



The effects of lupin (*Lupinus angustifolius*) addition to wheat bread on its nutritional, phytochemical and bioactive composition and protein quality



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ABSTRACT

The grain legume Australian sweet lupin (*Lupinus angustifolius*; ASL) is gaining international interest as a functional food ingredient; however its addition to refined wheat bread has been shown to decrease bread volume and textural quality, the extent of which is influenced by ASL variety. The present study evaluated the effects of ASL incorporation (20% of total flour) of the six commercial varieties; *Belara*, *Coromup*, *Gungurru*, *Jenabillup*, *Mandelup* and *Tanjil*, on the level of nutritional, phytochemical and bioactive composition and protein quality of refined wheat flour bread. Protein, dietary fiber, phenolic and carotenoid content, antioxidant capacity and protein digestibility corrected amino acid score (PDCAAS) were higher ($p < 0.05$), whereas available carbohydrate level was lower ($p < 0.05$) in ASL-wheat breads than the wheat-only bread, regardless of the ASL variety used. In addition, the blood-glucose lowering bioactive peptide γ-conglutin was detected in all ASL-wheat breads but not in wheat-only bread. The ASL variety used significantly ($p < 0.05$) affected the dietary fiber, fat, available carbohydrates and polyphenolic level, the antioxidant capacity and the PDCAAS of the ASL-wheat breads. These findings demonstrate the potential nutritional and health benefits of adding ASL to refined wheat bread and highlight the importance of selecting specific ASL varieties to maximise its nutritional attributes.

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1. Introduction

Bread is traditionally produced using wheat flour due to its gluten-forming proteins, gliadins and glutenins (Goesaert et al., 2005), that help provide the desired bread texture and volume. However, refining wheat reduces its nutritional quality through significant losses in protein, dietary fiber, vitamins, minerals and phytochemicals (Rosell, 2011). Non-wheat flours from cereals and grain legumes have been added to refined wheat flour bread to improve its nutritional value and meet consumer demands for healthier bread. Australian sweet lupin (ASL), so named due to its very low level of bitter alkaloids, is a

grain legume, underutilized as human food, that may enhance the nutritional profile of wheat bread.

Australian sweet lupin is a rich source of proteins and dietary fiber (Hall & Johnson, 2004), carotenoids (Wang, Errington, & Yap, 2008), phenolics (Oomah, Tiger, Olson, & Balasubramanian, 2006), and a range of vitamins and minerals. However, comparative studies on human nutrition and health related composition of different commercial ASL varieties are lacking. There is growing international interest in the use of ASL flour in baked goods (Hall & Johnson, 2004; Jayasena & Nasar-Abbas, 2011; Nasar-Abbas & Jayasena, 2012) due in part to its non-genetically modified and low phytoestrogen status (Sirtori, Arnoldi, & Johnson, 2005).

Studies show that substitution of wheat by lupin flour can significantly improve the protein and dietary fiber contents of wheat bread (Belski et al., 2011; Hall & Johnson, 2004; Mubarak, 2001). The amino acid profile of lupin has been reported to complement that of wheat, since it is higher in lysine but lower in sulfur-containing amino acids (e.g. methionine). Therefore, addition of ASL flour into wheat bread has the potential, not just to increase the protein content, but also improve the protein quality of the final product (Duodu &

Abbreviations: ASL, Australian sweet lupin; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; GAE, Gallic acid equivalents; HPLC, High performance liquid chromatography; IVPD, In vitro protein digestibility; LOX, Lipoxygenase; PDCAAS, Protein digestibility corrected amino acid score; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAC, Total antioxidant capacity; TBST, Tris-buffered saline/Tween; TCA, Trichloroacetic acid; TDF, Total dietary fiber; TE, Trolox equivalents; TPC, Total polyphenolic content

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Minnaar, 2011). However there is a lack of information on the protein digestibility, an important component of protein quality, of ASL–wheat bread.

Addition of ASL to refined wheat bread has been reported to decrease its glycemic index (Hall, Thomas, & Johnson, 2005) and ASL-containing foods have demonstrated potential through clinical trials to decrease risk factors for obesity (Lee et al., 2006), cardiovascular disease (Belski et al., 2011) and gastrointestinal problems (Johnson, Chua, Hall, & Baxter, 2006). Lupin also contains the protein, γ -conglutin, reported to have blood-glucose lowering properties in animal (Lovati et al., 2012) and human (Bertoglio et al., 2011) post-prandial studies.

We have shown in a previous study (Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2015) that addition of ASL flour, regardless of variety decreased volume and texture of refined wheat breads and that the extent of these effects was dependent on the ASL variety used. The present study aimed to assess the effects of addition of ASL flour from different varieties on the nutritional, phytochemical and bioactive composition and protein quality of wheat bread.

2. Materials and methods

2.1. Materials

The materials previously described in Villarino, Jayasena, Coorey, Chakrabarti-Bell, and Johnson (2014) were utilized in this study. Whole seeds of six ASL varieties, *Belara*, *Coromup*, *Gungurru*, *Jenabillup*, *Mandelup* and *Tanjil*, grown in the same agricultural region and harvest year, were provided by the Department of Agriculture and Food Western Australia. The seeds were dehulled (LH 5095 dehuller, Codema Inc., MN, USA), followed by air-induced and manual separation of the kernel from the seed coat and milling (Retsch ZM200, Retsch GmbH, Haan, DE) 100% through a sieve size of 250 μ m, then vacuum-packed in plastic bags and stored at ~ 10 °C until use. Two replicates of 1 kg flour samples were produced for each ASL variety. Details of other bread making ingredients (i.e. Western Australian bakers flour, yeast, bread improver, sugar, salt and vegetable) were described in Villarino et al. (2014).

2.2. Experimental design and bread making

Duplicate batches of seven types of bread buns were prepared using ASL–wheat composite flour from each of the six ASL varieties and one refined wheat flour-only control. Randomization of baking runs has been previously described (Villarino et al., 2014). The sponge and dough method (Villarino et al., 2014) was used in this study. For each batch, a 500 g dough was prepared that comprised of ASL (20%)–wheat (80%) composite flour (58.7% of total ingredients) and water (36.7%). The remainder of the ingredients were yeast (1.5%), vegetable fat (1.1%), bread improver (0.8%), salt (0.6%) and sugar (0.6% ingredients). The wheat-only bread was prepared similarly. Three buns were randomly selected from each treatment and freeze-dried prior to analysis.

2.3. Proximate and dietary fiber analyses

All analyses were conducted in at least duplicate. Proximate composition was determined using AOAC Methods (AOAC, 2008). Moisture content was determined using the AOAC oven drying method, 925.10; crude protein by Kjeldahl digestion and distillation ($N \times 5.7$ for wheat-only bread; $N \times 5.66$ for ASL–wheat bread) was measured according to the AOAC Method 920.87. Total dietary fiber (TDF) content was determined by the enzymatic gravimetric method using the Megazyme TDF Kit KTDFR (Bray, Co., Wicklow, Ireland). Crude fat was determined by petroleum ether extraction (Buchi E-816, Flawil, Switzerland) using AOAC Method 945.16 and ash was determined by

dry-ashing at 550 °C according to the AOAC Method 923.03. Total available carbohydrates were calculated by difference, i.e. $100 - (\% \text{ moisture} + \% \text{ protein} + \% \text{ fat} + \% \text{ ash} + \% \text{ TDF})$. All values were expressed as g/100 g dry basis (db).

2.4. Protein quality

2.4.1. Amino acid content

Amino acid profile of the breads was analyzed by the ChemCentre (Bentley, WA, Australia) using pre-column derivatization followed by high-performance liquid chromatography (HPLC) with UV detection. The bread samples were hydrolyzed with 6 N HCl for 24 h. After hydrolysis, the solution was diluted, filtered and neutralized with NaOH(aq) (Llames & Fontaine, 1994). All amino acids were determined individually, except for cysteine and cystine which were degraded to cysteic acid and were reported as a combined outcome based on AOAC Method 994.12 (AOAC, 2008). Tryptophan was not measured, due to the limitation of the available method for quantifying this amino acid. The amino acid content (mg/g protein) was calculated by dividing the individual amino acid content of the bread (mg/100 g db) with the protein content of the bread (g/100 g db).

2.4.2. Amino acid scoring

Amino acid scores were calculated according to FAO/WHO (1991), by dividing the amino acid content of the bread samples (mg/g protein) by the suggested reference pattern of amino acid requirements (mg/g protein) for pre-school children (2–5 years old) for nine essential amino acids plus tyrosine and cysteine as follows: Histidine, 19; isoleucine, 28; leucine, 66; lysine, 58; methionine + cysteine, 25; phenylalanine + tyrosine, 63; threonine, 34; valine, 35 (FAO/WHO, 1991).

2.4.3. In-vitro protein digestibility (IVPD) and protein digestibility corrected amino acid score (PDCAAS)

In-vitro protein digestibility of the breads was determined following the modified pepsin pancreatin digestion method (Akeson & Stahmann 1964; Faki, Venkatarama, & Desikachar, 1984). Protein digestibility of foods is frequently assessed using in-vivo rat experimental models as originally proposed by Sarwar and McDonough (1990). Aside from in-vivo methods, FAO/WHO also recommends the use of in-vitro methods using enzymes (FAO/WHO 1991).

Bread samples equivalent to approximately 50 mg protein were incubated at 37 °C with 0.75 mg pepsin (2500 units/mg activity; Chem-Supply, Gillman, SA, Australia) in 7.5 ml of 0.1 N HCl for 3 h. The solution was then neutralized with 3.75 ml of 0.2 N NaOH. Following this, 2 mg pancreatin (Chem-Supply, Gillman, SA, Australia) in 3.75 ml of pH 8.0 phosphate buffer was added and the sample incubated for 24 h at 37 °C. The undigested protein in 5 ml of digesta was then precipitated by addition of 25 ml of 10% TCA and the sample centrifuged for 30 min at $1000 \times g$ at room temperature. Nitrogen in the supernatant was determined using the Kjeldahl digestion and distillation method (Section 2.3). The IVPD was calculated according to Eq. (3.2). PDCAAS was determined by multiplying the IVPD with the limiting amino acid score (lowest score for an individual amino acid).

$$\text{IVPD (\%)} = \left(\frac{\text{Total nitrogen (g/100 ml)} - \text{nitrogen in the supernatant (g/100 ml)}}{\text{Total nitrogen g/100 ml}} \right) \times 100 \quad 3.2$$

2.5. Estimation of γ -conglutin

2.5.1. Extraction of proteins

Extractions of flour and bread proteins were performed according to the method of Capraro, Magni, Fontanesi, Budelli, and Duranti (2008). Two milligram of flour or 5 mg of bread were weighed into 1.5 ml

Eppendorf tubes and suspended in 100 µl of extraction solution comprising of 8 mol L⁻¹ urea, 20 mg/ml CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and 65 mmol L⁻¹ 1,4-dithiothreitol. Extraction was carried out with shaking at room temperature for 2 h. The slurry was centrifuged at 10,000 × g for 30 min at room temperature, and the supernatant containing the dissolved protein was stored at 80 °C until use.

2.5.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE of the extracted flour and bread proteins under reducing conditions was performed according to Wong, Pitts, Jayasena, and Johnson (2013) using NuPAGE Novex 10% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). The protein extracts were diluted with NuPAGE sample buffer (Invitrogen) to give 5 µg of protein in the 10 µl of final solution that was loaded onto the gel. Electrophoresis was performed with MES SDS running buffer (Invitrogen) at 200 V for 1 h until the electrophoretic front was approximately 1 cm from the bottom of the gel. Proteins were fixed and stained using 50 ml Bio Safe Coomassie G-250 stain (Bio-Rad Laboratories, Hercules, CA, USA). Destaining was performed by soaking the stained gels five times with deionized water. The molecular weights of the major peptide bands in the samples were estimated by comparison of their migration compared to bands of molecular weight markers (Prestained SDS-PAGE standards, broad range, Bio-Rad). The lupin protein subunits (α, β, γ and δ congenitins) were then tentatively identified by comparing their estimated molecular weights with literature values and subjectively quantified by visual assessment of band staining intensity.

2.5.3. Immunoblotting

Verification of the identity of γ-conglutin peptides was performed using immunoblotting based on the method of Foley and Singh (2002). Unstained SDS-PAGE gels (prepared as described in Section 2.5.2) were placed in transfer chambers filled with 1 L transfer buffer (100 ml of tris-base glycine solution [24 g tris-base and 112 g glycine per 1 L distilled water], 2.5% SDS, 200 ml of methanol and 700 ml of distilled water). The separated protein bands on the SDS-PAGE gels were transferred by electroblotting (Mini Trans-Blot® Cell, Bio-Rad Laboratories, Hercules, CA, USA) onto nitrocellulose filters (Amersham Hybond-C, GE Healthcare Australia Pty. Ltd., Rydalmere, NSW, Australia) overnight at 25 V and 4 °C with stirring. The nitrocellulose filter was then incubated with ~30 ml of a Tris-buffered saline/Tween (TBST) solution for 30 min at room temperature with gentle shaking. The TBST solution was prepared by mixing 50 mmol L⁻¹ tris(hydroxymethyl)aminomethane (pH 7.5), 200 mmol L⁻¹ NaCl solution and 0.05% (v/v) Tween-20. The nitrocellulose filter was then incubated in 20 ml of TBST blocking solution that containing 20 µl of a primary antibody overnight at room temperature with gentle shaking. The TBST blocking solution was prepared by adding 10% (w/v) skim milk powder (Diploma, Fonterra, Auckland, New Zealand), to the TBST solution. Synthesis of the synthetic peptides used in the development and production of the polyclonal serum

against γ-conglutin proteins were made by Mimotopes Pty Ltd (Clayton, VIC, Australia). Five milligrams of the synthesized peptide was conjugated to a keyhole limpet hemocyanin carrier and maleimidocaproyl-N-hydroxysuccinimide linker. Rabbit serum containing the polyclonal antibodies raised against γ-conglutin, was produced by IMVS Pathology, Veterinary Services Division (University of Adelaide, Adelaide, SA, Australia).

After the incubation with the primary antibody, the filter was washed (3×, 10 min each wash) with TBST solution. The nitrocellulose filter was then incubated in 20 ml TBST solution containing 0.4 µl secondary antibody (monoclonal anti rabbit IgG alkaline phosphatase conjugated [Sigma-Aldrich, St. Louis, MO, USA]) for 30 min at room temperature with gentle shaking. Following this, the filter was washed with TBST solution (3×, 10 min each wash). The nitrocellulose filter was then incubated with 1–2 ml of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Sigma-Aldrich, St. Louis, MO, USA) until intense bluish purple bands representing reactivity with γ-conglutin developed (~1 to 2 min).

2.6. Phytochemical analysis

2.6.1. Carotenoids

2.6.1.1. Extraction. Following the procedure of Fanning et al. (2010), bread samples (0.4 g) were mixed with 5 ml of acetone and vortexed. Ten milliliters of hexane and 5 ml of 10% NaCl(aq) was then added and re-vortexed and centrifuged at 5000 × g for 4 min at 4 °C. The top hexane layer containing the extracted carotenoids was transferred to clean tubes. A further 10 ml of hexane was added to the bottom aqueous layer, and the extraction was repeated. Further aliquots of hexane were used for extraction until the hexane layer was colorless. The combined hexane extracts were dried in a centrifugal evaporator prior to reconstitution in 2 ml of 50:50 (v/v) methanol/dichloromethane.

2.6.1.2. Analysis, identification, and quantification. The extracts were analyzed using HPLC as described by Fanning et al. (2010), using a diode YMC C30 Carotenoid Column, 3 µm, 4.6 × 250 mm (Waters, Milford, MA, USA) and a SPD-M10 A VP diode array detector (Shimadzu, Kyoto, Japan). Individual carotenoids (i.e. lutein, zeaxanthin, alpha-carotene and beta-carotene) were identified by comparison with retention times and absorption spectra of carotenoid standards. Standard curves were constructed for each carotenoid using concentrations ranging from 0.03 to 10 µg ml⁻¹. Carotenoid concentrations were expressed as µg g⁻¹ dry basis. Total carotenoids were calculated by summing the amount of individual carotenoids.

2.6.2. Phenolics and antioxidant capacity

2.6.2.1. Extraction. The extraction of breads was performed as described by Martinez-Villaluenga et al. (2009). One gram of sample was added to 10 ml of 80% methanol and shaken for 2 h at 37 °C. The mixture was centrifuged at 4000 × g for 10 min at 20 °C and the supernatant was

Table 1
Proximate and dietary fiber composition of ASL-wheat and wheat-only breads.¹

Bread	Protein ² (g/100 g)	Fat ² (g/100 g)	Ash ² (g/100 g)	Total dietary fiber ² (g/100 g)	Total available carbohydrates ² (g/100 g)
Belara-wheat	18.7 ± 0.6 ^{a*}	5.7 ± 0.6 ^{d*}	2.5 ± 0.4 ^a	15.8 ± 0.5 ^{bc*}	57.2 ± 0.3 ^{a*}
Coromup-wheat	19.3 ± 0.3 ^{a*}	4.4 ± 0.2 ^{c*}	2.3 ± 0.3 ^a	16.2 ± 0.6 ^{c*}	57.9 ± 0.5 ^{a*}
Gungurru-wheat	19.2 ± 0.8 ^{a*}	3.2 ± 0.2 ^{ab}	2.7 ± 0.4 ^a	14.8 ± 0.6 ^{ab*}	60.1 ± 0.4 ^{b*}
Jenabillup-wheat	19.1 ± 0.2 ^{a*}	3.5 ± 0.5 ^b	2.5 ± 0.3 ^a	14.6 ± 0.5 ^{a*}	60.3 ± 0.5 ^{b*}
Mandelup-wheat	18.9 ± 1.4 ^{a*}	2.6 ± 0.4 ^a	2.1 ± 0.4 ^a	15.1 ± 0.3 ^{ab*}	61.3 ± 1.1 ^{b*}
Tanjil-wheat	19.0 ± 0.7 ^{a*}	3.9 ± 0.6 ^{bc}	2.6 ± 0.4 ^a	14.6 ± 0.2 ^{a*}	59.5 ± 0.7 ^{b*}
Wheat-only	13.4 ± 0.4	3.4 ± 0.5	2.0 ± 0.3	9.2 ± 1.8	71.9 ± 0.2

¹ Means ± standard deviation (expressed as dry basis).

² Values within a column for ASL-wheat breads with different superscript letter denote significant difference (p < 0.05) using Duncan's test.

* Denotes significant difference (p < 0.05) from wheat-only bread using Dunnett's test.

Table 2Essential amino acid content of ASL–wheat and wheat-only breads.^{1,2}

Amino acid	Bread						
	Belara–wheat	Coromup–wheat	Gungurru–wheat	Jenabillup–wheat	Mandelup–wheat	Tanjil–wheat	Wheat-only
Histidine ³	25.4 ± 1.1 ^a	21.2 ± 1.1 ^a	24.0 ± 0.7 ^a	25.7 ± 1.5 ^a	24.9 ± 2.2 ^a	24.5 ± 1.1 ^a	25.7 ± 0.5
Isoleucine ³	43.8 ± 0.0 ^a	42.5 ± 0.5 ^{a*}	43.3 ± 0.0 ^a	43.0 ± 0.0 ^{a*}	43.1 ± 0.4 ^a	42.3 ± 0.4 ^{a*}	44.4 ± 0.5
Leucine ³	81.2 ± 1.1 ^e	78.5 ± 0.4 ^{bc*}	78.2 ± 0.0 ^b	79.5 ± 0.4 ^{cd*}	80.2 ± 0.4 ^{de}	76.8 ± 0.7 ^{a*}	84.3 ± 0.0
Lysine ³	28.0 ± 1.9 ^{a*}	26.9 ± 0.7 ^{a*}	23.4 ± 0.0 ^{a*}	26.8 ± 0.0 ^{a*}	28.1 ± 0.7 ^{a*}	22.4 ± 2.6 ^{a*}	17.2 ± 0.0
Methionine + cysteine ³	32.6 ± 0.8 ^{b*}	30.0 ± 1.5 ^{ab*}	28.1 ± 0.7 ^{a*}	31.2 ± 0.4 ^{b*}	31.5 ± 0.4 ^{b*}	32.4 ± 1.9 ^{b*}	38.4 ± 0.5
Phenylalanine + tyrosine ³	97.0 ± 1.9 ^{a*}	91.4 ± 2.6 ^{a*}	93.8 ± 1.5 ^{a*}	94.4 ± 2.2 ^{a*}	93.7 ± 0.7 ^{a*}	91.5 ± 1.5 ^{a*}	102.9 ± 1.1
Threonine ³	44.6 ± 0.4 ^e	41.9 ± 0.0 ^{b*}	40.6 ± 0.7 ^{a*}	43.3 ± 0.4 ^{cd*}	43.7 ± 0.4 ^{de*}	42.3 ± 0.4 ^{bc*}	45.5 ± 0.0
Valine ³	46.2 ± 0.4 ^{e*}	44.3 ± 0.4 ^{c*}	43.3 ± 0.0 ^{b*}	43.3 ± 0.4 ^{b*}	45.0 ± 0.0 ^{d*}	42.1 ± 0.0 ^{a*}	48.1 ± 0.5

¹ mg/g protein dry basis.² Means ± SD.³ Values within a row with different superscript denotes significant difference ($p < 0.05$) using Duncan's test.* Denotes significant difference ($p < 0.05$) with wheat flour using Dunnett's test.

filtered and stored at -20°C in the dark until analysis for total phenolic content (TPC) and antioxidant capacity (TAC).

2.6.2.2. Total phenolic content. The determination of TPC was performed as previously described by Adom and Liu (2002). Fifty microliters of extract was diluted with 650 μl ultrapure water, then 50 μl Folin-Ciocalteu reagent was added and the sample was neutralized with 500 μl of 7% sodium carbonate. After 90 min standing at room temperature, the absorbance was measured at 750 nm against a blank of 80% methanol. A calibration curve of 0–250 $\mu\text{g}/\text{ml}$ gallic acid (in 80% methanol) was used. The TPC was expressed as mg gallic acid equivalents (GAE)/g dry basis.

2.6.2.3. Total antioxidant capacity. Total antioxidant capacity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method modified from that of Martinez-Villaluenga et al. (2009). The sample extract (100 μl) was mixed with 250 μl of freshly prepared 0.6 mM DPPH in 80% methanol and 2 ml of 80% methanol then shaken for 40 min at room temperature in the dark. Absorbance was then measured at 517 nm against a blank comprised of 100 μl Trolox, 250 μl methanolic DPPH solution, and 2 ml 80% methanol. A standard curve of 0–150 $\mu\text{g}/\text{L}$ Trolox (dissolved in 80% methanol) was used. Antioxidant capacity of was expressed as μmol Trolox equivalents (TE)/g dry basis.

2.7. Statistical analysis

One-way analysis of variance was used to compare means of at least duplicate analyses per treatment followed by Duncan's multiple range test to separate the means when F was significant. Additionally, Dunnett's test was used as a post-hoc operation to compare mean values of wheat-only bread against those of each ASL–wheat bread. All tests

were performed using IBM SPSS Statistics V.21 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Proximate composition and dietary fiber

The proximate and total dietary fiber composition of the ASL–wheat and wheat-only bread are given in Table 1. Protein content of the ASL–wheat breads ranged from 18.7 to 19.3 g/100 db. All of the ASL–wheat breads had a significantly higher protein content than the wheat-only bread, increasing the protein content by ~42%. There was no varietal effect on the protein content of the ASL–wheat breads; possibly due to the relatively low level of ASL flour incorporation, since we have previously reported the ASL flour did differ in their protein content (Villarino et al., 2015).

The TDF content of the ASL–wheat breads ranged from 14.6 to 16.2 g/100 g db corresponding to an increase in fiber by approximately 75% from the wheat-only bread value. A small but statistically significant varietal effect on the total dietary fiber content of the ASL wheat-bread was observed, related to the differences in TDF of the ASL flours previously reported (Villarino et al., 2015).

The fat content of ASL–wheat breads ranged from 3.2 to 5.7 g/100 g db. Only ASL–wheat breads incorporating Belara and Coromup had slightly but significantly greater fat content than the wheat-only bread. There was a significant varietal effect on fat content of the ASL–wheat breads of which that incorporating Belara had the highest ($p < 0.05$) and that incorporating Mandelup the lowest ($p < 0.05$) fat content.

The ash contents of the ASL–wheat breads ranged from 2.1 to 2.7 g/100 g db which were not significantly different compared to wheat-only bread. No varietal effect ($p > 0.05$) was observed among the ASL–wheat breads.

Table 3Amino acid scores of the essential amino acids of ASL–wheat and wheat-only breads.^{1,2}

Amino acid	Bread						
	Belara–wheat	Coromup–wheat	Gungurru–wheat	Jenabillup–wheat	Mandelup–wheat	Tanjil–wheat	Wheat-only
Histidine	1.3 ± 0.1 ^a	1.1 ± 0.2 ^a	1.3 ± 0.0 ^a	1.4 ± 0.1 ^a	1.3 ± 0.1 ^a	1.3 ± 0.1 ^a	1.4 ± 0.0
Isoleucine	1.6 ± 0.0 ^a	1.5 ± 0.0 ^{a*}	1.5 ± 0.0 ^a	1.5 ± 0.0 ^{a*}	1.5 ± 0.0 ^a	1.5 ± 0.0 ^{a*}	1.6 ± 0.0
Leucine	1.3 ± 0.8 ^{e*}	1.2 ± 0.0 ^{bc*}	1.2 ± 0.0 ^{b*}	1.2 ± 0.0 ^{cd*}	1.2 ± 0.0 ^{de*}	1.2 ± 0.0 ^{a*}	1.3 ± 0.0
Lysine	0.5 ± 0.0 ^{a*}	0.5 ± 0.0 ^{a*}	0.4 ± 0.0 ^{a*}	0.5 ± 0.0 ^{a*}	0.5 ± 0.0 ^{a*}	0.4 ± 0.0 ^{a*}	0.3 ± 0.0
Methionine + cysteine	1.3 ± 0.0 ^{b*}	1.2 ± 0.1 ^{ab*}	1.1 ± 0.0 ^{a*}	1.2 ± 0.0 ^{b*}	1.5 ± 0.0 ^{b*}	1.3 ± 0.1 ^{b*}	1.5 ± 0.0
Phenylalanine + tyrosine	1.5 ± 0.0 ^{a*}	1.5 ± 0.0 ^{a*}	1.5 ± 0.0 ^{a*}	1.5 ± 0.0 ^{a*}	1.5 ± 0.0 ^{a*}	1.5 ± 0.0 ^{a*}	1.6 ± 0.0
Threonine	1.3 ± 0.0 ^{e*}	1.2 ± 0.0 ^{b*}	1.2 ± 0.0 ^{a*}	1.3 ± 0.0 ^{cd*}	1.3 ± 0.0 ^{de*}	1.2 ± 0.0 ^{bc*}	1.3 ± 0.0
Valine	1.3 ± 0.1 ^{e*}	1.3 ± 0.0 ^{c*}	1.3 ± 0.0 ^{b*}	1.3 ± 0.0 ^{b*}	1.3 ± 0.0 ^{d*}	1.2 ± 0.0 ^{a*}	1.4 ± 0.0

¹ Mean ± SD ($n = 2$).² Based on standard FAO/WHO/UNU (1991) 2–5 year old reference pattern (mg/g protein): Histidine-19; Isoleucine-28; Leucine-66; Lysine-58; Methionine + Cysteine-25; Phenylalanine + Tyrosine-63; Threonine-34; Valine-3.

Table 4

The in vitro protein digestibility and protein digestibility corrected amino acid score (PDCAAS) of ASL–wheat and wheat breads.¹

Bread	In vitro protein digestibility (%) ²	PDCAAS ²
<i>Belara</i> –wheat	82.1 ± 1.7 ^{a*}	0.40 ± 0.03 ^{c*}
<i>Coromup</i> –wheat	82.3 ± 4.2 ^{a*}	0.38 ± 0.01 ^{bc*}
<i>Gungurru</i> –wheat	81.0 ± 0.7 ^{a*}	0.33 ± 0.03 ^{ab*}
<i>Jenabillup</i> –wheat	83.6 ± 0.3 ^{c*}	0.38 ± 0.00 ^{bc*}
<i>Mandelup</i> –wheat	82.3 ± 0.6 ^{b*}	0.40 ± 0.01 ^{c*}
<i>Tanjil</i> –wheat	80.0 ± 0.0 ^a	0.31 ± 0.01 ^{a*}
Wheat-only	78.0 ± 0.0	0.23 ± 0.00

¹ Means ± SD.

² Values for ASL–wheat breads within a column with different superscript denotes significant difference ($p < 0.05$) using Duncan's test.

* Denotes significant difference ($p < 0.05$) from wheat-only bread using Dunnett's test.

The total available carbohydrates of ASL–wheat breads ranged from 59.5 to 61.3 g/100 g db. All of the ASL–wheat breads had significant lower levels than the wheat-only bread of ~17%. There was a significant varietal effect on available carbohydrates of the ASL–wheat breads of which that incorporating *Belara* and *Coromup* had the lowest ($p < 0.05$).

3.2. Protein quality of bread samples

3.2.1. Amino acid profile and amino acid scoring of bread samples

The essential amino acid contents of ASL–wheat and wheat-only breads are presented in Table 2. The levels of lysine in all ASL–wheat

breads (Table 2) were significantly higher than in the wheat-only bread. In contrast, all ASL–wheat breads had significantly lower levels of methionine + cysteine, phenylalanine + tyrosine, threonine (except *Belara*–wheat bread) and valine compared to those in wheat-only bread. The levels of isoleucine and leucine in *Coromup*–, *Jenabillup*– and *Tanjil*–wheat breads were significantly lower than those in wheat-only bread. The results highlight the complimentary effect of adding ASL, on the amino acid profile of wheat bread which is low in lysine but high in sulfur containing amino acids (Duodu & Minnaar, 2011).

Australian sweet lupin variety had a significant effect on leucine, methionine + cysteine, threonine and valine content of the ASL–wheat breads with *Belara*–wheat bread had the highest ($p < 0.05$) leucine, threonine and valine levels. *Coromup*– and *Gungurru*–wheat breads had the significantly lowest methionine + cysteine.

The essential amino acid scores of the ASL–wheat and wheat-only breads are presented in Table 3. The ASL–wheat breads (except *Belara*), had a significantly higher lysine score but lower scores for methionine + cysteine, phenylalanine + tyrosine, threonine and valine. The isoleucine and leucine scores in *Coromup*–, *Jenabillup*– and *Tanjil*–wheat breads were also significantly lower than those in wheat-only bread. The amounts of all the essential amino acids analyzed, except for lysine in all ASL–wheat breads were higher (amino acid scores > 1) compared to the standards for amino acids of ideal reference protein, appropriate for children ages 2–5 (which also covers the range appropriate for human adults) (FAO/WHO/UNU, 1985). Based on the essential amino acid scores (Table 3), the limiting amino acid is lysine for all ASL–

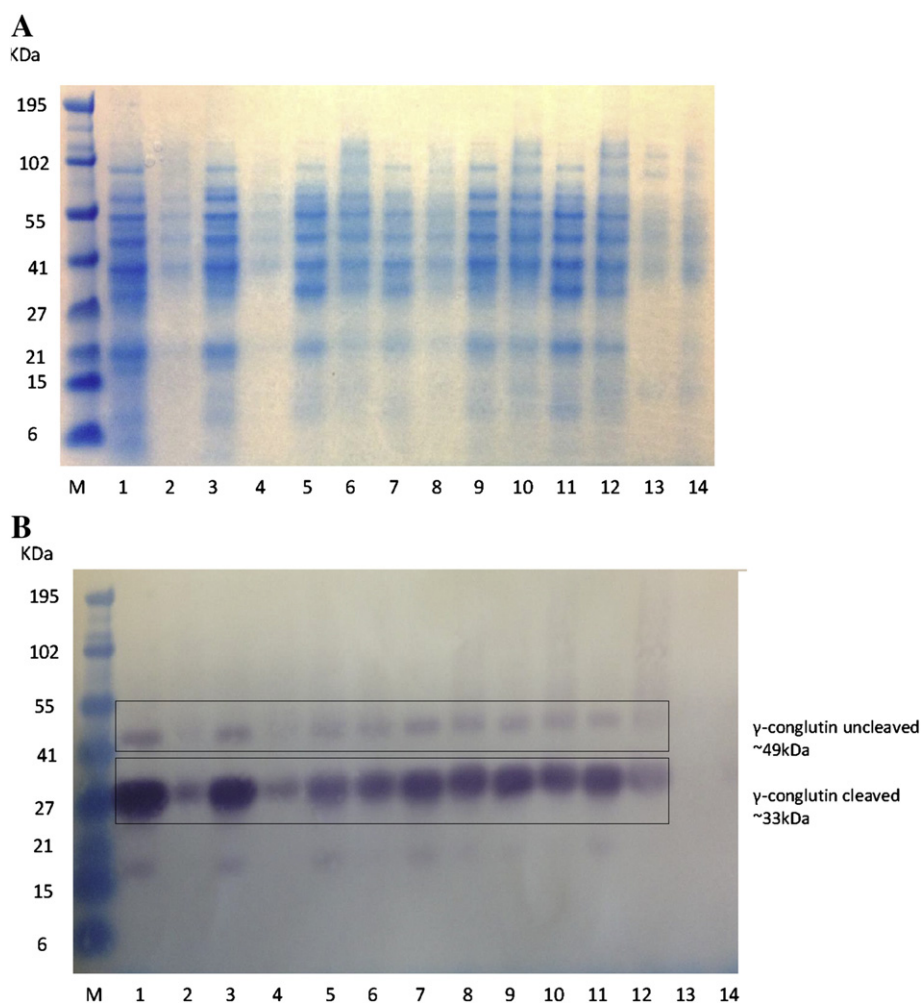


Fig. 1. Coomassie stained SDS-PAGE of replicated gel used for immunoblot (A) and immunoblot (B) of Australian sweet lupin (ASL) flour, wheat flour, ASL–wheat breads and wheat-only bread. M, molecular weight standards; 1, *Belara* flour; 2, *Belara*–wheat bread; 3, *Coromup* flour; 4, *Coromup*–wheat bread; 5, *Gungurru* flour; 6, *Gungurru*–wheat bread; 7, *Jenabillup* flour; 8, *Jenabillup*–wheat bread; 9, *Mandelup* flour; 10, *Mandelup*–wheat bread; 11, *Tanjil* flour; 12, *Tanjil*–wheat bread; 13, wheat flour; 14, wheat-only bread.

Table 5Carotenoid contents of ASL–wheat and wheat-only breads.¹

Bread	Lutein (μg/g) ²	Zeaxanthin (μg/g) ²	Alpha-carotene (μg/g) ²	Beta-carotene (μg/g) ²	Total carotenoids (μg/g) ²
<i>Belara</i> –wheat	0.6 ± 0.1 ^{b*}	0.2 ± 0.1 ^{b*}	0.7 ± 0.0 ^{a*}	1.1 ± 0.3 ^{c*}	2.5 ± 0.5 ^a
<i>Coromup</i> –wheat	0.5 ± 0.1 ^{ab}	0.1 ± 0.1 ^{ab}	0.6 ± 0.0 ^a	0.7 ± 0.1 ^{ab}	2.0 ± 0.3 ^a
<i>Gungurru</i> –wheat	0.3 ± 0.0 ^a	0.1 ± 0.0 ^a	0.6 ± 0.2 ^a	0.6 ± 0.0 ^{ab}	1.5 ± 0.1 ^a
<i>Jenabillup</i> –wheat	0.3 ± 0.0 ^a	0.1 ± 0.0 ^{ab}	0.5 ± 0.2 ^a	0.6 ± 0.2 ^b	1.5 ± 0.4 ^a
<i>Mandelup</i> –wheat	0.4 ± 0.2 ^{ab}	0.1 ± 0.0 ^{ab}	0.5 ± 0.1 ^a	0.7 ± 0.1 ^{ab}	1.8 ± 0.4 ^a
<i>Tanjil</i> –wheat	0.3 ± 0.1 ^a	0.1 ± 0.0 ^a	0.5 ± 0.1 ^a	0.6 ± 0.0 ^b	1.4 ± 0.1 ^a
Wheat-only	0.2 ± 0.0	0.025 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.9 ± 0.1

¹ Means ± standard deviation (expressed as dry basis).² Values for ASL–wheat breads within each column with different superscript letter denotes significant difference ($p < 0.05$) using Duncan's test.* Denotes significant difference ($p < 0.05$) from wheat-only bread using Dunnett's test.

wheat breads and wheat-only bread, therefore the scores for lysine were used to calculate the PDCAAS of the breads. A higher level of ASL addition would therefore be required to further improve the lysine score of the bread.

3.2.2. IVPD and PDCAAS

The IVPD and PDCAAS of the ASL–wheat bread samples (Table 4) ranged from 80% to 83.6% and 0.31 to 0.40, respectively. IVPD of wheat bread was significantly increased with addition of all ASL flours except that from *Tanjil*. Previous studies also demonstrated increases in protein digestibility when legume flours were added to baked products such as sourdough wheat bread (Anyango, de Kock, & Taylor, 2011) and gluten free cakes (Gularte, Gómez, & Rosell, 2012). The significant effect of ASL flour on the IVPD of wheat bread translated to a significant increase in the PDCAAS. These findings demonstrate that substitution of wheat flour with ASL flour at 20 g/100 g of composite flour can potentially increase the IVPD and PDCAAS of wheat bread by ~60% and ~50%, respectively, regardless of variety. The ASL variety significantly affected both the IVPD and PDCAAS of the breads, *Tanjil*–wheat bread having the significantly lowest values.

3.3. Identification and semi-quantitation of γ -conglutin

Photographic images of the SDS-PAGE gel and immunoblot of the flours and breads are presented in Fig. 1. The SDS-PAGE (Fig. 1(A)) shows bands in the ASL flour (lanes 1, 3, 5, 7, 9, 11) and ASL–wheat bread (lanes 2, 4, 6, 8, 10, 12) that correspond in molecular weights to the subunits of the potentially blood-glucose lowering bioactive peptide γ -conglutin (16 and 33 kDa) as well as those of the major lupin globulin proteins of α -conglutin (50 and 80 kDa) and β -conglutin (20 and 60 kDa) (Capraro et al., 2008). These bands are not visible in the wheat flour (lane 13) and wheat-only bread (lane 14). The band (33 kDa), corresponding to the molecular weight of the cleaved γ -conglutin is less intense in the ASL–wheat breads than in the ASL flours, which may be expected due to the ASL–wheat bread only containing only 20% lupin flour. In addition, the extraction of γ -conglutin

may be reduced in lupin–wheat bread due to its cross-linking with the gluten matrix (Islam, Ma, Yan, Gao, & Appels, 2011) or binding to the starch (Capraro et al., 2008), thus solubilization methods need careful optimization to ensure full extraction of this peptide from the bread matrix.

The immunoblots (Fig. 1(B)) all ASL flour (lanes 1, 3, 5, 7, 9, 11) and ASL–wheat breads (lanes 2, 4, 6, 8, 10, 12) have intense bands of reactivity with the γ -conglutin antibody at 33 kDa. A more dramatic reduction in intensities of this band of the *Belara*– and *Coromup*–wheat breads than the other ASL–wheat breads compared to their corresponding ASL flours was observed. This may have been due to the dilution effect of the wheat flour in the ASL–wheat breads in combination with reduced γ -conglutin extractability due to bread-making or breakdown to smaller peptide fragments in the cases of the *Belara*– and *Coromup*–wheat breads. The immunoblots of the ASL flour and ASL–wheat breads also display bands corresponding to the molecular weight of ~49 kDa that likely represents the uncleaved γ -conglutin. The immunoblots of the ASL flours also showed faint bands corresponding to a molecular weight of ~16 kDa that may represent non-specific binding or further processing of γ -conglutin.

Recently Bertoglio et al. (2011), demonstrated for the first time that administration of a γ -conglutin enriched preparation lowered post-prandial plasma glucose response in humans. However, it should be noted that the protein content of test powder used by Bertoglio et al. (2011) was only 44.8% of which only 47% was γ -conglutin, making it difficult to confirm if the hypoglycemic component was in fact the γ -conglutin. The SDS-PAGE and Western blotting used in the present study has limited ability to accurately identify and quantify the effects of bread making on the extractability and integrity of γ -conglutin. In light of this, more effective proteomics techniques based on mass spectrometry, such as HPLC-Chip-Multiple Reaction Monitoring (Resta, Brambilla, & Arnoldi, 2012) should be used in future studies.

3.4. Phytochemical composition

3.4.1. Carotenoids

The levels of carotenoids of the ASL–wheat breads (Table 5) were similar to that of the wheat-only bread, except for *Belara*–wheat bread which was significantly higher in lutein, zeaxanthin, beta-carotene alpha-carotene and total carotenoids. ASL variety had significant effect on the lutein, zeaxanthin and beta-carotene content of the ASL-breads, with for example the *Belara*–wheat bread having the highest ($p < 0.05$) level of beta-carotene.

The recovery of carotenoids in ASL–wheat breads compared to that of the raw composite flours (data not presented) were: lutein, 24%; zeaxanthin, 15%; alpha-carotene, 71%; beta-carotene, 48%; total carotenoids, 38%. According to Hidalgo et al. (2010), mixing and baking led to significant decreases in the carotenoid contents of refined einkorn and bread-wheat flours. During mixing, in the presence of water and oxygen, lipoxygenase (LOX) oxidises polyunsaturated fatty acids which in turn causes oxidation of carotenoids (Leenhardt et al., 2006). ASL has been shown to have high LOX activity (Yoshie-Stark, Bez, Wada, & Wasche, 2004) and this may have resulted in the low recovery

Table 6Total phenolic content and total antioxidant capacity of ASL–wheat and wheat-only breads.¹

Bread	Total phenolics (mg GAE/g) ²	Total antioxidant capacity (μmol TE/g) ²
<i>Belara</i> –wheat	0.7 ± 0.0 ^{b*}	1.2 ± 0.1 ^{ab}
<i>Coromup</i> –wheat	0.7 ± 0.0 ^{bc*}	1.6 ± 0.1 ^{c*}
<i>Gungurru</i> –wheat	0.6 ± 0.0 ^{a*}	1.1 ± 0.1 ^a
<i>Jenabillup</i> –wheat	0.7 ± 0.0 ^{bc*}	1.3 ± 0.1 ^{b*}
<i>Mandelup</i> –wheat	0.7 ± 0.1 ^{ab*}	1.3 ± 0.0 ^{b*}
<i>Tanjil</i> –wheat	0.8 ± 0.0 ^{c*}	1.5 ± 0.1 ^{c*}
Wheat-only	0.5 ± 0.0	1.0 ± 0.0

¹ Means ± standard deviation (expressed as dry basis).² Values for ASL–wheat breads within a column with different superscript letter denotes significant difference ($p < 0.05$) using Duncan's test.* Denotes significant difference ($p < 0.05$) from wheat-only bread using Dunnett's test.

rates of some of the carotenoids in the present study. In addition, the thermal process of baking can decrease carotenoid content (Namitha & Negi, 2010). ASL flour may be a useful source of carotenoids, however further research is required to develop processing approaches to reduce their losses during bread making.

3.4.2. Total phenolics content and total antioxidant capacity

The total phenolic content and total antioxidant capacities of the ASL–wheat breads (Table 6) were significantly higher than those of the wheat-only bread (except for the antioxidant capacity of the Gunguru–wheat bread). There was a significant effect of ASL variety on these measures with *Coromup*–wheat and *Tanjil*–wheat bread having the highest ($p < 0.05$) total antioxidant capacity. These results indicate that substitution of refined wheat flour by ASL flour can increase the polyphenolic content and antioxidant capacity of bread.

4. Conclusion

This study demonstrated that the nutritional, phytochemical and bioactive composition of refined wheat flour bread is significantly improved with addition of ASL flour, indicating that ASL–wheat bread may have useful nutritional and health functionality. Significant effects of ASL variety on the nutritional, phytochemical and bioactive composition of ASL–wheat bread were also revealed. Our previous study indicated that ASL variety also had a significant impact on the physical quality of wheat bread (Villarino et al., 2015). These combined findings underscore the importance of establishing varietal segregation of ASL for commercial flour supply to manufacture ASL–wheat bread of consistent quality, with maximum nutritional and health benefits combined with optimal volume and texture. Our findings suggest that the ASL varieties, *Belara*, *Coromup* and *Tanjil* may be added to wheat bread to give improved nutritional and phytochemical properties and protein quality combined with acceptable physical properties. There is now a need to optimise the processing and formulation factors in ASL–wheat bread manufacture to maximize the level of ASL incorporation whilst maintaining high consumer acceptability, to give a product with maximum potential nutritional and health benefits.

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