Final report

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AUSTRALIAN MEAT PROCESSOR CORPORATION

Kokumi flavour peptide production from beef offal co-products

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1.0 Executive summary

Offals are non-meat part co-products arising from meat processing/fabrication and are generated in large quantities daily. These co-products are considered low value products and pose a challenge to the industry to divert efforts into increasing their value to add to their business profit. While many of these co- products are edible for human consumption or pet food, they are generally processed to be used as animal feed, fertilizer or fuel. However, meat co-products are an excellent source of high nutritive value protein, minerals and vitamins and hence must be better diverted to contribute to alleviating the increasing global demand for protein. Most importantly, by adding value to them we can improve meat industry sustainability and profitability.

As a part of AgResearch internal SSIF- funded work (PRJ0416620, Objective 3: Flavour Creations), we optimised our enzymatic method to produce food-grade kokumi flavour rich extract for human consumption from a low value meat by-product, i.e. mechanically deboned meat. Hence, we hypothesized that our method can be applied to these meat offal co-products to produce kokumi extract for human consumption and add significant value to the business. We proposed a proof-of-concept study to investigate suitability of beef offal co-products liver and lung to produce a kokumi flavour rich extract enriched with kokumi taste substances i.e. Ɣ-glutamyl peptides and specific free amino acids.

Subsequently, identification and quantification of kokumi Ɣ-glutamyl di-peptides, free amino acids (FAA) were performed through mass spectrometry and HPLC, respectively. Furthermore, to compare the biological intensity of kokumi tastants in both extracts, we screened these against the kokumi tastant detecting receptor from the human tongue by performing *in vitro* cell based assays using the kokumi taste receptor assay (also called the calcium sensing receptor; CaSR). CaSR is activated by all kokumi taste substances in our taste buds to transmit information to the brain to perceive distinct kokumi sensory attributes. To validate our findings of enrichment of kokumi peptides and intensity in our *in vitro* CaSR activation assay through to the human sensory experience, we performed a Flash Profile (FP) sensory trial on offal-kokumi extract supplemented beef patties.

A significant increase in kokumi-Ɣ-glutamyl peptides and FAA in both liver (Liver-G) and lung (Lung-G) kokumi extract was achieved after the enzymatic treatment, compared to their non enzymatic treated water extract (Liver-W and Lung-W) counterparts, respectively. Most importantly, enrichment of the tripeptide, γ-Glu-Val-Gly (EVG) was detected only in the lung-G extract. This tripeptide is widely reported as the most potent kokumi peptide in imparting desired kokumi flavour attributes in foods (Kuroda and Miyamura, 2015, Kuroda and Mizukoshi, 2024). To the best of our knowledge, the current study is the first to report enrichment of EVG in any offal extract samples through an enzymatic method.

The enrichment of kokumi flavour extract with Ɣ-glutamyl peptides was successfully detected in our CaSR assay where both Liver-G and Lung-G sourced extracts activated kokumi-receptors in a dose dependent manner compared with their control water extracts. Comparatively, lung-kokumi extract showed higher potency/strength than liver in that it only needed 121 µg/mL instead of 181 µg/mL (for liver) to produce half maximal receptor activation. Additionally, Lung-G showed slightly higher efficacy (maximal ability to activate CaSR) compared to

Liver-G. The presence of EVG in Lung-G samples must have contributed to the stronger activation in comparison to Liver-G samples. Based on this information, when added to real food, Lung-G is predicted to produce a stronger kokumi sensation and more desirable flavour at a lower dose than Liver-G.

To validate our *in vitro* findings, the FP sensory method was employed as a rapid descriptive sensory analysis to identify flavour attributes of five beef patties incorporated with liver and lung kokumi rich extracts. The five patties were: non-supplemented beef patty control (control), patty with kokumi extract of liver (Liver-G) and patty with kokumi extract of lung (Lung-G), and their patties with their respective first step protease hydrolysates (Liver-P & Lung-P). In the FP trial, 7 sensory participants were asked to individually: (1) generate 5-6 attributes they felt best described the sensory properties of the 5 patties, and (2) rank the five patties in terms of intensity of each attribute they generated. The FP results showed the control patties were different from liver and lung protease extract as well as kokumi extract suggesting modification of flavour attributes. Notably, Lung-G supplemented patty was found to be different to Lung-P with enhanced and more intense desirable flavour attributes of juiciness and saltiness, indicating a strong effect of enriched kokumi substances. Conversely, Liver-G patties were not found to different than Liver-P, and both were affected by undesirable attributes of offal, tripe, metallic and grassy.

Based on our data we conclude that our enzymatic method is applicable to both liver and lung to produce kokumi rich extract powder through enrichment in ϒ-glutamyl peptides and free amino acids. The finding that Lung-G showed stronger kokumi intensity with high potency and efficacy when compared with Liver-G is most likely attributed to its enrichment in EVG, which was not found in Liver-G. These results were reflected in the FP sensory trial in which Lung-G supplemented beef patty emerged as distinct, with rich desirable flavour attributes of juiciness and saltiness.

2.0 Introduction

The meat industry, despite its large scale of operation, is reported to be one of the least profitable industries around the world. The substantial amount of offal co-products generated during meat processing often represents the difference between profit and loss. Additionally, because of the risk of microbial contamination, they represent a threat to the environment and human health, if not disposed of or processed properly. Offal co-products including non-meat components such as blood and skin have been estimated to account for between 54 and 56%, respectively, of live animal weight for cattle and may contribute up to 11.4% of the gross income (Toldrá et al., 2016). Underutilisation of offal co-products is therefore a significant revenue loss to the industry. Hence, there is an urgent need to apply innovative ways to valorise offal co-products for higher value and better environmental outcomes.

Among the most utilized offals such as blood, heart, kidney, Liver, brains and spleen, reported to be rich in nutrients such as essential amino acids, vitamins and minerals (Mullen and Álvarez, 2016) others like lung are largely overlooked despite being rich in useful proteins like collagen. This may be because traditional markets are limited, and future market demand appears uncertain due to the consumer perception of offal with off flavours, food safety concerns, regulatory barriers, and cultural/demographic acceptance factors. Therefore, while proposing valorisation methods of converting low value offal into high value products, we need to work on improving its flavour, consumer desirability and acceptability.

Kokumi is an emerging flavour concept and hailed as a sixth taste in the realm of culinary arena worldwide (Ahmad 2020). Originating in Japan, kokumi means “rich taste” and intriguingly, a pure kokumi substance does not taste of anything by itself, but rather heightens the other tastes (savoury, sweet and salty) and prolongs their flavour when added to foods.

Kokumi substances consist of very small pieces of protein called ϒ-glutamyl di- and tri-peptides that activate the kokumi-receptor (calcium sensing receptor; CaSR) in our taste buds to evoke sensation of complexity and balance of different taste sensations to produce distinct sensory attributes of mouthfeel, long-lasting aftertaste, and heartiness (Ahmad and Dalziel, 2020). Noteworthy to mention among ϒ-glutamyl peptides, a tripeptide, EVG has been recognised as the most potent kokumi compound confirmed through both *in vitro* CaSR assay and food applications where it produced desired kokumi attributes for enhanced flavour (Kuroda and Mizukoshi, 2024).

Although, kokumi ϒ-glutamyl peptides are found in nature, their extraction and purification are expensive, and tend not to be food grade. At AgResearch, we have developed a cost-effective enzymatic method (Ahmad et al., 2023, Kim, 2024, Kim et al., 2022) to produce food grade kokumi flavour compound-rich extract from meat-based by-products which are food ready and able to elevate its flavour and positively alter sensory attributes.

Despite being a reliable source of protein, there has not been a single study or report using offal as a source for production of kokumi flavour extract. Hence, we proposed a proof-of-concept study to determine the suitability of two bovine offal co-products, liver and lung, to produce kokumi flavour compounds (proteins and amino acids). We used our two-stage enzymatic method to modify hydrolysed small proteins from liver and lung into kokumi specific ϒ-glutamyl peptides. We quantified ϒ-glutamyl di-peptides and free amino acids in extracts to compare between liver and lung in terms of enrichment for kokumi taste substances. Subsequently, liver and lung kokumi rich extract was tested for its kokumi flavour intensity through our biological *in vitro* cell based kokumi taste receptor assay by determining their potential to activate CaSR in a dose dependent manner.

To confirm our *in vitro* findings, we tested its flavour potential in food by adding it to beef patty and carrying out rapid sensory trial by using flash profiling approach. Flash Profile is a simple and rapid sensory descriptive method based on free choice of attributes, a comparative evaluation of the samples, and quantification by the means of ranks for each attribute (Delarue, 2015). It aims at providing quick access to the relative positioning of a set of products. It was best suited for our study as it can provide initial information on comparative assessment

of sensory attributes of different products. In FP trial 7 participants were asked to provide free choice of five attributes, a comparative evaluation of the samples, and quantification by the means of ranks for each attribute. Apart from non-supplemented beef patty control (control), we assessed kokumi extract of Liver (Liver-G) and Lung (Lung-G) against their respective first step protease hydrolysates (Live -P & Lung-P). This provided vital information on the ability of these protein hydrolysates in modifying food flavours. Flash Profiling was used to create a descriptive sensory profile of liver and lung kokumi rich extract in a real food application. For the beef patty sensory test, we oven dried liver and lung kokumi extract and added it to beef (1%) patties before cooking them at 175 °C for 15 minutes and serving warm to the participants.

3.0 Project objectives

1. Preparation and yield assessment of oven dried kokumi rich extract of offal co-products bovine liver and lungs.
2. Quantification of kokumi taste peptides and relevant amino acids in kokumi extract.
3. Determine the kokumi flavour activity through *in vitro* taste receptor assay.
4. Conduct an in-house sensory test to identify kokumi sensory attributes of kokumi extract incorporated meat patties.
5. Final report writing and discussion with AMPC.

4.0 Methodology

The enzymatic method for cost effective production of liver and lung kokumi extract is outlined in Figure 1 & Figure 2. The extraction and kokumi enrichment method were developed as a part of an AgResearch funded project and the kokumi intensity responses were tested using an *in vitro* kokumi taste receptor assay established at AgResearch (Kim et al., 2022). The two-stage method involved a combination of specific enzymes that convert small proteins of offals into kokumi rich extract containing kokumi flavour substances, i.e. Ƴ-glutamyl peptides. The flavour potential of oven dried kokumi rich extract of liver and lung was tested in 1% (w/w) supplemented minced beef patties and their subsequent flash profiling sensory trial.

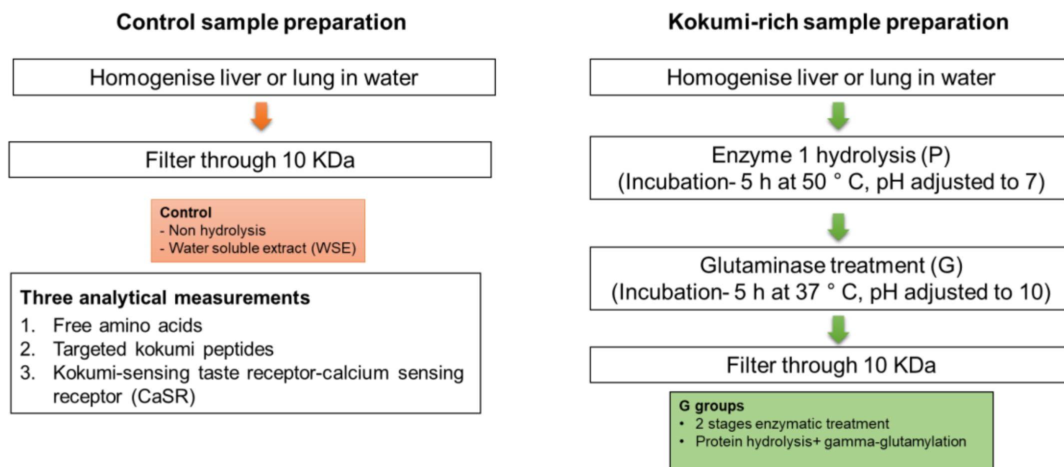

Figure 1. Sample preparation for control and offal kokumi extract from Liver and Lung.

4.1 Preparation of Liver and Lung kokumi rich extract and microbiological assessment for human consumption

Beef livers or lungs were minced, vacuum-packaged, and stored at -20 °C until use. Samples for analytical measurements were prepared in a non-food grade area (PC2 lab), while samples for sensory analysis were prepared in a food-grade product development laboratory. The thawed samples were homogenised with water (1:9 w/v) and then treated with a combination of two enzymes: 1) protease, for hydrolysing proteins into small proteins (amino acids and peptides), and 2) glutaminase, for reformulating the small proteins into kokumi-related peptides. After the two-stage enzyme processing, the samples for analytical measurements were filtered through a 10 kDa membrane, while the samples for sensory analysis were strained to remove visible particles and then dried in an oven at 60 °C. The yield of hydrolysates was calculated by determining the weight of the oven-dried hydrolysates as a percentage of total fresh weight of beef liver or lung used.

$$Dry\ matter\ yield\ (\%) = \frac{dry\ matter\ weight\ (g)\ \times\ 100}{\text{initial sample weight (g)}}$$

Microbiological tests were conducted for risk assessment for sensory analysis. The results showed that Bacillus cereus (<100 CFU/g), Escherichia coli (<3 CFU/g), Listeria monocytogenes (not detected), Salmonella species (not detected), and Staphylococcus aureus (<100 CFU/g) were within safe limits, indicating that the samples were food safe using this processing method.

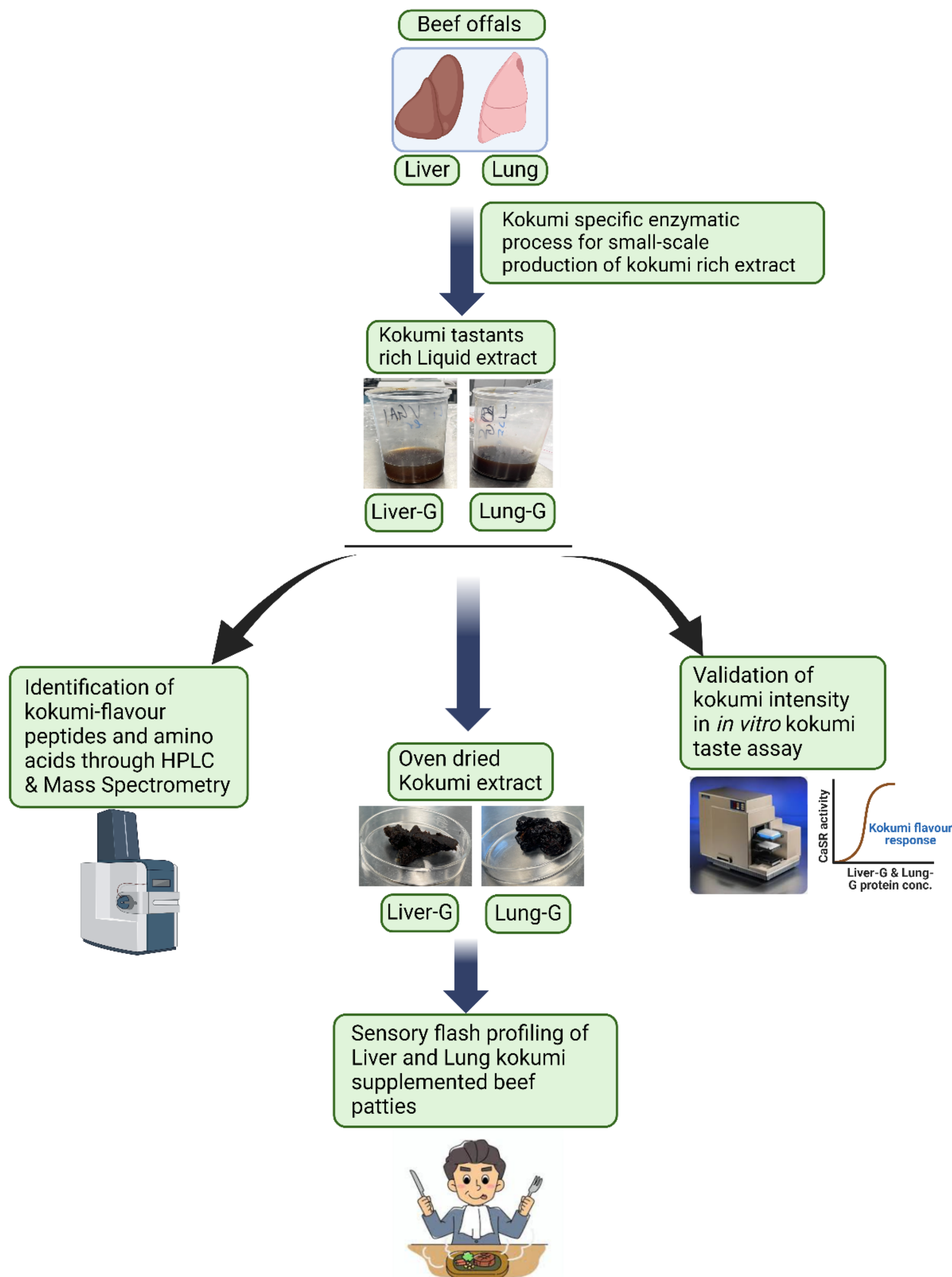

Figure 2. Schematic diagram showing research plan and methods employed for the project. Liver-G, kokumi peptide rich extract of liver; Lung-G, kokumi peptide rich extract of lung.

4.2 Quantitative analysis of free amino acids

The free amino acids in control water extract (W) and kokumi rich extracts of liver and lung were analysed based on the method published by our group (Kim, 2024, Kim et al., 2022).

Sample preparation: The soluble kokumi extracts of liver and lung were ultrafiltered through 10kDa molecular weight cut off filter and stored at -20 ˚C until analysed, then thawed & returned to ice. All sample preparations were performed by weight. 500 µl of vortexed sample was combined with 50 µl Nor-leucine internal standard (Sigma N1389, 10 µmol/ml in Lithium Loading Buffer pH 2.20) and 450 µl extraction solution containing 3.4 % 5-Sulfosalicylic acid. Samples were next vortexed for 10 seconds, then incubated on ice for 15 minutes, followed by 5 minute 10,000 Rcf centrifugation. The resultant pellet-free supernatant was removed via 45 micron syringe filter to HPLC vials for analysis.

The derivatised free amino acids were analysed by HPLC (LC-10AD VP, Shimadzu, Japan), 20Ai Ion Exchange Chromatography and Pickering Pinnacle PCX post-column derivatisation and UV/Vis detection (440/570nm) equipped with a PicoTag® column (3.9 mm × 300 mm, Waters Corporation, MA USA) according to the method described by White et al., (White et al., 1986). Analytical batches consisted of a blank, a mix of proteinogenic amino acid standards (Sigma AAS18, A0884, G3126), and a composite of physiological amino acid standards (Sigma A6407, A6282).

4.3 Relative quantitative analysis of γ-glutamyl peptides

The relative quantification of γ-glutamyl dipeptides and tripeptide EVG in water and kokumi rich extract of liver and lung was performed as published by our group (Kim, 2024, Kim et al., 2022). This involved extraction of peptides with methanol:water (1:1, v/v), reconstitution in acetonitrile:water (1:1, v/v) after drying, followed by separation, detection and semi-quantification using a ultra-high-performance liquid chromatography (UHPLC) system coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometer. An Ascentis Express HILIC UHPLC column (Sigma, USA) was used for peptide separation using a solvent gradient comprising 10 mM ammonium formate in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), at 400 µL/minute flow rate. Authentic standards of a dipeptide and glutathione were run to determine diagnostic fragments, and a full scan (m/z 55-1100) and multiple reaction monitoring (MRM) method was setup to detect γ-glutamyl dipeptides in negative ionization mode. Results were expressed as Log2 (sample peak area).

4.4 *In vitro* calcium sensing receptor activity assay to assess kokumi intensity

The methodology for the biological kokumi receptor assay, conducted using an *in vitro* CaSR assay to screen kokumi rich extracts of liver (Liver-G) and lung (Lung-G) after enzymatic treatment was performed as published previously (Ahmad et al., 2023, Kim, 2024, Kim et al., 2022). Non-enzymatic ground, homogenised and filtered water extract of liver (Liver-W) and lung (Lung-W) was used as a control. Total protein concentration was determined with a BCA assay kit (Thermo Fisher Scientific, MA USA). Mammalian stable cell lines CHO-K1-CaSR expressing human calcium sensing receptor and CHO-K1-Gα15 as a negative control (expressing Gα15 protein, but not CaSR) were purchased from Genscript, USA and cultured in Ham's F12, GlutamaxTM media (Gibco, TX, USA). The assay buffer was free of metal ions except 0.5mM $CaCl_2$. The cells were grown at 37°C

with 5% CO_2. CaSR activation was assessed by measuring intracellular calcium level with a Fluorescent Imaging Plate Reader assay (FLIPR, Molecular Devices, CA USA) as previously described (Kim et al. 2022, Ahmad et al., 2023). Briefly, when cells had reached 70–80% confluency, 100 µL of fluorescent calcium indicator dye (Molecular Devices, CA USA) was preloaded and incubated for 2 hr at 37 °C, and 5% CO_2 followed by the addition of serially diluted samples. Subsequently, fluorescence was recorded using a FlexStation® 3 Microplate Reader (Molecular Devices, CA, USA). The response was recorded as relative fluorescent unit (ΔRFU = maximum RFU – minimum RFU). To calculate EC50 of different dose response curves in the receptor assay, nonlinear regression analysis was performed using GraphPad Prism software (version 10). The EC50 was calculated by using the equation Y = 100*(X^HillSlope)/(EC50^HillSlope + (X^HillSlope)), where X and Y represent axis. CaSR activity was presented on a scale from 0 to 200 relative fluorescence units (ΔRFU) against concentration (mg/mL) of ultrafiltrates.

4.5. Sensory evaluation: Flash Profiling

Sensory evaluation was undertaken using a methodology known as Flash Profiling (Delarue, 2015). Flash Profiling is an established, quantitative method, used to describe/analyse the sensory properties of a set of food samples, in a single 60–90-minute session, with a small number of panellists (e.g. 5-8 people). The method is based on presenting panellists with food samples (in our case five beef patties, Figure 3), asking them to individually: (1) generate a list of sensory attributes that best describe and/or differentiate those samples, and (2) for each attribute they generate, rank those samples based on intensity. Multivariate statistics was then used to test if samples can be discriminated based on their sensory properties.

In this study, fresh minced beef (high fat content >18%) was purchased from a local grocery store. Five beef patties (i.e. five treatments) were prepared (Table 1): Control without extract, Lung-P (1% protein hydrolysate of lung), Lung-G (1% Ƴ-glutamyl peptide rich hydrolysate of lung), Liver-P (1% protein hydrolysate of liver), and Liver-G (1% Ƴ-glutamyl peptide rich hydrolysate of liver). The protein hydrolysate (P) group was prepared instead of the water-soluble fraction (W) to highlight the sensory differences between standard protein hydrolysis and our two-stage enzymatic method, as protein hydrolysis is commonly used to enhance flavour. Collecting the same mass of kokumi peptide rich G extract from water extract (W) is inefficient due to the low yield of W based on free amino acid concentrations. Liver-G showed 6-7 times higher and Lung-G showed 14-15 times higher concentrations than the W groups. Brine solution was prepared for each treatment. For the control, the brine (10% salt w/v) was used, and other treatments had an additional 10% of their extracts (w/v) added. The brine solution was mixed into the minced meat at 10% (w/w) that indicated 1% of salt was added, and then mixed using a mixer for 2 minutes. Each patty weighed 120 g and was stored in a chiller at 4 °C a day before sensory evaluation. The patties were cooked at 175 °C for 15 minutes until fully cooked. They were then wrapped in aluminium foil to keep them warmed during the session (which lasted 60 minutes) to ensure high food safety during consumption.

Seven panellists (m=5, f=2), aged 30-65 years, participated in the Flash Profiling session (Figure 3). All panellists had prior experience in sensory evaluation and had been working within the food science research group at AgResearch for at least 3 years. Each panellist received the five cooked beef patties at the same time, presented in a unique order, labelled by random three-digit codes. A knife and fork were provided to assist with evaluating the samples. Panellists were first asked to taste the patties in the order they were presented and write down 5-6 sensory attributes they feel best described and differentiated the samples. They were told the attributes could be taste, flavour, or texture attributes, but that hedonic (i.e. liking) attributes were not allowed. Panellists were then asked to rank the patties in terms of intensity (from weakest on left, to strongest on the right) for each attribute they chose. They did this at their own pace, retasting the samples as often as they needed. The session lasted approximately 60 minutes in total. The Flash profiling questionnaire each panellist was required to complete can be found in Appendix 1.

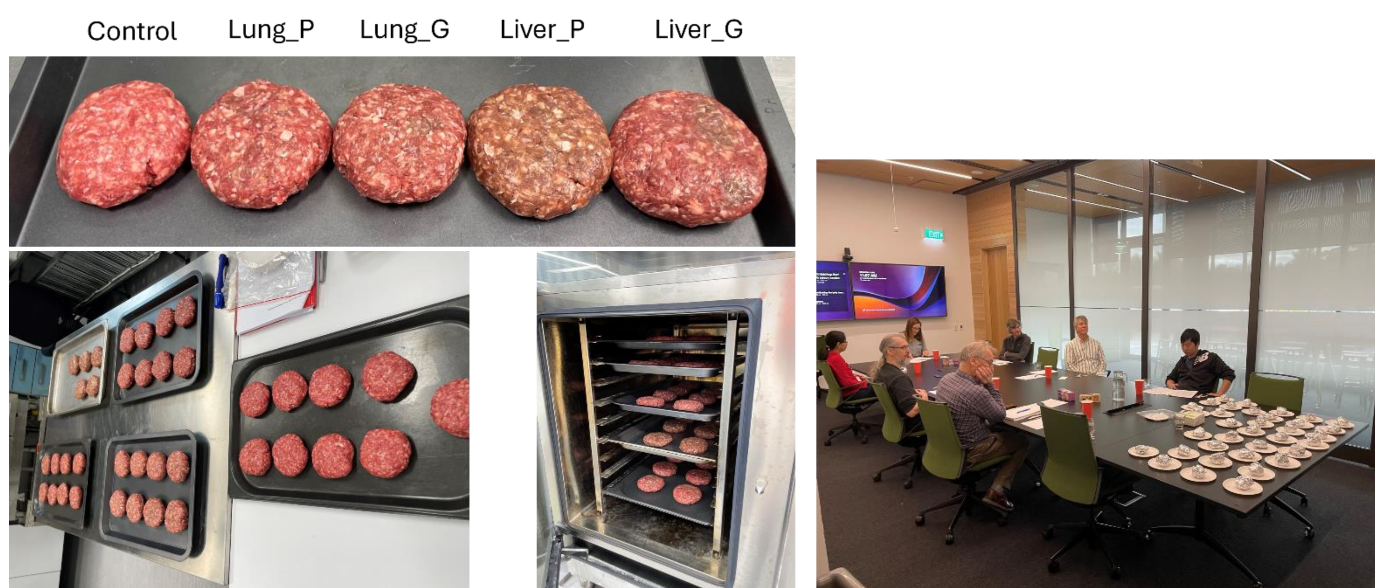

Figure 3. Photos of beef patties incorporated with extracts and sensory trial.

Table 1. Beef patty formulation with Liver or Lung extracts.

Ingredients	Formulation (unit: g)				
	Control	Lung-P	Lung-G	Liver-P	Liver-G
Mince beef *	1350	1350	1350	1350	1350
Water	150	135	135	135	135
Salt	15	15	15	15	15
Lung protein hydrolysate		15			
Lung protein hydrolysate+ γ-glutamylation			15		
Liver protein hydrolysate				15	
Liver protein hydrolysate+ γ-glutamylation					15

*Minced beef (>18% fat)

5.0 Results and Discussion

5.1 Yield and free amino acid analysis of kokumi rich extracts from Liver and Lung

The liver hydrolysates produced a higher yield of dry matter (11.22 %) compared to the lung hydrolysates (6.36 %), as expected. These yields were calculated based on the weight of the dried offal hydrolysate divided by the weight of the wet raw material. Alternatively, when expressed based on the dry matter of the raw materials, the yields were 30.8% for the lungs and 38.4% for the liver, respectively. This difference can be attributed to the variations in water and protein content between the two offals. According to the USDA FoodData Central database, beef lung contains 79.4 % moisture and 16.2 % protein, while beef liver contains 70.8 % moisture and 20.4 % protein. These differences in composition explain the higher yield obtained from the liver hydrolysates. (Beef lung: https://fdc.nal.usda.gov/fdc-app.html#/food-details/168628/nutrients and beef liver: https://fdc.nal.usda.gov/fdc-app.html#/food-details/169451/nutrients).

The results of the free amino acid analysis in water-soluble extract (W) and Gamma-glutamyl hydrolysates (G) from liver and lung are presented in Table 2 (quality, %) and Table 3 (quantification, µg/g of extract). Free amino acids can contribute to overall taste. In particular, several hydrophobic amino acids such as leucine, valine, isoleucine, and phenylalanine known to activate CaSR (Ahmad and Dalziel, 2020). In W groups, liver showed higher values of aromatic free amino acids compared to lung while in G samples, no differences were found. Glutamine is a precursor for the Gamma-glutamyl transferase reaction. In Table 3, although the quantity of glutamine in Lung-W (21.7 µg/mL) was lower than in Liver-W (25.9 µg/mL), the proportion of glutamine in Lung W (3.6%) was higher than that in Liver W (1.6%) suggesting lung could be a higher potential substrate source than the liver, based on the protein concentration.

High free amino acid concentrations in Liver W indicates a higher soluble protein content than in Lung W. However, the lung showed higher sensitivity to enzymatic hydrolysis product, resulting in a 16-fold increase in total free amino acid from 562 to 8,624 ug/mL compared to liver, which increased 5-6 fold from 1636 to 11,001 µg/mL. L-glutamic acid, known as an umami substance, was found at higher levels in the liver compared to lung samples (24.9% in liver versus 14.8% in lung in the W groups, and 12.1% in liver versus 8.7% in lung in the G groups). Glycine, glutamic acid, and taurine were the most abundant amino acids in Lung W. Notably, glycine, which is the primary amino acid in collagen (Reshan Jayawardena et al., 2022), may have been released by the natural hydrolysis of collagen into free amino acids.

Table 2. Free amino acid profile (%) of W and G extracts from beef liver and beef lung (n=3).

	Liver				Lung			
	W		G		W		GA	
	Mean	Std	Mean	Std	Mean	Std	Mean	Std
D,L-O-Phosphoserine	0.3	0.0	0.2	0.0	0.5	0.0	0.2	0.1
Taurine	2.9	0.0	0.3	0.0	14.9	0.7	1.0	0.0
O-Phosphoethanolamine	0.9	0.1	ND	-	ND	-	ND	-
L-Aspartic Acid	2.1	0.0	2.5	0.3	4.2	0.2	2.3	0.0
L-Threonine	3.8	0.4	5.0	0.4	1.5	0.0	4.8	0.2
L-Serine	4.6	0.0	5.0	0.4	3.3	0.6	4.8	0.2
L-Glutamic acid	24.9	0.4	12.1	0.8	14.8	6.6	8.7	1.2
L-Glutamine	1.6	0.0	0.2	0.3	3.6	4.8	1.2	0.7
L-Proline	3.3	0.0	4.0	0.1	3.3	0.2	2.5	0.1
Glycine	7.8	0.2	4.4	0.4	14.7	0.5	5.2	0.2
L-Alanine	6.7	0.1	6.6	0.3	5.4	0.3	7.6	0.1
L-a-Amino-n-butyric acid	0.2	0.0	0.0	-	0.3	0.0	0.1	0.0
L-Valine	4.4	0.1	6.8	0.2	3.1	0.1	6.8	0.1
L-Cystine	4.4	0.4	2.6	0.2	1.6	0.2	2.0	0.2
L-Methionine	1.7	0.2	2.8	0.1	1.2	0.1	2.5	0.1
L-Isoleucine	2.9	0.0	6.2	0.1	1.6	0.1	5.3	0.1
L-Leucine	6.9	0.0	11.2	0.4	4.4	0.2	11.7	0.3
L-Tyrosine	2.7	0.1	4.6	0.1	2.4	0.1	4.3	0.4
L-Phenylalanine	3.8	0.0	6.3	0.1	2.6	0.1	6.2	0.2
b-Alanine	0.6	0.1	0.1	0.0	ND	-	0.9	0.1
g-Amino butyric acid	0.1	0.0	0.1	0.1	0.1	0.0	ND	-
L-Tryptophan	0.9	0.1	1.8	0.2	0.5	0.2	1.5	0.2
Ethanolamine	0.1	0.0	0.1	0.0	1.3	0.2	0.2	0.0
Ammonia	1.0	0.0	0.6	0.1	1.6	0.1	0.4	0.1
L-Ornithine	3.6	0.1	4.5	0.1	3.7	0.2	0.2	0.0
L-Lysine	5.7	0.1	7.5	0.2	6.7	0.3	8.2	0.2
L-Histidine	2.0	0.0	2.3	0.1	1.9	0.0	2.8	0.0
L-Arginine	ND	-	0.2	0.0	1.2	0.6	7.5	0.2
Total	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0

Table 3. Free amino acid quantification (ug/mL of liquid extract) of W and G extracts from beef liver and beef lung (n=3).

	Liver				Lung			
	W		G		W		G	
	Mean	Std	Mean	Std	Mean	Std	Mean	Std
D,L-O-Phosphoserine	4.2	1.0	17.3	2.6	2.9	0.3	21.6	7.0
Taurine	46.7	3.5	35.9	6.0	88.6	10.4	87.1	7.0
O-Phosphoethanolamine	35.1	1.7	273.6	13.7	25.2	3.0	195.7	11.0
L-Aspartic Acid	62.8	7.9	558.0	113.9	8.8	1.3	410.8	1.4
L-Threonine	75.7	5.3	556.1	109.3	19.6	3.6	417.8	7.1
L-Serine			141.1	25.4			93.9	7.6
L-Glutamic acid	407.4	19.3	1340.7	281.1	87.0	38.1	744.6	77.4
L-Glutamine	25.9	1.6	23.2	28.6	21.7	29.6	103.1	58.1
L-Proline	53.3	4.2	443.1	51.4	19.5	2.4	217.2	12.1
Glycine	126.8	4.6	477.6	25.0	87.2	9.8	445.7	27.4
L-Alanine	110.1	6.1	727.0	76.9	32.2	3.7	651.8	25.0
L-a-Amino-n-butyric acid	3.2	0.5	3.4		1.5	0.3	7.9	1.1
L-Valine	71.7	6.1	748.9	83.6	18.5	1.7	586.8	24.6

L-Cystine	73.1	10.9	290.7	41.8	9.7	1.4	168.7	16.2
L-Methionine	28.4	3.7	312.9	53.0	7.0	1.0	215.9	11.8
L-Isoleucine	48.1	3.4	682.7	94.7	9.8	1.6	456.0	23.3
L-Leucine	112.6	7.2	1240.1	212.3	26.2	3.5	1010.4	64.5
L-Tyrosine	43.7	3.7	506.1	61.5	13.9	1.3	370.7	45.4
L-Phenylalanine	62.7	4.4	699.6	109.9	15.5	1.4	538.5	33.4
b-Alanine	9.3	0.5	7.6	1.2			75.9	9.6
g-Amino butyric acid	2.0	0.1	10.4	10.3	0.5	0.1		
L-Tryptophan	15.3	1.2	201.2	5.7	3.1	1.3	125.8	25.2
Ethanolamine	2.4	0.4	14.5	1.6	7.5	0.6	20.6	2.1
Ammonia	16.7	0.7	68.4	3.9	9.3	0.8	35.4	5.3
L-Ornithine	59.0	4.6	499.6	79.2	22.1	1.6	16.6	2.7
L-Lysine	93.0	7.1	827.8	104.0	40.0	4.9	707.6	41.5
L-Histidine	32.5	2.5	256.6	46.8	11.1	1.0	245.7	10.4
L-Arginine			18.1	7.4	7.2	3.7	645.7	30.1
Total	1636.9	106.1	11001.2	1544.2	592.6	54.4	8624.7	322.5

5.2 Kokumi- Ƴ-glutamyl peptide quantification of kokumi rich extracts from Liver and Lung

Twelve Ƴ-glutamyl dipeptides and one tripeptide (Ƴ-Glu-Val-Gly, EVG) were identified in this study. Both Liver-G and Lung-G showed significant enrichment of 12 γ-glutamyl dipeptides (Figure 4 A) and EVG (Figure 4 B). Liver W exhibited a few γ-glutamyl dipeptides at higher levels than Liver-G, likely due to natural formation by endogenous γ-transpeptidase enzymes present in the liver. Among the γ-glutamyl dipeptides, γ-Glu-Cys is the most potent agonist, followed by γ-Glu-Cal, γ-Glu-Ala, γ-Glu-Thr, γ-Glu-Leu, γ-Glu-Ile, γ-Glu-Ser, γ-Glu-Orn, γ-Glu-Met, γ-Glu-Asn, γ-Glu-Gly, and γ-Glu-Trp (Amino et al., 2016). EVG was present only in Lung-G extract, and was not detected in water extracts, confirming the efficient enzymatic γ-glutamylation of the hydrolysed proteins. EVG is the most potent agonist of CaSR identified to date, with a potency 20-10000 times higher than other Ƴ-glutamyl peptides (Amino et al., 2016), and thus induces a strong kokumi flavour. Beef lung is therefore appears to be a superior kokumi substrate than liver and might produce higher kokumi intensity in CaSR activation as well as in supplemented food.

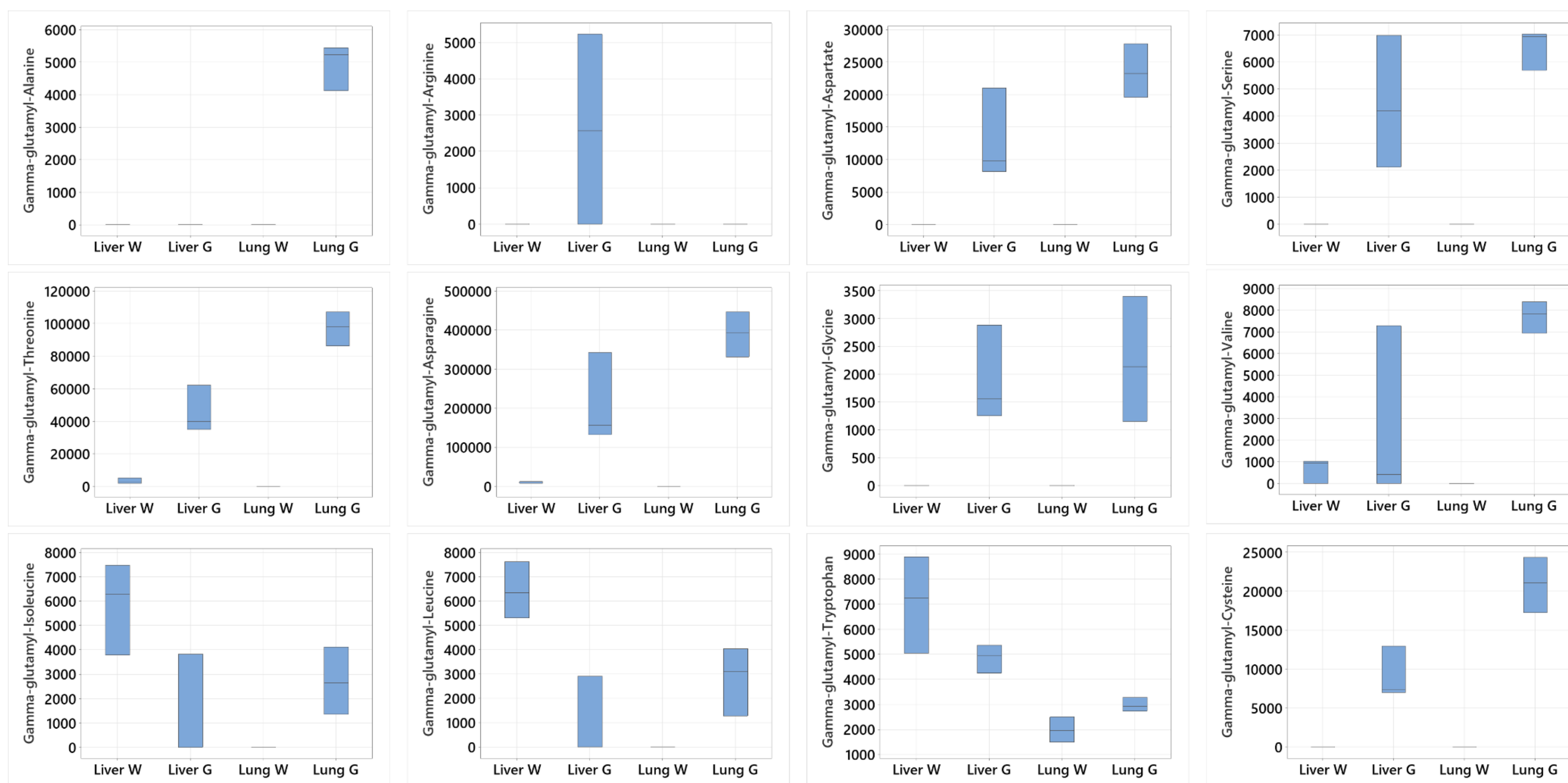

Figure 4 A. Relative concentrations of γ-Gamma-glutamyl dipeptides in water (W) and kokumi-gamma-glutamyl (G) extracts from beef liver and lung (mean ±SEM; n=3). Note that for the calculation, the peak area of each sample was divided by its total peptide concentration (mg/mL). Unit: Peak area/mg protein

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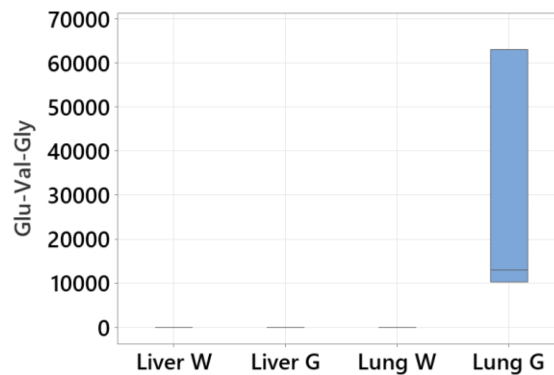

Figure 4 B. γ-Gamma-glutamyl-Valine- Glycine (Tripeptide, the most potent kokumi flavour peptide) relative concentration of water (W) and gamma-glutamyl-kokumi (G) extracts from beef liver and beef lung (n=3). Note that for the calculation, the peak area of each sample was divided by its total peptide concentration (mg/mL). Unit: Peak area/mg protein**.** Liver- G, liver kokumi extract; Lung-G, lung kokumi extract; Liver-W, Liver water extract; Lung-W, lung water extract.

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5.3 Kokumi intensity of kokumi rich extract from liver and lung through *in vitro* CaSR assay

Kokumi substances activate the calcium sensing receptor (CaSR) in our taste buds to evoke kokumi flavour sensation (Ahmad and Dalziel, 2020). The *in vitro* CaSR activation emerged as the most appropriate biological method available to reliably assess kokumi richness of flavour substances and also to compare different substrates to produce kokumi rich extract for food application (Ahmad et al., 2023, Kim, 2024, Kim et al., 2022). We applied 1mg/mL water soluble kokumi extract from liver (Liver-G) and Lung (Lung-G) in fold serial dilution on cells expressing human CaSR to determine their kokumi intensity or richness by measuring two parameters: potency and efficacy. Whereas potency is measured by lowest dosage required to have half maximal or 50% (EC50) activation of CaSR, efficacy represents maximal response at a particular dosage. Further these dosages are expected to produce similar kokumi flavour responses when added to food.

Both Liver-G and Lung-G activated CaSR in a dose dependent manner when compared to their water extracts (Liver-W, Lung-W) confirming the observed strong enrichment of Ɣ-glutamyl peptides when compared against water extract (Fig. 5). On comparing the extracts in terms of potency and efficacy, Lung-G showed stronger potency with EC50 approximately 121 µg (per mL of substrate) and slightly higher efficacy with maximal response approximately 200 relative fluorescent units (RFU) when compared to Liver-G with EC50 of 181µg (per mL of substrate) and efficacy of appx. 175 RFU (Fig. 5).

The stronger activation by Lung-G is most likely because of the presence of single tripeptide, EVG, as all other observed dipeptides were not significantly different between two groups. EVG is known to be the most potent kokumi peptide activator of CaSR when compared against other Ɣ-glutamyl peptides and hence able to enhance kokumi intensity when added to any extract or foods. Consequently, we infer that EVG enrichment in lung-G contributed to the high potency detected, as a lesser amount was needed to evoke half maximal activation of CaSR, while Liver-G (which was devoid of it) needed to be present at higher amounts a similar effect (Fig. 5). Nonetheless, to validate this hypothesis it is highly recommended to correlate *in vitro* dose response of CaSR activation with sensory perception of kokumi intensity in a food application. Although, *in vitro* cell based CaSR activation assay provides an indicative threshold of kokumi substances, this can vary considerably when tested in food applications because of complex food matrix and high variability of consumer sensory trial.

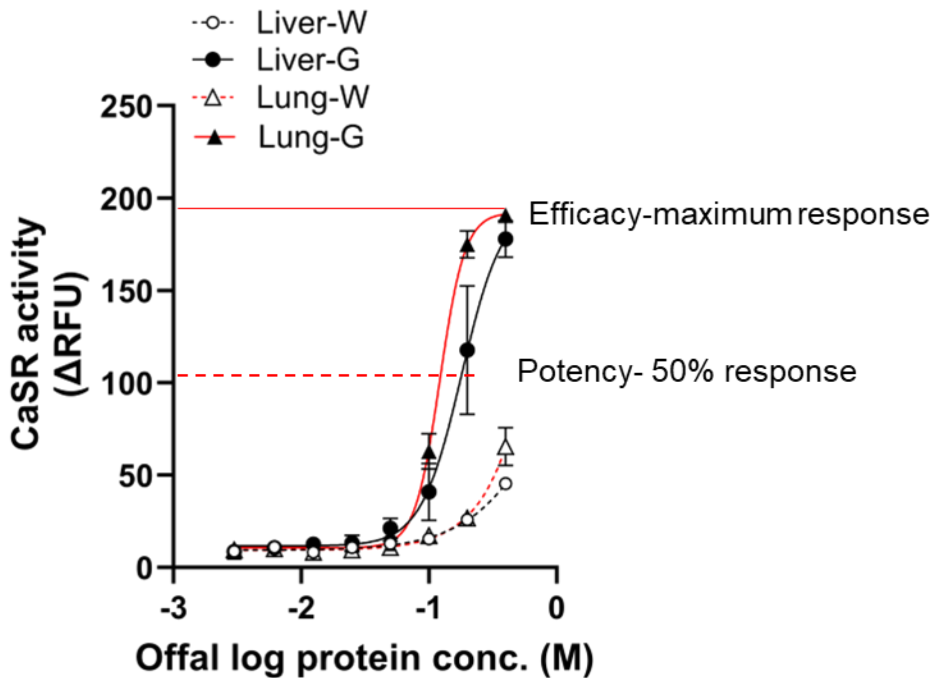

Figure 5. Dose response curves showing CaSR activity in relative fluorescence units (ΔRFU) against protein concentrations of liver and lung kokumi extract with control water extract. Mean±SE, n=3. Liver- G, liver kokumi extract; Lung-G, lung kokumi extract; Liver-W, Liver water extract; Lung-W, lung water extract. Red solid horizontal line shows efficacy (maximum RFU) and dotted horizontal red line shows half maximal or 50% CaSR response.

5.4 Flash profiling (sensory evaluation): beef patties supplemented with oven dried kokumi rich extract from liver and lung

By using the flash profile methodology, we sought to differentiate how kokumi extract of offal modifies the sensory profile of beef patties when compared against just hydrolysed extract (protease-A hydrolysis). Our kokumi extract production method comprises a first step of hydrolysing protein with protease-A (P group) which breaks down large proteins into small proteins and amino acids that serves as a substrate for second and final step of modifying them into kokumi specific Ƴ-glutamyl peptides (G-group).

Flash profiling results were first analysed using Generalized Procrustean Analysis (GPA). GPA obtains a common representation to compare overall differences in sensory properties between the five patties, based on the different attributes and rankings of those attributes used by the seven panellists. The extent of separation between the five patties shows how different the overall sensory properties were between samples (Fig 6A). Results show several interesting points. Firstly, it can be observed that the control or non-supplemented patties (purple) were well separated compared to the P and G supplemented patties for both Liver and Lung (Fig. 6A, yellow, green, teal, blue). More importantly, Lung-P (yellow) and Lung-G (green) showed large separation from each other, whereas Liver-P (teal) and Lung-G (blue) were in a very similar position (Fig. 6A). To understand what individual attributes from panellists were most closely related/associated to each of the five patties, Principal

Component Analysis (PCA) on all of the data was then undertaken (Fig. 6B). Results suggest that Lung-G was distinct from the other samples in terms of being the most juicy and salty sample, while Liver-P and Liver-G were the samples with the strongest offal/tripe/Livery flavours (Fig. 6B). It was unexpected that participants were unable to differentiate Liver-P and Liver-G samples. This may be because of strong off-flavour and metallic flavour in the kokumi rich liver extract (Liver-G), which hampered the impact of kokumi taste peptides. Although our kokumi extract is a crude mixture of kokumi rich flavour peptides and amino acids, it is not completely devoid of aromatic compounds and other minerals which might have affected the participants sensory perception in both patties containing Liver extracts. Noteworthy to mention we have kept free calcium concentration in *in vitro* assay at constant 0.5mM by using higher dilutions which was not feasible to do in sensory trial and indicates a limitation of the method.

Another informative observation was that during cooking a transparent, sticky film (which we suspect to be collagen) appeared to be released onto the baking dish from both Lung-G and Lung-P patties. This observation is perhaps not surprising, given that Lung is known to be rich in collagen (NUCKLES et al., 1990). Furthermore, the most abundant collagen amino acids i.e. glycine was found significantly higher in lung compared to Liver W. It is in fact possible the textural properties of the patties may have been modified slightly by the Lung-G and Lung-P extracts which will both have a high collagen content (i.e. this could be contributing to improved juiciness). However, the strong association of juiciness with Lung-G instead of Lung-P (Fig. 6B), suggests it is predominantly the kokumi flavour specific di and tri-peptides (present in the Lung-G extract but not the Lung-P extract) that are driving increased juiciness in Lung-G.

Notably, the enrichment of ϒ-glutamyl dipeptides and subsequent higher kokumi intensity detected in CaSR activation assay is the likely contributor desirable sensory attributes in FP trial when compared to Liver-G samples. Concomitantly, enrichment of EVG in Lung-G contributed to enhanced kokumi richness in both CaSR and sensory attributes of supplemented beef patties.

This is the first report of bovine lung being demonstrated as a source of EVG and utilized to produce a kokumi flavour extract that may increase the desirable sensory attributes in a real meat product. We suggest performing future investigations on Lung-G using larger scale consumer sensory trials at different concentration [low (0.25%), medium (0.5%) and high (1%)] to determine, with more accuracy, the impact of Lung-G on sensory properties and consumer acceptability, across a range of meat/food applications.

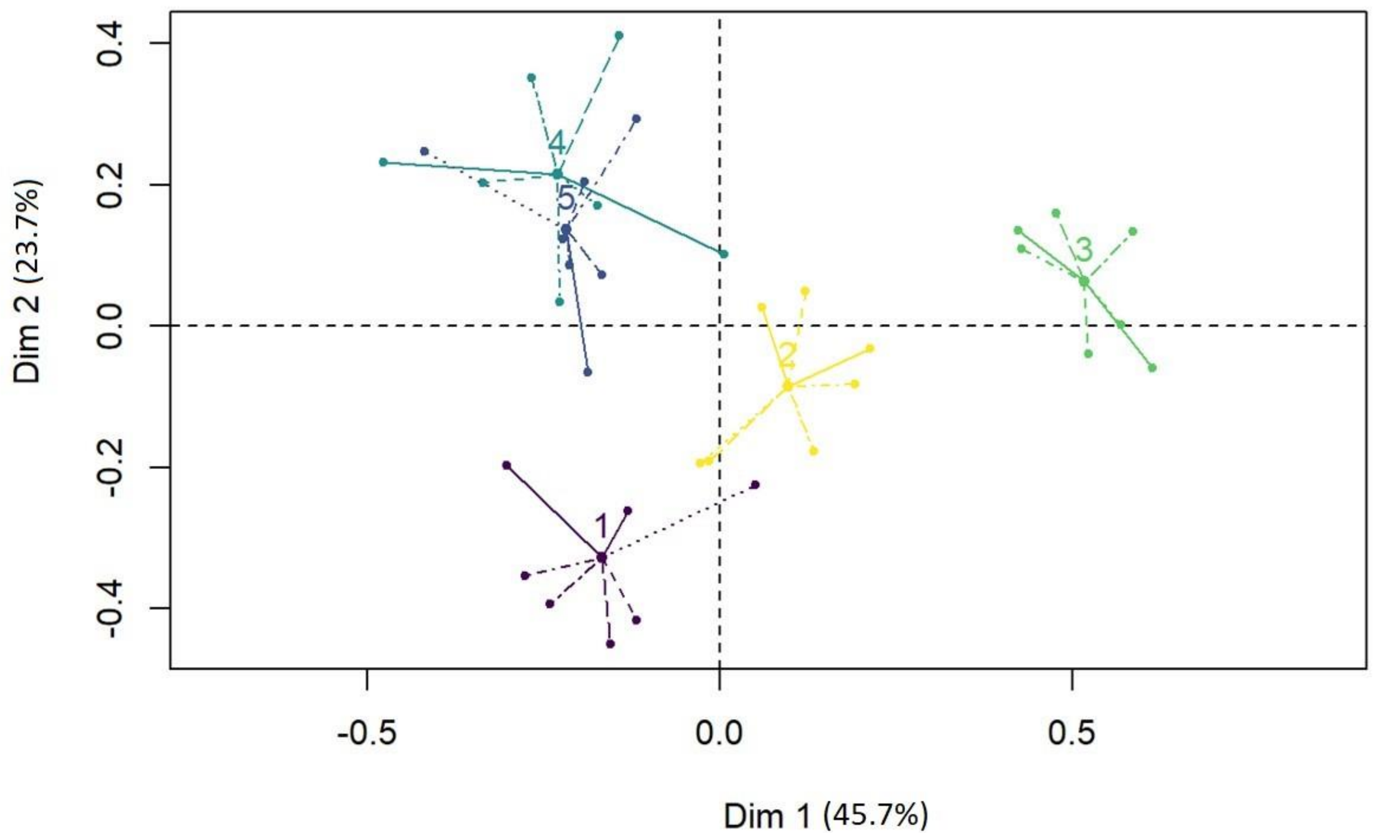

Figure 6 A. Flash Profiling sensory map (Generalized Procrustes Analysis (GPA) consensus plot). The extent of separation (distance) between each of the five patties (1= purple, 2=yellow, 3=green, 4=teal, 5=blue) shows how different the overall sensory properties were between each of the five patties. For each patty, the seven branches indicate how much consistency there was in ratings across the seven panellists.

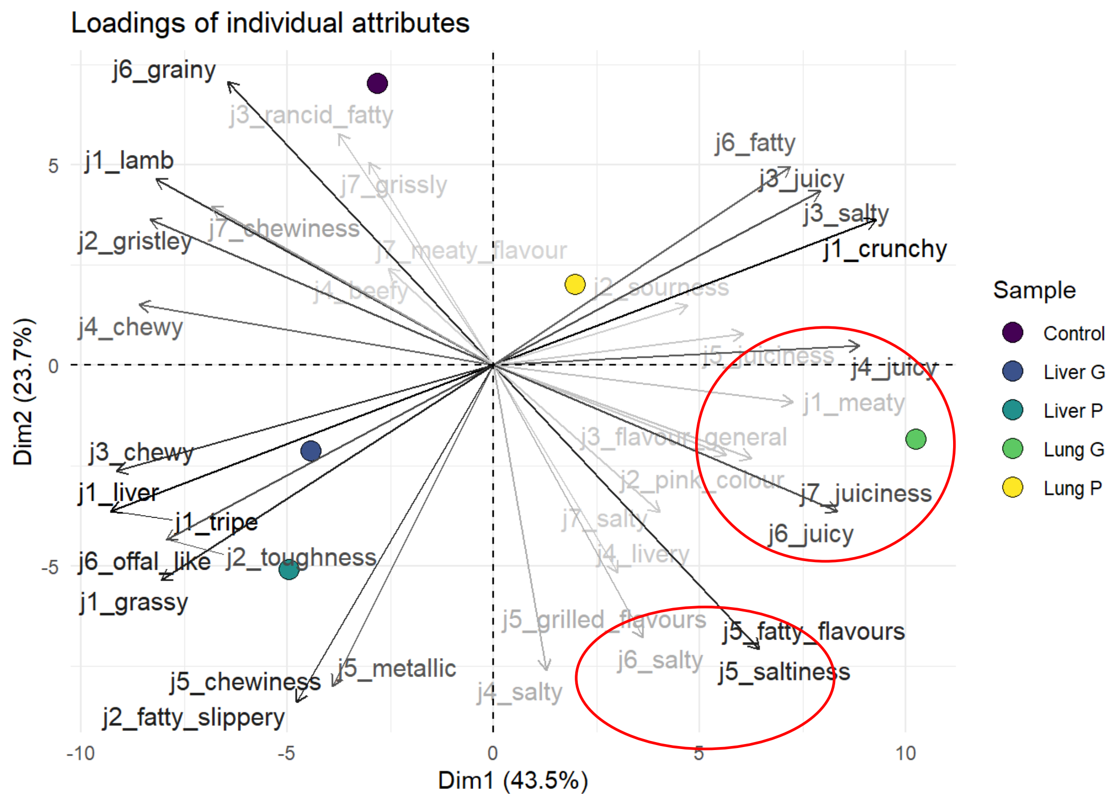

Figure 6 B. Principal component analysis plot of flash profiling results with all chosen attributes from participants (J1=participant 1, J2=participant 2 etc). The length and shade of the arrow demonstrates the weighting/influence each attribute had on the position of the product (a long black arrow indicates that this attribute was more meaningful in distinguishing between the samples compared to a shorter grey arrow). Red circle shows prominent attributes of Lung-G supplemented beef patties. Control- Beef patty with water extract; Liver- G- beef patty with liver kokumi extract; Liver-P- beef patty with Liver protease hydrolysate; Lung-G- beef patty with lung kokumi extract; Lung-P- beef patty with lung protease hydrolysate.

6.0 Conclusions / recommendations

Conclusions

Based on the data we conclude that a two-step enzymatic was efficient in producing kokumi rich extract of beef liver (Liver-G) and lung (Lung-G) enriched with ϒ-glutamyl di-peptides and free amino acids. Notably, only Lung-G showed enrichment of EVG, a recognized potent kokumi taste tri-peptide. Lung-G exhibited higher kokumi intensity in *in vitro* kokumi receptor assay than Liver-G predicting stronger kokumi richness and intensity on food application. This was validated in a sensory trial undertaken using Flash Profiling where, Lung-G stood out in producing the desirable attributes of juiciness and saltiness, when it was added to beef patties. To the best of our knowledge, this is the first report showing the use of bovine lung as an efficient substrate to produce a kokumi flavour extract rich in EVG that imparted desirable sensory attributes in a processed meat product.

Recommendations

- **Economic analysis of Lung-G kokumi extract:** To understand the financial feasibility and economic viability of Lung-kokumi extract we suggest carrying out techno-economic analysis of pilot scale production process of Lung-G kokumi extract in future project.
- **Investigation of collagen rich meat by-products for kokumi extract preparation.** Based on our data we hypothesize that collagen might serve as a compatible substrate to produce kokumi extract which further showed food favourable properties during its incorporation into foods. Therefore, other collagen rich meat by-products can be tested in future projects.
- **Maximizing utilisation of by-products:** After enzymatic hydrolysis, the hydrolysates are strained to remove residual connective tissues. Using collagenase in conjunction with the current enzyme method could help further reduce these residues, maximizing the utilization of collagen-rich by-products for kokumi production.
- **Large-scale consumer sensory study**: We suggest performing future investigations on Lung-G using larger scale consumer sensory trials to determine the impact of Lung-G on consumer acceptability, across a range of meat/food applications.

Notable observation:

High taurine and arginine content in Lung extract: Notably, we observed high taurine content in Lung-W (non-enzymatically treated water extract from lung; 5 times higher than Liver-W), and arginine in Lung-G (Lung-kokumi extract; 35-40 times higher than in Liver-G). This could be important as both taurine and arginine have been studied for their health benefits, and nutraceutical made from liver and lung are commercially available.

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7.0 Bibliography

AHMAD, R. & DALZIEL, J. E. 2020. G protein-coupled receptors in taste physiology and pharmacology. *Frontiers in pharmacology,* 11**,** 587664.

AHMAD, R., DALZIEL, J. E., NGUYEN, H. T., ROUNCE, J., DAY, L. & MAES, E. 2023. Investigation of free amino acids in lactic acid bacteria fermented milk and their ability to activate the calcium sensing receptor. *International Dairy Journal,* 141**,** 105568.

AMINO, Y., NAKAZAWA, M., KANEKO, M., MIYAKI, T., MIYAMURA, N., MARUYAMA, Y. & ETO, Y. 2016. Structure–CaSR–Activity Relation of <i>Kokumi</i> γ-Glutamyl Peptides. *Chemical and Pharmaceutical Bulletin,* 64**,** 1181-1189.

DELARUE, J. 2015. 6 - Flash Profile, its evolution and uses in sensory and consumer science. *In:* DELARUE, J., LAWLOR, J. B. & ROGEAUX, M. (eds.) *Rapid Sensory Profiling Techniques.* Woodhead Publishing.

KIM, J., AHMAD, R., DEB-CHOUDHURY, S., SUBBARAJ, A., DALZIEL, J. E. & KNOWLES, S. O. 2022. Generation and identification of kokumi compounds and their validation by taste-receptor assay: An example with dry-cured lamb meat. *Food Chemistry: X,* 13**,** 100218.

KIM, J., DEB-CHOUDHURY, S., SUBBARAJ, A., REALINI, CE., AND AHMAD, R. 2024. Comparative analysis of kokumi tastant intensity from mechanically deboned meat across three species through in vitro calcium sensing receptor activity. *International Journal of Food Science and Technology*.

KURODA, M. & MIYAMURA, N. 2015. Mechanism of the perception of “kokumi” substances and the sensory characteristics of the “kokumi” peptide, γ-Glu-Val-Gly. *Flavour,* 4**,** 1-3.

KURODA, M. & MIZUKOSHI, T. 2024. Identification and Quantification of the Kokumi Peptide, γ-Glu-Val-Gly, in Foods. *Kokumi Substance as an Enhancer of Koku: Biochemistry, Physiology, and Food Science.* Springer.

MULLEN, A. & ÁLVAREZ, C. 2016. Offal: types and composition.

NUCKLES, R. O., SMITH, D. M. & MERKEL, R. A. 1990. Meat By-product Protein Composition and Functional Properties in Model Systems. *Journal of Food Science,* 55**,** 640-643.

RESHAN JAYAWARDENA, S., MORTON, J. D., BEKHIT, A. E.-D. A., BHAT, Z. F. & BRENNAN, C. S. 2022. Effect of drying temperature on nutritional, functional and pasting properties and storage stability of beef lung powder, a prospective protein ingredient for food supplements. *LWT,* 161**,** 113315.

TOLDRÁ, F., MORA, L. & REIG, M. 2016. New insights into meat by-product utilization. *Meat science,* 120**,** 54-59.

WHITE, J., HART, R. & FRY, J. 1986. An evaluation of the Waters Pico-Tag system for the amino-acid analysis of food materials. *The Journal of Automatic Chemistry,* 8**,** 170.

8.0 Appendices

The author should any supporting documentation which has been referenced in the report. Each Appendix must be named and numbered.

8.1 Appendix 1: Flash profiling questionnaire

Attribute ranking Name:

Please taste samples in the following order:

Please generate 5-6 attributes you feel best describe the taste, flavour, or texture of the beef patties.

1

2

3

4

5

6

For each attribute you choose, please rank the 5 samples in terms of the intensity of that attribute on the following pages.

Attribute 1:

Lowest intensity Highest intensity

Comments:

Attribute 2:

Lowest intensity Highest intensity

Comments:

Attribute 3:

Lowest intensity   Highest intensity

Comments:

Attribute 4:

Lowest intensity Highest intensity

Comments:

Attribute 5:

Lowest intensity Highest intensity

Comments:

Attribute 6:

Lowest intensity Highest intensity

Comments: