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Research Paper

The role of legume peptides released during different digestion stages in modulating the bioaccessibility of exogenous iron and zinc: An in-vitro study

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ABSTRACT

The effects of legume protein fractions on Fe and Zn bioaccessibility remain equivocal to date, largely due to the protein's structure and the presence of anti-nutritional compounds. We administered Fe and Zn salts with legume concentrates consisting mainly of albumin or globulin from lupin, pea and faba to *in vitro* gastrointestinal digestion. Under the fasted intestinal state, faba globulins were found to enhance Fe^{2+} and Zn solubility compared to control salts without legume proteins. Meanwhile, other fractions had no effect or significantly lowered Fe and Zn solubility. Under the fed intestinal state, the presence globulins enhanced Fe solubility *versus* the control, where protein solubilization due to high bile concentration likely played a role in circumventing precipitation. The lupin albumin fraction significantly enhanced Fe^{2+} and Zn solubility, whilst other fractions generally reduced Zn solubility under fed state. Our results highlight the complex role of legume proteins towards Fe and Zn solubility.

1. Introduction

Dietary deficiencies in Fe and Zn are highly prevalent amongst lower socioeconomic populations, a group most represented in the developing world and segments of industrialized nations. Fe deficiency and its associated anaemia greatly increases the incidence of maternal and child mortality (Prentice et al., 2016), while severe cases of Zn deficiency can lead to compromised immunity, impaired growth and reduction in some organ function (Prasad, 2013).

Intervention programmes to enhance dietary Fe and Zn intake include biofortification of staple crops and foods fortification, as well as pharmaceutical supplementation (Das et al., 2013). However, mineral bioavailability often negates the success in correcting mineral deficiencies in such contexts. Lower socioeconomic populations tend to rely heavily on plant-based staples such as legumes, which present both physical and chemical barriers impeding the solubility of both endogenous and fortified minerals necessary for bioaccessibility to the body. Subsequently, both *in vitro* and *in vivo* studies in animals and humans have revealed mixed efficacy of Fe and Zn biofortified legumes as delivery vehicle, some with successes (e.g. Tako et al., 2011; Tako et al., 2015) while others have demonstrated no significant difference in bioaccessibility or bioavailability between non-fortified and biofortified legumes (e.g. Glahn, Wiesinger, & Lung'aho, 2020).

Two notable factors from legumes that hinder Fe and Zn bioaccessibility the legume protein composition and phytic acid[1] contents, both of which are co-associated in protein storage vacuoles of the cotyledon *in planta* (Raes et al., 2014). Legume proteins consist predominantly of peptides with multimeric structure and arranged in the β-sheet conformation (Carbonaro et al., 2015), which tend to be highly compact in structure by nature due to electrostatic driven self-association. Even as the native proteins dissociate during processing, they possess a tendency for re-association under varying ionic strength and pH (Ferreira et al., 2003). This dichotomy has implications not only for the protein solubility that may impact their digestibility, but also on the proneness to cation-driven aggregation that decreases cation solubility. This effect may induce a significant negative impact on benefit of mineral fortification with legumes in terms of bioaccessibility.

Augmenting this barrier to cation bioaccessibility by legume proteins

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[1] Phytic acid (PA).

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intrinsic properties is phytic acid, an inositol derivative coexisting with legume proteins. Phytic acid will have a high density of negatively charged phosphate groups under intestinal pH capable of forming insoluble complexes with divalent cations. PA plays an inhibitory, yet not fully understood, role in elemental bioavailability. It has been reported in several single-meal and long-term human studies that a PA: element molar ratio above 10:1 will inhibit Zn and Fe absorption and retention (Zhang et al., 2020). However, *in vitro* systems have shown that where the cations are in molar excess of PA, insoluble polymetallic co-precipitates can be rapidly developed (Crea et al., 2008). PA reduction in foods has been generally shown to enhance Fe and Zn absorption in humans. However, the effects of this phenomenon could be exclusive to where higher concentrations of the cation are involved, and where there is an absence of other components to nullify the antinutritional impact of phytate. A potential example of this in legumes was shown in a recent study by Glahn, Tako, Hart, Haas, Lung'aho, and Beebe (2017), who found that biofortified beans with high Fe and low PA had significantly lower *in vitro* Fe bioavailability compared to those with mid or lower levels of PA.

The co-existence of both PA and legume peptides can be a great source of discrepancy in the study of element bioaccessibility. Some legume peptic hydrolysates have been shown to effectively solubilise Fe and Zn in mung bean (Budseekoad et al., 2018; Fu et al., 2020) and chickpea (Torres-Fuentes et al., 2012)). However, some hydrolysates may also participate in the formation of insoluble ternary complexes with PA through a cationic bridge under physiological pH (Zhang et al., 2020). The modulatory effects may be dependent on the species and protein fraction. For example, semi-purified meals containing soy protein were found to inhibit Fe absorption in humans, with the effect imparted from the glycinin fraction only in the presence of PA, and the conglycinin fraction irrespective of PA content (Lynch et al., 1994). *in vitro* studies using fractionated legume proteins have generally focused on bioaccessibility of the endogenous minerals present, and showed that the globulin fraction contains more soluble Fe and Zn compared to albumins (Lombardi-Boccia et al., 2003; Lombardi-Boccia et al., 1998).

PA levels in legumes can be reduced with common domestic food processing approaches such as soaking or fermentation, although the extent of reduction can be small (Raes et al., 2014). Attempts at element fortification thus present a prospective scenario of a high element to PA ratio, which may substantiate the presence of insoluble PA-cation or ternary PA-protein-cation complexes.

In the current study, we examined the bioaccessibility of Fe and Zn when added to the albumin and globulin fractions from three legumes (faba bean, lupin, and pea) at the minimum physiologically relevant doses (5.58 and 6.54 mg, respectively) based on Australian Estimated Average Requirements (National Health and Medical Research Council, 2017). In a single dose, these quantities are expected to be in large molar excess of PA. Our findings provide insights towards the role of legume protein fractions as both prospective enhancers and inhibitors of exogenous Fe and Zn bioaccessibility, which are related to the state of digestion, Fe oxidation state, and the protein fraction involved.

2. Materials & methods

2.1. General reagents and equipment

Glyoxal bis(2-hydroxyaniline) was acquired from Spectrum Chemicals (Gymea, Australia). 2M Folin-Ciocalteu reagent, cetrimonium bromide ($C_{19}H_{42}BrN$, CTAB), sodium phytate ($C_6H_6Na_{12}O_{24}P_6$), gallic acid, HEPES buffer, $FeCl_3$ (97%), $FeCl_2{\cdot}4H_2O$, $ZnCl_2$, bovine bile, phosphorous buffered saline, dithiothreitol, *o*-phthalaldehyde, mucin, lyophilized bovine serum albumin, urea and *α*-amylase (from *Aspergillus oryzae*) were purchased from Sigma-Aldrich (Castle Hill, Australia). Pepsin and pancreatin were acquired from Thermo-Fisher Scientific (Scoresby, Australia). All other chemicals and organic solvents used were of analytical-grade or better. Deionised water (≤18 MΩ) used was produced using a Synergy UV Millipore System (Merck Millipore, Australia).

All UV-Visible spectrophotometric measurements were made on Multiskan™ GO from Thermo-Fisher Scientific (Scoresby, Australia). The incubator shaker used throughout the study is ZWYR-240 from Labwit Scientific (Shanghai, China). All centrifugation of samples were made on Eppendorf 5810R (Sigma-Aldrich, Castle Hill, Australia).

For all analyses of soluble protein, the bicinchoninic acid method (BCA) was used following a commercial kit with bovine serum albumin as the standard (Pierce BCA Protein Assay Kit, Thermo-Fisher Scientific, Australia).

2.2. Protein fractionation and analysis

Ground, dehulled field pea (*Pisum sativum* L.), faba bean (*Vicia faba* L. *var. major Harz*) and sweet lupin (*Lupinus angustifolius* L.) that have been passed through a 0.8 mm screen were kindly provided by Dr. Joe Panozzo and Dr. Jason Brand at Agriculture Victoria, Australia. The pulse powders were defatted by vigorous stirring with hexane (1:5, w/v) for 3 h at room temperature (RT). The mixture was centrifuged (2465×*g*, 10 min, 10°C) to decant hexane, and the defatted pellet was washed with 0.1 M KOH, dehydrated under the fume hood and stored at 4°C until used.

The Osborne sequential extraction method was employed to obtain the albumin and globulin fractions (Makeri et al., 2017). Defatted powders were suspended in distilled water (1:6, w/v) and stirred for 2 h at room temperature (RT) using a magnetic stirrer, rested for 20 min, and centrifuged at 2465×*g* for 20 min at 10°C. The supernatant was decanted as the albumin fraction, and the residue was extracted with the NaCl solution (1:6, w/v) for 2 h, using 0.5 M for the lupin and 1.0 M for the faba and pea fractions to yield the globulin proteins. The chosen salt concentration for the proteins' extraction was optimised in prior experiments using 0.25–1 M NaCl (data not shown). It is acknowledged that sequential extraction using water and salt can produce cross-contaminated proteins rather than pure albumins and globulins, due to intermediate solubility behaviours in these proteins (Rubio et al., 2014). However, the current study aimed to examine the effects of the water- and salt-soluble fractions of legume proteins as per household processing, rather than purified albumins and globulins.

The extracted protein fractions were dialysed at RT for 4 h followed by stirring overnight at 4°C with regular change of dialysis water to remove low molecular weight compounds, using cellulose tubing with 12 kDa cut-off (D-9652, Sigma-Aldrich). The dialyzed solutions were then filtered using a cellulose membrane (10311897, Whatman, Thermo-Fisher Scientific) to remove insoluble starch formed during dialysis. The soluble dialysed fraction for each protein was pooled and the proteins precipitated at their isoelectric points, which was pH 4 for lupin globulin, and pea and faba albumin and globulin, and pH 3 for lupin albumin. The residue was freeze-dried and stored at 4°C prior to reconstitution. The protein contents of these fractions were determined by the total nitrogen method (Dumas Combustion, Leco TruMac) and reported as w/w %.

2.3. Phytic acid analysis

Phytic acid in legume flours, as well as the dried protein extracts before and after dialysis, was extracted following the method of Gao et al. (2007). In this method, 0.5 g of sample was extracted with 10 mL of 2.4% HCl at RT using an incubator shaker for 24 h. The mixture was then centrifuged at 2465×*g* for 20 min at 10°C. A matrix cleaning step was performed by transferring the crude supernatant to 14 mL tubes containing 1 g NaCl and shaking at 26×*g* for 20 min to dissolve the salt. The mixture was allowed to settle at -20°C for 20 min, before being centrifuged at 2465×*g* at 10°C for 20 min.

The treated clear supernatant was quantitively collected and the PA content was determined using the spectrophotometric method as

described by Agostinho et al. (2016). In short, the optical density of the legume extracts were measured by mixing 1 mL of $CaCl_2$ solution (10 mg/L), 0.2 mL of glyoxalbis(2-hydroxyaniline) (1 mg/L in methanol), 1.8 mL of ethanol/methanol mixture (70:30 v/v), 1.0 mL of borate buffer solution (containing NaOH (5 g/L), sodium borate (5 g/L) and CTAB (1 mmol/L), pH 12.5) with 1 mL of sample or phytic acid standard in a 15 mL test tube. The analytical blank was prepared by replacing the PA solution with water, with a six-point calibration curve prepared from a sodium phytate stock solution (200 mg/L). The mixture was held for 20 min at RT and mixed before being pipetted in 250 μL aliquots onto a 96-well microplate (3367, Corning Costar, Sigma-Aldrich). The absorbance was measured at 500 nm, and PA contents of samples were determined from the standard calibration curve.

To validate the PA analysis method, the values obtained for the crude legume flours were calculated as a fraction of total phosphorous as analysed by ICP-OES using the method described below (Section 2.4.4). Comparisons were made to the range of values reported in the literature.

2.4. Total phenolic assay

Total phenolics were determined by the Folin-Ciocalteu method from the Biquochem Assay Kit Protocol (KB-03-006). In summary, aliquots (20 μL) of the sample (legume extract) or standard solution was pipetted onto a 96-well plate (3896, Corning, Sigma-Aldrich). To each well, 100 μL of Folin-Ciocalteu reagent (previously diluted to 1:10 in distilled water) and 80 μL of 2% Na_2CO_3 were added. The absorbance was colorimetrically measured at λ 750 nm after 30 min of incubation at 37 ^{o}C. The standard curve was constructed using gallic acid as a standard, and the final phenolic content was expressed as gallic acid equivalents (mg GAE g^{-1} dry weight).

2.5. Mineral chelation

2.5.1. Preparation of element-containing legume extract solutions

Freeze-dried concentrates of each legume were dissolved in 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer at pH 7 (Fe) or 8 (Zn) to a final concentration of 1% (w/v), by stirring overnight at 4°C. The optimal pH for solubility used for each mineral was predetermined by Eckert et al. (2014). Element solutions were prepared at 20 mM and 5 mL each were used. This was then added to the 1% protein concentrate (w:v) or control solutions of 0.1 M HEPES (10 mL) without proteins by stirring for 20 min to produce the solutions of element-containing legume extracts or control for *in vitro* digestion. Therefore, 5.58 mg Fe or 6.54 mg Zn with or without 100 mg of fractionated powders were subjected to the *in vitro* digestions (oral, oral-gastric or oral-gastric-intestinal).

2.5.2. Simulated oral digestion

A simulated saliva fluid (SSF) containing 0.15% NaCl, 0.02% $CaCl_2{\cdot}2H_2O$, 0.3% KH_2PO_4, 0.15% mucin and 0.01% bovine serum albumin was formulated based on human biochemistry reference values for both stimulated and non-stimulated saliva *in vivo* (S1). Before use, α-amylase was added to achieve 150 units/mL of the medium. The solution was stirred, adjusted to pH 6.8 and then preheated to 37 °C. The solutions of element-containing legume extracts or control (5 mL) were then mixed with an equal volume of salivary fluid (5 mL), and mixed using an incubator shaker at 37 °C for 3 min to mimic agitation in the mouth. The sample-to-fluid volume used is the same of that in the INFOGEST model (Brodkorb et al., 2019), whilst a different salivary fluid was utilised to account for the additional ionic salts present in natural saliva that may influence element solubility.

2.5.3. Simulated gastric and small intestinal digestion

A two-step digestion system adapted from our previous work (Zhang et al., 2018) was employed, with simulated gastric and intestinal fluids prepared per the original model. However, the current investigation employed separate endpoints at the end of gastric and intestinal digestion, respectively, to quantify the soluble elements by the end of each stage. Following oral digestion, 5 mL of simulated gastric fluid (containing HCl, pH 1.2) with pepsin (6.4 mg/mL of digestive fluid) was added to each sample, and incubated in the shaking incubator mentioned for 2 h at 37 °C at 2×*g*. Digestion was ceased by adjusting sample pH to 6.8 with 1 M NaOH. Digestion triplicates were subjected to centrifugation at 2465×*g* for 10 min to obtain the soluble fraction at the end of the gastric stage, whilst another set of triplicates continued onto intestinal digestion without centrifugation.

The intestinal electrolyte fluid containing 5 mM PBS, 0.4 M NaCl and 15 mM $CaCl_2$ was prepared and adjusted to pH 6.8 (±0.05). The electrolyte fluid was then gently stirred with added pancreatin (10 mg/mL of digestive fluid) and bile (0.5 or 30 mg/mL of digestive fluid). The two respective bile quantities are a proxy of the concentrations secreted by the gall bladder during fasting (absence of food) and fed (presence of food) conditions, where the range lies between 2 and 6.4 mM for fasted and 0.5–37 mM for fed. To each of the gastric digested replicates, the fraction was mixed with 7 mL of the prepared simulated intestinal fluid. Samples were incubated for 2 h (fasted) and 3 h (fed) at 37°C in a shaking incubator at 2×*g*, and digestion was terminated by placing samples in ice prior to centrifugation at 2465×*g* for 10 min. Digestion control samples without the addition of protein fractions to mineral salts were also subjected to the gastrointestinal model for comparison.

The soluble supernatant fraction after centrifugation from the gastric and intestinal digestion were subjected to analyses for soluble protein using the BCA method as above, degree of protein hydrolysis and soluble mineral content.

2.6. Proteolysis of proteins (Free α-amino groups)

The number of free α-amino groups from proteins prior and at the end of each phase of digestion was determined using an *o*-phthaldialdehyde (OPA) reagent as adapted from Nielsen et al. (2001) and Zhang et al. (2019). The OPA reagent contained 38.1 g/L sodium borate, 2 g/L SDS, 0.88 g/L DTT (99%) and 0.80 g OPA (97%) in 2% ethanol. An aliquot (10 μL) of sample was mixed with 240 μL OPA reagent and incubated for 2 min at room temperature before measuring the absorbance at 340 nm. The meqv. of serine released per gram of protein (mg Serine-NH_2) was calculated based on the following formula:

$$SerineNH_2 = \frac{Abs_{hydr} - Abs_{OPA}}{P} \times \frac{mM\ Serine}{Abs_{Serine} - Abs_{OPA}}$$

where Abs_{hydr} is the absorbance of the protein hydrolysate sample, Abs_{OPA} is the absorbance of the blank OPA reagent, and Abs_{serine} is the absorbance of the serine standard (0.1 mg/mL). P is the concentration of soluble protein in mg/mL as measured by the BCA method.

2.7. Inductively coupled plasma optical emission spectrometry (ICP-OES) analyses

2.7.1. Acid digestion

All samples were digested according to our previous work (Zhang et al., 2018). In summary, 2 mL from each soluble fraction of the gastric or intestinal digest was transferred into a 50 mL polypropylene tube (227261, Greiner Bio-One GmbH, Frickenhausen, Germany) containing 2 mL of 70% HNO_3 and 0.5 mL of 30% H_2O_2. For the analysis of phosphorous in crude samples, 0.3 g of the ground powder was used for the same quantity of HNO_3 and H_2O_2. The samples were then vortexed and pre-digested overnight at room temperature, prior to heating on an aluminium digestion block with Proportional-Integral-Derivative control (Custom made block, The University of Melbourne, Australia) for 30 min at 80 °C, and gradually increased to 125 °C for 120 min at 5 °C intervals. Tube caps were loosened to equalise pressure during the initial 30 min at 80 °C and tightened firmly before the 120 min of digest at

125 °C. Samples were diluted to an acid content of <5% using MilliQ-grade water (30 mL total) in correspondence with the 5% HNO_3 matrix of the calibration standards used.

2.7.2. ICP-OES specifications

ICP analyses were performed using a radial view Optima 8300 DV ICP-OES (PerkinElmer, Glen Waverley, Australia). Samples were injected using an automated sampler (S10, PerkinElmer, Glen Waverley) operated by the Syngistix for ICP 1.0 software. Calibration curves for elements were generated from multi-element standards (ICP-AM-17 and ICP-AM-12 Solution A, High-purity standards, Charleston, United States). Background correction was applied to all wavelengths (Fe at 259.94 nm, and Zn at 206.2 nm), with multiple emission lines used to check for spectral interference. Random and targeted repeat analysis were performed to provide confidence.

2.8. Statistical analysis

All statistical analyses were carried out at 95% confidence using Minitab 19 (Minitab Inc., Sydney, Australia). Uncertainty of replicate determinations was reported to 2 significant figures for reporting uncertainty in chemical analysis. For analyses of the mean difference between the solubility found with each legume protein fraction, a one-way ANOVA was performed for each element, with post-hoc comparisons made to the mineral control using Dunnett's method. To determine the source of variation in solubility in the presence of legume fractions, a separate multivariate ANOVA was performed for each element through the 'General Linear Model' function. One-way ANOVA with Fisher's LSD was also utilised to assess for differences in soluble protein or free amino groups between the fractions.

3. Results

3.1. Protien fractions, anti-nutritional, and phenolic compounds analyses

The phytic acid phosphate (PA-P) contents in the legumes supplied as powders were quantified by colorimetry and found to be 2.40 ± 0.15 mg/g for faba, 1.70 ± 0.082 mg/g for pea, and 3.22 ± 0.12 mg/g for lupin. Based on total phosphorous content as analysed by ICP-OES versus the PA-P content from colorimetric analyses, the percentage phosphorous contents in PA-P were 47.7, 69.1 and 59.8% in pea, lupin, and faba, respectively. These values are within the expected range of 45–72% for pea, 20–63% for lupin and 54–68% for faba reported by Humer & Zebeli (2015) and Selle et al. (2003).

Dialysis of the protein extracts was performed to reduce PA, phenolics and other low MW compounds to limit their interference with element binding in the digestion experiments. Dialysis reduced the phenolic content in all samples to <2 mg of GAE/g solids (data not shown), and to varying levels of PA-P. Based on 100 mg of fractionated legume powder, the amount of PA administrated in each dose within each digestion regime (gastric or gastric-intestinal) ranged from 193 μmol in lupin globulin, to 1.9 mmol in pea albumin. The quantities of Fe and Zn vastly exceed PA content in each sample (Table 1).

Table 1
The PA to metal ratio of exogenously added Fe and Zn in protein fractions for digestion.

Fraction	PA:Fe	PA:Zn
Lupin albumin	1:23	1:26
Lupin globulin	1:103	1:121
Faba albumin	1:16	1:15
Faba globulin	1:18	1:19
Pea albumin	1:11	1:13
Pea globulin	1:19	1:23

[1]PA content was calculated assuming 6 mol of phosphorus per mol of PA (28.2%) (Beiseigel et al., 2007).

The total protein of the legume protein concentrates as measured on the powdered concentrates using the Leco combustion method, was calculated using a Nitrogen conversion factor of 5.4 for legumes (Mariotti et al., 2008). Total protein of the fractionated powder measured as nitrogen content ranged from 55.71% in pea albumin, 68.41% in pea globulin, 78.49% in faba albumin, 76.8% in faba globulin, and 56.47% and 58.1% in lupin albumin and globulin, respectively. These values are equivalent to the same quantity in mg being administered in each dose.

3.2. Element bioaccessibility during simulated gastric-intestinal digestion of different legume protein fraction

3.2.1. Iron

In the current study, bioaccessibility was measured as solubility. Solubility reflects the soluble elements present in the supernatant of the digesta after centrifugation to remove the insoluble mineral precipitates, undigested proteins and protein bound minerals, which is measured by ICP-OES. Data in Figs. 1 and 2 show the solubility of ferrous (Fe^{2+}) and ferric (Fe^{3+}) salts, hence their bioaccessibility, with and without the presence of legume protein fractions as examined under fasted and fed intestinal conditions.

The ferric salt control, which was without added legume protein fractions, showed poor solubility during gastric digestion with only 0.85% of the loaded iron being soluble (47 μg out of the added 5580 μg) (Fig. 1). A significant enhancement in soluble Fe^{3+} was observed during gastric digestion in the presence of pea albumin (3601 μg), pea globulin (387 μg) and lupin globulin (657 μg) when compared to the salt control (Fig. 1-A). These values represented 65%, 6.9% and 12% of the original loaded iron, respectively. As digestion progressed, lower levels of soluble Fe^{3+} was detected during GI-fasted (Fig. 1-B) or GI-fed (Fig. 1-C) digestion indicating further loss through precipitation (GI-fasted: 40 μg, GI-fed: 13 μg). However, the presence of faba globulin fraction in the digest showed significantly ($P < 0.05$) higher levels of Fe^{3+} during GI-fasted (Fig. 1-B) when compared to the control (292 vs 40 μg). The inclusion of lupin globulin led to significantly ($P < 0.05$) higher levels of Fe^{3+} during GI-fed (Fig. 1-C), when compared to the control (48 vs 13 μg)

The ferrous salt showed higher solubility in the control samples (Fig. 2-A) during gastric digestion (2853 μg), retaining 51% of the of the initially loaded iron (5580 μg) at the start of the digestion. However, Fe^{2+} solubility declined during GI-fasted (Fig. 2-B) and GI-fed (Fig. 2-C) digestion states to lower values of 249 and 8.49 μg, respectively and 13 μg, respectively. Such decline is likely due to Fe^{2+} binding to insoluble bile and/or proteolytic peptide products.

All tested protein fractions reduced the soluble form of ferrous significantly ($P < 0.05$) during gastric (Fig. 2A) and GI-fasted (Fig. 2-B) digestion states in comparison with the control. However, the presence of any of the three legume globulins, or lupin albumin increased Fe^{2+} solubility (bioaccessibility) during GI-fed state (Fig. 2-C). For example, the recorded ferrous bioaccessibility values were 36 μg, 50 μg, 71 μg and 66 μg when using lupin albumin, lupin globulin, pea globulin and faba globulin, respectively as compared to 13 μg in the control.

3.2.1.1. Zinc. Mixed patterns of Zn solubility were observed during different digestion stages in the presence of legume protein fractions (Fig. 3). An initial load of 6540 μg Zn was added through the fortified salts. During gastric digestion, a significant ($P < 0.05$) reduction of mean soluble Zn from 1514 μg in the control to 855 and 917 μg in the presence of faba albumin and globulin, respectively (Fig. 3-A). However, during the fasted intestinal phase (Fig. 3-B), faba globulin and lupin globulin exhibited significantly ($P < 0.05$) higher mean Zn solubility (719 μg and 592 μg, respectively) compared to the control (359 μg).

Zn solubility under the fed digestion stage (Fig. 3-C) again showed mixed effects in the presence of legume protein fractions. For example, adding lupin albumin increased the amount of soluble Zn after digestion

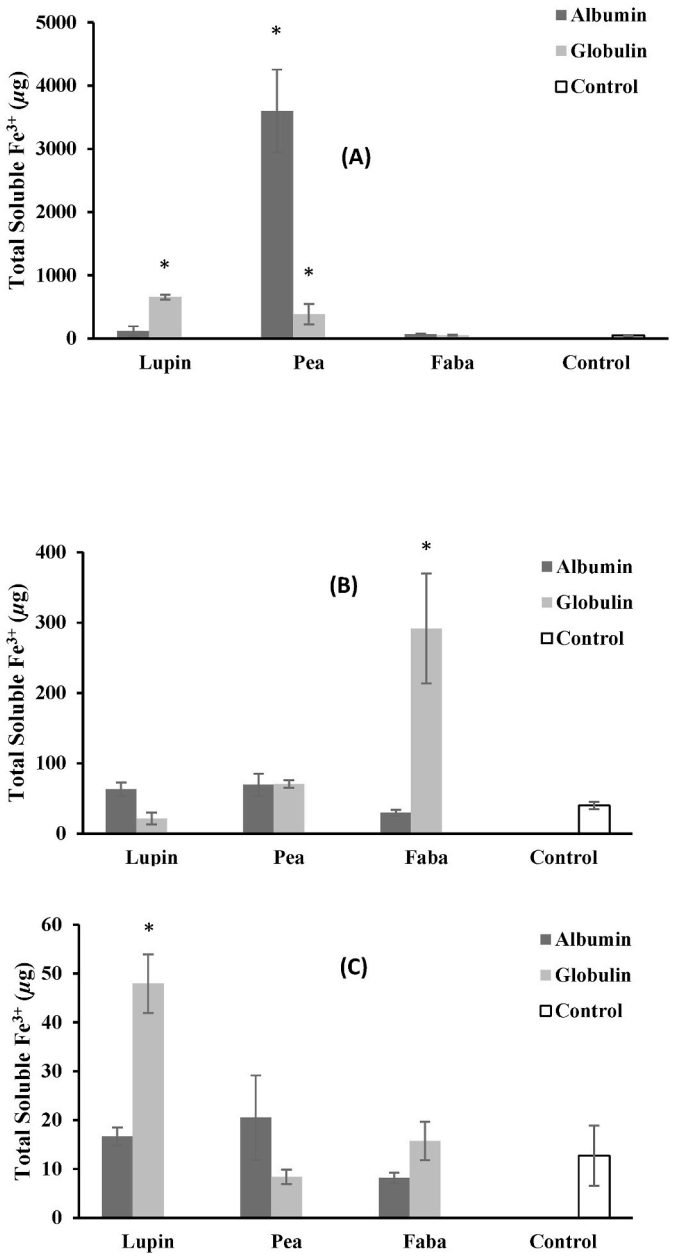

Fig. 1. Soluble Ferric (Fe^{3+}) from mineral salt control and mineral fortified legume protein fractions subjected to a) oral-gastric (G), b) sequential oral-gastric-fasted intestinal (GI-Fasted), or c) sequential oral-gastric-fed intestinal (GI-Fed) stages of digestion. Columns with an asterisk (*) are significantly different from that of the control mineral salt ($P < 0.05$). Results are expressed as means ± SEM (n = 3).

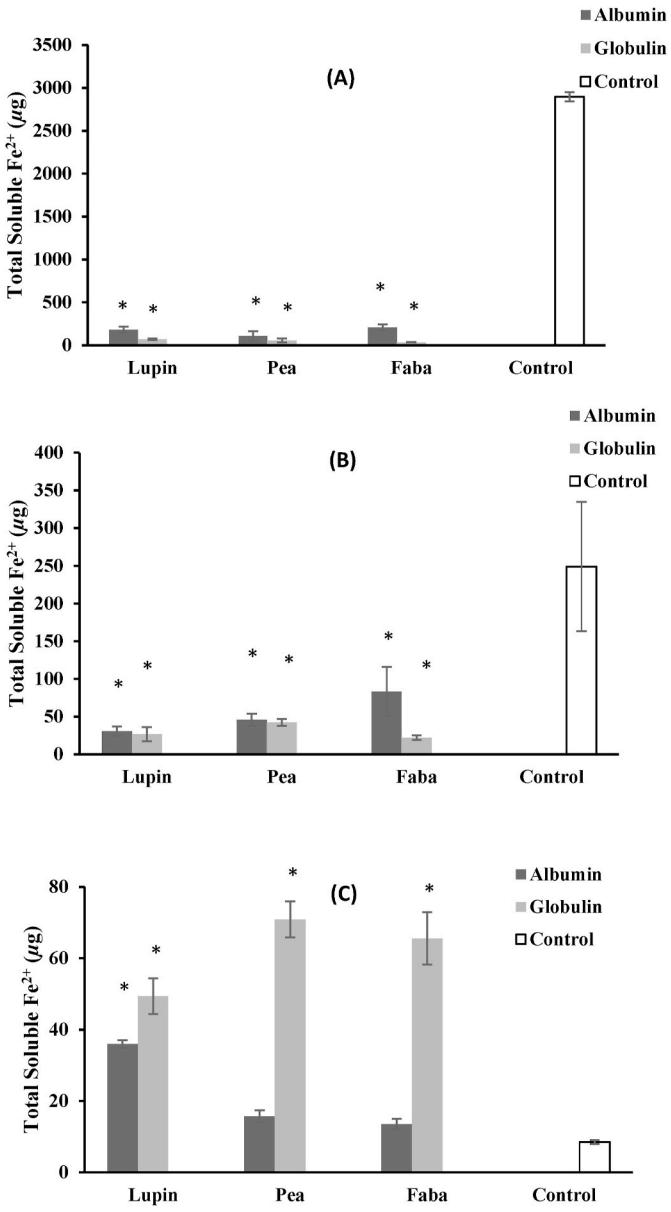

Fig. 2. Soluble Ferrous (Fe2+) from mineral salt control and mineral fortified legume protein fractions subjected to a) oral-gastric (G), b) sequential oral-gastric-fasted intestinal (GI-Fasted), or c) sequential oral-gastric-fed intestinal (GI-Fed) stages of digestion. Values with an asterisk (*) are statistically significantly different from that of the mineral salt control in each column ($P < 0.05$). Results are expressed as means ± SEM (n = 3).

when compared to the control (875 μg vs 443 μg). On the other hand, the presence of pea globulins significantly ($P < 0.05$) reduced Zn solubility from 443 μg in the control to 138 μg.

3.2.2. Overall patterns and source of variation

3.2.2.1. Iron. Results from multivariate ANOVA showed a significant ($P < 0.05$) three-way interaction between the legume source, protein fraction, and oxidation state on soluble Fe during all three stages of digestion (gastric, GI-fasted, GI-fed). This shows that although Fe solubility in each stage is dependent on the type of legume present, the solubility is also affected by the Fe oxidation state and type of protein.

During gastric digestion, albumins demonstrated significantly ($P < 0.05$) greater effect across groups in increasing overall mean Fe (both Fe^{2+} and Fe^{3+}) solubility compared to globulins (675 versus 168 out of the 5580 μg added). During this stage, the legume with the highest overall means of soluble Fe out of the added 5580 μg were pea (999 μg), followed by lupin (213 μg), and faba (51 μg). Opposing trends to the observations during gastric digestion were found following GI-fasted digestion. Faba had significantly ($P < 0.05$) higher overall soluble Fe (107 μg) compared to pea (57 μg) and lupin (35 μg) of the added 5580 μg. During GI-fed digestion, lupin (30 μg) had higher soluble Fe than faba (18 μg) and pea (21 μg). Significantly ($P < 0.05$) higher mean Fe solubility was observed in globulin compared to albumin during both digestion stages (GI-fasted: 79 versus 54 μg, GI-fed: 35 versus 11 μg, out of the added 5580 μg).

3.2.2.2. Zinc. The legume source had a significant ($P < 0.05$) effect on

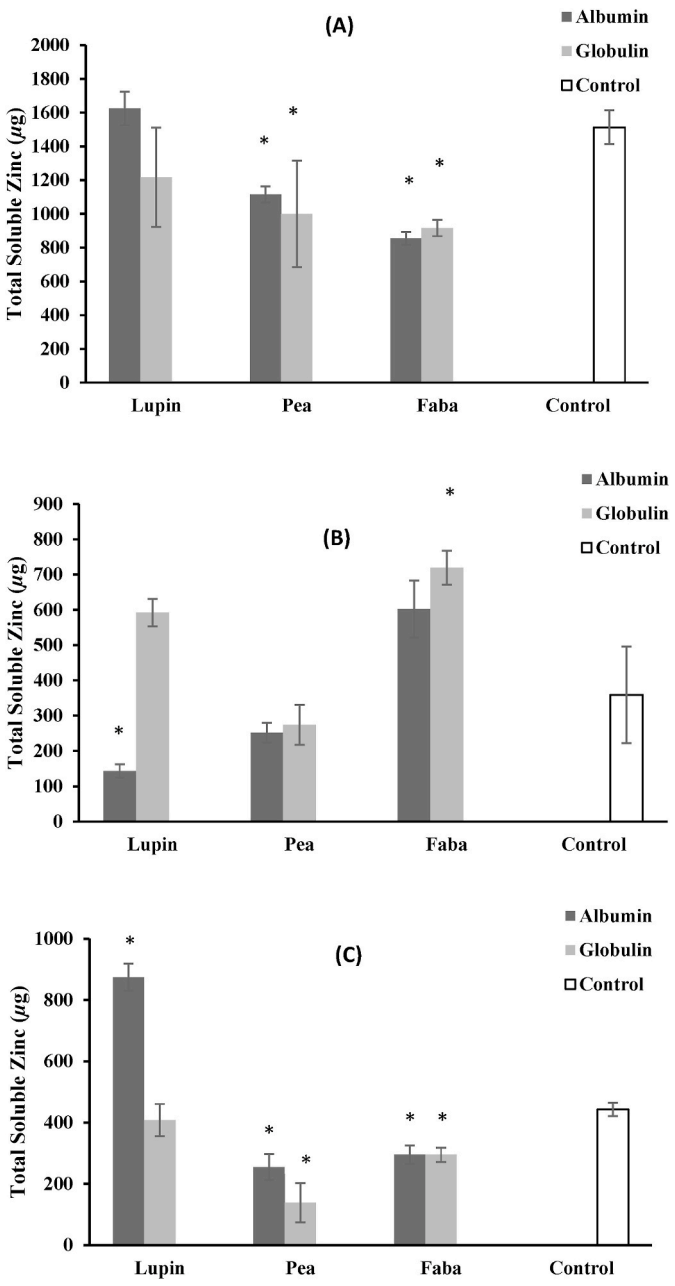

Fig. 3. Soluble zinc from mineral salt control and mineral fortified legume protein fractions subjected to a) oral-gastric (G), b) sequential oral-gastric-fasted intestinal (GI-Fasted), or c) sequential oral-gastric-fed intestinal (GI-Fed) stages of digestion. Values with an asterisk (*) are statistically significantly different from that of the mineral salt control in each column ($P < 0.05$). Results are expressed as means ± SEM (n = 3).

the solubility of Zn gastric, GI-fasted and GI-fed stages. Lupin showed significantly higher solubility than pea and faba during gastric stage (lupin: 1419 μg, pea: 1055 μg, faba: 884 μg out of the initial 6540 μg Zn loaded). This trend was also observed during fed intestinal digestion, where lupin demonstrated significantly higher mean solubility than faba and pea (lupin: 639 μg, faba: 292 μg, pea: 194 μg). There were no differences between the three legumes types during fasted intestinal digestion (faba: 429 μg, lupin: 420 μg, pea: 404 μg out of 6540 μg Zn loaded). However, protein type significantly affected the fasted intestinal phase, where solubility was higher with albumin compared to globulin (636 versus 200 μg out of 6540 μg Zn loaded). The two-way interaction between legume source and protein type was significant during the two intestinal stages ($P < 0.05$).

3.3. Soluble protein

The results of soluble protein are presented in Table 2. The proteins were gradually released as the soluble form into the digesta as simulated digestion progressed, with 1.51–8.57 mg of proteins released through the gastric digestion as soluble protein across all the protein fractions. By the end of the fasted state intestinal digestion, the legume fractions showed a range of 6.37–9.96 mg soluble proteins released, which is equivalent to 11.43–12.6% of the crude protein administered. Digestion under the fed state intensely increased the total soluble protein to a range of 19.08–47.25 mg per 100 mg fractionated protein powder.

The relationship between soluble protein and soluble Fe following simulated digestion appears nonlinear. Corresponding to the significant enhancement effect on soluble Fe^{3+}, concentrations of soluble protein are significantly higher in pea albumin and globulin during gastric phase compared to other protein fractions showing lower Fe^{3+} solubility (Table 2). Similarly, significantly higher levels of soluble Fe^{2+} (compared to the salt control) in the presence of legume globulins when digested during GI-fed, paralleled all legume globulins showing significantly greater soluble protein compared to the corresponding albumin (pea: 47.2 vs 29.4, lupin: 41.8 vs 21.3, faba: 40.6 vs 28.1 mg/100 mg fractionated powder). However, this effect was not consistently observed. For example, lupin and faba globulins demonstrated a positive effect on Fe^{3+} solubility as compared to the control during GI-fed and GI-fasted, respectively. Meanwhile, they did not show significantly higher levels of soluble protein in comparison to other legume fractions.

No distinct trends could be identified between soluble Zn and soluble protein in any of the digestion stages. The total soluble proteins from each fraction were not different by the end of GI-fasted. Whilst lupin albumin was the only fraction that enhanced Zn solubility during GI-fed, both lupin-derived fractions showed significantly higher total soluble proteins than other legume fractions by the end of GI-fed except pea globulin.

3.3.1. Proteolysis of proteins

Proteolysis may be blocked by strong element binding to the protein. To monitor this, the release of α-amino groups from proteins was measured through the digestion stages using the OPA assay. For both elements, a general pattern of time-dependent increase in α-amino groups was observed in all legume protein fractions, suggesting effective continuation of proteolysis as digestion progressed (Table 3). However, during the fed condition of intestinal digestion, some fractions demonstrated reduced α-amino group availability compared to fasted digestion, in serine equivalents on a per gram soluble protein basis. This includes both faba and pea proteins in the presence of Fe^{2+} (pea albumin: 8.4 vs 5.39, pea globulin: 5.85 vs 4.95, faba albumin: 8.4 vs 6.7, faba globulin: 9.17 vs 6.91 Ser-NH_2 meqv/g soluble protein), pea albumin (8.99 vs 5.794), lupin albumin (7.10 vs 5.25) and faba globulin (10.37 vs 5.6) in Fe^{3+}, and all proteins in the presence of Zn. Considering the dramatic increase in total soluble proteins during fed digestion in all samples, this still demonstrates a general trend of higher total number of α-amino groups (thus proteolysis) during this stage when compared to fasted intestinal digestion.

4. Discussion

Legume storage proteins play an uncertain role in Fe and Zn solubility, hence bioaccessibility. Whilst some evidence has emerged that some soluble Fe- and Zn- binding legume peptides may be exploited to enhance element bioaccessibility, it is unknown whether fortification with mineral salts at physiologically appropriate concentrations can evade the aggregation behaviour of legume storage proteins, and their gastrointestinal hydrolysis products promoted by cations and/or PA. In this investigation, we administered the equivalent of the lower end

Table 2
Total soluble protein (mg) released from the 100 mg administered fractionated protein powder in the supernatant of each sample at gastric and intestinal stages of digestion for each legume protein fraction. All samples were subjected to oral digestion and then carried over into gastric or gastric-intestinal intestinal digestion. Results are expressed as means ± SEM (n = 3). Means within each row followed by different superscript letters (a, b, c, d) are significantly different ($P < 0.05$).

Fortified mineral	Digestion stage	Soluble protein (mg/100 mg fractionated protein concentrate)					
		Pea		Lupin		Faba	
		Albumin	Globulin	Albumin	Globulin	Albumin	Globulin
Fe^{3+}	Gastric	6.62 ± 0.59 ^{a}	4.76 ± 0.33 ^{b}	3.94 ± 0.24 ^{c}	6.8 ± 1.1 ^{a}	4.23 ± 0.31^{bc}	4.15 ± 0.24 ^{bc}
	GI-Fasted	6.37 ± 0.54	8.05 ± 0.44	7.9 ± 1.7	8.29 ± 0.46	9.9 ± 2.1	6.73 ± 0.87
	GI-Fed	29.7 ± 2.8 ^{bc}	31.4 ± 2.9 ^{ab}	20.9 ± 1.7 ^{c}	38.1 ± 1.2 ^{a}	27.05 ± 0.33 ^{bc}	37.0 ± 1.9 ^{a}
Fe^{2+}	Gastric	6.08 ± 0.19	5.32 ± 0.28	7.0 ± 1.8	6.61 ± 0.45	7.01 ± 0.24	5.58 ± 0.62
	GI-Fasted	8.55 ± 0.21 ^{ab}	9.34 ± 0.14 ^{a}	8.4 ± 1.4 ^{abc}	9.46 ± 0.26 ^{a}	6.77 ± 0.18 ^{c}	7.16 ± 0.66 ^{bc}
	GI-Fed	29.4 ± 2.3 ^{b}	47.2 ± 1.3 ^{a}	21.3 ± 2.7 ^{b}	41.8 ± 1.9 ^{a}	28.1 ± 1.8 ^{b}	40.6 ± 1.5 ^{a}
Zn	Gastric	6.68 ± 0.62 ^{ab}	1.51 ± 0.20 ^{d}	4.5 ± 1.7 ^{c}	8.57 ± 0.27 ^{a}	5.6 ± 1.1 ^{bc}	4.10 ± 0.23 ^{c}
	GI-Fasted	7.67 ± 0.40	9.73 ± 2.04	7.9 ± 1.6	9.7 ± 1.8	6.49 ± 0.76	7.37 ± 0.44
	GI-Fed	19.0 ± 2.5 ^{d}	34.8 ± 1.5 ^{bc}	40.3 ± 4.6 ^{ab}	42.1 ± 1.2 ^{a}	19.6 ± 2.2 ^{d}	29.1 ± 3.5 ^{c}

Table 3
The number of free α-amino groups per gram of soluble protein, in the supernatant of each sample at gastric and intestinal stages of digestion for each legume protein fraction. α-amino groups as Ser-NH2 equivalents are expressed as means ± SEM (n = 3). Means within each row followed by different superscript letters (a, b, c, d) are significantly different ($P < 0.05$).

Fortified mineral	Digestion stage	Ser-NH_2 meqv/g soluble protein					
		Pea		Lupin		Faba	
		Albumin	*Globulin*	*Albumin*	*Globulin*	*Albumin*	*Globulin*
Fe^{3+}	Gastric	8.6 ± 1.0 ^{a}	4.01 ± 0.31 ^{b}	3.87 ± 0.19 ^{b}	3.12 ± 0.11 ^{b}	3.90 ± 0.34 ^{b}	4.23 ± 0.57 ^{b}
	GI-Fasted	8.99 ± 0.31 ^{ab}	8.10 ± 0.16 ^{a}	7.10 ± 0.33 ^{bc}	5.01 ± 0.12 ^{c}	5.13 ± 0.11 ^{b}	10.37 ± 0.42 ^{a}
	GI-Fed	5.794 ± 0.079	6.75 ± 0.14	5.25 ± 0.40	6.82 ± 0.77	7.655 ± 0.093	5.6 ± 1.0
Fe^{2+}	Gastric	3.007 ± 0.040 ^{b}	2.881 ± 0.017 ^{b}	2.63 ± 0.26 ^{b}	2.389 ± 0.078 ^{c}	3.41 ± 0.12 ^{a}	3.316 ± 0.060 ^{a}
	GI-Fasted	8.4 ± 2.3	5.85 ± 0.51	6.22 ± 0.30	4.86 ± 0.10	8.4 ± 1.5	9.17 ± 0.51
	GI-Fed	5.39 ± 0.41	4.95 ± 0.16	6.22 ± 0.28	4.64 ± 0.37	6.70 ± 0.93	6.91 ± 0.75
Zn	Gastric	2.172 ± 0.030 ^{cd}	6.63 ± 0.51 ^{a}	3.60 ± 0.76 ^{b}	1.17 ± 0.19 ^{d}	2.43 ± 0.13 ^{bcd}	3.28 ± 0.14 ^{bc}
	GI-Fasted	6.68 ± 0.39 ^{bc}	7.03 ± 0.40 ^{b}	5.782 ± 0.076 ^{cd}	5.01 ± 0.12 ^{d}	8.00 ± 0.30 ^{a}	8.078 ± 0.085 ^{a}
	GI-Fed	4.87 ± 0.53	5.11 ± 0.86	5.41 ± 0.31	4.68 ± 0.76	6.706 ± 0.066	5.843 ± 0.085

(6.54 mg for Zn, 5.58 mg for Fe) of the daily estimated average requirement (EAR) in Australia for adults (National Health and Medical Research Council, 2017). The vast majority of soluble Fe and Zn absorption takes place in the small intestine, where ionised Fe is known for its propensity to form inaccessible insoluble oxides and hydroxides species (Cremonesi et al., 2002). Meanwhile, Zn is known to bind both PA and legume oligomeric peptides with strong affinity at near small intestinal pH (~6.8) (Zhang et al., 2020). Whilst there have been no studies of extrinsically added minerals on the legume protein fractions we have examined to the best of our knowledge, a previous *in vitro* study on total soy protein found that the addition of Fe or Zn-sulphate at 15 mM (lower than the 20 mM in the current study) led to very little dialyzable quantities of both elements (Pérez-Llamas et al., 1997).

Gastric dissociation within the digestive tract is generally assumed to be important in Fe and Zn bioaccessibility within the small intestine, as this facilitates pH-induced release and ionisation (Betesh et al., 2015). However, our results suggest that the relationship between gastric and intestinal solubility was nonlinear in the presence of the legume fractions. Instead, the gastric-intestinal connection was dependent on the intestinal state, the protein involved, and oxidation state of Fe. Compared to the control mineral salt, the lower ferrous (Fe^{2+}) solubility in the presence of legume protein fractions during both gastric and GI-fasted digestion indicated that components derived from these fractions may have been bound to dissociated (Fe^{2+}) ions, but formed insoluble complexes under the conditions of both digestion stages. On the contrary, in the presence of the globulin fractions of all tested legumes under the fed state conditions the ferrous bioaccessibility was significantly ($P < 0.05$) enhanced (Fig. 2-C). These observations may indicate that the presence of large amounts of bile could facilitate and improve ferrous bioaccessibility.

Meanwhile in Fe^{3+}, despite observing a solubility enhancing effect in the presence of pea globulin, lupin globulin and pea albumin relative to the control during gastric digestion stage, none of these fractions yielded higher solubility during GI-fasted digestion stage. These fractions showed higher total solubilised proteins relative to other protein fractions during gastric but not during GI-fasted digestion stage. Therefore, it is conceivable that gastric solubilised Fe^{3+}-protein complexes underwent ligand exchange (dissociation) or ternary association with either PA/or other mineral salts upon the entry into the fasted intestinal phase. This behaviour coheres with that previously reported from pea phytoferritins, which forms a soluble complex with Fe^{3+} but tends to dissociate following gastric digestion (Perfecto et al., 2018). Since Fe^{2+} showed higher solubility in the control than Fe^{3+} in our study, this contributed to the observed differences in solubility between the two oxidation states.

The inverse relationship between Fe^{3+} and Zn solubility during gastric and intestinal phases found in some fractions, suggests the possibility that some soluble, element-binding peptides may be generated during intestinal rather than gastric digestion. However, it may also be due to changes in the iron-peptide interaction strength at the neutral pH conditions of intestinal digestion. For example, faba globulin was found in the current study to enhance both Fe^{3+} and Zn solubility during GI-fasted, yet did not show significantly higher levels of soluble Fe^{3+} or Zn than other fractions during gastric digestion. Recent studies on Fe^{2+}-casein complexes have also corroborated this discrepancy, where *in vitro* gastric dissociation was not found to be a requisite for solubility during intestinal digestion (Sabatier et al., 2020). Since a greater number of free α-amino groups was found in faba globulin during GI-fasted relative to others, the Fe^{3+} and Zn-solubilising properties of faba globulin may be related to the greater participation of peptides in element binding and solubilization from higher degree of hydrolysis of the protein. Soluble faba proteins hydrolysed by the intestinal enzymes pepsin and trypsin

have been previously reported to bind Fe^{2+} (Samaei et al., 2020), although the current study is the first to suggest its behaviour using simulated gastrointestinal digestion.

Our results reveal that the fed state of digestion with extended hydrolysis time and higher bile concentration can wield a biphasic impact on Fe and Zn solubility. As observed in the control mineral salt, high concentrations of bile in the fasted digesta mix decreased the solubility of the fortified elements (Figs. 1–3). The element insolubility was alleviated by the presence of lupin globulin for Fe^{3+}, most legume proteins for Fe^{2+}, and lupin albumin for Zn. On the other hand, the additional bile appears to have also increased the protein and minerals dissolution *via* enhanced solubility and/or hydration as previously described (Wang et al., 2011), as evident through the drastic enhancement in soluble protein for all fractions during this phase. Bile salts are known to be amphipathic in solution. Depending on its solubility product, they can transition into colloidal micelles above a critical concentration (CMC) or participate in salt precipitation. As such, the latter state appears to have been favoured in the mineral salt controls and most fractions containing Zn, which is also prone to co-precipitation with calcium phosphate in the digestive fluid (Feng et al., 2020). However, the presence of legume proteins likely promoted bile micellization to varying degrees under fed digestion, where bile concentrations may have been sufficient to reach the CMC. This surfactant effect, along with prolonged and enhanced protein hydrolysis due to bile (Gass et al., 2007), are likely responsible for the observed increase in legume proteins' solubility in the fed digesta mix with high level of bile salt.

We found a general pattern of higher Fe and Zn solubility in the presence of globulin as compared to albumins during small intestinal digestion, which included some enhancement effects during the fed state, and less inhibitory effects during the fasted state. Whilst we are not aware of any other studies that have examined digestion under the two intestinal states, this phenomenon was also recognized in previous studies that examined the bioaccessibility of endogenous Fe and Zn present in legumes. In a series of studies on *Phaseolus vulgaris* (Lombardi-Boccia et al., 1998, 2003), both the whole bean and the globulin fractions were found to have higher soluble Fe and Zn as compared to albumins by the end of *in vitro* intestinal digestion. The authors attributed the difference in Fe and Zn dialyzability between albumin and globulin to PA, and protein properties, respectively. Similar to our observations, lower PA levels were found in the globulin extracts that was linked to enhanced dialyzability. When the authors enzymatically dephytinized the albumin fraction, this led to a significant increase in dialyzability, especially in Zn. As comparable levels of PA were found in the current study (range: 1.63–15.8 mg/g versus 9.12 mg/g), it is likely that the application of enzymatic dephytinization would also enhance the Fe and Zn solubility of the fractions investigated, particularly given the high element:PA ratio employed in the current investigation.

The oxidation state of Fe is considered an imperative bioaccessibility factor in oral supplementation, where Fe^{2+} is commonly employed due to its higher solubility than Fe^{3+} at neutral intestinal pH, as well as the ability to be taken up directly without reduction by ferric reductase in the gut mucosa (Cremonesi et al., 2002). Our results demonstrated that although a higher quantity of Fe^{2+} was solubilised as compared to Fe^{3+} in the control mineral salts during fasted digestion, the presence of legume protein concentrates greatly inhibited Fe^{2+} solubility during GI-fasted to a similar extent regardless of oxidation state. During GI-fed, the presence of some legume proteins enhanced both Fe solubility by means of circumventing precipitation with bile, an effect that was significant in lupin globulin for Fe^{3+}, and in lupin albumin and all globulins for Fe^{2+}. This implies that the in the absence of high bile concentrations and the large mass of solubilised peptides during GI-fed, Fe^{2+} may not be advantageous over Fe^{3+} when administered with legume proteins. Some legume proteins and their hydrolysates are known to have bile-binding property (Yoshie-Stark & Wäsche, 2004), which may also lead to less precipitation between bile and iron. It is possible that the globulin fractions exert more bile-binding.

Overall, our results support that the solubility of Fe^{3+} iron may be enhanced in the presence of suitable enhancers, such as some globulin fractions identified in the current study. Fe^{3+}- protein ligand complexes can have important implications for food fortification, as the chemical reactivity of Fe^{3+} leads to detrimental organoleptic properties (Prentice et al., 2016). Whilst Fe^{3+} iron possesses lower solubility in its native form, its inert property could be more suitable for food fortification. Additionally, some albumin fractions, particularly from pea has also shown potential for Fe solubility enhancement. Pea albumin appeared to form stable soluble complexes with Fe^{3+} during gastric digestion that withstood centrifugation. However, its intestinal solubility remained low, which was likely due to the occurrence of PA-element-protein complexes associated with its high PA content. In the case of the current study, complete dephytinization would likely improve its low solubility during intestinal digestion. It is acknowledged that whilst examining *in vitro* solubility is only the prelude towards understanding mineral bioaccessibility in the presence of legume fractions, further validation *in vivo*, such using by animal models should be conducted.

5. Conclusion

In the current study, we administered Fe and Zn salts with legume protein concentrates, with the elements in large molar excess of the endogenous PA present in legumes. It was found the faba globulin fraction improved the *in vitro* bioaccessibility of fortified Fe^{3+} and Zn during the fasted state of intestinal digestion, whilst others had no effect or reduced mineral solubility. The presence some lupin fractions enhanced Fe bioaccessibility relative to the mineral salt control during fed stage intestinal digestion, including lupin globulins for Fe^{3+} and lupin albumin and all globulins for Fe^{2+}. This observation is likely related to the type or substantial quantity of proteins solubilised during this stage, which circumvented formation of insoluble complexes with bile. Zn bioaccessibility during the fed stage was also enhanced by lupin albumin, but not influenced or decreased by other protein fractions. The solubility advantage of using Fe^{2+} over Fe^{3+} was conditional in the presence of certain fractions, indicating that Fe^{3+} may be a potential food fortifier for its advantages in oxidative stability. The notion of elemental bioavailability as a result of non-specific interactions from the food matrix, rather than a specific component should continue to be recognized in the design of food-based fortification strategies.

Hypothesis

Peptides formed during *in-vitro* digestion of legume proteins may improve or inhibit the *in vitro* bioaccessibility of Fe and Zn mineral salt at high concentrations (20 mM)

CRediT authorship contribution statement

Yianna Y. Zhang: Writing – review & editing, Writing – original draft, Supervision, planned the study with significant contributions from, performed all the lab work and data collection. wrote the first draft of the manuscript and all supervisors read, revised and approved the final manuscript. **Regine Stockmann:** Writing – review & editing, Writing – original draft, Supervision, performed all the lab work and data collection, provided necessary advice, guidelines, and supervision in conducting the work. **Ken Ng:** Writing – review & editing, Writing – original draft, Supervision, performed all the lab work and data collection. provided necessary advice, guidelines, and supervision in conducting the work. **Said Ajlouni:** Writing – review & editing, Writing – original draft, Supervision, planned the study with significant contributions from, provided necessary advice, guidelines, and supervision in conducting the work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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