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Development of novel technology to manufacture blood derived functional ingredients

Stage II report – enzyme modulated blood protein flavour

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Executive summary

This project is targeted at adding value to blood, a by-product stream produced in abattoirs and rendering facilities, which at present is mainly used as a low value animal feed. Currently, the industry does not utilise this valuable source of high quality protein to its maximum potential. Value addition to blood presents Wagstaff Food Services with opportunities to produce ingredients which are physically functional, provide 'meaty/brothy' flavour attributes or physiological functionality.

This stage of the project was designed to capitalise on the potential opportunities presented in the flavour ingredient market by hydrolysing haem reduced blood to produce blood hydrolysates that provide a 'meaty/brothy' flavour.

Blood contains approximately 18% crude protein and consists of plasma and a red cell/corpuscles fraction. The cell fraction has ~35% solid content with ~33% protein corresponding to ~70% of protein in blood. The red cell fraction contains haemoglobin, a coloured iron containing protein used in oxygen transport. The iron in haemoglobin is held in the form of haem.

Commercially available enzymes approved for food application were chosen for the hydrolysis reactions. Eight blood protein hydrolysate samples using 6 different enzymes were produced following HACCP protocols that were developed as part of the project. The enzyme hydrolysis conditions were chosen based on the enzyme stability and activity data supplied by the enzyme suppliers.

The degree of hydrolysis of the haem reduced blood proteins subjected to enzymatic hydrolysis indicated that Umamizyme and Protex 6L were able to achieve a high degree of hydrolysis (>20%). Peptidase R and Fungal protease 31,000 achieved the lowest degree of hydrolysis (~3% and 4.4%, respectively). Protamex and Flavourzyme 500MG achieved degree of hydrolysis between 9.5 and 13.2%. Microbial analysis of the hydrolysate samples indicated that the samples were acceptable and suitable for human consumption.

The initial sensory screening of the eight samples resulted in the detection of a very strong liver flavour note in all samples. This flavour note was very strong and masked all other flavour attributes. Diluting the hydrolysates and mixing with other commercially available stocks were not able to overcome this strong liver flavour attribute. Due to this, the samples were not subjected to a trained sensory panel as any differences in sensory attributes would not have been detectable.

Meat flavour and bullion ingredients are generally formulated with combinations of various additives and hydrolysates from different protein sources. It is recommended that the blood hydrolysate samples be evaluated as protein hydrolysates to produce meat flavour and bullion ingredients. It is recommended that product development be conducted with companies specialising in developing formulated flavour ingredients.

In this project, the objective was to utilise and hydrolyse all the blood proteins to produce protein hydrolysates as flavour ingredients. It is recommended, as an alternative strategy, to hydrolyse plasma proteins on their own and evaluate the sensory properties of these hydrolysates.

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1 Project background and brief

This project is targeted at adding value to an abattoir and rendering by-product stream which at present is mainly used as a low value animal feed. Abattoirs produce large volumes of blood during the slaughter of animals. This is potentially a source of good quality protein for human consumption. Currently the industry does not utilise this valuable source of high quality protein to its maximum potential. Blood, if collected hygienically, could provide Wagstaff Food Services and in the long term, the Australian meat industry, with an additional income stream far in excess of that currently achieved through the animal feed market. The opportunities blood presents to Wagstaff Food Services and the meat industry spans from supplying flavour enhancing and meat flavouring ingredients for applications in soups and snack foods, to physically functional food ingredients suitable for use in processed food applications and physiologically functional food ingredients targeted to the health and well-being markets. In order to capture these opportunities the meat industry needs to develop cost effective technologies to collect blood hygienically and transform this low value co-product stream into value added ingredients that provide greater returns and help improve the sustainability of the industry.

Currently, the meat processing industry does not value-add to co-product streams as other food processing industries, notably the dairy industry. There are small companies that efficiently collect different organs and tissues and export these unprocessed co-products as commodities for value addition through extraction and separation processes. There are also a few companies that value-add at small scale by extracting specific components from plasma using combinations of solvents and heat precipitation methods, consequently denaturing the other components and not extracting maximum value from the plasma feed stream. Overall, the utilisation of coproducts such as bovine blood by the red meat industry to produce high value ingredients in Australia is not well developed compared to the practice globally, where a range of ingredients are manufactured. For example Proliant in the US manufactures and markets plasma proteins and an IgG enhanced protein fraction (NutrGammax and Immunolin respectively) as functional ingredients. Immunolin in particular is marketed as a nutraceutical and as a non-dairy source of IgG. Veos, a Belgian company markets the Vepro range of animal blood derived ingredients for human consumption including plasma and globin protein ingredients. In addition, Veos markets a Vepro red blood cell fraction as a natural red colouring agent for cured and cooked meat. This product has the advantage that no E-number is required when it is added to processed foods.

Blood contains approximately 18% crude protein and consists of plasma and red cell/corpuscles fraction. The solids content of the plasma fraction is ~9% with a protein content of ~8% which corresponds to ~30% of the protein in blood. The cell fraction consists of ~35% solid with ~33% protein corresponding to ~70% of protein in blood. The red cell fraction contains haemoglobin, a coloured iron containing protein used in oxygen transport. The iron is held in the protein in the form of haem.

This project aims to capitalise on the potential opportunities presented through valueaddition to bovine blood by developing cost effective technologies to manufacture functional food ingredients. Initially, this project will aim to develop cost effective technologies to capture two opportunities identified by Wagstaff Food Services; (1) flavour ingredients and enhancers for soups and snack foods, and (2) functional protein food ingredients. Once technologies for these two opportunities are established and commercialised, Wagstaff Food Services will be able to consider further projects in blood fractionation, notably strategic proteolytic hydrolysis and separation to manufacture bioactive blood-derived ingredients.

1.1 Target outcomes

The target outcomes of the project are:

· Experimental data on developing technology to remove haem from the red blood cell fraction

· Experimental data on methods to hydrolyse blood protein to produce meat flavour extracts

· Sensory analysis of the hydrolysates together with evaluation of sensory attributes

· Experimental data on the production of blood protein ingredients and their physical functionality in emulsification, foaming, solubility, gelation and viscosity.

These results will be included in a report detailing activities undertaken throughout the project including experimental procedures, results, conclusions and recommendations.

1.2 Project activities

Research Stage 1

Milestone 1: Research agreement between Wagstaff Food Services and CSIRO Food and Nutritional Sciences) signed. Commence literature review on haem removal and analytical methods to characterise haem and globin protein.

Target outcome

Develop technology for the removal of haem from haemoglobin at laboratory scale

Investigate physical and chemical methods at laboratory scale to remove haem from haemoglobin to produce a haem and a globin fraction. The processing aids used for haem removal will be food grade.

Milestone 2: Written report detailing laboratory scale experimental procedures to remove haem from haemoglobin and results of experiments to be submitted to Wagstaff Food Services.

Research stage 2

Target outcome

Hydrolysis of haem removed blood proteins for sensory evaluation

The haem depleted globin protein from stage 1 will be combined with plasma proteins and subjected to enzyme hydrolysis. Hydrolysis trials will be conducted using a maximum of six enzymes. A maximum of eight samples produced through enzyme hydrolysis will be evaluated using a trained sensory panel. The sensory panel will be trained with the hydrolysates to identify and define a descriptive vocabulary that will be used to profile the differences between the samples.

Samples of similar products currently in the market, e.g. stocks, soups, noodle mix will also be analysed for comparative purposes, up to a maximum of twelve samples including hydrolysates. The panel will be trained to consistently use this vocabulary to provide quantitative ratings of the perceived sensory attributes. The final vocabulary will describe all aspects of the samples, including appearance, aroma, flavour, texture/mouth-feel, and after-taste/after-feel. The trained panel will profile the samples in triplicate to obtain quantitative ratings of the sensory attributes for each product.

The descriptive sensory data will be analysed statistically to determine similarities and differences between samples. Data will be represented in univariate tables of means with multiple comparisons, and in PCA plots that illustrate how samples compare.

Milestone 3: Written report detailing

· Laboratory scale experimental procedures and results on hydrolysis of blood protein to produce meat flavour extracts.

· Results of sensory analysis of the hydrolysates together with data on sensory attributes.

· Sample up to 5kg of the hydrolysate selected through sensory analysis will be produced at pilot scale and supplied to Wagstaff.

Go/No-Go decision based on producing a hydrolysate sample with acceptable 'soupy/brothy' sensory attributes

Research stage 3

Target outcome

Develop technology to manufacture blood protein ingredients and assess their functionality

Milestone 4: A sample of up to 1kg of the protein ingredients will be supplied to Wagstaff.

Milestone 5: Written report detailing activities undertaken during the project including experimental procedures, results, conclusions and recommendations submitted to Wagstaff Food Services.

1.3 Objective of stage 2

The objective of stage 2 of the project was to hydrolyse haemoglobin reduced bovine blood using up to six different protease enzymes. The blood protein hydrolysates samples were to be tested by a trained sensory panel for evaluation as flavour ingredients and enhancers for soups and snack foods. This report details work carried out in stage 2.

2 Literature review on enzyme and blood derived flavour ingredients

Scientific literature and patents were sourced via electronic searches of the ISI Web of Knowledge database which includes includes 5700 major journals in 164 scientific disciplines. The search terms employed were: plasma, haem, globin, haemoglobin, degradation, enzyme and hydrolysis. The objective of the search was to gain information on relevant enzymatic hydrolysis technologies that have been developed and applied specifically in the meat industry and generally in the food industry.

2.1 Hydrolysis of proteins

Proteins or polypeptides are composed of a large number of amino acids arranged in a chain and folded into three dimensional structures. The amino acids are joined together by peptide bonds to

form protein chains. Proteins can be degraded to form smaller peptide chains through hydrolysis. Protein degradation or hydrolysis is achieved either with acids or by using proteolytic enzymes to selectively cleave peptide bonds located within the protein molecular structure. During hydrolysis, the peptide linkages are cleaved to produce degradation products such as peptides and amino acids. Some of these degradation products contribute to physical and physiological functional properties, and sensory properties.

A number of commercially available proteolytic enzymes have been investigated in the literature to hydrolyse haemoglobin or globin using enzymes such as pepsin, papain, Alcalase, and pancreatin. In these studies, the bitterness of the hydrolysates was tested using bitterness expert panels but the other sensory attributes of the hydrolysates were not tested. In addition to using enzymes, hydrolysis of proteins can also be achieved using acids. This method has the disadvantage that it may result in the loss and partial destruction of proteins and potentially form toxic compounds through undesirable side reactions (Aubes-Dufau *et al*, 1995). The activity of proteolytic enzymes is optimal at milder conditions, and at these conditions, the nutritional values of the proteins are maintained, hence enzymatic methods are superior techniques to achieve protein hydrolysis.

Hydrolysis of blood proteins has been carried out using pepsin at acid pH conditions and temperature of 40-43°C (Webster et al, 1982; Pi ot et al, 1988 &1992; Aubes- Dufau et al, 1995; Yu et al, 2006). In these examples, the hydrolysis of haemoglobin using pepsin resulted in the production of bitter peptides. The reduction in the bitter taste required further processing such as ultrafiltration, reversed phase chromatography and solvent extraction (Aubes-Dufau *et al*, 1995). Hydrolysis of haemoglobin using Alcalase, a commercially available protease also resulted in the production of bitter peptides. The hydrolysis was in alkaline conditions (pH 8.5) at 55°C (Hald-Christensen, 1979; Webster et al, 1982; Clark et al, 1987; Synowiecki et al, 1996).

Peptic globin hydrolysates have shown bioactivity by inducing ACE (angiotensin- Iconverting enzyme) inhibitory activity with IC50 values between 0.1 – 1.19 mg/ml (Yu *et al*, 2006). ACE has been reported to play an important role in regulating blood pressure.

2.2 Proteolytic Enzymes (Proteases)

Enzymes are high molecular weight proteins with long chains of amino acids linked together by peptide bonds and play a vital role in physiological processes. Enzymes such as pepsin and trypsin take part in breaking down food into simpler compounds in the digestive tract. Proteases, also referred to as peptidases, are enzymes that catalyse the breakdown of peptide bonds in proteins. Certain proteases are very specific in the peptide bonds that they cleave, while others are non-specific and could completely hydrolyse proteins to amino acids. Generally, there are two classes of protease enzymes, namely exopeptidases and endopeptidases. The exopeptidases cleave peptide bonds from the ends of the protein molecule, therefore they are likely to produce free amino acids while the endopeptidase cleaves peptide bonds within the protein molecule, thus producing smaller peptides rather than free amino acids.

Exopeptidases are further classified as carboxypeptidases as they hydrolyse amino acids on the carboxy end of the protein and aminopeptidases that hydrolyse amino acids at the amino end of the protein.

Proteases are further classified depending on their mechanism or specificity, such as;

· Serine or alkaline protease: these protease enzymes have a serine and histidine residue in their active sites. Optimum activity is at alkaline pH range. Trypsin is a typical example of a serine protease and many proteases derived from bacterial sources especially *Bacillus* (e.g. *Bacillus subtilis*) species and fungi (*Aspergillus oryzae*) tend to be serine or alkaline proteases.

· Cysteine (thiol or sulfhydryl): these protease enzymes have a cysteine residue in their active site. The pH range for optimum activity is generally broad but their activity is higher below pH 7. Plant-derived proteases generally tend to be cysteine proteases and typical examples are papain from papaya and bromelain from pineapple.

· Aspartic (acid proteases, carboxyl proteases): these proteases have carboxylic acid function, especially from aspartic acid residues. The optimum pH range for these proteases is in the acidic pH range. Chymosin is an example of this type of protease.

· Metallo proteases (neutral proteases): these proteases have metal atoms at their active site (e.g., zinc) with an optimal pH range around 7. A number of proteases from fungal and bacterial sources belong to this group.

Many commercial manufacturers supply the above mentioned groups of proteases with varying degrees of activity and purity. In addition, there are commercial supplies of mixtures of different groups of proteases that provide a wide range of activity with a specific substrate.

The activity of the protease is dependent on pH, reaction temperature, substrate and the pre-treatment the substrate receives before hydrolysis. Similarly, the stability of the enzyme is also dependent on temperature and pH. The operating conditions of the enzyme reaction are generally based on a compromise between stability and activity. Therefore, the peptide sequences that are produced by each protease would depend on many factors, such as hydrolysis temperature, pH, nature of the substrate and its concentration, reaction time, enzyme concentration and the biochemical characteristics of the enzyme.

Enzymes have their optimum operating conditions of temperature, pH and enzyme to substrate ratio and these optimum conditions are substrate-specific. In addition, each enzyme has its own inactivation conditions of temperature, heat hold time and pH. Combinations of these factors are used to successfully inactivate and terminate the enzyme reaction after completion of the hydrolysis reaction. With an enzyme being a larger molecule and hydrolysates consisting of smaller peptides, separating the enzyme from the peptides using membrane filtration is another method of terminating the enzyme reaction.

2.3 Meat flavour development

The flavour of meat is attributed to a heterogeneous system of precursors which interact and degrade during cooking, to produce compounds resulting in characteristic meat flavour. The precursors responsible for meat flavour include peptides, free sugars, glycopeptides, free nucleotides, nucleotide bound sugars, glycopeptides and organic acids (Ritter, 1978). It is generally accepted that meaty flavour is the result of non-enzymatic browning reaction between reducing sugars, amines, amino acids, peptides and lipids (Ritter, 1978), hence flavour development requires the presence of peptides, carbohydrates and sulphur compounds. In addition, metabolic activity prior to slaughter, post-mortem enzyme activity in the tissues and enzyme activity during the initial phase of cooking until protein denaturation, generates flavour precursors and compounds responsible for the development of meaty flavour.

Meat flavour is developed by a combination of peptides formed by the reaction mechanisms such as Millard and Strecker degradation reactions between the amines from proteins and carbonyl (sugars) compounds. These reactions result in the formation of numerous classes of complex heterocyclic aromatic and flavour compounds that are dependent on the amino acid composition of the reactants, the reaction temperature and time.

Direct predication of the sensory attributes of the products of these complex reactions is not possible. Determination of the sensory attributes of substrates subjected to different reaction conditions requires evaluating the reaction products using trained sensory panels.

3 Bovine blood hydrolysis – material and methodologies

3.1 HACCP and food safety plan

The project plan included subjecting the blood protein hydrolysates to sensory evaluation by a trained sensory panel. This required the development of a food safety hazard analysis and critical control point (HACCP) system prior to producing the hydrolysate samples. The HACCP procedure included hygienic methods of blood collection, transportation, processing and storage.

Procedures for hygienically collecting bovine blood was developed and implemented at the Wagstaff Food Services abattoir in Cranbourne, Victoria. Details of the collection procedure appear in Appendix A. A flow diagram of blood handling, red cell separation, haem reduction, enzyme hydrolysis and product storage used in developing the food safety plan is in Appendix B.

The detailed HACCP food safety plan is in Appendix C. The food safety plan was assessed by the CSIRO Food and Nutritional Sciences (FNS) Food Risk Assessment Team (FRAT) as food for human consumption. This assessment indicated a “high level risk” due to potential contamination of the blood by C*lostridium Perfringens* at collection. The temperatures and duration of enzyme treatments were deemed to provide suitable conditions for the growth of this microorganism to hazardous levels, a potential food safety risk. In addition, the enzyme inactivation temperature was not high enough to inactivate any spores of *C.* P*erfringens*, another potential food safety risk. Therefore the potential for *C. Perfringens* contamination during the whole process and subsequent growth was monitored by sampling and microbial testing before and after heat inactivation of the enzyme.

3.2 Blood collection and red cell preparation

Bovine blood was collected under hygienic conditions using a hollow knife by direct puncture into the artery after stunning. The blood was collected into sterile containers that contained concentrated sodium citrate solution, to which when blood was added, gave a final concentration of 0.33% (w/v). The blood was transported on ice slurry from Wagstaff, Cranbourne to FNS, Werribee, Victoria. The hygienic procedure followed during collection of blood is detailed in Appendix A.

The red cells were separated following Duarte’s method (Duarte et al, 1999). Whole blood stabilised in citrate was centrifuged at 5,000g, 100C for 15 minutes to separate the plasma from the cell fraction. The separated plasma and red cells were stored in an ice bath to maintain a temperature of <100C during processing to minimise microbial growth. The cell fraction was mixed with water (1:6, cell mass: water) and agitated for 1 hour on an ice slurry for erythrocyte haemolysis. The haemolysed cell fraction was adjusted to pH 1.5 with hydrochloric acid and stirred for 30 minutes on an ice slurry to achieve acidic deconjugation of the haem. The haemolysed and pH adjusted red cell fraction was centrifuged using food grade centrifuge bottles following the FRAT committee approved HACCP procedure (Appendix C) at a temperature of < 100C to separate the supernatant from the haem pellet. The supernatant after centrifugation was collected and stored in ice slurry and a sample (~25ml) was taken for analysis.

The pH adjusted red cell supernatant was mixed with the plasma and stored on ice slurry and a sample of the mixed plasma and supernatant were taken for analysis. The mixed plasma and supernatant were adjusted to pH 6.5 with the addition of sodium hydroxide and heated to 720C for 6 minutes to reduce the microbiological count before enzyme hydrolysis and also as a means to denature and expose the proteins for hydrolysis.

The temperature indicator used for measuring the temperature during the pilot trials was calibrated against a certified calibrated temperature indicator and the pH meter that was used for the trials was calibrated using pH standards.

3.3 Food grade enzymes used for blood protein hydrolysis

Proteolytic enzymes from the same fungal or bacterial sources but from different manufacturers tend to have different activities resulting in different peptide profiles in the protein hydrolysates. This is most likely due to the differences in the fermentation procedures, strains of the micro organism used to express the enzymes and the different separation and fractionation technologies used by different manufacturers resulting in different purities.

Based on past experience in using enzymes to produce protein hydrolysates as functional ingredients, the following enzymes approved for food manufacture in Australia were used to produce food grade blood protein hydrolysates for sensory evaluation.

Fungal protease 31,000 (Enzyme Solutions, Victoria, Australia) – enzyme derived from *Aspergillus Oryzae* with a combination of acid, neutral and alkaline protease demonstrating exo-peptidase and endo-peptidase activity.

Flavourzyme (Novozyme, Sydney, Australia) – enzyme with combined exo-peptidase and endo-protease activity, derived from A*spergillus Oryzae* and used for extensive hydrolysis of proteins for flavour development. Recommended by supplier to produce brothy meat protein hydrolysates.

Umamizyme (Amano Enzyme Inc., Japan) – enzyme with high exo-peptidase and endo-protease activity derived by fermentation of a selected strain of *Aspergillus Oryzae.*

Protex 6L (Genencor, International, Inc., supplied through Enzyme Solutions, Victoria, Australia) – enzyme with alkaline protease activity with predominantly endo-protease activity derived from *Bacillus Licheniformis*.

Peptidase R (Amano Enzyme Inc., Japan) – enzyme with high exo-peptidase activity and derived from R*hizopus Oryzae*

Protamex (Novozyme, Sydney, Australia) **–** neutral pH protease complex derived from *Bacillus.*

In previous sensory studies of dairy protein hydrolysates conducted at CFNS using trained sensory panels, Fungal Protease 31,000 and Flavourzyme produced hydrolysates with significant brothy flavour as the dominant flavour note. Flavourzyme with its combined exo-peptidase and endo-peptidase activity has been used with different substrates to produce hydrolysates without any bitter flavour attributes.

Therefore, these two enzymes were selected to produce bovine blood hydrolysates. Umamizyme also has exo-peptidase and endo-peptidase activity and is promoted as a proteolytic enzyme for hydrolysing meat proteins with minimal production of bitter peptides.

Protex is recommended by the manufacturer for hydrolysing most proteins, especially haemoglobin.

Peptidase R was selected due to its high exo-peptidase activity to reduce the formation of bitter peptides.

Protamex was selected for its neutral protease activity and is recommended by the manufacturer to be used to produce hydrolysed animal protein, meat and bone stock.

3.4 Enzyme hydrolysis procedure

Hydrolysis experiments were conducted at 500C for 4 hours using the pH conditions and enzyme dosage set out in Table 1. The reaction conditions were selected based on enzyme activity and stability data supplied by enzyme suppliers. At the end of the hydrolysis reaction period, the pH of the hydrolysate was adjusted to 6.5. In trial # FZS006 and UZS008 sucrose was added to the hydrolysate for the browning reaction between sugars, amino acids and peptides.

Any loss of water due to evaporation during the enzyme hydrolysis reaction was compensated for at the end of the reaction by adding water to achieve the original volume used for hydrolysis. Two samples were taken for microbiological analysis (100ml each) for *Clostridium perfringens* and total plate count. The temperature of the hydrolysates was increased to 850C and this temperature maintained for 6 minutes to inactivate the enzyme. Samples of enzyme inactivated hydrolysates were taken for analysis and aliquots of the remaining hydrolysate were stored in sterile containers at <-150C until they were used for sensory evaluation. The temperature profile during storage and transport of hydrolysate products to the sensory laboratory in Sydney was recorded using temperature sensors.

Table 1: Enzyme dosage and pH conditions used to produce samples of blood protein hydrolysates for sensory evaluation

Trial #	Enzyme	Hydrolysis pH	Enzyme dosage (g per L of mixed plasma and supernatant	Sucrose addition (g/L hydrolysate)
FP001	Fungal protease 31,000	8.0	0.25	
PX002	Protex 6L	9.0	0.25	
PM003	Protamex	7.0	0.375	
PR004	Peptidase R	7.0	0.375	
FZ005	Flavourzyme 500 MG	7.0	0.5	
FZS006	Flavourzyme 500 MG	7.0	0.5	7.5
UZ007	Umamizyme	7.0	0.5	
UZS008	Umamizyme	7.0	0.5	7.5

3.5 Analytical methods

3.5.1 Determination of protein and haem

Optical density method

Optical density ratio (ODR) method was used to estimate the ratio of protein and haem in the separated supernatants by measuring the absorbance at wavelengths between 220 to 700 nm. The absorbance of haem is between 375-420nm, and globin between 220-280nm (Sato et al, 1981). The ratio of the absorbance at 420nm/280nm was used to monitor changes in haem content.

Protein content

Protein content was determined using the Dumas combustion principle with a LECOFP- 2000. The protein content was estimated by multiplying the nitrogen content by the conversion factor of 6.25.

3.5.2 Degree of hydrolysis

In many hydrolysis reactions, the degree of hydrolysis (DH) is used as the controlling parameter. The degree of hydrolysis, which is a measurement of the extent of proteolytic degradation, is defined as the percentage of peptide bonds that are cleaved (Alder-Nissen, 1986). The degree of hydrolysis is

used for characterising the hydrolysis reaction and is determined by measuring the increase in free amino groups formed during the reaction. The free amino groups are measured using a modified method of Church et al. (1983). In this method, free a-amino groups released during hydrolysis react with o-phthalaldehyde (OPA) to form a yellow complex. The optical density of this complex is measured spectrophotometrically at 340 nm. Leucine is used as a standard to calculate the concentration of amino groups as leucine equivalents.

3.5.3 Microbiological testing

Enzyme hydrolysis of the blood was conducted at temperatures between 450C and 500C over a 4 hour period. After hydrolysis, the enzyme was inactivated by heating to 850C for 6 minutes. This time/temperature combination was unlikely to inactivate any C.*perfringens* spores present in the hydrolysate. Therefore, to determine microbial growth during enzyme hydrolysis, microbiological testing of the production samples was conducted before and after heat inactivation of the enzymes. Testing of the hydrolysate before heat inactivation was conducted to determine if enzyme hydrolysis at elevated temperature (45 – 500C) encouraged the growth of microorganisms which could produce enterotoxins that can cause illness after consumption of the hydrolysate.

Microbiological testing was carried out on 5 samples from each batch of blood hydrolysate as recommended by the Food Standard Code (Food Standards Australia New Zealand – Standard 1.6.1). The samples were tested for the following classes of microorganisms:

· Total plate count

· *E.coli*

· *Coliforms*

· *Listeria*

· *Salmonella*

· *Clostridium perfringens*

3.5.4 Sensory evaluation

Prior to subjecting the hydrolysate samples to a trained sensory panel, it was decided to initially screen the samples and determine their sensory attributes. The initial screening of the samples was conducted by three members of staff using the following tasting conditions:

· Neat hydrolysate at 400C

· Diluted hydrolysate at 400C

· Hydrolysate in combination with stock purchased from a supermarket and included:

- Continental beef powder
- Massel ultra vegetable stock cubes
- Vegeta gourmet stock
- Oxo beef flavour
- Campbell's vegetable stock

· Hydrolysate in combination with fresh vegetable stock produced in the sensory laboratory

In addition, Wagstaff Food Services supplied four commercial product samples as prototype products.

· Beef flavour RFB-X – Givaudan

· Rare beef flavour SSF 101-X – Givaudan

· 001703 Beef flavour (The product makers)

· 001559 Beef flavour (MSG free), (The product makers)

These products were complete formulations with additives incorporated into the product. These products were also tasted at 400C as a comparison to the hydrolysate samples.

4 Results and discussion

The objective of stage 2 of the project was to produce bovine blood hydrolysates using total blood proteins, different enzymes and reaction conditions. The resulting experimental hydrolysate samples were to be evaluated by a trained sensory panel to determine its sensory properties as a 'soupy/brothy' flavour ingredient.

4.1 Haemolysed red cell at pH 1.5 and plasma preparations

The ODR and protein content of the blood during processing is detailed in Table 2. The ODR of the supernatant at pH 1.5 was 1.72, which was lower than that of cell fraction after haemolysis at 2.67. Adjusting the pH of the red cells fraction to achieve acidic de-conjugation resulted in a 35% reduction in ODR indicating a reduction in the haem content. The ODR of the plasma was low at 0.13 indicating low haem content in the plasma.

The protein content (Table 2) in the blood was 19.6% and the protein content of the plasma and the red cell fraction were 7.0% and 31.6% respectively which is within the range published in the literature.

Table 2: Optical density ratio (ODR) and protein content of bovine blood fractions.

Sample	Optical density ratio	Protein %
Fresh blood	1.01	19.55
Red cell fraction	2.39	31.60
Plasma fraction	0.13	6.99
Red cells after haemolysis	2.67	32.62
Red cell supernatant at pH 1.5	1.72	23.54
Red cell supernatant and plasma	1.40	26.54

4.1.1 Protein, iron, haem contents and degree of hydrolysis in the blood hydrolysate samples

The %DH, ODR and protein contents of the hydrolysed samples are summarised in the Table 3. The protein contents in the hydrolysed samples varied between 21.4% and 26.6%. The ODR values of the hydrolysed samples were significantly low and ranged from 0.13 to 0.66 compared to the starting material at 1.4. This was mainly due to the reduction in the absorbance at 420nm (data not shown), which is indicative of further reduction in the haem content.

The degrees of hydrolysis of the samples from FP001 (Fungal protease 31,000) and PR004 (Peptidase R) were low (4.41% and 3% respectively), which demonstrated that the enzymes Fungal

protease 31,000 and Peptidase R was not efficient in cleaving the blood proteins under the experimental conditions compared to the other enzymes. The degrees of hydrolysis of the batches PX002 (Protex 6L), UZ007 (Umamizyme) and UZS008 (Umamizyme with sugar) were high (20%, 22.8% and 22.4%) which was approximately 3 to 6.5 fold higher than those of batch FP001and PR004. Enzymes Protex 6L and Umamizyme demonstrated the highest blood protein degradation capacity among all the enzymes used in the trials.

Table 3: ODR, %DH and protein contents in the hydrolysed blood samples.

Sample	Optical density ratio	Protein %	%DH
Red cell supernatant and plasma	1.40	26.54	
FP001	0.54	23.30	4.41
PX002	0.66	25.28	20.04
PM003	0.53	26.58	13.23
PR004	0.41	25.29	3.03
FZ005	0.32	21.39	9.57
FZS006	0.36	24.36	10.24
UZ007	0.48	22.01	22.82
UZS008	0.51	21.95	22.37

ODR – optical density ratio; DH – degree of hydrolysis

4.1.2 Microbiological analyses

The results of the microbiological analyses are detailed in Appendix D and indicated that the microbiological quality of the product during handling and processing was to food grade standards. The microbial testing of the hydrolysate before heat inactivation indicates that the total plate count was <50 cfu/g and C.*perfringens* was <10 cfu/g. After heat inactivation, the total plate count was <60 cfu/g, *C.perfringens* <10 cfu/g, *Listeria* and *Salmonella* was not detected per 25g, and *Coliform* and *E.coli* <0.3 MPN/g. The microbiology results before heat inactivation indicates that under the hydrolysis conditions the growth of organisms were at acceptable levels.

4.1.3 Sensory analysis

The eight hydrolysate samples were screened for sensory attributes on their own and in combination with other stock. The hydrolysate samples had a very strong flavour note which was best described as a ‘liver’ flavour. This flavour was very strong and was over powering any other flavour note(s) in the hydrolysate samples. Diluting the hydrolysate, mixing the hydrolysates with commercially available stocks or with vegetable stock made in the sensory laboratory were not able to overcome the very strong liver flavour.

Due to this overpowering liver flavour which could not be masked with other flavour ingredients, it was decided that subjecting the hydrolysates to a trained sensory panel would not generate any meaningful results that would identify any differences in sensory attributes due to changes generated by the different enzyme hydrolysis conditions. Therefore, the samples were not subjected to detailed sensory analysis using a trained panel.

The flavour of the four commercial samples supplied by Wagstaff Food Services had a very ‘brothy’ aroma but the samples on their own (neat and diluted hydrolysate at 400C) were also described as very unpleasant to taste.

5 Conclusions and recommendations

The sensory screening indicated that the sensory attributes of all eight hydrolysate samples, irrespective of the enzyme and the enzyme reaction conditions used, had no impact on the strong liver flavour. The strong liver flavour could not be masked by mixing with commercially available stocks. This did not enable the determination of differences in the sensory attributes of the eight samples.

In order to further develop the hydrolysates as potential flavour ingredients, it is recommended that these products be sent to flavour houses for further evaluation. These hydrolysates may be incorporate as a constituent of meat flavour ingredients.

The majority of the meat flavour and bullion ingredients in the market are formulated with a combination of additives and protein hydrolysates to provide the targeted flavour profile. Although the hydrolysate on its own was not palatable, with appropriate product development, these ingredients may be able to provide the ‘meaty’ flavour attributes for flavour and bullion ingredients.

Plasma proteins on their own are an excellent source of protein to develop protein hydrolysates. The plasma fraction, which is mainly composed of BSA and IgG, may provide acceptable sensory attributes and may be similar to whey protein hydrolysates. As an alternative strategy, it is recommended that the sensory attributes of plasma proteins hydrolysed on their own, be subjected to a sensory panel and tested for their sensory properties.

Another strategy that could be investigated further is to use different enzymes, especially plant-based protease enzymes as they may provide different peptide characteristics which may result in different sensory attributes.

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7 Appendices

7.1 Appendix A – Procedure for hygienic collection of bovine blood at Wagstaff food services

1. Plastic containers containing citrate and sterilised by autoclaving at 121°C for 15min will be provided by CSIRO Food and Nutritional Sciences.
2. CSIRO Food and Nutritional Sciences will also send sterilised sample bottles to collect blood for microbiological analysis by Wagstaff Food Services.
3. Blood should be collected from healthy animals fit for human consumption.
4. Ensure hollow knife is clean and has been sterilised before use.
5. Sterilise the sticking area if possible by removing a portion of the hide in the area of the neck where sticking will take place.
6. Collect bovine blood into containers using hollow knife.
7. Each container will have a solution of sodium citrate equivalent to 5g/L.
8. Fill container to 1000ml mark. Do not overfill container.
9. Seal container immediately after collecting the blood and invert gently three times to mix the sodium citrate solution with the blood.
10. Store container on ice slurry (at or below 4°C) immediately.
11. Take a sample of blood for microbiological analysis and store on ice.
12. Record time when sample collected to bottles
13. Despatch samples of blood on ice (at or below 4°C) to CSIRO Food and Nutritional Sciences, 671 Sneydes Road, Werribee, Vic., immediately after collection.

    Contact CSIRO Food and Nutritional Sciences when blood samples are despatched from Wagstaff.

    Contacts at CSIRO Food and Nutritional Sciences:

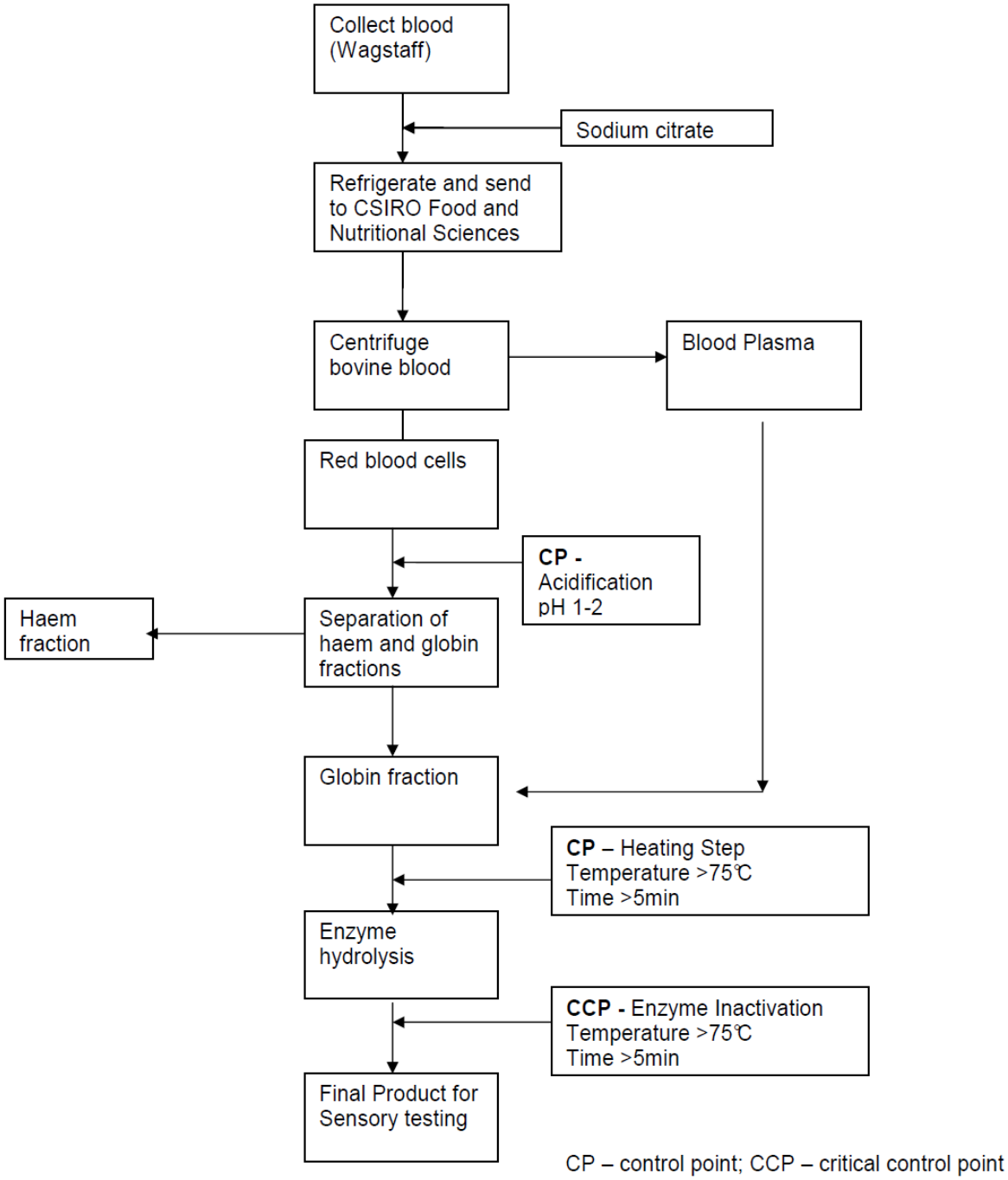
    Reception – 03 9731 3200

    Mr Kirthi De Silva – 03 9731 3319

    Ms Katherine Robinson – 03 9731 3352
14. Send certificate confirming that blood was collected from healthy animals fit for human consumption.
15. Send samples taken for microbial testing to Wagstaff microbiology testing laboratory.

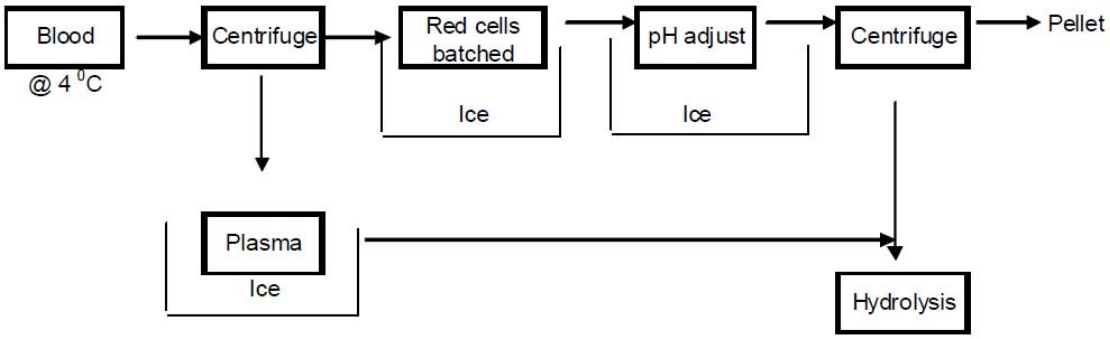
Send microbiology results to CSIRO Food and Nutritional Sciences when available.

7.2 Appendix B – Process flow chart of bovine blood processing from collection to final sensory ingredient



CP – control point; CCP – critical control point

Centrifugation and Batching of Blood



7.3 Appendix C - HACCP food safety plan

Food Science Australia

BOVINE BLOOD PROCESSING HACCP FOOD SAFETY PLAN

Issue Date: 20th February 2009 ; Current version date: 29th May 2009
Appendix: Bovine Blood - Process Flow Diagram

HAZARD
M-Microbiological
C-Chemical
P-Physical
R-Radioactive

RISK
A-High, likely to happen
B-Medium, could happen
C-Low, not likely to happen

RESULT
1-Critical, automatically results in unsafe product
2-Serious, probably result in unsafe product
3-Major, may result in unsafe product with potentially serious consequences
4-Minor, may result in unsafe product with no serious consequences
5-None, will not result in unsafe product

PRODUCT DESCRIPTION
Proteins from bovine blood

Step	CP/ CCP	Critical Operation	Potential Risk	HAZ	Risk	Control Point	Inspection Frequency	Person Responsible	Specification	Corrective Action	Records
1	CP	Origin of the raw material – bovine blood	Microbial growth, chemical & physical	M, C, P	B	Obtain documentation from supplier certifying that bovine blood is not contaminated, and supplier has their own HACCP food safety plan. Blood supplied from healthy animals fit for human consumption.	Before use	Wagstaff	Bovine blood supplied to FSA must be free of contaminants. Blood must be collected from healthy animals fit for human consumption (free from bovine spongiform encephalopathy).	Reject if certification not supplied. Check microbial levels of finished product before release.	P:\Protein\Wagstaff\Literature\HACCP
2	CP	Handling - blood collection	Microbial growth	M	B	Blood must be collected hygienically from animals and cooled to <4°C immediately.	Before use	Wagstaff	Wagstaff must use hygienic method for blood collection. Hollow knife method is regarded as hygienic.	Check microbial levels of finished product before release.	P:\Protein\Wagstaff\Literature\HACCP
3	CP	Cold storage at Wagstaff and transport	Microbial growth	M	B	Use refrigerated transport and store immediately upon arrival at Food Science Australia, Werribee.	Before use	Wagstaff	Use refrigerated transport < 4°C	Check microbial levels of finished product before release.	P:\Protein\Wagstaff\Literature\HACCP
4	CP	Receive blood at Food Science Australia	Microbial growth	M	B	Store blood at <4°C upon arrival at Food Science Australia.	Before use	Project Staff (Kirthi Desilva, Li hui Liu, and Katherine Robinson)	Store raw blood products in cool room.	Check microbial levels of finished product before release.	P:\Protein\Wagstaff\Literature\HACCP
5	CP	Centrifugation of bovine blood	Chemical & microbial contamination	C,M	B	Use food grade centrifuge bottles. Bottles autoclaved before use. Wipe bottles with disinfectant. Centrifuge at <10°C.	Visual inspection before use	Project Staff	Wipe centrifuge containers with Virkon disinfectant	Clean containers. Check microbial levels before product release.	P:\Protein\Wagstaff\Literature\HACCP

Step	CP/ CCP	Critical Operation	Potential Risk	HAZ	Risk	Control Point	Inspection Frequency	Person Responsible	Specification	Corrective Action	Records
6	CP	Acidification of bovine blood	Chemical	C	C	Bovine blood acidified to pH 1-2	Before release	Project Staff	Acid used for pH adjustment is food grade.	Reject if non-food grade acid used.	P:\Protein\Wagstaff\Literature\HACCP
7	CP	Separation of globin fraction from haem	Chemical & microbial contamination	C,M	B	All equipment in contact must be autoclaved before use. All chemicals must be food grade quality. Separate at <10°C.	Visual inspection before use	Project Staff	Use Food grade chemicals only	Clean containers. Check microbial levels of finished product before release.	P:\Protein\Wagstaff\Literature\HACCP
8	CP	Heating Step	Microbial	M	C	Heating above 70°C for >5min.	Before release	Project Staff	Temperature >72°C and time >5min	Check microbial levels of finished product before release.	P:\Protein\Wagstaff\Literature\HACCP
9	CP	Enzyme hydrolysis of globin fraction with blood plasma	Microbial contamination	M	B	All equipment must be autoclaved before use. All enzymes must be food grade quality.	Before use	Project Staff	Use Food grade enzyme only. Sanitise all equipment before use.	Check microbial levels of finished product before release.	P:\Protein\Wagstaff\Literature\HACCP
10	CP	Microbial Analysis	Microbial contamination	M	C	Determine microbial growth during hydrolysis.	Before release	Project Staff	E.coli Coliforms Listeria Salmonella Clostridium perfringens Total plate count	Reject samples if microbiological levels are not within specification.	P:\Protein\Wagstaff\Literature\HACCP
11	CCP	Enzyme inactivation	Microbial	M	C	Enzyme inactivation by heating above 75°C for >5min.	Before release	Project Staff	Temperature >75°C and time>5min Check temperature indicator against a calibrated temperature indicator.	Re-heat to achieve time/temperature. Check microbial levels of finished product before release.	P:\Protein\Wagstaff\Literature\HACCP
12	CP	Storage	Microbial contamination	M	B	Freeze all liquid samples at <-15°C.	Before release	Project Staff	Store liquid samples in freezer.	Check microbial levels of finished product before release.	P:\Protein\Wagstaff\Literature\HACCP
13	CP	Microbial Analysis	Microbial contamination	M	C	Use refrigerated transport.	Before release	Project Staff	E.coli Coliforms Listeria Salmonella Clostridium perfringens Total plate count	Reject samples if microbiological levels are not within specification.	P:\Protein\Wagstaff\Literature\HACCP
14	CP	Transport	Microbial contamination	M	C	Use refrigerated transport.	Before use	Project Staff at North Ryde	Store in freezer on arrival	Reject if integrity of packaging is damaged.	P:\Protein\Wagstaff\Literature\HACCP

7.4 Appendix D – Microbiological analysis of eight flavour ingredients produced using proteolytic enzymes

Microbiological testing of the production samples were conducted before and after heat inactivation of the enzymes. A sample was tested before heat inactivation to test for standard plate count and *Clostridium perfringens* and 5 samples from each batch of hydrolysates were tested after heat inactivation.

Before heat inactivation

Analysis	FZ005	FZ006	UZ007	UZ008	PR004	PM003	FP001	PX002
Standard plate count (cfu/g)	2.0×10^1	1.9×10^1	1.8×10^1	7.0×10^1	2.5×10^1	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Clostridium Perfringens (cfu/g)	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$

Samples (details of hydrolysis conditions are in Table 1): FP001 - Fungal protease 31,000; PX002 - Protex 6L; PM003 - Protamex; PR004 - Peptidase R; FZ005 - Flavourzyme 500 MG; UZ007 - Umamizyme

Cfu – colony forming units

MPN – most probable number

After heat inactivation – 5 samples per batch was tested for microbiology

Analysis	FZ005-1	FZ005-2	FZ005-3	FZ005-4	FZ005-5
Coliforms (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
E.coli (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
Listeria /25g	Absent	Absent	Absent	Absent	Absent
Salmonella /25g	Not detected	Not detected	Not detected	Not detected	Not detected
Clostridium perfringens (cfu/g)	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$
Standard plate count (cfu/g)	$<1 \times 10^1$	6×10^1	$<1 \times 10^1$	4×10^1	3×10^1

Analysis	FZ006-1	FZ006-2	FZ006-3	FZ006-4	FZ006-5
Coliforms (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
E.coli (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
Listeria /25g	Absent	Absent	Absent	Absent	Absent
Salmonella /25g	Not detected	Not detected	Not detected	Not detected	Not detected
Clostridium perfringens (cfu/g)	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$
Standard plate count (cfu/g)	1×10^1	$<1 \times 10^1$	2.5×10^1	1×10^1	1×10^1

Analysis	UZ007-1	UZ007-2	UZ007-3	UZ007-4	UZ007-5
Coliforms (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
E.coli (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
Listeria /25g	Absent	Absent	Absent	Absent	Absent
Salmonella /25g	Not detected	Not detected	Not detected	Not detected	Not detected
Clostridium perfringens (cfu/g)	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1
Standard plate count (cfu/g)	1.5 X 10^1	6 X 10^1	3.5 X 10^1	3 X 10^1	3.5 X 10^1

Analysis	UZ008-1	UZ008-2	UZ008-3	UZ008-4	UZ008-5
Coliforms (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
E.coli (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
Listeria /25g	Absent	Absent	Absent	Absent	Absent
Salmonella /25g	Not detected	Not detected	Not detected	Not detected	Not detected
Clostridium perfringens (cfu/g)	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1
Standard plate count (cfu/g)	1 X 10^1	<4.5 X 10^1	3.5 X 10^1	3.5 X 10^1	2.5 X 10^1

Analysis	PR004-1	PR004-2	PR004-3	PR004-4	PR004-5
Coliforms (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
E.coli (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
Listeria /25g	Absent	Absent	Absent	Absent	Absent
Salmonella /25g	Not detected	Not detected	Not detected	Not detected	Not detected
Clostridium perfringens (cfu/g)	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1
Standard plate count (cfu/g)	<1 X 10^1	<1 X 10^1	1.5 X 10^1	<1 X 10^1	1.7 X 10^1

Analysis	PM003-1	PM003-2	PM003-3	PM003-4	PM003-5
Coliforms (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
E.coli (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
Listeria /25g	Absent	Absent	Absent	Absent	Absent
Salmonella /25g	Not detected	Not detected	Not detected	Not detected	Not detected
Clostridium perfringens (cfu/g)	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1
Standard plate count (cfu/g)	<1 X 10^1	<1 X 10^1	<1 X 10^1	1.4 X 10^1	1 X 10^1

Analysis	FP001-1	FP001-2	FP001-3	FP001-4	FP001-5
Coliforms (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
E.coli (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
Listeria /25g	Absent	Absent	Absent	Absent	Absent
Salmonella /25g	Not detected	Not detected	Not detected	Not detected	Not detected
Clostridium perfringens (cfu/g)	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1
Standard plate count (cfu/g)	1 X 10^1	<1 X 10^1	1 X 10^1	1 X 10^1	1 X 10^1

Analysis	PX002-1	PX002-2	PX002-3	PX002-4	PX002-5
Coliforms (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
E.coli (MPN) / g	<0.3	<0.3	<0.3	<0.3	<0.3
Listeria /25g	Absent	Absent	Absent	Absent	Absent
Salmonella /25g	Not detected	Not detected	Not detected	Not detected	Not detected
Clostridium perfringens (cfu/g)	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1	<1 X 10^1
Standard plate count (cfu/g)	<1 X 10^1	<1 X 10^1	1 X 10^1	1 X 10^1	1 X 10^1

7.5 Appendix E – hygienic blood collection certification from Wagstaff

WAGSTAFF TRADING PTY LTD
ACN: 127 382 601 ABN: 44 722 080 312
1500 THOMPSONS ROAD, CRANBOURNE VIC 3977
PO BOX 110, CRANBOURNE VIC 3977
Phone: (03) 5996 0488 Fax: (03) 5996 0275

15 June 2009

Fax 03 9731 3390

Kirthi De Silva
Food Science Australia
671 Sneydes Rd
Werribee 3030
VICTORIA

Dear Sir,

RE: Bovine Blood Certificate

The bovine blood (fourteen litres) supplied 15 June 2009 was collected from animals that passed ante and posted mortem inspection

The blood was collected between 5.30am and 6.30am 15 June 2009 and placed on ice in transport eskies to achieve 4°C prior to shipment.

Temperature of blood at collection was 38°C and the ice slurry in esky was 0°C

Yours sincerely

W G Rijnbeek