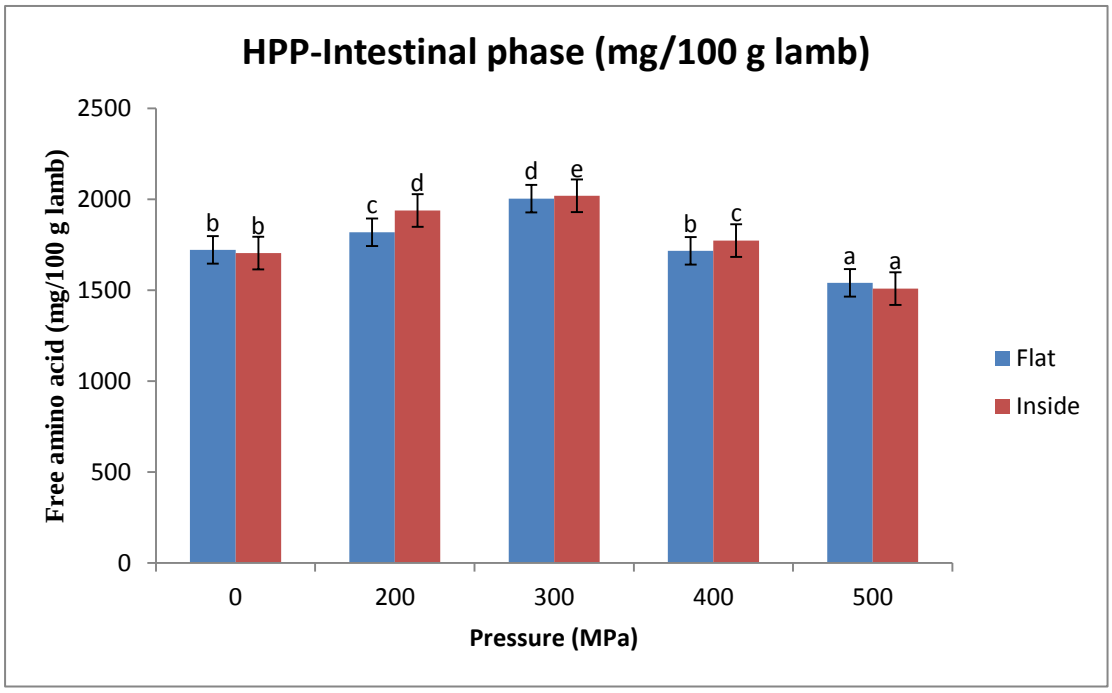


Figure 7 Total FAAs (mg/100 g) of high pressurized lamb flat and inside cuts are presented at different stages of *in vitro* digestion.

Mean values were plotted with error bars which represent standard deviations.

Figures a, b, c represent the results of before digestion, gastric phase and intestinal phase, respectively.

Different superscripts (^{a}, ^{b}, ^{c} and ^{d}) in the same digestion phase differ significantly

Advanced statistical analysis with mixed-linear model was used on the HPP samples, as shown in Table 14. Formula modules were tested for acceptability of a mixed-model formula for the collected data with the variables and the objectives of the study as suggested by Bates et al. (2015). The individual and total FAA results were plotted with actual data and logarithm numbers of FAAs to find out the trends of data and decide whether mixed-linear model would work on this study or not by R software (version 3.3.1). Preliminary plots have shown a better linear relation with log(FAA) as shown in Figure 8. Between a linear and a function of logarithm of FAA, the log model was chosen to describe the linear relations among the concentration of FAAs, the pressure applied and the time of digestion. Two formulae were tested to see which one fits linear better by calculating their REML (restricted maximum likelihood) Criterion Value, which the higher value represented the better linear (Bates, Mächler, Bolker, & Walker, 2015).

From Table 14, the formulae of the relation among log(total amino acid), digestion time and the pressure treatments in flat and inside cuts were expressed as follow in Table 10. The c values of log(total amino acids) at 300 MPa in flat and inside were the largest for 5.628015 and 5.374103, respectively, compared to the other pressures, indicating that the rate of FAA release was the fastest at that pressure. Most of the individual amino acids also showed the same tendency as the total FAAs. For example, the formula of log(LEU) and log(PHE) in flat were presented in Table 11. The c values of both log(amino acid) at 300 MPa were the biggest for 3.106189 and 2.997752, and were the smallest at 500 MPa for 2.260079 and 2.712227, respectively. It suggested that LEU and PHE were released the most at 300 MPa pressure treatment and the least at 500 MPa.

Table 10 Formulae of the relation among log(total amino acid), digestion time and the pressure treatments in flat and inside cuts after mixed-linear model analysis

Cut	Flat	Inside
Formula	log(Total)=c+0.3966563Hour	log(Total)=c+0.4307988Hour
P=0 MPa	c=5.431279	c=5.220691
P=200 MPa	c=5.518302	c=5.332974
P=300 MPa	c=5.628015	c=5.374103
P=400 MPa	c=5.470504	c=5.269195
P=500 MPa	c=5.331885	c=5.133198

Table 11 Formulae of the relation among log(LEU), digestion time and the pressure treatments in flat after mixed-linear model analysis

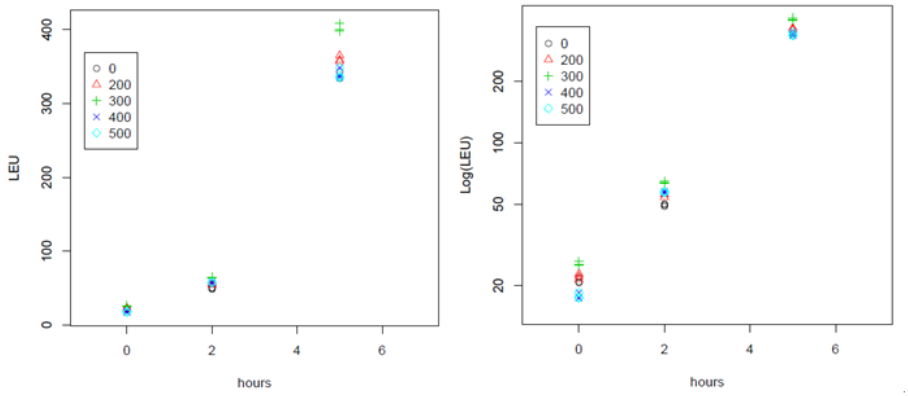
Cut	Flat	
Formula	log(LEU)=c+0.5749769Hour	Log(PHE)=c+0.5777366Hour
P=0 MPa	c=2.2919292	c=2.734632
P=200 MPa	c=2.989910	c=2.790078
P=300 MPa	c=3.106189	c=2.997752
P=400 MPa	c=2.914492	c=2.806899
P=500 MPa	c=2.260079	c=2.712227

The slope values of log(total amino acids) at 300 MPa in flat and inside were 0.3966563 and 0.4307988, respectively. The value of the slope indicates the rate of FAAs being released through digestion. For total FAAs, the inside's slope was higher than the flat's, which means the inside can liberate (or digest) FAAs faster than that of the flat, meaning higher digestibility. From the point view of individual amino acid in certain cuts, the slopes of TRP showed the highest values among all of amino acids in its corresponding cut. It suggested that TRP was liberated the fastest in flat and inside during digestion although its c values were relatively lower than most of the amino acids throughout all of pressure treatments (means it was released less from HPP). PHE and LEU were also shown to be liberated in faster rates in both cuts followed with TRP, TYR and LYS. On the other end of the order of rate, ALA was shown to be liberated the slowest in both cuts which the slopes were only 0.1285383 in the flat and 0.1250829 in the inside. Other amino acids such as THR, SER and PRO in the flat and THR and ASN in the inside were presented in slower rates compared to the others.

It was the first time that the mixed-linear model was applied to analyze the relationships among the digestibility of protein measured in the form of FAAs, digestion time and the pressures. The results from Table 14 have shown a high degree of consistency to the results in Table 12 which have proved to further support the

result that 300 MPa pressure was the most effective parameter of high pressure processing to improve the digestibility and nutritional value of lamb muscles. For the future research in this area, this statistical analysis method would be a useful tool to have a deeper understanding of the data which cannot always be picked up from ANOVA.

(a)



(b)

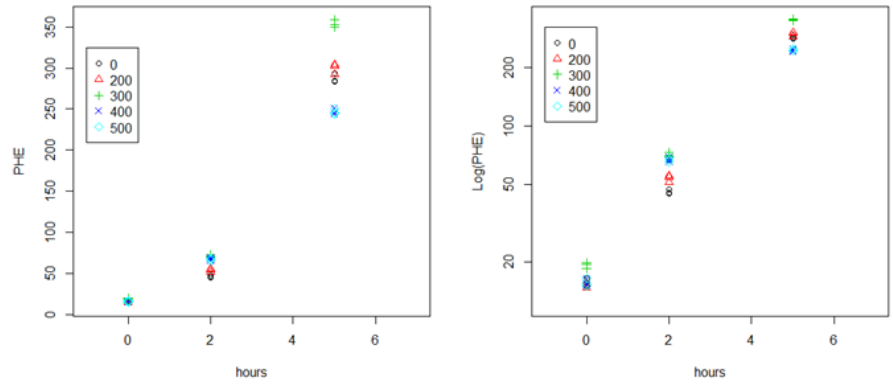


Figure 8 Two typical examples of the plots distribution of LEU (a) and PHE (b) in HPP flat cut. Figures on the left were plotted with the actual amino acid results, while the right ones were plotted with log(amino acid) results.

The existences of amino acids are of vital importance to regulate our body function. They can either act as neurotransmitters and facilitate the communication of brain with the nerve cells elsewhere in the body or enable vitamins and minerals to perform all their functions (Conley, 2012; Wu, 2009). Every amino acid has one's individual

duties in the human body, especially for the essential amino acids. Essential amino acids cannot be synthesized from our body and must come from protein foods, thus they are becoming more important than non-essential amino acids. These four essential amino acids, LEU, PHE, LYS and TYR, are not only the amino acids in the highest amount at the end of the intestinal phase, but also take the same level of responsibilities to human health. LEU, as well as ILE and VAL, help muscle recover after exercise, increase muscle mass and regulate blood sugar. It is invaluable when the body is stressed because it supplies the body with energy (Conley, 2012). In general, LEU could be understood as the main power source for our human activities. From this aspects, it looks reasonable that its concentration was the highest in the lamb samples and HPP released more LEU from meat proteins. In addition, PHE is a precursor to catecholamines that regulate peripheral and central nervous system. LYS is one of the important sources to synthesize carnitine to convert fatty acids into energy and produce hormones, enzymes and antibodies. Lacking of LYS in the body can lead to niacin deficiency and cause pellagra disease (Conley, 2012). Another amino acid in high concentration, TRP, influences our life in every second that it is the precursor of serotonin (a neurotransmitter) and melatonin (an antioxidant) which inhibit the production of inflammatory cytokines and superoxide to regulate mood and sleep (Conley, 2012; Wu, 2009). Other amino acids like MET and HIS, are necessary for the synthesis of haemoglobin structure and function, and glutathione to fight against free radicals. THR is responsible for creating other amino acids, assisting the production of collagen, which is vital for antibody production (Conley, 2012; Wu, 2009). These important and irreplaceable roles of essential amino acids in human health urge us to pay more attention on the consumption of protein foods. Apart from these, although non-essential amino acids can be synthesized from our body, it does not mean that we do not need any intakes from foods. For example, during exercise, muscle tissue breaks down and toxins are released. ALA is released from liver to metabolize them and remove them from the body (Fitday, 2016). Thus, the consumptions of non-essential amino acids from protein foods could be believed to

reduce the burden of our organs like liver and kidneys on releasing amino acids to maintain the body function. From this point of view, the increasing amounts of non-essential amino acids in lamb samples after HPP is also a satisfied contribution. As discussed above, 300 MPa of pressure treatment on lamb highly enhanced the possibility for the humans to obtain more FAAs from the same amount of lamb intake.

4.1.1.2 Effect of different cuts

Oh et al. (2016) reported a research on FAA contents of Hanwoo (*Bos taurus coreanae*) beef by cut and showed that the group of low preference cuts including brisket, topside and shank had higher total FAA contents than the group of high preference cuts like loin, tenderloin and rib. The topside contained the most FAAs, while the tenderloin contained the least. Another similar research was published by Samicho, Mutalib and Abdullah (2013) who studied amino acid composition of droughtmaster beef at wider ranges of cuts. The flat cut was found to contain higher amount of amino acid contents followed by topside and short rib. These findings proved that there should be some differences among red meat from different cuts, so should the lamb. Not a lot of work has been done to distinguish the FAA contents in different lamb cuts to date. Madruga et al. (2010) investigated FAAs in rump of goat meat which the three most abundant FAA compositions in goat rump were glycine, alanine and glutamine, whether in raw or cooked form. It is not accordant to the results in the current study, which the amount of glutamine was in the highest level in most of samples from both cuts before digestion, followed by alanine and glycine. As shown from Table 12 and Figure 7, flat cuts were shown to contain larger amount of total FAAs than inside cuts, prior to digestion, either in high pressure treated or untreated samples. This pattern had not changed even after the samples have gone through the peptic hydrolysis in the gastric phase of digestion. Dramatic changes were

found at the end of intestinal digestion that the inside samples under 200 MPa, 300 MPa and 400 MPa were significantly higher in total FAAs content than their corresponding samples in flat cuts. However, the presence of higher amount of total FAAs in the inside may still be hard to change the consumers' preferences of the inside cuts. As shown on Table 13, the amount of total essential amino acids in the 300 MPa treated flat sample after digestion reached 1471.28±21.92 mg/100 g lamb, which was about 73.39 % of the total FAAs. This was much higher than that of the inside cuts (1367.92±6.84 mg/100 g lamb), which occupied only 67.69 % of its total amino acids content. Considering the more important roles of essential amino acids in human health than non-essential amino acids, it is worth noting that although 300 MPa treated inside released more FAAs after digestion. Taking the essential amino acid contents into account, the flat is more recommendable compared to the inside.

Table 12 Concentration of the FAAs (mg/100 g lamb) of HPP processed lamb cuts at different digestion stages: before digestion (initial stage), gastric phase and intestinal phase of the *in vitro* digestion.

Before digestion													
	Flat							Inside					
Amino acid	Control	200MPa	300 MPa	400 MPa	500 MPa	P		Control	200MPa	300 MPa	400 MPa	500 MPa	P
VAL	9.19±0.29^{a}	10.25±0.49^{b}	12.78±0.39^{c}	11.17±0.82^{b}	12.39±0.67^{c}	***		7.17±0.94^{a}	9.53±0.36^{b}	10.66±0.72^{c}	8.53±0.25^{b}	7.42±0.22^{a}	**
LEU	21.12±0.69^{b}	22.15±0.72^{b}	25.63±0.54^{c}	17.80±0.61^{a}	17.31±0.62^{a}	***		17.22±0.71^{b}	19.87±0.11^{d}	18.86±0.36^{c}	14.77±0.26^{a}	19.96±0.63^{d}	***
ILE	6.91±0.17^{a}	9.07±0.25^{b}	9.15±0.31^{b}	6.90±0.14^{a}	7.38±0.62^{a}	***		6.64±0.44^{b}	8.53±0.40^{c}	6.75±0.13^{b}	5.03±0.14^{a}	6.72±0.09^{b}	***
PHE	15.56±0.72^{a}	15.22±0.44^{a}	19.16±0.69^{b}	15.63±0.67^{a}	14.85±0.70^{a}	***		11.82±0.76^{a}	13.77±0.16^{c}	15.12±0.56^{d}	12.64±0.55^{ab}	13.01±0.50^{bc}	**
MET	2.95±0.21^{b}	2.28±0.17^{a}	5.27±0.26^{d}	4.60±0.48^{c}	2.80±0.11^{b}	***		3.88±0.22^{a}	4.42±0.09^{b}	5.34±0.26^{c}	4.29±0.22^{b}	4.46±0.16^{b}	***
LYS	10.87±0.36^{b}	15.39±0.98^{c}	19.37±0.30^{d}	14.55±0.52^{c}	9.11±0.41^{a}	***		13.46±0.47^{b}	20.08±0.21^{d}	15.30±0.20^{c}	12.98±0.29^{b}	11.57±0.35^{a}	***
HIS	10.06±0.15^{ab}	8.52±0.51^{a}	13.80±1.31^{d}	11.84±0.83^{c}	11.67±1.28^{bc}	**		7.86±0.29^{a}	8.38±0.36^{a}	12.08±0.44^{b}	11.69±0.43^{b}	8.00±0.25^{a}	***
THR	13.22±0.46^{c}	14.91±0.60^{d}	16.39±0.47^{e}	10.79±0.25^{a}	12.30±0.20^{b}	***		9.23±0.65^{b}	12.75±0.43^{c}	13.30±0.73^{c}	9.87±0.36^{b}	8.21±0.37^{a}	***
TRP	1.06±0.10^{a}	1.82±0.36^{b}	1.22±0.08^{a}	1.06±0.06^{a}	0.89±0.17^{a}	*		0.64±0.03^{b}	0.85±0.05^{c}	0.32±0.03^{a}	1.20±0.06^{e}	1.00±0.03^{d}	***
ALA	39.31±0.99^{c}	43.36±0.62^{b}	48.84±0.99^{e}	29.64±0.66^{a}	35.43±1.30^{b}	***		31.08±0.21^{c}	34.71±0.99^{d}	38.15±0.85^{e}	27.59±0.45^{a}	29.27±0.75^{b}	***
GLY	18.35±0.77^{b}	16.44±1.45^{a}	20.13±0.85^{bc}	21.71±1.20^{c}	21.69±0.55^{c}	**		14.73±0.70^{a}	16.09±1.01^{b}	26.37±0.78^{c}	26.38±0.62^{c}	15.45±0.40^{ab}	***
TYR	10.57±0.41^{bc}	8.64±0.52^{ab}	12.02±0.49^{c}	10.95±1.10^{bc}	6.26±3.26^{a}	*		7.52±0.49^{a}	7.76±0.38^{a}	10.40±0.37^{d}	9.44±0.19^{c}	8.66±0.33^{b}	***
SER	9.51±0.42^{b}	16.19±0.54^{d}	16.20±0.68^{d}	11.75±0.15^{c}	6.68±0.21^{a}	***		9.03±0.53^{c}	11.80±0.41^{e}	8.10±0.25^{b}	9.74±0.34^{d}	7.21±0.24^{a}	***
ASN	2.70±0.26^{a}	6.05±0.60^{c}	5.78±0.79^{c}	3.75±0.30^{b}	3.02±0.08^{ab}	***		2.80±0.44^{b}	7.11±0.61^{d}	3.73±0.24^{c}	2.15±0.27^{ab}	1.85±0.19^{a}	***
PRO	6.54±0.30^{b}	4.93±0.35^{a}	6.51±0.33^{b}	8.12±0.46^{d}	7.35±0.50^{c}	***		4.82±0.25^{a}	4.98±0.39^{ab}	5.55±0.34^{b}	6.49±0.28^{c}	5.44±0.37^{b}	**
GLU	3.22±0.20^{a}	4.47±0.14^{c}	3.44±0.26^{a}	3.30±0.14^{a}	3.92±0.32^{b}	**		2.72±0.15^{a}	3.30±0.14^{b}	3.07±0.14^{ab}	5.23±0.23^{d}	3.79±0.42^{c}	***
ASP	2.50±0.37^{b}	3.19±0.40^{c}	1.84±0.16^{a}	4.27±0.52^{d}	1.91±0.27^{ab}	***		1.74±0.13^{b}	2.26±0.39^{c}	4.13±0.25^{e}	3.45±0.16^{d}	1.09±0.05^{a}	***
GLN	44.09±1.35^{a}	44.50±2.14^{a}	45.12±3.55^{a}	45.65±1.42^{a}	44.70±0.49^{a}	ns		37.03±0.57^{b}	45.48±0.39^{c}	50.21±0.74^{d}	44.87±1.05^{c}	30.98±0.48^{a}	***
ORN	1.00±0.06^{ab}	1.13±0.07^{b}	0.92±0.12^{a}	2.57±0.08^{d}	2.06±0.08^{c}	***		0.57±0.02^{a}	0.62±0.02^{a}	1.39±0.06^{c}	1.47±0.05^{d}	0.91±0.02^{b}	***
Total	228.74±5.96^{ab}	248.51±6.44^{c}	283.57±1.26^{d}	236.05±3.77^{b}	221.71±4.80^{a}	***		189.94±7.08^{a}	232.25±3.53^{c}	248.85±4.15^{d}	217.8±1.51^{b}	185.01±2.99^{a}	***

Gastric Phase													
	Flat						Inside					
Amino acid	Control	200MPa	300 MPa	400 MPa	500 MPa	P	Control	200MPa	300 MPa	400 MPa	500 MPa	P
VAL	18.60±0.62^{b}	23.76±0.60^{c}	29.71±1.14^{d}	17.93±0.59^{b}	11.89±0.26^{a}	***	11.43±0.68^{b}	13.57±0.21^{c}	10.77±0.08^{ab}	12.9±0.60^{c}	10.07±0.25^{a}	***
LEU	49.55±0.74^{b}	56.02±1.86^{c}	64.07±0.90^{d}	57.30±0.52^{c}	40.68±1.17^{a}	***	36.72±0.54^{b}	42.51±0.80^{d}	39.63±1.24^{c}	43.51±1.00^{d}	33.15±1.12^{a}	***
ILE	8.41±0.42^{a}	11.41±0.34^{b}	22.44±2.30^{c}	10.80±0.69^{b}	7.59±0.41^{a}	***	8.91±0.21^{c}	8.19±0.34^{b}	10.18±0.05^{d}	7.92±0.34^{b}	4.26±0.15^{a}	***
PHE	45.84±1.38^{a}	53.78±2.21^{b}	71.10±1.83^{d}	67.19±1.88^{c}	46.23±2.19^{a}	***	59.01±0.59^{e}	42.77±0.23^{b}	49.65±0.73^{d}	45.26±1.08^{c}	39.41±0.80^{a}	***
MET	27.75±1.42^{a}	40.87±1.08^{c}	53.39±0.77^{e}	44.06±0.91^{d}	30.95±1.41^{b}	***	19.96±1.57^{ab}	35.06±1.88^{d}	30.51±1.03^{c}	21.37±0.66^{b}	18.39±2.03^{a}	***
LYS	29.68±2.27^{b}	36.40±3.46^{c}	42.65±2.28^{d}	34.76±0.18^{c}	21.67±0.92^{a}	***	18.11±0.43^{b}	25.89±0.36^{e}	23.88±0.11^{d}	19.32±0.44^{c}	14.62±0.38^{a}	***
HIS	17.79±2.60^{b}	25.11±1.57^{c}	34.12±1.82^{d}	16.41±0.49^{b}	12.30±1.16^{a}	***	14.51±0.28^{c}	16.45±0.45^{d}	19.83±0.14^{e}	11.16±0.23^{a}	11.74±0.34^{b}	***
THR	21.42±0.18^{d}	19.75±0.37^{c}	17.16±0.70^{b}	20.08±0.48^{c}	15.60±0.41^{a}	***	10.55±0.73^{a}	16.41±0.69^{c}	16.88±0.67^{c}	12.99±0.10^{b}	11.04±0.38^{a}	***
TRP	10.63±0.43^{b}	13.50±1.47^{c}	16.50±0.88^{d}	12.81±0.15^{c}	7.63±0.90^{a}	***	6.73±0.93^{a}	11.46±0.47^{b}	12.36±0.43^{b}	6.00±0.21^{a}	5.91±0.54^{a}	***
ALA	40.77±1.60^{bc}	47.27±0.51^{d}	42.73±1.89^{c}	39.39±1.35^{b}	31.74±0.93^{a}	***	49.77±0.66^{e}	39.95±0.55^{b}	41.76±0.69^{c}	45.88±0.79^{d}	35.84±0.90^{a}	***
GLY	31.57±0.50^{c}	31.49±0.46^{c}	37.76±0.77^{d}	29.82±0.28^{b}	23.32±0.45^{a}	***	23.82±0.55^{c}	24.38±0.45^{cd}	20.58±0.58^{a}	24.81±0.50^{d}	22.25±0.42^{b}	***
TYR	26.21±1.55^{b}	35.87±0.89^{d}	43.23±1.56^{e}	32.48±0.91^{c}	22.34±1.40^{a}	***	18.97±0.34^{ab}	23.14±1.64^{c}	27.02±1.32^{d}	17.45±0.46^{a}	19.71±0.34^{b}	***
SER	25.73±0.82^{c}	26.05±0.96^{c}	11.15±0.65^{a}	23.32±1.09^{b}	29.53±0.40^{d}	***	15.96±0.53^{c}	15.41±0.35^{bc}	14.73±0.35^{b}	18.48±0.68^{d}	11.55±0.34^{a}	***
ASN	13.22±1.20^{b}	14.74±0.46^{c}	14.98±0.33^{c}	12.93±0.20^{b}	10.05±0.50^{a}	***	9.14±0.13^{b}	10.42±0.21^{c}	13.56±0.49^{d}	7.63±0.83^{a}	8.68±0.34^{b}	***
PRO	16.96±0.61^{e}	15.15±0.47^{d}	8.69±0.25^{a}	10.64±0.11^{b}	12.47±0.23^{c}	***	9.75±0.22^{a}	9.33±0.21^{a}	9.80±0.14^{ab}	10.25±0.22^{bc}	10.35±0.46^{c}	*
GLU	14.07±1.22^{b}	14.58±0.94^{b}	20.83±0.64^{c}	21.75±1.23^{c}	10.71±0.62^{a}	***	6.66±0.20^{a}	11.19±0.50^{c}	18.49±0.54^{d}	9.80±0.34^{b}	10.79±0.35^{bc}	***
ASP	16.97±0.30^{cd}	17.06±0.32^{d}	10.77±0.42^{a}	13.36±0.46^{b}	16.26±0.48^{c}	*	7.39±0.36^{b}	10.38±0.40^{c}	10.88±0.53^{c}	10.91±0.33^{c}	6.58±0.45^{a}	***
GLN	66.01±0.21^{bc}	63.56±1.31^{bc}	67.81±3.07^{c}	61.36±5.00^{ab}	58.16±1.48^{a}	ns	59.83±0.69^{c}	55.31±0.83^{a}	56.07±0.82^{a}	58.08±0.42^{b}	56.44±1.15^{a}	**
ORN	4.18±0.35^{b}	4.82±0.28^{c}	3.09±0.43^{a}	4.81±0.16^{c}	4.12±0.32^{b}	**	3.22±0.12^{b}	4.16±0.19^{c}	4.05±0.22^{c}	4.04±0.10^{c}	1.98±0.21^{a}	***
Total	485.36±13.31^{b}	551.20±5.80^{d}	612.19±2.61^{e}	531.22±1.07^{c}	413.24±7.48^{a}	***	390.42±2.75^{b}	416.00±1.72^{d}	430.63±3.46^{d}	387.77±2.41^{c}	332.77±7.06^{a}	***

Intestinal Phase													
	Flat						Inside						
Amino acid	Control	200MPa	300 MPa	400 MPa	500 MPa	P	Control	200MPa	300 MPa	400 MPa	500 MPa	P
VAL	50.25±0.77^{b}	55.56±0.58^{c}	58.96±1.09^{d}	50.13±1.51^{b}	42.83±0.55^{a}	***	35.07±0.66^{b}	42.92±0.53^{c}	49.22±1.05^{d}	34.90±0.95^{b}	31.64±1.24^{a}	***
LEU	337.04±5.04^{a}	360.37±4.09^{b}	402.33±5.42^{c}	340.14±7.20^{a}	341.38±2.10^{a}	***	312.34±1.48^{b}	369.93±10.23^{d}	340.27±7.17^{c}	304.94±7.97^{b}	257.11±10.10^{a}	***
ILE	37.70±1.22^{b}	38.56±4.25^{b}	43.06±3.56^{b}	38.35±0.33^{b}	29.86±3.37^{a}	*	29.46±1.23^{b}	34.44±0.43^{c}	47.59±1.50^{d}	33.77±1.03^{c}	24.79±1.01^{a}	***
PHE	287.29±5.57^{c}	299.74±6.81^{d}	353.98±4.41^{e}	246.79±3.52^{a}	277.69±4.33^{b}	***	256.87±2.50^{b}	291.05±2.44^{c}	337.54±5.07^{e}	308.98±4.06^{d}	240.34±2.30^{a}	***
MET	90.67±1.87^{c}	97.75±2.48^{d}	88.80±1.19^{c}	75.17±1.50^{b}	61.69±3.35^{a}	***	79.36±1.47^{d}	74.24±0.89^{c}	91.10±0.47^{e}	70.44±0.52^{b}	55.12±0.54^{a}	***
LYS	286.82±11.78^{c}	271.00±5.52^{b}	322.92±4.66^{d}	326.26±6.23^{d}	242.91±6.51^{a}	***	248.10±6.84^{b}	325.83±5.08^{e}	295.97±3.27^{d}	235.26±4.40^{a}	265.98±3.76^{c}	***
HIS	64.21±3.75^{a}	82.98±2.13^{b}	95.05±4.15^{c}	64.78±3.74^{a}	62.33±2.86^{a}	***	73.66±0.62^{c}	64.27±0.40^{b}	91.03±0.18^{e}	76.03±0.49^{d}	58.28±0.89^{a}	***
THR	30.72±0.54^{c}	28.18±1.30^{b}	34.47±0.85^{d}	34.83±1.47^{d}	22.33±0.98^{a}	***	32.24±6.01^{c}	27.64±0.77^{bc}	28.06±0.88^{bc}	26.44±1.32^{ab}	22.40±1.17^{a}	ns
TRP	54.58±4.03^{b}	69.51±2.35^{c}	71.70±3.03^{c}	48.81±1.34^{a}	54.50±3.13^{b}	***	68.86±0.45^{c}	56.07±1.80^{b}	87.14±0.70^{e}	72.06±1.49^{d}	47.21±2.72^{a}	***
ALA	73.38±2.02^{b}	74.97±2.94^{b}	83.18±0.76^{c}	73.15±1.77^{b}	56.46±1.81^{a}	***	61.87±0.69^{c}	74.45±1.71^{e}	65.39±1.16^{d}	54.33±0.99^{b}	47.28±1.68^{a}	***
GLY	61.13±0.69^{b}	64.24±0.93^{c}	66.30±2.21^{c}	61.63±1.01^{b}	48.64±1.49^{a}	***	61.64±0.54^{c}	60.27±1.04^{c}	66.44±1.39^{d}	56.75±2.02^{b}	49.08±1.34^{a}	***
TYR	191.46±3.54^{b}	213.14±5.20^{c}	229.93±6.70^{d}	210.31±3.59^{c}	172.07±5.55^{a}	***	196.36±0.85^{a}	243.2±5.07^{b}	289.51±1.76^{d}	256.40±7.21^{c}	192.58±2.50^{a}	***
SER	32.79±0.86^{c}	30.57±0.43^{b}	17.97±0.38^{a}	34.88±0.61^{d}	29.64±0.54^{b}	***	24.98±1.01^{b}	22.08±0.17^{a}	28.31±063^{c}	26.06±1.19^{b}	24.83±1.19^{b}	**
ASN	20.06±0.62^{a}	20.59±2.01^{a}	19.45±1.28^{a}	20.32±1.70^{a}	21.41±0.43^{a}	ns	27.79±0.73^{d}	21.34±0.37^{c}	18.04±0.51^{b}	15.26±0.73^{a}	18.59±0.43^{b}	***
PRO	19.69±0.21^{d}	21.22±0.28^{e}	14.33±0.38^{a}	16.92±0.41^{c}	15.28±0.40^{b}	***	15.38±0.44^{c}	14.85±0.27^{bc}	19.91±0.30^{d}	14.58±0.49^{ab}	13.95±0.51^{a}	***
GLU	28.64±1.63^{b}	36.65±1.58^{c}	36.32±1.53^{c}	27.38±0.85^{b}	17.78±1.27^{a}	***	29.59±0.85^{b}	36.56±1.04^{d}	37.23±0.83^{d}	31.65±0.81^{c}	25.42±1.23^{a}	***
ASP	19.92±0.84^{c}	19.10±0.91^{bc}	24.86±0.35^{d}	18.47±0.48^{b}	16.24±0.24^{a}	***	12.84±0.70^{b}	16.12±0.40^{c}	20.39±0.95^{d}	11.01±0.62^{a}	13.24±0.42^{b}	***
GLN	121.67±10.97^{ab}	118.72±10.20^{ab}	119.20±2.62^{ab}	126.54±4.80^{b}	110.55±1.97^{a}	ns	132.59±3.51^{c}	158.41±1.65^{e}	100.31±0.64^{a}	137.87±0.41^{d}	115.78±1.51^{b}	***
ORN	7.48±0.43^{c}	5.09±0.23^{a}	5.40±0.27^{a}	6.88±0.41^{bc}	6.59±0.27^{b}	***	5.46±0.31^{a}	6.20±0.41^{b}	7.47±0.43^{c}	7.03±0.29^{c}	6.04±0.24^{ab}	**
Total	1722.36±27.09^{b}	1819.33±23.90^{c}	2004.65±13.06^{d}	1717.73±15.71^{b}	1541.37±9.11^{a}	***	1704.45±9.85^{b}	1939.22±11.24^{d}	2020±7.73^{e}	1773.76±11.85^{c}	1509.67±16.93^{a}	***

Results are given as Mean ± S.D.

Values with different superscripts (^{a}, ^{b}, ^{c}, ^{d}, and ^{e}) in the same row of the same cut differ significantly within the same cut in five different pressure treatments.
Amino acids in bolded fonts represent essential amino acids, and those in normal fonts stand for non-essential amino acids.
$P < 0.0001$ presented as $***$ for level of significance; $P < 0.001$ presented as $**$ for level of significance; $P < 0.01$ presented as $*$ level of significance and ns meaning not statistically significant. Measurements were made in triplicate. GC analysis was made in triplicate for each of the sample tested.

Table 13 The total essential FAAs (mg/100 g lamb) and its percentage (%) in total FAAs of two HPP processed lamb cuts at different digestion stages: before digestion (initial stage), gastric phase and intestinal phase of the *in vitro* digestion

Flat								Inside						
Before digestion								Before digestion						
	Control	200MPa	300 MPa	400 MPa	500 MPa	P			Control	200MPa	300 MPa	400 MPa	500 MPa	P
EAA	90.94±2.23^{ab}	99.61±3.13^{c}	122.77±2.43^{d}	94.35±2.70^{b}	88.69±2.68^{a}	***		EAA	77.9±4.32^{a}	98.16±0.73^{b}	97.75±1.97^{b}	80.98±1.45^{a}	80.35±1.2^{a}	***
%	39.76^{a}	40.08^{a}	43.30^{b}	39.97^{a}	40.01^{a}	**		%	41.00^{c}	42.27^{d}	39.28^{b}	37.18^{a}	43.43^{e}	***
Gastric Phase								Gastric Phase						
	Control	200MPa	300 MPa	400 MPa	500 MPa	p			Control	200MPa	300 MPa	400 MPa	500 MPa	P
EAA	229.68±8.14^{b}	280.6±3.86^{c}	351.16±4.48^{d}	281.35±0.90^{c}	194.53±5.87^{a}	***		EAA	185.92±3.03^{b}	212.32±3.12^{c}	213.68±0.97^{c}	180.43±3.05^{b}	148.58±5.35^{a}	***
%	47.32^{a}	50.91^{b}	57.36^{d}	52.96^{c}	47.07^{a}	***		%	47.62^{b}	51.04^{c}	49.62^{c}	46.53^{b}	44.65^{a}	***
Intestinal Phase								Intestinal Phase						
	Control	200MPa	300 MPa	400 MPa	500 MPa	p			Control	200MPa	300 MPa	400 MPa	500 MPa	P
EAA	1239.27±21.25^{b}	1303.65±17.58^{c}	1471.28±21.92^{d}	1225.27±18.27^{b}	1135.51±14.02^{a}	***		EAA	1135.96±8.00^{b}	1286.39±12.97^{c}	1367.92±6.84^{d}	1162.81±8.04^{b}	1002.88±13.50^{a}	***
%	71.95^{a}	71.66^{a}	73.39^{b}	71.33^{a}	73.67^{b}	***		%	66.65^{b}	66.31^{b}	67.69^{c}	65.56^{a}	66.43^{b}	***

The total essential FAA results are given as Mean ± S.D.

EAA represents for essential amino acids.

Values with different superscripts (^{a}, ^{b}, ^{c}, and ^{d}) in the same row of the same cut differ significantly within the same cut in five different treatments.

$P < 0.0001$ presented as $***$ for level of significance; $P < 0.001$ presented as $**$ for level of significance; $P < 0.01$ presented as $*$ level of significance.

Table 14 Mixed-linear model of HPP flat and inside cuts

Amino acid	Flat				Inside			
	REML Criterion Value (FM1)	REML Criterion Value (FM2)	Closer model	Equation of the closer model	REML Criterion Value (FM1)	REML Criterion Value (FM2)	Closer model	Equation of the closer model
ALA	-31.1389	-32.9721	FM1	Log(ALA)=c+0.1285383Hour	-60.0438	-64.1248	FM1	Log(ALA)=c+0.1250829Hour
				P=0 MPa, c=3.589428				P=0 MPa, c=3.526138
				P=200 MPa, c=3.668992				P=200 MPa, c=3.548964
				P=300 MPa, c=3.705127				P=300 MPa, c=3.551799
				P=400 MPa, c=3.494330				P=400 MPa, c=3.425840
				P=500 MPa, c=3.406287				P=500 MPa, c=3.326508
GLY	-51.4423	-64.3174	FM1	Log(GLY)=c+0.2246724Hour	-4.7158	-10.6349	FM1	Log(GLY)=c+0.2307846Hour
				P=0 MPa, c=2.967685				P=0 MPa, c=2.808013
				P=200 MPa, c=2.949913				P=200 MPa, c=2.827815
				P=300 MPa, c=3.064147				P=300 MPa, c=2.922214
				P=400 MPa, c=3.000290				P=400 MPa, c=2.928848
				P=500 MPa, c=2.867946				P=500 MPa, c=2.752677
VAL	-19.8319	-20.6177	FM1	Log(VAL)=c+0.3142520Hour	-12.399	-12.4608	FM1	Log(VAL)=c+0.307169Hour
				P=0 MPa, c=2.299948				P=0 MPa, c=1.954600
				P=200 MPa, c=2.424275				P=200 MPa, c=2.131762
				P=300 MPa, c=2.577825				P=300 MPa, c=2.136398
				P=400 MPa, c=2.360824				P=400 MPa, c=2.033561
				P=500 MPa, c=2.260079				P=500 MPa, c=1.904267
LEU	-40.94	-44.2151	FM1	Log(LEU)=c+0.5749769Hour	-1.7493	-2.3475	FM1	Log(LEU)=c+0.5817801Hour
				P=0 MPa, c=2.2919292				P=0 MPa, c=2.734609
				P=200 MPa, c=2.989910				P=200 MPa, c=2.772393

				P=300 MPa, c=3.106189				P=300 MPa, c=2.755443
				P=400 MPa, c=2.914492				P=400 MPa, c=2.733985
				P=500 MPa, c=2.260079				P=500 MPa, c=2.722331
ILE	6.9852	6.78	FM1	Log(ILE)=c+0.3189007Hour	35.187	35.5215	FM1	Log(ILE)=c+0.335437Hour
				P=0 MPa, c=1.833998				P=0 MPa, c=1.700484
				P=200 MPa, c=2.010756				P=200 MPa, c=1.778370
				P=300 MPa, c=2.246487				P=300 MPa, c=1.851960
				P=400 MPa, c=1.912803				P=400 MPa, c=1.638347
				P=500 MPa, c=1.751856				P=500 MPa, c=1.485509
THR	-45.0354	-62.8361	FM1	Log(THR)=c+0.1592228Hour	-39.78	-65.4332	FM1	Log(THR)=c+0.2020203Hour
				P=0 MPa, c=2.644853				P=0 MPa, c=2.254660
				P=200 MPa, c=2.631079				P=200 MPa, c=2.285416
				P=300 MPa, c=2.676175				P=300 MPa, c=2.237638
				P=400 MPa, c=2.603836				P=400 MPa, c=2.328480
				P=500 MPa, c=2.440521				P=500 MPa, c=2.101199
SER	38.1071	30.9379	FM1	Log(SER)=c+0.1746495Hour	39.295	28.5208	FM1	Log(SER)=c+0.3598799Hour
				P=0 MPa, c=2.574326				P=0 MPa, c=1.337577
				P=200 MPa, c=2.672534				P=200 MPa, c=1.560566
				P=300 MPa, c=2.386958				P=300 MPa, c=1.407774
				P=400 MPa, c=2.610592				P=400 MPa, c=1.046480
				P=500 MPa, c=2.509047				P=500 MPa, c=1.096747
PRO	1.9193	-2.7406	FM1	Log(PRO)=c+0.1863741Hour	-42.4967	-48.3646	FM1	Log(PRO)=c+0.2067505Hour
				P=0 MPa, c=2.076953				P=0 MPa, c=1.732546
				P=200 MPa, c=2.007701				P=200 MPa, c=1.723169
				P=300 MPa, c=1.862937				P=300 MPa, c=1.809396

				P=400 MPa, c=1.990305				P=400 MPa, c=1.788557
				P=500 MPa, c=1.981061				P=500 MPa, c=1.747931
ASN	35.8782	29.2457	FM1	Log(ASN)=c+0.3100384Hour	-30.0418	-40.8932	FM1	Log(ASN)=c+0.1937317Hour
				P=0 MPa, c=1.526362				P=0 MPa, c=2.232609
				P=200 MPa, c=1.699291				P=200 MPa, c=2.422923
				P=300 MPa, c=1.683199				P=300 MPa, c=2.448972
				P=400 MPa, c=1.585299				P=400 MPa, c=2.260168
				P=500 MPa, c=1.509513				P=500 MPa, c=2.103674
ASP*	72.1734	71.7678	FM1	Log(ASP)=1.316235+0.379745Hour	56.4597	50.8976	FM1	Log(ASP)=c+0.3522754Hour
				P=0 MPa, c N/A				P=0 MPa, c=0.9170479
				P=200 MPa, c N/A				P=200 MPa, c=1.1365794
				P=300 MPa, c N/A				P=300 MPa, c=1.3757135
				P=400 MPa, c N/A				P=400 MPa, c=1.1638848
				P=500 MPa, c N/A				P=500 MPa, c=0.7700243
MET	88.2921	88.0718	FM1	Log(MET)=c+0.6058501Hour	35.5861	35.4795	FM1	Log(MET)=c+0.5445828Hour
				P=0 MPa, c=1.676076				P=0 MPa, c=1.671111
				P=200 MPa, c=1.676480				P=200 MPa, c=1.792974
				P=300 MPa, c=1.678481				P=300 MPa, c=1.842457
				P=400 MPa, c=1.677495				P=400 MPa, c=1.681080
				P=500 MPa, c=1.675426				P=500 MPa, c=1.611316
GLU	49.361	47.4476	FM1	Log(GLU)=c+0.3941001Hour	13.4789	106988	FM1	Log(GLU)=c+0.4335664Hour
				P=0 MPa, c=1.509655				P=0 MPa, c=1.137985
				P=200 MPa, c=1.605856				P=200 MPa, c=1.382844
				P=300 MPa, c=1.619402				P=300 MPa, c=1.502114
				P=400 MPa, c=1.575100				P=400 MPa, c=1.431622

				P=500 MPa, c=1.422347				P=500 MPa, c=1.312553
PHE	-54.4894	-54.9979	FM1	Log(PHE)=c+0.5777366Hour	-59.2478	-61.953	FM1	Log(PHE)=c+0.6131089Hour
				P=0 MPa, c=2.734632				P=0 MPa, c=2.600112
				P=200 MPa, c=2.790078				P=200 MPa, c=2.587970
				P=300 MPa, c=2.997752				P=300 MPa, c=2.698056
				P=400 MPa, c=2.806899				P=400 MPa, c=2.596472
				P=500 MPa, c=2.712227				P=500 MPa, c=2.494903
GLN	-108.3455	-109.9447	FM1	Log(GLN)=c+0.196826Hour	-35.9699	-52.6017	FM1	Log(GLN)=c+0.229512Hour
				P=0 MPa, c=3.792848				P=0 MPa, c=3.662834
				P=200 MPa, c=3.782010				P=200 MPa, c=3.737451
				P=300 MPa, c=3.798861				P=300 MPa, c=3.653305
				P=400 MPa, c=3.793075				P=400 MPa, c=3.712091
				P=500 MPa, c=3.751127				P=500 MPa, c=3.571999
ORN	39.0244	32.7349	FM1	Log(ORN)=c+0.2846892Hour	43.84	40.7789	FM1	Log(ORN)=c+0.3741441Hour
				P=0 MPa, c=0.4942206				P=0 MPa, c=-0.05379504
				P=200 MPa, c=0.4636529				P=200 MPa, c=0.06772683
				P=300 MPa, c=0.3091197				P=300 MPa, c=0.31518858
				P=400 MPa, c=0.7500182				P=400 MPa, c=0.31430473
				P=500 MPa, c=0.6423103				P=500 MPa, c=-0.03300941
LYS	3.4213	-2.1657	FM1	Log(LYS)=c+0.6232369Hour	61.0489	60.9254	FM1	Log(LYS)=c+0.6102296Hour
				P=0 MPa, c=2.366255				P=0 MPa, c=2.302041
				P=200 MPa, c=2.514607				P=200 MPa, c=2.421300
				P=300 MPa, c=2.685227				P=300 MPa, c=2.369401
				P=400 MPa, c=2.540920				P=400 MPa, c=2.299238
				P=500 MPa, c=2.168786				P=500 MPa, c=2.267793

HIS	2.1816	1.0751	FM1	Log(HIS)=c+0.3820457Hour	10.9986	10.9877	FM1	Log(HIS)=c+0.416454Hour
				P=0 MPa, c=2.233555				P=0 MPa, c=2.050356
				P=200 MPa, c=2.365348				P=200 MPa, c=2.064364
				P=300 MPa, c=2.641530				P=300 MPa, c=2.302165
				P=400 MPa, c=2.262364				P=400 MPa, c=2.094199
				P=500 MPa, c=2.158616				P=500 MPa, c=1.937286
TYR	-14.0658	-20.9552	FM1	Log(TYR)=c+0.6171744Hour	11.4994	11.2786	FM1	Log(TYR)=c+0.6690123Hour
				P=0 MPa, c=2.187974				P=0 MPa, c=1.898699
				P=200 MPa, c=2.256028				P=200 MPa, c=1.996511
				P=300 MPa, c=2.440053				P=300 MPa, c=2.134050
				P=400 MPa, c=2.2693879				P=400 MPa, c=1.989701
				P=500 MPa, c=1.915075				P=500 MPa, c=1.934402
TRP*	56.7459	56.4587	FM1	Log(TRP)=c+0.7654404Hour	64.4787	60.2244	FM1	Log(TRP)=-0.0762343+0.8813066Hour
				P=0 MPa, c=0.3904943				P=0 MPa, c N/A
				P=200 MPa, c=0.5915513				P=200 MPa, c N/A
				P=300 MPa, c=0.5611302				P=300 MPa, c N/A
				P=400 MPa, c=0.4076192				P=400 MPa, c N/A
				P=500 MPa, c=0.2881388				P=500 MPa, c N/A
Total AA	-126.77	-126.8511	FM1	Log(Total)=c+0.3966563Hour	-38.6594	-38.6692	FM1	Log(Total)=c+0.4307988Hour
				P=0 MPa, c=5.431279				P=0 MPa, c=5.220691
				P=200 MPa, c=5.518302				P=200 MPa, c=5.332974
				P=300 MPa, c=5.628015				P=300 MPa, c=5.374103
				P=400 MPa, c=5.470504				P=400 MPa, c=5.269195
				P=500 MPa, c=5.331885				P=500 MPa, c=5.133198

FM1 represents for the formula of (1|g). Its alternative formula is 1+(1|g). It means the random intercept with fixed mean.

FM2 represents for the formula of x+(x|g). Its alternative formula is 1+x+(1+x|g). It means correlated random intercept and slope.

The letter "g" represents for the grouping factors and "x" represents for a priori known offsets (Bates et al., 2015).

*: The c values of ASP of the flat and TRP of the inside were N/A (not available) in difference among all of pressure treatment after run by R.

4.1.2 Effect of PEF on digestibility of lamb

4.1.2.1 Effect of PEF and storage period on digestibility of lamb cuts

The FAA contents of digested PEF processed lamb cuts are shown in Table 15. Seven cuts, including bolar, cube roll, flat, full back, knuckle, rump and shank, were analyzed in triplicate with two types of control (non-PEF) samples (0 day and 7 days of storage at 4 °C) (C&0 and C&7) and two types of PEF samples (0 day and 7days of storage at 4 °C) (P&0 and P&7). The results are presented as Mean ± SD (mg/100 g lamb) of the amount of FAAs.

Significant differences ($P<0.001$) among the four samples (C&0, C&7, P&0 and P&7) were detected in all of the cuts at each stage of digestion. As shown in Table 15 and Figure 9, PEF treatment increases the total FAAs content during all of three stages of digestion in seven cuts compared to the corresponding control samples. The total FAA contents of P&0 and C&7 samples were higher than their corresponding C&0 samples. In general, prior to digestion, independent PEF treatment on 0 day of storage sample had no significant effect on the FAA content compared to the control one under 7 days of storage in most of the cuts except flat and knuckle. In the gastric phase, the FAA contents after peptic digestion in C&7 and P&0 samples were close, especially in bolar, cube roll and flat cuts, and no significant difference was found between them. Significant differences between C&7 and P&0 groups of samples appeared obviously in the end of the intestinal digestion stage, in all of seven cuts. Although PEF, regardless of storage days, plays positive roles in increasing the FAA compositions in lamb cuts overall, the FAA amounts of the samples under PEF processing after 0 days were lower than the C&7 samples in some cuts throughout three stages of digestion. This phenomenon was the most evident in the intestinal phase. For example, in flat cut, P&0 was 1646.93±38.54 mg/100 g lamb, while the concentration of C&7 was

much higher for 1804.74 ±72.85 mg /100 g lamb. In knuckle cut, C&7 contained 2154.07±57.29 mg of FAAs in 100 g lamb, but only 1864.02±61.27 mg of FAAs in 100 g lamb were quantified in P&0. In addition, P&7 resulted in the highest total FAAs amount for all of the treatments in bolar, cube roll, flat, full back, knuckle, rump and shank cuts which their concentrations were 1704.21±79.77, 1958.88±7.22, 2036.9±58.52, 2909.68±68.39, 2479.14±51.62, 2207.4±69.72, and 2174.88±48.69 mg/ 100 g lamb in the intestinal phase.

All of lamb cuts under PEF processing and frozen storage for 7 days have shown the highest level of total FAA contents after *in vitro* digestion processing. Most of the values of the FAAs throughout three sampling time point were higher than the other three samples (C&0, C&7 and P&0), indicating that combining PEF processing to a frozen storage for 7 days had better effects on improving the breaking down of lamb proteins to FAAs either prior to or post to digestion compared to the independent storage or PEF processing samples and non-treated control.

Among these individual amino acids in P&7 samples, the amount of LEU, ALA, and GLN were the highest in these cuts prior to digestion. During the *in vitro* digestion, only a slightly growth of ALA was found in the gastric and intestinal phase, while rapid increases of LEU and GLN were shown, particularly in the intestinal phase. Especially, the final concentration of LEU released from lamb protein was the highest one among all of the amino acids; even occupied around 20 % of the total FAA contents. Apart from LEU, the amount of PHE, LYS, TYR were another three amino acids which were released most at the end of the digestion. PHE and LYS were in the third and fourth place, preceded only by LEU and TYR. As what mentioned in HPP section, amino acids play important roles in maintaining humans' body functions by providing energy and also form the structural basis of chromosomes (Conley, 2012). Thus, the increases of FAA contents in P&7 samples after digestion, especially these essential amino acids in high concentrations, gave a positive signal that PEF and 7

days of storage increased nutritional value of lamb meat. Nevertheless, on the other side of the sort by amount, the lamb proteins released the least amount of ORN, ASP and ASN. The concentration of ORN did not even exceed 10 mg/100 g lamb at the end of the digestion among all of seven cuts. However, the results for non-treated control (C&0) was the opposite to P&7. Whether from the angle of total or individual amino acid content, the concentration of FAAs in C&0 was the lowest among these seven cuts during the whole digestion process. This suggests that the processing of frozen storage or PEF do have positive effects to improve the nutritional value of lamb muscles.

However, it is worth noting that different amino acids have their own specific tastes. For example, LEU has a bitter taste. ALA is the only amino acid tested with a truly sweet taste. The tastes of PHE and TRP are interesting to be mentioned that their tastes are quite different with different isomers. D-TRP and D-PHE were reported to have sweetness and D-TRP was 35 times sweeter than sucrose. L-TRP was the most bitter amino acid which is approximately half as bitter as caffeine and L-PHE was less bitter. In addition, the taste of LYS was described as no taste or barely perceptible taste (Solms, 1969). Based on these information, considering LEU, PHE and LYS being three amino acids in the highest concentration in P&7 samples, which contribute to significant changes on meat flavors, followed with the sensory attributes from TRP, a hypothesis could be raised that the flavour of P&7 lamb meat may have a higher bitterness followed with increasing in sweetness. A sensory evaluation is worth investigating in the future because only enhanced nutritional value would not be accepted by consumers. A further research on the development of cooking method as well as various ingredients used to improve the taste of P&7 meat is necessary for those meat manufacturers who want to utilize PEF and frozen stored meat.

Much work have been conducted to date, looking at the effect of PEF on the microstructure of food proteins. The most common explanation to support how PEF

works on proteins is the unfolding of proteins. Fernandez-Diaz et al. (2000) reported that a partial unfolding of ovalbumin (2% w/v) was induced by PEF treatments (electric field intensity of 27- 33 kV/cm, 50-400 pulses and pulse width of 0.3 μs), exposing all four S-H groups to the surface. A similar result was reported by Li et al. (2007) that PEF treatment (electric field intensity of 30 kV/cm, pulsed width 2μs, pulse frequency 600 pulse/s) caused partial unfolding of soybean protein isolate molecules and exposed internal S-H to form disulfide bridges. Pezez and Pilosof (2004) showed that the PEF processing (electric field intensity of 12.5 kV/cm with 40 μF of capacitance, pulse width of 2 ms, 1-10 pulses) denatured protein partially which both β-lactoglobulin and egg white were increasingly denatured by the increasing number of pulses.

Recently, a more detailed result was reported by Liu et al. (2014) on amino acid groups that a threshold of 30 kV/cm electric field intensity was found for the unfolding and reassembling of soy protein isolate's. The electric field intensity of 30 kV/cm led to a series of increases on amino acid groups, which includes the exposure of TYR and TRP, the vibration of C-H bending and C-N stretching inside a charged ARG (arginine), the vibration of CH_2 wagging in PRO and CH_2 in the midazole ring of HIS, and asymmetric H-C-H bending deformation vibration in CH2 and CH3 groups of aromatic and aliphatic amino acids. These results suggested that PEF processing induced an unfolding of soy protein molecules. When the electric field intensity of PEF treatment was increased from 30 to 50 kV/cm, the decreasing phenomenon were observed on all above amino acids because of the reassembly of unfolding protein (Liu et al., 2014). Although no work has been conducted to study the relationship between PEF and digestibility of meat protein, similar to the effect of HPP on protein, it can be speculated that the unfolded protein caused by PEF processing decreases the protein aggregation, thus increasing the protein digestibility. However, considering the electric field intensity of this study was 1-1.4 kV/cm, much lower than the threshold of 30 kV/cm reported by Liu et al. (2014), whether the

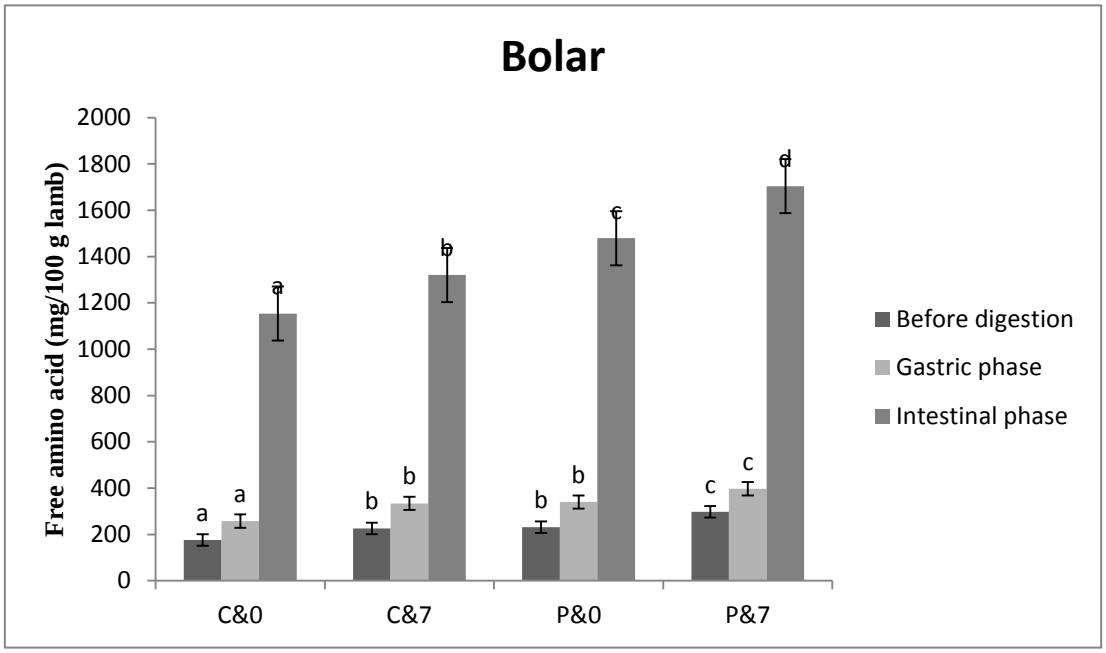
electric field intensity of PEF, as well as other parameters including pulse width, frequency and processing period used in this study was the most ideal one to increase the digestibility of lamb or not is still unknown. In addition, the degree of protein unfolding after PEF treatment under current processing parameters and the best PEF parameter to completely unfold protein are worth for further investigation. The best combination of PEF processing parameter for lamb digestibility requires further investigation.

In all of the cuts, the total FAA amounts of both C&7 and P&7 samples throughout the digestion were higher than their corresponding 0 day storage samples. It is obvious to find that freezing storage of 7 days had positive effects on improving the digestibility of lamb. This phenomenon can be explained from both physical and chemical angles. Leygonie, Britz and Hoffman (2012b) reported that ice crystals are formed between and within the fibres of lamb during freezing storage. The crystals physically damage the micro-structure of lamb. As water freezes out, the concentration of the remaining solutes such as protein, lipids, vitamins and minerals increase, destroying the balance of complex meat system. It indicates that the concentration of proteins available in frozen lamb is increased compared to the non-frozen lamb. In addition, the increase of protein concentration increases the susceptibility for protein denaturation. On the other hand, from the angles of chemistry, Wagner and Anon (1985) reported that the myofibrillar proteins of *Bovine semitendinosus* muscle were denatured after freezing regardless of the freezing rate, causing partial unfolding of the proteins. However, the effects of unfolded proteins on the digestibility of lamb appeared only in the intestinal phase. Sante′-Lhoutellier et al. (2008) reported that storage time had no significant effect on myofibrillar protein susceptibility of lamb *longissimus dorsi* to pepsin (in the gastric phase) while a significant increase in digestibility by trypsin and α-chymotrypsin (equivalent to pancreatin used in this study) was detected during the 7 days of storage. This fact is partly consistent to the results of this study which in the intestinal phase, the total

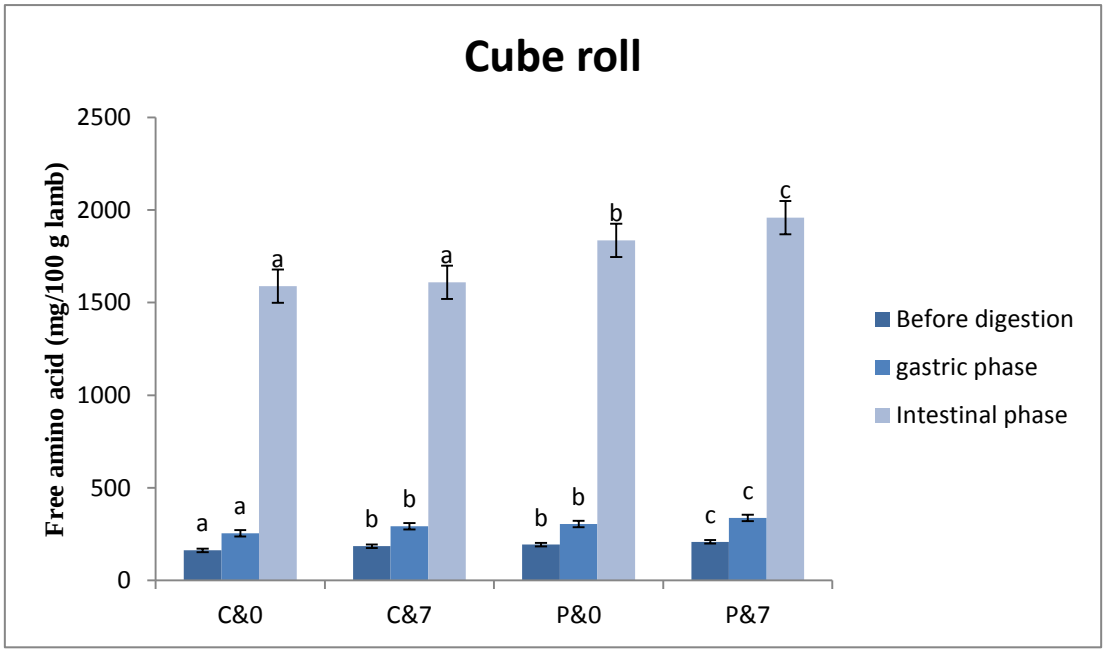
amino acids in the C&0 and C&7 were significantly different and the increases were more than 100 mg/ 100 g lamb in all of the cuts except in shank. Prior to digestion and in the gastric phase, significant differences of total amino acid contents between C&0 and C&7 were still detected. However, even though no significant effect was found in the gastric phase according to Sante′-Lhoutellier et al. (2008), slight increase after 7 days of storage was found, which is valuable to support current study to some extend that the increases seen in C&7 prior to digestion and in the gastric phase among all of the cuts were not as sharply as in the intestinal phase. Another research reported by Śmiecińska et al. (2015) showed a significant increase in the content of total protein of turkey meat after six weeks of storage, from 24.50% to 24.81%, while a decrease was found earlier from 24.50% 24.12% when it was stored for only two weeks long. It suggested that there might be some unstable or complex reaction taking place during a period of freezing storage. A more detailed research is required to fully explain the effect and mechanism of storage time on the nutritional value of protein in lamb. Apart from these, due to the total FAA contents of P&0 samples being larger than the C&7, it could be summarized that although both PEF and 7 days of frozen storage have enhancement to improve the digestibility of lamb proteins, PEF may play the dominant roles. Whatever, it is obvious that a combination of PEF with 7 days of storage is believed to have the best effects on improving the protein digestibility of frozen lamb.

Moreover, as what discussed in colour part (section 4.3), P&0 increased redness significantly compared to its corresponding C&0 sample in most of cuts, while P&7 sharply decreased the redness to a paler appearance compared to the C&7, which indicated an adverse effect of 7 days of frozen storage on the appearance of lamb cuts. It may highly influence consumers' acceptance to regard the meat in low quality although its nutritional value was increased (digestibility of FAAs increased).

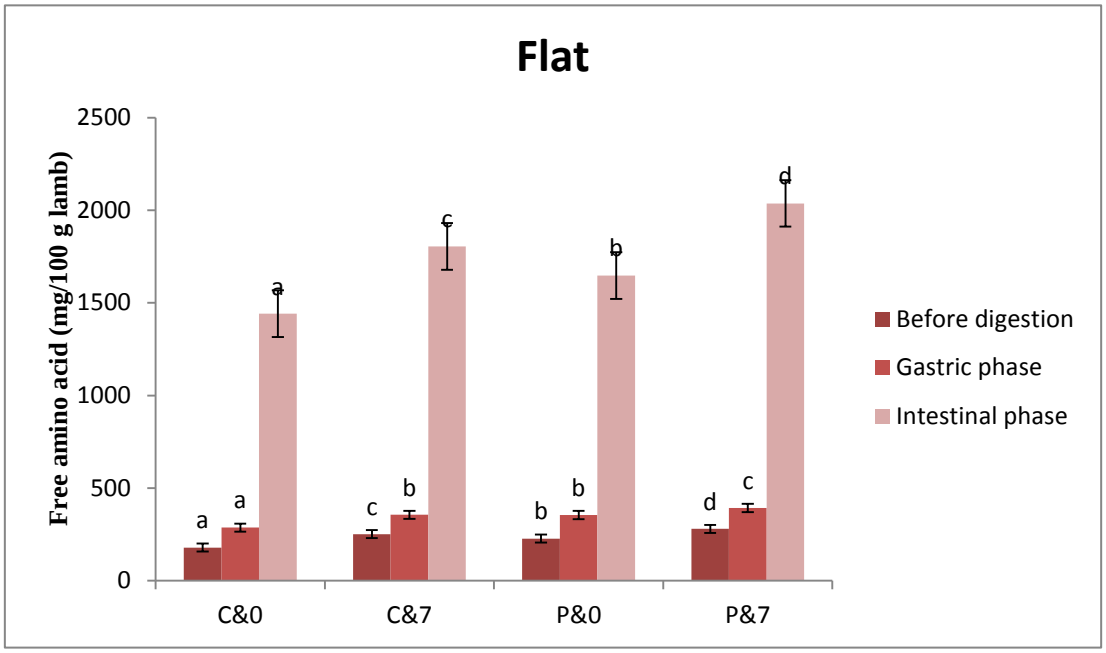
(a)


Bolar
Free amino acid (mg/100 g lamb)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
a
a
b
b
b
b
b
b
c
c
d
C&0
C&7
P&0
P&7
Before digestion
Gastric phase
Intestinal phase

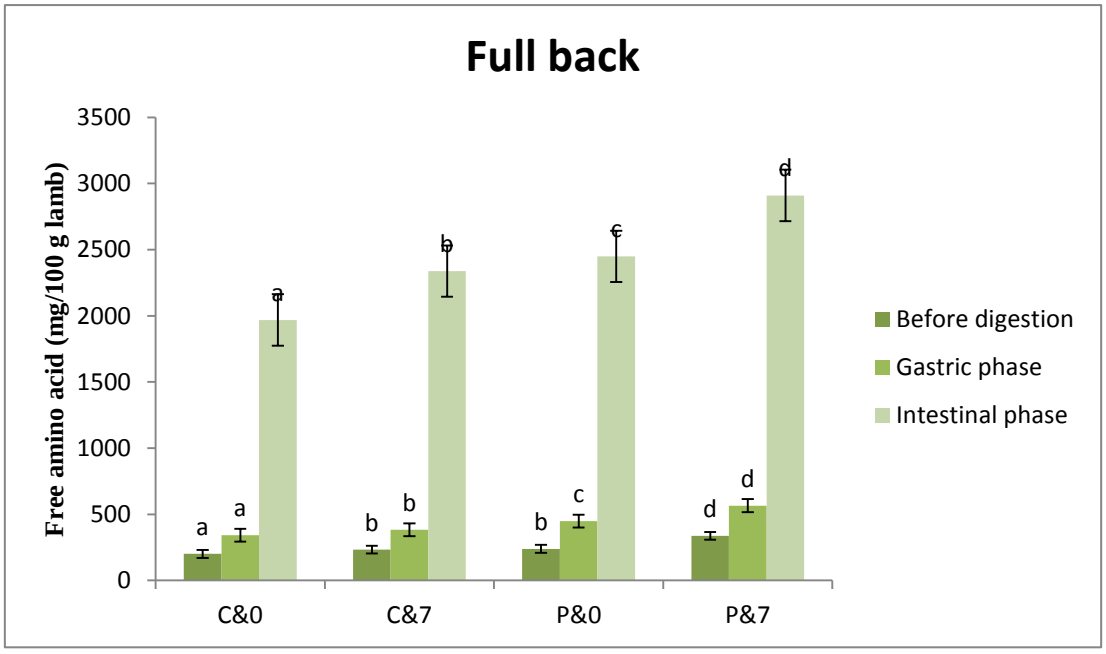
(b)


Cube roll
Free amino acid (mg/100 g lamb)
2500
2000
1500
1000
500
0
a
a
a
b
b
a
b
b
b
c
c
c
C&0
C&7
P&0
P&7
Before digestion
gastric phase
Intestinal phase

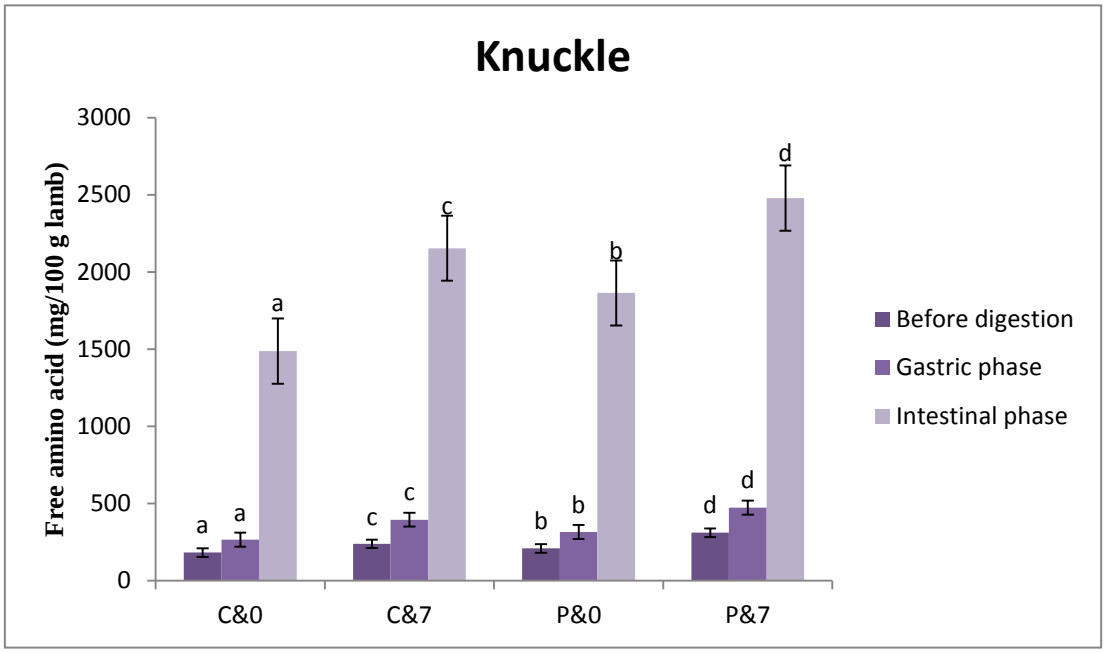
(c)


Flat
Free amino acid (mg/100 g lamb)
2500
2000
1500
1000
500
0
C&0
C&7
P&0
P&7
Before digestion
Gastric phase
Intestinal phase

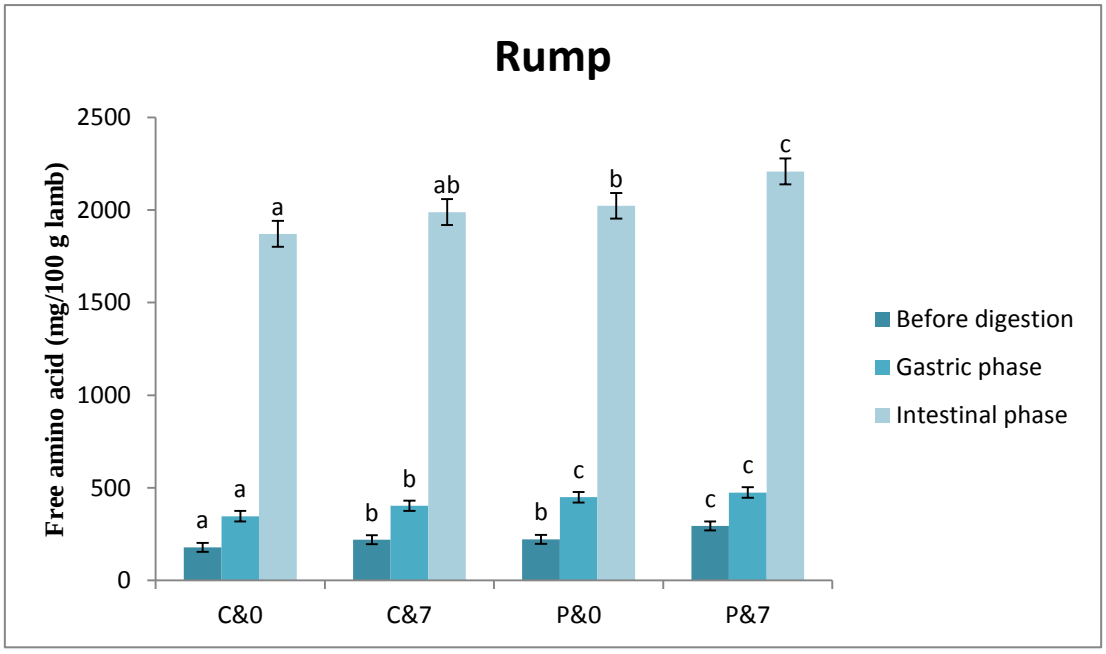
(d)


Full back
Free amino acid (mg/100 g lamb)
3500
3000
2500
2000
1500
1000
500
0
C&0
C&7
P&0
P&7
Before digestion
Gastric phase
Intestinal phase

(e)


Knuckle
Free amino acid (mg/100 g lamb)
3000
2500
2000
1500
1000
500
0
a a a
c c c
b b b
d d d
C&0
C&7
P&0
P&7
Before digestion
Gastric phase
Intestinal phase

(f)


Rump
Free amino acid (mg/100 g lamb)
2500
2000
1500
1000
500
0
a a a
b b ab
b c b
c c c
C&0
C&7
P&0
P&7
Before digestion
Gastric phase
Intestinal phase

(g)

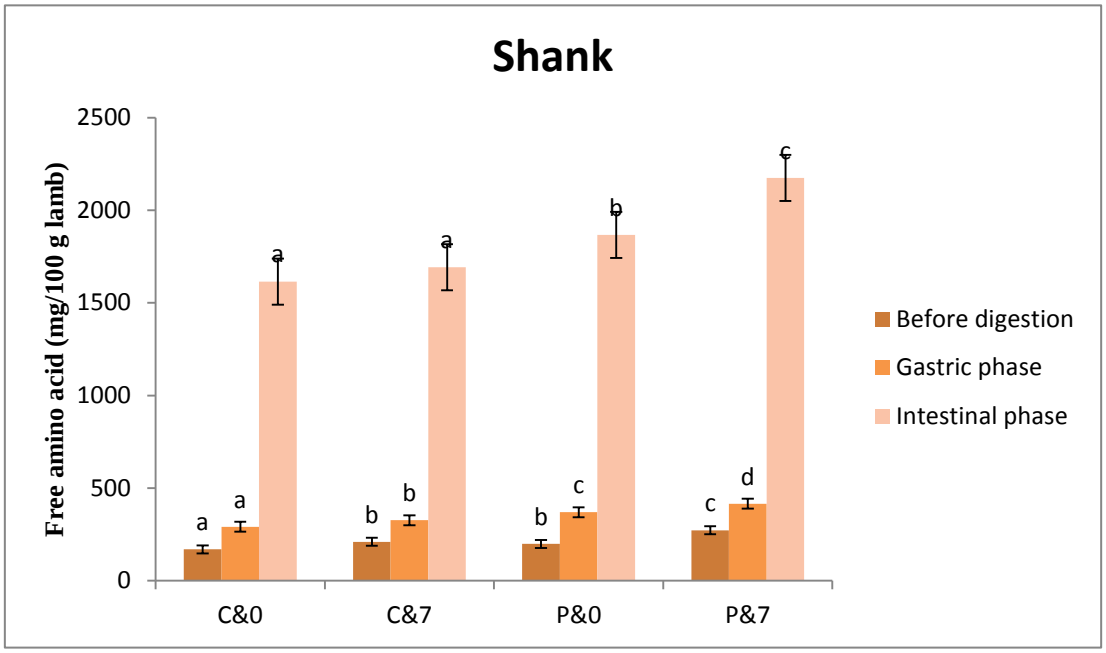


Figure 9 Total FAAs contents of three stages of digestion with the corresponding sample after PEF processing.

Mean values were plotted with error bars which represent standard deviations. Figure (a), (b),(c), (d), (e), (f) and (g) represent the results for PEF processed bolar, cube roll, flat, full back, knuckle, rump and shank cut, respectively. Different superscripts (^{a}, ^{b}, ^{c} and ^{d}) in the same digestion phase differ significantly

4.1.2.2 Effect of different cuts on digestibility of PEF treated lamb

The results of total FAA contents of P&7 samples from seven cuts during *in vitro* digestion are presented in Figure 10. Obvious increases in the total FAAs were found from the initial stage to the end of the digestion for all of the cuts. Full back and knuckle were two most nutritional cuts with the highest total FAA contents in all of three stages of digestion simulation after PEF and frozen storage. Prior to digestion, the FAAs contained in seven cuts were relatively close to one another which were approximately between 200 and 300 mg/100g lamb over all. Full back and knuckle contained the highest total FAA to begin with, which see 337.56±7.39 and 311.47±10.92 mg/100 g lamb. After peptic digestion, slight increases for 100-200

mg/100 g lamb were detected in these cuts. Full back was shown to have 565.52±5.97 mg of total FAAs in per 100 g lamb, which surprisedly increased for around 230 mg FAAs in per 100 g lamb from the initial stage. In the intestinal phase, significant differences of total FAAs were found in different cuts from Figure 10. The highest amount was found still in the full back, which was 2909.68±68.39 mg of total FAAs were released from 100 g sample. However, the cut containing lowest total FAAs in the intestinal phase was bolar, which only 1704.21±79.77 mg /100 g lamb was detected. More than 1200 mg/100 g lamb of the differences in total FAAs between full back and bolar were resulted from the most of individual amino acids, especially in LEU and PHE. The amount of LEU and PHE in bolar at the end of the intestinal phase were 342.52±12.78 and 296.45±12.79 mg/100 g lamb, respectively, which were far lower than those in the full back, for 566.01±14.62 and 547.68±18.27 mg/100 g lamb, respectively. The order of these seven cuts from the highest to the lowest total FAAs were listed as follow: Full back > knuckle > rump > shank > flat > cube roll > bolar. It indicates that PEF did not increase the nutritional value of expensive cuts (eg. cube roll) as much as that seen by cheaper cuts (eg. knuckle and rump). Hence, by using PEF, the nutritional value and digestibility of cheaper cuts can be improved and this can contribute towards adding "extra value" to the cheaper cuts of meat.

Moreover, total essential amino acids of the cuts during the whole *in vitro* digestion are shown in Table 16. The highest amount of total FAAs was shown in P&7 samples and they also contained the most of essential amino acids compared to the other treatment conditions. Consistent to the results of total FAAs, the order of cuts containing the most to the least of the essential amino acids in these cuts was the same as above. Full back contained the highest amount of essential FAAs, for 2031.94±26.35 mg/100 g lamb which was 69.85 % of total amino acids. Bolar still contained the least essential amino acids at the end of the digestion, however, its proportion of essential amino acids to total FAAs was 69.84 %, which was similar to that of the full back cut.

To sum up, as what was discussed above, PEF processing and frozen storage for 7 days both have significantly positive effects on improving the digestibility and nutritional value of all of seven lamb cuts. A combination of PEF technology and frozen storage is believed to be an optimal processing to enhance the nutritional quality of lamb. However, the most ideal parameter of PEF processing applied and the best storage time are still required for further study. In addition, owing to the unavoidable defects of PEF technology, the lamb samples were cooked during the processing which may limit the application of PEF processed lamb in meat industry.

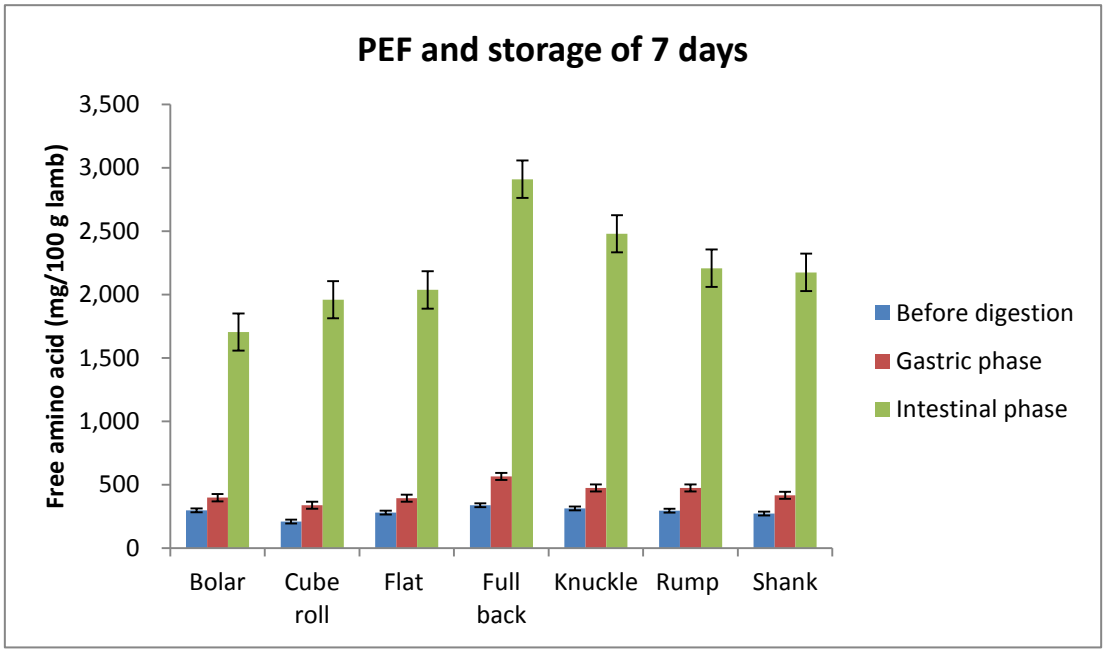


Figure 10 Total FAAs contents of seven cuts of lamb after PEF processing and 7 days of storage, during three *in vitro* digestion stages.

Mean values were plotted with error bars which represent standard deviations.

Table 15 Concentration of FAAs (mg/100 g lamb) of PEF processed lamb at different digestion stages: before digestion (initial stage), gastric phase and intestinal phase of the *in vitro* digestion. Table (a), (b), (c), (d), (e), (f) and (g) represent the results for PEF processed bolar, cube roll, flat, full back, knuckle, rump and shank cut, respectively.

(a) Bolar

Digestion	Before digestion					Gastric phase					Intestinal phase				
Treatment	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P
VAL	7.91±0.44^{a}	13.92±0.87^{b}	7.6±0.48^{a}	15.63±0.38^{c}	***	10.59±0.55^{a}	18.54±0.46^{c}	12.42±0.55^{b}	21.28±1.40^{d}	***	20.33±2.17^{a}	31.56±0.11^{c}	22.49±0.66^{a}	25.16±0.60^{b}	***
LEU	7.04±0.40^{a}	18.44±2.89^{c}	10.45±0.69^{b}	25.32±0.34^{d}	***	29.30±1.85^{a}	37.94±2.06^{c}	34.73±1.23^{b}	40.10±1.24^{c}	**	202.66±11.66^{a}	239.03±17.22^{b}	271.80±7.81^{c}	342.52±12.78^{d}	***
ILE	6.33±0.15^{a}	9.83±0.09^{c}	8.44±0.41^{b}	9.95±1.37^{c}	**	6.13±0.37^{a}	5.70±0.94^{a}	10.10±0.41^{b}	11.38±0.56^{c}	***	19.69±1.10^{a}	27.80±2.23^{b}	21.88±0.77^{a}	41.31±3.87^{c}	***
THR	4.75±0.52^{a}	9.65±0.55^{b}	11.90±0.82^{c}	13.50±1.01^{d}	***	8.95±1.09^{a}	11.72±1.58^{b}	12.66±0.59^{b}	12.47±0.31^{b}	*	12.40±0.29^{a}	16.83±0.48^{b}	16.27±0.78^{b}	12.44±0.93^{a}	***
MET	7.71±0.27^{a}	6.89±0.94^{a}	6.88±0.53^{a}	13.17±0.77^{b}	***	12.00±0.99^{a}	15.84±0.57^{b}	15.17±1.29^{b}	15.61±0.70^{b}	*	40.50±1.61^{a}	52.87±7.85^{b}	56.51±3.75^{b}	51.45±9.48^{ab}	ns
PHE	9.05±5.16^{a}	15.78±3.53^{bc}	11.38±0.63^{ab}	19.54±1.77^{c}	*	34.06±3.89^{a}	43.18±1.36^{b}	45.73±3.63^{b}	50.95±6.90^{b}	*	195.28±6.50^{a}	213.28±15.28^{a}	279.81±12.75^{b}	296.45±12.79^{b}	***
LYS	7.15±0.14^{a}	11.35±1.03^{a}	11.43±4.61^{a}	20.85±1.09^{b}	**	15.86±0.36^{a}	20.00±0.73^{b}	18.56±0.96^{b}	29.29±2.19^{c}	***	146.02±4.00^{a}	166.81±11.57^{b}	171.20±7.30^{b}	293.53±15.20^{c}	***
HIS	7.57±0.33^{a}	9.51±0.59^{b}	10.10±0.91^{b}	19.10±0.56^{c}	***	8.68±1.08^{a}	14.90±1.94^{b}	13.98±0.58^{b}	20.19±4.46^{c}	**	61.38±6.54^{a}	83.63±2.98^{b}	83.03±2.80^{b}	78.11±8.62^{b}	*
TRP	2.75±0.21^{a}	6.30±0.63^{c}	3.10±0.71^{ab}	3.72±0.32^{b}	**	4.17±0.16^{a}	7.17±0.59^{bc}	6.49±0.34^{b}	8.13±0.83^{c}	**	55.50±4.66^{a}	72.43±3.12^{c}	64.26±2.77^{b}	50.14±3.58^{a}	**
ALA	32.70±0.57^{a}	33.56±0.30^{a}	39.14±0.38^{b}	41.07±0.52^{c}	***	34.28±0.73^{a}	35.04±1.21^{a}	44.85±1.19^{b}	45.08±1.24^{b}	***	49.97±3.12^{b}	37.86±5.13^{a}	58.26±1.96^{c}	60.15±3.94^{c}	**
GLY	13.97±0.11^{a}	19.53±0.69^{b}	24.51±0.35^{d}	21.78±1.67^{c}	***	17.07±1.27^{a}	23.83±1.22^{c}	20.65±1.53^{b}	26.09±0.66^{c}	***	31.78±0.50^{a}	39.99±1.85^{b}	46.54±3.89^{c}	54.21±1.27^{d}	***
SER	16.43±2.09^{a}	7.25±0.18^{c}	20.95±0.38^{b}	10.09±0.34^{d}	***	7.38±0.72^{a}	13.01±2.67^{b}	10.80±0.48^{b}	18.11±0.22^{c}	***	19.86±0.43^{ab}	17.73±0.61^{a}	17.33±2.70^{a}	22.50±0.65^{b}	**
PRO	4.20±0.29^{a}	7.27±0.32^{b}	7.22±0.09^{b}	12.49±0.70^{c}	***	7.64±0.59^{a}	14.88±0.67^{c}	10.42±0.80^{b}	17.20±1.94^{d}	***	12.18±0.53^{c}	20.15±0.86^{b}	13.12±0.91^{a}	22.31±1.16^{c}	***
ASN	2.67±1.05^{a}	3.95±1.35^{a}	4.44±0.52^{a}	6.36±0.77^{b}	*	5.12±0.17^{a}	5.54±0.49^{a}	5.38±0.65^{a}	5.75±0.34^{a}	ns	6.67±0.68^{a}	12.87±0.73^{c}	8.36±0.54^{b}	15.80±0.79^{d}	***
ASP	3.25±0.80^{a}	5.74±0.14^{b}	3.63±1.24^{a}	7.64±0.68^{c}	**	6.00±0.35^{a}	6.83±0.35^{b}	7.12±0.55^{b}	9.53±0.03^{c}	***	7.61±0.29^{a}	7.73±0.33^{a}	8.34±0.45^{a}	10.44±0.54^{b}	**
GLU	13.78±1.22^{a}	13.66±0.74^{a}	19.14±0.61^{b}	15.73±1.88^{a}	*	15.56±0.53^{a}	16.9±0.92^{a}	21.58±1.14^{b}	16.57±1.33^{a}	**	16.12±0.87^{a}	20.29±1.88^{b}	17.59±0.49^{ab}	18.49±3.06^{ab}	ns
GLN	19.63±1.83^{a}	20.67±1.73^{a}	20.08±4.59^{a}	26.02±1.75^{b}	ns	20.53±1.91^{a}	22.47±0.35^{a}	28.23±5.41^{b}	24.67±1.39^{ab}	ns	51.75±3.32^{b}	45.45±4.67^{b}	45.41±4.79^{b}	35.84±5.90^{a}	ns
ORN	1.63±0.12^{a}	2.66±0.39^{b}	1.88±0.08^{a}	3.22±0.22^{c}	**	2.05±0.16^{a}	2.84±0.18^{b}	2.28±0.20^{ab}	3.65±0.49^{c}	**	3.94±0.37^{bc}	3.52±0.35^{ab}	2.97±0.27^{a}	4.55±0.41^{c}	*
TYR	7.18±2.93^{a}	9.54±0.55^{a}	8.94±1.41^{a}	12.69±0.15^{b}	ns	12.10±1.40^{a}	18.02±2.92^{b}	18.90±3.33^{b}	21.09±3.94^{b}	ns	200.23±9.75^{a}	210.19±5.08^{a}	273.97±11.38^{b}	268.81±14.51^{b}	***
Total	175.71±7.2^{a}	225.49±8.70^{b}	231.22±5.68^{b}	297.85±4.89^{c}	***	257.46±13.71^{a}	334.34±14.68^{b}	340.04±15.98^{b}	397.17±24.76^{c}	***	1153.87±42.76^{a}	1320.02±67.33^{b}	1479.16±51.74^{c}	1704.21±79.77^{d}	***

(b) Cube roll

Digestion	Before digestion					Gastric phase					Intestinal phase				
Treatment	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P
VAL	6.02±0.77^{a}	6.54±0.40^{a}	7.95±0.71^{b}	7.91±0.69^{b}	*	6.61±0.27^{a}	8.59±0.04^{b}	8.89±0.50^{b}	9.57±0.36^{c}	***	20.62±0.71^{a}	22.56±0.83^{a}	26.67±4.00^{b}	29.54±0.99^{b}	*
LEU	9.62±0.50^{a}	12.79±0.89^{b}	12.80±0.65^{b}	16.37±0.26^{c}	***	24.77±1.91^{a}	32.22±1.14^{b}	35.26±3.84^{b}	36.34±2.86^{b}	**	290.99±11.84^{a}	290.37±13.35^{a}	310.46±4.84^{a}	363.63±21.69^{b}	**
ILE	4.18±0.64^{a}	7.14±0.14^{b}	4.91±0.64^{a}	8.95±0.06^{c}	***	5.30±0.08^{a}	7.33±0.23^{b}	5.96±0.33^{a}	9.23±0.95^{c}	***	35.83±0.54^{a}	41.82±6.00^{ab}	41.07±1.40^{ab}	43.26±2.00^{b}	ns
THR	8.17±0.49^{a}	8.00±0.52^{a}	8.45±0.48^{a}	10.64±0.63^{b}	***	9.18±0.39^{a}	10.49±0.54^{b}	10.45±0.10^{ab}	11.99±1.21^{c}	*	12.46±0.80^{a}	19.11±1.36^{b}	19.40±2.09^{b}	18.86±4.21^{b}	ns
MET	5.44±0.18^{a}	5.14±0.30^{a}	7.37±0.76^{b}	5.50±0.46^{a}	*	11.05±1.55^{a}	15.38±0.67^{bc}	13.40±1.37^{b}	15.69±0.94^{c}	*	60.82±5.15^{a}	68.59±9.77^{a}	65.37±0.66^{a}	69.64±1.66^{a}	ns
PHE	9.81±0.05^{a}	11.92±0.33^{b}	15.13±0.80^{c}	12.09±0.39^{b}	***	38.44±0.80^{ab}	37.16±0.32^{a}	39.60±3.46^{ab}	40.67±0.95^{b}	ns	294.18±17.84^{ab}	268.43±12.74^{a}	317.31±11.66^{b}	347.37±14.26^{c}	**
LYS	6.75±0.58^{a}	14.25±0.63^{d}	9.21±0.96^{b}	12.60±0.98^{c}	***	10.00±0.06^{a}	20.62±1.84^{b}	20.22±0.18^{b}	19.90±1.24^{b}	***	242.55±7.50^{a}	241.61±11.50^{a}	270.93±11.64^{b}	301.17±9.97^{c}	**
HIS	7.14±0.52^{a}	11.72±0.64^{b}	8.00±0.48^{a}	10.96±0.77^{b}	***	9.53±0.74^{a}	14.25±1.29^{ab}	9.97±1.29^{a}	17.96±7.07^{b}	ns	74.59±6.43^{a}	83.41±9.07^{a}	98.07±5.99^{b}	99.75±6.88^{b}	*
TRP	1.71±0.28^{a}	3.15±0.11^{a}	1.93±0.19^{a}	2.84±1.55^{a}	ns	5.46±0.39^{a}	4.61±0.28^{a}	5.32±0.63^{a}	8.06±4.20^{a}	ns	90.74±6.93^{b}	79.09±6.71^{a}	104.14±2.43^{c}	108.13±3.34^{c}	**
ALA	22.29±0.94^{a}	33.69±1.76^{b}	24.46±0.82^{a}	36.16±1.73^{b}	***	27.33±0.26^{a}	35.67±1.29^{c}	33.15±1.32^{b}	40.05±1.20^{d}	***	38.25±0.78^{a}	47.41±1.56^{c}	42.13±2.78^{b}	50.03±0.90^{c}	**
GLY	18.15±0.75^{b}	15.30±0.38^{a}	21.70±1.45^{c}	16.68±0.97^{ab}	**	22.2±1.40^{a}	20.68±1.31^{a}	27.13±2.25^{b}	22.38±2.17^{a}	ns	39.96±4.42^{a}	42.44±2.00^{a}	44.92±2.98^{a}	45.52±3.33^{a}	ns
SER	5.52±0.60^{a}	6.68±0.56^{a}	12.37±0.99^{b}	9.88±1.11^{c}	***	7.20±0.70^{a}	12.73±0.99^{b}	13.59±0.82^{b}	17.44±0.66^{c}	***	12.41±0.59^{a}	15.26±0.52^{b}	12.87±2.18^{ab}	23.84±1.20^{c}	***
PRO	8.31±0.52^{a}	8.76±1.04^{a}	8.28±0.48^{a}	9.22±0.16a	ns	9.01±0.14^{a}	10.61±0.31^{bc}	9.54±0.49^{ab}	11.86±1.33^{c}	*	15.42±0.96^{a}	15.19±0.84^{a}	15.92±0.64^{ab}	16.87±0.49^{b}	ns
ASN	5.29±0.56^{ab}	5.20±0.18^{ab}	4.69±0.85^{a}	5.93±0.70^{b}	ns	6.31±0.33^{a}	6.53±0.14^{a}	6.51±0.28^{a}	8.66±0.64^{b}	**	9.07±0.44^{a}	8.16±1.00^{a}	8.14±0.95^{a}	11.16±0.44^{b}	**
ASP	4.75±0.35^{b}	4.80±0.13^{b}	2.73±0.72^{a}	4.40±0.07^{b}	**	5.15±0.47^{a}	8.62±0.29^{b}	9.54±1.66^{bc}	10.49±0.76^{c}	**	5.05±0.31^{a}	9.57±1.15^{b}	12.12±0.83^{c}	12.65±0.55^{c}	***
GLU	11.51±1.06^{a}	11.36±0.32^{a}	12.88±1.46^{ab}	14.22±0.19^{b}	ns	12.96±0.48^{a}	13.82±2.61^{a}	14.55±0.48^{a}	14.66±0.92^{a}	ns	17.35±1.19^{a}	24.78±9.51^{a}	18.17±1.73^{a}	18.67±1.61^{a}	ns
GLN	20.62±2.11^{a}	10.43±0.51^{c}	21.68±0.82^{c}	14.27±1.69^{b}	***	24.85±1.72^{c}	18.37±1.39^{a}	23.88±0.30^{c}	20.83±0.83^{b}	**	52.71±3.69^{a}	53.95±0.72^{a}	84.98±11.21^{b}	62.48±2.31^{a}	**
ORN	1.56±0.17^{a}	2.11±0.22^{b}	2.57±0.11^{c}	1.50±0.38^{a}	**	2.03±0.22^{a}	2.26±0.13^{a}	3.01±0.35^{b}	2.51±0.53^{ab}	ns	3.07±0.14^{a}	4.91±0.50^{b}	4.42±0.28^{b}	5.75±0.16^{c}	***
TYR	6.12±0.14^{a}	6.44±0.42^{a}	6.40±0.15^{a}	8.34±0.57^{b}	**	17.43±1.71^{ab}	12.52±1.16^{a}	14.68±0.96^{ab}	19.30±6.47^{b}	ns	272.01±15.23^{a}	269.60±19.86^{a}	337.86±17.33^{b}	330.55±4.78^{b}	**
Total	162.96±2.02^{a}	185.40±6.92^{b}	193.51±8.14^{b}	208.46±4.26^{c}	***	254.82±7.55^{a}	292.46±2.61^{b}	304.65±11.66^{b}	337.58±24.69^{c}	**	1588.09±41.41^{a}	1609.19±60.03^{a}	1834.95±25.71^{b}	1958.88±7.22^{c}	***

(c) Flat

Digestion	Before digestion					Gastric phase					Intestinal phase				
Treatment	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P
VAL	9.61±0.37^{a}	11.19±0.83^{b}	9.63±0.63^{a}	11.36±0.40^{b}	*	12.2±0.62^{a}	12.59±0.92^{a}	13.51±0.13^{a}	12.18±1.78^{a}	ns	22.31±0.73^{a}	26.96±1.47^{b}	29.73±2.19^{bc}	31.89±3.53^{c}	*
LEU	15.6±0.52^{a}	23.49±0.99^{b}	18.96±3.97^{a}	28.80±2.08^{c}	**	31.65±0.32^{a}	35.95±1.99^{b}	37.91±1.86^{b}	44.98±1.95^{c}	***	255.48±17.18^{a}	318.94±18.73^{b}	302.78±12.34^{b}	390.52±6.98^{c}	***
ILE	7.21±0.10^{a}	10.13±1.86^{b}	8.16±0.44^{a}	11.62±0.12^{b}	*	8.67±0.10^{a}	15.34±0.41^{c}	10.15±0.31^{b}	15.08±0.77^{c}	***	39.75±2.96^{a}	50.77±1.96^{b}	55.08±1.28^{c}	58.50±1.38^{c}	***
THR	10.67±0.54^{a}	12.34±1.02^{a}	11.75±0.29^{a}	12.46±1.70^{a}	ns	12.12±0.27^{a}	14.11±0.36^{b}	12.5±0.62^{a}	14.86±1.00^{b}	*	16.91±1.01^{a}	22.71±2.54^{b}	18.31±1.18^{ab}	15.86±2.29^{a}	*
MET	6.78±0.33^{a}	8.87±0.39^{c}	8.04±0.64^{b}	10.55±0.32^{d}	***	13.97±0.81^{a}	17.96±0.75^{b}	16.75±2.57^{ab}	21.10±1.18^{c}	*	54.59±4.93^{a}	71.87±10.63^{bc}	59.68±1.48^{ab}	74.62±6.14^{c}	ns
PHE	10.18±0.40^{a}	20.80±2.36^{c}	15.52±2.06^{b}	21.84±1.79^{c}	**	40.66±2.76^{a}	47.80±5.85^{ab}	51.42±2.71^{b}	45.91±5.91^{ab}	ns	227.68±11.15^{a}	342.02±11.76^{c}	277.25±9.71^{b}	373.11±25.17^{d}	***
LYS	7.42±0.33^{a}	19.23±0.99^{c}	14.66±0.58^{b}	18.91±0.77^{c}	***	14.04±0.44^{a}	17.92±0.46^{v}	16.22±1.41^{b}	20.62±0.49^{d}	***	213.81±16.70^{a}	203.36±7.33^{a}	250.91±23.09^{b}	312.42±19.05^{c}	**
HIS	10.08±1.43^{a}	12.51±1.26^{b}	14.37±1.27^{b}	12.79±0.67^{b}	ns	12.46±1.81^{a}	22.53±1.23^{c}	18.88±2.86^{b}	18.24±1.37^{b}	**	72.04±3.09^{a}	104.56±12.85^{b}	91.45±7.56^{b}	104.14±7.00^{b}	**
TRP	1.37±0.11^{a}	3.08±0.23^{b}	2.60±0.29^{b}	2.70±0.60^{b}	*	5.58±0.77^{a}	12.26±1.46^{c}	9.04±1.85^{b}	15.37±1.95^{d}	**	64.69±4.91^{a}	106.59±8.08^{b}	73.16±6.49^{a}	96.20±7.51^{b}	**
ALA	17.23±1.59^{a}	35.71±1.00^{c}	25.4±1.27^{b}	40.2±2.46^{d}	***	26.07±0.89^{a}	37.29±1.02^{c}	29.68±1.63^{b}	41.70±1.99^{d}	***	40.66±3.46^{a}	44.74±4.18^{ab}	50.14±3.18^{b}	50.62±2.47^{b}	ns
GLY	16.58±0.60^{a}	14.87±0.56^{a}	15.32±0.75^{a}	19.28±1.84^{b}	*	20.13±2.6^{a}	19.38±1.28^{a}	23.03±1.45^{a}	21.22±0.35^{a}	ns	33.77±0.91^{a}	38.65±0.27^{b}	42.81±1.93^{c}	41.4±2.83^{bc}	**
SER	6.15±1.09^{a}	8.96±1.46^{b}	8.97±0.53^{b}	9.93±1.99^{b}	ns	10.35±0.45^{a}	11.51±0.48^{a}	11.30±1.40^{a}	14.50±1.00^{b}	*	14.81±2.68^{a}	13.54±0.76^{a}	13.90±1.77^{a}	13.83±1.46^{a}	ns
PRO	9.00±0.21^{a}	10.41±0.20^{b}	11.17±0.47^{bc}	11.59±0.65^{c}	**	10.20±0.70^{a}	11.18±0.90^{ab}	15.99±1.52^{c}	13.12±1.96^{b}	*	12.37±1.97^{a}	15.55±0.92^{ab}	18.50±2.61^{b}	16.88±2.9^{b}	ns
ASN	6.36±0.59^{a}	7.19±0.80^{a}	9.00±0.64^{b}	6.66±0.57^{a}	*	7.83±1.57^{c}	6.09±0.58^{bc}	3.33±0.80^{a}	4.91±0.59^{ab}	*	13.69±1.73^{b}	14.75±1.39^{b}	18.03±0.59^{c}	9.83±0.74^{a}	**
ASP	4.75±0.22^{a}	4.77±0.61^{a}	7.74±0.34^{b}	10.72±0.78^{c}	***	5.57±0.10^{a}	5.68±0.99^{a}	6.12±0.06^{a}	7.17±0.22^{b}	ns	7.78±0.63^{b}	10.62±0.99^{c}	11.11±0.66^{c}	6.16±0.76^{a}	ns
GLU	7.69±0.71^{a}	11.01±0.39^{b}	11.41±0.92^{b}	14.06±1.21^{c}	ns	9.98±1.13^{a}	13.92±1.40^{b}	14.20±2.03^{b}	19.14±0.75^{c}	**	12.85±0.97^{a}	18.61±2.58^{b}	14.20±2.34^{a}	23.15±0.15^{c}	**
GLN	22.22±1.05^{a}	21.49±3.91^{a}	19.78±2.43^{a}	19.66±0.23^{a}	ns	23.49±1.19^{a}	25.04±1.08^{ab}	34.17±2.05^{c}	27.72±1.41^{b}	**	38.39±9.02^{a}	30.64±5.03^{a}	41.75±1.81^{a}	39.05±6.06^{a}	ns
ORN	1.52±0.16^{a}	2.08±0.41^{b}	2.22±0.13^{b}	2.93±0.12^{c}	**	2.31±0.24^{a}	2.77±0.05^{av}	2.84±0.34^{b}	3.18±0.38^{b}	ns	3.62±0.37^{a}	3.19±0.78^{a}	3.94±0.36^{a}	5.25±0.55^{b}	*
TYR	8.99±0.04^{a}	13.81±1.95^{b}	12.89±2.20^{b}	14.12±0.68^{b}	*	19.73±1.99^{a}	26.77±2.05^{b}	27.53±1.68^{b}	31.81±1.00^{c}	**	296.00±15.82^{a}	366.67±10.73^{b}	274.22±15.25^{a}	373.47±21.94^{b}	**
Total	179.40±4.03^{a}	251.93±11.64^{c}	227.58±9.13^{b}	280.18±7.20^{d}	***	287.00±12.41^{a}	356.07±8.32^{b}	354.58±13.17^{b}	392.8±15.01^{c}	***	1441.2±22.5^{a}	1804.74±72.85^{c}	1646.93±38.54^{b}	2036.9±58.52^{d}	***

(d) Full back

Digestion	Before digestion					Gastric phase					Intestinal phase				
Treatment	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P
VAL	14.49±2.67^{a}	13.88±0.91^{a}	11.93±0.83^{a}	18.27±0.18^{b}	*	12.63±1.26^{a}	13.75±0.43^{ab}	14.82±0.65^{b}	14.24±0.33^{b}	ns	27.23±0.20^{a}	30.37±2.26^{ab}	32.14±0.54^{b}	45.50±2.76^{c}	***
LEU	16.87±1.76^{a}	19.79±0.62^{b}	20.96±0.69^{b}	36.51±0.46^{c}	***	44.45±2.68^{a}	45.02±1.25^{a}	60.42±6.41^{b}	62.57±0.90^{b}	**	374.45±28.99^{a}	431.68±19.43^{b}	452.01±5.26^{b}	566.01±14.62^{c}	***
ILE	5.22±0.95^{a}	8.25±0.25^{n}	7.22±0.59^{n}	12.00±0.07^{c}	***	7.79±0.16^{a}	9.39±0.60^{b}	8.35±0.17^{a}	14.29±0.54^{c}	***	28.10±2.61^{a}	33.19±0.94^{b}	33.67±0.65^{b}	41.19±0.88^{c}	***
THR	6.46±0.79^{a}	9.83±0.61^{b}	10.48±0.70^{b}	15.43±0.61^{c}	***	9.36±0.90^{a}	11.9±0.50^{b}	21.57±0.57^{c}	21.46±0.85^{c}	***	15.35±0.72^{b}	12.73±1.23^{a}	19.60±0.60^{c}	20.66±1.02^{c}	***
MET	7.14±0.35^{a}	7.37±0.70^{a}	6.59±0.29^{a}	9.75±0.84^{b}	**	13.73±0.64^{a}	15.88±1.45^{a}	24.64±0.97^{b}	26.88±2.75^{b}	***	73.92±1.92^{a}	71.61±3.25^{a}	93.00±1.70^{b}	116.74±2.04^{c}	***
PHE	15.07±0.13^{a}	14.6±0.36^{a}	16.37±0.33^{b}	24.28±0.43^{c}	***	55.67±8.00^{a}	59.85±2.38^{ab}	66.48±5.73^{b}	83.24±1.04^{c}	**	383.14±13.88^{a}	459.44±12.42^{b}	482.61±11.93^{b}	547.68±18.27^{c}	***
LYS	12.05±0.47^{a}	15.6±1.17^{b}	14.92±1.24^{b}	20.38±0.40^{c}	***	20.55±1.47^{a}	21.82±0.28^{a}	26.95±3.26^{b}	26.98±0.38^{b}	*	246.31±11.36^{a}	292.38±12.06^{b}	336.14±12.72^{c}	369.31±19.95^{d}	***
HIS	12.00±0.69^{a}	12.14±0.59^{a}	12.14±0.36^{a}	19.94±0.74^{b}	***	17.30±2.63^{a}	23.55±1.95^{b}	23.96±1.50^{b}	34.12±0.74^{c}	***	97.94±3.68^{a}	125.49±14.54^{b}	128.98±10.59^{b}	159.70±6.55^{c}	**
TRP	3.18±0.22^{a}	3.22±0.31^{a}	3.32±0.28^{a}	3.50±0.11^{a}	ns	9.92±1.85^{a}	8.86±1.40^{a}	10.96±0.57^{a}	18.87±0.64^{b}	***	110.68±3.37^{a}	143.68±10.50^{b}	155.78±3.65^{bc}	165.16±13.51^{c}	**
ALA	28.37±2.01^{a}	34.96±0.35^{b}	33.25±0.28^{b}	52.40±2.83^{c}	***	34.35±1.32^{a}	37.86±3.03^{a}	44.68±2.10^{b}	49.60±0.80^{b}	***	43.87±4.05^{a}	53.84±0.57^{b}	53.81±1.01^{b}	78.67±1.69^{c}	***
GLY	9.35±0.65^{a}	16.79±0.25^{c}	13.32±0.60^{b}	28.17±1.55^{d}	***	13.95±0.53^{a}	17.55±1.64^{b}	29.10±0.59^{b}	47.46±0.84^{d}	***	42.11±1.02^{a}	59.88±0.33^{b}	59.74±1.51^{b}	83.31±7.36^{c}	***
SER	8.70±0.90^{a}	10.86±0.93^{b}	8.97±0.82^{a}	17.16±0.17^{c}	***	9.38±1.35^{a}	13.06±0.50^{a}	29.90±3.67^{b}	30.50±0.64^{b}	***	12.50±1.30^{a}	11.54±0.88^{a}	10.86±1.84^{a}	10.96±0.88^{a}	ns
PRO	10.27±0.9^{b}	10.58±0.71^{b}	7.01±0.8^{a}	15.66±0.55^{c}	***	15.19±2.63^{ab}	12.50±0.76^{a}	15.85±0.74^{b}	22.12±0.39^{c}	**	21.69±1.14^{b}	20.72±1.40^{b}	18.66±0.80^{a}	33.39±0.86^{c}	***
ASN	4.86±0.63^{a}	6.20±0.18^{b}	9.71±1.15^{c}	9.20±0.44^{c}	***	5.15±0.76^{a}	6.27±0.48^{a}	6.94±0.34^{a}	7.62±2.89^{a}	ns	6.90±0.90^{a}	20.53±4.62^{b}	6.64±1.24^{a}	23.68±1.39^{b}	***
ASP	5.00±1.29^{a}	7.10±1.16^{b}	5.44±0.18^{a}	5.00±0.24^{a}	ns	8.73±0.44^{bc}	9.01±0.24^{b}	7.86±0.99^{ab}	7.35±0.36^{a}	ns	10.70±1.11^{a}	13.32±2.05^{b}	15.41±0.53^{b}	21.60±0.87^{b}	***
GLU	6.34±0.36^{a}	9.43±0.99^{b}	16.63±0.54^{c}	19.95±1.06^{d}	***	9.09±1.09^{a}	14.28±1.01^{b}	9.08±1.39^{a}	13.42±0.61^{b}	**	19.35±3.10^{ab}	18.40±2.66^{a}	20.86±4.57^{ab}	24.53±1.37^{b}	ns
GLN	21.07±1.23^{a}	20.20±1.87^{a}	26.92±0.78^{b}	20.58±0.57^{a}	**	27.47±0.57^{ab}	29.38±4.50^{b}	23.57±0.65^{a}	35.9±1.92^{c}	*	38.26±2.64^{a}	43.94±4.98^{a}	63.83±1.29^{b}	45.97±7.39^{a}	**
ORN	0.88±0.24^{a}	1.33±0.13^{b}	1.95±0.28^{ac}	2.41±0.13^{d}	***	2.44±0.33^{a}	2.56±0.77^{a}	2.88±0.03^{a}	3.72±0.16^{b}	ns	3.17±0.94^{a}	3.28±0.29^{a}	4.83±2.12^{a}	8.61±0.26^{b}	*
TYR	13.35±0.01^{c}	11.10±0.65^{b}	11.74±0.49^{b}	6.97±0.29^{a}	***	24.8±0.81^{b}	29.99±1.66^{c}	19.73±0.89^{a}	45.18±1.57^{d}	***	412.97±28.00^{a}	491.61±10.50^{b}	460.68±21.30^{b}	547.01±29.80^{c}	**
Total	200.67±6.01^{a}	233.22±3.01^{b}	238.87±4.39^{b}	337.56±7.39^{c}	***	341.92±18.62^{a}	382.46±8.35^{b}	447.72±12.28^{c}	565.52±5.97^{d}	***	1968.64±25.10^{a}	2337.61±74.87^{b}	2449.25±33.49^{c}	2909.68±68.39^{d}	***

(e) Knuckle

Digestion	Before digestion					Gastric phase					Intestinal phase				
Treatment	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P
VAL	7.92±0.41^{a}	10.64±0.12^{c}	9.69±0.87^{b}	14.38±0.07^{d}	***	10.87±0.63^{a}	17.23±1.27^{b}	11.70±0.80^{a}	19.34±1.85^{b}	***	21.74±0.99^{a}	39.89±2.42^{c}	28.30±0.62^{b}	45.79±3.13^{d}	***
LEU	16.15±0.86^{a}	31.16±3.54^{b}	18.8±2.32^{a}	30.66±2.99^{b}	**	30.19±0.14^{a}	49.07±8.71^{b}	36.91±5.76^{a}	61.93±3.22^{c}	**	270.77±15.91^{a}	404.67±16.77^{c}	349.98±17.67^{b}	471.13±15.33^{d}	***
ILE	5.54±0.43^{a}	6.58±1.21^{ab}	8.24±1.02^{v}	12.77±1.23^{c}	**	6.96±0.68^{a}	8.40±0.18^{b}	10.66±0.24^{c}	19.79±0.30^{d}	***	21.80±0.31^{a}	41.06±1.91^{c}	30.13±4.61^{b}	41.28±2.45^{c}	***
THR	8.87±2.38^{a}	11.63±1.52^{a}	10.42±1.82^{a}	22.96±0.48^{b}	***	11.83±0.73^{a}	14.30±0.47^{b}	14.61±0.91^{b}	18.21±0.62^{c}	***	15.11±0.64^{a}	17.71±1.89^{a}	38.04±2.72^{c}	30.62±1.00^{b}	***
MET	4.67±1.49^{a}	9.65±1.81^{b}	6.35±0.99^{a}	12.84±0.90^{c}	**	14.67±1.26^{a}	19.77±2.78^{b}	18.00±0.84^{ab}	25.98±1.69^{c}	**	65.26±3.09^{a}	109.83±5.34^{d}	81.62±5.77^{b}	95.23±4.06^{c}	***
PHE	12.26±0.21^{a}	19.26±0.15^{b}	14.36±2.11^{a}	20.86±3.15^{b}	*	26.99±1.45^{a}	55.69±13.93^{b}	34.62±1.80^{a}	56.83±4.54^{b}	*	305.68±9.81^{a}	379.49±16.77^{b}	375.24±23.22^{b}	432.67±12.59^{c}	**
LYS	10.04±1.08^{a}	11.33±0.92^{a}	10.09±0.82^{a}	20.35±0.14^{b}	***	13.02±0.46^{a}	22.47±1.79^{b}	10.78±0.46^{a}	32.6±2.64^{c}	***	186.67±7.94^{a}	356.14±23.45^{c}	248.14±18.64^{b}	392.57±16.06^{d}	***
HIS	5.97±0.82^{a}	8.65±0.58^{b}	8.83±0.26^{b}	12.10±0.72^{c}	***	6.91±0.25^{a}	9.75±1.35^{a}	9.15±2.29^{a}	21.64±3.20^{b}	**	83.32±6.64^{a}	116.71±3.78^{b}	114.54±13.82^{b}	128.66±5.13^{b}	**
TRP	3.47±0.14^{ab}	3.96±0.37^{a}	3.32±0.19^{a}	3.98±0.37^{b}	ns	5.00±0.05^{a}	5.45±1.41^{a}	6.40±0.19^{ab}	7.92±1.36^{b}	ns	81.34±3.49	93.5±7.73	86.46±15.6	121.15±9.96	*
ALA	29.59±1.00^{a}	34.94±1.80^{b}	31.99±1.98^{ab}	40.86±3.33^{c}	**	33.37±1.47^{a}	43.93±1.32^{c}	39.74±1.22^{b}	54.14±1.34^{d}	***	39.94±4.38^{a}	52.16±1.35^{b}	48.86±1.71^{b}	78.49±3.68^{c}	***
GLY	12.79±0.29^{a}	14.55±1.13^{ab}	17.63±2.93^{bc}	20.46±1.78^{c}	**	15.92±0.28^{a}	21.93±2.14^{bc}	21.06±1.28^{b}	25.64±3.75^{c}	*	34.13±0.40^{a}	41.89±0.79^{b}	49.37±1.72^{c}	56.77±0.82^{d}	***
SER	6.61±0.56^{a}	7.42±0.94^{ab}	8.39±1.07^{b}	11.80±0.68^{b}	**	10.74±0.72^{a}	11.30±0.42^{a}	16.24±1.43^{c}	14.15±0.86^{b}	**	16.01±1.15^{a}	18.95±2.06^{a}	35.48±4.93^{b}	20.24±1.70^{a}	**
PRO	7.03±0.42^{a}	10.73±1.52^{b}	7.03±0.48^{a}	13.85±0.66^{c}	***	10.75±0.74^{a}	13.67±1.32^{b}	12.62±0.41^{ab}	17.83±1.94^{c}	**	12.62±0.93^{a}	22.55±0.98^{bc}	21.35±2.87^{b}	25.36±2.12^{c}	**
ASN	6.57±0.78^{a}	8.15±0.52^{b}	7.16±0.65^{ab}	11.14±1.12^{c}	**	9.9±2.25^{a}	8.96±1.18^{a}	12.49±2.42^{ab}	14.58±2.04^{b}	ns	13.53±1.23^{a}	13.68±0.85^{a}	19.86±1.36^{b}	22.34±3.21^{b}	**
ASP	5.57±1.19^{a}	6.22±0.20^{a}	6.85±0.44^{ab}	7.81±0.68^{b}	ns	6.77±0.63^{a}	7.98±0.37^{b}	8.14±0.72^{b}	9.89±0.38^{c}	**	9.12±0.42^{a}	13.52±0.76^{b}	14.06±1.19^{b}	7.46±1.01^{a}	***
GLU	7.33±0.57^{a}	8.00±0.45^{a}	10.68±1.57^{b}	11.47±1.95^{b}	ns	11.71±2.04^{a}	10.41±1.20^{a}	13.09±0.32^{a}	12.95±1.69^{a}	ns	28.98±1.67^{ab}	34.72±0.31^{b}	27.64±3.78^{a}	46.80±5.16^{c}	**
GLN	23.25±1.75^{b}	20.46±1.94^{ab}	17.74±2.16^{a}	23.79±3.78^{b}	ns	27.39±0.51^{b}	53.28±0.99^{d}	22.43±1.03^{a}	34.92±1.35^{c}	***	80.49±1.24^{a}	119.90±12.62^{c}	73.14±4.52^{a}	94.78±4.61^{b}	**
ORN	1.33±0.16^{a}	2.81±0.35^{b}	2.46±0.59^{b}	3.57±0.21^{c}	**	2.43±0.25^{a}	4.43±0.46^{a}	2.57±0.09a	2.26±0.29^{b}	***	4.46±0.09^{a}	6.00±0.10^{a}	6.31±1.50^{a}	5.72±0.57^{a}	ns
TYR	7.70±1.64^{a}	13.08±0.22^{b}	9.59±2.18^{a}	15.83±0.27^{c}	**	10.76±0.10^{a}	17.82±5.13^{ab}	15.50±0.84^{ab}	23.01±6.38^{b}	ns	196.51±12.28^{a}	259.57±12.14^{b}	215.53±10.57^{a}	362.06±12.96^{c}	***
Total	182.65±6.53^{a}	239.22±10.59^{c}	209.63±15.20^{b}	311.47±10.92^{d}	***	266.18±7.54^{a}	395.84±39.40^{c}	316.71±7.06^{b}	473.61±18.81^{d}	***	1487.48±57.58^{a}	2141.95±47.90^{c}	1864.02±61.27^{b}	2479.14±51.62^{d}	***

(f) Rump

Digestion	Before digestion					Gastric phase					Intestinal phase				
Treatment	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P
VAL	8.69±0.34^{a}	9.52±1.96^{a}	8.98±0.23^{a}	16.24±0.41^{b}	***	10.54±0.40^{a}	11.40±0.46^{a}	13.75±1.35^{b}	13.92±0.17^{b}	**	25.95±1.87^{a}	30.27±2.27^{ab}	30.95±3.67^{ab}	33.96±1.33^{b}	ns
LEU	16.38±1.27^{a}	25.21±2.89^{c}	21.92±0.39^{b}	27.44±1.38^{c}	**	37.96±1.25^{a}	42.27±2.73^{a}	52.36±2.46^{b}	49.82±3.67^{b}	**	345.1±14.45^{a}	355.7±15.31^{ab}	382.46±18.34^{b}	382.46±18.34^{b}	ns
ILE	5.69±0.35^{a}	6.20±0.30^{ab}	6.69±0.54^{b}	12.84±0.77^{c}	***	7.12±0.43^{a}	6.93±0.87^{a}	9.06±0.15^{b}	8.10±0.32^{b}	*	54.87±3.22^{b}	58.39±3.23^{b}	41.10±1.28^{a}	53.52±5.05^{b}	**
THR	5.67±0.57^{a}	10.18±0.84^{b}	5.65±0.19^{a}	18.94±1.52^{c}	***	8.55±0.27^{a}	14.42±1.83^{b}	14.87±0.56^{b}	21.18±0.77^{c}	***	11.84±2.23^{a}	17.10±0.83^{b}	19.43±1.78^{b}	21.54±1.94^{b}	**
MET	7.16±0.17^{a}	7.41±0.18^{a}	8.67±0.54^{b}	8.62±0.37^{b}	**	17.13±0.50^{a}	19.39±1.62^{ab}	23.86±1.63^{c}	21.99±1.51^{bc}	*	65.96±1.01^{a}	68.25±3.49^{a}	69.62±4.10^{a}	69.89±1.06^{a}	ns
PHE	12.1±0.24^{a}	18.55±1.32^{c}	16.23±0.61^{b}	22.64±0.96^{d}	***	55.62±6.12^{a}	57.48±3.87^{a}	68.39±4.04^{b}	62.26±4.34^{ab}	ns	307.75±8.07^{a}	382.1±14.56^{bc}	375.68±20.52^{b}	406.43±17.79^{c}	**
LYS	10.86±0.64^{a}	11.51±0.10^{ab}	15.03±1.82^{c}	12.94±0.89^{b}	*	16.87±0.71^{a}	20.68±0.59^{b}	23.78±1.68^{c}	25.14±1.61^{c}	**	333.40±17.34^{b}	283.57±8.93^{a}	280.88±8.21^{a}	315.51±18.33^{b}	*
HIS	9.90±0.52^{a}	9.54±0.16^{a}	11.42±1.94^{ab}	12.71±0.81^{b}	ns	13.92±0.58^{a}	21.30±1.07^{b}	22.65±0.54^{b}	24.37±0.72^{c}	***	92.07±5.26^{a}	100.93±2.42^{ab}	107.34±6.71^{b}	111.23±10.08^{b}	ns
TRP	1.53±0.08^{a}	3.63±0.32^{b}	1.94±0.24^{a}	4.96±0.15^{c}	***	8.86±1.35^{a}	11.97±1.62^{b}	12.63±0.91^{b}	15.76±0.53^{c}	**	104.26±7.99^{a}	98.01±6.21^{a}	100.35±5.11^{a}	123.15±11.31^{b}	ns
ALA	28.02±3.95^{a}	35.32±2.79^{b}	29.94±1.07^{a}	42.96±1.88^{c}	**	31.89±0.64^{a}	43.59±1.19^{b}	42.78±2.16^{b}	47.22±1.36^{c}	***	38.38±1.92^{a}	47.04±4.91^{b}	52.58±1.62^{b}	60.49±3.05^{c}	**
GLY	14.65±1.19^{a}	13.48±0.56^{a}	12.97±1.38^{a}	24.03±0.61^{b}	***	27.76±0.54^{b}	25.84±1.17^{a}	34.35±0.61^{c}	39.9±0.90^{d}	***	34.22±0.27^{a}	33.04±1.65^{a}	42.79±0.96^{b}	49.98±0.38^{c}	***
SER	7.81±1.25^{a}	10.69±1.12^{b}	7.73±1.76^{a}	14.89±1.54^{c}	**	10.95±0.79^{a}	14.10±0.85^{b}	14.86±1.72^{b}	22.79±1.05^{c}	***	14.22±1.38^{a}	18.52±2.12^{ab}	20.75±2.58^{b}	14.76±3.56^{a}	ns
PRO	6.15±0.12^{a}	7.92±0.13^{a}	7.91±1.42^{a}	15.18±0.49^{b}	***	10.69±0.59^{a}	11.80±0.57^{b}	11.44±0.70^{ab}	14.45±0.36^{c}	**	12.97±0.92^{bc}	11.61±0.59^{a}	12.36±0.87^{ab}	13.77±0.28^{c}	ns
ASN	5.55±0.65^{b}	6.08±0.47^{b}	4.46±0.56^{a}	3.82±0.19^{a}	*	7.97±1.03^{a}	8.18±0.07^{a}	10.60±0.76^{b}	8.67±0.17^{a}	*	10.56±1.10^{a}	17.30±1.90^{c}	14.13±0.96^{b}	8.57±0.98^{a}	**
ASP	4.77±0.92^{b}	2.42±0.59^{a}	8.53±0.20^{c}	3.23±0.39^{a}	***	7.04±0.28^{a}	6.45±0.43^{a}	8.41±1.20^{b}	7.41±0.61^{ab}	ns	12.55±0.88^{b}	10.43±1.30^{a}	15.21±0.60^{c}	16.09±0.57^{c}	**
GLU	7.86±0.72^{a}	8.59±1.22^{ab}	13.47±0.73^{c}	10.14±1.04^{b}	**	10.14±0.43^{a}	13.21±1.08^{b}	14.67±1.55^{b}	8.15±1.39^{a}	**	21.17±2.82^{a}	21.13±1.86^{a}	25.99±2.16^{b}	31.15±1.63^{c}	**
GLN	15.54±1.51^{a}	19.88±3.05^{ab}	25.61±5.78^{b}	25.38±0.55^{b}	ns	36.68±1.89^{b}	42.36±0.68^{c}	32.06±0.96^{a}	48.8±3.22^{d}	***	53.73±4.87^{a}	59.39±6.55^{a}	86.42±6.78^{b}	82.45±2.86^{b}	**
ORN	1.79±0.22^{a}	2.68±0.29^{b}	3.44±0.33^{c}	4.01±0.13^{d}	***	2.61±0.41^{a}	3.52±0.29^{b}	4.14±0.69^{b}	3.88±0.09^{b}	ns	5.07±0.14^{b}	4.37±0.03^{a}	6.80±0.16^{d}	6.48±0.22^{c}	***
TYR	8.32±0.18^{a}	11.78±1.39^{bc}	10.82±0.19^{b}	13.04±0.29^{c}	**	24.50±3.92^{a}	28.34±0.78^{ab}	34.80±4.37^{c}	30.97±1.64^{bc}	ns	326.48±8.79^{a}	371.32±16.36^{b}	337.34±15.20^{a}	406.69±9.38^{c}	**
Total	178.46±7.96^{a}	220.60±11.76^{b}	221.42±10.59^{b}	294.00±10.48^{c}	***	346.8±12.45^{a}	403.22±14.15^{b}	449.45±15.18^{c}	474.78±19.37^{c}	***	1870.55±46.05^{a}	1988.48±69.47^{ab}	2022.20±78.92^{b}	2207.40±69.72^{c}	*

(g) Shank

Digestion	Before digestion					Gastric phase					Intestinal phase				
Treatment	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P	C&0	C&7	P&0	P&7	P
VAL	7.38±0.75^{a}	8.21±0.97^{a}	10.43±0.94^{b}	12.06±0.36^{c}	**	12.30±1.23^{a}	11.84±0.67^{a}	13.16±0.47^{a}	14.84±0.41^{b}	*	17.64±2.19^{a}	24.13±0.74^{b}	19.36±0.69^{a}	31.95±2.30^{c}	***
LEU	6.94±0.51^{a}	14.45±0.12^{c}	9.89±0.53^{b}	16.42±1.36^{d}	***	27.35±0.46^{a}	33.02±2.27^{b}	42.90±0.80^{c}	45.45±2.03^{c}	***	273.48±15.74^{a}	302.84±15.80^{b}	335.46±15.54^{c}	357.83±9.64^{c}	**
ILE	4.66±0.07^{a}	6.43±0.22^{b}	8.44±0.39^{c}	10.14±0.78^{d}	***	8.55±0.48^{a}	8.87±0.48^{a}	11.73±0.39^{b}	16.17±1.97^{c}	***	27.20±1.56^{a}	25.54±2.83^{a}	23.90±4.61^{a}	29.35±4.25^{a}	ns
THR	2.21±0.21^{a}	7.49±0.61^{c}	4.49±0.46^{b}	12.28±0.74^{d}	***	6.29±0.24^{a}	10.65±1.58^{b}	11.30±0.77^{b}	10.67±0.44^{b}	**	8.75±0.31^{a}	10.52±0.70a	14.30±1.59^{b}	26.99±1.05^{c}	***
MET	8.33±0.14^{a}	10.63±0.44^{b}	10.83±0.32^{b}	13.24±0.76^{c}	***	14.11±1.17^{a}	13.78±0.40^{a}	17.54±0.21^{c}	16.25±0.48^{b}	**	49.07±2.47^{a}	53.99±1.85^{a}	62.95±4.42^{c}	64.88±3.35^{c}	**
PHE	8.83±1.66^{a}	14.98±1.07^{b}	8.28±0.55^{a}	16.26±1.49^{b}	***	44.34±4.02^{ab}	42.33±4.76^{a}	57.65±1.97^{b}	52.54±6.65^{bc}	ns	344.74±10.36^{a}	327.74±18.43^{a}	377.47±12.92^{b}	434.35±19.49^{c}	**
LYS	7.59±1.31^{a}	7.96±0.15^{a}	8.75±0.06^{a}	12.85±0.62^{b}	***	12.90±0.23^{a}	17.03±1.14^{c}	14.47±0.92^{b}	18.03±0.60^{c}	**	205.41±9.78^{a}	227.96±18.67^{ab}	231.27±10.90^{b}	224.23±12.55^{ab}	ns
HIS	10.41±0.36^{a}	12.51±0.52^{b}	11.17±0.32^{ab}	15.11±1.35^{c}	**	12.20±0.47^{a}	15.13±2.11^{b}	12.39±0.05^{a}	15.56±1.93^{b}	ns	76.21±5.68^{a}	81.65±8.79^{a}	81.78±9.53^{a}	108.84±6.31^{b}	*
TRP	4.27±0.22^{c}	2.27±0.31^{a}	5.33±0.45^{d}	3.42±0.16^{b}	***	5.66±0.12^{a}	7.33±1.26^{a}	6.77±0.22^{a}	6.77±2.16^{a}	ns	62.84±6.20^{a}	94.81±6.12^{b}	91.84±7.12^{b}	142.47±7.73^{c}	***
ALA	20.13±0.75^{a}	22.05±0.22^{a}	22.72±1.71^{a}	31.45±3.87^{b}	**	26.67±1.26^{a}	28.60±1.51^{a}	35.45±0.71^{b}	40.56±0.35^{c}	***	31.49±1.38^{a}	33.71±2.48^{a}	43.03±0.59^{b}	52.49±2.68^{c}	***
GLY	16.13±0.32^{a}	18.74±1.12^{ab}	17.67±3.89^{ab}	20.57±1.28^{b}	ns	20.54±0.21^{a}	21.64±2.42^{a}	26.72±1.90^{b}	29.31±0.70^{b}	**	30.78±1.05^{a}	36.83±0.59^{b}	41.26±0.93^{c}	46.21±0.47^{d}	***
SER	7.24±0.89^{a}	9.59±0.99^{ab}	10.21±2.38^{b}	12.91±0.44^{c}	*	9.16±0.30^{a}	11.68±0.77^{b}	15.60±0.49^{d}	13.51±0.89^{c}	***	11.42±0.39^{a}	14.06±2.37^{a}	18.81±2.60^{b}	17.86±1.39^{b}	*
PRO	6.06±0.63^{a}	7.25±0.63^{b}	8.29±0.86^{b}	12.31±0.24^{c}	***	8.74±0.34^{a}	11.86±1.75^{b}	9.68±0.54^{a}	15.52±0.57^{c}	**	12.91±1.72^{a}	14.44±0.67^{ab}	15.55±1.45^{b}	20.36±0.18^{c}	**
ASN	9.18±0.31^{a}	11.00±0.95^{ab}	9.15±0.84^{a}	12.75±1.83^{b}	ns	13.08±1.24^{b}	9.96±1.00^{a}	14.36±0.67^{bc}	15.72±0.35^{c}	**	17.20±1.33^{b}	11.52±1.43^{a}	20.54±1.66^{c}	24.15±0.51^{d}	***
ASP	6.52±0.57^{a}	8.17±0.06^{b}	6.88±0.29^{a}	6.85±0.62^{a}	*	7.48±0.73^{a}	9.90±0.10^{b}	8.62±0.14^{a}	13.31±1.10^{c}	***	10.59±1.56^{a}	12.31±2.04^{ab}	13.94±1.30^{b}	17.63±0.88^{c}	*
GLU	8.87±0.12^{a}	11.32±0.60^{b}	10.34±1.42^{ab}	20.53±1.07^{c}	***	10.75±0.65^{a}	13.28±1.29^{b}	13.24±0.76^{b}	22.89±1.51^{c}	***	19.68±2.17^{a}	19.74±2.46^{a}	27.78±4.35^{b}	42.42±0.47^{c}	***
GLN	26.14±2.47^{a}	27.23±0.75^{a}	24.45±2.01^{a}	32.88±1.84^{b}	*	29.99±3.42^{a}	35.19±1.41^{b}	34.26±2.50^{ab}	44.60±3.01^{c}	**	39.68±1.83^{a}	45.38±0.98^{b}	52.89±3.24^{c}	68.50±3.04^{d}	***
ORN	1.30±0.07^{a}	2.17±0.29^{b}	2.67±0.07^{c}	2.25±0.18^{b}	***	1.82±0.11^{a}	2.48±0.42^{b}	3.49±0.44^{c}	2.87±0.25^{b}	*	3.03±0.07^{a}	3.70±0.49^{b}	4.14±0.22^{b}	5.76±0.29^{c}	***
TYR	7.08±0.46^{a}	8.07±0.20^{b}	8.97±0.01^{c}	8.18±0.63^{b}	*	19.69±1.74^{a}	22.03±2.96^{a}	20.72±0.81^{a}	21.40±5.15^{a}	ns	372.23±9.39^{ab}	350.73±16.78^{a}	390.79±13.59^{b}	458.64±12.41^{c}	***
Total	169.28±3.06^{a}	210.5±4.93^{b}	198.96±8.35^{b}	272.43±11.74^{c}	***	291.62±8.94^{a}	326.80±11.24^{b}	370.05±8.66^{c}	415.95±19.58^{d}	***	1614.33±50.81^{a}	1691.62±82.21^{a}	1867.05±77.97^{b}	2174.88±48.69^{c}	***

Results are given as Mean ± S.D.

Values with different superscripts (^{a}, ^{b}, ^{c}, and ^{d}) in the same row of the same digestion phase differ significantly within the same cut in five different treatments.

Amino acids in bolded fonts represent essential amino acids, and those in normal fonts stand for non-essential amino acids.

$P < 0.0001$ presented as $***$ for level of significance; $P < 0.001$ presented as $**$ for level of significance; $P < 0.01$ presented as $*$ level of significance and ns meaning not statistically significant. GC analysis was made in triplicate for each of the sample tested.

Table 16 The total essential FAAs (mg/100 g lamb) and its percentage (%) in total FAAs of seven PEF processed lamb cuts at three different digestion stages: before digestion (initial stage), gastric phase and intestinal phase of the *in vitro* digestion

Cut		Before digestion					Gastric phase					Intestinal phase				
	Treatment	Control		PEF			Control		PEF			Control		PEF		
	Storage	0 day	7 days	0 day	7 days	P	0 day	7 days	0 day	7 days	P	0 day	7 days	0 day	7 days	P
Bolar	EAA	60.27±5.22^{a}	101.66±7.90^{c}	81.29±5.12^{b}	140.78±4.54^{d}	***	129.74±9.22^{a}	175.00±8.65^{b}	169.84±3.99^{b}	209.41±15.64^{c}	**	745.45±45.32^{a}	904.23±53.71^{b}	988.74±77.09^{b}	1188.74±67.32^{c}	**
	EAA %	34.26^{a}	45.04^{b}	35.15^{a}	47.26^{b}	***	50.36^{a}	52.33^{b}	49.99^{a}	52.70^{b}	ns	65.54^{a}	68.09^{b}	66.66^{a}	69.84^{c}	**
Cube roll	EAA	58.84±0.61^{a}	80.63±2.21^{c}	75.75±4.15^{b}	87.86±0.81^{d}	***	120.35±5.43^{a}	150.64±1.25^{bc}	149.08±11.20^{b}	169.4±16.38^{c}	*	1122.79±23.98^{A}	1114.99±43.73^{A}	1253.42±13.83^{B}	1381.35±15.91^{C}	***
	EAA %	36.11^{a}	43.5^{c}	39.13^{b}	42.16^{c}	***	47.22^{a}	51.51^{c}	48.89^{ab}	50.13^{bc}	*	70.71^{B}	69.29^{A}	68.31^{A}	70.52^{B}	*
Flat	EAA	78.91±1.11^{A}	121.62±4.18^{C}	103.68±7.24^{B}	131.02±6.27^{C}	***	151.36±6.00^{a}	196.45±6.25^{bc}	186.37±8.99^{b}	208.33±10.57^{c}	**	967.27±24.23^{a}	1247.78±58.74^{c}	1158.33±25.34^{b}	1457.26±45.62^{d}	***
	EAA %	43.99^{A}	48.29^{C}	45.54^{AB}	46.75^{BC}	ns	52.75^{a}	55.17^{b}	52.55^{a}	53.02^{a}	ns	67.11^{a}	69.13^{b}	70.34^{bc}	71.54^{b}	**
Full back	EAA	92.47±5.27^{a}	104.69±2.98^{b}	103.92±1.43^{b}	160.04±2.46^{c}	***	191.38±11.22^{a}	210.01±0.85^{b}	258.13±11.21^{c}	302.64±2.00^{d}	***	1357.12±49.38^{a}	1600.55±59.17^{b}	1733.93±20.09^{c}	2031.94±26.35^{d}	***
	EAA %	46.06^{bc}	44.88^{ab}	43.51^{a}	47.42^{c}	*	55.96^{bc}	54.93^{ab}	57.64^{c}	53.52^{a}	*	68.92^{a}	68.46^{a}	70.80^{b}	69.85^{ab}	ns
Knuckle	EAA	74.88±4.62^{a}	112.86±6.57^{c}	90.11±8.36^{b}	150.88±6.33^{d}	***	126.43±3.35^{a}	202.13±29.89^{b}	152.84±8.17^{a}	264.23±13.29^{c}	***	1051.70±40.43^{a}	1559±26.43^{c}	1352.43±51.87^{b}	1759.11±42.21^{d}	***
	EAA %	40.97^{a}	47.16^{c}	42.94^{b}	48.44^{c}	***	47.51^{a}	50.91^{b}	48.24^{ab}	55.78^{c}	**	70.71^{a}	72.79^{b}	72.55^{b}	70.95^{a}	**
Rump	EAA	77.99±2.38^{a}	101.76±5.56^{b}	96.53±3.09^{b}	137.33±5.27^{c}	***	176.57±10.88^{a}	205.84±10.89^{b}	241.34±9.17^{c}	242.55±12.44^{c}	**	1341.20±46.01^{a}	1394.32±43.98^{a}	1407.82±55.36^{a}	1516.96±65.92^{b}	ns
	EAA %	43.72^{a}	46.13^{b}	43.62^{a}	46.71^{b}	*	50.88^{a}	51.03^{a}	53.69^{b}	51.07^{a}	ns	71.69^{c}	70.13^{b}	69.62^{ab}	68.71^{a}	*
Shank	EAA	60.61±2.21^{a}	84.92±3.36^{b}	77.61±2.43^{b}	111.76±6.22^{c}	***	143.70±4.64^{a}	160.17±10.04^{a}	187.91±3.11^{b}	196.27±14.31^{b}	**	1065.33±43.94^{a}	1149.19±61.35^{ab}	1238.32±55.28^{b}	1420.88±36.88^{c}	**
	EAA %	35.81^{a}	40.33^{bc}	39.03^{b}	41.01^{c}	**	49.28^{b}	48.98^{b}	50.79^{b}	47.15^{a}	ns	65.98^{a}	67.93^{b}	66.32^{a}	65.33^{a}	*

The total essential FAA results are given as Mean ± S.D.

EAA represents for essential amino acids.

Values with different superscripts (^{a}, ^{b}, ^{c}, and ^{d}) in the same row of the same digestion phase differ significantly within the same cut in four different treatments.

$P < 0.0001$ presented as $***$ for level of significance; $P < 0.001$ presented as $**$ for level of significance; $P < 0.01$ presented as $*$ level of significance and ns meaning not statistically significant.

4.1.3 Effect of proteolysis on digestibility of HPP and PEF treated lamb cuts

Significant differences from initial to the end of digestion process with the corresponding samples of two HPP and seven PEF treated lamb cuts can be easily found from Table 12 and Figure 7, and Table 15 and Figure 9, respectively. Digestibility, measured in the form of FAAs, is likely to be related to proteolytic activities of specific enzymes in different phases. As what was introduced in Section 3.5.3, the samples were taken only from gastric phase and intestinal phase during digestion simulation. In oral phase, α-amylase was the only enzyme present, and it is known to be only involved in starch hydrolysis. Almost no protein or fat digestion occur in the mouth (Minekus, Alminger, et al., 2014). Thus, it was expected that there would be no significant difference of FAAs after oral food processing compared with prior to digestion. Due to α-amylase's negligible influence on the protein digestion, the effects of α-amylase on HPP and PEF processed lamb meat in oral phase were neglected and no sample was taken.

Except simulating human mouth chewing, the current project studied the digestion in stomach and small intestinal lumen. The protein digestion, also called acid-peptic hydrolysis, mainly begins in the stomach, where the acidic environment benefits on protein denaturation. Pepsin, a non-specific protease, is the only proteolytic enzyme in the gastric phase, existing in many isoforms (Berg, Tymoczko, & Stryer, 2002; Minekus, Alminger, et al., 2014). It was reported by Ulshen (1987) that pepsins are most active at peptide bonds such as VAL, LEU, PHE, TYR and GLU. Especially, between TYR and LEU, VAL and LEU, or between aromatic amino acids like PHE-PHE or PHE-TYR, pepsins play most pronounced effects (Krehbiel & Mattews, 2003). As shown in HPP results in Table 12, the values of such essential amino acids

as LEU and PHE were increased significantly and reached the highest values in the gastric phase, at approximately 200% to 300% respectively, when compared to before digestion. GLU, one of the non-essential amino acids, increased for about 600% prior to digestion, for example, from 3.44±0.26 to 20.83±0.64 mg/100 g lamb in flat 300 MPa sample although its concentration in lamb muscles was overall relatively low. Another non-essential amino acid found in high amount, TYR, was found in the highest concentration in 300 MPa treated flat sample, which increased from 12.02±0.49 to 43.23±1.56 mg/100 g lamb, for more than 300 % when compared to prior to digestion. Similar results can be found in PEF results in Table 15. For instance, the increases of LEU, PHE and TYR were found for around 200%, 300 % and 600 % in full back P&7. These significant increases were believed to be achieved from pepsin activity. Other residues like SER and TRP were also reported to be cleaved by pepsin fast, by Schmelzer et al. (2007), while several X-PRO cleavages, tryptophan-methionine, valine-methionine, and other cleavages between non-polar amino acids were only detected after 60 min of pepsin digestion, which means the rate of breaking down of these residues were limited to a certain extent.

After the digestion in stomach, the chyme enters the duodenum of the small intestine and is further broken down into smaller peptides and amino acids by the enzymes secreted from the pancreas and the small intestine. The enzymes involved in the digestion in the intestinal phase are pancreatic exopeptidases (trypsin, chymotrypsin, elastase), and pancreatic endopeptidases (carboxypeptidase A and B). Because of the difficulty in sourcing, I used pancreatin from porcine pancreas (3X, U.S.P., MP Biomedicals, LLC) instead of individual enzymes in this static *in vitro* digestion protocol, which was readily available and included all of the important pancreatic enzymes in different amounts. The amount of pancreatic enzymes was adjusted based on the activities of trypsin and chymotrypsin. Another two enzymes used in this protocol were bile extract from porcine, which plays important roles in the transport of lipolysis products and lipase from Candida antarctica B which works for lipid

degradation (Minekus, Alminger, et al., 2014). These did not contribute significantly on protein hydrolysis in the present study. In Table 12 and Table 15, the concentrations of such amino acids as LEU, PHE, TYR and TRP increased violently for about 5 to 8 times more than the ones in the gastric phase. Even the increases for more than 10 times were commonly found in many of the PEF samples. This may be due to the susceptibility of those linkage involving aromatic amino acid residues to chymotrypsin. Another highly increased amino acid in both HPP and PEF treated lamb samples, LYS, was reported to be easily hydrolyzed by trypsin because it catalyzes the cleaving of peptide bonds on the carboxyl side of basic cationic amino acids. Trypsin also plays most pronounced effects on ARG which was not able to be identified in this study but do exist in lamb meat. Although elastase is not as selective as another two pancreatic exopeptidases, it catalyzes the breakdown of peptide bonds of the carboxyl side of aliphatic amino acids such as ALA, LEU, ILE, GLY and VAL in general (Krehbiel & Mattews, 2003; Sitrin, 2014). Consequently, as a double effect under the catalysis of chymotrypsin and elastase, it is not strange that the amount of LEU was increased sharply and was found in the highest value among all of the individual amino acids at the end of the digestion in both HPP and PEF processed lamb samples. Apart from these, almost all of amino acids contain a carbonyl group, which is a good hydrogen-bond acceptor, with one exception that proline, containing an imino group, is a good hydrogen-bond donor. As a result, proline-containing peptides were reported to be resistant to be hydrolyzed by pancreatic enzymes, which was consistent to the results of current study, as shown in Table 12 and Table 15 (Berg et al., 2002; Sitrin, 2014). There was only a slight increase of proline observed at the end of the intestinal phase, which was less than 100 % of increase found in the intestinal phase when compared to the one at the end of gastric phase, in most of these samples.

As shown from Figure 7 and Figure 9, the amount of FAAs increased slightly at the end of gastric phase, while after another three-hour pancreatic proteolysis, it leaped to

a very high level. Cheng (2016) reported that the maximum yield of acid-peptic hydrolysis on breaking down the dietary proteins is 15 %. The rest of digestion is accomplished in the intestinal phase. In other words, the proportion of the FAA yield between the gastric phase and intestinal phase is maximum for 15%:85%, which equals to 1:5.6. Similar result of low efficiency in peptic degradation was shown by Restani et al. (1992). They investigated lamb meat and showed that the percentage of FAAs released during pepsin attach was about 10 %. In addition, pancreatic enzymes were reported to be more powerful on protein proteolysis than that of the pepsin, for approximately 40 % of FAAs. Another 60 % of amino acids would present oligopeptides of up to six amino acid residues (Krehbiel & Mattews, 2003; Sitrin, 2014). This could be supported in the present study as shown in Table 12 and Table 15. The proportions of the total FAAs in two phases were similar to Cheng (2016)'s results. For instance, in the HPP samples, the total FAAs content was about 2000 mg/100 g lamb in the intestinal phase and 450 mg/100g lamb in the gastric phase. The proportions between the gastric phase and intestinal phase of the flat control sample and inside 300 MPa sample were 1:3.5 and 1:4.9, respectively. In the PEF samples, the proportion for the full back P&7 was 1:5.1. The current study partially supports the argument by Sitrin (2014) that pepsin digestion is not as essential as intestinal digestion for efficient protein digestion. For example, from the view of medical, for a patient who has undergone the total gastrectomy operation, there is little impairment on protein intake. But conversely, the pepsin digestion could be vital for protein digestion and absorption to a matter of life and death for a patient with pancreatic disease and limited secretion of pancreatic proteases. However, although I have regarded FAAs concentration as a measure of the digestibility of protein, the peptides should be considered as the major digestive products, which Cheng (2016) reported that the peptides are found to be present three to four times more than that of the FAAs at the end of digestion.

It is interesting to note that the gastric secretion plays synergistic effects on peptides

and amino acids in both digestion stages, which in simple terms, peptides and amino acids release gastrin in G cells, and gastrin improves protein digestion (Cheng, 2016). Sitrin (2014) reported that not only does the proteolysis in the stomach contribute to the overall protein digestion, but also the peptides and amino acids generated are important stimulators to minimize the repetition of gastrin promoting gastric acid secretion to maintain pepsin activity that increases protein digestion. In addition, the amino acids absorbed into the blood stream stimulate the secretion of growth hormone, which eventually increases the cellular uptake of the amino acids are increased. In the intestinal phase, the gastric acid secretion is increased by vagovagal reflexes because of duodenal distension, stimulating the antral G and parietal cells in the duodenum and proximal jejunum. These cells are able to release gastrin, which can be promoted by the stimulation from peptides and/or amino acids. Moreover, the amino acids and peptides absorbed may circulate to the antral G cells following with a further increased gastrin secretion (Cheng, 2016). With a view to the synergistic effects between peptides/amino acids and gastric secretion which are not taken into the consideration in this static *in vitro* digestion model, more FAAs in the end of digestion could be expected to be digested and absorbed in the real human body. However, until now, this remains as an inference, and more studies are required to prove.

To sum up, both HPP and PEF technologies have proved to have positive effects to improve the nutritional value and digestibility of lamb muscles. PEF has significantly different effects on different cuts of lamb in terms of digestibility, and has shown to be highly selective on the microstructures of lamb muscles. This conclusion is not applicable to HPP because only two lamb cuts were analyzed. More cuts of lamb need to be investigated to study the effects of HPP on lamb microstructures from different muscles.

4.2 Moisture content

The moisture content of HPP and PEF lamb samples is presented in Table 17. HPP samples were analyzed both before cooking and after cooking for moisture. Only the moisture of cooked samples are presented for PEF samples due to the lamb samples being cooked inevitably during the PEF processing under the effects of electric fields.

Significant differences in moisture were found in HPP samples for both before and after cooking, as shown in Table 17a, while the impact of the pressures on different cuts were quite different. In flat, the highest moisture was found in both before and after cooking at 200 MPa pressure treatment; 72.24±0.04 % and 69.40±0.53 %, respectively. After an increase in moisture seen up to 200 MPa treatment, it started to decrease with the increase of pressures until 500 MPa. The differences between before cooking and after cooking were that before cooking, the moisture content of the 300 MPa (69.22±0.39 %) and 400 MPa (69.54±0.79 %) samples were higher than the control sample (65.38±0.60 %) significantly and it was only lower than the control one when the pressure was increased to 500 MPa (63.20±0.59 %). However, after cooking, only the value of 200 MPa was higher than the control and the others were lower.

For the inside cut, an interesting trend was found before cooking that the moisture content decreases with the increases of pressure from 0 MPa (control) to 400 MPa while an increase in moisture was observed when the pressure was increased from 400 MPa to 500 MPa and its value was close to the one of 200 MPa. After cooking, similar trend was found as in flat cut that 200 MPa contained the most moisture and with the increasing pressures, the moisture content decreased. However, the moisture contents of the inside cut under the pressure treatments from 200 MPa to 500 MPa were all higher than the control.

The moisture content is an important index on meat quality which influences the flavour and texture traits (Virgili, Parolari, Schivazappa, Bordini, & Borri, 1995) and juiciness arising from moisture, released by meat during chewing and from saliva (Hahaoua et al., 2016). Scarce information is available investigating the relation between moisture content and colour. Vieson et al. (2007) demonstrated that beef steaks from *gluteus medius* roasts under moisture enhancement treatment resulted in darker, less red and less yellow than the non-treated samples, while no significant difference was reported on lightness and yellowness from subjective color panel data which indicates that the differences in lightness and yellowness in beef steaks may not be visible to the consumers. But at least, it implied that the moisture content of meat muscle do have effects on meat colours. Besides, Vieson et al. (2007) also reported that enhancing the moisture tenderized beef steaks and improved the juiciness. Thus, the highest values of moisture content contributed by 200 MPa pressure treatment in both flat and inside cuts after cooking indicate that 200 MPa pressure is a good processing parameter to improve the sensory quality of lamb flat and inside.

However, the positive effect of 200 MPa on improving the moisture content of flat and inside cannot be extended to all of the pressure treatment because the decreases were found obviously in both cuts in higher pressures from 300 MPa to 500 MPa. Similar negative results were reported by Duranton et al. (2012) and Marcos, Kelly and Mullen (2010) that high pressure treatment of 500 MPa and 400 to 600 MPa reduced water retention capacity on pork *biceps femoris* muscle and Beef bovine *longissimus dorsi* muscle. However, an early research reported by Macfarlane (1973) showed opposite results that sheep ovine and bovine muscles under HPP of 103 MPa resulted in higher moisture content than non-pressurized sample. Besides, an interesting result was also reported by the same author that the juiciness was evaluated less in the sample which contained more moisture. Furthermore, the cooking loss is an important factor influencing the moisture content of lamb samples after cooking. It is ascribed to a temperature-induced, structural shrinkage,

contributing to fluid's being expelled from the meat (Omojola et al., 2014). It is worth noting that due to sous vide cooking that prevents evaporative loss of moisture during cooking to the most extend, being the cooking method used in this study (Baldwin, 2012), the moisture loss in current study after cooking was kept in a very low degree, for example, 2.84 %, 0.99 % and 3.80% of moisture loss in 200, 300 and 400 MPa sample in flat, respectively, compared to the 33-37 % of cooking loss of meat samples (sources of meat not available) from conventional heat cooking reported by Anwer et al. (2013), and 36.07 % of moisture loss of Muscovy drakes fillets after deep frying reported by Omojola et al. (2014). The decreases of moisture content may possibly result from the fats by serving as a barrier against moisture loss to preventing evaporation from lean (Smith & Carpenter, 1973). However, some strange moisture results were also found in the HPP table. There were some increases of moisture after cooking in both cuts. It is estimated that the increases may have resulted from the higher fat losses during cooking. Nikmaram, Yarmand and Emamjomeh (2011) reported that cooking has an influence on the magnitude of fat losses: the longer cooking time had higher fat loss. It might be one of the factors increasing the moisture loss of the samples. Although it is opposite to the theories we have known, it is not unrealistic if higher fat loss replaces the moisture being expelled from the meat, resulting increase of moisture content in meat samples per unit. Even though, no previous study was able to sufficiently explain or support this phenomenon. A study of the fat loss and moisture loss after cooking on HPP lamb samples could be applied in further study to answer this question.

However, 200 MPa has just been proved to be the best condition to improve moisture content in lamb flat and inside cuts among these four HPP treatments, whether it is the optimum pressure on moisture content is still uncertain. This should be investigated in further research. In addition, from the aspect of cuts, the extent of HPP on the changes of moisture content in flat was larger than that of the inside, which means that the inside cut has lower susceptibility to the effect of HPP moisture content compared to

flat. It suggests that the microstructure of inside cut has better capacity to hold moisture.

Significant differences of moisture content in the PEF processed lamb cuts in cooked samples were found. From Table 17b, it is obvious that the storage time played important roles in affecting the impact of PEF on lamb muscles, in terms of moisture. For those samples stored for 0 day, only the cube roll 's and rump's moisture contents have increased after PEF processing and the others' have decreased significantly. For those that were stored for 7 days, a contrasting result was found that the moisture contents of six lamb cuts were increased by PEF processing except that the full back cut was decreased from 65.01±0.05 % to 62.40±0.15 %, significantly. Previous studies have supported the decreasing phenomenon of PEF on moisture content in meat. O'Dowd et al. (2013) reported that the moisture content of beef *Semitendinosus* muscle decreased from 74.6 % to 73.4 % after PEF processing (electric field intensity of 1.9 kV cm−1, frequency of 65 Hz, 250 pulses and pulse width of 20 μs) compared to the control. The authors reported that it might be attributed to the greater weight loss (also analyzed in the study) observed in PEF processed samples which could have reduced the amount of water expressed. Similar results were reported by Bekhit et al. (2014) that a higher purge loss was found in beef topsides *Musculus semimembranosus* muscle after PEF processing (Voltage of 5 and 10 kV, frequencies of 20, 50 and 90 Hz), which indicates a decrease in moisture content. It might be resulted by the proteolysis that alters the ability of muscle to retain moisture so that their myofibril structure and ability to contain moisture were changed (Bekhit, Van De Ven, Suwandy, Fahri, & Hopkins, 2014; Pearce, Rosenvold, Anderson, & Hopkins, 2011). Moreover, from the aspect of storage time, it is reasonable to see the higher moisture content in C&0 than in C&7 because the water was frozen during the storage and its damage to the ultra-structure of lamb fibres did not allow to take moisture into the intracellular spaces (Leygonie et al., 2012b). It is not a good signal for the sensory attributes of meat quality. Leygonie, Britz and Hoffman (2012b) reported that the

toughness of the ostrich *M. iliofibularis* muscle was increased with increasing shear force due the shrinkage of the fibres, as water leached out of the meat during thawing so that the muscles fibres become less hydrated. Similar results were reported by Lagerstedt (2008) that the frozen beef *M. longissimus dorsi showed lower sensory scores in tenderness, juiciness and meat taste than the chilled one, which indicating a negative effect of frozen processing on beef muscles.* However, interesting results were still detected in knuckle and rump cuts that the values of C&7 were higher than C&0. In addition, regardless of the effects of PEF on lamb muscles, among these seven cut, the bolar contained the highest amount of moisture expressed after PEF processing no matter under 0 day or 7 days of storage, while the cube roll contained the least.

Table 17 Moisture Content of HPP lamb samples before and after cooking (a) and PEF lamb samples after cooking (b)

(a)

HPP						
Cut	Flat					
Process	Control	200 MPa	300 MPa	400 MPa	500 MPa	P
Before cook (%)	65.38 ± 0.60^{b}	72.24 ± 0.04^{d}	69.22 ± 0.39^{c}	69.54 ± 0.79^{c}	63.20 ± 0.59^{a}	***
After cook (%)	68.25 ± 0.52^{b}	69.40 ± 0.53^{b}	68.23 ± 1.00^{b}	65.74 ± 0.98^{a}	64.02 ± 1.75^{a}	**
Cut	Inside					
Process	Control	200 MPa	300 MPa	400 MPa	500 MPa	P
Before cook (%)	73.12 ± 0.11^{d}	72.03 ± 0.16^{c}	70.22 ± 0.56^{b}	69.14 ± 0.39^{a}	72.01 ± 0.40^{c}	***
After cook (%)	70.36 ± 1.08^{a}	73.72 ± 0.64^{c}	71.28 ± 0.49^{ab}	71.89 ± 1.09^{b}	70.79 ± 0.07^{ab}	*

(b)

PEF					
Process	Control (cooked)		PEF		P
Storage	0 day	7 days	0 day	7 days	
Bolar	68.67±0.16^{b}	61.43±1.05^{a}	67.99±0.34^{b}	68.20±0.46^{b}	***
Cube roll	59.78±0.77^{b}	58.82±0.18^{a}	61.46±0.46^{c}	61.04±0.18^{c}	**
Flat	67.16±1.75^{c}	63.81±1.05^{a}	66.46±0.69^{bc}	64.70±0.13^{ab}	ns
Full back	66.43±0.15^{d}	65.01±0.05^{c}	63.43±0.31^{b}	62.40±0.15^{a}	***
Knuckle	61.96±0.56^{ab}	62.68±0.32^{bc}	61.64±0.42^{a}	63.39±0.12^{c}	*
Rump	62.45±0.32^{a}	63.22±0.89^{ab}	64.10±0.33^{bc}	64.28±0.51^{c}	ns
Shank	68.95±0.56^{c}	63.74±0.31^{a}	62.76±0.94^{a}	67.71±0.40^{b}	***

Values with different superscripts (^{a}, ^{b}, ^{c} and ^{d}) in the same row differ significantly within the same cut in five different treatments.

P < 0.0001 presented as ∗∗∗ for level of significance; P < 0.001 presented as ∗∗ for level of significance; P < 0.01 presented as ∗ level of significance and ns meaning not statistically significant. Measurements were made in triplicate.

4.3 Colour

The instrumental colour of HPP and PEF processed lamb samples are presented in Table 18. For the same reason as the moisture analysis, the colour results of PEF samples before cooking could not be measured.

From Table 18a, the effect of pressure treatment on L^{*}, a^{*} and $b^{*}$was mostly significant (P<0.001), in both flat and inside cuts either before or after cooking while a^{*} values of inside after cooking was not significantly affected by the pressure treatment. The lightness, redness and yellowness of flat and inside cuts showed absolutely different relations to pressures. Non-pressurized flat sample showed a higher lightness than pressurized samples, of both before and after cooking. However, higher pressure treatment of 500 MPa promoted an increase in lightness which the

value was reaching similar to that from non-pressurized sample before cooking and higher after cooking. It is consistent to the results reported by Andres et al. (2004) on dry-cured Iberian ham under HPP of 200, 400, 600 and 800 MPa. In inside cut, pressurized samples showed an increase in lightness with the increase of pressures except that two unexpected decreases were found at 300 MPa before cooking and 500 MPa after cooking. This could be supported by Serra et al. (2007) that the pressurized *Biceps femoris* green ham (400 and 600 MPa) showed higher lightness than the control. The higher paleness of lamb meat through pressure processing may have resulted from the increase in protein denaturation, which a 'whiteness effect' is caused by globin denaturation and/or by heme displacement or release in the range of 200-350 MPa (Carlez, Veciana-Nogues, & Cheftel, 1995). Similar trends of redness in flat cut before and after cooking were found that the redness increases with the increase of pressures and decreases after 400 MPa before cooking and after 300 MPa after cooking. The redness values of pressurized inside samples were lower than the non-pressurized one before cooking. They were significantly decreased with pressure until 400 MPa, whereas 500 MPa treatment caused an increase in a^* value. This was similar to the results of Andres et al. (2007) that the redness of dry-cured Iberian ham decreased until 600 MPa and an increased was detected at 800 MPa. It could be explained as alteration of protein structure caused by pressurization, affecting the surface and structure properties. Carlez, Veciana-Nogues and Cheftel (1995) reported that redness (a*) of minced beef *Semimembranosus* decreased when treated above 350 MPa and it was caused by the oxidation of ferrous myoglobin to ferric metmyoglobin at or above 400 MPa. For yellowness, it increased with pressure until 400 MPa before cooking and 300 MPa after cooking in flat and decreased afterwards. A continuous decrease of yellowness was found in inside cut before cooking, which was similar to the findings by Tintchev et al. (2010) that the yellowness of smoked salmon decreased with increases of high pressure from 100 MPa to 600 MPa, and Jimenez-Colmenero et al. (1998) that the yellowness decreased with increases of high pressure from 0 MPa to 400 MPa with combination of 60 and 70 °C of thermal treatments in chicken batter

(mixture of *pectoralis major* and *pectoralis minor* muscles) and 60 °C in pork batter (mixture of *M. biceps femoris, M. semimembranosus, M. semitendinosus, M. gracilis* and *M. aductor*). Thus, Hogan et al. (2005) reported that the yellowness in meat varies considerably depending on the origin of the meat. The yellowness of meat is related to the fat due to the presence of β-carotene (a precursor of vitamin A) in the fat, which the greater the concentration of carotene in fat, the more intense is the yellowness (Johnson, 1991). From Table 18a, 200 MPa of flat and 300 MPa of inside show the highest b* value that reflects the higher concentration of fat contents. A hypothesis can be raised that the yellowness results indicated HPP of 200-300 MPa may have be effective to release more lipids or fatty acids from meat muscles. Further research on lipid or fatty acid contents of these HPP samples should be applied to prove this hypothesis.

As shown in Table 18b , the lightness of PEF treated lamb samples were affected by the specific microstructure of different cuts. Three cuts (bolar, cube roll and knuckle) of PEF treated samples were shown to increase in the lightness regardless of the storage time. The lightness of another three cuts (flat, full back and rump) were decreased by PEF processing. The lightness of PEF processed shank samples was increased in 7 days of storage sample but decreased in 0 day one. The redness values were decreased by PEF treatment for most of cuts over the storage times. The increases were only found in 0 day's storage of bolar, cube and flat cuts, and 7 days' storage of flat and shank cuts. The decreases of redness values in meat samples under PEF processing have been reported in many studies (Arroyo et al., 2015; Faridnia, Bekhit, Niven, & Oey, 2014; Faridnia, Bremer, Burritt, & Oey, 2016; Suwandy, Carne, Van De Ven, Bekhit, & Hopkins, 2015). The redness of meat is highly dependent on the meat structure, the concentration and the oxidation state of myoglobin present in the muscle (Suwandy et al., 2015). It has been believed to be a natural course of the meat pigment during the display , to be exposed to oxygen, and this causes the oxidation of oxymyoglobin (bright red colour) and deoxymyoglobin (dark red colour)

to metmyoglobin (brown colour) (Faridnia et al., 2016). Similar to the lightness, the effects of PEF on the yellowness of lamb samples were significantly affected by the muscle type. For 0 day of storage, the PEF processed flat, full back, knuckle and rump increased in yellowness significantly while bolar and cube roll decreased significantly. There was no significant effect on shank although a slight increase was observed. For 7 days' of storage, PEF significantly increased yellowness for most of cuts including the cube roll, full back, knuckle, rump and shank. It suggests that PEF have selective effects on increasing the fat content of lamb depending on the muscle of the cuts. Scarce information is available on lipids of PEF processed meat, hence more studies are required in this area.

From the angle of storage, a brighter appearance (increases of lightness) were found in bolar, cube roll, flat and knuckle after 7 days of storage. The redness deceased after freezing processing in almost all of the cuts except in full back. Based on these results, a paler effect (higher luminance and lower colour saturation) of frozen storage on lamb muscles could be shown. The denaturation of the globin moiety of the myoglobin is the important factor for the changes of meat colour after freezing which it leads to an increased susceptibility of myoglobin to autoxidation and subsequent loss of optimum colour presentation (Leygonie et al., 2012a). Farouk and Swan (1997) reported that the increased lightness may result from lipid oxidation. The lipid oxidation increases muscle protein denaturation, causing more light scattering. The less redness may have resulted by the greater protein denaturation at higher rigor temperature, which would allow a higher metmyoglobin concentration to lower a* values (Ledward, 1985). Akamittath, Brekke, and Schanus (1990) reported that lipid oxidation and discoloration occurred simultaneously in pork and turkey, indicating that increasing lipid oxidation increased lightness, as well as it decreased redness. In addition, the lack of redness enhanced yellowness, as reported by King and Whyte (2006), who showed partly consistent to results to the current study. Similar results

were reported by Farouk and Price (1994) that the enhancement in yellowness during storage was accompanied by decreases in redness in bovine muscles.

Khliji et al. (2010) reported that consumers' acceptability to lamb colour was related with the lightness (lighter lamb more preferred [$L^* \geq 34$]) but mainly with the redness (the reddest the most preferred [$a^* \geq 9.5$]) of the meat. Font-i-Furnols and Guerrero (2014) also summarized that red was the most preferred color, followed by purple and finally brown. Within red, bright red was preferred to pale red or dark red. The yellowness was reported not to affect the palatability of the cooked meat. But most consumers considered it objectionable due to it incorrectly suggests that the meat muscle may have come from an old, malnourished or unhealthy animal while fat is usually white or off white (Troy & Kerry, 2010), and the yellow color was associated with a high fat content, rancidity and lack of freshness (Kennedy, Stewart-Knox, Mitchell, & Thurnham, 2005). From this perspective, the 400 MPa treated flat sample and 200 MPa treated inside sample are believed to be the most efficient pressure level for HPP to be applied for lamb meats for consumers regardless of other quality attributes. In addition, the PEF results indicated that PEF treatment can be used without adverse effects on meat instrumental colour parameters for most of lamb cuts with no storage period, but due to PEF processing under 7 days of frozen storage decreasing redness sharply, especially in bolar, cube roll and knuckle, it may affect consumers' preference.

Table 18 Colour analysis of HPP lamb samples before and after cooking (a) and PEF lamb samples after cooking (b)

(a)

	HPP						
	Process	Control	200 MPa	300 MPa	400 MPa	500 MPa	P
Before cook (Flat)	L	50.52±0.37^{b}	39.60±2.06^{a}	48.95±3.31^{b}	43.81±0.08^{a}	51.05±0.10^{b}	***
	a	5.58±0.25^{a}	6.90±0.36^{b}	7.35±1.26^{b}	7.58±0.03^{b}	4.54±0.06^{a}	**
	b	16.71±0.71^{q}	16.90±0.86^{ab}	17.96±0.91^{bc}	19.83±0.10^{d}	19.03±0.12^{cd}	**
After cook (Flat)	L	47.38±0.78^{c}	46.70±0.50^{bc}	44.31±0.27^{a}	45.92±0.41^{b}	52.04±0.37^{d}	***
	a	4.91±0.04^{b}	5.90±0.14^{c}	7.61±0.31^{d}	5.22±0.05^{b}	3.99±0.16^{a}	***
	b	15.83±0.29^{a}	17.50±0.15^{b}	18.26±0.12^{b}	16.25±1.12^{a}	15.89±0.57^{a}	**
Before cook (Inside)	L	32.36±1.42^{b}	33.18±1.09^{b}	28.68±0.12^{a}	38.49±0.99^{c}	39.81±0.77^{c}	***
	a	12.50±0.35^{e}	9.90±0.07^{d}	8.66±0.13^{c}	5.73±0.20^{a}	6.24±0.23^{b}	***
	b	17.99±0.22^{c}	17.86±0.51^{c}	11.79±0.57^{a}	13.43±0.66^{b}	13.21±0.50^{b}	***
After cook (Inside)	L	42.87±0.44^{a}	43.85±0.69^{b}	45.29±0.17^{c}	47.95±0.46^{e}	46.67±0.47^{d}	***
	a	5.56±0.55^{a}	6.26±0.21^{b}	5.49±0.22^{a}	5.92±0.14^{ab}	5.46±0.28^{a}	ns
	b	15.06±0.39^{b}	17.57±0.19^{c}	14.09±0.08^{a}	15.48±0.04^{b}	14.47±0.32^{a}	***

(b)

	Process	Control		PEF		P
	Storage	0 day	7 days	0 day	7 days	
Bolar	L	33.08±0.67^{a}	34.58±1.00^{a}	38.78±0.99^{b}	41.68±2.00^{c}	**
	a	6.40±0.14^{b}	6.05±0.28^{b}	6.97±0.27^{c}	4.38±0.23^{a}	***
	b	12.10±0.69^{a}	12.33±0.39^{a}	15.38±0.20^{c}	13.83±0.21^{b}	***
Cube roll	L	33.61±0.62^{a}	34.07±0.88^{a}	34.45±2.18^{a}	41.03±0.76^{b}	**
	a	7.06±1.04^{bc}	6.35±0.81^{b}	8.23±0.24^{c}	3.01±0.06^{a}	***
	b	10.12±1.25^{a}	10.88±1.61^{a}	13.74±0.23^{b}	10.59±0.43^{a}	ns
Flat	L	36.52±0.35^{b}	36.79±0.71^{b}	32.30±1.96^{a}	36.62±0.27^{b}	*
	a	5.63±0.16^{b}	4.54±0.47^{a}	6.26±0.88^{b}	6.18±0.16^{b}	ns
	b	12.57±0.48^{b}	11.68±0.32^{b}	10.63±0.89^{a}	12.35±0.27^{b}	ns
Full back	L	37.57±0.53^{c}	35.46±0.89^{b}	35.91±1.49^{bc}	29.14±1.03^{a}	***
	a	7.84±0.35^{b}	8.31±0.70^{b}	5.74±0.48^{a}	6.22±0.54^{a}	**
	b	14.65±0.07^{c}	13.85±0.94^{bc}	13.40±0.41^{b}	11.66±0.56^{a}	*
Knuckle	L	35.72±0.96^{a}	37.30±1.21^{a}	35.96±0.18^{a}	41.33±1.38^{b}	**
	a	5.85±0.37^{b}	7.51±0.46^{c}	5.72±0.27^{b}	4.56±0.16^{a}	***
	b	13.16±0.48^{b}	14.54±0.87^{c}	11.48±0.82^{a}	13.64±0.30^{bc}	*
Rump	L	36.02±0.85^{c}	32.74±1.58^{b}	27.67±1.30^{a}	31.27±0.22^{b}	**
	a	6.20±0.16^{c}	5.75±0.14^{bc}	5.35±0.60^{ab}	5.08±0.12^{a}	ns
	b	12.97±0.15^{c}	11.67±0.43^{b}	9.43±0.78^{a}	9.41±0.33^{a}	***
Shank	L	38.65±2.27^{b}	33.72±0.95^{a}	34.44±1.58^{a}	35.46±1.24^{a}	ns
	a	6.19±0.28^{a}	6.03±0.32^{a}	5.81±0.53^{a}	6.90±1.08^{a}	ns
	b	12.41±0.97^{b}	12.86±0.42^{b}	12.69±0.40^{b}	9.01±0.26^{a}	**

Values with different superscripts (^{a}, ^{b}, ^{c}, ^{d} and ^{e}) in the same row differ significantly within the same

cut in five different treatments.

$P < 0.0001$ presented as $***$ for level of significance; $P < 0.001$ presented as $**$ for level of significance; $P < 0.01$ presented as $*$ level of significance and ns meaning not statistically significant. Measurements were made in triplicate

Chapter 5 Conclusion

In the present study, two HPP cuts (flat and inside) and seven PEF processed cuts (bolar, cube roll, flat, full back, knuckle, rump and shank) from New Zealand lamb were evaluated in term of *in vitro* digestibility and physiochemical properties to characterize whether HPP and PEF technologies increases the nutritional values of frozen lamb in terms of FAAs released from meat proteins.

HPP has been shown to have positive effects on increasing the digestibility, as shown by an increase of total FAAs at lower pressures from 0 MPa to 300 MPa which 300 MPa contributed the most and resulted the highest total FAA contents at the end of the intestinal phase and most of individual amino acids had similar trends to that of the total, while higher pressure processing from 300 MPa to 500 MPa decreased the amount of total FAAs. The effects of HPP on meat proteins are related to the impacts of the degrees of proteins' unfolding and aggregation on its digestibility under different pressures. Between two cuts, the inside contained higher total FAA contents after 300 MPa pressure processing than the flat at the end of the intestinal phase. The instrumental color of HPP lamb differed largely with different microstructure of the cuts. The moisture content of lamb under 200 MPa treatment contributed highest value after cooking in both cuts. More lamb cuts with different muscle structures should be studied for different effects of HPP on lamb cuts. Other kinds of meat, including pork, beef and chicken are also appropriate to study whether HPP have similar positive effect on improving their nutrition qualities.

PEF processing significantly increased the total amount of FAAs of frozen lamb in all three digestion phase. This can be commonly explained by PEF processing inducing proteins to unfold. However, not many articles have been reported to support this result. And higher electrical intensities are suggested to be investigated in the future to see whether they are helpful to increase the nutritional value of lamb. In addition, the

storage times (0 days and 7 days) had significantly different effects on total FAA contents as well, which 7 days of storage was able to release more FAAs after digestion. A combination of PEF processing and 7 days of storage contributed the highest value of total FAA contents at the end of the intestinal phase. Better parameters of PEF processing and storages times could be useful to be studied and developed in the future. The color and moisture content of PEF samples were significantly different in different muscle cuts with respect to the storage times.

Overall, HPP and PEF processing have shown satisfactory characteristics as meat preserving technologies. In particularly, favorable nutritional values and digestibility in processed lamb meats have been found. In the further research, the sensory evaluations of HPP and PEF processed lamb are required. There is no way that consumers accept the meat products with increased nutritional values and unwelcome sensory attributes. The data from this study will be useful to the New Zealand meat industry to increase the value of New Zealand lamb meat, even all of red meat, by processing them to not only extend shelf life but also increase nutritional values. With continuous development of meat preserving technologies, HPP and PEF processing certainly have a potential as commercial meat processing technologies to reach the global market.

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Appendix

1. Reagents and procedures of EZ Faast amino acid kit (GC-FID)

1.1 Reagents

Reagent	Ingredients	Volume
Reagent 1 Internal Standard Solution	Norvaline 0. 2 mM N-propanol 10%	50mL
Reagent 2 Washing Solution	N-propanol	90mL
Reagent 3A Eluting Medium Component I	Sodium Hydroxide	60mL
Reagent 3B Eluting Medium Component II	N-propanol	40mL
Regent 4 Organic Solution I	Chloroform	4 vials, 6mL each
Reagent 5 Organic Solution II	Iso-octane	50mL
Reagent 6 Acid Solution	Hydrochloric Acid 1N	50mL
SD 1, 2, & 3 Amino Acid Standard Mixtures	Please refer to section 4.7 in the manual	2 vials of each SD, 2mL each

3.0 Sample Preparation Procedure

3.1 Setup

The EZ:faast kit packaging has been designed as an efficient workstation. It holds a reagent tray, a vial rack, a pipette rack and a section for sorbent tips and vials. To speed up sample preparation it is recommend that the workstation be arranged as shown in figure 1a. By following directions and markings on the reagent box by breaking along perforations it can be transformed into a reagent tray. When the kit is not in use for several days, the reagent tray (figure 1b) may be conveniently removed and placed in the refrigerator.

3.2 Preparing the Eluting Medium

The volume of prepared Eluting Medium depends upon the number of samples to be analyzed during the day (200µL/sample). The eluting medium should be prepared fresh each day:

1. Use capped vials of appropriate size (not included) for preparation of the Eluting Medium.

2. Combine 3 parts Reagent 3A (Eluting Medium Component I) with 2 parts Reagent 3B (Eluting Medium Component II) in an appropriate sized vial (see Table 2, page 5, for reagent volumes based on number of samples). Mix briefly.
3. Store prepared eluting medium during the day at room temperature. Discard any unused mixture at the end of the day.

Workstation Arrangement - (Figure 1)

To speed up sample preparation it is recommended that the workstation be arranged as shown below.

Figure 1a

Figure 1b

Table 2 - For your convenience check the table below to determine the volume of Eluting Medium components needed depending on your number of samples:

Number of Samples	Reagent 3A Eluting Medium Component I	Reagent 3B Eluting Medium Component II
2	300µL	200µL
4	600µL	400µL
7	900µL	600µL
12	1.5mL	1.0mL
14	1.8mL	1.2mL
19	2.4mL	1.6mL
24	3.0mL	2.0mL
29	3.6mL	2.4mL
34	4.2mL	2.8mL
39	4.8mL	3.2mL
44	5.4mL	3.6mL
49	6.0mL	4.0mL

3.3 Sample Preparation by SPE and Derivatization

Prepare Eluting Medium first; refer to section 3.2 for preparation protocol. The freshly prepared Eluting Medium vial may be placed in one of the empty slots in the reagent tray.

1. For each sample, line up one glass sample preparation vial in the vial rack (Figure 2). Be aware of some variability in vial opening and sorbent tip dimensions, which may prevent the tip from reaching to the bottom of the sample preparation vial.

Note: *Droplets of liquid in SPE tip or spilled sorbent particles will not affect the precision of the assay in any way.*

Glass Vial Line Up - (Figure 2)

For each sample, line up one glass sample preparation vial in the vial rack.

Figure 2

2. Pipette 100µL sample (serum, plasma, urine or other), and 100µL Reagent 1 (Internal Standard Solution) into each sample preparation vial.

Caution: The pH of biological samples is usually around 7. After the addition of Reagent 1 (Internal Standard) the mixture has the correct pH for successful loading onto the SPE tip as described in the next step. With other samples make sure that the sample + Reagent 1 mixture has a pH between pH 1.5 and pH 6.0!

Note: *Samples with amino acid concentrations higher than 10mmol/L (10µmol/mL; e.g. dark colored urine) should be analyzed by pipetting only 50µL (or 25µL) sample in the sample preparation vial instead of 100µL. Concentrations recorded as a result of the GC analysis will be half (one quarter) of the actual concentrations for these samples. Conversely, when low concentrations of amino acids have to be quantified, the volume of sample to be prepared should be 200µL or more.* ***The total amount of amino acids present in the sample to be loaded onto the SPE tip should not exceed 1.2 µmols.***

3. Attach a sorbent tip to a 1.5mL syringe and loosen the syringe piston; immerse the tip and let the solution in the sample preparation vial pass through the sorbent tip by SLOWLY pulling back the syringe piston, in SMALL steps.

Caution: Do not quickly pull back the piston. Try to take at least one minute to pass low viscosity sample (such as urine or standard) through the sorbent tip. For very viscous samples like concentrated plasma, up to 200µL of water can be added to ease the sample transfer through the sorbent. The syringe should be capable of drawing all sample, and subsequent wash reagent into the barrel. Watch as the liquid accumulates inside the syringe barrel and move the piston only as the accumulation slows down. Urine passes relatively fast through the sorbent bed, while serum and plasma pass much slower. If you run out of piston range, detach the sorbent tip, expel the solution from the syringe barrel, then reattach the sorbent tip and proceed with sample preparation.

Note: *the sorbent tip should stay in the sample preparation vial through steps 3-9 (see figure 3) even while dispensing reagents. In case the sorbent tip cannot reach to the bottom of the vial, tilt the vial to about 45°, push the tip into the vial gently, and proceed with the SPE step.*

4. Pipette 200µL Reagent 2 (Washing Solution) into the same sample preparation vial. Pass the solution SLOWLY through the sorbent tip and into the syringe barrel. Drain the liquid from the sorbent bed by pulling air through the sorbent tip. Detach the sorbent tip, and leave it in the sample preparation vial, then discard the liquid accumulated in the syringe.

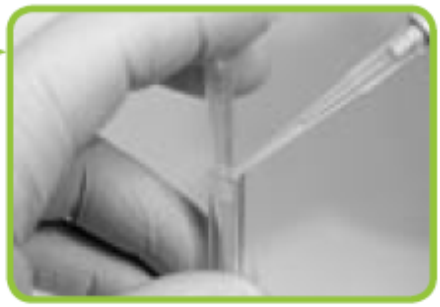
Note: *save the syringe, as it can be reused with many other samples. For convenience place it into the pipette rack.*

5. Pipette 200µL Eluting Medium (prepared fresh each day, section 3.2) into the same sample preparation vial.

Keep the Sorbent Tip in the Vial - (Figure 3)

Keep the sorbent tip in the sample preparation vial through steps 3-8, even while dispensing reagents.

Figure 3



6. Pull back the piston of a 0.6 mL syringe halfway up the barrel and attach the sorbent tip used in steps 3-6.
7. Wet the sorbent with Eluting Medium; watch as the liquid rises through the sorbent particles and stop when the liquid reaches the filter plug in the sorbent tip.
8. Eject the liquid and sorbent particles out of the tip and into the sample preparation vial. Repeat step 7 and 8 until the sorbent particles in the tip are expelled into the sample preparation vial. Only the filter disk should remain in the empty tip, see figure 4. Keep the syringe as it can be reused with many other samples.
9. Using the adjustable Drummond Dialamatic Microdispenser (included) transfer 50µL Reagent 4 into the sample preparation vial.

Caution: Avoid cross-contamination by not touching the inner wall of the sample vial with the tip of the Microdispenser. The piston will ensure proper transfer of liquids into the vial without the need of touching the vial wall. Use the same Microdispenser with both Reagents 4 and 5. There is no need to change Microdispenser tips or to wash between uses. Change Microdispenser tips only when broken.

Warning: Do not use regular pipettes and tips with Reagents 4 and 5 as they will contaminate the sample! Use the included Microdispenser for Reagents 4 and 5 ONLY!

Note: *for all subsequent sample preparation steps use a vortex mixer set in the touch (pulse) mode (to about 80% of max speed) for any mixing operations.*

10. Emulsify the liquid in the vial by repeatedly vortexing for about 5-8 seconds. During vortexing hold the sample vial firmly between fingers, and keep it straight as you push it onto the vortex plate. Do not let vial wobble, otherwise liquid may come out of the vial. Allow reactions to proceed 1 minute or more. The emulsion will gradually separate into two layers.

Note: *a longer reaction time than 1 minute at step 10 and at step 11, or later, at step 12, does not affect results.*

11. Re-emulsify the liquids in the vial by vortexing again for about 5 seconds. Allow the reaction to proceed for one additional minute or more.
12. Transfer with the Microdispenser 100µL Reagent 5 (50µL twice, for convenience) and mix for about 5 seconds. Let the reaction proceed for one more minute.
13. Pipette (DO NOT use the Microdispenser for this purpose!) 100µL Reagent 6, and vortex for about 5 seconds. The emulsion will separate into two layers again. The upper, organic layer contains amino acid derivatives to be analyzed by gas-chromatography (see GC set up and calibration in section 4). Sample this layer directly from the sample preparation vial or use a pasteur pipette to transfer part of it into an autosampler vial.

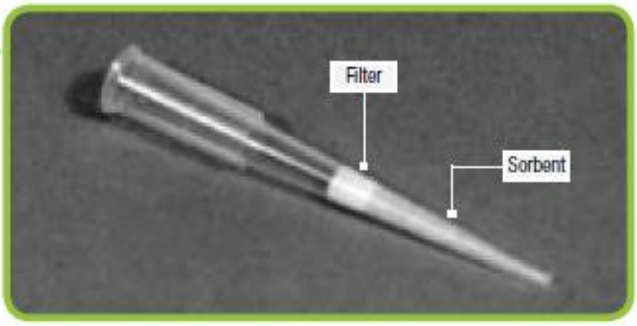
3.4 Optimizing Sample Preparation Time

For experienced users, sample preparation proceeds in 7-8 minutes per sample. This process can be further improved by preparing up to ten samples at a time. For example, at step 2 dispense Reagent 1 (and at later steps all other reagents) in ten vials successively, using the same pipette tip. At step 9, after dispensing Reagent 4, vortex 2-3 vials simultaneously. During each one minute wait at steps 10-12, prepare the next set of samples for SPE.

Sorbent Tip - (Figure 4)

Wet the sorbent with Eluting Medium and stop before it gets to the filter then eject the liquid and sorbent particles out of the tip.

Figure 4



4.0 Gas Chromatographic Analysis

4.1 Column For EZ:faast Free Amino Acid Analysis by GC

The Zebron ZB-AAA GC column comes without a cage. Connect the ends of the column in the usual manner; rest the column coil on the oven bracket. Keep the pieces of thermal thread spaced evenly around the column coil. The maximum column temperature is 320/340°C.

Caution: Always use safety glasses while installing the GC column.

4.2 Instrument Settings:

Constant Flow Mode GC-FID/NPD (recommended)	
Injection*	Split 1:15@250°C, 2µL (with hot needle, see section 4.6)
Carrier Gas	Helium 1.5mL/min **constant flow**
Oven Program	32°C/min from 110° to 320°C

Constant Pressure Mode GC-FID/NPD	
Injection*	Split 1:15 @ 250°C, 2.0µL
Carrier Gas	Helium, 8 psi (60 kPa) or Hydrogen 30 kPa
Oven Program	35°C/min from 110° to 320°C
Detector	320°C

*When using a Shimadzu GC instrument, please increase the injector temperature to 300°C

For your convenience we have included the GC method for the Agilent 6890 GC system on the reference CD included with the kit. To use the included method: copy the method folder into the appropriate method folder in your software .

4.3 Mode of Operation

For best resolution, a rate of 35°C/min is preferred with instruments operating in constant pressure mode only. Electronic Pressure Control (EPC) or Advanced Flow Controller (AFC) equipped instruments should be operated preferably in constant flow or constant velocity mode. With these instruments a temperature gradient of 30-32°C/min is fast enough to elute the least volatile derivatives (i.e. those of cystine and homocystine) with similar retention times to constant pressure mode. If the instrument is not equipped with the EPC option, you may use a pressure raise of 3kPa/min.

4.4 Liners

Use the best deactivated liners supplied by the instrument manufacturer. Good results were obtained with FocusLiners™ (included; Phenomenex P/N AG0-4680; fits Agilent and Varian 1177 injectors). In general, the liner should carry a plug of silanized quartz or pesticide grade glass wool.

4.5 Injection

- Split injection at a ratio of 1:10 to 1:20 is recommended
- Injection volumes of 1.5-2µL are optimal

Quasi-splitless injection mode will produce a 5 to 10 fold increase in sensitivity with some instruments. In this mode, the split valve should be closed for an initial 5 to 7 seconds. Before selecting this injection mode it should be checked experimentally that no significant discrimination of late eluting amino acid derivatives takes place in comparison with common split injection. Alternatively, instruments equipped with EPC/AFC can be operated with double initial head pressure for 6-10 seconds.

4.6 Sampling

Both autosampler and manual sampling can be performed. If manual sampling is preferred, hot needle injection is recommended to prevent discrimination of components with high boiling temperatures. With this technique the sample plug is completely drawn into the syringe barrel, leaving the needle empty. The needle is inserted and kept in the hot injector for about two seconds before injection.

4.7 Calibration Standards

For quantitation purposes, mixtures of amino acid standards should be prepared following the Sample Preparation by SPE and Derivatization procedure described in this manual in Section 3.3. Standard mixtures should be stored in the freezer as some amino acids are not stable in solution. Three vials of different standard mixtures are included in the kit:

SD1: 23 amino acids, 200 nmoles/mL each, as follows:

AAA	ASP	GLY	LEU	PHE	THR
ABA	ßAIB	HIS	LYS	PRO	TYR
alLE	C-C	HYP	MET	SAR	VAL
ALA	GLU	ILE	ORN	SER	

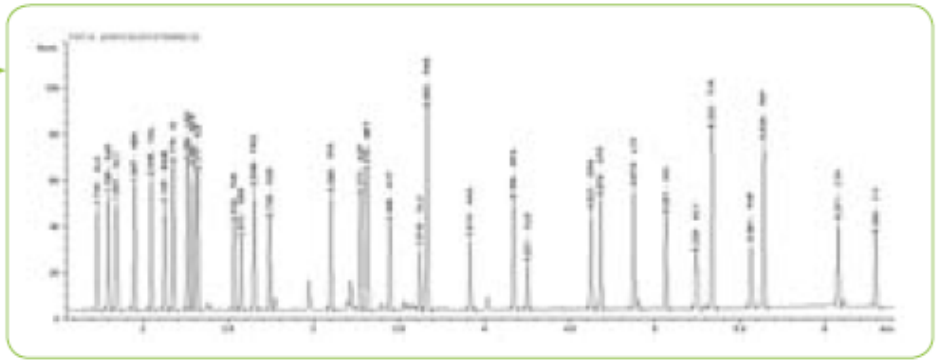
SD2: Complementary amino acids not stable in acidic solution, 200 nmoles/mL each, as follows:

ASN	GLN	TRP

SD3: Complementary urine amino acids, 200 nmoles/ mL each, as follows:

APA	CTH	GPR	HLY	PHP	TPR

Figure 5



A typical chromatogram of a mixture of all three amino acid standard solutions included in this kit. Column and instrumental settings as specified in section 4.1-4.2.