

# **Characterisation of bresaola products made from beef, veal, wagyu, mutton and lamb**

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# **Characterisation of bresaola products made from beef, veal, wagyu, mutton and lamb**

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## Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, ‘Characterisation of bresaola products made from beef, veal, wagyu, mutton and 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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## Abstract

Bresaola is a traditional Italian preserved meat product processed by salting and curing different cuts of lean bovine meat. Bresaola has been favoured by consumers across the globe owing to the characteristic flavour and low fat content (approximately 3-5%). New Zealand is one of the largest producers and exporters of sheep meat and an important contributor of bovine meat. Bresaola products made from New Zealand veal, wagyu, mutton and lamb were compared with the traditional beef products produced from New Zealand grown animals, in terms of physicochemical properties, sensory qualities and digestibility.

Cured products made from beef equivalents had higher protein content and lower intramuscular fat content except for wagyu meat, which was similar as those from sheep meat. Mutton and lamb bresaola had lower moisture content ( $< 40\%$ ) than the bresaola made from cattle meat. All of the products were significantly different ( $p < 0.05$ ) from each other in terms of instrumental texture quality. Wagyu and mutton bresaola showed significantly ( $p < 0.0001$ ) lower values in hardness ( $22.88 \pm 0.65$  and  $29.66 \pm 0.66$  kg, respectively) than the rest of products. Instrumental colour results of five products were found with significant variations ( $p < 0.0001$ ) in terms of redness ( $a^*$ ), lightness ( $L^*$ ) and yellowness ( $b^*$ ). Bresaola made from veal and wagyu contained more MUFAs ( $54.20 \pm 0.305$  and  $58.20 \pm 0.58\%$ , respectively) and wagyu had less PUFAs ( $3.04 \pm 0.04\%$ ) than other samples. Beef and lamb had a higher concentration of n-3 PUFAs ( $7.35 \pm 0.29\%$  and  $5.91 \pm 0.16\%$ , respectively) compared to veal, wagyu and mutton ( $3.58 \pm 0.06\%$ ,  $1.14 \pm 0.04\%$  and  $3.14 \pm 0.04\%$ , respectively). Lamb was rich in n-6 PUFAs ( $8.72 \pm 0.19\%$ ). A low value ( $< 1.5$ ) of the ratio of n-6 to n-3 fatty acids was detected in all five products, suggesting a favourable balance of two groups of fatty acids reflecting the positive effect on health and prevention of chronic disease. Free amino acids (17 amino acids, including 9 essential amino acids) were identified in bresaola products as the result of muscle proteolysis during curing process with the highest content of total free amino acids in mutton bresaola ( $2215.85$  mg/100 g dry matter). Over 50% of free amino acids found were essential amino acids in all five products.

In addition, consumers had significantly different ratings of acceptability for the five cured products, in terms of odour, flavour and overall liking ( $p < 0.05$ ), except for texture. Wagyu bresaola was the most favoured one for all of the sensory attributes tested,

and it was associated with juiciness and tenderness from the projective mapping. Mutton and lamb products were related with more of peppered flavour with sheep meat aroma.

Protein digestibility of bresaola products was evaluated by using a static *in vitro* digestion model. Simulated digestion for mouth, stomach and small intestine was completed over a 5 hr period. The highest digestibility was found in mutton bresaola, while the lowest was found in beef bresaola, releasing the amount of free amino acids (8.976 and 5.560 g/100 g protein, respectively) at the end of the simulated digestion procedure. The highest proportion (63.31%) of essential amino acids were found in wagyu meat after digestion.

Overall, the results have shown that dry-cured bresaola products made from New Zealand sheep meat have potential as a commercial product. They had similar qualities in the physicochemical, sensory, and digestibility perspectives to the bresaola made with beef. The findings from the present study may be helpful for giving greater value to New Zealand red meat and increasing the export of New Zealand food products.

## Chapter 1 Introduction

In many European countries, the demand for traditional food products has increased (Röhr, Lüddecke, Drusch, Müller, & Alvensleben, 2005; Wezemael, Verbeke, Kügler, Barcellos, & Grunert, 2010), along with the demand for convenience foods (Herrero et al., 2008; Paleari, Moretti, Beretta, Mentasti, & Bersani, 2003). A large variety of processed meat products are available as a type of convenience foods in the market across the world. They vary by different regions, cultures and religions. They can be classified into sausages, bacon, ham, salami, prosciutto (Italian ham) and bresaola (Italian beef ham).

Bresaola has originated from Valtellina in Italy, between 1450 and 1500. It is a type of preserved meat which is low in fats and calories, but contains high amounts of proteins, iron, vitamins and minerals (Calcinardi, 1962). Bresaola is produced from lean beef using dry-curing method, which includes salting, drying and ageing (Braghieri et al., 2009). The final product is thinly sliced and served uncooked. It is characterised as moderately tasty with delicate smell, and smooth and compact (Braghieri et al., 2009). Recently, there has been attempts to use different varieties of meats to develop new bresaola products, such as buffalo (Paleari et al., 2000), horse, wild boar, deer and goat meat (Paleari et al., 2003), donkey (Marino, Albenzio, Della Malva, Muscio, & Sevi, 2015; Marino, Malva, Gliatta, Muscio, & Sevi, 2010), and turkey (Dalzini et al., 2014).

As one of the largest producers and dominant exporter of sheep meat (lamb and mutton), and an important contributor to global beef market, red meat is the second largest merchandise next to milk and dairy products in New Zealand. The global production and consumption of sheep meat were not significant compared to beef, pork and poultry in the last decades (Kegalj, Krvavica, Vrdoljak, Ljubičić, & Dragaš, 2011; Ministry of Business, Innovation & Employment, 2014), owing to the characteristic flavour generated from odour-active compounds in the fat of sheep meat (Wong, Johnson, & Nixon, 1975; Yokoyama & Carlson, 1974). However, it has favourable technological properties for curing with high stability in colour and fast dehydration (Beriain, Iriarte, Gorraiz, Chasco, & Lizaso, 1997). Sheep meat is well-known for its nutritional value due to the high quality protein and content of healthy fat, like unsaturated fatty acids, particularly rich in conjugated linoleic acid which possesses unique and potent antioxidant activity (Williamson, Foster, Stanner, & Buttriss, 2005). Recently, some promising results have

been reported to improve the palatability of sheep meat by processing it into meat products, like sausages (Lu, Young, & Brooks, 2014; Prescott, Young, & O'Neill, 2001; Prescott, Young, Zhang, & Cummings, 2004). Still, there are lack of commercially available sheep meat derived products to meet the consumer demands.

The aim of the present study was to characterise bresaola products made from New Zealand cattle meat (beef, veal and wagyu) and sheep meat (mutton and lamb) in terms of nutritional profile, physicochemical, sensory properties and their digestibility. This study also anticipated to examining the feasibility of bresaola products made from sheep meat with a commercial potential.

This thesis is composed of five parts. Chapter two is a literature review summarising the past studies of the nutritional and organoleptic properties of meat and meat products. A brief review on human digestion system and available in vitro digestion models in the literature can also be found in Chapter two. Chapter three shows the materials and methods used in the project, including experimental design and statistical data analysis. In chapter four, results obtained from the study are presented and discussed. The final conclusion chapter summarises the main findings, explaining the research questions, and suggests avenues of future related research.

## Chapter 2 Literature review

### 2.1 Meat and meat derived products

Meat is defined by Food Standards Australia New Zealand (FSANZ) in Food Standard Code (Food Standards Australia New Zealand [FSANZ], 2002) as “the whole or part of the carcass of any buffalo, camel, cattle, deer, goat, hare, pig, poultry, rabbit or sheep, slaughtered other than in a wild state, but does not include eggs, or fetuses”. Recently, consumption of meat is becoming controversial due to the increasing incidence of chronic diseases, such as cancer, cardiovascular and metabolic diseases, having a possible association with over intake of meat (Biesalski, 2005; Ferguson, 2010). A healthy balanced diet recommended by New Zealand Ministry of Health (Ministry of Health, 2003), should include lean meat at moderate amount, together with starchy carbohydrates (breads, cereals, preferably whole grain), milk and milk products (preferably reduced or low-fat), and plenty of vegetables and fruits.

Meat is a concentrated source of several nutrients including protein, fat, essential amino acids, minerals, vitamin and other nutrients. As for red meat, it commonly includes meat from cattle, sheep and goat (i.e. beef, veal, lamb, mutton and goat meat), and is particularly characterised by containing high biological value protein and a range of fatty acids, such as omega-3 polyunsaturated fatty acids (PUFAs). The nutritional composition of meat differs significantly among different animal species (Pereira & Vicente, 2013; Williams, 2007), breeds (Arsenos et al., 2002; Enser, Hallett, Hewitt, Fursey, & Wood, 1996; Garcia et al., 2008), meat cuts (Pereira & Vicente, 2013; Reina, Sánchez del Pulgar, Tovar, López-Buesa, & García, 2013) and feeding regimens (Daley, Abbott, Doyle, Nader, & Larson, 2010; Leheska et al., 2008; Priolo, Micol, Agabriel, Prache, & Dransfield, 2002; Williams, 2007; Wood et al., 2008).

#### 2.1.1 Protein and amino acids in red meat

Protein, as the main component in meat, constitutes approximately 10 to 40% (Jiménez-Colmenero, Carballo, & Cofrades, 2001). It is an important source of human macronutrients, particularly rich in essential amino acids, and highly digestible (Biesalski, 2005). Dietary protein is an important contributor for the growth, maintenance and repairing of tissues (World Health Organization [WHO], 2007). Increasing the protein

content of diet would result in increased satiety and energy expenditure, because of the action of amino acids on the metabolic targets, and may also contribute to the body weight loss and maintenance (Hochstenbach-Waelen, Westerterp-Plantenga, Veldhorst, & Westerterp, 2009; Westerterp-Plantenga, Lemmens, & Westerterp, 2012).

According to the findings from New Zealand Adult Nutrition Survey (University of Otago and Ministry of Health, 2011), meat and meat products contribute 22% of protein intake in diet, compared to 11% from bread and 9% from milk. Consumption of a diet with high protein and low carbohydrate content whilst controlling energy intake has recently been associated with significant effect on weight loss and weight maintenance when compared with consuming a diet of similar energy intake that is low in protein (Layman, Clifton, Gannon, Krauss, & Nuttall, 2008; Paddon-Jones et al., 2008). Lean red meat is a concentrated dietary protein source. Modest increase in protein intake from lean red meat has been shown to lower blood pressure without increasing blood lipids and body weight (Hodgson, Burke, Beilin, & Puddey, 2006).

Protein content in meat can vary substantially depending on the species of the animal and the fat level. In general, content of protein decreases with increase in fat content. Red meat contains, on average, 20-24 g of protein per 100 g when raw, and 27-35 g of protein per 100 g when cooked (Williamson et al., 2005). Sheep meats, such as lamb, mutton and goat, have higher fat level with a decreased protein content compared to cattle meat including beef and veal (Williams, 2007; Williamson et al., 2005).

The nutritional quality of dietary protein is primarily dependent on whether the amount of nitrogen and amino acids provided is able to meet the requirement of metabolism, after being consumed and digested in human digestive system (FAO/WHO, 1989; Schaafsma, 2000). Proteins are built up from polypeptides composed of a large group of amino acids joined by peptide bonds. 20 of 190 amino acids are considered as the building blocks of proteins, while only nine of them are defined as indispensable or essential amino acids (EAAs) since they cannot be synthesized by human and must be supplied by diet (Pereira & Vicente, 2013). EAAs include histidine, lysine, threonine, methionine, phenylalanine, tryptophan, leucine, isoleucine and valine. Deficiency of even one of EAAs in the diet for a long term would result in the degradation of muscle proteins in the body. Another group of amino acids, known as semi-indispensable or semi-essential amino acids, consists of arginine, cysteine, glutamine, glycine, proline and tyrosine. These need to be exogenously supplied for the populations in specific



physiological conditions. For example, those under stress, in the states of muscle or organ recovery from disease or injury, and intensive growth of children and adolescents (Usydus, Szlinder-Richert, & Adamczyk, 2009). Cysteine and tyrosine could partly replace methionine and phenylalanine, respectively, where methionine can be converted to cysteine and phenylalanine can be converted to tyrosine under normal metabolic conditions (Boisen, Hvelplund, & Weisbjerg, 2000; National Health and Medical Research Council [NHMRC], 2006). The remaining amino acids, which are alanine, asparagine, aspartic acid, glutamic acid and serine, are recognized as dispensable or non-essential amino acids as they are synthesized by the human body in a sufficient amount.

The nutritional value of protein is determined by presence and absence of certain amino acids. Meat proteins are distinguished from other protein sources, such as plant proteins, because they contain all of the nine EAAs (Williamson et al., 2005). However, most of plant proteins would have shortage of at least one essential amino acid. For example, absence of lysine in wheat (Kies & Fox, 1970), tryptophan in corn and methionine in soybean (V. R. Young & Pellett, 1994). The problem can be solved by eating different plant-derived foods in combination, though a larger quantity would have to be consumed. Considering the aspects of the amount to be consumed, it would be more efficient to obtain higher quality proteins from meat than from the plant-based foods (NHMRC, 2006).

A scoring method, Protein Digestibility-Corrected Amino Acid Score (PDCAAS), was adopted and developed by FAO/WHO (1989) as a preferred method to indicate the protein quality in human nutrition. The basis of this method is by comparing the actual content of first limiting EAA acquired from the test protein with the same amino acid requirement by pre-school child, (FAO/WHO/UNU (1985)).

PDCAAS is calculated by (Schaafsma, 2000):

$$\text{PDCAAS (\%)} = \frac{\text{mg of limiting amino acid in 1 g of test protein}}{\text{mg of same amino acid in 1 g of reference protein}} \times \text{fecal true digestibility (\%)} \times 100$$

Higher PDCAAS score indicates higher digestibility of protein by means of a specific protocol with the better capability to provide amino acids for human nutrition. The maximum score is 1.0. Meat proteins show higher digestibility (around 94%) than

beans (78%) and whole wheat (86%) (National Research Council, 1989). Also, as for PDCAAS score, beef in particular, has a high score of around 0.9, compared to the scores of 0.5-0.7 for the most plant proteins (Schaafsma, 2000). The digestibility of proteins in human digestion system has a central impact on the amount of amino acids capable of being absorbed and utilised as human nutrients (Adibi & Mercer, 1973; Erickson & Kim, 1990).

### 2.1.2 Fat and fatty acids in red meat

Fat is the mixture of fatty acids mainly including saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Most of fatty acids can be synthesized inside the body except for some called essential fatty acids (EFAs), such as linoleic and alpha-linolenic acids, which must be obtained from the diet. Fat is an important source of EFAs (Williams, 2007). It is also a key dietary source of energy, which provides 37 kJ/g. This is considerably high compared to 16.7 kJ/g which can be obtained from carbohydrates and proteins (Ministry of Health, 2003). In addition, fat is able to carry fat-soluble vitamins, such as vitamin A, D, E and K, and promotes their absorption in human body.

Types of fats in carcass can be categorised into two groups: subcutaneous fats (below the skin, adipose tissues) and visceral fats. Early work on fats from meat mainly focused on the subcutaneous fats which are where the majority of fatty acids are located. However with the advances in butchery techniques, subcutaneous fats are largely removed during the dressing stage. Recently, there has been an increase in awareness of visceral fats (Biesalski, 2005; Ferguson, 2010). Visceral fats include intramuscular fats, which are known for marbling, and intermuscular fats. Intermuscular fats are those deposited around and between individual muscles. Intramuscular fats are those deposited between the muscle fibre bundles within a muscle, which appear as marbled. Intramuscular fats are the main contributor to tenderness and specific meat flavour, and enhance palatability (Ruiz-Carrascal, Ventanas, Cava, Andrés, & García, 2000).

Fat contents differ depending on the breeds of meat, feeding regimens, cuts and the degrees of trimming, which range from 3 to 25 g/100 g of food (Valsta, Tapanainen, & Männistö, 2005). Bovine meat commonly contains less fat than sheep meat (Enser et al., 1996; Williams, 2007), probably due to the butchery techniques and characteristics

associated with the animal species. In addition, the type of feeds has been shown to have impacts on the leanness of red meat. Pasture-fed animals generally produce leaner meat than the grain-fed or concentrate-fed animals (Leheska et al., 2008; Priolo et al., 2002). Higher proportion of PUFAs (alpha-linolenic acid) and more favourable Omega-6 (n-6): Omega-3 (n-3) FA ratio are also found in the meat from grass-fed ruminants (Enser et al., 1996). Beef and lamb, frequently consumed in Australia and New Zealand, are mainly grass-fed. These are favoured as a source of fatty acids and make the second greatest contribution to the intake of n-3 PUFA, after fish, in the diet (Williams, 2007).

Past concerns on dietary fat from red meat have gradually declined with adopting the trimming practices in meat processing from the 1980s. The reduction of fat was promoted by three factors; selective breeding and feeding practices, preference of leaner carcasses reinforced by marketing practices and meat classification systems, and advances in technology for trimming practices (Higgs, 2000). Since 1997 in New Zealand, the 'Quality Mark' requires the trimming of beef and lamb to no more than 5 mm external fats before they are allowed to enter the market. Trimming practices gave rise to a 30% decrease of fat content in the carcasses. This translates to an average of 7.4% fat for beef cuts, 15.3% for lamb cuts at retail shops according to Laugesen (2005). Fat content varies in different meat cuts, for example, meat cuts from Australia which have undergone a high level of trimming practices would include 4.7% (topside) and 1.9% (sirloin steak) for beef, and 4.3% (chump chop) and 3.2% (leg roast) for lamb (Williams, 2007).

With regards to the fat composition in red meat, great emphasis is placed on the saturated fat content. Excessive intake of saturated fat would increase the risk of cardiovascular disease and metabolic syndrome (Haffner, 2006). Increasing health concerns have been raised to reduce the intake of saturated fats in diet. However, not all of the saturated fats in red meat are associated with increasing the blood cholesterol level. The two primary SFAs in red meat are palmitic acid (16:0) and stearic acid (18:0). Stearic acid appears to be neutral in elevating the blood cholesterol level, since stearic acid will be partially converted to oleic acid *in vivo* (Bonanome & Grundy, 1988) and has not been shown to elevate blood cholesterol (Valsta et al., 2005). Palmitic acid has shown much less potential than myristic acid in elevating the blood cholesterol level (Higgs, 2000). The reduction of red meat consumption would not solely decrease the intake of fat or saturated fat in diet. For example, one tablespoon (approximate 15ml) of olive oil contains

more saturated fat (approximately 2 g SFAs) than 100 g of lean beef (approximately 1 g SFAs) (Valsta et al., 2005; Williams, 2007).

Well-balanced fatty acid profile is another factor to consider when choosing healthy dietary fat. The ratios of PUFA to SFA, MUFA to SFA and n-6 to n-3 FA have been widely used as an indicator for healthier dietary fat (Scollan et al., 2006). According to Haffner (2006), a higher PUFA to SFA ratio ( $\geq 0.4$ ) is desired to adverse the negative effects of saturated fat, hence decrease the risks of cardiovascular disease and metabolic syndrome. Consumption of higher levels of MUFAs, in conjunction with reduced levels of SFAs, is believed to prevent an increase in blood cholesterol levels (Boylston et al., 1995; Manuela A et al., 2011). As for the ratio of n-6 to n-3 FAs, a lower value less than 4.0 is desired according to Scollan et al. (2006). Therefore, the recommended intake of fatty acids by National Health and Medical Research Council Dairy (NHMRC, 2006) states that a higher level of PUFAs, especially n-3 FAs at an expense of n-6 FAs, and low level of SFAs should be taken. Source of fat from dairy is favoured over meat due to lower content of total SFAs. However, higher proportion of myristic acid and less MUFAs could lead to an unbalanced fatty acid profile than red meat (Enser et al., 1996; Higgs, 2000; Månsson, 2008).

### 2.1.3 New Zealand meat

Red meat (mainly cattle and sheep meat) is the second largest merchandise next to milk and dairy products in New Zealand. It is exported to more than 120 countries and contributed to the total export revenues of \$6.5 billion in 2013/2014 (Meat Industry Association of New Zealand [MIANZ], 2014).

New Zealand is the largest exporter of lamb and mutton across the globe. Over 90% of lamb and mutton produced in New Zealand are exported each year. The total volume of sheep meat exports in the year 2013/2014 increased by 10% to 402,661 tonnes, compared to 364,000 tonnes in 2003/2004 (MIANZ, 2004), which was worth the value of \$2.97 billion. New Zealand together with Australia accounts for around 70% of the world's lamb exports. With respect to the destinations, North Asia, dominated by China, took over the key market from European Union and became the fastest growing and the largest sheep meat export destination in 2013/2014. China has become New Zealand's

largest individual market, accounting for about 30% of lamb exports and 70% of mutton exports.

Beef production (offals not included) in New Zealand accounts for around 1% of the world production and contributes 8% to the global beef market. 90% of domestic beef production is exported. The total volume of beef (including veal) reached 379,885 tonnes in the year 2013/2014, which was worth the value of \$ 2.2 billion (MIANZ, 2014).

Wagyu cattle refer to the breed originally from Japan, which are famous for the production of nutty-flavoured and fat-marbled premium beef. Well known for the exceptional palatability, ease of farming and good fertility, New Zealand bred wagyu is growing to be innovative businesses both domestically and globally. Wagyu cattle raised in New Zealand are highly distinguishable by unique pasture dominant regimen with advantages in marbling, tenderness, flavour and higher omega-3 fatty acids over barn-fed in Japan, and grain-fed wagyu in other regions (Mountford, Piyasiri, & Warner, 2014). Competitive price with equivalent quality to USDA (U.S. Department of Agriculture) prime grade, grass-fed wagyu beef has gained the opportunity to increase the market shares are dominated by Japan, the U.S., and Australia. Direct global sales channels were opening up to market this specific-bred niche beef to the world.

#### 2.1.4 Consumption of sheep meat

Sheep meat, which includes lamb and mutton, is widely accepted by meat eaters across the world for sensorial or nutritional reasons, rather than religious or traditional reasons like in ancient times. There is a regional difference in production and consumption of sheep meat. Compared to beef, pork and poultry, both production and consumption of sheep meat are certainly low and there have not been significant increases in the last 50 years (Kegalj et al., 2011; Ministry of Business, Innovation & Employment, 2014). Usually sheep meat is marinated with strong aromatic spices like garlic (Smith & Young, 1991), ginger, pepper or rosemary. And then, smoking, grilling, roasting or stewing is applied to fresh or frozen form to neutralise the flavour of sheep meat. The characteristic “unpleasant” or “objectionable” flavour comes from two main sources in sheep meat, which are branched chain fatty acids (Wong et al., 1975) and skatole (3-methylindole) (Yokoyama & Carlson, 1974). 4-methyloctanoic acid and 4-

methylnonanoic acid are the branched chain fatty acids released from the esterified form in the sheep meat and contribute to the flavour during cooking or other type of meat processing. Skatole accumulates in the body of the sheep and milk fat, causing unpleasant flavour at high concentration (Lu et al., 2014).

The characteristic flavour of sheep meat is known to become more intense with the increasing age of the animal. It is also related to the process of curing or fermentation. An adult sheep that has been through more than two permanent incisors in wear is referred to as a mutton. Meat from a mutton is tougher in texture (O. A. Young, Reid, Smith, & Braggins, 1994) with higher concentration of unfavourable flavour components than the meat from a lamb (O. A. Young et al., 2006). Flavour or odour may be the primary attribute to the acceptability of lamb by consumers (Helgesen, Solheim, & Næs, 1997), while as for beef, it would be dominated by tenderness, and for pork and turkey, juiciness would be more critical (Bather, Brant, & Kunze, 1969). Therefore, the unfavourable sheep meat flavour accounts for the low consumer acceptability and low trade price of sheep meat. Sheep meat is rarely processed into other meat derivatives, like ham and sausages. With a few of sheep meat derived products that are available, they often do not meet the consumer expectations and stay low in demand. However, sheep meat has favourable technological properties for curing, for example, high stability in cured colour and high in dehydration rate, although the lesser sensorial satisfaction of this kind of meat persists (Beriaín et al., 1997).

Recently, an increasing number of studies have started to report on the production of sausages from lamb and/or mutton. Kukovics and Németh (2014) have investigated the use of selected lamb from various breeds and processing them into Wiener sausage. The flavour and odour were appreciated by all age and gender groups from various occupations (Kukovics & Németh, 2014), indicating a potential opportunity for commercialisation.

However, the acceptability of sheep meat products by consumers who generally do not consume sheep meat still requires extensive research. Recently, Lu et al. (2014) processed New Zealand mutton shoulder cuts into sausages and investigated the impact of nitrite and spices (garlic and rosemary oil) on increasing the acceptability of sheep meat sausages, particularly to unhabituated consumers. Flavours from spices showed positive effect on suppressing the characteristic flavours to appeal the unhabituated consumers, while nitrite mainly played a role in minimizing fat oxidation and extending

shelf life. The masking effect with spices has been previously reported by Baliga and Madaiah (1971), Bartholomew and Osuala (1986) and Li (2004).

The unfavoured flavour components are mainly stored in fat of sheep meat. Reducing the fat content in the end-product by selecting lean cuts or replacing the fat from sheep with the fat from pork or beef showed an increase in the palatability of processed sheep meat products (Anderson & Gillett, 1974; Bartholomew & Osuala, 1986; Wenham, 1974). Furthermore, the process of fermentation assisted by microbial enzymatic reaction has shown to overcome the objectionable flavour of sheep meat (Mangia, Murgia, Garau, Merella, & Deiana, 2008; W. H. Wu, Rule, Busboom, Field, & Ray, 1991).

However, other meat products that are similar to bresaola, like prosciuttos or ham, made from an entire piece of sheep or bovine meat cut were more rarely seen, both from scientific and commercial perspectives. Prosciutto is traditionally produced from pork, though beef is becoming a favourable alternative both in sensorial attributes and nutrition profile. Beef prosciutto from *Uzice* cattle showed a desirable sensory property with the characteristics of high content of protein and low in fat (Radovanović, Stamenković, & Saičić, 2004). Mutton prosciutto also commercially exists, with region-specific availability. With distinctive species-specific flavour combined with smoky aroma and salty taste, it seemed only appealed to habituated consumers of sheep meat (Operta & Tahmaz, 2010; Stamenkovic & Devic, 2006).

#### 2.1.5 Advantages of New Zealand red meat products

As a general rule, only lean meat cuts are suitable for dry-cured processing since rancidity from fats would develop quickly during the processing and storage, resulting in flavour deterioration. Bovine and sheep are generally pasture-fed without grain-finished in New Zealand, which makes them different in chemical composition, compared to the lamb from the United States. Lower proportion of intramuscular fat and higher moisture level with similar protein content have been found in New Zealand lamb (Lin, Cross, Johnson, Breidenstein, & Ono, 1988). Extensive studies to investigate the influence of feeding regimen on fat content of bovine (Alfaia et al., 2009; Garcia et al., 2008; Leheska et al., 2008; Ponnampalam, Mann, & Sinclair, 2006) and sheep meat (Priolo et al., 2002;

Watkins, Frank, Singh, Young, & Warner, 2013) are available in the literature. Meat obtained from grass rich or whole grass diet were lower in total or intramuscular fat content and cholesterol proportion than from grain or concentrate fed diet. Therefore, New Zealand bovine and sheep meat contains desirable quality for dry-cured meat manufacture from chemical and nutritional perspectives. The production of processed meat products like bresaola, could be used as a tool to add value to New Zealand sheep meat/bovine meat.

#### 2.1.6 Processed meat products

Processed meat refers to a product which has undergone treatments of preservation other than freezing, to improve the quality and flavour of meat (Sullivan, 2011). Processed meat products, as a source of dense energy and nutrients, are gaining attention in the global market (Paleari et al., 2003), as the demand for ready-to-eat meals with portion control increases (Herrero et al., 2008; Paleari et al., 2003). In particular, attention is being drawn to meat cuts of low trade prices or those that are difficult to sell, such as with the name flank, chuck, shin and leg. They are transformed into meat derivatives with added values, as processed meat products.

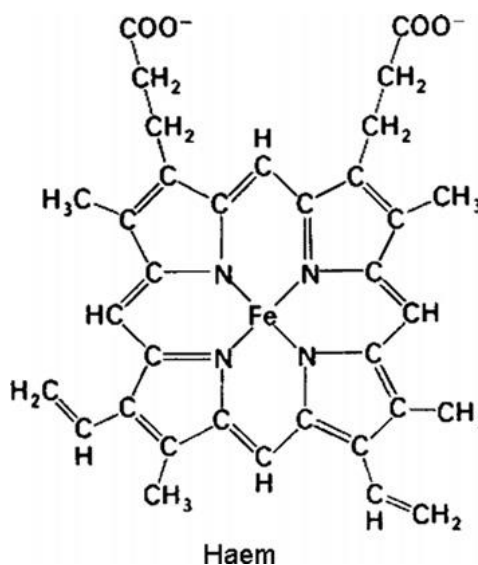
A large variety of processed meat products are available across the world. Provenance and cultural variations play a key role. For example, sausages, frankfurters, bacon, ham, salami, prosciutto (Italian ham), bresaola (Italian beef ham), corned beef and canned meat products. The methods of treatment mainly include curing, drying, smoking, cooking, and packaging (Toldrá, 2010). Curing is a traditional processing method aiming to enhance the shelf life of meat with the help of salt, acid (pickle), drying and/or smoking (Honikel, 2008). Nowadays, the term, 'cured meat', refers to fresh meat treated with salt, with or without nitrite and/or nitrate during the processing. On top of the purpose of preservation, obtaining desirable colour and flavour are also achieved with curing (Xiong & Mikel, 2001).

##### 2.1.6.1 Colour of cured meat products

The first impression consumers would have when it comes to meat and meat products is the appearance of the product. The appearance of meat is closely related to the palatability of meat based on colour, marbling and water holding capacity (Miller, Kerry, & Ledward, 2002). Particularly colour plays a critical role in ensuring customer



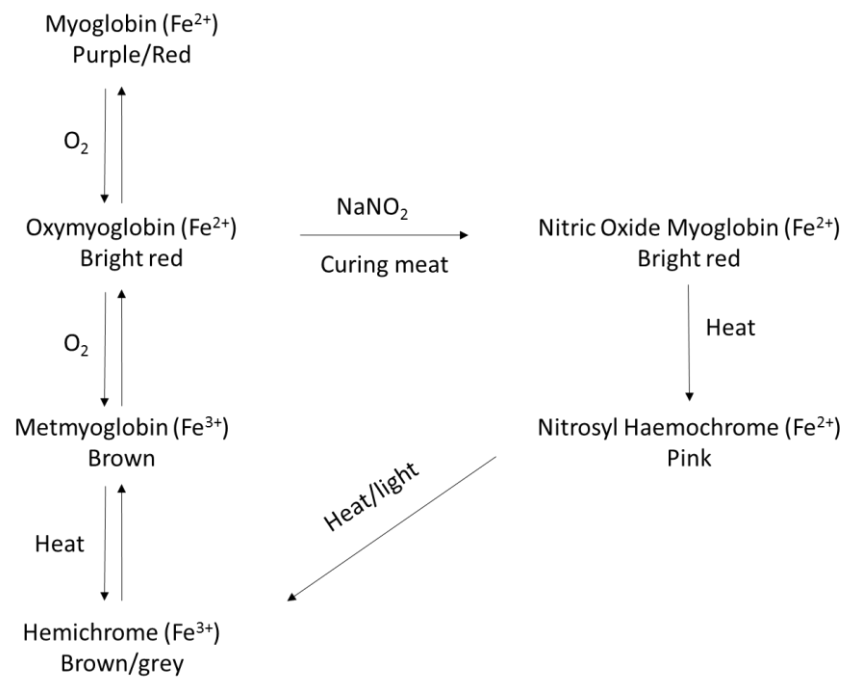
appeal and strongly contributes to the value of the product (Troy & Kerry, 2010). The pigment that contributes to the meat colour is myoglobin, a water-soluble protein that stores oxygen for aerobic metabolism in the muscle (Boles & Pegg, 2010). This pigment is very similar in nature to the blood pigment, haemoglobin. Both these compounds are made up of haem units which are combined with a basic protein called globin (Honikel, 2008). While haemoglobin is made up four haems and four globins, myoglobin is made up of the one haem and one globin (as shown in Figure 1).



**Figure 1 Haem in the myoglobin molecule (Honikel, 2008)**

The original colour of myoglobin is deep purplish red. The concentration of myoglobin differs from various muscles depending on the degree of activity, animal species, age, sex, diet, and environmental factors (Livingston & Brown, 1981). Generally, myoglobin content is higher in beef than lamb and pork, and it increases with the age of animals (Boles & Pegg, 2010). Myoglobin undergoes a series of reactions to different chemical states with changes in colour, as summarised in Figure 2. In the presence of oxygen, the ferrous ion ( $\text{Fe}^{2+}$ ), in the centre of porphyrin structure of myoglobin, binds to  $\text{O}_2$  molecule and it becomes bright (cherry) red. The state is called oxymyoglobin and favoured by consumers as the fresh meat colour. However, ferrous ion ( $\text{Fe}^{2+}$ ) in myoglobin and oxymyoglobin loses electrons and is further oxidized to ferric ion  $\text{Fe}^{3+}$ , to become metmyoglobin, which is brown in colour. The discoloration is caused by the several factors, including temperature, relative humidity, partial pressure of oxygen, light and lipid oxidation (Kannan, Kouakou, & Gelaye, 2001). Brown colour of metmyoglobin is commonly considered by consumers as loss of freshness or a chilled meat that has been

stored too long (Boles & Pegg, 2010; Troy & Kerry, 2010). However, these three forms of myoglobin can be interconverted depending on the conditions of storage, such as temperature, presences of oxygen, light and packaging. On the other hand, these pigments can be denatured during the cooking process and form irreversible brown colour of cooked meat, as a result of denatured metmyoglobin and hemichrome ( $\text{Fe}^{3+}$ ) (Livingston & Brown, 1981).



**Figure 2 Interchange of various myoglobin derivatives in meat and meat products**

The characteristic pink colour of cured meat products results from curing agents, nitrite or nitrate. Nitrite reacts with water to form nitrous acid and nitric oxide. This in turn reacts with myoglobin and becomes nitric oxide myoglobin with red colour. Once denatured from heat, nitric oxide myoglobin becomes nitrosyl haemochrome with pink colour, which is chemically more stable (Sullivan, 2011). Nitrosyl haemochrome is more stable than myoglobin, oxymyoglobin and metmyoglobin. However with the presence of oxygen, light and heat, denaturation may take place, causing the colour to turn green, grey or brown.

### **2.1.6.2 Texture of cured meat products**

Textural characteristics are one of the critical aspects of consumer acceptance for meat and meat products (Ruiz-Ramírez, Serra, Arnau, & Gou, 2005; Szczesniak, 2002). Tenderness is the most favourable textural quality of meat products. Tenderness in meat is defined as the state of being easily masticated, broken or cut (Bratzler, 1932). It is the impression perceived from the palate, which involves the ease of penetration by the teeth and how easily the meat breaks into fragments. Meat is often referred to as being tender and conversely, tough or lacking in tenderness. A wide variation in tenderness may occur between species, and even between different cuts of meat from the same carcass and the different methods of cooking and processing. The type and amount of connective tissue fibres present in meat have great impacts on the tenderness, because it requires further mastication to break down the fibres until it is ready to swallow, resulting in lack of tenderness. In addition, the level of proteolysis on key structural proteins and the extent of shortening of the muscle fibres also play key roles in the meat tenderness (Troy & Kerry, 2010).

The textural quality of processed meat products mainly depends on the characteristics of raw materials and technological parameters during processing (Serra, Ruiz-Ramírez, Arnau, & Gou, 2005; Virgili, Parolari, Schivazappa, Bordini, & Borri, 1995). Texture variations in relation to the characteristics of raw materials is associated with genetic origin of animals, sex, sexual maturity, pH and muscle structure which may differ in fat content (Lorenzo, Temperán, Bermúdez, Purriños, & Franco, 2011; Mendonza, Garcia, Casas, & Salgas, 2001; Ruiz-Carrascal et al., 2000; Tobin, O'Sullivan, Hamill, & Kerry, 2012) and proteolytic potential (Ruiz-Ramírez, Serra, et al., 2005; Virgili et al., 1995). Technological parameters including processing temperature (Arnau, Guerrero, & Sárraga, 1998), water activity (Ruiz-Ramírez, Serra, et al., 2005; Serra et al., 2005; Sullivan, 2011), processing time, relative humidity, addition of salt and treatment of nitrate or nitrite (Gil, Guerrero, & Sárraga, 1999; Molinero, Martínez, Rubio, Rovira, & Jaime, 2008; Ruiz-Ramírez, Arnau, Serra, & Gou, 2005; Ruiz, Ventanas, Cava, Timón, & García, 1998) also result in variations in texture of meat and meat products.

Curing processing involves an intense breakdown of muscle proteins by the action of proteases and microorganisms, and tenderises the meat texture. Proteolysis contributes to tenderisation by breakdown of the muscle structure (Rodríguez-Nuñez, Aristoy, & Toldrá, 1995; Soriano, Cruz, Gómez, Mariscal, & Ruiz, 2006). This is achieved in several

consecutive stages: (a) hydrolysis of major myofibrillar proteins by the action of calpains and cathepsins, (b) production of polypeptides that act as substrates for peptidases to generate small peptides and (c) a number of free amino acids released by the action of aminopeptidases (Toldrá, 2006). Besides the effect of proteolysis, dehydration during the drying process also contributes to the unique textural quality, and renders a firm texture of the final dry-cured product with shrinkage of sample shape and size. Water loss starts from the superficial layer of the product. Hence, higher extent of dehydration may occur at the outer surface. The common problem associated with dry-cured products is the formation of “crust”, a moisture gradient between the outer surface and the centre of products. It may cause the outer layer being more firm than the rest of the product (Ruiz-Ramírez, Serra, et al., 2005).

#### **2.1.6.3 Bresaola products**

In many European countries, the demand for traditional food products has increased (Röhr et al., 2005; Wezemael et al., 2010). Bresaola originated from Valtellina in Italy between 1450 and 1500. It is a Protected Geographical Indication (PGI) product with distinctive flavour. Bresaola is a type of preserved meat which is low in fats and calories, but contains high amounts of proteins, iron, vitamins and minerals (Calcinardi, 1962). As an air-dried and flavoured Italian ham-like product, bresaola is distinguished from other dry-cured products made from pork (prosciutto). Bresaola is produced by selecting lean beef from the best cuts of bovine hindquarter, such as topside, round and rump (Paleari et al., 2000).

The traditional method of bresaola production follows a strict rule. After visible fat is trimmed, meat is salted by massaging dried salt and natural flavours and then dried with constant air at appropriate humidity and temperature, and aged for at least 3 weeks (Braghieri et al., 2009).

Just like prosciutto, bresaola is thinly sliced to a thickness of 1 to 1.5mm and served uncooked. The flavour of bresaola can be characterised as, in general, moderately tasty with delicate smell, smooth and compact (Braghieri et al., 2009). Similar products from different regions are available across the globe. For example, “Bindenfleisch” or “Viande des Grisons” from Switzerland, “Brési” from France, and “Carne-de-sol” and “charqui” from northeastern and southern Brazil respectively.

The production and value of bresaola PGI have strongly increased since the 19th Century. The total production of bresaola increased from 8650 tons in 1999 to 17,000 tons with a value of € 280 million in 2009. 13% (2,200 tons) of the production was exported from Italy to a number of countries, such as Switzerland, France, Germany, the United Kingdom and Austria. Bresaola has a niche in both domestic and international markets (Marino et al., 2010). Until the end of year 2013, the export volume rose 6% and reached 2,956 tons compared to the previous year, which was worth the value of € 53,733 million. Demand for diversity in bresaola is increasing from consumers of distinctive cultural backgrounds and habits (Prescott, Young, O'Neill, Yau, & Stevens, 2002). Modern consumers, particularly of more developed and affluent countries, are more health conscious with higher expectation on the sensorial quality of foods. Flavour-oriented, with high in protein and low in fats and carbohydrates products have dramatically increased in recent years (Grunert, Bredahl, & Brunsø, 2004; Jiménez-Colmenero et al., 2001; Paleari, Bersani, Vittorio, & Beretta, 2002). As for meat products, increasing demands have been made on added nutritional value, being wholesome, fresh, lean and having adequate juiciness, flavour and tenderness (Dransfield, 2001).

The quality of cured meat products is mainly dependent on the variations arising from raw meat (species, regions, breeds and cuts), formulations and manufacturing methods (Marino et al., 2015). Distinctive nutritional and sensory profiles are expected in meat from different species. Beef and pork are dominant in traditional processed meat markets, such as, sausages, ham and bacon. There were other processed meat products made from different species which are favoured by particular regions, for example, horse bresaola and salami being popular in Italy. Recently, there has been an increasing number of commercial food products that transformed by-products to value-added food products. Meat from domestically abundant animal species, particularly those caused by over-production, the seconds quality and cuts of low trade price, are increasingly being processed into meat derived products with improved sensorial acceptability (Paleari et al., 2000; Paleari et al., 2003), with the aim to create or re-orient the market (Troy & Kerry, 2010).

Recent studies have attempted to use different varieties of meats to develop new bresaola products, such as buffalo (Paleari et al., 2000), horse, wild boar, deer and goat meat (Paleari et al., 2003), donkey (Marino et al., 2010; Marino et al., 2015), and turkey (Dalzini et al., 2014). Physicochemical and sensory properties of the new products have

been analysed. In comparison with the traditional bovine bresaola, results from the past studies have demonstrated similar sensorial properties between donkey and beef bresaola (Marino et al., 2010). Furthermore, bovine bresaola made from Podolian young bull (Braghieri et al., 2009) showed equivalent sensory quality with commercial bresaola but differences in dominant attributes that consumer preferred. From the study by Braghieri et al. (2009), a group of consumers preferred sensorial attributes of commercial beef bresaola, including marbling appearance and seasoned flavours with pepper and salt. While another group of consumers who favoured bull bresaola preferred the characteristics of sweetness and intensive odour. From the nutrition profile point of view, superior characteristics such as lower cholesterol content in buffalo and horse bresaola, higher protein and PUFAs content in donkey bresaola were detected. Elevated free amino acids were found in deer, wild boar and goat bresaola compared to bresaola of horse and bovine meat. However, among these products, the composition of essential amino acids (EAAs) was nearly as twice as high in all the other products compared to the bovine bresaola (Paleari et al., 2003). Therefore, it is possible to exploit different meats to produce bresaola-like products in generating new flavours.

## 2.2 Sensory characteristics of meat products

Consumers consider several characteristics of food products to determine its acceptance. These characteristics include sensory attributes (appearance, tastes, odour, texture and flavour), nutritional value and convenience (Mun˜oz, 1998). For most consumers of meat and meat products, the sensory properties are the most important product parameters associated with the purchase motivation and acceptance of the products (Resurreccion, 2004). The acceptability of a food can be estimated by consumer studies, and can then be combined with other sensory analyses, knowledge of consumer expectations, and product formulation constraints in determining the optimal design of food products (Lawless & Heymann, 1999). Consumer testing results can describe general tendencies of consumer preference (Braghieri et al., 2009). Flavour and texture are two primary sensory properties that are important in consumer perception of meat products.

### 2.2.1 Flavour of cured meat products

Flavour is a primary sensory quality which plays a principal role in the palatability of meat and meat products. The sensory perception of flavour is due to the simultaneous stimulation of human olfactory and taste systems and is triggered by odour-active volatiles, taste-active non-volatiles and the interaction of these compounds (Jurado, Garca, Timon, & Carrapiso, 2007; Piggott & Schaschke, 2001). Cured meat products have distinct flavour generated by the interplay of raw materials, differed by animal species and diets, and additives (spices or seasonings), and affected by processing techniques and aging time (Hierro, de la Hoz, & Ordonez, 2004; Lorenzo & Carballo, 2015; Ruiz et al., 1999). Sensory characteristics of processed meat products are influenced by enzymatic reactions including proteolysis and lipolysis, chemical reactions including lipid oxidation (Carrapiso, Ventanas, & Garca, 2002), Maillard condensation (Ventanas et al., 1992) and Strecker degradation (Hidalgo & Zamora, 2004) reactions. Proteolysis and lipolysis constitute the main biochemical reactions in the generation of flavour or flavour precursors (Toldra, 1998).

Numerous peptides and free amino acids produced from the enzymatic degradation of muscle proteins have been reported to contribute to meat taste during ageing, and the effect could be more pronounced for the dry-cured products that have undergone a long curing process (Rodrguez-Nunez et al., 1995; Toldra, Flores, & Sanz,

1997). Taste-active compounds would be released from the proteolysis, such as alanine (ALA), glycine (GLY) and serine (SER) which possess sweet taste, and glutamic acid (GLU) that contributes to “umami” taste (Jurado et al., 2007; Mau & Tseng, 1998). GLU is one of the amino acids with the highest impact for meat flavour due to the “umami” taste, which produces the so-called “mouth satisfaction” (Kurihara, 2009; Maga, 1994). In addition to the direct contribution of taste-active compounds, peptides and free amino acids also take part in the development of flavour as precursors of odorants associated with characteristic aroma of meat products (Hidalgo & Zamora, 2004; Jurado et al., 2007). For example, volatile aromatic compounds have been reported on dry-cured ham due to the further reactions of free amino acids, such as 2-methyl butanal (J. Ventanas et al., 1992) and 2-methyl propanal, sulphide compounds or thiols generated from Strecker degradations, and pyrazines from Maillard reactions with carbohydrates (Gianelli, Salazar, Mojica, & Friz, 2012; Toldrá & Flores, 1998).

Muscle and adipose tissue lipids are also subject to intense lipolysis during curing, generating free fatty acids by the action of lipases and further transformed to volatiles that contribute to odour (Toldrá, 1998). Most of the volatile compounds are the result of chemical or enzymatic oxidation of unsaturated fatty acids and further interactions with proteins, peptides and free amino acids released from proteolysis (Gianelli et al., 2012; Olivares, Navarro, & Flores, 2009). More than 260 volatiles have been found in dry-cured ham and most of them are hydrocarbons (Shahidi, 2012). Short-chain volatiles are those with less than six carbon atoms and generated from the hydrolysis of triglycerides and phospholipids. This group of aromatic compounds may contribute directly to the flavour of cured products. Another group of volatiles with more than six carbon atoms are the result of various chemical reactions that would have a great impact on the formation of cured meat flavour, such as aldehydes, alcohols, ketones,  $\gamma$ -lactones, esters, and other compounds like benzene derivatives, amines and amides (Carrapiso et al., 2002; Gianelli et al., 2012; Ruiz, Cava, Ventanas, & Jensen, 1998; Toldrá & Flores, 1998).

### 2.2.2 Texture of cured meat products

It is frequently accepted by consumers that texture is one of the most important sensory properties along with flavour that affects meat consuming quality and acceptability (Buscailhon et al., 1994). Texture is a multi-parameter attribute (Szczesniak,



2002). The textural characteristics of a food primarily derive from the structure (molecular, microscopic or macroscopic) of the food. The particle size, shape and orientation, moisture, and fat content are the main factors that influence textural characteristics (Bourne, 2002; Wilkinson, Dijksterhuis, & Minekus, 2000). The perception of texture is a complex procedure affected by several senses, such as vision, touch, hearing, and the pressure when masticating (Szczesniak, 2002; Wilkinson et al., 2000).

With the consumption of fresh meat, tender meat is preferred by most of consumers, particularly those in the middle age group (31-50 years) (Aaslyng et al., 2007; Bryhni et al., 2003). However, texture weakly influenced the acceptability and overall liking of Podolian beef bresaola (Braghieri et al., 2009) and dry-cured ham (Buscailhon et al., 1994; Ruiz, Garcia, Muriel, Andrés, & Ventanas, 2002). A study conducted by Braghieri et al. (2009) indicated that the acceptance of Podolian bresaola was driven by taste/flavour and appearance rather than texture. This could be due to the dynamic process of food breakdown that involved structure of the food, degree of mastication and lubrication, and perception time, which may differ significantly among individuals (Hutchings & Lillford, 1988; Wilkinson et al., 2000).

The influence of fat content on the textural properties of processed meat products have been reported in some studies (Braghieri et al., 2009; Cava, Ventanas, Ruiz, Andrés, & Antequera, 2000; Lorenzo et al., 2011; Pietrasik, 1999; Ruiz-Carrascal et al., 2000; Tobin et al., 2012). However, texture properties of meat products are affected by multiple factors other than fat level. These factors include tenderization during curing, degree of dehydration and origins of muscle. In general, hardness of meat products would decrease with the increase in fat content in dry-cured ham (Ruiz-Carrascal et al., 2000) and sausages (Barbut & Mittal, 1996; Bloukas, Paneras, & Papadima, 1997; Murphy, Gilroy, Kerry, Buckley, & Kerry, 2004; Tobin et al., 2012). Furthermore, the composition of unsaturated fat can further influence the hardness of meat products due to lower melting point, which is closely related to the decreased hardness perceived by consumers (Enser et al., 1996). Marbling is the visible fat (mainly intramuscular fat) present in the inter-fascicular spaces of a muscle and contributes directly to the visual characteristics of meat. There is also a favourable correlation between the marbling and eating qualities, such as juiciness, tenderness and flavour (Braghieri et al., 2009; Brewer, Zhu, & McKeith, 2001; Miller et al., 2002; Muriel, Ruiz, Martin, Petron, & Antequera, 2004).

### **2.2.2.1 Texture profile analysis**

Some texture testing instruments are able to simulate the human perception of various foods, but can only detect and quantify some mechanical parameters related to texture profile. Texture profile analysis (TPA) is an effective profiling method of texture description applicable to both sensory and instrumental measurements with good correlations (Breene, 1975; Szczesniak, 2002; Szczesniak, Brandt, & Friedman, 1963). Mechanical parameters can be quantified by means of compressing the test sample at least twice and giving rise to force-deformation curves. The results acquired can then be interpreted in terms of sensory perception. The mechanical parameters involved in the profiling method of solid and semi-solid foods are classified into two groups, primary and secondary parameters, as suggested by Civille and Szczesniak (1973); Szczesniak et al. (1963) (Table 1). Primary parameters consist of hardness, cohesiveness, viscosity, springiness and adhesiveness. Brittleness, chewiness and gumminess are included in the secondary parameters.

Extensive studies have characterised the textural properties of processed meat products using instrumental texture profile analysis as an objective method to evaluate the structural quality of dry-cured ham and loin (Costa, Bergamin Filho, Silveira, & Felício, 2008; Martinez, Salmerón, Guillén, & Casas, 2004; Ruiz-Ramírez, Arnau, et al., 2005; Ruiz-Ramírez, Serra, et al., 2005; Serra et al., 2005; Tabilo, Flores, Fiszman, & Toldra, 1999) and sausages (Herrero et al., 2007; Herrero et al., 2008; Lu et al., 2014; Pietrasik & Duda, 2000).

**Table 1 Definition of textural characteristics (Civille & Szczesniak, 1973; Szczesniak et al., 1963)**

Physical		Sensory
<b>Primary Properties</b>		
<i>Hardiness</i>	Force necessary to attain a given deformation	Force required to compress a substance between molar teeth (in the case of solids) or between tongue and palate (in the case of semi-solids).
<i>Cohesiveness</i>	Extent to which a material can be deformed before it ruptures.	Degree to which a substance is compressed between the teeth before it breaks
<i>Viscosity</i>	Rate of flow per unit force.	Force required to draw a liquid from a spoon over the tongue.
<i>Springiness</i>	Rate at which a deformed material goes back to its undeformed condition after the deforming force is removed.	Degree to which a product returns to its original shape once it has been compressed between the teeth
<i>Adhesiveness</i>	Work necessary to overcome the attractive forces between the surface of the food and the surface of the other materials with which the food comes in contact.	Force required to remove the material that adheres to the mouth (generally the palate) during the normal eating process
<b>Secondary properties</b>		
<i>Fracturability</i>	Force with which a material fractures; a product of high degree of hardness and low degree of cohesiveness.	Force with which a sample crumbles, cracks or shatters
<i>Chewiness</i>	Energy required to masticate a solid food to a state ready for swallowing; a product of hardness, cohesiveness and springiness.	Length of time (in sec) required to masticate the sample, at a constant rate of force application, to reduce it to a consistency suitable for swallowing.
<i>Gumminess</i>	Energy required to disintegrate a semi-solid food to a state ready for swallowing; a product of a low degree of hardness and a high degree of cohesiveness.	Denseness that persists throughout mastication; energy required to disintegrate a semi-solid food to a state ready for swallowing.

### 2.2.3 Projective mapping

Evaluation of the sensory quality of a food product via sensory characterization is the most powerful tool that has been extensively used for a wide range of food products (Lawless & Heymann, 2010). In the last decade, descriptive analysis (DA) has been a commonly accepted method favoured by sensory scientists for the purpose of sensory properties characterization, product development and marketing (Abdi & Valentin, 2007; Meilgaard, Carr, & Civille, 2006; Murray, Delahunty, & Baxter, 2001). Although this methodology is able to provide detailed, consistent and reproducible results, the requirement for highly trained assessors is tedious, expensive and time-consuming (Risvik, 1994; Varela & Ares, 2012). Hence there has been increasing interest in developing a valid alternative method that uses the perception of consumers to obtain sensory profile of a set of food products.

Projective mapping (PM) is one of the novel methodologies used as an alternative to traditional DA that has been increasingly utilised by sensory scientists in recent years (Hopfer & Heymann, 2013; Varela & Ares, 2012). It is a projective type method developed by Risvik, McEwan, Colwill, Rogers, and Lyon (1994), which involve simultaneous presenting of samples for each assessor in a single sensory session, placing samples on a bi-dimensional space according to the global differences and similarities among them. Results will be collected on bi-dimensional perceptual maps in a way that the more similar they are perceived by the assessor, the closer they should be on the provided space (Risvik et al., 1994; Risvik, McEwan, & Rødbotten, 1997). Configurations from projective mapping can provide similar product maps as those from descriptive data and similarity scaling but with better consistency over replications (Hopfer & Heymann, 2013; Louw et al., 2013; Pagès, 2005; Risvik, 1994). In addition, the importance of the product attributes can also be evaluated by performing projective mapping making it another advantage of projective mapping over descriptive analysis (Kennedy & Heymann, 2009).

Projective mapping has been reported to be a valid and simple sensory characterization method which can be performed by trained assessors and consumers (Varela & Ares, 2012; Vidal et al., 2014) on a wide range of food including both liquid and solid food products. Liquid foods include wine (Pagès, 2005; Perrin, 2009; Perrin et al., 2008; Ross, Weller, & Alldredge, 2012; Torri et al., 2013), powered drinks (Ares, Varela, Rado, & Giménez, 2011), citrus juices (Nestrud & Lawless, 2008), yoghurt (Cruz et al., 2013), soups (Risvik et al., 1997) and ice tea (Veinand, Godefroy, Adam, & Delarue, 2011).

With regards to solid or semi-solid food, as summarized in Table 2, most studies involved the sensory characterisation of food evaluated by using projective mapping method in comparison with other sensory methods such as DA, flashing profiling and sorting. Food products included chocolates (Kennedy & Heymann, 2009; Risvik et al., 1994), snack bars (Kennedy, 2010; King, Cliff, & Hall, 1998), cheese (Barcenas, Elortondo, & Albisu, 2004; Nestrud & Lawless, 2010), fruits and vegetables (Mielby, Hopfer, Jensen, Thybo, & Heymann, 2014; Nestrud & Lawless, 2010), fish nuggets (Albert, Varela, Salvador, Hough, & Fiszman, 2011), desserts (Cadoret & Lê, 2010; Vidal et al., 2014), bread (Normann, 2012), crackers (Vidal et al., 2014) and cheese pies (Marcano, Ares, & Fiszman, 2015). Results obtained from PM were found to be reliable

and similar to traditional descriptive methods in terms of different sample sizes and assessors.

**Table 2 Summary of Projective mapping (PM) studies on solid or semi-solid food products**

<i>Products (Sample amount)</i>	<i>Objective</i>	<i>Number of panellists</i>	<i>Statistical method</i>	<i>Findings</i>	<i>Reference</i>
Chocolates (5)	To compare maps obtained from PM, conventional profiling and dissimilarity scaling techniques	9 untrained subjects	GPA,RV coefficient used to compare methods	Higher consistency was obtained over repeated trials from PM compared with the CP and dissimilarity scaling	Risvik et al., 1994
Commercial Snack bars (18)	To compare maps obtained from sorting, structured & unstructured PM using different analysis.	2 untrained panels (24 judges, 1 for sorting, 1 for PM)	MDS, GPA used for unstructured PM and sorting; CA on structured PM; RV coefficient used to compare structured and unstructured PM methods	MDS or GPA for unstructured PM and sorting were more effective than CA analysis for structured PM; PM procedure makes it more easier for panellists to change their minds	King et al., 1998
Ewes milk cheese (8)	To compare maps obtained from PM by trained panel and naïve consumers	8 trained and 12 untrained panellists	Individual difference scaling to check for panel reproducibility and discrimination power; Correlation coefficient between replicate sessions	Trained panel had better performance quality index than consumer panel; overall both panel sample configurations followed similar trends	Barcenas et al., 2004
Milk and dark chocolates (14)	To compare results using PM and DA Untrained panel was used for PM. Once completed, the same panel was trained for DA and results were compared	3 groups (9, 9 and 8 subjects)	PCA, MFA used for PM and DA; RV coefficient used to compare methods and panels.	Untrained judges for PM provided equivalent maps as data obtained by DA; similarity among panels showed that overall the panellists perceived the product in a similar manner	Kennedy & Heymann, 2009
Granola bars (8)	To obtain maps and descriptions (terms) of berry flavoured granola bars using PM and evaluate the consistency of results obtained from 3 different sessions	1 untrained panel (15 subjects)	MFA, HMFA and PMFA used for data analysis; RV coefficient to compare results.	Maps showed similarity for all consumers; maps showed that the products were perceived similarly in terms of how the products were grouped.	Kennedy, 2010

<i>Products (Sample amount)</i>	<i>Objective</i>	<i>Number of panellists</i>	<i>Statistical method</i>	<i>Findings</i>	<i>Reference</i>
Apples (10) and cheeses (10)	To compare results obtained from PM and sorting	19 & 21 untrained panellists	MFA used for PM data; MDS used for sorting data.	PM was better suited than sorting; maps were similar for both PM and sorting; cluster analysis was easier to interpret for the napping configurations.	Nestrud & Lawless, 2010
Smoothies (8)	To present a new approach (sorted napping) that combined napping and categorization.	1 panel (24 subjects)	HMFA used for “sorted napping” data analysis. MFA used for tablecloths; MCA used for categorizations.	Combing PM with categorization using sorting task to group similar samples showed similar results from the subjects	Cadoret & Lê, 2010
Fish nuggets (9)	To compare and study the three sensory methods in case of hot served foods with contrasting textural layers	Conventional profiling- 10 subjects, flash profiling-10 semi-trained subjects & PM- 20 untrained subjects	MFA used for PM, GPA used for FP, PCA used for CP	Maps obtained by the three methods were well correlated; these methods could be used as a tool in consumer research with the use of an untrained panel	Albert et al., 2011
Bread (5) and yoghurt (5)	To characterize bread and yoghurt using the PM or Napping methods using an untrained consumer panel	7 panellists (5 women and 2 men)	MA, PCA	The partial Napping method is hard to introduce without guidance for consumers	Normann, 2012
Fruits and vegetables (32)	To evaluate the ability of DA, PM and sorting to describe differences among visual stimuli.	2 panels (11 assessors each)	MDS used for sorting data; MFA carried out on coordinates (x, y) of each samples recorded for each assessor and replicate	The separation level of samples obtained from PM and sorting is higher than DA.	Mielby et al., 2014

<i>Products (Sample amount)</i>	<i>Objective</i>	<i>Number of panellists</i>	<i>Statistical method</i>	<i>Findings</i>	<i>Reference</i>
Plain crackers (16) and vanilla milk dessert (8)	To evaluate global and individual reproducibility of projective mapping with consumers;  To evaluate the influence of the size of difference among samples	180 consumers for crackers, 4 groups of 42-48 consumers for desserts	MFA, RV coefficient used to compare results	Low individual reproducibility but a relatively high test-retest reproducibility of PM with consumers	Vidal et al., 2014
Cheese pies (8)	To compare the differences between global PM and partial PM based on texture and flavour.	3 groups of consumers (47 for global PM (G-PM), 53 for partial PM on flavour (F-PM), 61 for partial PM on texture (T-PM))	MFA carried out on coordinates of consumers; RV coefficient used to compare methods	No large differences in the vocabulary used for description of products between the two methods; T-PM and F-PM can provide more details than G-PM in each specific modality.	Marcano et al., 2015

PM, Projective Mapping; DA, Descriptive analysis; PCA, Principal component analysis; MFA, Multi-factor analysis; GPA, Generalized procrustes analysis; MDS, Multidimensional scaling; CA, Coordinate averaging; CP, Conventional profiling; RV, Regression vector; HMFA, Hierarchical multi-factor analysis; FP, Flash profile; RG, Repertory grid.



## 2.3 Digestibility study

### 2.3.1 Human digestive system

Human digestive system, also called digestive or gastrointestinal (GI) tract, is the essential pathway to transform foods to energy and nutrients for further utilisation by the human body. A series of connected organs constitute the system which starts from mouth, esophagus, stomach, small and large intestines, and anus. The digestion process is assisted by liver, pancreas, and gallbladder with secretions (Tortora & Derrickson, 2006).

Food is ingested and mechanically broken down by mastication to reduce the particle size and increase the surface area of foods to allow for faster enzymatic digestion. Small pieces of foods turn into bolus suitable for swallowing with the aid of saliva secreted by salivary glands. Early stage of digestion is started by the secretion of alpha-amylase (Vander, Sherman, & Luciano, 2001). The bolus travels through esophagus by peristalsis and enters stomach. With the aid of gastric secretions and peristalsis, the bolus turns into chyme. Proteins break down into peptides in the stomach with the action of pepsin, which is known as peptic proteolysis. Chyme is slowly released to small intestine. Further digestion and absorption of nutrients are accomplished in the small intestine with secretions from liver, pancreas (pancreatin), and gall bladder (bile). Peptides are further digested into individual amino acids in the small intestine and this is known as pancreatic proteolysis (Wickham, Faulks, & Mills, 2009). The presence of digestive enzymes, pH of the digestive organ and the presence of surfactants have a significant impact on the metabolism of ingested proteins. Unabsorbed materials move to large intestine and form feces (Tortora & Derrickson, 2006). Complex physicochemical and physiological events are involved in the digestion of foods. For example, conversion of starch-rich foods into oligosaccharides and monosaccharides mainly takes place in mouth and stomach (Noah et al., 1998), while the digestion of proteins (G. Wu, 2009) and lipids (Singh, Ye, & Horne, 2009) is primarily in stomach and small intestine.

#### 2.3.1.1 Stomach

Peptic digestion takes place in an acidic environment of pH range, 1.8 to 3.5 at 37 °C. Stomach acid, hydrochloric acid (HCl), is secreted by gastric parietal cells to initiate the conversion of pepsinogen into pepsin and maintain the acidic environment in the stomach. Pepsin is the most abundant gastric protease in the stomach which is

responsible for selective cleaving of proteins into mainly polypeptides, and a small amount of oligopeptides and amino acids (Restani, Restelli, Capuano, & Galli, 1992).

Furthermore, HCl also plays an essential role in the protein hydrolysis by denaturing or unfolding proteins for the easier contact with digestive enzymes. Proteins are made of polypeptides that are folded in a three-dimensional form of alpha helical coils and/or beta pleated sheet structure (NHMRC, 2006). Hence the structure of proteins, whether it is in quaternary, tertiary or secondary structure, will affect the digestibility. Peptic degradation accounts for 10 to 20 percent of protein digestion. It is the most active for the peptides bonded by phenylalanine, leucine, glutamine, valine and tyrosine (Krehbiel & Matthews, 2003; Monogioudi et al., 2011). Sites containing proline (PRO) and serine (SER) residues can also be cleaved (Fujioka & Scheraga, 1965; Schmelzer et al., 2007; Vance, LeBlanc, & London, 1997). Pepsin activity relates closely to the pH. The peptic activity is known to significantly increase from pH 1.5 and reach the maximal activity at pH 2. Then approximately 70 % of maximal activity has been shown to persist until it reaches pH 5. Loss of activity has been detected when the pH rose above 5 and irreversible inactivation occurred when the pH was raised above 7.5. These results were measured by in vitro methods reported in the studies reported by Piper and Fenton (1965) and Ville, Carrière, Renou, Laugier, and eacute (2001).

### **2.3.1.2 Small intestine**

Acidic gastric chyme is rapidly neutralised to pH of around 6 when it reaches duodenum in the small intestine, and then gradually the pH increases to about pH 7.4 in the terminal ileum (Fallingborg, 1999). The neutral environment is desirable for the activity of more powerful proteases secreted by pancreas and cells lining the small intestine. Protein digesta undergoes a complex digestion process by pancreatic enzymes, which include trypsin, chymotrypsin, elastase, and carboxypeptidases A and B (Krehbiel & Matthews, 2003). These enzymes catalyse the breakdown of proteins and polypeptides and liberate small peptide fragments with two to six amino acid residues, which accounts for approximately 60% of total proteolytic products. A large amount of free amino acids is also produced and it accounts for 40% of proteolytic products in the duodenum (Restani et al., 1992).

Each enzyme serves unique role in the process of proteolysis. Trypsin is responsible for the breakdown of the peptide bond linked with either or both of lysine

(LYS) and arginine (ARG). The linkage between aromatic amino acids is cleaved with the catalysis of chymotrypsin (Sitrin, 2014). As for the action of elastase, it generally targets the group of peptides bonded with aliphatic residues. The peptide residues produced from these enzymes release a number of terminal peptide bonds, which are passed on to lumen or mucosa and further hydrolysed by specific enzymes, carboxypeptidases A and B. Aromatic and non-polar amino acids with carboxy-terminals exposed to chymotrypsin and elastase further break down by carboxypeptidase A. Carboxypeptidase B is able to hydrolyse the carboxy-terminal exposed by the action of trypsin (Krehbiel & Matthews, 2003). Free amino acids (FAAs) released from proteins are absorbed via a distinctive carrier-mediated transport mechanism to the blood stream and utilised as energy and nutrients (Krehbiel & Matthews, 2003; Matthews, 1972).

### 2.3.2 *In vitro* digestion models for food application

For the purpose of assessing diet related questions, establishing *in vivo* feeding trials is still considered as the “gold standard” due to the capability of providing the most accurate results (Minekus et al., 2014). However, lengthy trials, labour, ethical restrictions and expenses encourage development of *in vitro* digestion models as an alternative screening tool of new foods or processing methods (Boisen & Eggum, 1991). The ideal *in vitro* models, in principle, would be a good alternative to human and animal models by offering rapid and accurate results of food and delivery systems with different structures and compositions (Hur, Lim, Decker, & McClements, 2011). The *in vitro* models allow a relatively large number of samples to be prepared and studied in parallel, at the site of interest and under expected conditions to produce reproducible results. However some compromises are still in demand for the models to accurately simulate the complex physicochemical and physiological events in the human digestion system (Hur et al., 2011; Wickham et al., 2009).

Extensive studies have been carried on the development of *in vitro* models and the application in a variety of food products. There are mainly three phases involved in the simulated models, which are oral, gastric and small intestinal phases as shown on Table 3. Sometimes large intestine is also included, in the form of a fermenter. These phases occur separately or in combination depending on the purpose of the study. Most of the studies for food application concentrate on evaluating the digestibility and bioaccessibility of macronutrients, such as proteins (Chen, Zhao, & Sun, 2013; Gatellier & Santé-Lhoutellier, 2009), carbohydrates (Stuknytė et al., 2014; Tamura, Singh, Kaur,

& Ogawa, 2015) and lipids (Hur, Lee, & Lee, 2015; Hur, Lim, Park, & Joo, 2009), and other bioactive compounds such as peptides (Ferranti et al., 2014; Schmelzer et al., 2007), minerals and trace elements (Garrett, Failla, & Sarama, 1999; Kulp, Fortson, Knize, & Felton, 2003; Purchas, Busboom, & Wilkinson, 2006).

The models can be divided into two groups; multi-compartmental dynamic systems and single static bioreactors. Dynamic models are constituted with multiple reactors to mimic the dynamic process of the human GI tract to study kinetics. For example, transport of digested meals, variable enzyme concentrations and pH changes over time (Minekus et al., 2014). Two of the most widely used and computerised dynamic models are ‘simulator of the human intestinal microbial ecosystem’ (SHIME) developed by Molly, Vande Woestyne, and Verstraete (1993), and ‘TNO gastrointestinal tract model’ (TIM) developed by Minekus, Marteau, and Havenaar (1995). More recently, some more sophisticated models have been developed to simulate the contraction forces and frequency that foods receive from the peristaltic movement. These models include ‘dynamic gastric model’ (DGM) developed by Thuenemann, Mandalari, Rich, and Faulks (2015), French INRA model developed by Ménard et al. (2014), ‘human gastric simulators’ (HGS) developed by Kong and Singh (2010), and ‘*in vitro* Physicochemical Upper Gastrointestinal System’ (IPUGS) developed by Yoo (2009).

Static models, also known as biochemical models, are the most widespread digestive systems which have been studied in a number of literature for decades (Argyri, Birba, Miller, Komaitis, & Kapsokefalou, 2009; Diaz, Vatter, & Mahoney, 2002; Farouk, Wu, Frost, Clerens, & Knowles, 2014; Ferranti et al., 2014; Howie, Calsamiglia, & Stern, 1996; Minekus et al., 2014; Saunders, Connor, Booth, Bickoff, & Kohler, 1973). Static models do not mimic the physiological processes that occur in *in vivo* settings, such as shearing, mixing, hydration, and varying conditions over time. The static digestion models expose the test material to simulated secretions of fixed concentrations, pH and temperature over a certain period of time (Boisen & Eggum, 1991). Conditions set for static models vary widely, in terms of concentrations and sources of enzymes, pH, time and stages of digestion, to suit the purpose of the study. In practical application, static models are able to provide a feasible protocol with low cost, and they are particularly relevant for large pre-screening approaches (Alminger et al., 2014; Minekus et al., 2014).

Several factors are accounted for the accuracy of results, such as sample characteristics, activities of digestive enzymes, ionic composition, pH, mechanical shear

and period of digestion time (Hur et al., 2011). Enzyme characteristics play a key role in the simulated digestion models. Enzyme activities are mainly affected by pH, temperature, concentration, stability, activators, inhibitors and incubation time (Boisen & Eggum, 1991). Choice of enzymes (Single- or multi-enzyme) and concentrations are dependent on the characteristics of foods and objectives of study. Single-enzyme has the advantage of producing more consistent results for predicting the digestibility of a single nutrient. For example, assessment of the digestibility of protein by using pepsin only, or lipids by using lipases only, or starch by using amylases only. However, nutrients interact with other nutrients and enzymes present in the human digestive system (Boisen & Eggum, 1991). The adoption of a mixture of enzymes seems more realistic to improve the *in vivo-in vitro* correlation. In early years, the pepsin-pancreatin system adopted by Akeson and Stahmann (1964) has shown a reasonable approximation of protein digestibility and high correlation with data obtained from *in vivo* studies. Due to the lack of data collected from *in vivo* studies, further research is still required to evaluate the *in vivo-in vitro* correlation and the validity of *in vitro* results (Emami, 2006; Souliman, Blanquet, Beyssac, & Cardot, 2006).

### 2.3.3 *In vitro* protein digestibility studies

A number of *in vitro* models, either static or dynamic, have been developed to assess the digestibility or the bioaccessibility of dietary protein from various meat sources, and this is summarized in Table 3. The bioavailability of bioactive compounds, including iron (Argyri, Komaitis, & Kapsokefalou, 2006; Diaz et al., 2002; Purchas et al., 2006; Sørensen, Sørensen, Søndergaard, & Bukhave, 2007; Storcksdieck, Bonsmann, & Hurrell, 2007), and amines (Kulp et al., 2003) as shown in Table 3, is of interest regarding the potential health benefits to human wellbeing. It is due to their roles as important micronutrients or in elevating the absorption of other bioactive compounds. For example, red meat (include bovine and sheep meat) is an important dietary source of iron, and particularly of the more bioavailable haem iron (Conrad & Umbreit, 2000). Muscle foods would also enhance the absorption of non-haem iron and known as “meat factor” (Diaz et al., 2002; Sørensen et al., 2007; Storcksdieck et al., 2007).

Amino acid profile, particularly essential amino acids, plays a principal role in evaluating the nutritive quality of a protein. FAAs released from enzymatic degradation of dietary protein human GI is of great health benefit, as discussed in previous sections. Regarding the digestibility of protein, the PDCCA score remains as the “gold standard”

(Schaafsma, 2000). However, the requirement for *in vivo* data is expensive and difficult to apply in many situations. In the recent years, measurement of low molecular compounds, such as bioactive peptides shown in Table 3 (Bordoni et al., 2014; Farouk et al., 2014; Marcolini et al., 2015; Paoletta et al., 2015; Storcksdieck et al., 2007) or free amino acids (Stuknytė et al., 2014) from the simulated digestive processes has been adopted to study the digestibility of dietary proteins. In the present study, the digestibility of proteins in bresaola products was evaluated by measuring the FAAs released during the simulated human digestion process.

**Table 3 Summary of *in vitro* digestion of proteins from different meat and meat products**

<i>Protein source</i>	<i>Models</i>	<i>Enzymes</i>	<i>Digestion Period</i>	<i>Objectives of the study</i>	<i>References</i>
<b>Animal by-product proteins</b>	Three-step <i>in vitro</i> static model in polyester bags	Pepsin	1 h	Evaluation of proteins	Howie et al., 1996
		Pancreatin	24 h		
<b>Chicken proteins</b>	Two-stage <i>in vitro</i> static model (Flask system)	Pepsin	2 h	Production of dialyzable iron	Diaz et al., 2002
		Pancreatin + bile salt	2 h		
<b>Cooked chicken</b>	Three-step <i>in vitro</i> static model in re-sealable plastic bag	Amylase	10 min	Effect of cooking methods on bioaccessibility of heterocyclic amines	Kulp et al., 2003
		pepsin	30 min		
		pancreatin	3.5 h		
<b>Sausage</b>	Both static and dynamic system	$\alpha$ -amylase + lysozyme	1 min	Evaluation survival or growth of E-Coli O157:H7 cells during the digestion of sausages in human gastrointestinal tract	Naim, Messier, Saucier, & Piette, 2004
		Mucin+pepsin	2 h		
		Pancreatin + bile salt	4 h		
<b>Lean beef and casein</b>	Two-stage static <i>in vitro</i> model in screw-cap vials	Pepsin	2 h	Effect of iron and red wine on antioxidant capacity in beef	Argyri et al., 2006
		Pancreatin + bile salt	2 h		
<b>longissimus</b>	Two-stage static <i>in vitro</i> model	Pepsin	2 h	Effect of cooking on iron and bioactive compounds	Purchas et al., 2006
<b>Lumborum muscle from beef</b>		Pancreatin + bile salt	1 h		
<b>Sarcoplasmic and myofibrillar protein from different animals sources (beef, chicken, cod, lamb and pork)</b>	Two-stage static <i>in vitro</i> model (beaker system)	Pepsin	2 h	Nitrogen and iron solubility and molecular weight distribution, recovery of <sup>59</sup> Fe;  Amino acid composition	Storcksdieck et al., 2007
		pepsin/pancreatin	2 h		

<i>Protein source</i>	<i>Models</i>	<i>Enzymes</i>	<i>Digestion Period</i>	<i>Objectives of the study</i>	<i>References</i>
<b>Pork</b>	Two-stage static <i>in vitro</i> model	Pepsin	1 h	Non-haem iron availability	Sørensen et al., 2007
		pepsin + pancreatin	1 h	Iron availability, iron-reducing capacity and molecular weight	
<b>Iberian dry Fermented sausages</b>	Two separated steps static system	Oxgall	0-8 h	Characterisation of the potential probiotic isolates	Ruiz-Moyano, Martín, Benito, Nevado, & de Guía Córdoba, 2008
		pancreatin	3 h		
<b>Myofibrillar protein from lamb meat</b>	Two-stage static model by supplying simulated digestive fluids at different time points	Pepsin	0-60 min	Digestibility of myofibrillar protein proteolytic activity	Santé-Lhoutellier, Engel, Aubry, & Gatellier, 2008
		Trypsin + $\alpha$ -chymotrypsin	0-30 min		
<b>Cooked beef round steak</b>	Two-stage static <i>in vitro</i> model in screw-cap vials	Pepsin	2 h	Concentration of bioavailable iron and development of dialyzability method	Argyri et al., 2009
		Pancreatin + bile salt	2 h		
<b>Beef protein</b>	Dynamic model with a semi-automatic procedure	Pepsin	120 min	Digestion of meat proteins, profile of peptides release with digestion time	Gatellier & Santé-Lhoutellier, 2009
		Trypsin + chymotrypsin	130 min		
<b>Processed meat (bresaola)</b>	Three-stage static <i>in vitro</i> digestion model	Amylase	5 min	Evaluation of protein bioaccessibility; proteolytic activity by measuring peptides formation	Bordoni et al., 2014
		Pepsin	1 h		
		Pancreatin + bile salt	5 h		
<b>Longissimus dorsi muscles from beef</b>	Two-stage <i>in vitro</i> dynamic digestion model system	Pepsin	0-90 min	Digestibility of beef at different inherent ultimate pH; Evaluation of peptide formation	Farouk et al., 2014
		Pancreatin	0-210 min		
<b>Processed meat (Bresaola)</b>	Static consecutive multi-enzymes model	$\alpha$ -amylase	5 min	Evaluating the release of potential bioactive peptides	Ferranti et al., 2014
		Pepsin	60 min		
		Pancreatin + bile	300 min		

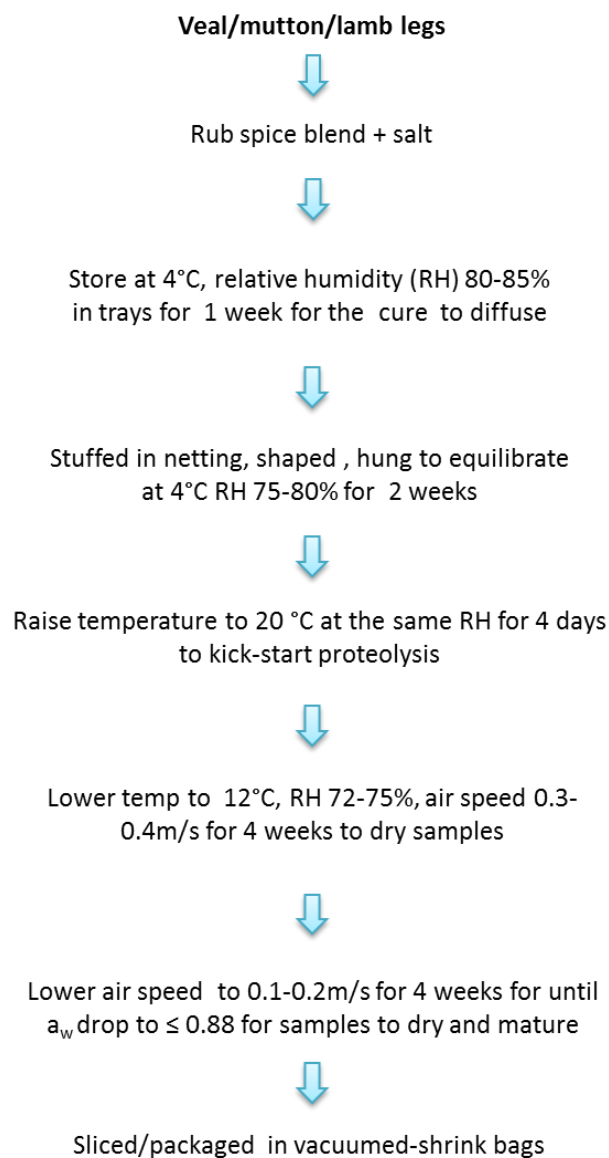


<i>Protein source</i>	<i>Models</i>	<i>Enzymes</i>	<i>Digestion Period</i>	<i>Objectives of the study</i>	<i>References</i>
<b>Myofibrillar proteins from Charolais heifer</b>	Dynamic <i>in vitro</i> digestion system	Pepsin	0-4 h	Digestibility of myofibrillar proteins by pepsin; Predicting <i>in vitro</i> digestion kinetics	Kondjoyan, Daudin, & Santé-Lhoutellier, 2015
<b>Cured beef meat (Bresaola)</b>	Three-stage static <i>in vitro</i> digestion model	$\alpha$ -amylase	5 min	Evaluation of bioaccessibility of bioactive dipeptides Carnosine	Marcolini et al., 2015
		Pepsin	60 min		
		Pancreatin + bile salt	300 min		
<b>Dry-cured ham</b>	Three-stage static <i>in vitro</i> digestion model	$\alpha$ -amylase + mucin	5 min	Maturation time on the digestibility of meat proteins and on the eventual release of bioactive sequences of peptides	Paolella et al., 2015
		Pepsin + mucin + BSA	2 h		
		Pancreatin + lipase + bile salt	2 h		

## Chapter 3. Materials and Methods

### 3.1 Sample acquisition

Bresaola products made from veal, mutton and lamb were provided by AgResearch Ltd, New Zealand. Veal bresaola was made from NZ Bobby calves legs (4 days old). Mutton and lamb bresaola were made from NZ mutton (3-4 years old) and lamb legs. The production protocol followed three main stages, salting, air-dry and maturing as summarised in Figure 3. The total preparation period took up to 12 weeks. Spice blend included sea salt (15 g); Horopito (3 g); Karengo (1g); Kawakawa (1g); black pepper (1 g); raw sugar (2 g); sodium nitrite (0.3 g) and ascorbate (0.6 g).



**Figure 3 Preparation protocol for veal, mutton and lamb bresaola samples**

Beef and wagyu bresaola were purchased from the local commercial cured meat producer Otello's, The Curing Co Ltd, New Zealand. Beef bresaola was made from 100% NZ lean beef loin, using hung, air-dry method. Wagyu bresaola was made from 100% NZ grass fed Wagyu beef, using air-dry method.

Five bresaola samples may differ in terms of stages of curing (length of curing), cuts (legs *vs.* loin) and storage time, due to two different origins of samples. All meat samples were kept vacuum packed and stored in a refrigerator (2-4 °C) until further analysis.

### 3.2 Physicochemical characterisation of bresaola

Physicochemical characterisation was carried out on the bresaola products made from five different sources of meat including beef, veal, wagyu, mutton and lamb. Nutritional composition, in terms of fatty acids profile, content of total intramuscular fat, total protein, moisture and ash, was assessed. Examination of physical properties included instrumental texture and colour measurements which were performed on all five samples. All the analyses were carried out in triplicate.

#### 3.2.1 Proximate analysis

##### 3.2.1.1 Extraction of intramuscular fat

'Mixed solvent extraction method' by Folch, Lees, and Sloane-Stanley (1957) was used with some modifications to extract intramuscular lipids from bresaola products.

2g of lyophilized ground bresaola sample was homogenized with solvent mixture made of chloroform/methanol (2:1, v/v) to a final volume of 40 ml (20 times the volume of the tissue sample) in a screw-capped bottle. 15-20 min of homogenization on a magnetic stirrer at 800 rpm (room temperature) was required to complete the extraction of lipids from the sample. The homogenate was filtered with a Whatman® filter paper to eliminate undissolved particles from the mixture. The homogenate was further washed with 0.2 volume (8 ml for 40 ml) of 0.9% sodium chloride solution. After vortexing for 30 seconds, lipid extract was recovered by centrifuging the mixture at 2000 rpm at 4 °C for 20 min to separate the two phases. Upper phase containing non-lipid contamination was removed by siphoning using a glass pipette. The interface was rinse three times with

methanol/water (1:1, v/v) without mixing the whole lower phase. Finally lower chloroform phase containing lipids was transferred into a round bottom flask and solvent in the mixture was evaporated under vacuum in a rotary evaporator until constant weight was obtained. The final weight of the round bottom flask was recorded to obtain the weight of extracted lipids.

### 3.2.1.2 Total protein analysis

Total protein content of bresaola products was determined using Kjeldahl method according to (AOAC, 2003). Three main steps were involved in this protocol, i) the kjeldahl nitrogen in the sample was converted to ammonia by metal-catalysed acid digestion; ii) the resulting ammonia was then separated from the sample by distillation. The ammonia released was captured in boric acid solution and transferred back to ammonium; iii) the nitrogen concentration within the receiving solution was determined by titrimetric method.

500 ± 0.1 mg of each lyophilized ground bresaola sample was accurately weighed, and then placed in a 250 mL kjeldahl digestion tube. A mixture of 7g of potassium sulphate and 500mg of copper sulphate was added as the kjeldahl catalyst. Another tube containing all the chemicals except the bresaola sample was used as a blank. Then 10 ml of concentrated sulphuric acid was added and the tube content was carefully mixed before placing on the Velp DK 20 heating block which was pre-heated to 420 °C. Samples were digested in the tube at 420°C for 60min. During the digestion, an extra 5-10ml of concentrated sulphuric acid was added to supplement the level of liquid in the digestion tube when dropped significantly. The tube was allowed to cool at room temperature for 5 minutes and 20 mL of distilled water was slowly added to avoid spilling of boiling liquid. The liquid in the test tube was clear and colourless.

After the kjeldahl digestion, the sample was distilled using a VelpUDK 139 distillation unit. The digestion tube with the digested sample was attached to the distillation unit. An automatic distillation process with 10M sodium hydroxide was performed. The released ammonia steam was trapped in the receiver filled with 20 mL of 4% (w/w) boric acid and 5-7 drops of mixed Kjeldahl indicator flask, The content was titrated with 0.1 M HCl. Mixed Kjeldahl indicator was made by mixing 1 part (volume) of 0.1% methyl red in 95% ethanol with 2 parts (volume) of 0.2% bromocresol green in 95% ethanol. All analyses were carried out in triplicate for each bresaola sample.

Total nitrogen content in bresaola products was calculated by the formula below:

$$\text{Nitrogen (g/100g)} = \frac{(V_1 - V_2) \times C_{HCl} \times 14.007}{W} \times 100$$

$V_1$  - titrated volume of standard acid for sample, mL;

$V_2$  - titrated volume of standard acid for reagent blank, mL;

$C_{HCl}$  - concentration of standard hydrochloric acid, mmol/mL;

14.007 = Molar mass of Nitrogen, mg/mmol

$W$  - sample weight, mg

The crude protein content of bresaola products was estimated by applying the commonly accepted nitrogen-protein conversion factor for meat meal, of 6.25 (Benedict, 1986). The formula is shown below:

$$\text{Crude Protein (g/100 g)} = \text{Nitrogen (g/100 g)} \times 6.25$$

### 3.2.1.3 Moisture content

Moisture content of bresaola products was measured using oven drying method according to AOAC 950.46B (AOAC, 2003). Bresaola samples were vacuum packed until the moisture analysis to avoid any water loss during the storage. Crucibles used for measurement were cleaned and pre-dried at 105°C to constant weight. The weight of each crucible was recorded and it was stored in a desiccator until use. About 5g of each bresaola sample was cut into slices of approximate 2mm thickness with a scalpel. Bresaola samples were spread out in the crucibles to facilitate the evaporation process. All samples were dried in a convection oven (SANYO Electric Biomedical Co. Ltd, Japan) at 105°C until constant weight was achieved. Crucibles were placed in the desiccator until they were completely cooled down to room temperature. Then the weight of crucibles with dried samples was measured. All samples were measured in triplicate.

Moisture content was obtained from the formula below:

$$\text{Moisture (g/100 g)} = \frac{(W_1 - W_2)}{W_1} \times 100$$

W<sub>1</sub>= Weight of sample before drying

W<sub>2</sub>= Weight of sample after drying

#### 3.2.1.4 Ash measurement

Ash content of bresaola products was assessed according to AOAC 920.153 (AOAC, 2003). Crucibles used for ash measurement were placed in a muffle furnace (Model 200, McGregor Kiln Furnace) at 550°C overnight to ensure all of the impurities on the surface were burned off. All crucibles were cooled down in a desiccator before weighing. Approximate 5g of each bresaola sample was weighed and put into a crucible and heated over low Bunsen flame until fumes were no longer produced. This was done to discard any organic materials in the samples. Crucibles were placed in the muffle furnace and heated at 550 °C overnight (approximate 15 hours) until ashing was complete. The door was carefully opened to avoid any loss of ash. The crucibles were carefully transferred from the muffle furnace into the desiccator to completely cool down prior to weighing. At the end of the cooling period, the desiccator cover was removed gradually by sliding to one side to prevent a sudden inrush of air. The cool crucible containing the ash was weighed. All of the samples and crucibles were weighed to 4 decimal places. Ash measurement of all samples was performed in triplicate.

Ash content was calculated by the formula below:

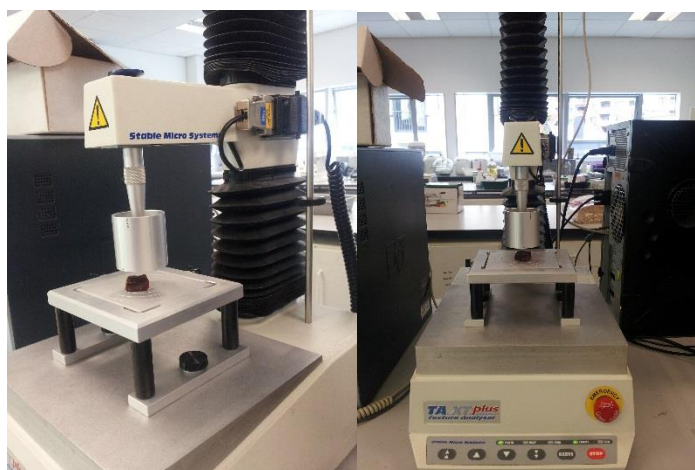
$$\text{Ash (g/100 g)} = \frac{\text{Weight of ash}}{\text{Initial weight of raw sample}} \times 100$$

Carbohydrates content was ignored due to the negligible amount of carbohydrates present in meat.

### 3.2.2 Texture profile analysis (TPA)

Texture Profile Analysis (TPA) was performed to determine the texture profile of bresaola in terms of hardness, cohesiveness, springiness, resilience, and chewiness. Gumminess was omitted because, according to Szczesniak (1995), it is a characteristic parameter for semisolid samples only, and should not be used on the same product along with chewiness which is specific for solid samples. Hardness was defined by the peak force during the first compression cycle and expressed in kg. Cohesiveness was calculated as the ratio of the area under the second curve to the area under the first curve. Springiness was defined as a ratio of time recorded between the start of the second area and the second probe reversal to the time recorded between the start of the first area and the first probe reversal. Resilience was defined as a ratio of the area during the withdrawal of the first penetration, divided by the area of the first penetration. Finally, chewiness, expressed in kg, was evaluated by multiplying hardness, springiness and cohesiveness.

The outer surface of all the bresaola samples was removed using a knife. A cube of 1cm\*1cm\*1cm was acquired from the same part of each sample (1 cm from the outer surface), to avoid the errors caused by the different textural characteristics of outer surface and the central part of cured meat. The dry-curing process would cause the crust formation, which could be defined as outer surface becomes irreversibly harder due to the water loss, while the central part is softer than the superficial layer (Ruiz-Ramírez, Serra, et al., 2005). Samples measured in this part of study could be characterised as moderate in hardness compared to rest parts of muscle.



**Figure 4 Photos of Stable Micro Systems TA.XT plus Texture analyser**

TPA of bresaola products was performed using a Stable Micro Systems TA.XT plus Texture analyser (Surry, UK) equipped with a Film Support Rig (HDP/FSR) on a heavy duty platform (HDP/90), as shown in Figure 4. Each sample cube was placed on the centre of base plate and beneath the p/50 cylindrical aluminium probe (50mm) in order to meet with a consistent flat surface. The probe was adjusted to 20mm distance away from the base plate for the ease of operation and saving time. Calibration was performed on the probe before the test and after each five analyses were carried out. Then the probe was programmed to slowly move down to a lower level in order to touch the sample, the probe pressed in two cycles with a 5s delay. The strain required for 50% compression of sample thickness was recorded using the following settings: pre-test speed: 10.0 mm/s, test speed: 0.5 mm/s, post-test speed: 10.0 mm/s, time 30s; load cell: 50 Kg; trigger force: auto-10g. Determinations were repeated 12 times per sample.

### 3.2.3 Instrumental colour analysis

A Hunter lab (45/0, Colourflex) was used to measure the colour of bresaola samples, as shown in Figure 5. Each bresaola at the part of 10mm from the edge was cut into a 10mm thickness slice for colour measurements. Slices of each sample were placed in a petri dish and then placed above the centre of lens of Chroma meter to ensure no light was emitted from lens. All the measurements were performed after correctly calibrating the Chroma meter. Measurements were taken shortly after cutting the slices to minimise oxidation and fading of colour. The colour coordinates of CIE 1976 lightness  $L^*$  (0 = black, 100 = white), redness  $a^*$  ( $-a^*$  = greenness and  $+a^*$  = redness), and yellowness  $b^*$  ( $-b^*$  = blueness,  $+b^*$  = yellowness) were determined at 3 different position of each sample (Braghieri et al., 2009), after using the standard Illuminant D65 to calibrate the instrument. And the values of colour saturation: chrome (C) (Liu, Scheller, Arp, Schaefer, & Frigg, 1996), and proportions of redness and yellowness: hue angle ( $H^\circ$ ) (McLellan, Lind, & Kime, 1995) were further calculated according to the formulae below:

$$Chroma (C) = ((a^*)^2 + (b^*)^2)^{1/2}$$

$$Hue\ angle\ (H^\circ) = arctangent\ (b^*/a^*)\ (a^* > 0)$$





**Figure 5 Photo of Hunter lab (45/0, Colourflex) Chroma meter**

### **3.2.4 Fatty acids profile**

Quantification of fatty acids profile in bresaola products was carried out according to (Juárez et al., 2008) with some modifications. Briefly, the method was performed by acid hydrolysis of lipids in lyophilized ground bresaola samples to release free fatty acids which was followed by in situ esterification to Fatty Acid Methyl Esters (FAMES) and their extraction with toluene for analysis by Gas-Chromatography.

#### Extraction of FAMES with toluene:

Visible fats and outer surface were trimmed with a knife, and freeze-dried for 48 hours to lyophilize them completely. All bresaola samples were ground under liquid nitrogen. A  $25 \pm 0.1$  mg of the ground sample was placed in a 10 ml test tube. 10  $\mu\text{l}$  of a  $2 \text{ g.L}^{-1}$  tridecanoic acid in toluene was added as an internal standard before a further 490  $\mu\text{l}$  of toluene and 750  $\mu\text{l}$  of freshly prepared 5% methanolic HCl were added. The mixture was mixed on a vortex. Then, the headspace of each tube was flushed with nitrogen before closing to prevent oxidation of fatty acid. Tubes were sealed and placed in a water bath at  $70^\circ\text{C}$  for two hours. Tubes were then cooled to room temperature. 1 ml of 6% aqueous  $\text{K}_2\text{CO}_3$  and 500  $\mu\text{l}$  of toluene were added and vortexed gently to mix. The mixture was centrifuged at 2000 rpm for 5 minutes and the organic phase was removed with a glass Pasteur pipette for further analysis of FAMES.

### FAMES analysis:

FAMES were separated and quantified on a Shimadzu GC2010 with a Flame Ionisation Detector (FID), a split injector and an AOC-20i auto-injector. Phenomenex Zebron ZB-WAX capillary column measuring 0.25 mm x 30 m x 0.25  $\mu\text{m}$  was used. Initial oven temperature was 140 °C, rising to 245 °C at a rate of 5 °C per minute and the temperature was held for 15 minutes. FAME peaks in samples were identified and quantified by comparison of their retention times with those produced from a mixture of 37 FAME standards (Supelco product 47885-U) obtained from Sigma Aldrich (Sydney, Australia) and serially diluted to five concentrations from 10 to 0.625 g.L<sup>-1</sup>.

### **3.2.5 Free amino acids analysis**

A diluted hydrochloric acid in rich alcohol-containing solution was used to extract free amino acids (FAA) available before hydrolysis from bresaola products according to Aristoy and Toldrá (2008) and Pérez-Palacios, Barroso, Ruiz, and Antequera (2015), with modifications. 1 g of lyophilized ground bresaola sample was weighed into a screw-capped bottle and homogenised with 25 mL of 0.1 M HCl in ethanol solution. This was made by diluting 2 parts of 0.5 M HCl in 8 parts of ethanol (v/v). Extraction was carried out in a shaking water bath (Model G76, New Brunswick Scientific co., INC, EDISON, N.J., U.S.A) at 40 °C for 2 hours. Supernatants contained FAA extracts were collected after centrifugation at 15,000 rpm for 10 min. All the FAA extracts were further analysed by using a commercial free amino acid kit (EZ:faast<sup>TM</sup>, Phenomenex®, USA) described in section 3.4.3 and Appendix 8 according to (Phenomenex, 2003).

## **3.3 Sensory evaluation of bresaola products**

Sensory experiments were performed at the sensory evaluation laboratory, Auckland University of Technology (Auckland, New Zealand) over three weeks. Consumer testing and projective mapping of five bresaola samples made from different sources of meat were carried out. Ethics application for this study was approved by the Auckland University of Technology Ethics Committee (AUTEC) on 29 October 2014 (Appendix 3).

Slices (1.5 mm) of bresaola products were obtained using a commercial slicing machine (Braghieri et al., 2009). Samples were packed in vacuum bags immediately after slicing and stored at 2-4 °C prior to evaluation. Samples were coded with three-digit randomized numbers and served in random order to avoid sample order and carry-over effects. Each slice of bresaola sample was placed separately in a 25 cm plastic container. Samples were served at room temperature (20-23 °C). Panellists were asked to drink filtered water at the beginning of the sensory evaluation and between samples to refresh their palate. Test samples were served to the panellists according to a randomised controlled trial. Analysis was conducted in individual test booths under red light to mask any variations in appearance and colour and to better focus on the attributes of flavour and texture. The FIZZ software (2.46B, Biosystems, 2010) was used to generate random three-digit codes, and to design the questionnaire and record results.

### **3.3.1 Consumer Testing**

Fifty-three panellists (aged 20-51 years old), who consumed meat and/or meat products at least once a month, assessed overall liking and the acceptability of bresaola products in terms of odour, flavour and texture using a 100 mm unstructured line scale ranging from extremely dislike to extremely like. A 1 min break was taken between samples. Panellists were provided with clean filtered water to rinse their mouth. Minimal information of the samples were provided to the panellists to avoid any bias on the rating. The FIZZ program recorded the information and sensory scores of panellists. The questionnaire used is attached in Appendix 1.

### **3.3.2 Projective mapping**

Fifteen regular meat consumers were invited to attend three projective mapping sessions at the Sensory suite once in a week over three consecutive weeks. Panellists were given verbal instructions of the sensory testing procedure and given a list of attributes agreed by the panellists at the start of the sensory session. Sensory attributes that described the samples in terms of odour, texture, taste, and flavour obtained from literature (Armero et al., 1999; Braghieri et al., 2009; Civille & Lyon, 1996; Ruiz et al., 1998) that panellists agreed on are summarized in Table 4.

A set of instructions (Appendix 2) was displayed on the computer terminals using a FIZZ programmed sensory projective mapping test (FIZZ Network v2.46C, Biosystemes). Panellists then tasted the randomised and coded bresaola samples in the sensory booths. Panellists grouped the samples according to their similarities and differences, with those grouped close together being more similar to each other. Additionally, they were asked to express descriptors and/or attributes that corresponded to their groupings. Products were positioned on the computer screen and sensory attributes associated with each product were keyed in by the panellists and recorded using the FIZZ Network v2.46C system. Analysis of results was performed using Multi-factorial Analysis (MFA) to obtain overall product maps, General Procrustes Analysis (GPA) was carried out to obtain overall product coordinates, and Principal Component Analysis (PCA) to obtain product and attribute biplots using Addinsoft XLSTAT-MX version 2011.5.01. Sensory attributes that occurred a minimum of five times across panellists per product were included in the PCA biplots.

**Table 4 Sensory attributes applied in projective mapping analysis**

<i>Attributes</i>		<i>Definition</i>
<b>Odour</b>	Fermented	Aroma associated with lactic or spoilage bacteria as in soured meat
	Rancid	Aroma associated with extremely oxidized fat or oil
	Sheep meat aroma	Aroma associated with the animal species (Lamb and mutton)
<b>Texture</b>	Tenderness	Effort required to compress the sample between molars
	Fibrousnesses	Extent to which fibres strands are perceived on chewing
	Fatty	Attribute relating to the perception of the quantity of fat during chawing
	Juiciness	Impression of lubrication during chewing of meat
	Chewiness	Time required to masticate sample into a state ready for swallowing
<b>Taste</b>	Saltiness	Describes the basic taste produced by dilute aqueous solutions of sodium chloride
	Sweetness	Describes the basic taste produced by dilute aqueous solutions of sucrose
	Bitterness	Describes the basic taste produced by caffeine
	Sourness	Describes the basic taste produced by dilute aqueous solutions of citric acid
	Peppered	Taste related to pepper
	Seasoned	Taste related to seasoning
	Aftertaste	Intensity and time of flavour perception after sample is swallowed
<b>Flavour</b>	Lamb flavour	Flavour associated with lamb
	Mutton flavour	Flavour associated with mutton

### 3.4 *In vitro* digestion of bresaola products

*In vitro* enzymatic digestion of the five bresaola products was the centre of this part of study. A standardised static digestion method according to (Ferranti et al., 2014; Minekus et al., 2014) was carried out on the five bresaola products with modifications. A consecutive three-stage digestion was simulated in a single bioreactor (screw-capped glass bottle) which constituted oral phase, gastric phase and intestinal phase. A multi-enzyme system composed of salivary  $\alpha$ -amylase (oral phase), pepsin (gastric phase) and pancreatin (intestinal phase) was tested for their enzymatic activity before the digestibility study, according to the standard protocols detailed in the subsequent sections. Compositions of free amino acids (FAA) prior to conducting enzymatic hydrolysis were determined. All digestibility trials were carried out in triplicate for each bresaola product.

#### 3.4.1 Enzyme activity assay

The activity of  $\alpha$ -amylase (EC 3.2.1.1) from the *Aspergillus oryzae* ( $\geq 150$  units/mg protein biuret, Sigma) was determined based on Bernfeld (1955) published by Sigma® (Appendix 4). Briefly, the assay was based on soluble potato starch: one unit liberates 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20 °C.

The activity of pepsin (EC 3.4.23.1) from porcine gastric mucosa (Sigma-Aldrich P-7000), was determined using a method based on the stop-point assay of haemoglobin degradation developed by (Anson, 1938) and published by Sigma® (Appendix 5). The assay was based on using bovine blood haemoglobin as a substrate: one unit produces a  $\Delta A_{280}$  of 0.001 per minute at pH 2.0 and 37 °C, and measured as TCA-soluble products.

Activity of pancreatin from porcine pancreas (Pancreatin 3X U.S.P., MP Biomedicals, LLC) was tested based on the proteolytic activity of trypsin and chymotrypsin. Assay of Trypsin (EC 3.4.21.4) activity (Appendix 6) is based on N $\alpha$ -Benzoyl-L-arginine ethyl ester (BAEE): one BAEE unit of trypsin activity will produce a  $\Delta A_{253}$  of 0.001 per minute with BAEE as substrate at pH 7.6 at 25 °C in a reaction volume of 3.20 ml. As for chymotrypsin (EC 3.4.21.1), enzymatic activity was determined based on N- benzoyl-L-tyrosine ethyl ester (BTEE) (Appendix 7): one unit hydrolyses 1.0 mmol of BTEE per minute at pH 7.8 at 25 °C.

### 3.4.2 Static *in vitro* digestion protocol

#### 3.4.2.1 Preparation of simulated digestion fluids

Simulated digestion fluids, including simulated salivary fluids (SSF), simulated gastric fluids (SGF) and simulated intestinal fluids (SIF), were prepared based on human *in vivo* data presented by Minekus et al. (2014) (Table 5). Simulated electrolyte stock solution was made by dissolving chemical constituents in deionized water (Table 6). Each of the simulated electrolyte stock solutions, referred to as SSF, SGF and SIF, was made up to x 1.25 concentrate to counterbalance the dilution from adding digestive enzymes, CaCl<sub>2</sub> and deionized water. 1 M NaOH and 6 M HCl were used to adjust pH during digestion.

**Table 5 Concentrations of electrolytes in final simulated digestion fluids used in the *in vitro* digestion model (Minekus et al., 2014).**

	SSF	SGF	SIF
Constituent	Conc. (mmol.L <sup>-1</sup> )	Conc. (mmol.L <sup>-1</sup> )	Conc. (mmol.L <sup>-1</sup> )
K <sup>+</sup>	18.8	7.8	7.6
Na <sup>+</sup>	13.6	72.2	123.4
Cl <sup>-</sup>	19.5	70.2	55.5
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	3.7	0.9	0.8
HCO <sub>3</sub> <sup>-</sup> , CO <sub>3</sub> <sup>2-</sup>	13.7	25.5	85
Mg <sup>2+</sup>	0.15	0.14	0.33
NH <sub>4</sub> <sup>+</sup>	0.12	1.0	-
Ca <sup>+</sup>	1.5	0.15	0.6

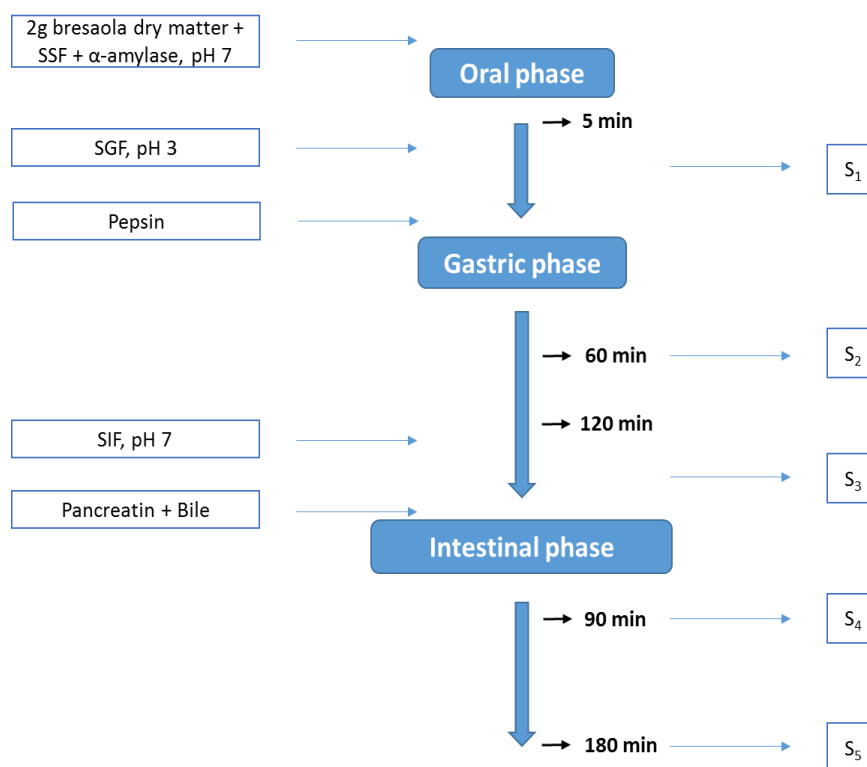
**Table 6 Components of the stock solutions of simulated digestion fluids used in the *in vitro* digestion model.**

Constituent	Stock conc. (mol.L <sup>-1</sup> )	SSF	SGF	SIF
		pH 7	pH 3	pH 7
		Vol. of stock (ml)	Vol. of stock (ml)	Vol. of stock (ml)
KCl	0.5	15.1	6.9	6.8
KH <sub>2</sub> PO <sub>4</sub>	68	3.7	0.9	0.8
NaHCO <sub>3</sub>	84	6.8	12.5	42.5
NaCl	117	-	11.8	9.6
MgCl <sub>2</sub> (H <sub>2</sub> O) <sub>6</sub>	30.5	0.5	0.4	1.1
(NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub>	48	0.06	0.5	-
*CaCl <sub>2</sub> (H <sub>2</sub> O) <sub>2</sub>	44.1	0.05	0.01	0.08

\*CaCl<sub>2</sub>(H<sub>2</sub>O)<sub>2</sub> is not added to the electrolyte stock solutions instead of adding to final mixture of simulated digestion fluid during digestion process.

### 3.4.2.2 Procedure of static *in vitro* digestion

The procedure of static *in vitro* digestion protocol is outlined in Figure 6. The stock solutions, enzyme solutions and deionized water used in the digestion process were incubated at 37 °C in a conventional oven until use. Three stages of digestion were carried out in a shaking water bath (Model G76, New Brunswick Scientific co., INC, EDISON, N.J., U.S.A) at 37 °C for 5 hours at the speed of 5 rpm.



**Figure 6 Flow diagram of a simulated static *in vitro* digestion method. SSF, SGF, SIG refer to Simulated Salivary Fluid, Simulated Gastric Fluid and Simulated Intestinal Fluid, respectively.**

Five samples were taken during the *in vitro* digestion process. 0.5 mL of sample (S<sub>1</sub>) was pipetted from the reactor at the end of the oral phase (5 min). The pH was adjusted to 2.0. 0.5 mL of sample (S<sub>2</sub>) was taken at 60 min mark from the beginning of the gastric digestion; 1 mL of sample (S<sub>3</sub>) was taken at the beginning of the intestinal phase. This was, after the pH adjustment to 7.0, but before the addition of pancreatin and bile; 1 mL of samples were taken at 90 and 180 min mark from the beginning of the intestinal digestion, S<sub>4</sub> and S<sub>5</sub>, respectively. In samples taken at check point S<sub>2</sub>, the pH was increased to 8.0 with 0.5 M sodium bicarbonate to stop peptic hydrolysis. Samples taken at check point S<sub>4</sub> and S<sub>5</sub> were acidified to pH 2 with 6 M hydrochloric acid to stop

pancreatic hydrolysis. All samples taken from the reactors were treated with snap-freezing in liquid nitrogen immediately to completely inactivate the enzymes and stored frozen at -20 °C until further analysis (section 3.4.3).

#### *3.4.2.2.1 Oral phase*

Lyophilized ground bresaola samples were used (Ferranti et al., 2014) for the digestion study for a homogenous feed mixture. 2 g of each sample was homogenized (in shaking water bath at 37 °C and the speed of 5 rpm) with 3.5 mL SSF electrolyte stock solution in a screw-capped glass bottle (50 mL) to create a thin paste-like consistency. 25 µL of 0.3 M CaCl<sub>2</sub> solution and 975 µL deionized water were added to the sample mixture, and the pH was adjusted to 7.0. Then 0.5 mL of salivary α-amylase solution (1500 U mL<sup>-1</sup>) was added to the SSF electrolyte stock solution to achieve 75 U.mL<sup>-1</sup> in the final mixture. Reactor was sealed and incubated in a shaking water bath for 5 min at 37 °C.

#### *3.4.2.2.2 Gastric phase*

Bolus from the oral phase was mixed with 7.5 mL SGF electrolyte stock solution, 5 µL of 0.3 M CaCl<sub>2</sub> solution and 695 µL deionized water. The pH was adjusted to 3.0 with 6 M HCl. The sample mixture was incubated in the shaking water bath at 37 °C. 1.6 mL pepsin solution (pepsin from porcine gastric mucosa, P-7000, Sigma-Aldrich) of 2500 U mL<sup>-1</sup> made up in SGF stock solution was added. Then the digestion reactor was further incubated for 120 min at 37 °C.

#### *3.4.2.2.3 Intestinal phase*

The final stage of digestion was to simulate the upper intestinal digestion. 15.5 mL of SIF electrolyte stock solution, 40 µL of 0.3 M CaCl<sub>2</sub> solution and 1.31 mL of deionized water were added to gastric chyme. The pH was adjusted to 7.0 with 0.5 M sodium hydroxide. Then 0.5 mL of pancreatin solution (80 mg.mL<sup>-1</sup>, pancreatin from porcine pancreas 3X U.S.P., MP Biomedicals, LLC) made up in SIF electrolyte solution and 2.5 mL of bile solution (38.4 mg.mL<sup>-1</sup>, bile extract porcine, Sigma) made up in deionized water were added into the digestion reactor. Intestinal digestion was performed in the shaking water bath at 37 °C for 180 min.



### 3.4.3 Amino acids profile

FAA extracts and digesta obtained from the five sampling points (Figure 6) were further analysed for the amino acids (AA) released before and after the simulated digestion. A commercial free amino acid kit (EZ:faast<sup>TM</sup>, Phenomenex®, USA) was used for profiling amino acids available in the digesta (Appendix 8). All steps, including the solid phase extraction (SPE) sample clean-up, elution from SPE sorbent, derivatisation, and analysis, were performed, as described in the kit's manual. 0.2 mM Norvaline in N-propanol solution was used as an internal standard.

AA derivatives were separated and quantified on a Shimadzu GC2010 GC with a Flame Ionisation Detector (GC-FID), a split injector and an AOC-20i auto-injector. The ZB-AAA GC column (10 m × 0.25 mm × 0.25 µm) included in the kit was used. The instrument settings used were recommended by the user manual with modifications to obtain better separation of individual amino acid.

1 µL of the derivatised samples recovered from digesta were injected at split ratio 1:15 at 300 °C into the column. Initial oven temperature was 50 °C. It raised to 120 °C at a rate of 50 °C per minute and it was held for 30 seconds. Temperature was again increased to 165 °C at a rate of 5 °C per minute. Once the temperature reached 165 °C, it was further increased at a rate of 20 °C per minute to reach 320 °C and it was held there for 1 minute. Nitrogen/Air with a column flow at 1.46 mL.min<sup>-1</sup> was used as carrier gas instead of helium, which was recommended by the manual. Amino acids in samples were identified and quantified by comparison of their retention times with those produced from a mixture of 26 AA standards included in the kit at four calibration levels, ranging from 400 to 50 nmol.mL<sup>-1</sup>. All peak areas in the chromatogram were corrected to the area and concentration of the internal standard peaks before any compositional calculations were made. The concentration of each amino acid identified in the samples was presented as mg of amino acid in 1 g of bresaola sample.

## Chapter 4. Results and Discussion

### 4.1 Physicochemical results

#### 4.1.1 Proximate analysis

As shown in Table 7, the proximate composition of the five bresaola products significantly differed from each other, in terms of protein, intramuscular fat (IMF), moisture and ash contents ( $p < 0.001$ ), by using one-way ANOVA and Turkey test.

**Table 7. Proximate composition of bresaola products (Mean  $\pm$  Standard Deviation). Values are presented as g per 100g of bresaola sample.**

	Protein	Intramuscular fat	Moisture	Ash
<b>Beef</b>	29.95 $\pm$ 0.24 <sup>a</sup>	5.22 $\pm$ 0.24 <sup>a</sup>	44.45 $\pm$ 0.42 <sup>a</sup>	6.26 $\pm$ 0.16 <sup>a</sup>
<b>Veal</b>	28.93 $\pm$ 2.32 <sup>a</sup>	4.43 $\pm$ 0.04 <sup>a</sup>	44.38 $\pm$ 0.41 <sup>a</sup>	5.66 $\pm$ 0.04 <sup>bc</sup>
<b>Wagyu</b>	28.54 $\pm$ 1.47 <sup>a</sup>	7.68 $\pm$ 0.29 <sup>b</sup>	48.58 $\pm$ 0.85 <sup>b</sup>	5.53 $\pm$ 0.10 <sup>c</sup>
<b>Mutton</b>	22.89 $\pm$ 1.44 <sup>b</sup>	8.38 $\pm$ 0.17 <sup>b</sup>	38.20 $\pm$ 0.26 <sup>c</sup>	4.90 $\pm$ 0.05 <sup>d</sup>
<b>Lamb</b>	22.43 $\pm$ 1.08 <sup>b</sup>	7.72 $\pm$ 0.27 <sup>b</sup>	35.23 $\pm$ 0.52 <sup>d</sup>	5.81 $\pm$ 0.01 <sup>b</sup>
<b>P-values</b>	***	***	***	***

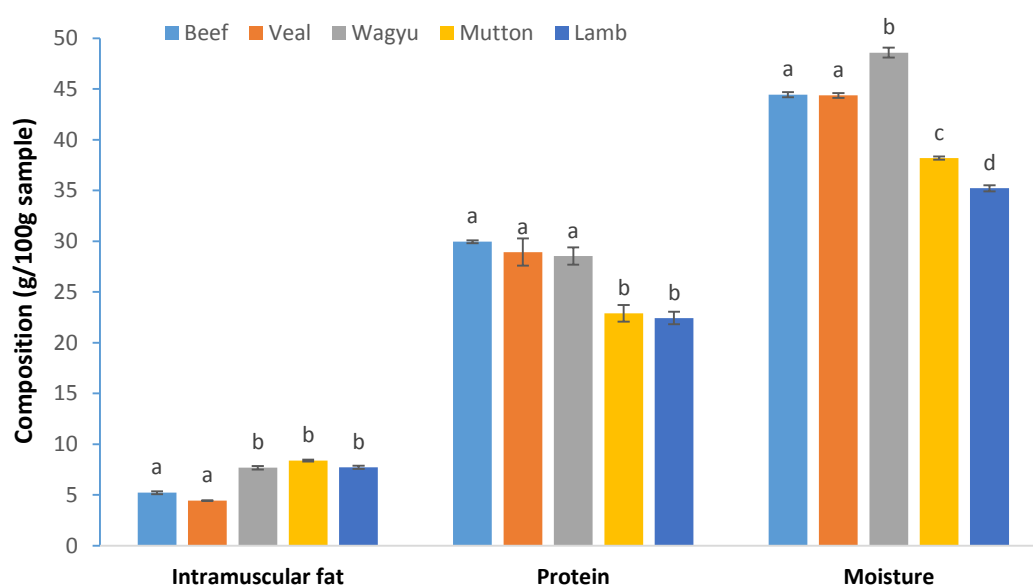
Values with different superscripts (a,b,c,d) in the same column differ significantly across the samples using one-way ANOVA and Tukey's test.

P < 0.0001 presented as \*\*\* for level of significance.

##### 4.1.1.1 Protein

With the increasing awareness of the importance of proteins in human diet, determination of the protein content is vital to characterise the nutritional value of a food product. Protein is one of the essential macronutrients for a healthy diet. It plays key roles in maintenance and repair of living cells and contributes towards growth (WHO, 2007). Meat and meat products are known to provide high content of dietary proteins which supply essential amino acids without limiting amino acids. In dry-cured meat products, generally, the protein content falls in the range of 20 to 40% depending on the water content, muscle types and the origin of animal (Paleari et al., 2003; Reina et al., 2013). In the present study, results were within the expected protein content in dry-cured meat products.

As shown in Table 7 and Figure 7, the protein contents of bresaola samples from beef, veal and wagyu were significantly higher than that of the mutton and lamb. The protein contents reported in the present study were in line with bresaola from different animal sources ranging from 20% to 40%, donkey (29.25%) (Marino et al., 2010) or (34.65%) (Marino et al., 2015), buffalo (29.79%) (Paleari et al., 2000), deer (44.6%), boar (39.3%), horse (39.7%) and goat (38.8%) (Paleari et al., 2003). They also compared their results with bresaola made from beef, which contained approximately 30% of protein content, and in line with the current findings (29.25%).



**Figure 7 Intramuscular fat, protein and moisture content of five bresaola samples.**  
Mean values are plotted with error bars representing standard error (S.E.)

#### 4.1.1.2 Intramuscular fat (IMF)

Fats are among the bioactive components that have received the most attention in the past years, in quantitative and qualitative terms, with respect to the development of healthier meat products (Marino et al., 2015). Concerns about the possibility of a potential risk on health from over-consumption of dietary fat have significantly increased. Therefore, health organizations across the world have been promoting guidelines about nutritional composition of foods and ways to lower the intake of dietary fat. Possible health concerns arising from high intake of dietary fats include chronic diseases, such as cancer, cardiovascular and metabolic diseases. However, consumption of moderate dietary fat is known to be beneficial to health. Fat is the key dietary source of energy and

the carrier of fat-soluble vitamins, such as vitamin A, D, E and K (Biesalski, 2005; NHMRC, 2006). A meat or meat product which is labelled as 'low-fat' meat generally contains less than 10% of total fat. When labelled as 'extra lean', it contains less than 5% total fat, according to the definitions by Food and Drug Administration (2009). Findings from the present study show that IMF content of bresaola products made from five animal sources fell in the category of 'low fat' meat products, and beef and veal bresaola fell in the 'extra lean' category (Table 7, Figure 7). Beef and veal bresaola products contained much lower level of IMF than the rest products. The low fat content of bresaola products is an important result from a nutritional point of view. It could be attributed to the selection of raw materials for bresaola products, which may have been from the lean part of carcass, such as rump, top side, and round. Furthermore, trimming practices before the production could have significantly lowered the fat content in final products. Results obtained from the present study were in correspondence to other reports on the fat content of bresaola products made from cow (4.51%) (Marino et al., 2015), boar (5.5%), bovine (4.9%) and goat (5.2%) (Paleari et al., 2003), but higher than those from buffalo (1.75%) (Paleari et al., 2000), donkey (2.85%) (Marino et al., 2015), deer (2.0%) and horse meat (3.4%) (Marino et al., 2015; Paleari et al., 2000; Paleari et al., 2003). The variations may have arisen from the use of different muscle types, difference in animal origins and processing conditions.

#### 4.1.1.3 Moisture

An important step involved in the production of dry-cured meat products is dehydration to lower the water content from approximately 70-80% in fresh meat to 40-50% in the dry-cured meat products (Paleari et al., 2003). Significant decrease of moisture content in processed meat product is essential for the purpose of lowering the water activity to extend shelf life and for preservation. In particular, palatability of the products, from sensory point of view (Virgili et al., 1995), which includes flavour and texture, is affected by the moisture content. Moisture content in the final products mainly depends on the processing parameters, such as humidity, temperature and ageing time.

Moisture contents in all of the samples fell below 50%. Values were higher in those made from cattle meat in comparison to the products from sheep meat (Table 7, Figure 7). Except for mutton and lamb bresaola with lower water level (< 40%), results from the present study corresponded well to the findings on bresaola made from different animal species, deer (45.8%), boar (48.2%), horse (49.2%), bovine (55.4%) and goat

(47.8%) (Paleari et al., 2003) and other dry-cured products, ham (50.96%) and shoulder (46.34%) (Reina et al., 2013). However, higher moisture level of approximately 60% has been reported on bresaola products made from donkey (Marino et al., 2015) and buffalo (Paleari et al., 2000). Differences in moisture content among bresaola samples may have been affected by different moisture level occurring at different anatomical locations, such as muscles in outer part that were dryer than those in the centre (Reina et al., 2013; Ruiz-Ramírez, Serra, et al., 2005) and the variations of muscle types from different animal origins (Paleari et al., 2003; Sutton, Hand, & Fitch, 1993).

#### 4.1.1.4 Ash

Ash content is an indicator of total amount of minerals within the food. Minerals, such as Ca, Na, K and Cl, are of significance to maintain health. Ash results obtained by ashing in muffle furnace at 550 °C overnight were in agreement with other bresaola products reported by other authors Marino et al. (2015), Paleari et al. (2000) and Paleari et al. (2003), with highest values fell in beef bresaola and lowest in mutton bresaola.

#### 4.1.1.5 Correlation analysis

Pearson correlation analysis was performed to determine the correlation between the nutritional contents among the bresaola samples. As expected, IMF content tended to increase with the reduction of moisture level ( $r = -0.42$ ) in wet samples. However, it is of interest to note that the protein content significantly increased in a linear manner with moisture composition ( $r = 0.894$ ,  $p = 0.041$ ). However, contradicting results have been reported by other authors, suggesting that a high lean muscle percentage (Chizzolini et al., 1996) and low fat content (Candek-Potokar, Monin, & Zlender, 2002), may cause the reduction of water content in the dry-cured products. With respect to the correlation between protein and IMF, water content showed no significant effect from the current findings, since protein content varied in a negative manner with the fat content ( $r = -0.75$ ).

### 4.1.2 Texture profile analysis (TPA)

Textural characteristics are crucial aspects of consumer acceptance of meat and meat products. It could be predicted from objective (instrumental texture analysis) and sensory perspectives (as discussed in section 2.2.2).

**Table 8 Textural profile of bresaola products (mean  $\pm$  S.D.)**

	Beef	Veal	Wagyu	Mutton	Lamb	<i>P</i> -values
<b>Hardness (kg)</b>	47.66 $\pm$ 0.16 <sup>a</sup>	34.19 $\pm$ 0.67 <sup>b</sup>	22.88 $\pm$ 0.65 <sup>c</sup>	29.66 $\pm$ 0.66 <sup>d</sup>	40.05 $\pm$ 0.32 <sup>e</sup>	***
<b>Cohesiveness</b>	0.59 $\pm$ 0.01 <sup>a</sup>	0.50 $\pm$ 0.04 <sup>b</sup>	0.51 $\pm$ 0.05 <sup>b</sup>	0.47 $\pm$ 0.02 <sup>b</sup>	0.49 $\pm$ 0.01 <sup>b</sup>	*
<b>Springiness</b>	0.32 $\pm$ 0.01 <sup>a</sup>	0.35 $\pm$ 0.01 <sup>a</sup>	0.37 $\pm$ 0.03 <sup>ab</sup>	0.45 $\pm$ 0.02 <sup>c</sup>	0.40 $\pm$ 0.02 <sup>bc</sup>	***
<b>Resilience</b>	0.32 $\pm$ 0.01 <sup>a</sup>	0.26 $\pm$ 0.01 <sup>bc</sup>	0.29 $\pm$ 0.02 <sup>ab</sup>	0.22 $\pm$ 0.02 <sup>c</sup>	0.25 $\pm$ 0.01 <sup>b</sup>	**
<b>Chewiness (kg)</b>	8.92 $\pm$ 0.48 <sup>a</sup>	5.90 $\pm$ 0.70 <sup>b</sup>	4.22 $\pm$ 0.27 <sup>c</sup>	6.33 $\pm$ 0.14 <sup>b</sup>	7.84 $\pm$ 0.40 <sup>a</sup>	***

Values with different superscripts (a,b,c,d,e) in the same row differ significantly across the samples using one-way ANOVA and Tukey's test.

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

**Table 9 Correlation coefficients (r value)\* between TPA texture parameters, moisture content and fat content of five bresaola products**

	Cohesiveness	Springiness	Resilience	Chewiness	Moisture	Lipid	Protein
<b>Hardness</b>	0.65	-0.47	0.43	0.97*	-0.32	-0.47	0.12
<b>Cohesiveness</b>		-0.84	0.91*	0.53	0.46	-0.52	0.72
<b>Springiness</b>			-0.89*	-0.24	-0.64	0.77	-0.89*
<b>Resilience</b>				0.28	0.61	-0.41	0.76
<b>Chewiness</b>					-0.49	-0.25	-0.10

The correlation analysis between TPA texture parameters, moisture content and fat content was carried out on the mean scores using Pearson correlation analysis.

Correlation coefficients with “\*” labelled are at significant level ( $p < 0.05$ ).

Instrumental texture profile analysis (TPA) is a good tool and has been extensively used due to the good correlation with sensory results, to assess textural properties of processed meat products, such as dry cured ham (Costa et al., 2008; Lorenzo & Carballo, 2015; Lorenzo & Laura, 2013; Tabilo et al., 1999) and sausages (Andrés, García, Zaritzky, & Califano, 2006; Crehan, Hughes, Troy, & Buckley, 2000; Pietrasik, 1999; Tobin et al., 2012). The textural quality of processed meat products mainly depends on the

characteristics of raw materials and technological parameters during processing (Serra et al., 2005; Virgili et al., 1995). Texture variations in relation to raw material characteristics could be associated with genetic origin of animals, sex and sexual conditions, pH and muscle structures which may differ in fat content and proteolytic potential (Ruiz-Ramírez, Serra, et al., 2005; Virgili et al., 1995). Furthermore, technological parameters, including processing temperature, time, relative humidity, addition of salt and treatment of nitrate or nitrite (Gil et al., 1999; Molinero et al., 2008; Ruiz-Ramírez, Arnau, et al., 2005), may also contribute towards the textural quality.

Textural properties of bresaola processed from five different animals were characterised by quantifying TPA on TA.XT texture analyser. TPA was designed to emulate the sensory chewing experience and give rise to multiple textural characteristics in a single measurement (refer to section 3.2.2 for parameters and settings). The correlation analysis between TPA parameters, moisture, lipid and protein contents was carried out on the mean scores using Pearson's regression model.

#### 4.1.2.1 Hardness

As shown in Table 8, statistical significance ( $P < 0.0001$ ) was found on all of the textural attributes across the five different samples. In particular, all samples differed significantly ( $p < 0.0001$ ) from each other in terms of hardness, which was generated from the force required to reach 50% compression of the sample thickness. Beef bresaola was the hardest among all samples, while mutton and wagyu bresaolas were much softer. Increase in hardness of dry-cured meat products could be attributed to the dehydration during the dry-curing process. Normally the curing process starts from 60-70% water content in fresh meat and continues until 30-40% water content is reached in end products. This causes shrinkage of sample size, contributing to a firm structure and greater instrumental hardness. A negative but not significant correlation ( $r = -0.32$ ) between moisture content and hardness also suggests the phenomenon detected on hardness. Increase in hardness values caused by decrease in moisture content after dehydration has been reported on dry-cured ham reported by Ruiz-Ramírez et al. (2005), Lorenzo and Laura (2013), Lorenzo (2014) and Lorenzo and Carballo (2015).

Furthermore, intramuscular fat content could have affected the hardness. Hardness was prone to inversely relate to the fat content ( $r = -0.47$ , Table 9) among different bresaola products, in agreement with studies on dry-cured ham (Ruiz-Carrascal

et al., 2000) and sausages (Barbut & Mittal, 1996; Bloukas et al., 1997; Murphy et al., 2004; Tobin et al., 2012). Weak correlation found in the present study between lipid content and hardness could be attributed to the low fat content in bresaola products compared to ham and sausages from other studies. The same phenomenon was also reported by Válková, Saláková, Buchtová, and Tremlová (2007).

Large variability in hardness across the five samples could have been caused by different degradation patterns of myofibrillar protein and collagen, as suggested by Monin et al. (1997), Tabilo et al. (1999), Toldrá (2006), and Lorenzo and Laura (2013). Proteolysis during dry-curing is known to contribute to tenderization by breaking down the muscle structure to polypeptides, small peptides and amino acids. In other studies, total protein content has shown a significant correlation with hardness, for dry-cured sausages (Lorenzo & Laura, 2013; Pietrasik, 1999) and ham (Cheng & Sun, 2005; Válková et al., 2007). However, the correlation was weakly supported in the present study ( $r = 0.115$ , Table 9).

#### 4.1.2.2 Cohesiveness

Cohesiveness indicates the ability of a food to maintain the integrity of structure after applying compressive deformation (Breene, 1975). It is a factor related to both compressing time and force used during the analysis. Cohesiveness was calculated from force and time used in the second deformation cycle divided by in the first compression. Values of cohesiveness fell in the range of 0 to 1, where higher values indicate higher structural integrity. Five bresaola samples have shown similar cohesiveness but with significantly higher value for beef bresaola ( $p < 0.01$ , Table 8).

Cohesion of bresaola products was prone to be higher with decreased fat content ( $r = -0.52$ , Table 9), which has been seen on processed meat products (Pietrasik, 1999). Furthermore, cohesiveness seemed to positively vary with the protein content ( $r = 0.72$ , Table 9), which was expected. This could be explained by the presence of myofibrils in the proteins, which is responsible for maintaining the structural integrity as a key subunit of muscle (Koohmaraie, 1996). Better structural integrity seemed to maintain higher internal tension between proteins and therefore require more energy and more intensive force to rupture sample structure at second penetration cycle, and result in a higher cohesiveness.



#### 4.1.2.3 Springiness

Another textural attribute related to the structural integrity of food is springiness, which describes how a food can regain its original position between two cycles of chewing. A delay time of 5 seconds used in this study could potentially increase the time to spring back. Consumption of solid food requires a continuous physical deformation on its structure until it is ready to swallow. Bresaola made from cattle meat had significantly lower values of springiness ( $p < 0.0001$ ) compared to those made from sheep meat (Table 8). Mutton bresaola was prone to have better elasticity than the rest samples, with the highest value of springiness,  $0.45 \pm 0.02$ .

Fat and moisture contents have shown no significant effect ( $p > 0.1$ ) on springiness. However, springiness has shown an increasing trend ( $r = 0.77$ , Table 9) with the fat content, and an inverse correlation ( $r = -0.64$ , Table 9) to the moisture content. Similar trends of the correlation between springiness and level of fatness have also been found on dry-cured meat products (Mendonza et al., 2001; Pietrasik, 1999; Pietrasik & Duda, 2000; Tobin et al., 2012). However, contradictory results have been reported by Colmenero, Barreto, Mota, and Carballo (1995) and Crehan et al. (2000). The level of fatness had lesser impact on the textural properties of bresaola compared to that of the proteins. This could be due to the presence of a much higher concentration of proteins in the samples compared to fat. Although Pietrasik (1999) suggested that the springiness was not affected by the protein content, an inverse correlation was found between the protein content and the springiness with  $r$  value of  $-0.89$  ( $p = 0.044$ , Table 9). The effect of proteins on springiness seemed to be attributed to the degradation of proteins during the curing process which may decrease the springiness as suggested by other authors on dry-cured ham (Costa et al., 2008; Ruiz-Ramírez, Arnau, Serra, & Gou, 2006).

#### 4.1.2.4 Resilience

Resilience, involved in the measurement of TPA, is defined as instant springiness, or the ability to spring back within a single compressive deformation (Breene, 1975). Greater resilience ( $p < 0.001$ ) was observed on bresaola from beef, veal and wagyu, as  $0.32 \pm 0.01$ ,  $0.26 \pm 0.01$  and  $0.29 \pm 0.02$ , respectively. This means bresaola from cattle meat had better capability of recovering to original positions during the withdrawing of compressive force than the bresaola made from sheep meat. Resilience of bresaola samples seemed not significantly to be affected by the variations of protein, fat and water content ( $p > 0.05$  Table 9). However, resilience increased with increases in protein ( $r =$

0.76) and water levels ( $r = 0.61$ ), and the reduction of fat content ( $r = -0.41$ ). The positively linear correlation between the protein content and resilience can be explained by the presence of myofibrils, which are key structural proteins present in meat that helps to maintain the structure and spring back upon compression. Similar effect of fat level on resilience has been reported on sausages by Andrés et al. (2006).

#### 4.1.2.5 Chewiness

Chewiness is the integrated textural property, generated by multiplying hardness with cohesiveness and springiness. It indicates the resistance level of a food to breakdown upon mastication to the state, ready for swallowing, which is known as bolus. Chewiness is a complementary parameter to hardness. Higher values of chewiness indicate more energy required to breakdown the food structure. Beef and lamb bresaola were significantly higher ( $p < 0.0001$ ) in chewiness than the rest of the products,  $8.92 \pm 0.48$  and  $7.84 \pm 0.40$ , respectively. Wagyu bresaola had the lowest value of  $4.22 \pm 0.27$ , indicating that the least effort and chewing cycles are required until it becomes ready for swallowing.

Protein, fat and water content seemed to have no significant effect on chewiness in the present study, though a negative trend was found for all three factors ( $r = -0.10$ ,  $-0.25$ ,  $-0.49$ , respectively). The negative correlation between fat and chewiness has been suggested by Pietrasik (1999) and Andrés et al. (2006), however, disparate results have been reported by Bloukas & Paneras (1996) and Cofrades, Carballo, & Jiménez-Colmenero (1997).

#### 4.1.2.6 Correlation analysis between textural attributes

As shown in Table 9, Pearson correlation analysis of five textural attributes indicated that the variations of resilience were significantly in a linear manner with cohesiveness ( $r = 0.91$ ,  $p = 0.03$ ), while inversely correlated with springiness ( $r = -0.89$ ,  $p = 0.045$ ). Resilience was highly correlated with cohesiveness suggesting a food with better cohesion may have a greater resilience due to the better structural integrity. While the negative correlation between springiness and resilience, indicated a greater springiness on bresaola samples if a sufficient time delay was applied between each penetration. Changes in chewiness were consistent with the results in hardness ( $r = 0.965$ ,  $p = 0.008$ ). Furthermore, it is of interest to note that springiness was likely to be negatively

related to cohesiveness ( $r = -0.84$ ) and hardness ( $r = -0.47$ ), while hardness varied with similar tendency as cohesiveness ( $r = 0.65$ ) and resilience ( $r = 0.43$ ).

### 4.1.3 Instrumental colour

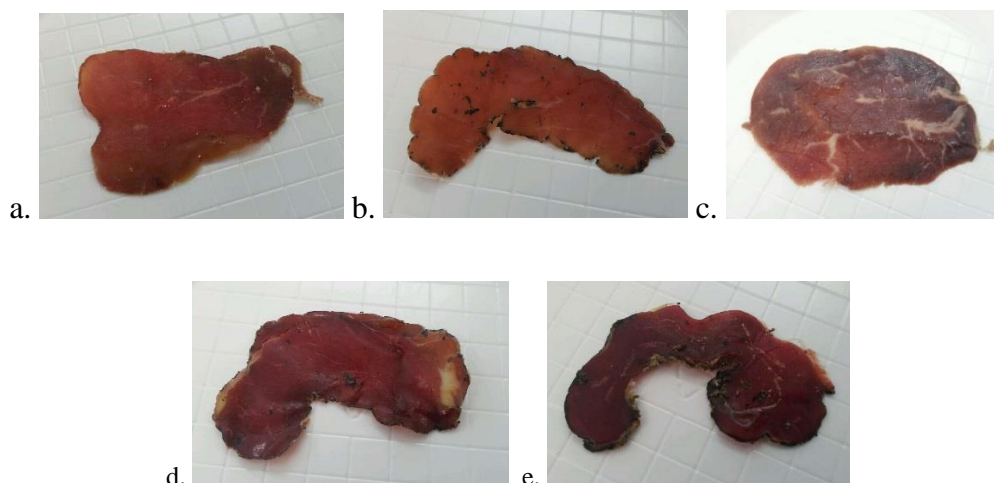
The appearance of meat deals with the visual identification and palatability of quality meat based on colour, marbling, and water holding capacity. The colour of a meat product plays an essential role in ensuring customer appeal and strongly contributes to product value and quality. Consumers associate bright red colour, the colour of pigment oxymyoglobin with meat freshness (Calnan, Jacob, Pethick, & Gardner, 2014).

**Table 10 Instrumental colour analysis of bresaola products (mean  $\pm$  S.D.)**

	Beef	Veal	Wagyu	Mutton	Lamb	<i>P</i> -values
<b>L*</b>	26.92 $\pm$ 0.06 <sup>a</sup>	27.99 $\pm$ 0.40 <sup>b</sup>	24.44 $\pm$ 0.09 <sup>c</sup>	24.41 $\pm$ 0.58 <sup>c</sup>	23.23 $\pm$ 0.08 <sup>d</sup>	***
<b>a*</b>	7.53 $\pm$ 0.01 <sup>a</sup>	12.30 $\pm$ 0.25 <sup>b</sup>	4.38 $\pm$ 0.04 <sup>c</sup>	7.17 $\pm$ 0.32 <sup>a</sup>	7.23 $\pm$ 0.05 <sup>a</sup>	***
<b>b*</b>	6.99 $\pm$ 0.17 <sup>a</sup>	10.46 $\pm$ 0.19 <sup>b</sup>	4.09 $\pm$ 0.08 <sup>c</sup>	3.86 $\pm$ 1.17 <sup>c</sup>	3.98 $\pm$ 0.24 <sup>c</sup>	***
<b>C</b>	10.27 $\pm$ 0.12 <sup>a</sup>	16.15 $\pm$ 0.16 <sup>b</sup>	6.01 $\pm$ 0.08 <sup>c</sup>	8.15 $\pm$ 0.23 <sup>d</sup>	8.25 $\pm$ 0.11 <sup>d</sup>	***
<b>H°</b>	42.87 $\pm$ 0.67 <sup>a</sup>	40.38 $\pm$ 0.96 <sup>b</sup>	43.04 $\pm$ 0.26 <sup>a</sup>	28.30 $\pm$ 1.68 <sup>c</sup>	28.83 $\pm$ 1.56 <sup>c</sup>	***

Values with different superscripts (a,b,c,d,e) within the same row differ significantly across the samples using one-way ANOVA and Tukey's test.

P < 0.0001 presented as \*\*\* for level of significance.



**Figure 8 Pictures of sliced bresaola (1.5 mm thickness). a to e represent beef, veal, wagyu, mutton and lamb bresaola, respectively.**

Factors associated with meat colour include the chemical status of heme pigment (mainly myoglobin), muscle structure, and environmental conditions. Muscles differ greatly in the concentrations of myoglobin, which may result in the colour variations

among different muscle sources from various animals (Boles & Pegg, 2010; Nam & Ahn, 2003). Colour development is also influenced by the processing, and storage conditions such as pH, humidity, and temperature. In cured meat products, treatments with nitrites/nitrates in the curing process stabilise and secure the favourable pink colour produced by the pigment nitrosyl hemochrome. In this study, bresaola products were cured by rubbing sodium nitrite onto the surface of meat cuts from five different animal sources before the initiation of curing. Nitrite firstly react with water to form nitrous acid and nitric oxide, which would combine with myoglobin to form nitric oxide myoglobin, and subsequently a more stable pigment nitrosyl hemochrome will be formed. The cured meat products were vacuum packed and stored at low temperature (2-4 °C) prior to analysis to protect products from the negative action of oxygen, temperature and light on pigments. The measurement of instrumental colour in this study was carried out quickly after meat were unpacked and cut into slices to minimise colour variation caused by exposure to oxygen.

As shown in Table 10, three parameters of colour,  $L^*$ ,  $a^*$ , and  $b^*$ , were directly calculated from the Chroma meter, Hue angle ( $H^\circ$ ) and colour saturation, or Chroma (C) were calculated from  $L^*$ ,  $a^*$ , and  $b^*$  values. ANOVA results for all the colour values varied significantly with the five bresaola products ( $P < 0.0001$ ).

#### 4.1.3.1 Lightness

Lightness ( $L^*$ ) is considered to be the main indicator of meat quality and the subcutaneous fat content of processed meat products (Ferreira, Fernandes, & Yotsuyanagi, 1994; García-Esteban, Ansorena, Gimeno, & Astiasarán, 2003). Values of  $L^*$  range from 100 (white) to 0 (black). Higher  $L^*$  values indicate a higher light reflectance, which would result in the presence of lighter (white) colour.

$L^*$  values were lower for all five bresaola samples ( $L^* = 20-30$ ), compared to results reported on buffalo bresaola ( $L^* = 70$ ) (Paleari et al., 2000), young bull bresaola (Braghieri et al., 2009) and commercial beef bresaola ( $L^* = 40$ ) (Rød, Hansen, Leipold, & Knøchel, 2012). This could be attributed to the long dry-curing time (8 weeks, as shown in Figure 3), which resulted in low moisture content (30-40%) as shown in Table 7. Bresaola products measured in this study were dry-cured as long as 8 weeks. Loss of water content in the sample would result in the increase of salt and pigments like myoglobin concentrations that may result in decreased lightness. The effects of long

curing period, low moisture content and increase in salt content that result in the loss of lightness of dry-cured products have also been reported on other dry-cured meat products (Estévez, Morcuende, & Cava, 2003; Molinero et al., 2008; Pérez-Alvarez et al., 1997; Pérez-Alvarez, Sayas-Barberá, Fernández-López, & Aranda-Catalá, 1999).

Generally higher fat content and lower protein composition favour the appearance of lighter colours (higher lightness and lower redness values) of processed meat products due to the increase in the overall light scattering properties of fat, and decrease in myoglobin protein associated with red colour of meat products. In the present study, correlation analysis carried out on the five products showed a significantly inverse ( $r = -0.96$ ,  $p = 0.011$ ) correlation between lightness and fat content. However, variations in lightness were too small (only approximate 1% difference) to be significantly important in the present study.

**Table 11 Correlation coefficients (r values)\* between instrumental colour parameters and proximate composition of bresaola products from different meat sources**

	<b>a*</b>	<b>b*</b>	<b>C</b>	<b>H°</b>	<b>Moisture</b>	<b>Intramuscular fat</b>	<b>Protein</b>
<b>L*</b>	0.72	0.94*	0.84	0.59	0.65	-0.96*	0.77
<b>a*</b>		0.87	0.98*	-0.05	0.04	-0.78	0.19
<b>b*</b>			0.96*	0.44	0.51	-0.97*	0.64
<b>C</b>				0.16	0.24	-0.89*	0.39
<b>H°</b>					0.94*	-0.60	0.98*

The correlation analysis between TPA texture parameters, moisture content and fat content was carried out on the mean scores using Pearson correlation analysis.

C refers to Chroma, H° refers to hue angle.

Correlation coefficients with “\*” label are at significance level ( $p < 0.05$ ).

#### 4.1.3.2 Redness

The most dominant colour of cured meat products is red. Differences in a\* value can have a great impact on product colour. According to Zarubica, Miljković, Purenović and Tomić (2005), a\* values (values range from 80 to -80, red to green) is the most sensitive parameter in colour measurement and is an indicator of stability and quality of

meat products. Redness was expected to be greater in low fat processed meat products due to the concentration of the lean meat.

In the present study, redness of bresaola products were lower than results reported on commercial beef bresaola (Rød et al., 2012) and bull bresaola (Braghieri et al., 2009). Higher redness ( $12.30 \pm 0.25$ , Table 10) was detected from veal bresaola which was not commercially available in local markets. Bresaola processed from beef, mutton and lamb showed similar redness. However, wagyu bresaola showed significantly lower redness compared to other bresaola products. Redness of processed meat samples could be associated with the protein and fat content. Pigment proteins may be present at higher concentration when a meat product contains more total proteins. However in the present study there was no significant correlation between redness and protein content ( $r = 0.19$ , Table 11). Redness was negatively related to fat content ( $r = -0.78$ , Table 11) as expected but not significant. Veal bresaola which had a lower composition of fat (4.43%) tended to be redder than other products. Other authors have also reported similar correlations between fat content and redness of processed meat products (Gimeno, Ansorena, Astiasarán, & Bello, 2000; Lorenzo et al., 2011; Pietrasik, 1999; Tobin et al., 2012).

The variations in  $a^*$  values among the five samples could also be attributed to the oxidation of pigment nitrosyl hemochrome to form pigment hemichrome that results in brown/grey colour during the drying period and storage time. This would result in the loss of lightness. Discoloration associated with longer drying time of dry cured beef has been reported by Rubio et al. (2006) and Gök, Obuz, and Akkaya (2008) despite being vacuum packed. However, Válková et al. (2007) suggested increased redness of processed meat products will make products less acceptable by consumers probably due to the perception that artificial colouring was added.

#### 4.1.3.3 Yellowness/Chroma/Hue

Another chromatic component  $b^*$  shows yellow-blue characteristics (values range from 80 to -80). The hue angle  $H^\circ$  is expressed in degrees ( $H^\circ = 0$  corresponds to red,  $H^\circ = 90$  to yellow,  $H^\circ = 180$  to green,  $H^\circ = 270$  to blue).  $H^\circ$  indicates development of colour from red to yellow, and larger angles indicate a less red product. Colour saturation indicates the colour intensity and vividness of colour compared to a neutral gray of the same  $L^*$  with higher values indicating a more vivid colour. Bresaola made from beef and veal were more yellow (higher  $b^*$  values) and had higher colour intensity (higher C values)

than the other three products. Bresaola made from wagyu meat had a similar value of yellowness to those made with mutton and lamb ( $4.09 \pm 0.08$ ,  $3.86 \pm 1.17$ ,  $3.98 \pm 0.24$ , respectively, Table 10). This sample also had the lowest colour saturation and a higher hue angle than rest of the samples. Bresaola processed from cattle meat had significantly higher in hue angle than products made from sheep meat. However, disparate results of the C and H° values have been reported by other authors on different bresaola products (Paleari et al., 2000), that reported better colour saturation and lower hue angle in comparison to results in the present study.

Water, fat and protein content could further affect b\*, C and H° values of bresaola products. Increase in fat content of processed meat products, in general, could contribute to higher yellowness as reported in other studies (Lorenzo et al., 2011; Pietrasik & Duda, 2000). However, a significant inverse correlation ( $r = -0.97$ ,  $p = 0.006$ ) was found between yellowness value and fat content. The effects of fat and protein content on b\* values have also been reported by Lorenzo and Laura (2013). Different muscle types from different animals, and different lipid oxidation level and amount of pigment oxymyoglobin may influence b\* values as suggested by Pérez-Alvarez et al. (1999) and Cavalleiro et al. (2013). Pearson correlation analysis also revealed significant correlation between yellowness and lightness values ( $r = 0.974$ ,  $p = 0.017$ ) among the five samples. Chroma was inversely correlated with intramuscular fat content ( $r = -0.89$ ,  $p = 0.043$ ).



#### 4.1.4 Fatty acids profile

For decades, fatty acid composition of meat has been studied but still it receives particular attention in research due to its implications for human health (Enser et al., 1996; Raes, De Smet, & Demeyer, 2004; Riediger, Othman, Suh, & Moghadasian, 2009; Simopoulos, 1991). Rather than solely concentrating on the quantity of total fat intake, intake of a well-balanced fatty acids is recommended for health, with a higher proportion of polyunsaturated fatty acids (PUFAs), especially of Omega-3 (n-3) fatty acids at the expense of Omega-6 (n-6) fatty acids, and a lower proportion of saturated fatty acids (SFAs) (NHMRC, 2006).

**Table 12** A table showing % composition of fatty acids present in IMF of bresaola products. Values are presented as mean  $\pm$  S.D.

	Beef	Veal	Wagyu	Mutton	Lamb	P-values
<b>14:0</b>	1.41 $\pm$ 0.22 <sup>a</sup>	2.39 $\pm$ 0.09 <sup>b</sup>	1.21 $\pm$ 0.02 <sup>ac</sup>	0.29 $\pm$ 0.02 <sup>d</sup>	0.94 $\pm$ 0.04 <sup>c</sup>	***
<b>16:0</b>	24.31 $\pm$ 0.25 <sup>a</sup>	19.13 $\pm$ 0.10 <sup>b</sup>	28.01 $\pm$ 0.73 <sup>c</sup>	25.63 $\pm$ 0.07 <sup>d</sup>	21.44 $\pm$ 0.08 <sup>e</sup>	***
<b>16:1 (n-7)</b>	2.25 $\pm$ 0.03 <sup>a</sup>	2.17 $\pm$ 0.08 <sup>a</sup>	6.09 $\pm$ 0.08 <sup>b</sup>	1.59 $\pm$ 0.04 <sup>c</sup>	0.25 $\pm$ 0.01 <sup>d</sup>	***
<b>17:0</b>	3.08 $\pm$ 0.12 <sup>a</sup>	0.13 $\pm$ 0.01 <sup>b</sup>	0.29 $\pm$ 0.01 <sup>c</sup>	0.69 $\pm$ 0.01 <sup>d</sup>	0.42 $\pm$ 0.03 <sup>c</sup>	***
<b>17:1</b>	1.00 $\pm$ 0.02 <sup>a</sup>	0.17 $\pm$ 0.01 <sup>b</sup>	0.65 $\pm$ 0.01 <sup>c</sup>	0.32 $\pm$ 0.01 <sup>d</sup>	0.03 $\pm$ 0.01 <sup>e</sup>	***
<b>18:0</b>	15.68 $\pm$ 0.08 <sup>a</sup>	16.88 $\pm$ 0.21 <sup>b</sup>	9.24 $\pm$ 0.13 <sup>c</sup>	21.31 $\pm$ 0.12 <sup>d</sup>	21.63 $\pm$ 0.28 <sup>d</sup>	***
<b>18:1 (n-9)</b>	40.61 $\pm$ 0.12 <sup>a</sup>	51.86 $\pm$ 0.26 <sup>b</sup>	51.47 $\pm$ 0.55 <sup>b</sup>	43.33 $\pm$ 0.13 <sup>c</sup>	40.66 $\pm$ 0.07 <sup>a</sup>	***
<b>18:2 (n-6)</b>	1.91 $\pm$ 0.31 <sup>a</sup>	3.04 $\pm$ 0.14 <sup>b</sup>	1.87 $\pm$ 0.06 <sup>a</sup>	3.65 $\pm$ 0.02 <sup>c</sup>	8.63 $\pm$ 0.19 <sup>d</sup>	***
<b>18:3 (n-3)</b>	5.40 $\pm$ 0.28 <sup>a</sup>	0.35 $\pm$ 0.01 <sup>b</sup>	0.64 $\pm$ 0.02 <sup>b</sup>	2.03 $\pm$ 0.04 <sup>c</sup>	3.99 $\pm$ 0.13 <sup>d</sup>	***
<b>20:3 (n-3)</b>	1.27 $\pm$ 0.16 <sup>a</sup>	1.34 $\pm$ 0.04 <sup>a</sup>	0.14 $\pm$ 0.01 <sup>b</sup>	0.35 $\pm$ 0.01 <sup>c</sup>	0.52 $\pm$ 0.01 <sup>c</sup>	***
<b>20:4 (n-6)</b>	2.70 $\pm$ 0.03 <sup>a</sup>	0.65 $\pm$ 0.03 <sup>b</sup>	0.02 $\pm$ 0.01 <sup>c</sup>	0.04 $\pm$ 0.01 <sup>cd</sup>	0.09 $\pm$ 0.01 <sup>d</sup>	***
<b>20:5 (n-3)</b>	0.68 $\pm$ 0.07 <sup>a</sup>	1.89 $\pm$ 0.06 <sup>b</sup>	0.36 $\pm$ 0.01 <sup>c</sup>	0.76 $\pm$ 0.01 <sup>a</sup>	1.41 $\pm$ 0.05 <sup>d</sup>	***
<b>SFAs</b>	44.48 $\pm$ 0.21 <sup>a</sup>	38.53 $\pm$ 0.38 <sup>b</sup>	38.76 $\pm$ 0.62 <sup>b</sup>	47.93 $\pm$ 0.12 <sup>c</sup>	44.43 $\pm$ 0.36 <sup>a</sup>	***
<b>MUFAs</b>	43.56 $\pm$ 0.09 <sup>a</sup>	54.20 $\pm$ 0.30 <sup>b</sup>	58.20 $\pm$ 0.58 <sup>c</sup>	45.25 $\pm$ 0.10 <sup>d</sup>	40.93 $\pm$ 0.07 <sup>e</sup>	***
<b>PUFAs</b>	11.96 $\pm$ 0.29 <sup>a</sup>	7.27 $\pm$ 0.11 <sup>b</sup>	3.04 $\pm$ 0.04 <sup>c</sup>	6.82 $\pm$ 0.03 <sup>b</sup>	14.93 $\pm$ 0.35 <sup>d</sup>	***
<b>n-3</b>	7.35 $\pm$ 0.29 <sup>a</sup>	3.58 $\pm$ 0.06 <sup>b</sup>	1.14 $\pm$ 0.04 <sup>c</sup>	3.14 $\pm$ 0.04 <sup>b</sup>	5.91 $\pm$ 0.16 <sup>d</sup>	***
<b>n-6</b>	4.61 $\pm$ 0.28 <sup>a</sup>	3.69 $\pm$ 0.16 <sup>b</sup>	1.89 $\pm$ 0.06 <sup>c</sup>	3.68 $\pm$ 0.03 <sup>b</sup>	8.72 $\pm$ 0.19 <sup>d</sup>	***
<b>n-6:n-3</b>	0.63 $\pm$ 0.06 <sup>a</sup>	1.03 $\pm$ 0.06 <sup>b</sup>	1.66 $\pm$ 0.10 <sup>c</sup>	1.17 $\pm$ 0.02 <sup>b</sup>	1.48 $\pm$ 0.01 <sup>d</sup>	***
<b>PUFA:SFA</b>	0.27 $\pm$ 0.01 <sup>a</sup>	0.18 $\pm$ 0.01 <sup>b</sup>	0.08 $\pm$ 0.01 <sup>c</sup>	0.14 $\pm$ 0.01 <sup>d</sup>	0.33 $\pm$ 0.01 <sup>e</sup>	***
<b>MUFA:SFA</b>	0.98 $\pm$ 0.01 <sup>a</sup>	1.41 $\pm$ 0.02 <sup>b</sup>	1.50 $\pm$ 0.04 <sup>c</sup>	0.94 $\pm$ 0.01 <sup>ab</sup>	0.92 $\pm$ 0.01 <sup>b</sup>	***

Values with different superscripts (a,b,c,d,e) in the same row differ significantly across the samples using one-way ANOVA and Tukey's test.

SFA, MUFA, PUFA and n refers to saturated fatty acids, monounsaturated fatty acid, polyunsaturated fatty acid and Omega, respectively.

P < 0.0001 presented as \*\*\* for level of significance.

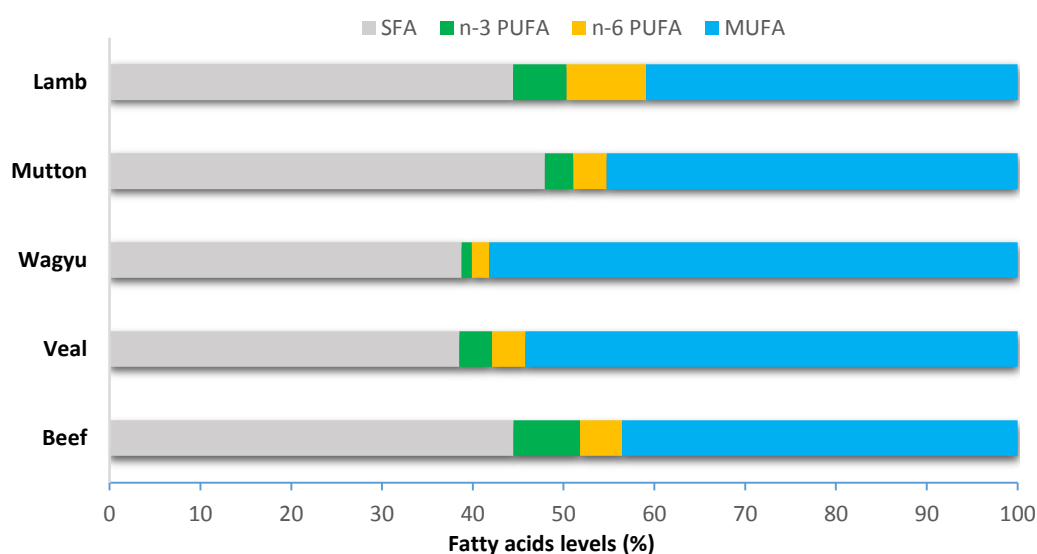
As shown in Table 12, the fatty acid profiles of bresaola made from five different animal species significantly differed from each other ( $p < 0.0001$ ). Cured products of veal and wagyu bresaola showed a lower value of SFAs, and a significant increase in monounsaturated fatty acids (MUFAs), as shown in Figure 9.

There is an increasing concern regarding the health benefits of PUFAs in the recent years, such as reducing cardiovascular disease and inflammatory disease, and promising effects of anticancer, antioxidant and antiaging (Kouba & Mourot, 2011; Raes et al., 2004; Wood et al., 2008). PUFAs were found to be at a higher level in the sheep meat products. From the current study, % PUFA was the highest in lamb bresaola, which was  $14.93 \pm 0.35\%$ . PUFAs were at an intermediate level in the bresaola made from beef and veal,  $11.96 \pm 0.29\%$  and  $7.27 \pm 0.11\%$ , respectively, and they were extremely reduced in the wagyu products,  $3.04 \pm 0.04\%$ . The lamb bresaola contained almost five times more of PUFAs than that of the wagyu bresaola. From the findings, a ratio of PUFA to SFA was calculated. This is widely used as an indicator for healthier dietary fat, when the ratio is 0.4 or above (Scollan et al., 2006). According to Haffner (2006) a higher PUFA to SFA ratio is desired to adverse the negative effects of saturated fat, hence decreasing the risks of cardiovascular disease and metabolic syndrome. Fats from ruminants generally have higher SFA content and a low PUFA to SFA ratio compared to fats from non-ruminants due to hydrogenation of dietary unsaturated fatty acids in the rumen (French et al., 2000). The ratios of PUFA to SFA were all significantly different from one another. They fell below 0.4 for all of the samples. However, the highest ratio was seen from lamb bresaola, at  $0.33 \pm 0.01$ , reflecting the highest % of PUFAs.

PUFAs are composed of two major FAs: Omega-3 (n-3) FA and Omega-6 (n-6) FA. Beef ( $7.35 \pm 0.29\%$ ) and lamb ( $5.91 \pm 0.16\%$ ) products had significantly higher level of n-3 FA than other species, also resulting in an increased proportion of total PUFA content. n-3 FA was the lowest in wagyu bresaola just being over 1% ( $1.14 \pm 0.04\%$ ). The ratio of n-6 FA to n-3 FA received an increasing attention as a balanced intake of these two FAs may exert enhanced health benefits and prevention of chronic disease (Simopoulos, 2002). A low n-6 to n-3 ratio of less than 4.0 is desired according to (Scollan et al., 2006). All of the cured products in the present study showed low (around 1.0) n-6 to n-3 ratios. Furthermore, anthropological and epidemiological studies by Simopoulos (1991, 1999, 2006) and Simopoulos, Leaf and Salem Jr (1999) indicated that the humans evolved on a diet with a ratio of n-6 to n-3, at approximate 1.0. From the findings, it is

evident that the bresaola products made from five animal species contained a well-balanced PUFA composition. PUFAs also play an important role in the sensory profile of processed meat products. With higher degree of unsaturation, PUFAs are more prone to oxidation during the curing process, generating volatile compounds that contribute towards the undesirable oxidative flavour notes (Pastorelli et al., 2003; Toldrá & Flores, 1998).

Compositions of alpha-linolenic acid (18:3, n-3), linoleic acid (18:2, n-6) and long chain PUFA Eicosapentaenoic acid (EPA, 20:5, n-3) are of particular significance since these cannot be synthesized in mammals and must be supplied from diet. They are defined as essential fatty acids (EFAs). A significant difference was found in the three EFAs. Lamb showed the highest C18:2 (n-6), of  $8.63 \pm 0.19\%$ , and wagyu was the lowest, with  $1.87 \pm 0.06\%$ . However for C18:3 (n-3), the highest percentage was seen in beef,  $5.40 \pm 0.28\%$ . Veal and wagyu both showed insignificant difference between one another, both being lower than 1%,  $0.35 \pm 0.01\%$  and  $0.64 \pm 0.02\%$ , respectively. For EPA, the meat product of veal showed the highest level of  $1.89 \pm 0.06\%$ , compared with the products of the other species.



**Figure 9 Fatty acids composition (%) of bresaola products made from five animal species**

For SFAs, palmitic acid (16:0) and stearic acid (18:0) were the two dominating SFAs for all of the samples. A higher proportion of C16:0 was found for the wagyu,  $28.01 \pm 0.73\%$ , while a significantly higher level of C18:0 was found in sheep meat;  $21.31 \pm 0.12\%$  for mutton and  $21.63 \pm 0.28\%$  for lamb. Mutton bresaola showed the highest

amount of total SFAs (Table 12, Figure 9), which contributes to the characteristic odour of mutton.

MUFAs are distinguished from the other fatty acid classes on the basis of having only one double bond. According to the position of the hydrogen atoms around the double bond, MUFA can be a *cis*- or a *trans*-isomer. High intake of *trans* fatty acids (TFA), particularly those from partially-hydrogenated fat, has been found to exert undesirable effect on serum lipid profile than SFAs, and may increase the risk of coronary heart disease (Mozaffarian & Clarke, 2009; Vijver et al., 2000). *Cis*-configuration is the naturally occurring configuration in vegetable oils and adipose tissues of animals. The MUFAs found in the five products were *cis*-MUFA with the principal forms of palmitoleic acid (16:1, n-7) and oleic acid (18:1, n-9). Wagyu products have shown the highest content of C16:1 than other species,  $6.09 \pm 0.08\%$ . This was almost 24 times higher than that found in the lamb bresaola. Oleic acid (18:1, n-9) was predominant in bresaola made from veal and wagyu meat with over 50% of the total FA content,  $51.86 \pm 0.26\%$  and  $51.47 \pm 0.55\%$ , respectively. It also contributed for the higher concentration of MUFAs in the cured products of veal and wagyu. In addition, the wagyu beef had significantly higher MUFA to SFA ratio than other products, of  $1.50 \pm 0.04$ . Consumption of higher levels of MUFAs, in conjunction with reduced levels of SFAs, is believed to prevent an increase in blood cholesterol levels (Boylston et al., 1995; Manuela A et al., 2011).

Many factors are associated with the variations seen in FA composition in cured meat products, including factors arising from different raw materials (genetic and dietary factors), and processing techniques. Dry-curing process may have a major impact on the FA composition due to the presence of high amount of free fatty acids (FFAs) released from lipolysis. FFAs are more prone to oxidation than those in the ester forms (Sampels, Pickova, & Wiklund, 2004). Furthermore, FA composition is affected by the level of fatness. The contents of SFAs and MUFAs tend to increase faster with increasing level of fatness than they do with the content of PUFAs, resulting in a decrease in PUFA to SFA ratio (De Smet, Raes, & Demeyer, 2004). Present study confirmed the positive effect of the level of fatness on SFA and PUFA contents, (*r* values of 0.66 and 0.19, respectively), while contradicting results were found on MUFA and ratio of PUFA to SFA. This could be attributed to the low fat content of bresaola products and the species factors. On the other hand, dietary feeds may have played a more important role than the

fat content in explaining the variations of the PUFA to SFA ratios. Grass-based diets, in general, would give rise to a higher level of PUFAs, while have less effect on SFA content (Daley et al., 2010; Wood et al., 2008).

In summary, PUFA was the lowest in wagyu and the highest in lamb. Beef was rich in n-3 PUFAs and lamb had the highest composition of n-6 PUFAs. MUFA was the highest in wagyu and the lowest in lamb. SFA was the highest in mutton and lowest in wagyu.

#### 4.4.5 Free amino acids analysis

Processed meat products have long been known as highly nutritious foods with distinctive flavours arising from the presence of certain FAAs produced by proteolysis during the curing process (Lorenzo & Laura, 2013; Toldrá et al., 1997; Toldrá, 1998). Dry-cured meat products undergo a series of curing practices (Figure 3, section 3.1), such as salting, ripening and drying, which would result in an extensive degradation of protein by the action of muscle protease (Toldrá, 1998).

**Table 13 Composition of free amino acids extracted from freeze dried bresaola samples (mean  $\pm$  S.D., mg/100g dry matter)**

	Beef	Veal	Wagyu	Mutton	Lamb	P-values
<b>ALA</b>	159.36 $\pm$ 2.98 <sup>a</sup>	228.85 $\pm$ 0.53 <sup>b</sup>	188.18 $\pm$ 6.80 <sup>c</sup>	245.53 $\pm$ 1.52 <sup>d</sup>	237.27 $\pm$ 1.35 <sup>bd</sup>	***
<b>ASP</b>	46.54 $\pm$ 0.37 <sup>a</sup>	27.76 $\pm$ 3.52 <sup>b</sup>	5.62 $\pm$ 2.18 <sup>c</sup>	37.59 $\pm$ 6.74 <sup>ab</sup>	26.97 $\pm$ 6.33 <sup>b</sup>	*
<b>GLU</b>	149.46 $\pm$ 1.32 <sup>a</sup>	131.01 $\pm$ 5.55 <sup>a</sup>	100.36 $\pm$ 1.45 <sup>b</sup>	211.35 $\pm$ 8.59 <sup>c</sup>	107.20 $\pm$ 1.34 <sup>b</sup>	***
<b>GLY</b>	103.63 $\pm$ 3.08 <sup>ab</sup>	113.58 $\pm$ 2.26 <sup>ab</sup>	98.21 $\pm$ 5.64 <sup>a</sup>	146.87 $\pm$ 4.08 <sup>c</sup>	122.45 $\pm$ 9.22 <sup>b</sup>	*
<b>HIS#</b>	45.69 $\pm$ 1.93 <sup>a</sup>	70.69 $\pm$ 0.95 <sup>b</sup>	71.30 $\pm$ 3.43 <sup>b</sup>	95.65 $\pm$ 8.46 <sup>c</sup>	67.99 $\pm$ 4.23 <sup>b</sup>	*
<b>ILE#</b>	84.13 $\pm$ 0.33 <sup>a</sup>	261.13 $\pm$ 1.33 <sup>b</sup>	70.33 $\pm$ 4.72 <sup>a</sup>	198.27 $\pm$ 8.75 <sup>c</sup>	249.60 $\pm$ 2.11 <sup>b</sup>	***
<b>LEU#</b>	115.19 $\pm$ 6.53 <sup>a</sup>	149.08 $\pm$ 1.32 <sup>bc</sup>	155.03 $\pm$ 2.28 <sup>bc</sup>	168.74 $\pm$ 8.54 <sup>c</sup>	139.41 $\pm$ 5.23 <sup>b</sup>	*
<b>LYS#</b>	140.58 $\pm$ 6.67 <sup>a</sup>	138.88 $\pm$ 7.78 <sup>a</sup>	133.09 $\pm$ 8.87 <sup>a</sup>	196.36 $\pm$ 8.89 <sup>b</sup>	122.37 $\pm$ 5.70 <sup>a</sup>	*
<b>MET#</b>	46.16 $\pm$ 2.37 <sup>a</sup>	44.16 $\pm$ 3.98 <sup>a</sup>	55.15 $\pm$ 3.37 <sup>a</sup>	47.16 $\pm$ 4.61 <sup>a</sup>	41.45 $\pm$ 1.67 <sup>a</sup>	ns
<b>ORN</b>	74.54 $\pm$ 2.26 <sup>a</sup>	43.00 $\pm$ 0.53 <sup>b</sup>	31.65 $\pm$ 1.63 <sup>c</sup>	46.00 $\pm$ 4.31 <sup>b</sup>	41.41 $\pm$ 0.91 <sup>b</sup>	**
<b>PHE#</b>	125.21 $\pm$ 8.00 <sup>a</sup>	138.56 $\pm$ 0.62 <sup>a</sup>	127.22 $\pm$ 6.13 <sup>a</sup>	145.24 $\pm$ 6.18 <sup>a</sup>	124.10 $\pm$ 8.77 <sup>a</sup>	ns
<b>PRO</b>	83.21 $\pm$ 5.50 <sup>a</sup>	121.73 $\pm$ 4.11 <sup>b</sup>	63.72 $\pm$ 9.63 <sup>a</sup>	133.40 $\pm$ 2.01 <sup>b</sup>	117.89 $\pm$ 5.22 <sup>b</sup>	**
<b>SER</b>	14.61 $\pm$ 1.43 <sup>a</sup>	80.87 $\pm$ 1.15 <sup>b</sup>	94.60 $\pm$ 9.22 <sup>bc</sup>	107.32 $\pm$ 8.51 <sup>c</sup>	95.65 $\pm$ 5.66 <sup>bc</sup>	**
<b>THR</b>	93.99 $\pm$ 8.39 <sup>a</sup>	91.02 $\pm$ 0.64 <sup>a</sup>	96.07 $\pm$ 5.68 <sup>a</sup>	88.99 $\pm$ 3.88 <sup>a</sup>	88.57 $\pm$ 6.41 <sup>a</sup>	ns
<b>TRP#</b>	33.07 $\pm$ 2.57 <sup>b</sup>	34.49 $\pm$ 2.84 <sup>a</sup>	36.89 $\pm$ 5.59 <sup>a</sup>	52.26 $\pm$ 0.79 <sup>b</sup>	35.54 $\pm$ 0.32 <sup>a</sup>	*
<b>TYR#</b>	70.03 $\pm$ 3.95 <sup>a</sup>	46.58 $\pm$ 5.52 <sup>b</sup>	61.18 $\pm$ 3.01 <sup>ab</sup>	62.71 $\pm$ 6.31 <sup>ab</sup>	46.76 $\pm$ 4.26 <sup>b</sup>	0.015
<b>VAL#</b>	169.39 $\pm$ 4.80 <sup>a</sup>	196.95 $\pm$ 4.36 <sup>bc</sup>	213.99 $\pm$ 6.27 <sup>c</sup>	186.89 $\pm$ 7.10 <sup>b</sup>	167.84 $\pm$ 7.52 <sup>a</sup>	*
<b>Total FAAs</b>	1615.62 $\pm$ 43.60 <sup>a</sup>	1956.62 $\pm$ 7.50 <sup>b</sup>	1629.06 $\pm$ 36.51 <sup>a</sup>	2215.85 $\pm$ 12.07 <sup>c</sup>	1857.73 $\pm$ 2.41 <sup>b</sup>	***
<b>Essential AAs (%)</b>	829.45 $\pm$ 18.71 <sup>a</sup> (53.35%)	1080.54 $\pm$ 21.78 <sup>b</sup> (56.32%)	924.17 $\pm$ 8.30 <sup>c</sup> (57.68%)	1153.27 $\pm$ 27.94 <sup>b</sup> (53.14%)	995.06 $\pm$ 12.93 <sup>c</sup> (54.30%)	***

Values with different superscripts (a,b,c,d) in the same row differ significantly across the samples using one-way ANOVA and Tukey's test.

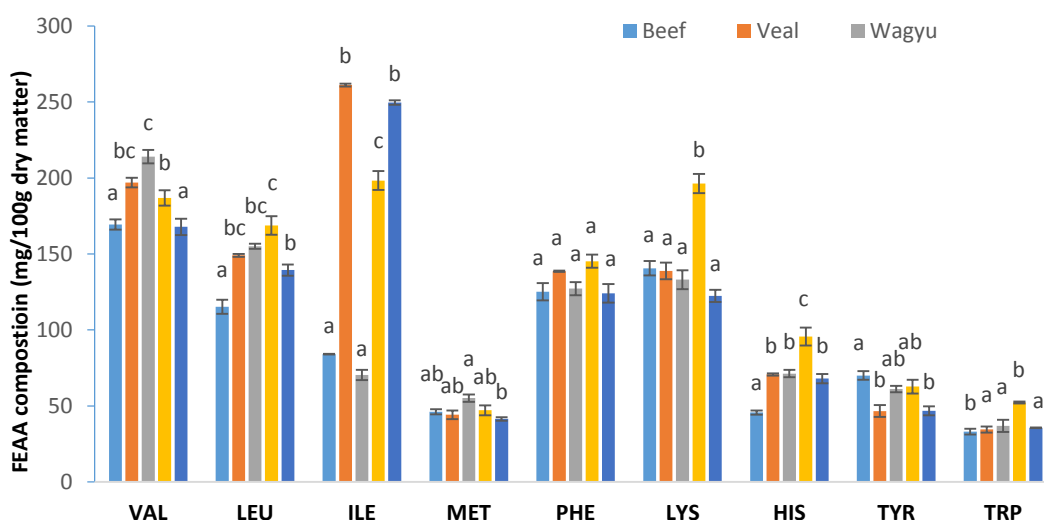
P < 0.0001 presented as \*\*\* for level of significance; P < 0.001 presented as \*\* for level of significance; P < 0.01 presented as \* level of significance and ns meaning not statistically significant.

Names of amino acids with “ # ” refer to essential amino acids. FAA stands for Free Amino Acids.

% Essential AA was calculated by EAAs/Total FAAs.

Bresaola made from five different animal species all had high content of free amino acids (FAAs). Seventeen amino acids, including all of nine essential amino acids

were found in all of the samples (as shown in Table 13 with “#” and Figure 10) and quantified automatically by GC-FID (ZB-AAA GC column, 10 m × 0.25 mm × 0.25 µm, section 3.5.4). The FAAs have been identified in dry-cured meat products in the past. However minor variations arising from analytical methods and animal species have been seen in other studies (Cordoba et al., 1994; Jurado et al., 2007; Marino et al., 2015; Paleari et al., 2003). A statistical difference in total FAAs was found from the current study, with the highest level in the mutton bresaola ( $2215.85 \pm 12.07$  mg/100g dry matter) and a significantly ( $p < 0.0001$ ) lower level in bresaola made from beef and wagyu meat,  $1615.62 \pm 43.60$  and  $1629.06 \pm 36.51$  mg/100g dry matter, respectively. Another author Paleari et al. (2003) also reported the differences of FAA composition (mg/100 g sample) in bresaola and cured products made from different animal species, including deer ( $2539 \pm 289.80$ ), boar ( $2315 \pm 238.73$ ), horse ( $2047 \pm 129.60$ ), bovine ( $1338 \pm 76.80$ ) and goat ( $2562 \pm 290.02$ ). This could be due to the species factor and differences in processing techniques. Multiple factors involved in the curing process may have significant effect on proteolysis of muscle proteins, including content of salts and water, pH, temperature, curing time, muscle enzymes (protease) activity, and action of microorganisms (Lorenzo & Laura, 2013; Ruiz-Ramírez et al., 2006; Toldrá et al., 1997). Numerous FAAs are released and accumulated in the final dry-cured products. Besides the effect on increasing the nutritional values, proteolysis also contributes to the characteristic texture and flavour of cured meat (Jurado et al., 2007; Martín, Antequera, Ventanas, Benitez-Donoso, & Córdoba, 2001), as discussed in the TPA study (section 4.1.2) analysis and sensory analysis (section 4.2).



**Figure 10 Composition of free essential amino acids (FEAAs) extracted from bresaola products (mg/100g dry matter). Mean values are plotted with error bars representing S.E.**

It is of particular significance to mention essential amino acids (EAAs) with regards to the nutritional value of meat products (Figure 10). A well-balanced amino acid mixture would have a higher biological value, which refers to the available content of essential amino acids (Oser, 1959), and could produce a higher thermogenic response and energy expenditure than the amino acid mixture with a lower biological value (Hochstenbach-Waelen et al., 2009; Westerterp-Plantenga et al., 2012). EAAs took up over 50% of total FAAs with a significant difference ( $p < 0.0001$ ) between the five cured products, which was along with the results reported by Paleari et al. (2003). A significantly lower amount of EAAs was found in the beef and wagyu products and this may have resulted from different curing time and species of the animal meat. Paleari et al. (2003) also found a low concentration of EAAs in bovine bresaola compared with goat, horse and venison. It is interesting to mention wagyu bresaola had the highest proportion of EAA to the total FAAs content, even though it had lower amounts of EAA.

Considering the FAAs individually, alanine (ALA), valine (VAL) and lysine (LYS) were the most abundant FAAs present in all of the five products, and each of them accounted for approximately 10% of total FAA content, whereas aspartic acid (ASP) appeared at the lowest concentration ( $< 2\%$ ). A relatively high amount ( $> 100$  mg/100 g dry matter) of flavour-active AAs, including ALA, glycine (GLY) and serine (SER) which possess sweet taste, and glutamic acids (GLU) which contributes to “umami” taste (Jurado et al., 2007; Mau & Tseng, 1998), was present. The results of FAAs correlate well with the sensory characteristics of bresaola products. For example, “sweetness” and “sourness” perceived by the panellists (section 4.22) may have resulted from the presence of GLU. GLU is one of the AAs with the highest impact for meat flavour due to the “umami” taste which produces the so-called “mouth satisfaction” (Kurihara, 2009; Maga, 1994). The presence of taurine (TAU), cysteine (CYS), and arginine (ARG), has been previously reported in dry-cured products (Marino et al., 2015; Paleari et al., 2003). However, they could not be identified in the current study due to the limitation of the amino acids analysis kit used (Phenomenex, 2003), and this phenomenon was also suggested in a study of cured meat by using EZ: faast amino acids kit (Trani et al., 2010), or other analytical methods (Cordoba et al., 1994; Jurado et al., 2007; Martin et al., 2001). Asparagine (ASN) and Glutamine (GLN), two of twenty common amino acids in proteins, were not reported due to the amount of these amino acids was too small to be quantified by GC-FID within the calibration range. The absence of these ASN and GLN were also



reported by Paleari et al. (2003) on the amino acids profile of bresaola made from different animal species.

Mutton meat had higher content of LYS than other animal species. Lysine, is one of the limiting AAs in plant proteins, particularly in cereal crops (Yu et al., 2004). It has been known that LYS is among the first of the amino acids to be attacked when there is any reaction between individual amino acids in the proteins of meat, and the nitrite used in the curing process (Wilder & Kraybill, 1947). LYS is strongly correlated with the length of curing time but with disparate results (Lorenzo & Laura, 2013; Ruiz et al., 1999; Toldra', 1998; Virgili, Saccani, Gabba, Tanzi, & Bordini, 2007). However, the positive effect of curing time on the concentration of isoleucine (ILE), suggested in the studies by (Cordoba et al., 1994; Lorenzo & Laura, 2013; Virgili et al., 2007), could also be supported in the present study that veal, mutton and lamb bresaola have shown a significant increase in ILE content. These products may have gone through a longer post-ripening time compared with beef and wagyu bresaola purchased from local stores.

All five bresaola products were further studied as part of digestibility evaluation to analyse the FAA profiles in the simulated human digestion process, as described in section 3.4.2, and discussed in section 4.3.

## 4.2 Sensory evaluation

Physicochemical characteristics of bresaola samples made from different animal species were analysed in comparison to traditional beef bresaola. Results indicated that all bresaola products differed significantly ( $p < 0.05$ ) in terms of instrumental textural properties and nutritional compositions. Five cured products were further subjected to consumer testing and projective mapping to evaluate product acceptability and identify sensory attributes related to consumer preference.

### 4.2.1 Consumer testing

Consumer perception of meat products is closely associated with sensory properties, with flavour (or taste) at the highest priority, followed by odour and texture (Møller, Hinrichsen, & Andersen, 1998). Eating satisfaction is one of the most important characteristics by which consumers judge meat quality after purchase (Grunert et al., 2004). Consumer testing results of 53 panellists (most between the ages of 20-31 years old) were determined for bresaola products in terms of odour, texture, flavour and overall liking and summarized in Table 14.

**Table 14 Consumer testing of bresaola products (mean  $\pm$  S.D.)**

	Beef	Veal	Wagyu	Mutton	Lamb	<i>P</i> -values
<b>Odour</b>	4.54 $\pm$ 2.41 <sup>a</sup>	4.19 $\pm$ 1.95 <sup>a</sup>	5.90 $\pm$ 1.92 <sup>b</sup>	4.04 $\pm$ 1.76 <sup>a</sup>	3.48 $\pm$ 2.02 <sup>a</sup>	***
<b>Texture</b>	5.75 $\pm$ 2.04	5.05 $\pm$ 2.00	5.76 $\pm$ 2.35	4.89 $\pm$ 1.84	4.94 $\pm$ 2.08	ns
<b>Flavour</b>	5.76 $\pm$ 2.12 <sup>a</sup>	4.55 $\pm$ 2.02 <sup>b</sup>	6.17 $\pm$ 2.18 <sup>a</sup>	4.27 $\pm$ 2.19 <sup>b</sup>	4.01 $\pm$ 2.24 <sup>b</sup>	***
<b>Overall liking</b>	5.72 $\pm$ 2.10 <sup>a</sup>	4.48 $\pm$ 1.88 <sup>b</sup>	6.30 $\pm$ 2.11 <sup>a</sup>	4.28 $\pm$ 2.16 <sup>b</sup>	4.05 $\pm$ 2.29 <sup>b</sup>	***

Values with different superscripts (a,b) in the same row differ significantly across the samples using one-way ANOVA and Tukey's test.

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

Most samples had scores of around 5 with respect to the different attributes, which indicates that all five bresaola products were acceptable by consumers. Analysis of variance was performed on the data. There was a significant difference ( $p < 0.0001$ ) between original beef bresaola and bresaola products made from New Zealand veal, wagyu, mutton and lamb in terms of odour, flavour and overall liking, except for texture ( $p = 0.056$ ). Similarly results from a study on donkey and beef bresaola (Marino et al.,

2010) showed no significant difference in consumer acceptance between the bresaola products. This could be attributed to the proteolysis of dietary muscles during the curing process that breaks down proteins into low molecular weight polypeptides and free amino acids, and results in tenderization of cured meat products (Toldrá & Flores, 1998).

As for the acceptability of odour, wagyu bresaola was most favoured by consumers with a mean rating at  $5.90 \pm 1.92$ . Wagyu and beef bresaola were separated from other products ( $p < 0.0001$ ) and received higher acceptance in terms of flavour and overall liking. Low liking ratings of odour of mutton and lamb bresaola could be attributed to the characteristic odour associated with sheep meat, which was commonly considered as unfavourable by unhabituated consumers (Sink & Caporaso, 1977; Watkins et al., 2013). This finding is also supported by data from projective mapping (Figure 11) that showed sheep bresaola to be associated with sheep meat aroma.

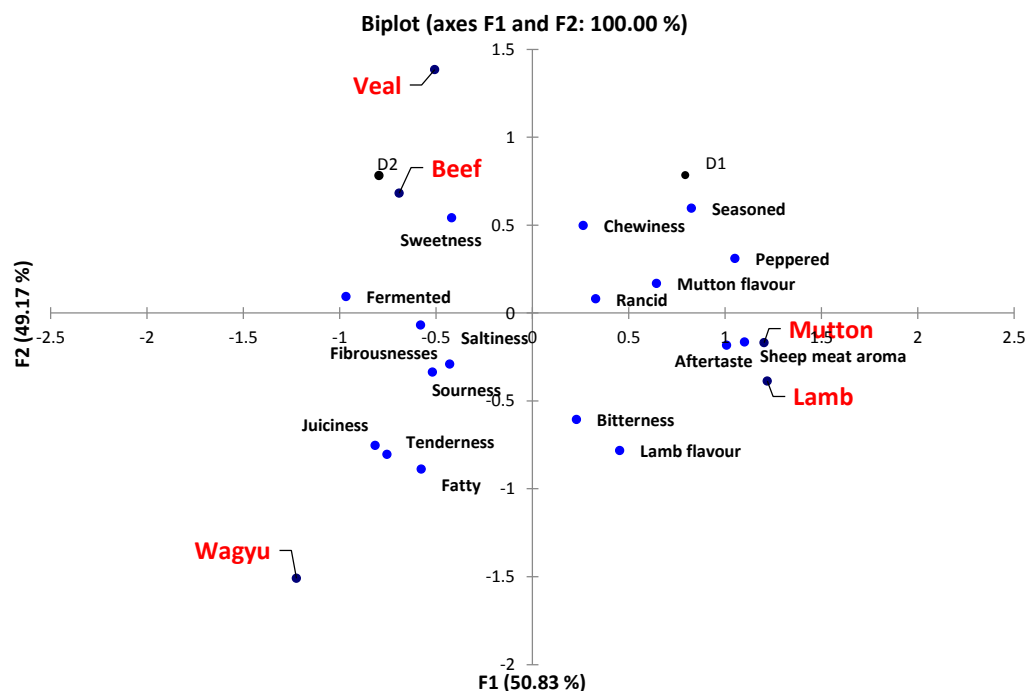
#### **4.2.2 Projective mapping**

Projective mapping, as a sensory profiling technique, studies the relationship among multiple products in terms of sensory attributes, and results are presented in a visual manner (Nestrud & Lawless, 2010). It is also a useful tool to perform a qualitative market research on the characteristics of food products in terms of quality properties such as sensory perception and packaging (Risvik et al., 1994).

A total of 15 panellists participated in the projective mapping study on five bresaola products over the period of three weeks. The RV coefficients between data from three sessions were assessed by multifactor analysis (MFA) as shown in Appendix 9, to evaluate the repeatability of panellists' performance in three duplicate trails. Performances of all the panellists in this study were acceptable ( $RV > 0.5$ ). Four panellists scored poorly for one of the three sensory trials ( $RV < 0.5$ ). Panellists' performance improved with time as indicated by the RV coefficient over the three trials. In trials 1 and 2, the RV coefficients of 86% panellists were more than 0.5, and in the third trial, 100% agreement were found among panellists.

Principal component analysis (PCA) was performed with the aim to study the similarities and differences between bresaola products made from different species in terms of sensory perception. Factor scores of observations and loading of variables were plotted in a biplot, as shown in Figure 11. Five observations as shown in the biplot referred to five bresaola products (Beef, veal, wagyu, mutton and lamb) considered in this

study. Variables referred to attributes generated by panellists for characterising sensory properties of the five samples.



**Figure 11 Principal Component Analysis of bresaola samples over the combined three sensory trials.**

In general, five products were clearly distributed into three groups. Bresaola from mutton and lamb had high positive scores along principal component (PC) 1 and were associated with peppered, sheep meat aroma and mutton aroma. The volatile compounds such as trace amount of branched chain fatty acids (Wong et al., 1975) and skatole (3-methylindole) (Yokoyama & Carlson, 1974) included in sheep fat may contribute to the predominant sheep meat smell.

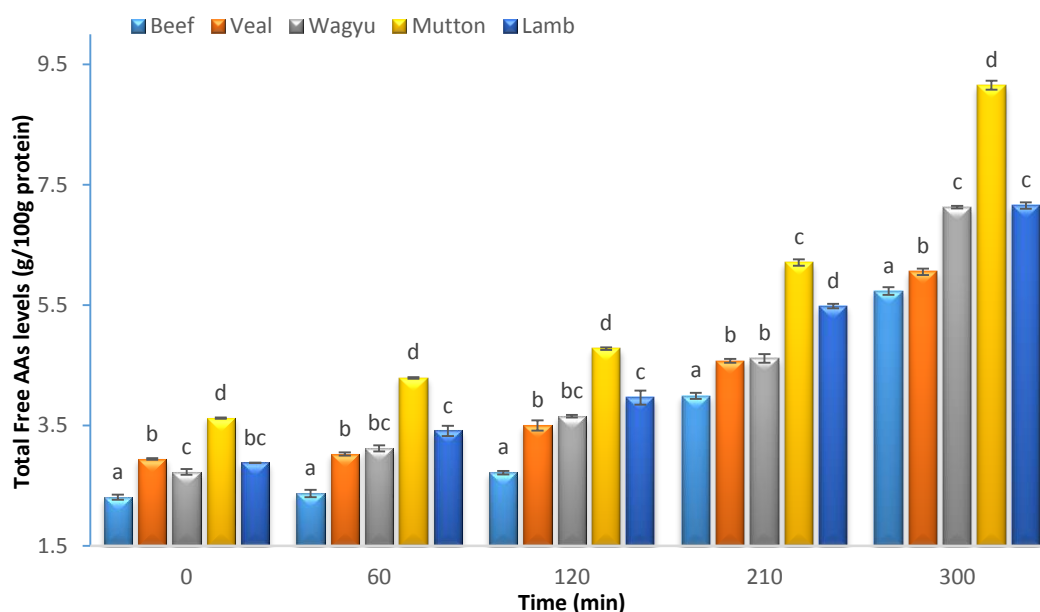
Wagyu that high negative scores was separated from beef and veal bresaola along PC 2 that corresponded to juiciness, tenderness and fatty. Regarding PC 2, the more representative attributes were fatty, tenderness, seasoned and sweetness. These attributes were well defined by panellists and considered as representative sensory characteristics of the bresaola products in the present study. Wagyu meat is noted for its extensive marbling appearance, favourable nutritional value and characteristic tenderness and flavour (Boylston et al., 1995; De Smet et al., 2004). Wagyu bresaola used in this study was processed from New Zealand grass-fed wagyu meat with a superior flavour. Fatty taste of wagyu bresaola could be attributed to the high grade of marbling. Mountford,

Piyasiri, and Warner (2014) suggested that as beef marbling increased, oily mouth coating became more intense and sour taste decreased. They also reported that grass-fed wagyu was more tender, juicy and lower in chewiness as shown in our study.

Juiciness is an important sensory quality of meat products (Hutchings & Lillford, 1988). It is generally related to the moisture and fat content presented (Ruiz-Carrascal et al., 2000) in the meat. Wagyu bresaola had higher moisture level than the other products, as shown in the results of proximate analysis, which could directly contribute to juiciness. In addition, fatty acids profile could also influence sensory quality of dry-cured products. Lower proportion of PUFA and higher of oleic acid could contribute to juiciness, and increase consumer preference (Ruiz-Carrascal et al., 2000), as confirmed in the present study. Wagyu bresaola contained lowest PUFA level and highest oleic acid concentration in comparison with other species according to the results of fatty acids analysis (Table 12).

### 4.3 Digestibility of bresaola products

Dietary protein is an important contributor for the growth, maintenance and repairing tissues. Meat proteins are distinguished from plant proteins by their ability to supply substantial amount of essential amino acids (EAAs). Ingested proteins would undergo a series of proteolysis and break down into amino acids, which are absorbed in the small intestine for further utilization in the human body. Therefore, the level of free amino acids (FAAs) released from a certain amount of proteins by proteolysis could act as an indicator of protein digestibility (Stuknytė et al., 2014). A static *in vitro* digestion protocol (section 3.4) was used to evaluate the digestibility of dietary proteins from bresaola products. FAAs were examined from the digested samples collected from five sampling points over a course of five hours (Figure 6), results as shown in Table 15 and Figures 12, 14.

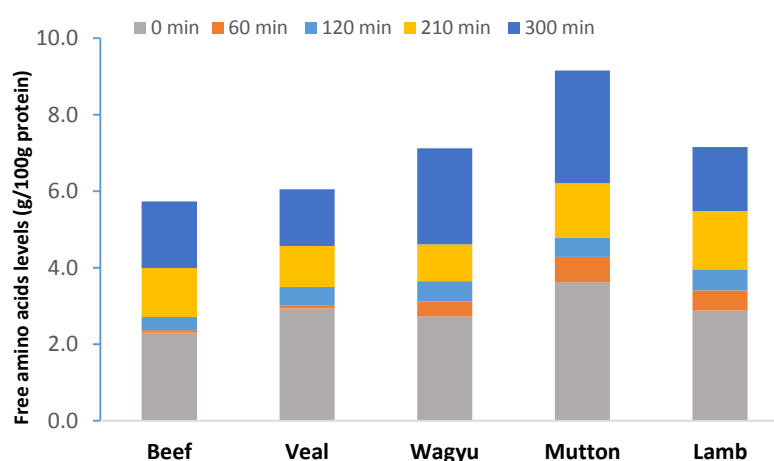


**Figure 12 Total FAAs of bresaola products from 5 time points of *in vitro* digestion.** 0 min” refers to the initial time point of digestion (free amino acids). “60 min” and “120 min” refer to the sampling point of S2 and S3 in Figure 6, section 3.4.2, respectively. “210 min” and “240 min” refer to the sampling point of S4 and S5 in Figure 6, section 3.4.2, respectively. Mean values are plotted with error bars representing S.E.

Processed meat products have long been known as highly nutritious foods the presence of certain FFAs produced by proteolysis during the curing process (Lorenzo & Laura, 2013; Toldrá et al., 1997; Toldra', 1998). As shown in the FAA profile (Table 13),

FAAs were present in all of the raw bresaola samples to start with. The amount of FAAs of all bresaola samples increased steadily throughout the five hour digestion period. After the oral phase, which involved immersing the lyophilized bresaola sample in artificial saliva containing alpha amylase, the level of FAAs did not change (data not shown). This was expected as  $\alpha$ -amylase has negligible influence over the digestion of peptide linkages present in proteins. Artificial saliva contained electrolytes which also did not contribute towards protein digestion. Lingual lipase was omitted in the artificial saliva used. Hence no significant difference in total FAAs was seen between the initial feed material and the sample collected after the oral phase.

Besides simulating the human gastric digestion, the present study also simulated the upper duodenum digestion with pancreatin extracts from porcine. The enzyme activity was determined according to the activity of trypsin and chymotrypsin. Each pancreatic enzyme has a unique and complementary action on breakdown of proteins and protein fractions into smaller peptides and free amino acids. Trypsin is responsible for the breakdown of the peptide bond linked with carboxyl side of basic amino acids, such as LYS and ARG (Krehbiel & Matthews, 2003). The linkage between aromatic amino acids, such as PHE, TYR and histidine (HIS) would be cleaved with the catalysis of chymotrypsin (Sitrin, 2014). As shown in Table 15, higher values of amino acids PHE, TYR and HIS in the intestinal phase, at the approximately 300%, 400% and 200% respectively of the values in gastric phase, may also be an indicator of this selective breakdown of protein by chymotrypsin.



**Figure 13** FAAs (g/100g protein) of the five bresaola samples collected from the digestion protocol. Each bar represents the total FAAs present at the given time point in the digestion period, which were 0, 60, 120, 210 and 300 min.

Other enzymes also play an important role in the protein degradation, which include pancreatic exopeptidases elastase, and endopeptidases carboxypeptidase A and B (Krehbiel & Matthews, 2003). However these were omitted from the *in vitro* digestion protocol due to difficulty in sourcing. Sitrin (2014) suggested that proline (PRO)-containing peptides would be resistant to pancreatic hydrolysis. This was supported in the present study of bresaola products, as shown in Table 15. There was no considerable change detected on PRO content at the end of the intestinal phase compared with the gastric phase.

There was a slight increase of FAAs at the end of the gastric phase and a considerably raised amount of FAAs found at the end of the intestinal phase of simulated digestion, as shown in Figure 12 and Table 15. This could be explained by the peptic degradation of proteins being less effective and generally accounted for approximately 10-20% of proteolysis in the human stomach, while pancreatic enzymes are more powerful than gastric pepsin and are known to contribute for approximately 40% of FAAs production (Krehbiel & Matthews, 2003; Restani et al., 1992). From human physiology textbook written by Vander et al. (2001), it is known that about 60% of amino acids would remain as oligopeptide fractions, containing up to six amino acid residues, after the pancreatic digestion in the humans.

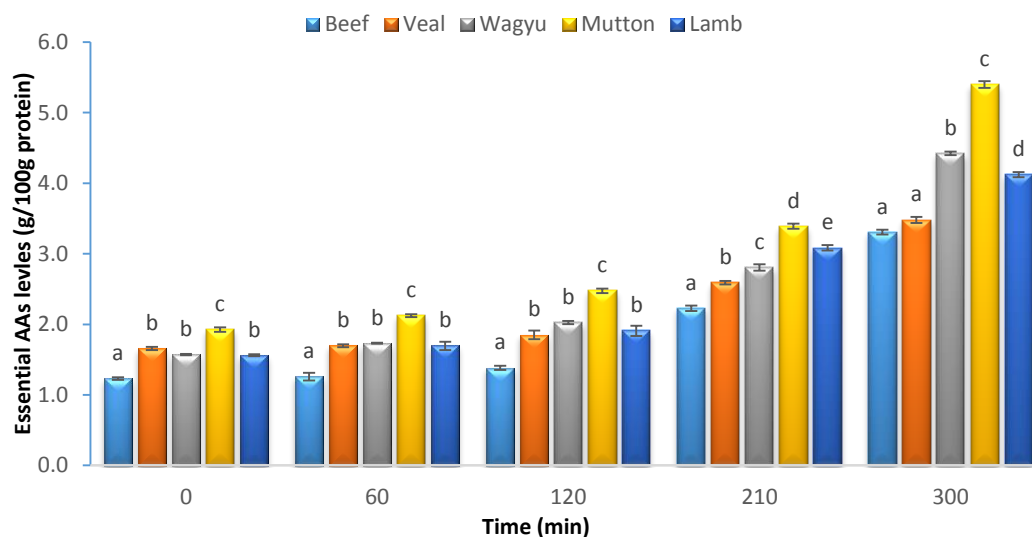
Bresaola sample made from mutton meat has shown the highest level of FAAs at each of the five sampling time points and the values of the FAAs throughout the digestion period were significantly distinguished from other animal species ( $p < 0.05$ ), indicating that proteins in mutton bresaola were digested more extensively and faster. However, for the beef bresaola, results were the opposite. The FAAs present in the digested samples for the beef bresaola have always been the lowest among the five different bresaola samples. Regarding the amount of FAAs released during the first 90 minutes of the intestinal phase, 1.43 and 1.52 g/100 g protein were detected from mutton and lamb samples, respectively, as shown at 210 min in Figure 13. However for the second half of the intestinal phase, the release of amino acids from wagyu was high (2.51 g/100 g protein), showing a similar value to that of the mutton (2.95 g/100 g protein). This phenomenon could be attributed to the hydrolysis and oxidation of proteins during the curing process and storage. As discussed in the previous chapter (section 2.1.6.2), dietary proteins are prone to be hydrolysed during the curing process, which may involve unfolding of the three-dimensional structure of protein quaternary or tertiary structure,



exposing amino acid residues to ease further breakdown by the digestive enzymes (Branden, 1999; Rodríguez-Núñez et al., 1995; Toldrá et al., 1997). In the present study, different products could have been at different stages of proteolysis prior to the digestion simulation, as indicated by the significant difference of amino acids profile shown in Table 15 and Figure 13. Other authors measured the formation of peptides to predict the hydrolysis of protein during the dry-curing process. Results suggested a great number of polypeptides with a wide range of molecular mass were produced from dry-cured ham (Rodríguez-Núñez et al., 1995) and sausages (Soriano et al., 2006). This method could have been adopted to better understand the degree of polypeptide digestion from the bresaola samples.

Another factor associated with the quality of dry-cured meat products is the oxidation of muscle protein, which could have a considerable impact on the digestibility of final products. S. Ventanas, Estevez, Tejeda, and Ruiz (2006) suggested the ripening of dry-cured loin would induce the oxidation of muscle proteins and result in the raise of carbonyl compounds. Accompanied by the oxidation of lipids, muscle proteins are prone to oxidative reactions initiated by oxidizing lipids, metal ions and other pro-oxidants generated during processing (Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008; Fuentes, Ventanas, Morcuende, Estévez, & Ventanas, 2010). Oxidative degradation of muscle proteins involves modification of the amino acid side chains, with the formation of carbonyl compounds and Schiff bases and protein aggregation by polymerization between modified amino acids, and result in the loss of amino acids (Lund, Heinonen, Baron, & Estevez, 2011; Xiong, 2000). A negative correlation between protein aggregation, formation of carbonyl compounds and pepsin activity during *in vitro* proteolysis of muscle proteins have been suggested by Santé-Lhoutellier, Astruc, Marinova, Greve, and Gatellier (2008). The intermolecular cross-links and the formation of aggregation may disturb the recognition of proteins by protease, causing changes in protein hydrophobicity and solubility, and in turn, decreases the protein digestibility (Morzel, Gatellier, Sayd, Renerre, & Laville, 2006). However, contradicting results on the variation of protein digestibility associated with protein oxidative reactions were also indicated in the study on fresh meat (lamb) at different oxidative stage and digested by pepsin and pancreatic enzymes (trypsin and  $\alpha$ -chymotrypsin) (Santé-Lhoutellier, Engel, et al., 2008). There was no significant but negative correlation between the production of carbonyl compounds and peptic hydrolysis by measuring protein fractions using SDS-PAGE. While a considerably favourable correlation with pancreatic degradation of

myofibrillar proteins was shown, this could also be explained by different levels of oxidation having inverse effect on digestibility of proteins. Other authors suggested low levels of oxidation inducing subtle changes in protein structure which favoured their recognition by proteases and proteolytic susceptibility initially increased with oxidation (Davies, 2001; Grune, Jung, Merker, & Davies, 2004).



**Figure 14 Total EAAs (g/100 g protein) of bresaola products from 5 sampling points of *in vitro* digestion. Mean values are plotted with error bars representing S.E.**

In evaluating EAAs released from *in vitro* digestion (Figure 14), mutton bresaola was distinctively higher in EAAs throughout the digestion period. Regarding the proportion of EAAs in total FAAs, as shown in Table 15, all samples underwent a slight decrease during the peptic degradation, and was significantly raised during the intestinal phase with the proteolysis of pancreatic enzymes. It is interesting to note that, as shown in table 15, wagyu bresaola was found to have consistently higher concentration of EAAs in total FAAs than mutton bresaola, which had the highest amount of EAA (as shown in Figure 14), and the other cured products at all the sampling points. It could be explained by selective cleavage of protease at different digestive phases. Pepsin, the primary digestive enzyme in human stomach, is an aspartic protease with broad substrate specificity (Monogioudi et al., 2011). The cleavage sites include mainly valine (VAL), phenylalanine (PHE), glutamic acid (GLU) and leucine (LEU), but sites containing proline (PRO), tyrosine (TYR) and serine (SER) residues can also be cleaved (Fujioka & Scheraga, 1965; Schmelzer et al., 2007; Vance et al., 1997). Its most pronounced effect

is the peptide sites between LEU-VAL, TYR-LEU, or between the aromatic amino acids such as PHE-PHE or PHE-TYR (Krehbiel & Matthews, 2003; Ulshen, 1987).

**Table 15 Comparison of FAAs (g/100g protein) between five bresaola products at different digestion stages: undigested (initial) stage (0 min, FAAs), at the end of the gastric phase (120 min, S<sub>3</sub>) and at the end of the intestinal phase (300 min, S<sub>5</sub>) of the *in vitro* digestion process. Values are presented as Mean ± S. D.**

	Undigested products						Gastric phase						Small intestinal phase					
	Beef	Veal	Wagyu	Mutton	Lamb	P- values	Beef	Veal	Wagyu	Mutton	Lamb	P- values	Beef	Veal	Wagyu	Mutton	Lamb	P- values
ALA	0.237±0.004 <sup>a</sup>	0.351±0.001 <sup>b</sup>	0.320±0.012 <sup>c</sup>	0.410±0.003 <sup>d</sup>	0.373±0.002 <sup>b</sup>	***	0.239±0.008 <sup>a</sup>	0.366±0.005 <sup>bc</sup>	0.339±0.019 <sup>b</sup>	0.453±0.015 <sup>d</sup>	0.401±0.016 <sup>c</sup>	**	0.328±0.004 <sup>a</sup>	0.449±0.001 <sup>b</sup>	0.510±0.010 <sup>c</sup>	0.736±0.017 <sup>d</sup>	0.542±0.003 <sup>c</sup>	***
GLY	0.154±0.005 <sup>a</sup>	0.174±0.003 <sup>ab</sup>	0.167±0.010 <sup>ab</sup>	0.245±0.007 <sup>c</sup>	0.192±0.014 <sup>b</sup>	*	0.153±0.008 <sup>a</sup>	0.194±0.001 <sup>bc</sup>	0.173±0.001 <sup>b</sup>	0.247±0.003 <sup>d</sup>	0.205±0.009 <sup>c</sup>	***	0.278±0.011 <sup>a</sup>	0.279±0.004 <sup>a</sup>	0.342±0.011 <sup>b</sup>	0.434±0.016 <sup>c</sup>	0.305±0.009 <sup>ab</sup>	**
VAL	0.251±0.007 <sup>a</sup>	0.302±0.007 <sup>bc</sup>	0.364±0.011 <sup>d</sup>	0.312±0.012 <sup>c</sup>	0.264±0.012 <sup>ab</sup>	*	0.266±0.005 <sup>a</sup>	0.313±0.002 <sup>b</sup>	0.432±0.016 <sup>c</sup>	0.383±0.005 <sup>d</sup>	0.316±0.017 <sup>b</sup>	**	0.441±0.006 <sup>a</sup>	0.404±0.013 <sup>b</sup>	0.615±0.007 <sup>cd</sup>	0.587±0.007 <sup>c</sup>	0.626±0.005 <sup>d</sup>	***
LEU	0.171±0.010 <sup>a</sup>	0.229±0.002 <sup>b</sup>	0.264±0.004 <sup>c</sup>	0.282±0.014 <sup>c</sup>	0.219±0.008 <sup>b</sup>	**	0.198±0.016 <sup>a</sup>	0.242±0.006 <sup>ab</sup>	0.289±0.009 <sup>bc</sup>	0.331±0.011 <sup>c</sup>	0.224±0.014 <sup>a</sup>	*	0.623±0.005 <sup>a</sup>	0.583±0.009 <sup>b</sup>	0.899±0.014 <sup>c</sup>	0.986±0.012 <sup>d</sup>	0.648±0.007 <sup>a</sup>	***
THR	0.140±0.012 <sup>a</sup>	0.140±0.001 <sup>a</sup>	0.164±0.010 <sup>a</sup>	0.149±0.006 <sup>a</sup>	0.139±0.010 <sup>a</sup>	ns	0.117±0.004 <sup>a</sup>	0.147±0.007 <sup>ab</sup>	0.211±0.021 <sup>c</sup>	0.180±0.007 <sup>bc</sup>	0.154±0.010 <sup>ab</sup>	*	0.222±0.013 <sup>a</sup>	0.248±0.004 <sup>b</sup>	0.299±0.003 <sup>c</sup>	0.280±0.008 <sup>bc</sup>	0.303±0.010 <sup>c</sup>	*
SER	0.022±0.002 <sup>a</sup>	0.124±0.002 <sup>b</sup>	0.161±0.016 <sup>bc</sup>	0.179±0.014 <sup>c</sup>	0.150±0.009 <sup>bc</sup>	**	0.046±0.001 <sup>a</sup>	0.159±0.008 <sup>b</sup>	0.179±0.003 <sup>b</sup>	0.234±0.018 <sup>c</sup>	0.167±0.005 <sup>b</sup>	***	0.072±0.001 <sup>a</sup>	0.209±0.002 <sup>b</sup>	0.249±0.003 <sup>c</sup>	0.293±0.003 <sup>d</sup>	0.227±0.003 <sup>c</sup>	***
ILE	0.125±0.000 <sup>a</sup>	0.401±0.002 <sup>b</sup>	0.120±0.008 <sup>a</sup>	0.331±0.015 <sup>c</sup>	0.392±0.003 <sup>b</sup>	***	0.112±0.007 <sup>a</sup>	0.459±0.008 <sup>b</sup>	0.265±0.013 <sup>c</sup>	0.469±0.014 <sup>b</sup>	0.532±0.001 <sup>d</sup>	***	0.344±0.005 <sup>a</sup>	0.722±0.010 <sup>b</sup>	0.339±0.016 <sup>a</sup>	0.740±0.012 <sup>b</sup>	0.760±0.011 <sup>b</sup>	***
PRO	0.124±0.008 <sup>a</sup>	0.187±0.006 <sup>b</sup>	0.108±0.016 <sup>a</sup>	0.223±0.003 <sup>b</sup>	0.185±0.008 <sup>b</sup>	**	0.121±0.009 <sup>a</sup>	0.197±0.001 <sup>b</sup>	0.116±0.004 <sup>a</sup>	0.206±0.014 <sup>b</sup>	0.184±0.014 <sup>b</sup>	*	0.291±0.009 <sup>a</sup>	0.238±0.009 <sup>b</sup>	0.183±0.019 <sup>c</sup>	0.273±0.003 <sup>ab</sup>	0.259±0.009 <sup>ab</sup>	*
ASP	0.069±0.001 <sup>a</sup>	0.043±0.005 <sup>a</sup>	0.010±0.004 <sup>b</sup>	0.063±0.011 <sup>a</sup>	0.042±0.010 <sup>a</sup>	*	0.119±0.011 <sup>a</sup>	0.093±0.004 <sup>b</sup>	0.079±0.001 <sup>b</sup>	0.117±0.002 <sup>a</sup>	0.072±0.003 <sup>b</sup>	*	0.203±0.005 <sup>a</sup>	0.116±0.009 <sup>b</sup>	0.115±0.003 <sup>b</sup>	0.156±0.003 <sup>c</sup>	0.182±0.011 <sup>ac</sup>	**
MET	0.069±0.004 <sup>a</sup>	0.068±0.006 <sup>a</sup>	0.094±0.006 <sup>b</sup>	0.079±0.008 <sup>ab</sup>	0.065±0.003	0.015	0.084±0.017 <sup>a</sup>	0.088±0.007 <sup>ab</sup>	0.120±0.016 <sup>ab</sup>	0.138±0.009 <sup>b</sup>	0.085±0.012 <sup>b</sup>	0.024	0.168±0.003 <sup>a</sup>	0.177±0.002 <sup>a</sup>	0.263±0.001 <sup>b</sup>	0.293±0.011 <sup>b</sup>	0.216±0.014 <sup>c</sup>	***
GLU	0.222±0.002 <sup>a</sup>	0.201±0.009 <sup>ab</sup>	0.171±0.002 <sup>bc</sup>	0.353±0.014 <sup>d</sup>	0.168±0.002 <sup>c</sup>	***	0.281±0.014 <sup>a</sup>	0.337±0.005 <sup>b</sup>	0.388±0.014 <sup>c</sup>	0.669±0.011 <sup>d</sup>	0.710±0.014 <sup>d</sup>	***	0.687±0.012 <sup>a</sup>	0.825±0.006 <sup>b</sup>	0.751±0.007 <sup>c</sup>	1.265±0.013 <sup>d</sup>	0.970±0.016 <sup>c</sup>	***
PHE	0.186±0.012 <sup>a</sup>	0.213±0.001 <sup>ab</sup>	0.217±0.010 <sup>ab</sup>	0.242±0.010 <sup>b</sup>	0.195±0.014 <sup>a</sup>	0.018	0.200±0.004 <sup>a</sup>	0.230±0.019 <sup>a</sup>	0.282±0.009 <sup>bc</sup>	0.325±0.013 <sup>c</sup>	0.238±0.015 <sup>ab</sup>	*	0.620±0.014 <sup>a</sup>	0.540±0.008 <sup>b</sup>	0.776±0.009 <sup>c</sup>	0.909±0.007 <sup>d</sup>	0.636±0.004 <sup>a</sup>	***
ORN	0.111±0.003 <sup>a</sup>	0.066±0.001 <sup>bc</sup>	0.054±0.003 <sup>c</sup>	0.077±0.007 <sup>bc</sup>	0.065±0.001 <sup>b</sup>	***	0.130±0.005 <sup>a</sup>	0.093±0.011 <sup>b</sup>	0.080±0.004 <sup>b</sup>	0.104±0.001 <sup>ab</sup>	0.085±0.007 <sup>b</sup>	*	0.170±0.005 <sup>a</sup>	0.106±0.014 <sup>b</sup>	0.114±0.010 <sup>b</sup>	0.141±0.007 <sup>ab</sup>	0.107±0.007 <sup>b</sup>	*
LYS	0.209±0.010 <sup>a</sup>	0.213±0.012 <sup>a</sup>	0.227±0.015 <sup>a</sup>	0.328±0.015 <sup>b</sup>	0.192±0.009 <sup>a</sup>	*	0.251±0.019 <sup>a</sup>	0.234±0.013 <sup>a</sup>	0.269±0.002 <sup>a</sup>	0.404±0.003 <sup>b</sup>	0.232±0.018 <sup>a</sup>	**	0.377±0.011 <sup>a</sup>	0.391±0.001 <sup>a</sup>	0.580±0.013 <sup>b</sup>	0.824±0.013 <sup>c</sup>	0.472±0.002 <sup>d</sup>	***
HIS	0.068±0.003 <sup>a</sup>	0.108±0.001 <sup>b</sup>	0.121±0.006 <sup>b</sup>	0.160±0.014 <sup>c</sup>	0.107±0.007 <sup>b</sup>	*	0.087±0.008 <sup>a</sup>	0.118±0.009 <sup>ab</sup>	0.141±0.005 <sup>b</sup>	0.192±0.005 <sup>c</sup>	0.118±0.011 <sup>ab</sup>	**	0.124±0.009 <sup>a</sup>	0.165±0.011 <sup>b</sup>	0.231±0.007 <sup>c</sup>	0.283±0.010 <sup>d</sup>	0.183±0.004 <sup>b</sup>	***
TYR	0.104±0.006 <sup>a</sup>	0.071±0.008 <sup>b</sup>	0.104±0.005 <sup>a</sup>	0.105±0.011 <sup>a</sup>	0.073±0.007 <sup>b</sup>	0.012	0.116±0.003 <sup>ab</sup>	0.096±0.015 <sup>a</sup>	0.144±0.016 <sup>b</sup>	0.128±0.002 <sup>ab</sup>	0.096±0.012 <sup>a</sup>	0.027	0.451±0.009 <sup>a</sup>	0.353±0.010 <sup>b</sup>	0.516±0.012 <sup>c</sup>	0.535±0.011 <sup>c</sup>	0.414±0.022 <sup>a</sup>	**
TRP	0.049±0.004 <sup>a</sup>	0.053±0.004 <sup>a</sup>	0.063±0.010 <sup>a</sup>	0.087±0.001 <sup>b</sup>	0.056±0.001 <sup>a</sup>	*	0.066±0.001 <sup>a</sup>	0.072±0.008 <sup>a</sup>	0.084±0.002 <sup>a</sup>	0.106±0.007 <sup>b</sup>	0.068±0.004 <sup>a</sup>	*	0.161±0.008 <sup>a</sup>	0.146±0.012 <sup>a</sup>	0.205±0.002 <sup>b</sup>	0.241±0.001 <sup>c</sup>	0.166±0.007 <sup>a</sup>	**
Total	2.308±0.060 <sup>a</sup>	2.943±0.018 <sup>b</sup>	2.728±0.067 <sup>c</sup>	3.623±0.014 <sup>d</sup>	2.878±0.004 <sup>bc</sup>	***	2.588±0.041 <sup>a</sup>	3.439±0.119 <sup>b</sup>	3.591±0.032 <sup>bc</sup>	4.686±0.031 <sup>d</sup>	3.886±0.165 <sup>c</sup>	***	5.560±0.089 <sup>a</sup>	5.948±0.076 <sup>b</sup>	6.987±0.030 <sup>c</sup>	8.976±0.106 <sup>c</sup>	7.016±0.075 <sup>c</sup>	***
EAA	1.231±0.028 <sup>a</sup>	1.658±0.033 <sup>b</sup>	1.573±0.014 <sup>b</sup>	1.925±0.047 <sup>c</sup>	1.563±0.020 <sup>b</sup>	***	1.382±0.042 <sup>a</sup>	1.853±0.087 <sup>b</sup>	2.026±0.033 <sup>b</sup>	2.476±0.044 <sup>c</sup>	1.908±0.104 <sup>b</sup>	**	3.309±0.048 <sup>a</sup>	3.480±0.059 <sup>a</sup>	4.423±0.035 <sup>b</sup>	5.400±0.069 <sup>c</sup>	4.122±0.051 <sup>d</sup>	***
EAA %	53.35% <sup>ab</sup>	56.32% <sup>c</sup>	57.68% <sup>c</sup>	53.14% <sup>ab</sup>	54.30% <sup>ab</sup>	*	53.37%	53.86%	56.40%	52.83%	49.09%	*	59.51%	58.50%	63.31%	60.15%	58.75%	***

Values with different superscripts (a,b,c,d,e) in the same row differ significantly across the samples.

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.

## Chapter 5. Conclusion

In the present study, five bresaola products processed from New Zealand cattle meat (beef, veal and wagyu) and sheep meat (mutton and lamb) were evaluated in term of the nutritional profile, physicochemical and sensory properties and *in vitro* digestibility, to investigate the feasibility of developing value-added meat products like “bresaola” from different animal species.

Bresaola made from beef equivalents were higher in protein content but lower in intramuscular fat content except for wagyu meat compared to that of the sheep meat. Similar intramuscular fat level of approximately 7-8% in wet samples has been found in bresaola from wagyu as those from the sheep meat. Mutton and lamb had lower moisture content ( $< 40\%$ ) than the other products. All the products were significantly different ( $p < 0.05$ ) from each other in terms of instrumental texture quality (hardiness, cohesiveness, springiness, chewiness and resilience) from the texture profile analysis. The difference may have risen from the use of different animal species and different level of tenderization during the dry-curing process. Regarding the colour of the cured meat, all five products differed significantly ( $p < 0.05$ ) in lightness ( $L^*$ ), redness ( $a^*$ ) and yellowness ( $b^*$ ). The variations may have been caused by the concentration and status of myoglobin.

Fatty acids and free amino acids are of great significance in cured meat products. They play an important role in flavour (taste and odour) development. Bresaola made from veal and wagyu contained high composition of MUFAs ( $58.20 \pm 0.58\%$ ) but low content of PUFAs ( $3.04 \pm 0.04\%$ ). Beef and lamb had a higher concentration of n-3 PUFAs ( $7.35 \pm 0.29\%$  and  $5.91 \pm 0.16\%$ , respectively) compared to veal, wagyu and mutton ( $3.58 \pm 0.06\%$ ,  $1.14 \pm 0.04\%$  and  $3.14 \pm 0.04\%$ , respectively). Lamb was rich in n-6 PUFAs ( $14.93 \pm 0.35\%$ ). A low value ( $< 1.5$ ) of the ratio of n-6 to n-3 fatty acids was detected in all five products, suggesting a favourable balance which may exert enhanced health benefits and prevention of chronic disease. Free amino acids of 17 different amino acids were identified in bresaola products as the result of muscle proteolysis under the action of protease and microorganisms during curing process. A higher concentration of amino acids ( $2215.85 \pm 12.07$  mg/100 g dry matter) was found in mutton bresaola with lower value of essential amino acids content (53.14%). Over 50% of total amino acids found in the products were essential amino acids which are of great significance since they can only be supplied from diets.

Consumers had significantly different ( $p < 0.05$ ) ratings of acceptability among five cured products in terms of odour, flavour and overall liking, except for the texture ( $p > 0.05$ ). Wagyu bresaola was the most preferred choice by consumers regarding all of the sensory attributes, followed by beef, veal and sheep meat products. Panellists participated in the projective mapping were able to differentiate the five products with 18 sensory attributes. They associated wagyu bresaola with juiciness and tenderness with fermented odour, and for mutton and lamb products, peppered flavour with sheep meat aroma was associated.

A static *in vitro* digestion model, as a simple and speedy method, was used to examine the digestibility of proteins from dry-cured bresaola. Mutton bresaola has shown the highest content (8.976 g/100 g protein) of total amino acids released after digestion. Mutton and lamb had similar rate of digestibility. When the amount of FAAs released during the first 90 minutes of the intestinal phase was measured, 1.43 g and 1.52 g were detected from mutton and lamb samples, respectively. However for the second half of the intestinal phase, the release of amino acids from wagyu was high (2.51 g), showing a similar value to that of the mutton (2.95 g). Wagyu products had a higher proportion of essential amino acids to total amino acids. Differences in the amino acid profile at different stages of digestion were found among five samples. This could be associated with the impact of animal species and the different levels of protein oxidation. For the first time, digestibility of bresaola products was assessed in terms of the release of FAAs after *in vitro* digestion.

In this study, bresaola products were made from different animal species, undergone different stages of curing and storage time, which may affect the results, such as the variations in colour and texture properties, free amino acids content and the digestibility. Further studies looking at the interaction of protein oxidation and protein digestibility would be useful to explain the data. Static models can provide feasible and reproducible information about the digestibility of food products. But this protocol cannot mimic the physiological processes that occur *in vivo*. Adopting Protein digestibility-corrected amino acid score (PDCAAS), which is known as a “gold standard” of measuring protein digestibility, would be desirable to evaluate the digestibility of dietary protein from bresaola products in *in vivo* settings. Additionally, *in vivo* - *in vitro* correlation study could also be performed.

Overall, bresaola products produced from different animal species (veal, wagyu, mutton and lamb) have shown acceptable characteristics as cured meat products. In

particular, superior sensory qualities in wagyu bresaola and favourable nutritional values and digestibility in sheep meat products have been found. The data from this study will be useful to the New Zealand meat industry to increase the value of New Zealand red meat by producing a cured meat product made of sheep meat. With continuous development of the dry-curing techniques and new flavours, bresaola made from sheep meat certainly has a potential as a commercial cured meat product to reach the global market.

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# Appendices

## 1. Instruction and questionnaire for consumer testing

- Please indicate your gender

☐ Male

☐ Female

- Please indicate your age group

☐ Under 20

☐ 20-29

☐ 30-39

☐ 40-49

☐ Above 50

- Are you a vegetarian or culturally sensitive to the consumption of any of following red meat products?

☐ Beef

☐ Veal

☐ Wagyu

☐ Mutton

☐ Lamb

☐ None

If you tick any of the meat products, please do not proceed with the test.

- Are you allergic to any of following red meat products? Or advised by doctor not to consume red meat?

☐ Beef

☐ Veal

☐ Wagyu

☐ Mutton

☐ Lamb

☐ None

If you tick any of the meat products, please do not proceed with the test.

- How often do you consume meat or meat products?

- ☐ Never
- ☐ Once a month
- ☐ Twice a month
- ☐ Once a week
- ☐ More than once a week

➤ **Consumer testing section**

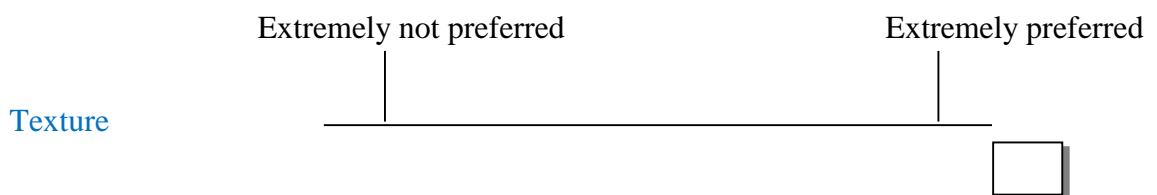
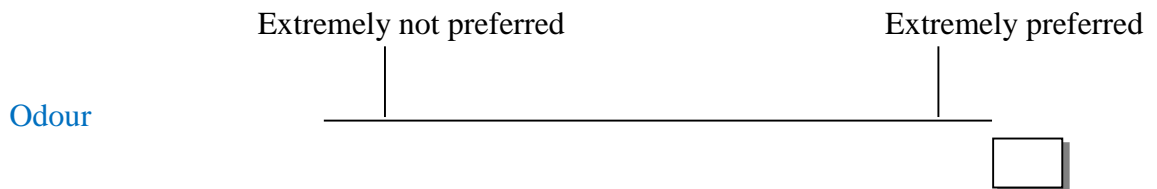
❖ **Instruction:**

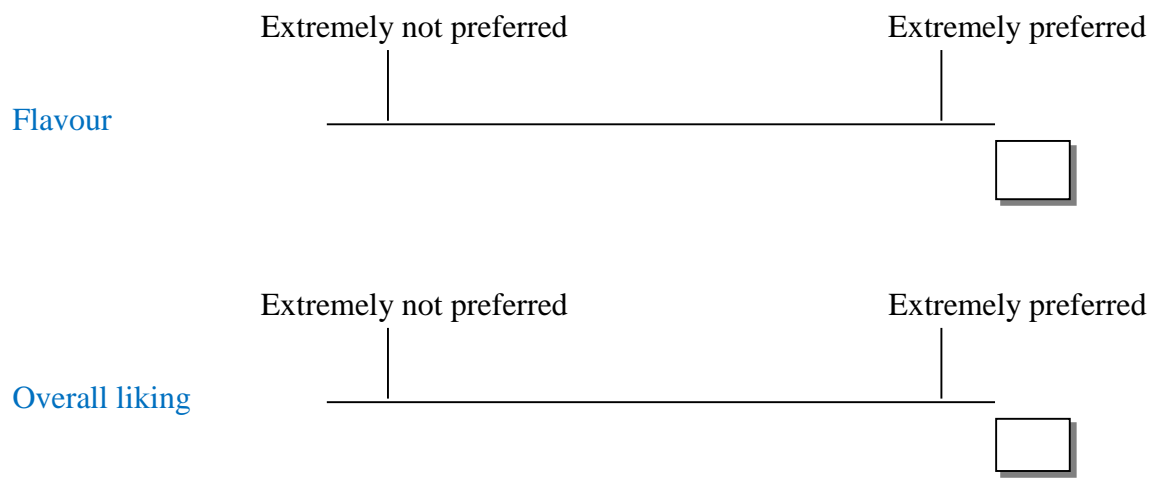
- ✓ Please rinse your mouth with the filtered water provided before every testing.
- ✓ Please rate the sample by clicking on the line scale given depending on the perceived preference
- ✓ Please take 30 seconds break after every testing.

- Example:



- Please rate the sample corresponding the 3-digits sample code





## 2. Instruction and questionnaires for projective mapping

### ➤ **Projective mapping section**

#### ❖ Instruction:

- ✓ Please position those samples in the map according to their similarities.
- ✓ Please rinse your mouth with the filtered water provided before every testing.
- ✓ Please take 30 seconds break after every testing.



### 3. Letter of Approval from AUT Ethics committee (AUTEC)



A U T E C  
S E C R E T A R I A T

29 October 2014

Michelle Yoo  
Faculty of Health and Environmental Sciences

Dear Michelle

Re Ethics Application: **14/308 Sensory test of bresaola products (a series of air dried meat products made from beef, lamb, mutton and veal).**

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

Your ethics application has been approved for three years until 29 October 2017.

As part of the ethics approval process, you are required to submit the following to AUTEC:

- A brief annual progress report using form EA2, which is available online through <http://www.aut.ac.nz/researchethics>. When necessary this form may also be used to request an extension of the approval at least one month prior to its expiry on 29 October 2017;
- A brief report on the status of the project using form EA3, which is available online through <http://www.aut.ac.nz/researchethics>. This report is to be submitted either when the approval expires on 29 October 2017 or on completion of the project.

It is a condition of approval that AUTEC is notified of any adverse events or if the research does not commence. AUTEC approval needs to be sought for any alteration to the research, including any alteration of or addition to any documents that are provided to participants. You are responsible for ensuring that research undertaken under this approval occurs within the parameters outlined in the approved application.

AUTEC grants ethical approval only. If you require management approval from an institution or organisation for your research, then you will need to obtain this.

To enable us to provide you with efficient service, please use the application number and study title in all correspondence with us. If you have any enquiries about this application, or anything else, please do contact us at [ethics@aut.ac.nz](mailto:ethics@aut.ac.nz).

All the very best with your research,

A handwritten signature in black ink, appearing to read 'Kate O'Connor'.

Kate O'Connor  
Executive Secretary

**Auckland University of Technology Ethics Committee**

Cc: Renyu Zhang [zhangrenyu01@126.com](mailto:zhangrenyu01@126.com)

#### 4. Enzyme activity protocol for $\alpha$ -Amylase (EC 3.2.1.1)

Based from SSSTAR01. Refer to CR SOP DEK ENZ

### 1. OBJECTIVE

To standardize a procedure for determining the enzymatic activity of  $\alpha$ -Amylase.

### 2. SCOPE

This procedure applies to all products that have a specification for  $\alpha$ -amylase.

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### 3. DEFINITIONS

3.1 Purified Water - water from a deionizing system, resistivity  $\geq 18\text{M}\Omega\cdot\text{cm}$  @ 25°C

3.2. Unit Definition - One unit will liberate 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20°C.

3.3. STD = Maltose Standard

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### 4. DISCUSSION

Starch +  $\text{H}_2\text{O}$   $\xrightarrow{\alpha\text{-Amylase}}$  Reducing Groups (Maltose)

### 5. RESPONSIBILITIES

It is the responsibility of trained Analytical Services laboratory personnel to follow this procedure as written.

### 6. SAFETY

Refer to Material Safety Data Sheets (MSDS) for hazards and appropriate handling precautions.

### 7. PROCEDURE

#### 7.1 CONDITIONS:

T = 20°C, pH = 6.9,  $A_{540\text{nm}}$ , Light path = 1 cm

#### 7.2 METHOD:

Spectrophotometric Stop Reaction

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#### 7.3 REAGENTS:

7.3.1 20 mM Sodium Phosphate Buffer with 6.7 mM Sodium Chloride, pH 6.9 at 20°C (**Buffer**)

Prepare 100 ml in purified water using Sodium Phosphate, Monobasic, Anhydrous, Sigma-Aldrich Product Number S0751 and Sodium Chloride, Sigma Product No S9888. Adjust to pH 6.9 at 20°C with 1 M NaOH.

7.3.2 1.0% (w/v) Soluble Starch Solution (**Starch**)

Prepare 25 ml in Reagent 7.3.1 using Starch Potato Soluble, Sigma-Aldrich Product Number S2630. Facilitate solubilization by heating the starch solution in a glass beaker directly on a heating/stir plate using constant stirring. Bring to boil and maintain the solution at this temperature for 15 minutes. Allow the starch solution to cool to room

temperature with stirring. Return the starch solution to its original volume (25 ml) by the addition of purified water and dispense aliquots for assay with stirring.

### 7.3.3 Sodium Potassium Tartrate Solution

Dissolve 12.0 g of Sodium Potassium Tartrate, Tetrahydrate, Sigma-Aldrich Product Number S2377, in previously heated 8.0 ml of 2 M NaOH, 50°C - 70°C. Heat directly on a heating/stir plate with constant stirring to dissolve. **DO NOT BOIL.**

### 7.3.4 96 mM 3,5-Dinitrosalicylic Acid Solution

Prepare 20 ml in purified water, 50°C - 70°C, using 3,5-Dinitrosalicylic Acid, Sigma-Aldrich Product Number D0550. Heat directly on a heating/stir plate with constant stirring to dissolve. **DO NOT BOIL.**

### 7.3.5. Color Reagent Solution (**Clr Rgt Soln**)

To 12 ml of purified water, 50°C - 70°C, slowly add Reagent 7.3.3 followed by Reagent 7.3.4. If not completely dissolved, the reagents should dissolve when mixed. The solution should be stored in an amber bottle at room temperature. The Color Reagent Solution is stable for 6 months.

### 7.3.6 0.2% (w/v) Maltose Standard (**STD**)

Prepare 10 ml in purified water using Maltose, Monohydrate, Sigma-Aldrich Product Number M5885 .

### 7.3.7 $\alpha$ -Amylase Solution (**Enzyme**)

7.3.7.1 Immediately before use, prepare a solution containing 1 unit/ml of  $\alpha$ - Amylase in 20°C purified water.

7.3.7.2 If test enzyme requires a dilution scheme, the first dilution and subsequent dilutions should be in cold purified water until the last dilution, and the last dilution should be in 20°C purified water.

## 7.4 ASSAY

7.4.1 Pipette (in milliliters) the following reagents into suitable containers:

	<u>Test1</u>	<u>Test2</u>	<u>Test3</u>	<u>Blank</u>
Reagent 7.3.2 (Starch)	1.00	1.00	1.00	1.00

7.4.2 Mix by swirling and equilibrate to 20°C. Then add:

Reagent 7.3.7 (Enzyme)	0.50	0.70	1.00	-- --
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7.4.3 Mix by swirling and incubate for exactly 3.0 minutes at 20°C. Then add:

Reagent 7.3.5	1.00	1.00	1.00	1.00
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(Clr Rgt Soln)
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7.4.4 Cap with a vented cap and place in a boiling water bath. The add:

Reagent			--	
7.3.7	0.50	0.30	--	1.00
(Enzyme)			-	

7.4.5 Boil for exactly 15 minutes, then cool on ice to room temperature, approximately 3 minutes, and add:

7.4.6

Purified water	9.00	9.00	9.00	9.00
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7.4.7 Mix by inversion and record the  $A_{540\text{nm}}$  for both the Test and Blank using a suitable spectrophotometer.

7.4.8 Due to the short enzymatic incubation time of three minutes, each test lot must be run one at a time.

7.5. Standard Curve:

7.5.1 A standard curve is made by pipetting (in milliliters) the following reagents into suitable containers:

	<u>Std</u> <u>1</u>	<u>Std</u> <u>2</u>	<u>Std</u> <u>3</u>	<u>Std</u> <u>4</u>	<u>Std</u> <u>5</u>	<u>Std</u> <u>6</u>	<u>Std</u> <u>7</u>	<u>Std</u> <u>Blank</u>
Reagent 7.3.6 (STD)	0.0 5	0.2 0	0.4 0	0.6 0	0.8 0	1.0 0	2.0 0	-----
Purified Water	1.9 5	1.8 0	1.6 0	1.4 0	1.2 0	1.0 0	----- -	2.00
Reagent 7.3.5 (Clr Rgt Soln)	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0	1.00

7.5.2 Place in a boiling water bath for exactly 15 minutes, then cool on ice to room temperature and add:

Purified water	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
	0	0	0	0	0	0	0	0

7.5.3 Mix by inversion and record the A<sub>540nm</sub> for the Standards and Standard Blank using a suitable spectrophotometer.

## 7.6 CALCULATIONS

### 7.6.1 Standard Curve

$$\Delta A_{540nm} \text{ Standard} = A_{540nm} \text{ Std} - A_{540nm} \text{ Std Blank}$$

Plot the  $\Delta A_{540nm}$  of the Standards vs milligrams of Maltose. Calculate and record the slope, y-intercept, and linear regression(r-square).

### 7.6.2 Sample Determination

$$\Delta A_{540nm} \text{ Sample} = A_{540nm} \text{ Test} - A_{540nm} \text{ Test Blank}$$

Determine the milligrams of Maltose liberated using the Standard Curve.

7.6.3	Units/Ml enzyme =	<u>mg of Maltose released(df)</u> (1)
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df = Dilution Factor

1 = Volume (in milliliter) of enzyme used

7.6.4	Units/mg solid =	<u>units/mL enzyme</u> mg solid/mL enzyme
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7.6.5	Units/mg protein =	<u>units/mL enzyme</u> mg protein/mL enzyme
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## 7.7 FINAL ASSAY CONCENTRATION

In a 2.00 ml reaction mix, the final concentrations are 8 mM sodium acetate, 0.50% (w/v) starch and 1 unit  $\beta$ -amylase.

## 5. Enzyme activity protocol for Pepsin (EC 3.4.23.1)

### Description

This procedure may be used for determination of Pepsin activity using hemoglobin as the substrate. It is a spectrophotometric stop rate determination.

Unit Definition: One unit of Pepsin will produce a  $\Delta A_{280}$  of 0.001 per minute at pH 2.0 at 37 °C measured as trichloroacetic acid (TCA)-soluble products using hemoglobin as the substrate. (Final volume = 16 ml, light path = 1 cm.)

### Precautions

Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

### Reagents and Equipment Required

1.0 M Hydrochloric Acid (Catalog No. 318949)

Hemoglobin from bovine blood (Catalog No. H2625)

6.1 N [ $\sim$ 100% (w/v)] Trichloroacetic acid solution (Catalog No. T0699)

### Preparation Instructions

Use ultrapure water ( $\geq 18 \text{ M}\Omega\text{cm}$  resistivity at 25 °C) for the preparation of reagents.

10 mM HCl (Hydrochloric Acid)

– Dilute 1.0 M Hydrochloric Acid (Catalog No. 318949) 100-fold with ultrapure water.

2.5% (w/v) Hemoglobin Stock Solution

– Prepare a 25 mg/ml solution in ultrapure water using Hemoglobin from bovine blood (Catalog No. H2625). Stir vigorously, creating a vortex, for a minimum of 10 minutes at 37 °C. Then filter through a polypropylene column with a coarse filter (90–130  $\mu\text{m}$ ).

Substrate [2.0% (w/v) Hemoglobin solution]

– Transfer 80 ml of the filtered 2.5% (w/v) Hemoglobin Stock Solution into a suitable container. Adjust the pH of this solution to 2.0 at 37 °C using 5 M HCl. Bring the final volume to 100 ml with ultrapure water.

TCA Solution [5% (w/v) Trichloroacetic Acid]

– Dilute 6.1 N [~100% (w/v)] 6.1 N [~100% (w/v)] Trichloroacetic acid solution (Catalog No. T0699) 20-fold with ultrapure water.

Enzyme Solution (Pepsin)

– Prepare a 1 mg/ml stock solution in cold (2–8 °C) 10 mM HCl. If insoluble material is present, allow the stock solution to sit on ice until dissolved. Allow up to 1 hour for the Pepsin to completely dissolve. When the Pepsin has dissolved or after 1 hour, whichever occurs first, dilute the 1 mg/ml stock solution to 0.01–0.05 mg/ml in cold 10 mM HCl.

## Procedure

1. Pipette the Substrate into suitable glass vials.

Reagent	Blank (ml)	Test (ml)
Substrate	5.00	5.00

2. Place vials in a thermostatted water bath and equilibrate to 37 °C for ~10 minutes, then add:

Reagent	Blank (ml)	Test (ml)
Enzyme Solution	–	1.00

3. Mix by swirling and incubate at 37 °C for *exactly* 10 minutes, then add:

Reagent	Blank (ml)	Test (ml)
TCA Solution	10.0	10.0
Enzyme Solution	1.00	–

4. Mix by swirling and incubate at 37 °C for an additional 5 minutes.

5. Filter the Blank and Test mixtures through a 0.45 µm syringe filter. Record the A<sub>280</sub> versus air for each filtered solution for each vial.

## Results

### Calculations

$$\text{Units/ml enzyme} = \frac{(A_{280} \text{ Test} - A_{280} \text{ Blank}) (df)}{(10) (1.0) (0.001)}$$

where:

df = Dilution factor

10 = Assay incubation time in minutes

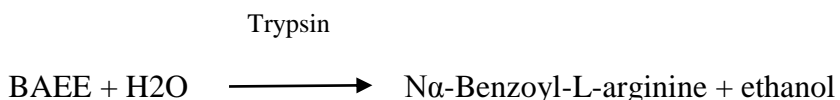
1.0 = Volume of Enzyme Solution (ml) added

0.001 =  $\Delta A_{280}$  per unit of Pepsin per unit definition

## 6. Enzyme activity protocol for Trypsin (EC 3.4.21.4)

### Description

This procedure is for products with a specification for Trypsin activity using N $\alpha$ -Benzoyl-L-arginine ethyl ester (BAEE) as a substrate. The procedure is a continuous spectrophotometric rate determination ( $A_{253}$ , Light path = 1 cm) based on the following reaction:



where:

BAEE – N $\alpha$ -Benzoyl-L-arginine ethyl ester

Unit Definition – One BAEE unit of trypsin activity will produce a  $\Delta A_{253}$  of 0.001 per minute with BAEE as substrate at pH 7.6 at 25 °C in a reaction volume of 3.20 ml.

### Precautions

Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

### Reagents and Equipment Required

Sodium phosphate, monobasic (Catalog No. S0751)

N $\alpha$ -Benzoyl-L-arginine ethyl ester (BAEE, Catalog No. B4500)

1 M Hydrochloric acid (Catalog No. 318949)

### Preparation Instructions

Use ultrapure water ( $\geq 18 \text{ M}\Omega\text{cm}$  resistivity at 25 °C) for the preparation of reagents.

Buffer (67 mM Sodium Phosphate Buffer, pH 7.6 at 25 °C) – Prepare a 8.04 mg/ml solution using sodium phosphate, monobasic (Catalog No. S0751) in ultrapure water. Adjust to pH 7.6 at 25 °C with 1 M NaOH solution.

Substrate Solution (0.25 mM N $\alpha$ -Benzoyl-L-arginine ethyl ester) – Prepare a 0.086 mg/ml solution using N $\alpha$ -Benzoyl-L-arginine ethyl ester (BAEE, Catalog No. B4500) in Buffer.

HCl Solution (1 mM Hydrochloric Acid) – Prepare a 1,000-fold dilution of 1 M Hydrochloric acid solution (Catalog No. 318949) in ultrapure water.

Enzyme Solution (Trypsin) – Immediately before use, prepare a solution containing 425-575 units/ml of Trypsin in cold (2-8 °C) HCl Solution.

### Procedure

In a 3.20 ml reaction mix, the final concentrations are 62.8 mM sodium phosphate, 0.23 mM N $\alpha$ -Benzoyl- L-arginine ethyl ester, 0.031-0.063 mM hydrochloric acid, 42.5-115.0 units of trypsin.

1. Pipette the following reagents into suitable quartz cuvettes:

Reagent	Blank (ml)	Test (ml)
Substrate Solution	3.00	3.00
HCl Solution	0.200	0.125

2. Mix by inversion and equilibrate to 25 °C using a suitably thermostatted spectrophotometer. Then add:

Reagent	Blank (ml)	Test (ml)
Enzyme Solution	–	0.075

Note: Final volume in each cuvette must be 3.2 ml per unit definition.

3. Immediately mix by inversion and record the increase in A<sub>253</sub> for 5 minutes. Using a 1 minute time period and a minimum of 4 data points, obtain the  $\Delta A_{253}/\text{minute}$  using the maximum linear rate for both the Blank and Test.

## Results

### Calculations

1.

$$\text{BAEE units/ml enzyme} = \frac{(\Delta A_{253}/\text{minute Test} - \Delta A_{253}/\text{minute Blank}) \times (\text{df})}{(0.001) \times (0.075)}$$

Where:

df = dilution factor

0.001 = The change in  $A_{253}$ /minute based on unit definition

0.075 = volume (ml) of test sample used in assay

Note: The total volume in the cuvette is not used in the calculation since the unit definition is based on 3.2 ml.

2.

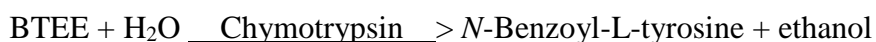
$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$



## 7. Enzyme activity protocol for Chymotrypsin

### Description

This procedure is for products with a specification for Chymotrypsin activity. It is not to be used to assay Insoluble Chymotrypsin such as Catalog No. C9134. The procedure is a continuous spectrophotometric rate determination ( $A_{256}$ , Light path = 1 cm) based on the following reaction:



where:

BTEE – *N*-Benzoyl-L-tyrosine ethyl ester

Unit Definition – One unit of chymotrypsin will hydrolyze 1.0  $\mu\text{mole}$  of BTEE per minute at pH 7.8 at 25 °C.

### Precautions

Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

### Reagents and Equipment Required

Trizma<sup>®</sup> Base (Catalog No. T1503)

*N*-Benzoyl-L-Tyrosine Ethyl Ester (Catalog No. B6125)

Methanol (Catalog No. M1775)

Calcium chloride, dihydrate (Catalog No. C3881)

Hydrochloric acid solution (Catalog No. 318949)

### Preparation Instructions

Use ultrapure water ( $\geq 18 \text{ M}\Omega\text{cm}$  resistivity at 25 °C) for the preparation of reagents.

Buffer (80 mM Tris HCl Buffer, pH 7.8 at 25 °C)

- Prepare a 9.69 mg/ml solution in ultrapure water using Trizma<sup>®</sup> Base (Catalog No. T1503). Adjust the pH of this solution to 7.8 at 25 °C.

#### BTEE Solution (1.18 mM *N*-Benzoyl-L-Tyrosine Ethyl Ester)

- Weigh 37 mg of *N*-Benzoyl-L-Tyrosine Ethyl Ester (Catalog No. B6125) into a 100 ml Class A volumetric flask. Add 63.4 ml of Methanol (Catalog No. M1775) and mix by swirling. Bring the final volume of the solution to 100 ml using ultrapure water. Invert the flask several times to ensure complete mixing.

#### CaCl<sub>2</sub> Solution (2 M Calcium Chloride)

- Prepare a 294 mg/ml solution in ultrapure water using Calcium chloride, dihydrate (Catalog No. C3881).

#### HCl Solution (1 mM Hydrochloric Acid)

- Prepare a solution by diluting 0.10 ml of 1.0 M Hydrochloric acid solution (Catalog No. 318949) to 100 ml with ultrapure water in a 100 ml Class A volumetric flask. Mix by inversion and place on ice.

#### Enzyme Solution (Chymotrypsin)

- Immediately before use, prepare a solution containing 2-5 chymotrypsin units per milliliter in cold (2-8 °C) HCl Solution.

## Procedure

In a 3.00 ml reaction mix, the final concentrations are 38 mM Tris, 0.55 mM *N*-Benzoyl-L-Tyrosine Ethyl Ester, 30% (v/v) Methanol, 53 mM Calcium Chloride, 0.03 mM Hydrochloric Acid, and 0.2-0.5 units of Chymotrypsin.

1. Pipette the following reagents into suitable quartz cuvettes:

Reagent	Blank (ml)	Test (ml)
Buffer	1.42	1.42
BTEE Solution	1.40	1.40
CaCl <sub>2</sub> Solution	0.08	0.08

2. Mix by inversion and equilibrate to 25 °C using a suitably thermostatted spectrophotometer.

3. Add the following to the cuvettes:

Reagent	Blank (ml)	Test (ml)
HCl Solution	0.10	–
Enzyme Solution	–	0.10

4. Immediately mix by inversion and record the increase in  $A_{256}$  for 3-5 minutes.
5. Obtain the  $\Delta A_{256}/\text{minute}$  for both the blank and test reactions using the maximum linear rate over a one minute interval using at least 4 points.

## Results

### Calculations

1.

$$\text{Units/ml enzyme} = \frac{(\Delta A_{256}/\text{minute Test} - \Delta A_{256}/\text{minute Blank}) \times (3) \times (\text{df})}{(0.964) \times (0.10)}$$

Where:

3 = volume (ml) of reaction mix

df = dilution factor

0.964 = millimolar extinction coefficient of BTEE at 256 nm

0.10 = volume (ml) of test sample used in assay

2.

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

## 8. Reagents and procedures of EZ: Faast amino acids kit (GC-FID)

### 1.1 Reagents

Reagent	Ingredients	Volume
<b>Reagent 1</b> Internal Standard Solution	Norvaline 0.2 mM N-propanol 10%	50mL
<b>Reagent 2</b> Washing Solution	N-propanol	90mL
<b>Reagent 3A</b> Eluting Medium Component I	Sodium Hydroxide	60mL
<b>Reagent 3B</b> Eluting Medium Component II	N-propanol	40mL
<b>Reagent 4</b> Organic Solution I	Chloroform	4 vials, 6mL each
<b>Reagent 5</b> Organic Solution II	Iso-octane	50mL
<b>Reagent 6</b> Acid Solution	Hydrochloric Acid 1N	50mL
<b>SD 1, 2, &amp; 3</b> 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 recommended 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.



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.

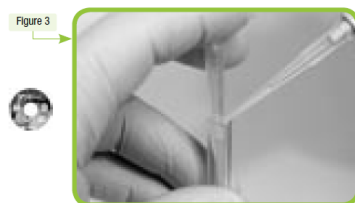
4. Pipette 200µL Reagent 2 (Washing Solution) into the same sample preparation vial. Pass the solution SLOWLY through the sorbent tip and into the syringe barrel. Drain the liquid from the sorbent bed by pulling air through the sorbent tip. Detach the sorbent tip, and leave it in the sample preparation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



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.

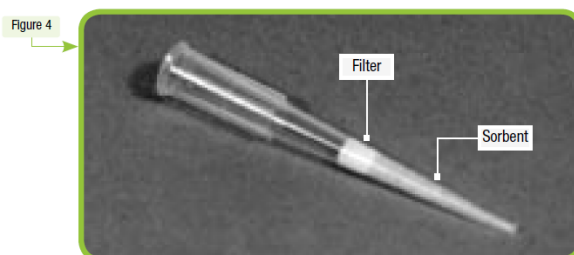
**Caution:** Avoid cross-contamination by not touching the inner wall of the sample vial with the tip of the Microdispenser. The piston will ensure proper transfer of liquids into the vial without the need of touching the vial wall. Use the same Microdispenser with both Reagents 4 and 5. There is no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

### 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.



**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.

## 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 <b>constant flow</b>
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:

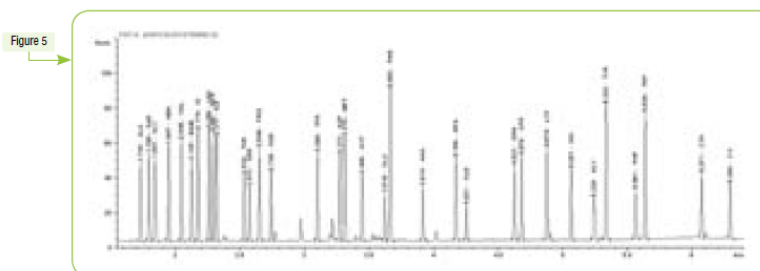
AAA	ASP	GLY	LEU	PHE	THR
ABA	BAIB	HIS	LYS	PRO	TYR
alILE	C-C	HYP	MET	SAR	VAL
ALA	GLU	ILE	ORN	SER	

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
-----	-----	-----	-----	-----	-----



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.

## 9. The RV coefficient between projective maps and multifactor analysis

**Table 16 RV coefficient between projective maps and multifactor analysis (MFA) for week one where 86% of panellists scored > 0.5. Panellists are identified as N1 to N15. Values shown in red indicate poor fit with MFA.**

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	MFA
N1	1.000	0.490	0.796	0.092	0.742	0.424	0.891	0.524	0.502	0.629	0.719	0.585	0.644	0.579	0.231	0.880
N2	0.490	1.000	0.092	0.407	0.186	0.299	0.489	0.063	0.323	0.585	0.370	0.606	0.523	0.194	0.254	0.576
N3	0.796	0.092	1.000	0.177	0.638	0.631	0.838	0.494	0.485	0.350	0.824	0.344	0.584	0.807	0.227	0.826
N4	0.092	0.407	0.177	1.000	0.037	0.723	0.177	0.167	0.132	0.240	0.569	0.053	0.595	0.432	0.299	0.489
N5	0.742	0.186	0.638	0.037	1.000	0.140	0.595	0.879	0.601	0.518	0.487	0.266	0.346	0.358	0.249	0.700
N6	0.424	0.299	0.631	0.723	0.140	1.000	0.573	0.162	0.140	0.133	0.784	0.257	0.674	0.881	0.121	0.680
N7	0.891	0.489	0.838	0.177	0.595	0.573	1.000	0.312	0.290	0.414	0.828	0.689	0.639	0.768	0.160	0.858
N8	0.524	0.063	0.494	0.167	0.879	0.162	0.312	1.000	0.677	0.363	0.399	0.056	0.418	0.205	0.144	0.584
N9	0.502	0.323	0.485	0.132	0.601	0.140	0.290	0.677	1.000	0.679	0.326	0.010	0.373	0.145	0.414	0.615
N10	0.629	0.585	0.350	0.240	0.518	0.133	0.414	0.363	0.679	1.000	0.395	0.053	0.356	0.176	0.375	0.624
N11	0.719	0.370	0.824	0.569	0.487	0.784	0.828	0.399	0.326	0.395	1.000	0.376	0.863	0.799	0.090	0.872
N12	0.585	0.606	0.344	0.053	0.266	0.257	0.689	0.056	0.010	0.053	0.376	1.000	0.443	0.299	0.258	0.518
N13	0.644	0.523	0.584	0.595	0.346	0.674	0.639	0.418	0.373	0.356	0.863	0.443	1.000	0.509	0.045	0.791
N14	0.579	0.194	0.807	0.432	0.358	0.881	0.768	0.205	0.145	0.176	0.799	0.299	0.509	1.000	0.111	0.716
N15	0.231	0.254	0.227	0.299	0.249	0.121	0.160	0.144	0.414	0.375	0.090	0.258	0.045	0.111	1.000	0.387
MFA	0.880	0.576	0.826	0.489	0.700	0.680	0.858	0.584	0.615	0.624	0.872	0.518	0.791	0.716	0.387	1.000

**Table 17 RV coefficient between projective maps and multifactor analysis (MFA) for week three where 86% of panellists scored > 0.5. Panellists are identified as N1 to N15. Values shown in red indicate poor fit with MFA.**

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	MFA
N1	1.000	0.529	0.592	0.644	0.012	0.197	0.433	0.336	0.040	0.268	0.203	0.589	0.517	0.454	0.850	0.689
N2	0.529	1.000	0.593	0.470	0.665	0.589	0.067	0.275	0.152	0.098	0.576	0.631	0.530	0.282	0.571	0.733
N3	0.592	0.593	1.000	0.515	0.268	0.131	0.207	0.161	0.545	0.379	0.201	0.747	0.523	0.556	0.574	0.728
N4	0.644	0.470	0.515	1.000	0.046	0.337	0.354	0.112	0.037	0.058	0.338	0.731	0.312	0.285	0.856	0.629
N5	0.012	0.665	0.268	0.046	1.000	0.554	0.065	0.429	0.095	0.144	0.631	0.056	0.278	0.265	0.147	0.490
N6	0.197	0.589	0.131	0.337	0.554	1.000	0.409	0.383	0.071	0.178	0.792	0.297	0.146	0.181	0.272	0.576
N7	0.433	0.067	0.207	0.354	0.065	0.409	1.000	0.760	0.353	0.701	0.563	0.239	0.184	0.505	0.359	0.644
N8	0.336	0.275	0.161	0.112	0.429	0.383	0.760	1.000	0.356	0.605	0.508	0.205	0.540	0.751	0.196	0.691
N9	0.040	0.152	0.545	0.037	0.095	0.071	0.353	0.356	1.000	0.734	0.066	0.405	0.148	0.477	0.048	0.470
N10	0.268	0.098	0.379	0.058	0.144	0.178	0.701	0.605	0.734	1.000	0.187	0.119	0.120	0.450	0.134	0.534
N11	0.203	0.576	0.201	0.338	0.631	0.792	0.563	0.508	0.066	0.187	1.000	0.285	0.139	0.179	0.343	0.632
N12	0.589	0.631	0.747	0.731	0.056	0.297	0.239	0.205	0.405	0.119	0.285	1.000	0.646	0.542	0.614	0.740
N13	0.517	0.530	0.523	0.312	0.278	0.146	0.184	0.540	0.148	0.120	0.139	0.646	1.000	0.806	0.272	0.645
N14	0.454	0.282	0.556	0.285	0.265	0.181	0.505	0.751	0.477	0.450	0.179	0.542	0.806	1.000	0.286	0.732
N15	0.850	0.571	0.574	0.856	0.147	0.272	0.359	0.196	0.048	0.134	0.343	0.614	0.272	0.286	1.000	0.674
MFA	0.689	0.733	0.728	0.629	0.490	0.576	0.644	0.691	0.470	0.534	0.632	0.740	0.645	0.732	0.674	1.000

**Table 18 RV coefficient between projective maps and multifactor analysis (MFA) for week two where all of panellists scored > 0.5. Panellists are identified as N1 to N15.**

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	MFA
N1	1.000	0.144	0.407	0.335	0.139	0.212	0.050	0.119	0.118	0.429	0.381	0.579	0.740	0.385	0.181	0.541
N2	0.144	1.000	0.121	0.118	0.234	0.612	0.752	0.218	0.516	0.342	0.648	0.304	0.304	0.622	0.819	0.704
N3	0.407	0.121	1.000	0.937	0.218	0.478	0.073	0.598	0.265	0.258	0.172	0.448	0.584	0.143	0.185	0.584
N4	0.335	0.118	0.937	1.000	0.364	0.410	0.072	0.620	0.406	0.318	0.068	0.446	0.490	0.237	0.143	0.590
N5	0.139	0.234	0.218	0.364	1.000	0.066	0.662	0.519	0.853	0.620	0.290	0.576	0.133	0.304	0.045	0.623
N6	0.212	0.612	0.478	0.410	0.066	1.000	0.550	0.318	0.045	0.307	0.497	0.093	0.328	0.032	0.663	0.570
N7	0.050	0.752	0.073	0.072	0.662	0.550	1.000	0.427	0.631	0.533	0.686	0.373	0.185	0.313	0.604	0.721
N8	0.119	0.218	0.598	0.620	0.519	0.318	0.427	1.000	0.503	0.637	0.261	0.223	0.485	0.029	0.364	0.641
N9	0.118	0.516	0.265	0.406	0.853	0.045	0.631	0.503	1.000	0.580	0.351	0.638	0.183	0.599	0.222	0.717
N10	0.429	0.342	0.258	0.318	0.620	0.307	0.533	0.637	0.580	1.000	0.501	0.375	0.333	0.211	0.405	0.717
N11	0.381	0.648	0.172	0.068	0.290	0.497	0.686	0.261	0.351	0.501	1.000	0.613	0.236	0.440	0.671	0.720
N12	0.579	0.304	0.448	0.446	0.576	0.093	0.373	0.223	0.638	0.375	0.613	1.000	0.303	0.582	0.077	0.690
N13	0.740	0.304	0.584	0.490	0.133	0.328	0.185	0.485	0.183	0.333	0.236	0.303	1.000	0.384	0.443	0.621
N14	0.385	0.622	0.143	0.237	0.304	0.032	0.313	0.029	0.599	0.211	0.440	0.582	0.384	1.000	0.496	0.601
N15	0.181	0.819	0.185	0.143	0.045	0.663	0.604	0.364	0.222	0.405	0.671	0.077	0.443	0.496	1.000	0.655
MFA	0.541	0.704	0.584	0.590	0.623	0.570	0.721	0.641	0.717	0.717	0.720	0.690	0.621	0.601	0.655	1.000